ON PERHEXILINE AND ITS APPLICATION TO MYOCARDIAL PROTECTION DURING CARDIAC SURGERY

by

NIGEL EDWARD DRURY

A thesis submitted to
The University of Birmingham
for the degree of
DOCTOR OF PHILOSOPHY

Department of Cardiovascular Medicine
College of Medical and Dental Sciences
The University of Birmingham

January 2012

ABSTRACT

Perhexiline is an anti-anginal drug that is thought to shift myocardial metabolism from β-oxidation of fatty acids to glucose utilisation. An associated improvement in energy efficiency may be beneficial in ischaemia-reperfusion as an adjunct to established techniques for myocardial protection during cardiac surgery. In this thesis, I conduct a prospective double-blind randomised placebo-controlled trial of oral perhexiline in patients undergoing coronary artery surgery, obtaining samples of serum, right atrium and left ventricle. I measure the concentration of perhexiline using high performance liquid chromatography and find that although highly concentrated in the heart, it may not have reached steady-state in the ventricular myocardium. I perform enzymatic colourimetry and ultra-high resolution mass spectrometry to detect changes in carbohydrate and lipid metabolism; however, the myocardial metabolic profiles of patients on perhexiline are indistinguishable from controls. On analysing the results of the clinical trial, I find no improvement in the primary endpoint, the incidence of a low cardiac output episode, or any secondary outcomes. I conclude that preoperative oral perhexiline does not improve clinical markers of myocardial protection and despite significant accumulation in the myocardium, it has no significant effect on the measurable metabolic profile of the heart at the time of surgery.

Dedicated to the memory of Colin Gordon Drury,

an inspiration who worked tirelessly to support his children

ACKNOWLEDGEMENTS

This thesis would not have been possible without the advice, support & collaboration of many individuals across the UK and beyond to whom I am eternally grateful. Firstly, I would like to thank my four supervisors, who have played prominent roles through different stages of the evolution, conduct and analysis of its components, taken an electronic red pen to previous drafts and coaxed me in the right direction:

Professor Domenico Pagano, Consultant Cardiothoracic Surgeon at the Queen Elizabeth Hospital Birmingham & Principal Investigator of the CASPER trial

Professor Michael P. Frenneaux, former BHF Chair of Cardiovascular Medicine, now Regius Professor of Medicine at the University of Aberdeen

Professor Nick Freemantle, former Chair of Clinical Epidemiology & Biostatistics, now Chair at University College London, and

Professor Mark R. Viant, Chair of Metabolomics at the University of Birmingham.

I am grateful to several charitable bodies for funding different aspects of my research: the *British Heart Foundation*, who funded the clinical trial in Birmingham including my salary for three years through a project grant; the *Sussex Heart Charity* who sponsored a research nurse for one year to assist with data collection in Brighton; the *Wellcome Trust*, who subsidised my collaboration with Adelaide through a training award; and the *Queen Elizabeth Hospital Birmingham Charity*, who paid my salary whilst I was working in Adelaide.

I am indebted to the enthusiasm and dedication of many colleagues in facilitating the conduct of the clinical trial. At the Queen Elizabeth Hospital Birmingham – *Consultant Cardiac Surgeons*: Mr Stephen J. Rooney, Mr Timothy R. Graham, Mr Ian C. Wilson, Mr C. Jorge G. Mascaro, Mr Uday Dandekar and Professor Robert S. Bonser; *Consultant Cardiac Anaesthetists*: Dr Peter Townsend, Dr Deborah Turfrey, Dr Tessa Oelofse, Dr David Riddington, Dr Mark Wilkes, Dr Harjot Singh, Dr Suneel Desai, Dr Muzaffar Faroqui, Dr Tariq Hoth, Dr Anwar Karim and Dr Craig McGrath; *Surgical Registrars*: Mr Thomas Barker, Mr Sunil Bhudia, Mr J. Saravana Ganesh, Miss Deborah Harrington, Mr Mauro Iafrancesco, Mr Abdul Itrajky, Mr Mahmoud Loubani, Miss Ashvini Menon, Mr Calvin Ng, Miss Vanessa Rogers, Mr Kirk Santo and Mr Selvaraj Shanmuganathan; *Clinical Perfusion*: Nick Trimmer, Sue Matthews, Ramesh Gohil, Natalie Phillips, Kevin Smith and colleagues; *Cardiac theatres*: Veda Grey and colleagues; *Cardiac Intensive Care*: Pete Vance and colleagues; *Research & Development*: Dr Chris Counsell and colleagues; *Clinical Biochemistry*: Dr Bob Cramb and colleagues; *Pharmacy*: Mary Kotadia, Rosemarie Seadon and colleagues; *Administration*: Kay Fletcher, Samantha Thomas, Lesley Melvin, Yvonne Smith, Viv Barnett, Ann Howells and Ian Shakespeare; *Data and Safety Monitoring Board* members: Dr Paul Jordan and Dr Graham Lipkin.

At the Royal Sussex County Hospital, Brighton – *Consultant Cardiac Surgeons*: Mr Michael E. Lewis, Mr Jonathan A. J. Hyde and Mr Andrew S. Cohen; *Consultant Cardiac Anaesthetists*: Dr Robert Kong and colleagues; *Research Nurses*: Emma Gardner, Ailie Mackenzie and Nina Cooter; *Research & Development*: Scott Harfield; and many other staff in perfusion, theatre, pharmacy and clinical biochemistry.

At the University of Birmingham – *Cardiovascular Medicine*: Dr Lynne K. Williams, Dr Roger M. Beadle and Dr Khalid Abozguia; *Statistics*: Dr Sayeed Haque. At the Basil Hetzel Institute and Queen Elizabeth Hospital, Adelaide – *Clinical Pharmacology*: Dr Ian S. Westley and colleagues; *Cardiology*: Dr Doan T. Ngo, Dr. Jenny Kennedy, Marilyn Black, Irene Stafford and colleagues. In the Discipline of Surgery at the University of Adelaide: Professor Guy J. Maddern. At the Toxicology Unit, Llandough Hospital: Dr Alun D. Hutchings and Emma Bennett. At Bilcare GCS: Julie Lippiett, Nia Stone and Kerrie Evans. At Sigma Pharmaceuticals: Con Kouskouris and colleagues. At Marken UK: Tim Saint and Lina Koulaouzos. I am grateful to Daniel R. Nelson for his Russian-English translation of articles for the systematic review in Chapter 2.

All clinical trials rely on the selfless cooperation of patients to give informed consent and play their part in the protocol. Without the hundreds of participants who took that leap of faith, my voyage into translational research would have soon run aground.

In particular, I would like to thank Mr David W. Quinn and Mr Aaron M. Ranasinghe for establishing the foundations for conducting large clinical trials of myocardial protection at the Queen Elizabeth Hospital, Birmingham and Dr David Green for his work on the anaesthetic protocol and assistance in expanding the trial to Brighton. I would like to extend my appreciation to Clare Palmer, Paul Yates, Maureen Haycock and Sarah Faulkner for their help in facilitating the day-to-day management of the trial. I am also grateful for the advice of my mentor, Mr Timothy J. Jones at Birmingham Children's Hospital and for the unwavering support of Miss Nicole Breitenfeldt and my mother, Rosalind F. Drury over many years.

Whilst many have contributed to the success of this project, there are a few to whom I owe the most gratitude. In Birmingham, my friend and colleague Mr Neil J. Howell was instrumental in setting up the clinical trial, securing funding and expanding the scope of research in our department. Mr Eshan L. Senanayake picked up the ball and ran with it, enabling completion of the trial whilst I was working in Adelaide. Dr Melanie J. Calvert was a constant source of statistical help and advice throughout the trial and its analysis. Dr David Hauton gave frequent and valuable advice on writing this thesis and in the laboratory, he and Stephanie Baxter facilitated the biochemical experiments. Dr Ralf J. Weber performed the complex metabolomic analysis of the invaluable ventricular biopsies for which I am greatly indebted. At the University of Adelaide, Professor John D. Horowitz, Associate Professor Benedetta C. Sallustio and Dr John Licari made me most welcome in their exquisite laboratory for two months, nurtured the development of my hypotheses and guided me through the techniques to achieve my goals; I hope and believe that with these roots, our international collaboration will continue to grow and flourish for many years to come.

Finally, I am fortunate to have the love, support and inspiration of Dr Clare J. Taylor, my fiancée and a shining example of a promising clinical academic, for whom I hope to return these sentiments on the road ahead.

PERSONAL CONTRIBUTION

The foundation of this thesis is a clinical trial of perhexiline in patients undergoing cardiac surgery conceptualised by Professor Domenico Pagano and Professor Michael P. Frenneaux. I was appointed to the post of British Heart Foundation Clinical Research Fellow at the University of Birmingham following a competitive application process in late 2006. From February 2007, I was responsible for every aspect of trial management including the logistical and financial administration of grants from the British Heart Foundation and the Wellcome Trust.

I was personally responsible for the conduct or oversight of all aspects of the trial protocol including recruitment, randomisation, prescribing of the trial drug, liaising with administrative staff regarding scheduling of trial patients and management of trial tablets. I provided a 24-hour patient helpline for nearly three years and managed any reported potential side-effects. I was accountable for the collection, processing and storage of all data, human blood and tissue samples in accordance with the Data Protection Act 1998 and the Human Tissue Act 2004. I was responsible for the early postoperative management of patients on the Intensive Care Unit and remained with each patient until at least four hours following surgery to ensure adherence to the trial protocol; in addition, I held educational seminars for the medical and nursing staff to promote the theory behind the trial and their understanding of the protocol. I entered all trial data into an electronic database for analysis, cleaned the data and submitted monthly summary data from both sites to the UK Clinical Research Network database. I chaired and presented data to the blinded endpoints committee.

Chapter 3: Methods

The trial methodology was an evolution of previous trials on myocardial protection conducted in the department over the preceding decade. The original protocol and BHF grant application were written by my predecessor Mr Neil J. Howell who also obtained ethical and MHRA approval for conduct of the trial at QEH, Birmingham. I submitted a substantial amendment to obtain ethical and regulatory approval to expand the trial to the new trial site in Brighton and secured sponsor and local Trust approval. The randomisation schedule and power calculations were devised by Dr Melanie J. Calvert and Professor Nick Freemantle prior to my appointment but I developed the statistical analysis in conjunction with Dr Calvert. I completed Good Clinical Practice training to comply with the NHS Research Governance framework and was responsible for reporting serious adverse events to the sponsor and MHRA.

Chapter 4: Results

I recruited three hundred (91.7%) of the 327 patients randomised during the trial. The remainder of the patients in Birmingham (8, 2.4%) were enrolled by my successor, Mr Eshan L. Senanayake who completed the trial whilst I was working in Adelaide. All 19 (5.8%) of the trial patients enrolled at RSCH, Brighton were recruited by Sr. Emma Gardner or Sr. Ailie Mackenzie under my supervision; I made five visits to Brighton and was in frequent email and telephone contact to facilitate recruitment.

Chapter 5: Perhexiline pharmacokinetics in the myocardium

I developed the concept of studying myocardial pharmacokinetics in collaboration with Professor John D. Horowitz and Associate Professor Benedetta C. Sallustio. I

collected all of the human samples and arranged the specialist courier to Adelaide. During my Wellcome Trust Training Award, I performed all of the perhexiline extractions, chromatography and data analysis. I was taught the practical techniques by Dr John Licari under the supervision of A/Prof Sallustio.

Chapter 6: Metabolic preconditioning with perhexiline

I performed the serum and tissue carbohydrate analysis. The tissue lipid analysis including histology was performed by both I and Stephanie Baxter, as part of her undergraduate BMedSci project under my supervision. Some of the remaining serum analyses, which I had not completed, were performed by Dr David Hauton.

In metabolomics, Dr Ralf J. Weber prepared the samples, performed FT-ICR MS and analysed the data as part of his post-doctoral research. Whilst I attended the laboratory, assisted with the experiments and participated in discussions at all stages of the study design and analysis, these highly specialised techniques remain beyond my level of laboratory and statistical modelling training.

Chapter 7: Results of the clinical trial

Statistical analysis of the main trial outcomes was performed by Dr Melanie J. Calvert in her role as trial statistician; however I independently repeated all of the calculations contained in this thesis, except the mixed model for the primary endpoint and the propensity scoring, and performed additional analyses including a detailed evaluation of the baseline cardiac index and a Forest plot.

PERSONAL GRANTS & AWARDS

The clinical trial was funded by a project grant from the British Heart Foundation. In addition, I have been awarded several grants relating to my doctoral research:

Wellcome Trust Value in People Training Award 2010 – £4,000

To visit the Basil Hetzel Institute for Translational Health Research at the University of Adelaide, South Australia to conduct research on the myocardial pharmacokinetics of perhexiline in February-March 2010.

Queen Elizabeth Hospital Birmingham Charity Research Grant 2010 – £6,330

To pay my salary during my research visit to Adelaide.

University of Birmingham Overseas Travel Fund Award 2011-12 – £500

From the School of Clinical & Experimental Medicine to present the results of the clinical trial at the prestigious Scientific Sessions of the American Heart Association in Orlando, FL, November 2011.

ABSTRACTS & PUBLICATIONS

The work submitted in this thesis has been presented at one national and one international meeting to date. I have also given two invited lectures on this work.

Drury NE, Howell NJ, Senanayake E *et al*. The effect of Perhexiline on myocardial protection during coronary artery surgery: a two-center randomized double-blind placebo-controlled trial. Presented at the Scientific Sessions of the AHA, Orlando, FL, November 2011, abstract published in *Circulation* 2011; 124: A15650.

Drury NE, Howell NJ, Senanayake E *et al*. The effect of Perhexiline on myocardial protection during coronary artery surgery: a two-centre randomised double-blind placebo-controlled trial. Presented at SCTS Annual meeting, London, March 2011.

Drury NE. Improving myocardial protection during cardiac surgery. By invitation to the Grand Round, New Cross Hospital, Wolverhampton, November 2010.

Drury NE. Improving myocardial protection during cardiac surgery. By invitation to the Department of Cardiology, The Queen Elizabeth Hospital, Adelaide, March 2010.

A combined manuscript on the results of the clinical trial and the effect of perhexiline on the myocardial metabolome has been prepared and is awaiting submission. Further papers on the pharmacokinetics of perhexiline in the myocardium and population modelling of perhexiline pharmacokinetics are in preparation.

CONTENTS

ILLUSTRATIONS

TABLES

ABBREVIATIONS

ACC	acetyl-CoA carboxylase
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AMPK	adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
ASR	annual safety report
ATP	adenosine triphosphate
AUC	area under the curve
BHF	British Heart Foundation
C_{Px}	concentration of perhexiline
$C_{OH\text{-}Px}$	concentration of hydroxyperhexiline
CABG	coronary artery bypass grafting
CK-MB	creatine kinase muscle-brain type isoenzyme
CONSORT	Consolidated Standards of Reporting Trials
CPB	cardiopulmonary bypass
CPT	carnitine palmitoyltransferase
CTA	clinical trial authorisation
CV	coefficient of variation
CVP	central venous pressure
DCA	dichloroacetate
DiM	difference in means
DSMB	Data and Safety Monitoring Board
ECG	electrocardiogram
ETC	electron transport chain
F2,6-BP	fructose 2,6-bisphosphate
FAD	flavin adenine dinucleotide
FBPase2	fructose 2,6-bisphosphatase
FDR	false discovery rate
FFA	free fatty acid

FT-ICR	Fourier transform ion cyclotron resonance
G3P	glyceraldehyde 3-phosphate
G6P	glucose 6-phosphate
GDP	guanosine diphosphate
GIK	glucose-insulin-potassium
GSK-3β	glycogen synthase kinase 3 beta
GTP	guanosine triphosphate
HPLC	high performance liquid chromatography
IC_{50}	half maximal inhibitory concentration
ICH	International Conference on Harmonisation
ICU	intensive care unit
IMP	investigational medicinal product
IQR	interquartile range
IRS	insulin receptor substrates
K_m	Michaelis constant
LCOE	low cardiac output episode
LDH	lactate dehydrogenase
LVEF	left ventricular ejection fraction
m/z	mass-to-charge ratio
MACE	major adverse cardiac event
MAP	mean arterial pressure
MAP kinase	mitogen-activated protein kinase
MCD	malonyl co-A decarboxylase
MHRA	Medicines and Healthcare products Regulatory Agency
mPTP	mitochondrial permeability transition pore
MS	mass spectrometry
NAD^+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide reduced form
NADPH	nicotinamide adenine dinucleotide phosphate reduced form
NMR	nuclear magnetic resonance
OCT	optimal cutting temperature
OR	odds ratio

PAWP	pulmonary artery wedge pressure
PCA	principal components analyses
PCI	percutaneous coronary intervention
PCr	Phosphocreatine
PD	pharmacodynamic
PDH	pyruvate dehydrogenase
PDK	pyruvate dehydrogenase kinase
PFK	phosphofructokinase
PGC-1α	peroxisome proliferator-activated receptor gamma co-activator 1-alpha
PI3K	phosphatidyl-inositol-3-kinase
PK	pharmacokinetic
PLS-DA	partial least squares discriminant analyses
PPARα	peroxisome proliferator-activated receptor alpha
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
QC	quality control
QEH	Queen Elizabeth Hospital, Birmingham
QP	Qualified Person
R	regression coefficient
RISK	reperfusion injury salvage kinase
ROS	reactive oxygen species
RSCH	Royal Sussex County Hospital, Brighton
SAE	serious adverse event
SAFE	survivor activating factor enhancement
SIM	selected ion monitoring
SUSAR	suspected unexpected serious adverse reaction
TAG	triacylglycerol
TQEH	The Queen Elizabeth Hospital, Adelaide
TRX	thioredoxin reductase
TXNIP	thioredoxin-interacting protein
UCP	uncoupling protein
Vo_2 max	maximal oxygen consumption
WMD	weighted mean difference

1. INTRODUCTION

1.1 Myocardial protection: the key to open heart surgery

> *'I became aware that surgeons obviously would never be able to correct or cure [many] heart conditions unless they were able to stop the circulation of the blood through the heart, open it and operate in a bloodless field under direct vision.'*
>
> (Bigelow, 1984)

In his memoirs, Wilfred G. Bigelow reflects on his time with Alfred Blalock at the Johns Hopkins Hospital, Baltimore in 1946-47. In conjunction with Helen B. Taussig and Vivien T. Thomas, Blalock had developed a method for palliating complex intracardiac anomalies by forming a surgical shunt between the systemic and pulmonary circulations; this allowed deoxygenated blood to reach the lungs without passing through the right side of the heart (Blalock and Taussig, 1945). Prior to the introduction of this operation, life expectancy in such conditions was often limited to early childhood. Bigelow recognised both the importance of this pioneering 'blue baby operation' and the limitations of extra-cardiac palliation. To achieve anatomical correction, surgeons would need to venture inside the heart and this would require a totally new approach. Back in Toronto, he set to work on testing his hypothesis for making open heart surgery possible using hypothermia (Bigelow et al., 1950c). In his laboratory, anaesthetised dogs were cooled to 20 degrees Celsius by a blanket containing coils of circulating refrigerant. At this temperature, he found that total body oxygen consumption was reduced to 15% of baseline with a corresponding reduction in cardiac output (Bigelow et al., 1950b). The heart could be excluded from the circulation for up to 15 minutes by occluding the venae cavae and azygos vein,

enabling incision of the right auricle, intra-cardiac exploration and resuturing (Bigelow et al., 1950a). The animals were then revived to normal body temperature in a bath; in those that survived, apparently normal activity was resumed within a few days with no evidence of end-organ injury. These experiments demonstrated that by reducing metabolism, total body hypothermia had the potential to protect the heart, brain and other vital organs whilst allowing safe access to the cardiac chambers to repair defects. Bigelow presented his findings to the American Surgical Association meeting at Colorado Springs in April 1950 and his audience included two young surgeons from the University Hospital in Minneapolis, F. John Lewis and C. Walton Lillehei. Inspired by the potential application of hypothermia to cardiac surgery, they returned to Minnesota and began their own investigations (Miller, 2000).

By 1952, satisfied with his laboratory experiments on dogs, Lewis was ready to use systemic hypothermia on a patient with congenital heart disease. On 2nd September, assisted by Richard L. Varco, Lillehei and others, a five year old girl was cooled down to 28 degrees Celsius over two hours using rubberised blankets (Lewis and Taufic, 1953). Lewis opened the chest, isolated and snared the caval and azygous veins, then clamped the pulmonary artery and aorta, thereby isolating the heart and stopping the circulation. He opened the right atrium and identified the pathological lesion, an atrial septal defect measuring two centimetres; in a time when a diagnosis was often difficult to make pre-mortem, the surgeons were thankful that it had proven correct. The defect was repaired with direct suture, the atrial incision closed and the clamps released in 5½ minutes. The heart began to contract spontaneously and with some manual encouragement, rapidly regained its normal rhythm. By modulating

metabolism using hypothermia, Lewis and colleagues had introduced the application of myocardial protection to clinical practice. Eleven days later, the world's first successful open heart surgery patient was discharged home and a new epoch in cardiac surgery had begun.

Amidst the excitement, Lillehei was sceptical that total body hypothermia alone would permit more complex and time-consuming repairs (Miller, 2000). He was embarking on a new and altogether more controversial strategy of circulatory support – cross-circulation. In earlier studies, by occluding the venae cavae, it had been shown that a dog could survive for up to two hours with a cardiac output as low as 10% of normal followed by a full recovery, termed 'the azygous principle' (Andreasen and Watson, 1952). By connecting the great vessels of a patient to the femoral arteries of a much larger blood-matched donor, such as a parent, with intervening arterial and venous pumps, the recipient could be maintained on low-flow perfusion from the donor to enable opening of the heart and repair of structural defects (Warden et al., 1954). Between March 1954 and May 1955, Lillehei and colleagues used this technique of controlled cross-circulation to operate on 45 children including the first successful repairs of a ventricular septal defect, an atrioventricular septal defect and Tetralogy of Fallot (Lillehei et al., 1955a, Lillehei et al., 1955b). This seminal achievement was acknowledged by the Lasker Award for Clinical Medical Research in 1955 (Gott and Shumway, 2004). Remarkably, 28 (62%) children were discharged home and 20 (44%) are alive today without significant limitations related to their original condition (Lillehei et al., 1986, Moller et al., 2009). Meanwhile, others were using alternative biological strategies for extracorporeal support; in Toronto, William T. Mustard

employed the freshly extricated lungs of rhesus monkeys as an artificial oxygenator, positioned inside bell jars and connected to a pump (Mustard and Thomson, 1957). Alas, the outcomes were almost universally poor and many surgeons were now working towards the holy grail of early cardiac surgery – a heart-lung *machine*.

In order for such a device to be safe and effective, three factors would need to be overcome: predictable and reversible anticoagulation to prevent clot formation in the machine or excessive postoperative bleeding; a method for pumping blood without significant haemolysis; and a means of oxygenating the circulating blood whilst removing carbon dioxide (Stoney, 2009). By the middle of the last century, heparin and protamine were readily available, and several pumps used by the dairy sector could be adapted for the task; the major problem however was an artificial oxygenator. This challenge was taken up by numerous teams around the world through innovation, sharing of ideas and collaboration with industry, a feature that would become a driving force in the development of the speciality; by the time that the Space Race took off, the race to mechanise cardiac surgery was well underway.

Its early leader was John H. Gibbon Jr. at the Jefferson Medical College in Philadelphia. Supported by engineers and funding from IBM during 1946-53, he developed a vertical screen oxygenator fed by roller pumps, culminating in the first successful heart operation using cardiopulmonary bypass (Gibbon, 1954). However, the machine was complicated, expensive, labour-intensive and at times temperamental; three of the first four patients died in the operating room and after 19 years of laboratory development, Gibbon imposed a year-long moratorium on the

clinical use of his machine (Romaine-Davis, 1991). Inspired by Gibbon, Clarence Crafoord, Viking Olov Björk and Åke Senning at the Karolinska Institute, Stockholm had been working on a rotating disc oxygenator with engineers at the AGA Company and in July 1954, successfully removed a left atrial myxoma (Crafoord et al., 1957). Meanwhile at the Mayo Clinic in Rochester, John W. Kirklin and colleagues were modifying Gibbon's complex vertical screen oxygenator (Kirklin et al., 1956) and just 60 miles away in Minneapolis, Richard A. DeWall was developing a more simple bubble oxygenator system (DeWall et al., 1956). In May 1955, Lillehei switched from controlled cross-circulation to the DeWall bubble oxygenator and within a year, a sterile disposable version of the device was on the market (Lillehei et al., 1956). This inexpensive yet effective device enabled many medical centres to start a cardiac surgery programme and in the words of Denton A. Cooley (Lillehei et al., 1986):

"Walt Lillehei brought the can opener to the cardiac surgery picnic."

Through the 1960s, the bubble oxygenator with an integrated defoaming chamber and heat exchanger, emerged as the dominant technology. By the mid-1980s, microporous hollow-fibre membrane oxygenators became commercially available and remain the most common in use around the world today.

Whilst the advent of cardiopulmonary bypass (CPB) was a major milestone in the development of open heart surgery, other problems remained: opening the contracting heart had the potential for fatal massive air embolism; operating on the beating heart was technically difficult; and blood return through the coronary sinus and pulmonary veins often obscured the operative field (Cordell, 1995). Attention

now turned to developing methods for the elective and reversible arrest of the heart whilst the bypass machine maintained circulation to the brain and other vital organs. The key to open heart surgery would be allowing a safe period of cardiac arrest during cessation of coronary blood flow by minimising the effects of ischaemia, a concept known today as myocardial protection.

In this thesis, I explore the metabolic pathways involved in generating energy for maintaining homeostasis and performing the contractile function of the myocardial cell, and the effect of ischaemia and subsequent reperfusion on these processes. I then focus on how the resulting injury can be minimised in the field of cardiac surgery through current and novel techniques, including the application of metabolic drug therapies. I present clinical and laboratory data obtained through a trial of the metabolic agent perhexiline in patients undergoing coronary artery bypass graft (CABG) surgery on CPB to evaluate its potential application to myocardial protection. I also use the opportunity to perform the first direct studies in humans on the myocardial pharmacokinetics of perhexiline and its effects on myocardial metabolism.

1.2 Metabolism and the myocardium

'And what is a man without energy? Nothing – nothing at all.'

(Twain, 1860)

1.2.1 Adenosine triphosphate and the transfer of free energy

All living things require a continual input of free energy for three major purposes: the performance of mechanical work in muscle contraction and cellular movement, the active transport of molecules and ions, and the synthesis of macromolecules from simple precursors. The metabolic pathways that drive these processes are highly efficient and have been remarkably conserved throughout evolution.

Adenosine triphosphate (ATP) is the principal immediate donor of free energy in biological systems and is continuously formed and consumed. The triphosphate unit of ATP contains two phosphoanhydride bonds which release large amounts of free energy when hydrolysed to adenosine diphosphate (ADP) or adenosine monophosphate (AMP). The energy charge of a cell is the ratio of high-energy phosphoanhydride bonds to the mole fraction of ATP, ADP and AMP. If the energy charge falls, AMP levels rise and stimulate *AMP-activated protein kinase* (*AMPK*), a master regulator of intracellular metabolism. Allosteric activation of *AMPK* increases the cellular energy level by inhibiting synthetic, ATP-consuming pathways and stimulating catabolic, ATP-generating pathways including glycolysis and β-oxidation of fatty acids; *AMPK* thereby acts as a 'fuel gauge' for the cell (Hue and Rider, 2007).

As the turnover of ATP is so high, it is not a useful mode of energy storage. In order to sustain contractile activity, muscle contains a reservoir of high-phosphoryl groups

in the form of phosphocreatine (PCr) which can readily transfer a ~P group to ATP catalysed by *creatine kinase*. PCr therefore acts as a short-term high-energy phosphate buffer enabling maintenance of a high ATP concentration during bursts of intense energy demand such as the initiation of exercise. Longer term energy storage is afforded by macromolecules such as glycogen and triglycerides that can be mobilised in response to energy needs.

Hans Krebs (1957) described three stages in the generation of energy from the oxidation of foodstuffs: the digestion of complex macromolecules into smaller units; the conversion of these small molecules into a few simple units, principally the acetyl group of acetyl-CoA; and finally, the entry of these intermediates into the citric acid cycle and electron transport chain. The evolutionary endurance of these metabolic pathways is due to their efficiency in extracting the maximum yield of free energy through complete oxidation. The main substrates of metabolism are carbohydrates, fats and proteins although energy may also be derived from other molecules including ketones, lactate and alcohol.

1.2.2 Glucose metabolism

Complex carbohydrates from the diet are digested into monosaccharides and stored as glycogen mainly in the liver and skeletal muscle. The concentration of glucose in the blood is maintained at a relatively constant level by glycogenolysis and gluconeogenesis in the liver which exports glucose into the circulation for use in other tissues. Glucose is a polar molecule that moves into the cell by facilitated diffusion via passive glucose transporters in the plasma membrane. The GLUT1 isoform is

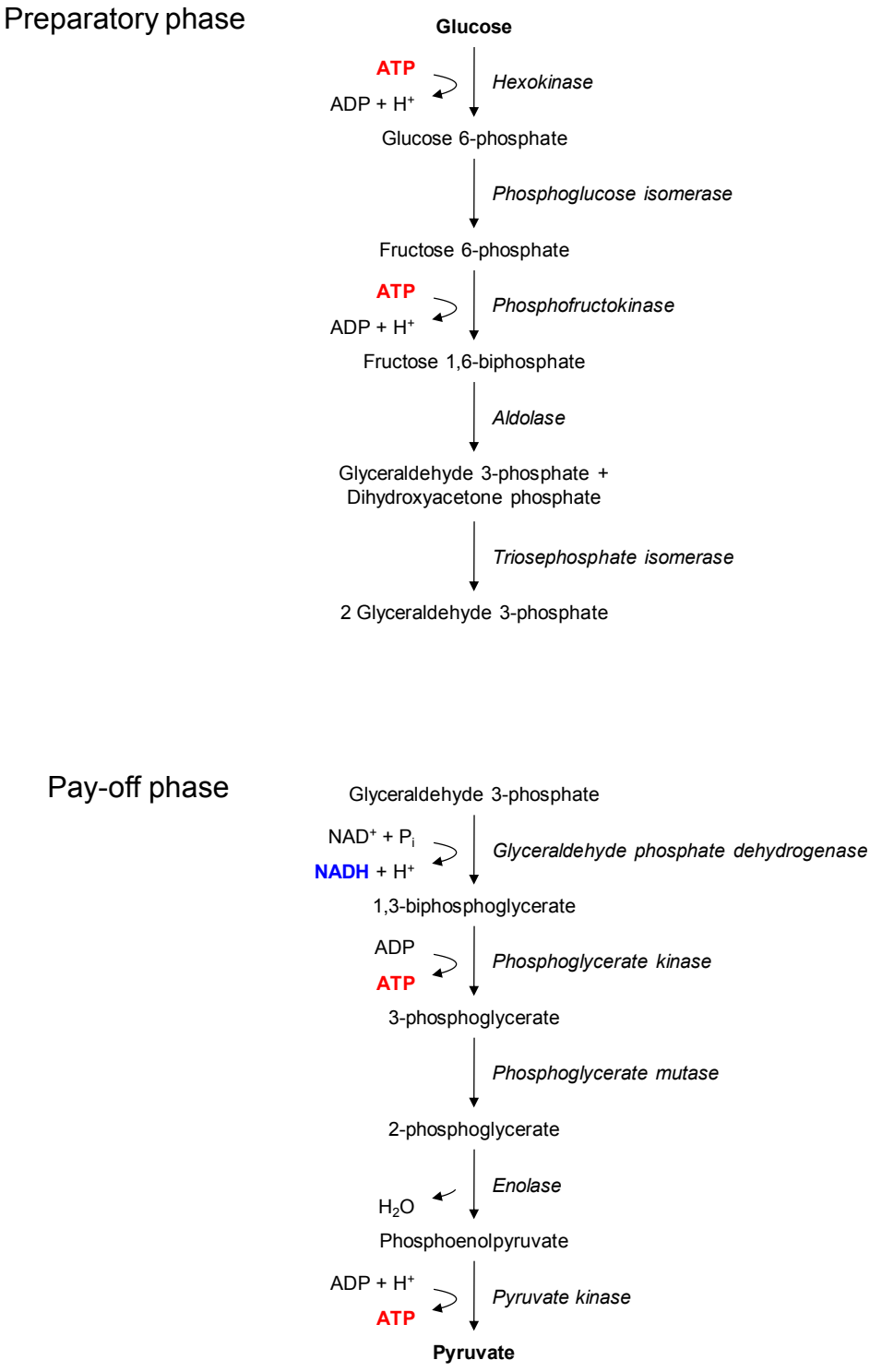
responsible for the low-level basal glucose uptake down a thermodynamic gradient required to sustain respiration in all cells. GLUT4 is the main glucose transporter expressed on myocardial cells and is stimulated by insulin, catecholamines and ischaemia, recruiting an increased number of transporters to the plasma membrane from intracellular vesicles (Depre et al., 1999).

Once inside the cell, glucose is phosphorylated to glucose-6-phosphate (G6P) by *hexokinase* and directed into one of four alternative pathways in the cytosol: storage as glycogen, hexosamine biosynthesis, the pentose phosphate pathway or glycolysis (Bouche et al., 2004). Glycogen granules are a medium-term energy reserve that can be rapidly mobilised to meet a sudden demand for G6P; in muscle cells, a lack of *glucose-6-phosphatase* activity prevents glucose resynthesis or export, so G6P liberated from glycogen is obliged to enter one of the other metabolic pathways within the cell. The hexosamine biosynthetic pathway produces glucosamine and subsequently N-acetylglucosamine, a key regulator of post-translational nuclear and cytoplasmic protein activity in addition to being a precursor in the synthesis of glycosaminoglycans, proteoglycans and glycolipids (Hanover et al., 2010). The pentose phosphate pathway is primarily an anabolic process producing 5-carbon sugars but is also the major source of reduced nicotinamide adenine dinucleotide phosphate (NADPH), a reducing equivalent used in biosynthesis and oxidative-reduction reactions including protection against oxidative stress. However, the majority of cytosolic G6P enters the glycolytic pathway to produce pyruvate.

1.2.3 Glycolysis

The glycolytic (Embden-Meyerhof) pathway consists of two phases: a *preparatory phase* in which energy-absorbing steps facilitate the splitting of glucose into two molecules of glyceraldehyde-3-phosphate (G3P); and a *pay-off phase* where each G3P is oxidised to pyruvate and energy is harvested (figure 1). The end products of glycolysis are two pyruvate, two reduced nicotinamide adenine dinucleotide (NADH) and a net gain of two ATP, independent of the availability of oxygen.

The pathway has two major roles: the oxidation of glucose to generate ATP and the provision of building blocks for synthetic reactions, and is regulated to meet these two major cellular needs. The reactions catalysed by *hexokinase*, *phosphofructokinase* (*PFK*) and *pyruvate kinase* are irreversible and thereby act as sites of control through reversible allosteric binding, phosphorylation and transcription. *PFK* is the key regulatory site for the rate of glycolysis. Allosteric control is elicited by a high concentration of ATP and reversed by AMP so that activity increases when the ATP/AMP ratio falls i.e. glycolysis is stimulated as the energy charge drops. Another key regulator of *PFK* activity is fructose 2,6-bisphosphate (F2,6-BP), the production and degradation of which are catalysed by *phosphofructokinase 2* (*PFK2*) and *fructose bisphosphate 2* (*FBPase2*) respectively, a bifunctional enzyme which responds to glucose availability via feedforward stimulation (Rider et al., 2004). Thereby, when glucose is abundant, the enzyme is dephosphorylated, activating *PFK2* and inhibiting *FBPase2*, which leads to a rise in F2,6-BP, stimulation of *PFK* and the consequent acceleration of glycolysis. In addition, *PFK* is inhibited by a fall in pH thereby preventing excessive lactate formation and a further fall in pH.



As each glucose produces two G3P, glycolysis leads to 2 pyruvate, 2 NADH and a net gain of 2 ATP.

Figure 1. The glycolytic pathway.

The fate of pyruvate is determined by the relative availability of oxygen. When oxygen is limited, pyruvate is reduced by *lactate dehydrogenase* (*LDH*) to produce lactate at the expense of consuming one NADH. Therefore, in the conversion of glucose to lactate, there is no net oxidation-reduction; the NADH formed in the oxidation of glyceraldehyde 3-phosphate is consumed in the reduction of pyruvate. This enables glycolysis to continue under anaerobic conditions, generating low levels of ATP which are crucial for cell viability prior to the return of an oxygen-rich environment. Lactate produced during anaerobic metabolism enters the bloodstream and is transported to the liver where it is reoxidised to pyruvate and then glucose via the gluconeogenic pathway. This process, known as the Cori cycle, enables a shift of the metabolic burden to the liver through the recycling of lactate. On the other hand, during aerobic conditions, the heart is a net importer of lactate which is converted to pyruvate, generating one NADH and thereby improving the redox status of the cell. Pyruvate is transported into the mitochondria by the monocarboxylase transporter where it is converted to acetyl-CoA or oxaloacetate and enters the citric acid cycle.

1.2.4 Fatty acid metabolism

Fatty acids are stored as triglycerides, uncharged esters of glycerol that are a highly concentrated source of energy; one gram of anhydrous fat stores more than six times as much energy as one gram of hydrated glycogen and is therefore the major reservoir of metabolic energy. Storage of triacylglycerol (TAG) occurs principally in the cytoplasm of adipose cells and is mobilised by hydrolysis to glycerol and fatty acids by *lipases*. Adrenaline, noradrenaline, glucagon and ACTH promote lipolysis through the activation of *adenylate cyclase* whilst insulin inhibits adipose cell *lipases*.

Free fatty acids (FFA) are released into the bloodstream and taken up into cells by transporters. The myocardium also has a small endogenous store of triglycerides with a high rate of turnover, providing an important source of fatty acids for oxidative metabolism when the exogenous supply is reduced (Saddik and Lopaschuk, 1991).

On the outer mitochondrial membrane, fatty acids are activated by esterification to acyl-CoA, a reaction catalysed by *fatty acyl-CoA synthetase* that consumes one ATP. Activated long chain fatty acids are shuttled across the mitochondrial membranes by the *carnitine-palmitoyltransferase* (*CPT*) enzyme system (figure 2). Acyl-CoA is conjugated to carnitine by *CPT-1* located on the outer mitochondrial membrane and then shuttled into the matrix by a *translocase*. On the inner mitochondrial membrane, *CPT-2* liberates acyl-CoA allowing carnitine to be returned to the cytosol by the *translocase* in exchange for another acyl-carnitine (Lopaschuk et al., 2010).

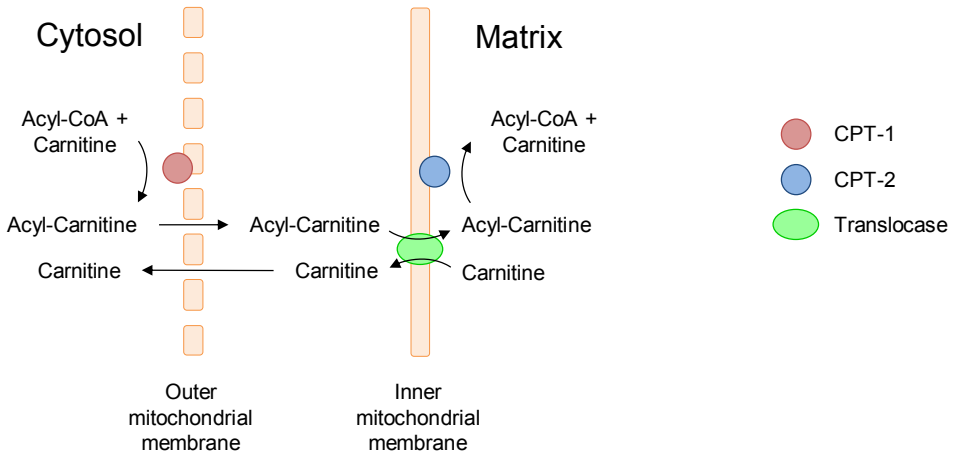


Figure 2. The carnitine-palmitoyltransferase system.

Energy is derived from fatty acids through β-oxidation in the mitochondrial matrix. A saturated molecule of acyl-CoA is degraded by a recurring series of four reactions: oxidation by *acyl-CoA dehydrogenase* and flavin adenine dinucleotide (FAD), hydration, oxidation by nicotinamide adenine dinucleotide (NAD^+) and thiolysis by another molecule of coenzyme A (figure 3). Each sequence shortens the fatty acyl chain by two carbon atoms and produces one $FADH_2$, one NADH and an acetyl-CoA. Therefore, one molecule of palmitate, a C_{16}-chain fatty acid, yields eight molecules of acetyl-CoA (C_2), seven $FADH_2$ and seven NADH to enter the citric acid cycle and electron transport chain. Odd-chain fatty acids are a minority and generate propionyl-CoA (C_3) and acetyl-CoA in the final round of degradation; propionyl-CoA is converted into succinyl-CoA and enters the cycle (Lopaschuk et al., 2010).

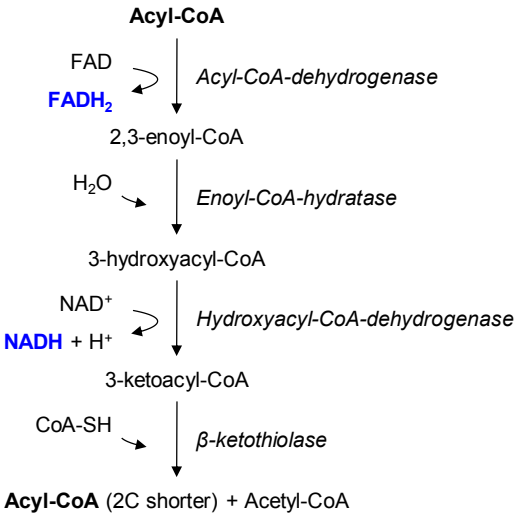


Figure 3. The β-oxidation of fatty acids.

Fatty acid metabolism in the heart is closely regulated and responsive to energy demand. *CPT-1* is the key determinant of long-chain fatty acid uptake into the mitochondria for β-oxidation and is potently inhibited by allosteric binding of malonyl-CoA. The concentration of malonyl-CoA is dependent on the balance between its synthesis by *acetyl-CoA carboxylase* (*ACC*) and its degradation by *malonyl-CoA decarboxylase* (*MCD*). *ACC* activity in the heart is regulated by *AMPK*, the cellular fuel sensor, which increases β-oxidation during times of energy need and applies the malonyl-CoA brake when demand is reduced. ACC is also under indirect hormonal control, activated by insulin but inhibited by adrenaline (Lopaschuk et al., 2010).

1.2.5 Citric acid cycle

Also known as the tricarboxylic acid or Krebs cycle, the citric acid cycle is the central metabolic pathway in all living beings that use oxygen in cellular respiration and is the final common pathway for the oxidation of fuel molecules – carbohydrates, fatty acids and amino acids. This series of reactions takes place in mitochondria, organelles that are believed to have originated as phagocytosed respiring bacteria but became domesticated within eukaryotic cells in a symbiotic energy-producing relationship. Unlike glycolysis, the citric acid cycle can only operate under aerobic conditions; whilst molecular oxygen does not directly participate in the cycle, it is essential for the regeneration of NAD^+ and FAD by accepting electrons in the electron transport chain.

The oxidative decarboxylation of pyruvate (C_3) to form acetyl-CoA (C_2) in the mitochondrial matrix is the link between glycolysis and the citric acid cycle. Catalysed by the *pyruvate dehydrogenase* (*PDH*) complex, it is the key irreversible rate-limiting

step for glucose oxidation, regulating the flow of carbohydrate-derived acetyl-CoA into the cycle. The activity of *PDH* is stringently controlled through phosphorylation and dephosphorylation by the enzymes *pyruvate dehydrogenase kinase* (*PDK*) and *PDH-phosphatase* respectively. *PDK* deactivates *PDH*, reducing glucose oxidation in response to increases in NADH/NAD$^+$, acetyl-CoA/CoA or ATP/ADP ratios i.e. it is turned off when the energy charge in the cell is high and biosynthetic intermediates are abundant (Goodwin et al., 1998).

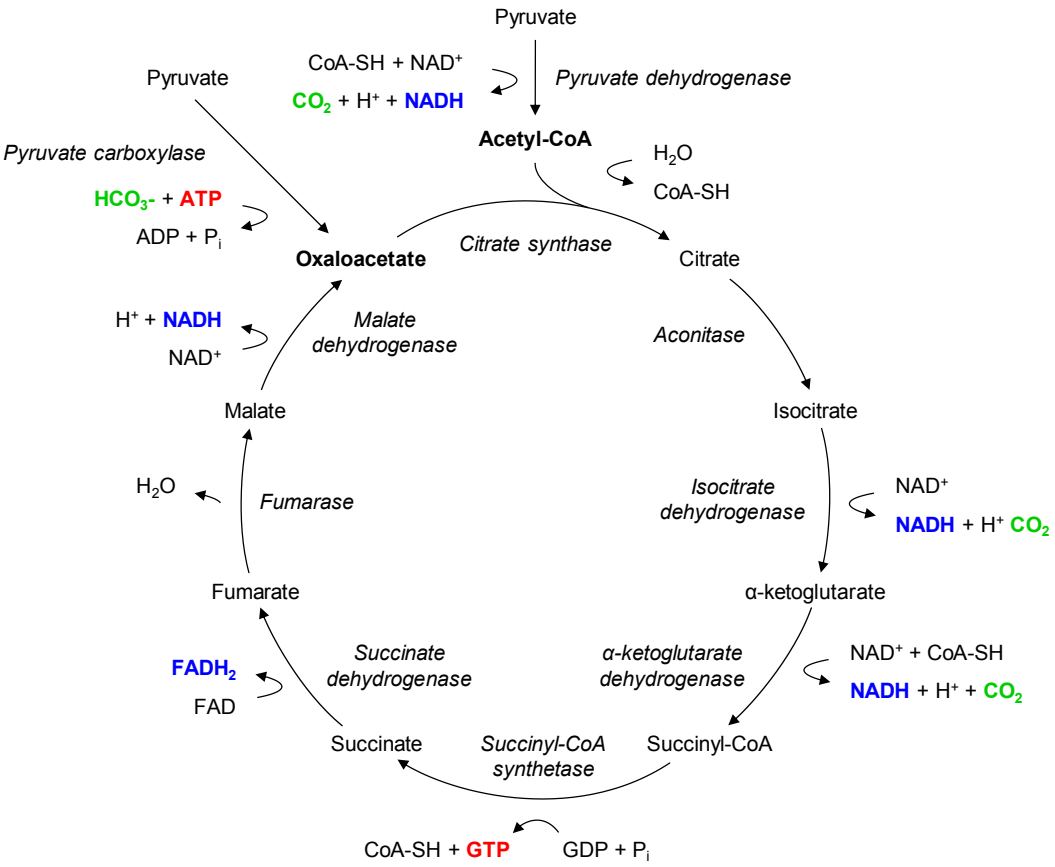


Figure 4. The citric acid cycle.

Acetyl-CoA (C_2) enters the citric acid cycle by condensing with oxaloacetate (C_4) to form citrate (C_6). Through a series of reactions (figure 4), oxaloacetate is regenerated with the liberation of energy in the form of intermediates; in effect, oxaloacetate is a catalyst for the cycle as it participates in the overall reaction but is not consumed by it. The net product of one molecule of acetyl-CoA entering the cycle is two molecules of CO_2, one GTP, three reduced NADH and one $FADH_2$. Of note, the two carbon atoms that enter the cycle as acetyl-CoA are not the same two that are released as CO_2 in that round.

The citric acid cycle is precisely regulated to meet the energy needs of the cell. *Pyruvate carboxylase* is activated by acetyl-CoA to covert pyruvate into oxaloacetate which increases the rate of the cycle. Whilst most of the acetyl-CoA that enters the cycle is derived from fatty acids, it is the production of oxaloacetate from glycolysis-derived pyruvate that determines flux through the cycle and therefore the rate of energy production (Jitrapakdee et al., 2008). The activities of *citrate synthase*, *isocitrate dehydrogenase* and the *α-ketoglutarate dehydrogenase* complex are also diminished by a high energy charge and several steps in the cycle require NAD^+ or FAD carriers which are only abundant when energy charge is low.

In the fasted state, when the effects of insulin are absent and plasma glucose is low, oxaloacetate in the liver is consumed to form glucose via the gluconeogenic pathway and is unavailable for condensation with acetyl-CoA derived from fatty acid oxidation to enter the citric acid cycle. In these conditions, acetyl-CoA is diverted into formation of ketone bodies – acetoacetate and β-hydroxybutyrate. Initially regarded as waste

products released into the blood, it is now known that ketone bodies are normal fuels of respiration and quantitatively important sources of energy; indeed the heart uses acetoacetate in preference to glucose (Kodde et al., 2007). Acetoacetate is activated by the transfer of coenzyme A from succinyl-CoA and then cleaved by *thiolase* to produce two molecules of acetyl-CoA which can enter the cycle. Ketone bodies may be regarded as a transportable form of acetyl units that are exported from the liver during fasting for oxidation elsewhere.

Anaplerosis is the replenishment of the pool of biosynthetic intermediates in the citric acid cycle. Whilst α-ketoglutarate and oxaloacetate are amino acid precursors, surplus amino acids may also be used as a metabolic fuel by conversion to pyruvate, acetoacetate, acetyl-CoA or other cycle intermediates. Overall, intermediates must be replenished by anaplerosis else the cycle will rapidly halt due to a lack of oxaloacetate (Owen et al., 2002).

1.2.6 Electron transport chain

The NADH and $FADH_2$ formed during glycolysis, fatty acid oxidation and the citric acid cycle are energy-rich molecules that have a high electron transfer potential. The inner mitochondrial membrane is impermeable to NADH and so electrons from glycolytic NADH enter the mitochondria via the malate-aspartate shuttle (figure 5). The net effect of the shuttle is purely redox, regenerating NAD^+ and enabling electrons from cytosolic NADH to participate in the electron transport chain. Oxidative phosphorylation is the complex process through which ATP is formed as a result of the transfer of electrons to oxygen by a series of electron carriers (Rich, 2003).

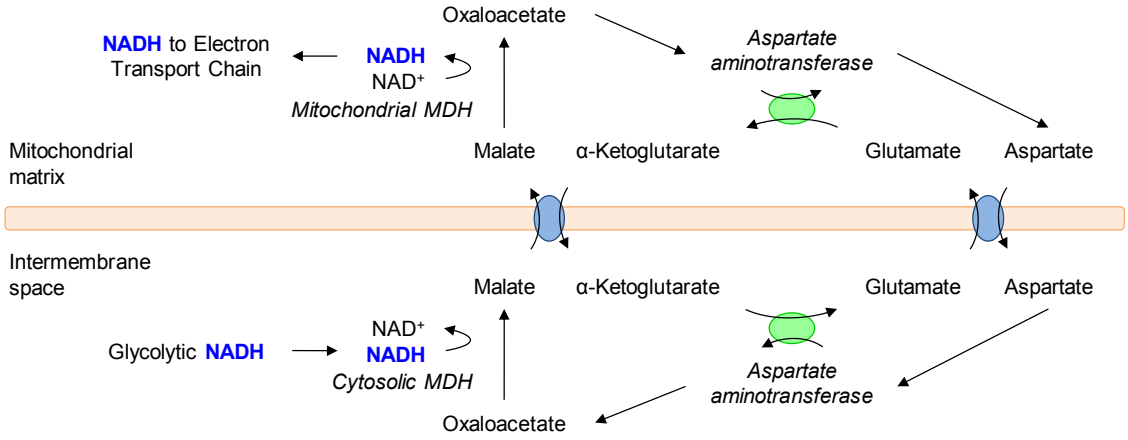


Figure 5. The malate-aspartate shuttle.

According to Mitchell's (1961) Nobel prize-winning chemiosmotic hypothesis, it occurs across the inner mitochondrial membrane and is the major source of ATP production in aerobic organisms. In the first phase of oxidative phosphorylation, electron-motive force is converted into proton-motive force. Electrons are transferred from NADH to O_2 through a chain of three large protein complexes: *NADH dehydrogenase* (Complex I), *cytochrome bc_1 complex* (III) and *cytochrome c oxidase* (IV) (figure 6). $FADH_2$ enters the pathway via *succinate dehydrogenase* (II) and *ubiquinone* (Q) after the first proton pumping site and therefore produces less ATP than NADH. The flow of electrons within these transmembrane complexes leads to the pumping of protons across the inner mitochondrial membrane from the matrix to the intermembrane space. In the second phase, the potential of the transmembrane proton concentration gradient is converted into phosphoryl potential in the form of high-energy bonds. The flow of protons back into the mitochondrial matrix through the proton channel core of *F_0F_1-ATP-synthase* is coupled to the formation of ATP from ADP and inorganic phosphate.

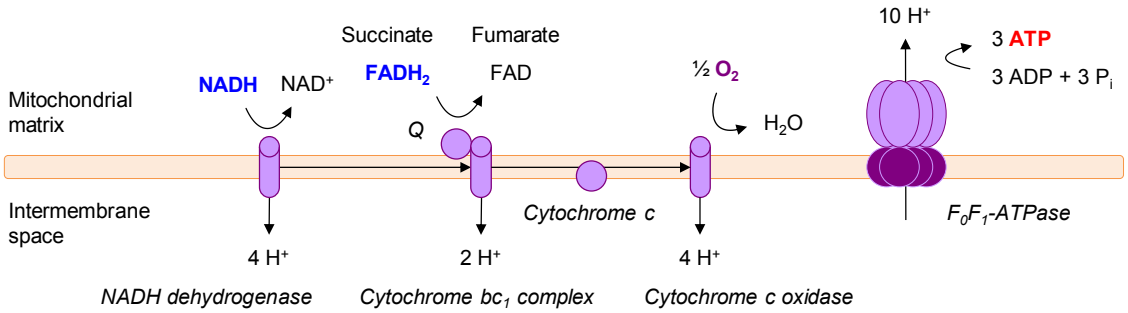


Figure 6. The electron transport chain.

The most important factor determining the rate of oxidative phosphorylation is the concentration of ADP and it is thereby coupled to ATP utilisation and energy charge, termed respiratory control. ATP generated by oxidative phosphorylation is exported from the mitochondria and donates a ~P to creatine to generate PCr and recycle ADP. The PCr shuttle is thought to enable the regeneration of ATP at sites of energy utilisation throughout the cell such as myofibrils and sarcoplasmic reticulum. During oxidative phosphorylation, small quantities of potentially destructive reactive oxygen species (ROS), such as the superoxide anion, are produced in proportion to the rate of oxygen consumption. Leakage of activated oxygen from the mitochondria may cause damage to cell components including DNA, lipids and proteins through production of peroxides and free radicals. Other enzymes such as *xanthine oxidase* and *NADPH oxidase* may also produce ROS. Under normal conditions, these are detoxified by antioxidants: *superoxide dismutase*, *catalase* and the glutathione and thioredoxin systems (Inoue et al., 2003). An imbalance between the production and removal of ROS or inability to repair associated damage is termed oxidative stress.

In recent years, the yield of ATP synthesis from the electron transport chain has been re-evaluated (Rich, 2003). It is now apparent that one complete revolution of the motor element of *ATP synthase* requires ten H^+ and generates 3 ATP whilst an additional proton is consumed in transporting each ATP to the cytosol via *ATP-ADP translocase*; hence the net synthesis of cytoplasmic ATP from ADP should operate on a ratio of 13:3 H^+/ATP. It has been estimated that ten protons are pumped out of the matrix for each NADH and six protons for each $FADH_2$ donating a pair of electrons to the transport chain. Consequently, it is predicted that NADH generates 3 x 10/13 ≈ 2.3 cytosolic ATP and oxidation of succinate via $FADH_2$ produces 3 x 6/13 ≈ 1.4 ATP per electron pair donated. The complete oxidation of one molecule of glucose to CO_2 through glycolysis, the citric acid cycle and oxidative phosphorylation therefore generates a net yield of approximately 30 ATP, of which 26 are harvested via the electron transport chain. On the other hand, one molecule of the fatty acid palmitate (C_{16}) produces a net yield of around 106 ATP through β-oxidation, the citric acid cycle and oxidative phosphorylation, demonstrating the greater caloric value of fats than carbohydrates. However, it is important to note that the energy derived from substrates is not equal. The oxidation of fatty acids requires approximately 12% more oxygen by stoichiometry than that of glucose to generate an equivalent amount of ATP due to an increased $FADH_2$ to NADH ratio; in terms of oxygen consumption, glucose is a more efficient fuel (Liedtke, 1981). Whilst almost all ATP is generated through the same final common pathways, the yields and costs of oxidation have significant implications on substrate selection especially when oxygen is limited.

1.2.7 Myocardial substrate utilisation

The human heart consumes up to 5kg of ATP per day, more than any other organ of the body per gram of tissue. Myocardial energy reserves are limited, so ATP must be continually produced by the catabolism of carbon compounds. Metabolism is tightly controlled at many levels to produce sufficient ATP to meet the immediate contractile and homeostatic demands of the heart. A complex and integrated signalling pathway has evolved to regulate supply precisely and respond to changes in demand for energy. The heart is a true omnivore, metabolising a variety of competing substrates from the bloodstream (figure 7). Under normal aerobic conditions, it generates 70% of its ATP from the β-oxidation of fatty acids, 10-30% from the uptake and oxidation of glucose and the remainder from lactate, pyruvate, ketones and amino acids (Taegtmeyer, 2007). The relative utilisation of substrates is determined by availability, blood supply, cardiac workload, hormonal control and the regulation of metabolic genes at a transcriptional level resulting in the altered expression of receptors, transporters, enzymes and regulators as an adaptive response (Taegtmeyer, 2002).

The glucose-fatty acid cycle, or Randle (1963) cycle, is the primary local regulatory mechanism for determining substrate oxidation. An abundance of FFA undergoing β-oxidation increases the ratios of acetyl-CoA/CoA and NADH/NAD^+ which stimulate *PDK* to inhibit the *PDH* complex and decrease the entry of pyruvate into the citric acid cycle. Hence, FFA are preferentially oxidised over glucose and are the dominant substrate for myocardial metabolism. A counter balance is achieved through the potent inhibition of *CPT-1* by malonyl-CoA, thereby decreasing the uptake of FFA into the mitochondria and reducing β-oxidation (Ussher and Lopaschuk, 2008).

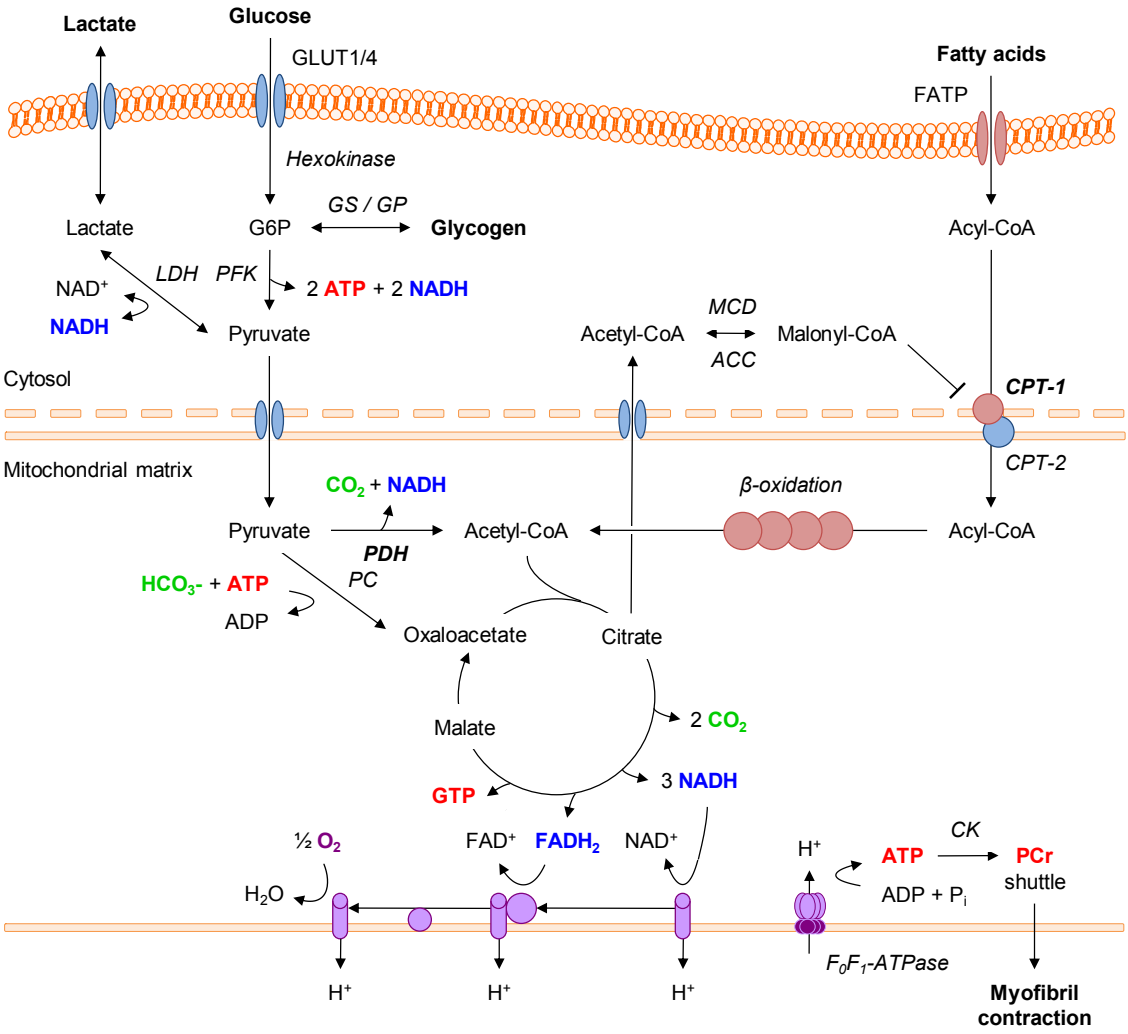


Figure 7. An overview of myocardial substrate metabolism.

Mitochondrial ATP synthesis is dependent upon the electrochemical proton gradient across the inner membrane. Uncoupling proteins (UCP) 1-5 are membrane transport proteins that enable re-entry of protons into the matrix without the generation of ATP. UCP3 is a transporter that exports excess fatty acid anions out of the matrix; once in the intermembrane space, the anion can associate with a proton, re-esterify with CoA and be transported back into the matrix. This has the benefit of recycling CoA but at a cost of ATP and a net leak of protons from the matrix (Lopaschuk et al., 2010). Futile

cycling of fatty acid intermediates also contributes to non-contractile consumption of ATP, reducing the net energy yield from fatty acids and decreasing cardiac efficiency.

