Myocardial Energy Metabolism in Patients Undergoing Cardiac Surgery.

Mr Yama Shoaib Haqzad

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"The heart is more than just a pump. It is also an organ that needs energy from metabolism. A metabolic disease, ischemia, should ideally be treated by metabolic therapy"

"Characterizing and quantifying myocardial energy metabolism [and its genetic underpinning] is the first step in recognizing what we do not know, and lays the groundwork for discovering its metabolic and functional importance

Declaration

I confirm that the thesis is my own work; and that all published or other sources of material consulted have been acknowledged in notes to the text or the bibliography.

I confirm that the thesis has not been submitted for a comparable academic award.

Acknowledgements:

The path towards this dissertation has been challenging and its completion is thanks in large part to the special people who supported and stuck with me along the way.

First of all, I would like to express my immeasurable appreciation and deepest gratitude to my supervisor, Professor Mahmoud Loubani, for giving me the opportunity, funding and encouragement to complete my Medical Doctorate at Castle Hill Hospital in collaboration with Hull York Medical School. Your advice on both research and my future career has been invaluable. Secondly, this project would not have been completed without the unwavering support, guidance and dedicated supervision of my mentor and friend Dr James Hobkirk. He has been influential at every stage of my research from its inception to its completion.

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A statement of personal contribution:

I came up with the idea behind the first chapter. I collected data, analyzed the data and presented the results nationally and internationally at conferences. The first chapter led to the concept of the second chapter. The second and third chapters were conceived with the help of Dr James Hobkirk, Professor Mahmoud Loubani and Professor Sean Carroll. With the help of Dr James Hobkirk, I obtained ethical approval for the studies. I recruited patients for both of the studies (chapter 2 and 3) and consented them the day before surgery and at least a week before patient information was given to them. I collected and processed the samples on the day of the operation. This included measuring, fixing and freezing for analysis in future. I performed the simulated ischaemia and re oxygenation of samples for 10 patients, each lasting at least 28 hours of continuous monitoring and sampling for LDH release. I performed the cell viability studies on all samples with the help of Dr James Hobkirk and Dr Huw Jones.

Epicardial fat profiling was conducted as a collaborative project with Professor Tchernof, Laval University, Canada. I performed the data analysis and presentation with the help of Dr James Hobkirk. I was helped by Dr Agata Burska, university of Leeds, UK to perform gene expression analysis and presentation. The measurement of IMCL forms part of an important collaborative partnership with Professor Hesselink from the University of Maastricht. I recruited, collected and processed samples in the UK. I visited Maastricht where the measurements took place. I analyzed and presented the data with the help of Dr James Hobkirk.

Abbreviations:

ACC	Acetyl CoA Carboxylase
ACS	Acute Coronary Syndrome
ADP	Adenosine diphosphate
ACSLC	Acyl-CoA synthetase long chain
ATP	Adenosine triphosphate
AHA	American Heart Association
Apo B	Apolipoprotein B
ATGL	Adipose Tissue Triglyceride Lipase
AVR	Aortic Valve Replacement
CABG	Coronary Artery Bypass Surgery
CAD	Coronary Artery Disease
CD36	Cluster of differentiation-36
Cer-S	Ceramide synthetase
CHREBP_1	Carbohydrate Response Element Binding Protein-1
CK	Creatine Kinase
CPT-1	Carnitine Palmitoyl Transferase-1
CT	Computed Tomography
CVD	Cardiovascular Disease
ELISA	Enzyme Linked Immunosorbent Assay
EPF	Epicardial fat
FAS	Fatty Acid Synthetase
FFA	Free Fatty Acids
GLUT-1	Glucose Transporter-1
GLUT-4	Glucose Transporter-4
hFABP	Heart Specific Fatty Acid binding Protein
HDL-c	High Density Lipoprotein Cholesterol
HIF-1	Hypoxia Inducing Factor-1

HSL	Hormone Sensitive Lipase
IGT	Impaired Glucose Tolerance
ICD	Insulin controlled Diabetes
I/R	Ischaemia and Reperfusion
IMCL	Intramyocardial content of lipid
IRS-1	Insulin Receptor Substrate-1
IRS-2	Insulin Receptor Substrate-2
LDL	Low Density Lipoprotein
LDL-c	Low Density Lipoprotein cholesterol
LPL	Lipoprotein Lipase
LV	Left Ventricle
RV	Right Ventricle
MAG-1	Monoacylglycerol Lipase
MetS	Metabolic Syndrome
MI	Myocardial Infarction
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
MRS	Magnetic Resonance Spectroscopy
OGTT	Oral Glucose Tolerance Test
PCI	Percutaneous Coronary Intervention
PET	Positron Emission Tomography
PDH	Pyruvate Dehydrogenase
PDK	Pyruvate Dehydrogenase Kinase
Pi	Phosphocreatine
PPAR	Peroxisome Proliferator Activated Receptor
RA	Right Atrium
SPT	Serine Palmitoyltransferase
S1P	Sphingosine-1-phosphate
SREBP1c	Sterol Regulatory Element Binding Protein-1c

TCD	Tablet Controlled Diabetes
T2D	Type 2 Diabetes
TC	Total Cholesterol
TG	Triglycerides
VDR	Vitamin D Receptor
VLDL	Very low Density Lipoprotein
VLDR-r	Very low Density Lipoprotein receptor
WHO	World Health Organisation

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Abstract:

In chapter 1, short and long term outcomes were assessed in diabetic phenotypes. The phenotypes have been propensity matched for factors that significantly contribute to outcomes. Tablet controlled diabetics have worse short and long term outcomes compared to a non-diabetic. This is important as T2D status (those not on insulin) are not considered in EUROSCORE. Refining surgical risk based on more discrete classifications of continuous variables is required.

In chapter 2, EPF was dysfunctional and is highly associated with EPF lipolysis in T2D and this may overload the T2D heart with FFA that exceeds its ability to oxidise. Identifying EPF volume may be a sensitive surrogate for LV IMCL and abnormal cardiac energy metabolism. The LV IMCL was higher in T2D versus non-T2D. VDR was significantly associated with IMCL and the majority of IMCL was located close to the myofibrillar T-tubule. The role that VDR/IMCL relationship plays in cellular lipogenesis and calcium metabolism and action potential propagation requires further study. This may be an important mechanism linking abnormal energy metabolism with poor contractile function. We identified that serum β-hydroxybutyrate is a predictor of IMCL. Promoting ketone oxidation (due to its oxygen efficiency) prior to surgery may be protective against ischaemia and reperfusion (I/R) induced myocardial damage.

In chapter 3, RA tissue was exposed to ischaemia and reperfusion in two vitamin D (50nmol/L and 100 nmol/L) doses. Genes controlling energy metabolism were assessed after experimental I/R. Cell viability and injury markers confirmed cell viability. FFA oxidation and PDK was decreased and insulin sensitivity, glucose transport and glucose oxidation increased. None of the gene expression profiles were significant when comparing 50 to 100 nmol/L. This shift in energy substrate <FFA and >glucose oxidation may be beneficial in the I/R period to protect the heart from cellular damage.

Overview of Studies

This thesis includes three chapters.

Chapter 1 (ULTIMATE—OUTCOME) is an epidemiological propensity matched study constituting over 8,000 patients. The study was undertaken to compare short and long-term outcomes between patients with type II diabetes (T2D) (both on insulin and tablet controlled) and those without T2D undergoing cardiac surgery. The outcomes include short and long-term morbidity (post-op wound-infections, renal failure, 30 day and 18 year mortality, readmission with MI or CVA). This is important as patients with T2D have been shown to have worse outcomes when compared to those without diabetes. Therefore, the role of T2D and glycaemic control in defining pre-surgical risk and outcomes needs further clarification, especially in T2D patients who are controlled by tablet or by insulin. These patients may represent significantly different diabetic phenotypes and may therefore pose different risk of coronary artery bypass graft (CABG) surgery. Understanding how T2D treatment and glycaemic control is incorporated into pre-surgical risk scores is discussed.

Chapter 2 (ULTIMATE-1) is an exploratory cross sectional study and was undertaken to investigate gene expression controlling cardiac energy metabolism and intra-myocardial content of lipid (IMCL) between patients with T2D and without T2D. In chapter one we establish that diabetic patients have worst short and long term outcomes compared to non-diabetic patients. In chapter 2 we will explore this further at molecular level by looking at the gene expressions that control energy metabolism between diabetic and non-diabetic patients. This is important as T2D have dysregulated cardiac energy metabolism and are susceptible to diabetic cardiomyopathy and heart failure. Cardiac energy metabolism is a topical and contemporary debate and may constitute a therapeutic target to improve outcomes post-surgery. ULTIMATE-1 recruited thirty patients undergoing a CABG operation. In ULTIMATE-1 we sought to investigate gene profiles involved in cardiac energy metabolism in the left ventricle (LV) and epicardial adipose tissue (EPF). There are a number of possible gene candidates that may provide insight into the imbalance between energy storage and oxidation

in tissue specific depots. Therefore, an exploratory gene array was designed to assess a number of mRNA gene profiles between tissues. Furthermore, we assessed a quantitative measure of LV IMCL, measured via electron microscopy, and EPF morphological characteristics (adipocyte number and size). CD68 macrophage labelling was also undertaken to provide a measure of dysfunctional EPF in a sub-sample of patients. These tissue/gene analyses were combined with clinical characterization data, including drug history, EUROSCORE, blood lipid-lipoprotein subclass analyses, FFA's (NEFA), h-FABP, βhydroxybutyrate, systemic insulin resistance (HOMA-IR), serum vitamin D and NTProBNP.

There are a number of sub-studies within ULTIMATE-1. These are both highly novel and proved somewhat challenging. Firstly, we sought to overcome ethical constraints in obtaining human LV biopsies from patients undergoing cardiac surgery. Reporting on the efficacy and safety of these LV biopsies is important to broaden and extend the research outlined in this thesis. ULTIMATE-1 is one of the largest cohorts of LV biopsies from patients undergoing CABG surgery in the UK, and the first with corresponding EPF tissue. This uniqueness allows interrogation between distinct human tissue depots. We also sought to provide further understanding in the potential 'cross-talk' between the LV and EPF. One evolving hypothesis is that EPF tissue work synergistically to protect the LV from lipid overflow and subsequent increase in IMCL and progression to myocardial lipotoxicity. In contrast, an increase in EPF lipolysis may be detrimental to the LV by supplying FFA that exceed LV mitochondrial capacity to oxidise. This will be undertaken by comparing selected genes controlling uptake and oxidation in both EPF and LV in both patients with T2D and those without T2D. Individual genes and other routine blood based markers will be associated with LV IMCL. Establishing a potential biomarker of abnormal cardiac energy storage and utilization (reflected by IMCL) may be used to screen patients who may have worse outcomes due to abnormal energy metabolism and correspondingly poor contractile recovery post CABG induced ischaemia and reperfusion (I/R) injury.

Chapter 3 (ULTIMATE-2) is an experimental, ex-vivo intervention study was conducted to evaluate the effectiveness of a non-CTIMP approach to modify cardiac energy metabolism

during I/R. High circulating levels of free fatty acids are observed during and following cardiac surgery. Over-reliance on fatty acid oxidation has a detrimental effect on functional recovery of hearts following surgery induced myocardial I/R. FFA inhibition of glucose oxidation contributes to impaired myocardial contractility. Therefore, subtly shifting energy substrate selection (i.e. lowering the reliance on FFA oxidation and increasing glucose oxidation following I/R may improve cardiac functional recovery. Vitamin D dosing may also be a promising strategy to attenuate ischaemia and reperfusion injury in patients undergoing CABG. Current experimental evidence is mainly focused on heart specific cell models and mouse studies which include VDR gene activators and/or silencers (siRNA's). Therefore, in ULTIMATE-2 we sought to investigate the effects of supplementary and clinically relevant dose of vitamin D (100nmol/L/ or 40ng/ml) in ten patients undergoing CABG on modulating glucose and lipid metabolism (i.e. pyruvate dehydrogenase kinase, glucose transporters (GLUT's), peroxisome proliferator related receptors (PPAR's) and carnitine palmitoylacyltransferase-1 (CPT-1) after being exposed to experimental I/R. The findings of this study may provide an impetus to undertake appropriately designed randomised multi-centre controlled trials in vitamin D supplementation in patients requiring cardiac surgery for the purpose of altering cardiac energy metabolism during I/R and improving outcomes.

Aims

Conceptualising the thesis as part scientific and part clinical is of paramount importance. The broad elements of the review of literature are to provide clinically meaningful concepts, interpretations and plausible treatment options. Therefore, the following clinical themes shall be discussed in chapters 1-3.

CHAPTER 1:

SHORT AND LONG-TERM OUTCOMES FOLLOWING CABG SURGERY IN DIABETIC PHENOTYPES

KEY CLINICAL QUESTIONS:

1) What defines surgical risk in T2D?
2) Can risk profiles be improved in T2D?

CHAPTER 2:

THE GENE PROFILES IN T2D COMPARED TO NON-T2D PATIENTS IN LV IMCL AND EPF DYSFUNCTION:

KEY CLINICAL QUESTION:

1) Is cardiac energy metabolism important to a practicing cardiologist and/or a cardio-thoracic surgeon and what are the consequences of abnormal cardiac energy metabolism?
2) How can I assess if my patient has abnormal cardiac energy metabolism prior to surgery?

CHAPTER 3:

EX-VIVO CARDIAC ENERGY METABOLISM IN RESPONSE TO VITAMIN D & EXPERIMENTAL ISCHAEMIA AND REPERFUSION

KEY CLINICAL QUESTION:

1) What are the treatment options that may be prescribed to correct abnormal cardiac energy metabolism pre-operatively, during surgery and post-surgery?

CHAPTER 1:

SHORT AND LONG-TERM OUTCOMES FOLLOWING CABG SURGERY IN T2D PHENOTYPES

CHAPTER 1:

REVIEW OF LITERATURE

Prevalence of cardiovascular disease and coronary artery bypass surgery:

According to data from the British Heart Foundation annual report on mortality suggests that cardiovascular diseases (CVD) causes twenty-six percent of all deaths in the UK. That is nearly 160,000 deaths each year. In particular, coronary heart disease (CHD) is the leading cause of death worldwide in developed and developing countries. In the UK 2.3 million (1.4 million males) people are living with CHD. In the UK, one in seven men and one in eleven women die from CHD. CHD is responsible for nearly 70,000 annual deaths in the UK each year. Most deaths from CHD is caused by an acute myocardial infarction (MI). The annual number of hospital admissions due to acute MI was in excess of 200,000. Mortality rates from acute MI have decreased from 70% in the 1960's to around 30% in 2015. Given the effective treatment options for CHD, there are 7 million people living with CVD in the UK. The burden of CVD requires considering given improved survival rates from CVD events. When survival rates are considered with a growing and ageing population the burden is truly worrying. The estimated costs to the UK economy (including premature death, disability) is estimated to be £19 billion annually. The financial ramifications of CVD are showing no signs of abatement either. Health costs may also vary considerably based on economical calculation models, health-related inflation, improvements in prospective screening, improved/early diagnosis and trends in societal lifestyles (nutrition, alcohol, stress, exercise, smoking and pollution).

Treatments for Coronary Artery Disease: PCI vs. CABG?

An acute coronary syndrome (ACS) occurs when there is a reduction in the blood supply to the myocardium; typically including unstable angina and myocardial infarction. There are two coronary revascularisation techniques used to treat stenosed coronary arteries; coronary artery bypass surgery (CABG) and percutaneous coronary intervention (PCI). PCI involves stenting (metal mesh scaffolding) which restores coronary lumen diameter and blood flow. During CABG surgery, the narrowed artery is bypassed using a vein or artery. Table 1 shows a decreasing number of CABG surgeries in the UK. This contrasts PCI which has shown a sharp increase; the ratio of PCI to CABG is now over 5:1. The reasons are likely to be multi-factorial and beyond the scope of this thesis.

Figure 1: Annualised numbers of CABG versus PCI procedures in the UK (1977-2014).

Table 3.6 Number of CABGs and PCIs, United Kingdom 1977 to 2014

	Coronary artery bypass surgery (CABG)	Percutaneous coronary interventions (PCI)
1977	2,297	
1978	2,653	
1979	2,918	
1980	4,057	
1981	5,130	
1982	6,008	
1983	8,332	
1984	9,433	
1985	10,667	
1986	10,767	
1987	11,521	
1988*	11,113	
1989	12,648	
1990	14,431	
1991	15,659	9,933
1992	19,241	11,575
1993	21,031	12,937
1994	22,056	14,624
1995	22,475	17,344
1996	22,160	20,511
1997	25,639	22,902
1998	25,083	24,899
1999	24,733	28,133
2000	25,127	33,256
2001	24,663	38,992
2002	25,277	44,913
2003	25,461	53,261
2004	25,160	62,780
2005	23,412	70,142
2006	23,623	73,692
2007	25,372	77,373
2008	22,846	80,331
2009	19,766	83,130
2010	17,822	87,676
2011	17,972	88,692
2012	17,119	92,445
2013	16,791	92,589
2014	17,513	96,143

Notes: *Operations performed in NHS hosptials and selected private hospitals are included.*
Source: *British Cardiovascular Intervention Society (2017). BCIS Audit returns . Personal communication. The Society for Cardiothoracic Surgery in Great Britain & Ireland (2017). . Accessed in May 2015* http://bluebook.scts.org/#ActivityRates

Studies have established that CABG is a better treatment option with lower adverse clinical outcomes compared with PCI in patients with or without diabetes (Bundhun et al., 2015, Bundhun et al., 2016, Hlatky et al., 2009). The FREEDOM trial recruited 1900 diabetic patients (insulin and tablet controlled) and randomized to PCI or CABG. The primary endpoint, comprised of all-cause mortality, myocardial infarction and stroke were significantly lower in the CABG group (Dangas et al., 2014, Farkouh et al., 2012). The BARI Trial (Chaitman et al., 1997) showed 5 year cardiac mortality in patients with tablet controlled diabetes (TCD) and multi-vessel disease to be significantly higher in PCI group compared to the CABG group. Subgroup analysis of SYNTAX II trial showed that PCI resulted in higher rate of major adverse cardiac events and repeat revascularization at 5 years follow up compared to CABG (Kappetein et al., 2013). However, diabetic patients have an increased risk of morbidity and mortality following CABG surgery when compared to a non-T2D patient (Eagle and Guyton, 2005, Charlesworth et al., 2003). Several studies (Leavitt et al., 2004, Kubal et al., 2005) have shown a higher incidence of stroke, renal failure and sternal wound infections in diabetic groups following CABG surgery. The PATCH trial illustrated a greater risk of readmission for any cause (+44%) and for cardiovascular related issues (24%) in diabetic patients compared to non-T2D patients following CABG (Herlitz et al., 1996, Whang et al., 2001).

Surgical risk in T2D patients:

There is considerable heterogeneity in post-surgical outcomes for T2D patients undergoing CABG surgery. Subgroup analysis in the BARI (Bypass Angioplasty Revascularization Investigation) trial shows that cardiac mortality was higher (5.8% vs 4.7%) in T2D versus non-T2D patients respectively (Alderman et al., 1997, Brooks et al., 2007). Non-cardiac mortality was significantly higher in the T2D group (12.2% vs 4.8%). Thus, worse long-term survival after CABG in T2D patients could be due to the diabetes related comorbidities. Cardiac surgery outcomes are largely dependent on pre-operative risk scores and clinical evaluation. The risk scores have evolved over time. In the 1980's, risk stratification models in cardiac surgery came into existence because of the rise in operative mortality in cardiac surgery due to increasing risk profile of patients.

Risk modelling should also aim to ascertain the likelihood of an abnormal outcome in the long-term. Various score systems have been developed to predict mortality using patient derived data such as age, gender, unstable angina, pulmonary hypertension, left ventricular ejection fraction, myocardial infarction, Asthma/COPD, Creatinine >200 / Dialysis, history of cerebrovascular accident, peripheral vascular disease, pre-operative ionotropic /mechanical support, operative urgency and concomitant operations (Geissler et al., 2000). Procedures such as coronary artery bypass surgery which is cost intensive, have received great interest with regard to cost benefit analysis and comparison of mortality rates among institutions.

However, there are considerable differences between risk models and scores with regard to their design and validity. The initial risk models used data from single centres or single population cohort which meant they were not applicable to the rest of the world or even to different ethnic groups within their own population. The most widely applied risk scores for cardiac procedures are the Parsonnet Score (Parsonnet et al., 1989), The Cleveland Score (Higgins et al., 1992), the Society of Thoracic Surgeons score (Anderson, 1994), EURO SCORE I (Nashef et al., 1999, Nashef et al., 2012, Roques et al., 1999). Subsequent models such as EURO SCORE II (Nashef et al., 2012) were designed to address both internal and external validation by using data from different hospitals around Europe. But even then, the validity of EURO SCORE II is questionable for populations outside Europe. There still remains difficulty to predict risk for individual patients especially in the high-risk groups. Further, studies show that the observed mortality diverges from the prediction line in higher risk groups (Nilsson et al., 2004). This problem can be overcome by including more patients with higher risk score to improve the risk algorithm. Thus, larger international cardiac surgery databases are needed to produce a more accurate risk algorithm(Wyse and Taylor, 2002).

The Society of Thoracic Surgeons (STS) risk score is primarily used in North America. It began collecting data in 1989 and currently includes 4.5 million patients (Anderson, 1994). The predictive model based on this dataset is periodically updated following reanalysis of the data.

STS risk score is highly effective in determining the risk of all-cause mortality in both the short and the longer- term(Puskas et al., 2012). The STS score offers risk prediction for numerous outcomes (i.e. stroke, myocardial infarction, renal failure and hospital stay). EURO SCORE I (Nashef et al., 1999, Roques et al., 1999) was developed between 1995 and 1999 using a European database of 19,000 cardiac surgical patients in 8 European countries to assess risk for cardiac surgery patients. EURO SCORE tends to overestimate the risk of mortality for low risk patients while underestimating risk of mortality for high risk patients (Kunt et al., 2013). Other limitations of EURO SCORE I include its failure to take into account creatinine clearance (which is more accurate measure of renal function) rather than creatinine (Nashef et al., 2012). EURO SCORE I was revised in 2012 to the new EURO SCORE II to make it applicable to a wider variety of cardiac surgical procedures and to incorporate fewer variables than the STS Score. EURO SCORE II incorporated data from over 20,000 patients in 43 countries worldwide and is the most recent risk assessment system developed using widely available clinical variables and operation-related factors. A fundamental change in EURO SCORE II is the definition of mortality used, i.e. in- hospital mortality rate versus 30 days post-surgery mortality in EURO SCORE I and the inclusion of diabetes (in those requiring insulin).

The long-term outcomes for T2D patients are significantly worse than a non-diabetic patient undergoing CABG surgery (Alderman, 1997, Alderman et al., 1997, Brooks et al., 2007). However, there are variations in outcomes even in diabetic patients, suggesting that these patients are not a homogenous group. For example, there are significant phenotypes of diabetes; type 1 (insulin controlled), type 2 (tablet controlled, insulin controlled and diet controlled) which all confer different risks and comorbidities. Likewise, in heart failure, there are significant phenotypic variations (diastolic HF, systolic HF) with different aetiologies, diagnostic strategies and treatments options. Therefore, chapter 1 was undertaken to compare outcomes between insulin controlled diabetes (ICD), tablet controlled diabetes (TCD) and non-diabetic (ND) patients undergoing cardiac surgery in a propensity matched study. Our highly calibrated, precise matching algorithm was designed to ensure that the observed outcomes between the groups are closely related to their T2D and non T2D status rather than

other variables that significantly alter outcomes post CABG surgery. The outcomes include short and long-term morbidity (post-op wound-infections, renal failure, respiratory failure, hospital stay), 30 day and 18 year mortality, readmission with MI or CVA.

CHAPTER 1:

METHODS

Data was obtained retrospectively on all consecutive patients undergoing first time isolated CABG surgery within the department of cardiothoracic surgery, Castle Hill Hospital, Cottingham, Kingston upon Hull, UK from April 1999 to April 2017. Patient data was obtained using the nationally standardised cardiothoracic (SCTS, Dendrite) database. This database requires all consultant cardiac surgeons performing procedures to provide information on patients' demographic and peri-operative variables. The data collection system based on specifications from the Society for Cardiothoracic Surgeons in the UK/Ireland (SCTS), includes multi- step data cleaning process and is frequently audited and validated to ensure data accuracy. The cardiothoracic database is used by SCTS for publication of risk-adjusted CABG mortality for individual surgeons and hospitals. In addition, records of death were obtained from the NHS Spine, a tool that captures mortality data across the UK within 24 hours of it being reported.

All isolated, first time CABG surgery patients were divided into the following three clinical sub-groups:

1- Insulin controlled diabetes (ICD)
2- Tablet controlled diabetes (TCD)
3- Non diabetes (ND)

Re- admission of patients for myocardial infarction (MI) and cerebrovascular accident or transient ischaemic attack (CVA) was obtained from the hospital records using appropriate medical coding system, as per international classification of diseases (Pavillon and Maguin, 1993). In- hospital mortality and morbidity (renal impairment, neurological impairment, myocardial infarction, respiratory failure, sternal wound infection and atrial fibrillation together with long term survival (up to 18 years follow up) and re- admission with a major cardiovascular event (MACE); comprising myocardial infarction and cerebrovascular accident were compared between the propensity matched groups described below.

Statistical analysis were performed using IBM SPSS 24.0 software (SPSS Inc., Chicago, IL, USA). Propensity score matching was employed to match the following patients:

1- ICD vs ND

2- TCD vs ND

3- ICD vs TCD

All pre-operative variables in the European System for Cardiac Operative Risk Evaluation (EUROSCORES I – our primary risk calculator) and several intraoperative variables (bypass time, cross clamp times and number of coronary grafts) were used to match patients 1:1 with a matching calliper of 0.02 for close matching in order to reduce bias (Lunt, 2014). The overall balance test (Hansen & Bowers, 2010) showed good matching based on preoperative and intraoperative demographics. Kaplan-Meier survival curves were constructed to represent the result of survival analysis. Continuous variables were represented as mean (± standard deviation) and categorical variables were expressed as percentages. Student's *t*-test was used for continuous outcomes and the χ^2 test for categorical outcomes. Matched outcomes were evaluated with a paired *t*-test for continuous outcomes and a paired McNemar's test for categorical outcomes.

CHAPTER 1:

RESULTS

In total, 8378 patients underwent first time isolated coronary artery bypass surgery between April 1999 and April 2017. Of these, 137 patients' data were incomplete and therefore not included in the final analysis. The remaining 8241 patients comprised of 6302 ND patients versus 1939 diabetic patients (618 ICD, 919 TCD and 402 diet controlled diabetes). The diet controlled diabetes patients (n=402) were excluded. Therefore, the total number of patients included in the final analysis was 7839. In this cohort, 2804 patients had 2 or less number of grafts while 5035 patients had 3 or more grafts. The majority of patients had single mammary artery with long saphenous vein as conduits for bypass grafting. There was a small subgroup of patients with bilateral mammary arteries and/ or radial artery and/or isolated vein grafts as conduits.

Peri-operative variables in unmatched groups:

Patient characteristics for pre-operative and intra-operative variables are provided in Table 1. All pre-operative and intra operative variables showed a statistically significant association with the three group of patients (ICD, TCD, ND). The post-operative outcomes in unmatched groups are given in Table 2. Mean total length of stay, AF, new dialysis, sternal wound infections and incidence of stroke and in-hospital mortality were significantly higher in both of the diabetic treatment sub-groups compared to ND patients.

Table 1: Pre-operative and intra-operative data for the three unmatched groups (mean ± SD, or proportion %).

	ICD (618)	TCD (919)	ND (6302)	p value
Age (mean +/-SD)	64.9 (9.1)	66.9 (8.5)	65.7 (9.2)	**<0.001**
Female gender %	29.4	18.0	19.9	**<0.001**
Elective surgery %	61.7	66.8	66.8	**<0.001**
New York Heart Association classification (III and IV) %	31.7	21.9	17.9	**<0.001**
CCS angina class (3 and 4) %	54.8	46.5	44.1	**<0.001**
Good (>50%) ejection fraction %	58.1	66.1	74.7	**<0.001**
Atrial Fibrillation %	6.8	7.6	5.0	**<0.001**
Hypertension %	84.8	84.2	68.0	**<0.001**
COPD %	23.0	16.4	22.5	**<0.001**
On Dialysis %	1.6	0.4	0.4	**<0.001**
Creatinine > 200 mmol/L	4.9	1.8	0.7	**<0.001**
Previous CVA %	6.1	4.2	2.5	**<0.001**
Peripheral vascular disease %	22.2	17.6	10.9	**<0.001**
Body Mass Index kg/m2	30.6 (4.9)	30.3 (4.8)	28.2 (4.3)	**<0.001**
2 grafts or less %	32.5%	31.5	36.5	**<0.001**
EuroScore I (mean +/-SD)	4.4 (3.2)	4.1 (2.8)	3.5 (2.6)	**<0.001**
Log EuroScore (mean +/-SD)	5.8 (8.8)	4.8 (6.5)	3.8 (5.2)	**<0.001**
Cross clamp time (mins) (mean +/-SD)	37.0 (17.8)	41.3 (18.3)	36.3 (18.4)	**<0.001**
Bypass time (mins) (mean +/-SD)	68.6 (27.9)	74.7 (30.3)	66.8 (31.6)	**<0.001**

Number in the parenthesis represent +/- Standard deviation. The values in bold indicate statistical significance. The number in each box represents percentage of the total at the top of each column. CCS: Canadian Cardiovascular Society grading of angina pectoris; COPD: Chronic Obstructive Pulmonary Disease; CVA: Cerebrovascular Accident.

Table 2: Post-operative (mean ± SD, or proportion %) outcomes in unmatched groups.

	ICD (618)	TCD (919)	ND (6302)	p value
Total stay (days) +/-SD	14.3 (14.7)	12.4 (12.2)	10.5 (8.1)	**<0.001**
Reopen for bleeding %	3.1	4.2	3.3	0.31
Atrial Fibrillation %	35.5	39.7	32.5	**<0.001**
New Dialysis %	5.7	1.7	0.8	**<0.001**
MI %	1.6	0.5	1.3	0.08
Sternal wound infection %	14.3	7.7	6.4	**<0.001**
CVA %	2.0	2.8	1.2	**<0.001**
In-hospital mortality %	4.2	2.0	1.6	**<0.001**
Long- term mortality %	18.2	17.9	17.4	0.09
Re- admission with MI %	17.8	22.4	20.3	0.08
Re- admission with CVA %	2.1	1.7	2.1	0.82

Number in the parenthesis represent +/- Standard deviation. The values in bold indicate statistical significance. CVA: Cerebrovascular Accident; MI: Myocardial Infarction.

Peri-operative variables in matched patients:

Propensity score matching was employed to match all three groups of patients by age, gender, operative priority, operative date category (time group), European System for Cardiac Operative Risk Evaluation (EuroScore 1 – Our primary risk calculator), bypass time and cross clamp time.

After 1:4 propensity score matching, the following groups were constructed for comparison of outcomes:

1- 532 ICD compared to 1812 ND

2- 871 TCD compared to 2645 ND

3- 288 ICD compared to 401 TCD

The pre and intra operative characteristics of three matched groups are presented in Table 3.Table 3: Matched pre-and intra operative characteristics between the three groups (mean ± SD, or proportion %).

