A metabolomic analysis of cardiac metabolism in a human model of early myocardial ischaemia

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Abstract

Introduction: Acute myocardial ischaemia and the transition from reversible to irreversible myocardial injury are associated with abnormal metabolic patterns. Advances in metabolomics have extended our capabilities to define these metabolic perturbations on a metabolome-wide scale.

Objectives: This study was designed to identify changes in cardiac metabolism during the first 5 minutes following early myocardial ischaemia in humans, using an untargeted metabolomics approach.

Methods: Peripheral venous samples were collected from 46 patients in a discovery study (DS; 25 patients) and a validation study (VS; 21 patients). Coronary sinus venous samples were collected from 7 patients (4 for DS, 3 for VS). Acute cardiac ischaemia was induced by transient coronary occlusion during percutaneous coronary intervention (PCI). Blood samples were collected at baseline (prior to PCI), and at 1 and 5 mins following coronary occlusion. Samples were analyzed using Ultra Performance Liquid Chromatography-Mass Spectrometry (UPLS-MS) in an untargeted metabolomics approach.

Results: The study observed changes in the circulating levels of metabolites at 1 and 5 minutes following transient coronary ischaemia. Both DS and VS identified 54 and 55 metabolites respectively as significantly changed when compared to baseline levels. These include a range of metabolites which have previously been shown to be detrimental to cardiac function such as arachidonic acid, lysophosphatidylcholine, carnitine, and tryptophan, as well as metabolites which are known to have a cardio-protective effect, like docosahexaenoic acid. A further multivariate analysis to UPLS-MS serum data

was performed to explore the effects of co-morbidities that may also have an influence on the metabolite profile. Principal component analysis, projection to latent structures and orthogonal projection to latent structures, discriminative analysis was used to test the statistical significance of the variables. Lysophosphatidylcholine, and 2-hydroxybutyric acid were noted to be influential metabolites with statistically significant models obtained for diabetes, body mass index, coronary versus peripheral collection site, and antianginal medications. Analysis revealed that none of the comorbidities influenced the metabolite pattern induced by ischaemia, suggesting myocardial specific metabolite perturbation as a consequence of controlled reversible myocardial ischaemia.

Conclusion: The study provides novel insights into cellular changes occurring in a human model of controlled early myocardial ischaemia. Distinct classes of metabolites were shown to be involved in the rapid cardiac response to ischaemia opening new avenues of research into diagnostic and therapeutic targets in those suffering acute cardiovascular events.

Keywords: acute myocardial ischaemia, cardiac metabolism, metabolomics

Declaration

I declare that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

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Dedication

I dedicate this thesis to my loving parents Mr. Mathew. K. Chacko and Mrs. Annamma Chacko, Mr. C.C Joseph and Mrs. Rosamma Joseph, my beloved wife Betsy Sanoj and children Sneha, Bevan and Johan and my brothers Lenin Chacko, Manoj Chacko and Bishop Joshua Mar Ignathios. It would have been impossible for me to have reached this level without their sacrifices and constant encouragement.

I also wish to dedicate this thesis to Professor Trevor Greenhough, who kindly extended his support and encouragement, which enabled me to complete and submit my research work.

Thank you all for giving me this luxury of education

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List of abbreviations

Abbreviation	Expansion
ACS	Acute coronary syndrome
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
AAs	Amino acids
AA	Arachidonic Acid
ANP	Atrial natriuretic peptide
APCI	Atmospheric pressure chemical ionisation
ACE-i	Angiotensin converting inhibitor
ARB	Angiotensin receptor blocker
BHF	British heart foundation
BNP	B-type natriuretic peptide
BMI	Body mass index
CHD	Coronary heart disease
CVD	Cardiovascular disease
CRP	C-reactive protein
CPT	Carnitine palmitoyl transferase
CK-MB	Creatine kinase myocardial band
CKD	Chronic kidney disease
CS	Coronary sinus
CVA	Canonical variance analysis
DS	Discovery study
DM	Diabetes mellitus
DHA	Docosahexaenoic acid
ESI	Electron spray ionisation
ETC	Electron transport chain
ECG	Electrocardiogram
FFA	Free fatty acid
FA	Fatty acid
FADH	Flavin adenine dinucleotide
FAT	Fatty acid translocase
FABP	Fatty acid binding protein
FAB	Fast atom bombardment
GLUT	Glucose transporter
GIK	Glucose insulin potassium

GC	Gas chromatography
HF	Heart failure
HPLC	High performance liquid chromatography
IHD	Ischaemic heart disease
IMS	Intermembrane space
ISMN	Isosorbide mononitrate
LPC	Lysophosphatidylcholine
LCAC	Long chain acylcarnitine
LV	Left ventricle
LDH	Lactate dehydrogenase
LC	Liquid chromatography
LAD	Left anterior descending
MALDI	Matrix assisted laser desorption
MI	Myocardial infarction
MS	Mass spectrometry
NADH	Nicotinamide adenine dinucleotide
NSTEMI	Non-ST elevation myocardial infarction
NMR	Nuclear magnetic resonance
OPLS	Orthogonal partial least square
PCI	Percutaneous coronary intervention
PCA	Principal component analysis
PC	Principal component
PLS	Partial least square
QC	Quality control
ROS	Reactive oxygen species
RMSE	Root mean square error
RCA	Right coronary artery
SMI	Silent myocardial infarction
STEMI	ST elevation myocardial infarction
TP	Time point
TCA	Tricarboxylic acid
TOF	Time of flight
UPLS	Ultra-performance liquid chromatography
UA	Unstable angina
VS	Validation study
VLDL	Very low-density lipoproteins

Publications

1. Sanoj Chacko, Mamas A. Mamas, Magdi El-Omar, David Simon, Sohaib Haseeb, Farzin Fath-ordoubadi, Bernard Clarke, Ludwig Neyses and Warwick B. Dunn. Perturbations in cardiac metabolism in a human model of acute myocardial ischaemia. *Metabolomics. 2021; 17(9):76.*

2. Sanoj Chacko, Sohaib Haseeb, Benedict M Glover, David Wallbridge, and Alan Harper. The role of biomarkers in the diagnosis and risk stratification of acute coronary syndrome. *Future Sci OA. 2018; 4(1): FS0251*.

3. Sanoj Chacko, Yumna B Haseeb, Sohaib Haseeb. Metabolomics work flow and analytics in systems biology. *Curr Mol Med. 2021: 10.2174.*

4. Sanoj Chacko, Mamas A. Mamas, Magdi El-Omar, Farzin Fath-ordoubadi, Bernard Clarke, Ludwig Neyses, Warwick B. Dunn, Adrian Baranchuk, and Alan Harper. Impact of comorbidities in perturbations in cardiac metabolism during early myocardial ischaemia. Drafted for submission to *Current Problems in Cardiology* (In progress).

Chapter 1. Introduction

1.1 Overview of normal cardiac metabolism and the mechanism of cardiac metabolic alterations in acute coronary syndrome

1.1.1 Cardiovascular disease

Cardiovascular diseases (CVDs) are a group of disorders that affect the heart and the blood vessels. Atherosclerosis is an insidious pathologic process that causes disease of the coronary, cerebral, and peripheral arteries and the aorta. Atherosclerosis involves several interrelated processes, including endothelial dysfunction, inflammation, vascular smooth muscle activation, and oxidative stress leading to build up of lipids and immune cells within the wall of the arteries (1). The process begins with fatty streaks, progressing to plaque formation and culminating in plaque rupture or erosion that triggers thrombotic events that can occlude downstream blood vessels triggering ischemic events that are responsible for almost all cases of acute CVD (2). CVDs comprise coronary heart disease (CHD; manifested by angina pectoris, myocardial infarction and heart failure), cerebrovascular disease, (manifested by transient ischaemic attack and stroke) and peripheral vascular disease presenting with limb claudication. CVD is the leading cause of morbidity and mortality worldwide (3). The last two decades has seen a substantial increase in morbidity due to CVD particularly in the low and middle-income nations (4). It represents the most common non-communicable disease globally, responsible for an estimated 17.8 million deaths in 2017 (5).

CHD accounts for approximately half of the total cases of CVD. Epidemiological data from the Framingham heart study estimated that for a person aged 40 years, the lifetime risk of developing CHD is 49% in men and 32% in women (6). A person's lifetime risk of CHD is known to progressively increase as the number and severity of risk factors increase (7). In the face of the enormous public health burden imposed by CHD, the data suggest a substantial increase in the prevalence of CHD risk factors, such as diabetes, obesity and metabolic syndrome (8, 9). The 2019 edition of the British Heart Foundation (BHF) mortality statistics has shown that CVD causes more than a quarter (27%) of all deaths in the UK in men and women. This accounts for more than 160,000 deaths annually, of which 43,000 are premature (under the age of 75). In addition, CHD was one of the leading causes of death responsible for 63,000 deaths per year (Figure 1 & 2) (10).

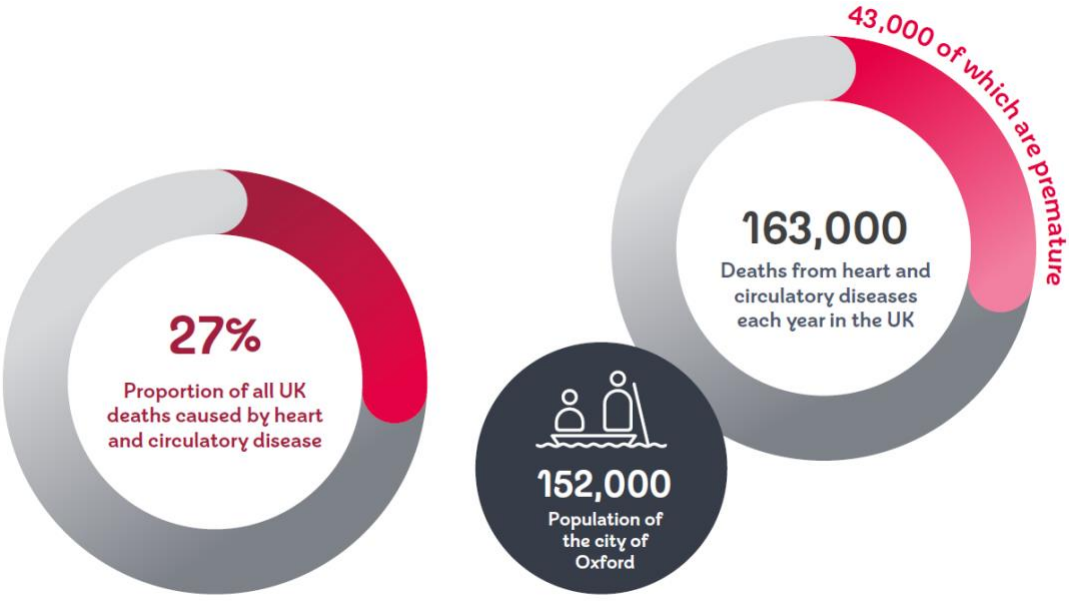

Figure 1: BHF Mortality Statistics 2019 showing 163,000 deaths each year due to CVD, of which 43,000 deaths are premature, contributing to 27% of all deaths in the UK. Figure reproduced from (10).

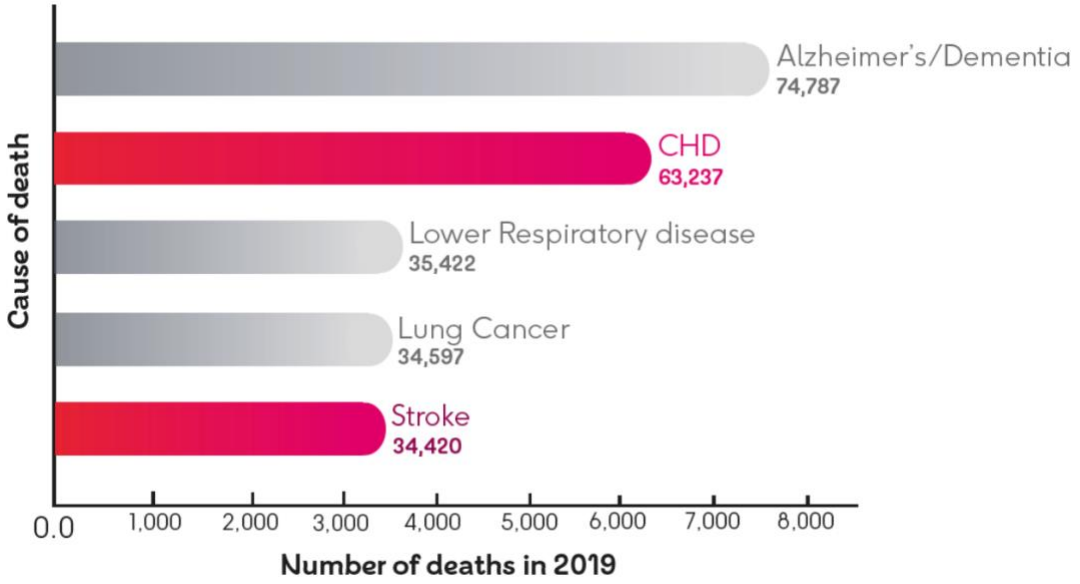

Figure 2: BHF Mortality Statistics 2019 showing the top 5 causes of death, with CHD projected as the second common cause of death and CVD (combined CHD and stroke), as the number one cause of death in the UK. Figure reproduced from (10).

Despite advances in the health care system, morbidity and mortality secondary to CHD continue to pose a major challenge. This is because advanced CHD can exist with minimal or no symptoms, with the first clinical manifestation often being myocardial infarction or sudden cardiac death (11). In addition, for those who presents with symptoms, suboptimal diagnosis and delay in management are known to adversely influence the outcome (12).

1.1.2 Acute Coronary Syndrome (ACS)

Myocardial ischaemia occurs when blood flow to the myocardium is reduced and ischemia of prolonged duration results in myocardial infarction (13). Ischaemia can range in its extent from low flow to total coronary occlusion and

can be of short to long duration. ACS is a broad term used in clinical practice which represents three primary presentations that form a part of the continuum of ACS that can be classified as:

1) ST elevation myocardial infarction (STEMI), when presenting with ST segment elevation in the ECG with symptoms of ischaemia and rise in cardiac biomarkers.

2) Non-ST elevation myocardial infarction (NSTEMI), when presenting with symptoms of cardiac ischaemia and elevated cardiac biomarkers in the absence of ST segment elevation, but may have either ST depression, T inversion or equivocal changes.

3) Unstable Angina (UA), when patients have symptoms of cardiac ischaemia at rest in the absence of biomarker elevation but are at increased risk of future cardiac events.

Although the diagnosis of these different types of ACS have traditionally relied upon a combination of ischaemic symptoms, ECG changes and elevation in serum biomarkers (14, 15), often these symptoms are either atypical or absent. For example, around one third of the patients arriving at the emergency department with confirmed myocardial infarction may not have chest pain (16). Similarly ECG changes which aid with early diagnosis may be nonspecific or even absent in around 37% of the patients (17). As a result, in these cases diagnosis relies upon the presence of certain serum biomarkers, specifically cardiac troponins (elaborated later in the discussion), and their dynamic pattern of rise and fall (18). However, despite improved laboratory assays for cardiac specific biomarkers and revised definitions for ACS, early detection of coronary

ischemia in some patients remains a major challenge. Therefore, the proportion of people dying from an acute myocardial infarction remains high and the concomitant rise in morbidity secondary to heart failure (60%) remains a global burden (19). It is well known that ACS is accompanied by significant metabolic alterations causing myocyte death and dysfunction. Alterations in cardiac metabolism thus contributes to the pathophysiology of myocardial ischaemia. To understand the pathological changes in cardiac metabolism, it is first necessary to consider the normal cardiac metabolism.

1.1.3 Cardiac Metabolism

The human heart consumes more energy than any other organ; it beats around 100,000 times and pumps approximately 10 tons of blood through the body every day. Failure to meet this demand results in mechanical failure of the heart (20). Thus, the requirement of energy to fuel optimal heart function is immense. The heart is capable of using all classes of energy substrates including carbohydrates, lipids, amino acids and ketone bodies for the production of adenosine triphosphate (ATP) in mitochondria (21). Cardiac myocytes have the highest mitochondria content of all cells, accounting for around a third of the cellular volume. These organelles are capable of generating >95% of the ATP used by the heart, largely fueled by the metabolites of fatty acid and carbohydrate metabolism (22).

Under physiological conditions the heart metabolism is predominantly aerobic in nature. As such it depends upon the adequate delivery of oxygen and oxidizable metabolic substrates to meet the heart’s energy demands. Approximately 70% of the ATP production is achieved from free fatty acid (FFA), 20% from carbohydrate metabolism and the remaining through the

oxidation of ketones, lactate and amino acids (23). Cardiac metabolism has three main components (Figure 3); substrate utilization, oxidative phosphorylation and ATP transfer and utilization. Substrate utilization involves the cellular uptake of FFA and glucose, and breakdown of these components by beta oxidation and glycolysis respectively. The resultant intermediate metabolites of these initial reactions enter into the tricarboxylic acid (TCA) cycle where they are used to create a large quantity of reduced nicotinamide adenine dinucleotide (NADH). Oxidative phosphorylation uses the NADH produced from the TCA cycle to produce ATP through powering the mitochondrial electron transport chain and ATP synthase. Mitochondrial creatine kinase catalyzes the transfer of the high-energy phosphate bond in ATP to creatine to form phosphocreatine (PCr) and adenosine diphosphate (ADP). PCr, a smaller molecule than ATP, rapidly diffuses from the mitochondria to the myofibrils, where myofibrillar creatine kinase catalyzes the reformation of ATP from ADP and PCr. The free creatine, formed by the removal of phosphate from PCr, diffuses back to the mitochondria (24).

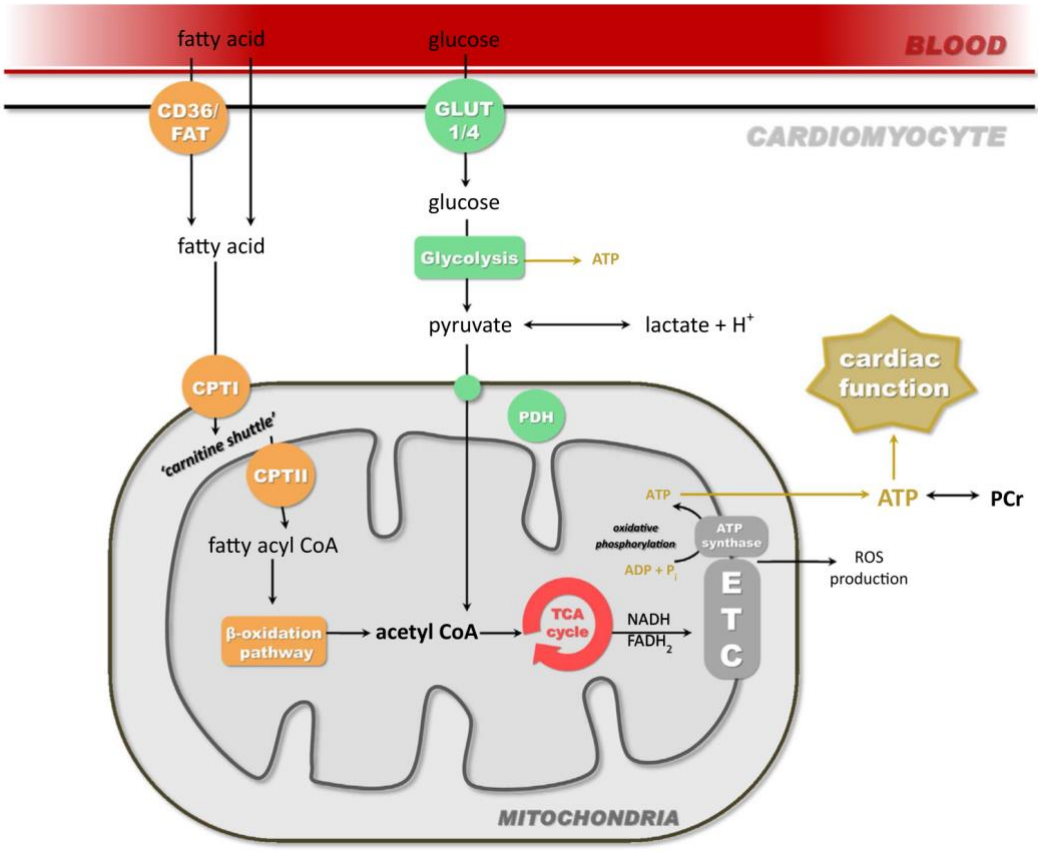

Figure 3: A summary of the processes involved in ATP generation in healthy cardiomyocytes. Fatty acids and glucose are the key substrates of cardiac metabolism. Fatty acids and glucose enter the cardiomyocyte via fatty acid translocase (FAT) and glucose transporters (GLUT). This figure demonstrates the various enzymatic pathways involved in ATP production in cardiomyocytes and subsequent closely coupled interactions of the TCA cycle and electron transport chain (ETC) in the mitochondria. Figure reproduced from (24).