An acute increase in cardiac workload, during exercise or inotropic stimulation, requires a substantial rise in ATP generation. This need is initially met through stores of PCr and then by the preferential oxidation of intracellular glycogen, lactate and glucose (Goodwin et al., 1998). If the high energy requirement remains, triglycerides mobilised from adipose tissue in response to catecholamine stimulation provide an additional substrate. This metabolic reserve forms a link between coronary flow reserve (*supply*) and contraction (*demand*) (Kassiotis et al., 2008).

Substrate utilisation is influenced by circulating hormones, most notably insulin and the catecholamines, through α- and β-receptor stimulation. Adrenergic hormones stimulate cellular ATP production through the oxidation of all available substrates. Local glycogenolysis and recruitment of glucose transporters is enhanced whilst stimulation of lipolysis in adipose cells and gluconeogenesis in the liver increase the availability of circulating fatty acids and glucose. Insulin, on the other hand, has a glucose-selective effect by stimulating glucose uptake, glucose oxidation and glycogen synthesis but inhibiting fatty acid oxidation. The activated insulin receptor initiates phosphorylation cascades including the *insulin receptor substrates (IRS)*, *phosphatidyl-inositol-3-kinase (PI3K)*, *glycogen synthase kinase 3* (*GSK-3*) and *Akt* which mediates many of its metabolic effects. There is emerging evidence that circulating adipokines, such as leptin, may be important modulators of myocardial metabolism particularly via promotion of fatty acid oxidation (Karmazyn et al., 2008).

Mitochondrial oxidative energy metabolism is also regulated at a gene transcription level. Peroxisome proliferator-activated receptor γ co-activator-1 (PGC-1α) is a master regulator of myocardial metabolism that functions to increase β-oxidation of fatty acids, inhibit pyruvate oxidation via the citric acid cycle and promote mitochondrial biogenesis (Finck and Kelly, 2007).

Therefore, in addition to substrate and oxygen availability, myocardial metabolism is subject to multiple levels of control including workload (seconds → minutes), hormones (minutes → hours) and gene transcription (hours → days). Whilst these regulators have independent mechanisms of action, their effects are interlinked.

1.3 Ischaemia and reperfusion

'Myocardial reperfusion: a double-edged sword?'

(Braunwald and Kloner, 1985)

In the healthy heart, over 95% of the energy generated is derived from mitochondrial oxidative phosphorylation, with the remainder from glycolysis. The optimal metabolic and contractile function of the myocardium therefore requires a continuous supply of oxygen and substrates; if this is reduced or interrupted, the efficient production of ATP is inhibited. The consequences that result depend upon the degree and persistence of the metabolic insult. In chronic heart failure, impairment of substrate metabolism contributes to contractile dysfunction and ventricular remodelling; this maladaptive response is characterised by downregulation of fatty acid oxidation, increased glycolysis and an impaired reserve for mitochondrial oxidative flux (Stanley et al., 2005). On the other hand, ischaemia and subsequent reperfusion precipitate an acute metabolic challenge that threatens cell survival.

1.3.1 Ischaemia

In low-flow ischaemia, PCr stores are rapidly exhausted and the falling energy charge of the cell activates *AMPK* to promote anaerobic energy production and suppress energy-requiring biosynthetic processes. *AMPK* promotes the utilisation of glucose by promoting glucose uptake and activating *PFK* to accelerate the rate of glycolysis in an attempt to make up for the reduction in oxidative ATP production. However, it also inhibits *acetyl-CoA carboxylase* leading to a fall in malonyl-CoA levels and a subsequent increase in the uptake and β-oxidation of FFA (Hopkins et al., 2003). In ischaemia, catecholamine-induced lipolysis causes a dramatic rise in circulating FFA

levels which dominate competition for residual oxidative capacity but have a detrimental effect on the ischaemic myocardium (Liedtke, 1981); this is exacerbated during cardiac surgery as the administration of heparin also increases plasma FFA. A relative increase in the β-oxidation of FFA inhibits the *pyruvate dehydrogenase* complex, uncoupling glycolysis from the citric acid cycle; the fate of pyruvate is shifted from oxidation to lactate production, enabling ATP production to continue through anaerobic metabolism at the expense of contributing to cellular acidosis and further reducing myocardial contractile function (Depre et al., 1999). High levels of FFA are associated with a fall in myocardial metabolic efficiency of around 30% (Myrmel et al., 1992), significantly more than the 12% predicted by stoichiometry alone (Liedtke, 1981). This oxygen-wasting effect is proposed to occur via the activation of PPARα, upregulation of the expression of mitochondrial uncoupling proteins and dissipation of the electrochemical gradient (Murray et al., 2004). In addition, accumulation of β-oxidation intermediates has been shown to induce diastolic dysfunction during ischaemia (Kennedy et al., 2000).

During total ischaemia, glucose uptake cannot be increased although mobilisation of glycogen stores enables a temporary increase in glycolytic lactate production. Despite stimulation of *AMPK*, oxidation of fatty acids and glucose stops due to lack of oxygen. The accumulation of long-chain acyl-carnitines contributes to the initiation of cell-to-cell electrical uncoupling and predisposes to arrhythmias (Yamada et al., 1994). The low level of cytosolic ATP produced during ischaemia is primarily used to maintain cellular ion homeostasis in the face of worsening acidosis. Progressive loss of ATP is accompanied by degradation of adenine nucleotides and a massive rise in

the concentration of inorganic phosphate. If total ischaemia persists, glycolysis halts due to the accumulation of lactate and protons and the inability to regenerate NAD^+. As cytosolic phosphorylation potential falls further, there is failure of ATP-dependent Ca^{2+} pumps in the plasma membrane and sarcoplasmic reticulum (Crompton, 1999). Intracellular acidosis activates plasma membrane Na^+-H^+ exchange, leading to increased intracellular sodium; this subsequently drives the Na^+-Ca^{2+} membrane pump with a marked rise in cytosolic calcium (figure 8). A lack of ATP, intracellular acidosis and calcium overload results in cessation of contractile function and without restoration of myocardial perfusion, cell death is inevitable within 40-60 minutes.

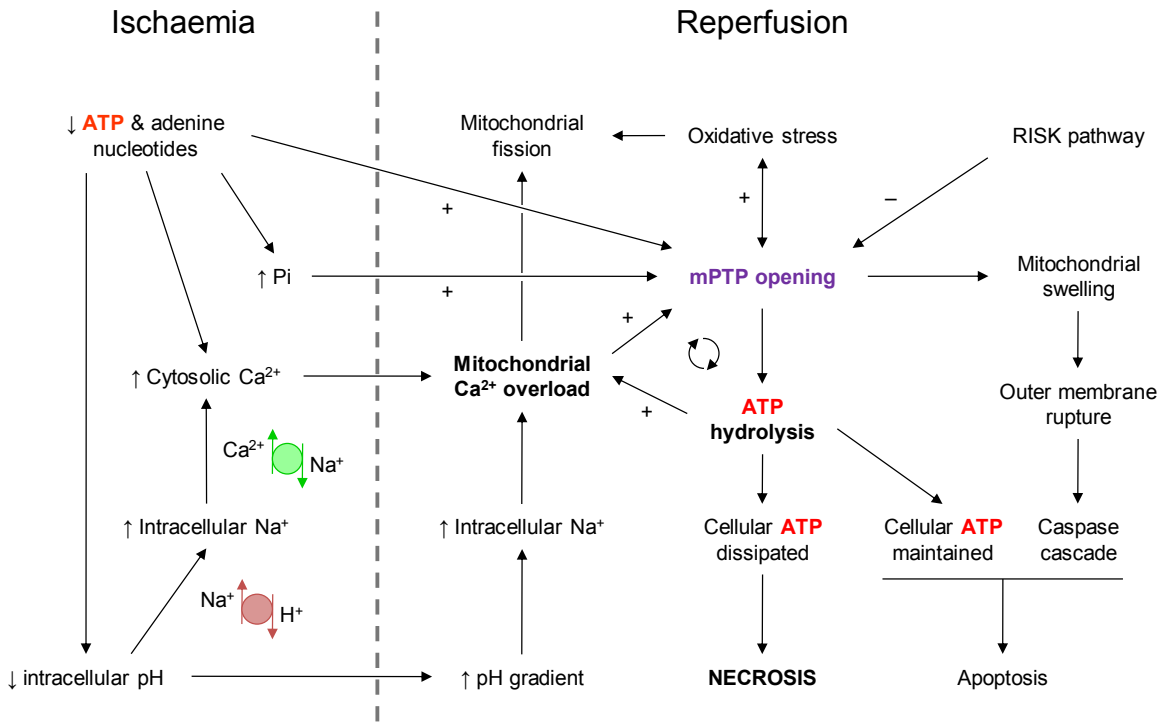


Figure 8. An overview of ischaemia-reperfusion injury.

1.3.2 Reperfusion

Clearly reperfusion of the ischaemic myocardium is the prerequisite for limiting cell death and the potential return of contractile function. However reperfusion may have unwanted effects of its own and cause additional cellular damage, known as reperfusion injury. The extent of myocardial injury is therefore a composite of that caused by ischaemia and reperfusion; this has clinical importance since interventions targeting the reperfusion phase may be implemented when there has already been a significant period of ischaemia, such as in a patient presenting with a myocardial infarction (Kharbanda, 2010).

During reperfusion of the ischaemic heart, the rate of glycolysis remains elevated but FFA dominate oxidative energy production at the expense of pyruvate (Liu et al., 1996). Activation of *AMPK* persists into reperfusion, inhibiting *ACC* and leading to low levels of malonyl-CoA. *CPT-1*-mediated uptake and β-oxidation of FFA is accelerated to meet the energy demand of the cell but at the expense of continued *PDH* complex inhibition. Despite the restoration of glucose availability, glycolysis remains uncoupled from the citric acid cycle and increased production of lactate and protons worsens the cellular acidosis and decreases cardiac efficiency. Washout of the acidic extracellular environment during reperfusion further increases the pH gradient across the plasma membrane, promoting a second wave of rising intracellular sodium and further calcium overload in the cytosol and mitochondria (Crompton, 1999). The increased requirement for ATP to restore ischemia-induced alterations in ion homeostasis also contributes to the impairment in contractile function during reperfusion.

The return of molecular oxygen to ischaemic tissue leads to oxidative stress with the formation of ROS including superoxide anions ($\bullet O_2^-$), hydroxyl radicals ($\bullet OH^-$) and hydrogen peroxide (H_2O_2). Reperfusion causes an immediate burst of $\bullet O_2^-$ production due to the build up of one-electron donors in the respiratory chain during the preceding ischaemia; mitochondria are therefore especially vulnerable to oxidative stress on reperfusion (Lenaz, 1998). The release of ROS rapidly exceeds the capacity of endogenous antioxidant scavenging systems resulting in damage to mitochondrial and cellular components through lipid peroxidation, protein oxidation and DNA mutations. ROS also stimulate an acute inflammatory response with activation, adherence and chemotaxis of leukocytes, activation of complement and direct endothelial damage.

Over the last two decades, opening of the mitochondrial permeability transition pore (mPTP) has emerged as the central hypothesis for the triggering of cell death during ischaemia-reperfusion injury (Crompton, 1999). The mPTP is a non-selective, high conductance channel that forms at sites where the inner and outer mitochondrial membranes converge although its exact structure is yet to be defined. Assembly and opening of the mPTP during reperfusion is triggered by mitochondrial calcium overload and promoted by the rise in inorganic phosphate, loss of ATP and protective adenine nucleotides, and increased oxidative stress (figure 8). By rendering the inner mitochondrial membrane freely permeable to protons, widespread opening of the mPTP dissipates the transmembrane proton concentration gradient, uncoupling oxidative phosphorylation such that mitochondria *hydrolyse* rather than *synthesise* ATP (figure 9). The ATP-generating capacity of the cell is overwhelmed and a vicious

cycle of impaired energy metabolism, further loss of calcium homeostasis and progressive pore opening leads to necrotic cell death. If the ischaemic insult is less severe and mPTP opening is more limited, enabling the cell to maintain sufficient ATP for viability, rupture of the outer mitochondrial membrane may trigger a caspase cascade, leading to apoptotic cell death (Crompton, 1999).

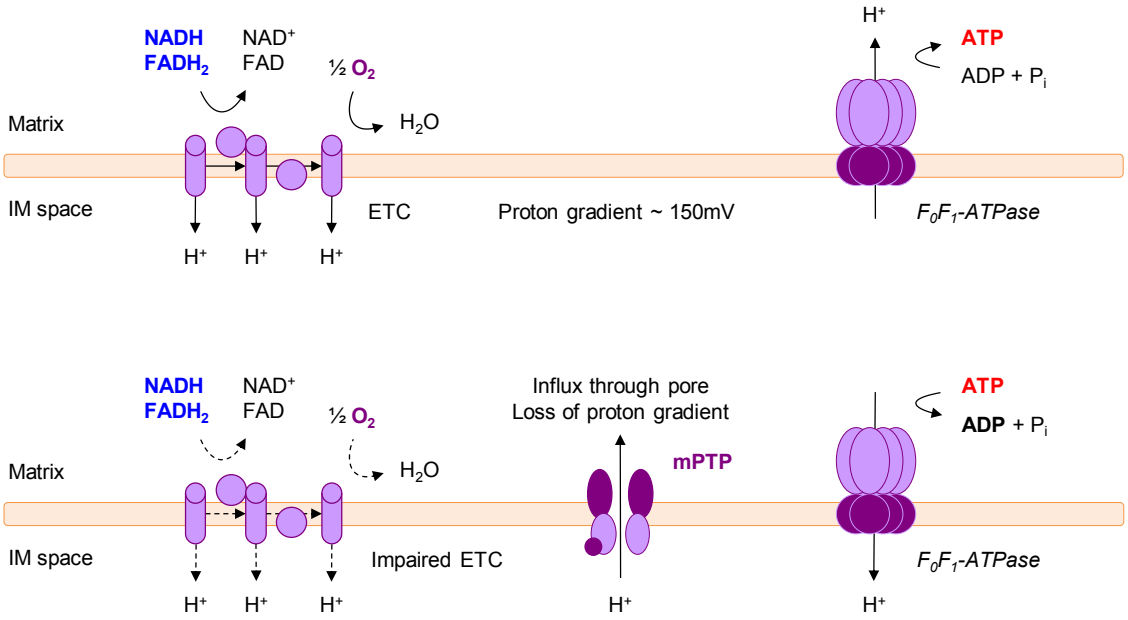


Figure 9. The mitochondrial permeability transition pore and ATP hydrolysis.

Recent recognition that apoptosis contributes to lethal reperfusion injury led to the emergence of the Reperfusion Injury Salvage Kinase (RISK) pathway, a series of pro-survival anti-apoptotic protein kinases which include *Akt*, *Erk1/2*, *PKC* and *GSK-3β* (Hausenloy and Yellon, 2007). Activation of the RISK pathway is thought to be the core component of a common cardioprotective pathway which converges through inactivation of *GSK-3β* to inhibit formation of the mPTP and protect against

reperfusion injury (Gomez et al., 2008). An alternative intrinsic mechanism, the Survivor Activating Factor Enhancement (SAFE) pathway, has also been described in which the pro-inflammatory cytokine TNFα paradoxically activates the *JAK/STAT3* signalling pathway to promote cell survival (Lecour, 2009). These pathways represent potential therapeutic targets for attenuating the cellular effects of ischaemia-reperfusion in the heart and other organs.

1.3.3 Ischaemia-reperfusion injury in cardiac surgery

Most cardiac surgery involves a period of elective myocardial ischaemia; as such, this represents both a clinical challenge and a practical opportunity to examine the effects of ischaemia-reperfusion on the human myocardium. A prolonged period of ischaemia results in myocardial necrosis with permanent loss of contractile function and release of large quantities of intracellular markers, such as creatine kinase and troponins, into the circulation. A short period of total or low-flow ischaemia invariably causes temporary contractile dysfunction which persists after blood flow and ATP production are restored. This post-ischaemic hangover is termed *myocardial stunning* as it ultimately leads to biochemical, physiological and clinical recovery (Braunwald and Kloner, 1982). The degree of clinical dysfunction due to myocardial stunning can be assessed by measuring cardiac contractility in the early postoperative period, either as absolute cardiac output or the frequency of a low cardiac output episode. As impairment of the stunned myocardium is reversible upon adrenergic stimulation (Ellis et al., 1984), the quantity of inotropic support required is also a marker of the effects of ischaemia-reperfusion injury. The clinical application of these markers of ischaemia-reperfusion injury will be discussed further in Chapter 3, section 2.7.

1.4 Myocardial protection: principles and practice

'Be still my heart; thou hast known worse than this'

(Homer, *c.*850 BC)

The term *myocardial protection* refers to the strategies used to prevent or attenuate the myocardial dysfunction that occurs during or after cardiac surgery due to an ischaemic insult (Mentzer et al., 2008). It provides a window of opportunity for permissive ischaemia during which interventions can be performed without significant irreversible damage to the myocardium on reperfusion. Whilst this principle was recognised by Bigelow and exploited by Lewis almost 60 years ago, it soon became clear that systemic hypothermia alone would be insufficient for more challenging intra-cardiac procedures and focused solutions would be required to prolong the tolerable ischaemic period.

1.4.1 Cardioplegia: the solution to myocardial protection?

In July 1955, Denis G. Melrose and colleagues at the Postgraduate Medical School in London published an experimental report in *The Lancet* on elective cardiac arrest using potassium citrate. Adult dogs were maintained with either a heart-lung machine or systemic hypothermia whilst the venae cavae, pulmonary artery and aorta were ligated. A solution of concentrated potassium citrate was injected into the aortic root, entering the coronary arteries and rapidly arresting the heart in diastole. A simulated intracardiac procedure was performed during which the heart remained pink and serial coronary sinus samples indicated that little oxygen was being extracted. However, reperfusion almost inevitably resulted in ventricular fibrillation and resumption of normal contraction was unreliable. Using lower concentrations of

potassium citrate diluted in blood, Melrose found improved consistency of recovery in his experimental model, concluding:

> *'The oxygen consumption of the quiescent heart is very low, and at normal body-temperature, cessation of the coronary circulation for over fifteen minutes does not endanger such a heart. Although a great deal of further work remains to be done, this method may offer an opportunity for useful surgery on the motionless heart, without the danger of air embolism.'*

In a further study, Bentall and Melrose (1957) demonstrated that lactate concentrations in coronary effluent following potassium arrest were about one-third of those after arrest without potassium citrate; although not performing contractile work, the arrested heart remained metabolically active. Three years after the initial report, Gerbode and Melrose (1958) published their collaborative work, successfully using potassium citrate arrest in 34 patients undergoing open heart surgery in Stanford, California. The technique gained in popularity and several groups reported similar results including Sealy and co-workers (1959) who coined the term '*cardioplegia*'. However, others experienced frequent dysrrhythmias and severe ventricular dysfunction following its use and the technique was widely abandoned following a report from the US National Heart Institute (McFarland et al., 1960) in which 79% of patients who had died after open heart procedures using potassium citrate arrest were found to have histological evidence of focal myocardial necrosis.

Over the next 15 years, several alternatives to chemical arrest were utilised for myocardial protection: intermittent aortic occlusion with direct coronary perfusion, cross-clamping with induced fibrillation, topical hypothermia, and normothermic ischaemia. However, these techniques were limited by subendocardial ischaemia, a

short period of ‘safe’ ischaemic arrest, flooding of the operative field and non-uniform cooling of the heart (Shiroishi, 1999). Most significantly, Cooley and associates (1972) coined the term ‘*stone heart*’ for an ischaemic contracture of the left ventricle due to severe depletion of myocardial ATP which led to the death of a number of patients following normothermic arrest (Hearse et al., 1977).

Although the use of potassium citrate in clinical practice had ceased in the early 1960s, a few researchers continued to investigate the potential of chemical cardioplegia. Bernhard Hoelscher (1967) in Berlin concluded that the damaging effects of Melrose solution were not due to the potassium ion rather the calcium- and magnesium-chelating action of the citrate ion leading to intracellular and extracellular oedema. Another German pioneer, Hans J. Bretschneider (1975) from the University of Göttingen, developed an alternative cardioplegia that used a low sodium, calcium-free solution to prevent excitation and therefore contraction. In Aarhus, Denmark, Tyge Søndergaard and colleagues (1975) used a modified form of Bretschneider solution in 100 patients and reported superior myocardial protection to the coronary perfusion technique. However, much of Bretschneider’s original work was published in the German language literature and went largely unnoticed elsewhere.

Across the Atlantic in New York, William A. Gay Jr. and Paul A. Ebert (1973) were re-exploring the use of potassium-induced arrest. Using an isolated, supported dog heart model, they achieved arrest by continuous coronary perfusion with a normothermic mixture of blood and an isotonic high-potassium chloride solution also containing sodium chloride, glucose and bicarbonate. They demonstrated a 75-85%

reduction in oxygen consumption in the arrested hearts compared with those that were spontaneously beating but nonworking, paced or in ventricular fibrillation. Following one hour of potassium-induced arrest, resuscitated hearts demonstrated only a mild reduction in contractile function whilst those exposed to normothermic ischaemia could not be resuscitated. Their improvement over the Melrose method was attributed to the balanced osmolality of their solution and the replacement of citrate ions with chloride.

Meanwhile at St. Thomas' Hospital, London, a buffered potassium-based cardioplegia solution was also being refined and in 1976, David J. Hearse and colleagues published their landmark study on the isolated rat heart in *Circulation*. Their Solution No.1 comprised 4°C Ringer's solution with the addition of 16mM potassium chloride, 16mM magnesium chloride and 1mM procaine hydrochloride. In fact, the components of this solution form the basis of crystalloid cardioplegia still in use today; co-author Mark V. Braimbridge later wrote (1992):

> *'Many surgeons have said to me since that it was this article which first persuaded them that cardioplegia was the way to go.'*

By the end of the 1970s, cold crystalloid potassium-based cardioplegia was established as the dominant method for myocardial protection in cardiac surgery (Shiroishi, 1999). Later studies used detailed dose-response curves to determine the composition of the optimal cardioplegic solution, leading to the introduction of St. Thomas' Hospital Solution No.2 which was demonstrated to be superior to the original (Ledingham et al., 1987). The technique enabled surgeons to induce a rapid depolarised arrest of the heart that was readily reversible and preserved contractile

function in the post-ischaemic period. Reliable myocardial protection using cardioplegia was a catalyst for the industrial age of cardiac surgery. Coronary artery bypass grafting and valve replacement surgery were being performed with increasing frequency at a rapidly growing number of centres and improved organ protection enabled the practical application of thoracic transplantation, maintaining a viable extracorporeal organ *en route* between donor and recipient.

1.4.2 The metabolic basis of cardioplegia and hypothermia

Like every organ, the heart is able to withstand a certain period of time of interrupted substrate and oxygen supply without suffering irreversible injury, termed *ischaemic tolerance*. The central principle of myocardial protection is to safely extend this period by reducing the metabolic demand of the myocardium to facilitate surgical intervention. Other than the inevitable loss of energy as heat, the vast majority of ATP generated in the myocardium is utilised for mechanical work with the remainder for the maintenance of cellular homeostasis (Taegtmeyer, 2007). Resting the heart in diastole whilst maintaining the systemic circulation with cardiopulmonary bypass therefore provides a rationale for lowering myocardial energy demand; in addition, hypothermia reduces the rate of chemical reactions and hence energy consumption in biological systems according to the Arrhenius equation (Levine, 2005).

The landmark studies of Gerald D. Buckberg and associates at the University of California, Los Angeles demonstrated that myocardial oxygen consumption was reduced by almost 90% in the normothermic arrested heart (1.10ml/100g/min) compared with perfused full beating (8.7), empty beating (5.59) or fibrillating (6.50)

hearts (Hottenrott et al., 1974, Buckberg et al., 1977). In addition, hypothermia led to a further stepwise reduction in oxygen consumption of the arrested hearts to 0.83ml/100g/min at 32°C, 0.59 at 28°C and 0.31 at 22°C (figure 10). The oxygen consumption of the cooled, empty, arrested heart may therefore be reduced to less than 3.5% of baseline requirements.

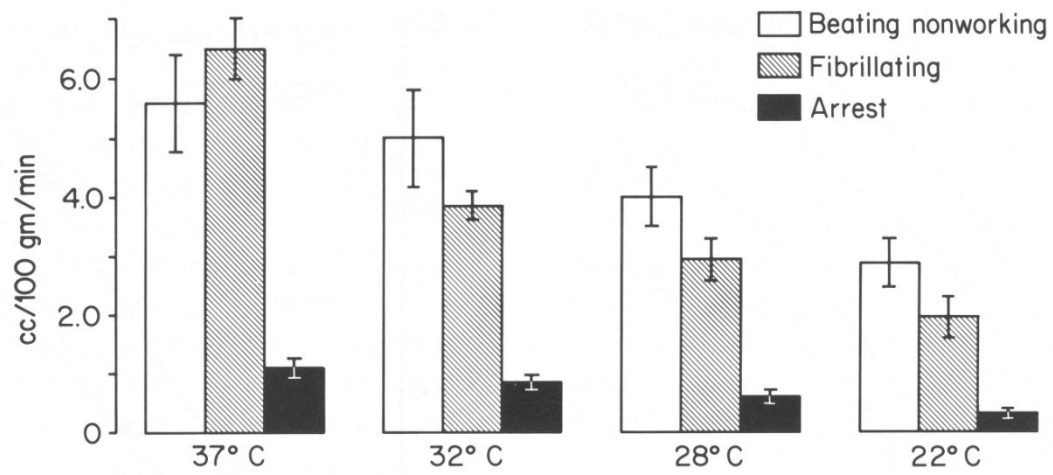


Figure 10. Left ventricular myocardial oxygen uptake.[1]

Buckberg (1987) defined a number of key principles for optimising elective cardiac arrest with cardioplegia in order to reduce ischaemia-reperfusion injury. Firstly, arrest should occur promptly after the onset of ischaemia to avoid rapid energy depletion by ischaemic electromechanical work. Secondly, lowering the temperature of the solution further reduces the metabolic rate and may be supplemented by direct topical cooling. Thirdly, the enrichment of cardioplegia with substrates for aerobic and

[1] Reprinted from The Journal of Thoracic and Cardiovascular Surgery, 73(1), Buckberg GD, Brazier JR, Nelson RL, Goldstein SM, McConnell DH, Cooper N. Studies of the effects of hypothermia on regional myocardial blood flow and metabolism during cardiopulmonary bypass. I. The adequately perfused beating, fibrillating and arrested heart, 87-94, Copyright 1977, with permission from Elsevier.

anaerobic metabolism including oxygen, glucose and citric acid cycle intermediates, particularly glutamate and aspartate, promotes anaplerosis and enhances substrate utilisation. Fourthly, the use of a buffer, such as bicarbonate or tromethamine, to maintain optimal extracellular pH despite increased anaerobic generation of protons, promotes metabolic recovery. Fifthly, the addition of membrane stabilisers (e.g. procaine) and oxygen radical scavengers (e.g. co-enzyme Q_{10}) decreases the harmful effects of reperfusion. Finally, controlling extracellular osmolality and oncotic pressure reduces the extent of myocardial oedema and maintain cellular integrity.

According to these principles, further refinements have since been made to enhance the efficacy of potassium cardioplegia. Buckberg's group found that replacing the crystalloid solution with whole blood as the vehicle for delivery improved experimental and clinical myocardial protection (Follette et al., 1978). Blood offers a number of theoretical advantages including augmented oxygen delivery, ideal oncotic pressure, effective buffering capacity with *carbonic anhydrase*, endogenous free-radical scavengers and less haemodilution when a large volume of cardioplegia is used (Barner, 1991). However, the largest randomised controlled trial of cold blood versus cold crystalloid cardioplegia (Ovrum et al., 2004) reported no significant difference in myocardial protection for patients undergoing CABG. On the other hand, a recent meta-analysis (Guru et al., 2006) found that:

> *'Blood cardioplegia provides superior myocardial protection as compared with crystalloid cardioplegia, including lower rates of low output syndrome and early CK-MB increase, whereas the incidence of myocardial infarction and death are similar. On balance, and despite the limitations of this investigation, the evidence supports the use of blood rather than crystalloid cardioplegia for coronary surgery.'*

Nelson and colleagues (1976) demonstrated the importance of intermittent reinfusion of cardioplegia to compensate for washout from non-coronary collateral flow. Solorzano and associates (1978) promoted the use of retrograde cardioplegia perfusion via the coronary sinus as an alternative to antegrade administration via the aortic root. Teoh *et al*. (1986) demonstrated that a '*hot shot*' terminal dose of warm blood cardioplegia improves metabolic and functional recovery following protection with cold blood cardioplegia. Another recent meta-analysis (Fan et al., 2010), comparing the effects of warm versus cold cardioplegia, concluded:

> *'Using warm cardioplegia for myocardial protection during heart surgery resulted in similar incidences of clinical events, significant improvement in postoperative cardiac index and reduction in postoperative enzyme release as compared with cold cardioplegia.'*

However, definitive evidence in the form of contemporary, prospective, multi-centre, randomised controlled trials demonstrating survival advantage for different compositions, temperatures or routes of delivery is lacking. Hyperkalaemic arrest remains the mainstay of myocardial protection in the modern era; a survey of practice in the UK found that of surgeons performing CABG on CPB, 56% use cold blood cardioplegia, 14% use warm blood, 14% use crystalloid and just 16% do not use cardioplegia at all, preferring cross-clamp fibrillation (Karthik et al., 2004). It has been estimated that more than four tonnes of depolarising potassium are passed through the coronary arteries of 800,000 patients undergoing cardiac surgery in 2500 centres worldwide each year (Dobson, 2010).

1.4.3 Myocardial protection into the 21st century

Outcomes following cardiac surgery continue to improve in the British Isles and across the developed world. Between 2001 and 2008 the mortality rate for isolated CABG in the UK & Ireland fell from 2.3% to 1.5%, despite an increase in patients' risk profiles due to age and co-morbidities (Bridgewater et al., 2009); medium term survival following CABG also increased to over 90% at 5 years after surgery. Currently, 17% of surgical revascularisation in the UK & Ireland is performed on the beating heart, without the use of cardiopulmonary bypass or cardioplegic arrest, so called 'off-pump' surgery with apparently similar outcomes. There has also been a recent take-off in transcatheter aortic valve implantation (TAVI) as a beating-heart alternative to open aortic valve replacement in symptomatic patients who are deemed too high risk for conventional surgery (Vahanian et al., 2008). So if the outcomes of cardiac surgery are now so good and there is a move towards performing some common procedures without stopping the heart at all, why is there a need to further improve strategies for myocardial protection?

Following cardiac surgery using standard techniques for myocardial protection, around one-in-three patients have a period of clinical dysfunction due to myocardial stunning, most of whom require inotropes to support the heart during its recovery (Arnold et al., 1985, Quinn et al., 2006). An episode of non-fatal low cardiac output syndrome in the early postoperative period has been shown to be associated with a significant reduction in late survival following CABG (Fremes et al., 2000). The need for inotropic support is also associated with prolonged length of stay, morbidity and mortality, in addition to added cost and resource utilisation, and has been identified

as a risk factor for reduced quality of life ten years after surgery (Herlitz et al., 2005). Inadequate myocardial protection may lead to permanent injury, diffuse necrosis resulting in fibrosis, remodelling and long-term impairment of ventricular function.

The population undergoing cardiac surgery is becoming older, with more complex patterns of heart disease and more frequent co-morbidities, such as diabetes mellitus, hypertension and renal impairment (Bridgewater et al., 2009). The myocardium is often poorly functioning with a low ejection fraction and is more vulnerable to a low cardiac output episode in the early postoperative period. These patients increasingly require more urgent and complicated surgery, involving multiple valve procedures frequently in combination with revascularisation. There is also a move towards complex valve repair rather than replacement for mitral pathology and concomitant procedures such as radiofrequency ablation for atrial fibrillation. Such factors contribute towards increasing the length of ischaemic arrest and the risk of inadequate myocardial protection. At the other end of the spectrum, there has been a move in congenital cardiac surgery towards earlier intervention to repair complex lesions in neonates and infants. Metabolism in the immature heart exhibits marked differences in substrate utilisation, insulin sensitivity, calcium handling and antioxidant defences (Doenst et al., 2003b); therefore adult-derived solutions to myocardial protection may not be optimal for the paediatric heart (Allen, 2004). In cardiac transplantation, improved protection of organs may also extend the acceptable cold ischaemic time, increasing geographical flexibility and enabling better harmonisation of organ supply and demand (Banner et al., 2008).

In summary, current techniques for myocardial protection using cardioplegia and hypothermia are not a panacea for prolonged, recoverable ischaemia but extend the metabolic window of opportunity. Recent data demonstrate that length of cardioplegic arrest remains an independent predictor of mortality in contemporary practice (Doenst et al., 2008). The need for longer periods of ischaemia in more metabolically vulnerable patients necessitates further research to improve protective strategies.

1.4.4 Novel techniques to improve myocardial protection

Current research strategies to attenuate ischaemia-reperfusion injury in cardiac surgery fall into two categories: innovative mechanisms to achieve arrest through cardioplegia and adjuvant therapies to improve myocardial protection. The search for an alternative to hyperkalaemic cardioplegia has resurfaced due to concerns over the effect of depolarised arrest on ionic imbalances, endothelial injury, coronary spasm and myocardial stunning; apart from lowering oxygen demands through electromechanical arrest, there is accumulating evidence that hyperkalaemia *per se* offers little or no *direct* cardioprotection (Dobson, 2010). Several agents that arrest the heart in a polarised state, maintaining resting myocyte membrane potential, have been studied in experimental models. Dobson and Jones (2004) proposed using adenosine and lidocaine in a normokalaemic cardioplegic solution, whilst in the UK, Chambers and colleagues have investigated the use of tetrodotoxin, a selective sodium channel blocker (Snabaitis et al., 1997), and esmolol, an inhibitor of calcium and sodium channels (Bessho and Chambers, 2002), demonstrating improved myocardial protection in isolated rat hearts. However, none of these agents have yet been adequately tested in randomised controlled clinical trials.

Over the last two decades, the focus of most translational research groups has been on identifying adjuncts to be used alongside the established clinical methods of hyperkalaemic arrest and hypothermia. Three main areas have been investigated: induction of endogenous protective mechanisms through local or remote ischaemic conditioning; pharmacological mimicking of endogenous protection; and targeting other components of the ischaemia-reperfusion cascade (Kharbanda, 2010).

The concept of ischaemic conditioning proposes that brief periods of ischaemia lead to the induction of endogenous protective mechanisms that diminish the consequences of a subsequent more prolonged ischaemic insult. It was first described in a canine model (Murry et al., 1986) and later demonstrated to be preserved in humans undergoing cardiac surgery (Yellon et al., 1993). A recent meta-analysis of 933 patients from 22 trials found that ischaemic preconditioning was associated with significant reductions in ventricular arrhythmias, inotrope requirements and critical care stay (Walsh et al., 2008). The authors concluded that ischaemic preconditioning may provide additional myocardial protection over cardioplegia alone. Lately, there has been growing interest in remote ischaemic preconditioning in which the initial ischaemic stimulus is applied to a distant organ, such as a limb, to improve protection of the target organ (Przyklenk et al., 1993). This technique has additional advantages in being non-invasive, simple to apply and otherwise benign, and early clinical studies suggested a significant reduction in myocardial injury and inotrope requirements in children (Cheung et al., 2006) and adults (Hausenloy et al., 2007) undergoing cardiac surgery. However, the largest trial published to date on 162 patients from our group in Birmingham found no

improvement in early postoperative haemodynamics or troponin release (Rahman et al., 2010) raising questions as to how much additional benefit is provided by preconditioning over adequate cardioplegic arrest.

The exact pathway that mediates cardioprotection through ischaemic preconditioning remains unclear. It is thought that the ischaemic stimulus generates autocoids such as adenosine, bradykinin and opioids which recruit pro-survival kinase signalling pathways though activation of their receptors in the target tissue (Hausenloy and Yellon, 2008). These cascades converge through the inactivation of GSK-3β to inhibit opening of the mPTP and improve cell survival. A number of pharmacological agents have been investigated for their ability to trigger these cellular cardioprotective pathways including adenosine, bradykinin, opioids, atrial natriuretic peptide, cyclosporine A, high-dose HMG-CoA reductase inhibitors and volatile anaesthetic agents, such as isoflurane (Venugopal et al., 2009, Kharbanda, 2010). Many agents targeting other cellular processes have also been investigated, either via systemic therapy or supplementation of cardioplegia, with some experimental but limited clinical success. These include antioxidants (N-acetylcysteine, aprotinin, allopurinol, vitamin E), substrate supplements (L-glutamate, phosphocreatine), modulators of ionic flux (cariporide, levosimendan) and nitric oxide donors (nitrite infusion).

The failure to translate novel cardioprotective strategies from bench to bedside is hugely disappointing and has been attributed to the inadequacy of animal models and inappropriate design of clinical trials, amongst others (Hausenloy et al., 2010).

2. MODULATING MYOCARDIAL METABOLISM

2.1 Metabolic therapy for myocardial protection

'Metabolism – the lost child of Cardiology.'

(Taegtmeyer, 2000)

The first principle for the manipulation of myocardial metabolism is that inducing a shift in substrate utilisation from fatty acids to glucose will increase the efficiency of energy production and decrease the potentially harmful effects of fatty acid oxidation during ischaemia-reperfusion (Taegtmeyer, 2002). Physiological or pharmacological suppression of FFA uptake and/or β-oxidation stimulates myocardial glucose utilisation, in part by reducing the suppression of *PDH* complex and maintaining the entry of glycolytic pyruvate into the citric acid cycle. Coupling of glycolysis to glucose oxidation is increased, the production of lactate and protons is reduced and the efficiency of ATP production per mole of oxygen is improved. The enhanced energetic status of the cell leads to downregulation of *AMPK* and *PPARα*, with reduced expression of mitochondrial uncoupling proteins and diminished proton leakage across the inner mitochondrial membrane (Lopaschuk et al., 2010). The impact of ischaemia on the cell is thereby reduced translating into enhanced recovery of cardiac function on reperfusion.

In recent years, a second principle of metabolic protection has emerged through the activation of anti-apoptotic pro-survival pathways (Opie and Sack, 2002). It is thought that key glycolytic enzymes may induce protection against ischaemia-reperfusion by activation of the RISK pathway via the *PI3K-Akt* cascade, leading to inactivation of

GSK-3β and inhibition of mPTP formation (Hausenloy and Yellon, 2004). This common cardioprotective mechanism has been implicated in other therapeutic strategies although its relative importance in metabolic therapy, compared with direct improvements in oxidative efficiency, is yet to be established.

A number of pharmacological agents have been developed that act at various points in the metabolic pathways to alter the supply of substrates to the heart, decrease the mitochondrial uptake or β-oxidation of fatty acids, or promote the preferential oxidation of glucose (figure 11). These metabolic therapies have mainly been studied in patients with acute or chronic myocardial ischaemia and heart failure (Lee et al., 2004, Abozguia et al., 2006). However, their established and proposed mechanisms of action suggest that they may have a role in myocardial protection against ischaemia-reperfusion injury as adjuncts to cardioplegia and hypothermia. Rather than further reducing metabolic demand *per se*, inducing a shift in myocardial metabolism may enable these requirements to be met more efficiently from available energy sources whilst upregulating innate cardioprotective pro-survival pathways. An increase in ischaemic tolerance would reduce cellular injury on reperfusion, enhance recovery of contractile function and improve clinical outcomes.

In this chapter, I review the evidence for the potential role of glucose-insulin-potassium (GIK) and other metabolic agents in myocardial protection, including a systematic review, provide a detailed discussion on the clinical and experimental use of perhexiline, and propose a series of hypotheses on perhexiline, its uptake into the heart, its effect on myocardial metabolism and its application to myocardial protection during cardiac surgery to be tested in the remainder of this thesis.

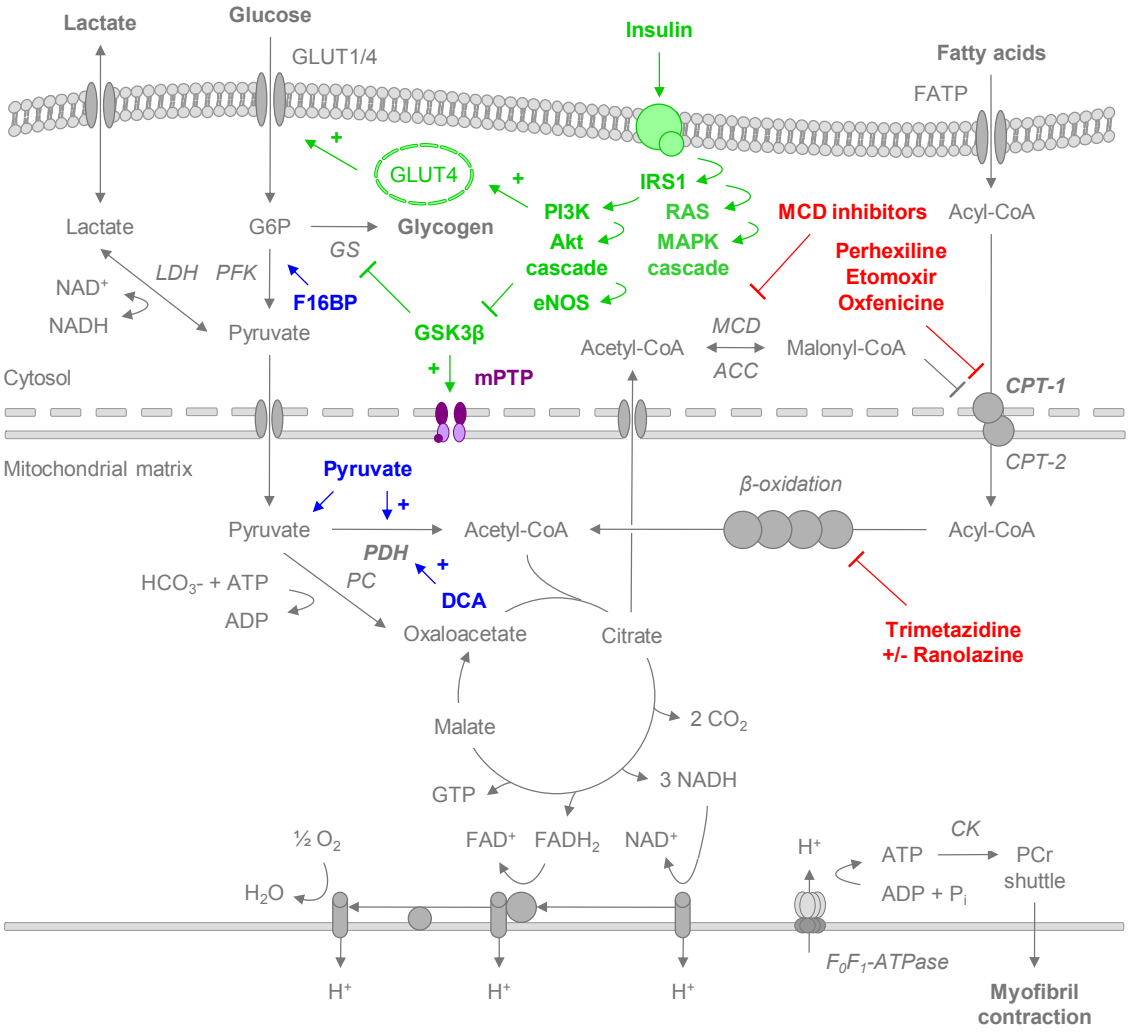


Figure 11. Cellular sites of action of metabolic therapies.

2.1.1 Glucose-Insulin-Potassium

The most extensively studied therapeutic strategy in myocardial metabolism is altering the availability and use of substrates using insulin, usually in the form of GIK solution. This was first used in the treatment of acute myocardial infarction (Sodi-Pallares et al., 1962) and in low cardiac output syndrome after cardiac surgery not responding to other therapies (Braimbridge et al., 1969).

The mechanism of action of insulin on the myocardium has not been fully elucidated; it seems that multiple metabolic and non-metabolic mechanisms have complimentary effects but their relative importance is yet to be established (Doenst et al., 2003a).

- *Increased glycogen content*: GIK has been shown to increase myocardial glycogen content which may then be used for anaerobic ATP production during ischaemia; however, the rate of glycogen turnover rather than content may be more important in ischaemic tolerance (Goodwin et al., 1995).
- *Decreased free fatty acid metabolism*: Insulin suppresses lipolysis in adipose tissue, reducing circulating levels of FFA, acyl-carnitine accumulation in the cytosol and peroxide formation due to incomplete oxidation. It also inhibits FFA oxidation through activation of *acetyl-CoA-carboxylase*, increasing malonyl-CoA and thereby inhibiting mitochondrial uptake of acyl-CoA for β-oxidation (Gamble and Lopaschuk, 1997). Reduced uncoupling of glycolysis and glucose oxidation enhances ATP production per mole of oxygen consumed and decreases anaerobic lactate formation.
- *Increased glycolytic ATP production*: Insulin promotes glucose uptake via GLUT4 and cytosolic ATP production through glycolysis which may be used to maintain basal cell function. However, during low-flow ischaemia, this also increases lactate production and intracellular acidosis which may negatively impact on contractile function (Neely and Morgan, 1974); low-flow may be sufficient to remove the waste products but remains a conceptual obstacle to the use of GIK in ischaemia.
- *Anaplerosis*: Citric acid cycle intermediates are depleted in ischaemia, impairing the turnover rate of the cycle and reducing energy production. During reperfusion,

GIK enables more effective replenishment of cycle intermediates via anaplerosis, a process which is particularly active in the myocardium (Taegtmeyer et al., 1997).

- *Pro-survival pathways*: Insulin activates *MAP kinases* and the pro-survival RISK pathway, via *PI3K-Akt* and the suppression of *GSK-3β*, thereby modulating the acute inflammatory response and reducing apoptosis in ischaemia-reperfusion (Sack and Yellon, 2003). It has also been shown to activate the Nrf2-antioxidant response element pathway and reduce oxidative stress (Wang et al., 2012).
- *Other non-metabolic mechanisms*: Insulin is a known systemic vasodilator, lowering peripheral vascular resistance and improving cardiac output, but has also been shown to have a direct inotropic effect on the heart (Doenst et al., 1999).

Early randomised trials in the pre-thrombolysis/primary PCI era suggested that GIK may have been beneficial in reducing in-hospital mortality following myocardial infarction (Fath-Ordoubadi and Beatt, 1997). However, the CREATE-ECLA trial, the largest randomised controlled trial of high-dose GIK in patients with ST-elevation myocardial infarction, recruited over 20,000 patients from 470 centres worldwide and showed no clinical benefit, including subgroups with diabetes or heart failure (Mehta et al., 2005). A subsequent meta-analysis declared that GIK therapy is not beneficial in the modern management of acute myocardial infarction (Mamas et al., 2010). However, it has been suggested that the neutral outcome may reflect the competing benefits of GIK and harm of systemic hyperglycaemia (Kloner and Nesto, 2008).

Patients undergoing cardiac surgery are in a state of starvation, generate a stress response with endogenous catecholamine release and are administered a large

bolus of heparin which liberates FFA during CPB (Wittnich et al., 1984); insulin resistance during the early postoperative period is also common. As myocardial ischemia due to aortic cross-clamping is an elective event, the potential benefits of a metabolic shift, suppression of lipolysis and increased glucose availability with GIK may be instituted prior to the ischaemic insult and continued to meet the increased requirements of reperfusion. Therefore GIK may be particularly useful in the setting of improving myocardial protection during cardiac surgery.

Two meta-analyses have been performed to examine the use of GIK in cardiac surgery. Bothe and colleagues (2004) found 11 randomised trials comprising 468 patients that investigated the recovery of contractile function as a primary endpoint. Six studies found a significant improvement in cardiac index with GIK, one was neutral and four did not have comparable statistical analyses. The authors estimated a weighted-mean of relative improvement in postoperative recovery of cardiac function of 11.4% with GIK versus controls; sub-group analysis found that continuing GIK following surgery increased the relative improvement from 6.1% to 19.5%. Similarly, Fan and associates (2011) identified 33 randomised controlled trials with 2113 patients undergoing cardiac surgery. GIK was associated with significantly fewer myocardial infarctions (RR 0.63, 95% CI 0.42-0.95, p 0.03), lower inotrope requirements (RR 0.66, 95% CI 0.45-0.96, p 0.03), higher postoperative cardiac index (WMD 0.43L/min/m^2, 95% CI 0.31-0.55, p<0.001) and reduced length of stay on ICU (WMD -7.96 hours, 95% CI -13.36--2.55, p 0.004). They concluded that:

> *'GIK significantly reduced myocardial injury and improved haemodynamic performance in patients undergoing cardiac surgery.'*

However, both meta-analyses were limited by the weaknesses of the published trials included: low number of patients, historical nature of some trials, potential publication bias, and marked heterogeneity of GIK regimes, including dose, timing, duration of therapy and patient groups. In addition, as a load-sensitive parameter, cardiac index is affected by other cardiac physiological variables which were not often reported.

Our group in Birmingham has reported two of the largest randomised controlled trials of systemic GIK as an adjunct to myocardial protection in cardiac surgery. The MESSAGE trial (Quinn et al., 2006) examined the use of perioperative GIK infusion versus placebo in 280 non-diabetic patients undergoing CABG. The infusion of GIK contained 70 IU/L of insulin, commenced at 0.75ml/kg/hr at induction of anaesthesia and continued until six hours after ischaemia. GIK significantly improved early cardiac indices ($p<0.01$) and reduced the incidence of low cardiac output episodes ($p=0.02$) and inotrope requirements ($p<0.01$) versus controls. In addition, there was a decrease in peak troponin-I release with GIK ($p=0.03$) but no difference in area-under-the-curve up to 72 hours after ischaemia (p 0.27). The authors concluded that:

> *'Systemic GIK has beneficial cardiovascular and myocardial protective effects... even in the presence of hyperglycaemia. It can thus be considered an effective, inexpensive and safe adjunctive myocardial protective technique.'*

More recently, the HINGE trial (Howell et al., 2011) reported the use of a similar regime in a randomised controlled trial of 217 non-diabetic patients with left ventricular hypertrophy undergoing aortic valve replacement +/- CABG. We found that perioperative GIK was associated with a significant reduction in the incidence of low cardiac output episodes ($p<0.01$) and inotrope usage ($p<0.01$) but there was no difference in the release of troponin-T (DiM at 6 hours -0.01, 95% CI -0.09-0.07, p

0.81). Left ventricular biopsies obtained after the administration of GIK but before ischaemia demonstrated a substantial increase in the phosphorylation of *AMPK* and *Akt* and a significant rise in protein O-linked β-N-acetylglucosamination, confirming that insulin directly modulates myocardial metabolism and upregulates pro-survival pathways in these patients. Further unpublished data (Howell, 2010) showed that GIK significantly increased pre-ischaemia atrial glycogen content and markedly suppressed plasma FFA concentrations during reperfusion compared with controls.

An alternative approach to the use of insulin in myocardial protection involves the use of insulin-enhanced cardioplegia solution. However, despite encouraging results from smaller, non-randomised studies, the Insulin Cardioplegia trial randomised 1127 patients but found no benefit in high-risk patients undergoing isolated CABG (Rao et al., 2002). The cellular effects of insulin at the onset of ischaemia are limited compared with the administration of systemic GIK which also augments pre-ischaemic myocardial glycogen stores, suppresses plasma FFA and increases substrate availability during reperfusion to meet the heightened energy demands and the replenishment of citric acid cycle intermediates via anaplerosis.

To date, GIK has not been widely adopted for routine clinical use in cardiac surgery; this is likely to be due not only to the perceived lack of evidence but also the burdensome nature of its administration and monitoring against a backdrop of improving outcomes. There have been no large multicentre randomised controlled trials and most studies that have been reported were weakened by low numbers of patients, heterogeneous inclusion criteria and trial protocols, and inadequate study

design, often with a lack of randomisation. There remains no standardised protocol for the administration of GIK in myocardial protection with variation in total insulin dose up to one hundred-fold in reported trials (Bothe et al., 2004). The mechanisms of action of insulin are not fully established and remain controversial. Furthermore, there is concern over the potential negative effects of hyperglycaemia with systemic GIK, which has been shown to be an independent risk factor for worse outcomes after cardiac surgery (Doenst et al., 2005), and the reported lack of benefit in the modern management of acute myocardial infarction (Kloner and Nesto, 2008).

Our experience in Birmingham reflects potential practical issues with the routine use of GIK. In addition to the reported benefits, intensive management of blood glucose was obligatory and vasoconstrictor requirements were markedly increased; the trial setting also enabled a greater level of medical input in the early postoperative period. High-dose insulin regimes are used routinely in a few centres with expertise (Doenst et al., 2003a) but their widespread application is not practical. There is a need for another metabolic agent with a similar efficacy to insulin but without its limitations.

2.1.2 Other metabolic therapies

Numerous drugs have been found to modulate myocardial metabolism by acting at different potential sites within the metabolic pathways, as previously shown in figure 11. The common product is a shift from β-oxidation of fatty acids to the preferential oxidation of glucose and is achieved principally via several mechanisms of action: reduced uptake of fatty acids into the mitochondria, direct inhibition of β-oxidation or facilitated entry of pyruvate into the citric acid cycle.

Carnitine palmitoyltransferase-1 (*CPT-1) inhibitors*: perhexiline, etomoxir, oxfenicine, amiodarone. Located on the outer membrane, *CPT-1* is the first component and rate-limiting step for the uptake of long-chain fatty acids into mitochondria (figure 2). Pharmacological inhibition of *CPT-1* reproduces the physiological action of malonyl-CoA by reducing access of fatty acids to the mitochondrial matrix for β-oxidation and represents a powerful point of control for determining myocardial substrate utilisation. Etoxomir, an irreversible *CPT-1* inhibitor, was shown to protect isolated working rat hearts from fatty acid-induced ischaemic injury with improved functional recovery (Lopaschuk et al., 1988). However, long-term inhibition of *CPT-1* leads to the cellular accumulation of phospholipids; etomoxir showed early promise in a study of patients with heart failure but was withdrawn from the market after the premature cessation of a larger trial due to liver dysfunction (Holubarsch et al., 2007). There is currently no clinical data for the use of oxfenicine whilst amiodarone is only a weak *CPT-1* inhibitor, principally used for its Class III anti-arrhythmic properties. On the other hand, perhexiline is the most potent *CPT-1* inhibitor available for clinical use and will be discussed in greater detail in section 2.2.

Malonyl-CoA decarboxylase (MCD) inhibitors: Malonyl-CoA is the intrinsic inhibitor of fatty acid uptake through potent inhibition of mitochondrial *CPT-1*. Activation of *AMPK* during ischaemia and persisting into reperfusion leads to a marked decline in malonyl-CoA and the excessive oxidation of fatty acids. Therefore, the malonyl-CoA axis represents a novel therapeutic target for reducing β-oxidation (Ussher and Lopaschuk, 2008), although at present, no agents are available for clinical use.

Carnitine biosynthesis inhibitors: Mildronate is a reversible inhibitor of *γ-butyrobetaine hydroxylase*, a key enzyme in the biosynthesis of carnitine. The lower availability of free carnitine leads to a fall in *CPT* activity, mitochondrial uptake and β-oxidation of long-chain fatty acids. It is not currently licensed for therapeutic use in the UK.

Fatty acid oxidation inhibitors: trimetazidine, ranolazine. Widely used across Europe as an anti-anginal agent, trimetazidine has been shown to inhibit *long-chain 3-ketoacyl-CoA thiolase*, a key enzyme in the β-oxidation pathway (Kantor et al., 2000) although its mechanism of action remains controversial (MacInnes et al., 2003). Whilst its downstream site of action is theoretically much less potent than *CPT-1* inhibition, it is not associated with tissue phospholipidosis in chronic use. Several studies have shown that pre-treatment with trimetazidine reduces injury and improves recovery of cardiac function after ischaemia-reperfusion in animal models; a potential role in myocardial protection has been strengthened by evidence for its enhancement of pro-survival pathways (Khan et al., 2010), prevention of apoptosis (Ruixing et al., 2007) and inhibition of mPTP opening (Argaud et al., 2005). However, the administration of trimetazidine-enriched cardioplegia without pre-treatment offered no haemodynamic or metabolic improvement in a swine model of myocardial protection (Silveira Filho et al., 2008).

Ranolazine may also be a partial fatty acid inhibitor but its primary effect is now established as inhibition of late sodium channels (Hale et al., 2008). However, in the isolated rat heart model, it has been shown to have therapeutic potential as an additive to cardioplegia (Hwang et al., 2009).

Pyruvate dehydrogenase kinase (PDK) inhibitors: Dichloroacetate (DCA) facilitates entry of pyruvate into the citric acid cycle for oxidation by indirect activation of *PDH* complex. In an isolated rat heart model, DCA has been shown to be protective in the recovery of post-ischaemic ventricular function (Taniguchi et al., 2001). However, optimal protection was afforded by the combination of DCA, insulin and N^6-cyclohexyladenosine, an adenosine A_1 receptor antagonist and partial inhibitor of glycolysis (Gandhi et al., 2008); recovery of function was inversely proportional to H^+ production during reperfusion. DCA is currently only used in the treatment of inherited mitochondrial disorders to prevent lactic acidosis by improved glycolytic coupling.

Several metabolic intermediates have also shown promise either as additives to cardioplegia e.g. pyruvate (Knott et al., 2006), fructose-1,6-biphosphate (Riedel et al., 2004) or with intravenous loading e.g. L-glutamate (Bitzikas et al., 2005). However, this approach has intrinsic weaknesses: non-selective supplementation requires huge quantities of substrate to significantly affect target tissue concentration and may have metabolic side-effects; cardioplegia additives are delivered to the myocardium but still depend upon either uptake along a concentration gradient or an efficient membrane transport system; increased anaerobic metabolism during ischaemia may worsen cellular acidosis; and neither strategy increases substrate supply on reperfusion.

2.1.3 Metabolic therapy in cardiac surgery: a systematic review

Numerous drugs directly modulate cellular metabolic pathways and have been shown to ameliorate ischaemia-reperfusion in animal models. Either alone or in combination, they may have a role as adjuncts to myocardial protection during cardiac surgery. In order to establish the current evidence supporting metabolic agents other than insulin and metabolite supplements, I performed a systematic review of the literature.

Methods

Data sources: A systematic literature search of Medline (1950-July 2011), Embase (1982-July 2011) and Cochrane Library 2011 was performed. To achieve maximum sensitivity of the search and identify all relevant studies, a combination of terms were used including metabolism, myocardial protection, heart surgery, ischaemia, perhexiline, etomoxir, oxfenicine, trimetazidine, mildronate, dichloroacetate and malonyl CoA decarboxylase. No language restrictions were placed. The search also included online trial registries and reference lists of relevant review articles.

Study selection: All studies assessing the use of a pharmacological agent, other than insulin/GIK, with a confirmed or proposed metabolic action in myocardial ischaemia-reperfusion were included. Studies were excluded if the subjects were non-human, there was no comparative group, the drug was not given before or during ischaemia, the intervention was not cardiac surgery, or the outcome measures did not include clinical, physiological or biochemical markers of myocardial function or injury.

Data extraction and assessment: Eligibility was assessed in a standardised manner by a single reviewer. Included studies were assessed for their characteristics and quality indicators using the Jadad score (Jadad et al., 1996).

Results

The identification, screening, eligibility and inclusion of studies is documented in a flow diagram (figure 12) according to the PRISMA statement (Moher et al., 2009). Sixty three records were screened of which 55 were excluded: 41 involved non-human subjects, 13 did not involve cardiac surgery and one reference was an unpublished abstract. Eight full-text articles were assessed for eligibility and all were included in the review (table 1), seven used trimetazidine and one evaluated mildronate and trimetazidine; no comparable studies on perhexiline, etomoxir, oxfenicine, dichloroacetate or malonyl CoA decarboxylase inhibitors were identified.

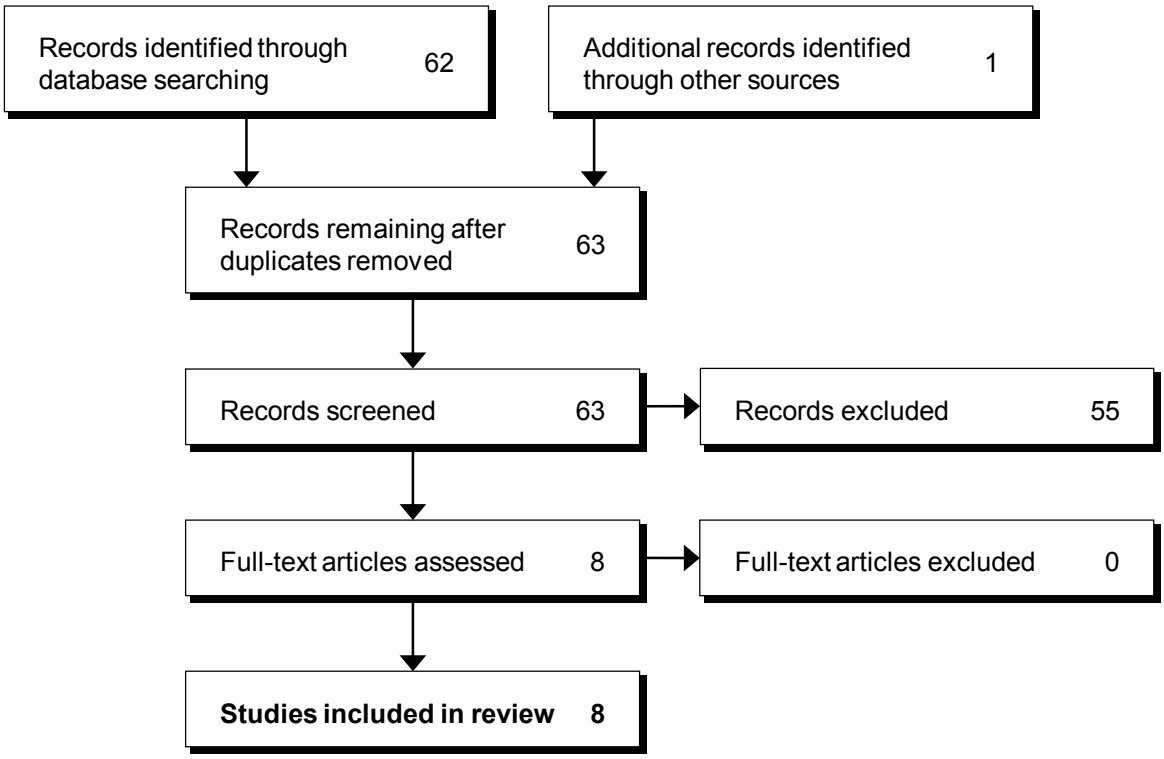


Figure 12. PRISMA flow diagram of study selection.