	ICD (532)	ND (1812)	p-value +/- Standardized Differences	TCD (871)	ND (2645)	p-value +/- Standardized Differences	ICD (288)	TCD (401)	p-value +/- Standardized Differences
Age (mean +/-SD)	65.2 (8.7)	65.9 (9.4)	0.13 (-0.077)	66.6 (8.5)	66.3 (9.4)	0.50 (0.0334)	65.9 (8.5)	66.7 (8.0)	0.19(-0.096)
Female gender %	24.1	23.6	0.81	18.0	18.3	0.45	19.8	17.2	0.42
Elective surgery %	66.4	67.1	0.93	69.0	67.9	0.15	66.3	65.3	0.75
Operation date 1999-2004 %	42.1	44.3	0.24	28.6	29.2	0.45	28.8	27.9	0.81
Operation date 2005-2010 %	29.7	31.1	0.31	33.5	34.6	0.35	34.0	36.4	0.12
Operation date 2011-2017 %	26.2	26.6	0.82	37.8	36.2	0.74	37.2	35.7	0.23
Good/Fair (>30%) ejection fraction %	94.1	95.8	0.12	92.8	85.1	**<0.001**	93.4	90.5	**<0.001**
Atrial Fibrillation %	7.1	5.2	0.38	7.1	5.4	**<0.001**	5.9	7.0	0.85
COPD %	21.2	22.4	0.32	16.1	17.8	0.42	17.4	16.0	0.68
On Dialysis %	3.6	0.8	**<0.001**	0.2	0.2	0.12	2.1	0.5	**<0.001**
Previous Stroke %	1.7	1.2	0.22	1.6	1.2	0.40	2.8	2.0	0.61
Peripheral vascular disease %	18.4	11.4	**<0.001**	16.6	11.9	**<0.001**	20.8	19.0	0.73
BMI kg/me	30.6 (4.6)	28.0 (4.2)	**<0.001 (0.5902)**	30.2 (4.8)	28.2 (4.2)	**<0.001 (0.4434)**	30.8 (4.6)	30.1 (4.8)	0.96 (0.1489)
EuroScore (mean +/-SD)	3.8 (2.6)	3.6 (2.5)	0.08 (0.0784)	3.9 (2.6)	3.6 (2.5)	0.09 (0.1176)	4.2 (3.1)	4.2 (2.9)	0.98 (0.0000)
Log EuroScore (mean +/-SD)	4.2 (5.6)	3.7 (4.2)	0.07 (0.1010)	4.0 (4.8)	3.7 (4.4)	0.07 (0.0651)	5.2 (7.4)	4.9 (6.8)	0.62 (0.0422)
Cross-clamp time (mean +/-SD)	36.9 (17.9)	36.5 (17.9)	0.66 (0.0223)	40.5 (18.1)	39.0 (18.4)	0.11 (0.0821)	39.5 (17.0)	40.0 (17.3)	0.73 (-0.029)

Bypass time (mean +/-SD)	68.1 (28.1)	66.7 (28.9)	0.31 (0.0491)	73.1 (28.8)	71.4 (30.7)	0.08 (0.0571)	72.4 (26.0)	72.8 (28.1)	0.86 (-0.014)

Number in the parenthesis represent +/- Standard deviations. The values in bold indicate statistical significance. COPD: Chronic Obstructive Pulmonary Disease; BMI: Body Mass Index;

After propensity score matching, the matched variables were comparable between the three groups.

Variables that were not used in the matching process, were comparable between the three groups except the differences in established renal impairment, peripheral vascular disease, BMI and category of ejection fraction. We did not match for all the variables in order to avoid risk of over matching between the groups.

Post-operative outcomes in the matched groups:

Table 4: Post-operative outcomes between the three matched groups (mean ± SD, or proportion %).

	ICD (532)	ND (1812)	P value	TCD (871)	ND (2645)	P value	ICD (288)	TCD (401)	P value
Reopen for bleed/tamponade %	3.0	3.3	0.45	4.0	3.1	0.19	2.8	4.7	0.23
Post- op AF %	34.2	31.3	0.63	37.1	33.9	**<0.001**	30.0	37.2	**<0.001**
New dialysis %	4.7	0.9	**<0.001**	1.7	0.6	**<0.001**	3.8	1.2	**<0.001**
Raised Creatinine (>200 µmol/L) %	9.6	3.6	**<0.001**	7.0	4.3	**<0.001**	11.8	9.7	**<0.001**
Respiratory complications (ARDS, infection, effusion) %	25.6	21.9	**<0.001**	25.7	22.7	**<0.001**	28.0	26.1	0.54
Post -op MI	1.5	1.0	0.26	0.5	1.1	0.08	2.4	0.7	0.10
Sternal wound infection requiring debridement %	3.6	1.3	**<0.001**	0.9	0.9	0.42	3.1	1.5	0.26
Sternal wound infection superficial %	8.8	5.8	**<0.001**	6.7	5.4	**<0.001**	9.4	7.9	0.25
Gastrointestinal Bleed/ischaemia %	1.4	1.4	0.91	0.8	1.3	0.24	0.3	0.5	0.31
CVA %	2.2	1.2	0.14	2.9	1.2	**<0.001**	2.4	2.9	0.77
Multisystem failure %	2.6	1.8	**<0.001**	0.7	0.7	1.00	2.4	1.0	0.22
Total stay in hospital (days) (mean +/-SD)	13.5 (13.3)	10.6 (8.0)	**<0.001**	11.8 (11.0)	10.7 (8.2)	**0.008**	14.1 (14.5)	13.0 (14.4)	0.33
In- hospital mortality %	3.8	1.7	**<0.001**	1.7	1.2	0.31	4.5	2.0	0.07
Long- term mortality %	16.9	17.2	0.87	14.6	14.9	0.87	14.2	14.7	0.48
Re- admission with MI %	17.7	19.0	0.13	22.4	19.6	**<0.001**	17.0	21.7	**<0.001**
Re- admission with CVA %	2.3	2.0	0.73	1.8	2.1	0.78	2.4	1.7	0.36

Number in the parenthesis represent +/- Standard deviation. The values in bold indicate statistical significance. The number in each box represents percentage of the total at the top of each column. ARDS: Acute Respiratory Distress Syndrome; MI: Myocardial Infarction; CVA: Cerebrovascular Accident; AF: Atrial Fibrillation

ICD versus ND:

There were statistically significant higher rates of requirement for new dialysis (4.7% vs 0.9%, p <0.001), respiratory complications (25.6% vs 21.9%, p<0.001), superficial sternal wound infection (8.8% vs 5.8%, p<0.001), deep sternal wound infection requiring debridement (3.6% vs 1.3%, p<0.001), multisystem failure (2.6% vs 1.8%, p<0.001), prolonged hospital stay (13.5 vs 10.6 days, p <0.001) and in hospital morality (3.8 vs 1.7%, p<0.001) in the ICD group compared to the ND group, respectively. There were no significant differences in the post- operative MI/CVA, re admission with MI/CVA and long term survival between ICD and ND, respectively (Figures 2, 3 and 4).

TCD versus ND:

Patients with TCD had statistically significant higher rates of requirement for new dialysis (1.7% vs 0.6%, p<0.001), post-operative AF (37.1% vs 33.9%, p<0.001), respiratory complications (25.7% vs 22.7%, p<0.001), superficial sternal wound infection (6.7% vs 5.4%, p<0.001), immediate post-operative CVA (2.9% vs 1.2%, p<0.001) and readmission with MI (22.4% vs 19.6%, p<0.001) (Figure 3) compared to ND group. Moreover, TCD patients had prolonged hospital stay (11.8 vs 10.7 days, p<0.001) compared to ND patients. There were no significant differences in multisystem failure, immediate post op MI, sternal wound infection requiring debridement, respectively . Likewise, readmission with CVA, short and longer- term mortalities were comparable between the two matched groups (Figure 2 and 3).

ICD versus TCD:

Patients in ICD group had significantly higher rates of requirement for new dialysis (3.8% vs 1.2%, p<0.001). However, patients in TCD group had statistically significant higher rates of post-operative AF (37.2% vs 30.0, p<0.001), and re admission with MI (21.7% vs 17%, p<0.001) compared to ICD group. The ICD group had higher rates of post op MI, sternal

wound infection, multisystem failure, prolonged hospital stays and in hospital mortality compared to TCD group. However, these differences were not statistically significant.

To support the propensity matching conducted within this study, a number of further traditional analyses including cox regression for variables with censored end-points including short and long-term mortality. The cox results are comparable to the PM. ICD have a higher risk of in hospital mortality RR 1.39 (3.8% vs. 1.7%) compared to ND. Long-term cox mortality results were in agreement with PM data (see table 4).

Mortality data

In hospital-mortality (n=146):

Group	HR (95% CI)	p-value
No diabetes	Reference	
Tablet diabetes	0.82(0.49,1.38)	0.46
Insulin diabetes	1.39 (0.88,2.21)	0.15

Post-discharge dead (n=1330):

Group	HR (95% CI)	p-value
No diabetes	Reference	
Tablet diabetes	1.18 (0.97,1.43)	0.09
Insulin diabetes	0.86 (0.68,1.09)	0.23

*Adjusting for age, sex, risk score, operative priority, operative time, clamp time, bypass time.

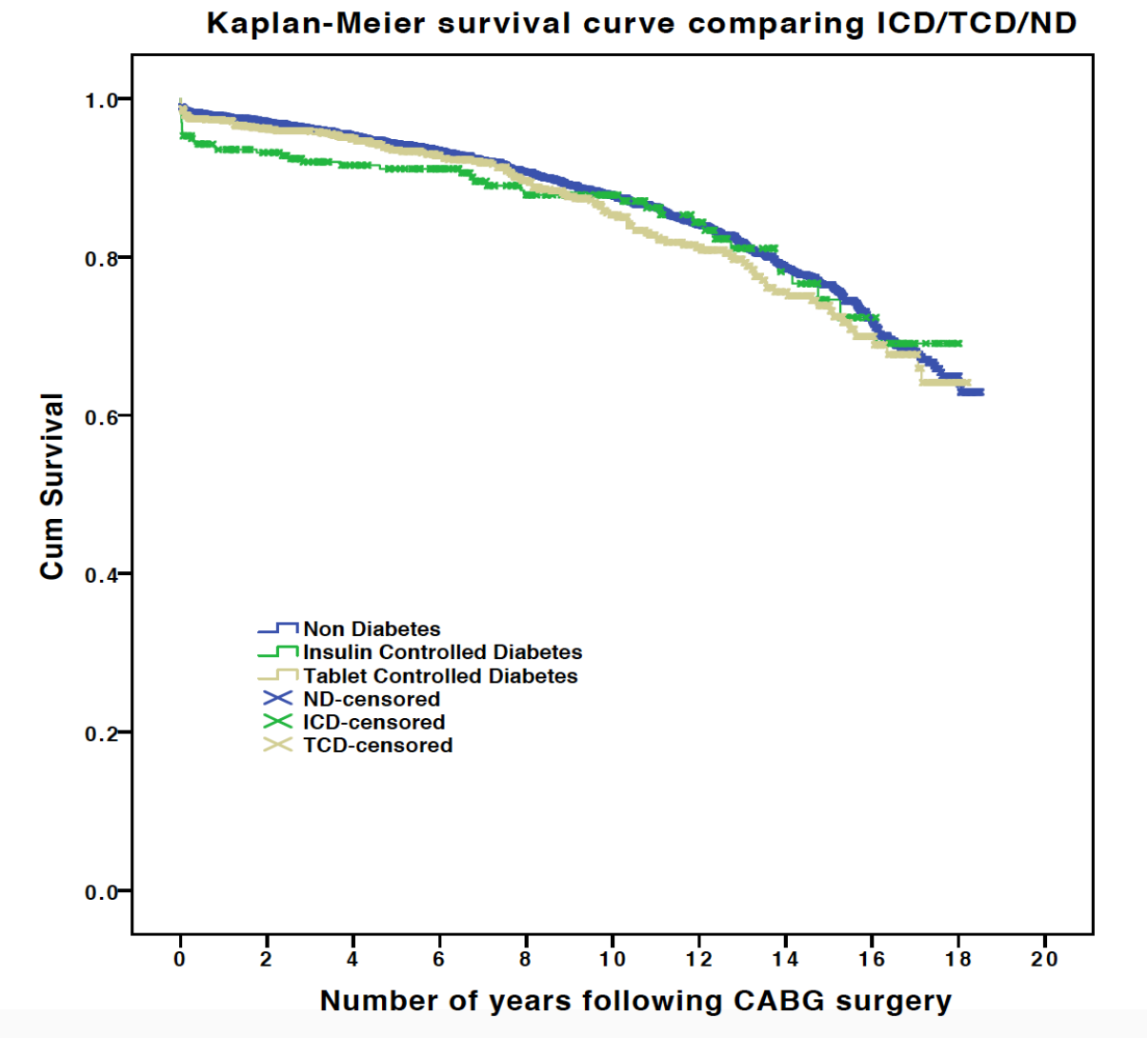


Figure 2; Long-term survival between diabetic treatment sub-groups following CABG surgery.

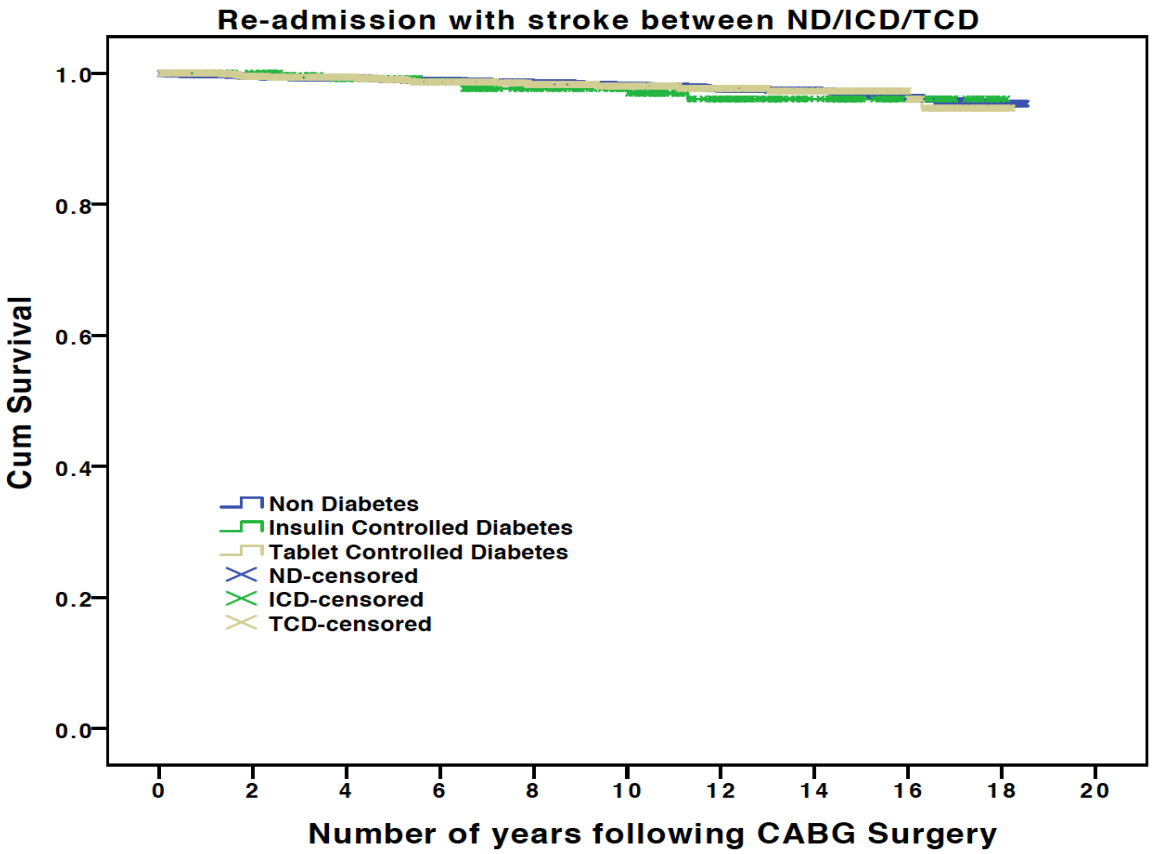


Figure 3: Readmission with cerebrovascular accident between diabetic treatment sub-groups following CABG surgery.

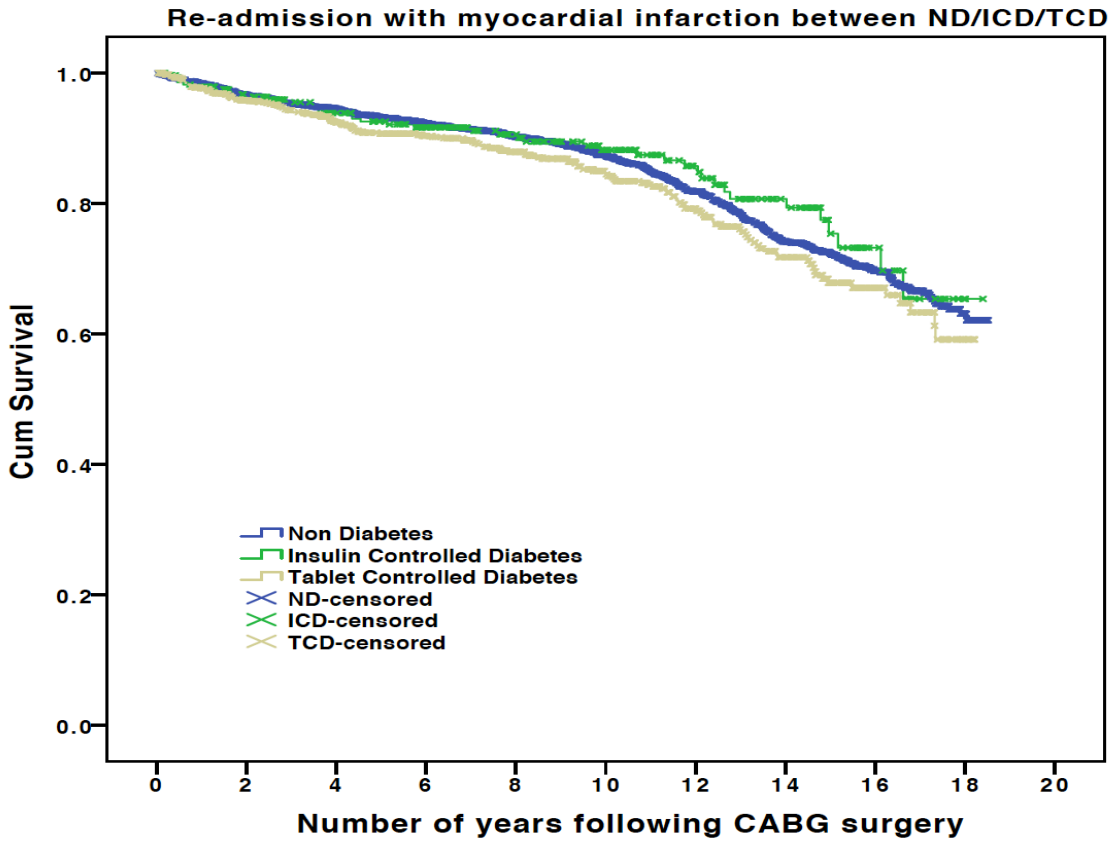


Figure 4: Re-admission with myocardial infarction between unmatched diabetic treatment sub-groups following CABG surgery.

CHAPTER 1:

DISCUSSION:

In this chapter, the main aim is to evaluates post CABG surgical outcomes in the phenotypes of diabetes. This study is one of the largest epidemiological studies that has been undertaken in diabetic phenotypes. In addition, there is a long follow-up and a wide spectrum of post-surgical outcomes. Furthermore, the patients were propensity matched for a number of pre-operative and intra-operative variables that contribute significantly to risk and post-surgical outcomes. In that sense, these data investigate the outcomes that were more specifically associated with diabetic phenotype rather than other surgical factors like aortic cross clamp time, age etc.

Given the inherent variation in diabetes, is it pragmatic to treat diabetics as one? Is there a difference in surgical outcome in the tablet controlled T2D patients? Currently, increased risk (and elevation in EUROSCORE) is for T2D on insulin. EUROSCORE II does not consider the T2D patients not requiring exogenous insulin at heightened risk. This is despite the adjusted odds of death OR 4.41 (1.44 – 13.42) in those with HbA1c above 8.6% illustrated in a retrospective study of over 3000 consecutive isolated CABG patients (Halkos et al., 2008). The T2D non-insulin group will likely exhibit significant within group heterogeneity based on therapy optimization (metformin only, gliclazide, SGLT-2, GLP-1 etc). When considering the T2D per se (y/n) it does not raise the EUROSCORE II (Nashef et al., 2012) in patients undergoing cardiac surgery.

Therefore, should glycaemia in non-insulin treated T2D patients be optimized prior to cardiac surgery by their GP or endocrinologist? Should HbA1c be measured by cardiothoracic surgeons (not GP) in clinics and be considered as part of pre-surgical risk? Optimizing T2D patients prior to CABG surgery is the focus of a recently registered clinical trial http://www.isrctn.com/ISRCTN10170306. Similar to renal function, should HbA1c be considered in discrete classifications (6-7%, 7-8%, 8-9%, >9%). How EUROSCORE score can reflect these (0.25, 0.5, 0.75 and 1 point for each classification respectively) requires further evaluation and validation using large, multicenter, international databases. From a clinical perspective, it may be prudent to start routinely measuring HbA1c (data acquisition) and commence analyses on short term outcomes. Our findings show that there was a significant

groups effect in surgical outcomes and these were both statistically and clinically significant. We were limited to ascertain the differential outcomes based on HbA1c derived glycaemic control in the whole cohort as a continuous variable or within specific groups, i.e. in the TCD where there may be high HbA1c variability. Risk scoring is an evolving cycle of data acquisition, evaluation and validation. Clinical adoption of risk markers needs simplicity, for example, (yes/no) but may also require sensitive markers of risk (those that are strongly related to adverse outcomes) for optimal risk prediction. In particular, improved thresholds for these risks may require addressing, i.e. at what threshold constitutes risk? A good example is glycaemic control versus a phenotypic classification of diabetes (type 1, insulin controlled T2D, tablet controlled and diet controlled) which all confer different risks and comorbidities. Likewise, in heart failure, there are significant phenotypic variations (diastolic HF, systolic HF) and levels of NTproBNP with different aetiologies, diagnostic strategies and treatments options.

In other studies, comparing phenotypes of diabetes post cardiac surgery, patients were not propensity matched, relying on statistical adjustment (Carson et al., 2002, Alserius et al., 2006). Study by (van Straten et al., 2010) propensity matched for age and sex in over 10,000 Dutch patients undergoing CABG surgery. Their 10 year survival results favored non T2D patients. However, matching only for age and sex did not eliminate the significant differences in pre and intra operative variables in their patients' groups that may have affected survival.

The role of diabetes (notably insulin- dependent diabetes) was associated with increased hospital mortality in CABG surgery during the EUROSCORE re-evaluation(Nashef et al., 2012). In contrast, TCD was not significantly associated with increased risk. Interestingly, the diet-controlled diabetics had better outcomes than non- diabetics. This may be in part to an improved personal care, diet and physical activity. In the EUROSCORE II risk model, diabetics on insulin, there was an odds ratio of 1.43 for operative mortality compared to non-diabetics. In comparison, in the original STS database of isolated CABG, diabetics not on insulin had

hazards ratio of 1.01; increasing to 1.30 on patients receiving insulin therapy compared to non-diabetics (Shroyer et al., 1998).

The predictive ability of pre-operative diabetic treatment subgroups among patients undergoing isolated CABG has received limited consideration. However, both diagnosis of diabetes and treatment method were recently incorporated into the latest STS final risk models for nine end-point outcomes, including operative mortality, stroke, renal failure, prolonged ventilation, mediastinitis/deep sternal wound infection and major morbidity or mortality composite, derived from an extensive (>439,000) cohort undergoing isolated CABG surgery (O'Brien et al., 2018).

A meta-analysis by (Munnee et al., 2016) included 64,152 T2D patients (23781 ICD and 40371 TCD) undergoing CABG surgery. Significantly higher short term mortality (OR: 1.47; 95% CI: 1.33 – 1.61, P < 0.00001) and major adverse events (OR: 1.66; 95% CI: 1.48–1.87, P<0.00001) were observed in ICD compared to TCD. There were significantly higher rates of long term mortality, (OR: 1.23, 95% CI: 1.02 – 1.49, P = 0.03; major adverse events OR: 1.50, 95% CI: 1.07 – 2.12, P = 0.02; and stroke OR: 1.39, 95% CI: 1.22 – 1.59, P < 0.00001, respectively) in ICD compared to TCD patients after CABG surgery. Nonetheless, studies that were included in this study had significant pre and intra operative variabilities between ICD and TCD groups. The studies included were 3 RCTs which were sub-group analyses of major RCTs (SYNTAX trial and FREEDOM trial) (Banning et al., 2010, Kappetein et al., 2013, Dangas et al., 2014). The remaining eight observational studies were not propensity score matched for pre and intra operative variables. However, most of those studies tried to adjust for some of those variables that affect mortality and morbidity using multivariate analysis (Carson et al., 2002, Alserius et al., 2006, Lawrie et al., 1986).

Insulin controlled diabetic patients typically have had longer duration of diabetes and may have higher comorbidities compared to diabetics managed by diet or tablet alone (Bundhun et al., 2015, Munnee et al., 2016). However, diet or TCD patients are not part of the current risk assessment system in EUROSCORE II. Studies have demonstrated that elevated HbA1c, in

different surgical specialties, is an independent risk factor for post-operative morbidities and mortality (O'Sullivan et al., 2006, Karimian et al., 2018, Liu et al., 2011). In a systematic review by (Tennyson et al., 2013), pre-operative elevated HbA1C is a stronger predictor of morbidities and mortality post CABG surgery irrespective of previous diagnosis of diabetes. In particular, the mortality risk for CABG surgery quadrupled at HbA1C of >8.6% (>70mmol/mol). The authors proposed that these patients' operations should be delayed until good glycaemic control is achieved in elective situations (Tennyson et al., 2013). (Halkos et al., 2008) studied over 3500 patients undergoing isolated CABG. They noted that an elevated HbA1C level predicted in hospital mortality after CABG (odds ratio 1.40 per unit increase, p=0.019). For each unit increase in HbA1C, there was a significantly increased risk of MI and deep sternal wound infection. A retrospective study of 1474 non-diabetic patients, showed that 30 day mortality in patients with pre-operative HbA1c >6.0% (46mmol/mol) was significantly higher than those <6.0% (46mmol/mol) [OR 1.53, CI (1.24–1.91), *P* = 0.0005 per unit increase in HbA1c (Hudson et al., 2010). Robich et al showed that pre-operative HbA1c level is predictive of long term survival with risk of death increasing by 13% with every unit increase in HbA1c (Adj HR = 1.13, CI 1.07 – 1.19, p<0.001) (Robich et al., 2019). All these studies are suggesting that HbA1c as a good predictor for post-operative outcomes post cardiac surgery. Unfortunately, our patients did not have routine HbA1c levels checked prior to their surgery and we acknowledge this as a limitation. However, HbA1c is unlikely to make significant difference to the outcome (above and beyond the assigned diabetic treatment group as 'treatment group' is typically determined by HbA1c. HbA1c cut-offs would potentially be a valuable addition to EUROSCORE (rather than solely insulin) and could redefine pre and post-surgical risk in diabetic groups.

Summary:

In this chapter, the surgical outcomes in different diabetic phenotypes has been presented, utilising data collected in our centre and compared to limited published literature. In our propensity score matched study of isolated first time CABG patients, we observed a significant increase in several post-operative morbidities, such as renal failure, respiratory failure,

gastrointestinal complications and prolonged length of stay in patients with diabetes (both ICD and TCD). TCD also exhibited increased risk of reopening for bleeding /tamponade, atrial fibrillation, stroke and readmission with MI compared to ND patients. Furthermore, ICD patients had an increased risk of sternal wound infection requiring debridement, multi-system failure and in hospital mortality compared to TCD patients. The rate of readmission with MI was significantly higher in the TCD group. However, long term survival post-CABG with up to 18 years follow up did not show significant differences between the ICD and TCD and non-diabetic groups. Our highly calibrated, precise matching algorithm was designed to ensure that patients had comparable risk profiles in each group irrespective of their diabetic status. Therefore, the observed outcomes between the groups are closely related to their diabetic and non-diabetic status rather than other variables (pre-operative morbidities, intra operative bypass or cross clamp times). The comparable long-term mortality between non diabetics and diabetics (insulin and tablet) could be due to their treatment approaches and time in hyperglycaemic states. Therefore, in this propensity matched cohort of patients, diabetes does not seem to influence long term mortality. Given the findings described above, showing that all diabetics with elevated HbA1c, are associated with a greater risk than non-diabetics when undergoing a CABG procedure, we question whether future scoring systems should incorporate HbA1c regardless of current treatment i.e. take pre surgical HbA1c as a risk marker.

These findings suggest that risk stratification (in relation to glycaemic control) in T2D patients not requiring exogenous insulin may need to be optimized. Although the underlying pathology in diabetes has received considerable attention (decreased vessel patency/calcification, glycation, fibrosis, susceptibility for wound infection, poorer renal function), the role of abnormal cardiac energy metabolism, i.e. how the heart produces ATP for contractile function has received considerably less attention. Without ATP (even with coronary artery integrity and flow) the heart would cease to contract. Understanding (ab)normal cardiac energy metabolism and lipid accumulation is therefore of utmost importance. Optimising cardiac energy metabolism (supply and utilisation of substrates) may be a therapeutic target. A paradigm shift in thinking may be the answer, after all, "the heart is more than just a pump.

It is also an organ that needs energy from metabolism. A metabolic disease, ischemia, should ideally be treated by metabolic therapy". We therefore study in details the left ventricular intra-myocardial lipid accumulation and cardiac energy metabolism gene expression profiles between type 2 diabetic patients and non-diabetic patients in chapter 2 of this thesis.

Conclusion:

This study shows that a previous diagnosis of diabetes is an independent risk factor for increased risk of post-operative morbidities, including sternal wound infection, renal failure, respiratory failure, prolonged hospital stays, readmission with MI and in hospital mortality after CABG surgery. We have also shown that TCD patients have a higher incidence of AF, new dialysis, respiratory complications, sternal wound infection, CVA, total length of stay and readmission with MI compared to ND patients. This suggests that these non-insulin treated patients do confer additional risk and warrants further evaluation of HbA1c and cardiovascular disease risk markers to prevent post-surgical morbidities, recurrent MI and a possible transition into heart failure. This leads us to chapter 2 of this thesis where an exploratory cross sectional study was undertaken to investigate gene expression controlling cardiac energy metabolism and intra-myocardial content of lipid (IMCL) between patients with T2D and non-diabetics.