1.1.4 Glucose Metabolism

Under normal physiological conditions, glucose metabolism accounts for 20% of the myocardial oxygen demand. The primary determinant of glucose utilization is the rate of its uptake into the cells, which is regulated by specific glucose transporters (GLUT 1 and GLUT 4). GLUT 1 is distributed in most cells, and is involved in the transport of glucose under resting conditions. GLUT 4 (also known as insulin-sensitive glucose transporter) is primarily expressed in both cardiac and skeletal muscle as well as adipocytes. Under resting conditions, GLUT 4 is localized intracellularly in vesicles. During increased

energy demands such as exercise or augmented muscle contractions, GLUT 4 is translocated to the plasma membrane where it is able to facilitate greater glucose transport (25, 26). Once taken up into the cell, glucose enters the glycolytic pathway, undergoing a series of reactions to form pyruvate and a small amount of ATP.

1.1.5 Glycolysis

Glycolysis occurs in the cytoplasm and does not require a source of oxygen. The glycolytic pathway consists of ten enzyme-catalyzed reactions as shown in Figure 4. The first five steps of glycolysis are energy investing reactions. For each molecule of glucose entering the pathway, two ATP molecules are consumed producing two glyceraldehyde-3-phosphates and two ADP. The net reaction for the first five steps of glycolysis can be summarized as follows:

$$\text{Glucose} + 2\ \text{ATP} \Rightarrow 2\ \text{Glyceraldehyde-3-phosphate} + 2\ \text{ADP}.$$

The second half of the glycolytic pathway transforms each glyceraldehyde-3-phosphate into pyruvate. This part of glycolysis is an energy-generating process, as four molecules of ATP and two molecules of NADH are produced. The net reaction for the second half of the glycolytic pathway is derived as follows:

$$2\ \text{Glyceraldehyde-3-phosphate} + 2\ \text{Pi} + 4\ \text{ADP} + 2\ \text{NAD}^+ \Rightarrow 2\ \text{Pyruvate} + 4\ \text{ATP} + 2\ \text{NADH} + 2\ \text{H}^+ + 2\ \text{H}_2\text{O}$$

The process of glycolysis is regulated according to the cells need for ATP and NADH. Although glycolysis involves several enzymatic reactions, the third step of the glycolytic pathway, catalyzed by the enzyme phosphofructokinase,

represents the central target for regulation of glycolysis. The activity of phosphofructokinase is negatively regulated by ATP, whilst the ADP breakdown product, adenosine monophosphate (AMP), is a positive regulator of this enzyme. If ATP falls, ADP levels increase two-fold in response to accelerating the ATP supply and meeting the demand. Thus, a relatively small decrease in ATP levels can markedly stimulate glycolysis. Similarly, high ATP levels inhibit phosphofructokinase, the key rate-limiting enzyme in glycolysis (27).

Myocardial ischaemia results in an increase in the rate of glycolysis, however, the fate of the pyruvate produced in glycolysis is determined by the availability of oxygen. In aerobic conditions, pyruvate enters the TCA cycle, whilst in the absence of oxygen pyruvate forms lactate. During mild ischaemia, fatty acid oxidation rates decrease but remain an important source of energy. With moderate level of ischaemia (~50% reduction in the mean coronary blood flow) (28) pyruvate oxidation is impaired (29). Hence depending upon the severity of ischaemia there is a switch from lactate uptake to lactate production by the heart (30). Accumulation of NADH and lactate inhibits glyceraldehyde-3-phosphate dehydrogenase, one of the major regulatory steps of glycolysis in ischaemia (31).

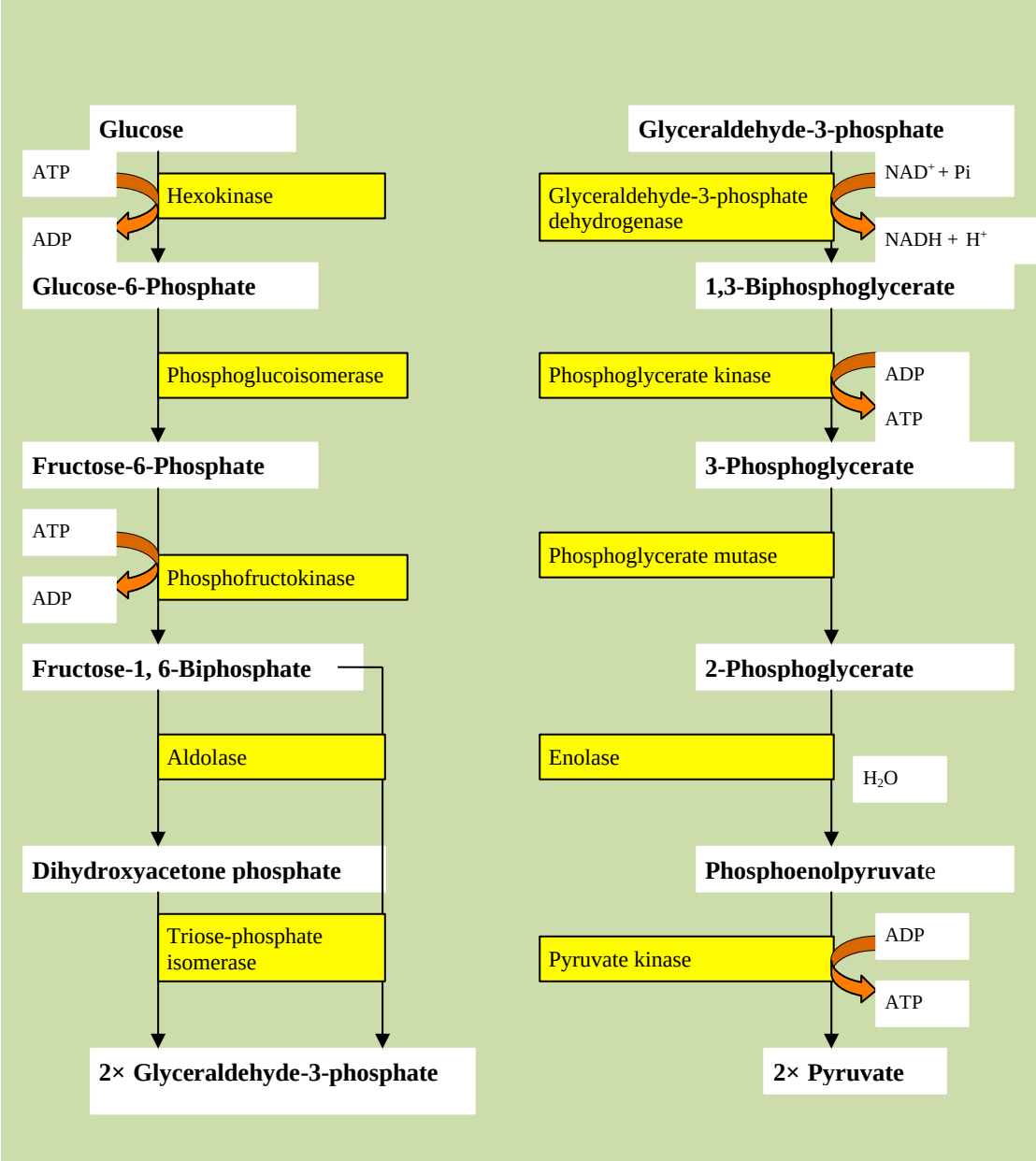

Figure 4: **The enzymatic reactions of glycolysis.** The first five steps produce 2 molecules of glyceraldehyde-3-phosphate from one molecule of glucose. In the second half of the pathway each glyceraldehyde-3-phosphate is catalyzed separately. Thus, for each molecule of glucose two molecules of pyruvate are produced.

1.1.6 Tricarboxylic acid (TCA) Cycle

The TCA cycle, also known as citric acid cycle or the Krebs cycle, constitutes an epicenter in cellular metabolism as multiple substrates feed into the cycle, thus serving as the final common pathway for the oxidation of carbohydrates, lipids and proteins inside the mitochondria. Pyruvate from glycolysis is converted into acetyl-CoA, whilst beta-oxidation of fatty acids also forms this metabolite (see Section 1.1.7). In contrast, amino acids can be converted into pyruvate and other metabolites of the TCA cycle (see section 1.1.8). The cycle starts with the reaction between acetyl-CoA and oxaloacetate. The completion of the TCA cycle, through a series of eight reactions as summarised in Figure 5, generates three molecules of NADH, one molecule of flavin adenine dinucleotide ($FADH_2$), one molecule of ATP and two molecules of CO_2 (32). The resulting NADH and $FADH_2$ are then oxidized by the mitochondrial electron transport chain to generate energy by oxidative phosphorylation in the mitochondria. The TCA cycle and oxidative phosphorylation are tightly coupled since the oxidation of NADH and $FADH_2$ is required for the TCA cycle to continue functioning. Therefore, a tightly regulated pathway and its constant feedback with oxidative phosphorylation is critical for the stability of the cells.

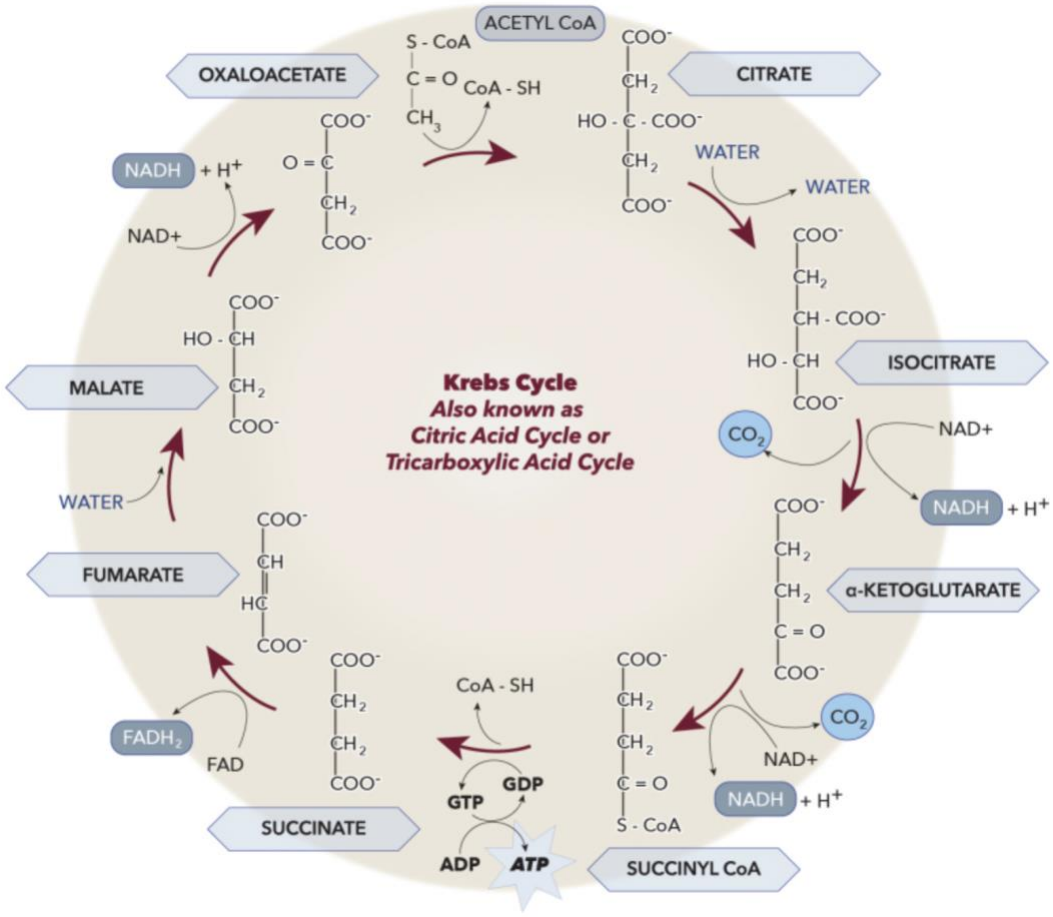

Figure 5: The TCA cycle is a series of chemical reaction to generate energy through the oxidation of acetyl-CoA derived from carbohydrate, proteins and fatty acids. The cycle starts with the reaction of acetyl-CoA and oxaloacetate and completion of the cycle generates one molecule of ATP, three molecules of NADH, two molecules of CO_2, one molecule of $FADH_2$. Figure reproduced from (32).

1.1.7 Oxidative Phosphorylation

Oxidative phosphorylation is a process by which the energy released during oxidation of NADH and $FADH_2$ is used for the synthesis of ATP. This process is performed by the mitochondrial electron transport chain (ETC) and ATP synthase (Figure 6). The ETC transports protons (H^+) from the mitochondrial matrix to the intermembrane space (IMS). The ETC is composed of clusters of five protein complexes (CI-CV) integrated into the inner mitochondrial membrane. The TCA cycle in the mitochondrial matrix supplies NADH and

$FADH_2$, each of which donates a pair of electrons to the ETC via CI and CII respectively. The electrons from CI or CII are utilized to reduce ubiquinone (Q) to ubiquinol (QH_2). Ubiquinol is oxidized by CIII allowing one electron at a time to continue the process through cytochrome C (Cyt-C). For every electron transferred to Cyt-C, two protons are pumped into the IMS. Cyt-C transports electrons to CIV where a molecule of oxygen acts as a terminal electron acceptor and is reduced to water. The reduction of oxygen to water results in pumping of 4 protons to the IMS. The Complex II does not span the inner membrane and does not participate in proton translocation. However, in response to the electron transport, a total of ten protons, two from Complex III and four from each of Complex I and Complex IV, are pumped from the matrix into the IMS. The generated electrochemical gradient provides a source of potential energy that is used for the synthesis of ATP from ADP by the enzyme ATP synthase. Oxidative phosphorylation is the greatest generator of ATP of each substrate, such that an additional 36 ATP can be produced from the breakdown of a single glucose molecule, and more than twice this amount from a fatty acid molecule (33). Mitochondrial oxygen consumption is required for ATP generation and cell survival is threatened when the cells are deprived of oxygen. Consequently, all cells have the ability to sense oxygen and to activate an adaptive process that would enhance the likelihood of survival in anticipation to the limited availability of oxygen. The ETC acts as an oxygen sensor by releasing reactive oxygen species (ROS) in response to hypoxia (34).

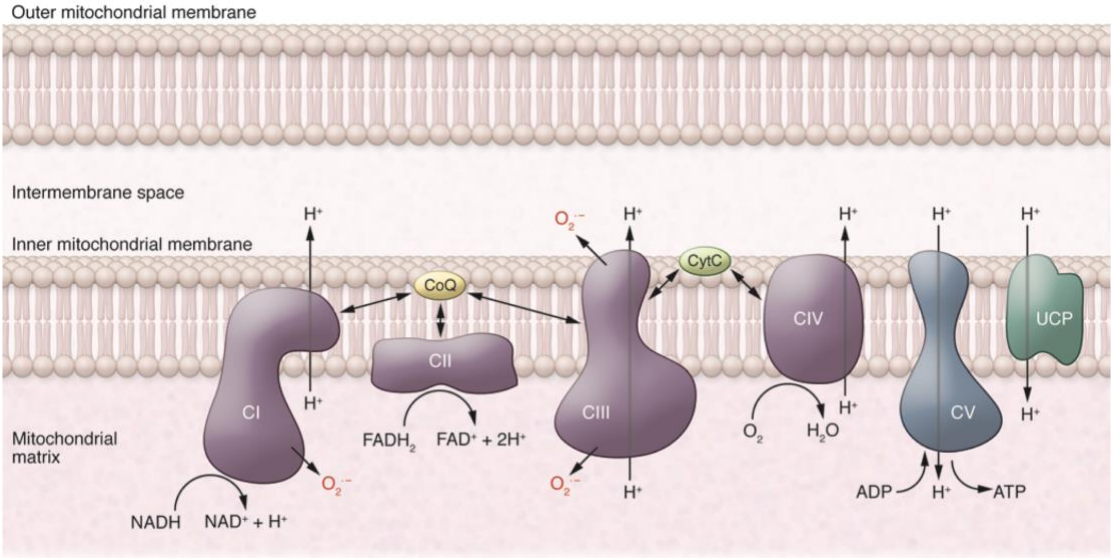

Figure 6: ATP is generated by oxidative phosphorylation mediated by the four respiratory chain complexes (C1- CIV) and ATP synthase (CV) located in the inner mitochondrial membrane. The energy released by the electron transfer from NADH and $FADH_2$ to O2 is used to pump H through C1, CIII and CIV. The proton gradient across the inner mitochondrial membrane drives ATP production via ATP synthase. The reentry of protons to the mitochondrial matrix through uncoupling proteins (UCP), leads to ATP synthesis (33).

1.1.8 Lipid Metabolism

Lipids are a heterogeneous group of compounds, which have many key biological functions, such as acting as structural components of cell membranes, serving as energy substrate and participating in signaling pathways (35). Lipids are divided into different categories as shown in Figure 7 (36)

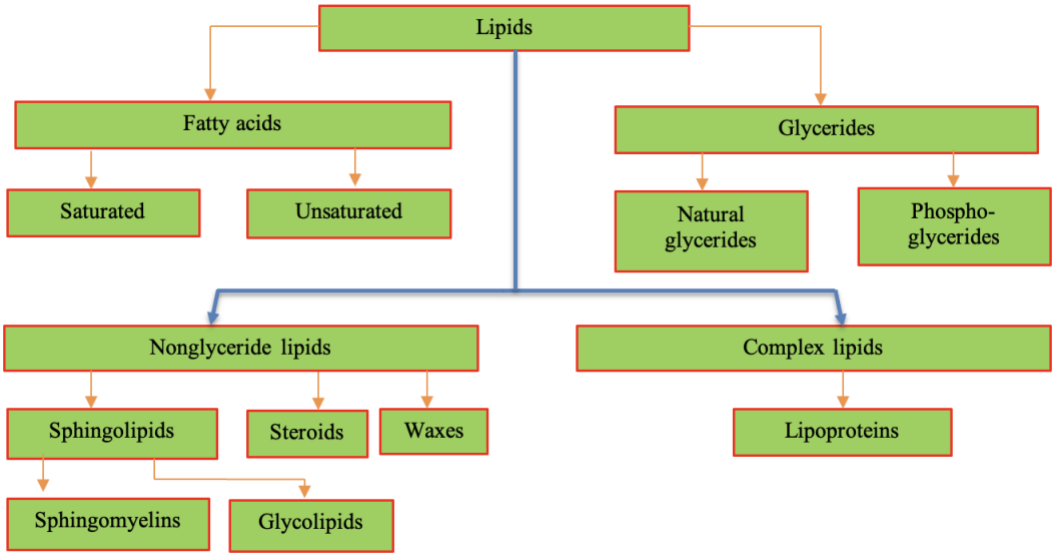

Figure 7 Classification of Lipids: Lipids are classified as; fatty acids, glycerides, nonglyceride lipids and complex lipids. Fatty acids are classified as saturated and unsaturated fatty acids depending on the presence or absence of covalent bonds respectively. Glycerides form the most abundant lipid and are classified into natural glycerides (triglycerides) and phospholipids (common lipid found in the cell membrane). Nonglyceride lipids consists of sphingolipids, steroids and waxes. Complex lipids are lipoprotein complexes which contain lipids and proteins (chylomicrons, high density lipoproteins, low density lipoproteins and very low-density lipoproteins).