Study	Drug	Language	Random	Patients	Jadad
Fabiani *et al*. (1992)	Trimetazidine	English	Yes	19	1
Vedrinne *et al*. (1996)	Trimetazidine	English	Yes	30	4
Tünerir *et al*. (1999)	Trimetazidine	English	Yes	40	2
Iskesen *et al*. (2006)	Trimetazidine	English	Yes	24	1
Gordeev *et al*. (2007)	Mildronate & Trimetazidine	Russian	Yes	119	1
Iskesen *et al*. (2009)	Trimetazidine	English	Yes	30	1
Lopatin *et al*. (2009)	Trimetazidine	Russian	Yes	306	2
Martins *et al*. (2011)	Trimetazidine	English	Yes	60	5

Table 1. Characteristics of studies included in the systematic review.

Studies were mostly published in the English language but contained few patients (median 35, range 19-306) and were of low quality (mean Jadad score 2.1). In seven studies, the trial drug was given orally for at least two weeks preoperatively; in the other, intravenous loading was given at the time of surgery with supplemental trimetazidine in the cardioplegia, which was also given in one of the oral studies (table 2). None of the studies detected any significant improvement in perioperative haemodynamics with metabolic therapy. Two studies reported an improvement in left ventricular systolic function following surgery; one also found a reduction in areas of hypokinesia with trimetazidine ($p<0.01$) and mildronate ($p<0.01$) compared with controls but no difference between the active treatments (Gordeev et al., 2007). However, the other study continued trimetazidine for *three years* after surgery and

only detected an improvement in ejection fraction after one year and exercise testing after three years which may reflect the long-term efficacy of the drug in heart failure rather than any improvement in myocardial protection during surgery (Lopatin and Dronova, 2009). Likewise, another study found that pre-treatment with trimetazidine improved cardiac function before but not after surgery (Martins et al., 2011). Four studies reported a reduction in troponin and/or CK-MB release with trimetazidine, suggesting less myocardial ischaemia-reperfusion injury. Two studies observed a significant decline in plasma malondialdehyde, a product of lipid peroxidation and marker of oxidative stress, with trimetazidine although another found no difference. One study found that it also preserved systemic endogenous antioxidant capacity.

Study	**Drug**	**Route**	**Timing**	**Outcomes**
Fabiani *et al*. (1992)	Trimetazidine	oral & CP	3 weeks	↔ CI, ↓ MDA
Vedrinne *et al*. (1996)	Trimetazidine	iv & CP	Intra-op	↔ CI, ↔ MDA
Tünerir *et al*. (1999)	Trimetazidine	oral	3 weeks	↓ troponin-T, ↔ CI
Iskesen *et al*. (2006)	Trimetazidine	oral	2 weeks	↓ MDA, ↑ SOD, ↑ GP
Gordeev *et al*. (2007)	Mildronate	oral	2 weeks	Both ↑ LVEF, ↑ local
	Trimetazidine	oral	2 weeks	contractility, ↓ RWMA
Iskesen *et al*. (2009)	Trimetazidine	oral	2 weeks	↓ troponin-T,↓ CK-MB
Lopatin *et al*. (2009)	Trimetazidine	oral	2 weeks	↑ LVEF, ↓ CK-MB
Martins *et al*. (2011)	Trimetazidine	oral	2 weeks	↓ troponin-T, ↔ LVEF

CI, cardiac index; CK-MB, creatine kinase isoenzyme; CP, cardioplegia; GP, glutathione peroxidise; LVEF, left ventricular ejection fraction; LVSWI, left ventricular stroke work index; MDA, malondialdehyde; RWMA, regional wall motion abnormalities; SOD, superoxide dismutase.

Table 2. Interventions and results of studies included in the systematic review.

Conclusions

In a systematic review of the literature to identify the current evidence supporting the use of metabolic therapies other than insulin as adjuncts to myocardial protection in cardiac surgery, I found little proof of benefit to support their use. Only eight studies applicable to cardiac surgery were identified; these were often small, poorly designed and reported, reflected in low Jadad scores, with heterogeneity of trial patients, protocols and endpoints and invariably underpowered to detect clinically important outcomes. Only one study demonstrated a significant improvement in any measure of cardiac function in the early postoperative period (Gordeev et al., 2007) whilst four found a reduction in clinical markers of myocardial injury. Two studies identified a reduction in markers of oxidative stress but none examined the effect of metabolic therapies on the pro-survival pathways implicated in the mechanism of action of insulin. Indeed, in the entire literature on metabolic therapies other than GIK, only two studies recruited more than one hundred patients undergoing surgery.

All studies used trimetazidine (*Vastarel*), a widely available anti-anginal agent developed and marketed by Les Laboratoires Servier, France. One study also investigated mildronate, a drug produced by Grindex in Latvia but currently used little outside Russia and the Baltic States. Trimetazidine showed potential in reducing troponin release following surgery but given the size of the studies and the lack of haemodynamic and other outcome data, there is insufficient evidence to support its routine use. Furthermore, there is currently no clinical evidence for the application of other metabolic therapies, including perhexiline, to improve myocardial protection against ischaemia-reperfusion injury during cardiac surgery.

2.2 Perhexiline

'New tricks for an old drug.'

(Frenneaux, 2002)

2.2.1 The rise and fall of carnitine-palmitoyltransferase inhibition

Developed by Richardson-Merrell Pharmaceuticals Limited in the late 1960s, perhexiline was first marketed during the 1970s as an anti-anginal agent (Ashrafian et al., 2007). It proved to be highly effective for the symptomatic relief of exertional angina pectoris and was particularly useful as mono-therapy in patients who were either unsuitable or failed to respond to beta-blockers (Burns-Cox et al., 1971). Early experimental and clinical studies suggested that whilst perhexiline led to coronary vasodilatation and increased coronary blood flow, it was not associated with intolerable reductions in blood pressure or heart rate. Perhexiline was introduced to the United Kingdom in 1975 and by the end of that decade, had become widely used across Europe with particular popularity in France.

Alas, reports of serious adverse events soon began to emerge describing severe peripheral neuropathy (Lhermitte et al., 1976) and potentially fatal hepatotoxicity (Pessayre et al., 1979). By August 1983, 80 cases of liver failure and 131 cases of neuropathy associated with perhexiline had been reported to the UK authorities (Shah, 2006). Similarly in France, it accounted for 8.9% of all hepatic adverse drug reactions between 1976 and 1980 (Albin et al., 1981) but no correlation was established between perhexiline use and increased mortality in a large observational study, although length of follow-up was limited (Killalea and Krum, 2001). Toxicity appeared in a small number of patients on long-term therapy; in a report of two

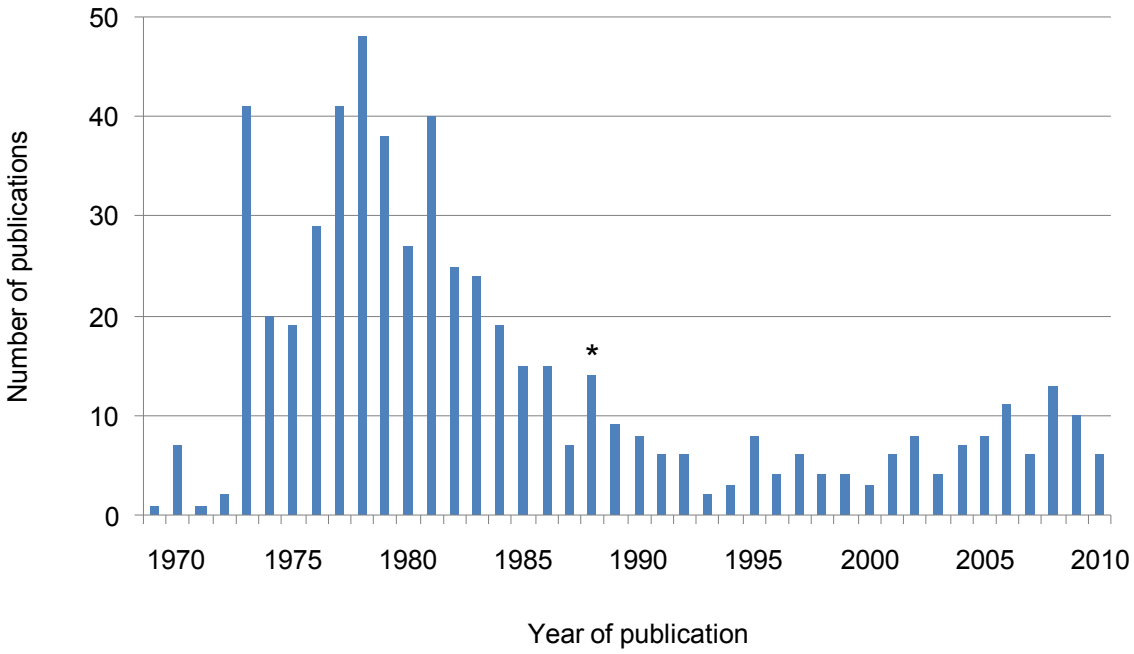
patients who died from cirrhosis after receiving perhexiline, Pessayre and colleagues (1979) concluded that:

> *'Prolonged administration of perhexiline maleate may induce... ultrastructural and histochemical lesions resembling those of phospholipidosis.'*

It later emerged that the severe prolonged inhibition of mitochondrial β-oxidation leads to microvesicular steatosis and liver failure (Fromenty and Pessayre, 1995). Patients with neuropathy were found to have higher plasma levels of perhexiline with a prolonged half-life (Singlas et al., 1978) whilst impaired drug metabolism in those with side-effects suggested a genetic susceptibility to toxicity (Shah et al., 1982).

Perhexiline was withdrawn in the United Kingdom in 1985 and then worldwide in 1988 (Shah, 2006) with the exception of Australia and New Zealand, where it remains licensed for the treatment of patients with refractory angina conditional upon therapeutic monitoring (Inglis and Stewart, 2006). Clinical use and research interest in perhexiline has persisted, most notably at The Queen Elizabeth Hospital, Adelaide through defining the safe therapeutic range, developing dosing regimens, establishing methods for therapeutic drug monitoring, and improving the understanding of its pharmacokinetic and pharmacogenomic properties (Horowitz et al., 1986, Stewart et al., 1996, Davies et al., 2006a). Today, perhexiline maleate (PEXSIG) is manufactured by Sigma Pharmaceuticals Ltd. in Baulkham Hills, New South Wales, Australia and is marketed internationally by Swedish Orphan Biovitrium AB, a Swedish based pharmaceutical company specialising in emerging late stage development of orphan and speciality drugs for rare diseases.

Over the last 40 years, research interest in perhexiline has waxed and waned. On searching Medline for the period 1969-2010, there were 575 publications in peer-reviewed journals featuring the term *perhexiline* in the title, abstract or text. Plotted against year of publication, there has been a bimodal frequency distribution (figure 13). The greatest interest occurred during the late 1970s and early 1980s with a peak of 48 publications in 1978 but declined such that following its withdrawal in 1988, the number of reports per annum did not reach double figures again until 2006. A second wave of publications has been driven by Professor John D. Horowitz (21 papers since 2000) and Associate Professor Benedetta C. Sallustio (9 papers) in Adelaide, South Australia and Professor Michael P. Frenneaux now in Aberdeen (8 papers), all of whom have been instrumental in facilitating the work contained in this thesis.

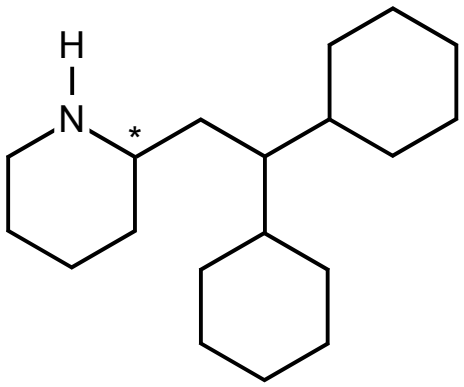


* international withdrawal of perhexiline

Figure 13. Frequency of publications on perhexiline in peer-reviewed journals.

2.2.2 Chemistry

Perhexiline, or 2-(2,2-dicyclohexylethyl) piperidine, is prepared for pharmaceutical use as the maleate salt ($C_{19}H_{36}N^{+}.C_4H_3O_4^{-}$), containing a racemic mixture of the (+) and (–) enantiomers.



* chiral carbon

Figure 14. The chemical structure of perhexiline.

It consists of a CH-CH_2 backbone with two saturated cyclohexane rings (hydrophobic) and a pyridine group (hydrophilic), making the whole molecule amphipathic. Each of the rings assumes a chair formation but it is chiral due to asymmetry of the second carbon of the piperidine ring (figure 14). Attempts to predict the biological actions of perhexiline based on its chemical structure have not been fruitful (Dawson et al., 1986).

2.2.3 Mechanisms of action

Early work with a canine right heart perfusion model using intravenous perhexiline found that whilst left ventricular work (blood pressure x cardiac output) decreased due to a fall in both blood pressure and heart rate, left ventricular efficiency (Δ work / Δ myocardial oxygen consumption) was increased suggesting that perhexiline

improves metabolic efficiency (Cho et al., 1970). In human studies, Pepine and colleagues (1974) assessed haemodynamic and metabolic changes with atrial pacing after two weeks of treatment with oral perhexiline. They showed that perhexiline increased cardiac output, improved lactate utilisation and enhanced myocardial oxygen extraction without altering coronary flow, suggesting a shift in cardiac metabolism from predominately fatty acids towards the more efficient utilisation of carbohydrates (Vaughan Williams, 1980). Consistent with this hypothesis, *ex vivo* rat liver preparations treated with perhexiline showed inhibition of mitochondrial β-oxidation of fatty acids (Deschamps et al., 1994).

In a landmark paper, Kennedy and colleagues (1996) from the University of Adelaide performed classical pharmacological inhibition studies in which rat heart and liver homogenates were isolated and treated with perhexiline. The kinetics of mitochondrial fatty acid transport were examined in the presence of varying concentrations of perhexiline and confirmed it to be a potent inhibitor of *CPT-1*. Indeed, perhexiline was more potent at inhibiting cardiac than hepatic *CPT-1* with a half-maximal inhibitory concentration (IC_{50}) of 77μmol/L and 148μmol/L in heart and liver mitochondria, respectively. They also found that unlike malonyl-CoA, the principle endogenous inhibitor of *CPT-1*, which acts at a cytoplasmic regulatory site, perhexiline also appeared to act via a protected site facing the intermembrane space. However, unlike etomoxir, perhexiline is a partial inhibitor of fatty acid oxidation with competitive inhibition of *CPT-1*, implying that it may be overcome by high cellular levels of fatty acids. They concluded that the anti-anginal properties of perhexiline were due to improved metabolic efficiency through *CPT-1* inhibition. It was also

proposed that this may be protective in myocardial ischaemia, since other *CPT-1* inhibitors had been shown to markedly delay the uncoupling effects of ischaemia by reducing the accumulation of long-chain acyl-carnitines (Yamada et al., 1994).

Perhexiline was found to be concentrated at least 20-fold in rat heart homogenate compared with plasma due to its amphipathic nature (Kennedy et al., 1996). Similarly, Deschamps *et al*. (1994) had previously reported the progressive uptake of perhexiline into isolated mouse liver mitochondria, reaching a concentration 20-fold higher than in the incubation medium; it was proposed that similar to other cationic amines with lipophilic moieties, protonated perhexiline enters the matrix by diffusion along the mitochondrial membrane potential. These studies suggest a cumulative rise in the relative concentrations of perhexiline across the compartments from plasma to cytoplasm to mitochondrial matrix. Therefore, the concentration required for the inhibition of *CPT-1* in a broken cell preparation may be achieved *in vivo* at its mitochondrial active site at therapeutic plasma concentrations.

Kennedy and colleagues (2000) also provided evidence from a perfused rat heart model that perhexiline inhibits *CPT-2*. Unlike oxfenicine, perhexiline had no effect on ischaemia-induced long-chain acyl-carnitine accumulation by simultaneous inhibition of the enzymes responsible for its formation (*CPT-1*) and degradation (*CPT-2*). Perhexiline inhibited lactate release and increased acetyl-CoA concentrations in the myocardium under normal flow conditions, which may improve oxidative coupling of glucose during ischaemia by increasing substrate availability for the citric acid cycle. Whilst it was found to enhance diastolic function during ischaemia, perhexiline failed

to improve contractility during reperfusion compared with controls. The authors were surprised by this lack of benefit in reperfusion and speculated that it may be related to the incomplete nature of the ischaemia and the short length of exposure to perhexiline in their model.

In a further study, Unger *et al*. (2005) found that acute exposure to perhexiline did not inhibit palmitate oxidation in either isolated cardiomyocytes or working rat hearts. Following 24 hours of pre-treatment with perhexiline, cardiac work and efficiency improved by approximately 30% but without any effect on fatty acids oxidation; only by 48 hours was palmitate oxidation reduced. The dissociation between metabolic and efficiency effects led the authors to conclude that:

> *'...perhexiline increases myocardial efficiency by a mechanism that is largely or entirely independent of its effects on CPT.'*

The delayed metabolic effect was attributed to gradual myocardial accumulation of perhexiline in order to reach inhibitory levels in the cytosol and mitochondrial matrix and supports the concept of cumulative drug uptake and tissue loading.

Perhexiline has also been shown to have anti-inflammatory effects via the inhibition of superoxide formation by phagocytic NADPH oxidase in neutrophils (Kennedy et al., 2006) and to potentiate platelet responsiveness to nitric oxide via an increase in cyclic GMP (Willoughby et al., 2002). The relative importance of these mechanisms in the clinical effects of perhexiline therapy is yet to be established.

2.2.4 Pharmacokinetics

The decline in the use of perhexiline was not due to a lack of efficacy; on the contrary, it was effective in the relief of angina but its highly variable metabolism led to severe toxicity in a small number of patients. Perhexiline is well absorbed orally, with a large volume of distribution but variable systemic bioavailability (Wright et al., 1973). The major determinant of its oral clearance is hepatic metabolism with only a fraction of the dose eliminated unchanged in the urine. The primary metabolites of perhexiline are *cis*-OH-perhexiline and *trans*-OH-perhexiline, which in turn may be further oxidised to secondary dihydroxy derivatives. Formation of both *cis*- and *trans*-OH-perhexiline is catalysed by *cytochrome P450 2D6* (*CYP2D6*), a member of the microsomal mixed-function oxidase system (Davies et al., 2007); the saturability and genetic polymorphism of this enzyme has been identified as the main determinant of perhexiline clearance (Sallustio et al., 2002). Accordingly, the half-life of perhexiline may show a several hundred-fold variation between patients from just a few hours to over a month. On the other hand, there is relatively little intraindividual variability in the clearance of OH-perhexiline metabolites and so the ratio of the metabolites to the parent drug in the blood represents a useful clinical measure of perhexiline metabolism. This metabolic ratio ($C_{OH\text{-}Px}/C_{Px}$) allows the separation of patients according to phenotypic metaboliser status, enabling the prediction of dosing requirements to maintain a steady-state within the therapeutic range and therefore prevention of the long-term effects of toxicity (table 3) (Sallustio et al., 2002).

Metaboliser status	C_{OH-Px}/C_{Px} ratio	Predicted daily dose (mg/day)
Ultra-rapid	>20	300-500
Extensive	2.5-20	100-250
Intermediate	0.3-2.49	50-100
Poor	<0.3	10-25

C_{OH-Px} indicates concentration of OH-perhexiline; C_{Px}, concentration of perhexiline.

Table 3. Perhexiline metaboliser status, metabolic ratio and predicted dose.

Therapeutic drug monitoring has become the cornerstone of perhexiline therapy over the past 25 years. It has enabled patients to be maintained on effective therapy whilst preventing the toxicity previously associated with chronic use. The therapeutic range has been defined as a plasma perhexiline concentration of 0.15-0.6mg/L. Patients on long-term therapy undergo regular surveillance of plasma levels with dose titration according to a defined protocol (Horowitz et al., 1986, Lee et al., 2004). Personalised dosing may be used to account for variability in perhexiline pharmacokinetics between poor metabolisers with low clearance and high bioavailability and ultra-rapid metabolisers with high clearance and low bioavailability. Recent studies have also found differences in the stereoselective metabolism of perhexiline (+) and (–) enantiomers between metaboliser groups (Inglis et al., 2007, Davies et al., 2008) which may suggest a role for monitoring of the enantiomers rather than the current undifferentiated perhexiline concentration.

2.2.5 Pharmacogenomics

The human CYP2D6 gene, part of the P450 gene superfamily, is comprised of nine exons and resides on the long arm of chromosome 22. It is thought to have emerged around 400 million years ago as tetrapods took to the land and required new enzymes to detoxify ingested plant metabolites (Gonzalez and Nebert, 1990). P450 gene evolution has resulted in significant polymorphism, leading to marked variability in individual responses to drugs and toxins. Poor metabolisers of perhexiline possess two CYP2D6 null alleles and have no detectable enzymatic activity; intermediate metabolisers express low level residual function with at least one partially deficient allele. At the other end of the spectrum, ultra-rapid metabolisers have alleles carrying multiple copies of the gene resulting in excessive expression (Zanger et al., 2004).

The prevalence of poor metabolisers is 7-10% in European Caucasians and 1-2% in African or East Asian populations (Gardiner and Begg, 2006). Many mutations of the CYP2D6 gene are recognised to activate or inactivate enzyme function (Ingelman-Sundberg et al., 2011) and genotyping for the common variants has an accuracy of 99% for differentiating poor and extensive metaboliser phenotypes (Sachse et al., 1997). However, it has proven less useful for predicting future dosing as it excludes non-genetic factors affecting metabolism, including other drugs e.g. SSRIs, and does not account for alternative lower-affinity pathways, such as *CYP3A*. Therefore CYP2D6 genotyping is not a replacement for therapeutic drug monitoring but may be used to identify poor metabolisers who are at greater risk of toxicity (Barclay et al., 2003, Davies et al., 2006a). On this basis, perhexiline has been suggested as a withdrawn drug that may be ‘rescued’ by genotype-based prescribing (Shah, 2006).

2.2.6 Adverse effects

It has been known for many years that the toxic effects of perhexiline therapy are concentration-dependent. In poor metabolisers, the lack of *CYP2D6* leads to steadily rising plasma levels with a standard dosing regimen. In intermediate metabolisers, there is a disproportionate change in plasma concentration compared to a change in dose; the unbound perhexiline concentration exceeds the K_m for *CYP2D6* within the clinical range, saturating its metabolism and predisposing to toxicity (Davies et al., 2007). Early transient side-effects are related to acute elevation of plasma perhexiline concentration above 0.6mg/L and occur in 10%-28% of patients depending on dose (Philpott et al., 2004). Symptoms are principally nausea, dizziness and headache but may also include vomiting, diarrhoea, tremor, lethargy and insomnia; acute hypoglycaemia has also been reported in patients with diabetes mellitus (Sigma Pharmaceuticals, 2009).

Chronic toxicity may occur after at least three months of continuous perhexiline therapy in susceptible individuals and is concentration-dependent. Serious adverse events are uncommon but include peripheral neuropathy, hepatitis, extrapyramidal dysfunction, muscle weakness, ataxia and weight loss (Sigma Pharmaceuticals, 2009). Elevation of serum liver enzymes is common and must be monitored with long-term therapy. Sensory, motor and autonomic neuropathies have been reported with evidence of reduced nerve conduction velocity, denervation atrophy and demyelination. At a cellular level, these adverse effects are thought to be due to excessive inhibition of *CPT-1*, impairment of mitochondrial energy metabolism and upstream cytoplasmic accumulation of long-chain fatty acids (Ashrafian et al., 2007).

Toxicity can be prevented in long-term use by therapeutic drug monitoring and adjustment of dosing to achieve plasma levels within the therapeutic range (0.15-0.6mg/L) (Horowitz et al., 1986, Cole et al., 1990). This strategy has been shown to maintain efficacy whilst avoiding almost all serious adverse effects (Horowitz et al., 1995) in poor metabolisers over several years.

2.2.7 Clinical use

Perhexiline was originally marketed as a treatment for chronic angina pectoris and was found to be highly effective in patients who were either unsuitable or refractory to other agents, resulting in fewer angina attacks, improved exercise tolerance and reduced consumption of glyceryl trinitrate (Burns-Cox et al., 1971, White and Lowe, 1983, Pilcher et al., 1985); although all of these early studies were limited by adverse effects. By dose adjustment according to plasma levels, Horowitz and colleagues (1986) were able to demonstrate a marked improvement in symptoms without long-term toxicity. Cole *et al*. (1990) found perhexiline to be an effective therapy in patients with refractory angina who were unsuitable for coronary revascularisation; similarly, it was associated with marked improvement in symptoms in patients with severe aortic stenosis deemed unsuitable for aortic valve replacement (Unger et al., 1997).

In recent years, metabolic manipulation with perhexiline has been shown to improve symptoms, left ventricular ejection fraction (LVEF) and maximal oxygen consumption (Vo_2 max) in patients with chronic heart failure (Lee et al., 2005) and to enhance exercise capacity and ameliorate cardiac energetic impairment in symptomatic hypertrophic cardiomyopathy (Abozguia et al., 2010). Whilst patients on perhexiline

have previously undergone cardiac surgery (appendix 9.1), no studies to date have assessed whether it improves clinical markers of myocardial protection.

Perhexiline is currently licensed for the treatment of refractory angina in Australia and New Zealand, subject to therapeutic monitoring of patients (Sigma Pharmaceuticals, 2009). Whilst effective in the treatment of stable angina, unstable angina and acute coronary syndromes (Willoughby et al., 2002), its use is limited due to its narrow therapeutic index and variable pharmacokinetics. However, particularly at The Queen Elizabeth Hospital, Adelaide, it has been used off-label in patients with other cardiac conditions including diabetic or hypertrophic cardiomyopathy. In the United Kingdom, perhexiline is prescribed on a named-patient basis only and is restricted to a few specialist centres, often in the context of clinical trials (Phan et al., 2009). Currently, therapeutic drug monitoring of perhexiline is only performed in two centres.

2.3 Summary and hypotheses

Myocardial protection enables the prolongation of ischaemic tolerance through the use of cardioplegia and hypothermia to produce elective cardiac arrest and facilitate surgery. However non-fatal perioperative events including low cardiac output episode due to inadequate protection significantly reduce late survival (Fremes et al., 2000). Modulation of metabolism offers a means to reduce ischaemia-reperfusion injury and improve function in the early postoperative period. GIK has been shown to improve postoperative haemodynamics and reduce myocardial injury in a meta-analysis of 33 trials including 2113 patients (Fan et al., 2011); several potential mechanisms of action have been identified but their relative importance is unclear. However, it is not widely used in cardiac surgery today due to the lack of definitive randomised multi-centre trials, the need for a standardised protocol and concerns over perioperative blood glucose control. In a systematic review of the literature, I found little evidence for the use of other metabolic therapies in myocardial protection.

Perhexiline has been used to treat ischaemic heart disease and chronic heart failure. It is thought to induce a metabolic shift from fatty acids to glucose, increase efficiency of ATP production per mole of oxygen and improve coupling of glycolysis to glucose oxidation, resulting in a net reduction of myocardial proton burden during ischaemia. The available experimental evidence suggests that it may have a role in reducing myocardial ischaemia-reperfusion injury but this has never been tested in a clinical trial and so its application to cardiac surgery is unknown.

Coronary artery bypass graft surgery remains the predominant heart operation in Europe (Bridgewater et al., 2010) and the vast majority are still performed on cardiopulmonary bypass; hence it represents the largest pool of patients in whom to study myocardial protection and makes the findings widely and directly applicable to the greatest number of patients. The period of elective ischaemia with aortic cross-clamping provides both a model for assessment of the effect of metabolic therapies on ischaemia-reperfusion and unique access to myocardial tissue to directly study the pharmacology of drugs in humans.

In this thesis, I propose to test the following hypotheses:

1. *Preoperative oral loading with perhexiline maleate as an adjunct to cold blood cardioplegia during ischaemic arrest in patients undergoing CABG will improve markers of myocardial protection (Chapter 7). This will be reflected by a decreased incidence of low cardiac output episodes, reduced need for inotropic support and lower release of troponin in the perioperative period compared with controls.*

I conduct a prospective, two-centre, double-blind, randomised, placebo-controlled trial of preoperative oral perhexiline in patients undergoing CABG (Chapter 3) using validated clinical markers of myocardial stunning and ischaemia-reperfusion injury to evaluate its application to myocardial protection.

2. *Cardiac indices will also be higher in the perhexiline group prior to ischaemia due to improved metabolic efficiency (Chapter 7). This will be reflected by changes in*

the myocardial metabolic profile favouring glucose metabolism and glycolytic flux through pyruvate to enter the citric acid cycle (Chapter 6). The short length of therapy will bring the benefits of CPT inhibition on metabolic efficiency without the potential risks of lipid accumulation caused by long-term inhibition of β-oxidation.

I use two independent laboratory techniques, traditional enzymatic colourimetry and state-of-the-art Fourier transform ion cyclotron resonance mass spectrometry, to assess the effect of perhexiline therapy on carbohydrate and/or lipid metabolism in the atrial and ventricular myocardium prior to ischaemia.

3. *At least 80% of patients will be within the therapeutic range for serum perhexiline at the time of surgery with a standard dosing regimen of 200mg bis die for three days then 100mg bis die until surgery (Chapter 4). Patients may fall outside of the therapeutic range due to variable metabolism, side-effects or non-compliance.*

Following an evidence-based loading regime, I determine the serum concentration of perhexiline at the time of surgery and examine factors that may have influenced attainment of therapeutic levels including length of therapy and metaboliser status.

4. *Perhexiline is more concentrated in the human myocardium than in the serum reflecting cumulative drug uptake and tissue loading as previously shown in the rat heart (Chapter 5). Serum and myocardial levels will be closely correlated but may be reduced by insufficient length of therapy to reach steady-state or high serum concentrations with saturation pharmacokinetics.*

I analyse atrial and ventricular biopsies obtained from patients in the trial using high performance liquid chromatography to determine the tissue concentration of perhexiline, correlate with serum levels and examine factors that may affect drug uptake. This work was conducted through a Wellcome Trust Training Award to visit the University of Adelaide, South Australia.

5. *The concentration of perhexiline in the myocardium following at least one week of therapy will be sufficient to significantly inhibit CPT-1 corresponding to inhibitory concentrations observed in the rat heart (Chapter 5)* (Kennedy et al., 1996).

I compare these novel human findings with those from studies of perhexiline in the rat heart to assess whether the concentration is likely to be sufficient for *CPT-1* inhibition

The subsequent chapters of this thesis are structured to address these hypotheses along a biological pathway from oral drug administration, attainment of a therapeutic serum concentration, uptake into the myocardium, modulation of myocardial metabolism and improvement in clinical markers of myocardial protection (figure 15).

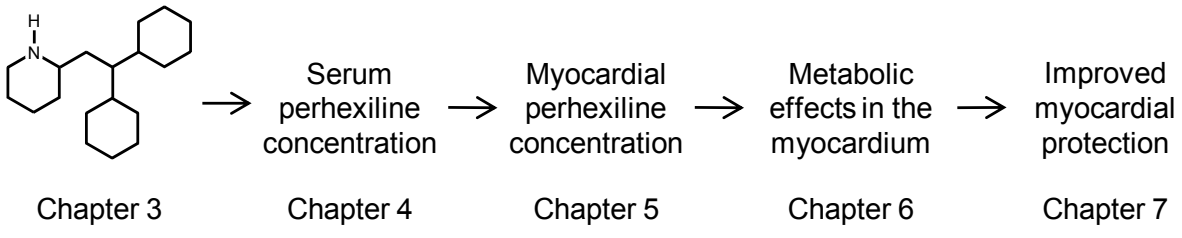


Figure 15. Structure of the subsequent chapters of this thesis.

3. METHODS

Randomised controlled trials, alone or as part of a systematic review, provide the highest level of evidence for the effectiveness of an intervention and form the bedrock of evidence-based medicine (OCEBM, 2009). The International Conference on Harmonisation (www.ich.org) has developed tripartite guidelines for the scope, design, conduct, analysis and reporting of trials through a process of scientific consensus between regulatory authorities and the pharmaceutical industry in the United States, Europe and Japan. These international standards have been widely adopted into law to regulate clinical trials, maintain quality and ensure patient safety.

In this chapter, I review the key ICH principles of clinical trial methodology including identifying research questions, avoiding bias, the intention-to-treat principle and the Consolidated Standards of Reporting Trials (CONSORT) statement. I relate these principles to the design, conduct and analysis of the CASPER trial, a multi-centre trial of perhexiline in myocardial protection on which this thesis is founded.

3.1 Principles of clinical trial methodology

The efficacy and safety of medicinal products should be demonstrated by clinical trials which follow the ethical and methodological principles of the Declaration of Helsinki (World Medical Association, 2008) and Good Clinical Practice (ICH, 1996). Such studies can be classified according to their objectives and timing during the process of drug development (table 4) (ICH, 1997).

Type of study	Objectives	Study examples
Human Pharmacology	• Assess tolerance • Describe PK and PD • Explore drug metabolism • Estimate drug activity	• Dose-tolerance • Single or multi-dose • Drug interactions
Therapeutic exploratory	• Explore targeted indications • Estimate dosage • Provide basis for design of confirmatory studies	• Well-defined population with pharmacological or surrogate endpoints of clinical measures • Dose-response
Therapeutic confirmatory	• Demonstrate efficacy • Establish safety profile • Risk-benefit analysis • Establish dose-response	• Controlled efficacy • Parallel dose-response • Clinical safety • Large simple trials
Therapeutic use	• Refine understanding of risk-benefit especially in special populations • Identify uncommon adverse drug reactions • Refine dosing proposals	• Studies of comparative effectiveness • Mortality/morbidity • Additional endpoints • Pharmacoeconomics • 'Real world' trials

PK indicates pharmacokinetics; PD, pharmacodynamics.

Table 4. Classification of clinical drug studies according to objectives.

In addition, clinical studies can be described according to the temporal phase of drug development and whilst these typically track the objectives, they do not represent a fixed order for the conduct of research (ICH, 1997).

- *Phase I* starts with the initial administration of the drug to humans and therefore are most typically Human Pharmacology studies. They are used to estimate the safety and tolerability of the drug, its pharmacokinetic (PK) and pharmacodynamic (PD) profile and may involve healthy volunteers.
- *Phase II* studies are often therapeutic exploratory in which the primary objective is to investigate efficacy of the drug in patients. These studies are usually conducted in a selected group of relatively homogeneous patients and are used to determine potential endpoints, doses, regimens and target populations for *Phase III* trials.
- *Phase III* is usually considered to start with the initiation of studies which demonstrate or confirm therapeutic benefit. These trials aim to confirm the safety and efficacy data from earlier phases and form the basis of marketing approval. They may also explore the drug's use in a wider population, for a long period of time, in different stages of disease or in combination with other drugs.
- *Phase IV* relates to therapeutic use and therefore begins with regulatory approval for a specific indication. These post-marketing studies may explore drug interactions, dose response, cost-benefit or acquisition of additional safety or epidemiological data.

When appropriately designed, conducted and reported, randomised controlled trials represent the gold standard in evaluating healthcare interventions. However, complete, clear and transparent information on methodology and findings has often

been inadequately reported, leading to the CONSORT statement in 1996, updated in 2001 and most recently in 2010 (Schulz et al., 2010). These guidelines include a checklist and flow diagram for the reporting of trials and therefore indirectly provide an impetus for improving trial design and conduct.

3.1.1 Scope of a clinical trial

Clinical trials should pose an important, clear and precise research question and all aspects of the trial should be conducted in order to safely and effectively answer this question. The hypothesis should be derived from a background of evidence supporting both the rationale for and the plausibility of the trial. Confirmatory trials test a pre-defined hypothesis as the primary objective in order to provide strong scientific evidence regarding the efficacy of a treatment (ICH, 1998). Adherence to protocol and standard operating procedures is therefore particularly important and any deviations should be documented, explained and potential effects scrutinised.

The trial population should mirror the target population to enable generalisation of the results to the wider community, termed external validity. The inclusion and exclusion criteria should be considered to achieve a balance between maintaining homogeneity and restricting applicability including age, sex, ethnicity, co-morbidities and access to healthcare of the participants. Whilst no clinical trial can be wholly representative of future users due to the impact of geographical, temporal and logistical variables, the influence of such factors should be considered and minimised.

The primary endpoint should be a measurable variable which provides the most clinically relevant and convincing evidence directly related to the primary hypothesis of the trial (ICH, 1998). It should be an objective, reliable and validated measure of treatment benefit in the patient population and conform to the accepted norms and standards of the relevant field of research. The primary endpoint must be precisely defined and justified in the trial protocol and should be used to estimate the sample size. Secondary variables are used either to support the findings of the primary endpoint or as measurements of effects related to defined secondary objectives.

3.1.2 Avoiding bias

The ethical and scientific validity of a clinical trial is dependent upon its freedom from influences that may affect its design, execution or reporting. Bias may compromise a trial's internal validity, the accuracy of its conclusions about the effect of the intervention on the trial subjects, or external validity, the applicability of the study to the wider population (Paradis, 2008). The two key techniques for avoiding the effects of bias in clinical trials are randomisation and blinding.

Randomisation is the process of assigning participants to a treatment group such that the groups differ only by the intervention and the play of chance; if chance is found to be implausible by statistical analysis, then any observed differences in outcome can be attributed to the intervention. Whilst it does not guarantee a perfect balance between groups, it removes *selection bias* by obviating systematic differences due to known or unidentifiable factors other than the intervention and provides a sound statistical basis for the quantitative evaluation of treatment effect (ICH, 1998). The

CONSORT statement recommends that sequence generation, allocation concealment and implementation be reported for all randomised trials (Schulz et al., 2010). There are several accepted mechanisms for generating randomisation sequences including simple, block, stratified and dynamic random allocation. Minimisation is a method of adaptive stratified sampling that aims to minimise the imbalance between treatment groups for a number of identified prognostic factors (Pocock and Simon, 1975); however it is controversial as the allocation of the next subject by the algorithm is dependent upon the allocation of previous subjects in a trade-off between balance and uncertainty (McEntegart, 2003). Concealment is the process used to implement the random allocation sequence such as sealed envelopes or a computer programme. Implementation refers to the people involved in generating the sequence, enrolling participants and assigning them to interventions.

The process of randomisation facilitates blinding, the concealment of treatment assignment from one or more parties. It is intended to limit conscious or subconscious bias arising from the influence which the knowledge of treatment may have on the conduct and interpretation of a trial including recruitment and allocation of subjects, their subsequent care, the attitudes of subjects to treatments, assessment of endpoints, handling of withdrawals and exclusion of data from analysis (ICH, 1998). The gold standard is a double-blind trial in which neither the participant nor the investigators are aware of the allocated treatment arm until all such opportunities for bias have passed; if maintained, any potential *expectation biases* from patients or researchers are removed. If interim or safety analyses are performed, adequate standard operating procedures must be established to prevent

dissemination of treatment codes and compromise of internal validity. In order to maintain blinding, treatments must be indistinguishable in appearance, taste and immediately apparent consequences. Therefore, particularly in surgical interventions, blinding of one or both parties may be difficult or even impossible to achieve.

Comparative trials in which patients are required to actively participate, such as reliably taking the drug under investigation, are also exposed to *compliance bias*. Differences in subject adherence to the protocol may be affected by an increased rate of side-effects in the treatment group and therefore precipitate *withdrawal bias*. An evaluation of patient compliance should be included in the protocol and actual usage documented if possible; a planned subgroup analysis may therefore be used to assess any impact on outcomes.

Performance bias is an additional factor of particular importance in surgical trials. No two surgeons perform a procedure in exactly the same way nor does a surgeon repeat the same operation in an identical manner each time (Paradis, 2008). Whilst this potentially reduces internal validity by creating differences between patients in the same treatment group, it improves external validity by reflecting real-world variability in practice between surgeons and institutions. In a randomised trial, any detrimental effects may be controlled by including surgeon as a minimisation factor in the randomisation procedure, balancing any potential impact on the groups.

3.1.3 Clinical trial design

The most common trial design is parallel groups in which subjects are randomised to one of two or more arms with each arm allocated a different treatment. Other designs include crossover, where all participants experience all arms of the trial in a randomised sequence and so act as their own controls, and factorial design, in which subjects are randomly allocated to combinations of treatments to examine their additive effects and positive or negative interactions (ICH, 1998). A multicentre trial enables an increased number of potential participants from a wider population, thereby improving both the efficiency of recruitment and the generalisation of the findings. However, it must be conducted according to a common protocol across participating sites with appropriate training of personnel and monitoring of conduct.

The number of subjects participating in a clinical trial should have sufficient statistical power to provide a reliable answer to the primary research question. The sample size is determined by specifying: a primary variable, the test statistic, the null hypothesis, the probability of erroneously rejecting the null hypothesis (type I error), the probability of erroneously failing to reject the null hypothesis (type II error) and the approach to dealing with treatment withdrawals and protocol violations (ICH, 1998). In confirmatory trials, assumptions of treatment effect are usually based on published data from earlier trials or the minimum effect that has clinical relevance. Conventionally the probability of type I error is set at one-sided 2.5% or less whilst the probability of type II error is set at 10% or 20% in order to reduce the risk of incorrect and so misleading conclusions.

3.1.4 Conducting a clinical trial

Obtaining informed consent is a statutory requirement for clinical trials. A favourable opinion must be obtained from a recognised body such as a NHS Research Ethics Committee for the use of the proposed Patient Information Sheet and Consent form, using clear, non-technical language. The seeking of consent in particular must adhere to the ethical principles of Good Clinical Practice (ICH, 1996). Consent must be given freely after the subject is informed of the nature, significance, implications and risk of the research and should be recorded in writing. The subject should be aware that they may decline participation or withdraw at any time. Special protection is afforded to vulnerable groups including children, prisoners and those without the mental capacity to give legal consent.

In the UK, clinical trials are regulated by *The Medicines for Human Use (Clinical Trials) Regulations 2004 (SI 1031)* as amended, which implements the European *Clinical Trials Directive (2001/20/EC)* (MHRA, 2011). Accordingly, clinical trials of medicinal products in human subjects require authorisation by the competent authority, in addition to a favourable opinion from an ethics committee. The Medicines & Healthcare products Regulatory Authority (MHRA) is the governmental agency responsible for assessing applications from sponsors to conduct clinical trials of medicinal products and for issuing a clinical trial authorisation (CTA). All authorised trials are registered on the European Clinical Trials Database (EudraCT, 2011). During the conduct of a trial, the sponsor is responsible for safety reporting via the submission of Suspected Unexpected Serious Adverse Reactions (SUSARs) and Annual Safety Reports (ASRs) to the MHRA.

The updated Declaration of Helsinki states that (World Medical Association, 2008):

> *'Every clinical trial must be registered in a publically accessible database before recruitment of the first subject'*

Registries, such as the US National Institutes of Health (www.ClinicalTrials.gov) or the International Standard Randomised Controlled Trial Number Register (ISRCTN) (www.controlled-trials.com), contain a minimum registration dataset defined by the World Health Organisation. This ensures that researchers, clinicians, funding bodies and potential participants are aware of current trials to inform decision-making, identify gaps in knowledge and avoid unnecessary duplication of research.

During a clinical trial, there are two forms of monitoring: oversight of the quality of the trial, and comparing treatment outcomes through interim analyses. Monitoring the quality of the trial conduct includes ensuring adherence to the protocol, accuracy and completeness of data collection, the meeting of planned accrual targets, keeping patients in the trial and the appropriateness of design assumptions. This role may be performed by the sponsor or an independent group. On the other hand, any interim analyses of efficacy or safety data prior to the formal completion of the trial require access to unblinded comparative information and must be performed by an external independent group, the Data and Safety Monitoring Board (DSMB). The schedule of interim analyses and the *modus operandi* of the DSMB, including guidelines for stopping the trial early, should be clearly documented in the protocol; any deviation from this plan may flaw the results of the trial and weaken confidence in the conclusions drawn.

3.1.5 Analysis of a clinical trial and the intention-to-treat principle

The principal features of the statistical analysis should be prespecified in the protocol including the way in which anticipated problems will be handled. The statistical analysis plan contains more technical and detailed procedures for executing the analysis of the primary and secondary endpoints and other data. The plan should be reviewed and updated if required following review of the dataset but prior to unblinding. The protocol should address anticipated problems prospectively, including how these may affect trial data, and specify procedures aimed at minimising irregularities in study conduct that may impair the analysis, including protocol violations, withdrawals and missing values (ICH, 1998). The frequency and type of all such irregularities should be documented in the clinical trial report and their potential influence of the trial results described.

The intention-to-treat principle aims to analyse all randomised subjects in the groups to which they were originally allocated. Preservation of the initial randomisation is important to preserve an unbiased treatment allocation, the prognostic balance between the groups and confidence in the statistical analysis (Nuesch et al., 2009). However, there are some circumstances in which the exclusion of randomised subjects from the primary analysis is valid, avoiding bias and minimising random error (Fergusson et al., 2002); these include eligibility violations due to human error, failure to take at least one dose of the trial medication and the lack of any outcome data post-randomisation. Any such exclusions must always be justified and should not be influenced by knowledge of the assigned treatment. The CONSORT statement urges transparent reporting of the flow of participants through the various stages of a

trial, including a description of withdrawals and losses to follow-up with reasons for exclusions from the analysis (Schulz et al., 2010). In contrast, per protocol analyses allow exploration of the sensitivity of conclusions, particularly in superiority trials where intention-to-treat analysis alone may be seen as a conservative strategy and is more susceptible to type II error (ICH, 1998). Other statistical considerations include handling missing values, which represent a potential source of bias, identifying and dealing with outliers, selection of appropriate statistical tests, modelling and confidence intervals, and management of trial data.

3.1.6 Reporting of a clinical trial

The methodology and findings of all clinical trials should be publically reported according to prespecified criteria and standards. The CONSORT reporting criteria (Schulz et al., 2010) include the need for clear and detailed information on the hypotheses, trial design, participants, interventions, outcomes, sample size, randomisation, blinding, statistical methods, recruitment, baseline data, analysis, limitations, generalisability, interpretation, registration and funding. In particular, all subjects entered into the trial should be accounted for in the report, whether or not they were included in the analysis, through a CONSORT flow diagram which documents the progress of participants through the stages of enrolment, allocation, follow-up and analysis. Subjects excluded from the analysis, lost to follow up, withdrawn from treatment, or with a severe protocol violation should be identified and a detailed description provided, including reasons and relationship to the treatment and outcomes (ICH, 1998). The use of descriptive statistics is encouraged to illustrate clearly the important features of demographic and prognostic variables.

Efficacy and safety data should be presented through planned analyses with limited reliance on *ad hoc* analyses. The results of statistical tests should be presented precisely rather than with reference to critical thresholds to facilitate the inclusion of trial data in subsequent meta-analyses.

The International Committee of Medical Journal Editors has published Uniform Requirements for Manuscripts Submitted to Biomedical Journals (ICMJE, 2010). This document aims to reduce *citation bias*, the incomplete reporting of trial outcomes associated with statistical significance, *publication bias*, the selective reporting of positive trials favouring an intervention, and overlapping or redundant publication, through prospective trial registration and the use of reliable literature sources and standards for trial reporting.

3.2 Improving myocardial protection during Coronary Artery Surgery with Perhexiline: the CASPER trial

A prospective, double-blind, randomised, placebo-controlled trial was conducted at two University teaching hospitals in the United Kingdom – the Queen Elizabeth Hospital (QEH), part of the University Hospitals Birmingham NHS Foundation Trust, and the Royal Sussex County Hospital (RSCH), a member of the Brighton & Sussex University Hospitals NHS Trust. The protocol was approved by the Cambridgeshire 1, South Birmingham and Brighton East Research Ethics Committees (REC: 06/Q0104/141) and the UK Medicines & Healthcare products Regulatory Authority. The trial was registered with clinicaltrials.gov (NCT: 00845364), the European Clinical Trials Database (EudraCT: 2006-003164-62) and the UK Clinical Research Network (UKCRN: 5812). It was funded by grants from the British Heart Foundation (BHF: PG/06/044/20703) and Sussex Heart Charity, and sponsored by University Hospitals Birmingham NHS Foundation Trust (UHB: RRK3217). In this section, I discuss the design, conduct, endpoints and proposed analysis of the CASPER trial in relation to the ICH principles of clinical trial methodology and Good Clinical Practice.

3.2.1 Inclusion & exclusion criteria

The criteria for inclusion in this trial were: adult patients undergoing first-time, isolated, CABG for multi-vessel coronary artery disease; therefore, patients with single vessel disease, proposed combined procedures (such as valve surgery, radiofrequency ablation or left ventricular remodelling) or previous cardiac surgery were not included.

The following exclusion criteria were applied (Sigma Pharmaceuticals, 2009):

- *Known hypersensitivity to perhexiline*
- *History of porphyria*: Perhexiline is deemed unsafe in porphyria as it may precipitate a symptomatic attack.
- *Hepatic impairment*: Perhexiline is cleared from the body via extensive hepatic metabolism by the *cytochrome P450 2D6* mixed-function oxidase system which is readily saturable within the clinical dose range. Evidence of impaired liver function is therefore a documented contraindication to prevent rapid drug toxicity.
- *Renal impairment*: The metabolites of perhexiline are mainly excreted in the urine. Patients with significant renal impairment, defined as serum creatinine > 200 µmol/L, were therefore excluded.
- *Recent amiodarone therapy*: In addition to its Class III anti-arrhythmic effects, amiodarone is a partial inhibitor of cardiac mitochondrial *CPT-1* and may therefore exert cardioprotective effects via the same mechanism as perhexiline (Kennedy et al., 1996). It also inhibits hepatic *cytochrome P450* isoenzymes, reducing the systemic clearance of perhexiline and predisposing to toxicity.
- *Diabetes mellitus*: Perhexiline should be administered with caution in diabetic patients as it may precipitate hypoglycaemia, particularly with concurrent use of insulin or a sulfonylurea. Fasting blood sugar levels fall by around 30% over the first three days and most trial patients were starting therapy unsupervised in the community.
- *Peripheral neuropathy*: Perhexiline toxicity has been linked with denervation atrophy and demyelination; patients with pre-existing disease were excluded.

- *Pregnancy or breast-feeding*: There is limited data on the use of perhexiline in pregnancy and it is not known whether it is excreted in breast milk. Any effects on the foetus and newborn infant are also unknown.
- *Atrial fibrillation*: The irregularity of heart rate and variation in stroke volume in atrial fibrillation produces inconsistent and irreproducible measurements of cardiac output as determined with a pulmonary artery catheter by the thermodilution technique. In addition, the loss of coordinated atrial transit diminishes left ventricular end-diastolic volume in the absence of ventricular dysfunction. Therefore atrial fibrillation would be likely to introduce both type I and type II errors in the measurement of clinical endpoints through actual and observed alterations in cardiac function.
- *Emergency surgery*: Defined as requiring potentially life-saving surgery prior to 8am the following day, these patients had insufficient time for adequate loading with perhexiline and therefore could not be included.
- *Ethical considerations*: The application for ethical approval did not seek to include patients lacking capacity to consent for themselves or those in the custody of HM Prison Service; these patients were therefore excluded from the trial.
- *Significant deviation from protocol required on clinical grounds*: A small number of patients have known co-morbidities that would mandate significant deviations from the trial protocol. For example, sickle-cell disease (or trait) and cryoglobulinaemia are contraindications to the cardiopulmonary bypass, cardioplegia and cardiac output measurement techniques used in this trial. Any such patients were therefore identified and excluded on an individual basis.

Patients taking Selective Serotonin Reuptake Inhibitors (SSRIs), such as fluoxetine or paroxetine, were not excluded from the trial. Cases of perhexiline toxicity have been reported with co-administration of these agents due to competition for *cytochrome P450 CYP2D6* microsomal enzymes (Alderman et al., 1997); however the low dose and short duration of perhexiline therapy used in this trial were deemed not to be prohibitory to concurrent use (Frenneaux, 2007).

3.2.2 Patient recruitment

Suitable patients were identified from the NHS waiting lists of nine Consultant Cardiac Surgeons involved in the trial through two referral pathways: those referred for routine elective surgery and those warranting urgent inpatient surgery, defined as requiring definitive intervention during the current hospital admission. The latter included many patients who had recently been admitted to hospital due to a myocardial infarction; these patients often require further investigations and optimisation prior to surgery, providing a window of opportunity for recruitment and instigation of trial therapy. If the time between the opportunity for recruitment to the trial and the predicted date of surgery was less than five days, the patient was not approached unless surgery was subsequently delayed beyond this time. Patients who were referred through the normal NHS pathway but were later invited for surgery at a private institution as part of a waiting list initiative were either excluded or withdrawn due to the lack of sponsor/ethical approval for this hospital as a trial site. Patients were invited to participate in the trial with a site-specific patient information sheet (appendix 9.2) and given at least 24 hours to consider their involvement. All patients gave written informed consent prior to being enrolled (appendix 9.3).

3.2.3 Randomisation

Once written informed consent had been obtained, patients were randomised to either the perhexiline or placebo arm of the trial. A password-protected, encrypted Microsoft Access database containing the randomisation code was designed by Dr Melanie J. Calvert and Professor Nick Freemantle and used to generate a random allocation sequence. The randomiser allocated treatment in a 1:1 ratio and used minimisation as a method of adaptive stratified sampling in order to reduce any imbalance between treatment groups for three identified factors: consultant surgeon; operation status (elective or urgent); and left ventricular systolic function (good ≥ 50%, moderate 30-49%, poor < 30% according to standard criteria). The patient details were entered by myself (or later Mr Senanayake) and the programme allocated a number corresponding to the bottle of tablets to be supplied to the patient; it also had the facility to allocate a second bottle in the same arm of the trial to the same patient whilst maintaining allocation concealment. The database was reviewed at regular intervals by Dr Calvert who remained unblinded to ensure that it was functioning satisfactorily; she also had access to the allocation sequence as a mechanism for breaking the code if required in the case of a serious adverse event.

3.2.4 Investigational Medicinal Product

Perhexiline and placebo tablets (Sigma Pharmaceuticals, Baulkham Hills, Australia) were manufactured in accordance with standards of Good Manufacturing Practice equivalent to those applied in the European Union and supplied with a Certificate of Analysis. All tablets were identical in appearance: white to off-white, 8.5mm in diameter and labelled ‘PEXSIG’. Perhexiline has a provisional shelf-life of two years

(Sigma Pharmaceuticals, 2009) and was therefore imported into the UK in batches to undergo Qualified Person (QP) release (Bilcare GCS, Crickhowell, UK). The Investigational Medicinal Product (IMP) was dispensed into bottles of 34 tablets and labelled consecutively between 1 and 600 according to the randomisation schedule. Bottles were stored under standard conditions in the hospital pharmacy until allocated to a named patient.

A standardised one-size-fits-all loading and maintenance regime was employed. Each patient received 200mg *bis die* for three days followed by 100mg *bis die* until surgery. This dosing regime was developed in cooperation with Professor John D. Horowitz at The Queen Elizabeth Hospital (TQEH), Adelaide who has the world's largest clinical experience with perhexiline. It had previously been used in over one thousand patients with a 10% incidence of transient nausea but patient compliance of over 95% and no serious adverse events related to toxicity (appendix 9.4). It was therefore deemed the most suitable for prompt, effective yet tolerable drug loading in the community. However, if a patient had not had surgery by 31 days after commencing the IMP, it was discontinued; including the loading period, this equated to two full bottles of tablets.

Initially, it was intended that interim plasma perhexiline levels would be measured in patients who took the IMP for more than two weeks prior to their date of surgery. This would have enabled dosing adjustment by an independent, unblinded colleague to maximise the number of patients within the therapeutic range at the time of surgery. A similar number of patients in the placebo group would also have been tested to

maintain blinding. However, the logistical limitations of the test availability limited its application; perhexiline therapeutic drug monitoring is only performed at two centres in the UK and the assay is only run once per week. There was insufficient funding to establish the assay *ad hoc* in Birmingham. Therefore, interim monitoring was not used rather dose adjustment was guided by patient-reported nausea and/or dizziness which have been shown to be strongly predictive of the accumulation of perhexiline beyond the therapeutic range during short-term therapy (Stewart et al., 1996).

Patients were provided with access to a dedicated 24-hour mobile telephone helpline for advice and support. If patients reported potential side-effects from the trial therapy, they were offered up to three options in sequence until an acceptable solution was found: to continue at the current dose if tolerable and see whether the symptoms settled with time; to half the dose to one tablet per day and see whether the symptoms settled with time; to stop the trial medication. Routine telephone follow-up enquiries were made to ensure that symptoms had settled.

On admission for surgery, all patients were specifically questioned regarding the occurrence of potential side-effects and deviation from the dosing regimen in a standard, open manner to avoid an observer-expectancy effect. Even if the patient had stopped the IMP, either following discussion or spontaneously, they continued in the trial unless they specifically declined and withdrew. Compliance with trial therapy was assessed retrospectively by taking blood at the time of surgery and measuring serum perhexiline levels in all patients at the end of the trial.

On the morning of surgery, the IMP was taken as usual at approximately 07:00, along with pre-medication for patients on the morning operating list. Trial patients underwent surgery on both morning and afternoon operating lists and therefore blood for perhexiline levels was taken either between 08:00-09:00 (C_1-C_2) or 13:00-15:00 (C_6-C_8). As perhexiline and OH-perhexiline have a long half-life in humans, the within-day variability in plasma perhexiline concentrations is small, with a mean 16% difference between peak (around C_4) and trough (C_0) levels in steady-state patients (Jones et al., 2004). The timing of surgery would therefore not be expected to significantly affect the measurement of perhexiline concentrations.

3.2.5 Perioperative protocols

Anaesthesia, cardiopulmonary bypass, myocardial protection and surgical techniques were standardised. The protocols were similar to those used in previous trials in our department (Quinn et al., 2006, Ranasinghe et al., 2006, Howell et al., 2011) in order to promote process familiarity and thereby reduce human error.

Anaesthesia: A standard pre-medication of temazepam, ranitidine and metoclopramide was given. Anaesthesia was induced using intravenous etomidate, fentanyl and pancuronium and maintained with propofol, alfentanil and supplementary isoflurane; other volatile anaesthetic agents were not permitted. Invasive monitoring lines were inserted to obtain radial artery, central venous and pulmonary artery pressure readings. Phenylepherine was administered in aliquots to maintain a mean arterial pressure of 60-70mmHg. Intravenous antibiotics and a bolus of mannitol were given prior to sternotomy according to the unit protocol. An anti-

fibrinolytic agent, either aprotinin or tranexamic acid, was given at the surgeon's request. Anticoagulation was achieved with heparin prior to the institution of CPB and reversed with protamine after the termination of CPB. Standard monitoring was performed throughout including haemodynamic variables, urine output, blood gas analysis and measurement of activated clotting time.

Operative procedure: Coronary artery bypass graft surgery was conducted in a standard manner. After transfer to the operating theatre, the surgical checklist was completed, the patient was prepped and draped, and the chest opened through a median sternotomy. Internal mammary artery, radial artery and saphenous vein conduits were harvested simultaneously as required. Cardiopulmonary bypass between the right atrium and the ascending aorta was instituted at 34°C with a roller pump, hollow-fibre membrane oxygenator and asanguineous prime. An aortic cross-clamp was applied to the proximal ascending aorta and intermittent antegrade cold blood St. Thomas' Hospital Solution No.2 cardioplegia (Martindale Pharma, Brentwood, UK) given via the aortic root for myocardial protection: 12ml/kg for induction and 6ml/kg for maintenance at intervals of approximately 20 minutes. Supplemental retrograde or 'hot shot' terminal cardioplegia were not permitted. Distal anastomoses were constructed during a single cross-clamp period (*ischaemic time*) and proximal anastomoses were made with partial aortic occlusion following removal of the aortic cross-clamp. After rewarming with a 37°C maximal heat-exchanger, CPB was discontinued at 36-37°C nasopharyngeal temperature. In the event of difficulty separating from CPB or marked haemodynamic instability, subjective and objective measures of ventricular function were made and inotropic support instituted at the

discretion of the operating surgeon. Intraoperative ventricular tachyarrhythmias were treated with internal cardioversion. Intravenous glycopyrollate, atropine and atrial or dual chamber epicardial pacing wires were used to achieve a target heart rate of 70 to 110 beats per minute. Drains were placed in the mediastinum and open pleural cavities. Once haemodynamic stability and haemostasis had been achieved, the chest was closed with sternal wires in a standard manner and the patient transferred to the Cardiac Intensive Care Unit (ICU).

Postoperative care: The following target parameters were used to guide patient management in the early postoperative period: heart rate of 70-110 beats per minute in sinus rhythm, paced AAI, DDD or VVI, in order of preference; mean arterial pressure (MAP) of 65-85mmHg; central venous pressure (CVP) of 8-12mmHg; pulmonary artery wedge pressure (PAWP) of 12-16mmHg; and a cardiac index ≥ 2.2L/min/m^2. These parameters were adjusted on an individual patient basis according to intraoperative observations of changes in haemodynamic variables particularly in response to fluid volume. If the cardiac index remained < 2.2L/min/m^2 despite correction of heart rate, filling pressures and appropriate vasoconstriction with phenylepherine, the patient was deemed likely to be in a state of low cardiac output and inotropic support was commenced. The first-line inotrope used in this trial was an infusion of dopamine at a dose of 5μg/kg/min; subsequent escalation of inotropic support was at the discretion of the clinical team but would usually involve adrenaline or enoximone as required. Any decision on chest reopening for suspected cardiac tamponade, cardiac arrest or excessive bleeding was taken by the clinical team and the findings documented.

If the patient was deemed to be in a state of vasodilatation despite a phenylepherine infusion reaching its therapeutic ceiling, noradrenaline was used as the second-line vasoconstrictor followed by vasopressin. Hypertension with a sustained MAP > 85mmHg was treated with glyceryl trinitrate infusion once potential causes such as inadequate sedation or analgesia were excluded. Atrial arrhythmias were managed with stepwise correction of electrolyte imbalance, amiodarone and DC cardioversion.

Other clinical variables and management targets were as standard for the unit and defined in the clinical protocol including urine output ≥ 0.5ml/kg/hr, serum potassium 4.5-5.5mmol/L, haemoglobin ≥ 8g/dL and blood glucose 4-7mmol/L, with insulin commenced on a sliding-scale if above 10mmol/L. Arterial blood gas analyses were performed regularly and extubation was driven by a nurse-led protocol. Routine Intensive Care and hospital discharge criteria were used and applied at the discretion of the clinical team. The patient's involvement in the trial ceased once discharged from the immediate care of our department to home, convalescence, another department or hospital, or upon death, whichever occurred sooner.