CHAPTER 2:

GENE PROFILES IN T2D COMPARED TO NON-T2D PATIENTS IN LV IMCL AND EPF DYSFUNCTION:

CHAPTER 2:

REVIEW OF LITERATURE:

Cardiac energy metabolism:

In chapter one we establish that diabetic patients have worst short and long term outcomes compared to non-diabetic patients. In chapter 2 we will explore the gene profiles that play a role in controlling energy metabolism between diabetic and non-diabetic patients. This is important as T2D have dysregulated cardiac energy metabolism and are susceptible to diabetic cardiomyopathy and heart failure. The heart is a truly magnificent organ, beating forty-two million times per year and over three billion times in an average lifespan. In a diabetic heart there is cardiac remodeling that can lead to diabetic cardiomyopathy (DCM), which affects up to 60% of T2D patients. DCM leads to myocardial hypertrophy, stiffening, fibrosis (mainly by advanced glycation), defects in excitation-contraction coupling and calcium handling and diastolic dysfunction (Schannwell et al., 2002). EPF surrounding the heart is also significantly higher in T2D versus non-T2D ((Gaborit et al., 2012) and in obese T2D versus lean T2D (Levelt et al., 2016) which produces pro-inflammatory adipocytokines and may oversupply the heart with FFA. Metabolic dysfunction precedes both structural and functional impairments in T2D (McGavock et al., 2007) suggesting a unique opportunity to intervene in the pathology of DCM. In a mouse model study, correction of metabolic derangements, i.e. altering energy substrate metabolism (and lipid overflow/accumulation) prevented the development of DCM (Bugger and Abel, 2009). Myocardial energy metabolism is a under recognized and unappreciated entity. Identifying patients with derangements in myocardial energy metabolism is therefore essential and altering myocardial energy metabolism in human patients may be a promising therapeutic target to improve outcomes.

The heart consumes> 30kg of adenosine triphosphate (ATP) daily, the molecular currency of intracellular energy transfer. All cellular processes rely on ATP-dependent pathways. ATP formation via metabolism of lipid, carbohydrate, protein, ketones and lactate is inextricably linked with cardiac function and performance. Impaired energy production and utilization leads to dysfunctional cellular processes that affect Ca2+ handling and contractile function. Energy availability (supply) and workload (demand) are in continual flux. Surprisingly, the heart has a limited capacity for energy storage. The heart must therefore respond

proportionately to dynamic fluctuations in physiological demands and fuel delivery as observed in the post-prandial period and during exercise.

Metabolic regulatory pathways vary in nature i.e. enzymatic (e.g. lipoprotein lipase or pyruvate dehydrogenase kinase activity, metabolic intermediates that control FFA oxidation (e.g. PPAR-alpha/delta, malonyl CoA), and cell signal transduction that sense energy depravations (e.g. AMPK) events. The energy regulating pathways in the normal and failing heart occur at the level of gene expression. Therefore, abnormalities in cardiac energy metabolism (supply and utilization) may be explored by genetic profiles. These profiles provide molecular mechanistic information that is hindered in other technologies, i.e. MRI/MRS imaging. Cardiac energy metabolism is incredibly complex in health; in disease there is further complexity as adaptive (and maladaptive) process become apparent.

Energy Metabolism: Key concepts:

It is important to provide a succinct overview of the profound complexities of cardiac energy metabolism. The myocardium is non-voluntary (in contrast to skeletal muscle) and has a high proportion of slow twitch muscle fibres. Mitochondria occupy one-third of the cell volume in each cardiomyocyte (Kolwicz et al., 2013). Oxidation of fatty acids (FFAs) and glucose in mitochondria accounts for the majority of ATP generation in the healthy adult heart. FFA supply about 70% of total ATP production through oxidative phosphorylation. Myocardial FFAs are derived from chylomicrons and VLDL. The main pathophysiological underpinnings of FFA supply are increased adipocyte lipolysis, decreased adipocyte lipogenesis (fatty acid trapping) and increases in hepatic de-novo lipogenesis (large VLDL secretion) These abnormalities stem from systemic insulin resistance, not solely in T2D but also in pre-diabetic states, such as metabolic syndrome.

FFA are oxidized in the mitochondrial matrix by the process of βeta-oxidation. Glucose undergoes glycolysis into pyruvate or lactate (in aerobic and anaerobic conditions respectively). Pyruvate is oxidized by the pyruvate-dehydrogenase (PDH) complex, localized within the inner mitochondrial membrane in to acetyl CoA (Figure 5). Acetyl-CoA enters the

tricarboxylic acid (TCA) cycle. Flavin adenine dinucleotide (FADH2) and NADH are generated via substrate flux through the β-oxidation and the TCA cycle. The reducing equivalents enter the electron transport chain that promotes ATP synthesis.

Figure 5: A simplified overview of energy metabolism in the cardiomyocyte.

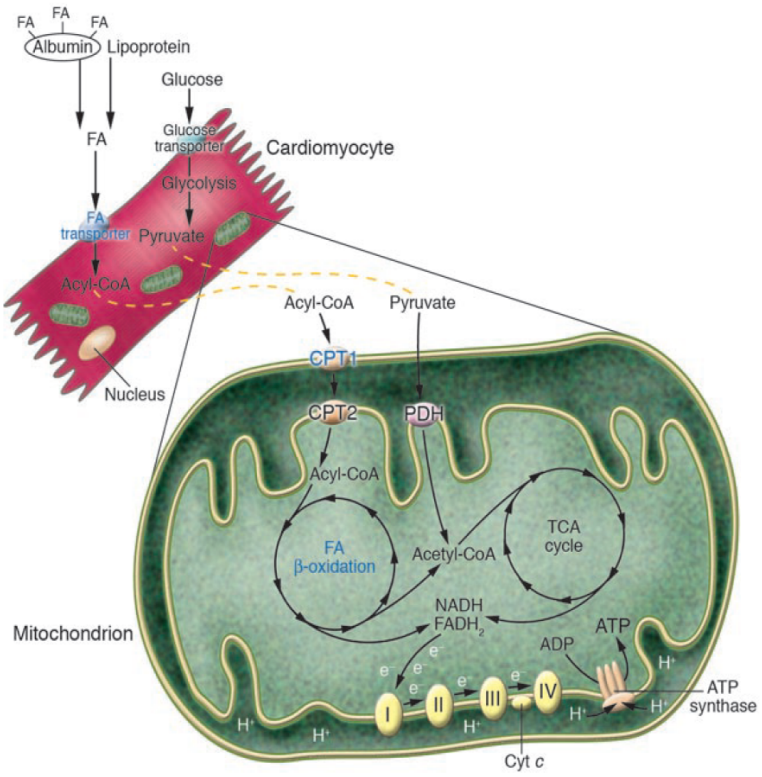


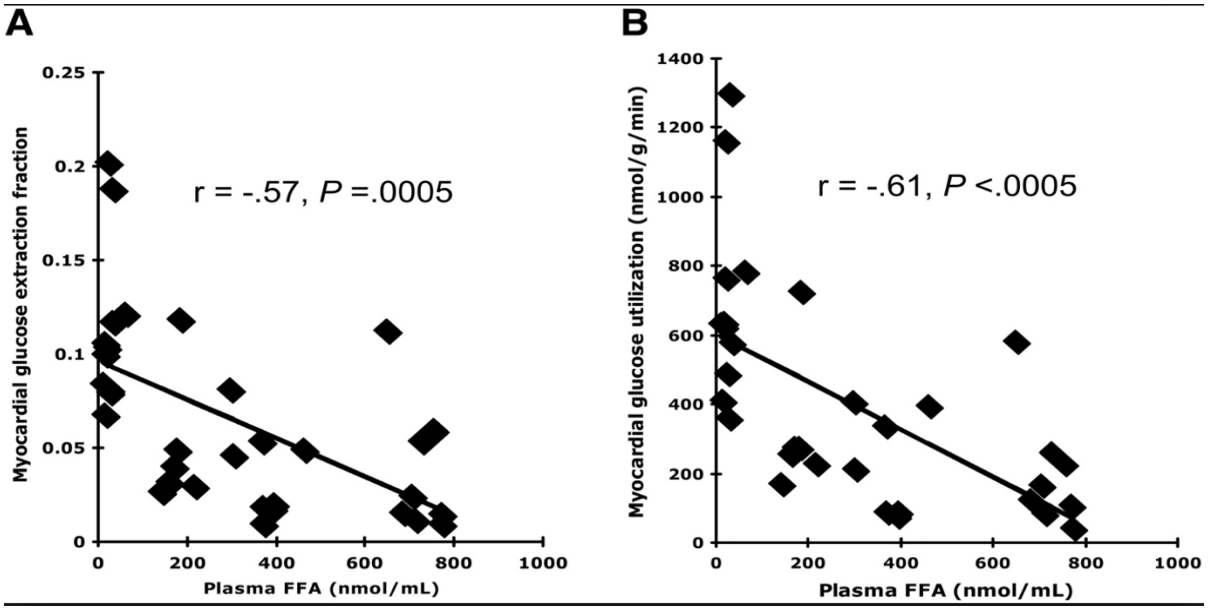
Figure 1
Pathways involved in cardiac energy metabolism. FA and glucose oxidation are the main ATP-generating pathways in the adult mammalian heart. Acetyl-CoA derived from FA and glucose oxidation is further oxidized in the TCA cycle to generate NADH and $FADH_2$, which enter the electron transport/oxidative phosphorylation pathway and drive ATP synthesis. Genes encoding enzymes involved at multiple steps of these metabolic pathways (i.e., uptake, esterification, mitochondrial transport,and oxidation) are transcriptionally regulated by PGC-1α with its nuclear receptor partners, including PPARs and ERRs (blue text). Glucose uptake/oxidation and electron transport/oxidative phosphorylation pathways are also regulated by PGC-1α via other transcription factors, such as MEF-2 and NRF-1. Cyt *c*, cytochrome *c*.

Myocardial metabolic flexibility:

Metabolic flexibility is the ability to utilize various energy substrates for ATP synthesis, including fatty acids, glucose, lactate, amino acids and ketones. This flexibility or plasticity is observed when a particular substrate is increased, for example, during long-term fasting or exercise. For example, lactate is preferentially oxidized during exercise to attenuate the exercise induced decline in cellular pH (Goodwin and Taegtmeyer, 2000). Prolonged fasting or high fat/low carbohydrate diet increases ketogenesis and circulating ketones and cardiac utilization of ketone for ATP synthesis is increased (Wentz et al., 2010). In the isolated perfused hearts, the reduction in FFA and glucose oxidation was observed with the addition of lactate or ketone bodies, indicating a substrate preference (Stanley et al., 2003). Apart from substrate availability, other mechanisms contribute to metabolic flexibility by regulating gene expression and proteins involved in fatty acid oxidation by the peroxisome proliferator–activated receptor (PPAR)/PPARγ coactivator-1 α (PGC1α) (Lehman et al., 2000) and the phosphorylation and activation of PDH (Sugden and Holness, 2003) These play a key role in shift of substrate oxidation between glucose and FFA in the heart. These regulatory concepts are important, especially in ischaemic hearts where a switch from FFA oxidation to glucose oxidation may of clinical significance, due primarily to lower oxygen requirements for glucose oxidation compared to FFA oxidation. Metabolic reserve is defined as the available potential energy that is accessible in response to an increase in cardiac work. Metabolic reserve in the T2D heart resembles an 'engine oversupplied with fuel' (FFA delivery). Despite an oversupply of FFA and energy substrates the diabetic heart is energetically compromised, reflected by myocardial PCr and ATP concentrations, as measured by MRS.

(Scheuermann-Freestone et al., 2003).

Figure 6: Myocardial glucose extraction in response to increasing FFA concentrations:



(Peterson et al., 2008).

Excess substrates may be sequestered into storage pools (such as intracellular lipid droplets) and are uncoupled from mitochondrial metabolism. Therefore, the mechanisms of energy supply, delivery and utilization are integral to our broader understanding of impairments in cardiac energy metabolism. Approaches to mitigate the pathological process are essential. The next section will provide a succinct overview of some of the most important metabolic pathways that control myocardial energy metabolism.

Energy supply: Lipid metabolism

Cardiomyocytes are highly dependent on fatty acids to synthesis sufficient ATP for contractile function. Fatty acids are either bound to albumin (NEFA's) or esterified with glycerol into triglyceride (TG) in circulating chylomicrons and lipoproteins (van der Vusse et al., 2000). Utilisation of FA's require lipoprotein lipase (LPL) to lipolyse FA's from the TG molecule in post prandial states and via the enzymes (monoacylglycerol lipase (MAG), adipose tissue triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) (Goldberg, 1996). FFA delivery via chylomicrons and VLDL in the heart are essential for the supply of ATP generating substrates (Hauton et al., 2001) & (Niu et al., 2004). The fatty acid translocase (FAT) or CD36 is an important mechanism in FFA uptake (Abumrad et al., 1993). In humans, impairments of FFA uptake are observed with CD36 mutations (Hirano et al., 2003)

In addition to CD36, intracellular FA transport requires heart-type fatty acid-binding protein (h-FABP) (Binas et al., 1999). h-FABP-null cardiomyocytes exhibit reduced uptake of palmitate when electrically stimulated, even though the capacity for oxidation remains unchanged (Schaap et al., 1999). Thus, binding of FAs to FABPs is a crucial step for their intracellular transport and utilisation. Unlike short chain FFA's, long chain FFAs cannot cross the outer mitochondrial membrane and consequently rely upon a carnitine-dependent transport system involving carnitine palmitoyl transferase 1 and 2 (CPT1 and 2), and carnitine acyltransferase (CAT). CPT I occupies a central role in the metabolism and is the rate-limiting step in fatty acid oxidation. The most important regulatory mechanism of fatty acid transfer into the mitochondrial matrix is the inhibitory effect of malonyl-CoA, a powerful inhibitor of CPT 1, thereby reducing fatty acid oxidation.

Glucose metabolism

Glucose metabolism is initiated by stimulating the IRS/PI3K, AKT insulin signaling pathway, GLUT- translocation and cellular glucose uptake. Glucose uptake is regulated by the glucose concentration and the ability of glucose transport across the sarcolemma. GLUT 1 is important for the uptake of glucose in response to ischaemia and increased cardiac work under basal conditions (insulin independent mechanism)(Young et al., 1997). GLUT 4 is insulin dependent and can be increased by contractile work (Till et al., 1997), ischaemia (Young et al., 1997). Within the cell, glucose is rapidly converted through the process of glycolysis, a series of enzymatic reactions that result in the formation of pyruvate and can be shuttled into lactate (in anaerobic conditions) or is converted into acetyl CoA to enter the tricarboxylic cycle (TCA). Pyruvate dehydrogenase Complex (PDH) is a multi-enzyme complex situated in the mitochondrial matrix. It determines whether pyruvate (derived primarily from glucose or amino acids) enters the TCA cycle for oxidation and is the key irreversible step in carbohydrate oxidation. (Bowker-Kinley et al., 1998). PDH is associated with two enzymes that perform regulatory roles. It is activated through dephosphorylation by PDH phosphatase and inactivated through phosphorylation by PDH kinase (PDK) (Randle et al., 1964, Holness and Sugden, 2003). The rate of pyruvate oxidation is therefore dependent on the concentration of PDP and PDK and degree of PDH phosphorylation.

The electron transport chain:

Nutrients in food (FFA's and glucose and amino acids) release metabolic energy as they are catabolized down into 2-carbon units and CO_2 in the citric acid cycle. The reducing equivalents that are produced undergo a series of redox reactions in the electron transport system, ultimately converting O_2 to H_2O, thereby creating a proton gradient between mitochondria and cytosol. The energy is stored in the high-energy phosphate bonds of ATP. At complex V

(ATP synthase), the ion channel facilitates proton movement back into the mitochondrial matrix, releasing energy that drives the phosphorylation of ADP to ATP. However, nutrients release differing amounts of energy depending on their degree of oxidation and thus produce differing amounts of ATP per mole of substrate.

Energy transfer:

Normal cardiac function requires tight ATP production for myocardial contraction via sarcomere and opening of ion channels (Opie, 1992). Central to energy transfer is the creatine kinase (CK) system located in the mitochondria. CK generates phosphocreatine (Pi) via the phosphorylation of creatine. ATP releases one of its high energy phosphates for energy use and Pi transfers a high energy phosphate to ADP that reconstitutes ATP. In myocardial ischaemia there is a reduction in Pi concentration compared to ATP. This imbalance may impact the ability to reconstitute ATP from ADP. Therefore, energy status (determined chemically) is typically determined by the [ATP/ADP (Pi)] ratio. Cardiac energetics as measured by Pi/ATP ratio via MRS (a marker of the inability to replenish ADP to ATP) and a 50% reduction in CK activity (a lowering of Pi formation via inability to phosphorylate creatine) is a highly significant predictor of mortality in heart failure (Neubauer et al., 1997).

Energy sensing:

The enzyme AMP kinase, coded by the AMPK gene plays a vital role in energy sensing, ensuring a match between supply and demand. AMPK is upregulated in heart failure, I/R, and fasting conditions. (Dyck and Lopaschuk, 2006) provides a comprehensive review of AMPK.

Myocardial lipid accumulation:

Lipid droplets (LDs) are intracellular storage depots of triglyceride in the heart (Greenberg et al., 2011). The formation and breakdown of LD's can affect the risk of metabolic disorders such as diabetes (Meex et al., 2009). FFA delivery/uptake which exceeds the cellular requirement (i.e. for stability, integrity and function) and ATP generation through mitochondrial oxidative phosphorylation are stored in LD's. However, some evidence suggests that uptake and turnover are reciprocally regulated (Carley et al., 2013) and in T2D patients there is increased

turnover, as observed via upregulation of PPAR –alpha expression (O'Donnell et al., 2006). LD's are regulated via a number proteins (perilipins) and lipases, such as ATGL. Perilipins coat the LD membrane and are associated with ATGL TG lipolysis (Miyoshi et al., 2007). In ATGL KO mice there is lipid accumulation and severe cardiomyopathy. LpL on the surface of cardiomyocytes increases lipid uptake and cardiomyopathy also ensues (Yagyu et al., 2003). Genetic KO of CD36 (an FFA intracellular transporter) corrects myocardial lipid accumulation when PPAR-alpha was overexpressed (Yang et al., 2007). It appears that LD TG accumulation is highly amenable to alterations in its TG content via numerous factors such as fasting (FFA supply) and prolonged exercise. There is also evidence that cellular stress, including inflammation and oxidative stress, can also induce LD biogenesis (Younce and Kolattukudy, 2012). LD's also accumulate triglyceride in ischaemia which may be cellular protective and an adaptive process because mitochondrial dysfunction triggers LD biogenesis (Lee et al., 2013) which appears to be a very efficient adaptive process. Despite the variable uptake and utilization rates, the evidence is that cardiomyocytes do not store appreciable quantities of TG in LD's (approximately 0.5-2%).

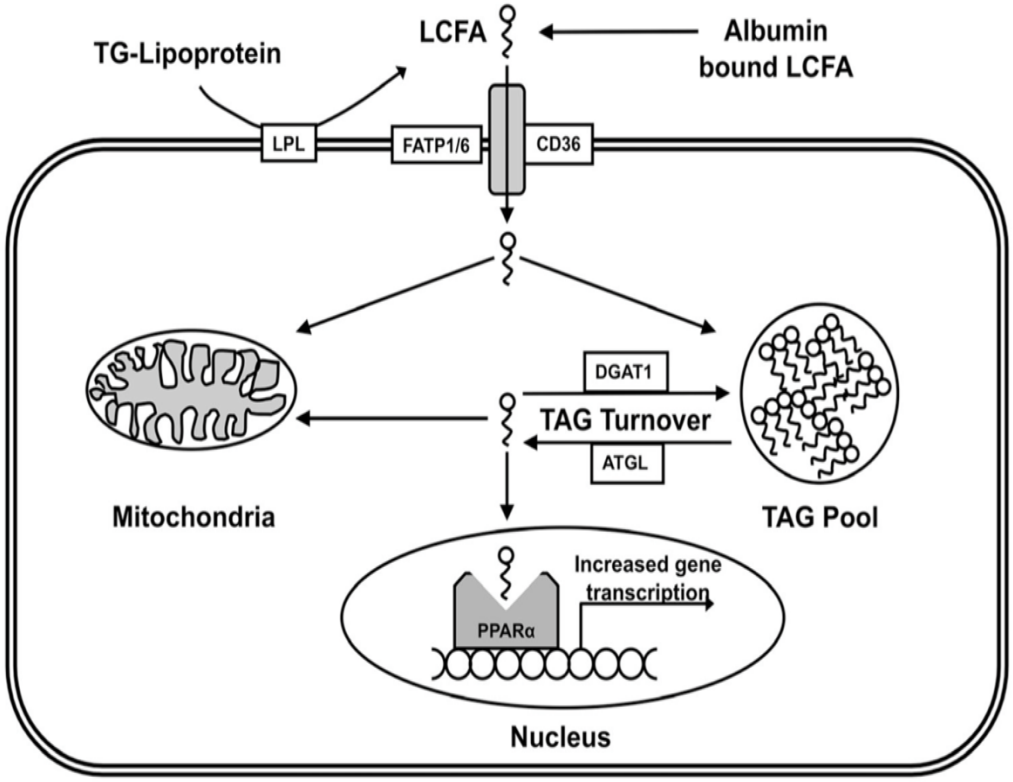


Figure 7: A simplified overview of cardiomyocyte lipid uptake and storage. Essentially FFA delivery (from TG rich lipoproteins/NEFA or from EPF lipolysis) and uptake mediated via LPL, FABP-3, VLDL-r and CD36 can enter storage pools (lipid droplets) or be oxidized in the mitochondria. Mitochondrial dysfunction and a decrease in FFA oxidation (<PPAR-alpha) is postulated to increase LD accumulation (>IMCL). Therefore, the >in IMCL may be dependent on gene expression controlling metabolic pathways (supply and uptake) and mitochondrial function. Factors that decrease the supply of FFA to the myocardium such as improved insulin resistance might be a clinical therapeutic target that may promote improved mitochondrial function and number.

Cardiac lipotoxicity:

FFA's and in particular long-chain fatty acids are the predominant source of ATP in the healthy adult heart. A mismatch between the uptake and oxidation of FFAs can result in cardiac pathology. Therefore, an intricate balance is required. An accumulation of FFA's can lead to a

conversion into toxic lipid species, for example, ceramides. Ceramide can be generated either through *de novo* synthesis requiring serine, palmitoyl-CoA and another fatty acyl-CoA species, or via recycling of more complex sphingolipid species, principally sphingomyelin. *De novo* synthesis of ceramide begins with the condensation of palmitoyl-CoA with serine, catalysed by the rate limiting enzyme, serine palmitoyl transferase (SPT). Ceramide synthases are also involved in the *de novo* synthesis pathway of ceramides by joining sphinganine to a long-chain acid. Additional sources of intracellular ceramide are hydrolysis of membrane SM via sphingomyelinases. The only exit for ceramide is via the formation of sphingosine-1-phosphate (S1P) from sphingosine and subsequent cleavage via sphingosine-1-phosphate lyase. The balance between ceramide, sphingosine, and S1P may hold an important role in determining cell fate. For example, studies have suggested that ceramide accumulation in cardiomyocytes may be positively associated with apoptosis in vivo(Bielawska et al., 1997). Indeed, several key studies have demonstrated that ceramide can induce pore formation in mitochondrial membranes and mitochondrial fission (Parra et al., 2008), thus allowing intra-membrane cytochrome -c release, and activation of the apoptotic cascade (Wang, 2000, Wang et al., 2000).

Lipid storage depots: Intra-myocardial lipid content:

Measuring dynamic substrate fluxes into specific metabolic pathways is now achievable due to the emergence of PET, PET/computed tomography, SPECT, SPECT/computed tomography, and now PET/magnetic resonance (MR 31P and 1H-MRS). Experimental work using PET can investigate disappearance of radio-labelled tracers (FDG-glucose) and MRS can detect the ATP, ADP and Pi which provides the most energetically accurate marker of metabolic status in the myocardium. These technologies are expensive and not readily available in the routine clinical environment but can do quantify IMCL in research settings.

Imaging and biopsy quantification of IMCL:

Establishing correlation and agreement between tissue triglyceride and imaging derived triglyceride is important for validation purposes. (Szczepaniak et al., 2003) undertook a validation study in Zucker rats that compared the H-MRS acquired Myocardial-TG with tissue triglycerides over a range of 5-13 μmol/g). Results confirmed a highly significant correlation ($r=0.94$, $p<0.001$). These results concur with other studies in rats (Madden et al., 1993).

The reproducibility of H-NMR has also been evaluated and proved highly reproducible in three separate measurements (2-hours apart) and one measurement 5 days later. Finally, myocardial triglycerides was highly associated with cardiac concentricity (LV mass/LV volume), $r=0.6$, $p<0.0001$). These initial validation findings supported the clinical application of myocardial triglycerides measurement non-invasively. Follow-on studies evaluated myocardial triglycerides in a variety of patient's groups and controls. These studies aimed to assess the degree of lipid accumulation and to link these to cardiac function. The sections below summarises the relationship of IMCL and EPF with cardiac function.

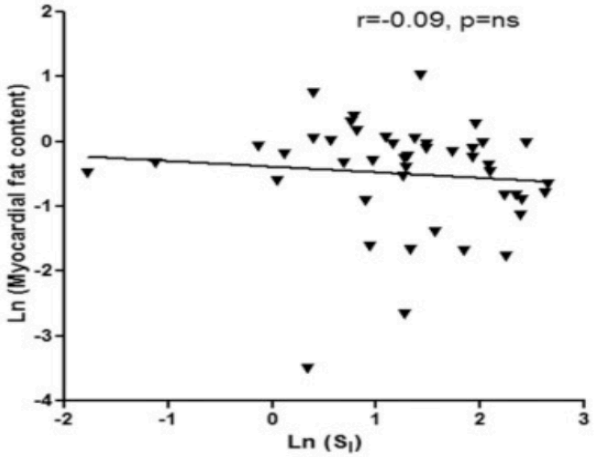
IMCL and cardiac function:

Nyman et al (2013) measured myocardial lipid content and epicardial fat (EPF) via cardiac magnetic resonance in individuals with and without metabolic syndrome (MetS). Unsurprisingly, IMCL was significantly different between MetS+ and MetS- (0.90%, versus 0.43%) and there was a high degree of variability in both groups MetS+ (0.31-2.33%) compared to (0.14-1.39%) in the MetS- group). There was a significant positive correlation between EPF and IMCL ($r=0.27$) and a negative correlation between IMCL with LV global index ($r=-0.22$) and LV diastolic function ($r=-0.42$). Interestingly, the abnormalities in cardiac dimensions and functions were more highly associated with EPF than IMCL. In regression models only, age and EPF were predictive of LV mass, LV stroke volume, peak filling rate and LV end-systolic volume. This suggests that EPF accumulation is a very important clinical surrogate of IMCL accumulation and cardiac diastolic dysfunction in the absence of overt CVD (Nyman et al., 2013).

Garborit et al (2012) conducted a similar study by measuring both EPF and IMCL in very heterogeneous patients without CVD but in lean (n-33), obese (n-30), metabolically obese (n-17) and T2D (n-13) individuals. The EPF volume was independently related to age, BMI and waist to thigh ratio (a good surrogate for intra-abdominal fat) and these accounted for 87% of the EPF variance. The IMCL was independently related to age and BMI accounting for 57% of the EPF variance. EPF (93 $\pm$ 8 ml vs. 199 $\pm$ 26ml) and IMCL (0.7 $\pm$ 0.4% vs. 1.2 $\pm$ 0.6%) was significantly higher in those with MetS. Similarly, those with obesity had higher EPF (141 $\pm$1 8 ml vs. 79 $\pm$ 7ml) and IMCL (1.0 $\pm$ 0.1% vs. 0.6 $\pm$ 0.61%) compared to the lean group. The severity of obesity < or >40 BMI units had no discernible differences on both EPF or IMCL, suggesting that these are more related to metabolic obesity. In fact, the correlation coefficient between visceral (intra-abdominal fat) and EPF was r=0.79, whereas the r value was much lower (albeit significant) for IMCL (r=0.35). When EPF and IMCL was inserted into multivariate model, only IMCL was significantly associated with EDV, ESV and SV. However, insulin resistance that predispose to dysregulated lipid metabolism and supply of FFA to the myocardium were more related to EPF than IMCL ($p<0.0001$). (Gaborit et al., 2012).

Levelt et al (2016) conducted a multi-organ, multi imaging study and compared obese (BMI kg/m2) to lean (23kg/m2) T2D patients. Despite higher glucose (9.5 $\pm$ 3.3 versus 8.1 $\pm$ 3.0 mmol/L) and systemic insulin resistance (5.45 $\pm$ 5.6 versus 1.26 $\pm$ 0.70) in the obese T2D patients, the IMCL was similar between the T2D groups (1.22 $\pm$ 0.91 % versus 1.14$\pm$ 0.66), as was the energetic profiling pCR/ATP ratio (1.64 $\pm$ 0.32 versus 1.75 $\pm$ 0.29). The LV systolic and diastolic function as measured by peak strain was higher in the lean T2D (+23% in systole and +21% in diastole) suggesting that factors beyond BMI and insulin sensitivity were partly responsible for the higher IMCL in the hearts of lean T2D (Figure 8). It also suggests a mismatch between IMCL and cardiac function, as the lean T2D had higher IMCL 1.14 $\pm$ 0.66 versus 0.48 $\pm$0.28% in the controls but had cardiac function that was not statistically different to the lean non-T2D controls (Levelt et al., 2016). It may be speculated that genetic variations in FFA uptake and utilization mechanistic pathways (i.e. mitochondrial function) may be an important consideration when investigating the IMCL/cardiac function relationship.

Figure 8: Relationship between IMCL and insulin sensitivity (measured by the frequently sampled IV glucose tolerance test (Muniyappa et al., 2015).



Epicardial adipose tissue:

IMCL is relatively low in healthy and diseased individuals, typically less than 2%. In contrast, the Epicardial fat (EPF) can store considerable FFA. EPF is the myocardial visceral fat deposit which accumulates between the pericardium and the myocardium. EPF is predominantly located in the atrioventricular and interventricular grooves and right ventricular lateral wall (Iacobellis and Willens, 2009). The EPF is in direct contact with the myocardium and can affect the function of the underlying myocardium. EPF shares many of the pathophysiological properties of other visceral fat deposits such as intra-abdominal fat. Adipocytokines secreted by EPF are also secreted by intra-abdominal adipose tissue including anti-inflammatory and pro-inflammatory markers (Baker et al., 2006). EPF increases in metabolically obese states, and the excessive FFA mobilization from dysfunctional insulin resistant adipocytes are converted into triglycerides and accumulate in EPF.

EPF has various physiological roles including local distribution and regulation of vascular flow by vasocrine mechanisms(Yudkin et al., 2005); immune barrier, thereby protecting the myocardium and coronary arteries from inflammatory and pathogenic substances (Schaffler and Scholmerich, 2010); mechanical protection of the coronary arteries and local source of FFA for the myocardium during of periods of high-demand (Sacks et al., 2011) and as a repository for excessive FFA. Therefore, the EPF may have a more significant physiologically role than LD in protecting the myocardium from FFA and from lipotoxic precursors. In certain situations, such as dysfunctional, hypertrophied and inflamed EPF, triglyceride infiltration into cardiomyocytes may also occur resulting in IMCL accumulation. MRS has demonstrated the strong correlation ($r=0.79$, $p=0.01$), ($r=0.52$, $p=<0.0001$) and $r=0.27$, $p=<0.05$ between EVE thickness and triglyceride concentration in the myocardium respectively (Malavazos et al., 2010) (Gaborit et al., 2012, Nyman et al., 2013). Therefore, there has been increasing interest in EPF, especially in relation to EPF's role in energy storage and mobilization and secretion of adipocytokines that may have deleterious effects in the myocardium.