Fatty acids (FA) are the main constituents of lipids, which serve as an important source of energy for the heart. FA are either supplied in the diet or synthesized from acetyl Co-A in the cytoplasm. Based on the presence and number of carbon-to-carbon double bonds, they are classified as saturated FA when the FA contain no carbon-to-carbon double bonds (palmitic acid, stearic acid, lauric acid and myristic acid) and unsaturated FA when the FA contain one or more carbon-to-carbon double bonds (linoleic acid, α-linolenic acid, arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid and oleic acid). While being an important source of energy, studies have also shown that high levels of circulating FA may be toxic to the acutely ischaemic myocardium. The relation between high plasma FA concentrations and ACS has been well recognised

(37), In addition, higher incidence of arrhythmia and death has been observed with high circulating concentrations of FA within the first twelve hours of ACS (38). A five -year follow-up study showed that an increased plasma FA in healthy men predicted subsequent development of CHD (39). Another 22-year follow-up study showed that an increase in concentrations of FA at baseline were related to subsequent sudden cardiac death (40). However, not all FAs are detrimental, and there have been consistent reports of the beneficial effects of polyunsaturated n-3 FA such as docosahexaenoic acid and eicosapentaenoic acids in reducing cardiovascular events (41). By reducing the inflammatory response, improving endothelial function, regression of atherosclerotic plaque, and decreasing platelet aggregation, n-3 FA have shown to favorably influence people at risk of CHD (42). The evidence therefore suggests that whilst also being an important source of energy to heart, FA have been proven to have both detrimental and protective effects in acute myocardial ischaemia and can thus play an important role in the outcome of patients with CHD. It is therefore essential to understand the basic metabolism of FA and how these changes during ACS.

1.1.9 Fatty Acid Metabolism

Oxidation of FA is a major means of metabolic energy production, providing over 60% of the total ATP synthesized in the body. Synthesis and degradation of fatty acids occurs by multiple steps in the cytoplasm and mitochondria respectively. Both pathways are similar in that two carbon atoms are either added (synthesis) or removed (degradation) in each round of a cyclical process.

FAs are transported in the plasma and supplied to the heart as either FFA bound to albumin or as triacylglycerols contained in chylomicrons or very-low-density lipoproteins (VLDL; 41). Both sources contribute significantly to overall fatty acid supply to the cardiac myocyte. They enter the cardiac myocyte either by passive diffusion or through a protein carrier-mediated pathway. These protein carriers include fatty acid translocase (FAT)/CD36, fatty acid binding protein (FABP), and fatty acid transport protein (FATP;(43, 44). In addition, VLDL receptors have been demonstrated to be expressed in the heart (45) and uptake of VLDL by this route has been proposed to be a possible source of myocardial FA transport (46). The FFA concentration in plasma typically ranges under normal conditions between 0.2-0.6 mM (47). However, these levels can increase dramatically to over 2 mM during severe stress, such as myocardial ischaemia (48).

FA that arrive at the cell surface are taken up and used for energy production in the mitochondria. The first step in the oxidation of a fatty acid is its conversion to fatty acyl-CoA by the enzyme acyl-CoA synthetase. The fatty acyl-CoA cannot pass through the mitochondrial membrane; therefore, it requires carnitine as a transporter (Figure 8). The acyl group of the acyl-CoA is transferred to carnitine by carnitine palmitoyl-transferase I (CPT I) on the outer mitochondrial membrane. The acylcarnitine is then exchanged for carnitine across the inner mitochondrial membrane by acyl carnitine translocase. Finally, the fatty acyl group is transferred back to CoA by carnitine palmitoyl-transferase II (CPT II). Once a fatty acyl-CoA enters the mitochondrion, it enters into a sequence of reactions called β-oxidation, to produce energy (49).

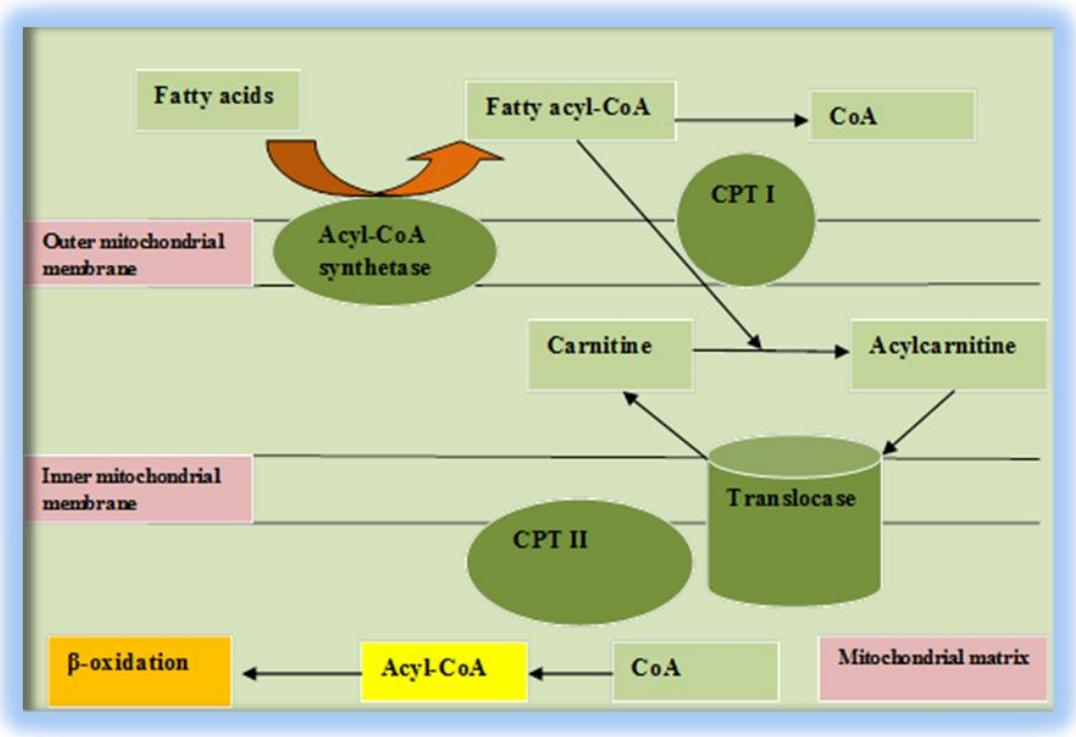

Figure 8: The transport of acyl-CoA into the mitochondria by carnitine. The figure shows a series of reactions how carnitine transports the fatty acyl-CoA to pass through the mitochondria, to enter the chain of β-oxidation. The chain of reactions is mediated by enzymes; acyl-CoA synthetase, carnitine palmitoyl-transferase 1(CPT-I), translocase and carnitine palmitoyl-transferase II (CPT-II)

1.1.10 β-Oxidation

Fatty acids that enter the mitochondria are taken up and used for energy production by a process called β-oxidation. It is a sequence of four reactions: dehydrogenation, hydration, oxidation and thiolysis, through which acetyl-CoA is produced (Figure 9). The acetyl-CoA thus produced enters into the TCA cycle and oxidative phosphorylation to generate ATP.

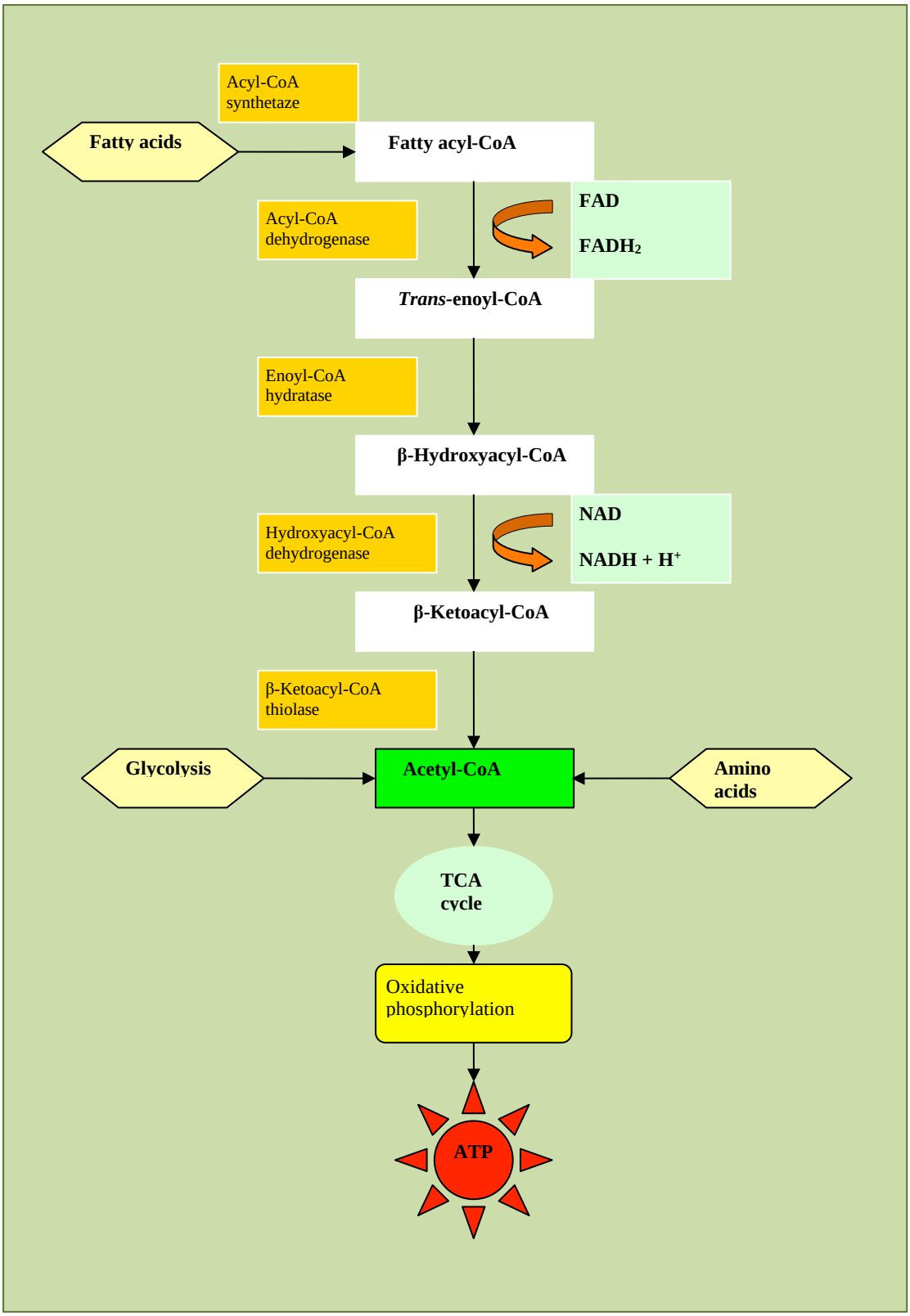

Figure 9: Sequence of four reactions in β-oxidation**:** dehydrogenation, hydration, oxidation and thiolysis resulting in ATP production.

1.1.11 Amino acid Metabolism

Amino acids (AAs) are the fundamental constituents of plant, microbe and human body proteins. As shown in Figure 10, they are classified as essential AAs (those not produced in sufficient enough quantities in the body and therefore must be obtained from nutrition), non-essential AAs (synthesized sufficiently by the body), and conditional AAs (becomes essential under certain conditions). The heart is a metabolic omnivore, as it is capable of using glucose, FAs and AAs as metabolic substrates. It is well known that the primary metabolic substrate for cardiomyocytes under normal conditions are FAs. However, during hypoxia, FAs cannot be broken down into acetyl CoA and glycolysis becomes the primary source of ATP production, but this yields far less energy. In these circumstances, AA become more important as a fuel source (50). Glucose and FA requires oxygen for ATP production and sufficient production of acidic by-products. AA is of particular interest in this regard due to their potential for nonoxidative metabolism. Glutamate and glutamine are key due to their ready conversion to alpha-ketoglutarate, which not only participates and maintains the TCA cycle, but also provides cellular energy through substrate-level phosphorylation during the conversion to succinate. The ability to produce ATP directly from glutamine and glutamate, makes these AAs important for ischaemic myocardium. However, glutamate can produce only a small amount of ATP through this mechanism (one GTP and one NADH). Ischaemic tissues suffer from several issues of supply and demand: 1) inadequate substrate supply 2) energetic demands and 3) excess acid and ROS production (51). This is particularly relevant under conditions of prolonged ischaemia, when cardiomyocytes must derive energy from substrates that are

independent of oxygen supply. AAs are now becoming more widely appreciated as cardioprotective substrates as they play a central role in supporting cardiac metabolism during cardiac ischaemia. Although it is well known that the heart is capable of metabolizing a wide variety of substrates, its substrate preference under ischaemic conditions is not well understood and is sensitive on the relative concentration of the available substrates. During hypoxia, the heart is unable to metabolize FAs and ketone bodies into acetyl-CoA, forcing the myocardium to utilize metabolic substrates such as AAs that do not require oxidation.

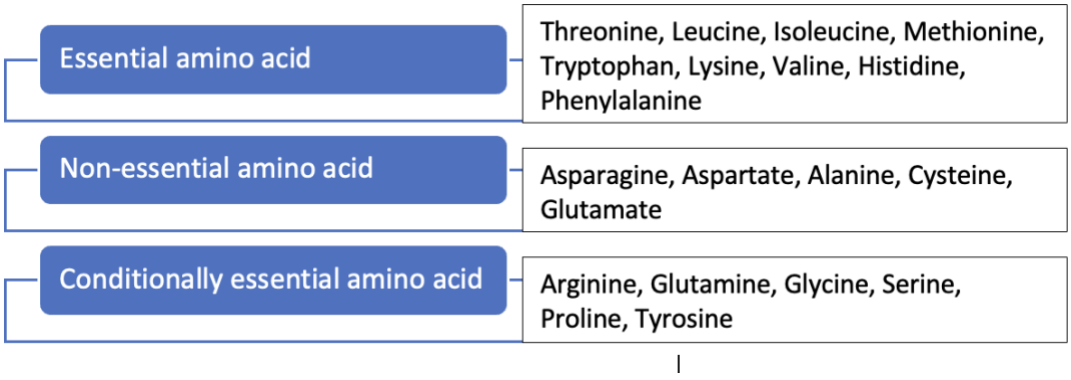

Figure 10: Classification of different type of amino acids based on their synthetic capacity.

1.1.12 Cardiac Metabolic Alterations in Myocardial Ischaemia

The myocardium depends on oxygen to sustain oxidative phosphorylation, the only metabolic process that is capable of providing ATP at the rate required to maintain normal myocardial contraction. A normal healthy heart adapts to its increasing workload through increasing its myocardial oxygen consumption as well as glucose, lactate and fatty acid oxidation (52, 53). In CHD there is a mismatch between oxygen demand and supply, resulting in myocardial

ischaemia. Hence during ischaemia, the oxidative phosphorylation cannot continue, resulting in decreased ATP production causing the myocardium to fail. This causes acceleration of glycolysis and a switch to lactate production (54). In this way, accelerated glycolysis becomes the initial response to ischaemia and oxygen deprivation (55). This is thought to be a natural protective mechanism of the heart to maintain the cell integrity and function, and this has been demonstrated in a number of different animal models of cardiac ischaemia (56, 57).

Depending upon the severity of the ischaemic insult, the patterns of glycolysis vary during myocardial ischaemia. In the healthy myocardium, the glucose taken up by myocardial tissue is converted to pyruvate and ultimately to carbon dioxide and water. Under these conditions there is no glycogen breakdown as sufficient glucose is available for the cells (58). However, in severe cardiac ischaemia, restriction in blood supply resulting from the arterial occlusion compromise the supply of glucose to tissue, leading to glycogenolysis as a route for glycolytic drive. Thus, glycogen becomes the major source of glucose for glycolysis, while pyruvate is entirely converted into lactate (59). This causes accumulation of glycolytic end-products such as lactate. Accumulation of lactate and the subsequent intracellular acidosis results in inhibition of phosphofructokinase, which is the key rate-limiting enzyme in glycolysis. Thus glycolysis, which is the only remaining source of ATP production, also begins to slow. Glycolysis is not as efficient as oxidative phosphorylation, producing only four molecules of ATP for one molecule of glucose. Therefore, the energy store is limited and rapidly depleted, causing irreversible myocyte injury if the insult is left uncorrected (60). These metabolic changes have been shown in

experimental animals such as in the under perfused dog's heart, in which reduced glucose uptake, increased glycogen breakdown and lactate output was observed (61, 62).

FFAs are the preferred metabolic substrate of myocardium under normal physiological conditions providing 60-70% of the energy requirements (63). The focus on FFAs as a major myocardial fuel started with the finding in 1961 that FFAs inhibited glucose oxidation in isolated heart (64). This inhibitory effect of FFAs on glucose metabolism was further expanded by Randle *et al*., (65) and Kurien *et al.,* who subsequently demonstrated high plasma FFA levels in patients with ACS (66). In the early phase of ACS, chest pain, dyspnea and distress provoke increased sympathetic drive and catecholamine surge resulting in increased mobilization of FAA from the adipocytes. The myocardial uptake of FFAs depends on the circulating concentrations of plasma FFAs, and consequently in ACS there is an increased uptake of FFA by the cardiomyocytes. Once within the cardiomyocytes, FFAs are converted to acetyl-CoA leading to accumulation of NAD^+ and acetyl-CoA leading to profound inhibition of β-oxidation (67). In contrast, the other steps of fatty acid metabolism are only slightly inhibited, therefore metabolism in the cytoplasm continues. This results in the accumulation of intermediates like long chain acyl-CoA and acylcarnitine, which has two consequences. First, glucose oxidation and its protective effects are suppressed by inhibition of the key enzyme pyruvate dehydrogenase. Second, the accumulation of toxic intermediates leads to increased myocardial ischaemic injury and life-threatening arrhythmias (68, 69). FFAs have been shown to be detrimental to the myocardium during ischaemia (70). Normally there is a close correlation between cardiac work and

fatty acid oxidation, with fatty acid oxidation increasing and decreasing in parallel with increases and decreases in cardiac work (71). However, following severe ischaemia in rat hearts, fatty acid oxidation rates are high, even though mechanical function is markedly depressed, suggesting that normal control of fatty acid oxidation is altered in the post-ischaemic period (72, 73). There is substantial evidence from animal experiments that high circulating FFA levels could be detrimental to the function and rhythm of the ischaemic heart. Injection of long chain FA directly into the venous blood of animals can cause cardiac arrest (74) and atrioventricular conduction disturbances (75). Octanoate, a short chain FA caused atrioventricular block in isolated rat hearts (76). In intact anesthetized dogs, balloon inflation in the coronary artery followed by administration of intralipid (a triglyceride emulsion) raised blood FFA levels and caused ectopic beats, ventricular tachycardia and fibrillation (77). These results therefore suggest that changes in lipid metabolism during cardiac ischaemia may exacerbate the pathological outcomes of these cardiovascular events.