3.2.6 Trial investigations

Clinical data for each patient was prospectively recorded on a specific data collection sheet including demographics, risk factors, medications, perhexiline therapy, operative data, cardiac output studies, blood gas analyses, inotrope and vasoconstrictor requirements, intra-aortic balloon pump usage, volume requirements, arrhythmias, intensive care data, blood results, complications, discharge data and any significant deviations from the trial protocol.

Cardiac output studies: A pulmonary artery catheter enabled invasive assessment of haemodynamic variables and the thermodilution technique was used to measure cardiac function. Baseline readings were obtained with the patient under general anaesthesia but prior to sternotomy. Following weaning from CPB, further measurements were taken before and approximately ten minutes after the administration of protamine, and then at two, four, six, nine and 12 hours after reperfusion of the myocardium, marked by the release of the aortic cross-clamp. At each time point, the following variables were recorded: heart rate, rhythm, cardiac output, mean systemic blood pressure, central venous pressure, mean pulmonary artery pressure and pulmonary artery wedge pressure.

Blood samples: Preoperative baseline samples for serum perhexiline (plain tube) and troponin-T (clot activator & gel separator) were obtained in the anaesthetic room after insertion of the arterial line. Postoperative blood for troponin-T was drawn at six, 12 and 24 hours after removal of the aortic cross-clamp. Blood taken during the night-time was refrigerated at 4°C and processed the next morning. Sample tubes were centrifuged at 3000rpm for five minutes, the supernatant decanted into cryotubes and frozen at -80°C in our remotely-monitored ultra-low temperature freezers at QEH, Birmingham and RSCH, Brighton. Perhexiline and OH-perhexiline concentrations in serum were measured by HPLC using a previously described technique (Sallustio et al., 2002) by the Clinical Pharmacology Laboratory, TQEH, Adelaide. Serum troponin was measured using the Elecsys Troponin-T assay (Roche Diagnostics, Burgess Hill, UK) by the Department of Clinical Biochemistry, QEH, Birmingham.

Tissue biopsies: Atrial and ventricular biopsies were obtained during surgery at QEH, Birmingham and promptly snap-frozen in liquid nitrogen. The right atrial appendage was truncated during venous cannulation for CPB; in selected patients, it was divided in two: one portion was snap-frozen, the other mounted on a cork disk, embedded in *Tissue-Tek* Optimum Cutting Temperature (OCT) compound (Sakura, Netherlands) and frozen in isopentane pre-cooled to approximately -40°C with dry ice. Transmural Tru-Cut needle (Allegiance, McGaw Park, IL) biopsies of the left ventricular free-wall between the left anterior descending artery and the first diagonal branch were taken at three time points: *pre-ischaemia* (on CPB but before application of the aortic cross-clamp), *end ischaemia* (before cross-clamp removal or local reperfusion through an anastomosed internal mammary artery graft) and *reperfusion* (approximately 10-15 minutes after cross-clamp removal). Once frozen, all samples were stored at -80°C.

Electrocardiograms: Serial standard 12-lead electrocardiogram (ECG) tracings were obtained at four time points: preoperative baseline, within two hours of arrival on ICU, and on the first and fourth postoperative days.

3.2.7 Outcome measures

The primary endpoint was *an episode of low cardiac output* in the first six hours after removal of the aortic cross-clamp, defined as hypotension (MAP < 65mmHg) with a cardiac index < 2.2L/min/m^2 in the presence of adequate filling pressures (CVP 8-12mmHg or PCWP 12-16mmHg) and heart rate (> 75bpm) where systolic blood pressure < 90mmHg and/or inotropic support ± intra-aortic balloon pump for > 60 minutes was required to maintain such a clinical picture. A blinded endpoints

committee was convened at regular intervals to adjudicate on borderline cases, comprising Professor Pagano, Mr Rooney, Mr Ranasinghe, Mr Howell and myself.

The secondary endpoints were:

- *Cardiac index* at six and 12 hours after removal of the aortic cross-clamp: absolute values and corrected for change in cardiac index from baseline.
- Prevalence of *inotrope usage* in the first six and 12 hours after removal of the aortic cross-clamp
- *Cardiac troponin-T release* in the first 24 hours: peak ranked according to quintile and area under the concentration-time curve (AUC)
- *ECG evidence of new myocardial injury*, defined as the presence of new Q waves of ≥ 2mm in depth in two contiguous leads, new bundle branch block or loss of R wave progression, analysed by Dr Lynne K. Williams, a blinded cardiologist
- *Length of stay* in the ICU and in the hospital

The haemodynamic endpoints of low cardiac output and cardiac index were chosen as surrogate measures of cardiac contractility to reflect the degree of clinical recovery of myocardial function and stunning. Following cardiac surgery, around one-in-three patients have a period of detectable dysfunction due to myocardial stunning, most of whom require inotropes to support the heart during its recovery (Arnold et al., 1985). An episode of non-fatal low output syndrome in the early postoperative period has also been shown to be associated with a significant reduction in late survival following CABG (Fremes et al., 2000). By improving metabolic efficiency and the resilience to ischaemia-reperfusion, this trial aims to reduce myocardial stunning and

improve contractile function in the perioperative period. As the nadir in early cardiac contractility invariably occurs within the first six hours of reperfusion (Hearse et al., 1981), this was chosen as the timeframe for the primary endpoint. Cardiac output is the product of heart rate and stroke volume, which is itself influenced by preload, afterload and contractility. By optimising these variables (with the exception of contractility unless clinically necessary) by pacing, intravascular fluid replacement or vasoconstriction to achieve predetermined targets, comparison of cardiac function could be made between patients and groups. These endpoints for myocardial stunning were used in our previously published trials of myocardial protection during cardiac surgery in Birmingham (Quinn et al., 2006, Howell et al., 2011) and in a meta-analysis of GIK (Bothe et al., 2004), and have therefore been both internally and externally validated.

The identification of perioperative myocardial injury is complicated despite recent consensus guidelines on diagnosis of myocardial infarction (Thygesen et al., 2007):

> *'Biomarker values more than five times the 99th percentile of the normal reference range during the first 72 hours following CABG, when associated with the appearance of new pathological Q-waves or new left bundle-branch block (LBBB), or angiographically documented new graft or native coronary artery occlusion, or imaging evidence of new loss of viable myocardium should be considered as diagnostic of a CABG-related myocardial infarction (type 5 MI).'*

Cardiac troponin-T has been shown to be superior to CK-MB for the prediction of complications following cardiac surgery (Januzzi et al., 2002). However, interpretation of troponin release is more complex than in other causes of myocardial injury. The concentration of serum troponin in the early postoperative period is almost universally elevated compared to baseline; it is released in response to irreversible myocardial

necrosis but rather than the acute regional infarction that occurs with coronary occlusion, it usually represents diffuse myocardial injury (Januzzi, 2011). Diagnosed according to the consensus guidelines, myocardial infarction occurs in just 2% of patients following CABG (Mohammed et al., 2009) although the AUC for serum troponin at 24 hours has been shown to correlate strongly with the mass of new myocyte necrosis on delayed-enhancement cardiac magnetic resonance (Pegg et al., 2011). Recent studies have shown that postoperative troponin release is a strong independent predictor of ICU stay, major adverse cardiac events (MACE), and short-, medium- and long-term survival after cardiac surgery (Croal et al., 2006, Lurati Buse et al., 2009). In clinical practice, the use of multiple troponin assays with considerable heterogeneity in analytical sensitivity, precision and reference ranges, has led to difficulties in determining a threshold for significant myocardial injury (Mohammed et al., 2009). Januzzi and colleagues (2002) found that a troponin-T level in the highest quintile within a population was the strongest predictor of outcomes, avoiding the need for an absolute cut-off for internal comparisons. In our trial, the troponin-T subunit was measured up to 24 hours following release of the aortic cross-clamp as this approach is supported by the evidence described and the assay was already in routine clinical use at QEH, Birmingham.

ECG changes following cardiac surgery are common and not necessarily diagnostic for ischaemia (Yokoyama et al., 2000). However, when new pathological Q waves appear in territories other than those identified before surgery, myocardial infarction should be considered, particularly if associated with elevated biomarkers, new wall motion abnormalities or haemodynamic instability (Thygesen et al., 2007).

3.2.8 Power calculations and statistical analysis

It was hypothesised that the preoperative use of perhexiline would reduce the incidence of a low cardiac output episode (LCOE) compared with controls. The sample size was estimated by extrapolating pooled data from our recently completed MESSAGE and MESSAGE-2 trials (Quinn et al., 2006, Ranasinghe et al., 2006) in which patients undergoing CABG were randomised to GIK or placebo; a reduction of 50% in the incidence of LCOE was detected in the GIK (19%) group compared with controls (37%). To achieve a similar difference in incidence between groups, with α 0.05 and 1-β 0.9, required a total of 280 patients randomised 1:1 between groups and provided sufficient statistical power to examine all secondary outcome measures.

A statistical analysis plan (appendix 9.5) was developed before starting the trial and refined during blinded review in accordance with ICH Statistical Principles for Clinical Trials (ICH, 1998). Analysis of the main outcome measures was performed according to the intention-to-treat principle on all eligible subjects who had data on the primary outcome; patients who were randomised, dosed but failed to undergo surgery on CPB and reach the collection of outcome data were excluded. A planned exploratory analysis of the primary outcome compared patients in the perhexiline arm who had reached the lower threshold of the therapeutic range (0.15mg/L) at the time of surgery with propensity score-matched controls. Propensity scoring was based on age, ethnicity, weight, days of trial therapy, left ventricular function and priority of surgery and an optimal matching approach was applied using the *dist* macro for SAS (Bergstralh and Kosanke, 2003).

Analysis was performed using SAS (version 9.2, SAS Institute Inc., Cary, NC) and SPSS software (version 17.0, SPSS Inc., Chicago, IL). Continuous data was assessed for normality and presented as mean (standard error of the mean) or median (interquartile range). Normally distributed data were analysed by Student's t-test or by analysis of variance (ANOVA) whilst skewed data were analysed with Mann-Whitney U-test. Categorical data were analysed by Fisher's exact test. The primary analysis was conducted using a non-linear mixed model. All statistical tests were two-sided unless stated and deemed to be statistically significant at $p < 0.05$. Analyses were undertaken stratified for baseline left ventricular function and elective/urgent status; Consultant Surgeon was accounted for as a random effect. In the event of death before assignment of the primary endpoint, a LCOE was assigned. Missing data for all other outcomes was identified and excluded from the analysis. An outline of the statistical tests used in this thesis is contained in appendix 9.6.

3.2.9 Data and Safety Monitoring Board and adverse event reporting

An independent Data and Safety Monitoring Board (DSMB) comprising Professor Freemantle (Chair), Dr Jordan and Dr Lipkin met at predetermined milestones in recruitment. Data were reviewed on the primary and safety endpoints after 50, 125 and 225 patients had undergone surgery and been discharged. A truncated modified O'Brien-Fleming alpha spending plan was used to preserve the overall one-sided type I error rate for efficacy at the 0.025 level. A non-symmetrical power function was used for safety to increase the likelihood of stopping early for harm (figure 16). The following were defined as Serious Adverse Events (SAE) and reported to the DSMB within 48 hours of diagnosis: death, stroke, renal failure requiring replacement

therapy, and any additional surgical procedures, including reopening of the chest, laparotomy or limb amputation, with the exception of tracheostomy. A mechanism was in place for the reporting of suspected unexpected serious adverse reactions (SUSAR) to the MHRA via their eSUSAR website (www.esusar.mhra.gov.uk). Annual safety reports (ASR) were submitted to the MHRA and Research Ethics Committee.

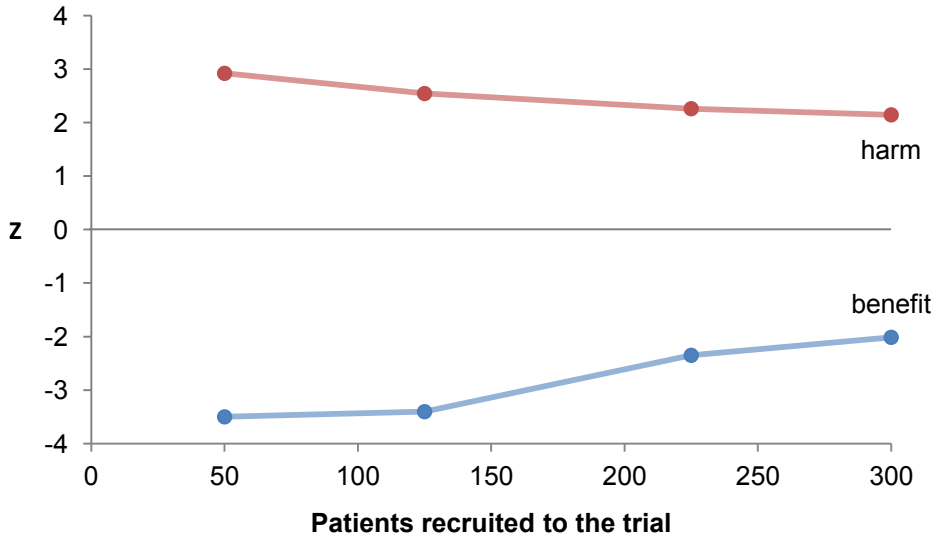


Figure 16. Stopping boundaries to control alpha for benefit and harm.

In this chapter, I have described the ICH principles for conducting a randomised controlled clinical trial through its scope, design, administration, analysis and reporting. I related the application of these standards to the conduct of the CASPER trial, a multi-centre trial of perhexiline in myocardial protection, on which this thesis is founded. I justified the selection of inclusion & exclusion criteria, IMP dosing and primary & secondary endpoints. Finally, I explained the patient pathway through the trial, the use of perioperative protocols, the acquisition of blood and biopsies, the analysis of clinical data and procedures for the monitoring of patient safety.

4. RESULTS

The CASPER trial was given ethical and regulatory approval in January 2007. Recruitment commenced at the Queen Elizabeth Hospital, Birmingham in February that year and the first patient underwent surgery in May. Following approval of a substantial amendment, the trial was expanded to a second site at the Royal Sussex County Hospital, Brighton from April 2009. The final patient completed the trial pathway in April 2010 after the target recruitment of at least 280 eligible patients had been successfully passed.

In this chapter, I describe the recruitment of patients to the trial across both sites, document the baseline and operative demographics of the trial population and discuss the serum concentration of perhexiline at the time of surgery in relation to its pharmacokinetics. The myocardial pharmacokinetics, biochemical and metabolomic effects of perhexiline, and the clinical outcome measures of the trial will be discussed in subsequent chapters.

4.1 Trial recruitment and CONSORT flow diagram

The CONSORT flow diagram (figure 17) documents the number of trial participants who were enrolled, randomly assigned, received intended treatment and analysed for the primary outcome. At each stage, there were a number of exclusions and losses, the justifications for which are as follows:

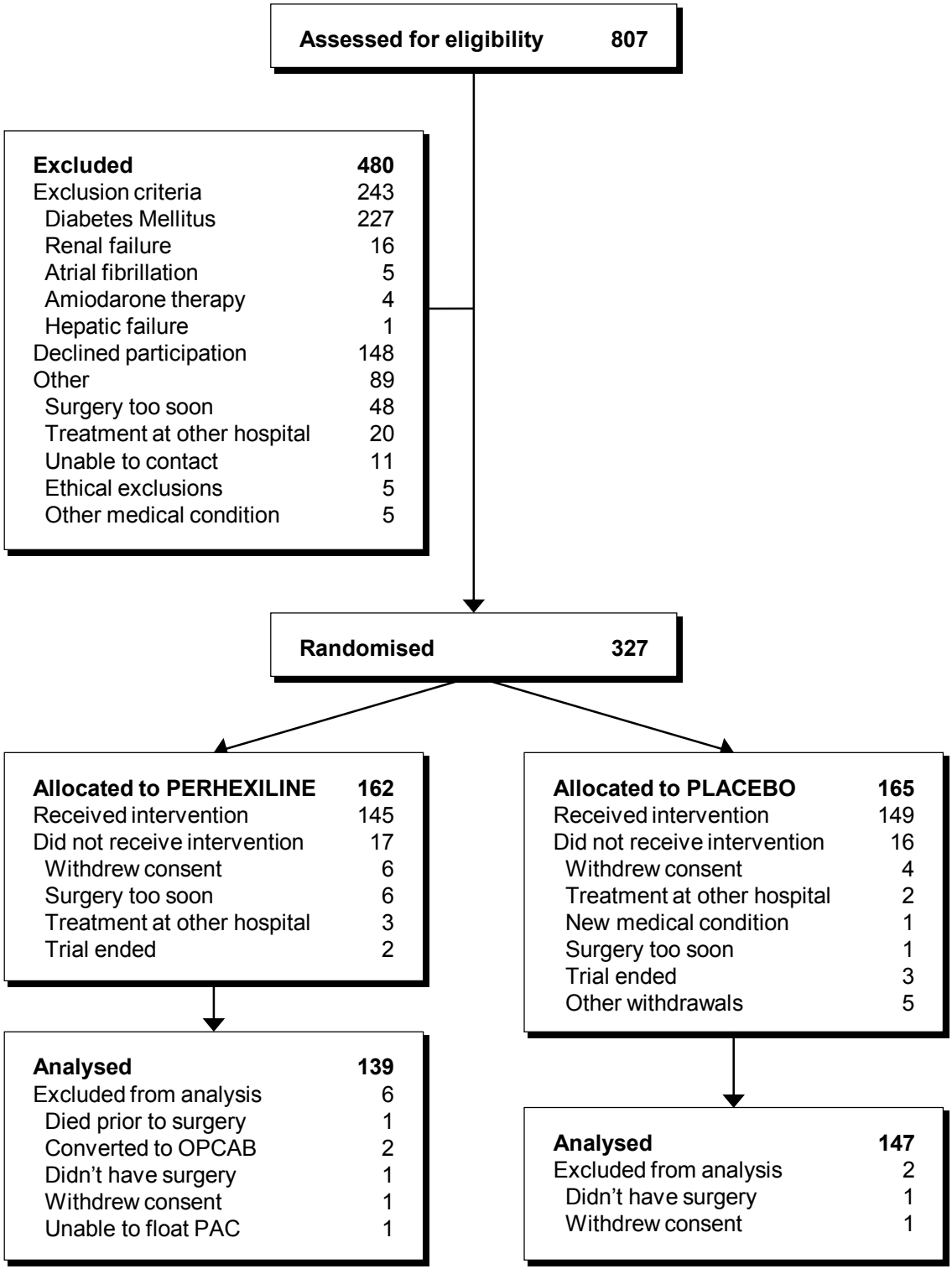


Figure 17. CONSORT flow diagram of trial recruitment.

Eight hundred and seven patients met the inclusion criteria and were assessed for eligibility. Of these, 480 patients were excluded – 243 (30.1%) met one or more of the exclusion criteria, including ten patients who had both diabetes and renal impairment, 148 (18.3%) declined participation in the trial and 89 (11.0%) were excluded for other reasons: 48 patients underwent surgery at short notice before it was possible for them to be recruited to the trial; 20 patients underwent surgery at a non-NHS hospital (BMI The Priory Hospital, Edgbaston) as part of an initiative to reduce NHS waiting times; 11 patients were not able to be contacted by telephone or post prior to admission for surgery; five patients fell outside the scope of ethical approval for the trial (three with severe learning difficulties, two from an HM Prison); and five patients had other medical conditions that were judged to necessitate significant deviation from the protocol (three with Sickle-cell disease or trait, one with Cryoglobulinaemia, one with Hermansky-Pudlak syndrome, a platelet storage pool defect).

Three hundred and twenty seven patients were randomised – 162 to receive perhexiline and 165 to receive placebo – of whom 33 did not receive the intervention.

- In the perhexiline group, 145 patients received the intervention and 17 did not: six patients withdrew consent after randomisation but before starting the IMP; six patients had surgery at short notice prior to starting the IMP; three patients underwent surgery at a non-NHS hospital without starting the IMP; and two patients were undergoing further investigations with no proposed date for surgery when the trial ended.

- In the placebo group, 149 patients received the intervention and 16 did not: Four patients withdrew consent after randomisation but before starting the IMP; two patients underwent surgery at a non-NHS hospital without starting the IMP; one patient developed a new medical condition and did not proceed to surgery; one patient had surgery at short notice prior to starting the IMP; three patients were undergoing further investigations with no proposed date for surgery when the trial ended; and five patients were excluded for non-medical reasons (three patients withdrawn by Consultant Surgeons for personal reasons and two patients had their care transferred to a Consultant who was not participating in the trial).

Two hundred and ninety four patients received the intervention, of whom eight were subsequently excluded from the analysis.

- In the perhexiline group, 139 patients were included in the analysis and six were not: one patient died as an inpatient prior to surgery (see below); two patients were converted to off-pump coronary artery bypass (OPCAB) surgery at the discretion of the operating surgeon and so did not undergo aortic cross-clamping; one patient did not proceed to surgery as planned following further discussion on the risk-benefits of an operation; one patient withdrew consent after starting the IMP but before surgery; and in one patient, investigators were unable to float the pulmonary artery catheter and therefore unable to measure cardiac output.
- In the placebo group, 147 patients were included in the analysis and two were not: one patient did not proceed to surgery as planned; and one patient withdrew consent after starting the IMP but before surgery.

One patient died prior to surgery whilst taking the IMP and this was reported to the sponsor and ethics committee as a SAE. The patient had been admitted with a non-ST elevation myocardial infarction and poor left ventricular function, was referred for urgent surgical revascularisation and was expeditiously recruited to the trial. However, two days prior to his scheduled surgery, he suffered a VF arrest on the ward, precipitating emergency transfer to the catheterisation laboratory. Despite immediate treatment, he was not able to be resuscitated and died from a further myocardial infarction.

Two hundred and eighty six patients were included in the intention-to-treat analysis, representing 87.5% of those randomised and 97.3% of those receiving the intervention. These represent all consenting patients who underwent cardiac surgery with a period of ischaemia-reperfusion in whom the primary outcome was measurable, analysed in the original groups to which they were randomly assigned, regardless of the need for additional procedures or whether or not they actually took the IMP. Baseline patient demographic and operative variables are shown in tables 5 and 6, respectively. Five trial patients underwent an additional procedure: in the perhexiline group, one patient had a mitral valve replacement after failed repair; in the control group, three patients had a mitral valve repair and one had an aortic valve replacement.

	Control (n=147)	Perhexiline (n=139)
Age (years), median (IQR) *	65.7 (60.2-73.6)	66.1 (59.4-73.2)
Male, n (%) *	134 (91.2)	128 (92.1)
Race, n (%)		
Caucasian	136 (92.5)	133 (95.7)
South Asian	10 (6.8)	5 (3.6)
Black	1 (0.7)	1 (0.7)
Body Mass Index (kg/m^2), median (IQR)	27.8 (25.4-30.7)	28.0 (25.1-30.3)
CCS angina score, n (%)		
0	7 (4.8)	12 (8.6)
I	14 (9.5)	9 (6.5)
II	71 (48.3)	70 (50.4)
III	43 (29.3)	39 (28.1)
IV	12 (8.2)	9 (6.5)
NYHA functional class, n (%)		
I	59 (40.1)	58 (41.7)
II	79 (53.7)	68 (48.9)
III	9 (6.1)	13 (9.4)
IV	0 (0)	0 (0)
Previous MI, n (%)	48 (32.7)	49 (35.3)
Previous coronary stenting, n (%)	10 (6.8)	13 (9.4)
Left main stem disease ≥ 50%, n (%)	47 (32.0)	54 (38.9)
Left ventricular function, n (%) *		
Good, ≥ 50%	122 (83.0)	118 (84.9)
Moderate, 30-49%	24 (16.3)	20 (14.4)
Poor, <30%	1 (0.7)	1 (0.7)
Urgent non-elective, n (%)	23 (15.7)	19 (13.7)
Hypertension, n (%)	89 (60.5)	90 (64.8)
Hypercholesterolaemia, n (%)	109 (74.2)	94 (67.6)
Family history of IHD, n (%)	60 (40.8)	56 (40.3)
Smoking status on admission, n (%)		
Non-smoker	55 (37.4)	38 (27.3)
Ex-smoker	76 (51.7)	87 (62.6)
Current smoker	16 (10.9)	14 (10.1)
Pulmonary disease, n (%) *	12 (8.2)	17 (12.2)
Extra-cardiac arteriopathy, n (%) *	10 (6.8)	14 (10.1)
Neurological dysfunction, n (%) *	4 (2.7)	2 (1.4)
Unstable angina, n (%) *	1 (0.7)	1 (0.7)

Recent MI, ≤ 90 days, n (%) *	17 (11.6)	19 (13.7)
Pre-operative IABP, n (%) *	0 (0)	1 (0.7)
Medications, n (%)		
Aspirin	139 (94.6)	131 (94.2)
Clopidogrel	38 (25.9)	35 (25.2)
ACE inhibitor or ARB	96 (65.3)	90 (64.8)
Statin	139 (94.6)	129 (92.8)
β-adrenergic antagonist	121 (82.3)	99 (71.2)
Calcium channel antagonist	47 (32.0)	48 (34.5)
Long-acting oral nitrate	47 (32.0)	46 (33.1)
Potassium channel blocker	41 (27.9)	32 (23.0)
Diuretic	22 (15.0)	24 (17.3)
Number of anti-anginal agents, n (%)		
None	2 (1.4)	3 (2.2)
One	65 (44.2)	68 (48.9)
Two	55 (37.4)	48 (34.5)
Three	19 (12.9)	15 (10.8)
Four or more	6 (4.1)	5 (3.6)
Haemoglobin (g/dL), median (IQR)	14.2 (13.4-14.9)	14.4 (13.5-15.2)
Creatinine (μmol/L), median (IQR) *	98 (85-110)	105 (91-119)
eGFR, MDRD (ml/min), median (IQR)	68.9 (61.0-81.4)	64.8 (54.8-74.5)
EuroSCORE, median (IQR)		
Additive EuroSCORE	3 (1-4)	3 (1-4)
Logistic EuroSCORE	1.82 (1.07-2.94)	1.82 (1.23-3.06)

CCS, Canadian Cardiovascular Society; NYHA, New York Heart Association; MI, myocardial infarction; IHD, ischaemic heart disease; IABP, intra-aortic balloon pump; ACE, angiotensin converting enzyme; ARB, angiotensin II receptor blocker; eGFR, estimated glomerular filtration rate; MDRD, Modification of Diet in Renal Disease; EuroSCORE, European System for Cardiac Operative Risk Evaluation.
* factored into EuroSCORE calculation

Table 5. Baseline patient demographics.

	Control (n=147)	Perhexiline (n=139)
Number of grafts, mean (SD)	3.29 (0.82)	3.30 (0.73)
IMA graft used, n (%)	136 (92.5)	132 (95.0)
Incomplete revascularisation, n (%)	13 (8.8)	12 (8.6)
Additional procedure, n (%)	4 (2.7)	1 (0.7)
Operation performed by trainee, n (%)	50 (34.0)	38 (27.3)
CPB time (min), median (IQR)	110 (90-134)	109 (93-131)
Reinstitution of CPB, n (%)	6 (4.1)	3 (2.2)
XC time (min), median (IQR)	57 (45-72)	56 (45-70)
Cardioplegia (ml), mean (SD)	1999 (520)	2018 (484)
VF/VT on reperfusion, n (%)	3 (2.0)	3 (2.2)
Anti-fibrinolytic agent, n (%)	100 (68.0)	97 (69.8)
Volume of cell-salvaged blood (ml), mean (SD)	499 (355)	487 (328)
Intra-aortic balloon pump used, n (%)	21 (14.3)	10 (7.2)
Pre-op for unstable angina	0 (0)	1 (0.7)
Pre-op elective in theatre e.g. LMS, poor LV	6 (4.1)	3 (2.2)
Pre-CPB non-elective e.g. ischaemia, unstable	0 (0)	2 (1.4)
Before off CPB, anticipating problems	4 (2.7)	1 (0.7)
Post-CPB, emergency e.g. LCOE, post-arrest	8 (5.4)	2 (1.4)
On ICU, emergency e.g. LCOE, post-arrest	3 (2.0)	1 (0.7)

IMA indicates internal mammary artery; CPB, cardiopulmonary bypass; XC, aortic cross-clamp; VF, ventricular fibrillation; VT, ventricular tachycardia; LMS, left main stem coronary stenosis; LV, left ventricle; ICU, intensive care unit; LCOE, low cardiac output episode.

Table 6. Intraoperative variables.

4.2 Serum perhexiline concentration at the time of surgery

The therapeutic range for perhexiline has been defined as a serum concentration of 0.15-0.6mg/L (Horowitz et al., 1986). Therapeutic drug monitoring to maintain serum levels within this target range confers clinical benefit without toxicity (Cole et al., 1990). In this trial, the IMP was prescribed according to a standardised one-size-fits-all loading and maintenance regime: 200mg *bis die* for three days followed by 100mg *bis die* until surgery. In over one thousand patients taking perhexiline, this regime has a previously reported incidence of transient nausea of 10% but patient compliance of over 95% and no serious toxicity (appendix 9.4). Patients commenced the IMP at least five days before the planned date of surgery to set a minimum level of perhexiline exposure in the treatment group and thereby maximise the number of patients in the therapeutic range.

Serum perhexiline concentration at the time of surgery was determined in 280/286 (97.9%) patients analysed in the trial using high-performance liquid chromatography (HPLC). Samples were processed in batches by the routine therapeutic drug monitoring service provided by the Department of Clinical Pharmacology at The Queen Elizabeth Hospital, Adelaide. Serum perhexiline was measured in 135/139 (97.1%) patients in the treatment group. The median serum concentration was 0.26mg/L (IQR 0.14-0.48) with a range of 0 to 2.19mg/L and was not normally distributed (Kolmogorov-Smirnov test, D=0.18, $p<0.001$). 98 (72.6%) patients were above the lower threshold of the therapeutic range (0.15mg/L), with 19 (14.1%) patients also above the upper threshold (0.6mg/L). In the placebo group, 145/147

(98.6%) patients were assessed and all had a serum perhexiline level of zero, confirming the difference between the groups.

Metaboliser phenotype was determined by the serum OH-perhexiline to perhexiline concentration ratio ($C_{OH\text{-}Px}/C_{Px}$) and classified according to published criteria (table 3) (Sallustio et al., 2002, Zanger et al., 2004). Of 132 patients, eight (6.1%) were *poor* (mean C_{Px} 1.26mg/L), 28 (21.2%) were *intermediate* (mean C_{Px} 0.65mg/L), 87 (65.9%) were *extensive* (mean C_{Px} 0.25mg/L) and nine (6.8%) were *ultra-rapid* (mean C_{Px} 0.06mg/L) metabolisers (figure 18); the other three patients in the treatment group in whom it was measured had no detectable serum perhexiline and therefore their metaboliser phenotype remains unknown.

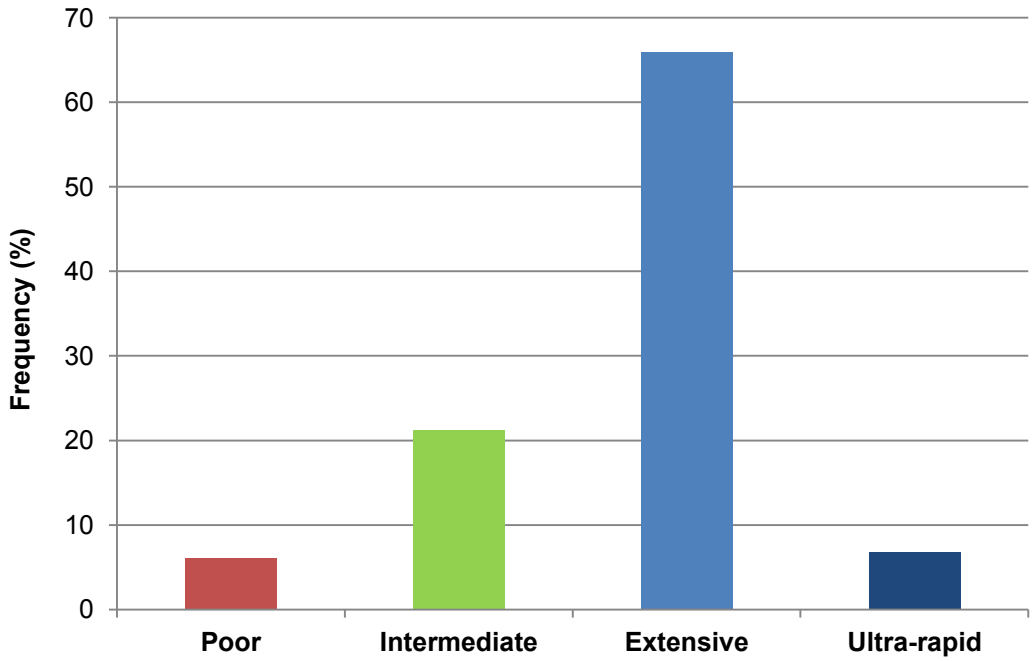


Figure 18. Frequency distribution of patients within metaboliser groups.

This distribution of metabolic ratios is broadly reflective of the local population, with a reported poor metaboliser prevalence of 7-10% in European Caucasians and 1-2% in those of African or East Asian decent (Gardiner and Begg, 2006). Serum perhexiline and metaboliser status are not independent variables and therefore it was unsurprising that the Spearman's rank coefficient was found to be -0.75 ($p<0.001$), suggesting a strong correlation. The log transformation of serum $C_{OH\text{-}Px}/C_{Px}$ was calculated and found to have a bimodal distribution in the population (figure 19) with the lowest ratios to the left representing the poor metabolisers; these findings are similar to those of previous studies on the pharmacokinetics of perhexiline (Sallustio et al., 2002).

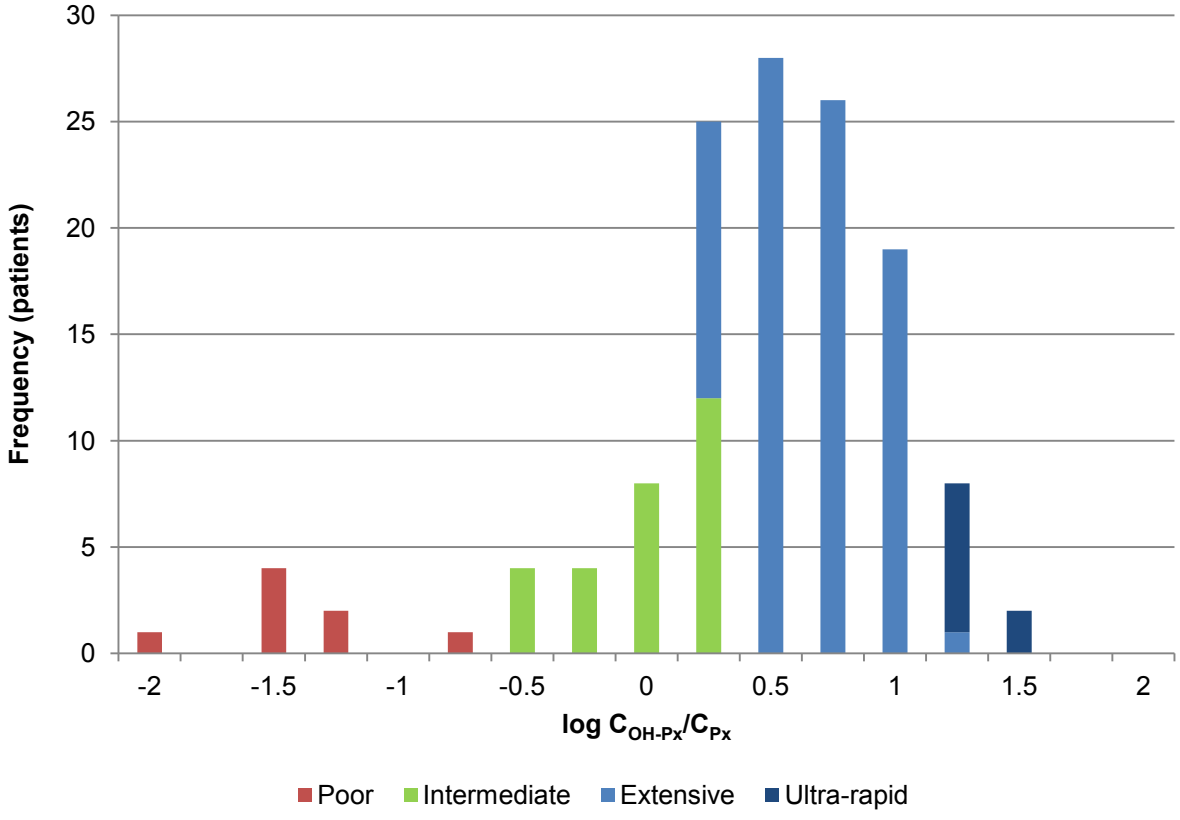


Figure 19. Frequency distribution of serum log $C_{OH\text{-}Px}/C_{Px}$ in 132 patients.

During the trial, 18 patients reported potential side-effects consistent with perhexiline toxicity: nausea (12), dizziness (8), headache (3), vomiting (2) and altered sensation in legs (1) (table 7). Six of 139 (4.3%) patients in the perhexiline group reported non-compliance with the protocol due to side-effects: four stopped the IMP prior to surgery and two reduced the dose. At the time of surgery, six patients were above, seven patients within and only two patients below the therapeutic range; of note, three patients reporting side-effects were found to be in the control group, all of whom had continued as per protocol. Overall, 15/139 (10.8%) patients in the perhexiline group reported side-effects prior to surgery with the one-size-fits-all regime, of whom five were poor metabolisers (5/8, 63%). On the other hand, three poor metabolisers denied any new symptoms despite serum perhexiline levels more than twice the upper limit of the therapeutic range. No patients withdrew from the trial due to side-effects and no such symptoms persisted beyond discharge from hospital.

There were twelve other known deviations from the IMP protocol, four of which were in patients randomised to perhexiline: three patients were no longer taking the IMP at the time of surgery due to delays in rescheduling their operations; one patient was not given the IMP consistently due to confusion among the nurses on a hospital ward (C_{Px} zero, $C_{OH\text{-}Px}$ 0.6mg/L). Furthermore, two other patients reported taking the IMP as per protocol but were found to have a perhexiline level of zero with either a trace or no OH-perhexiline suggesting a lack of compliance.

ID	Symptoms	Reported deviation	C_{Px} (mg/L)	Phenotype
19	Nausea	Stopped for 3 days	0.08	Extensive
24	Nausea	Reduced for 5 days	0.28	Extensive
42	Nausea & vomiting	Stopped for 3 days	0.46	Poor
49	Severe dizziness	Continued	1.46	Intermediate
67	Dizziness	Reduced for 5 days	1.18	Poor
68	Nausea & headache	Continued	0.29	Extensive
73	Dizziness	Continued	0.34	Intermediate
87	Nausea	Continued	0.07	Extensive
88	Dizziness & nausea	Stopped for 4 days	0.59	Poor
91	Nausea & vomiting	Continued	0.64	Poor
117	Burning sensation in legs	Continued	0	Control
153	Dizziness & nausea	Continued	1.36	Poor
154	Headache, nausea, dizziness	Stopped for 3 days	0.43	Intermediate
177	Headache	Continued	0	Control
185	Nausea	Continued	0	Control
229	Dizziness	Continued	1.75	Intermediate
234	Nausea & dizziness	Continued	0.47	Extensive
268	Nausea	Continued	1.58	Intermediate

C_{Px} indicates serum concentration of perhexiline.

Table 7. Patient-reported side-effects and deviations from protocol.

In this trial, it was intended that the length of therapy with the IMP would be at least five days (ten doses), including three days at the loading dose, to enable subjects receiving perhexiline to reach the therapeutic range. The maximum length of therapy was 31 days (62 doses) corresponding to two bottles of 34 tablets each. Overall, 46 (16%) of the 286 trial patients exceeded 28 doses and required a second bottle, 19 of whom were in the perhexiline arm. Of the 130 patients in the treatment group who reported still taking the IMP and in whom serum perhexiline was measured, 120 (92.3%) had taken ten or more consecutive doses prior to surgery. The remaining ten patients had taken nine (7, 5.4%), eight (1, 0.8%) or seven (2, 1.5%) doses. Median length of therapy was 9.5 days (19 doses) with a range of 3.5 to 30.5 days (7-61 doses) and was not normally distributed (D=0.16, p<0.001) but rather skewed towards one week of therapy (figure 20).

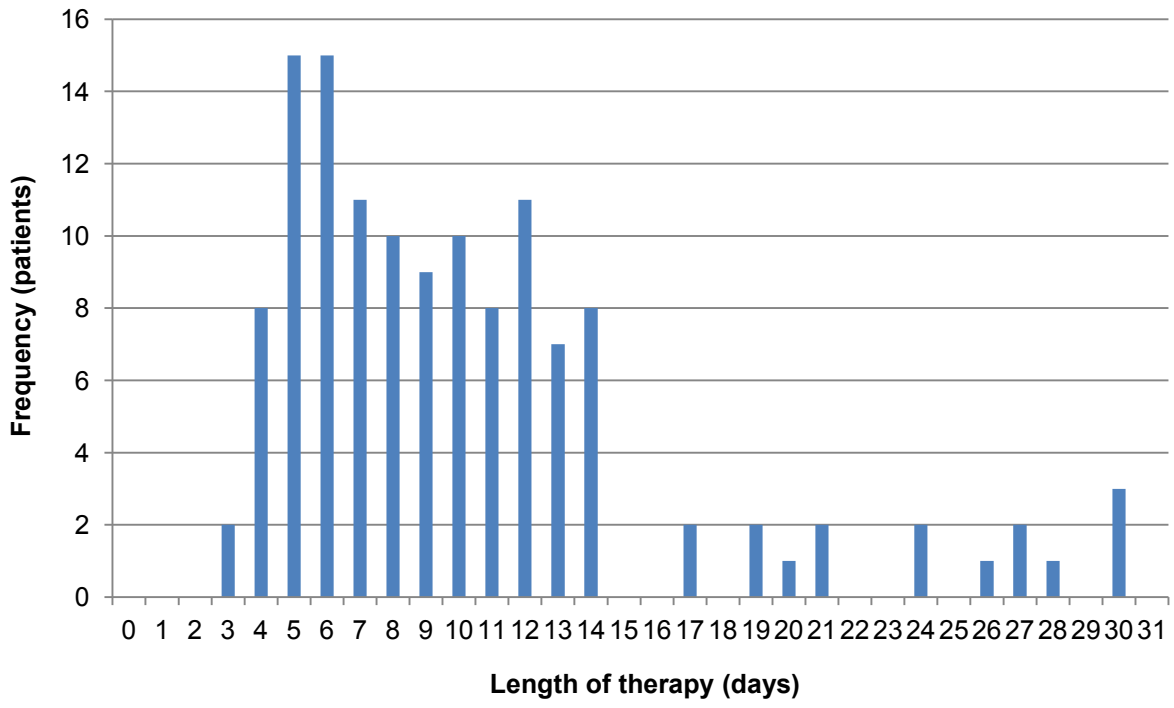
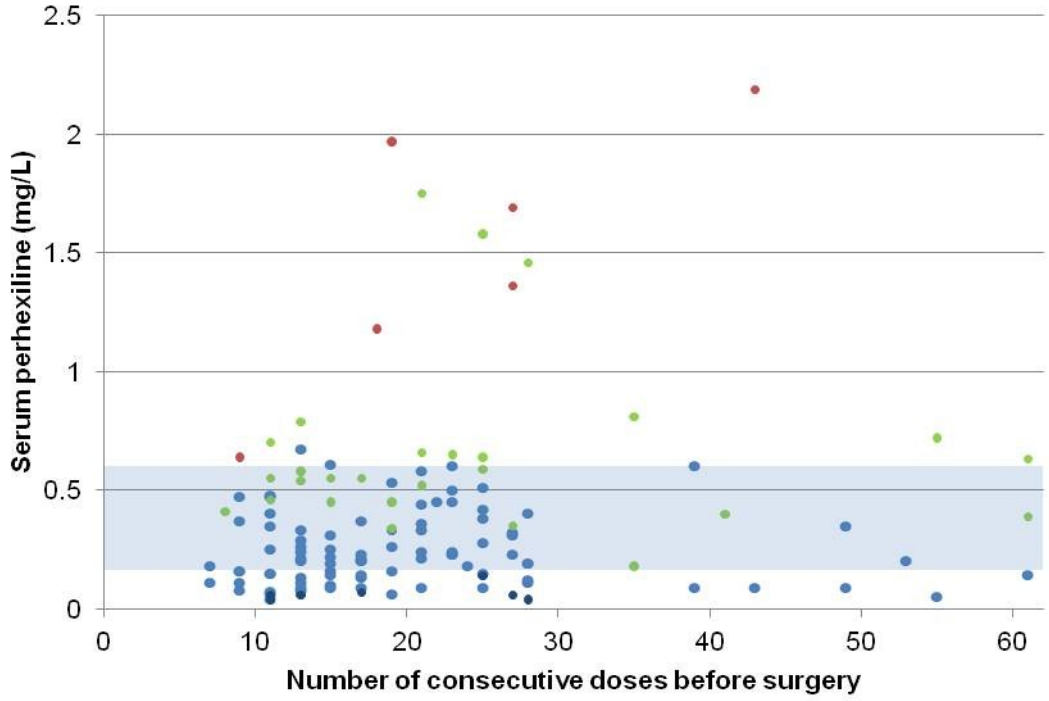


Figure 20. Frequency distribution of length of therapy in the perhexiline group.

The relationship between serum perhexiline and length of therapy was measured in the 130 perhexiline patients using Spearman's rank correlation with an r_s coefficient of 0.13 (p 0.14), suggesting a very weak correlation. The analysis was repeated in the 87 extensive metabolisers and showed no correlation, with a coefficient of 0.03 (p 0.80). This suggests that after a minimum period, length of therapy did not significantly affect serum perhexiline concentration, which was strongly influenced by other factors such as metaboliser status and compliance with treatment. Figure 21 highlights the distribution of serum perhexiline measurements across the metaboliser groups with all poor metabolisers above and all ultra-rapid metabolisers below the therapeutic range (0.15 to 0.6mg/L).



Shading indicates therapeutic range. ● Poor, ● Intermediate, ● Extensive, ● Ultra-rapid metabolisers.

Figure 21. Plot of serum perhexiline versus length of therapy in 130 patients.

Overall, 37 (27.4%) patients randomised to perhexiline were found to be below the therapeutic range at the time of surgery: nine patients were ultra-rapid metabolisers, with metabolic ratios from 20.2 to 37.0; two patients had reported side-effects, one of whom had stopped the IMP, the other had apparently continued; one patient was not given the IMP due to nursing errors; and two patients are highly likely to have been non-compliant as both C_{Px} and $C_{OH\text{-}Px}$ were negligible. Serum perhexiline was not available in the three patients who were no longer taking the IMP due to rescheduling delays. Of the remaining 23 patients, three had taken the IMP for less than five days (ten doses) but the median length of therapy for this cohort was eight days (17 doses). All were classified as extensive metabolisers with metabolic ratios ranging from 5.7 to 18.3. There is no clear explanation for the sub-therapeutic concentrations in these patients but it may be related to undeclared non-compliance, particularly in those with a low metabolic ratio, or other as yet unidentified factors. An exploratory analysis of the primary endpoint will evaluate whether sub-therapeutic serum levels impacted on the clinical effect of perhexiline.

In this chapter, I have detailed the recruitment of patients to the CASPER trial in a CONSORT flow-diagram, justified the reasons for excluding patients from the trial and documented the baseline and operative demographics of the trial population. I have reported the measurement of serum perhexiline in relation to metaboliser status, side-effects, protocol deviations and length of therapy. I have also sought to explain why more than one quarter of the perhexiline group were found to be sub-therapeutic at the time of surgery.

5. PERHEXILINE PHARMACOKINETICS IN THE MYOCARDIUM

5.1 Introduction

In clinical practice, the principles of pharmacokinetics determine the dosage of each drug prescribed and its effects on an individual patient. If the dose is too low, it will be ineffective; if too high, it may cause side effects due to toxicity and reduce patient compliance leading to inefficient treatment and complications. Most drugs reach their active site through untargeted delivery in the circulation and the concentration in the blood is determined by several pharmacokinetic parameters, principally absorption, distribution and clearance. However, in order for a drug to be effective, it must reach its target tissue in sufficient concentration to influence a biological process either via stimulation or inhibition. The half maximal inhibitory concentration (IC_{50}) is a measure of the effectiveness of a drug and indicates the concentration required at the site of action to reduce the activity of the process by 50% *in vitro*. The IC_{50} can be used to predict the effect of a particular drug or to compare the potency of two antagonists.

Drug distribution to a tissue depends upon its perfusion, drug binding, regional pH, partitioning and the permeability of cell membranes. Many drugs have an intracellular site of action and enter the cell either by passive transcellular diffusion or by active carrier-mediated transport across the membrane. Factors affecting drug uptake into the myocardium include tachycardia, sepsis, shock and circulatory volume overload, which all increase uptake, and ischaemia which inhibits drug uptake (Horowitz and Powell, 1986). A model of drug uptake into the myocardium has been proposed

(Lullmann et al., 1979), comprising four compartments: the freely accessible extracellular space, a low-capacity lipid barrier (the sarcolemma) and two larger capacity compartments within the cell: cell water and intracellular binding sites (figure 22). Binding to macromolecules such as proteins, phospholipids or nucleic acids may lead to its progressive accumulation, reaching a concentration many times higher than in plasma. Some drugs require substantial tissue uptake before reaching IC_{50} and their effects are delayed according to the rate of intracellular accumulation.

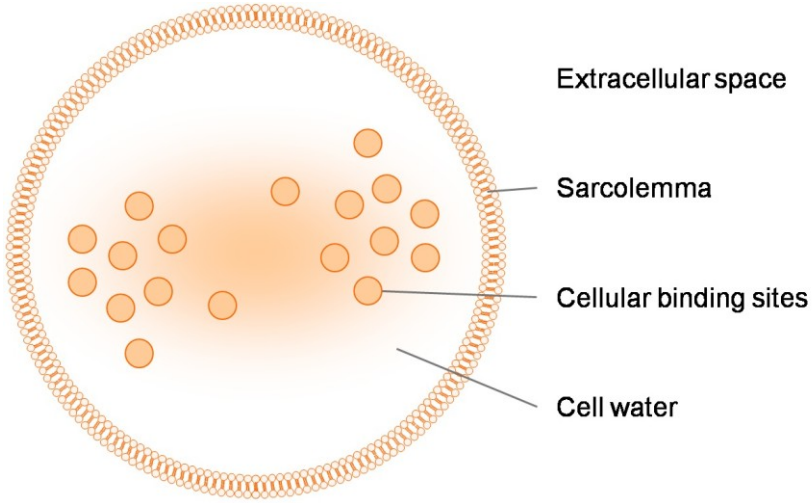


Figure 22. Compartments of drug uptake into the myocardium.

Historically, there have been difficulties in balancing the clinical effectiveness of perhexiline with significant toxicity due to marked inter-individual variation in its pharmacokinetics, principally differences in elimination by genetic polymorphisms of *CYP2D6*. Severe adverse events can be avoided by therapeutic drug monitoring to maintain the plasma concentration within a defined therapeutic range (Horowitz et al., 1986). Perhexiline has been shown to accumulate in the rat myocardium, reaching

levels 20-fold higher than in the corresponding plasma and to inhibit mitochondrial uptake of fatty acids via *CPT-1* in a concentration-dependent manner with an IC_{50} of 77μmol/L in rat heart homogenate (Kennedy et al., 1996). However, some biological effects of perhexiline are delayed and this has been attributed to a gradual tissue accumulation to reach an intracellular concentration that significantly inhibits *CPT-1* activity (Willoughby et al., 2002, Unger et al., 2005). Due to the practical difficulties in obtaining myocardial biopsies from patients who have traditionally been treated with perhexiline, no previous studies have addressed its uptake by the human heart.

High-performance liquid chromatography (HPLC) is a chromatographic technique used in analytical chemistry to identify, quantify and purify the individual components of a mixture of compounds (Snyder et al., 2010). A small volume of the mixture is dissolved in a stream of mobile phase which is pumped through a densely packed column containing the stationary phase. The constituents of the mixture travel at different speeds depending on their differential partitioning between phases causing them to separate. The detection sensitivity and separation selectivity of an HPLC column depend upon its internal diameter, particle size, pore size and pump pressure. Retention time within the column depends upon the strength of interactions with the stationary phase, the ratio of solvents used, the flow rate of the mobile phase and temperature; these variables may be modified to enhance mixture separation. A detector measures changes in the absorption and emission of ultraviolet light near the far end of the column to provide a retention time of each constituent and generate a plot of analyte peaks against time. The area under each peak is used to quantify the concentration of the analyte in the sample.

In this chapter, I measure the concentration of perhexiline in biopsies of human atrial and ventricular myocardium obtained from patients in the treatment arm of the CASPER trial using HPLC. I correlate these findings with serum perhexiline levels at the time of surgery and examine factors that may affect steady-state uptake into the myocardium. Finally, I compare the human pharmacokinetic findings with data from an animal model to assess how the ventricular concentration of perhexiline *in vivo* in patients corresponds to that required for *CPT-1* inhibition in the rat myocardium.

5.2 Materials & methods

The experiments described in this chapter were conducted in the laboratory of Associate Professor Benedetta C. Sallustio at the Basil Hetzel Institute, University of Adelaide using techniques previously validated in an animal model (Licari, 2010) adapted from a published method (Davies et al., 2006b). Serum and tissue samples obtained at QEH, Birmingham were shipped to Adelaide on dry-ice in two batches by Marken UK, specialists in cold-chain logistics. I performed all of the experiments described in this chapter during my Wellcome Trust Research Training Award visit. In this section, I describe the laboratory methods including the reagents and standards, tissue specimens, perhexiline extraction technique and chromatography used to determine the concentration of perhexiline in the atrial and ventricular myocardium.

5.2.1 Reagents and standards

The following reagents were used in the extraction of perhexiline and HPLC:

- Methanol, n-hexane and ethyl acetate (all Merk-Hipersolv grade)
- HPLC glass-distilled water
- 2.0M NaOH: 8.0g of NaOH dissolved in 100mL of distilled water.
- 0.5M NaOH: 5.0g of NaOH dissolved in 250mL of distilled water.
- 10% Ethyl acetate in n-Hexane: To a 250mL measuring cylinder, 25mL of ethyl acetate was added and topped up to full volume with n-hexane, stored at 2-8°C.
- 0.05% (R)-(-)-1-(1-Napthyl)ethyl isocyanate (derivitising agent) in acetone: 5 µL of 1-NEIC dissolved in 10mL of acetone in a nitrogen environment, stored at 2-8°C.
- 0.1M HCl: 4.3ml of concentrated HCl was added to 500mL of distilled water.

The following standard stock solutions were used:

- *Perhexiline racemic stock* (100mg/L): 7.09mg of perhexiline maleate (Sigma, MW 393, equivalent to 5mg of perhexiline base, MW 277) was weighed and transferred to a 50mL volumetric flask. 5mL of methanol was added to completely dissolve the solid, made up to volume with 0.1M HCl and mixed well to ensure solution.
- *Perhexiline standard solution* (10mg/L, 0.5mg/L): In a 50mL volumetric flask, 5mL of racemic perhexiline 100mg/L solution was added and made up to full volume with 0.1M HCl to give a 10mg/L racemic stock solution. 1mL batches were aliquoted into disposable glass culture tubes, capped and stored frozen. On the day of the assay, one tube was thawed at 37°C. To prepare a 0.5mg/L racemic stock solution, after mixing well, it was diluted 1 in 20 with 0.1M HCl.
- *Prenylamine stock solution* (10mg/L, internal standard): 10mg of prenylamine (Sigma) was weighed out and transferred to a 100mL volumetric flask. 10mL of methanol was added to dissolve the solid and made up to volume with 0.1M HCl. It was diluted 1 in 10 with 0.1M HCl to give a 10mg/L stock solution, stored at 2-8°C.
- *Quality control specimens*: Fresh perhexiline 100mg/L racemic stock solution was prepared as above. To make a 1.50mg/L racemic (0.75mg/L enantiomers) QC batch, 300µL of 100mg/L stock was added to a 20mL volumetric flask and made up to volume with control right atrial homogenate. To make a 0.80mg/L racemic (0.40mg/L enantiomers) QC batch, 160µL of 100mg/L stock was added to a 20mL volumetric flask and made up to volume with control atrial homogenate. To make a 0.06mg/L racemic (0.03mg/L enantiomers) QC batch, 120µL of 10mg/L stock was added to a 20mL volumetric flask and made up to volume with control atrial homogenate. 700µL of each QC solution was transferred to Eppendorf tubes.

5.2.2 Tissue specimens

As described previously, atrial and ventricular biopsies were obtained during surgery and promptly snap-frozen in liquid nitrogen. The right atrial appendage was truncated during venous cannulation for CPB. Transmural Tru-Cut needle (Allegiance, McGaw Park, IL) biopsies of the left ventricular free-wall between the left anterior descending artery and the first diagonal branch were taken at three time points but only the pre-ischaemia biopsy (on CPB but before application of the aortic cross-clamp) was used in this experiment. Once frozen, all samples were stored at -80°C until analysis, except during shipping to Adelaide on dry-ice.

Right atrial biopsies were mechanically digested in 0.15M phosphate buffer solution (pH 6.0) using a homogeniser and a tissue grinder to form a suspension; a fine tissue grinder alone was used for the left ventricular biopsies which had far lower masses. Atrial and ventricular biopsies from patients in the perhexiline arm constituted the experimental groups. Atrial biopsies from patients in the control arm, with proven zero serum perhexiline concentrations, were also prepared for generating standard curves and quality control (QC) samples.

5.2.3 Perhexiline extraction

Biopsies were analysed in batches of approximately 25 samples. A standard curve and quality control samples were prepared for each batch using 0.5mL of control right atrial homogenate in 15mL screw-cap disposable glass tubes (table 8). Prenylamine was added to all samples, except the blank, as an internal standard to enable reverse quantification of sample perhexiline concentration.

No.	Perhexiline (mg/L)	0.1M HCl	Control atrium	Racemic Px stock		Prenylamine
				10mg/L	0.5mg/L	10mg/L
1	2.00	-	500	200	-	50
2	1.00	100	500	100	-	50
3	0.50	150	500	50	-	50
4	0.20	180	500	20	-	50
5	0.10	-	500	-	200	50
6	0.05	100	500	-	100	50
7	0.02	160	500	-	40	50
8	0.01	180	500	-	20	50
9	0	200	500	-	-	50
10	Blank	250	500	-	-	-
11+	?	200	QC and patient specimens			50

HCl indicates hydrochloric acid; Px, perhexiline; QC, quality control. All volumes in µL.

Table 8. Preparation of HPLC standard curve, control and patient specimens.

For each QC solution and patient specimen, 0.5mL of homogenised tissue was used. 50µL of 10mg/L prenylamine was added to all tubes except Blank (No.10) and vortexed briefly. 50µL of 2M NaOH was added to all tubes and vortexed briefly. 4mL of a solution of 10% ethyl acetate in n-hexane was added and mixed on a horizontal shaker for 15 minutes at 100opm. All tubes were centrifuged for 15 minutes at 2500rpm and 10°C. The aqueous layer was frozen in a dry ice/ethanol bath and discarded whilst the organic layer was transferred to 5mL disposable glass tubes. Samples were evaporated at room temperature in an evacuated centrifuge.

To the dry residue, 200µL of derivitising agent (1-NEIC in acetone) was added and vortexed briefly then incubated at room temperature for 5 minutes. 200µL of 0.5M NaOH was added and vortexed briefly then incubated at room temperature for 5 minutes. 3mL of 10% ethyl acetate in n-hexane was added, the tubes capped and vortexed for 5 minutes. All tubes were centrifuged for 3 minutes at 2500rpm and 10°C. The aqueous layer was frozen in a dry ice/ ethanol bath and discarded whilst the organic layer was quickly transferred to clean 5mL disposable glass tubes. Samples were evaporated at room temperature in an evacuated centrifuge. The dry residue in all tubes was reconstituted in 150µL of a mixture of 82% methanol and 18% water and 100µL injected into the HPLC column.

5.2.4 Chromatography

Analyses were conducted on an Agilent 1100 series HPLC apparatus (Agilent Technologies, Forest Hill, VA, Australia) operated by Chemstation for LC 3D software, as previously reported (Davies et al., 2006b). The hardware consisted of a model G1322A degasser, a model G1311A pump generating flow at 1ml/min, a model G1313A autosampler and a model G1321A fluorescence detector with excitation and emission wavelengths of 218 and 334nm, respectively. Resolution of the diasteromers was achieved using a Merck Purospher RP-18E (5µm, 125mm x 4mm) column at ambient temperature. With gradient elution, the mobile phase was composed of 82% methanol and 18% water for the first 11.5 minutes, then increased to 86% methanol in a linear gradient over 1 minute, maintained at this concentration for a further 15.5 minutes, then reduced back to 82% methanol over 1 minute and continued at this concentration for the remaining 3 minutes of each sample run time.

The calibration curves of the concentrations of each enantiomer against the peak area ratios between the analyte and the first peak of the internal standard were constructed using linear regression (Davies et al., 2006b). Precision and accuracy were evaluated using the coefficient of variation (CV) and the bias of the measured concentration versus the known spiked concentration, respectively; a CV and bias of +/- 15% was considered acceptable. Inter-assay quality control was assessed by analysing aliquots from three separate QC pools spiked with 0.03, 0.4 and 0.75mg/l of perhexiline enantiomers. Due to the lack of UV absorbance and fluorescence of underivatised perhexiline and the unavailability of pure derivatised perhexiline, it was not possible to determine the absolute extraction efficiency of this method.

5.2.5 Statistical analysis

Data is presented as mean (standard deviation) or median (interquartile range). The concentration of perhexiline is reported as the sum of the two enantiomers measured. The normality of data was assessed using the Kolmogorov-Smirnov test. Spearman's rank test was used to correlate non-parametric data. The relationship between variables was modelled using simple and multiple linear regression in SPSS. An outline of the statistical tests used in this thesis is contained in appendix 9.6.