EPF measurement:

Echocardiography, computed tomography and magnetic resonance imaging have been used to evaluate EPF, but variations between methodologies limit their comparability. Anatomical landmarks should always be used for the measurements, such as the position of the interventricular septum and the aortic annulus EPF thickness should be measured on the right ventricular free wall in at least two locations, over three cardiac cycles, although at what time in the cardiac cycle is the most suitable for measuring EPF thickness is contentious. Some recommend the measurement during systole to prevent possible deformation by EPF compression during diastole and others suggest in diastole, to coincide with other imaging modalities (CT and MRI). Not with standing measurement issues, the mean values described for EPF thickness in systole were 9.5mm and 7mm in a sample of obese and overweight men and women, respectively (Iacobellis et al., 2008). When measured in diastole, Jeong et al (2007) found a mean value of 6.4 mm (1.1 to 16.6 mm) in more than 200 patients undergoing PCI (Jeong et al., 2007). Nelson et al (2011) found a mean of 4.7 ± 1.5 mm in 356 asymptomatic patients (Nelson et al., 2011). There is a suggestion that EPF thickness >5mm is a risk factor for CVD. MRI is considered the gold standard for the assessment of ventricular volumes, mass and EPF volume. The reproducibility of EPF volume measurement is superior to EPF thickness measurement (coefficient of variability of 5.9% for volume and 13.6% for thickness), however, it is technically more difficult (Fluchter et al., 2007). The mean volume of EPF found in the MESA community-based study ranges from 68 ± 34 mL in women to 124 ± 50 mL in men (Ding et al., 2008). In a study including patients from the Framingham cohort, the mean EPF volume was 110 ± 41 mL in women and 137 ± 53 mL in men (Rosito et al., 2008).

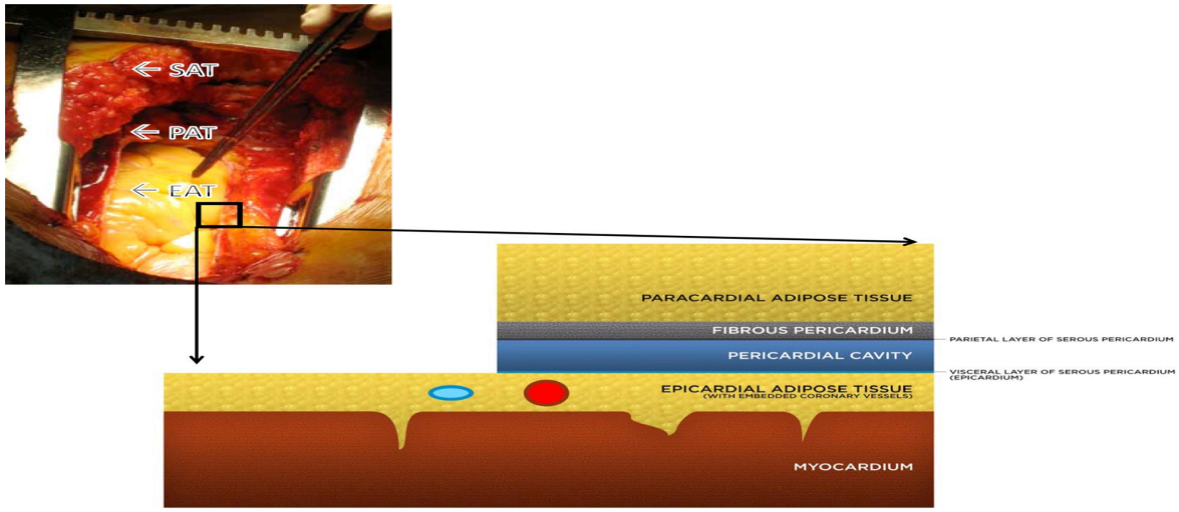


Figure 9: EPF in situ during open heart surgery:

EPF & clinical surrogates:

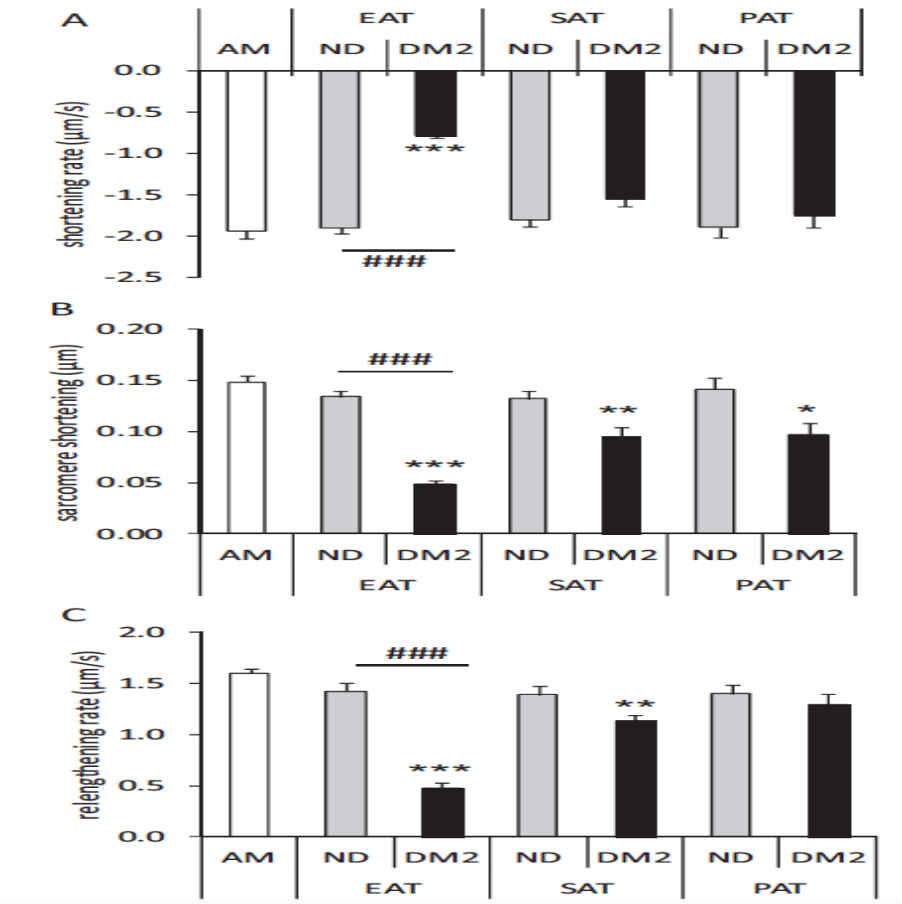
Despite the technology to assess EPF accurately, there is no guideline supported rationale for its measurement despite a strong relationship between EPF and cardiac function. There is an association between EPF and coronary artery calcium (CAC) score (Nakanishi et al., 2017) and the presence of ischaemia detected by single-photon emission computed tomography (SPECT), myocardial perfusion imaging (Possner et al., 2016). Sade et al (2009) reported that coronary microvascular dysfunction (in the absence of obstructive coronary lesions), is more closely associated with EPF than any traditional risk factor of atherosclerosis including insulin resistance, intra-abdominal obesity, and hypertension. (Sade et al., 2009).

A recent study investigating coronary arteries through optical coherence tomography found a strong association between the amount of pericoronary fat and markers of plaque instability (Ito et al., 2013). Cetin et al. (2013) suggested that increased EPF might produce a deterioration in diastolic function by decreasing coronary flow reserve even before clinically evident ischemia (Cetin et al., 2013). In the MESA (Multi-Ethnic Study of Atherosclerosis) cohort, EPF was associated with CAD (relative risk for increase of one standard deviation in EPF = 1.26, 95% CI: 1.01-1.59) even after adjustment for major cardiovascular risk factors (Ding et al., 2009).It is speculated that the increase in EPF and FFA mobilization and uptake in the

myocardium may cause other deleterious effects, such as predisposing to AF (Wong et al., 2011) and interfering with diastolic and systolic functions (EDV/ESV% and SV) even in obese and asymptomatic individuals. It was noteworthy that EPF was not a significant predictor of these cardiac functional markers when myocardial TG was inserted into the regression model alongside EPF (Gaborit et al., 2012). However, IMCL is more technically challenging and costly.

There is no agreed consensus on EPF volumes or thickness corresponding to functional (healthy) and dysfunctional (unhealthy) EPF. An excellent study by Gruelich et al. evaluated the contractile function in adult rat cardiomyocytes when incubated with EPF from human patients with and without T2D. The results illustrated the deleterious effects of EPF only (not subcutaneous or pericardial AT) from T2D patients in sarcomere shortening, cytosolic Ca+, and reduction in SERCA by 50% (regulator of myocardial Ca+ metabolism) and decreased myocardial insulin sensitivity. The EPF-T2D appears to secrete AT specific mediators that have a negative inotropic effect in cardiomyocytes, such as activin-A and angiopoietin-2(Greulich et al., 2012). Similar experiments conducted in corresponding human EPF and LV are lacking but clearly warranted to confirm these findings. Further research combining imaging and biopsy approaches should be conducted to identify factors originating from EPF that may be targeted to improve coronary blood flow in addition to current established medications.

Figure 10: Sarcomere function in rat cardiomyocytes incubated in EPF from T2D patients versus non-T2D patients.



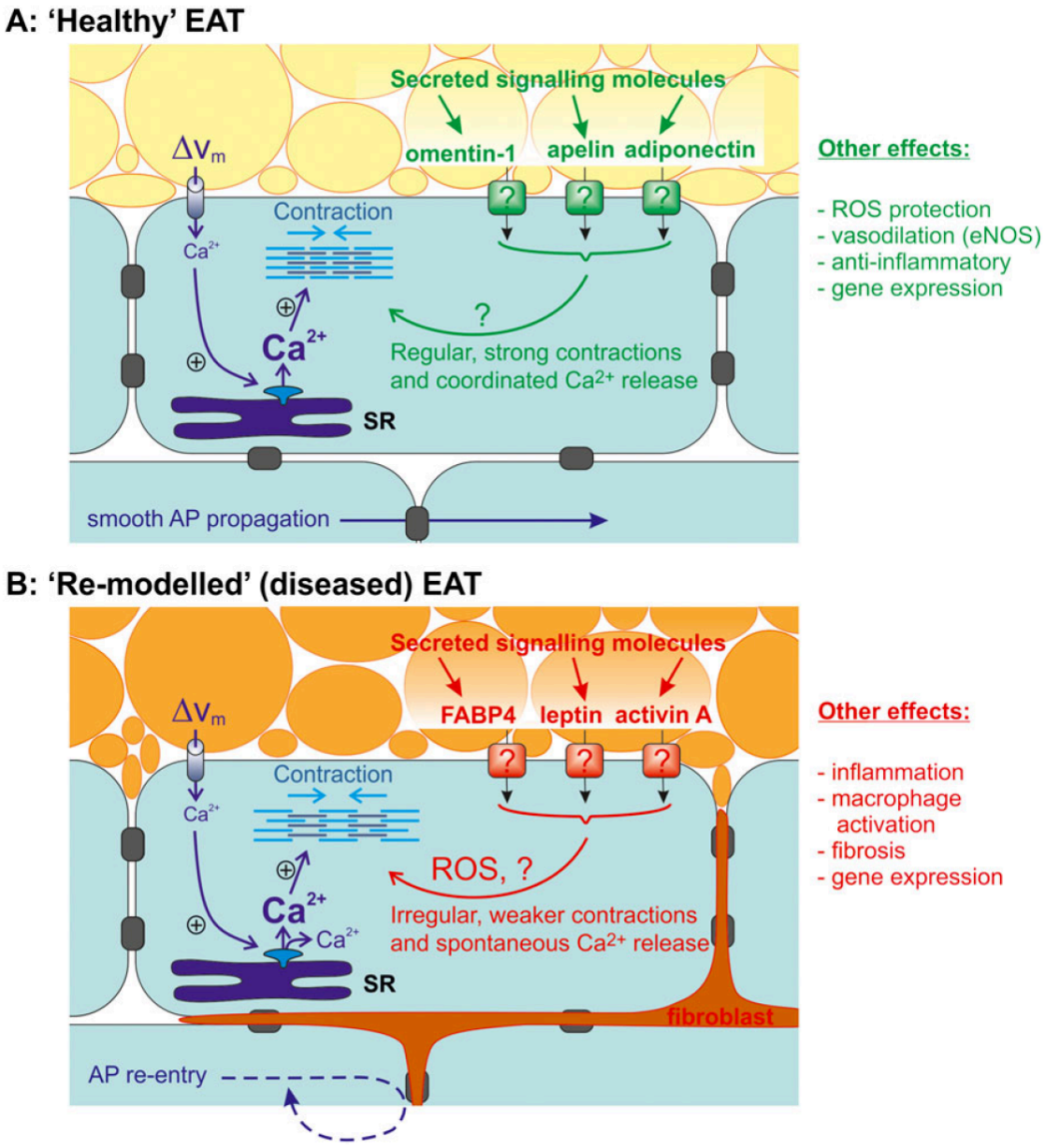


Figure 11: Model of potential interactions between cardiac excitation-contraction coupling and EPF. Adipokines related excitation-contraction coupling of cardiac myocytes. EPF can become remodeled and EPF secreted adipokines, which can activate fibrosis, hindering the smooth propagation of action potentials. EPF also increases intracellular ROS production, pro-inflammatory macrophages and a change the pattern of gene expression.

CHAPTER 2:

METHODS:

ULTIMATE 1: Patient recruitment:

As a tertiary referral centre, this cardiothoracic centre attracts patients from East Yorkshire (Hull, Scarborough, York and surrounding area) and Northern Lincolnshire (Grimsby, Scunthorpe and the surrounding area). This cohort of patients are mostly Caucasians (White = 29, Asian =1). Patients were referred by the cardiologists from the District General Hospitals in the surrounding area. All patients were assessed in the pre-assessment clinic 2-4 weeks prior to surgery. The pre-assessment clinic performs and documents full history including past medical or surgical problems, comprehensive examination and routine laboratory tests such as bloods (full blood count, biochemical profile, clotting factors and diabetic status) and ECG. Patients awaiting CABG have routine angiograms and echocardiograms within the last 6 months prior to surgery. T2D patients had to satisfy the WHO (World Health Organization 2006 recommendation) criteria for diagnosis of diabetes.

1- Diabetes symptoms (e.g. polyuria, polydipsia plus:

- a random venous plasma glucose concentration ≥ 11.1 mmol/l or
- a fasting plasma glucose concentration ≥ 7.0 mmol/l (whole blood ≥ 6.1 mmol/l) or
- two hour plasma glucose concentration ≥ 11.1 mmol/l two hours after 75g anhydrous glucose in an oral glucose tolerance test (OGTT).

The patients were initially approached at the pre assessment appointment. Patient information sheets were given to them to read prior to consenting. The risks (including risk of bleeding) and benefits of the study were clearly explained to the patients. Patients were encouraged to discuss participation with family members before making a fully informed decision. Patients were approached again the day before the operation and only consented if they agreed to be included in the study. This was documented (with date and time) in the patients' medical records for the operating surgeon and the theatre staff. Patients were given clear instructions that they could withdraw from the study at any time. A decision to participate or withdraw from the study was not going to affect their planned surgery in any way. Consent was only obtained by GCP (Good Clinical Practice) trained, cardiothoracic registrar or consultant surgeon approved by the principle investigator. Everyone involved in

the conduct of clinical research is competent to perform their task, qualified by education, training and experience. This is a requirement of the Research Governance Framework for Health and Social Care 2005, the policy covering all research in the NHS in England, and in law for those people working on clinical trials.

The inclusion and exclusion criteria is outlined below. The rationale was to be as liberal as possible therefore to obtain a representative group of patients.

Inclusion criteria

Be on stable statin therapy.

Undergoing routine/urgent CABG surgery

Type 2 diabetes mellitus (in T2D cohort)

Provide informed written consent and be willing to sign to that effect.

Aged 40-80 years

Exclusion criteria

Be on medication known to alter FFA utilisation/oxidation (insulin, steroids, perhexilene, trimetazidine and acipimox.

Safety measurements

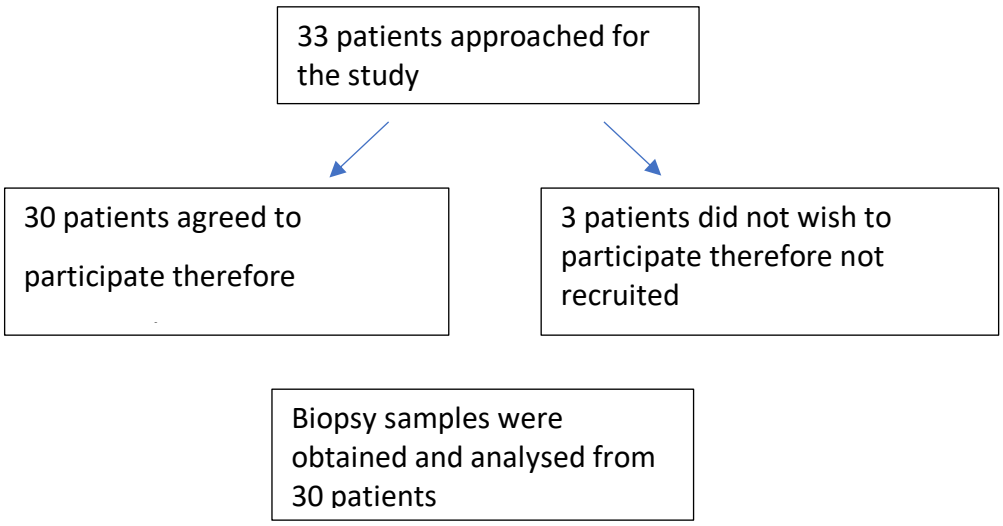
The collection and reporting of data on trial specific adverse events and trial specific serious adverse events was in accordance with International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) GCP and the Research Governance Framework 2005.

Ethical considerations:

The programme of study conformed to the requirements of the National Research Ethics Services committee of West Midlands, South Birmingham with reference number 14/WM/0109 (See appendix 1-3). The main ethical issues included informed consent, autonomy to participate in or withdraw from the study, causing no harm to the participants, complications associated with right atrial and left ventricular biopsies, anonymity and data confidentiality. All participants gave informed verbal and written consent to participation in

the study prior to taking biopsy. Participants were verbally informed of the purpose and nature of the procedures and were given an opportunity to ask questions. Participants were also informed that they did not have to participate and that they did not have to give any reason for not participating in the study. Their planned surgery was not affected by their decision either way. All data was coded anonymously with an ULTIMATE number (ULTIMATE-1-30 ULTIMATE-1 and 31-40 (ULTIMATE-2). A case reporting form (CRF) was completed for each patient with demographics, drug history, Allergy status, past medical /surgical history with the results of blood samples, echocardiography, angiograms and ECGs. 33 patients, that met the inclusion and exclusion criteria above, were approached at the pre assessment clinic during September 2014 and February 2016. EUROSCORE was not an exclusion and there was a large range of scores (0.9-20.1).

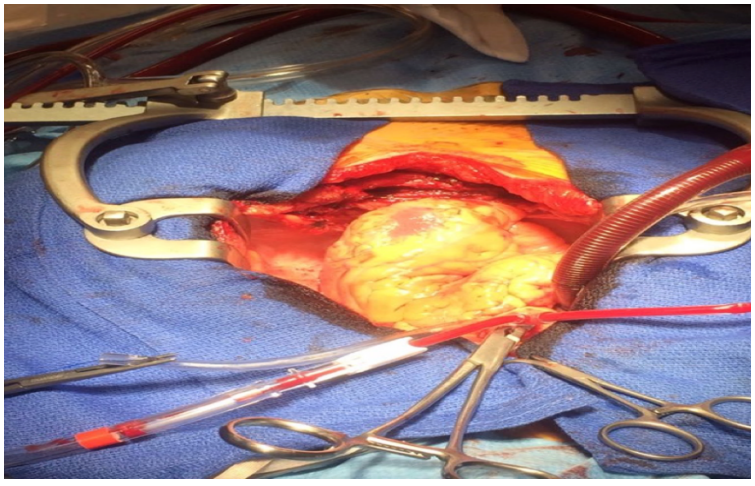
Only 30 patients agreed to be included in the study. One biopsy sample was taken from the LV lateral to the LAD whilst on cardio-pulmonary bypass circuit but prior to cardioplegia administration. A single time point was chosen as this study was cross-sectional and not assessing any form of intervention. EPF was taken on the surface of the right ventricle. This is an exploratory cross sectional and pilot study and power calculations were not undertaken.



Biopsy Samples:

The sterility of the tissues were maintained at all times. Epicardial was taken on the surface of the right ventricle. The left ventricle was biopsied following administration of cardioplegia and lateral to the left anterior descending artery using true cut biopsy needles. The biopsy sites were over sewn to ensure haemostasis. The tissue initially washed in dH2o to remove blood. The tissue was divided in 3-5 pieces and snap-frozen in OCT and 2-methyl-butane, transferred to red (epicardial) and yellow (left ventricular) capped aliquots and kept frozen in liquid nitrogen in the flask before transferring to a -80 degrees freezer. A 50ml blood sample was also obtained during the surgical procedure (prior to heparinisation) to measure a number of biomarkers of metabolic control and other cardiovascular risk factors. The bloods were left to equibrilate at room temperature then were centrifuged for 15 minutes at 3000 rpm. Plasma/serum (1ml) was harvested by pipette into individual aliquots. All aliquots were anonymized with the respective ULT number. All tissues were then transferred to an alarmed freezer at -80 degrees Celsius in the Daisy Building, Castle Hill Hospital.

Figure 12: One patient consented for photography during surgery



Blood analyses:

Serum samples were analyzed for [Total cholesterol], [HDL], and [Triglycerides]. Plasma samples from blood collection tubes containing Sodium Fluoride were analyzed for [Glucose]. Both serum and plasma samples were analyzed using a clinical chemistry analyser (Pentra 400, Horiba ABX, Northampton, United Kingdom). Quality control (QC) checks and calibration were performed before analysis. The analyser's pipetting accuracy was checked first by running 15 separate dilutions of the "qualitest" solution containing Potassium Dichromate; QC passed with a CV of ≤1.0%. Next, a calibration for each marker was performed which provided a standard curve. The calibration was then checked using two solutions containing a known normal and pathological concentration of each marker to be analyzed. Analysis of each sample was performed using the SOP recommended by the manufacturer. A total of 250µl of serum or plasma was used for the analysis of each sample. The analyser uses <20µl per test Duplicate tests for each marker were performed with the average of the two duplicates reported.

L**ipoprotein subclasses:**

Serum samples were analyzed with the *AXINON*® *lipoFIT*®-S100 test system. 630µl of serum were used and gently mixed with 70 µl of an internal standard (with reference substances, NaN3 and D2O). From this solution 600 µl were transferred into 5 mm NMR tubes with barcode labeled caps. All 1H NMR spectra were recorded at a temperature of 310K on a shielded 600 MHz Bruker Avance III HD spectrometer (Bruker) with a 5 mm triple resonance TXI probe head including deuterium lock channel and a z-gradient coil. Lipoprotein analysis was conducted via deconvolution of the broad methyl group signal at about 0.9-0.8 ppm.

NTproBNP:

The assay is a sandwich technique where sample, biotinylated polyclonal NTproBNP specific antibody and polyclonal NTproBNP antibody labelled with a ruthenium complex react to form a sandwich complex. After the addition of Streptavidin coated microparticles the complex

becomes bound to the solid phase. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell. Application of a voltage to the electrode then induces the chemiluminescence.

Insulin:

The ultrasensitive Insulin assay is a simultaneous one-step immunoenzymatic ("sandwich") assay. Plasma 320ul is added to a reaction vessel along with mouse monoclonal anti-insulin alkaline phosphatase conjugate and paramagnetic particles coated with mouse monoclonal anti-insulin antibody. The serum or plasma insulin binds to the antibody on the solid phase, while the conjugate reacts with a different antigenic site on the insulin molecule. After incubation in a reaction vessel, materials bound to the solid phase are held in a magnetic field while unbound materials are washed away. Then, the chemiluminescent substrate Lumi-Phos* 530 is added to the vessel and light generated by the reaction is measured with a luminometer. The light production is directly proportional to the concentration of insulin in the sample.

Myoglobin, h-FABP, NEFA and ß-hydroxybutyrate

Serum Myoglobin (MYO), heart-type fatty acid-binding protein (h-FABP) were measured by immunoturbidimetric assay and serum non-esterified fatty acids (NEFA) were determined by colorimetric assay using a RX daytona auto-analyzer with calibrator, control and reagents (Randox Laboratories Ltd, Crumlin, County Antrim, UK). Serum beta Hydroxybutyrate(ß-HB) level was estimated by Abcam's assay Kit (Colorimetric , ab83390) with a BioTek EL800 Microplate reader (BioTek® Instruments, Inc. Highland Park, Winooski, Vermont USA).

Epicardial adipocyte profiling:

The EPF morphological assessments were conducted as a collaborative project with Professor Tchernof, Laval University, Canada. The purpose of the project was to investigate a EPF size, number and macrophage infiltration (an acknowledged marker of adipocyte dysfunction) with EPF gene profiles in surgical patients. Paraffin embedded EPF samples were transported to Laval analyzed according to a well-established and validated laboratory technique. The simplified protocol is described below.

EPF cell size/number

To measure adipocyte diameter and macrophage infiltration, a portion of each fat sample was fixed in 5% formalin at room temperature for 48 hours and then processed for standard paraffin embedding. Five micrometer slices were mounted on slides for each patient. Slides were de-parrafined using a standard protocol and stained with hematoxylin and eosin (H&E) by the pathology service of the Quebec Heart and Lung Institute. Digital images of total sample area were obtained using Zeiss Z1 inverted microscope (Carl Zeiss, Oberkochen, Germany) at 20x magnification. From the total sample, 5 digital sections were randomly chosen and the mean adipocyte area was measured for at least 100 adipocytes. Adipocytes diameter measurements were performed using ImageJ software as described by (Laforest et al., 2017, Laforest et al., 2018).Briefly, the digital images were converted into black and grey 8-bit image. A threshold was applied to contrast the adipocyte membrane. The digital images were then transformed into binary images. The area of each complete cell (defined as the cell showing complete membrane) on the image was measured. The diameter of each cell (µm) was calculated from the area value with the following formula: D= $2\sqrt{(A/\pi)}$.

Macrophage infiltration analysis

Immunohistochemical detection of total macrophages, defined here as CD68+ cells, was performed using Mouse and Rabbit specific HRP/DAB detection kit (Abcam 64264, Cambridge, UK). Immunostaining was performed using the standard company protocol with small

modifications. First, antigen retrieval was performed with citrate buffer at 95°C prior the blocking step. The primary antibody used was an Anti-CD68 antibody (1:50, rabbit monoclonal to CD68, Abcam, Cambridge, UK). Staining revelation was performed with a 10 minutes incubation with diaminobenzidine (DAB) and slides were counterstained using Gill No.1 hematoxylin solution during 3 minutes. Digital slides of total tissue were captured at 20x magnification using zeiss Z1 inverted microscope (Carl Zeiss, Oberkochen, Germany). Adipocytes and CD68+ cells were counted in 5 randomly selected areas using ZEN-lite 2.3 digital imaging software. The number of macrophage was normalized with the number of adipocytes (expressed as percentage of macrophages) as in (Michaud et al., 2016). At least 100 adipocytes were counted for every patient.

Table 4: EPF analysis

	No. Adipocyte	#photo	Area pixel	Area µm	Diameter µm
	1	ULT-30_1920m30.tif	39002	8268	57.9
	2	ULT-30_1920m30.tif	54832	11624	68.6
	3	ULT-30_ 1920m30.tif	91342	19365	88.6
	4	ULT-30_ 1920m30.tif	37457	7941	56.7
	5	ULT-30_1920m30.tif	77361	16401	81.5
	6	ULT-30_1920m30.tif	38551	8173	57.6
	7	ULT-30_1920m30.tif	56817	12045	69.9
	8	ULT-30_1920m30.tif	82508	17492	84.2
	9	ULT-30_1920m30.tif	104080	22065	94.6
	10	ULT-30_1920m30.tif	79193	16789	82.5
	11	ULT-30_1920m30.tif	32428	6875	52.8
	12	ULT-30_1920m30.tif	72208	15308	78.8
	13	ULT-30_1920m30.tif	20482	4342	42.0
	14	ULT-30_1920m30.tif	98705	20925	92.1
	15	ULT-30_1920m30.tif	39989	8478	58.6
	16	ULT-30_1920m30.tif	56001	11872	69.4
	17	ULT-30_1920m30.tif	41820	8866	59.9
	18	ULT-30_1920m30.tif	99276	21047	92.4
	19	ULT-30_1920m30.tif	28663	6077	49.6
	20	ULT-30_1920m30.tif	71257	15106	78.2

Gene expression:

To ensure consistency and reproducibility of published data the 'Minimum Information for publication of Quantitative real time PCR Experiments' (MIQE) guidelines (Bustin et al., 2009) recommend that the justification of choice and number of reference genes should be an essential part of all RT–PCR studies, helping to guarantee the normalization of resulting data. The reference gene ('endogenous control gene') should be one whose expression is stable in all samples, regardless of tissue type, disease state and disease progression and/or treatment (Dheda et al., 2005, Bonefeld et al., 2008, Ayakannu et al., 2015). The aim of this study was to

find the most suitable reference gene for gene expression studies in human right atrium, epicardial adipose tissue and left ventricle biopsies for the ULTIMATE study.

TaqMan Low Density Endogenous Control Panel

TaqMan Low Density Endogenous Control Panel was used (ThermoFisher Scientific) to establish best endogenous control for this study. Each microfluidic card (or low density array LDA) contains 16 human TaqMan Gene Expression Assays. Each LDA has a three well replicate for each assay, and eight ports for loading of complementary DNA (cDNA) and TaqMan Universal Master Mix (ThermoFisher Scientific) in a final volume of 100ul. The 384-well micro fluid cards were run in the following PCR program: 50 °C for 2 min, 94 °C for 10 min, and 40 cycles of 97 °C for 30 s followed by 60 °C for 1 min on Applied Biosystems 7900HT Real-Time PCR System.

Table 5 . TaqMan Endogenous Control Assays

Gene	Full gene name	Assay ID
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	Hs99999905_m1
18S	Eukaryotic 18S rRNA	Hs99999901_s1
ACTB	actin, beta	Hs99999903_m1
B2M	beta-2-microglobulin	Hs99999907_m1
GUSB	glucuronidase, beta	Hs99999908_m1
HMBS	hydroxymethylbilane synthase	Hs00609297_m1
HPRT1	hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome)	Hs99999909_m1
IPO8	importin 8	Hs00183533_m1
PGK1	phosphoglycerate kinase 1	Hs99999906_m1
POLR2A	polymerase (RNA) II (DNA directed) polypeptide A, 220kDa	Hs00172187_m1
PPIA	peptidylprolyl isomerase A (cyclophilin A)	Hs99999904_m1
RPLP0	ribosomal protein, large, P0	Hs99999902_m1
TBP	TATA box binding protein	Hs99999910_m1
TFRC	transferrin receptor (p90, CD71)	Hs99999911_m1
UBC	ubiquitin C	Hs00824723_m1
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	Hs00237047_m1

Analysis

Gene expression was measured by quantitative real time PCR and expression stability was analyzed with the RefFinder (Xie et al., 2012). RefFinder is a user-friendly web-based comprehensive tool developed for evaluating and screening reference genes from extensive experimental datasets. It integrates the currently available major computational programs (geNorm (Vandesompele et al., 2002), Normfinder (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004), and the comparative delta Ct method (Silver et al., 2006)) to compare and rank the tested candidate reference genes. Based on the rankings from each program, it assigns an appropriate weight to an individual gene and calculated the geometric mean of their weights for the overall final ranking. All software's generate a stability value of which a lower value indicates increased stability in gene expression.