In the cardiomyocyte, the long chain acylcarnitine (LCAC), is generated as the breakdown product of acetyl-CoA mediated by CPT1. The primary function of LCAC is to traverse the mitochondrial membrane, which is impermeable to acetyl-CoA. This allows acetyl-CoA to be regenerated within the mitochondrial matrix and to enter β-oxidation. This is a bidirectional process meaning that LCAC can be generated within the mitochondria and exported into the circulation (78, 79). Cardiac muscle has a relatively high LCAC content due to its preference for fatty acid metabolism (80). Relative to normal conditions, total LCAC content has been found to increase 3- to 70-fold in the ischaemic myocardium depending on the severity and duration of hypoxia (81, 82). LCAC

is shown to inhibit the enzymes that catabolize lysophosphatidylcholine (LPC) resulting in intracellular accumulation of LPC, predisposing to ventricular arrhythmias (83). The other possible mechanism for this accumulation of LPC is the inhibition of the membrane bound lysophospholipase activity brought about by the abrupt decrease in cytosolic pH observed in ischaemic myocardium (84)**.** *In vitro* studies have demonstrated that LPC is rapidly released from the ischaemic myocardium, and have been implicated as possible biochemical mediators of arrhythmogenesis. LPC has been shown to accumulate in the tissues within the ischaemic zones as well as the effluents leaving from these areas (85, 86). Several animal models of ischaemia have shown that LPC causes cardiac myocyte injury and induces ventricular arrhythmias (87, 88). Inhibition of the production of LCAC has been shown to prevent LPC accumulation, thus preventing lethal ventricular arrhythmias (89). Treatment with CPT I inhibitors (like oxfenicine) prior to onset of hypoxia prevents membrane and cytosolic LCAC accumulation in early ischaemia and the occurrence of electrophysiological derangements (90). These data therefore suggest that localized increases in LPC concentration may also potentiate the initial damage elicited by myocardial infarction.

During myocardial ischaemia there has also been shown to be a significant reduction in the biosynthesis of phosphatidylcholine, due to the diminished conversion of choline to phosphocholine, as a result of severe reduction in ATP levels (91). Phosphatidylcholine, which is the principle phospholipid in the mammalian heart, is an essential building block of all biological cell membranes. The maintenance of the appropriate composition and content of phospholipids in the membrane is essential to ensuring cellular integrity and

membrane function. Phosphatidylcholine is synthesized via the cytidine diphosphocholine (CDP-choline) pathway (92). Choline is actively taken up by the heart and rapidly phosphorylated by ATP to phosphocholine, mediated by the enzyme choline kinase. The requirement of ATP in the CDP-choline pathway suggests that the intracellular levels of ATP will affect the rate of biosynthesis of phosphatidylcholine. Hence during myocardial ischaemia, reduced formation of phosphatidylcholine affects the integrity and function of cell membranes, contributing to myocyte injury (93).

From the available literature it is evident that the mechanical dysfunction in ischaemic heart disease is associated with an energetic deficit often generated by disturbances in one or more metabolic pathways of ATP production. Additionally, it is apparent that the metabolic pathways are sensitive to any insult, such as ischaemia, and attempt to adapt by alterations in the relative activity in these pathways to try to protect cardiomyocyte integrity.

When a coronary artery becomes occluded during ACS, the threatened myocardium rapidly undergoes several metabolic changes that result in significant cellular dysfunction and myocyte injury. Both experimental and clinical studies have shown that the ischaemic myocardium becomes more dependent on the glucose for ATP production. In addition, the potential role of therapies in beneficially altering the metabolic patterns in the early-phase of myocardial ischaemia has evolved as an area of promise. In this regard, the metabolic phenotype of ACS is similar to the failing myocardium observed in heart failure - energy deprivation, excess circulating FFAs, and suppression of glucose. The principle of metabolic therapy for heart failure has been extensively analysed with benefit from metabolically modulating drugs such as

ranolazine, trimetazidine and perhexiline (94). It is therefore essential to focus on metabolic therapies that may help to mediate the change from fatty acid to glucose metabolism.

1.1.13 Targeting Metabolic Pathways:

In heart failure (HF), myocardial energy efficiency is reduced due to the adverse metabolic effects of long-term sympathetic adrenergic system activation and the resulting increased myocardial oxygen consumption (95-97). Beneficial effect of therapies that could potentially counteract the sympathetic adrenergic system activation and the shift in myocardial substrate use from FFA to glucose oxidation have been studied (98). Metabolic therapies in HF have shown that β-adrenoreceptor blockage, by reducing the circulating levels of FFA and by altering the metabolic pattern from the use of FFA to glucose (99), can result in dramatic improvement in the left ventricular function in both ischemic and non-ischaemic cardiomyopathies (100). Wallhaus *et al*., studied the effect of carvedilol therapy on myocardial FFA and glucose metabolism in patients with HF using positron emission tomography. The study showed that administration of carvediol over a 3-month period to patients with chronic HF resulted in a 57% decrease in the rate of myocardial fluoro-6-thiaheptadecanoic acid (FTHA) uptake, consistent with a marked inhibition of myocardial FFA oxidation (101). The potential explanation for this observation is due to the decrease in the activity of myocardial CPT 1, a key enzyme involved in the mitochondrial FFA uptake (102). No significant change was observed in the myocardial glucose use, consistent with a relative switch in myocardial substrate from FFA to glucose utilisation (103). A further study by Panchal *et al*., in a canine model of

HF demonstrated a 28% reduction in the activity of CPT 1 after treatment with metoprolol (104). Decreased CPT 1 activity by beta-blockers could account for a significant lowering of myocardial FFA oxidation. This provides a potential mechanism for improved energy efficiency seen in patients with HF who are treated with beta-blockers. These metabolic effects of beta-blockers may contribute to its remarkable clinical success in the therapy of HF. Perhexiline, a reversible CPT-1 inhibitor has also shown to be beneficial as a treatment for heart failure putatively by reducing mitochondrial FFA transport (105). In a randomized, double-blinded study of 56 optimally-medicated HF patients over 8 weeks, perhexiline improved symptoms, peak exercise oxygen uptake, and LV function (relative improvement of ≈40%) (106).These studies in HF demonstrate that through a better understanding of changes in cellular metabolism we can improve treatments and outcomes for patients with cardiovascular disease.

Similar understanding of how myocardial ischemia affects cardiomyocyte metabolism could help us identify new methods for reducing the ischemic damage caused, and thus reduce death and disability elicited by these pathologies. Glucose uptake and glycolytic ATP production are important determinants of viability during ACS. Studies examining the effects of treatments which could increase glucose utilization by cardiac muscle have also shown the ability to improve outcome. Metabolic therapy with glucose-insulin-potassium (GIK) have gained immense attention in ACS. For instance, the administration of GIK in the acute phase of myocardial infarction have shown to inhibit FA and promote glucose metabolism (107). This effect was associated with a protective effect on the ischaemic myocardium, by preventing

the accumulation of LCAC. A meta-analysis of all GIK trials in ACS have showed a mortality reduction of 28% (108). The Diabetes Mellitus Insulin-Glucose Infusion in Acute Myocardial Infarction (DIGAMI) trial, a randomized placebo-controlled clinical trial of GIK therapy, also showed a significant reduction in mortality in patients with ACS treated with glucose and insulin (109). This effect was thought to occur as insulin stimulates myocardial glucose metabolism and inhibits FA metabolism (110). Additionally, experimental studies have shown that insulin given alone improves the contractile function of the heart during myocardial ischaemia (111). In patients with chronic ischaemic cardiomyopathy with a left ventricular ejection fraction of <45%, GIK lessened ventricular dysfunction (112). A large Canadian observational study in diabetic patients with HF found that metformin (an insulin sensitizer) but not sulfonylurea therapy (which increase insulin secretion from the pancreas) was associated with reduced all-cause mortality (113) . Parenteral application of Glucagon-like peptide, an endogenous insulin-sensitizer, improves myocardial glucose uptake and LV function in canine and human HF (114). Invasive strategies to modify the metabolic pathways in HF have demonstrated that by reduction of the inefficiency of uncoordinated ventricular contractions, cardiac resynchronization therapy increases glucose metabolism to improve cardiac efficiency and cardiac energetics (115).

These data therefore demonstrated that stimulation of glucose metabolism and reduction of FFA in the ischaemic tissue by targeting therapies remains a viable option in the management of HF, arrhythmias and sudden cardiac death, a feared complication in patients with ACS. In addition to the therapeutic benefits,

the breakdown products of these metabolic pathways could serve as a potential diagnostic or prognostic biomarker for ACS.

1.1.14 Alterations in plasma metabolite concentration as novel biomarkers of cardiac ischaemia

The term biomarker was introduced in 1989, and is defined as measurable and quantifiable biological substances which are used as an indicator of a specific biologic state relevant to a specific disease process (116). A biomarker may be measured as a biosample (blood, urine, or tissue), it may be a recording obtained from a person (ECG), or an imaging test (echocardiogram or CT scan). In 2001 the Biomarker Definitions Working Group standardized the definition of a biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biologic process, pathogenic process or pharmacologic responses to therapeutic intervention” (117). Biomarkers can thus indicate a variety of health or disease characteristics and can be classified as an antecedent biomarkers (identifying the risk of developing as illness), screening biomarkers (screening for subclinical disease), diagnostic biomarkers (recognising overt disease), staging biomarkers (categorising disease severity), or prognostic biomarkers with a potential to predicting future disease course and response to therapy (118). Biomarkers play an important and indisputable role in the diagnosis and management of patients with ACS. It also provides information that can reflect the pathophysiology and may be useful in directing and delivering treatment strategies appropriately to improve the patient care. The overall expectations of a cardiac specific biomarker is to enhance the ability of the clinician to optimally manage the patients. For example, in a patient presenting to the emergency department with acute chest

pain, a biomarker may help reliably differentiate patients with ACS (e.g. troponin), acute pulmonary embolism (D-Dimer or ventilation-perfusion scan) or an aortic dissection (CT scan), in a timely manner. Furthermore, in patients with established diagnosis of ACS, specific biomarkers may reflect different components of the pathophysiology of ACS: For instance, troponins are markers of myocardial necrosis, C-reactive protein and myeloperoxidase reflect inflammatory process of the cardiac tissues and natriuretic peptides reflect neurohormonal activation and haemodynamic stress (119). Similarly, in patients presenting with STEMI, a biomarker may be able to assess the likelihood of the extent of myocardial damage (e.g. troponin) and progression to heart failure (e.g. B-type natriuretic peptide).The emergence of cardiac biomarkers has not only provided unique insight into the disease process, but are also now an essential criterion in the diagnosis of ACS (120).

Over many years there have been considerable advances which led to comprehensive understanding of the pathophysiology and biomolecular basis of CHD. As a consequence of this, the research for novel biomarkers of cardiovascular disease has been assisted by this new knowledge. Furthermore, new technologies such as metabolomics, now allow large-scale screening of blood samples in a short period of time, providing more scope for researchers to apply these techniques according to clinical demand. However, the rapid growth in the number of potential novel biomarkers has emphasized the importance of their evaluation before being absorbed into clinical practice, by thorough validation to establish their value beyond the existing markers (121). An ideal biomarker should convincingly demonstrate its value and utility in helping with diagnosis, disease stratification and prognostication, beyond

that of the existing markers and should contribute to the drive towards better patient care. Benchmarks of a perfect biomarker and the principles of a disease-specific biomarker are shown in Figure 11 & 12 below respectively. Assessment of the clinical potential of a novel biomarker may be based on three fundamental questions: 1) Can the clinician measure it? 2) Does it add new information? and 3) Does it help the clinician to manage patients? To be clinically useful, analytic methods must be readily available that allow reliable measurement, prompt turnaround time, and reasonable cost (122). The most important criterion regarding the clinical value of a candidate biomarker is the consistency and strength of the association between the biomarker and the disease of interest. Biomarkers have a variety of clinical applications that may enhance the care of patients with CVD (123). Broadly, they may be used for a) diagnosis of an acute or chronic clinical illness; b) early detection of a subclinical disease; c) risk stratification of patients with a disease; d) selection of appropriate therapeutic intervention; and e) monitoring response to therapy (124).

So far, the best evidence base for an effective marker of CVD disease lies with assessment of plasma concentration of troponins, creatine kinase myocardial band (CK-MB), myoglobin and lactate dehydrogenase (discussed below). However, many other potential biomarkers have emerged and are under intense research.

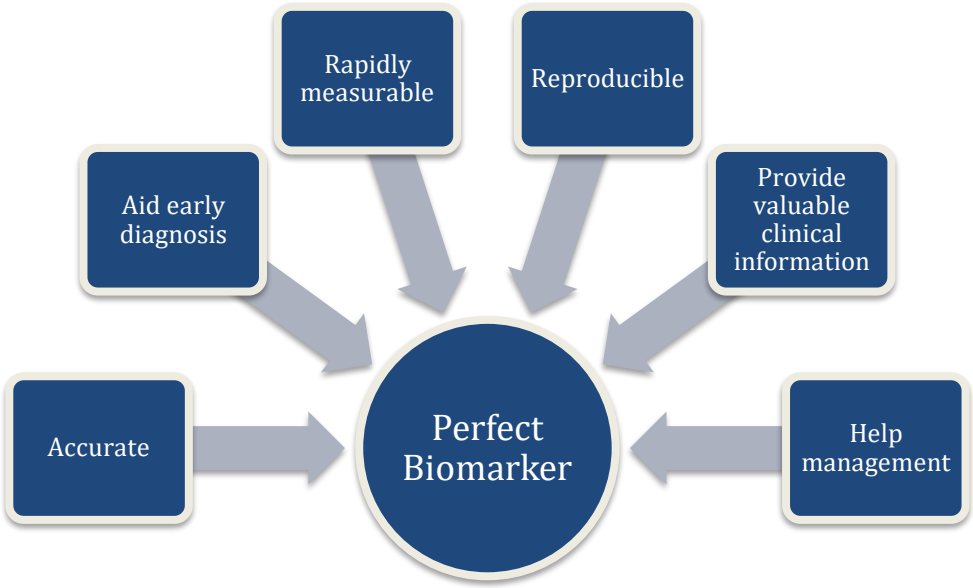

Figure 11: Represents the benchmark of a perfect biomarker. A perfect biomarker will be of clinical value only if it is accurate and reproducible. For screening biomarkers, high sensitivity and specificity and predictive values and low costs are important. For diagnostic biomarkers, in addition to the aforementioned characteristics, rapid sustained elevation, high tissue specificity (eg, troponins indicating myocardial origin), release proportional to disease extent and assay features conductive to point-of-care testing is crucial.

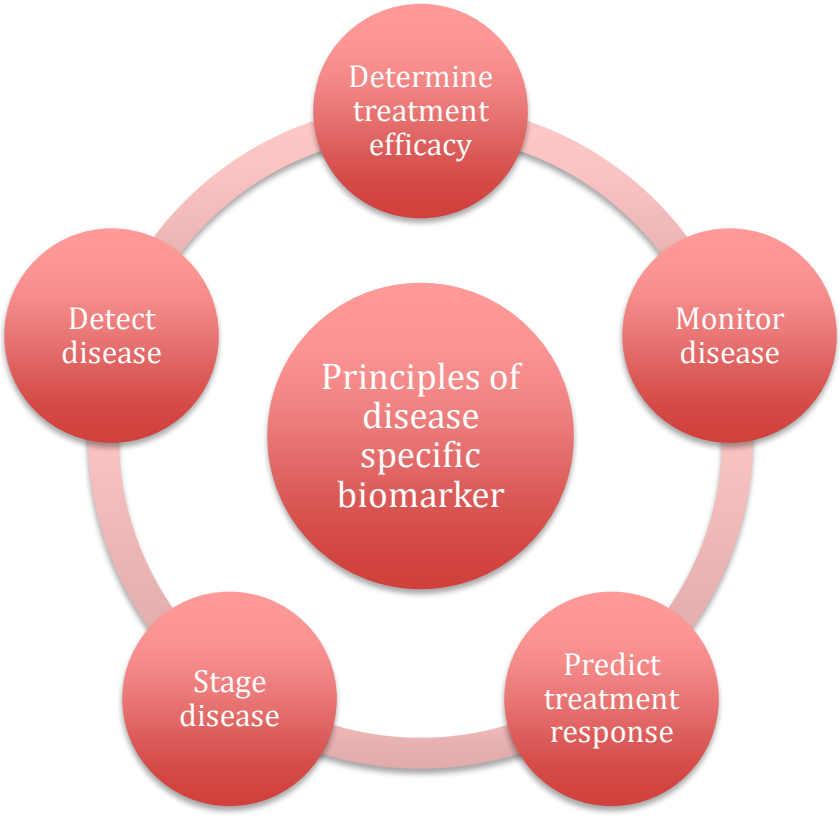

Figure 12: Represents the fundamental principles of disease specific biomarkers. For biomarkers particularly monitoring disease progression or response to therapy, narrow intraindividual variation and tracking with disease progression or response to therapy is more important than sensitivity or specificity, as patients serves as their own controls (e.g. B-type natriuretic peptide in chronic HF). Cost may be less important for prognostic markers as only patients with disease are tested.

1.1.15 The role of biomarkers in the diagnosis of acute coronary syndrome

Cardiac biomarkers play a major role in the diagnosis of ACS, particularly in situations where other diagnostic evidence is lacking, such as atypical cardiac chest pain or non-specific ECG changes. Several biomarkers have emerged as useful diagnostic tools for ACS, such as Troponin, CK, CK-MB, Lactate dehydrogenase and Myoglobin (125). The various time courses of biomarker

response are shown in Table 1. However, because of their increased specificity compared to other markers, serum troponins are currently the preferred biomarker for the diagnosis of myocardial injury.

MARKERS	ONSET	PEAK	DURATION
Troponin	3-12 hours	18-24 hours	10 days
CK-MB	3-12 hours	18-24 hours	36-48 hours
Lactate dehydrogenase	6-12 hours	24-48 hours	6-8 days
Myoglobin	1-4 hours	6-7 hours	24 hours

Table 1: Time course of Biomarker response in Myocardial Infarction. Troponin and CK-MB are detected early when compared to LDH. Myoglobin is detected earlier than other biomarkers but remains elevated only for 24 hours.

1.1.15.1 Troponins

Troponins are cardiac regulatory proteins which control the calcium-mediated interaction of actin and myosin which underlies myocardial contraction (126). The troponin complex consists of three subunits namely, troponin T (TnT) which binds to the tropomyosin, troponin I (TnI) which binds to actin and inhibits actin-myosin interaction and troponin C (TnC) which binds Ca^{2+}.

Troponins are currently the gold standard test for the detection of ACS. Plasma Troponin levels are elevated at 3 to 6 hours after ACS, and can remain elevated up to 10 days beyond the event. These temporal dynamics helps not only with diagnostic precision, but also permits late diagnosis (127). Troponin concentration above 99th percentile of normal is considered as the diagnostic criteria for myocardial infarction, according to the European Society of Cardiology and American College of Cardiology consensus recommendation

(128) and most studies support that the diagnostic accuracy is maximised using this criterion (129). It has good sensitivity and specificity for detecting myocardial necrosis and has also emerged as a powerful predictor of prognosis (130). The correlation of the degree of troponin elevation with mortality has been shown in major trials like Thrombolysis in Myocardial Infarction (TIMI) IIIB trial (Figure 13). The study showed troponin levels were measured at baseline in 1404 patients with UA or NSTEMI, the degree of troponin rise correlated with 42-day mortality and more over there was a progressive increase in risk with higher troponin levels (131).