5.3 Results

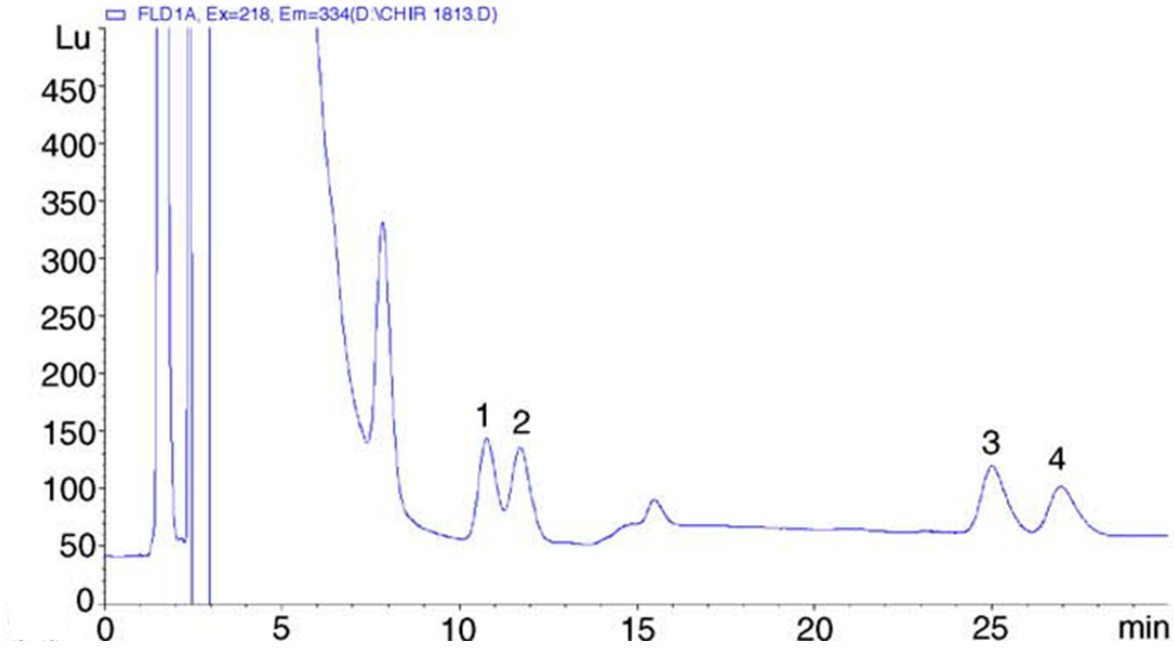
The concentration of perhexiline in right atrial wall biopsies was measured in 94/139 (67.6%) patients in the treatment group (table 9), for whom serum perhexiline was also available; the mean mass of atrial tissue analysed was 85mg (range 10-458mg). Pre-ischaemia left ventricular biopsies from 28/139 (20.1%) patients in the treatment group, in whom serum and atrial perhexiline had also been determined, were analysed; the mean mass of ventricular specimens was 3.6mg (range 1.6-15mg). Poor metabolisers were excluded as they were unlikely to have reached steady-state.

Variable	**Perhexiline (n = 94)**
Serum perhexiline, median (IQR), mg/L	0.24 (0.12-0.44)
Serum OH-perhexiline, median (IQR), mg/L	1.18 (0.96-1.52)
Metaboliser status, n (%)	
Intermediate	18 (19.1)
Extensive	68 (72.3)
Ultra-rapid	8 (8.5)
Length of therapy, median (IQR), d	8.5 (6.5-12.5)
Age, mean (SD), y	64.3 (8.9)
Weight, mean (SD), kg	84.2 (15.0)
Pre-treatment heart rate, mean (SD)	60 (10)
Pre-ischaemia cardiac index, mean (SD), L/min/m^2	2.13 (0.60)
Preoperative medications, n (%)	
ACE inhibitor or ARB	60 (63.8)
Statin	87 (92.6)
β-adrenergic antagonist	66 (70.2)
Calcium channel antagonist	34 (36.2)
Long-acting oral nitrate	32 (34.0)
Potassium channel blocker	20 (21.3)

ACE, angiotensin converting enzyme; ARB, angiotensin II receptor blocker.

Table 9. Characteristics of 94 patients providing atrial biopsies for analysis.

Analysis of each biopsy using HPLC generated a plot of UV light against retention time with identifiable peaks representing the concentration of perhexiline (figure 23).



Peaks 1 & 2 correspond to the diasteromers of derivatised prenylamine, the internal standard. Peaks 3 & 4 correspond to the diasteromers of derivatised (+) and (–) perhexiline, respectively; the sum of these two peaks gives the total concentration of perhexiline in the specimen (Davies et al., 2006b).

Figure 23. Representative chromatogram of a patient sample.

5.3.1 Atrial perhexiline concentration

The median atrial concentration was 6.02mg/Kg (IQR 2.70-9.06) with a range of 0.74 to 28.6mg/Kg and was not normally distributed (D=0.16, p<0.001). Paired atrial and serum perhexiline concentrations were compared using Spearman's rank correlation finding an r_s coefficient of 0.91 (p<0.001), suggesting a very strong correlation. Linear regression was performed using atrial perhexiline as the dependent variable and serum perhexiline as the independent variable. The R value of 0.87, R^2 of 0.76 and *F-ratio* of 279 (p<0.001) suggest that serum perhexiline is highly predictive of atrial

concentration, explaining 76% of the variation in measured atrial concentration (figure 24). Unsurprisingly, atrial perhexiline and metaboliser status were also strongly correlated with an r_s of -0.71 (p<0.001). The median atrial-serum perhexiline ratio was 21.5 (IQR 18.1-27.1) with a range from 4.9 to 55.6 and was not normally distributed (*D*=0.10, p=0.03).

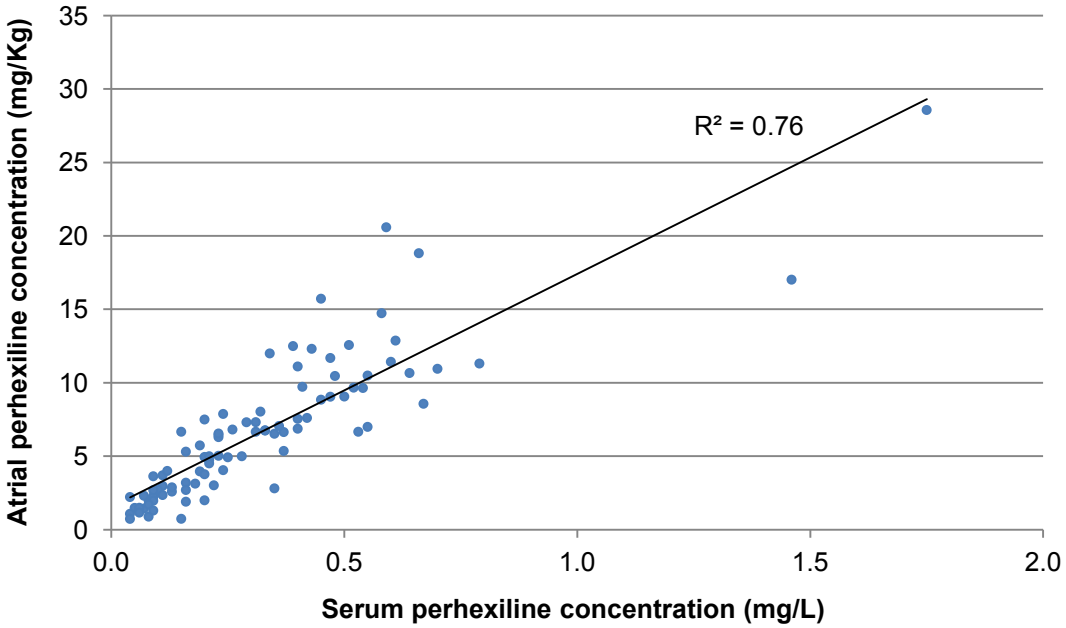


Figure 24. Plot of atrial against serum perhexiline concentrations.

Stepwise multiple linear regression was performed using serum perhexiline, OH-perhexiline, length of therapy, age, weight, heart rate, left ventricular function, pre-sternotomy cardiac index and concurrent mediations as potential predictors of atrial concentration. A model containing serum perhexiline (β 0.82, p<0.001), age (β 0.14, p<0.01), heart rate (β -0.17, p<0.01) and weight (β -0.15, p<0.01) achieved an *R* value of 0.91, R^2 of 0.82 and *F-ratio* of 99.2 (p<0.001); it should be noted that serum perhexiline alone has already been shown to have an R^2 of 0.76. Length of therapy

had no impact on the model suggesting that similar to serum perhexiline, atrial drug concentration was not significantly affected by the length of therapy beyond five days and that most patients had reached a steady-state of atrial drug uptake.

5.3.2 Ventricular perhexiline concentration

The median ventricular concentration was 10.0mg/Kg (IQR 5.76-13.1) with a range of 3.05 to 35.7mg/Kg and did not fit a normal distribution model (D=0.19, p<0.01). Median length of therapy in the 28 patients with ventricular biopsies was 9.5 days (IQR 7.0-14.0). Linear regression was used to assess the impact of serum perhexiline on the measured concentration in the ventricular myocardium with an R value of 0.85, R^2 of 0.73 and *F-ratio* of 69.1 (p<0.001) (figure 25). Repeating the analysis for the predictive value of atrial concentration found an R of 0.88, R^2 of 0.77 and *F-ratio* of 86.6 (p<0.001) (figure 26). The ventricular-serum perhexiline ratio had a median of 34.9 (IQR 24.5-55.2) with a range of 16.7 to 89.3. The ventricular-atrial ratio had a median of 1.67 (IQR 1.39-2.22) with a range from 0.9 to 5.0.

Stepwise multiple linear regression was used to identify predictors of the ventricular concentration. Unsurprisingly, serum perhexiline concentration (β 0.71, p<0.001) remained the leading predictor but the model was improved by the addition of length of therapy (β 0.26, p<0.01) and OH-perhexiline (β -0.23, p 0.02), achieving an R value of 0.92, R^2 of 0.85 and *F-ratio* of 43.8 (p<0.001). The presence of length of therapy in the model suggests that unlike perhexiline uptake into the atrium, the concentration in the ventricular myocardium had not yet reached steady-state by the time of surgery. Therefore, the concentration measured in these ventricular biopsies may not reflect that achievable with a longer period of therapy.

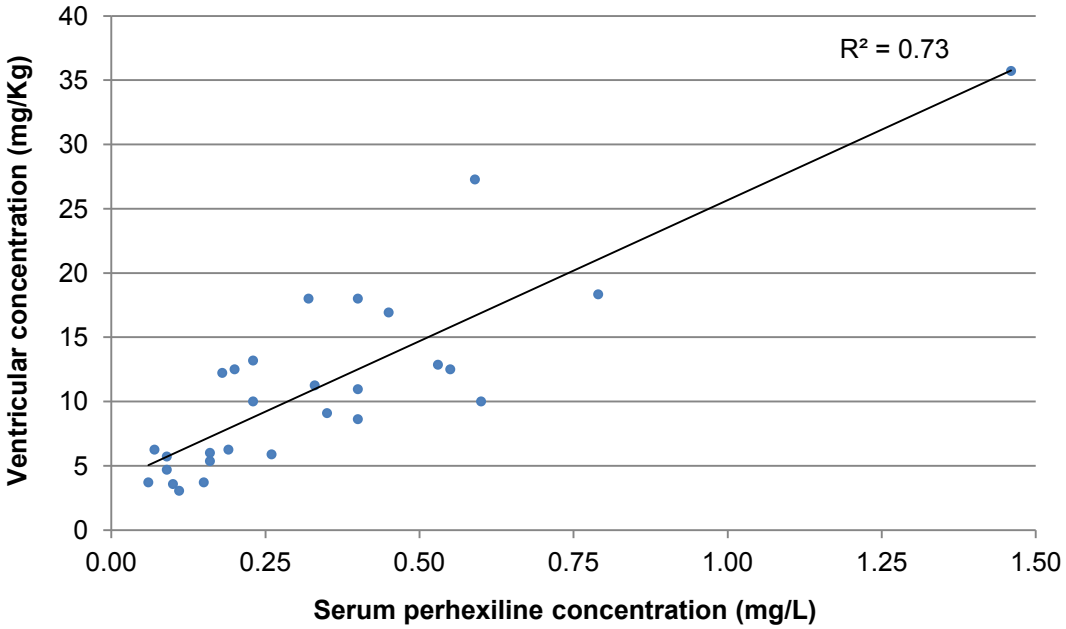


Figure 25. Plot of ventricular against serum perhexiline concentrations.

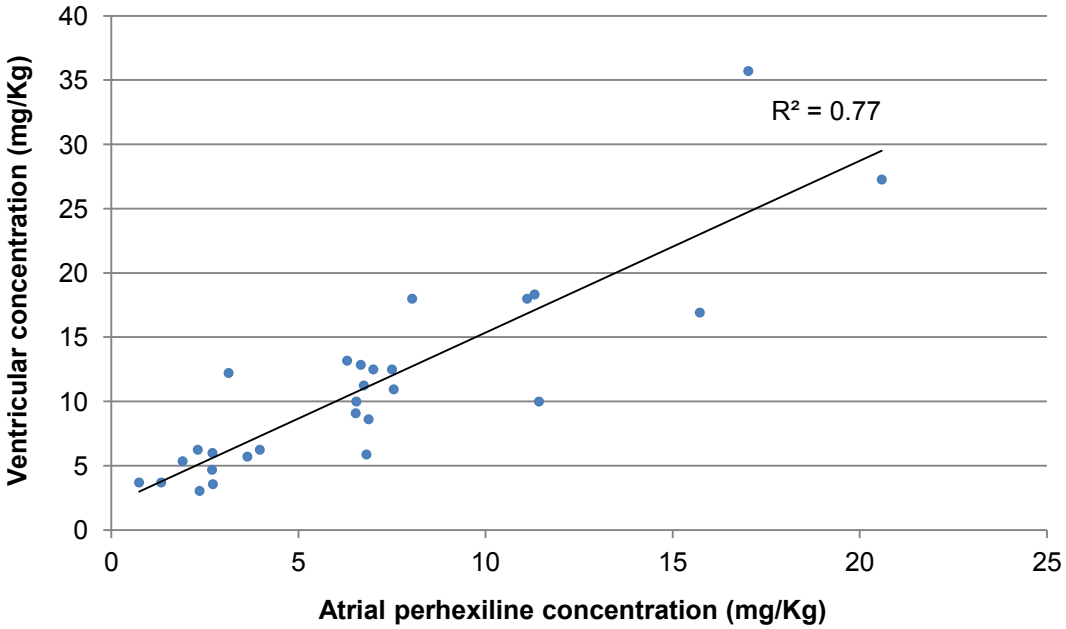


Figure 26. Plot of ventricular against atrial perhexiline concentrations.

5.4 Discussion

This study is the first to measure the myocardial concentration of perhexiline in humans. In patients undergoing cardiac surgery, I have demonstrated that it is highly concentrated in the human myocardium compared with serum, approximately 20-fold in the right atrium and 35-fold in the left ventricle, after median nine days of therapy. These observations reflect closely the 20-fold accumulation previously observed following acute exposure to perhexiline in the rat (Kennedy et al., 1996). Despite a low serum concentration, there is progressive accumulation of perhexiline in the myocardium, potentially leading to a high tissue concentration at its site of action.

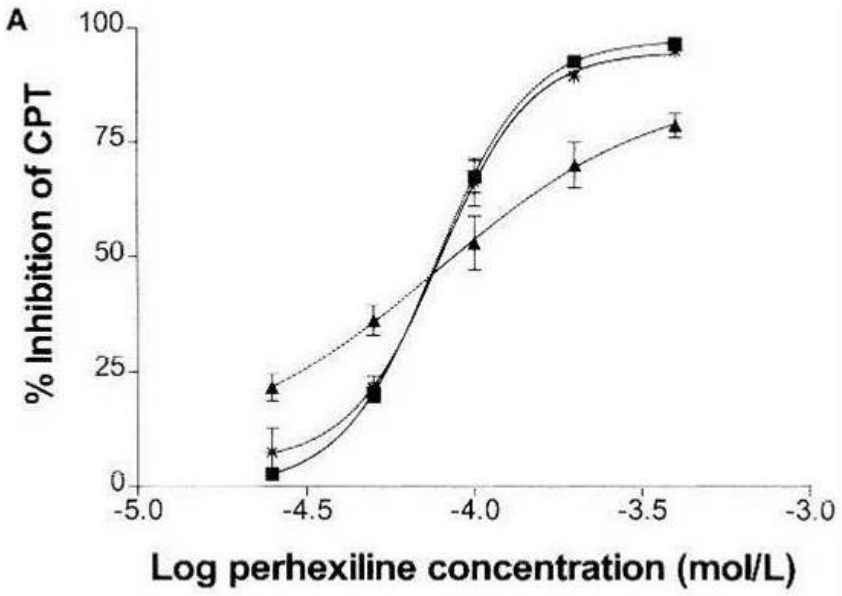
On multivariate analysis, serum perhexiline concentration was the strongest predictor of myocardial concentration confirming that drug uptake is concentration-dependent (Horowitz and Powell, 1986). Uptake of perhexiline appeared to have linear kinetics implying that the mechanism is not saturable within the therapeutic range and that either it is not carrier-mediated or the unbound serum concentration of perhexiline is well below the K_m of any transporters. Atrial concentration was also correlated with increasing age and inversely with heart rate and weight, although the relationship with weight may be mediated by lower serum levels. Increasing concentration with age is a novel finding and likely to reflect unmeasured factors such as myocardial uptake, efflux, metabolic capacity, protein binding and serum:myocardial partitioning. On the other hand, the inverse correlation with heart rate was an unexpected finding; it has previously been suggested that myocardial drug uptake is increased by tachycardia (Lullmann et al., 1979) although increased myocardial mechanical work may have been a confounding factor.

The hypothesis that cumulative drug uptake in the myocardium may be influenced by mechanical work (Lloyd and Taylor, 1976, Lullmann et al., 1980) is supported by the finding that perhexiline was more concentrated in the ventricle than in the atrium. A similar observation has been reported in patients on long-term digoxin therapy undergoing mitral valve surgery in whom the drug concentration was 41-times higher in atria and 77-times higher in left ventricular papillary muscle than in plasma (Carruthers et al., 1975). Both the serum and atrial perhexiline concentration were strongly predictive of the ventricular level; the slightly higher predictive value of the atrial concentration (R^2 0.77) is likely to reflect one or more unidentified factors that affect myocardial drug uptake *per se* and were therefore already taken into account. However, length of therapy was found to be a predictor of the ventricular but not the atrial perhexiline concentration. Despite greater drug accumulation in the ventricle, it may not have yet reached a steady-state following a median 9.5 days of therapy. This suggests that the rate of perhexiline uptake into the myocardium of the ventricle may be protracted, with a larger volume of distribution and slower drug metabolism than in the atrium and delayed equilibration between serum and tissue. With a longer period of exposure, an even higher concentration may have been achieved in the ventricular myocardium and improved correlation with atrial levels.

Little is known regarding the uptake of drugs into the human myocardium at steady-state; most studies have examined changes in compartments following acute drug administration (Horowitz and Powell, 1986). Gradual accumulation in the myocardium may be due to high tissue binding with or without an active cellular uptake mechanism. The availability of intracellular binding sites may be affected by disease

states e.g. ischaemia, other drugs and metabolites, including OH-perhexiline, and contribute to inter-individual variability in perhexiline uptake and efficacy.

Whilst this study has shown that perhexiline is markedly concentrated in the human myocardium compared with serum, is it sufficient to significantly inhibit *CPT* function? In the rat heart, Kennedy *et al*. (2000) examined the effect of perhexiline on *CPT-1* and -*2* inhibition at varying concentrations (figure 27) and found the IC_{50} in isolated cardiac mitochondria to be 77µmol/L (21.3mg/L) with no significant differences between intact mitochondrial and homogenate preparations.



Intact mitochondria (■——■), pellet (✶——✶) and supernatant (▲——▲) preparations after Tween-20.

Figure 27. Concentration response curve for the inhibition of *CPT-1* in the rat.[2]

[2] Reprinted from Kennedy JA, Kiosoglous AJ, Murphy GA, Pelle M, Horowitz JD. Effect of Perhexiline and Oxfenicine on Myocardial Function and Metabolism during Low-Flow Ischemia/Reperfusion in the Isolated Rat Heart. Journal of Cardiovascular Pharmacology, 36(6), 794-801, Copyright (2000), with permission from Wolters Kluwer Health.

In the current study, only 2/28 (7.1%) patients had a ventricular perhexiline concentration above 77µmol/L (21.3mg/L), one of whom had significantly toxic serum levels and the other was at the upper limit of the therapeutic range. This raises the possibility that the myocardial concentration of perhexiline may have been insufficient to significantly inhibit *CPT-1* at the time of surgery, although there are a number of important caveats. The degree of interspecies preservation of enzyme function, binding sites and susceptibility to perhexiline inhibition is unknown. There were also important methodological differences between the *in vitro* rat mitochondria and the *ex vivo* human homogenate affecting the exposure of *CPT-1* on the outer mitochondrial membrane to perhexiline. These include the availability of cellular binding sites, the proportion of unbound drug in the myocardium, the length of exposure to perhexiline, the solvent extraction technique and potential drug compartmentalisation within the sarcolemma, cytosol and mitochondria. Total tissue concentration may not reflect drug concentration at its site of pharmacological action. On the other hand, the concentration-dependent inhibition of *CPT* was similar across the intact mitochondria, pellet and supernatant preparations with no significant difference in IC_{50} values (Kennedy et al., 2000). The concentration of perhexiline measured in the ventricular biopsies from trial patients was in the same order of magnitude as that used in the rat studies. Indeed as it falls close to the steep section of the sigmoid log curve, small changes in concentration would lead to large changes in the degree of enzyme inhibition. Direct comparisons of perhexiline concentrations and IC_{50} between models should therefore be regarded as hypothesis-generating rather than definitive evidence.

Further improving our understanding of drug uptake into the myocardium will provide insights into the relationship between efficacy and toxicity, inform drug design, enable targeted delivery in disease states such as ischaemia and facilitate an individualised approach to therapeutics. It also provides a platform for physiologically-based pharmacokinetic modelling (PBPK), a mathematical technique for assessing system dynamics that utilises a multi-compartment model to improve understanding of the interactions of a drug and its metabolites across compartments.

In this chapter, I have demonstrated the utility of myocardial biopsies for the elucidation of steady-state drug uptake in the heart. I have shown that perhexiline is highly concentrated in the myocardium and whilst there is a strong correlation with plasma levels, other factors such as heart rate and age may modulate the uptake process. However, it appears that the ventricular concentration of perhexiline had not reached steady-state by the time of surgery using the trial dosing regimen; the concentration may have risen further with a longer period of exposure. Whilst the level achieved in the ventricular myocardium was in the correct order of magnitude for *CPT-1* inhibition according to the rat model, it remains unknown whether at steady-state, the concentration would be sufficient to significantly affect the flux through cellular metabolic pathways in humans.

6. METABOLIC PRECONDITIONING WITH PERHEXILINE

6.1 Introduction

The myocardium is the most metabolically active tissue in the body for weight. Its major source of ATP is mitochondrial oxidative metabolism which is tightly controlled to meet the contractile and homeostatic needs of the heart. As discussed previously, energy is derived from a variety of competing substrates taken up from the blood (Taegtmeyer, 2007). Substrate utilisation is primarily regulated locally by the glucose-fatty acid cycle whereby the availability of fatty acids undergoing β-oxidation inhibits the *PDH* complex, decreasing the entry of pyruvate into the citric acid cycle and leading to preferential oxidation of fatty acids over glucose. Malonyl-CoA acts as the endogenous regulator, decreasing mitochondrial uptake of fatty acids through potent *CPT-1* inhibition (Ussher and Lopaschuk, 2008). In ischaemia, a rise in circulating FFA further uncouples glycolysis from glucose oxidation and the fate of pyruvate is switched to lactate production, maintaining ATP production via anaerobic metabolism at the expense of worsening acidosis and reduced contractile function (Depre et al., 1999). Upon reperfusion, β-oxidation of FFA is accelerated to meet the increased energy demands of the cell to the detriment of glucose oxidation. High levels of circulating FFA during reperfusion have been shown to contribute to myocardial ischaemia-reperfusion injury through *PDH* complex inhibition (Lopaschuk et al., 1994) leading to worsening cellular acidosis, calcium overload and oxidative stress which may precipitate cell death (Crompton, 1999) and impairment of contractile function.

Pharmacological modulation of myocardial metabolism aims to induce a shift in substrate utilisation from fatty acids to glucose, increasing oxidative energy efficiency and decreasing the harmful effects of β-oxidation during ischaemia-reperfusion (Taegtmeyer, 2002). Reduced suppression of the *PDH* complex maintains the entry of glycolytic pyruvate into the citric acid cycle, decreases lactate and proton production, and downregulates mitochondrial uncoupling proteins thereby restoring the efficacy of oxidative phosphorylation and electron transport (Lopaschuk et al., 2010). The enhanced energetic status of the cell is postulated to activate pro-survival pathways and reduce the impact of ischaemia, translating into enhanced recovery of cardiac function during reperfusion. GIK has been shown to enhance myocardial protection during cardiac surgery (Fan et al., 2011) and many metabolic mechanisms have been proposed including a reduction in circulating FFA and their β-oxidation, glycogen loading, increased glycolytic ATP production, repletion of depleted energy cycles by anaplerosis and improved efficacy of ATP production from glucose oxidation (Doenst et al., 2003a). Indeed, our group has shown that perioperative GIK significantly increases pre-ischaemia atrial glycogen content and markedly suppresses plasma FFA concentrations during reperfusion (Howell, 2010).

Perhexiline has been shown to inhibit mitochondrial *CPT* in rat heart mitochondria, leading to reduced uptake and β-oxidation of long-chain fatty acids (Kennedy et al., 1996). In a subsequent study using the Langendorff isolated perfused heart model, Kennedy and colleagues (2000) found that perhexiline inhibited the release of lactate from the myocardium during normal flow but had no effect of pre-ischaemic glycogen content. Tissue levels of malonyl-CoA were similar to controls despite an increase in

acetyl-CoA with perhexiline (216nmol/g v 143 nmol/g, $p<0.05$) which may represent increased availability of substrate for the citric acid cycle. There was no significant difference in myocardial long-chain acyl-carnitine consistent with the simultaneous inhibition of both *CPT-1* and *-2* with perhexiline. Whilst this study demonstrated reduced diastolic dysfunction during ischaemia, perhexiline had no beneficial effect on the recovery of myocardial systolic function on reperfusion and concerns were raised over whether the length of pre-exposure to perhexiline had been sufficient to achieve a high level of cellular accumulation and maximise *CPT* inhibition.

In the previous chapter, I demonstrated that after a median 8.5 days (IQR 6.5-12.5) of treatment, there had been uptake and accumulation of perhexiline in the myocardium of patients undergoing surgery to within the same order of magnitude required for the effective inhibition of *CPT* in the rat. According to its proposed mechanism of action, *CPT* inhibition by perhexiline should produce significant changes in myocardial carbohydrate and lipid metabolism. Preoperative treatment is proposed to enable short-term metabolic optimisation prior to ischaemia by maintaining the oxidative coupling of glucose to improve myocardial protection without the potential risks of lipid accumulation associated with long-term therapy. The fate of glucose may be assessed by its storage (glycogen), flux through glycolysis (G6P, pyruvate) and anaerobic metabolism (lactate), and lipid metabolism by the concentration of myocardial triacylglycerol and phospholipid. Substrate availability prior to ischaemia is determined by serum glucose, lactate, lipids and ketones whilst the lipid profile during reperfusion has been shown to affect metabolic and functional recovery (Lopaschuk et al., 1994).

In this chapter, I perform laboratory experiments on human tissue to directly examine the impact of perhexiline on myocardial metabolism. I use traditional biochemical methods to measure the effect of perhexiline on carbohydrate and lipid intermediates in serum and atrial myocardium and the effect of surgery on the serum lipid profile. In collaboration with colleagues in Biosciences, I analyse the metabolomic profile of left ventricular biopsies obtained prior to ischaemia from patients on perhexiline therapy compared with controls using state of the art ultra-high resolution mass spectrometry.

6.2 Traditional biochemical analysis

6.2.1 Materials & methods

The experiments described in this section were conducted in collaboration with Dr David Hauton at the Institute for Biomedical Research, University of Birmingham. The concentrations of carbohydrates and lipids in human tissue and serum samples were determined principally by enzymatic colourimetry. In this technique, the analyte is degraded by a specific enzyme to produce an intermediate which reacts with an additive to form a coloured complex; the resulting colour absorbance of the solution is proportional to the concentration of the analyte according to Beer-Lambert's law:

Absorbance (*A*) = *molar absorptivity* (ε) x *path length* (*l*) x *molar concentration* (*c*)

in which path length and absorptivity are known. Absorbance was measured using a Labsystems Multiskan spectrophotometer (Thermo Fisher Scientific, Loughborough, UK) at predefined wavelengths. All solvents and reagents were of analytical grade.

Tissue and serum specimens

Serum samples and atrial biopsies were obtained during surgery from trial patients at QEH, Birmingham. Serum samples were collected at induction of anaesthesia and six hours after removal of the aortic cross-clamp. The right atrial appendage was truncated during venous cannulation for CPB and any macroscopic epicardial fat removed; in selected patients, it was divided in two: one portion was promptly snap-frozen in liquid nitrogen, the other mounted on a cork disk, embedded in *Tissue-Tek* Optimum Cutting Temperature (OCT) compound (Sakura) and frozen in isopentane pre-cooled to approximately -40°C with dry ice. All samples were stored at -80°C.

Serum biochemical analyses

Fasted serum samples collected at induction of anaesthesia were tested for glucose (Thermo-Electron, Melbourne, Australia), insulin (Mercodia, Uppsala, Sweden), FFA (Wako Chemicals, Neuss, Germany), TAG and β-hydroxybutyrate (Randox, Crumlin, UK) using commercial assay kits according to the instructions. Serum samples collected during reperfusion at six hours after removal of the aortic cross-clamp were analysed for FFA and TAG.

Myocardial glycogen

Tissue glycogen content was determined by isolation using conventional alkali digestion and ethanol precipitation, followed by hydrolysis and spectrophotometric assay of the glucose product (Lavery et al., 2007). Briefly, right atrial biopsies were pulverised under liquid nitrogen, an aliquot of 50-100mg tissue weighted into an Eppendorf tube and 200μL of 2M KOH added. The tissue/alkali mixture was heated at 70°C for two hours and shaken occasionally to aid digestion. After cooling, 1ml (five volumes) of absolute ethanol was added to promote the precipitation of glycogen, left on ice for 30 minutes then centrifuged for 2 minutes at 13,000rpm. To recover the glycogen, the supernatant was decanted off, leaving a pellet which was allowed to dry before 200μL of distilled water was added and mixed thoroughly to redissolve. The washing process was repeated twice before redissolving the pellet in 500μL of acetate buffer containing 100mM sodium acetate at pH 4.5. To this solution, 50μL of *α-amyloglucosidase* (50mg/ml) from *Aspergillus niger* (Sigma-Aldrich, Poole, UK) in acetate buffer was added and left overnight at room temperature to digest the glycogen into free glucose units.

To a 96-well plate, 6µL of each sample or standard and 450µL of *glucose oxidase* reagent (Thermo-Electron) were added in duplicate and mixed thoroughly. After 10 minutes, glucose concentrations were determined by enzymatic colourimetry with absorbances read at 490nm. Standard curves were constructed and the glucose concentration of each sample calculated using its linear regression as a reference. Concentrations were expressed as glucose units from glycogen per mass of tissue.

Myocardial carbohydrate metabolites

The extraction of carbohydrate metabolites was performed by the method of Hohorst (1963). Briefly, 30-50mg of right atrial tissue was weighted into Eppendorf tubes and 400µL of 0.6M perchloric acid added. Samples were homogenised using glass beads (100µL equivalent volume) in a Mini-Beadbeater (BioSpec Products, Bartlesville, OK) in short bursts to prevent sample overheating, neutralised with 330µL of 0.5M KOH (pH ~8.0), centrifuged for two minutes at 5000rpm and the supernatant recovered. Metabolite concentrations were determined using enzymatic colourimetric techniques as outlined below with absorbances read on a spectrophotometer at 340nm. Standard curves were constructed and concentrations calculated using its linear regression as a reference. Concentrations were recorded per unit mass of tissue.

Tissue pyruvate and lactate were estimated using the methods of Neville and Gelder (1971). Briefly, for pyruvate 50µL of supernatant or standard were added in duplicate to a 96-well plate. To each well were added 200µL of 0.1M tris-HCl buffer solution, 50µL of NADH solution (5mg/ml) and 10µL of *lactate dehydrogenase* solution (10mg/ml) and mixed well. The assay was run at room temperature to completion of

NADH consumption. For lactate, 50µL of supernatant or standard were added in duplicate to a 96-well plate. 200µL of NAD solution (10mg/ml) in buffer and 10µL of *lactate dehydrogenase* solution (10mg/ml) were added to each well and mixed well. The assay was run at room temperature to completion of NADH production.

Tissue glucose-6-phosphate (G6P) concentration was estimated using the method described by Hohorst (1963). Briefly, 200µL of supernatant or standard were added in duplicate to a 96-well plate. 200µL of NADP solution (10mg/ml) in buffer and 10µL of *glucose-6-phosphate dehydrogenase* solution (10mg/ml) were added to each well, mixed thoroughly and run at room temperature to completion of NADPH production.

Myocardial lipids

Lipids were extracted from atrial biopsies using the method described by Folch *et al*. (1957). In brief, 50-100mg of right atrium was weighed out, ground to a fine powder by pestle and mortar under liquid nitrogen, added to 20ml of Folch solvent (chloroform/methanol in a 2:1 ratio) in an extraction tube and shaken vigorously. 5ml of distilled water was added to break the solvent phases, mixed well and the solution left to separate into two phases. The lower, solvent fraction was recovered, transferred to a clean dry test tube and evaporated to dryness under a stream of inert gas in a fume cupboard. The residue was resuspended in 500µL of absolute ethanol and the concentrations of various lipids determined as follows.

The triacylglycerol (TAG) content was estimated by the Trinder method using a commercial assay (Randox) according to the kit instructions. Briefly, 10µL of lipid

suspension or standard were added in duplicate to a 96-well plate with 200μL of reagent, containing *lipases*, *glycerol kinase*, *glycerol-3-phosphate oxidase* and *peroxidase*. TAG concentrations were determined by the method of Jacobs & Vandemark (1960) using enzymatic colourimetry with absorbances read at 492nm, using the standard as a reference and recorded per unit mass of tissue.

The phospholipid content of atrial samples was estimated using a commercial assay (Sentinel, Milan, Italy) according to the kit instructions. Briefly, 5μL of lipid suspension or standard were added to 500μL of enzyme reagent, containing *phospholipases*, *choline oxidase* and *peroxidase*, in Eppendorf tubes and 200μL aliquots transferred in duplicate to a 96-well plate. Phospholipid concentrations were determined using enzymatic colourimetry with absorbances read at 520nm, using the standard as a reference and recorded per unit mass of tissue.

Histological sectioning and staining for lipids

Atrial biopsies embedded in OCT were transferred to a cryostat maintained at -20°C. Samples were mounted perpendicular to the cutting blade and sequential 10μm sections were carefully cut from a representative depth to provide the maximum surface area of atrial muscle. Sections were applied to polylysine-coated histology slides in duplicate and stored at -20°C until analysis. Myocardial lipid content was estimated by Oil Red O staining using a method adapted from Koopman *et al*. (2001). Briefly, sections were air dried for 30 minutes, outlined with a wax pen and fixed by immersion in 3.7% formalin (Sigma-Aldrich) for 30 minutes. Slides were rinsed in phosphate buffer solution (PBS) thrice for 30 seconds each and laid flat. 200μL of Oil

Red O stain (Oil Red O 300mg/L in 60% triethyl phosphate) was layered onto the specimens and incubated at room temperature for 15 minutes. Slides were rinsed again in PBS thrice for 30 seconds then in water for 10 minutes and cover slips applied with 50% glycerol. An AxioScope darkfield fluorescent microscope (Zeiss, Germany) with a monochromator and Texas Red filter was used to view the slides. Digital images were captured of four different x400 magnification fields per slide with AxioCam and the area of lipid staining was quantified by RGB colour thresholding using ImageJ image processing software (NIH, Bethesda, MD).

Statistical analysis

Data is presented as mean (standard deviation) or median (interquartile range). Mean values of the treatment and control groups were compared using independent Student's t-tests. Spearman's rank correlation was used to compare non-parametric data. All analyses were conducted blinded to the group allocation. An outline of the statistical tests used in this thesis is contained in appendix 9.6.

6.2.2 Results

Serum analyses

Biochemical analyses were performed on serum from 35 patients: 16 in the treatment group, 19 in the control group; the perhexiline group had a median serum perhexiline level of 0.37mg/L (IQR 0.19-0.62) after 12.5 days of therapy (IQR 10.5-14.0). There were no differences in mean glucose, insulin, β-hydroxybutyrate or TAG pre-ischaemia at induction of anaesthesia although FFA were found to be significantly higher in the perhexiline group than in the control group ($p<0.001$) (table 10).

	Control (n=19)	**Perhexiline (n=16)**	**p value**
Glucose, mmol/L	6.81 (1.75)	6.91 (2.17)	0.88
Triacylglycerol, mmol/L	0.14 (0.07)	0.16 (0.10)	0.45
Free fatty acids, mmol/L	0.50 (0.13)	0.75 (0.08)	**<0.001**
β-hydroxybutyrate, mmol/L	0.57 (0.25)	0.67 (0.46)	0.44
Insulin, nmol/L	0.27 (0.11)	0.32 (0.13)	0.32

Table 10. Serum metabolite and insulin concentrations pre-ischaemia.

At six hours after release of the aortic cross-clamp, the difference in serum FFA had been lost: 0.73mmol/L (SD 0.15) in the perhexiline group and 0.70mmol/L (SD 0.21) in the control group (p 0.64). Serum TAG was approximately 7-fold higher than pre-ischaemia ($p<0.001$) but again there was no difference between groups at six hours: 1.06mmol/L (SD 0.53) in the perhexiline group versus 1.10mmol/L (SD 0.38) in the

control group (p 0.79) (figure 28). There was a strong negative correlation between serum TAG concentration at six hours and age (r_s -0.53, p 0.002) but no correlation with body mass index (r_s -0.01, p 0.95).

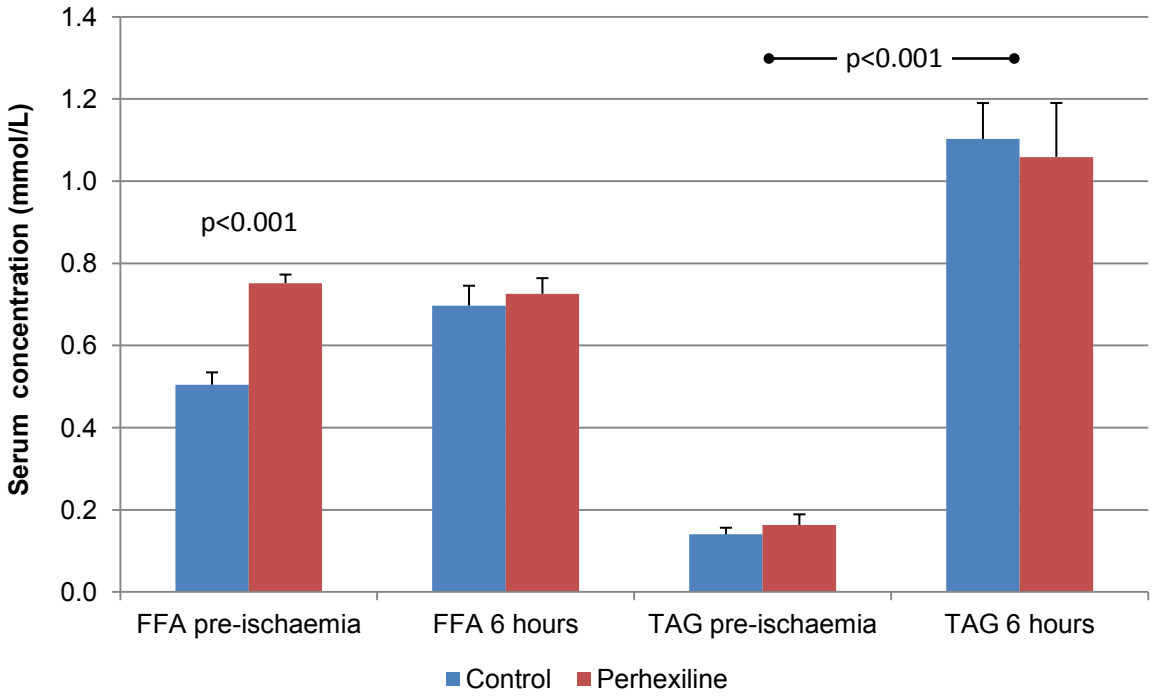


Figure 28. Serum lipid profile pre-ischaemia and at six hours into reperfusion.

Myocardial carbohydrates

Atrial glycogen concentration was measured in atrial biopsies from 101 patients: 50 in the treatment group, 51 in the control group; those in the perhexiline group had a median serum perhexiline level of 0.31mg/L (IQR 0.13-0.50) after 8.5 days of therapy (IQR 6.5-11.5). Groups were remarkably similar with a mean glycogen concentration of 4.74µmol/100g (SD 1.74) in the perhexiline group versus 4.73µmol/100g (SD 1.63) in the control group (p 0.97). There was no correlation between atrial glycogen and serum perhexiline (r_s 0.02), days of therapy (r_s 0.02), body mass index (r_s 0.05) or

baseline cardiac index (r_s 0.13, p 0.18) although there was a moderate negative correlation with age (r_s -0.37, p<0.001).

The concentration of carbohydrate metabolites was measured in right atrial biopsies from 18 patients: seven in the treatment group, 11 in the control group; those in the perhexiline group had a median serum perhexiline level of 0.45mg/L after 12.5 days of therapy. There were no significant differences in the mean concentrations of lactate (Px: 47.2μmol/g, SD 16.3; control: 45.9μmol/g, SD 26.1, p 0.89), pyruvate (Px: 11.0μmol/g, SD 2.85; control: 12.5μmol/g, SD 6.53, p 0.53) or G6P (Px: 6.54μmol/g, SD 1.33; control: 6.62μmol/g, SD 2.97, p 0.93) between groups (figure 29). There was also no difference in the lactate-pyruvate ratio between groups: 4.65 (SD 0.91) in the perhexiline group versus 3.93 (SD 0.44) in the control group (p 0.50).

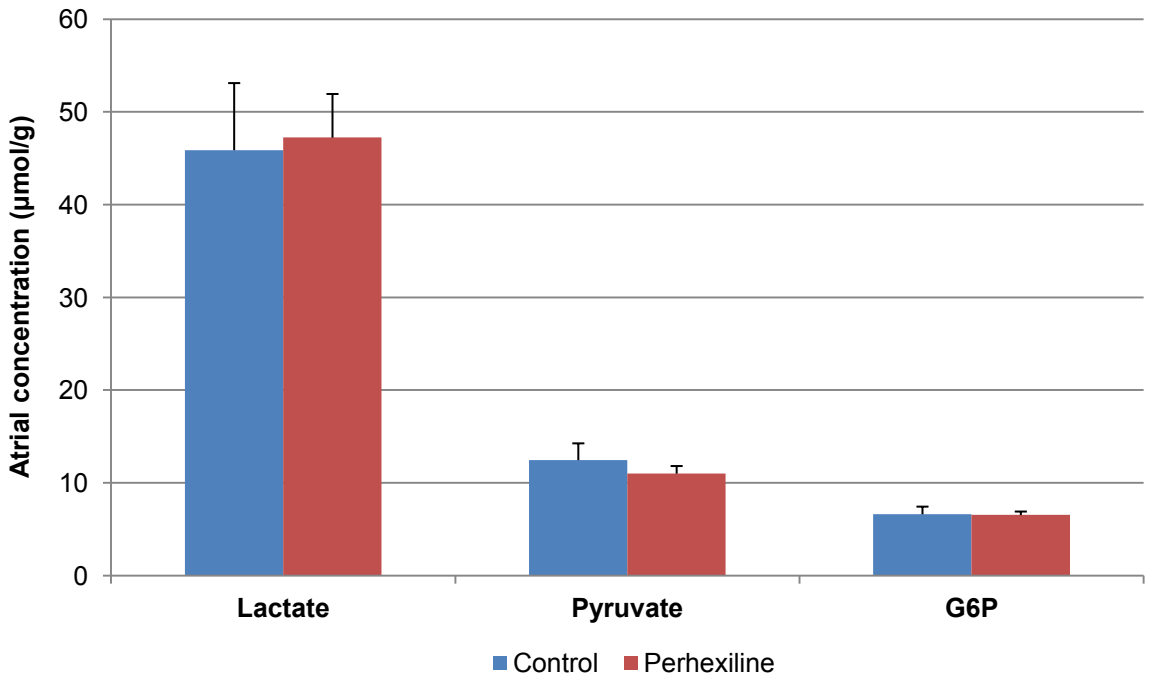


Figure 29. Lactate, pyruvate and G6P concentrations in right atrium.

Myocardial lipids

TAG and phospholipids were measured in atrial biopsies from 25 patients: 12 in the perhexiline group, 13 in the control group; the perhexiline group had a median serum perhexiline level of 0.35mg/L (IQR 0.14-0.58) after 12 days (IQR 10.5-13.5). There were no significant differences in the mean concentrations of TAG (Px: 31.2μmol/g, SD 18.1; control: 45.4μmol/g, SD 34.8, p 0.21) or phospholipid (Px: 12.9μmol/g, SD 8.5; control: 15.0μmol/g, SD 8.8, p 0.54) (figure 30). There was no correlation with serum perhexiline (r_s -0.34, p 0.28) although the sample size was small (n=12).

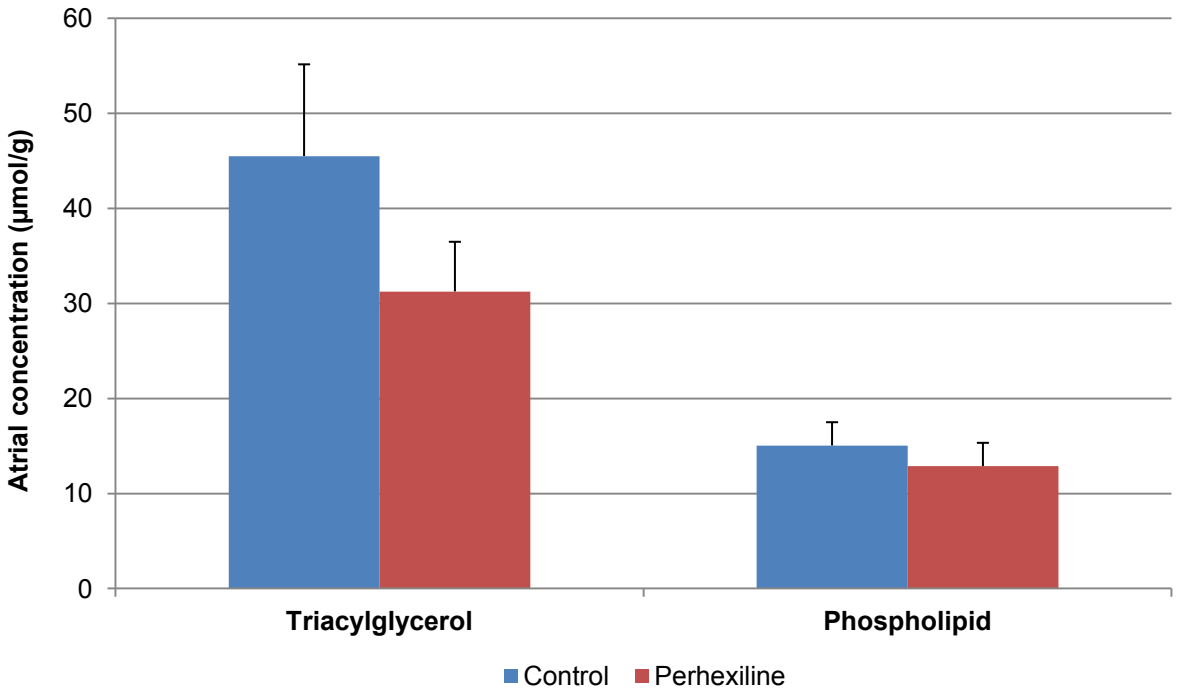
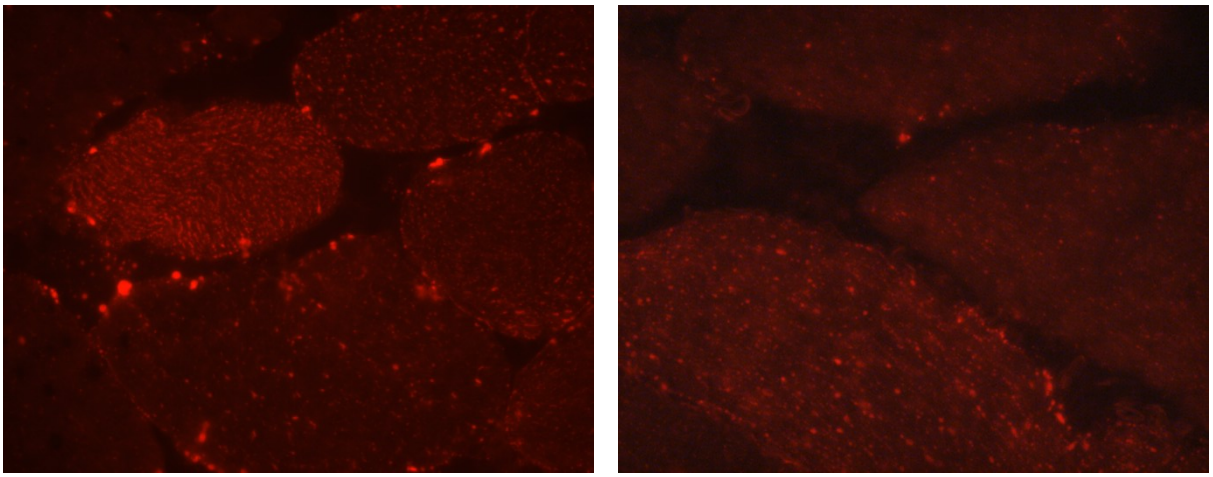


Figure 30. Triacylglycerol and phospholipid concentrations in right atrium.

Histology was performed on atrial biopsies frozen in OCT from 13 patients: five in the perhexiline group, eight in the control group (figure 31). There was no significant difference between groups in the percentage area of tissue staining for lipid at x400 magnification: 3.1% (SD 0.75) in the perhexiline group versus 4.7% (SD 3.11) in the

control group (p 0.20) (figure 32). However, there was marked inter-individual variation, particularly in the control group with a 7-fold difference between the highest and lowest percentages of tissue staining. In addition, on light microscopy, lipid droplets were often visualised on the surface of the sections suggesting that there may have been contamination from residual epicardial adipose tissue.



a b

Figure 31. Oil Red O staining of (a) control and (b) perhexiline atrium (x400).

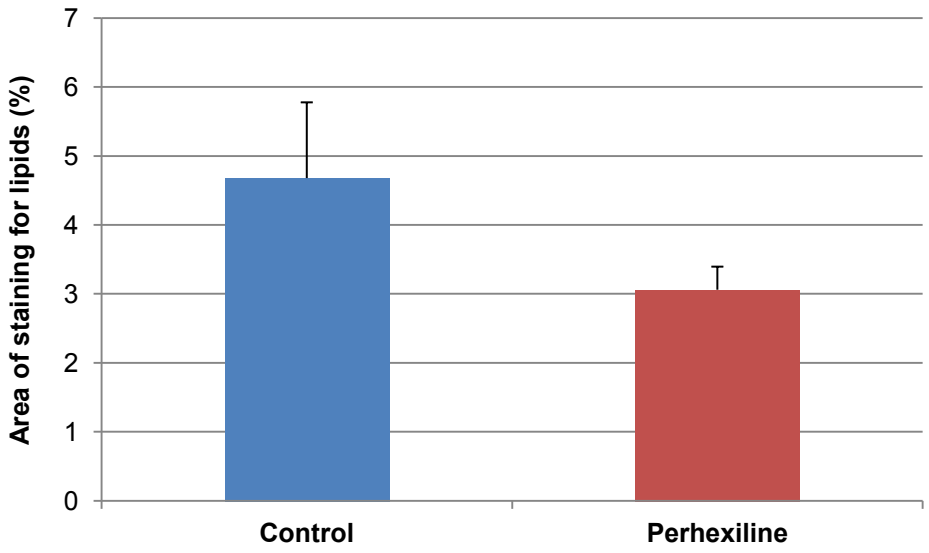


Figure 32. Area of Oil Red O staining of control and perhexiline atrium.

6.3 Metabolomic analysis

6.3.1 Metabolomics: understanding the biochemical network

Metabolomics is the measurement of small molecules within a cell, tissue, biofluid or organism and represents a paradigm shift in metabolic research (Griffin et al., 2011), attempting to profile the complexity of metabolic networks rather than focusing on single pathways. It tells a story downstream of other functional-genomic approaches (figure 33), providing a snap-shot of cellular physiology to demonstrate actual changes in biological systems rather than their mechanisms of regulation. Over the last decade, metabolomics has rapidly grown in importance for the understanding of systems biology and the discovery of new biomarkers of disease.

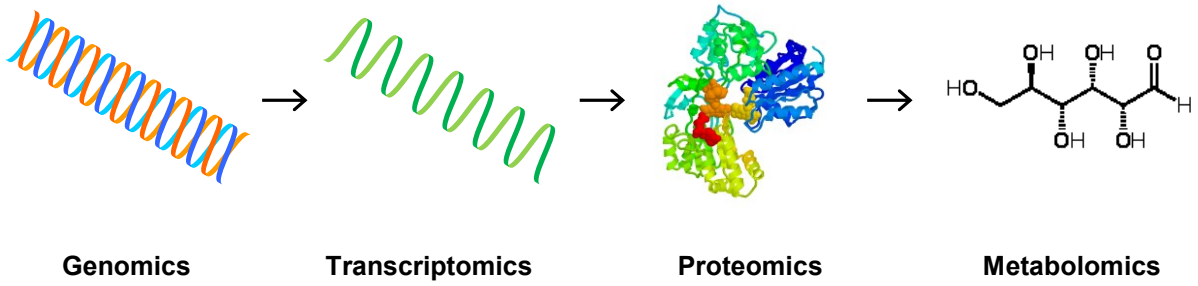


Figure 33. The spectrum of functional-genomic analyses.

The Human Metabolome Database already contains over 8,500 metabolites that are found in human tissues and biofluids at concentrations over 1 μmolar (Wishart et al., 2009). No single analytical tool can measure all of the metabolites that exist over a wide range of molecular masses, polarities and concentrations. The two most commonly used techniques are nuclear magnetic resonance (NMR) spectroscopy

and mass spectrometry (MS). NMR utilises the property of certain nuclei, including ^{1}H, ^{13}C and ^{31}P, to spin when placed inside a magnetic field; the frequency at which a nucleus resonates is determined by coupling with adjacent nuclei and its chemical environment enabling a high specificity for determining chemical structure. Whilst it is a fast, simple test with high analytical reproducibility, NMR lacks sensitivity for metabolites at low biological concentrations. On the other hand, MS has an ionisation source, an ion trap, a mass analyser and a detector to measure the mass-to-charge ratio (*m/z*) of positive or negative ions in the mixture. Highly sensitive and specific, it generates a vast quantity of data in the form of a mass spectrum (figure 34) with each peak representing the relative abundance of molecules with a particular *m/z*.

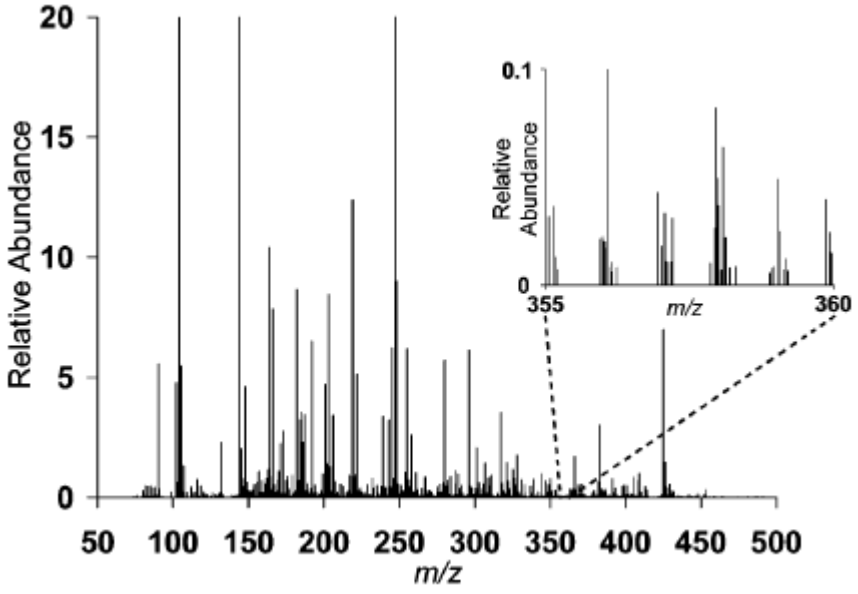


Figure 34. Representative FT-ICR mass spectrum with SIM-stitching method.[3]

[3] Reprinted with permission from Southam AD, Payne TG, Cooper HJ, Arvanitis TN, Viant MR. Dynamic range and mass accuracy of wide-scan direct infusion nanoelectrospray Fourier Transform Ion Cyclotron Resonance Mass Spectrometry-based metabolomics increased by spectral stitching method. Analytical Chemistry, 79(12), 4595-4602. Copyright 2007 American Chemical Society.

Fourier transform ion cyclotron resonance (FT-ICR) MS measures mass by detecting the image current produced by ions cyclotroning in a magnetic field and is a particularly powerful tool for analysing complex metabolite mixtures due to its ultra-high mass resolution and accuracy (Southam et al., 2007). In principle, this enables the empirical formula of many low molecular weight metabolites to be unambiguously identified based upon mass alone. Positive and negative ions can be evaluated but to detect molecules with functional groups that readily lose a proton, e.g. carbohydrates and oligonucleotides, negative ion mode is used. Direct infusion nanoelectrospray is a technique that has high reproducibility of the *m/z*-dependent axis, shorter analysis times and substantially reduced differential ionisation of metabolites. There are only a few laboratories in the world that currently have expertise in these technologies.

FT-ICR mass spectra require extensive data processing. Multiple narrow overlapping spectra are collected and analysed in selected ion monitoring (SIM) mode; spectra are then combined by SIM-stitching to produce a spectrum with high mass accuracy across a wide mass range (Southam et al., 2007). This technique generates a single spectral fingerprint for each biological sample, facilitating peak characterisation, data handling and multivariate analysis. Other stages of data processing include filtering, normalisation, false discovery rate (FDR) control and peak identification through an algorithm enhanced with knowledge of metabolic pathways (Weber and Viant, 2010).

In this section, I report an overview of the techniques used to analyse the myocardial metabolome of pre-ischaemia left ventricular biopsies and compare the negative ion mode profiles of patients in the perhexiline and placebo groups of the clinical trial.

6.3.2 Methods

The experiments described in this section were conducted in the laboratory of Professor Mark R. Viant in the School of Biosciences at the University of Birmingham. Methods for the metabolomic analysis of human myocardial biopsies using a state-of-the-art FT-ICR mass spectrometer have been refined by our group to reduce mass variation and signal-to-noise ratio and to improve confidence of metabolite peak identification.

Tissue specimens

Transmural Tru-Cut needle (Allegiance, McGaw Park, IL) biopsies of the left ventricular free-wall between the left anterior descending artery and the first diagonal branch were taken at three time points, as described previously, but only the baseline pre-ischaemia biopsy (on CPB but before application of the aortic cross-clamp) was used in this experiment. Biopsies were promptly snap-frozen in liquid nitrogen and stored at -80°C until analysis.

Metabolite extraction

Metabolites were extracted using a methanol:water:chloroform solvent system (Wu et al., 2008). A blank was prepared using the same extraction method except that no biopsy material was added. Samples were dried using a centrifugal concentrator (Thermo Savant, Holbrook, NY) and stored at -80°C. Each dried polar extract was re-dissolved in 60µL of 80:20 methanol:water containing 20mM ammonium acetate for negative ion analysis, vortexed and centrifuged at 10,000-g prior to MS. QC samples were prepared by pooling an equal aliquot of each sample.

Mass spectrometry

MS analyses were conducted using a hybrid 7-Tesla linear ion trap FT-ICR mass spectrometer (LTQ FT Ultra, Thermo Fisher Scientific, Germany) equipped with a Triversa chip-based nanoelectrospray ion source (Advion Biosciences, NY) using conditions as described previously (Weber et al., 2011). Three mass spectra were collected for each sample using a selected-ion-monitoring (SIM) stitching method from *m/z* 70 to 740 in negative ion mode (Southam et al., 2007, Weber et al., 2011), processed (Payne et al., 2009, Hrydziuszko and Viant, 2011), normalised and generalised-log transformed as reported previously (Dieterle et al., 2006, Parsons et al., 2007). This produced a peak intensity matrix representing the metabolic profile of each extracted biopsy. Using MI-Pack software (Weber and Viant, 2010), *m/z* measurements were putatively annotated.

Statistical analysis

Student's t-tests were conducted on the non-generalised log-transformed peak intensity matrix, using an FDR of 5% to correct for multiple testing, to determine if individual peaks changed significantly between the two groups (Benjamini and Hochberg, 1995). In addition, principal components analyses (PCA) and partial least squares discriminant analyses (PLS-DA) were conducted to discover metabolic differences between the groups (Nicholson et al., 2002). Internal cross-validation with venetian blinds was applied to assess for over-fitting of the optimal PLS-DA model. An outline of the statistical tests used in this thesis is contained in appendix 9.6.

6.3.3 Results

Polar extracts from pre-ischemia left ventricular biopsies were analysed from 43 patients: perhexiline n=22, control n=21. All biopsies from the perhexiline group were from patients above the lower end of the therapeutic range (median 0.36mg/L, IQR 0.24-0.58). The median spectral relative standard deviation (RSD), a benchmark to assess reproducibility in metabolomics (Parsons et al., 2009), for each sample analysed in triplicate by MS was relatively small and consistent across all samples (mean 11.6%, SD 1.6%).

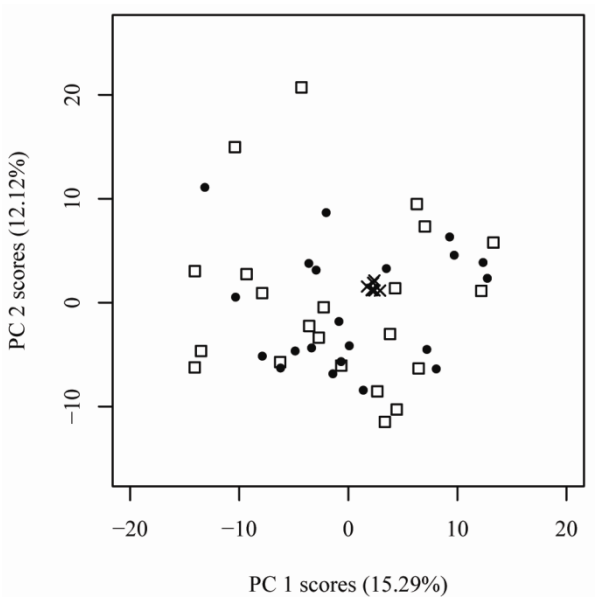
After data processing, the final intensity matrix consisted of 4039 peak intensity measurements for each sample. All peaks were examined using univariate statistics to determine if any changed intensity significantly in response to perhexiline therapy (table 11). No significant peak intensity changes were found with FDR 5% correction.