Results:

We conducted a thorough analysis of a panel of 16 candidate reference genes for samples tested in this study (epicardial adipose tissue, right atrium and left ventricle). Comparison between candidates led us to the identification IPO8 as the most accurate reference gene for our clinical specimens. Further analysis showed that there were no statistically significant differences in IPO8 expression between tissue types and with excellent expression stability. In addition, we demonstrate that the commonly used genes GAPDH and HPRT1 are inappropriate to normalize data derived from biopsies collected for this study.

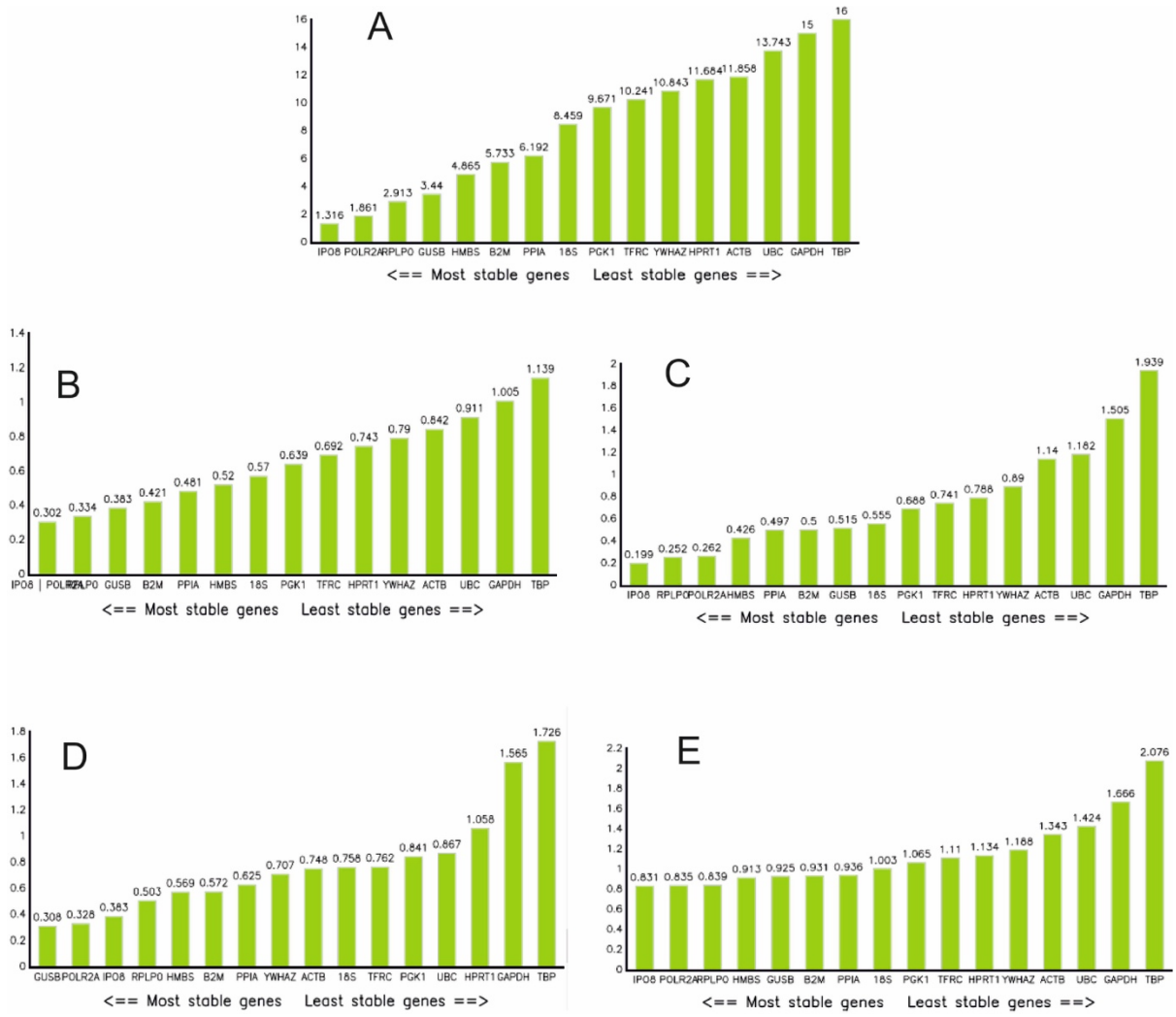


Figure 13. Gene ranking results from A) RefFinder, B) geNorm , C) Normfinder, D) BestKeeper, E) comparative delta Ct method.

RNA extraction:

Collected tissues were homogenised using Lysing Matrix D (MP Biomedicals) tubes containing beads and 700ul Qiazol lysis reagent (Qiagen) on Hybaid RiboLyser. RNA was extracted using miRNeasy Mini Kit (Qiagen). NanoDrop Spectrophotometer ND-1000 (Labtech International) and ND1000 v3 software was used to quantify the total RNA concentration, RNA was stored at -80°C until cDNA conversion.

Reverse transcription:

Reverse transcription reaction was carried out using High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) to cDNA in 20ul reaction following the manufacturer's instructions to give a 1:1 conversion ratio of input RNA to cDNA. For endogenous control panel 50ng and custom made cards 400 of RNA were reversed-transcribed.

Real-Time quantitative Polymerase Chain Reaction (RT-qPCR).

Gene expression was measured using TaqMan Low Density Arrays (TLDA) (ThermoFisher Scientific) which were custom loaded by the company with 48 pre-selected genes of interest (GOI) (Appendix 3) on the 384-well micro fluid cards. Each patients sample was mixed with TaqMan Universal PCR Master Mix (ThermoFisher Scientific) to a final volume of 100µL which was loaded on the TLDA. Best endogenous control gene was selected using TaqMan Low Density Endogenous Control Panel containing 16 human most used controls (Appendix 3) for the 3 different tissue types used in this study (epicardial adipose tissue, right atrium and left ventricle). PCR cycling program was applied to run all TLDAs: 50 °C for 2 min, 94 °C for 10 min, and 40 cycles of 97 °C for 30 s followed by 60 °C for 1 min on Applied Biosystems 7900HT Real-Time PCR System. The expression of each gene was analyzed using RQ Manager v1.1.2. Importin 8 gene (IPO8) was selected as the most stable (as described) and accurate reference gene for this study and was used for normalization. Cycle threshold values (Ct) were used to calculate relative expression of each GOI to IPO8(endogenous control)according to the formula: ΔCt = Ct (GOI) - Ct (IPO8). Next fold changes from ΔCt values were calculated following formula: Fold change (FC) = $2^{-\Delta Ct}$

Measurement of Intramyocardial lipid content (IMCL):

The measurement of IMCL forms part of an important collaborative effort with Professor Hesselink from the University of Maastricht. The research group focuses predominantly on skeletal muscle IMCL and lipid droplet morphology (number and size) and structure (lipid droplet surface proteins (perilipins) that act as gatekeepers for lipid droplets. The purpose of the project was to investigate an objective marker of IMCL with cardiac energy metabolism gene profiles in surgical patients. Samples were transported to Maastricht on 25kg of dry ice and analyzed according to a well-established and validated laboratory technique. The simplified protocol is described below.

Five µm thick sections were cut and mounted on glass slides. Each glass slide contained a subject from the three different groups to minimize bias originating from putative variation in staining intensity. The whole procedure (staining, imaging and quantification) was performed blinded with regard disease state. In addition, as an internal control for staining quality a skeletal muscle sample from an endurance trained athlete (always the same biopsy) was mounted as well on every single glass slide. Sections were fixated for 30 minutes with 3.7% formaldehyde/PBS. After rinsing, sections were blocked with blocking buffer (150 mM NaCl, 20 mM Tris pH 6.8 and 2% BSA) for 45 minutes. Subsequently, sections were permeabilized with 0.25% Triton TX-100/PBS. Sections were rinsed for 5 minutes with PBS and incubated for 1 hour with primary antibodies against laminin (L9393, Sigma,

MO, USA) and desmin (sc-58745, Santa Cruz, CA, USA) both diluted 1:25 in 0.05% Tween/PBS. Subsequently, Bodipy 493/503 (D3922, Molecular Probes, Leiden, The Netherlands) and appropriate secondary antibodies conjugated with AlexaFluor-405 (AF405) and AlexaFluor-647 (AF647) were incubated for 90 minutes at 37ÅãC. Sections were mounted in Mowiol and covered with coverslips #1. Until imaging, sections were stored in the dark at room temperature. Sections were imaged on a Leica TCS SP8 confocal microscope with a 100x 1.4 N.A. oil immersion objective. Images contained 2048 x 2048 pixels and 4 slices with 0.17 µm in between for deconvolution purposes. For measuring lipid area fraction, LD size and number a 1.6x optical zoom was used resulting in a pixel size of 35.5 nm x 35.5 nm. For imaging subcellular distribution of LDs (in direct vicinity of the t-tubular system or in in contact with

contractile elements) a 5x optical zoom was used. This resulted in a pixel size of 11.4 nm x 11.4 nm. For imaging laminin-AF405 the 405 laser line was used. For imaging Bodipy 493/503 and desmin-AF647 the white laser line was used. For imaging Bodipy 493/503 a wavelength of 488 nm and for desmin-AF647 a wavelength of 647 nm were used for excitation. All fluorophores were detected with a photomultiplier tube. Emission was detected between 415 nm –460 nm, 500 nm – 530 nm and 657 nm – 750 nm for respectively laminin-AF405, Bodipy 493/503 and desmin-AF647. Acquired images were deconvoluted with Huygens Professional software (Scientific Volume Imaging B.V., Hilversum, The Netherlands) to improve signal-to-noise ratio. Total fat content measured as lipid area fraction, LD size and number were measured with a custom written macro in ImageJ (Schneider et al., 2012)

CHAPTER 2:

RESULTS

Table 6: Basic demographics and peri-operative variables for patients recruited into ULTIMATE 1:

Variables		**N**	**%**
Sex	Male	23	77%
	Female	7	23%
Grafts	0	0	0%
	1	0	0%
	2	10	33%
	3	17	57%
	4	3	10%
LV_Hypertrophy	No	28	93%
	Yes	2	7%
Dyspnoea status (NYHA)	None	2	7%
	Mild	3	10%
	Moderate	22	73%
	Severe	3	10%
Angina status (CCS)	None	1	3%
	Mild	2	7%
	Moderate	18	60%
	Severe	9	30%
Hypertension	No	3	10%
	Yes	27	90%
Smoker	Never	8	28%
	Ex-Smoker	18	60%
	Current-Smoker	4	12%
Statin	Yes	30	100%
Ezetimibe	No	26	87%
	Yes	4	13%
Beta_blocker	No	4	13%
	Yes	26	87%
ACE_i	No	16	53.5%
	Yes	14	46.5%
ARB	No	25	83%
	Yes	5	17%
Diuretic	No	24	80%
	Yes	6	20%
Calcium_CB	No	23	77%
	Yes	7	23%
Antiarrhythmic	No	29	97%
	Yes	1	3%
Anti_angina	No	17	57%
	Yes	13	43%
Antiplatelet	No	0	0%
	Yes	30	100%
Metformin	No	20	67%
	Yes	10	33%

Table 7: Patient characteristics in ULTIMATE-1

	CABG n-20)	**CABG-T2D (n-10)**	**Sig.**
Age (years)	66.1 ± 7.7	62.3 ±10.7	0.28
EuroScore	3.7 ± 2.4	4.5 ± 3.0	0.43
Logistic EuroScore	3.6 ± 3	6.6 ± 7.5	0.13
BMI Kg/m^2	30.5 ± 5.86	34.3 ± 5.2	0.11
NTproBNP (ng/L)	432 ± 593	743 ± 1330	0.38
Creatinine μM/L	89.2 ± 20.3	89.3 ± 21.6	0.99
eGFR (ml/min/1.73^2)	72.1 ± 20.3	81.2 ± 20.8	0.35
Urea mmol/L)	6.2 ± 2.1	6.6 ± 3.3	0.72
Potassium (mmol/L)	4.47 ± 0.27	4.40 ± 0.34	0.55
Sodium(mmol/L)	138 ± 3	137 ± 1	0.15
Haemoglobin (g/L)	136 ± 16	138 ± 12	0.76
Platelets 10*9/L	238 ± 52	273 ± 74	0.14
Glucose (mmol/L)	5.82 ± 0.61	8.47 ± 2.13	<0.001
Insulin (IU/ml)	7.1 ± 6.7	8.4 ± 6.0	0.62
HOMA-IR (arbitary units)	1.94 ± 2.03	3.13 ± 2.02	0.14
HOMA-B (arbitary units)	19.88 ± 19.97	17.06 ± 16.16	0.70
Total cholesterol (mmol/L)	3.31 ±0.68	3.33 ± 0.79	0.94
LDL-cholesterol (mmol/L)	2.07 ±0.49	2.07 ±0.60	0.99
SD-LDL particles (n)	460 ±136	506 ± 104	0.39
HDL-cholesterol (mmol/L)	0.92 ± 0.23	0.78 ± 0.13	0.08
Large-HDL particles	3528 ± 1438	3083 ±594	0.41
Triglycerides (mmol/L)	1.56 ± 1.05	2.39 ± 1.11	0.057
Large VLDL (n)	5.96 ± 6.12	13.04 ± 10.89	<0.05
NEFA (mmol/L)	0.99 ± 1.00	0.85 ± 0.19	0.66
hFABP (ng/ml)	7.0 ± 3.3	7.1 ± 4.1	0.95
β-hydroxybutyrate (mmol/L)	0.17 ± 0.15	0.21 ± 0.15	0.49
Vitamin D (nmol/L)	43.1 ± 16.1	43.1 ± 26.9	0.99

Patient recruitment:

There have been numerous myocardial biopsy studies conducted in the RA and EPF whereas LV biopsies are much more scarce due to the inherent risk of the procedure, the ethical considerations and recruitment. In this study, the risks of the LV biopsy were mitigated by carefully excluding those at risk of bleeding. The patients who had a LV biopsy were all discharged with no complications due to the procedure. The ethical approvals were granted in a timely fashion after via NHS REC proportionate review. Lastly, of all patients approached for an LV biopsy only one patient refused due to severe generalized procedural anxiety.

Our recruited cohort comprised of men (77%). The grafts were mainly 2 or 3 vessel (90%). The T2D patients had a higher EUROSCORE and NTproBNP but they were statistically non-significant compared to non T2D. Renal function were comparable as were serum electrolytes. The only significant differences between T2D and non-T2D was glucose ($P<0.001$) and TG contained within large VLDL particles ($P<0.05$). NEFA, β-hydroxybutyrate and hFABP and vitamin D were comparable between T2D and non-T2D patients.

Table 8: Genes in **EPF** in patients with and without T2D.

Gene	T2D (n-10)	Non-T2D (n-20)	% difference (T2D vs. Non-T2D	Sig.
VDR	5.75 + 1.91	5.62 + 1.16	+2%	0.95
HIF-1	1.64 + 0.38	2.02 + 0.24	-19%	0.39
AMPK	0.05 + 0.01	0.35 + 0.22	-86%	0.38
GLUT-1	0.11 + 0.01	0.09 + 0.01	+22%	0.52
GLUT-4	0.84 +0.23	1.83 +0.46	-54%	0.16
PI3-Kinase	0.22 + 0.05	0.36 + 0.05	-39%	0.08
IRS-1	0.21 + 0.07	0.15 + 0.03	+40%	0.44
PDK	5.13 + 1.39	4.85 + 0.65	+6%	0.84
PDH	6.47 + 1.46	5.20 + 0.53	+24%	0.33
CPT-1	1.08 + 0.10	0.75 +0.06	+44%	<0.01
PPAR-alpha	0.93 +0.19	1.13 +0.14	-18%	0.40
ACC	2.39 + 0.55	2.60 + 0.34	-8%	0.74
ACS	7.84 + 2.28	14. 00 +2.24	-44%	0.10
PGC-1	0.06 + 0.01	0.19 + 0.01	-68%	0.41
Cardiolipin	0.96 + 0.36	1.88 + 0.31	-49%	0.08
FABP-3	0.07 + 0.01	0.05 + 0.01	+40%	0.17
CD-36	11.97 + 1.94	18.18 + 2.55	-34%	0.13
LPL	23.56 +6.79	55.71 + 10.36	-58%	<0.05
VLDL-r	2.67 + 0.71	4.8 + 0.71	-44%	0.07
MAG-1	22.57 + 6.17	12.22 + 1.75	+85%	<0.05
ATGL	10.18 + 2.52	11.33 + 2.02	-10%	0.73
FAS	10.9 + 1.90	10.03 + 1.84	+9%	0.77
SREBP-1c	1.76 + 0.66	2.47 + 0.49	-29%	0.41

Table 9: EPF profiling:

EPF characteristic	T2D (n-7)	Non-T2D (n-8)	Relative % difference	Sig
EPF size	73.8 ± 7.9	66.1 ± 13.2	+11.6	0.22
EPF number	108.8 ±8.9	106.7 ± 9.3	+2%	0.66
EPF-CD68 (%)	36.6 ± 7.2	29..2 ± 15.3	+25.3%	0.26

There was strong correlation between insulin resistance (HOMA-IR derived) and EPF dysfunction (r=0.466, p=0.08, n-15). Age and NT proBNP were negatively correlated with EPF CD68 (r=-0.340, p=0.21 and r=-0.536, p=0.039, respectively.

AMPK mRNA (cell energy sensor) was suppressed in T2D which indicates that the EPF cells were not in an energy compromised state. Furthermore, other gene profiles in T2D indicated a suppression of lipogenesis as observed by the downregulation of the following gene (SREBP-1c/-29%, acetylCoA carboxylase/-8%, and acetyl CoA synthase/-44%). The genes involved in FFA transport/uptake were also significantly downregulated (CD36/-34%, LpL/-58%, and VLDL-r/-44%). In contrast, there was upregulation of the lipolytic gene MAG-1/+85%. CPT-1 was upregulated which suggests that FFA can pass into the mitochondrial matrix. EPF PGC-1, PPAR-a and cardiolipin mRNA expression were all lower in T2D which indicates a lower degree of FFA oxidation, via the electron chain transport.

Table 10: EPF genes in T2D patients with decreased expression compared to non-T2D.

Gene	T2D (n-10)	Non-T2D (n-20)	% difference (T2D vs. Non-T2D	Sig.
AMPK	0.05 + 0.01	0.35 + 0.22	-86%	0.38
ACC	2.39 + 0.55	2.60 + 0.34	-8%	0.74
ACS	7.84 + 2.28	14. 00 +2.24	-44%	0.10
CD-36	11.97 + 1.94	18.18 + 2.55	-34%	0.13
LPL	23.56 +6.79	55.71 + 10.36	-58%	<0.05
VLDL-r	2.67 + 0.71	4.8 + 0.71	-44%	0.07
SREBP-1c	1.76 + 0.66	2.47 + 0.49	-29%	0.41
PGC-1	0.06 + 0.01	0.19 + 0.01	-68%	0.41
Cardiolipin	0.96 + 0.36	1.88 + 0.31	-49%	0.08
PPAR-alpha	0.93 +0.19	1.13 +0.14	-18%	0.40

Table 11: EPF genes in T2D patients with increased expression compared to non-T2D

Gene	T2D (n-10)	Non-T2D (n-20)	% difference (T2D vs. Non-T2D	Sig.
VDR	5.75 + 1.91	5.62 + 1.16	+2%	0.95
GLUT-1	0.11 + 0.01	0.09 + 0.01	+22%	0.52
IRS-1	0.21 + 0.07	0.15 + 0.03	+40%	0.44
PDK	5.13 + 1.39	4.85 + 0.65	+6%	0.84
PDH	6.47 + 1.46	5.20 + 0.53	+24%	0.33
CPT-1	1.08 + 0.10	0.75 +0.06	+44%	<0.01
FABP-3	0.07 + 0.01	0.05 + 0.01	+40%	0.17
MAG-1	22.57 + 6.17	12.22 + 1.75	+85%	<0.05

The EPF characteristics were statistically non-significant despite relatively large relative differences, notably for EPF CD68 (25.3%). When all subjects were combined both T2D and non-T2D, only MAG-1 was highly correlated with EPF CD68 ($r=0.517$, $p=0.029$) and contributed to 20.6% of the variance in EPF-CD68 macrophage infiltration ($p<0.05$).

Figure 14: Scatterplot of MAG-1 and EPF CD68:

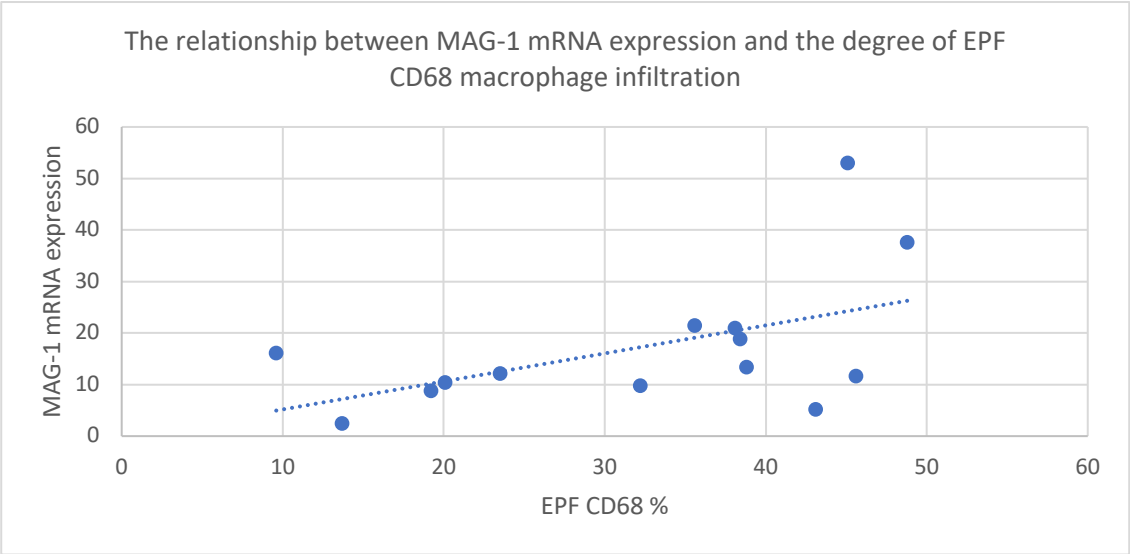


Figure 15: Microscopic illustration of EPF adipocytes including macrophage infiltration

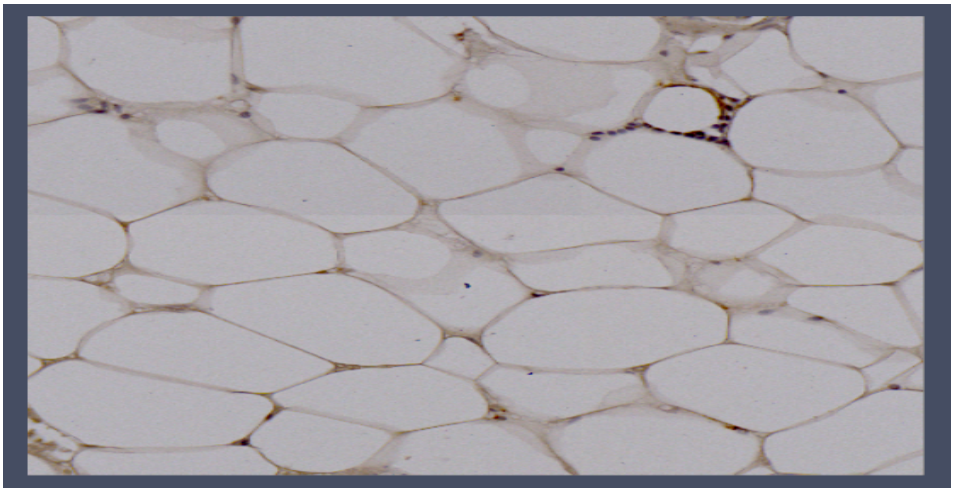


Table 12: Genes expression in **LV** tissue between patients with and without T2D.

Gene	T2D	Non-T2D	% difference	Sig.
VDR	0.85 +0.35	0.30 + 0.06	+183%	<0.05
HIF-1	3.84 + 0.72	2.99 +0.25	+28%	0.18
AMPK	5.38 + 0.99	4.61 + 0.42	+17%	0.41
GLUT-1	0.38 +0.15	0.49 +0.05	-22%	0.20
GLUT-4	4.44 +0.88	5.53 +0.76	-20%	0.39
PI3-Kinase	1.10 + 0.22	1.13 + 0.11	-3%	0.90
IRS-1	0.32 +0.03	0.25 +0.02	+28%	<0.05
PDK	13.80 + 2.33	5.23 + 1.00	+164%	<0.001
PDH	23.47 +2.12	20.26 +1.67	+16%	0.26
CPT-1	1.10 + 0.19	0.79 + 0.08	+39%	0.09*
PPAR-alpha	2.15 +0.15	2.28 + 0.16	-6%	0.61
PPAR-delta	0.79 + 0.14	0.67 + 0.07	+18%	0.43
ACC	0.56 + 0.08	0.48 +0.04	+17%	0.35
MCD	7.61 +1.04	5.75 +0.51	+32%	0.08*
ACS	5.05 +0.72	4.12 +0.55	+23%	0.26
PGC-1	3.05 +0.50	2.61 + 0.20	+17%	0.61
Cardiolipin	2.91 + 0.42	3.51 +0.33	-17%	0.29
FABP-3	194 + 24	205 + 20	-5%	0.76
CD-36	34.66 + 5.19	28.04 +2.73	+24%	0.22
LPL	33.1 + 5.47	38.4 +3.37	-14%	0.39
VLDL-r	7.81 +2.62	4.04 +0.45	+93%	0.07*
MAG-1	5.6 + 0..55	5.5 +0.41	+2%	0.89
ATGL	3. 53 + 1.25	3.55 +0.75	-0.5%	0.99
FAS	0.16 + 0.03	0.16 + 0.02	0%	0.99
SREBP-1c	0.13 +0.03	0.15 + 0.03	-13%	0.79
SPT-1	0.29 + 0.04	0.29 +0.02	0%	0.90
Cer-synthase	0.08 +0.02	0.04 + 0.01	+100	0.07*
Caspase-9	0.26 + 0.03	0.29 +0.02	-10%	0.42
Beclin-1	2.61 + 0.31	3.13 + 0.29	-17%	0.27
UCP-3	0.13 + 0.04	0.06 +0.04	+117%	<0.05

Results

In table 10 the mRNA expression of genes of T2D patients were compared with non-T2D. Despite the high relative differences, only a few genes were statistically significant between groups (although four genes were approaching statistical significance). The genes with the highest expression were FABP-3, CD36, LPL, PDH,PDK, MCD, VLDL-r, MAG-1 and AMPK. Other

genes were not as highly expressed (e.g. VDR, GLUT's, CPR-1 and UCP-3) but this does not mitigate their importance.

Table 13: LV genes in T2D patients with increased expression compared to non-T2D

Gene	T2D	Non-T2D	% difference	Sig.
VDR	0.85 +0.35	0.30 + 0.06	+183%	<0.05
HIF-1	3.84 + 0.72	2.99 +0.25	+28%	0.18
AMPK	5.38 + 0.99	4.61 + 0.42	+17%	0.41
IRS-1	0.32 +0.03	0.25 +0.02	+28%	<0.05
PDK	13.80 + 2.33	5.23 + 1.00	+164%	<0.001
PDH	23.47 +2.12	20.26 +1.67	+16%	0.26
CPT-1	1.10 + 0.19	0.79 + 0.08	+39%	0.09*
PPAR-delta	0.79 + 0.14	0.67 + 0.07	+18%	0.43
ACC	0.56 + 0.08	0.48 +0.04	+17%	0.35
MCD	7.61 +1.04	5.75 +0.51	+32%	0.08*
ACS	5.05 +0.72	4.12 +0.55	+23%	0.26
PGC-1	3.05 +0.50	2.61 + 0.20	+17%	0.61
CD-36	34.66 + 5.19	28.04 +2.73	+24%	0.22
VLDL-r	7.81 +2.62	4.04 +0.45	+93%	0.07*
MAG-1	5.6 + 0..55	5.5 +0.41	+2%	0.89
Cer-synthase	0.08 +0.02	0.04 + 0.01	+100	0.07*
UCP-3	0.13 + 0.04	0.06 +0.04	+117%	<0.05

Table 14: LV genes in T2D patients with decreased expression compared to non-T2D

Gene	T2D	Non-T2D	% difference	Sig.
GLUT-1	0.38 +0.15	0.49 +0.05	-22%	0.20
GLUT-4	4.44 +0.88	5.53 +0.76	-20%	0.39
PI3-Kinase	1.10 + 0.22	1.13 + 0.11	-3%	0.90
PPAR-alpha	2.15 +0.15	2.28 + 0.16	-6%	0.61
Cardiolipin	2.91 + 0.42	3.51 +0.33	-17%	0.29
FABP-3	194 + 24	205 + 20	-5%	0.76
LPL	33.1 + 5.47	38.4 +3.37	-14%	0.39
ATGL	3. 53 + 1.25	3.55 +0.75	-0.5%	0.99
FAS	0.16 + 0.03	0.16 + 0.02	0%	0.99
SREBP-1c	0.13 +0.03	0.15 + 0.03	-13%	0.79
SPT-1	0.29 + 0.04	0.29 +0.02	0%	0.90
Caspase-9	0.26 + 0.03	0.29 +0.02	-10%	0.42
Beclin-1	2.61 + 0.31	3.13 + 0.29	-17%	0.27

IMCL:

Total IMCL was the highest in the CAD-T2D group (Figure-16) but the difference was not significant (0.45±0.38% and 0.73±0.44% for CAD and CAD-T2D; p=0.152). The stepwise increase in total IMCL from non-CAD to CAD-T2D was accounted for by a stepwise increase in the number of LDs (Figure 16) 0.04 ± 0.03 #/µm2, 0.07 ± 0.03 #/µm2 for CAD and CAD-T2D; p=0.212) rather than in size 0.102 ± 0.01µm2, 0.103 ± 0.01 µm2 for CAD and CAD-T2D; p=0.877. Total IMCL was highly variable across both groups.