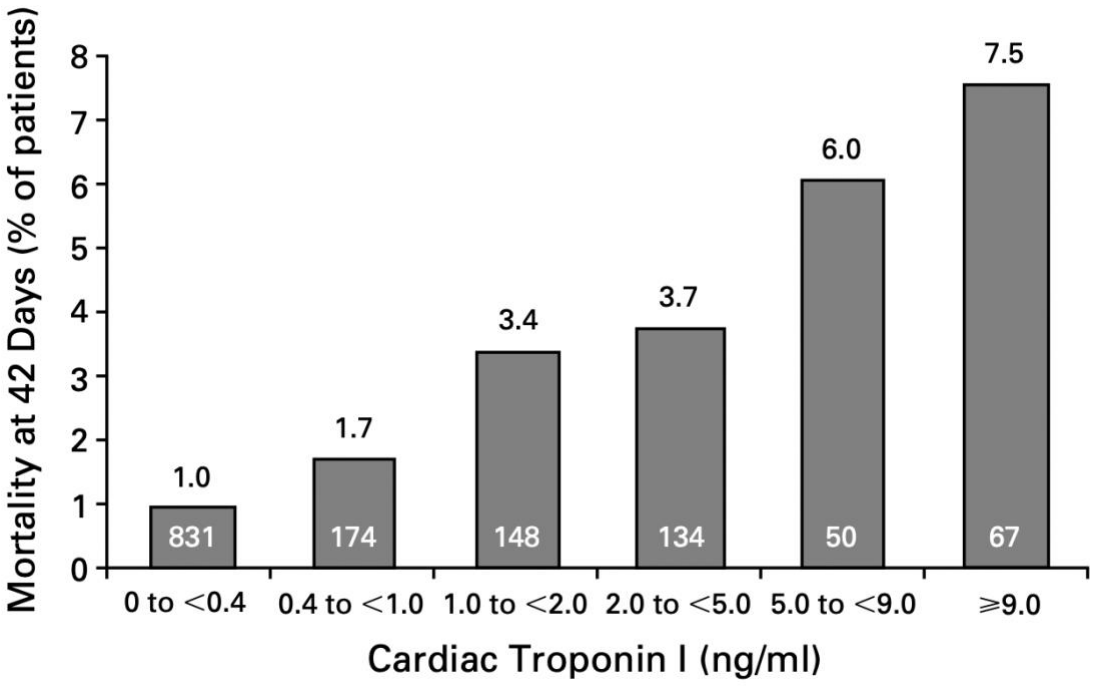

Figure 13: Mortality rates at 42 days are shown for ranges of cardiac troponin rise measured at baseline. The number at the bottom of each bar are the numbers of patients with cardiac troponin I levels in each range and the numbers above the bars are percentages, Figure reproduced from (131)

Fragmin During Instability in Coronary-Artery Disease (FRISC) trial studied 917 patients with NSTEMI (Figure 14), and showed that elevated troponin

measured within 24 hours of presentation was associated with an increased incidence of death at a mean follow up of 37 months (132).

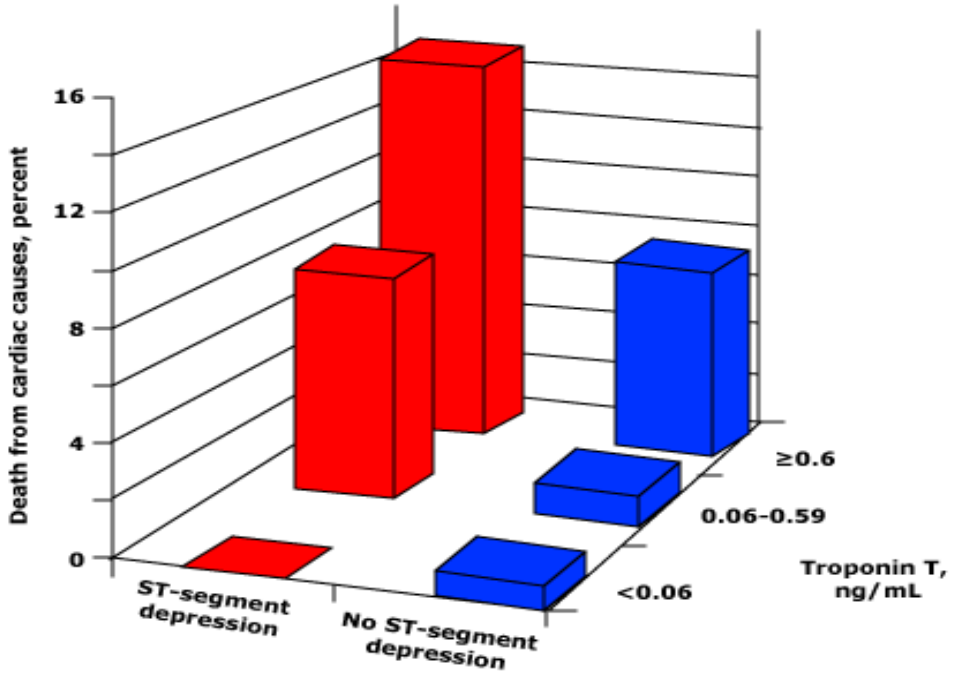

Figure 14: Incidence of death from cardiac causes at two years according to the presence or absence of ST-segment depression on the admission ECG and the maximal troponin T levels during the first 24 hours after enrollment. Figure reproduced from (132).

Despite its strengths as a biomarker in the management of ACS, troponins have a number of limitations. Firstly, the normal cut-off point for myocardial infarction diagnosis is about 0.01 ng/ml, yet most of the routine laboratory assays do not reliably measure levels below 0.03 ng/ml. Therefore, patients with troponin concentration between 0.01 ng/ml and 0.03 ng/ml, who are currently classified as having myocardial infarction according to the criteria, are potentially undetected by current tests and are miscategorised as a low risk group (133). Secondly, the immunoassay used to measure troponin can produce false results in some people which could be difficult to identify (134). Thirdly, it is

crucial to identify high risk patients early, who may benefit from particular treatment strategies, who are otherwise at increased risk of adverse outcome. Troponins are unable to detect myocardial ischemia in the absence of necrosis and hence unable to yield early diagnosis, as they only become elevated 3-12 hours after myocardial injury. Therefore they are only able to indicate irreversible myocardial injury (135). A biomarker which could detect reversible injury might provide a potential therapeutic benefit. Lastly, although troponin provides improved specificity for the detection of myocardial injury, its elevation is not completely selective for ACS. Troponin elevation is also known to occur in a variety of other clinical conditions, such as moderate-to-severe pulmonary embolism (136) with acute right heart overload, heart failure (137) and myopericarditis (138). The troponins are more challenging to interpret for the diagnosis of ACS in patients with chronic kidney disease (CKD). This is because stably elevated troponin levels are commonly observed in CKD patients in the absence of clinical evidence of ACS. (139-141). The cause of low-level troponin elevations in patients with CKD is possibly related to chronic myocardial injury or other underlying structural heart disease rather than epicardial coronary artery disease. However, it is not clear whether chronic troponin elevation is related to decreased renal clearance or increased cardiac release (142). Despite this, the troponin elevations are usually more modest and transient in these disorders, in contrast to the larger, dynamic, and more prolonged elevation with acute myocardial injury (143).

1.1.15.2 Higher-sensitivity troponin

With recent advances in technology, high sensitivity troponins have emerged as an important biomarker with added advantage in early diagnosis and risk stratification in patients with acute coronary syndrome. High sensitivity troponin assays detect concentrations of the same protein that conventional assays are aimed at detecting, just in much lower concentrations. According to the 2012 expert consensus, a high-sensitivity assay should have a coefficient of variance of <10% at the 99th percentile value, and a concentration below the 99th percentile should be detectable above the assay's limit of detection for >50% of healthy individuals in the population of interest (144). Its improved sensitivity has allowed us to detect concentrations that are tenfold lower than those detected with the standard assay and its first low level elevations of troponin detectable within 90 to 180 mins of index cardiac event (145, 146). The high sensitivity troponins share the same prognostic ability as the earlier generation troponins. It is a strong prognostic marker for cardiovascular death and recurrent ischaemic events. A community-based prospective study of 1499 individuals, showed that detectable troponin levels were observed in 820 participants with history of hypertension, diabetes and CKD and was associated with a subsequent increased risk of cardiovascular events and all-cause mortality (147). A recent study further showed a strong association between baseline concentration of high-sensitivity troponin and the risk of ACS and HF among patients with diabetes and stable CAD (148).

Despite the increase in accuracy and early detection of ACS with high sensitivity assay, there are still a few problems. A nominal level of high sensitivity troponin at baseline is seen in apparently healthy subjects, and also

the presence of biological variability over time can lead to misinterpretation of the results. These changes could be due to circadian rhythms, seasonal changes or a random biological fluctuation around an inherent set point that may be specific to an individual (149). Due to the variability of this biomarker over time and with detectable levels in healthy individuals, further studies are needed to assess how to apply this test in a more reliable manner. As the sensitivity and precision of troponin assay have improved, false positive results have become less of a problem. However, given that the differential diagnosis is significantly influenced by positive troponins, positive results should be closely correlated with clinical presentation. While the new assay has increased its sensitivity for cardiac myocyte necrosis, they are not specific for the aetiology of the cardiac cell death and there are a lot of debate on its role of detection of early myocardial ischaemia.

1.1.15.3 Creatine kinase

Creatine kinase and its MB isoenzyme (CK-MB) was the preferred marker of cardiac injury for several years. It has high specificity for cardiac tissue injury particularly in patients with ischaemic symptoms, in the absence of skeletal muscle damage. Increases in CK usually begin four to six hours after the onset of MI, peaks at 18 to 24 hours and returns to baseline levels by 36 to 48 hours (150). Total CK elevation two-fold above normal with a simultaneously elevated CK-MB is required for the diagnosis of an MI. However, the important drawback is the prevalence of false positive results, which can result from skeletal muscle damage, hypothyroidism, renal failure and pulmonary embolism (151, 152). CK is therefore comparatively less sensitive and specific than troponin in the diagnosis of an ACS. Several trials in the past have compared troponin with

CK-MB, a review of around 30,000 patients in the multicentre, Can Rapid Risk Stratification of Unstable Angina Patients Suppress Adverse Outcomes with Early Implementation of the ACC/AHA (CRUSADE) trial, showed that troponin was more sensitive and has enhanced prognostic value than CK-MB (153). Similarly in a report of over 10,000 patients with ACS from the multicentre GRACE registry showed that the in-hospital mortality was highest when both troponin and CK-MB were positive (7.7 percent), intermediate in troponin-positive/CK-MB-negative patients (3.9 percent), and lowest in patients in whom both markers were negative and those who were troponin-negative/CK-MB-positive (1.7 and 2.3 percent, respectively) (154). Although high sensitivity troponins are currently the gold standard biomarker for the diagnosis of ACS, CK-MB, when measured in parallel with troponins, may add incremental value as a multimarker, for the purpose of predicting disease and patient outcomes.

1.1.15.4 Lactate Dehydrogenase (LDH): Lactate dehydrogenase is a tetramer and has five isoenzymes. Heart, kidney, stomach and red cells are important sources of LDH1 and skeletal muscle and liver contains LDH5 (155). LDH was commonly used in the past in combination with CK-MB to diagnose acute MI. The levels rise approximately 10 hours after acute MI, peaks at 24-48 hours and remains elevated for 6-8 days (156). LDH was mainly used in the past to help with the diagnosis of late presentation MI, however as the troponin remains elevated for a considerable length of time, it can identify these cases and moreover since troponins are more specific than LDH and there are no added advantages over troponin, LDH is no longer used in the diagnosis of an acute MI (157).

1.1.15.5 Myoglobin is a heme protein that is rapidly released from the injured tissue and has a short half-life of around nine minutes (158). In the past it was postulated to be useful as an adjuvant to troponins for early diagnosis of ACS. However, there is little advantage for the use of myoglobin as a marker of early myocardial injury with the emergence of sensitive troponin assays (159). In addition, the low specificity and more rapid rises and fall of myoglobin during MI results in the staccato pattern causing clinical confusion (160).

1.1.16 Role of Biomarkers in the Risk Stratification of ACS

Risk stratification of patients with ACS is an important part of the management strategy in delivering the most appropriate patient care. Risk stratification begins upon the initial presentation of ACS and is then a continuous process to help predict those who are at increased risk of recurrent ischemic events, arrhythmia or death. It is not only aimed at identifying patients who require early coronary intervention, but also predicting at risk patients following discharge. In the Euro Heart Survey, which involved over 10,000 patients with ACS from 103 hospitals, the 30-day mortality was higher in patients with ACS with undetermined ECG (13.3%) when compared to STEMI (8.4%) and NSTEMI (3.5%) (161). In the Global Use if Strategies To Open Occluded Arteries in Acute Coronary Syndromes (GUSTO-IIb) trial, one of the largest trial conducted in early 1990, involving 12,142 patients with ACS, the one year mortality rate was significantly high in NSTEMI (11.1%) when compared to STEMI (9.6%) (162). Although, the basis of the steeper increase in long-term mortality among patients who are asymptomatic or with a nondiagnostic ECG when compared to patients with STEMI is not entirely clear, it is important to appreciate that only

symptomatic ischaemia was identified during hospitalization (163). We know that silent ischaemia and its frequency and duration bear a direct correlation to prognosis (164). A better prognostic triage and selective therapeutic strategies for such patients might be possible with the use of novel cardiac biomarkers acquired early in the course of the event. Although there are well established and validated ACS risk scoring system such as TIMI score (165) and GRACE score (166), several studies have shown that biomarkers have an additional role and can independently predict adverse outcome. In this respect troponin and CK-MB have established themselves as prognostic markers in addition to their diagnostic value. Several other biomarkers such as natriuretic peptides and C-reactive protein have been proven to provide valuable prognostic information in ACS (167). Moreover, a lot of interest has developed around novel markers like matrix metalloproteinase, myeloperoxidase, heart-type fatty acid binding protein and high sensitivity troponin.

1.1.16.1 Natriuretic peptides

Plasma natriuretic peptides are increasingly being recognized as an important prognostic marker in patients with ACS and HF. Brain natriuretic peptide (BNP), as the name indicates, was initially identified in the brain but is primarily released from the ventricles of the heart. Atrial natriuretic peptide (ANP), is primarily released form the atria in response to atrial stretch. In ACS and HF, the cardiac neurohormonal system is activated and circulating plasma levels of BNP, ANP and their prohormones (N-proBNP and N-proANP) are elevated. Increased secretion of these hormones may partially counteract the effects of endothelin and angiotensin II, thus limiting the degree of sodium retention and

vasoconstriction. In normal subjects, the plasma BNP concentrations are approximately 10 pmol/L. A BNP level of >100 pmol/L provides the diagnosis of HF with reasonable accuracy (168). The levels are observed to be higher in older individuals and women (169) and lower in obese individuals (170). In the acute phase of myocardial infarction, the intracardiac pressure rises and depending on the extent of myocardial damage, the concentration of BNP and ANP rises rapidly and correlates directly with right atrial pressure, pulmonary capillary wedge pressure and left ventricular end diastolic pressure (171). The multicenter Biomarkers in Acute Congestive Heart Failure (BACH) trial evaluated the N-terminal portion of ANP, known as proANP and compared with BNP in 1640 patients attending the emergency department with shortness of breath. The study showed proANP was not inferior to BNP for the diagnosis of acute HF (172). Although the plasma ANP levels emerged as an important prognostic tool of myocardial infarction, in the Sleep Apnea Cardiovascular Endpoints trial and Cooperative North Scandinavian Enalapril Survival 11 trial, the multivariate analysis that included patients with left ventricular dysfunction showed that the independent predictive value of plasma ANP levels was markedly reduced (173). Furthermore, studies showed that BNP is more useful than ANP in risk stratification of patients with ACS (174). The reason being, in normal subjects, the circulating concentration of BNP is 20 percent lower than ANP, but exceeds that of ANP in patients with HF. This wider range of concentrations makes measurement of BNP more useful than ANP in clinical practice (175).

BNP is currently used as the gold standard laboratory test for the diagnosis of heart failure. BNP levels rise rapidly over the first 24 hours following ACS and

then tend to stabilize (176). When measured one to seven days after ACS, BNP elevation identified patients at risk for LV dysfunction, HF and death (177). It was therefore hypothesized that early intervention in ACS patients with elevated BNP may be beneficial. However, a report from the Treat Angina with Aggrastat and Determine Costs of Therapy with Invasive or Conservative Strategies (TACTICS)–TIMI 18 study did not support this, and showed that there was no difference in the benefit of the early invasive strategies in patients with and without BNP elevation (178). In contrast, the FRISC II study showed mortality benefits with early intervention in patients with elevated BNP (179, 180). One possible reasons for this discrepancy is the fact that the clinical decision-making and therapeutic implications of BNP in ACS is still in its infancy. For example, the optimum timing of measurement is not yet clear and in addition, the specific threshold values may be affected by age, sex, and neurohormonal activation. It is clear that in patients with suspected ACS, BNP adds important and unique prognostic information. Two clinical trials which involved more than 2000 patients with non-ST-elevation ACS, oral glycoprotein IIb/IIIa Inhibition with Orbofiban in Patients with Unstable Coronary Syndrome (OPUS-TIMI) and TACTICS-TIMI 18 trials, showed that simultaneous assessment of BNP along with troponin and C-reactive protein at the time of presentation proved to be a powerful predictor of cardiovascular morbidity and mortality (181)

1.1.16.2 C-reactive protein

C-reactive protein (CRP) is an acute phase protein that is produced predominantly by the hepatocytes under the influence of interleukin (IL)-6 and

tumor necrosis factor-alpha (182) Inflammation in an important factor in the development of atherosclerosis and ongoing inflammation increases the vulnerability of an atherosclerotic lesion to rupture causing ACS (183). Data from the different epidemiologic studies have shown significant association between CRP and prevalence of atherosclerosis and cardiovascular events (184) The CRP assays are broadly separated into high sensitivity CRP (hs-CRP) and traditional assays (185). The hs-CRP assay detects plasma concentrations of CRP as low as 0.3mg/L when compared to the traditional assay which has a detection limit of 3 to 5 mg/L. Hence hs-CRP is used to assess cardiovascular risk because it is able to quantify CRP within the normal range seen in asymptomatic patients (186). In 2003, the American Heart Association and the Centre's for Disease Control and Prevention issued guidelines for the use of hs-CRP [43]. This higher sensitivity is required for risk stratification of cardiovascular disease (187), whereby levels less than 1, 1 to 3 and >3 mg/L re considered as low, moderate and high risk respectively (188). Levels of hs-CRP greater than 3 mg/L have been shown to predict recurrent coronary events, poor outcome in patients with unstable angina and also thrombotic complications following percutaneous coronary angioplasty. The Framingham Offspring Study evaluated CRP levels of 3006 asymptomatic patients and followed up for an average of 12 years. Patients with a CRP greater than 3mg/L had a significantly higher cardiovascular event rate and CHD-related mortality risk (189). In the Prevention of Events with Angiotensin-Converting Enzyme (PEACE) trial, a large study involving 3771 patients with stable CHD, it was shown that patients with higher baseline hs-CRP levels had an increased rate of cardiovascular events when compared to those subjects

who had levels less than 1 mg/L (190). This assay has also been shown to provide useful prognostic information in patients with NSTEMI. In the GUSTO IV ACS Trial over 7000 patients with NSTEMI were evaluated. The study showed that raised CRP and troponin had independent and complimentary prognostic significance (191). Similarly raised CRP has also proved to be useful to predict adverse outcome in patients after acute MI (192). These studies have therefore shown that hs-CRP has emerged as a useful screening tool as it can strongly and independently predict risk of developing ACS among apparently healthy individuals. More importantly it has also been demonstrated to add prognostic information at all levels of risk as determined by the Framingham Risk Score (193).

1.1.17 Limitations of currently-used biomarkers

Improvements in our understanding of the pathophysiology of atherosclerosis and ACS have led to a surge in the development of biomarkers to characterize these processes and to predict prognosis. Each biomarker discussed here has limitations. An ideal biomarker is one in which there is an easily measurable increase that clearly aligns with the diagnosis or a predictable outcome. cTn appears to be the most sensitive and specific biomarker among all other diagnostic biomarkers of ACS. However, cTn is a marker of myocardial necrosis, meaning that it reflects irreversible myocyte death. In addition, single measurement of cTn on arrival to the hospital is insufficient because a single measurement of cTn will not detect 10% to 15% of patients at-risk of ACS (194).The optimal timing for obtaining a sample for measurement of biomarkers is another limitation. cTn and CK-MB levels rise within 3 to 4 hours after the

onset of symptoms. Although these biomarkers appear to be sensitive, the temporal rise in the serum concentration of these biomarkers do not permit an early detection (1-3 hours) of myocardial injury (195) and does not support maximal sensitivity of these markers until 6 hours after the onset of symptoms (196).