Number of *m/z* measurements	**p value criteria**		
	<0.01	<0.05	<0.5
Before FDR correction at 5%	4	19	142
After FDR correction at 5%	0	0	0

m/z indicates mass-to-charge ratio; FDR, false discovery rate.

Table 11. *m/z* measurements changing significantly following Student's t-test.

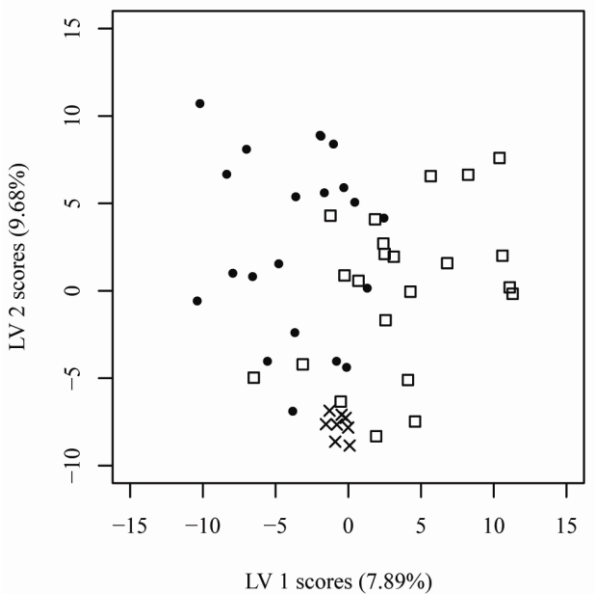
Multivariate PCA was used to reduce the dimensionality of the data and visualise the metabolic similarities and differences between groups; the PCA scores plot showed no differential grouping along the PC1 and PC2 axes suggesting that there was no metabolic response to perhexiline (figure 35). Clustering of the eight QC samples demonstrates the consistency in MS instrument performance over time.



Perhexiline (□) and control (●) left ventricular extracts and quality control (x) samples.

Figure 35. PCA scores plot from analysis of negative ion mass spectra.

In addition, PLS-DA was conducted to maximise separation of the metabolic profiles. Control and treatment samples were minimally separated and the associated mean classification error rates of the model for predicting class membership were high at 43% and 47%, respectively, again suggesting no metabolic differences between groups (figure 36). More than 200 *m/z* measurements in the FT-ICR MS dataset were assigned to at least one putative named metabolite including ATP, creatine, phosphocreatine, glycolytic and citric acid cycle intermediates (appendix 9.7).



Perhexiline (□) and control (●) left ventricular extracts and quality control (x) samples.

Figure 36. PLS-DA scores plot from analysis of negative ion mass spectra.

6.4 Discussion

The metabolic effects of perhexiline were first documented in a rat heart model and appear to involve a metabolic shift from β-oxidation of fatty acids to glucose utilisation through inhibition of *CPT-1* (Kennedy et al., 1996). In patients, metabolism has been assessed indirectly using non-invasive (^{31}P magnetic resonance spectroscopy) (Abozguia et al., 2010) and invasive (coronary sinus catheter) (Beadle et al., 2011) techniques to determine cardiac energetics, respiratory quotient and mechanical efficiency. However, the studies presented in this chapter are the first to directly examine the effect of perhexiline on the human myocardium. Indeed, I believe that this analysis of left ventricular biopsies is the first to investigate the effects of any drug on the human myocardium using MS metabolomics.

On serum analysis, the concentration of FFA at the time of anaesthesia was found to be significantly higher in patients treated with perhexiline compared with controls with no differences in TAG, glucose, β-hydroxybutyrate or insulin. At six hours into reperfusion, there was no difference between groups for serum FFA or TAG but the concentration of TAG was seven-fold higher than pre-ischaemia. Cardiac surgery is known to precipitate a marked rise in FFA due to endogenous and exogenous catecholamine release and the administration of heparin. Exposure of the post-ischaemic heart to high levels of FFA during reperfusion inhibits the *PDH* complex and uncouples glucose oxidation (Lopaschuk et al., 1994). The marked rise in serum TAG at six hours represents the lag phase of FFA release with uptake by the liver, re-esterification and export back into the blood. Perhexiline did not appear to suppress

the serum surge in FFA during reperfusion and indeed, pre-ischaemia levels were higher than in controls. As perhexiline is a competitive inhibitor of *CPT-1*, any potential inhibition may have been reduced even before ischaemia and would almost certainly have been overwhelmed during reperfusion.

In the atrial myocardium, there were no significant differences in pre-ischaemia glycogen, lactate, pyruvate, glucose-6-phosphate, triacylglycerol or phospholipid between groups. However, significant variation within the groups suggests that the concentration of metabolites is determined by factors other than perhexiline therapy, despite likely reaching steady-state atrial concentrations. These results concur with another recent study conducted in Birmingham. In a double-blind trial, cardiac energetics and substrate utilisation were assessed in patients with non-ischaemic dilated cardiomyopathy using ^{31}P magnetic resonance spectroscopy (Beadle et al., 2011). Perhexiline was found to significantly improve the myocardial PCr/ATP ratio and NYHA functional class compared with controls. However, preliminary invasive data suggests no improvement in efficiency or change in respiratory quotient across the myocardium between the aorta and the coronary sinus (Beadle, 2011).

Furthermore, on studying the polar metabolome of the left ventricle prior to ischemia using ultra-high resolution MS, I found that the metabolic profiles of patients on perhexiline therapy were indistinguishable from controls. This metabolomic analysis demonstrates that perhexiline has no significant effect on the myocardial metabolome including any of the intermediates of energy transfer, glycolysis or the citric acid cycle (appendix 9.7).

The experiments presented in this chapter independently demonstrate that in patients with ischaemic heart disease undergoing cardiac surgery following treatment with perhexiline, there is no evidence of a shift in myocardial metabolism. The lack of upregulation of glucose pathways or cellular accumulation of lipid intermediates suggests that there was no significant inhibition of *CPT-1* in the heart. Rather than its primary mechanism of action, *CPT-1* inhibition may occur only after prolonged accumulation of the drug in the myocardium which was not evident following a short period of therapy; any metabolic effects of perhexiline may therefore contribute only to its toxic rather than its therapeutic effects. The possibility that perhexiline exerts anti-ischaemic effects predominantly via a *CPT*-independent pathway has been raised previously. Unger *et al*. (2005) observed a temporal dissociation between its effects on cardiac efficiency and metabolism in the rat. Other potential mechanisms of action include inhibition of neutrophil superoxide formation (Kennedy et al., 2006) and potentiation of platelet responsiveness to nitric oxide (Willoughby et al., 2002).

In this chapter, I found that perhexiline does not appear to suppress the surge in serum FFA associated with reperfusion in cardiac surgery. I observed no significant difference in the concentration of any carbohydrate or lipid intermediates in the right atrial myocardium prior to ischaemia with perhexiline. I presented novel human data suggesting that treatment with perhexiline has no significant effect on the MS-visible polar myocardial metabolome *in vivo* at therapeutic serum concentrations. These findings support the suggestion that perhexiline acts via a pathway that is largely or entirely independent of myocardial *CPT-1* inhibition (Unger et al., 2005). Further evidence is required to elucidate its mechanism of action in the human heart.

7. Results of the Clinical Trial

Three hundred and twenty seven patients undergoing coronary artery bypass graft (CABG) surgery at two centres were recruited to the trial and randomised to perhexiline or placebo between February 2007 and April 2010. Of these, 286 patients for whom data on the primary endpoint was available, were included in the analysis on an intention-to-treat basis: 139 in the treatment arm and 147 in the control arm. In the treatment arm, 98/135 (72.6%) patients were shown to be within or above the therapeutic range for serum perhexiline at the time of surgery; in the remaining four patients, it was not measured. Perhexiline was not detected in any patients in the control arm, confirming the difference between the groups.

In this chapter, I document the clinical findings of the trial according to the primary and secondary endpoints as defined in the statistical analysis plan (appendix 9.5). I perform an exploratory analysis to examine the primary endpoint in patients above the lower threshold of the therapeutic range against propensity score-matched controls, thereby excluding those who were sub-therapeutic. I assess the impact of serum perhexiline concentration on cardiac index, review the safety endpoints used to monitor the trial and report other postoperative outcomes. Finally, I discuss the impact of these findings on the use of perhexiline in patients undergoing cardiac surgery and explore potential explanations for the results of the trial, in particular the potential effect of perhexiline on the pre-ischaemia cardiac index.

7.1 Primary outcome

The primary endpoint was *an episode of low cardiac output* in the first six hours after removal of the aortic cross-clamp, defined as hypotension (mean arterial pressure < 65mmHg) with a cardiac index < 2.2L/min/m^2 in the presence of adequate filling pressures (CVP 8-12mmHg or PCWP 12-16mmHg) and heart rate (> 75bpm) where systolic blood pressure < 90mmHg and/or inotropic support ± intra-aortic balloon pump for > 60 minutes was required to maintain such a clinical picture; this was adjudicated in borderline cases by a blinded endpoints committee.

An episode of low cardiac output was diagnosed in 102 (35.7%) of the 286 patients analysed; however, there was *no significant difference* in incidence between the two groups: 51 (36.7%) in the perhexiline arm and 51 (34.7%) in the control arm (OR 0.92, 95% CI 0.56 to 1.50, p 0.74). In the exploratory analysis, patients in the treatment arm who were above the lower threshold of the therapeutic range for serum perhexiline (≥ 0.15mg/L) at the time of surgery were compared with propensity score matched controls (n=97 in each group) (table 12). There was *no significant difference* in the incidence of low cardiac output episodes in these groups: 38 (39.2%) in the perhexiline arm and 31 (32.0%) in the control arm (OR 0.73, 95% CI 0.40 to 1.33, p 0.30). A further analysis was performed to assess the effect of perhexiline on the incidence of an episode of low cardiac output in several prespecified sub-groups and found no significant heterogeneity between groups (figure 37).

	Control (n=97)	Perhexiline (n=97)
Age (years), median (IQR)	65 (59-72)	66 (60-73)
Weight (kg), median (IQR)	80 (72-88)	80 (72-88)
Serum perhexiline (mg/L), median (IQR)	0	0.39 (0.23-0.56)
Left ventricular function, n (%)		
Good, ≥ 50%	79 (81.4)	81 (83.5)
Moderate, 30-49%	17 (17.5)	15 (15.5)
Poor, <30%	1 (1.0)	1 (1.0)
Urgent surgery, n (%)	14 (14.4)	12 (12.4)

Table 12. Propensity score-matched groups for exploratory analysis.

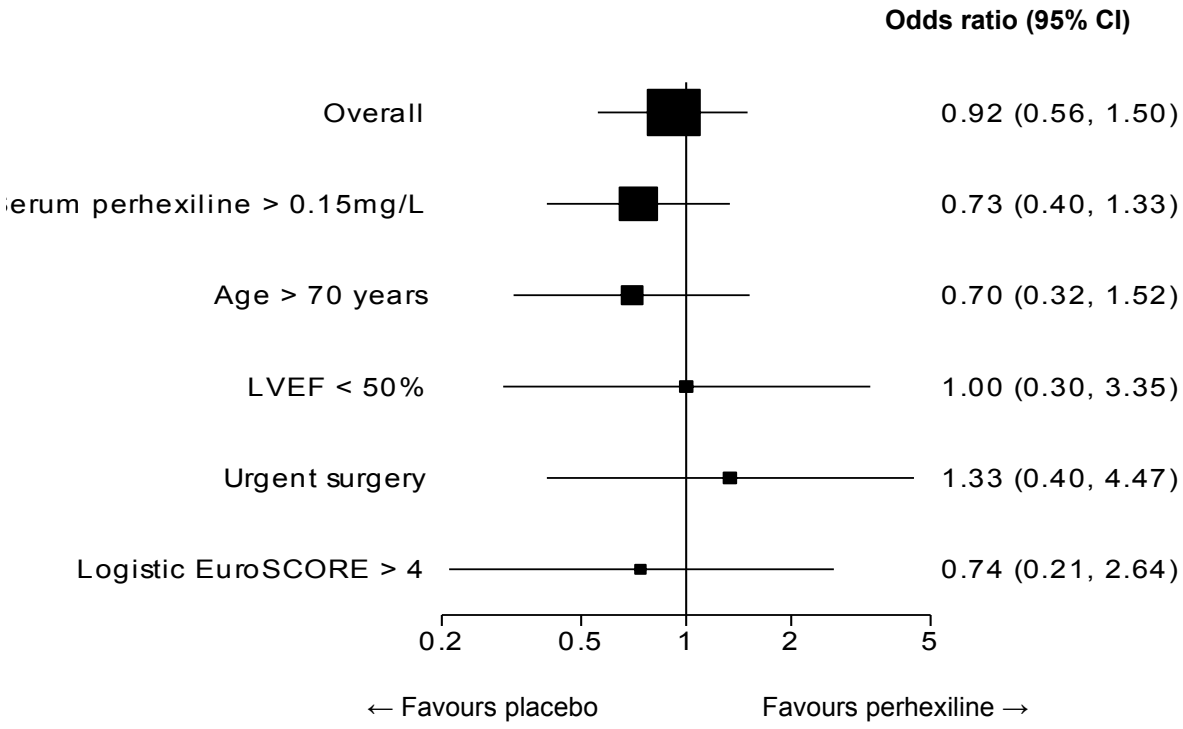


Figure 37. Forest plot for sub-group analyses of the primary outcome.

7.2 Secondary outcomes

7.2.1 Cardiac Index

Cardiac output was measured before ischaemia and at intervals following release of the aortic cross-clamp using a pulmonary artery catheter and indexed to body surface area (figure 38). At six hours following reperfusion, the mean cardiac index was *significantly lower* in the perhexiline group (2.51L/min/m^2, SD 0.43) than in the control group (2.70L/min/m^2, SD 0.54) (Difference in Means 0.17, 95% CI 0.05 to 0.29, p 0.005). By 12 hours, there was no difference between the groups: 2.73L/min/m^2 (SD 0.54) in the perhexiline arm versus 2.79L/min/m^2 (SD 0.48) in the control arm (DiM 0.58, 95% CI -0.06 to 0.18, p 0.34).

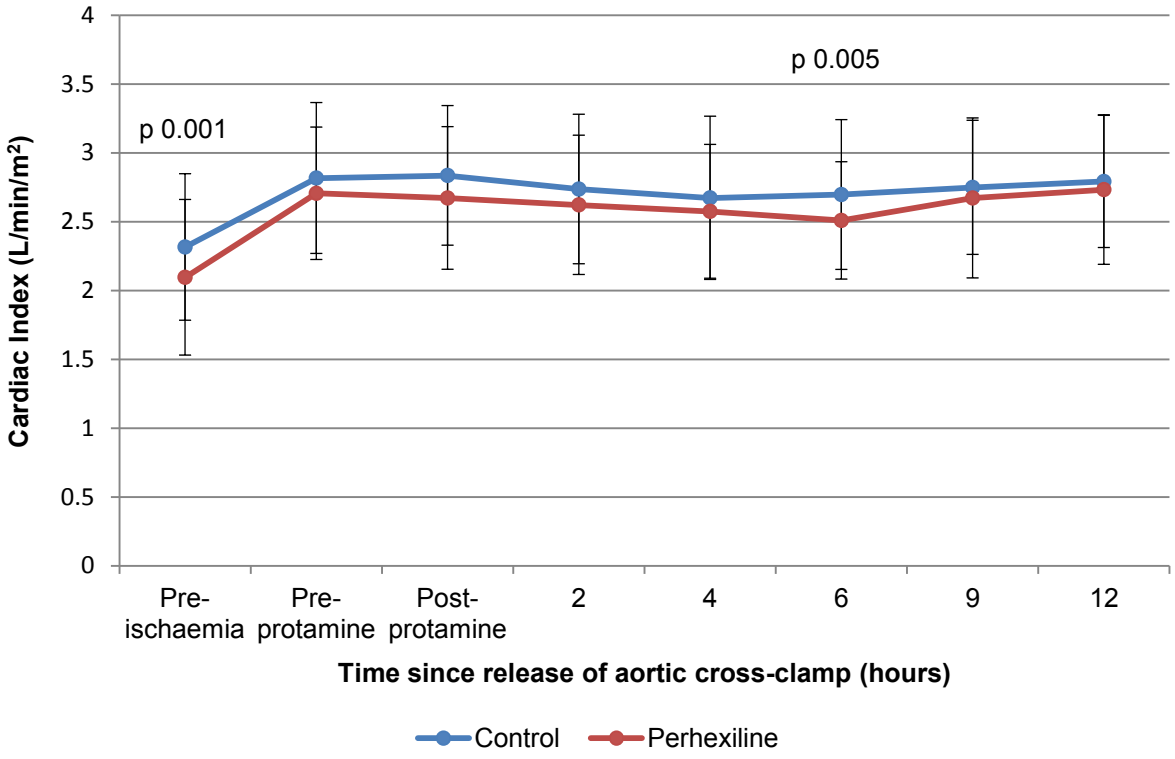


Figure 38. Mean cardiac index during the period of haemodynamic monitoring.

Pre-ischaemia	Control (n=145)	Perhexiline (n=135)	DiM (95% CI)	p value
Heart rate (beats/min)	58 (10.8)	56 (9.0)	1.94 (-0.41-4.28)	0.11
CVP (mmHg)	11 (3.9)	10 (3.9)	0.92 (-0.003-1.83)	0.05
PAWP (mmHg)	13 (4.4)	12 (4.3)	1.08 (0.06-2.10)	**0.04**
MAP (mmHg)	74 (10.2)	72 (9.3)	1.79 (-0.52-4.09)	0.13
Cardiac index (L/min/m^2)	2.31 (0.53)	2.09 (0.57)	0.22 (0.09-0.35)	**0.001**

6 hours of reperfusion	Control (n=144)	Perhexiline (n=135)	DiM (95% CI)	p value
Heart rate (beats/min)	94 (7.4)	93 (6.9)	0.61 (-1.08-2.30)	0.48
CVP (mmHg)	9 (3.0)	9 (3.4)	-0.19 (-0.94-0.55)	0.61
PAWP (mmHg)	10 (3.4)	9 (3.2)	0.77 (0-1.55)	**0.05**
MAP (mmHg)	71 (7.7)	72 (8.7)	-1.02 (-2.96-0.92)	0.30
Cardiac index (L/min/m^2)	2.70 (0.54)	2.51 (0.43)	0.17 (0.05-0.29)	**0.005**

12 hours of reperfusion	Control (n=142)	Perhexiline (n=135)	DiM (95% CI)	p value
Heart rate (beats/min)	93 (7.9)	93 (6.9)	0.54 (-1.23-2.31)	0.55
CVP (mmHg)	9 (3.1)	8 (2.9)	0.56 (-0.17-1.24)	0.14
PAWP (mmHg)	10 (3.0)	9 (2.9)	0.43 (-0.26-1.12)	0.22
MAP (mmHg)	74 (9.2)	73 (9.5)	0.56 (-1.65-2.77)	0.62
Cardiac index (L/min/m^2)	2.79 (0.48)	2.73 (0.54)	0.58 (-0.06-0.18)	0.34

CVP, central venous pressure; PAWP, pulmonary artery wedge pressure; MAP, mean arterial pressure.

Table 13. Haemodynamic variables before and after ischaemia/reperfusion.

However, the cardiac index measured prior to ischaemia was also found to be *significantly lower* in the perhexiline group (2.09L/min/m^2, SD 0.57) than the control group (2.31L/min/m^2, SD 0.53) (DiM 0.22, 95% CI 0.09 to 0.35, p 0.001). Using repeated-measures with pre-ischaemia cardiac index as a covariate, cardiac index was not significantly affected by perhexiline therapy, *F* (3.57, 896.9) = 1.02, p 0.40. *No significant relationship* between serum perhexiline concentration and pre-ischaemia cardiac index was observed on univariate analysis (β -0.05, 95% CI -0.30 to 0.20, p 0.69). There was little difference between the groups in terms of heart rate, filling pressures (CVP and PAWP) or afterload (MAP); there was a trend towards lower filling pressures in the perhexiline group but this was statistically significantly (p≤0.05) only for the PAWP pre-ischaemia and at six hours (table 13).

7.2.2 Inotrope usage after reperfusion

The use of inotropic support in the perioperative period was indicated if the cardiac index remained < 2.2L/min/m^2 despite correction of other haemodynamic variables to within predetermined limits according to the trial protocol, although the decisions on commencement and escalation of inotropes were ultimately at the discretion of the clinical team. There was *no difference* in the prevalence of inotropic support in the first six hours after reperfusion: perhexiline 39/139 (28.1%) versus control 36/147 (24.5%) (OR 0.84, 95% CI 0.49 to 1.44, p 0.52). However, by 12 hours, inotrope use was *significantly more frequent* in the perhexiline group (67, 48.2%) versus the control group (50, 34.0%) (OR 0.55, 95% CI 0.34 to 0.89, p 0.015). Of note, there were *no differences* between groups in the dose requirements for phenylepherine (p 0.26), noradrenaline (p 0.12) or insulin (p 0.43) in the first 12 hours.

7.2.3 Serum troponin-T and ECG evidence of new myocardial injury

Troponin-T is an established marker of irreversible myocyte necrosis and a valuable predictor of complications after cardiac surgery. ECG changes are common although the appearance of new pathological Q waves is suggestive of myocardial infarction. In this trial, there was *no difference* between groups in peak troponin-T concentration in the first 24 hours: 0.78ng/ml (SD 0.71) in the perhexiline patients versus 0.89ng/ml (SD 0.92) in the control patients (DiM 0.11, 95% CI -0.09 to 0.30, p 0.28). Similarly, there was *no difference* in the number of patients in the highest quintile of peak troponin-T (≥1.18ng/ml): 25/134 (18.7%) in the perhexiline group versus 32/143 (22.4%) in the control group (p 0.46). There was also *no significant difference* in area under the concentration-time curve in the first 24 hours between groups: perhexiline 3.98ng.hr/ml (SD 3.79) versus control 4.71ng.hr/ml (SD 5.32) (p 0.12) (figure 39).

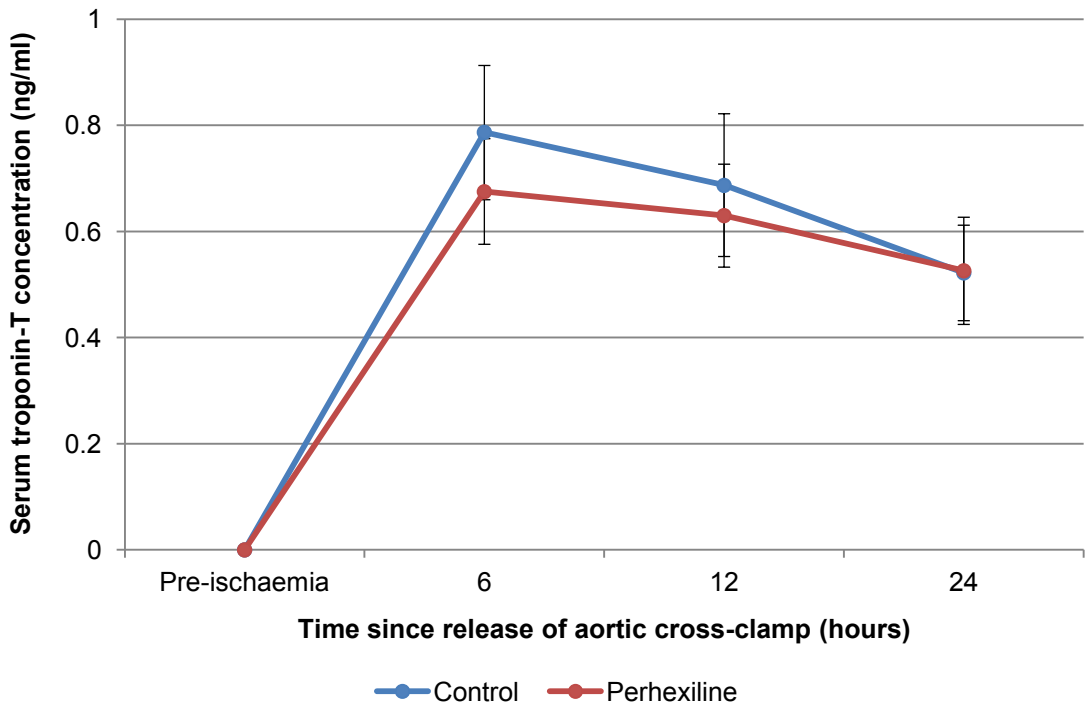


Figure 39. Mean concentration-time curves for the release of troponin-T.

There was *no difference* in the frequency of ECG changes consistent with new myocardial injury: 23/139 (16.6%) in the perhexiline arm versus 25/147 (17.0%) in the control arm (OR 1.02, 95% CI 0.55 to 1.92, p 0.94); nor the composite endpoint of a rise in troponin-T or ECG evidence of injury: 50/139 (36.0%) in the perhexiline arm versus 55/147 (37.4%) in the control arm (OR 1.06, 95% CI 0.65 to 1.73, p 0.82).

7.2.4 Length of stay

There was *no significant difference* in mean length of stay on the Intensive Care Unit between the perhexiline group (102 hours, SD 150) and the control group (105 hours, SD 134) (DiM 3.37, 95% CI -29.9 to 36.7, p 0.84). Likewise, there was *no difference* in mean hospital length of stay: perhexiline 9.2 days (SD 6.9) versus control 9.9 days (SD 9.8) (DiM 0.73, 95% CI -1.27 to 2.73, p 0.47).

7.3 Safety outcomes

The safety of the trial was reviewed at regular intervals by the Data & Safety Monitoring Board (DSMB). The primary and secondary endpoints were evaluated in addition to Serious Adverse Events: death; stroke; renal replacement therapy; and any additional surgical procedures, including chest reopening but with the exception of tracheostomy. A non-symmetrical power function was used to increase likelihood of stopping early for harm. Following all three scheduled DSMB meetings, the board were content for the trial to continue as planned. No extraordinary DSMB meetings were held and the overall frequency of SAEs during the trial is shown in table 14.

	Control (n=147)	**Perhexiline (n=139)**
Death, n (%)	2 (1.4)	2 (1.4)
Stroke, n (%)	1 (0.7)	3 (2.2)
Renal replacement therapy, n (%)	2 (1.4)	3 (2.2)
Chest reopening follow surgery, n (%) *	13 (8.8)	9 (6.5)
Bleeding, n (%)	11 (7.5)	6 (6.5)
LCOE/tamponade, n (%)	1 (0.7)	1 (0.7)
Arrest, n (%)	1 (0.7)	2 (1.5)
Use of CPB, n (%)	1 (0.7)	1 (0.7)
Other operation, n (%)	1 (0.7)	0

LCOE, low cardiac output episode; CPB, cardiopulmonary bypass. * Not significant Fisher's exact test.

Table 14. Frequency of serious adverse events reported during the trial.

7.4 Other outcomes

	Control (n=147)	Perhexiline (n=139)
Neurological injury		
Type I – stroke or TIA, n (%)	2 (1.4)	3 (2.2)
Type II e.g. confusion, disorientation, n (%)	10 (6.8)	15 (10.8)
Any respiratory complication, n (%)	34 (23.1)	33 (23.7)
Pneumonia, n (%)	14 (9.5)	15 (10.8)
Reintubated, n (%)	4 (2.7)	2 (1.4)
Tracheostomy, n (%)	4 (2.7)	4 (2.9)
Peak creatinine (μmol/L), median (IQR)	102 (90-119)	109 (94-128)
AKIN score for change in renal function		
0	126 (85.7)	121 (87.1)
1	16 (10.9)	13 (9.4)
2	0	2 (1.4)
3	5 (3.4)	3 (2.2)
Any abdominal complication, n (%)	4 (2.7)	5 (3.6)
Gastrointestinal bleed, n (%)	2 (1.4)	2 (1.4)
Prolonged paralytic ileus, n (%)	2 (1.4)	0
Any treated infective episode, n (%) *	25 (17.0)	27 (19.4)
Sternal wound infection		
Superficial infection/dehiscence, n (%)	4 (2.7)	5 (3.6)
Deep infection requiring surgery, n (%)	1 (0.7)	0
Leg wound complication, n (%)	7 (4.8)	2 (1.4)
Atrial fibrillation/flutter, n (%) *	66 (44.9)	55 (39.6)
Chest tube drainage at 12 hours (ml), mean (SD)	704 (448)	700 (498)
Transfusion of blood (units), mean (SD)	2.0 (2.6)	2.3 (2.6)
Discharge destination		
Home, n (%)	138 (93.9)	130 (93.5)
Convalescence, n (%)	6 (4.1)	5 (3.6)
Other hospital or department, n (%)	1 (0.7)	2 (1.4)

TIA, transient ischaemic attack; AKIN, Acute Kidney Injury Network * Not significant Fisher's exact test

Table 15. Postoperative outcomes and complications.

7.5 Discussion

In this prospective, double-blind, randomised, placebo-controlled trial conducted at two centres in the United Kingdom, preoperative administration of oral perhexiline to patients undergoing non-emergent surgical revascularisation on cardiopulmonary bypass with cold blood cardioplegic arrest did not improve clinical markers of myocardial protection. The recruitment target of at least 280 patients randomised in a 1:1 ratio to perhexiline or placebo was met. There was no difference in the primary outcome, the incidence of a low cardiac output episode and this lack of effect persisted once patients with a sub-therapeutic serum perhexiline concentration were removed from the analysis. With the exception of cardiac index at six hours, no significant differences were seen for the secondary endpoints, safety outcomes or other postoperative variables.

Cardiac output is the product of heart rate and stroke volume, which itself is determined by preload, afterload and contractility; by clinically optimising heart rate, filling pressures and mean arterial pressure, cardiac index becomes a surrogate measure of contractility. Therefore, any divergence in cardiac index between the groups following ischaemia-reperfusion should reflect the degree of clinical recovery of myocardial function and stunning. A significant difference in cardiac index at six hours, a secondary endpoint, was found but favoured the control group. A similar difference in cardiac index was present pre-ischaemia and once this was taken into account as a covariate in ANOVA, the difference at six hours became non-significant. Whilst the other haemodynamic variables (heart rate, CVP and MAP) were within

protocol limits, pulmonary artery wedge pressure (PAWP) was consistently a mean of 9-10mmHg in both groups, below the generalised, pre-defined target range of 12-16mmHg; there was a statistically significant difference in PAWP at six hours but this is unlikely to have been clinically important as the difference in means was only 1mmHg. The observation that the difference in cardiac index between groups was present pre-ischaemia suggests two possibilities:

The difference in cardiac index was due to chance, bias or confounding.

Countering this argument are the strength of the statistical findings and aspects of study design intended to enhance validity. The low p value of 0.001 means that there is 1 in 1000 probability that the observed difference between the groups is due to chance; a large number of patients (n=286) were entered into the trial and the baseline demographics of the two groups appear similar. To avoid selection bias and confounding factors, patients were randomly allocated using minimisation, an adaptive stratified sampling method to reduce imbalance; this included left ventricular function although it was not precisely quantified on echocardiogram prior to commencing trial therapy. Cardiac output was measured using a validated, reproducible technique, other physiological variables determining cardiac output were actively controlled and both trial participants and investigators were blinded to address measurement bias. The trial was analysed on an intention-to-treat basis, including all 286/294 (97.3%) patients who started the IMP in whom the primary endpoint was available, in order to reduce analysis bias. Perhaps most importantly, no difference between groups was observed for the primary endpoint, other secondary outcomes, safety endpoints or any other postoperative variable.

The difference in cardiac index was due to a negative inotropic effect of perhexiline.

Despite a weak calcium channel inhibitory effect (Horowitz et al., 1986), perhexiline has not previously been found to be negatively inotropic although no previous studies involved anaesthesia or surgery. In a working rat heart model, Unger and colleagues (2005) demonstrated that pre-exposure to perhexiline led to a significant *increase* in heart rate (20%, $p<0.05$), cardiac output (31%, $p<0.01$) and cardiac work (29%, $p<0.05$). In a randomised controlled trial of short-term perhexiline therapy in patients with chronic heart failure, Lee *et al*. (2005) found improvements in left ventricular ejection fraction and resting/peak stress myocardial function. Recent data from our department using magnetic resonance and echocardiography to assess the effect of perhexiline in patients with non-ischaemic cardiac failure has also shown an improvement in cardiac energetics but not ventricular function (Beadle, 2011). Furthermore, if perhexiline was responsible for reducing cardiac output in the study population, it would be expected to be exposure-dependent but no correlation was observed between serum perhexiline concentration and pre-ischaemia cardiac index. High levels of serum FFA are known to reduce cardiac efficiency (Murray et al., 2004) and as shown in the previous chapter, FFA concentrations prior to ischaemia were significantly higher in the perhexiline group (0.75mmol/L, SD 0.08) than in the control group (0.50mmol/L, SD 0.08). However, on linear regression, serum FFA did not predict pre-ischaemia cardiac index (R^2 0.09, *F-ratio* 3.28, p 0.08).

A convincing explanation for the finding of a lower mean pre-ischaemia cardiac index with perhexiline remains elusive. Cardiac index remained lower in the perhexiline group at six hours but by 12 hours, the difference was lost. This late convergence

may be explained by a higher frequency of inotrope use in the perhexiline group. The two outcomes are not independent as institution of inotropic support was prompted by a low cardiac index and its use leads to an elevation in the observed contractility; the increased use of inotropes between six and 12 hours may have been a delayed response to the nadir in cardiac index around six hours (figure 38).

The release of troponin-T was consistently lower in the perhexiline group when assessed by peak value, number of patients in the highest quintile, repeated-measures ANOVA and area under the concentration-time curve; however, none of these measures were significant. A previous trial of myocardial protection in our department (REMOTE) was powered to detect a reduction in AUC for troponin-T in a similar cohort of patients and required 120 subjects (Rahman et al., 2010). Therefore, it is unlikely that the failure to reach significance for troponin-T in this trial was due to it being underpowered. All other secondary endpoints demonstrated no significant difference between groups including predefined safety outcomes (table 14) or other complications (table 15).

In the trial protocol, all outcome measures reflected potential differences between the groups in the early postoperative period and data collection was completed upon death or discharge from the Department of Cardiothoracic Surgery. It did not address any long-term benefits that perioperative perhexiline may bestow upon ventricular dysfunction. Whilst the trial was not designed to measure long-term outcomes, any significant effect on myocardial protection would seem unlikely given that no advantage was discernible in the early postoperative period.

This study is the first high quality, adequately-powered trial to examine the role of a preoperative oral direct metabolic modulator on clinical outcomes of ischaemia-reperfusion in the setting of myocardial protection during cardiac surgery. Our previous trial of GIK in patients undergoing CABG used a similar perioperative protocol and outcome measures but found higher cardiac indices, a lower incidence of LCOE, lower inotrope usage and reduced early release of troponin in the treatment group (Quinn et al., 2006); these findings supported the results of two meta-analyses which found an improvement in perioperative haemodynamics in patients treated with GIK (Bothe et al., 2004, Fan et al., 2011). In this trial, perhexiline failed to improve any of the clinical markers of recovery in myocardial function after cardioplegic arrest. Our findings do not support the use of preoperative perhexiline as an adjunct to myocardial protection in patients undergoing coronary artery bypass graft surgery.

In this chapter, I reported the clinical results of the CASPER trial, including a statistical analysis of the primary, secondary, safety and other outcomes. I demonstrated that the lack of effect of perhexiline was not influenced by patients who failed to reach the lower threshold of the therapeutic range using propensity score-matched controls. I also found that serum perhexiline concentration had no direct impact on cardiac index either before or after ischaemia-reperfusion. I discussed the endpoint analysis but failed to explain why the pre-ischaemia cardiac index was significantly lower in the perhexiline group. In the final chapter of my thesis, I will draw together the results of the clinical trial with those of preceding chapters to present a coherent biological argument on why the trial proved negative.

8. CONCLUSIONS

> *'Surgery of the heart has probably reached the limits set by nature to all surgery; no new method, and no new discovery, can overcome the natural difficulties that attend a wound of the heart.'*
>
> (Paget, 1896)

Over the last sixty years, cardiac surgery has evolved; it is no longer an experimental, last-ditch option for fatal diseases performed by courageous pioneers (Miller, 2000); it has progressed through the age of discovery, when artificial valves were crafted in the garage or hand-sewn at the kitchen table (Starr, 1986); and it has seen the industrial revolution of coronary surgery to successfully treat the twentieth century epidemic of ischaemic heart disease in the developed world. Today cardiac surgery offers widely-accessible, reproducible, evidence-based and increasingly safe treatment options. It drives a global market of multi-billion dollar companies producing a range of precision engineered products that have been implanted millions of times (Gott et al., 2003). Surgical revascularisation remains the gold-standard for the treatment of multi-vessel coronary artery disease (Kolh et al., 2010) whilst advances in transplantation, mechanical assist and surgery for congenital heart disease offer improving survival for otherwise incurable conditions. But even 116 years after Paget's premature epitaph for the speciality, significant challenges remain across the spectrum of cardiac surgery. Myocardial protection research was vogue in the early decades but with the advent of effective cardioplegia, enthusiasm cooled. The changing scope of surgical procedures and demographics of the patients has made it clear that this is not a problem solved; preserving the ischaemic heart remains an important discipline of scientific endeavour.

In this thesis, I have traced the historical development of cardiac surgery and the quest for effective myocardial protection. I have outlined the metabolic pathways that produce cellular energy, their substrates and regulators. I have explored the rapidly evolving field of ischaemia-reperfusion injury and the development of protective strategies from hypothermia to cardioplegia to ischaemic conditioning. I have evaluated the clinical use of metabolic therapies with a systematic review of the literature. I have recalled the troubled story of perhexiline, its long road to redemption and its potential role in myocardial protection. And I have drawn together hypotheses to examine its pharmacokinetics in the heart, directly elucidate its metabolic effects in humans and test its novel clinical application in patients undergoing cardiac surgery.

In this final chapter, I focus on my hypotheses and review the evidence generated in this thesis to assess whether they can be supported or refuted. I draw conclusions from each of the experiments performed to determine how this work adds to the literature on perhexiline and its application to myocardial protection during cardiac surgery. I reflect on my experience of conducting a randomised controlled trial and discuss the limitations of the clinical and laboratory experiments presented. Finally, I present areas of future study, building on the foundation of this thesis to adapt the concepts and techniques gained to address new research questions.

8.1 The pharmacokinetics of perhexiline

'At least 80% of patients will be within the therapeutic range for serum perhexiline at the time of surgery with a standard dosing regimen of 200mg bis die for three days then 100mg bis die until surgery (Chapter 4). Patients may fall outside of the therapeutic range due to variable metabolism, side-effects or non-compliance.'

At the time of surgery, only 72.6% of patients in the treatment group were above the lower threshold of the therapeutic range for serum perhexiline, despite following expert advice on the optimal loading and maintenance dosing regimen. High inter-individual variation in the metabolism of perhexiline was one factor, with all of the ultra-rapid metabolisers found to be sub-therapeutic (6.8%). Some patients had either known or suspected non-compliance with therapy (6.5%), often due to dosing errors or side-effects, particularly in the poor metabolisers. A small proportion of patients (7.7%) had less than five days of therapy and three (2.2%) had long finished the IMP by the time of their delayed surgery. However, in the remaining patients, there was no clear explanation for falling below the therapeutic range.

'Perhexiline is more concentrated in the human myocardium than in the serum reflecting cumulative drug uptake and tissue loading as previously shown in the rat heart (Chapter 5). Serum and myocardial levels will be closely correlated but may be reduced by insufficient time to reach steady-state or high serum concentrations with saturation pharmacokinetics.'

The experiments conducted during my research fellowship in Adelaide are the first to measure the myocardial concentration of perhexiline in humans. I confirmed that perhexiline is highly concentrated in the atrial and ventricular myocardium of patients. There was a strong correlation with serum levels but other factors such as heart rate and age may modulate drug uptake and accumulation. The ventricular concentration was significantly higher than that in the atrium suggesting that contractile work may also affect compartmentalisation. However, it appears not to have reached steady-state by the time of surgery and this may have impacted on any metabolic effects and therefore any potential benefit in myocardial protection.

'The concentration of perhexiline in the myocardium following at least one week of therapy will be sufficient to significantly inhibit CPT-1 corresponding to the inhibitory concentrations observed in the rat heart (Chapter 5).'

Despite apparently not reaching steady-state, the level achieved in the left ventricular myocardium appears to have been in the correct order of magnitude for *CPT-1* inhibition according to the studies in the rat heart by Kennedy *et al*. (1996, 2000). However, *CPT-1* inhibition may not represent a clinically important mechanism of action in the human myocardium even at steady-state and therapeutic plasma levels.

8.2 The metabolic effects of perhexiline

'Improved metabolic efficiency with perhexiline will be reflected by changes in the myocardial metabolic profile favouring glucose metabolism and glycolytic flux through pyruvate to enter the citric acid cycle (Chapter 6). The short length of therapy will bring the benefits of CPT inhibition on metabolic efficiency without the potential risks of lipid accumulation caused by long-term inhibition of β-oxidation.'

On analysis of myocardial biopsies obtained from patients during the trial, using both traditional biochemical techniques and state of the art FT-ICR MS metabolomics, I found no evidence for a metabolic switch from fatty acids to glucose with perhexiline. There was neither an increase in carbohydrate intermediates nor lipid accumulation within the myocardium. Indeed, the polar negative ion metabolomic profiles of patients on perhexiline and placebo were indistinguishable; perhexiline had no significant effect on the myocardial metabolome including the intermediates of energy transfer, glycolysis or the citric acid cycle. This lack of upregulation in glucose metabolism may account for the absence of clinical benefit in myocardial protection and suggests that the mechanism of action of perhexiline *in vivo* in humans may not be primarily metabolic. These findings are supported by recent data from another double-blind trial in Birmingham in which no difference in cardiac efficiency or respiratory quotient across the myocardium was found following four weeks of dose-adjusted perhexiline therapy (Beadle, 2011).

It is conceivable that *CPT* inhibition may be responsible for the side-effects and long-term toxicity of perhexiline rather than its therapeutic anti-ischaemic effects. A chronic rise in the tissue perhexiline concentration may lead to progressive inhibition of β-oxidation and accumulation of TAG, phospholipids and other products of fatty acid metabolism, including the pro-apoptotic ceramide. Lipotoxicity is known to have deleterious effect on cardiac function and has been implicated in the pathogenesis of diabetic cardiomyopathy (Brindley et al., 2010). Whilst it was reassuring to find that there was no rise in myocardial lipid content in the trial, the maximum length of therapy was only 31 days. For patients undergoing cardiac surgery at least, the promise of short-term gain without long-term risk from perhexiline therapy was obviated by the lack of actual clinical benefit.

8.3 The role of perhexiline in myocardial protection

'Preoperative oral loading with perhexiline maleate as an adjunct to cold blood cardioplegia during ischaemic arrest in patients undergoing CABG will improve markers of myocardial protection (Chapter 7). This will be reflected by a decreased incidence of low cardiac output episodes, reduced need for inotropic support and lower release of troponin in the perioperative period compared with controls. Cardiac indices will also be higher in the perhexiline group prior to ischaemia.'

In the CASPER trial, preoperative administration of oral perhexiline as an adjunct to cold blood cardioplegia failed to improve any of the clinical markers of myocardial protection, providing no evidence to support its routine use in patients undergoing non-emergent coronary artery bypass graft surgery. Perhexiline has been shown to be an effective agent in the treatment of angina pectoris (Cole et al., 1990) and heart failure (Lee et al., 2005) but appears to offer no protection against the detrimental effects of ischaemia-reperfusion. The only previous study of perhexiline in this setting found improvement in diastolic dysfunction during ischaemia but similarly no benefit in recovery of systolic function on reperfusion in a rat model (Kennedy et al., 2000).

So why was the CASPER trial a negative trial? A large number of patients were recruited (n=286) and it was adequately powered to detect its primary and secondary endpoints but found absolutely no difference between groups (OR 0.92, 95% CI 0.56-1.50, p 0.74). An exploratory analysis with propensity score-matched controls confirmed that the finding was not significantly weakened by sub-therapeutic patients.

The mean pre-ischaemia cardiac index was found to be lower in the treatment group but even when taken into account, there was no difference in postoperative myocardial contractility. It is apparent that perhexiline did not significantly inhibit *CPT-1* at the time of surgery in these patients either due to not reaching steady-state in the ventricular myocardium or because that is not its mechanism of action; no change was demonstrated in myocardial metabolism prior to ischaemia using two different biochemical approaches. Any potential inhibition of *CPT-1* may have been negated by the higher serum FFA levels prior to ischaemia in the perhexiline group. In any case, this would almost certainly have been overcome by the surge of FFA entering the myocardium on reperfusion which was not suppressed by perhexiline therapy, evidenced by the similarly raised TAG profiles at six hours.

In conclusion, this trial does not detract from the principal that metabolic drugs have a role in improving myocardial protection during cardiac surgery rather perhexiline is not such an agent. In contrast, GIK has multiple metabolic and non-metabolic actions including increased availability of glucose, suppression of plasma fatty acids during reperfusion and activation of pro-survival pathways. The search for a metabolic agent with similar efficacy to insulin but without its limitations continues.

8.4 Reflections on conducting a clinical trial

The CASPER trial was a phase III clinical trial intended to demonstrate a therapeutic benefit, confirm the safety of perhexiline in patients undergoing cardiac surgery and provide another '*new trick for an old drug*' (Frenneaux, 2002). The trial design, endpoints and power were derived from previous local trials of GIK, an agent with a long history of use in myocardial protection that had been successful, positive and published. In retrospect, it would have been prudent to have first conducted proof-of-concept studies with non-clinical endpoints; these could have included an animal model of ischaemia-reperfusion building upon the initial work of Kennedy *et al*. (2000). If these preliminary studies had demonstrated biologically plausible benefits, they should have been followed by a phase II study to improve dosing of patients to achieve therapeutic serum levels, explore its use in patient sub-groups such as those with diabetes and provide endpoint data to enable a validated power calculation based on the same drug in the same circumstances. It is likely that these studies would have led to the same conclusion as the multi-centre trial but perhaps at greater efficiency of time, money, effort and patient participation.

8.4.1 Recruitment

The conduct of a clinical trial is a complex process and unanticipated difficulties are likely to arise in every trial. Recruitment is a common problem; most publically funded trials fail to meet their original recruitment target (Campbell et al., 2007), requiring either an extended recruitment period or reconsideration of variables in the power calculation (Carter, 2004). Projected recruitment may have been unconsciously

overambitious in order to secure funding whilst procedural delays may be protracted and beyond the researchers' control. Poor recruitment may increase the probability of a type II error, increase costs due to extending the period of the trial, and prolong uncertainty over the effectiveness of the treatment (Watson and Torgerson, 2006). Recruitment efficiency may be improved by the establishment of a research network with experienced clinical and management personnel, familiarity with trial processes, a programme of education for trialists and an awareness of the existence and limitations of the pool of potential trial participants (Barnard et al., 2010).

The CASPER trial is the largest randomised controlled trial in cardiac surgery to have been conducted at our institution and the first to involve another centre. However, recruitment of patients represented a major challenge and was constrained by the throughput of suitable cases. The MESSAGE trial took 32 months to recruit 280 patients undergoing CABG with six Consultant Surgeons from 2000 to 2002 (Quinn et al., 2006). However, the number of patients undergoing CABG in the department has been gradually declining over the subsequent decade from 569 in 2000, to 468 in 2006 and now to 318 in 2010; amongst these, the proportion of patients with diabetes increased from 19.6% in 2000 to 25.4% in 2010 (Barnett, 2011). Initially only four of the six Surgical Consultants agreed to contribute patients due to simultaneous trials recruiting patients undergoing CABG within the department (Rahman et al., 2010); later two more Consultants, one newly appointed, agreed to collaborate.

Of the 564 patients who fulfilled the inclusion/exclusion criteria, only 286 (50.7%) progressed through to be included in the final analysis. The major reason was that

148 (26.2%) suitable patients undergoing heart surgery declined to participate in a trial that aimed to make their surgery safer. There are many reasons why patients refuse to participate in trials including inconvenience, financial and protocol issues (Brintnall-Karabelas et al., 2011). However in the year prior to commencing the CASPER trial, a catastrophic incident related to a drug trial in the UK was widely reported. During a phase I safety trial at Northwick Park Hospital in London, all six healthy volunteers administered TGN1412, a monoclonal antibody, rapidly developed multi-organ failure and required admission to Intensive Care (Boseley, 2006). The negative publicity surrounding drug trials *per se* at this time is likely to have made patient recruitment more difficult. Indeed, perhexiline itself has a chequered history and was once withdrawn on safety grounds (Shah, 2006). On searching Google with the term *perhexiline*, the highest ranked webpage is a Wikipedia (2011) article in which the first paragraph states:

'It was outlawed in many countries due to its adverse effects...'

Despite being the fifth randomised controlled trial on myocardial protection to be undertaken at QEH, Birmingham, this trial was the first to require intervention prior to arrival in the anaesthetic room; previous trials had used either an infusion of GIK (MESSAGE, MESSAGE-2, HINGE) or inflation of a blood pressure cuff (REMOTE) to induce metabolic or ischaemic preconditioning, respectively. A lower recruitment rate in the CASPER trial may have been expected as it required the active participation of patients to take the IMP at home prior to surgery rather than the passive engagement of an additional infusion whilst under anaesthesia. It soon became clear that this trial would take longer than the 3 years of secured funding to complete at a single site.

There are several centres in the West Midlands who could have been approached to form a local research network. However, from May 2008, discussions took place to expand the trial to a second site at RSCH, Brighton; whilst geographically distant, two of the Consultants in Brighton had trained in Birmingham and were familiar with the MESSAGE trials. Following a visit in late 2008, I submitted a substantial amendment to obtain ethical, MHRA, sponsor and local NHS Trust approval to expand the trial. Recruitment began at the new site in March 2009 and the first patient underwent surgery the following month. I made numerous visits to Brighton during the trial, attending preliminary meetings and collecting data on the first two patients. Over the next 12 months, local recruitment was coordinated by two research nurses; however, only 15 patients were successfully recruited and proceeded through to analysis. Figure 40 illustrates the number of patients recruited from the two centres during each month of the trial and table 16 documents the contribution of each Consultant.

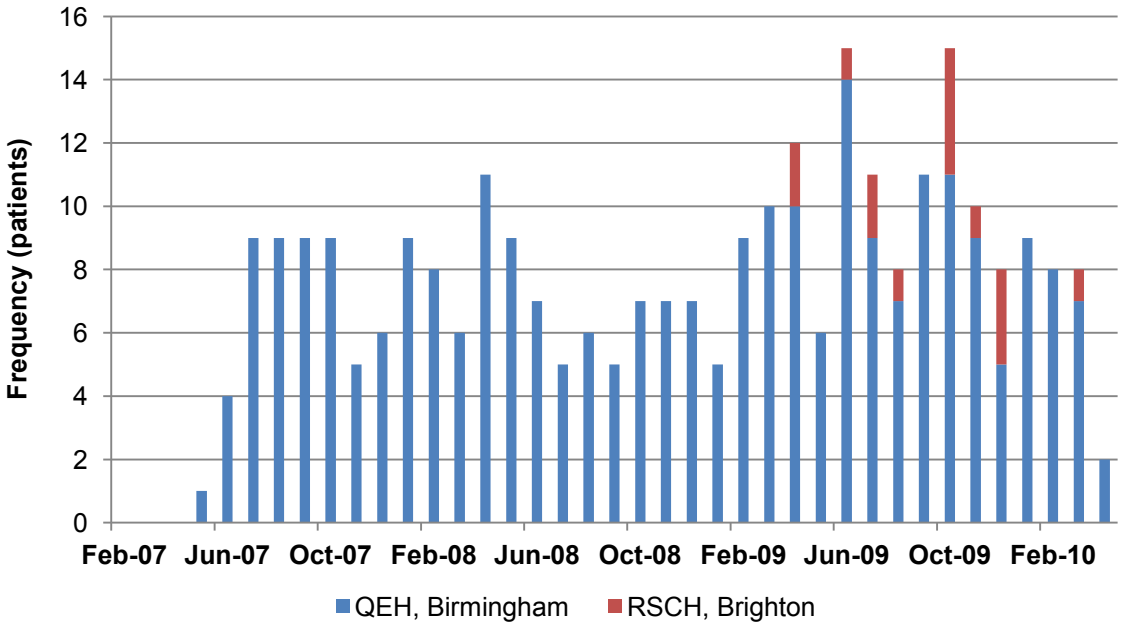


Figure 40. Number of patients in the trial undergoing surgery each month.

Centre	Consultant	Cases	Months	Cases/month
QEH	Pagano, D	78	36	2.17
	Rooney, SJ	68	38	1.79
	Graham, TR	54	38	1.42
	Wilson, IC	38	38	1.00
	Mascaro, CJG	28	16	1.75
	Dandekar, U	5	6	0.83
RSCH	Lewis, ME	10	12	0.83
	Hyde, JAJ	4	6	0.66
	Cohen, AS	1	3	0.33
Both sites	All Consultants	286	38	7.53

QEH, Queen Elizabeth Hospital, Birmingham; RSCH, Royal Sussex County Hospital, Brighton.

Table 16. Patients completing the trial contributed by each Consultant Surgeon.

8.4.2 Investigational Medicinal Product

In Birmingham, several issues slowed the progress of the trial. There were delays in obtaining perhexiline from Australia; a large batch of matching placebo tablets were already available in the UK but it took more than three months for the IMP tablets to be imported, undergo testing and QP release, be bottled and labelled according to the randomisation code and be available for allocation, delaying the start of the trial. As perhexiline has a shelf-life of 24 months (Sigma Pharmaceuticals, 2009), two further batches of tablets had to be ordered during the trial; whilst hold-ups in this process never halted recruitment once underway, it was outside of our control.

8.4.3 Clinical scheduling of cases

The scheduling and rescheduling of patients for surgery presented many difficulties. The allocation of patients to forthcoming operating lists often changed for clinical, logistic, bureaucratic, social or other unknown reasons. Naturally, some patients had their surgery expedited due to a change in their clinical condition. However, patients were often scheduled at short notice to cover gaps on operating lists; this led to the loss of several randomised patients from the trial and many others who may have considering participating (48, 8.5% of suitable patients). It also meant that I had to contact some patients regarding a decision on participation and deliver the IMP to their homes so that they could start the tablets that day to enable inclusion with at least five days of therapy prior to surgery. On the other hand, last minute cancellations led to the frequent issuing of a second bottle of tablets to provide a 17-day extension to the window of IMP therapy. Alas, five patients had their surgery delayed beyond (or indeed *well beyond*) this period, meaning that they were no longer taking the IMP at the time of surgery but had to be included on an intention-to-treat basis; three of these patients were in the perhexiline arm. This situation arose due to the limited period of therapy defined in the protocol, driven by a lack of locally available therapeutic monitoring, combined with recurrent scheduling difficulties.

In addition, the NHS Trust introduced a four-month waiting list initiative, as part of a government drive to reduce waiting times in 2007. As these cases were performed at a private hospital which fell beyond the ethical, regulatory and sponsor approval for conducting the trial, any patients conscripted to this initiative were lost; unfortunately, this removed 25 suitable patients, five of whom had already been randomised.

These hindrances reflect the administrative constraints of the NHS but overall their impact lessened during the trial as I became more familiar with the practices of those involved and took steps to predict future scheduling. The conduct of the trial would have benefitted from the involvement a Clinical Trials Unit, with knowledgeable staff and well-oiled infrastructure, rather than a single-handed approach. However, given the absence of trial managerial or administrative support, my lack of previous trial experience and the constraints outlined, I am proud that the trial was completed. Personally, my understanding of the design, conduct and analysis of clinical trials has vastly improved during this process and I aim to build upon this experience.

8.5 Limitations of the work presented

8.5.1 Chapter 3: Methods

In the analysis, although the groups were randomised and seemingly well-matched at baseline, the finding of a lower mean pre-ischaemia cardiac index in the perhexiline group remains unexplained with neither probability nor biological explanations being satisfactory. This conundrum could have been avoided by including a quantitative measure of ventricular function, such as transthoracic echocardiography, prior to trial therapy in the protocol to differentiate unbalanced groups from treatment effect.

Cardiac index is a load sensitive measure of contractility. In the protocol, this was taken into consideration by targets for CVP and PAWP to maintain consistency of ventricular loading conditions. During the trial, my presence in theatre, at the bedside during the first six hours and active involvement in the perioperative care of patients attempted to ensure optimisation of haemodynamic variables. Whilst this seems to have been accomplished with only a minor difference in PAWP between groups at six hours (table 13), it is not certain that this was achieved for every individual patient.

Measurement of serum troponin-T occurred up to 24 hours after aortic cross-clamp release. No significant difference in area under the concentration-time curve was found (p 0.10) but there was a trend towards lower troponin release in the perhexiline group and both groups were well above baseline values. It is unknown whether a difference in AUC may have been found if collection had continued to 72 hours, although it is unlikely as the mean 24 hour values were almost identical. However, a

recent reanalysis of troponometrics from the MESSAGE trials suggests that 72 hour AUC is the best predictor of long-term outcomes (Ranasinghe et al., 2011).

8.5.2 Chapter 4: Results – Serum perhexiline concentration

The aim of the clinical trial was to evaluate whether preoperative metabolic therapy with perhexiline as an adjunct to cardioplegia and hypothermia improves myocardial protection during cardiac surgery. However, as discussed, 27.4% of patients in the treatment group were below the established therapeutic range for serum perhexiline. This reflects the inherent weakness of using a one-size-fits-all dosing regimen for a drug with a narrow therapeutic index and high inter-individual pharmacokinetic variability. The proportion of sub-therapeutic patients would have been reduced by therapeutic drug monitoring and dose adjustment during the preoperative period, as intended in the original study design; however, logistical issues relating to the location and frequency of the assay were prohibitive. Plasma monitoring may have enabled therapy to have been continued beyond 31 days such that the three patients for whom surgery was significantly delayed may have been able to continue safely. A significant number of sub-therapeutic patients could have led the trial to become underpowered for the primary endpoint; reassuringly, the propensity score-matched analysis excluding such patients confirmed the lack of therapeutic benefit.

8.5.3 Chapter 5: Perhexiline pharmacokinetics in the myocardium

The analysis of human myocardial biopsies obtained during surgery is limited by the clinical and ethical restrictions of *primum non nocere*. The ventricular biopsies were very small with a mean mass of only 3.6mg (range 1.6-15mg) due to operator

difficulties with the technique and clinical unease over the removal of left ventricular myocardium. Despite preparation in just 200μL of phosphate buffer, the measured perhexiline concentration was near the limit of detection with the standard curve using HPLC. The small size of the biopsies may also have increased the influence of tissue contamination from any areas of fibrosis within the specimen. Only 28 biopsies were used in this analysis as they were also required for other planned experiments.

The degree of perioperative ischaemia at the site of each biopsy was unknown. All patients had flow-limiting atherosclerotic disease affecting the proximal left anterior descending artery. As biopsies were taken from this territory, it is possible that some were of hibernating myocardium. Chronic ischaemia is known to reduce drug uptake such that a poorly perfused segment may be regarded as a peripheral compartment and may not be representative of the true second compartment (Horowitz and Powell, 1986); this may partly explain the inter-patient variability in ventricular to serum ratio. In addition, as only one pre-ischaemia biopsy was taken per patient, there may be regional differences in myocardial drug content that were undetected.

The total myocardial drug content measured in homogenate may not reflect the true concentration of free drug at the site of action due to tissue binding and retention in lipid membranes. The tentative comparison with the IC_{50} of *CPT-1* with perhexiline in the rat model makes many assumptions including interspecies conservation of *CPT-1* affinity for perhexiline and response to inhibition. Indeed, the concentrations are not directly comparable due to different preparation techniques. As discussed in chapter 5, these extrapolations are only intended to be hypothesis-generating.

8.5.4 Chapter 6: Metabolic preconditioning with perhexiline

During reperfusion, the serum lipid profile was only evaluated at six hours after aortic cross-clamp removal; therefore the expected rise in FFA was witnessed only by the subsequent rise in TAG re-exported from the liver. Measurement of FFA and TAG at more regular time intervals would have provided a greater insight into lipid metabolism during the early stages of reperfusion: at release of aortic cross-clamp, ten minutes, 30 minutes, one hour, two hours, six hours and 12 hours.

There was significant inter-individual variation in some myocardial metabolites and a relatively small number of samples were processed. Surprisingly, there was a trend towards *lower* atrial TAG in the perhexiline group on both the assay (p 0.21) and histology (p 0.20); a larger cohort of samples may have reached significance. It would also have been informative to measure *CPT-1* activity in the myocardial biopsies but the resources to process 'hot' samples were not available locally during the trial.

In the MS metabolomic analysis, it is important to note that we did not measure the entire metabolome of the human myocardium; only the negative ion mode of the polar fraction was evaluated in order to focus on the intermediates of carbohydrate metabolism. Positive ion mode and lipidomic analyses were not performed and hence any perturbations in peptide or lipid intermediates with perhexiline were not assessed (Griffin et al., 2011). The ventricular biopsies used in this analysis were also limited by the same factors as noted previously: small mass, extent of chronic ischaemia, unknown degree of representation and the suggestion that despite therapeutic serum levels, the concentration of perhexiline may not have reached steady-state.

8.6 Future research

Over the last five years, I have gained valuable research skills and an improved understanding of clinical and translational research, including extensive experience in the design and conduct of a clinical trial. Building on the foundation of this thesis, I am pursuing a number of avenues of research into areas either directly related to this work or using the same concepts and techniques to address diverse yet important and novel research questions.

8.6.1 Myocardial protection

The HYPER trial is a prospective, multi-centre, double-blind, randomised, placebo-controlled trial of preoperative oral perhexiline in patients with left ventricular hypertrophy undergoing aortic valve replacement for aortic stenosis, with or without concomitant CABG, on cardiopulmonary bypass with cold blood cardioplegic arrest (NCT: 00989508). As hypertrophy is associated with cardiac energetic impairment and metabolic downregulation (Neubauer, 2007), the myocardium is more vulnerable to ischaemia-reperfusion and may derive greater benefit from improved metabolism. I am a co-investigator on this trial which began recruitment in October 2009, examining the same clinical endpoints for myocardial protection as our previous trials. A sub-study is also underway using ^{31}P magnetic resonance spectroscopy to assess metabolic changes with perhexiline in these patients prior to surgery. In light of the clinical and metabolic findings of the CASPER trial, the futility of continuing with the HYPER trial is currently being discussed by the independent DSMB.