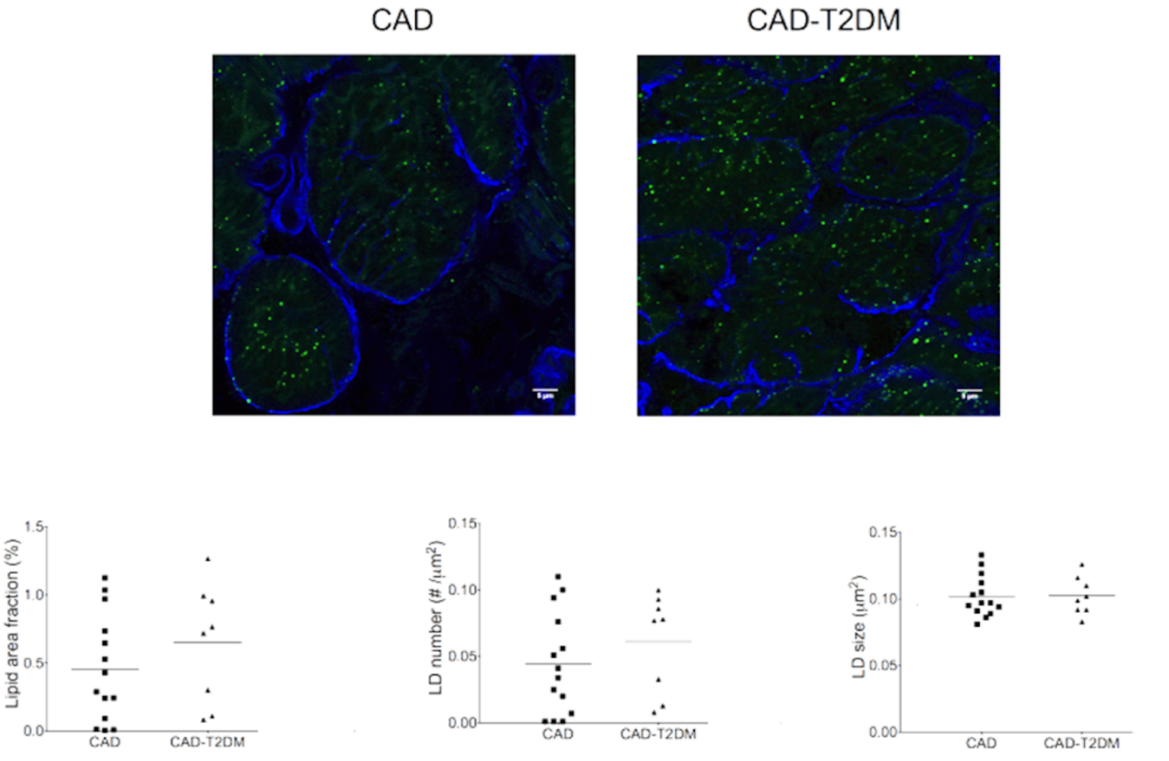


Figure 16): Morphology of intracellular cardiac lipid storage. Representative confocal immunofluorescent images of cardiac lipid storage of CAD and CAD-T2D.
Quantification of lipid area fraction (left), lipid droplet number (middle) and lipid droplet size (right)

IMCL location within cardiomyocytes:

LD location was examined in cardiomyocytes sectioned cross-sectionally as well as and longitudinally. In cardiomyocytes cut cross sectionally, LD location was studied in relation to the t-tubular system whereas in cardiomyocytes cut longitudinally, LD location was studied relative to the Z-lines (as indicated by staining of the Z-line interconnecting intermediate filament desmin). In the cross-sectionally cut cardiomyocytes LDs in the CAD groups were more prominent in near vicinity of the t-tubules, whereas in the non-CAD group, LDs were dispersed throughout the cardiomyocytes (data not shown). In longitudinally cut cardiomyocytes LDs were located between the contractile elements (Figure 17).

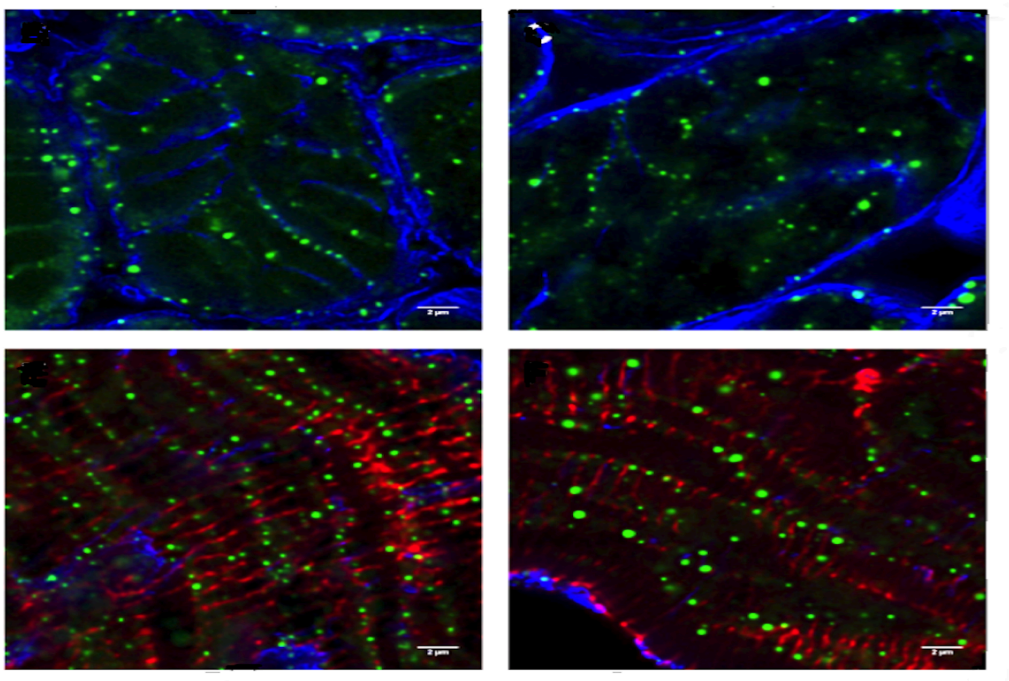


Figure 17: Confocal immunofluorescence images of LD location in reference to T-tubuli and Z-lines (as indicated by staining of the Z-line interconnecting intermediate filament desmin). LDs (green) and T-tubuli (blue) of CAD (top left panel) and CAD-T2D (top right panel).
Both CAD groups store LDs in the vicinity of the T-tubuli. LDs (green), T-tubuli (blue) and desmin (red) of CAD (bottom left panel) and CAD-T2D (bottom right panels).

Predictors of IMCL:

1: Gene mRNA profiles IMCL:

The aims of this study were to investigate the associations between LV IMCL accumulation and gene profiles (mRNA expression). Genes significantly correlated to the IMCL were included in the regression model. These data suggests that only VLDLR-r (r=0.460, p=0.036), VDR (r =0.487, p=0.030) are strongly correlated to IMCL. Upon regression analysis only VDR expression was an associated with IMCL (r=0.478) and accounted for 19.5% (r^2 0.195) of the variance in IMCL (p=0.030).

2: Blood biomarker and IMCL:

In addition to the mRNA gene expression, it is important to establish a simple, accessible clinical blood-based surrogate for IMCL when specialized imaging is neither practical or feasible. This may provide a good indication of abnormal cardiac metabolism (susceptibility to increased lipid storage) or in which patient may require metabolic optimization prior, during or after surgery. Therefore, regression analyses were conducted with IMCL as the dependent variable. Correlation between potential predictors and IMCL was undertaken. Only β-hydroxybutyrate approached statistical significance r—0.378, p=0.08 (n-22). The following variables TG (r=0.17, p=0.46), NEFA (r=-025, p=0.29), hFABP (r=0.18, p=0.45) were not strongly correlated. Regression analyses showed that β-hydroxybutyrate was associated with IMCL (r=-0.378) and accounted for 10.0% (r^2 0.102) of the variance in IMCL p=0.08).

CHAPTER 2:

DISCUSSION

EPF mRNA profiles

EPF is associated IMCL, abnormal coronary blood flow, microvascular dysfunction and LV contractile impairments. In addition, EPF can pose problems with viewing and accessing coronary arteries during CABG surgery. EPF volume via imaging is typically higher in T2D compared to non-T2D and in lean T2D versus obese T2D (Levelt et al., 2016, Gaborit et al., 2012).

It is important to understand the pathophysiology of EPF accumulation as this provides a clear picture of appropriate treatment strategies. Under normal physiological conditions, subcutaneous adipose tissue and skeletal muscle can control the flux of FFA/glucose during the post-prandial period. In insulin resistant and hypertrophied adipocytes, the buffering capacity or 'FFA trapping' becomes impaired and VLDL/chylomicrons return to the liver highly lapidated due to insufficient LPL action. Simultaneously, in insulin resistant skeletal muscle glucose cannot be stored efficiently to glycogen (mediated via impairments in GLUT-4 and glycogen synthase) and glucose is shuttled to the liver. The liver converts this glucose to triglyceride in a process called de novo lipogenesis. The liver then secretes large VLDL-1 particles into the circulation to undergo delipidation via LPL, an enzyme that cleaves FFA off the triglyceride molecule. When there is insulin resistance at subcutaneous adipocytes this increases the storage at ectopic sites (liver, pancreas and EPF). The underlying pathophysiological basis is insulin resistance at both the skeletal muscle and subcutaneous adipose tissue. Our data shows a strong correlation between insulin resistance (HOMA-IR derived) and EPF dysfunction ($r=0.466$, $p=0.08$, n-15).

These tissues are the preferred storage sites for triglycerides and glucose. EPF is the myocardial manifestation of impaired triglyceride storage in subcutaneous adipocytes and skeletal muscle. Approaches to improve the functionality of these tissue will have a beneficial effects on whole body ectopic lipid accumulation. Thus, factors that impact on the storage and release of FFA is clearly important. For example, in a recent multi-organ, MRI, MRS imaging study, the EPF volume (they did not assess EPF dysfunction) was assessed in a lean T2D group and an obese T2D group. EPF was significantly higher in the more metabolically obese T2D

cohort when compared to the normal weight T2D group (96 ± 40 cm^3 vs. 71± 21 cm^3). The lower EPF in the lean T2D was related to obesity (10 BMI units lower in lean T2D), glycaemic control (insulin 50% lower in lean T2D) and hepatic fat (50% lower in lean T2D). The data highlight the benefit of optimizing metabolic function in T2D as cardiac LV diastolic and systolic function was decreased by 25% despite comparable IMCL. Interestingly, in the lean non-T2D patient had a BMI comparable to the lean T2D, but glucose was normal 5.0 ± 0.5 versus 8.1 ± 3.0 in the lean T2D. When IMCL between the two lean groups was assessed (EPF not measured in the lean non-T2D group) the lean non-T2D had low IMCL (0.48±0.28 versus 1.14 ±0.66). This suggests that insulin resistance is the key metabolic driver predisposing to accumulation of EPF and IMCL.

In the current study, EPF biopsy was taken and gene profiles were measured in combination with a marker of EPF dysfunction. In hindsight, measuring EPF volume would have provided useful information to combine EPF volume with EPF dysfunction and gene profiles. This is acknowledged as a major limitation. The purpose of investigating gene profiles between T2D and non T2D is to establish the balance between lipogenesis and lipolysis and the genes controlling these processes. As discussed previously, EPF has a dual role, 1) being a repository for surplus FFA acids as a partial protector of myocardial lipid accumulation 2) as a supplier of FFA during periods of high cardiac workload. Therefore, the balance between lipogenesis and lipolysis is very important, notably in metabolic disease. The T2D heart resembles an 'engine oversupplied with fuel' (FFA delivery from EPF). Despite an oversupply of FFA and substrates the heart in both T2D and in heart failure is energetically compromised (as observed by decreases in Pi/ATP ratio and CK concentrations). However, it is conceivable that excess EPF lipolysis, i.e. an oversupply of FFA via lipolysis in the short term may exacerbate mitochondrial capacity to oxidise the liberated FFA, thus causing cellular stress and further worsening of mitochondrial dysfunction and ATP production. It is also conceivable that EPF adapts by inhibiting lipogenesis to prevent further lipid accumulation. It is unclear at what stage or severity of accumulation this may occurs or if a feedback or cross talk mechanism exists. The tissue cross talk hypothesis has received increasing attention, especially adipose tissue and skeletal muscle (Samdani et al., 2015).

In our study gene profiles in non-T2D versus T2D patients were assessed and indicated a suppression of lipogenesis as observed by the downregulation of the following gene (SREBP-1c/-29%, acetylCoA carboxylase/-8%, and acetyl CoA synthase/-44%). The genes involved in FFA transport/uptake were also significantly downregulated (CD36/-34%, LpL/-58%, and VLDL-r/-44%). In contrast, the monoacylglycerol lipase (MAG-1) gene involved in the last step of triglyceride lipolysis was upregulated by 85% compared to non-T2D. These gene profiles suggest either a protective mechanism/adaptive process to prevent further EPF accumulation or may be reflective of FFA oversupply. There is very limited data but in one study the genes controlling glucose and lipid metabolism have been shown to be impaired in EPF. In particular, glucose transport, as well as FFA uptake and storage are decreased, whereas lipolysis is augmented in CHF patients (Burgeiro et al., 2016).

Although it is becoming clearer what predisposes EPF accumulation it is unclear and what volume the EPF becomes less cardioprotective and more cardio damaging. Is there an EPF threshold (volume or thickness) that dictates EPF health? In a study including patients from the Framingham cohort, the mean EPF volume was 110 ± 41 mL in women and 137 ± 53 mL in men (Rosito et al., 2008). Although this is a representative sample in relatively healthy individuals, it doesn't necessarily provide a EPF measure of a lean, young and highly fit individual, or a true control. It is important to point out that in young (mean age 26.2 ± 4.4 years), lean (BMI 22.8 ± 2.9) and normoglycaemic (4.59 ± 0.44)individuals the mean EPF volume (expressed as ml/5mm of EPF) is 8.6 ± 2.4 (De Larochelliere et al., 2014). This suggests that in insulin sensitive, metabolically normal individuals the heart does not rely on EPF as a source of FFA to power contractility.

Can EPF expand similarly to subcutaneous adipose tissue in metabolically normal individuals and remain healthy? Alternatively, is the health of the EPF a result of the systemic metabolic state regardless of thickness or volume. For example, can a patient have a relatively low EPF volume but be very dysfunctional? This is similar to individuals who are insulin resistant at a

relatively low level of BMI/waist circumference, like SE Asians. One approach of classifying EPF health is the degree of macrophage infiltration. Greater macrophage infiltration in EPF a powerful sign of adipocyte dysfunction and essentially renders it proinflammatory (M1 phenotype versus an M2 phenotype) (Lumeng et al., 2007). To the best of knowledge, there are no specific data investigating at what EPF volumes (imaging derived) correspond with EPF dysfunction. In one study the association of EPF volume and EPF derived adipocytokines were evaluated in relation to the degree of coronary artery stenosis. EPF Samples were obtained from 50 patients who underwent elective CABG (CAD group) and 50 patients who underwent valve surgery (non-CAD group) All patients underwent a coronary angiography. As expected, amongst the 50 CAD subjects, one patients had single vessel disease, eight had double-vessel disease, and forty-one had triple vessel disease (defined as >75% coronary luminal stenosis). In contrast, the non-CAD subjects, forty-seven patients had no coronary luminal stenosis, two had <25% and one had <50% stenosis. A number of multivariate models were conducted to investigate the association of clinical markers to the degree of EPF volume. Interestingly, well recognized CVD risk marker such as LDL-c, blood pressure, smoking were not associated with EPF volume. Only coronary artery disease was strongly associated with EFP volume ($P<0.001$). Additional analyses indicated that EPF dysfunction (EPF CD68, Il-beta) and EPF derived adiponectin as measured from EPF biopsy located over the left anterior descending artery) were the only markers that were associated with degree of coronary artery disease, contributing to 75.6% of the variance (EPF CD68 $p=0.01$, IL-beta $p=0.03$, and adiponectin $P<0.0001$). No other risk factors added to the prediction including LDL-c, blood pressure, smoking, diabetes, sex, age or EPF volume or intra-abdominal fat (Shimabukuro et al., 2013). These data suggest that adiponectin secretion by EPF is a key factor in EPF health and coronary artery disease. Adiponectin is highly associated with insulin sensitivity.

These data support other research conducted by (Hirata et al., 2011) who in addition to measuring CAD severity (via gensini score) measured EPF CD68 and macrophage polarization (M2/M1 ratio) and mRNA of pro-inflammatory genes (IL-6, TNF-alpha). The results showed that EPF CD68 and macrophage polarization, i.e. susceptibility towards pro-inflammatory

phenotype were increased in CAD patients (both $p<0.05$). There were significant correlation between mRNA expression of TNF-alph, IL-6 were strongly associated ($p<0.05$) with M1 proinflammatory macrophages. These data suggest an imbalance between the protective adipocytokines (involved in insulin sensitization and anti-inflammatory action) secreted by EPF and the damaging adipocytokines (pro-inflammatory) might be a crucial determinant in the development of coronary artery disease during the progressive stages of insulin resistance.

In this study, EPF CD68 macrophage labelling was undertaken in sub-group of patients (n-15) as a validated measure of EPF dysfunction. MAG-1 was strongly correlated to EPF CD68 ($r=0.517$, $p=0.029$) and contributed to 20.6% of the variance in EPF-CD68 macrophage infiltration. In these data, MAG-1 was significantly and negatively correlated to EPF cardiolipin synthase mRNA expression (r-0.434 $p=0.03$) which suggests that FFA delivery exceeds the electron transport chain capacity to oxidise. Cardiolipin is important in the function of the ETC, as it binds cytochrome *c*, an electron carrier, to the inner mitochondrial membrane, permitting efficient electron transfer between complexes III and IV (ATP synthesis) reducing the mitochondrial energy production capacity. Recently, angiopoietin-like protein 4 was induced in skeletal muscle by excessive FFAs (Staiger et al., 2009). It could be speculated that increased EPF lipolysis in T2D (as observed in our study) may be a source of myocardial angiopoietin-like protein 4) a potent inflammatory molecule which has been shown to induce impairments in sarcomere shortening, Ca+ transport and myocardial insulin sensitivity (Greulich et al., 2012) A reduction in EPF volume and improvement in EPF health represents a viable therapeutic target for the prevention, diagnosis and management of coronary blood flow. It is currently unknown if decreasing EPF thickness/volume and EPF health (inflammatory status) results in changes in IMCL and/or improvements in FFA metabolism in EPF and the LV.

Adipose tissue hypoxia is postulated to explain the development of inflammation. Adipose tissue hypoxia induces pro-inflammatory phenotype of macrophages. In our data, there was no correlation of hypoxia- inducing factor (HIF-1) expression with the degree of EPF CD68 (r-0.09, p=0.75). In subcutaneous adipocytes the degree of hypoxia (or reduction of adipose tissue blood flow) significantly predicts the degree of inflammation, an effect mediated by insulin sensitivity (Karpe et al., 2002). Given that EPF shares its blood supply with the coronary arteries, it is plausible that EPF is ischaemic in patients undergoing CABG. This may be the result of an oxygen shunting to the coronary arteries, an adaptive hierarchical mechanism to preserve myocardial oxygen supply and function rather than supply EPF with sufficient oxygen.

Treating EPF dysfunction:

Many intervention studies have proven that EAT is flexible and is a modifiable. Given IMCL-EPF relationship it may seem prudent to modify the volume/thickness of EPF but also transform the EPF into a less inflammatory form of EPF. There are a number of approaches that can lower EPF including to optimizing metabolic function through weight loss, through dietary manipulation (nutritional quality/ and calorie intake) and exercise. A recent systematic review identified eight studies that showed a statistically significant reduction in EPF with dietary changes (mainly calorie restriction). The reduction in EPF (measured by various imaging modalities were strongly correlated with the degree of weight loss. In contrast, exercise was not associated with changes in EPF. This may reflect that diet habits are more amenable to change when compared to initiating and maintaining an exercise program in older, inactive and metabolically dysfunctional individuals(Rabkin and Campbell, 2015). Recent evidence suggests that vitamin D may be protective to EPF(promoting its anti-inflammatory properties) and contribute to a healthier EPF. In 54 vitamin D deficient men (10.85 ng/mL) there was an increased expression of VDR and pro-inflammatory markers including MCP-1, TNFa, IL-6) in EPF. There was also no correlation between serum vitamin D and EPF thickness(Dozio et al., 2015).

The significance of increased FFA lipolysis from EPF in patients with T2D remains to be fully elucidated especially in relation to the direct impact that EPF FFA lipolysis has on directly stimulating pro-inflammatory adipocytokines which appear to have a direct and detrimental effect on the myocardium (acute effects of sarcomere shortening and Ca+ metabolism) in mice. The role of EPF dysfunction (and related adipocytokine secretion) and its contribution to abnormalities in LV energy metabolism is unknow in human patients. EPF secretion of pro-inflammatory markers may directly influence coronary artery inflammation, the earliest manifestation of coronary artery atherosclerosis. Thus, EPF volume may play a role in stratification of CVD risk and could serve as a therapeutic target even in a symptomatic individuals as the volume of EPF and the pro-inflammatory nature of EPF is amenable to change, especially by diet and exercise. Altering a patient's diet (quality and quantity of calories) and physical activity are likely to cause weight loss (especially in the visceral depots), improve insulin sensitivity and cellular functionality. Further research is clearly warranted, especially the role of EPF in T2D/or metabolically compromised patients undergoing cardiac surgery in preoperative assessments. Routinely measuring EPF via echocardiography or MRI (if available) and surgical EPF biopsies may provide additional information on the role of EPF and EPF health in T2D and non-T2D patients undergoing CABG because EPF is not an innocent bystander in CABG surgery- it is hindrance which can compress the coronaries and limit access to complete grafts quickly and safely. It is not an inert storage depot, it secretes metabolites and pro-inflammatory molecules that significantly contribute to atherosclerotic progression and most likely contribute to surgical risk and outcomes.

IMCL and predictors

In this study IMCL (via lipid droplet size and number) and location in human LV biopsies in T2D and non-T2D patients undergoing CABG was undertaken. Potential predictors (both individual genes and some blood-based markers) of IMCL was also undertaken. Predictors are vitally important as these can then be used in pre-surgical risk profiling in EUROSCORE (like insulin controlled diabetes). Generally speaking, risk scores should be amenable to change and reflect advances in medical science. In chapter 1, the role of HbA1c (an easily measurable, inexpensive marker) in risk stratification and outcome was discussed in those not currently not deemed at increased risk (i.e. not on insulin therapy). Routinely measuring a predictor of risk or a marker of a dysfunctional pathway is a pragmatic approach. This is nothing new in cardiology. A well-recognised example of improved risk and outcome marker was the shift from echocardiographic LV function (EF) to a blood based NTproBNP measure.

IMCL can be measured accurately and non-invasively by contemporary imaging techniques such as MRI, MRS. The information generated from these technologies can be combined with real-time changes in cardiac function and performance. Given the role of cardiac energy metabolism to global cardiac function, it seems logical to assess if individual genes or blood markers can be used to predict the accumulation of LV IMCL. In chapter 2, we sought to investigate gene expression controlling cardiac energy metabolism and IMCL between patients with and without T2D in tissue biopsies. Biopsies can provide mechanistic information, such as gene expression, enzymatic and protein quantification. These provide additional information beyond that of imaging technologies. We also sought to investigate a number of proposed blood based biomarkers to predict the degree of IMCL, as biopsies and many imaging technologies are expensive and laborious and require specialist facilities and personnel to conduct and analyse the scans for clinical application.

IMCL:

Studies examining human LV IMCL or specifically LD size, number and location) in the human heart by microscopy are scarce. Total intramyocardial lipid content (IMCL) is the product of LD size and number. IMCL was higher in T2D compared to non-T2D, but was not statistically significant, perhaps due to small numbers and therefore lack of statistical power. The stepwise increase in cardiac fat content originates from LD numbers (i.e. LD hyperplasia) rather than LD size (i.e. LD hypertrophy). This hyperplasia rather than hypertrophy may result primarily due to the accelerated triglyceride turnover as observed in diabetic rat hearts ((O'Donnell et al., 2006). In 48 hours, fasted mice the number of lipid droplets increased without effects on lipid droplet size (Suzuki et al., 2002) an observation which mimics the observations in our study of increased IMCL originating from increased LD number rather than size. A higher number of LDs were observed in obese subjects with dilated cardiomyopathy compared to their lean counterparts LD size, however, was not assessed (Saito et al., 2013). Jointly, these data may indicate that in the early stages of cardiac dysfunction, lipid droplets increase in number and that an increase in size is warranted to exert negative effects on the heart such as more profound lipotoxicity. For example, an increase in cardiac mitofusion-2 to facilitate the fusion of smaller LD's into larger LD's is necessary for oxidative stress-induced heart muscle cell apoptosis in heart failure (Shen et al., 2007). Therefore, the predominance of smaller LD's may reflect the relative inability to undergo mitofusion and protect the heart from cell lipotoxic damage in the earlier stages of cardiac dysfunction.

The subcellular distribution of LD may be important in cardiomyocytes because in skeletal muscle, storage of LD's in vicinity of the sarcolemma has been associated with insulin resistance (Nielsen et al., 2010). Similar to skeletal myocytes, cardiomyocytes have invaginations of the sarcolemma towards the core of the muscle fibre, a system referred to as the t-tubular system. The t-tubular system is essential for a rapid propagation of the cardiac action potential throughout the cardiac muscle fiber. The connectivity of the t-tubular system with the calcium containing sarcoplasmic reticulum (SR) facilitates swift entry (and reuptake) of Ca2+ to and from the cardiomyocyte resulting in contractility (Brette and Orchard, 2003). It is unknown if the location of cardiac LDs plays a role in cardiac dysfunction. One human study

examined the location of cardiac LDs relative to the t-tubules in a non-failing donor heart (Sulkin et al., 2014). This study reported that ~29% percent of the LDs were located in the subsarcolemmal region. Of the remaining intermyofibrillar LDs, 59% was within a distance of 0.5 µm from a t-tubule. Our data suggest that in both groups the LDs were located near the t-tubules. Understanding the consequence of LD accumulation in vicinity of the t-tubular system is clearly important. It may be hypothesized that LD's may interfere with propagation of the action potential and hence compromise cardiac contractility. Testing this hypothesis is required. In subsequent experiments, IMCL (combination of LD size and number) was investigated with respect to mRNA profiles. VDR expression was an associated with IMCL (r=0.478) and accounted for 19.5% (r^2 0.195) of the variance in IMCL (p=0.030). It may be speculated that lipid droplets sequester vitamin D and Ca+. This is partially supported by a study of (Tishkoff et al., 2008) who observed that the VDR is located at the T-tubular structure. Vitamin D has a major role in Ca+ metabolism and it can only be speculated that LD alter Ca+ movement in the mitochondria and the SR. It may also be speculated that vitamin D is lipid soluble and vitamin D has been shown to promote lipogenesis. The association of VDR to IMCL (lipid contained in lipid droplets) may reflect the lipophilic uptake of vitamin D. This postulated uptake of vitamin D into intracellular lipid droplet may promote Ca+ uptake. It has been shown that a rise in adipocyte cytosolic Ca+ promotes de novo lipogenesis and lipid storage via the expression of fatty acid synthase (FAS) (Zemel et al., 2000). Our data show that VDR was highly correlated with FAS (r=0.561, p=0.007). Thus, the VDR/IMCL relationship may reflect both lipid storage and calcium sequestration. The role of vitamin D in Ca+ metabolism is shown in mice that lack 1α-OH enzyme (the enzyme that inhibits the activation of vitamin D). In this KO mouse model Ca2+ handling abnormalities were observed but treatment with active vitamin D3 significantly attenuated the defective Ca2+ handling (Choudhury et al., 2014).

Therefore, if there is vitamin D mediated Ca+ storage in lipid droplets it is plausible that this may affect Ca+ action in the mitochondria and/or SR. There is no definitive proof, rather it is solely hypothesis generating as we did not measure aspects of calcium handling (SERCA 2a

gene) and we cannot localize the VDR to any particular cellular location. However, immunohistochemistry and western blot analysis may provide further insights.

In summary, IMCL was not significantly higher between T2D and non-T2D patients, perhaps due to low statistical power. LV IMCL is dominated by LD number (hyperplasia) rather than LD size (hypertrophy). In addition, the lipid droplets were located in vicinity of the t-tubular system. The subcellular location of LV lipid droplets in T-tubular location requires further examination but it is plausible that LD's interfere with propagation of the action potential and hence compromise cardiac contractility.. The role of VDR expression with IMCL also requires further study in relation to the impact on Ca+ metabolism at the mitochondria and SR and cardiac contractility, especially in relation to vitamin D supplementation.

IMCL and β-hydroxybutyrate: A clinically adoptable marker of IMCL?

In chapter 2, there was an inverse correlation between and IMCL and serum β-hydroxybutyrate. Therefore, high concentrations of β-hydroxybutyrate is associated with a low LV IMCL. In contrast, age, sex, LDL-c, glucose, insulin , creatinine, NTproBNP or other clinical markers related to FFA transport or metabolism including hFABP, NEFA, or TG were not correlated to β-hydroxybutyrate. Furthermore, when β-hydroxybutyrate was associated with gene mRNA profiles it was most strongly and negatively correlated to PAPR-delta (r= -0.354, p=0.059, PDH r= -0.318, p=0.093 and PPAR-alpha (r-0.262, p=0.171). Although these are not statistically significant, it suggests a down regulation in FFA and glucose oxidation, suggesting an energy substrate preference. Dietary induced increases of β-hydroxybutyrate showed significant down-regulation of PDK which inactivates PDH and inhibits glucose oxidation (Peters et al., 1998). In addition, PPAR down regulation by β-hydroxybutyrate has also been observed in the liver. The regulation of ketogenesis and ketolysis and the molecular mechanisms that determine the ketogenic rate remain incompletely understood and further studies are warranted. This area β-hydroxybutyrate research in human hearts is very embryonic and is the focus of a recent brief review in *Circulation Research* (Lopaschuk and

Ussher, 2016). Understanding the role of β-hydroxybutyrate metabolism in context of cardiac energy metabolism is important, especially during stressful times such as CABG and I/R.

Circulating total ketone body concentrations in healthy adult humans are normally approximately 100 and 250μM, rise to ~1 mM after 24 hr of fasting, and to over 20mM in diabetic ketoacidosis (Wildenhoff et al., 1974). The human liver produces up to 300g of ketone bodies per day which contribute between 5% and 20% of total energy expenditure in fed, and fasted, and states (Balasse and Fery, 1989). Biochemically, the synthesis of ketone bodies occurs when β-oxidation derived acetyl-CoA exceeds citrate synthase activity and/or oxaloacetate availability for condensation to form citrate. Ketone production is highly associated with a decrease in long-chain acyl CoA species. This is particularly important as acyl-CoA synthetase (ACSL) activates long chain fatty acid synthesis. ACSL is important in channeling FFA towards for triglyceride synthesis and storage into intramyocellular lipid droplets. ACSL overexpression increased IMCL 12-fold and induced apoptotic pathways, cardiac hypertrophy, left ventricular dysfunction, and heart failure in mice (Chiu et al., 2001). The administration of the ketone bodies, including β-hydroxybutyrate and acetoacetate has been shown to exert highly protective effects against rare mitochondrial diseases by promoting mitochondrial biogenesis, increasing complex I activity, ATP synthesis and improving the NADH/NAD ratio (hydrogen availability) (Frey et al., 2017) (Ahola-Erkkila et al., 2010). In combination with improved aspects of mitochondrial function, the oxidation of ketones are energetically efficient. The ATP yield of pyruvate is 10 which contrast that of β-hydroxybutyrate which produces 13 ATP (Burgess et al., 2008). This increases the efficiency of the heart by ~30% compared with oxidation of pyruvate (Sato et al., 1995) which may be highly beneficial in periods of acute stress, for example I/R.