1.1.18 How could a metabolite screen be better?

The traditional approach of biomarker discovery has been ineffective as this usually focuses on one or few potential candidate biomarkers at a time. This led to the low rate of biomarker discovery with clinical utility. The pathophysiological changes in ACS are influenced by many factors, including comorbidities, genetic and environmental factors. The recent advances in genomics, transcriptomics, proteomics and metabolomics have revolutionized the field of biomarker discovery. The advances in bioinformatics coupled with cross-disciplinary collaborations (e.g. biologists, clinicians, chemists, computer scientist and physicists), have enhanced the ability to retrieve, characterize and analyse large amount of data with precision. Changes in the plasma concentration of metabolites in the human body are rapid and reflects the physiological state of the body very closely. High-throughput platforms in metabolomics has evolved as a powerful platform for more global approach to discover the mechanism and molecular fingerprints of ACS. A “systems biology” approach that investigates multiple components of a disease network may provide better insights into disease diagnosis, prognosis and management (197). Metabolomics provide the ability to monitor the rapid and dynamic

changes in the body that may closely reflect the disease process and provide opportunities for novel biomarker discovery.

1.2 A 'systems biology' approach and the application of metabolomics in cardiovascular disease

1.2.1 Metabolites and the Human Metabolome

Metabolites are organic molecules with a molecular weight of <1500 Da, which take part in reactions required for growth, maintenance and normal function of the human body (198). In humans, these metabolites can be exogenous (dietary or drug related), endogenous (substrates, intermediates and final products of chemical reactions), and derived from the effect of the microbiome. Metabolites include carbohydrates, peptides, lipids, nucleotides, amino acids, organic acids and other classes of small molecules (199). The collection of metabolites present in a cell or an organism is collectively called a metabolome. This global collection of metabolites that eventually occurs as the end result of the combined effects of all the different metabolic processes in the body, acts as a final avenue to explore the effects of changes in gene function and cellular processes. The entire chain of this cellular processes is shown in Figure 15 (200). Although the exact size of the human metabolome remains uncertain, at present it is estimated that it consists of approximately 3000 endogenous metabolites (201).

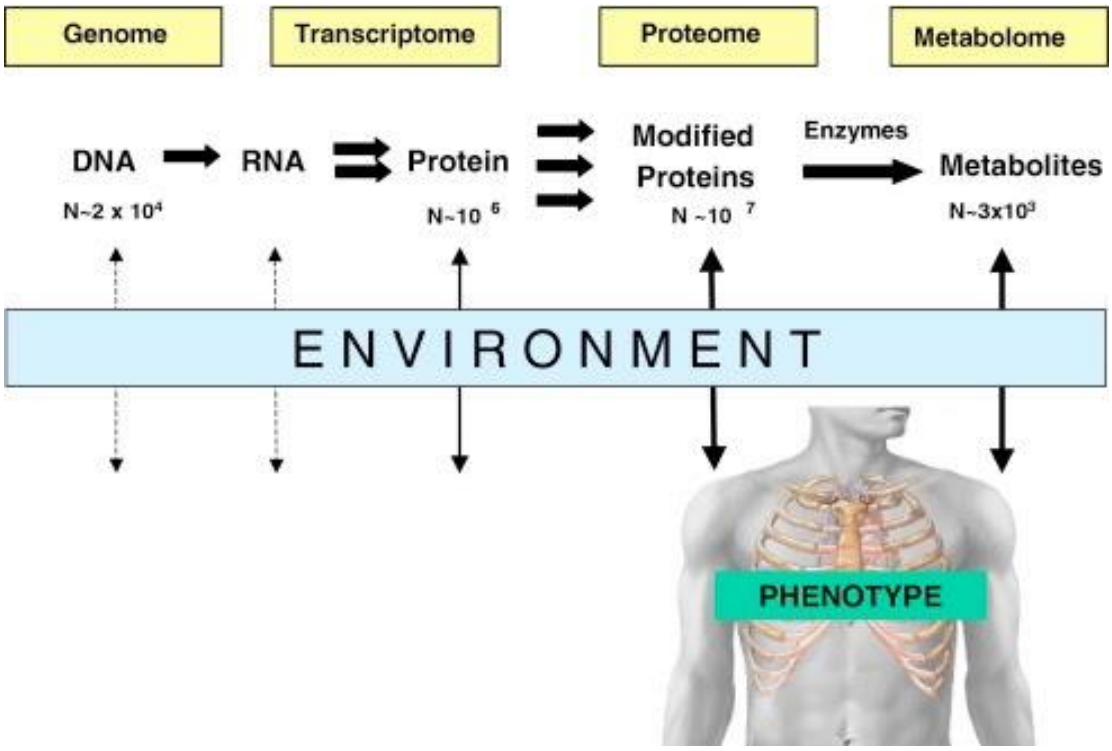

Figure 15: The entire chain of transcriptional and translational regulation of cellular processes with metabolites as the final avenue. Figure reproduced from (200).

1.2.2 History of Metabolomics

Metabolomics began to form in the 1970s when Arthur Robinson and Linus Pauling investigated urine vapour and found that the chemical constituents of the urine contained useful information for monitoring human health (202). However due to the lack of adequate technologies and knowledge, the information available was not sufficient enough to understand the interaction of metabolites, its link to the human genome and its response to genetic and environmental variations. In this context the publication of the first draft sequence of the human genome by Venter *et al*. in 2001 was a major milestone in large-scale analysis of the biological events occurring within the human body (203). This was then followed by the rapid expansion of different omics

approaches to gain information from the genome sequence, such as analysis of genetic variation (genomics), gene expression (transcriptomics), protein expression (proteomics) and their metabolites (metabolomics). In 1998, Oliver and colleagues provided the original definition of the metabolome (204). Since 2000, with advances in technologies and more comprehensive understanding of the human metabolome, the application of metabolomics has become widely adopted into current biomedical research, as shown in Figure 16. (205).

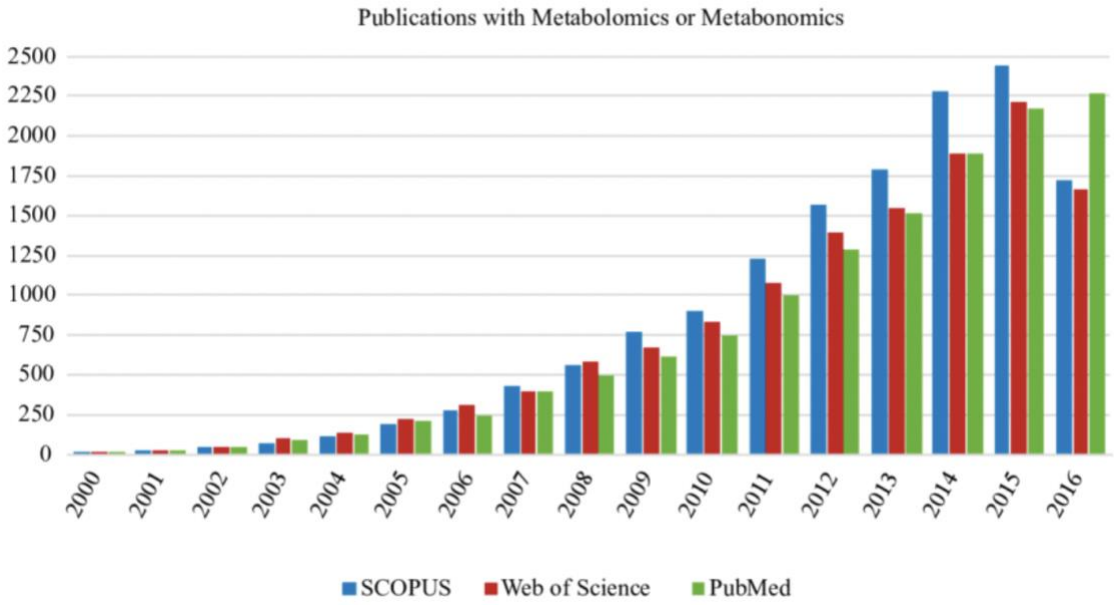

Figure 16: Rapid expansion of metabolomics with growing number of publications. Figure reproduced from (205) .

1.2.3 Metabolomics

Metabolomics is a growing field which detects the unique chemical fingerprints of a metabolome and analyzes different patterns of metabolite profiles produced by the active living cell, tissue or organism and compares them to disease/dysfunctional states (206)**.**

Metabolomics have gained attraction as it offers several advantages over transcriptomics, genomics or proteomics:

1) The metabolome is the final avenue of the cellular processes and reflects more closely the phenotype of an organism (207).

2) The metabolome is numerically small therefore more tractable, for example the baker's yeast (Saccharomyces cerevisiae) has 584 identifiable metabolites and more than 6600 genes whereas the number of proteins are in millions (208).

3) Changes in the flux through metabolic pathways can cause substantial changes in the concentration of the metabolites, even when alterations in the concentration of genome or proteins are minimal or even undetectable**.**

4) Metabolomics is a high–throughput strategy and is less expensive compared to proteomics or genomics (209).

The advantage of metabolomics lies in its ability to profile large number of metabolites at the same time instead of analyzing individual components. Through this, metabolomics is developing as a research area with immense potential for use in disease detection, diagnosis, risk stratification, prognosis

and prediction of therapeutic response. By incorporating the use of bioinformatics, metabolomics can identify changes in cellular response occurring during disease processes in humans. In the post-genomic era, metabolomics is therefore emerging as an area of great significance for both translational and basic biological research.

The flow of biological information from genome to metabolome can be bi-directional and these two areas are intimately connected to each other. Although genome, proteome and metabolome are closely connected in the biological system, the rapid changes in the metabolites in response to a pathological state or physiologic response to environmental variation, does not always directly correlate with slower changes observed in either gene or protein expression. Hence metabolomics is unique in providing data about rapid alterations in cellular response levels at a given time in biological samples.

Perturbation of metabolites are influenced by several factors including, disease process, treatment strategies and environmental factors. Furthermore, metabolites are chemically diverse in their solubility, stability, volatility and reactivity and thus may be invariably lost at different levels of analytic work flow. Consequently, no single method can capture and analyze the entire metabolome at once. Hence several extraction methods were developed to identify and quantify specific classes of metabolite. Metabolomics work flow involving a series of processes from sample preparation to metabolite identification and validation is shown in Figure 17 (210).

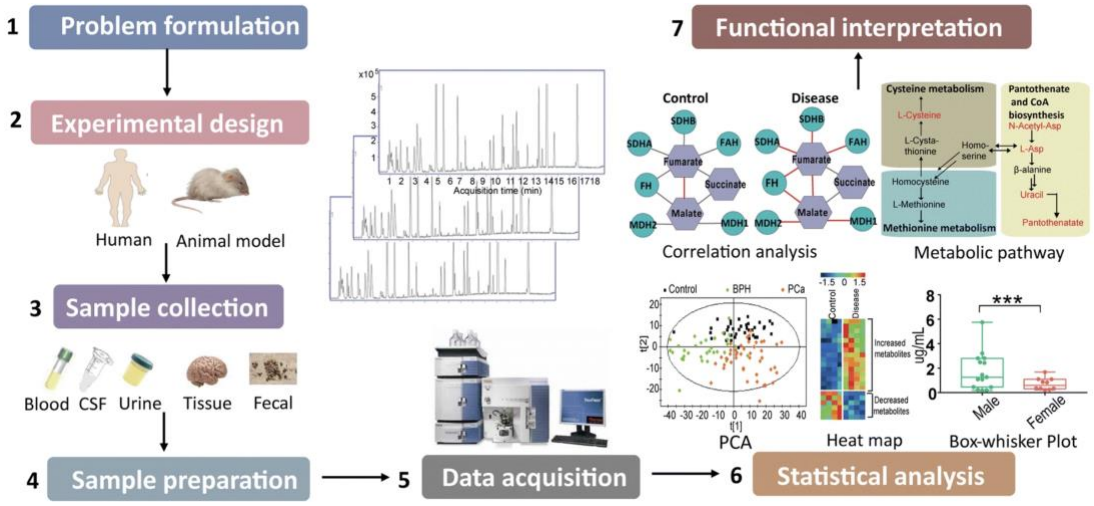

Figure 17: Metabolomic Work Flow: The typical analytical work flow begins with the biological question followed by developing experimental design, sample collection, sample preparation, data acquisition, statistical analysis and clinical interpretation. Figure reproduced from (210).

The pipeline of metabolomics in biomarker discovery involves an initial discovery phase, that aims to establish the disease metabolic signature and identify key metabolic pathways altered in response to the disease. This is followed by an in-depth quantitative analysis on validated compounds and finally a targeted diagnostic assay is focused on a subset of biomarker of interest (Figure 18).

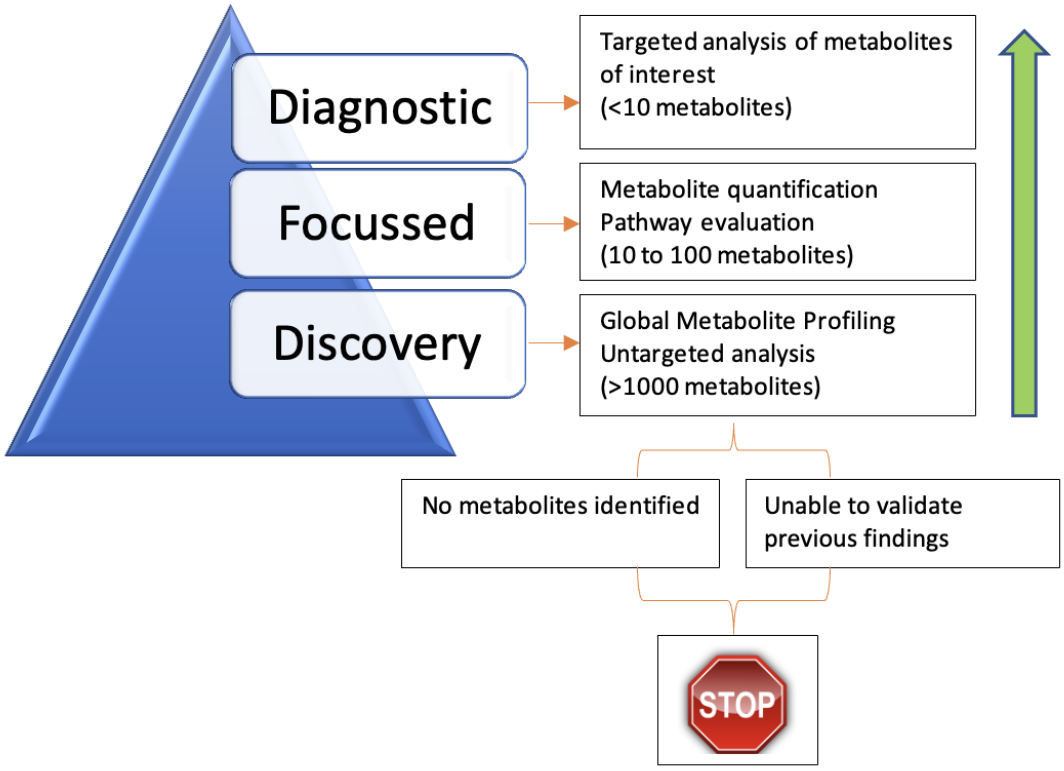

Figure 18: Stages of Metabolomics- Discovery phase involves global metabolite profiling in an untargeted approach. If no metabolites are identified or unable to validate previous findings then the experiment stops. If metabolites are identified and further analysis confirms the finding then the next step in the pipeline is a focused phase of metabolite quantification followed by the final diagnostic phase where targeted analysis of few metabolites of interest is performed.

1.2.4 Mass Spectrometry (MS)

MS is a powerful and highly sensitive analytic tool that can be used to determine the chemical composition of a sample and supply data which are essential to assigning the chemical structure of the components by identification of small molecules by measuring their molecular weight and chemical fragmentation patterns. MS measures the weight of the molecular ions, which may be native molecules, derivatives or fragments, and uses a prior knowledge to assign a

putative molecular structure. It is essentially a technique of weighing molecules based upon the motion of charged particle in an electric or magnetic field. A typical MS consists of a number of functional components as shown in Figure 19 below (211). Samples are first introduced through an inlet system and separated as appropriate. The ions are created from the sample in an ion source operating at high vacuum pressures or at atmospheric pressure, where positively or negatively charged ions are generated. As the ions are short lived and reactive, their formation and movements are conducted in vacuum. The high vacuum minimizes ion/molecule reactions, scattering and neutralization of ions. The ions are then subsequently separated according to their mass to charge ratio (*m/z*) in the mass analyser. The ions are then detected as electrical signals in proportion to their abundance and charge as they emerge from the mass analyzer. The signals of the molecular ions are generated using a mass detector, and a calibrated mass spectrum of the profile of the sample is generated.

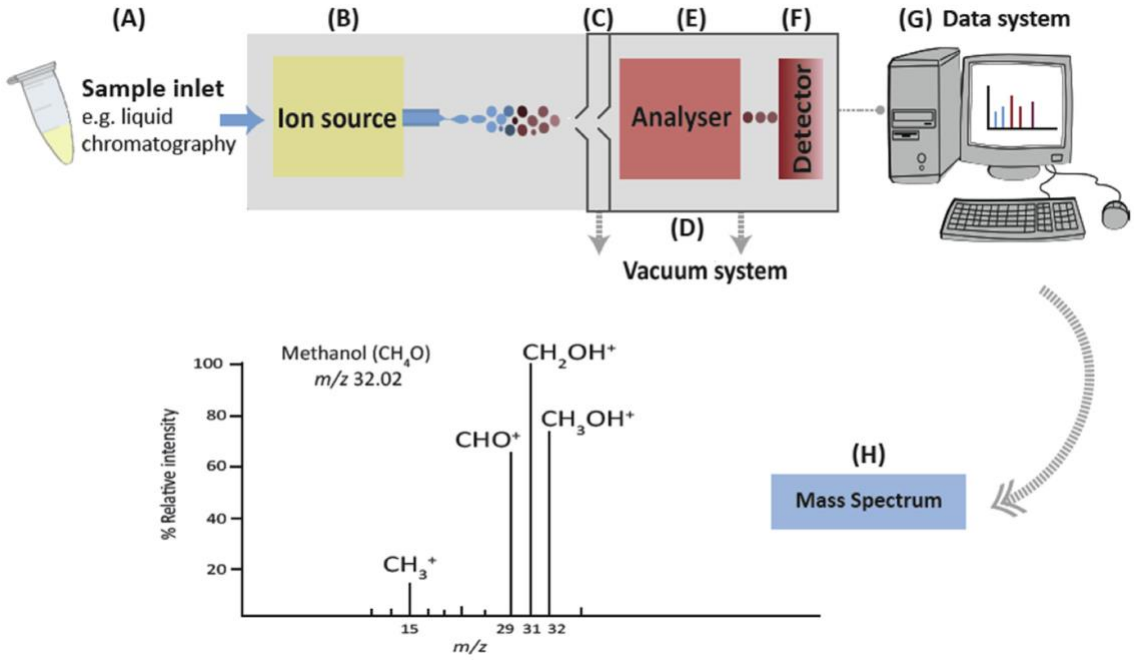

Figure 19: Functional Unit of MS: Molecules are introduced into the inlet (A), Ions are created in the ion source (B), Ions pass through the specialized vacuum interface (C) and to vacuum system (D), Ions enter the mass analyser (E) and detector (F). Signals passed into the data system (G) producing mass spectrum of the molecule (H). Figure reproduced from (211).

1.2.5 Chromatography

Chromatography is an important biophysical technique that enables the separation of the components of a given sample by the difference of each compound's speed through the column. The separation process is achieved by distributing the components of a mixture between two phases, a stationary phase and a mobile phase. Based on the polarity of these phases, they are classified as normal-phase chromatography (nonpolar mobile phase and polar stationary phase) and reversed-phase chromatography (polar mobile and nonpolar stationary phase). The reversed-phase chromatography is more popular because of its reproducibility and predictable retention times and wide applicability to various classes of metabolites. The factors influence this

separation process include molecular characteristics of the compound, analytes relative affinity for the mobile phase versus stationary phase and the difference in the molecular weights (212). Based on these differences, some components of the mixture stay longer in the stationary phase and they move slowly, while others pass rapidly into the mobile phase resulting in spatial separation of metabolites as they leave the system (213). The stationary phase in chromatography, is a solid phase or a liquid phase coated on the surface of solid phase. The mobile phase flowing over the stationary phase is a gaseous or liquid phase. Based on the type of mobile phase, chromatography is broadly termed as gas chromatography, if the mobile phase is a gas, and liquid chromatography if the mobile phase is liquid (214). The process of chromatographic separation if shown in Figure 20 below.