As an alternative, we are investigating the potential application of oral fumarate to myocardial protection. In a murine model, both inactivation of the *fumarate hydratase* (Fh11) gene and oral supplementation with dimethylfumarate resulted in significant attenuation of myocardial injury following a period of ischaemia-reperfusion with improved haemodynamics and reduced troponin release (Ashrafian et al., 2012). Fumarate is both a strategic intermediate in the citric acid cycle and a potent activator of the Nrf2-antioxidant response element signalling pathway, a key mechanism in the regulation of cellular oxidative stress (Linker et al., 2011). We are currently applying for funding for a clinical trial of determine whether oral dimethylfumarate prior to cardiac surgery improves clinical markers of myocardial protection, using the same outcome measures as the CASPER trial but with a more heterogeneous patient population. We will also analyse left ventricular biopsies to determine changes in the expression of the Nrf2 signalling pathway and the MS-visible polar metabolome.

8.6.2 Mechanism of action of perhexiline

Unger and colleagues (2005) demonstrated that the increased efficiency derived from perhexiline is in part independent of *CPT* inhibition and it has been suggested that this may be due to modulation of cellular oxidative stress; trimetazidine has been shown to decrease global markers of oxidative stress following ischaemia-reperfusion associated with cardiac surgery (Iskesen et al., 2006). The thioredoxin (TRX) system is a ubiquitous thiol oxidoreductase system that regulates cellular reduction-oxidation status (World et al., 2006). It plays an essential role by limiting oxidative stress, directly via antioxidant effects and indirectly by influencing key signalling molecules such as thioredoxin-interacting protein (TXNIP). There is accumulating evidence that

TXNIP plays a pivotal role in cardiovascular disorders, functioning as a sensor for biochemical and oxidative stress. It has been shown in a working rat heart model that ischaemia-reperfusion down-regulates TRX expression and that hearts over-expressing TRX had improved post-ischaemic recovery (Turoczi et al., 2003). These studies implicate a protective role for endogenous TRX. On the other hand, TXNIP inhibits TRX by binding to its catalytic site and thereby preventing reduction of oxidised cellular proteins; in practice, levels of TXNIP expression generally control net TRX activity (Junn et al., 2000). In collaboration with Dr Doan T. Ngo and Professor John D. Horowitz at the University of Adelaide, I am studying the effect of perhexiline on the thioredoxin and AMPK/PGC-1α systems in serial left ventricular biopsies obtained during the CASPER trial. Using Western blot analysis of protein expression, we have found a significant reduction in TXNIP and increases in AMPK and PGC-1α expression with perhexiline, suggesting that the effects may contribute to the beneficial impact of perhexiline on redox stress and energetic impairment. This preliminary work was presented at the annual meeting of the American Heart Association in Orlando, FL in November 2011 (Ngo et al., 2011).

In further proposed work, the collection of 'hot' biopsies from patients undergoing cardiac surgery with perhexiline could be used to replicate the rat heart experiments of Kennedy *et al*. (1996). The inhibition of *CPT-1* in human myocardial homogenate and isolated mitochondria may be correlated with the *ex vivo* myocardial perhexiline concentration to clarify whether there is indeed inter-species preservation of *CPT-1*. Preliminary work suggests that this may soon become feasible locally.

8.6.3 Pharmacokinetics

The study of myocardial pharmacokinetics described in this thesis is being extended to measure the relative concentrations of perhexiline enantiomers and determine whether myocardial uptake is enantiomer selective; it has previously been suggested that the (+) and (–) enantiomers have different pharmacokinetic profiles (Davies et al., 2008). We also intend to use population modelling techniques to further evaluate factors that influence the pharmacokinetics of perhexiline.

The measurement of drug levels in a target tissue and correlation with cellular effects is widely applicable to understanding the pharmacokinetics of other drugs. I have designed a novel study applying these principles to the use of immunosuppressant therapy following heart transplantation (NCT: 01423552). In collaboration with Professor Robert S. Bonser in Birmingham and Associate Professor Benedetta C. Sallustio and colleagues at the University of Adelaide, we will measure the relative concentrations of ciclosporin and mycophenolate in whole blood, isolated T-lymphocytes and transvenous endomyocardial biopsies to correlate relative drug levels, pharmacogenomics and pharmacodynamics with clinical and pathological evidence of rejection in patients following heart transplantation in Birmingham. This study aims to improve our understanding of interindividual variations in the clinical pharmacology of these drugs using pharmacokinetic and pharmacodynamic endpoints to optimise the dosing of immunosuppressive therapy and outcomes in these vulnerable patients.

8.6.4 Metabolomics

At the University of Birmingham, we are fortunate to have one of the most advanced metabolomic platforms in the world under the directorship of Professor Mark R. Viant in the School of Biosciences. An ultra-high resolution, high accuracy tool for the detection of biological molecules, FT-ICR mass spectrometry is a valuable addition to the armamentarium of cardiac surgery research. I intend to harness this new technology to improve our understanding of myocardial metabolism in normal, ischaemic, reperfused, hypertrophied and cyanotic hearts in order to generate new hypotheses on how they may be better protected.

9. APPENDICES

9.1 Letter from Mr James R. Edwards, RAH Adelaide, 9th December 2005

9.2 Patient Information Sheet, version 3a, 17th September 2008

University Hospital Birmingham NHS
NHS Foundation Trust

PATIENT INFORMATION

Support of the heart during Coronary Artery Surgery with Perhexiline

Principal Investigator: Mr D Pagano, Consultant Cardiothoracic Surgeon

AN INVITATION TO PARTICIPATE IN RESEARCH

The heart surgery team at the Queen Elizabeth Hospital, Birmingham is inviting patients to participate in research aimed at making coronary artery 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 patients' progress. We include a simple and non-technical summary of the reasons for the study and what it will involve for you over and above your routine treatment if you take part.

If you are being approached in the outpatient clinic, we would like you to take this patient information sheet home with you to read and consider participating in this study. At the Pre-admission clinic, the doctor will ask you whether you wish to participate and to sign a consent form. You can only be included in this clinical trial if you give your express permission in the form of signed consent. On admission for your operation, a heart surgical research doctor will be available to discuss the study further and answer any questions.

If you are already an inpatient on the ward, a member of the heart surgical team will be happy to discuss the study with you once you have had at least 24 hours to read through this information sheet.

Questions? Please contact Mr Nigel Drury, Clinical Research Fellow in Cardiac Surgery via the Queen Elizabeth Hospital, Birmingham switchboard on **0121 472 1311**.

WHAT IS THE STUDY ABOUT?

This study is about trying to improve the treatment of patients undergoing coronary artery surgery. To perform this heart operation requires the help of a heart-lung machine, which supports your body's circulation whilst the surgeon bypasses the diseased blood vessels. 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 the operation. We are seeking ways to improve on these established 'protective techniques' by preparing the heart before surgery with a drug that switches the fuel of the heart from less efficient fatty acids to more efficient sugars.

In summary, taking the drug perhexiline before your operation has the potential to benefit the way your heart works during periods of strain during and immediately after your surgery.

HOW HAS THIS CLINICAL TRIAL BEEN DESIGNED?

This study is a multi-centre double-blind randomised placebo-controlled trial. This means that if you agree to take part, you would be allocated by chance to receive *either* the drug perhexiline *or* a placebo – a tablet that looks the same but has no medicinal properties. The tablets are coded so that neither you nor the heart surgical team will know which one you are receiving. The code is revealed once all the patients in the trial have been completed. By giving the tablets in this way, we can determine the benefits of using them in patients undergoing coronary artery surgery.

HOW WILL WE MEASURE THE EFFECTS OF THIS TABLET?

To detect changes in heart function, we need to take measurements of performance before, during and after surgery to show whether the treatment has made a difference. To do this we will make use of small monitoring catheters (plastic tubes) inserted into blood vessels. These tubes are routinely used in two out of three patients who are not in the trial. These catheters are placed in position in blood vessels whilst you are under anaesthetic (asleep) and are usually removed on the first or second day following surgery. We will record measurements from these catheters during this period at specific time points. We will insert an additional sampling catheter into your heart during your operation to monitor blood in the veins of your heart. This catheter is normally used as an additional way to deliver protection to the heart in patients at high-risk for surgery; in your case, it will be used only to withdraw a sample of blood from your heart veins. It will be removed at the end of the use of the heart-lung

machine. We will also use a special camera to look at how the heart is working. This will be placed in the gullet whilst you are asleep and used to measure the function of the heart before the operation. We will also look at how the heart is working later on the intensive care unit. This camera will be removed from the gullet before you wake up. It is now used routinely in the majority of patients requiring heart valve surgery and in those who require more heart support in the intensive care unit in our hospital.

MEASURING THE POSSIBLE BENEFICIAL EFFECTS OF THE TABLET 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 monitoring lines already mentioned. The total amount of additional blood taken for this study is about a cupful (approximately 60ml or 2fl oz).

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 moved to different positions. The scan will take approximately 20-40 minutes to complete and will be performed once before surgery and once after.

Imaging the heart using a transoesophageal echocardiogram: This is a similar test to the one above (transthoracic echocardiography) but instead the ultrasound probe is placed in the gullet. It allows the doctors to look at the heart during the operation as the previous test cannot be used at this stage. This is performed in most patients undergoing heart surgery but extra pictures will be taken to analyse the heart function in more detail. This will be placed whilst you are asleep and removed before you are woken up.

The removal of tiny samples of the heart muscle known as biopsies: This will help tell us how the perhexiline tablet improves the way the heart works. We would aim to obtain 3 heart muscle biopsies, all whilst you are asleep under the anaesthetic, one each at the beginning, middle and end of being on the heart-lung machine. 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. Although there is a hypothetical risk of bleeding in taking this biopsy, we have performed more than 1500 biopsies in patients at the time of writing this information leaflet with no complications.

WHAT WILL I HAVE TO DO?

If you decide to participate, 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 have been given this written information sheet and asked to read it at your leisure at home or on the ward, if you are already an inpatient. Before your surgery, in either the pre-operative assessment clinic or on the ward, a member of the research team will visit so you can ask any questions. If you give signed consent, we will then randomise you to receive either the tablet or the placebo and we will give you the medication with a date to start taking it. Before the operation you may be asked to have one of the scans listed above.

In the anaesthetic room of the operating theatre, the Anaesthetist will give you a general anaesthetic. Once you are asleep, the Anaesthetist 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 also 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 samples and monitoring tests will remain. The last blood test will be performed at 24 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 60 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 giving patients the tablet perhexiline will benefit them as an individual – this study is investigating that question. It is hoped that this study will provide information that will benefit future patients undergoing cardiac surgery. In particular it will help us design more suitable treatments for patients who have a very weak heartbeat before surgery and are at high risk of complications after surgery. In the future and after more research, the use of this tablet could significantly improve their recovery from heart surgery.

WHAT ARE THE RISKS?

Previous studies with perhexiline have shown that it is safe. Some patients may feel a bit sick after the first tablet but that usually passes very soon. If these tablets continue to make you feel sick, you can simply stop taking them without harm and your surgery will proceed as planned. If you stop taking these tablets, we would be grateful if you could contact the hospital to the Heart Surgery Research team know – telephone number on page 7.

In some patients who had taken the tablet for long periods of time (many months or years), a very small number developed some numbness or muscle weakness and/or mild liver changes including going yellow (jaundice) and loss of appetite. It has since been shown that this can be avoided by monitoring the level of the drug in the blood. These side effects do not occur when people take the tablet for just a few weeks and so will not affect you. However, if you have any concerns, we would ask you to contact one of the Heart Surgery Research team at the Queen Elizabeth Hospital, Birmingham on the contact number on page 7. You can also contact your GP, who will be given more information about the tablet and the trial if you agree to participate.

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 15,000. The only additional invasive procedure performed on you is the biopsy of the heart muscle. There is a very small risk of bleeding following this procedure. However, we have performed approximately 1,500 biopsies so far and this complication has never happened.

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?

All the study data will be collected by the Heart Surgery Research team. It will be stored as paper files and on a hospital computer which are kept in a locked office. 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 the

research findings. All data is coded and confidential, ensuring that your identity will not be revealed at any time. All necessary measures will be taken to keep your data safe and confidential and to comply with the Data Protection Act. Only the Heart Surgery Research team and the Research & Development office will have access to this data. Following completion of this trial, the data will be kept for 15 years and then destroyed.

WHAT HAPPENS TO THE SAMPLES?

All of the biopsies will be stored in a coded form in a locked laboratory and only the Heart Surgery Research team will have access to these samples. The samples will be analysed to look at how much perhexiline is taken up by the heart. We will also look at what effect this has on the heart in terms of how it works and how much energy it can store. The biopsies are very small and the simple act of analysing them will destroy the sample.

WHO ELSE IS TAKING PART?

We will recruit 300 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 the measurements, you will be in either the theatre or the ICU where trained staff are at hand at all times. Your safety during and after surgery is paramount, and takes precedence over any research.

In the event that something does go wrong and you are harmed during the research study, there are no special compensation arrangements. If you are harmed due to someone's negligence, you may have grounds for a legal action for compensation against University Hospital Birmingham NHS Foundation Trust but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will be available to you. Further information can be obtained from the Patient Advice & Liaison Service (PALS) which is available Monday to Friday 9am to 5pm on 0121 627 8820.

WHAT HAPPENS AT THE END OF THE STUDY?

At the end of the study, your 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 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. Contact QE Hospital switchboard on **0121 472 1311** and ask them to telephone Mr Nigel Drury, Clinical Research Fellow. If you are on the ward, ask one of the nurses to contact Mr Drury. If he is unavailable, ask for either Mr Howell (Clinical Lecturer) or Mr Pagano (Consultant Surgeon).

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. We will give you the tablets, which will be either perhexiline or the placebo, and instructions on when to take them. You will need to take one or more tablets twice per day prior to your operation – in the morning and in the evening. We will also write to your GP to let them know that you have agreed to join this study and giving them more details. You will be listed for surgery as normal. You are free to withdraw from the study after initially consenting without giving reason and without prejudice to your continuing care or the standard care of any future treatment.

WHO IS ORGANISING AND FUNDING THIS RESEARCH?

This research has been organised and developed by the Heart Surgery Research teams at the Queen Elizabeth Hospital, Birmingham and the Royal Sussex County Hospital, Brighton. It is being funded by the **British Heart Foundation**.

WHO HAS REVIEWED THIS STUDY?

This study was given a favourable ethical opinion for conduct in the NHS by the Cambridgeshire 1 Research Ethics Committee in Cambridge, UK.

THANK YOU

Finally the Heart Surgery Research team would like to thank you for taking the time to consider this research proposal.

PROPOSED STUDY INVOLVEMENT

This summary flow chart demonstrates what will happen if you consent to join this study:

When you were seen by your Surgeon for the first time to discuss your operation, you will have been invited to join this trial and given this information leaflet.

↓

In your free time, we would like you to read this leaflet and consider joining the study.

↓

In a few weeks, in the pre-operative assessment clinic (or on the ward if you are already an inpatient), you will be seen by a member of the Research team.

↓

If you are happy to join this trial, we will ask you to give written consent to join the trial. You will then be randomised to receive either the perhexiline tablets or the placebo (the dummy tablets). Neither you nor the research team will know which one you are given. You will then be given a date for your operation and a date to start taking the tablets. An information leaflet and instructions will be included with your tablets.

↓

On your admission to the hospital for your surgery, you will be seen by a member of the Research team. You may then be invited to have an extra scan of the heart, as detailed in this leaflet, depending upon what scans you have already had.

↓

You will then be taken to theatre for your operation as normal.
After you have been put to sleep by the anaesthetist, we will do some blood tests and measure how well your heart is working.

↓

After your operation you will be taken to the Intensive Care Unit as normal.
We will then make further tests of how well your heart is working.

↓

After the operation, you will be have a several extra blood tests, not using a needle but from the special monitoring lines that will be in place. Before you are discharged home, we will take an extra heart tracing and you may be asked to have another scan.

9.3 Consent form, version 3a, 17th September 2008

University Hospital Birmingham NHS
NHS Foundation Trust

CONSENT FORM

Support of the heart during Coronary Artery Surgery with Perhexiline

Principal Investigator: Mr D Pagano, Consultant Cardiothoracic Surgeon

Please *initial* boxes

I confirm that I have read and understand the information sheet (Version 3a, dated 17/09/2008) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	☐
I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.	☐
I understand that relevant sections of any of my medical notes and data collected during the study, may be looked at by responsible individuals from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	☐
I understand that blood and tissue samples will be kept for the purpose of research and I give permission for these samples to be taken and stored.	☐
I agree to my GP being informed of my participation in the study.	☐
I agree to take part in the above study.	☐

Name of Patient	Signature	Date
Researcher	Signature	Date

9.4 Letter from Prof John D. Horowitz, TQEH Adelaide, 2nd March 2007

9.5 Statistical Analysis Plan, version 2, 5th July 2010

STATISTICAL ANALYSIS PLAN

Efficacy

Primary Outcome
Incidence of Low Cardiac Output Episode (LCOE)

Secondary Outcomes
Cardiac index
Inotrope usage
Peak cardiac troponin-T
ECG evidence of new myocardial injury
Composite endpoint of either peak troponin-T or ECG evidence of new myocardial injury
Length of stay in the Intensive Care Unit and Hospital

Safety Outcome Measures

Postoperative death
Stroke with residual deficit
Requirement for renal replacement therapy
Reoperation
Chest drainage at 12 hours
Treated infection episodes
Respiratory index (PaO_2/FiO_2) on arrival in Intensive Care and 12 hours postoperatively

STATISTICAL METHODS AND DATA CONSIDERATIONS

Analysis will be carried out using SAS version 9.2 All statistical tests will be two-sided, and deemed to be statistically significant if $p<0.05$. No adjustments for multiplicity will be made. All analyses will be undertaken stratified for LV function at baseline and elective/urgent surgery. Consultant surgeon will be accounted for as a random effect. The only data which will be formally analyzed are those which could potentially be affected by which treatment group they are assigned to. Demographic and other baseline data will not be formally analyzed, other than descriptively.

The two treatment groups of patients in this study will be identified in tables and listings as:
Perhexiline
Control

Handling of missing and incomplete data

Primary efficacy variable
In the event of death before assessment of primary endpoint, LCOE will be assigned.

Secondary efficacy variables
Missing secondary endpoints will be excluded from the analysis.

PRIMARY OUTCOME MEASURE AND ESTIMATION OF SAMPLE SIZE

LCOE after cardiac surgery is an indicator of inadequate myocardial protection and contributes to postoperative morbidity and mortality. For the purpose of this study LCOE is defined as:

Hypotension (mean arterial pressure < 65mmHg) with cardiac index $\leq$ 2.2L/min/m^2 in the presence of adequate filling pressures (CVP 8-12mmHg, PCWP 12-16mmHg) and heart rate (> 75bpm) where systolic blood pressure < 90mmHg and/or inotropic support $\pm$ intra-aortic balloon pump for > 60 minutes is required to maintain such a clinical picture within the first six hours after removal of the aortic X-clamp.

We hypothesise that the use of perhexiline will reduce the incidence of LCOE compared to control and the sample size has been estimated by extrapolating data from our recently completed MESSAGE trials, in which a reduction of 50% in incidence in LCOE was detected in the GIK group compared with control **[difference between incidence in groups 0.19 versus 0.37 to achieve similar difference with an α 0.05% and 1-β 0.9]**.

This requires a total of 280 patients, randomised 1:1 between treatment and placebo which will also provide sufficient statistical power to examine the range of important secondary endpoints outlined below.

ANALYSIS

Primary efficacy variable

The primary objective is to evaluate the effects of perhexiline on the incidence of LCOE. The primary analysis will be a non-linear mixed model accounting for baseline LV function and elective/urgent surgery with surgeon as a random effect. Odds ratio 95% CI and p value will be presented.

The presence of LCOE is adjudicated by the blinded CASPER Endpoints Committee.

Exploratory analysis. The predictive value of the following baseline characteristics (where feasible) will be examined: age, ventricular function, urgent surgery and logistic EuroSCORE. A parsimonious statistical model describing the predictive value of the listed parameters will be estimated, using a stepwise model building procedure. The linearity in response of continuous variables will be examined and model fit assessed using the AIC.

Efficacy evaluable population. This will consist of the all-patients-randomised population who received therapy (perhexiline or placebo) and underwent surgery on CPB.

Exploratory analysis of the primary outcome for those that reached the lower limit of the therapeutic serum perhexiline. Based on a model which identifies predictors of LCOE, a propensity score will be generated. Patients with a sub-therapeutic plasma perhexiline level and matched controls (based on propensity score) will be removed and an exploratory analysis of treatment effect in those reaching the lower end of the therapeutic range for plasma perhexiline at the time of surgery (0.15 mg/L or above) undertaken.

Secondary outcome measures

Cardiac index: to detected an increase of 0.3 L/min/m^2 in cardiac index in the first 6 hours from release of aortic cross clamp in the treatment group.

1. Cardiac index at six hours will be analysed using generalised linear modelling with baseline cardiac index, elective/urgent surgery, baseline LV function and treatment (perhexiline/control) as covariates and surgeon as a random effect.

2. Cardiac index up to 12 hours will be analysed using generalised linear modelling with cardiac index (baseline, pre, post, 2, 4, 6, 9 & 12 hours) as repeated measures, adjusted to baseline, and accounting for elective/urgent surgery, LV function and perhexiline as covariates and surgeon as a random effect.

3. Exploratory repeated measures analysis accounting for time-dependent inotrope use.

Inotrope usage: assessed by incidence of use based on predetermined protocols and total dosage/kg in the first 6 and 12 hours postoperatively. Incidence and dose will be assessed:

1. In the first six hours after removal of aortic X-clamp (also includes on CPB).

2. In the first 12 hours after removal of aortic X-clamp.

The incidence of inotrope use in each time period will be assessed using a non-linear mixed model accounting for urgency of surgery and LV function with surgeon as a random effect.

Peak cardiac troponin-T: in the first 24 hours following release of the aortic cross clamp

1. Linear model with baseline troponin-T as a covariate accounting for LV function, urgency of surgery and with surgeon as a random effect (using peak troponin-T at any time point).

2. Repeated measures analysis – baseline, 6 hours, 12 hours, 24 hours.

ECG evidence of new myocardial injury: assessed by a blinded Cardiologist according to standard criteria using the presence of new Q waves, new bundle branch block or loss of R wave progression. The presence of ECG evidence of new myocardial injury will be assessed using a non-linear mixed model (logistic) accounting for baseline LV function and elective/urgent surgery with surgeon as a random effect.

Length of stay: this will include both intensive care and hospital stay within the Department of Cardiothoracic Surgery. The effect of perhexiline on mean length of stay (days) will be assessed using a non-linear mixed model (Poisson distribution) accounting for baseline LV function and elective/urgent surgery with surgeon as a random effect.

Safety outcome measures: will be monitored by the DSMB and include postoperative death, stroke with residual deficit, requirement for renal replacement therapy, reoperation, chest drainage at 12 hours, treated infection episodes, respiratory index (PaO_2)/FiO_2 on arrival in Intensive Care and 12 hours postoperatively.

Should any patient report the same event more than once, this event will only be counted once and summarised at the highest observed severity in relationship to study drug. Adverse events that occur in at least 5% of patients will be formally tested using Fisher's exact test.

9.6 Statistical tests used in this thesis

Analysis of variance (ANOVA) is a linear model used to test whether there is a difference between two or more means. For example, the mean cardiac index at six hours was significantly lower in the treatment group (2.51L/min/m^2, SD 0.43) than in the control group (2.70L/min/m^2, SD 0.54) (DiM 0.17, 95% CI 0.05 to 0.29, p 0.005).

Area under the concentration-time curve (AUC) is a technique to describe the summative concentration of a molecule over a period of time and is often used in pharmacokinetics to estimate drug exposure. It may also represent the release of a biomarker in response to a stimulus; for example, there was no significant difference in AUC for troponin-T between the treatment group 3.98ng.hr/ml (SD 3.79) and the control group 4.71ng.hr/ml (SD 5.32) (p 0.12) in the 24 hours following reperfusion.

False discovery rate (FDR) *control* is a method used in multiple hypothesis testing to correct for multiple comparisons and reduce the expected proportion of falsely rejected null hypotheses. For example, all 4039 peaks in the final metabolomic intensity matrix were examined using univariate statistics and a FDR of 5% applied.

Fisher's exact test is used to detect the significance of any deviation from a null hypothesis with small samples of categorical data, usually in the form of a 2 x 2 contingency table. For example, there was no difference between the incidence of postoperative atrial fibrillation between the treatment group (55/139, 39.6%) and the control group (66/147, 44.9%) (p 0.40).

Kolmogorov-Smirnov test determines whether a sample is significantly different from a normally distributed population. A significant value indicates deviation from normality; for example, the concentration of serum perhexiline in the treatment group was not normally distributed (D=0.18, p<0.001).

Partial least squares discriminant analysis (PLS-DA) is a multiple linear regression tool that enables discrimination between groups by the levels of continuous predictor variables and can therefore be used to maximise separation of groups. For example, metabolomic profiles of the control and treatment samples were minimally separated and the associated mean classification error rates for predicting group membership were 43% and 47%, respectively, suggesting no difference between groups.

Power is the probability that a test will correctly reject the null hypothesis and is used to estimate the minimum sample size required for the test to detect an effect of a particular size. It is a trade-off between the probability of a false negative (β, type II error) and the probability of a false positive (α, type I error). For example, by extrapolating the 50% reduction in incidence of the primary endpoint, a low cardiac output episode, from previous trials, it was determined that to achieve a similar difference between groups, with α 0.05 and 1-β 0.9, the CASPER trial required at least 280 patients randomised in a 1:1 ratio.

Principal component analysis (PCA) is a multivariate technique to identify the linear components of a set of variables and reduce its dimensionality. For example, PCA was used to visualise the metabolomic similarities and differences between groups.

Regression is a technique for modelling the relationship between a dependent variable and one or more independent variables and in some circumstances, unlike correlation, it can infer causation. For example, linear regression was used to assess the impact of serum perhexiline on the concentration in the ventricular myocardium with an *R* value of 0.85, R^2 of 0.73 and *F-ratio* of 69.1 ($p<0.001$).

Repeated measures ANOVA is an analysis of variance in which a variable has been measured several time on the same participants. For example, with pre-ischaemia cardiac index as a covariate, cardiac index during reperfusion was not significantly affected by perhexiline therapy, F (3.57, 896.9) = 1.02, p 0.40.

Spearman's rank correlation (r_s) is a standardised non-parametric measure of the strength of the relationship between two variables. For example, the correlation between serum perhexiline and length of therapy was r_s 0.13 (p 0.14), suggesting that length of therapy did not significantly affect serum perhexiline concentration.

Student's t-test establishes whether the difference between two means is significantly different from zero in a normal distribution. An independent test is used when the means are collected from unrelated samples of continuous data; for example, FFA were significantly higher in the perhexiline group 0.75mmol/L (SD 0.08) than in the control group 0.50mmol/L (SD 0.13) ($p<0.001$). In regression, it is used to test whether the standardised regression coefficient β is significantly different from zero; there was no significant relationship between serum perhexiline concentration and pre-ischaemia cardiac index (β -0.05, 95% CI -0.30 to 0.20, p 0.69).

9.7 Putative metabolite identification of peaks detected in mass spectra

Only peaks that were putatively annotated to at least one named metabolite using the *transformation mapping* approach are listed (Weber and Viant, 2010). None of the metabolite assignments reported can be regarded as definitive as they do not fulfil the Metabolomics Standards Initiative for identification (Sumner et al., 2007).

Observed		Putative Identification				
m/z	Average intensity [1]	Empirical formula	Ion form [2]	Theoretical mass (Da)	Error (ppm) [3]	Putative metabolite name(s) [4]
						4-Aminobutanoate
102.05601	5462.3	C4H9NO2	[M-H]-	102.05605	-0.42	N,N-Dimethylglycine
103.04003	4956.8	C4H8O3	[M-H]-	103.04007	-0.37	(R)-3-Hydroxybutanoate
104.03527	1718.6	C3H7NO3	[M-H]-	104.03532	-0.46	D-Serine', 'L-Serine
105.01929	95319.1	C3H6O4	[M-H]-	105.01933	-0.42	D-Glycerate
109.04069	7535.2	C5H6N2O	[M-H]-	109.04074	-0.43	Imidazole-4-acetaldehyde
113.02197	1491.3	C3H8O3	[M+Na-2H]-	113.02201	-0.39	Glycerol
114.05602	2688.4	C5H9NO2	[M-H]-	114.05605	-0.29	D-Proline, L-Proline
115.00366	4805.8	C4H4O4	[M-H]-	115.00368	-0.21	Fumarate, Maleic acid
						4-Methylaminobutyrate
116.07168	8315.2	C5H11NO2	[M-H]-	116.07170	-0.19	5-Aminopentanoate, Betaine
117.01931	14311.3	C4H6O4	[M-H]-	117.01933	-0.2	Succinate
						(R)-Lactate, (S)-Lactate,
125.00107	9964.8	C3H6O3	[M+Cl]-	125.00110	-0.21	D-Glyceraldehyde, Glycerone
125.00107	9964.8	C4H8O2	[M+K-2H]-	125.00104	0.27	(R)-Acetoin, Butanoic acid
125.03562	5027.5	C5H6N2O2	[M-H]-	125.03565	-0.25	Imidazole-4-acetate, 'Thymine
127.01672	2744.7	C3H8O3	[M+Cl]-	127.01675	-0.21	Glycerol
127.01672	2744.7	C4H10O2	[M+K-2H]-	127.01669	0.26	(R,R)-Butane-2,3-diol
						5,6-Dihydrothymine, gamma-
127.05127	372519.9	C5H8N2O2	[M-H]-	127.05130	-0.25	Amino-gamma-cyanobutanoate

						1-Pyrroline-4-hydroxy-2-carboxylate, 4-Oxoproline, 5-Oxo-D-proline, 5-Oxoproline, L-1-Pyrroline-3-hydroxy-5-carboxylate
128.03528	655414.2	C5H7NO3	[M-H]-	128.03532	-0.29	
						2,5-Dioxopentanoate, 2-Methylmaleate, 4,5-Dioxopentanoate
129.01931	8144.9	C5H6O4	[M-H]-	129.01933	-0.18	Mesaconate
						(S)-4-Amino-5-oxopentanoate, 2-Amino-4-oxopentanoic acid, 5-Amino-2-oxopentanoic acid, 5-Aminolevulinate, L-Glutamate 5-semialdehyde, cis-4-Hydroxy-D-proline,
130.05094	5129	C5H9NO3	[M-H]-	130.05097	-0.21	trans-4-Hydroxy-L-proline
130.06217	241162.8	C4H9N3O2	[M-H]-	130.06220	-0.24	Creatine
						(3R)-beta-Leucine, 6-Aminohexanoate,
130.08732	59580.2	C6H13NO2	[M-H]-	130.08735	-0.25	L-Isoleucine, L-Leucine
131.04619	16585.6	C4H8N2O3	[M-H]-	131.04622	-0.2	L-Asparagine
						(2R,4S)-2,4-Diaminopentanoate,
131.08257	4120.4	C5H12N2O2	[M-H]-	131.08260	-0.24	D-Ornithine, L-Ornithine
132.0302	94521.3	C4H7NO4	[M-H]-	132.03023	-0.25	D-Aspartate, L-Aspartate
						(R)-Malate, (S)-Malate,
133.01422	48717.7	C4H6O5	[M-H]-	133.01425	-0.22	3-Dehydro-L-threonate
134.04719	8941.2	C5H5N5	[M-H]-	134.04722	-0.21	Adenine
135.02987	10600.7	C4H8O5	[M-H]-	135.02990	-0.21	Threonate
135.0312	2324.2	C5H4N4O	[M-H]-	135.03123	-0.26	Hypoxanthine
136.0516	2490.4	C6H7N3O	[M-H]-	136.05164	-0.26	Isoniazid
						(3S)-3,6-Diaminohexanoate, (3S,5S)-3,5-Diaminohexanoate, 2,5-Diaminohexanoate,
145.09822	5757.4	C6H14N2O2	[M-H]-	145.09825	-0.22	D-Lysine, L-Lysine
156.02779	658.2	C4H9NO4	[M+Na-2H]-	156.02783	-0.24	4-Hydroxy-L-threonine

157.0273	1097.1	C8H8O2	[M+Na-2H]-	157.02710	1.28	4-Hydroxyphenylacetaldehyde
161.04552	15205	C6H10O5	[M-H]-	161.04555	-0.18	(2R,3S)-2,3-Dimethylmalate, (S)-2-(Hydroxymethyl)glutarate, 2-Dehydro-3-deoxy-D-fuconate, 2-Dehydro-3-deoxy-L-rhamnonate, 2-Deoxy-scyllo-inosose, L-Rhamnono-1,4-lactone
163.06114	89290.6	C6H12O5	[M-H]-	163.06120	-0.36	6-Deoxy-L-galactose, L-Fuculose, L-Rhamnofuranose, L-Rhamnose, L-Rhamnulose
164.07168	5482.5	C9H11NO2	[M-H]-	164.07170	-0.14	D-Phenylalanine, L-Phenylalanine
165.04029	625	C5H10O6	[M-H]-	165.04046	-1.05	D-Xylonate, L-Arabinonate
166.03886	10141.5	C4H9N3O2	[M+Cl]-	166.03888	-0.11	Creatine
166.03886	10141.5	C5H11N3O	[M+K-2H]-	166.03882	0.25	4-Guanidinobutanal
166.97508	4082.3	C3H5O6P	[M-H]-	166.97510	-0.13	3-Phosphonopyruvate, Phosphoenolpyruvate
166.97508	4082.3	C4H4O5	[M+Cl]-	166.97528	-1.18	Oxaloacetate, trans-2,3-Epoxysuccinate
166.97508	4082.3	C5H6O4	[M+K-2H]-	166.97522	-0.82	2,5-Dioxopentanoate, 2-Methylmaleate, 4,5-Dioxopentanoate, Mesaconate
167.01165	720.5	C4H9O5P	[M-H]-	167.01149	0.98	Butanoylphosphate
167.01165	720.5	C5H8O4	[M+Cl]-	167.01166	-0.07	(S)-2-Acetolactate, 2-Acetolactate, 3-Hydroxy-3-methyl-2-oxobutanoic acid
167.01165	720.5	C6H10O3	[M+K-2H]-	167.01160	0.29	(S)-3-Methyl-2-oxopentanoic acid, 4-Methyl-2-oxopentanoate, Adipate semialdehyde, beta-Ketoisocaproate
167.02104	7583.2	C5H4N4O3	[M-H]-	167.02106	-0.15	Urate

						2-Oxo-4-hydroxy-5-aminovalerate, D-Glutamate, DL-Glutamate, L-4-Hydroxyglutamate semialdehyde, L-Glutamate, L-threo-3-Methylaspartate,
168.02779	43985.4	C5H9NO4	[M+Na-2H]-	168.02783	-0.22	O-Acetyl-L-serine
168.04311	4676.8	C6H13NO2	[M+K-2H]-	168.04324	-0.75	(3R)-beta-Leucine, 6-Aminohexanoate, L-Isoleucine, L-Leucine
168.99074	2437.2	C3H7O6P	[M-H]-	168.99075	-0.07	D-Glyceraldehyde 3-phosphate, Glycerone phosphate
168.99074	2437.2	C4H6O5	[M+Cl]-	168.99093	-1.1	(R)-Malate, (S)-Malate, 3-Dehydro-L-threonate
168.99074	2437.2	C5H8O4	[M+K-2H]-	168.99087	-0.75	(S)-2-Acetolactate, 2-Acetolactate, 3-Hydroxy-3-methyl-2-oxobutanoic acid
169.02726	551.5	C5H10O4	[M+Cl]-	169.02731	-0.31	(R)-2,3-Dihydroxy-3-methylbutanoate, 2,3-Dihydroxy-3-methylbutanoate
169.02726	551.5	C9H8O2	[M+Na-2H]-	169.02710	0.95	4-Hydroxycinnamyl aldehyde, trans-Cinnamate
170.02387	2023.4	C5H5N5	[M+Cl]-	170.02390	-0.16	Adenine
171.00639	21316.7	C3H9O6P	[M-H]-	171.00640	-0.07	sn-Glycerol 3-phosphate
171.00639	21316.7	C4H8O5	[M+Cl]-	171.00658	-1.09	Threonate
171.00639	21316.7	C5H10O4	[M+K-2H]-	171.00652	-0.74	(R)-2,3-Dihydroxy-3-methylbutanoate, 2,3-Dihydroxy-3-methylbutanoate
171.02748	9568.7	C5H10O5	[M+Na-2H]-	171.02749	-0.08	D-Lyxose, D-Ribose,D-Ribulose, D-Xylose, D-Xylulose, L-Arabinose, L-Lyxose, L-Ribulose, L-Xylulose
171.0427	636237.6	C9H10O2	[M+Na-2H]-	171.04275	-0.28	4-Coumaryl alcohol, Phenylpropanoate
172.00175	1069.5	C4H9NO4	[M+K-2H]-	172.00177	-0.09	4-Hydroxy-L-threonine

173.00916	3776.7	C6H6O6	[M-H]-	173.00916	-0.02	Dehydroascorbate, cis-Aconitate
173.09312	567.9	C7H14N2O3	[M-H]-	173.09317	-0.27	N-Acetylornithine
173.10438	5872.4	C6H14N4O2	[M-H]-	173.10440	-0.11	D-Arginine, L-Arginine
174.0884	1065	C6H13N3O3	[M-H]-	174.08842	-0.09	L-Citrulline
175.0248	1250.6	C6H8O6	[M-H]-	175.02481	-0.08	(4S)-4,6-Dihydroxy-2,5-dioxohexanoate, 5-Dehydro-4-deoxy-D-glucuronate, Ascorbate, D-Glucuronolactone, L-xylo-Hexulonolactone
177.04043	549	C6H10O6	[M-H]-	177.04046	-0.19	2,4,6/3,5-Pentahydroxycyclohexanone 2-Dehydro-3-deoxy-D-gluconate, 2-Deoxy-5-keto-D-gluconic acid, 5-Deoxy glucuronic acid, D-Galactono-1,4-lactone, D-Glucono-1,5-lactone, L-Galactono-1,4-lactone, L-Gulono-1,4-lactone
179.0115	3779.7	C6H8O4	[M+Cl]-	179.01166	-0.9	2,3-Dimethylmaleate, 2-Methyleneglutarate Methylitaconate
179.0561	16787.6	C6H12O6	[M-H]-	179.05611	-0.08	D-Fructose, D-Fuconate, D-Galactose, D-Glucose, D-Mannose, D-Tagatose, L-Galactose, L-Rhamnonate, L-Sorbose, alpha-D-Glucose, beta-D-Fructose, beta-D-Glucose, myo-Inositol
179.05735	1052.4	C7H8N4O2	[M-H]-	179.05745	-0.56	1,7-Dimethylxanthine, Theobromine
180.06661	7890.5	C9H11NO3	[M-H]-	180.06662	-0.04	3-Amino-3-(4-hydroxyphenyl)propanoate, L-Tyrosine
181.07151	685826.3	C6H14O6	[M-H]-	181.07176	-1.4	D-Sorbitol, Galactitol, Mannitol
184.00177	6718.1	C3H8NO6P	[M-H]-	184.00165	0.65	O-Phospho-L-serine

						2-Oxo-4-hydroxy-5-aminovalerate, D-Glutamate, DL-Glutamate, L-4-Hydroxyglutamate semialdehyde, L-Glutamate, L-threo-3-Methylaspartate,
184.00177	6718.1	C5H9NO4	[M+K-2H]-	184.00177	0.02	O-Acetyl-L-serine
184.98564	5791.1	C3H7O7P	[M-H]-	184.98567	-0.14	2-Phospho-D-glycerate, 3-Phospho-D-glycerate
184.98564	5791.1	C4H6O6	[M+Cl]-	184.98584	-1.09	(R,R)-Tartaric acid, meso-Tartaric acid
184.98564	5791.1	C5H8O5	[M+K-2H]-	184.98578	-0.77	(R)-2-Methylmalate, (S)-2-Methylmalate, 2-Dehydro-3-deoxy-D-xylonate, 2-Dehydro-3-deoxy-L-arabinonate, D-Xylonolactone, D-erythro-3-Methylmalate, L-Arabinono-1,4-lactone
186.11354	44783.5	C9H17NO3	[M-H]-	186.11357	-0.15	N2-Acetyl-L-lysine
187.00142	7015.3	C5H10O5	[M+K-2H]-	187.00143	-0.06	D-Lyxose, D-Ribose,D-Ribulose, 'D-Xylose, D-Xylulose, L-Arabinose, L-Lyxose, L-Ribulose, L-Xylulose
187.00593	698.7	C6H6N4S	[M+Na-2H]-	187.00599	-0.3	6-Methylmercaptopurine
187.03787	2754.2	C5H12O5	[M+Cl]-	187.03788	-0.04	L-Arabitol, Ribitol, Xylitol
187.03787	2754.2	C9H10O3	[M+Na-2H]-	187.03766	1.1	3-(2-Hydroxyphenyl)propanoate, 3-(3-Hydroxy-phenyl)-propanoic acid, 3-Methoxy-4-hydroxyphenylacetaldehyde
188.05641	708.6	C7H11NO5	[M-H]-	188.05645	-0.2	L-2-Amino-6-oxoheptanedioate, N-Acetyl-L-glutamate
188.957	720.9	C3H5O6P	[M+Na-2H]-	188.95705	-0.25	3-Phosphonopyruvate, Phosphoenolpyruvate
189.04047	3389	C7H10O6	[M-H]-	189.04046	0.03	2,4-Dihydroxyhept-2-enedioate, 3-Dehydroquinate

189.0517	2695.2	C6H10N2O5	[M-H]-	189.05170	0.02	N-Carbamyl-L-glutamate
189.07685	9257.5	C8H14O5	[M-H]-	189.07685	0.01	(R)-3-((R)-3-Hydroxybutanoyloxy)butanoate
189.08807	959.2	C7H14N2O4	[M-H]-	189.08808	-0.06	LL-2,6-Diaminoheptanedioate, meso-2,6-Diaminoheptanedioate
189.98882	2741.4	C3H8NO5P	[M+Na-2H]-	189.98868	0.73	2-Amino-3-phosphonopropanoate
190.07209	1667.2	C7H13NO5	[M-H]-	190.07210	-0.04	2-Amino-3,7-dideoxy-D-threo-hept-6-ulosonic acid
191.01972	42163.2	C6H8O7	[M-H]-	191.01973	-0.05	(4R,5S)-4,5,6-Trihydroxy-2,3-dioxohexanoate, 5-Dehydro-4-deoxy-D-glucarate, Citrate, Isocitrate
191.05612	12923	C7H12O6	[M-H]-	191.05611	0.03	Quinate
192.98839	2227.1	C3H9O6P	[M+Na-2H]-	192.98835	0.22	sn-Glycerol 3-phosphate
193.03539	1757.2	C6H10O7	[M-H]-	193.03538	0.06	2-Keto-D-gluconic acid, 3-Dehydro-L-gulonate, D-Fructuronate, D-Galacturonate, D-Glucuronate, D-Tagaturonate
193.07176	7304.9	C7H14O6	[M-H]-	193.07176	-0.02	1-O-Methyl-myo-inositol, 3-O-Methyl-myo-inositol
194.05469	926.2	C6H11N3O3	[M+Na-2H]-	194.05471	-0.11	5-Guanidino-2-oxopentanoate
194.06703	1752.4	C6H13NO6	[M-H]-	194.06701	0.09	2-Amino-2-deoxy-D-gluconate
195.043	1060.9	C8H14O3	[M+K-2H]-	195.04290	0.5	2-n-Propyl-4-oxopentanoic acid, 3-Oxovalproic acid
195.05104	3437.7	C6H12O7	[M-H]-	195.05103	0.06	D-Altronate, D-Gluconic acid, D-Mannonate
195.05237	1247.2	C7H8N4O3	[M-H]-	195.05236	0.03	1,7-Dimethyluric acid, 3,7-Dimethyluric acid
196.98578	630.8	C5H6O6	[M+Cl]-	196.98584	-0.31	D-4-Hydroxy-2-oxoglutarate
196.98578	630.8	C6H8O5	[M+K-2H]-	196.98578	-0.01	2-Formylglutarate, 2-Oxoadipate, 3D-(3,5/4)-Tri-hydroxycyclohexane-1,2-dione

						(4S)-4,6-Dihydroxy-2,5-dioxohexanoate, 5-Dehydro-4-deoxy-D-glucuronate,Ascorbate, D-Glucuronolactone,
197.00679	3507.5	C6H8O6	[M+Na-2H]-	197.00676	0.16	L-xylo-Hexulonolactone
						3-Hydroxyvalproic acid, 4-Hydroxyvalproic acid,
197.05862	1211.3	C8H16O3	[M+K-2H]-	197.05855	0.35	5-Hydroxyvalproic acid
199.00137	1302.8	C4H9O7P	[M-H]-	199.00132	0.27	D-Erythrose 4-phosphate
						(2R,3S)-2,3-Dimethylmalate, (S)-2-(Hydroxymethyl)glutarate, 2-Dehydro-3-deoxy-D-fuconate, 2-Dehydro-3-deoxy-L-rhamnonate, 2-Deoxy-scyllo-inososе,
199.00137	1302.8	C6H10O5	[M+K-2H]-	199.00143	-0.31	L-Rhamnono-1,4-lactone
						2,4,6/3,5-Pentahydroxycyclohexanone, 2-Dehydro-3-deoxy-D-gluconate, 2-Deoxy-5-keto-D-gluconic acid, 5-Deoxy glucuronic acid, D-Galactono-1,4-lactone, D-Glucono-1,5-lactone, L-Galactono-1,4-lactone,
199.02248	2872.5	C6H10O6	[M+Na-2H]-	199.02241	0.36	L-Gulono-1,4-lactone
199.03779	116504.7	C10H10O3	[M+Na-2H]-	199.03766	0.64	Coniferyl aldehyde
						6-Deoxy-L-galactose, L-Fuculose, L-Rhamnofuranose,
199.03779	116504.7	C6H12O5	[M+Cl]-	199.03788	-0.44	L-Rhamnose, L-Rhamnulose
199.99664	1270.7	C5H9NO5	[M+K-2H]-	199.99668	-0.2	L-erythro-4-Hydroxyglutamate
200.03301	2277.1	C6H13NO4	[M+K-2H]-	200.03307	-0.28	2-Deoxy-scyllo-inosamine
						D-Phenylalanine,
200.04837	962.3	C9H11NO2	[M+Cl]-	200.04838	-0.05	L-Phenylalanine
200.05408	9870.2	C6H13NO5	[M+Na-2H]-	200.05404	0.19	D-Glucosamine
201.01696	1158.4	C5H10O6	[M+Cl]-	201.01714	-0.9	D-Xylonate, L-Arabinonate

201.01696	1158.4	C6H12O5	[M+K-2H]-	201.01708	-0.6	6-Deoxy-L-galactose, L-Fuculose, L-Rhamnofuranose, L-Rhamnose, L-Rhamnulose
201.01696	1158.4	C9H8O4	[M+Na-2H]-	201.01693	0.16	2-Hydroxy-3-(4-hydroxyphenyl)propenoate, 3-(4-Hydroxyphenyl)pyruvate, Caffeate, trans-2,3-Dihydroxycinnamate
201.03229	2192.3	C13H8O	[M+Na-2H]-	201.03218	0.53	Fluoren-9-one
201.03229	2192.3	C9H10O3	[M+Cl]-	201.03240	-0.53	3-(2-Hydroxyphenyl)propanoate, 3-(3-Hydroxy-phenyl)-propanoic acid, 3-Methoxy-4-hydroxyphenylacetaldehyde
201.03808	16997.8	C6H12O6	[M+Na-2H]-	201.03806	0.11	D-Fructose, D-Fuconate, D-Galactose, D-Glucose, D-Mannose, D-Tagatose, L-Galactose, L-Rhamnonate, L-Sorbose, alpha-D-Glucose, beta-D-Fructose, beta-D-Glucose, myo-Inositol
201.05355	4583.3	C10H12O3	[M+Na-2H]-	201.05331	1.18	Coniferyl alcohol
203.08264	14621.7	C11H12N2O2	[M-H]-	203.08260	0.19	L-Tryptophan
204.01994	934.7	C4H12NO4P	[M+Cl]-	204.01980	0.69	Phosphodimethylethanolamine
204.04331	3352.3	C8H11NO3	[M+Cl]-	204.04330	0.07	Pyridoxine
204.04331	3352.3	C9H13NO2	[M+K-2H]-	204.04324	0.36	3-Methoxytyramine
204.93102	1138.4	C3H5O6P	[M+K-2H]-	204.93098	0.17	3-Phosphonopyruvate, Phosphoenolpyruvate
204.99815	1103.8	C5H4N4O4	[M+Na-2H]-	204.99792	1.1	5-Hydroxyisourate
205.03543	15956.3	C7H10O7	[M-H]-	205.03538	0.25	(2S,3R)-3-Hydroxybutane-1,2,3-tricarboxylate, Homoisocitrate
205.96285	2556.5	C3H8NO5P	[M+K-2H]-	205.96262	1.12	2-Amino-3-phosphonopropanoate
206.96766	35241.3	C3H7O7P	[M+Na-2H]-	206.96761	0.23	2-Phospho-D-glycerate, 3-Phospho-D-glycerate
206.98315	746.2	C3H9O6P	[M+Cl]-	206.98308	0.34	sn-Glycerol 3-phosphate'

208.96238	1936.4	C3H9O6P	[M+K-2H]-	208.96228	0.46	sn-Glycerol 3-phosphate
208.98591	39463.6	C6H6O6	[M+Cl]-	208.98584	0.33	Dehydroascorbate, cis-Aconitate
208.98591	39463.6	C7H8O5	[M+K-2H]-	208.98578	0.61	2-Hydroxy-5-methyl-cis,cis-muconate, 2-Hydroxyhepta-2,4-dienedioate, 2-Oxo-5-methyl-cis-muconate, 2-Oxohept-3-enedioate, 3-Dehydroshikimate
209.02213	12081.9	C7H10O5	[M+Cl]-	209.02223	-0.46	(2S)-2-Isopropyl-3-oxosuccinate, Shikimate
209.05867	875.4	C8H14O4	[M+Cl]-	209.05861	0.28	2-Propylglutaric acid
209.06981	600.7	C7H14N2O3	[M+Cl]-	209.06984	-0.17	N-Acetylornithine
209.08114	2265.9	C6H14N4O2	[M+Cl]-	209.08108	0.3	D-Arginine, L-Arginine
210.02852	170043.1	C4H10N3O5P	[M-H]-	210.02853	-0.06	Phosphocreatine
210.02852	170043.1	C6H11N3O3	[M+K-2H]-	210.02865	-0.61	5-Guanidino-2-oxopentanoate
210.03845	3842	C7H11NO5	[M+Na-2H]-	210.03839	0.27	L-2-Amino-6-oxoheptanedioate, N-Acetyl-L-glutamate
211.00143	2352.8	C6H8O6	[M+Cl]-	211.00149	-0.29	(4S)-4,6-Dihydroxy-2,5-dioxohexanoate, 5-Dehydro-4-deoxy-D-glucuronate, Ascorbate, D-Glucuronolactone, L-xylo-Hexulonolactone
211.00143	2352.8	C7H10O5	[M+K-2H]-	211.00143	-0.01	(2S)-2-Isopropyl-3-oxosuccinate, Shikimate
211.03368	1909.3	C6H10N2O5	[M+Na-2H]-	211.03364	0.18	N-Carbamyl-L-glutamate
211.04918	15755.7	C7H14N2O3	[M+K-2H]-	211.04905	0.62	N-Acetylornithine
212.03311	3282.8	C10H9NO3	[M+Na-2H]-	212.03291	0.93	5-Phenyl-1,3oxazinane-2,4dione
212.05412	1806	C7H13NO5	[M+Na-2H]-	212.05404	0.37	2-Amino-3,7-dideoxy-D-threo-hept-6-ulosonic acid
212.98075	3530.4	C4H7O8P	[M-H]-	212.98058	0.79	2-Oxo-3-hydroxy-4-phosphobutanoate
212.98075	3530.4	C6H8O6	[M+K-2H]-	212.98070	0.25	(4S)-4,6-Dihydroxy-2,5-dioxohexanoate, 5-Dehydro-4-deoxy-D-glucuronate, Ascorbate, D-Glucuronolactone, L-xylo-Hexulonolactone

						(4R,5S)-4,5,6-Trihydroxy-2,3-dioxohexanoate, 5-Dehydro-4-deoxy-D-glucarate,
213.00173	2033.9	C6H8O7	[M+Na-2H]-	213.00167	0.26	Citrate, Isocitrate
213.03811	1266.7	C7H12O6	[M+Na-2H]-	213.03806	0.24	Quinate
						3-Carbamoyl-2-phenylpropionaldehyde, 4-Hydroxy-5-phenyltetrahydro-
214.04862	116610.9	C10H11NO3	[M+Na-2H]-	214.04856	0.27	1,3-oxazin-2-one
214.04862	116610.9	C5H14NO6P	[M-H]-	214.04860	0.09	sn-glycero-3-Phosphoethanolamine
214.04862	116610.9	C6H13NO5	[M+Cl]-	214.04878	-0.73	D-Glucosamine
214.98975	3280.7	C5H10N2O3S	[M+K-2H]-	214.98982	-0.33	Cys-Gly
214.9961	734.1	C4H9O8P	[M-H]-	214.99623	-0.61	4-Phospho-D-erythronate
						2,4,6/3,5-Pentahydroxycyclohexanone, 2-Dehydro-3-deoxy-D-gluconate, 2-Deoxy-5-keto-D-gluconic acid, 5-Deoxy glucuronic acid, D-Galactono-1,4-lactone, D-Glucono-1,5-lactone, L-Galactono-1,4-lactone,
214.9961	734.1	C6H10O6	[M+K-2H]-	214.99635	-1.15	L-Gulono-1,4-lactone
						2-Keto-D-gluconic acid, 3-Dehydro-L-gulonate, D-Fructuronate, D-Galacturonate,
215.01737	4629.4	C6H10O7	[M+Na-2H]-	215.01732	0.22	D-Glucuronate, D-Tagaturonate
215.02714	634.1	C13H8O	[M+Cl]-	215.02692	1.04	Fluoren-9-one
						5-Hydroxyconiferaldehyde,
215.03278	91480	C10H10O4	[M+Na-2H]-	215.03258	0.94	Ferulate
						2-C-Methyl-D-erythritol
215.03278	91480	C5H13O7P	[M-H]-	215.03262	0.76	4-phosphate

						D-Fructose, D-Fuconate, D-Galactose, D-Glucose, D-Mannose, D-Tagatose, L-Galactose, L-Rhamnonate, L-Sorbose, alpha-D-Glucose, beta-D-Fructose,
215.03278	91480	C6H12O6	[M+Cl]-	215.03279	-0.05	beta-D-Glucose, myo-Inositol
215.05363	1349.5	C7H14O6	[M+Na-2H]-	215.05371	-0.37	1-O-Methyl-myo-inositol, 3-O-Methyl-myo-inositol
216.02799	4357.8	C6H13NO5	[M+K-2H]-	216.02798	0.04	D-Glucosamine
216.02799	4357.8	C9H9NO4	[M+Na-2H]-	216.02783	0.75	2-Carboxy-2,3-dihydro-5,6-dihydroxyindole, Dopaquinone
216.13542	767.4	C9H19N3O3	[M-H]-	216.13537	0.25	gamma-L-Glutamylputrescine
217.01213	1572.9	C6H12O6	[M+K-2H]-	217.01200	0.61	D-Fructose, D-Fuconate, D-Galactose, D-Glucose, D-Mannose, D-Tagatose, L-Galactose, L-Rhamnonate, L-Sorbose, alpha-D-Glucose, beta-D-Fructose, beta-D-Glucose, myo-Inositol
217.04253	791.3	C13H10O	[M+Cl]-	217.04257	-0.17	Fluoren-9-ol
218.10343	8973.3	C9H17NO5	[M-H]-	218.10340	0.15	Pantothenate
219.00654	1197.1	C9H10O4	[M+K-2H]-	219.00652	0.11	3-(2,3-Dihydroxyphenyl)propanoate, 3-(4-Hydroxyphenyl)lactate, Homovanillate, cis-3-(3-Carboxyethenyl)-3,5-cyclohexadiene-1,2-diol
219.97849	3886	C3H8NO6P	[M+Cl]-	219.97833	0.73	O-Phospho-L-serine
222.94162	6725.9	C3H7O7P	[M+K-2H]-	222.94155	0.32	2-Phospho-D-glycerate, 3-Phospho-D-glycerate
245.07017	6032.6	C10H14N2O3	[M+Cl]-	245.06984	1.33	2,6-Dihydroxypseudooxynicotine
63.96846	15672	C5H10NO7P	[M+K-2H]-	263.96810	1.37	L-Glutamyl 5-phosphate
302.06573	9744.5	C10H13N5O4	[M+Cl]-	302.06616	-1.41	Adenosine, Deoxyguanosine

306.0761	478523.4	C10H17N3O6S	[M-H]-	306.07653	-1.41	Glutathione
308.06946	2138.7	C16H17NO3	[M+K-2H]-	308.06945	0.03	(S)-Norcoclaurine
308.09828	4051.6	C11H19NO9	[M-H]-	308.09871	-1.39	N-Acetylneuraminate
311.22236	2738.6	C18H32O4	[M-H]-	311.22278	-1.36	(7S,8S)-DiHODE, 8(R)-HPODE
326.01567	63391.4	C9H12N3O7P	[M+Na-2H]-	326.01596	-0.89	2',3-Cyclic CMP
326.99966	5927.7	C9H11N2O8P	[M+Na-2H]-	326.99997	-0.96	2',3-Cyclic UMP
328.04489	3199.4	C10H12N5O6P	[M-H]-	328.04525	-1.09	2',3-Cyclic AMP, 3',5-Cyclic AMP
335.04271	10082.4	C10H20O7P2	[M+Na-2H]-	335.04310	-1.16	(+)-Bornyl-diphosphate, Geranyl diphosphate
343.12408	5427	C12H24O11	[M-H]-	343.12459	-1.48	Melibiitol
344.0268	1559.3	C9H14N3O8P	[M+Na-2H]-	344.02652	0.8	3-CMP, CMP
369.0207	5620.6	C9H17O12P	[M+Na-2H]-	369.02044	0.71	2-(alpha-D-Mannosyl)-3-phosphoglycerate
373.03914	1314.7	C12H16O12	[M+Na-2H]-	373.03885	0.78	4-(4-Deoxy-alpha-D-gluc-4-enuronosyl)-D-galacturonate
384.9939	1695.7	C9H17O12P	[M+K-2H]-	384.99437	-1.23	2-(alpha-D-Mannosyl)-3-phosphoglycerate
386.01629	18092.6	C9H15N3O10P2	[M-H]-	386.01600	0.76	dCDP
389.00022	6867.6	C10H13N2O11P	[M+Na-2H]-	389.00037	-0.38	Orotidine 5-phosphate
389.01254	4007.2	C12H16O12	[M+K-2H]-	389.01279	-0.63	4-(4-Deoxy-alpha-D-gluc-4-enuronosyl)-D-galacturonate
400.01021	1097.6	C10H14N5O7PS	[M+Na-2H]-	400.00983	0.95	6-Thioguanosine monophosphate
402.99445	48074.1	C9H14N2O12P2	[M-H]-	402.99493	-1.19	UDP
406.95553	2818.2	C7H16O13P2	[M+K-2H]-	406.95523	0.74	D-glycero-D-manno-Heptose 1,7-bisphosphate, Sedoheptulose1,7-bisphosphate
407.99836	4460	C9H15N3O10P2	[M+Na-2H]-	407.99794	1.03	dCDP
417.31395	842.7	C28H44O	[M+Na-2H]-	417.31388	0.16	3-Keto-4-methylzymosterol, 5,7,24(28)-Ergostatrienol, 5-Dehydroepisterol, Ergosterol, Vitamin D2
426.02083	1828916.5	C11H20NO12P	[M+K-2H]-	426.02092	-0.22	Acetylneuraminate 9-phosphate
440.95039	6666.7	C9H14N2O12P2	[M+K-2H]-	440.95081	-0.95	UDP

447.9827	9786	C10H15N5O9P2	[M+K-2H]-	447.98311	-0.92	dADP
455.09698	4283.9	C17H21N4O9P	[M-H]-	455.09734	-0.8	FMN
462.06713	8018.7	C14H18N5O11P	[M-H]-	462.06677	0.78	N6-(1,2-Dicarboxyethyl)-AMP
463.96836	8167.1	C10H14N5O10PS	[M+K-2H]-	463.96851	-0.33	Adenylyl sulfate
484.04894	14280.2	C14H18N5O11P	[M+Na-2H]-	484.04872	0.46	N6-(1,2-Dicarboxyethyl)-AMP
496.09594	7874.9	C16H27N4O8P2S	[M-H]-	496.09521	1.47	2-Methyl-1-hydroxypropyl-ThPP
500.02272	5669.7	C14H18N5O11P	[M+K-2H]-	500.02265	0.13	N6-(1,2-Dicarboxyethyl)-AMP
504.94246	1827.6	C9H15N2O15P3	[M+Na-2H]-	504.94321	-1.48	UTP
505.98839	5903.7	C10H16N5O13P3	[M-H]-	505.98848	-0.17	ATP, dGTP
527.95021	11173.6	C10H16N5O12P3	[M+K-2H]-	527.94945	1.45	dATP
543.94407	23546.1	C10H16N5O13P3	[M+K-2H]-	543.94436	-0.53	ATP, dGTP
558.06425	4178.7	C15H23N5O14P2	[M-H]-	558.06440	-0.28	ADP-ribose
565.04748	11914.7	C15H24N2O17P2	[M-H]-	565.04775	-0.48	UDP-D-galactose, UDP-glucose
580.04626	5302.8	C15H23N5O14P2	[M+Na-2H]-	580.04635	-0.15	ADP-ribose
587.02954	26051	C15H24N2O17P2	[M+Na-2H]-	587.02970	-0.27	UDP-D-galactose, UDP-glucose
588.07499	2797.2	C16H25N5O15P2	[M-H]-	588.07497	0.03	GDP-L-fucose
596.01996	2716.4	C15H23N5O14P2	[M+K-2H]-	596.02029	-0.55	ADP-ribose
603.00377	12665.6	C15H24N2O17P2	[M+K-2H]-	603.00364	0.22	UDP-D-galactose, UDP-glucose
604.06991	853.3	C16H25N5O16P2	[M-H]-	604.06988	0.04	GDP-L-galactose, GDP-L-glucose, GDP-mannose
606.07387	10431.6	C17H27N3O17P2	[M-H]-	606.07430	-0.71	UDP-N-acetyl-D-galactosamine, UDP-N-acetyl-D-glucosamine, UDP-N-acetyl-D-mannosamine
611.14414	73610.7	C20H32N6O12S2	[M-H]-	611.14469	-0.9	Glutathione disulfide
613.14004	7208.6	C20H31N4O16P	[M-H]-	613.14000	0.07	CMP-N-acetylneuraminate
628.05573	17769.1	C17H27N3O17P2	[M+Na-2H]-	628.05625	-0.82	UDP-N-acetyl-D-galactosamine, UDP-N-acetyl-D-glucosamine, UDP-N-acetyl-D-mannosamine
633.12603	63138.8	C20H32N6O12S2	[M+Na-2H]-	633.12664	-0.96	Glutathione disulfide
635.12197	7328.8	C20H31N4O16P	[M+Na-2H]-	635.12194	0.04	CMP-N-acetylneuraminate
649.10006	34790.8	C20H32N6O12S2	[M+K-2H]-	649.10057	-0.79	Glutathione disulfide

1. Average intensity across all samples (n=22 perhexiline, n=21 control).
2. Ion form in which metabolite occurs in mass spectrum i.e. $[M-H]^-$, $[M+Cl]^-$, $[M+Na-2H]^-$, $[M+K-2H]^-$.
3. Error between observed and theoretical masses, presented as parts per million of theoretical mass.
4. Derived from the transformation mapping approach for metabolites (Sumner et al., 2007).