A recent randomized controlled trial of empagliflozin, a SGLT in diabetic patients was cardio-protective, improved cardiac energy substrate efficiency and cardiac function, an effect directly attributable to the increases in β-hydroxybutyrate (Ferrannini et al., 2016). The role of ketones is also receiving considerable interest in the failing myocardium. Bedi et al (2016)

measured a whole series of lipid intermediates via mass spectroscopy in failing non-ischaemic (n-22) and non-failing, non-T2D, non-ischaemic, non-obese, (n-20) hearts. The results showed that serum β-hydroxybutyrate was significantly higher in the failing heart, but myocardial β-hydroxybutyrate was lower. This suggests increased myocardial utilisation because succinyl-CoA:3-oxoacid CoA transferase (SCOT) mRNA was significantly increased which is the rate limiting enzyme in β-hydroxybutyrate oxidation (Bedi et al., 2016). The energy efficiencies of ketones combined with evidence that β-hydroxybutyrate is an inhibitor of FFA lipolysis, mediated by the nicotinic acid receptor thereby suppressing FFA release from adipocytes. The degree of suppression at specific β-hydroxybutyrate concentrations remains to be elucidated and compared to the suppression of FFA lipolysis via nicotinic acid drugs, such as acipimox (Taggart et al., 2005). The impact of β-hydroxybutyrate on EPF accumulation/dysfunction is also unknown.

Increasing β-hydroxybutyrate during I/R contributes to myocardial protection by decreasing production of mitochondrial reactive oxygen species (mtROS), by increasing expression of antioxidant enzymes through histone deacetylase (HDAC) inhibition. In rats, injection of β-hydroxybutyrate increased activities of SOD (a mitochondrial antioxidant) that has been shown to increase antioxidant defense in the heart (Nagao et al., 2016). In an excellent study, cardiomyocyte specific deletion of ketone metabolism (via SCOT gene) was evaluated in the myocardial response to injury induced by aortic constriction. In SCOT KO mice (therefore unable to use ketones) there was significant ROS production, mitochondrial damage and myofibril structural impairments and a correspondingly decreased LV ejection fraction. These data suggest that ketone metabolism may be an important mechanism to suppress the myocardial damage after aortic constriction (Schugar et al., 2014). Therefore, the role of β-hydroxybutyrate may be beneficial in the context of cardiac surgery. A higher concentration of serum β-hydroxybutyrate may be clinically beneficial I/R because of improved mitochondrial function, anti-oxidant potential and energy metabolism.

All of our patients had circulating serum β-hydroxybutyrate concentrations that were less than 0.4 mM, which is below the range for even nutritional ketosis (defined by β-hydroxybutyrate 0.5->0.5mmol/L). Therefore, we were unable to compare nutritionally ketogenic >0.5mM versus more ketogenic patients, >1mM or higher. β-hydroxybutyrate concentration is higher in T2D patients and there appears to be considerable heterogeneity within both T2D cohort and non-T2D cohorts. For example, in patients having cardiac catheterization the 25^{th} and 7^{5th} percentile in β-hydroxybutyrate was 56-142 μmol/L in T2D and 20-86μmol/L in non-T2D (Mizuno et al., 2017). This heterogeneity is clearly evident in our data (mean 0.18± 0.15, range 0.06-0.62). There are a number of metabolic factors that control ketogenesis which may be important, including insulin and glucagon but insulin was not correlated to β-hydroxybutyrate in our data. One factor that may control β-hydroxybutyrate concentration is cardiac dysfunction. For example, cardiac utilization of ketones (β-hydroxybutyrate and acetoacetate) assessed by sampling blood at the coronary sinus and aortic root was significantly correlated to BNP (P=0.013) (Mizuno et al., 2017). We did not see any correlation between NTproBNP and β-hydroxybutyrate (r=0.094) but this is serum β-hydroxybutyrate and does not assess cardiac utilization per se. In our study although patients are fasted, the fasting period can be variable, especially for PM cases. This could be important consideration for the variance in β-hydroxybutyrate. However, it does not interfere with the robustness of the cross sectional metabolic association with IMCL and β-hydroxybutyrate. When IMCL was split by the median (0.477%) there was a 0.10 (91% increase in serum β-hydroxybutyrate) in the non-accumulators compared to the accumulators. This may suggest that inherent flexibility towards ketone formation and oxidation (and lowering of IMCL) may be cardio-protective as higher IMCL is associated with worsening cardiac function (Nyman et al., 2013, Gaborit et al., 2012).

The impact of ketone utilization needs to be studied in context of CABG induced I/R. There needs to be further research in larger number of patients to increase statistical power. This is realistic as surgical patients are hospital inpatients and dietary intake can be manipulated and controlled in the short term. However, it is unknown if the minimum 6 hour fast or 12 hour

fast is sufficiently long enough to promote ketosis but β-hydroxybutyrate measurement is inexpensive and adoptable for use in clinical contexts. The long term effects of ketosis in heart tissue protection is not known as such experiments require tissue pre-post intervention. In the current study, we did not document fasting time prior to surgery but all patients were >8 hours. Fasting may not be the only approach, higher fat diets may be a plausible option thereby increasing patient palatability. Investigating the longer term benefits of ketosis in surgical patients is a very promising area of research especially during acute and stressful times. In a PET and insulin clamped study, healthy men were administered acutely with β-hydroxybutyrate to a serum concentration of 3.8 mmol/L and saline over two separate study days. FFA and glucose uptake and oxidation were assessed after both administrations. Glucose uptake decreased by approximately 50% by β-hydroxybutyrate (304±97nmol/g/min in saline compared to 156±62 nmol/g/min in the β-hydroxybutyrate infusion (P<0.01), whereas no effects were observed in FFA uptake or oxidation. In the β-hydroxybutyrate assessments heart rate increased by 25% and myocardial blood flow by 75% where these changes were not observed during the saline administration (Gormsen et al., 2017). The role that long term ketosis plays in tissue protection is focused on animal models. In mice on a long-term ketogenic diet there was a decrease in liver injury markers (Douris et al., 2015) but no long term data exists for the myocardial protection afforded by ketosis.

In summary our data suggests that sub-ketotic β-hydroxybutyrate is associated with a lowering of IMCL. A higher β-hydroxybutyrate concentration may provide a surrogate marker of lower IMCL/ and be a marker of a more efficient use of circulating energy substrates i.e. ketones versus FFA or indeed glucose. It is inexpensive and clinically adoptable Inducing ketosis is an option in the pre surgical period. If the clinical findings of improved cardiac function (heart rate and myocardial blood flow) can be observed in surgical patients post CABG it may be a major advancement in post-surgical care.

CHAPTER 3:

EX-VIVO CARDIAC ENERGY METABOLISM IN RESPONSE TO VITAMIN D & EXPERIMENTAL ISCHAEMIA AND REPERFUSION

CHAPTER 3:

REVIEW OF LITERATURE:

Pathophysiology of I/R:

The consequence of myocardial ischaemia is the negative effects on mitochondrial oxidative metabolism. As a consequence, cardiomyocyte ATP concentration decreases. There is a decrease in electron transport chain flux and a decrease in NAD from NADH (a lack of hydrogen transport). The decrease in the ATP/ADP ratio stimulates glycolysis and glucose uptake. Given the increase in anaerobic glycolysis during ischaemia, pyruvate is not oxidized in the mitochondria to acetyl CoA but converted to lactate and a decrease in cellular pH occurs, thereby increasing acidity. The decrease in cellular pH attenuates the ability to maintain Ca+ homeostasis. ATP requirements at the sarcoplasmic Ca+ pump are increased during ischaemia mediated declines in pH. Energy derived from ATP breakdown is diverted to preferentially to chemical work, i.e. Ca+ transport to the sarcoplasmic reticulum rather than contractile work (Schonekess et al., 1996). Surgery induced I/R injury can lead to clinically observed arrhythmia, myocardial stunning, low cardiac output, and myocardial infarction. There is autopsy detected histologic evidence of I/R in up to 45% of patients who die post operatively (Weman et al., 2000). Furthermore, biochemical evidence of myocardial injury is associated with adverse events after cardiac surgery (Klatte et al., 2001) (Costa et al., 2001).

Therefore, there is a need to reduce the clinical consequences of I/R. Duration of ischaemia and aortic cross clamp time is linked strongly to lower ischaemic injury. In addition, altering the conditions of reperfusion can impact on myocardial recovery—this includes gradual reperfusion as a cardioprotective strategy(Okamoto et al., 1986). Ischaemic conditioning via intermittent aortic cross clamping has been shown to improve arrhythmias and inotropic support (Walsh et al., 2008). Other large randomized trials investigating I/R protection are generally negative including the PRIMO-CABG II pexelizumab trial (immune modulator), the RED-CABG acadesine trial (mitochondrial opening), the MEND CABG trial (prevention of Ca+ overload), and EXPEDITION trial (prevention of Ca+ overload) (Verrier et al., 2004, Investigators et al., 2008, Mentzer et al., 2008, Newman et al., 2012). Another possible approach is the use of dichloroacetate (DCA). The protective effects of DCA have been attributed to stimulation of glucose oxidation with improved coupling between glycolysis and

glucose oxidation (thereby decreasing lactate production and preventing cellular acidosis). The protective effects of DCA were found when administered during reperfusion, due to activation of PDH and partial inhibition of FFA metabolism. Data from McVeigh and Lopaschuk demonstrated that beneficial effects of DCA were completely lost if given prior to ischaemia (McVeigh and Lopaschuk, 1990). This has been supported by a number of studies where one arm was treated with DCA on reperfusion and the other arm exposed to DCA either prior to ischaemia, during ischaemia or throughout the whole study period. In addition, DCA given to rats in the long term (7 days) caused massive myocardial lipid accumulation which suggest that DCA may be an acute modifier in myocardial stress (such as I/R) rather than a long term treatment option (Jones et al., 2014). Some of these methodological differences may impact outcomes so careful consideration of the timing of dose, duration of dosing and the actual dose are warranted to optimize treatment outcomes.

Contemporary treatments are therefore required. There is accumulating evidence for the beneficial effects of vitamin D in auto-immune diseases, infections, osteoporosis, cancer, blood pressure, hepatic steatosis and a host of other inflammatory conditions. The prevalence of vitamin D deficiency in both healthy and diseased populations is shockingly high. Vitamin D deficiency is defined as a serum 25(OH)D concentration of <50 nmol/L (20 ng/mL). Vitamin D status has been extensively studied worldwide. Vitamin D deficiency affects an estimated 1 billion people worldwide, with vitamin D insufficiency affecting almost 50% of the population (Holick, 2007) Vitamin D deficiency is prevalent across all geographic regions and age groups (Garland et al., 2009). A meta-analysis conducted by (Schottker et al., 2014) investigated the association between serum 25(OH)D concentrations and all-cause mortality. They concluded that despite levels of 25(OH)D varying with different countries, sex, and season, the association between 25(OH)D level and all-cause mortality was consistent. These findings were confirmed by (Saliba et al., 2012) who concluded that all-cause mortality was independently and inversely associated with serum 25(OH)D levels less than 50 nmol/l. In a study comprising >4,400 cardiac surgical patients vitamin D was measured and 38.0% had deficient 25(OH)D values (<30 nmol/L), 32.3% had insufficient values (30-49.9 nmol/L). Only

3.1% had values >100 nmol/L which is deemed sufficient (see figure x). In multivariable-adjusted logistic regression models, the odds ratio of MACCE at deficient, insufficient 25(OH)D levels was 2.23 [95% CI: 1.31-3.79], 1.73 (95% CI: 1.01-2.96) respectively, compared with 25(OH)D levels of 75-100 nmol/L. Multivariable-adjusted 6- and 12-month mortality were higher in patients with deficient 25(OH)D levels compared with patients with 25(OH)D levels of 75-100 nmol/L (Zittermann et al., 2013).

Figure 18: The vitamin D concentrations distribution in N=4418 patients undergoing CABG surgery (taken from (Zitterman et al 2013)

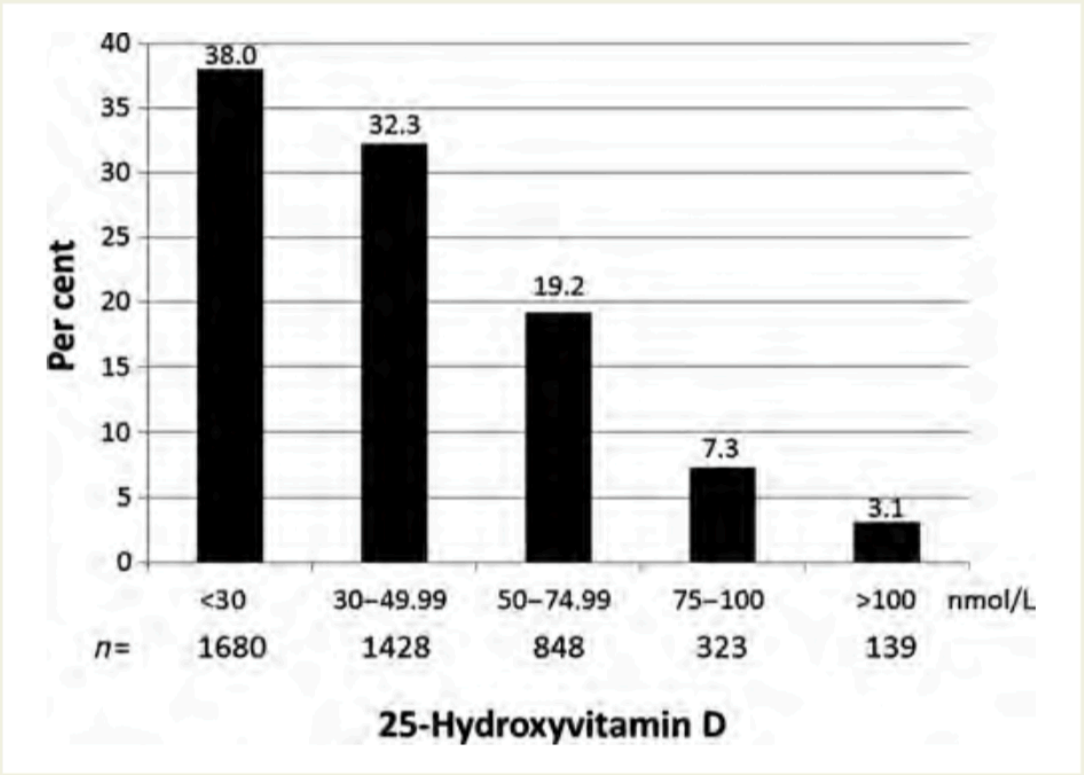


Figure 1 Numbers and percentages of cardiac surgical patients in different 25-hydroxyvitamin D categories.

Emerging evidence that vitamin D exerts beneficial effects in skeletal muscle. Vitamin D enhances muscle contractibility and myosin heavy chain expression (Okuno et al., 2012), muscle cell differentiation, proliferation and myogenesis in cultured cells and in humans (Garcia et al., 2013, Girgis et al., 2014, Ceglia et al., 2013). These actions may, in part, be indirect by way of vitamin D's effect on calcium metabolism and muscle functionality, mediated via the VDR. VDR is expressed in cardiomyocytes, endothelial cells and vascular smooth muscle cells (Chen et al., 2011, Zehnder et al., 2002). In ventricular cardiomyocytes isolated from neonatal rat hearts, calcitriol (the active form of vitamin D) regulated the number of cells entering the synthesis phase of the cell cycle, therefore affecting subsequent maturation and differentiation (O'Connell et al., 1997). In addition, animal models with VDR KO exhibited increased ventricular mass, higher levels of atrial natriuretic peptide and cardiac metalloproteinases which are linked with myocardial fibrosis. These changes eventually lead to impaired diastolic and diastolic function (Simpson, 2011, Xiang et al., 2005, Wu et al., 1996). Moreover, rats fed vitamin D deficient diet show a higher systolic pressure and serum creatine phosphokinase (creatine breakdown) that parallels the decreases in calcium levels. All these negative effects are corrected by vitamin D treatment (Weishaar et al., 1990, Mancuso et al., 2008).

Fat soluble vitamins (A D E and K) and the water soluble vitamins (B complex and C) are essential for growth, reproduction and homeostasis. These vitamins have essential roles for maintenance of normal metabolism. Due to compelling evidence linking vitamin D deficiency to cardiovascular risk, we sought to investigate its role in cardiac energy metabolism. Studies with supplemental vitamin A has shown positive effects on I/R and oxidative stress (Tao et al, 2019) but did not on establish its association with energy metabolism. However, we acknowledge that future studies are needed to study the effects of different doses of other vitamins, alone and in combination on cardiac energy metabolism, and reducing oxidative stress in patients undergoing CABG.

CHAPTER 3:

METHODS:

ULTIMATE-2: Protocol

Ten (n-10) non-T2D CABG patients were approached and consented that aligned to the recruitment protocol in ULTIMATE-1 during February 2016 to September 2016. Patients in ULTIMATE-2 were not consented on the basis of acquiring an EPF or LV biopsy. The only biopsy was of the RA appendage. Pre-operative bloods were acquired and processed according to ULTIMATE-1 protocol. All equipment and surfaces were aseptic, and all flasks used to house RA biopsies were autoclaved prior to surgery. Media (500ml) was freshly prepared on the day of surgery. Penstrep (5ml/500ml of media), FCS 10% (50ml/500ml of media), amphotericin (500ul/500ml of media), and glutamine 1% (5ml/500ml) was added to media. A 1mM vitamin D stock was prepared prior to surgery in 0.1% ethanol. Aliquots of 50nmol/L and 100nmol/L vitamin D was added to flasks (50ml media plus 25ul and 12.5ul vitamin D 1mM stock respectively). In this cohort of patients, the mean serum vitamin D levels prior to experiment was 51.3 nmol/L. Therefore, the 50nmol/L flask was used as control in order to keep the tissue at a steady state of vitamin D. The 100nmol/L flask was used as intervention as it was double the amount of average vitamin D concentration for this cohort of patients. Our rationale was to establish the effect of modifying vitamin D to a sufficient concentrstion. Ideally, a non-exposed control should have been part of the experimental methodology. To ensure consistency in experimental conditions the flasks were pre-warmed to 37 degrees in 21% oxygen (BOC gases). Baseline media samples from each flask was taken in small aliquots for the determination of LDH and oxygen concentration) as quality control. The RA appendage was biopsied (max tissue weight of 250mg) and transported in control media (no vitamin D) to the laboratory. RA tissue was gently stripped of visible fat and split equally into small pieces (approximately 50 mg +/- 10mg) over a sterile flat surface on ice. Each biopsy is weighed individually and then split into two pieces. The baseline sample was 1) snap frozen in 2-metyl butane and liquid nitrogen and 2) incubated with MTT reagent (Promega Inc.) for 30 minutes at 37 degrees then stored at -80 degrees. All flasks containing RA were then exposed for 24 hours in media at 37degrees and 21% oxygen through tubing connected to BOC purchased gases. During the 24-hour period samples of media were taken to assess LDH release at 1, 2, 4, 8, 18, and 24 hours. Furthermore, media was analyzed for partial pressure of oxygen (p02)

at 2, 8 and 24 hours. At 23 hours, new flasks with fresh media (with equivalent of 50 and 100 nmol/L) of vitamin D was pre-bubbled for 1 hour with 5% CO2 and 95% nitrogen to ensure ischaemic media. Tissue samples were then gently transferred into the new ischaemic flasks for 90 minutes of ischaemia at time point 24 hours. After 90 minutes the media was exposed to reperfusion by 21% 02 for 2 hours. The model described aimed to replicate the clinical exposure during CABG. LDH, oxygen and lactate concentration were measured at 30 and 90 minutes of ischaemia and at 60 and 120 minutes of reperfusion. At the end of the reperfusion period, samples were snap frozen in 2-metylbutane and liquid nitrogen and 2) incubated with MTS reagent (Promega Inc.) for 30 minutes at 37 degrees then stored at -80 degrees.

Cell viability assessments:

Tissue viability was assessed by the reduction of 3-[4,5-dimethylthiaxol-2-y]-2-5 diphenyltetrazolium bromide (MTS) to a blue formazan product at the end of the experimental condition i.e. at the end of ischaemia and reperfusion. The tissue was homogenized in 2 ml of TRIS buffer using a pestle and mortar on ice. The homogenate was then centrifuged at lOOOg for 10 minutes and 0.2ml of the supernatant was dispensed into a 98-well flat-bottom microtiter plate ELISA plate. After this, the absorbance of the blue formazan formed was measured on a plate reader at 550nm and the results in the experimental condition expressed as relative to baseline untreated RA sample.

The RNA from the RA samples were then extracted as per manufacturer's instruction (see section x). Other aspects of the methodology (biopsy protocol, blood tests and gene expression) are identical and the methodology in ULTIMATE_1 should be referred to.

CHAPTER 3: RESULTS

Table 15 : Patient characteristics in ULTIMATE-2

Variables		N	%
Sex	**Male**	8	80%
	Female	2	20%
Grafts	**0**	0	0%
	1	0	0%
	2	2	20%
	3	6	60%
	4	2	20%
LV_Hypertrophy	**No**	10	100%
	Yes	0	0%
Dyspnoea status (NYHA)	**None**	0	0%
	Mild	2	20%
	Moderate	6	60%
	Severe	2	20%
Angina status (CCS)	**None**	0	0%
	Mild	0	0%
	Moderate	5	50%
	Severe	5	50%
Hypertension	**No**	2	20%
	Yes	8	80%
Smoker	**Never**	2	20%
	Ex-Smoker	6	60%
	Current-Smoker	2	20%
Statin	**Yes**	10	100%
Ezetimibe	**No**	9	90%
	Yes	1	10%
Beta_blocker	**No**	3	30%
	Yes	7	70%
ACE_i	**No**	9	90%
	Yes	1	10%
ARB	**No**	10	100%
	Yes	0	0%
Diuretic	**No**	8	80%
	Yes	2	20%
Calcium_CB	**No**	10	100%
	Yes	0	0%
Antiarrhythmic	**No**	8	80%
	Yes	2	20%
Anti_angina	**No**	5	50%
	Yes	5	50%
Antiplatelet	**No**	0	0%
	Yes	10	100%
Metformin	**No**	10	100%
	Yes	0	0%

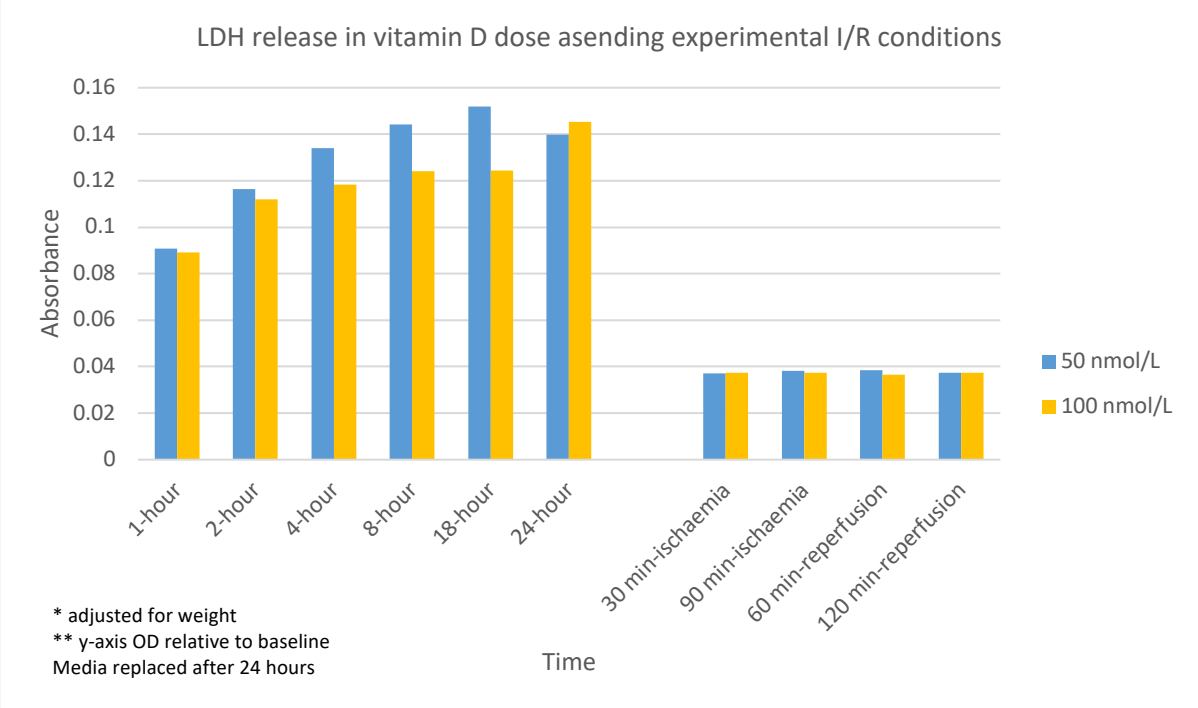
Table 16: Patient characteristics in ULTIMATE-2

Clinical characteristic	**CABG n-10**
Age (years)	64.6 ± 8.5
BMI (kg/m^2)	27.6 ± 3.9
Creatinine (μM/L)	89.5 ± 19.1
eGFR (ml/min/1.73^2)	77.7 ± 11.8
Urea (mmol/L)	6.0 ± 1.7
Potassium (mmol/L)	4.5 ± 0.3
Sodium (mmol/L)	137 ± 4
Haemoglobin (g/L)	136 ± 14
Platelets (10*9/L	232 ± 39
Glucose (mmol/L)	5.72 ± 0.63
Total cholesterol (mmol/L)	3.79 ±0.42
HDL-cholesterol (mmol/L)	1.04 ± 0.18
Triglycerides (mmol/L)	1.98 ± 0.71
NEFA (mmol/L)	0.86 ± 0.19
hFABP (ng/ml)	9,65 ± 15.9
β-hydroxybutyrate mmol/L)	0.16 ± 0.10
Myoglobin (ng/ml)	178 ± 218
Vitamin D (nmol/L)	51.3 ± 20.0

Table 12 shows our patient cohort recruited into this study. Patients were predominantly men (80%) and the majority were having a 3 vessel bypass (60%). All patients were overweight but non-T2D. All patients were mildly dyslipidemia (triglycerides and HDL-c) but on maximally tolerated dose of statin. Mean vitamin D concentration was 51.3nmol/L which corresponded to our comparison dose in the I/R experiments.

Cell viability experiments:

LDH release during 24 hours vitamin D priming and during ex-vivo stimulated I/R, expressed as relative to baseline media sample (no vitamin D, no sample). There was a maximal increase in LDH (1-hour to 8-hours (75%) in 50nmol/L and (1-hour to 24-hours (63%) in 100nmol/L). There was no change in LDH relative to baseline sample during the I/R exposure.



Figures 19: LDH release during 24 hours vitamin D priming and during ex-vivo stimulated I/R,

MTS:

Cell viability (MTS) experiments confirmed cell viability with a decrease on 17% and 21% post I/R in 50nmol/L compared to 100nmol/L when expressed to baseline (i.e. untreated baseline RA sample).

Summary:

These data show that not only were cells viable (approximately 20% decrease in viability) but also illustrated that vitamin D 100nmol/L appeared to decrease LDH release under identical conditions when compared to 50nmol/L.

Table 17: FFA and glucose and toxic lipid metabolism gene expression following experimental I/R.

Gene	50nmol/L IR exposed Mean+ SE	100nmol/L IR exposed Mean+ SE	Relative change (100 vs. 50 nmol/L) (%)	Sig.
VDR	**0.60 (0.23)**	**0.69 (0.21)**	**+15%**	**0.25**
HIF-1	**7.02 (1.12)**	**7.49 (1.54)**	**+7%**	**0.68**
AMPK	**0.56 (0.20)**	**0.72 (0.22)**	**+29%**	**0.59**
GLUT-1	**3.05 (0.69)**	**3.66 (0.58)**	**+20%**	**0.45**
GLUT-4	**0.21 (0.09)**	**0.27 (0.06)**	**+29%**	**0.62**
PI3-Kinase	**0.80 (0.11)**	**0.88 (0.15)**	**+10%**	**0.46**
IRS-1	**0.04 (0.01)**	**0.11 (0.04)**	**+175%**	**0.11**
PDK	**1.06 (0.25)**	**0.99 (0.16)**	**-7%**	**0.75**
PDH	**4.98 (0.75)**	**5.39 (0.80)**	**+8%**	**0.65**
CPT-1	**0.31 (0.06)**	**0.26 (0.03)**	**-16%**	**0.43**
PPAR-alpha	**0.76 (0.14)**	**0.65 (0.11)**	**-15%**	**0.44**
PGC-1	**0.56 (0.22)**	**0.67 (0.16)**	**+20%**	**0.69**
FABP-3	**17.10 (7.02)**	**21.51 (7.26)**	**+26%**	**0.57**
CD-36	**0.87 (0.23)**	**0.84 (0.21)**	**-3%**	**0.81**
LPL	**0.34 (0.10)**	**0.34 (0.11)**	**No change**	**0.92**
VLDL-r	**0.93 (0.20)**	**1.21 (0.30)**	**+31%**	**0.38**
SPT-1	**2.37 (0.29)**	**3.90 (1.13)**	**+65%**	**0.16**
Cer-synthase	**0.06 (0.01)**	**0.17 (0.07)**	**+183%**	**0.18**
Beclin-1	**2.60 (0.44)**	**1.87 (0.28)**	**-28%**	**0.15**

Results:

Table 13 shows the expression of genes controlling glucose uptake and FFA oxidation following experimental I/R in two vitamin D doses. None of the changes observed by increasing vitamin D from 50 to 100nmol/L were statistically significant, which could be attributed to low statistical power. The mRNA expression profiles measured after I/R in two experimental conditions show a decrease in FFA oxidation; PPAR-alpha (-15%) and CPT-1 (-16%) and PDK (-7%). Furthermore, there was an increase in insulin signaling (IRS-1 (+175%) and PI3K (+10%), glucose transport (GLUT 1 (+20%)+ GLUT 4 (+29%), and PDH (+8%). As a result of this subtle shift in genes controlling energy substrate metabolism (<FFA oxidation and >glucose oxidation) an observed increase in genes controlling toxic lipid metabolism were observed (SPT-1 (+65%) and ceramide synthase +183%). Beclin-1 expression was down-regulated in response to 100 nmol/L vitamin D, which suggests that a positive effect on inhibiting cell autophagy (cell death).

CHAPTER 3:

DISCUSSION:

In ULTIMATE 2, the experiments were very much proof of principle, exploratory and hypothesis generating. Our experiments were designed to replicate CABG surgery to have more clinical applicability. There are no data specifically investigating vitamin D induced alterations in myocardial energy substrate utilization in human tissues upon exposure to I/R. This highlights the novelty of these experiments. Tissue exposure to 100nmol/L of vitamin D showed some subtle and favourable alterations in genes controlling FFA and glucose uptake and oxidation after experimental I/R when compared to 50nmol/L. Our MTS and LDH release experiments also indicted cell viability. We did not assess the independent effects of ischaemia and reperfusion and the gene profiles are reflective of the conditions at 2 hours post reperfusion. Therefore, expressed genes are not indicative at 30 minutes of reperfusion which seems the normal protocol for rat perfused heart studies (Kantor et al., 2000, Heather et al., 2013). It is likely that there will be a time course effect on restoration of FFA and glucose oxidation. It is therefore, highly plausible that our observed effects at 2 hours <FFA oxidation and <glucose oxidation may have of been of higher magnitude at 30 minutes. Our experiments were controlled in that our ischaemic and reperfusion media corresponded to approx. Po2 of 6-7% and approx. 21% respectively. We did not measure metabolites at the tissue level or radiolabel FFA/glucose to investigate metabolic pathways (glycolysis and oxidation) specifically.