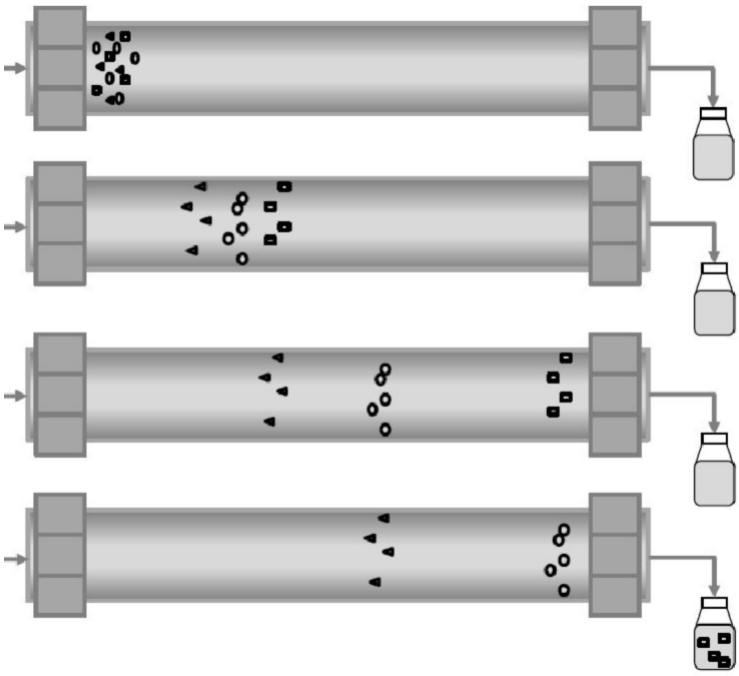

Figure 20: Chromatographic separation technique shows that the components of the mixture once injected travels at a different speed depending on their molecular characteristics and interaction with the phase. The four blocks in the

figure are a schematic representation of different time points before the components reaches the analyzer. Figure reproduced from (215).

1.2.5.1 Gas Chromatography (GC)

The GC was first employed in 1950s and has since then undergone significant development and is now widely used in the analysis of volatile and thermally stable substances. GC operates by introduction of a liquid sample into a heated injector, where rapid vaporization and mixing with the carrier gas occurs, which is followed by temporospatial separation of metabolites on the GC column and subsequent detection (216).

1.2.5.2 Liquid Chromatography (LC)

LC is applicable for the analysis of thermally labile molecules with a wider range of molecular weights extending from low molecular weight molecules (< 350 Da), that are detectable by GC to samples of macromolecules in the megadalton size range. The modern high-performance liquid chromatography (HPLC) and LC-MS platforms have been used to provide mass and structural information on large biomolecules and biomolecular complexes with molecular weights in the range of kilodaltons to megadaltons (217). Due to its high sensitivity and wide range of applicability, LC has expanded rapidly over these years. LC operates by the passage of a liquid mobile phase through a stainless-steel column packed with the stationary phase, usually silica gel. The components of the mixture are differentially attracted between the two phases and are thus separated (218).

1.2.5.3 Ionisation techniques

Several ionisation methods have been used with MS, such as electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), fast atom bombardment (FAB), and matrix-assisted laser desorption ionization (MALDI). The method of ionisation used depends on the sample under investigation and the type of MS being used. Although each has its own advantages and disadvantages, ESI and MALDI are the most commonly used soft ionisation techniques that are able to ionize analytes with little or no fragmentation (219). Furthermore, ESI and APCI are readily interfaced with high-performance liquid chromatography (HPLC), therefore both are commonly used for LC-MS (220). The choice of the ionisation method can substantially alter the data obtained by MS. Therefore, to choose the appropriate ionization method, an understanding of the mechanism governing each technique is required. The basics of the general principles of the most commonly used ionisation techniques are discussed below.

1.2.5.4 Electrospray Ionisation

In ESI, the sample is introduced in a solution at a typical flow rate of 1-1000 µL/min into a capillary or spray needle with a high voltage applied. This produces charged droplets that are sprayed out of the needle tip into the atmosphere by nebulization. The charged droplets pass through a curtain of heated inert gas (e.g. nitrogen) for complete solvent evaporation. The ions formed at atmospheric pressure then pass through the mass analyser (221).

ESI is suitable for a wide range of compounds with high to moderate polarity and also with varying molecular weight. However, ion suppression is a major

drawback, because the presence of a high concentration of nonvolatile compounds may alter the droplet formation and evaporation, which in turn inhibits the ion release (222). Therefore, this technique is widely used for the study of relatively volatile organic molecules. To overcome ion suppression, several strategies are applied; samples are subjected to purification, chromatographic conditions are modified, amount of sample being introduced to the ionization chamber is reduced (223).

1.2.5.5 Atmospheric Pressure Chemical Ionisation:

APCI is a soft ionisation technique and it produces ions with little excess energy. The sample is introduced at a flow rate of 200- 2000 µL/min into a pneumatic nebulizer that sprays the solution under atmospheric pressure. The droplets then pass through a heated quartz vaporization chamber. After vaporization of the solvent, the gas-phase molecule of the analyte pass through a discharge electrode where ionization occurs (224). This technique has the advantage of producing ions with less or no fragmentation yielding a spectrum in which molecular species are easily recognized and can be used to determine molecular mass. APCI is mainly applied to analytes of low to medium polarity with moderate molecular weight of up to 1500 Da. Unlike ESI, APCI has good sensitivity at high flow rates and has better tolerance to salts and buffers(225). APCI and ESI uses the same principle but the main difference is based on the mechanism of ionization. In ESI the analyte is ionized in the liquid phase and then released into the gas phase, but in APCI, the analyte is introduced to the gas phase in natural form to be ionized via chemical reaction. APCI does not

involve droplet formation as in ESI, therefore ion suppression factors related to the droplet properties are not an issue to APCI (226).

1.2.5.6 Atmospheric Pressure Photoionization

APPI is a modified is a modified form of APCI, where the discharge electrode is replaced by a photon-emitting lamb (227). In APPI, to selectively ionize the analyte, the emitted photon energy should be higher than the ionization energy of the analyte and lower than that of air and the used solvent such as methanol or water. Such selectivity makes APPI less susceptible to ion suppression and salt buffer effects than APCI and ESI (228). In addition, APPI is very useful for the ionization of nonpolar compounds that are not ionizable by APCI or ESI.

1.2.5.7 Matrix-assisted laser desorption ionisation (MALDI)

MALDI was first introduced in 1988. It is now a firmly established method and is characterized by relatively easy sample preparation and has large tolerance to contamination by salts, buffers etc. It has become a widespread analytical tool for peptides, proteins and carbohydrates. In the first step the sample solution is diluted in a solution of matrix, and a small volume of the combined solution (1μl or less) is deposited into the sample plate and the sample is allowed to dry. In the second step, by using intense beam of laser pulse over a short period of time, rapid heating of the crystals causes ablation of bulk portion of the dried sample surface and expansion of the matrix into a gas phase, pulling intact analyte in the expanding matrix. Ionisation reaction occurs at any time during this process and the most common mechanism involves proton

transfer, either in the solid phase before laser desorption or in the gas phase after desorption (229).

1.2.6 Mass Analysers

Once the ions are produced they are separated according to their mass-to-charge ratio using a mass analyser. The physical property of the ions that is measured by the mass analyser is their mass-to-charge ratio (*m/z*) where *m* is the mass of the ion and *z* is the charge. There are several types of analysers in practice and almost all analysers use electric and magnetic fields either combined or alone, however the basic difference between the various common types depends on the manner in which such fields are used for ion separation.

1.2.6.1 Time-of Flight (TOF)

TOF analysers operates by accelerating ions through a high voltage (230). TOF analyser separates ions and measure their *m/z* based on the time they take to pass from the ion source to the detector. Ions which are formed in the source are pushed down the flight tube through the application of high accelerating potential. The flight tube is 1-2m in length and the ions are separated in the field free region of the tube without the use of either electric or magnetic fields. Since the length of the flight tube and the accelerating voltage are fixed, the time it takes for the ions to reach the detector depends only on their mass and charge. Therefore, ions with similar m/z should reach the detector at the same time, provided that all ions receive the same initial kinetic energy. However due to the spatial distribution of the ions in the ion source and depending upon the proximity of individual ions to the applied electric field, all ions may not receive

the same initial kinetic energy. Despite this TOF analyzer can acquire spectra very quickly with high sensitivity and mass accuracy allowing analysis of the small molecules with precision (231).

1.2.6.2 Quadrupole Analysers

The concept of quadrupole analyser was first reported by Paul and Steinwedel in 1950s. This consists of four rods arranged in parallel where those opposite to one another are electrically charged. Ions are accelerated out of the source between the rods, which follow the path in the field by oscillating towards and away from the rods and it reach the detector depending upon the m/z ratio. Quadrupole has the advantage of their compact size, low cost of construction and fast scanning ability (232).

1.2.6.3 Orbitrap Analysers

The Orbitrap mass spectrometer (also called the electrostatic Fourier Transform mass spectrometer) has inbuilt electrodes between which ions are manipulated by a combination of electrostatic and centrifugal forces resulting in orbital oscillation of ions around the central electrode depending upon their mass- to- charge (m/z) ratio. When compared to other mass spectrometers it has the advantage of detecting metabolites with high mass resolution and accuracy (233).

1.2.6.4 Ion Cyclotron Resonance

When a magnetic field is applied the ions travel in a curved path. If the ion velocity is low and if the field energy is intense then the radius of the path

becomes small and the ions can be trapped on a circular path in the magnetic field. This is the basic principle of ion cyclotron resonance. The ion cyclotron frequency depends on the mass and charge of the ion and the m/z ratio of an ion can be determined by measuring its cyclotron frequency. Ions with lower m/z have higher cyclotron frequencies and ions with higher m/z have low cyclotron frequencies. Thus depending on the m/z ratio the ions are detected (234).

1.2.7 Nuclear Magnetic Resonance Spectroscopy (NMR)

The scientific theory of NMR is based on the observation that certain nuclei (such as hydrogen, phosphorus or carbon) possess a nuclear magnetic moment (also known as spin), that allows them to interact with the magnetic field (235). NMR experiments rely on the property of these nuclei to align themselves preferentially in one direction when placed in a uniform magnetic field. When these nuclei are excited by a radiofrequency pulse at their resonance frequency their axis is tipped off and when the radiofrequency pulse is no longer on, the nuclei relax back to their original position. During this process they emit energy in the form of radiofrequency signal, which is then detected and displayed as a spectrum, which comprises of a series of peaks, where the area of each peak represents the amount of that compound in the sample (236)**.** NMR spectroscopy is thus the study of the interaction of electromagnetic radiation with matter, by which the physical, chemical and biological properties of a given sample can be obtained, without the need for invasive chemical analysis. Due to its high selectivity, analytical reproducibility, non-destructive nature, simplicity of sample preparation, and high-throughput application, NMR can be applied for large-scale epidemiological studies and

routine clinical analysis, as most of the metabolites have a measurable proton (237). However, when compared to MS, NMR has the limitation of small coverage of metabolome due to the lower sensitivity and spectral overlap issues (238).

1.2.8 Metabolomic Platforms

Due to the huge diversity of chemical compounds in a human metabolome, it is practically impossible to measure the metabolome using a single analytic technique. Therefore, application of multiple analytic platforms is required to increase the coverage of the metabolome. The commonly used analytic platforms are liquid chromatography-mass spectrometry (LC/MS), gas chromatography-mass spectrometry (GC/MS), capillary electrophoresis coupled to mass spectrometry (CE/MS) and nuclear magnetic resonance (NMR; (239).

1.2.8.1 Selecting a Metabolomic Platform

The criteria for selecting metabolomic platform depend on three factors:

1. Hypothesis: If the study hypothesis is to look at a specific aspect of metabolome, e.g. lipid metabolism, then it may be reasonable to select a platform that can accurately quantify the lipids and related metabolites.
2. Wider metabolomic coverage: If a hypothesis is not formed and not much is known about the expected metabolome, then it may be reasonable to apply non-targeted strategies.
3. Sample availability: Small sample amount means limited options in terms of available platform.

1.2.8.2 Analytic Strategies

The analytic strategies can be broadly divided into two approaches

1. Untargeted approach: This approach aims to measure the metabolome as broadly as possible. It is a relative quantification of a large range of metabolites, which were previously known and unknown to have a role in the disease process, in a given sample. This method not only characterizes the metabolites covered in targeted analyses but also helps to detect previously unknown or poorly characterized metabolites. This is a hypothesis generating experiment because it is used when a hypothesis is not formed and not much is known about the expected metabolome. It requires powerful analytic tools for identification of a wide range of metabolites (240).
2. Targeted approach: Targeted analysis refers to metabolomic analysis that focuses on specific known metabolites and uses internal standards for absolute quantification. A pre-defined subset of metabolites are chosen and a particular analytical method optimized for that subset of metabolites are used. This strategy is used for quantification of a small number of known related metabolites for hypothesis testing (241).

1.2.9 Metabolomics and its Application in Human Disease

The heart is the most metabolically demanding organ in the body and its metabolic perturbations leads to measurable changes in the metabolome of the body fluids. Therefore, identifying these metabolite changes in plasma will allow us to understand the downstream effects of the perturbed cellular pathways during cardiac ischaemia. Several advances in the “omics” technology platform

such as UPLS-MS, NMR spectroscopy, have allowed us to determine the patterns of changes at metabolome level, in CHD.

1.2.9.1 Myocardial Ischaemia

Surendran *et al*., (2019) performed a non-targeted metabolomic analysis of plasma from 27 STEMI patients who had undergone primary percutaneous intervention. Peripheral venous blood samples were obtained from patients at four different time intervals including the time at the arrival in the hospital, 2 hours post angioplasty, 24 hours post angioplasty and 48 hours post angioplasty. Using a LC-MS platform, a metabolomic analysis was performed. This revealed significant perturbation in plasma FFA concentrations, such as arachidonic acid, docosahexaenoic acid, eicosatrienoic acid and lysophospholipids. The peak concentration of these metabolites was noted at time point zero and progressively decreased following angioplasty over the 48-hour period (242).

Based on the mechanism of presentation of ACS, the international guidelines recognize two different types of MI - thrombotic (type 1) due to thrombotic occlusion of the coronary artery, and non-thrombotic (type 2) MI most often due to demand ischaemia (243). DeFilippis *et al*., (2017), analyzed the plasma samples of 11 thrombotic MI, 12 non-thrombotic MI and 15 patients with stable CHD. Plasma samples were collected at three different time points including baseline (prior to angiogram at the time of enrollment), six hours after enrollment and 3 months from the time of enrollment. Plasma samples were analyzed using untargeted approach using UPLC-MS platform. The unique metabolic changes associated with acute phase of thrombotic MI as compared

to acute phase of non-thrombotic MI and stable CAD, consisted mainly of lipids, lysophospholipids, 2-hydroxybutyrate, and amino acids including glycine, histidine, asparagine, n-acetylphenylalanine and histidine (244).

Sabatine *et al.*, (2005) performed a targeted analysis of 477 metabolites using HPLC and MS, for the first time demonstrating the feasibility of applying metabolomics to the study of human subjects with acute myocardial ischaemia. They prospectively enrolled 36 patients who underwent myocardial perfusion imaging following exercise stress testing. Patients with >5% reversible perfusion defect were selected as cases and those without perfusion defect were selected as controls. Blood samples were taken from the patients immediately before, immediately after, and 4 hours after stress testing. Metabolic perturbations detected predominantly involving amino acids such as γ-aminobutyric acid, citrulline and argininosuccinate. Sabatine and colleagues also demonstrated statistically significant changes in circulating levels of metabolites belonging to the TCA cycle, such as oxaloacetate and citric acid, which as discussed previously play a central role in oxidative phosphorylation in the myocardium (245). However, the concentration of metabolites could also have been influenced by metabolites released from skeletal muscle during the exercise test. In addition this study only utilized a targeted analysis approach looking at a total of 477 metabolites therefore there may have been changes in other metabolite classes and pathways which were not detected in this study.

Lewis *et al*., (2008) performed a targeted metabolite profiling of the peripheral blood samples, in a human model of planned myocardial infarction. A total of 36 patients undergoing planned myocardial infarction using alcohol septal ablation for hypertrophic obstructive cardiomyopathy (HOCM) were compared

with patients undergoing elective diagnostic coronary angiography. The results of this initial study was then validated with a cohort of patients who had spontaneous myocardial infarction. Applying an LC-MS platform, Lewis *et al.*, identified purine degradation products (hypoxanthine and xanthines), pyrimidine metabolites (malonic acid and aminoisobutyric acid), metabolites involved in the TCA pathway (succinic acid, aconitic acid and malic acid) and amino acids (threonine, taurine and glutamic acid) (246). The study by Lewis and colleagues had a number of limitations. Firstly it only involved subjects who sustained irreversible myocardial damage rather than early ischaemia. Secondly HOCM itself is associated with significant changes in cardiac metabolism – as studies in the past have shown that glucose metabolism and oxidative phosphorylation are impaired in these patients (247). Moreover, in cardiac hypertrophy there is increased ventricular mass. Considerable oxygen deprivation of the inner myocardial tissue is observed due to lack of vascular flow in this area, which leads to chronic hypoxia. Under these circumstances oxidative metabolism is usually significantly diminished (248). The metabolic alterations observed may also have been influenced by the injection of alcohol down the septal arteries which is an unphysiological process.

Lin *et al.*, (2009) used Ultraperformance liquid chromatography (UPLC) and Time-of-flight (TOF) and studied peripheral venous blood sample from 14 patients with silent myocardial ischaemia (SMI). All patients underwent exercise stress test and SMI was defined by >1mm ST segment depression in males and >2mm ST segment depression in females within 8 mins of the test. The control subjects were 25 randomly selected age and gender matched healthy volunteers. The commonly-used biomarkers used to evaluate myocardial

infarction such as Troponin T, CK-MB, and LDH did not show any significant changes in the study population with SMI when compared to the control subjects, and were not useful in differentiating the two groups. However, metabolomic analysis showed that there were significant alterations in lipid metabolism, with changes in membrane phospholipids (Lysophosphatidylcholine, Phosphatidylcholine), and down regulation of the product of phosphocholines in the SMI group (249). These results were similar to those found in animal ischaemia models. In isolated hamster hearts ischaemia resulted in 51% reduction in the biosynthesis of phosphocholines. Phosphocholines are the principle component of the phospholipids, and are the essential building block of the cell membrane. These results suggest that changes in phospholipids during SMI may reflect membrane destruction and that this could be used as a tool to screen patients with SMI.

Barba *et al.*, (2008) used NMR-based metabolomics to analyze serum samples of 31 patients with exertional angina. The study was conducted in patients with stable coronary artery disease without history of previous MI. All patients underwent exercise stress myocardial perfusion scan, of which 22 patients had exercise induced ischaemia and 9 patients did not have ischaemia. Venous blood samples were taken from all patients immediately before the stress test. Serum NMR metabolomic analysis of the patients revealed significant changes in the lipid and amino acid metabolism, which differentiated patients with reversible ischaemia and others. They suggested that metabolomic analysis can thus be used as a screening tool and to risk stratify patients with coronary risk factors (250). The patients in this study were stable patients with exertional angina and thus by definition they do not have ischaemia at rest. As the blood

samples were taken at rest prior to the stress test therefore the metabolic changes observed where highly unlikely to reflect true myocardial ischaemia.

Teul *et al*., (2011) performed both a targeted- and non-targeted metabolomics analysis in plasma of patients with ACS (251). Using GC-MS, plasma samples taken at time of admission to hospital with chest pain, as well as four days, two months and six months post ACS, were analysed using an untargeted approach. This study again revealed that metabolites predominantly involved in the TCA cycle were significantly altered. Targeted analysis of the fatty acids in the same samples showed that plasma samples taken at admission had the maximum rise when compared to the samples collected at four days and six months post cardiac event. However, the patients in this study had documented evidence of myocardial necrosis and hence this probably better represents a model of myocardial infarction, and not early myocardial ischaemia.