REFERENCES

Abozguia, K., Clarke, K., Lee, L., et al. (2006) Modification of myocardial substrate use as a therapy for heart failure. **Nat Clin Pract Cardiovasc Med***,* 3: (9): 490-498.

Abozguia, K., Elliott, P., McKenna, W., et al. (2010) Metabolic modulator perhexiline corrects energy deficiency and improves exercise capacity in symptomatic hypertrophic cardiomyopathy. **Circulation***,* 122: (16): 1562-1569.

Albin, H., Pere, J.C., Begaud, B., et al. (1981) [Adverse hepatitis effects and imputability (author's transl)]. **Therapie***,* 36: (4): 477-482.

Alderman, C.P., Hundertmark, J.D. and Soetratma, T.W. (1997) Interaction of serotonin re-uptake inhibitors with perhexiline. **Aust N Z J Psychiatry***,* 31: (4): 601-603.

Allen, B.S. (2004) Pediatric myocardial protection: where do we stand? **J Thorac Cardiovasc Surg***,* 128: (1): 11-13.

Andreasen, A.T. and Watson, F. (1952) Experimental cardiovascular surgery. **Br J Surg***,* 39: (158): 548-551.

Argaud, L., Gomez, L., Gateau-Roesch, O., et al. (2005) Trimetazidine inhibits mitochondrial permeability transition pore opening and prevents lethal ischemia-reperfusion injury. **J Mol Cell Cardiol***,* 39: (6): 893-899.

Arnold, J.M., Braunwald, E., Sandor, T., et al. (1985) Inotropic stimulation of reperfused myocardium with dopamine: effects on infarct size and myocardial function. **J Am Coll Cardiol***,* 6: (5): 1026-1034.

Ashrafian, H., Czibik, G., Bellahcene, M., et al. (2012) Fumarate is cardioprotective via activation of the Nrf2 antioxidant pathway. **Cell Metab***,* 15: (3): 361-371.

Ashrafian, H., Horowitz, J.D. and Frenneaux, M.P. (2007) Perhexiline. **Cardiovasc Drug Rev***,* 25: (1): 76-97.

Banner, N.R., Thomas, H.L., Curnow, E., et al. (2008) The importance of cold and warm cardiac ischemia for survival after heart transplantation. **Transplantation***,* 86: (4): 542-547.

Barclay, M.L., Sawyers, S.M., Begg, E.J., et al. (2003) Correlation of CYP2D6 genotype with perhexiline phenotypic metabolizer status. **Pharmacogenetics***,* 13: (10): 627-632.

Barnard, K.D., Dent, L. and Cook, A. (2010) A systematic review of models to predict recruitment to multicentre clinical trials. **BMC Med Res Methodol***,* 10: 63.

Barner, H.B. (1991) Blood cardioplegia: a review and comparison with crystalloid cardioplegia. **Ann Thorac Surg***,* 52: (6): 1354-1367.

Barnett, V. 2011 **Personal Communication**

Beadle, R.M. 2011 **Personal Communication**

Beadle, R.M., Williams, L.K., Abozguia, K., et al. (2011) Metabolic manipulation in chronic heart failure: study protocol for a randomised controlled trial. **Trials***,* 12: 140.

Benjamini, Y. and Hochberg, Y. (1995) Controlling the false discovery rate - a practical and powerful approach to multiple testing. **J R Stat Soc Ser B-Methodol***,* 57: (1): 289-300.

Bentall, H.H. and Melrose, D.G. (1957) Elective cardiac arrest: lactic acid production in the arrested heart. **J Physiol***,* 135: (2): 38-39P.

Bergstralh, E. and Kosanke, J. (2003) "dist macro for SAS". Rochester, MN, Mayo Clinic

Bessho, R. and Chambers, D.J. (2002) Myocardial protection with oxygenated esmolol cardioplegia during prolonged normothermic ischemia in the rat. **J Thorac Cardiovasc Surg***,* 124: (2): 340-351.

Bigelow, W.G. (1984) **Cold Hearts: the story of hypothermia and the pacemaker in heart surgery.** Toronto, ON: McClelland & Stewart.

Bigelow, W.G., Callaghan, J.C. and Hopps, J.A. (1950a) General hypothermia for experimental intracardiac surgery; the use of electrophrenic respirations, an artificial pacemaker for cardiac standstill and radio-frequency rewarming in general hypothermia. **Ann Surg***,* 132: (3): 531-539.

Bigelow, W.G., Lindsay, W.K. and et al. (1950b) Oxygen transport and utilization in dogs at low body temperatures. **Am J Physiol***,* 160: (1): 125-137.

Bigelow, W.G., Lindsay, W.K. and Greenwood, W.F. (1950c) Hypothermia; its possible role in cardiac surgery: an investigation of factors governing survival in dogs at low body temperatures. **Ann Surg***,* 132: (5): 849-866.

Bitzikas, G., Papakonstantinou, C., Lazou, A., et al. (2005) The supportive value of pre-bypass L-glutamate loading in patients undergoing coronary artery bypass grafting. **J Cardiovasc Surg (Torino)***,* 46: (6): 551-557.

Blalock, A. and Taussig, H.B. (1945) The surgical treatment of malformations of the heart in which there is pulmonary stenosis or pulmonary atresia. **JAMA***,* 128: 189-202.

Boseley, S. (2006) "Six men in intensive care after drug trial goes wrong". **The Guardian.** London, Guardian News & Media Ltd.

Bothe, W., Olschewski, M., Beyersdorf, F., et al. (2004) Glucose-insulin-potassium in cardiac surgery: a meta-analysis. **Ann Thorac Surg***,* 78: (5): 1650-1657.

Bouche, C., Serdy, S., Kahn, C.R., et al. (2004) The cellular fate of glucose and its relevance in type 2 diabetes. **Endocr Rev***,* 25: (5): 807-830.

Braimbridge, M.V. (1992) "European origins of cardioplegia". In Engelman, R.M. & Levitsky, S. (Eds.) **A textbook of cardioplegia for difficult clinical problems***.* Mount Kisco, Futura 9-16.

Braimbridge, M.V., Clement, A.J., Brown, A.H., et al. (1969) Triple Starr valve replacement. **Br Med J***,* 3: (5672): 683-688.

Braunwald, E. and Kloner, R.A. (1982) The stunned myocardium: prolonged, postischemic ventricular dysfunction. **Circulation***,* 66: (6): 1146-1149.

Braunwald, E. and Kloner, R.A. (1985) Myocardial reperfusion: a double-edged sword? **J Clin Invest***,* 76: (5): 1713-1719.

Bretschneider, H.J., Hubner, G., Knoll, D., et al. (1975) Myocardial resistance and tolerance to ischemia: physiological and biochemical basis. **J Cardiovasc Surg (Torino)***,* 16: (3): 241-260.

Bridgewater, B., Gummert, J., Walton, P., et al. (2010) **Fourth EACTS Adult Cardiac Surgical Database Report 2010. Towards global benchmarking.** Henley-on-Thamas: Dendrite Clinical Systems Ltd.

Bridgewater, B., Keogh, B.E., Kinsman, R., et al. (2009) **Sixth National Adult Cardiac Surgery Database Report 2008. Demonstrating Quality.** Henley-on-Thames: Dendrite Clinical Systems Ltd.

Brindley, D.N., Kok, B.P., Kienesberger, P.C., et al. (2010) Shedding light on the enigma of myocardial lipotoxicity: the involvement of known and putative regulators of fatty acid storage and mobilization. **Am J Physiol Endocrinol Metab***,* 298: (5): E897-908.

Brintnall-Karabelas, J., Sung, S., Cadman, M.E., et al. (2011) Improving recruitment in clinical trials: why eligible participants decline. **J Empir Res Hum Res Ethics***,* 6: (1): 69-74.

Buckberg, G.D. (1987) Strategies and logic of cardioplegic delivery to prevent, avoid, and reverse ischemic and reperfusion damage. **J Thorac Cardiovasc Surg***,* 93: (1): 127-139.

Buckberg, G.D., Brazier, J.R., Nelson, R.L., et al. (1977) Studies of the effects of hypothermia on regional myocardial blood flow and metabolism during cardiopulmonary bypass. I. The adequately perfused beating, fibrillating, and arrested heart. **J Thorac Cardiovasc Surg***,* 73: (1): 87-94.

Burns-Cox, C.J., Chandrasekhar, K.P., Ikram, H., et al. (1971) Clinical evaluation of perhexiline maleate in patients with angina pectoris. **Br Med J***,* 4: (5787): 586-588.

Campbell, M.K., Snowdon, C., Francis, D., et al. (2007) Recruitment to randomised trials: strategies for trial enrollment and participation study. The STEPS study. **Health Technol Assess***,* 11: (48): iii, ix-105.

Carruthers, S.G., Cleland, J., Kelly, J.G., et al. (1975) Plasma and tissue digoxin concentrations in patients undergoing cardiopulmonary bypass. **Br Heart J***,* 37: (3): 313-320.

Carter, R.E. (2004) Application of stochastic processes to participant recruitment in clinical trials. **Control Clin Trials***,* 25: (5): 429-436.

Cheung, M.M., Kharbanda, R.K., Konstantinov, I.E., et al. (2006) Randomized controlled trial of the effects of remote ischemic preconditioning on children undergoing cardiac surgery: first clinical application in humans. **J Am Coll Cardiol***,* 47: (11): 2277-2282.

Cho, Y.W., Belej, M. and Aviado, D.M. (1970) Pharmacology of a new antianginal drug: perhexiline. I. Coronary circulation and myocardial metabolism. **Chest**, 58: (6): 577-581.

Cole, P.L., Beamer, A.D., McGowan, N., et al. (1990) Efficacy and safety of perhexiline maleate in refractory angina. A double-blind placebo-controlled clinical trial of a novel antianginal agent. **Circulation**, 81: (4): 1260-1270.

Cooley, D.A., Reul, G.J. and Wukasch, D.C. (1972) Ischemic contracture of the heart: "stone heart". **Am J Cardiol**, 29: (4): 575-577.

Cordell, A.R. (1995) Milestones in the development of cardioplegia. **Ann Thorac Surg**, 60: (3): 793-796.

Crafoord, C., Norberg, B. and Senning, A. (1957) Clinical studies in extracorporeal circulation with a heart-lung machine. **Acta Chir Scand**, 112: (3-4): 220-245.

Croal, B.L., Hillis, G.S., Gibson, P.H., et al. (2006) Relationship between postoperative cardiac troponin I levels and outcome of cardiac surgery. **Circulation**, 114: (14): 1468-1475.

Crompton, M. (1999) The mitochondrial permeability transition pore and its role in cell death. **Biochem J**, 341 (Pt 2): 233-249.

Davies, B.J., Coller, J.K., James, H.M., et al. (2006a) The influence of CYP2D6 genotype on trough plasma perhexiline and cis-OH-perhexiline concentrations following a standard loading regimen in patients with myocardial ischaemia. **Br J Clin Pharmacol**, 61: (3): 321-325.

Davies, B.J., Coller, J.K., Somogyi, A.A., et al. (2007) CYP2B6, CYP2D6, and CYP3A4 catalyze the primary oxidative metabolism of perhexiline enantiomers by human liver microsomes. **Drug Metab Dispos**, 35: (1): 128-138.

Davies, B.J., Herbert, M.K., Coller, J.K., et al. (2008) Steady-state pharmacokinetics of the enantiomers of perhexiline in CYP2D6 poor and extensive metabolizers administered Rac-perhexiline. **Br J Clin Pharmacol**, 65: (3): 347-354.

Davies, B.J., Herbert, M.K., Culbert, J.A., et al. (2006b) Enantioselective assay for the determination of perhexiline enantiomers in human plasma by liquid chromatography. **J Chromatogr B Analyt Technol Biomed Life Sci**, 832: (1): 114-120.

Dawson, B.M., Katz, H. and Glusker, J.P. (1986) Perhexiline is a 2-(2,2-dicyclohexylethyl)piperidine maleate. **Acta Crystallogr**, 42: 67-71.

Depre, C., Vanoverschelde, J.L. and Taegtmeyer, H. (1999) Glucose for the heart. **Circulation**, 99: (4): 578-588.

Deschamps, D., DeBeco, V., Fisch, C., et al. (1994) Inhibition by perhexiline of oxidative phosphorylation and the beta-oxidation of fatty acids: possible role in pseudoalcoholic liver lesions. **Hepatology**, 19: (4): 948-961.

DeWall, R.A., Gott, V.L., Lillehei, C.W., et al. (1956) Total body perfusion for open cardiotomy utilizing the bubble oxygenator; physiologic responses in man. **J Thorac Surg**, 32: (5): 591-603.

Dieterle, F., Ross, A., Schlotterbeck, G., et al. (2006) Probabilistic quotient normalization as robust method to account for dilution of complex biological mixtures. Application in 1H NMR metabonomics. **Anal Chem**, 78: (13): 4281-4290.

Dobson, G.P. (2010) Membrane polarity: A target for myocardial protection and reduced inflammation in adult and pediatric cardiothoracic surgery. **J Thorac Cardiovasc Surg**, 140: (6): 1213-1217.

Dobson, G.P. and Jones, M.W. (2004) Adenosine and lidocaine: a new concept in nondepolarizing surgical myocardial arrest, protection, and preservation. **J Thorac Cardiovasc Surg**, 127: (3): 794-805.

Doenst, T., Borger, M.A., Weisel, R.D., et al. (2008) Relation between aortic cross-clamp time and mortality--not as straightforward as expected. **Eur J Cardiothorac Surg**, 33: (4): 660-665.

Doenst, T., Bothe, W. and Beyersdorf, F. (2003a) Therapy with insulin in cardiac surgery: controversies and possible solutions. **Ann Thorac Surg**, 75: (2): S721-728.

Doenst, T., Richwine, R.T., Bray, M.S., et al. (1999) Insulin improves functional and metabolic recovery of reperfused working rat heart. **Ann Thorac Surg**, 67: (6): 1682-1688.

Doenst, T., Schlensak, C. and Beyersdorf, F. (2003b) Cardioplegia in pediatric cardiac surgery: do we believe in magic? **Ann Thorac Surg**, 75: (5): 1668-1677.

Doenst, T., Wijeysundera, D., Karkouti, K., et al. (2005) Hyperglycemia during cardiopulmonary bypass is an independent risk factor for mortality in patients undergoing cardiac surgery. **J Thorac Cardiovasc Surg**, 130: (4): 1144.

Ellis, S.G., Wynne, J., Braunwald, E., et al. (1984) Response of reperfusion-salvaged, stunned myocardium to inotropic stimulation. **Am Heart J**, 107: (1): 13-19.

EudraCT (2011) **European Clinical Trials Database** [online]. https://eudract.ema.europa.eu/ London European Medicines Agency [Accessed June 2011 2011]

Fabiani, J.N., Ponzio, O., Emerit, I., et al. (1992) Cardioprotective effect of trimetazidine during coronary artery graft surgery. **J Cardiovasc Surg (Torino)**, 33: (4): 486-491.

Fan, Y., Zhang, A.M., Xiao, Y.B., et al. (2010) Warm versus cold cardioplegia for heart surgery: a meta-analysis. **Eur J Cardiothorac Surg**, 37: (4): 912-919.

Fan, Y., Zhang, A.M., Xiao, Y.B., et al. (2011) Glucose-insulin-potassium therapy in adult patients undergoing cardiac surgery: a meta-analysis. **Eur J Cardiothorac Surg**, 40: (1): 192-199.

Fath-Ordoubadi, F. and Beatt, K.J. (1997) Glucose-insulin-potassium therapy for treatment of acute myocardial infarction: an overview of randomized placebo-controlled trials. **Circulation**, 96: (4): 1152-1156.

Fergusson, D., Aaron, S.D., Guyatt, G., et al. (2002) Post-randomisation exclusions: the intention to treat principle and excluding patients from analysis. **BMJ**, 325: (7365): 652-654.

Finck, B.N. and Kelly, D.P. (2007) Peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1) regulatory cascade in cardiac physiology and disease. **Circulation**, 115: (19): 2540-2548.

Folch, J., Lees, M. and Sloane Stanley, G.H. (1957) A simple method for the isolation and purification of total lipides from animal tissues. **J Biol Chem**, 226: (1): 497-509.

Follette, D.M., Mulder, D.G., Maloney, J.V., et al. (1978) Advantages of blood cardioplegia over continuous coronary perfusion or intermittent ischemia. Experimental and clinical study. **J Thorac Cardiovasc Surg**, 76: (5): 604-619.

Fremes, S.E., Tamariz, M.G., Abramov, D., et al. (2000) Late results of the Warm Heart Trial: the influence of nonfatal cardiac events on late survival. **Circulation**, 102: (19 Suppl 3): III339-345.

Frenneaux, M.P. (2002) New tricks for an old drug. **Eur Heart J**, 23: (24): 1898-1899.

Frenneaux, M.P. 2007 **Personal Communication**

Fromenty, B. and Pessayre, D. (1995) Inhibition of mitochondrial beta-oxidation as a mechanism of hepatotoxicity. **Pharmacol Ther**, 67: (1): 101-154.

Gamble, J. and Lopaschuk, G.D. (1997) Insulin inhibition of 5' adenosine monophosphate-activated protein kinase in the heart results in activation of acetyl coenzyme A carboxylase and inhibition of fatty acid oxidation. **Metabolism**, 46: (11): 1270-1274.

Gandhi, M., Finegan, B.A. and Clanachan, A.S. (2008) Role of glucose metabolism in the recovery of postischemic LV mechanical function: effects of insulin and other metabolic modulators. **Am J Physiol Heart Circ Physiol**, 294: (6): H2576-2586.

Gardiner, S.J. and Begg, E.J. (2006) Pharmacogenetics, drug-metabolizing enzymes, and clinical practice. **Pharmacol Rev**, 58: (3): 521-590.

Gay, W.A., Jr. and Ebert, P.A. (1973) Functional, metabolic, and morphologic effects of potassium-induced cardioplegia. **Surgery**, 74: (2): 284-290.

Gerbode, F. and Melrose, D. (1958) The use of potassium arrest in open cardiac surgery. **Am J Surg**, 96: (2): 221-227.

Gibbon, J.H., Jr. (1954) Application of a mechanical heart and lung apparatus to cardiac surgery. **Minn Med**, 37: (3): 171-185; passim.

Gomez, L., Paillard, M., Thibault, H., et al. (2008) Inhibition of GSK3beta by postconditioning is required to prevent opening of the mitochondrial permeability transition pore during reperfusion. **Circulation**, 117: (21): 2761-2768.

Gonzalez, F.J. and Nebert, D.W. (1990) Evolution of the P450 gene superfamily: animal-plant 'warfare', molecular drive and human genetic differences in drug oxidation. **Trends Genet**, 6: (6): 182-186.

Goodwin, G.W., Arteaga, J.R. and Taegtmeyer, H. (1995) Glycogen turnover in the isolated working rat heart. **J Biol Chem**, 270: (16): 9234-9240.

Goodwin, G.W., Taylor, C.S. and Taegtmeyer, H. (1998) Regulation of energy metabolism of the heart during acute increase in heart work. **J Biol Chem**, 273: (45): 29530-29539.

Gordeev, I.G., Liusov, V.A., Il'ina, E.E., et al. (2007) [Derangements of contractility of left ventricular myocardium in patients subjected to coronary bypass surgery. Methods of their correction]. **Kardiologiia***,* 47: (2): 22-24.

Gott, V.L., Alejo, D.E. and Cameron, D.E. (2003) Mechanical heart valves: 50 years of evolution. **Ann Thorac Surg***,* 76: (6): S2230-2239.

Gott, V.L. and Shumway, N.E. (2004) Cross-circulation: a milestone in cardiac surgery. **J Thorac Cardiovasc Surg***,* 127: (3): 617.

Griffin, J.L., Atherton, H., Shockcor, J., et al. (2011) Metabolomics as a tool for cardiac research. **Nat Rev Cardiol***,* 8: (11): 630-643.

Guru, V., Omura, J., Alghamdi, A.A., et al. (2006) Is blood superior to crystalloid cardioplegia? A meta-analysis of randomized clinical trials. **Circulation***,* 114: (1 Suppl): I331-338.

Hale, S.L., Shryock, J.C., Belardinelli, L., et al. (2008) Late sodium current inhibition as a new cardioprotective approach. **J Mol Cell Cardiol***,* 44: (6): 954-967.

Hanover, J.A., Krause, M.W. and Love, D.C. (2010) The hexosamine signaling pathway: O-GlcNAc cycling in feast or famine. **Biochim Biophys Acta***,* 1800: (2): 80-95.

Hausenloy, D.J., Baxter, G., Bell, R., et al. (2010) Translating novel strategies for cardioprotection: the Hatter Workshop Recommendations. **Basic Res Cardiol***,* 105: (6): 677-686.

Hausenloy, D.J., Mwamure, P.K., Venugopal, V., et al. (2007) Effect of remote ischaemic preconditioning on myocardial injury in patients undergoing coronary artery bypass graft surgery: a randomised controlled trial. **Lancet***,* 370: (9587): 575-579.

Hausenloy, D.J. and Yellon, D.M. (2004) New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway. **Cardiovasc Res***,* 61: (3): 448-460.

Hausenloy, D.J. and Yellon, D.M. (2007) Reperfusion injury salvage kinase signalling: taking a RISK for cardioprotection. **Heart Fail Rev***,* 12: (3-4): 217-234.

Hausenloy, D.J. and Yellon, D.M. (2008) Remote ischaemic preconditioning: underlying mechanisms and clinical application. **Cardiovasc Res***,* 79: (3): 377-386.

Hearse, D.J., Braimbridge, M.V. and Jynge, P. (eds.) (1981) **Protection of the ischemic myocardium: cardioplegia***,* New York: Raven Press.

Hearse, D.J., Garlick, P.B. and Humphrey, S.M. (1977) Ischemic contracture of the myocardium: mechanisms and prevention. **Am J Cardiol***,* 39: (7): 986-993.

Hearse, D.J., Stewart, D.A. and Braimbridge, M.V. (1976) Cellular protection during myocardial ischemia: the development and characterization of a procedure for the induction of reversible ischemic arrest. **Circulation***,* 54: (2): 193-202.

Herlitz, J., Brandrup-Wognsen, G., Caidahl, K., et al. (2005) Determinants for an impaired quality of life 10 years after coronary artery bypass surgery. **Int J Cardiol***,* 98: (3): 447-452.

Hoelscher, B. (1967) Studies by electron microscopy on the effects of magnesium chloride-procaine amide or potassium citrate on the myocardium in induced cardiac arrest. **J Cardiovasc Surg (Torino)**, 8: (2): 163-166.

Hohorst, H.J. (1963) "Glucose 6-phosphate". In Bergmeyer, H.-U. (Ed.) **Methods of Enaymatic Analysis**. New York, Academic Press 134-138.

Holubarsch, C.J., Rohrbach, M., Karrasch, M., et al. (2007) A double-blind randomized multicentre clinical trial to evaluate the efficacy and safety of two doses of etomoxir in comparison with placebo in patients with moderate congestive heart failure: the ERGO (etomoxir for the recovery of glucose oxidation) study. **Clin Sci (Lond)**, 113: (4): 205-212.

Homer (*c.*850 BC) **The Odyssey**. Cambridge: Cambridge University Press.

Hopkins, T.A., Dyck, J.R. and Lopaschuk, G.D. (2003) AMP-activated protein kinase regulation of fatty acid oxidation in the ischaemic heart. **Biochem Soc Trans**, 31: (Pt 1): 207-212.

Horowitz, J.D., Button, I.K. and Wing, L. (1995) Is perhexiline essential for the optimal management of angina pectoris? **Aust N Z J Med**, 25: (2): 111-113.

Horowitz, J.D. and Powell, A.C. (1986) Myocardial uptake of drugs and clinical effects. **Clin Pharmacokinet**, 11: (5): 354-371.

Horowitz, J.D., Sia, S.T., Macdonald, P.S., et al. (1986) Perhexiline maleate treatment for severe angina pectoris--correlations with pharmacokinetics. **Int J Cardiol**, 13: (2): 219-229.

Hottenrott, C., Maloney, J.V., Jr. and Buckberg, G. (1974) Studies of the effects of ventricular fibrillation on the adequacy of regional myocardial flow. I. Electrical vs. spontaneous fibrillation. **J Thorac Cardiovasc Surg**, 68: (4): 615-625.

Howell, N.J. 2010 **Personal Communication**

Howell, N.J., Ashrafian, H., Drury, N.E., et al. (2011) Glucose-Insulin-Potassium Reduces the Incidence of Low Cardiac Output Episodes After Aortic Valve Replacement for Aortic Stenosis in Patients With Left Ventricular Hypertrophy: Results From the Hypertrophy, Insulin, Glucose, and Electrolytes (HINGE) Trial. **Circulation**, 123: 170-177.

Hrydziuszko, O. and Viant, M.R. (2011) Missing values in mass spectrometry based metabolomics: an undervalued step in the data processing pipeline. **Metabolomics**, 1-14.

Hue, L. and Rider, M.H. (2007) The AMP-activated protein kinase: more than an energy sensor. **Essays Biochem**, 43: 121-137.

Hwang, H., Arcidi, J.M., Jr., Hale, S.L., et al. (2009) Ranolazine as a cardioplegia additive improves recovery of diastolic function in isolated rat hearts. **Circulation**, 120: (11 Suppl): S16-21.

ICH (1996) **Guideline for Good Clinical Practice E6(R1)** [online]. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Efficacy/E6_R1/Step4/E6_R1__Guideline.pdf [Accessed 5th June 2011 2011]

ICH (1997) **General considerations for clinical trials E8** [online]. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Efficacy/E8/Step4/E8_Guideline.pdf [Accessed 5th June 2011 2011]

ICH (1998) **Statistical principles for clinical trials E9** [online]. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Efficacy/E9/Step4/E9_Guideline.pdf [Accessed 5th June 2011 2011]

ICMJE (2010) **Uniform requirements for manuscripts submitted to biomedical journals** [online]. http://www.icmje.org/urm_full.pdf [Accessed 5th June 2011 2011]

Ingelman-Sundberg, M., Daly, A.K. and Nebert, D.W. (2011) **CYP2D6 allele nomenclature** [online]. http://www.cypalleles.ki.se/cyp2d6.htm Human Cytochrome P450 (CYP) Allele Nomenclature Committee [Accessed 17/09/2011

Inglis, S.C., Herbert, M.K., Davies, B.J., et al. (2007) Effect of CYP2D6 metabolizer status on the disposition of the (+) and (-) enantiomers of perhexiline in patients with myocardial ischaemia. **Pharmacogenet Genomics**, 17: (5): 305-312.

Inglis, S.C. and Stewart, S. (2006) Metabolic therapeutics in angina pectoris: history revisited with perhexiline. **Eur J Cardiovasc Nurs**, 5: (2): 175-184.

Inoue, M., Sato, E.F., Nishikawa, M., et al. (2003) Mitochondrial generation of reactive oxygen species and its role in aerobic life. **Curr Med Chem**, 10: (23): 2495-2505.

Iskesen, I., Kurdal, A.T., Eserdag, M., et al. (2009) Trimetazidine may protect the myocardium during cardiac surgery. **Heart Surg Forum**, 12: (3): E175-179.

Iskesen, I., Saribulbul, O., Cerrahoglu, M., et al. (2006) Trimetazidine reduces oxidative stress in cardiac surgery. **Circ J**, 70: (9): 1169-1173.

Jacobs, N.J. and Vandemark, P.J. (1960) The purification and properties of the alpha-glycerophosphate-oxidizing enzyme of Streptococcus faecalis 10C1. **Arch Biochem Biophys**, 88: 250-255.

Jadad, A.R., Moore, R.A., Carroll, D., et al. (1996) Assessing the quality of reports of randomized clinical trials: is blinding necessary? **Control Clin Trials**, 17: (1): 1-12.

Januzzi, J.L. (2011) What is the role of biomarker measurement after cardiac surgery? **Minerva Anestesiol**, 77: (3): 334-341.

Januzzi, J.L., Lewandrowski, K., MacGillivray, T.E., et al. (2002) A comparison of cardiac troponin T and creatine kinase-MB for patient evaluation after cardiac surgery. **J Am Coll Cardiol**, 39: (9): 1518-1523.

Jitrapakdee, S., St Maurice, M., Rayment, I., et al. (2008) Structure, mechanism and regulation of pyruvate carboxylase. **Biochem J**, 413: (3): 369-387.

Jones, T.E., Morris, R.G. and Horowitz, J.D. (2004) Concentration-time profile for perhexiline and hydroxyperhexiline in patients at steady state. **Br J Clin Pharmacol**, 57: (3): 263-269.

Junn, E., Han, S.H., Im, J.Y., et al. (2000) Vitamin D3 up-regulated protein 1 mediates oxidative stress via suppressing the thioredoxin function. **J Immunol**, 164: (12): 6287-6295.

Kantor, P.F., Lucien, A., Kozak, R., et al. (2000) The antianginal drug trimetazidine shifts cardiac energy metabolism from fatty acid oxidation to glucose oxidation by inhibiting mitochondrial long-chain 3-ketoacyl coenzyme A thiolase. **Circ Res***,* 86: (5): 580-588.

Karmazyn, M., Purdham, D.M., Rajapurohitam, V., et al. (2008) Signalling mechanisms underlying the metabolic and other effects of adipokines on the heart. **Cardiovasc Res***,* 79: (2): 279-286.

Karthik, S., Grayson, A.D., Oo, A.Y., et al. (2004) A survey of current myocardial protection practices during coronary artery bypass grafting. **Ann R Coll Surg Engl***,* 86: (6): 413-415.

Kassiotis, C., Rajabi, M. and Taegtmeyer, H. (2008) Metabolic reserve of the heart: the forgotten link between contraction and coronary flow. **Prog Cardiovasc Dis***,* 51: (1): 74-88.

Kennedy, J.A., Beck-Oldach, K., McFadden-Lewis, K., et al. (2006) Effect of the anti-anginal agent, perhexiline, on neutrophil, valvular and vascular superoxide formation. **Eur J Pharmacol***,* 531: (1-3): 13-19.

Kennedy, J.A., Kiosoglous, A.J., Murphy, G.A., et al. (2000) Effect of perhexiline and oxfenicine on myocardial function and metabolism during low-flow ischemia/reperfusion in the isolated rat heart. **J Cardiovasc Pharmacol***,* 36: (6): 794-801.

Kennedy, J.A., Unger, S.A. and Horowitz, J.D. (1996) Inhibition of carnitine palmitoyltransferase-1 in rat heart and liver by perhexiline and amiodarone. **Biochem Pharmacol***,* 52: (2): 273-280.

Khan, M., Meduru, S., Mostafa, M., et al. (2010) Trimetazidine, administered at the onset of reperfusion, ameliorates myocardial dysfunction and injury by activation of p38 mitogen-activated protein kinase and Akt signaling. **J Pharmacol Exp Ther***,* 333: (2): 421-429.

Kharbanda, R.K. (2010) Cardiac conditioning: a review of evolving strategies to reduce ischaemia-reperfusion injury. **Heart***,* 96: (15): 1179-1186.

Killalea, S.M. and Krum, H. (2001) Systematic review of the efficacy and safety of perhexiline in the treatment of ischemic heart disease. **Am J Cardiovasc Drugs***,* 1: (3): 193-204.

Kirklin, J.W., Donald, D.E., Harshbarger, H.G., et al. (1956) Studies in extracorporeal circulation. I. Applicability of Gibbon-type pump-oxygenator to human intracardiac surgery: 40 cases. **Ann Surg***,* 144: (1): 2-8.

Kloner, R.A. and Nesto, R.W. (2008) Glucose-insulin-potassium for acute myocardial infarction: continuing controversy over cardioprotection. **Circulation***,* 117: (19): 2523-2533.

Knott, E.M., Sun, J., Lei, Y., et al. (2006) Pyruvate mitigates oxidative stress during reperfusion of cardioplegia-arrested myocardium. **Ann Thorac Surg***,* 81: (3): 928-934.

Kodde, I.F., van der Stok, J., Smolenski, R.T., et al. (2007) Metabolic and genetic regulation of cardiac energy substrate preference. **Comp Biochem Physiol A Mol Integr Physiol***,* 146: (1): 26-39.

Kolh, P., Wijns, W., Danchin, N., et al. (2010) Guidelines on myocardial revascularization. **Eur J Cardiothorac Surg***,* 38 Suppl: S1-S52.

Koopman, R., Schaart, G. and Hesselink, M.K. (2001) Optimisation of oil red O staining permits combination with immunofluorescence and automated quantification of lipids. **Histochem Cell Biol***,* 116: (1): 63-68.

Krebs, H.A. and Kornberg, H.L. (1957) **Energy transformations in living matter**. Berlin: Springer-Verlag.

Lavery, G.G., Hauton, D., Hewitt, K.N., et al. (2007) Hypoglycemia with enhanced hepatic glycogen synthesis in recombinant mice lacking hexose-6-phosphate dehydrogenase. **Endocrinology***,* 148: (12): 6100-6106.

Lecour, S. (2009) Activation of the protective Survivor Activating Factor Enhancement (SAFE) pathway against reperfusion injury: Does it go beyond the RISK pathway? **J Mol Cell Cardiol***,* 47: (1): 32-40.

Ledingham, S.J., Braimbridge, M.V. and Hearse, D.J. (1987) The St. Thomas' Hospital cardioplegic solution. A comparison of the efficacy of two formulations. **J Thorac Cardiovasc Surg***,* 93: (2): 240-246.

Lee, L., Campbell, R., Scheuermann-Freestone, M., et al. (2005) Metabolic modulation with perhexiline in chronic heart failure: a randomized, controlled trial of short-term use of a novel treatment. **Circulation***,* 112: (21): 3280-3288.

Lee, L., Horowitz, J. and Frenneaux, M. (2004) Metabolic manipulation in ischaemic heart disease, a novel approach to treatment. **Eur Heart J***,* 25: (8): 634-641.

Lenaz, G. (1998) Role of mitochondria in oxidative stress and ageing. **Biochim Biophys Acta***,* 1366: (1-2): 53-67.

Levine, R.D. (2005) **Molecular Reaction Dynamics**. Cambridge: Cambridge University Press.

Lewis, F.J. and Taufic, M. (1953) Closure of atrial septal defects with the aid of hypothermia; experimental accomplishments and the report of one successful case. **Surgery***,* 33: (1): 52-59.

Lhermitte, F., Fardeau, M., Chedru, F., et al. (1976) Polyneuropathy after perhexiline maleate therapy. **Br Med J***,* 1: (6020): 1256.

Licari, J. 2010 **Personal Communication**

Liedtke, A.J. (1981) Alterations of carbohydrate and lipid metabolism in the acutely ischemic heart. **Prog Cardiovasc Dis***,* 23: (5): 321-336.

Lillehei, C.W., Cohen, M., Warden, H.E., et al. (1955a) Direct vision intracardiac surgical correction of the tetralogy of Fallot, pentalogy of Fallot, and pulmonary atresia defects; report of first ten cases. **Ann Surg***,* 142: (3): 418-442.

Lillehei, C.W., Cohen, M., Warden, H.E., et al. (1955b) The direct-vision intracardiac correction of congenital anomalies by controlled cross circulation; results in thirty-two

patients with ventricular septal defects, tetralogy of Fallot, and atrioventricularis communis defects. **Surgery**, 38: (1): 11-29.

Lillehei, C.W., Dewall, R.A., Read, R.C., et al. (1956) Direct vision intracardiac surgery in man using a simple, disposable artificial oxygenator. **Dis Chest**, 29: (1): 1-8.

Lillehei, C.W., Varco, R.L., Cohen, M., et al. (1986) The first open-heart repairs of ventricular septal defect, atrioventricular communis, and tetralogy of Fallot using extracorporeal circulation by cross-circulation: a 30-year follow-up. **Ann Thorac Surg**, 41: (1): 4-21.

Linker, R.A., Lee, D.H., Ryan, S., et al. (2011) Fumaric acid esters exert neuroprotective effects in neuroinflammation via activation of the Nrf2 antioxidant pathway. **Brain**, 134: (Pt 3): 678-692.

Liu, B., Clanachan, A.S., Schulz, R., et al. (1996) Cardiac efficiency is improved after ischemia by altering both the source and fate of protons. **Circ Res**, 79: (5): 940-948.

Lloyd, B.L. and Taylor, R.R. (1976) Influence of myocardial mechanical activity and coronary blood flow on myocardial digoxin uptake. **Cardiovasc Res**, 10: (4): 487-493.

Lopaschuk, G.D., Collins-Nakai, R., Olley, P.M., et al. (1994) Plasma fatty acid levels in infants and adults after myocardial ischemia. **Am Heart J**, 128: (1): 61-67.

Lopaschuk, G.D., Ussher, J.R., Folmes, C.D., et al. (2010) Myocardial fatty acid metabolism in health and disease. **Physiol Rev**, 90: (1): 207-258.

Lopaschuk, G.D., Wall, S.R., Olley, P.M., et al. (1988) Etomoxir, a carnitine palmitoyltransferase I inhibitor, protects hearts from fatty acid-induced ischemic injury independent of changes in long chain acylcarnitine. **Circ Res**, 63: (6): 1036-1043.

Lopatin, I.M. and Dronova, E.P. (2009) [Clinical-pharmacoeconomic aspects of trimetazidine modified release use in patients with ischemic heart disease undergoing coronary artery bypass grafting]. **Kardiologiia**, 49: (2): 15-21.

Lullmann, H., Timmermans, P.B., Weikert, G.M., et al. (1980) Accumulation of drugs by guinea pig isolated atria. Quantitative correlations. **J Med Chem**, 23: (5): 560-565.

Lullmann, H., Timmermans, P.B. and Ziegler, A. (1979) Accumulation of drugs by resting or beating cardiac tissue. **Eur J Pharmacol**, 60: (4): 277-285.

Lurati Buse, G.A., Koller, M.T., Grapow, M., et al. (2009) 12-month outcome after cardiac surgery: prediction by troponin T in combination with the European system for cardiac operative risk evaluation. **Ann Thorac Surg**, 88: (6): 1806-1812.

MacInnes, A., Fairman, D.A., Binding, P., et al. (2003) The antianginal agent trimetazidine does not exert its functional benefit via inhibition of mitochondrial long-chain 3-ketoacyl coenzyme A thiolase. **Circ Res**, 93: (3): e26-32.

Mamas, M.A., Neyses, L. and Fath-Ordoubadi, F. (2010) A meta-analysis of glucose-insulin-potassium therapy for treatment of acute myocardial infarction. **Exp Clin Cardiol**, 15: (2): e20-24.

Martins, G.F., Siqueira Filho, A.G., Santos, J.B., et al. (2011) Trimetazidine on ischemic injury and reperfusion in coronary artery bypass grafting. **Arq Bras Cardiol**.

McEntegart, D.J. (2003) The pursuit of balance using stratified and dynamic randomization techniques: an overview. **Drug Information Journal**, 37: 293-308.

McFarland, J.A., Thomas, L.B., Gilbert, J.W., et al. (1960) Myocardial necrosis following elective cardiac arrest induced with potassium citrate. **J Thorac Cardiovasc Surg**, 40: 200-208.

Mehta, S.R., Yusuf, S., Diaz, R., et al. (2005) Effect of glucose-insulin-potassium infusion on mortality in patients with acute ST-segment elevation myocardial infarction: the CREATE-ECLA randomized controlled trial. **JAMA**, 293: (4): 437-446.

Melrose, D.G., Dreyer, B., Bentall, H.H., et al. (1955) Elective cardiac arrest. **Lancet**, 269: (6879): 21-22.

Mentzer, R.M.J., Jahania, M.S. and Lasley, R.D. (2008) "Myocardial Protection". In Cohn, L.H. (Ed.) **Cardiac Surgery in the Adult**. 3rd ed. New York, McGraw-Hill 443-464.

MHRA (2011) **Clinical trials for medicines: Implementation of the Clinical Trials Directive in the UK** [online]. http://www.mhra.gov.uk/Howweregulate/Medicines/Licensingofmedicines/Clinicaltrials/Legislation/ImplementationofClinicalTrialsDirectiveintheUK/index.htm London Crown Copyright [Accessed June 2011 2011]

Miller, G.W. (2000) **King of Hearts: The true story of the maverick who pioneered open heart surgery.** New York, NY: Crown.

Mitchell, P. (1961) Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. **Nature**, 191: 144-148.

Mohammed, A.A., Agnihotri, A.K., van Kimmenade, R.R., et al. (2009) Prospective, comprehensive assessment of cardiac troponin T testing after coronary artery bypass graft surgery. **Circulation**, 120: (10): 843-850.

Moher, D., Liberati, A., Tetzlaff, J., et al. (2009) Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. **BMJ**, 339: b2535.

Moller, J.H., Shumway, S.J. and Gott, V.L. (2009) The first open-heart repairs using extracorporeal circulation by cross-circulation: a 53-year follow-up. **Ann Thorac Surg**, 88: (3): 1044-1046.

Murray, A.J., Anderson, R.E., Watson, G.C., et al. (2004) Uncoupling proteins in human heart. **Lancet**, 364: (9447): 1786-1788.

Murry, C.E., Jennings, R.B. and Reimer, K.A. (1986) Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. **Circulation**, 74: (5): 1124-1136.

Mustard, W.T. and Thomson, J.A. (1957) Clinical experience with the artificial heart lung preparation. **Can Med Assoc J**, 76: (4): 265-269.

Myrmel, T., Forsdahl, K. and Larsen, T.S. (1992) Triacylglycerol metabolism in hypoxic, glucose-deprived rat cardiomyocytes. **J Mol Cell Cardiol**, 24: (8): 855-868.

Neely, J.R. and Morgan, H.E. (1974) Relationship between carbohydrate and lipid metabolism and the energy balance of heart muscle. **Annu Rev Physiol**, 36: 413-459.

Nelson, R.L., Fey, K.H., Follette, D.M., et al. (1976) Intermittent infusion of cardioplegic solution during aortic cross-clamping. **Surg Forum**, 27: (62): 241-243.

Neubauer, S. (2007) The failing heart--an engine out of fuel. **N Engl J Med**, 356: (11): 1140-1151.

Neville, J.F., Jr. and Gelder, R.L. (1971) Modified enzymatic methods for the determination of L-(+)-lactic and pyruvic acids in blood. **Am J Clin Pathol**, 55: (2): 152-158.

Ngo, D.T., Drury, N.E., Frenneaux, M., et al. (2011) How does perhexiline modulate myocardial energetics and ameliorate redox stress? **Circulation**, 124: Abstract 14461.

Nicholson, J.K., Connelly, J., Lindon, J.C., et al. (2002) Metabonomics: a platform for studying drug toxicity and gene function. **Nat Rev Drug Discov**, 1: (2): 153-161.

Nuesch, E., Trelle, S., Reichenbach, S., et al. (2009) The effects of excluding patients from the analysis in randomised controlled trials: meta-epidemiological study. **BMJ**, 339: b3244.

OCEBM, L.o.E.W.G. (2009) **Levels of evidence** [online]. http://www.cebm.net/index.aspx?o=1025 Oxford Centre for Evidence-Based Medicine [Accessed June 2011 2011]

Opie, L.H. and Sack, M.N. (2002) Metabolic plasticity and the promotion of cardiac protection in ischemia and ischemic preconditioning. **J Mol Cell Cardiol**, 34: (9): 1077-1089.

Ovrum, E., Tangen, G., Tollofsrud, S., et al. (2004) Cold blood cardioplegia versus cold crystalloid cardioplegia: a prospective randomized study of 1440 patients undergoing coronary artery bypass grafting. **J Thorac Cardiovasc Surg**, 128: (6): 860-865.

Owen, O.E., Kalhan, S.C. and Hanson, R.W. (2002) The key role of anaplerosis and cataplerosis for citric acid cycle function. **J Biol Chem**, 277: (34): 30409-30412.

Paget, S. (1896) **The Surgery of the Chest**. Bristol: John Wright.

Paradis, C. (2008) Bias in surgical research. **Ann Surg**, 248: (2): 180-188.

Parsons, H.M., Ekman, D.R., Collette, T.W., et al. (2009) Spectral relative standard deviation: a practical benchmark in metabolomics. **Analyst**, 134: (3): 478-485.

Parsons, H.M., Ludwig, C., Gunther, U.L., et al. (2007) Improved classification accuracy in 1- and 2-dimensional NMR metabolomics data using the variance stabilising generalised logarithm transformation. **BMC Bioinformatics**, 8: 234.

Payne, T.G., Southam, A.D., Arvanitis, T.N., et al. (2009) A signal filtering method for improved quantification and noise discrimination in fourier transform ion cyclotron resonance mass spectrometry-based metabolomics data. **J Am Soc Mass Spectrom**, 20: (6): 1087-1095.

Pegg, T.J., Maunsell, Z., Karamitsos, T.D., et al. (2011) Utility of cardiac biomarkers for the diagnosis of type V myocardial infarction after coronary artery bypass grafting: insights from serial cardiac MRI. **Heart***,* 97: (10): 810-816.

Pepine, C.J., Schang, S.J. and Bemiller, C.R. (1974) Effects of perhexiline on coronary hemodynamic and myocardial metabolic responses to tachycardia. **Circulation***,* 49: (5): 887-893.

Pessayre, D., Bichara, M., Degott, C., et al. (1979) Perhexiline maleate-induced cirrhosis. **Gastroenterology***,* 76: (1): 170-177.

Phan, T.T., Shivu, G.N., Choudhury, A., et al. (2009) Multi-centre experience on the use of perhexiline in chronic heart failure and refractory angina: old drug, new hope. **Eur J Heart Fail***,* 11: (9): 881-886.

Philpott, A., Chandy, S., Morris, R., et al. (2004) Development of a regimen for rapid initiation of perhexiline therapy in acute coronary syndromes. **Intern Med J***,* 34: (6): 361-363.

Pilcher, J., Cooper, J.D., Turnell, D.C., et al. (1985) Investigations of long-term treatment with perhexiline maleate using therapeutic monitoring and electromyography. **Ther Drug Monit***,* 7: (1): 54-60.

Pocock, S.J. and Simon, R. (1975) Sequential treatment assignment with balancing for prognostic factors in the controlled clinical trial. **Biometrics***,* 31: (1): 103-115.

Przyklenk, K., Bauer, B., Ovize, M., et al. (1993) Regional ischemic 'preconditioning' protects remote virgin myocardium from subsequent sustained coronary occlusion. **Circulation***,* 87: (3): 893-899.

Quinn, D.W., Pagano, D., Bonser, R.S., et al. (2006) Improved myocardial protection during coronary artery surgery with glucose-insulin-potassium: a randomized controlled trial. **J Thorac Cardiovasc Surg***,* 131: (1): 34-42.

Rahman, I.A., Mascaro, J.G., Steeds, R.P., et al. (2010) Remote ischemic preconditioning in human coronary artery bypass surgery: from promise to disappointment? **Circulation***,* 122: (11 Suppl): S53-59.

Ranasinghe, A.M., Quinn, D.W., Pagano, D., et al. (2006) Glucose-insulin-potassium and tri-iodothyronine individually improve hemodynamic performance and are associated with reduced troponin I release after on-pump coronary artery bypass grafting. **Circulation***,* 114: (1 Suppl): I245-250.

Ranasinghe, A.M., Quinn, D.W., Richardson, M., et al. (2011) Which troponometric best predicts midterm outcome after coronary artery bypass graft surgery? **Ann Thorac Surg***,* 91: (6): 1860-1867.

Randle, P.J., Garland, P.B., Hales, C.N., et al. (1963) The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. **Lancet***,* 1: (7285): 785-789.

Rao, V., Christakis, G.T., Weisel, R.D., et al. (2002) The insulin cardioplegia trial: myocardial protection for urgent coronary artery bypass grafting. **J Thorac Cardiovasc Surg***,* 123: (5): 928-935.

Rich, P.R. (2003) The molecular machinery of Keilin's respiratory chain. **Biochem Soc Trans***,* 31: (Pt 6): 1095-1105.

Rider, M.H., Bertrand, L., Vertommen, D., et al. (2004) 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase: head-to-head with a bifunctional enzyme that controls glycolysis. **Biochem J***,* 381: (Pt 3): 561-579.

Riedel, B.J., Gal, J., Ellis, G., et al. (2004) Myocardial protection using fructose-1,6-diphosphate during coronary artery bypass graft surgery: a randomized, placebo-controlled clinical trial. **Anesth Analg***,* 98: (1): 20-29, table of contents.

Romaine-Davis, A. (1991) **John Gibbon and his heart lung machine.** Philadelphia, PA: University of Pennsylvania Press.

Ruixing, Y., Wenwu, L. and Al-Ghazali, R. (2007) Trimetazidine inhibits cardiomyocyte apoptosis in a rabbit model of ischemia-reperfusion. **Transl Res***,* 149: (3): 152-160.

Sachse, C., Brockmoller, J., Bauer, S., et al. (1997) Cytochrome P450 2D6 variants in a Caucasian population: allele frequencies and phenotypic consequences. **Am J Hum Genet***,* 60: (2): 284-295.

Sack, M.N. and Yellon, D.M. (2003) Insulin therapy as an adjunct to reperfusion after acute coronary ischemia: a proposed direct myocardial cell survival effect independent of metabolic modulation. **J Am Coll Cardiol***,* 41: (8): 1404-1407.

Saddik, M. and Lopaschuk, G.D. (1991) Myocardial triglyceride turnover and contribution to energy substrate utilization in isolated working rat hearts. **J Biol Chem***,* 266: (13): 8162-8170.

Sallustio, B.C., Westley, I.S. and Morris, R.G. (2002) Pharmacokinetics of the antianginal agent perhexiline: relationship between metabolic ratio and steady-state dose. **Br J Clin Pharmacol***,* 54: (2): 107-114.

Schulz, K.F., Altman, D.G. and Moher, D. (2010) CONSORT 2010 statement: updated guidelines for reporting parallel group randomised trials. **BMJ***,* 340: c332.

Sealy, W.C., Young, W.G., Jr., Brown, I.W., Jr., et al. (1959) Potassium, magnesium, and neostigmine for controlled cardioplegia: a report of its use in 34 patients. **J Thorac Surg***,* 37: (5): 655-659.

Shah, R.R. (2006) Can pharmacogenetics help rescue drugs withdrawn from the market? **Pharmacogenomics***,* 7: (6): 889-908.

Shah, R.R., Oates, N.S., Idle, J.R., et al. (1982) Impaired oxidation of debrisoquine in patients with perhexiline neuropathy. **Br Med J (Clin Res Ed)***,* 284: (6312): 295-299.

Shiroishi, M.S. (1999) Myocardial protection: the rebirth of potassium-based cardioplegia. **Tex Heart Inst J***,* 26: (1): 71-86.

Sigma Pharmaceuticals (2009) "PEXSIG: Perhexiline maleate". Croydon, Victoria, Australia.

Silveira Filho, L.d.M., Petrucci, O., Jr., Carmo, M.R., et al. (2008) Trimetazidine as cardioplegia addictive without pre-treatment does not improve myocardial protection: study in a swine working heart model. **Rev Bras Cir Cardiovasc***,* 23: (2): 224-234.

Singlas, E., Goujet, M.A. and Simon, P. (1978) Pharmacokinetics of perhexiline maleate in anginal patients with and without peripheral neuropathy. **Eur J Clin Pharmacol**, 14: (3): 195-201.

Snabaitis, A.K., Shattock, M.J. and Chambers, D.J. (1997) Comparison of polarized and depolarized arrest in the isolated rat heart for long-term preservation. **Circulation**, 96: (9): 3148-3156.

Snyder, L.R., Kirkland, J.J. and Dolan, J.W. (2010) **Introduction to Modern Liquid Chromatography**. 3rd edition.Hoboken, NJ: John Wiley & Sons.

Sodi-Pallares, D., Testelli, M.R., Fishleder, B.L., et al. (1962) Effects of an intravenous infusion of a potassium-glucose-insulin solution on the electrocardiographic signs of myocardial infarction. A preliminary clinical report. **Am J Cardiol**, 9: 166-181.

Solorzano, J., Taitelbaum, G. and Chiu, R.C. (1978) Retrograde coronary sinus perfusion for myocardial protection during cardiopulmonary bypass. **Ann Thorac Surg**, 25: (3): 201-208.

Sondergaard, T., Berg, E., Staffeldt, I., et al. (1975) Cardioplegic cardiac arrest in aortic surgery. **J Cardiovasc Surg (Torino)**, 16: (3): 288-290.

Southam, A.D., Payne, T.G., Cooper, H.J., et al. (2007) Dynamic range and mass accuracy of wide-scan direct infusion nanoelectrospray fourier transform ion cyclotron resonance mass spectrometry-based metabolomics increased by the spectral stitching method. **Anal Chem**, 79: (12): 4595-4602.

Stanley, W.C., Recchia, F.A. and Lopaschuk, G.D. (2005) Myocardial substrate metabolism in the normal and failing heart. **Physiol Rev**, 85: (3): 1093-1129.

Starr, A. (1986) The thoracic surgical industrial complex. **Ann Thorac Surg**, 42: (2): 124-133.

Stewart, S., Voss, D.W., Northey, D.L., et al. (1996) Relationship between plasma perhexiline concentration and symptomatic status during short-term perhexiline therapy. **Ther Drug Monit**, 18: (6): 635-639.

Stoney, W.S. (2009) Evolution of cardiopulmonary bypass. **Circulation**, 119: (21): 2844-2853.

Sumner, L.W., Urbanczyk-Wochniak, E. and Broeckling, C.D. (2007) Metabolomics data analysis, visualization, and integration. **Methods Mol Biol**, 406: 409-436.

Taegtmeyer, H. (2000) Metabolism--the lost child of cardiology. **J Am Coll Cardiol**, 36: (4): 1386-1388.

Taegtmeyer, H. (2002) Switching metabolic genes to build a better heart. **Circulation**, 106: (16): 2043-2045.

Taegtmeyer, H. (2007) "Fuelling the heart: multiple roles for cardiac metabolism". In Willerson, J.T.;Cohn, J.N.;Wellens, H.J.J. & R., H.D. (Eds.) **Cardiovascular Medicine**. 3rd ed. London, Springer-Verlag 1157-1175.

Taegtmeyer, H., Goodwin, G.W., Doenst, T., et al. (1997) Substrate metabolism as a determinant for postischemic functional recovery of the heart. **Am J Cardiol**, 80: (3A): 3A-10A.

Taniguchi, M., Wilson, C., Hunter, C.A., et al. (2001) Dichloroacetate improves cardiac efficiency after ischemia independent of changes in mitochondrial proton leak. **Am J Physiol Heart Circ Physiol**, 280: (4): H1762-1769.

Teoh, K.H., Christakis, G.T., Weisel, R.D., et al. (1986) Accelerated myocardial metabolic recovery with terminal warm blood cardioplegia. **J Thorac Cardiovasc Surg**, 91: (6): 888-895.

Thygesen, K., Alpert, J.S. and White, H.D. (2007) Universal definition of myocardial infarction. **Eur Heart J**, 28: (20): 2525-2538.

Tunerir, B., Colak, O., Alatas, O., et al. (1999) Measurement of troponin T to detect cardioprotective effect of trimetazidine during coronary artery bypass grafting. **Ann Thorac Surg**, 68: (6): 2173-2176.

Turoczi, T., Chang, V.W., Engelman, R.M., et al. (2003) Thioredoxin redox signaling in the ischemic heart: an insight with transgenic mice overexpressing Trx1. **J Mol Cell Cardiol**, 35: (6): 695-704.

Twain, M. (1860) "Letter to Orion Clemens".

Unger, S.A., Kennedy, J.A., McFadden-Lewis, K., et al. (2005) Dissociation between metabolic and efficiency effects of perhexiline in normoxic rat myocardium. **J Cardiovasc Pharmacol**, 46: (6): 849-855.

Unger, S.A., Robinson, M.A. and Horowitz, J.D. (1997) Perhexiline improves symptomatic status in elderly patients with severe aortic stenosis. **Aust N Z J Med**, 27: (1): 24-28.

Ussher, J.R. and Lopaschuk, G.D. (2008) The malonyl CoA axis as a potential target for treating ischaemic heart disease. **Cardiovasc Res**, 79: (2): 259-268.

Vahanian, A., Alfieri, O.R., Al-Attar, N., et al. (2008) Transcatheter valve implantation for patients with aortic stenosis: a position statement from the European Association of Cardio-Thoracic Surgery (EACTS) and the European Society of Cardiology (ESC), in collaboration with the European Association of Percutaneous Cardiovascular Interventions (EAPCI). **Eur J Cardiothorac Surg**, 34: (1): 1-8.

Vaughan Williams, E.M. (1980) **Antiarrhythmic action and the puzzle of perhexiline**. London ; New York: Academic Press.

Vedrinne, J.M., Vedrinne, C., Bompard, D., et al. (1996) Myocardial protection during coronary artery bypass graft surgery: a randomized, double-blind, placebo-controlled study with trimetazidine. **Anesth Analg**, 82: (4): 712-718.

Venugopal, V., Ludman, A., Yellon, D.M., et al. (2009) 'Conditioning' the heart during surgery. **Eur J Cardiothorac Surg**, 35: (6): 977-987.

Walsh, S.R., Tang, T.Y., Kullar, P., et al. (2008) Ischaemic preconditioning during cardiac surgery: systematic review and meta-analysis of perioperative outcomes in randomised clinical trials. **Eur J Cardiothorac Surg**, 34: (5): 985-994.

Wang, X., Tao, L. and Hai, C.X. (2012) Redox-regulating role of insulin: The essence of insulin effect. **Mol Cell Endocrinol**, 349: (2): 111-127.

Warden, H.E., Cohen, M., Read, R.C., et al. (1954) Controlled cross circulation for open intracardiac surgery: physiologic studies and results of creation and closure of ventricular septal defects. **J Thorac Surg**, 28: (3): 331-341; discussion, 341-333.

Watson, J.M. and Torgerson, D.J. (2006) Increasing recruitment to randomised trials: a review of randomised controlled trials. **BMC Med Res Methodol**, 6: 34.

Weber, R.J.M., Southam, A.D., Sommer, U., et al. (2011) Characterization of isotopic abundance measurements in high resolution FT-ICR and Orbitrap mass spectra for improved confidence of metabolite identification. **Anal Chem**, 83: (10): 3737-3743.

Weber, R.J.M. and Viant, M.R. (2010) MI-Pack: Increased confidence of metabolite identification in mass spectra by integrating accurate masses and metabolic pathways. **Chemometrics and Intelligent Laboratory Systems**, 104: (1): 75-82.

White, H.D. and Lowe, J.B. (1983) Antianginal efficacy of perhexiline maleate in patients refractory to beta-adrenoreceptor blockade. **Int J Cardiol**, 3: (2): 145-155.

Wikipedia (2011) **Perhexiline** [online]. http://en.wikipedia.org/wiki/Perhexiline [Accessed 5th September 2011

Willoughby, S.R., Stewart, S., Chirkov, Y.Y., et al. (2002) Beneficial clinical effects of perhexiline in patients with stable angina pectoris and acute coronary syndromes are associated with potentiation of platelet responsiveness to nitric oxide. **Eur Heart J**, 23: (24): 1946-1954.

Wishart, D.S., Knox, C., Guo, A.C., et al. (2009) HMDB: a knowledgebase for the human metabolome. **Nucleic Acids Res**, 37: (Database issue): D603-610.

Wittnich, C., Dewar, M.L. and Chiu, R.C. (1984) Myocardial protection: heparin-induced free fatty acid elevation during cardiopulmonary bypass and its prevention. **J Surg Res**, 36: (6): 527-531.

World, C.J., Yamawaki, H. and Berk, B.C. (2006) Thioredoxin in the cardiovascular system. **J Mol Med (Berl)**, 84: (12): 997-1003.

World Medical Association (2008) **Declaration of Helsinki: Ethical principles for medical research involving human subjects** [online]. http://www.wma.net/en/30publications/10policies/b3/ October 2008 Seoul, South Korea [Accessed 5th June 2011 2011]

Wright, G.J., Leeson, G.A., Zeiger, A.V., et al. (1973) Proceedings: The absorption, excretion and metabolism of perhexiline maleate by the human. **Postgrad Med J**, 49: Suppl 3:8-15.

Wu, H., Southam, A.D., Hines, A., et al. (2008) High-throughput tissue extraction protocol for NMR- and MS-based metabolomics. **Anal Biochem**, 372: (2): 204-212.

Yamada, K.A., McHowat, J., Yan, G.X., et al. (1994) Cellular uncoupling induced by accumulation of long-chain acylcarnitine during ischemia. **Circ Res**, 74: (1): 83-95.

Yellon, D.M., Alkhulaifi, A.M. and Pugsley, W.B. (1993) Preconditioning the human myocardium. **Lancet**, 342: (8866): 276-277.

Yokoyama, Y., Chaitman, B.R., Hardison, R.M., et al. (2000) Association between new electrocardiographic abnormalities after coronary revascularization and five-year cardiac mortality in BARI randomized and registry patients. **Am J Cardiol***,* 86: (8): 819-824.

Zanger, U.M., Raimundo, S. and Eichelbaum, M. (2004) Cytochrome P450 2D6: overview and update on pharmacology, genetics, biochemistry. **Naunyn Schmiedebergs Arch Pharmacol***,* 369: (1): 23-37.