Given the predominant role of FFA in generating ATP for contractile function albeit at a higher oxygen cost, approaches that target FFA metabolism may be good candidates for mitigating the FFA induced cellular dysfunction observed during reperfusion. Over-reliance on FFA oxidation depresses the hearts functional recovery post ischemia. It is now accepted that FFA inhibition of glucose oxidation is associated with cardiac dysfunction during reperfusion, especially at the area of ischaemic risk (Ussher et al., 2012). Our data show that aspects of insulin signaling were positively affected by vitamin D (50-100nmol/L) and this improved glucose transport (GLUT 1 and 4), and lowered PDK, thereby activating PDH. Increasing glucose uptake/oxidation is beneficial in improving functional outcomes and the area at ischaemic risk. Most work in this area of I/R alterations in energy substrate utilization have been undertaken in perfused rat hearts, implementing gene KO (such as PDK and malonyl coA decarboxylase

(MCD) inhibitors. Other studies have assessed the effects of clinically available medicines (such as trimetazidine and etomoxir).

Lopaschuk et al (2012) stimulated PDH activity by administering dichloroacetate (DCA) in rats and this reduced infarct size following temporary ligation of the left anterior descending coronary artery. In rats with PDK KO (and therefore high PDH activity) DCA had no additional effect on infarct size. In mice KO for MCD an increase in glucose oxidation (via inhibition of FFA oxidation) reduced infract size from 39.5 $\pm$ 4.7% (in the wild-type rat) to 10.8 $\pm$ 3.8% in the MCD KO). Therefore, medicines that activate PDH or inactivate MCD may be beneficial in the context of cardiac surgery but require more clinical testing.

We have shown that in addition to the gene expression changes attributed to glucose metabolism that FFA was suppressed by vitamin D (50-100nmol/L). In particular, PPAR-alpha and CPT-1 reduced by 16 and 15% respectively. This is deemed beneficial as FFA oxidation inhibits glucose oxidation and contributes to the heart's functional recovery. Our data suggest this effect extended relatively long into reperfusion, an effect that may contribute to sustained cardio protection post CABG surgery.

(Heather et al., 2013) in elaborate experiments showed the dynamic and reciprocal nature of energy substrate delivery/utilization in rat hearts exposed to I/R only (i.e. no intervention to alter energy metabolism). They measured the movement of CD36 (FFA transporter) and GLUT-4 (glucose transporter) at the cell membrane and showed that during ischemia CD36 FFA delivery and FFA oxidation was suppressed by 35 and 95% respectively. In contrast, GLUT-4 translocation was increased by 90% and glycolysis by 86% which corresponded to approximately 50% increase in PDH and a 7-fold increase in intracellular lactate. Upon 30 minutes reperfusion, FFA oxidation rates were comparable to pre-ischaemic levels but glycolytic rate recued to around 30% of pre-ischaemic levels. This indicates that in reperfusion, glucose oxidation is decreased, and this corresponded to a decrease in PDH from 1.4 to 0.4 μmol/min/gram wet weight (71%) (Heather et al., 2013). Therefore, strategies to increase PDH upon reperfusion may a pragmatic approach to increases glucose oxidation and decrease FFA oxidation. In our experiments glucose uptake and glucose oxidation was enhanced during and up to 2-hours of reperfusion, which suggests that vitamin D may be a useful therapy to activate

PDH and/or promote lipid uptake in lipid droplets (discussed in the following sections). (Kantor et al., 2000)investigated the efficacy of trimetazidine, a drug classically used as an anti-anginal to alter energy substrate utilization in isolated and perfused rat hearts. In untreated hearts perfused with 1.2 or 0.4mmol/L of FFA palmitate there was a significantly lower glucose oxidation rate (347 $\pm$ 58 nmol/g versus 1889 $\pm$ 119 nmol/L/g, respectively. Glycolysis rates were not affected by increasing FFA concentrations, which supports that FA's (and not glycolytic rates) are inhibitors of glucose oxidation. Despite this inhibition of glucose oxidation by addition of 1.2mmol/L FFA palmitate, trimetazidine decreased glycolysis by 10% from control to 10 μmol/L dose. In contrast, glucose oxidation increased 101% from control to 10 μmol/L dose and FFA oxidation which was associated with a 37% increase in PDH. Trimetazidine decreased FFA oxidation, mainly by inhibiting the enzyme mitochondrial long-chain 3 ketoacyl CoA thiolase in the mitochondria. These data suggest excessive FFA (particular long chain FFA) delivery into the heart may be detrimental for cardio protection, and PDH activation (Kantor et al., 2000).

I/R injury and vitamin D:

Some specific studies investigating the role of vitamin D in mitigating cellular damage post I/R has recently been published in abstract form. Our work investigated an underlying cause of oxidative stress (i.e. abnormal energy substrate metabolism) combined with makers of cellular consequences such as apoptosis and autophagy (Beclin-1). This combination of cause and effect, in human tissue and at therapeutic doses adds strength to our experiments.

In one study, thirty-two mice were randomly divided into 4 groups: (1) sham group, (2) ischemia and reperfusion (IR) group, (3) vehicle-treated group, and (4) vitamin D-treated group receiving vitamin D 1 mcg/kg once daily before I/R. Vitamin D markedly improved LV function vitamin D supplemented mice but not in other groups. Plasma pro-inflammatory markers IL-1β, IL-6, and TNF-α, MCP-1, cardiac troponin was measured in myocardial tissue after mouse sacrifice. Plasma level of cardiac troponin-I as marker of cardiac injury was significantly reduced in the vitamin D group. Moreover, the effects of vitamin D was associated with reductions in both pro-inflammatory expression following I/R (Yousif et al., 2017).

In another small rat-based study, the combination of vitamin D and resveratrol following myocardial ischemia-reperfusion (IR) was assessed. Ligation of coronary artery was performed (n = 6 per group) comprising of 1 (IR only group), vitamin D (0.1 μg/kg/day)+ IR), 1 mg/kg/day of resveratrol (resveratrol + IR) or a combination (vitamin D + resveratrol + IR) for 14 days. MI size was measured by cellular staining. In the vitamin D + resveratrol + IR group the mean infarct size was 17.6 ± 3.5%, which was significantly less than that in the Vitamin D + IR, and Res + IR groups ($p < 0.001$). Combination of vitamin D and resveratrol increased transcription of catalase by 119 ± 37 % (an enzyme in protecting the cell from oxidative damage). This study showed that combination of vitamin D and resveratrol is an effective strategy for protecting against I/R damage (Safari et al., 2015).

Yao et al. (2015) conducted a comprehensive series of experiments investigating the efficacy of calcitriol (the active form of vitamin D) in myocardial protection during I/R in rats. The authors incorporated VDR siRNA and VDR overexpression to illustrate the importance of the VDR. Functional assessments were undertaken including micro/PET and echocardiography and markers of ROS, apoptosis and autophagy were measured but no direct measures of

cardiac energy metabolism were undertaken. VDR expression was upregulated after acute (I/R), and

VDR acted as a novel self-defensive and cardio-protective receptor against I/R injury. I/R-induced decline of LVEF was highly significant, LVEF 63.01% and in calcitriol group versus 46.59% in the VDR deficient vehicle group. VDR activation reduced oxidative stress and inhibited apoptosis and autophagy dysfunction (cell death). These data provide new insights into the cardioprotective mechanisms of VDR in the myocardium (Yao et al., 2015).

As stated high FFA concentrations are observed during MI induced ischaemia and during and following cardiac surgery (Svensson et al., 1990, Lopaschuk et al., 1994). These FFA can lead to insulin resistance which further worsens substrate utilization, <PDH activity. EPF lipolysis can lead to further FFA delivery in stressful states. The T2D heart also resembles an 'engine oversupplied with fuel and is also insulin resistant. In a very interesting study investigated the effects of vitamin D to improve FFA induced insulin resistance in cultured muscle cells. Compared with a non FFA vehicle-treated group, FFA treatment in muscle cells was associated with 70.6% reduction in insulin-mediated uptake of glucose, and a five-fold (data not shown in paper) decrease in IRS-1. In cells exposed to identical FFA in addition to vitamin D improved the FFA-induced inhibition of glucose uptake in a dose- dependent fashion (with maximal uptake at 10nmol/L) ($p<0.001$) and time-dependent manner (0-48 hours ($p<0.01$) . The time that vitamin D exerted its maximal effect was between 24-36 hours. This was accompanied by a high significant increase in IRS-1 ($P<0.001$). Vitamin D also inhibited the FFA-induced reduction in the myotube (cell fusion) diameter by 35.3% ($p < 0.001$), suggesting that vitamin D may attenuate declines in muscle atrophy. It summary, the deleterious effects of high FFA concentrations can be mitigated by vitamin D, and this effect occurs 24-36 hours and at a dose/time course that may adoptable by a surgical team (Zhou et al., 2008).

FFA oxidation and IMCL in I/R:

In our experiments, there was an increased expression of enzymes that promote toxic lipid accumulation (SPT-1- 2.37 fold expression (50nmol/L) to 3.90 fold expression at 100nmol/L). This may be highly important as *de novo* synthesis of ceramide begins with the condensation of palmitoyl-CoA with serine, catalyzed by the rate limiting enzyme, serine palmitoyl transferase (SPT-1). Ceramide synthases are also involved in the *de novo* synthesis pathway of ceramides by joining sphinganine to a long-chain acid. Unfortunately, we were unable to measure LD (as in chapter 2). It is speculated that LD/IMCL would be positively associated with SPT-1 and ceramide synthase in I/R conditions. However, that does not necessarily translate into tissue accumulation which have been associated with apoptosis. In fact, very low levels of ceramides or spingolipids are found in right atrial tissue acquired during CABG (Baranowski et al., 2010).

In one cell based study, the effects of vitamin D on myocardial insulin signaling was assessed in relation to lipid partitioning in skeletal muscle cells. C2C12 myotubes were treated with calcitriol (100nmol) or vehicle control for 96 hours. Compared to vehicle-treated myotubes, Vitamin D increased insulin signaling. Vitamin D increased total ceramides (1.8 fold) and DAG by 46.9 fold. Vitamin D increased total IMCL but decreased the proportion of lipid within myotubes. Vitamin D increased mRNA content of genes involved in lipid droplet packaging (perilipin-2, 2.07 fold) and lipolysis (ATGL 1.80 fold). Vitamin D alters myocellular lipid partitioning and lipid droplets which may favour lipid turnover and partially explain improvements in insulin sensitivity. In contrast lipid accumulation in non-lipid droplet storage depots can promote insulin resistance. These data suggest that lipid droplets are an acute storage depot that may ultimately protect the mitochondria from excessive FFA flux (Jefferson et al., 2017). These findings were supported by Barba et al (2009) that showed that IMCL or LD accumulation may protect against cell death during/R, by providing a depot for FFA delivery and FFA that exceeds the capacity for oxidation. In these experiments, the addition of FFA to isolated adult rat cardiomyocytes or to HL-1 atrial cells (with and without LD's) resulted in the accumulation of lipid in those cells with LD's, detected by a combination fluorescence microscopy and NMR. Cell death (via LDH release) was significantly decreased in cells

containing LD (40% reduction compared with cells that contained no LD, *P*=0.02). LD accumulation was inversely correlated with cell death (*r*=0.68, *P*= 0.0003). Cells with LD also showed less Ca^{2+} overload than control cells. These results suggest that LD exert a cellular cardioprotective effect during I/R by sequestering FFA and Ca^{+} (Barba et al., 2009). Therefore, acute lipid storage in LD's during I/R is a cardio-protective mechanism whereas accumulation in ischaemic states may reflect highly dysfunctional mitochondria and systemic metabolic dysfunction.

Patient selection for metabolic modulation undergoing CAG/I/R.

All patients will have variable degrees of I/R injury based on a number of factors including cross clamp time and underlying metabolic control. Recent interesting data suggests that the duration of diabetes is may be an important consideration when assessing susceptibility to I/R injury. Isolated perfused hearts were subjected to 40 minutes of ischemia and 120 minutes of reperfusion. IR injury was assessed by cellular staining and myocardial glucose metabolism was evaluated.T2D mice were more susceptible for IR injury compared to controls. Long term T2D suffered increased damage, while I/R injury was decreased in the short-term T2D group. Metabolomic analyses demonstrated that I/R damage was associated with dysfunctional mitochondrial glucose metabolism (inability to activate PDH) during ischemia and early reperfusion. These findings may be a clinical consideration in T2D patients as cardioprotective interventions may be more efficacious in long standing T2D patients (Povlsen et al., 2013).

Clinically altering FFA and glucose oxidation in patients undergoing CABG

To the best of our knowledge, there is currently no evidence of the beneficial effects of vitamin D in altering myocardial energy metabolism during I/R. However, outcomes may be better if patients were metabolically primed, insulin sensitivity improved, and metabolic flexibility enhanced. Clinically speaking, should surgeons utilize metabolic modulators prior, during or post-surgery to mitigate I/R damage. There are a number of options, from attenuating FFA supply to inhibiting FFA oxidation and activating PDH.

Nicotinic acid (acipimox) act on adipocytes to suppress adipose tissue lipolysis (Stirling et al., 1985). Acipimox therefore lowers plasma FFA levels, reduces plasma insulin levels and insulin resistance and improves glucose tolerance. LV mechanical work, myocardial perfusion and intramyocellular lipids have been well characterized in non-diabetic patients with idiopathic dilated cardiomyopathy (Tuunanen et al., 2006) and non-CVD T2D patients administered acipimox (Wolf et al., 2016). In both studies, acute administration of acipimox (250mg BD vs. 250mg OD) reduced FFA mobilization from adipose tissue (plasma derived FFA) by 84% and 80% and respectively. In the T2D patients (Wolf et al, 2016) receiving 250mg OD acipimox, IMCL decreased by 42% which correlated with changes in FFA (r=0.70) and had a deleterious effect on cardiac systolic (EF -13% and diastolic (EDV -42%) function. Similarly, in IDCM patients, suppression of FFA by 84% resulted in a decrease in myocardial FFA uptake by 82% and an 18% increase in FFA oxidation. These observed changes resulted in a lower stroke volume (-8%), cardiac output (11%), LV power (-10%) and cardiac efficiency (-11%). The relative contribution of glucose metabolism was not assessed in either acipimox study so no inferences about relative proportions of substrates could be ascertained. In conclusion, excessive FFA metabolism due to high adipose tissue FFA lipolysis, results in impaired cardiac function. On the other hand, insufficient cardiac FFA metabolism due to suppression of adipose tissue lipolysis results in a depletion of IMCL (and likely decrease in toxic lipids, mitochondrial dysfunction, ROS production) and impaired cardiac function. Therefore, it is likely that there is an intricate balance between FFA concentrations and cardiac function. Kerr et al (2017) suggests a “Goldilocks” zone that is ‘just right’ to support cardiac contractile function (Kerr et al., 2017). Future trials may consider dosing strategies to optimize FFA release. Of interest, newer FFA mobilizing drugs are being developed, such as adipose tissue triglyceride lipase (ATGL) inhibitors.

In a randomized controlled trial in 46 patients with symptomatic hypertrophic cardiomyopathy, perhexiline increased cardiac energetic status (improvement of the Pi/ATP ratio) leading to augmented LV diastolic filling (Abozguia et al., 2010). Perhexiline decreased fatty acid β-oxidation and increased glucose oxidation by PDH activation (Yin et al., 2013).Drury et al (2013) observed no clinical benefit of perhexiline in protecting the heart

from low cardiac output post cardiac surgery (Drury et al., 2013).Trimetazidine has shown to increase cardiac energetic status (Pi/ATP ratio) in patients with LV systolic dysfunction by 33%. Vedrinne et al (1996) showed significantly lower lactate levels in patients receiving trimetazidine, suggesting a shift from glycolysis to glucose oxidation (Vedrinne et al., 1996). Ranasinghe et al (2006) randomized 48 patients to either 5% dextrose or glucose/insulin/potassium (GIK) during ischaemia and for 6 hours post-surgery. There was a significant difference in LV SERCAa2 expression between the two groups, suggesting an improvement in LV ca+ metabolism and contractile function post CABG surgery (Ranasinghe et al., 2006). More recently, the glucose lowering drugs (DDP-4 and GLP-1) have been studied on myocardial glucose uptake. The results appear promising but require further clinical trials in larger numbers of patients and in patients undergoing CABG (Gejl et al., 2014, Witteles et al., 2012).

Conclusion:

In this study we have shown that the genes controlling glucose oxidation were upregulated and the genes controlling FFA oxidation were down regulated when the media vitamin D concentration increased from 50nmol/L to 100nmol/L. We observed an upregulation in toxic lipid species gene profiles which may be a consequence of IMCL lipid accumulation. In contrast, Beclin-1 expression was down-regulated in response to 100 nmol/L vitamin D, which suggests a positive effect on inhibiting cell autophagy (cell death). These changes persisted at 2 hours into reperfusion. Understanding the time course changes of vitamin D induced alterations in FFA/glucose oxidation would allow insights into dosing strategies. The changes in energy metabolism genes may be beneficial in the functional recovery of the heart after CABG induced I/R. Increasing the therapeutic dose of vitamin D to 150nmol/L (60-80ng/ml) may further improve energy substrate selection.

Further histochemical and microscopical analyses combined with gene/protein markers of Ca+ metabolism and contractility (SERCA2a) would have provided a clear understanding of vitamin D's beneficial effect on muscle tissue function and damage. Larger patient numbers would have strengthened the statistical power. Vitamin D may be a therapeutic and protective agent that could be used in cardio-thoracic surgery. The findings of this study may provide an impetus to undertake further laboratory based studies, and perhaps appropriately designed randomized multi-centre controlled trials in vitamin D supplementation in patients requiring cardiac surgery.

THESIS SUMMARY:

This thesis has highlighted a number of novel concepts and findings that may influence a cardiac surgeon. Firstly, the contentious issue of T2D related surgical risk has received close interrogation in diabetic phenotypes. Our findings show that tablet controlled (but non-insulin) diabetics do considerably worse on a number of short and long term outcomes when compared to a non-diabetic. Currently, T2D patients are not at increased risk based on EUROSCORE points. A need to refine surgical risk based on more discreet classifications of continuous variables is required to improve risk stratification and initiate pre-surgical patient optimization i.e. HbA1c. In chapter 2, the notion that EPF is an inert fat depot has been refuted. Rather it is a paracrine organ that partially protects the heart from FFA oversupply but also is amenable to dysfunction which may correspond to worsening LV contractile function. In T2D patients, EPF is dysfunctional and is highly associated with EPF lipolysis and this may overload the T2D heart with FFA that exceeds its ability to oxidise. Identifying EPF volume may be a sensitive surrogate for LV IMCL and abnormal cardiac energy metabolism. The LV IMCL was higher in T2D versus non-T2D. VDR was significantly associated with IMCL and the majority of IMCL was located close to the T-tubule near the myofibrils. The role that VDR/IMCL relationship plays in calcium metabolism and action potential propagation needs further research. This may be an important mechanism linking abnormal energy metabolism with poor contractile function and diabetic cardiomyopathy. Furthermore, we identified that serum β-hydroxybutyrate (a ketone) is a predictor of IMCL. Promoting ketosis prior to surgery may optimize energy metabolism in a more oxygen efficient way and be protective against I/R induced myocardial damage and ROS production. In chapter 3, there were subtle alterations in energy metabolism, such that FFA oxidation was decreased and insulin sensitivity and glucose transport increased. This shift is beneficial in the post I/R period when FFA supply exceed the capacity to oxidise and glucose is more oxygen sparing. This shift is speculated to protect the cardiomyocytes from cellular damage which is a common feature post cardiac surgery. With further research, vitamin D may be an adjunctive treatment option for mitigating I/R complications in patients that are typically highly deficient in vitamin D.

LIMITATIONS & FUTURE DIRECTIONS:

No research is devoid of limitation or weaknesses. It is therefore, imperative that these are considered for the interpretation and for future experimentation. In chapter 1, phenotypes of diabetes (insulin controlled vs, tablet controlled) were considered in relation to post-surgical outcomes. The argument presented was based on the risk of differences in glycaemia in T2D patients but those not currently on insulin therapy. In our T2D cohort it was not possible to look at the difference in risk in HbA1c sub-groups in this heterogenous group of T2D patients as our data was retrospective and we were hindered by the lack of HbA1c measurements. We were also limited in our ability to ascertain duration of diabetes as this may be highly predictive of mortality (especially in the insulin treated group).

As in all surgical (and biopsy) led studies, no control group can be recruited. Therefore, all the patients consented were by virtue, diseased. Although an inclusion/exclusion criterion was employed to maximise recruitment, it may have been worthwhile to recruit more highly polarized groups, i.e. based on the severity of ischaemia, myocardial function or plasma glucose. In hindsight, a comprehensive patients characterisation was somewhat lacking. Although NTproBNP was measured as a marker of cardiac dysfunction, further echocardiographic measurements to ascertain cardiac function was not collected due a technical/software error. Echocardiographic data via ToE at time of surgery was collected but data was lost due to a hardware and software upgrade in our Trust. This is acknowledged as a major weakness and prevented the association between IMCL/gene profiles with functional and specific cardiac function. The inability to associate IMCL with measures of diastolic and systolic function is a major limitation. Furthermore, measuring and quantifying EPF volume or thickness could have provided an estimation of myocardial visceral AT.

There are some inherent limitations that may have provided further insights and confirmed some of the observed associations. Assessing cardiac energy metabolism is highly dynamic and it may be best studied as part of a multi-methodological approach. For example, including cardiac imaging, such as PET or MRS would have allowed IMCL of a control heart, such as a lean, fit, non –T2D participant [as described in section x]. Imaging derived IMCL could have been used as a comparator to 1) compare IMCL measured by imaging and by biopsy. This

would have been highly novel. Due to lack of external funding, and reliance of collaboration we were limited in undertaking IMCL in all samples and in recruiting a larger cohort of patients. Many studies summarized within this thesis have comparable patient numbers to our data. However, to compare well defined subgroups may have provided some useful insights. The genome is breathtakingly complex, in relations to splice variants, post transcriptional modifications and epigenetic factors. In hindsight, there were additional genes that might have provided further insight into the consequences of abnormal energy metabolism, i.e. genes controlling ATP synthesis and nucleotide metabolism, calcium handling, sarcomere shortening, glycolysis, ketone metabolism and oxidative stress. It is plausible that stored cDNA could be used to undertake further gene expression work and stored tissue for protein quantification (e.g. SERCA, VDR).

Newer technologies to assess cellular (commercially bought cardiomyocytes or cells harvested from human surgical patients) energy metabolism can be undertaken, such as SEAHORSE (Agilent Technologies). The SEAHORSE can assess cellular respiration (oxidative phosphorylation) and a surrogate of glycolysis (via extracellular acidification rate) when exposed to inhibitors (oligomycin, FCCP) when exposed to molecules that may alter energy metabolism. This may have provided more real-time, dynamic measures of energy metabolism, which may give insights into myocardial energy plasticity in response to a specific modulator.

In ULTIMATE-2 the doses of vitamin D (the active form calcitriol) were conservative with a top dose of 100nmol which corresponds to 40ng/ml). Vitamin D recommendations vary (with toxic levels only exceeding 150ng/ml or 375nmol/L. However, the rationale was to investigate doses that were above the expected mean of the patients undergoing CABG but within concentrations that may have clinical translation i.e. expected concentration post supplementation in our patients. Clearly, assessing higher doses in a greater number of patients may have given greater insights into the potential of vitamin D for I/R protection of human hearts. The mean vitamin D of patients was relatively low (deemed deficient) and it may have been useful to select patients with highly polarized vitamin D concentrations, i.e. highly deficient and sufficient, despite the latter proving quite elusive. These ex-vivo

experiments were very much proof of principle, hypothesis generating and assessing the role of vitamin D in relation to whole body genomics/proteomics pathways may be the next step- to build a clear picture of vitamin D mediated metabolic pathways in targeted tissues.

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Appendix 1: Participant Information Sheet

Hull and East Yorkshire Hospitals NHS
NHS Trust

Participant Information Sheet *(Version 2.0)*

Full title: Molecular markers of fat use and storage in epicardial and myocardial tissue in patients with and without coronary artery disease undergoing cardiac surgery.
Short title: Molecular markers of fat use and storage in myocardial tissue.

Name of Researchers: Mr Mahmoud Loubani, Dr James P Hobkirk, Professor Sean Carroll, Professor Andre Tchernof, Professor Patrick Schrauwen.

Introduction
We would like to invite you to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it would involve for you. Please take the time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. If there is anything that is not clear, or if you would like more information, please ask us. Take time to decide whether or not you wish to take part. The research is being carried out by the Cardiothoracic Surgeons at the Department of Cardiac Surgery, under the guidance of Mr Mahmoud Loubani and Dr James P Hobkirk.

What is the purpose of the study?
The human heart relies on fat as a source of energy to function optimally, but this requires a good supply of oxygen. In situations where oxygen is compromised (like in some forms of heart disease) the heart can benefit from switching to more sugar as an energy source, which uses less oxygen. However, we do not know what the consequences of altering this energy source and how it might affect the accumulation of fat or sugar in the heart muscle and adipose tissue surrounding the heart in humans. We already know abnormal energy use in the heart can affect heart-pumping function and can cause

heart muscle cell death. In this study, we want to investigate what factors that are found in both heart muscle and heart fat tissue that control energy usage. This information is very important to potentially provide new treatments and evaluate existing ones. To answer our questions, we need to investigate this in patients who have a good supply of oxygen to the heart, i.e. those having aortic valve replacements and those who have a bad supply of oxygen to the heart, i.e. those having a coronary artery bypass.

Why have I been chosen?
You have been chosen because you are having routine cardiac surgery at Castle Hill Hospital. You will either be undergoing a coronary artery bypass graft or having an aortic valve replacement.

Do I have to take part?
No, the choice to take part is entirely yours. Only when you feel satisfied that you have been given enough information about the study and you would like to participate, will you be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?
The CT surgery will proceed normally as discussed with your consultant surgeon. Apart from two exceptions, all the procedures being carried out are required as part of the surgical procedure itself. During cardiac surgery the surgeon has to access the area of the heart that needs fixing, so a very small amount of heart muscle (what we call the right atrium) and fat tissue (on the outer casing of the heart) would normally be discarded. This is routine procedure. In this study, this tissue would be retained for analysis in the laboratory. Taking a small biopsy of another part of the heart muscle (what we call the left ventricle) poses a very small risk but the cardiac surgeons have done many hundreds of these procedures. The amount of heart muscle is very small 0.2-0.5% of total heart weight. We would also retain this tissue and analyse it in the laboratory. All previous work in this area shows this amount has absolute no negative effects on heart function and recovery from surgery. We would also take a 50 ml blood sample, which is less than 1% of your total blood volume. Your body is very efficient at replenishing blood volumes. There are no follow-up visits for the sole purpose of this study and after the surgical procedure your involvement ends. You will be monitored post-surgery through standard NHS post-surgical care**.**

What are the possible benefits of taking part?
There will be no direct benefits to you for volunteering in this study. However, the information we obtain from this study may aid the development of new treatments (and understand the consequences of existing therapy) involved in improving heart function and life expectancy in those with heart disease in the future.

Are there any risks to taking part in the study?
Cardiac surgery does have risks, and these will have been discussed at your pre-operative assessment and consultations with the surgical team. During surgery when the surgeon has to access the area of the heart that needs fixing, a very small amount of heart muscle (what we call the right atrium) and fat tissue (on the outer casing of the heart) would normally be discarded. This is routine procedure. Taking

a small biopsy of another part of the heart muscle (what we call the left ventricle) poses a very, very small risk but the cardiac surgeons have done many hundreds of these procedures. In fact, a recent report has shown the complication rate was extremely low <0.2% in over 4500 patients undergoing this procedure. The amount of heart muscle is very small 0.2-0.5% of total heart weight. All previous work in this area shows this amount has absolutely no negative effects on heart function and recovery from surgery.

Expenses and payments
There will be no formal payment for volunteering in the study.

Will my taking part in the study be kept confidential?
Yes. The blood samples and tissue analysis results that are collected will be anonymous when the results are studied. All information will be confidential and treated in accordance with the Data Protection Act 1998.

What will happen to the results of the study?
The results will be published in appropriate medical journals. However, individual people will not be identified, and complete anonymity will be maintained in line with Trust policy and the Data Protection Act 1998.

What if something goes wrong?
We do not anticipate any problems with the study. If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (01482 675372). However, in the unlikely event that this occurs you will be covered under the NHS compensation scheme, and details on our complaint procedure can be obtained on our trust website (http://www.hey.nhs.uk).

Ethical considerations
This study has been given a favourable opinion by the NRES Committee West Midlands- South Birmingham. The research is organised by Mr Mahmoud Loubani and Dr James P Hobkirk and sponsored by the Cardiothoracic Charitable Funds. Problems or concerns can be discussed with Dr James P Hobkirk (01482 463979) or (07894 264660) for out of office hours. If you would like to talk to someone independent of the study team about taking part you can contact the Hull and East Yorkshire Hospitals NHS Trust Patient Advice Liasion Service (PALS) can be contacted on (01482) 623065 or via email pals@hey.nhs.uk

Thank you for taking time to read the details of our research project.

This information sheet and a copy of the consent form that you have signed are for you to keep for future reference.

Appendix 2: Patient consent form

CONSENT FORM ***(Version 2.0).***

Patient identification number:

Full title: Molecular markers of fat use and storage in epicardial and myocardial tissue in patients with and without coronary artery disease undergoing cardiac surgery.
Short title: Molecular markers of fat use and storage in myocardial tissue.
Name of Researchers: Mr Mahmoud Loubani, Dr James P Hobkirk, Professor Sean Carroll, Professor Andre Tchernof, Professor Patrick Schrauwen.

Please write your Initials all the boxes **to show that you have read, understood and where needed had the meaning of the points explained to you by a member of the research team.**

1.I confirm that I have read and understand the Participant Information Sheet *Version 2.0 dated 27th March 2014* for the above study and have had the opportunity to ask questions, and I am prepared to take part in the above study. ☐

2.I understand and agree that the anonymised tissue samples collected in the study will be transferred to a laboratory in the Netherlands and Canada for analysis. ☐

3.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. ☐

4.I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from regulatory authorities or 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 ☐

Name of the Patient	Signature	Date and time
……	…….	……..
Name of Researcher	Signature	Date and time
……	…….	……

One for patient, one for researcher, one to be kept with source documents.

Appendix 3: REC approval:

NHS
Health Research Authority

National Research Ethics Service

NRES Committee West Midlands - South Birmingham
HRA NRES Centre Manchester
3rd Floor
Barlow House
4 Minshull Street
Manchester
M1 3DZ

Telephone: 0161 625 7815
Facsimile: 0161 625 7299

01 April 2014

Mr Mahmoud Loubani
Consultant Cardiothoracic Surgeon
Hull and East Yorkshire NHS Trust
Castle Hill Hospital

Dear Mr Loubani

Study title:	**Molecular markers of fat use and storage in pericardial and myocardial tissue in patients with and without coronary artery disease undergoing cardiac surgery.**
REC reference:	**14/WM/0109**
Protocol number:	**v1.0**
IRAS project ID:	**136220**

Thank you for your email of 28 March 2014, responding to the Proportionate Review Sub-Committee's request for changes to the documentation for the above study.

The revised documentation has been reviewed and approved by the sub-committee.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the Co-ordinator Dr Ashley Totenhofer, nrescommittee.westmidlands-southbirmingham@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).