Yang *et al.*, (2013), experimented on animal models of myocardial infarction by ligating the left anterior descending coronary artery of adult rats and analyzed the infarcted myocardial tissue using NMR and HPLC. This showed significant reduction in the levels of taurine (56.5%) after 5 mins of ligation. Indicating that taurine could be a potential marker of early myocardial infarction (252). Taurine is the most abundant free amino acid in the mammalian hearts and it has a number of functions, including the regulation of intracellular calcium balance, anti-inflammatory actions and immune regulation (253). It has been demonstrated that cardiac muscle lacks the ability to synthesize taurine and majority of the taurine in the cardiac tissue is accumulated by uptake from blood (254).

1.2.10 Common Analytic Strategies

The study of many biological processes at the molecular level involves understanding how biological molecules behave dynamically. The three-dimensional shape of these molecules, usually determine the chemical action they perform. Both the stable or native shape of the molecule and dynamic deviations from it are important to understand how they interact with each other. The resulting, simplified representation has to be useful to classify the different conformations along one or more "directions" or "axes" that provide enough discrimination between them.

1.2.10.1 Principal Component Analysis (PCA)

PCA is a simple non-parametric statistical method of extracting relevant information from a complex data. It is one of the oldest and most widely used analytic technique. Its idea is simple - by transforming the data into fewer dimensions, PCA summaries and simplifies the complexity in high-dimensional date while retaining trends and patterns (255). The technique explores and visualizes the data set by emphasizing variation and bringing out strong patterns in the dataset. This is accomplished by identifying vectors, called principal components, along which the variation in the data is maximal. In simple terms, principal components are the directions where there is the most variance, the directions where the data is most spread out. By using these few components, each sample can be represented by relatively few numbers instead of by values for thousands of variables. The samples can then be plotted, making it possible to visually assess similarities and differences between samples and determine whether samples can be grouped. What PCA

does is that it takes the cloud of data points and rotates it such that the maximum variability is visible. For example, if we have some bottles of wines, we can describe each wine by its color, strength, smell, how old it is and so on and we can group them in the cellar based on these characteristics. But most of these wines will share similar properties. So, in order to differentiate one from another we need to look at properties that strongly differ across the wines. Let's say our original dataset has two variables, x and y (Figure 21).

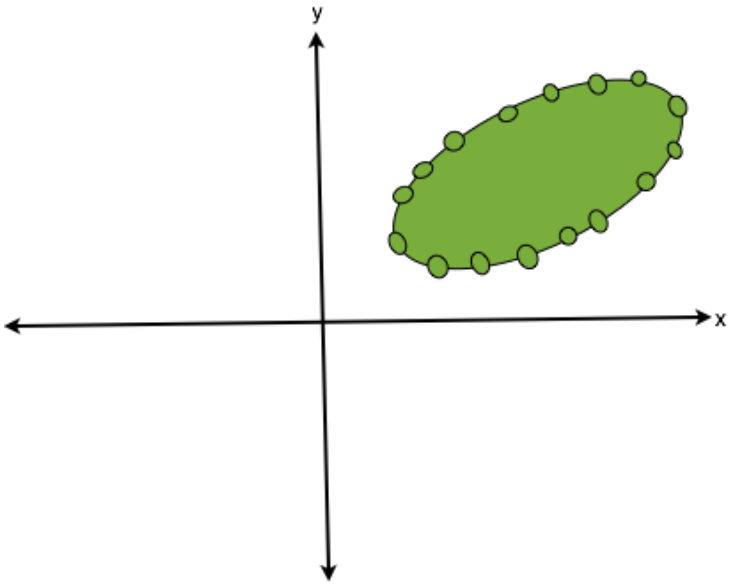

Figure 21: Original data set with two variable x and y.

We want to identify the first principal component (PC1), which has the highest variance. Graphically if we draw a line in the oval with the maximum variance then that is PC1 (Figure 22).

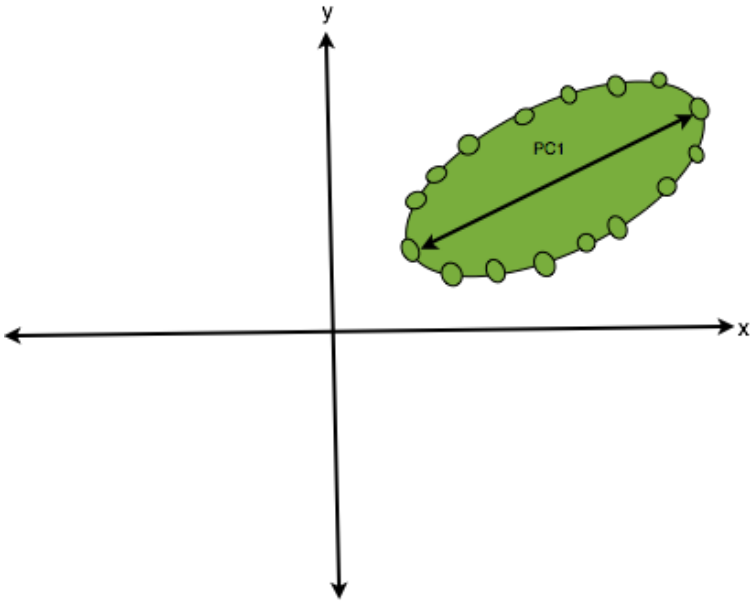

Figure 22: Identifying principal component 1 by drawing a line with the maximum variance.

If we then want to project the data into PC1 only (i.e. reduce the data from a two-dimensional to a one-dimensional dataset) it will collapse the dataset into a single line, by projecting into that line which is the PC1 (Figure 23).

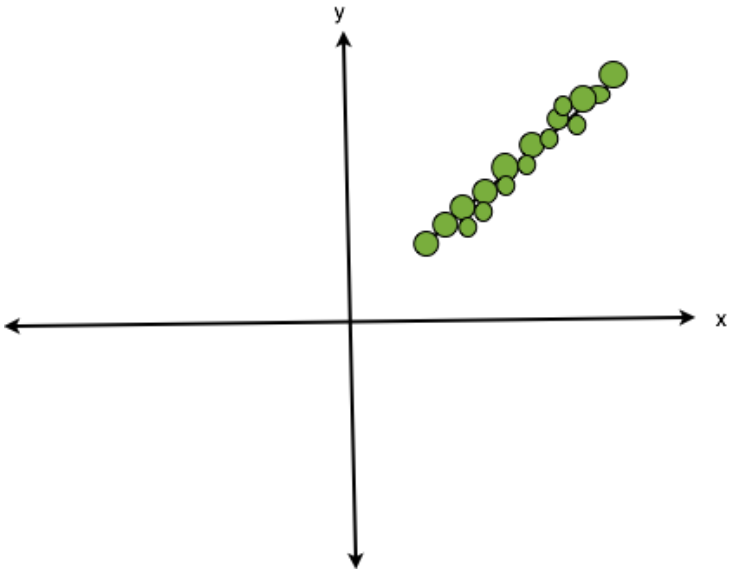

Figure 23: By reducing the two dimensional to one-dimensional dataset, we project the dataset into a single line.

We can see that we have lost some information in the transformation although we kept the most important axis. The second principal component (PC2), must be orthogonal to PC1, which does its best to capture the variance in the data that is not captured by the PC1 (Figure 24).

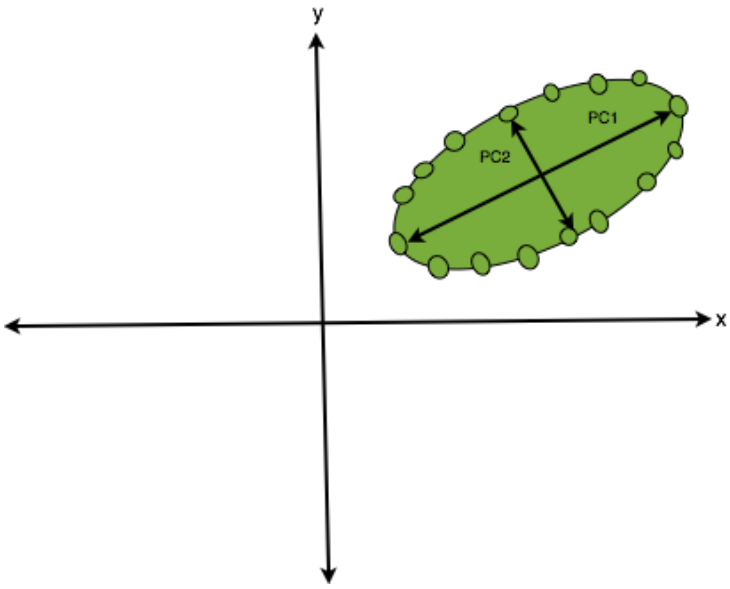

Figure 24: The second principal component (PC2) is the orthogonal axis to the first principal component (PC1). This captures the maximum variance in data not captured by PC1.

Through projecting the original dataset into the first two principal components, no information would be lost. Thus, this process has transformed the original two-dimensional dataset into a new two-dimensional dataset and the new dataset will look as shown in Figure 25. The PC1 will be the new x-axis and PC2 will become the new y-axis. Thus, we rotate the data to fit these new axes.

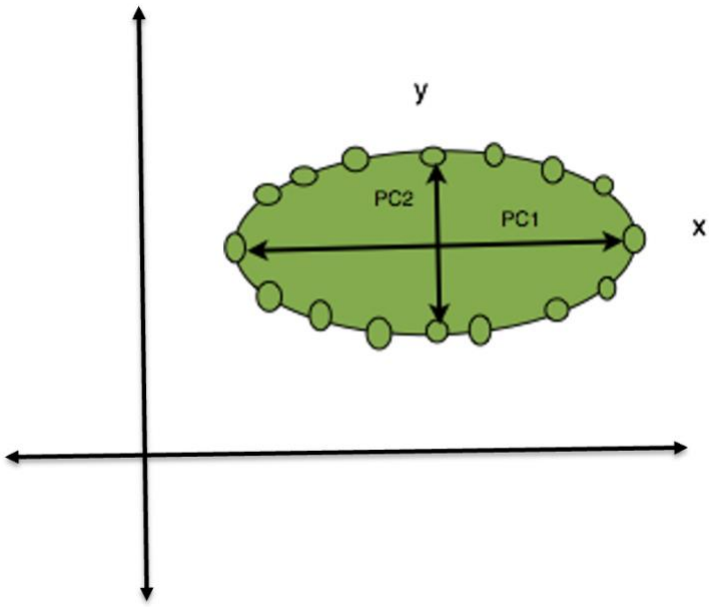

Figure 25: We transform the original two-dimensional dataset into new two-dimensional dataset. PC1 and PC2 will become the new x and y axis respectively. PCI has the highest possible variance and PC2 has the second most variance and so on.

The data is being visualized from a different angle which is more intuitive and where there is more information. The number of principal components is less than or equal to the number of original variables. The PC1 has the highest possible variance, the PC2, has the second most variance and so on.

Eigenvectors and eigenvalues exist in pairs; every eigenvector has a corresponding eigenvalue. The eigenvector indicates the direction of these new axes (PC), and the eigenvalue indicates how much variance there is in that direction. The smallest eigenvalues correspond to the thinnest directions having the least variation. Hence the eigenvalue magnitude indicates how much of data's variability is explained by its eigenvector.

1.2.10.2 Measuring Correlation

Values close to +1 indicate a high degree of positive correlation, values close to -1 indicate a high degree of negative correlation, values close to 0 indicate poor correlation and 0 indicates no correlation at all. In addition, correlation is also measured by clustering of the dataset. More clustering of the dataset indicates a closer correlation and less clustering indicates weak correlation (Figure 26)

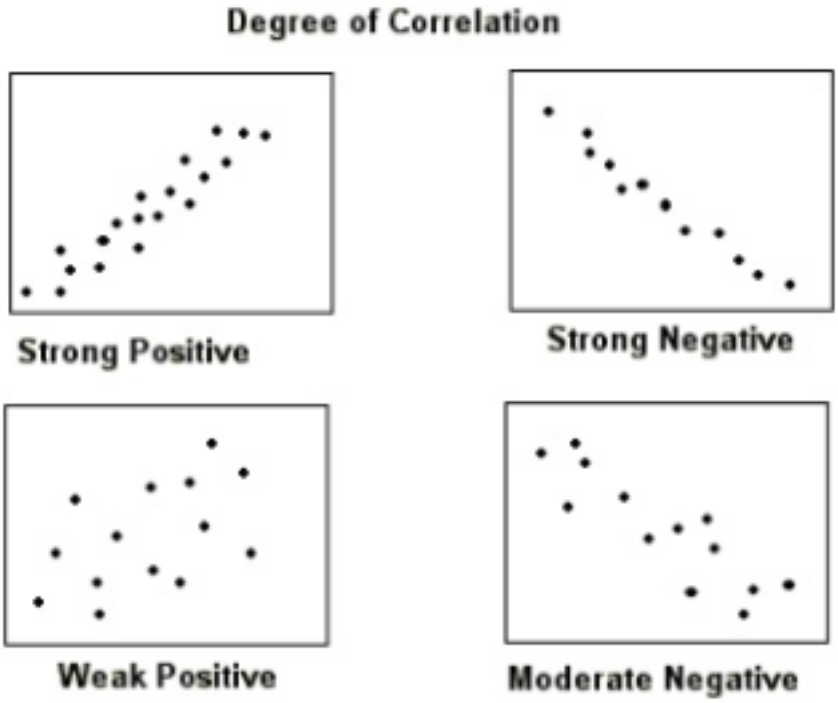

Figure 26: Degree of correlation indicated by clustering of the dataset. More clustering indicates a strong correlation (top two) and less clustering (bottom right) and more scattered (bottom let) indicates weaker correlations respectively.

1.2.10.3 PCA methodology

There are five steps in performing PCA.

1) Standardizing the scale of data. Because of standardization, all PC's will have a mean 0. The standard deviation of each component will be the square root of the eigenvalue

2) Calculate covariance. This is the measure of how two variables move together. The interpretation of PC is based on finding which variables are most strongly correlated with each component, i.e, which of the numbers are larger in magnitude, the farthest from 0 in either positive or negative direction.

3) Work out Eigenvectors and eigenvalues. The PC1 will be the new X axis and PC2 will become the new Y axis. Thus, the data can be rotated to fit these new axes. Eigenvectors indicate the direction of these new axes (PC) and eigenvalues give you the lengths. Each eigenvector will correspond to an eigenvalue and the smallest eigenvalues correspond to the thinnest directions having the least variation. Hence the eigenvalue magnitude indicates how much of data's variability is explained by its eigenvector.

4) Re-orient data. Since eigenvectors represent the direction of the new axes, we need to multiply the original data by eigenvectors to re-orient the data onto the new axes.

5) Plot re-oriented data

1.2.10.4 Projection of latent structures (PLS)

To understand PLS, it is important to know about the principles of linear regression models. Linear regression is a method of assessing the relationship between a dependent variable (y) and one or more independent variables (x_1,

$x_2 \ldots x_n$). For one independent variable, this process is called simple linear regression and for more than one independent variable, it is called multiple linear regression. A dependent variable represents the variable whose outcome is already being studied or is known, and an independent variable that represents the input or potential reasons for potential variation. For example, in a study of how different doses of drugs affects the symptom severity. Here the independent variable is the different doses and the dependent variable is the symptom severity.

PLS is an extension of the multiple linear regression model. It is a method for constructing predictive models when the factors are many and highly collinear. The emphasis of PLS is on predicting the response and not necessarily trying to understand the relationship between the variables. The overall goal is to use the factors to predict the responses in the population. For example, one could estimate a person's weight as a function of the person's height and gender. To do this it is possible to use linear regression to measure the respective regression coefficients from a sample of data, measuring height, weight and observing the patient's gender. For many data analysis, estimates of the linear relationships between variables are adequate to describe the observed data and to make reasonable predictions for new observations. This is achieved indirectly by extracting latent variables T and U from factors and responses respectively. The extracted factors T also referred as x-scores are used to predict y-scores U and subsequently the predicted y-scores are used to construct predictions for the responses (Figure 27).

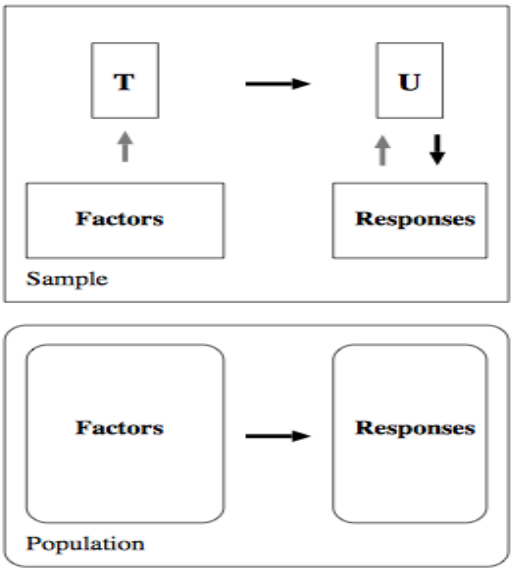

Figure 27: Shows a schematic outline of PLS method

x-scores are chosen to explain as much as factor variation as possible and y-scores to explain as much predicted Y-variation as possible. In principle, PLS is a robust form of redundancy analysis seeking directions in the space that are associated with high variation in the responses, biasing them together towards directions that are accurately predicted.

1.2.10.5 Root Mean Square Error (RMSE)

RMSE is the square root of the variance of the residuals. It is used in Ordinary Least Square (OLS) regression analysis to evaluate the model fit. It is based on two sums of squares: Sum of Squares Total (SST) and Sum of Squares Error (SSE). SST measures how far the data are from the mean and SSE measures how far the data are from the models predicted values. It indicates the absolute fit of the model to the data and how close the observed data points are to the models predicted value. As the square root of the variance, RMSE can be interpreted as the standard deviation of the unexplained variance. Lower

values of RMSE indicates better fit and is a good measure of how accurately the model predicts the response.

1.3 Research Objectives

The general purpose of the research was to define changes in cardiac metabolism during early myocardial ischaemia and to explore the perturbations of metabolites at cellular level using metabolomics. To understand the pathophysiology of acute coronary ischaemia and explore the metabolites that are involved in the rapid response to ischaemia that may provide insights into diagnostic and therapeutic targets in patients with coronary artery disease.

The specific research objectives were as follows:

1. Create a robust human model of early myocardial ischaemia:
    a) Recruit 25 patients with single vessel coronary artery disease as a discovery cohort in the preliminary study.
    b) Validate the preliminary study with a second group of 25 patients with matched patient characteristics.
2. Characterize the cardiac metabolic perturbation during early myocardial ischaemia using metabolomic analysis.
3. Understand the metabolic pathway and pathophysiology involved in early myocardial ischaemia.
4. Perform subgroup analysis of the common comorbidities in the patients to further characterize the metabolic perturbations.

Chapter 2 Materials and methods

2.1 Materials and methods used for the metabolomic profiling of the human model of acute myocardial ischaemia.

2.1.1 Ethical approval and consent procedures

This study complies with the Declaration of Helsinki and was approved by the Manchester Research Ethics Committee (Ref: 04/Q1406/58) and Central Manchester and Manchester Children's University Hospitals NHS Trust Research and Development (PIN:10562). Eligible patients were contacted and provided with patient study information sheet. Patients were given sufficient time (minimum 24 hours), prior to the clinic appointment. The study was discussed during the clinic visit when the patients have opportunity to clarify any concerns and accept or decline to participate in the study. A written consent was obtained for all those who expressed their wish to participate in the study.

2.1.2 Study Design and Venue

This is a prospective cohort study, based in a tertiary care centre at The Manchester Heart Centre (MHC), Central Manchester University Hospitals NHS Foundation Trust (CMFT). Participants underwent assessment during their scheduled visit in the preadmission clinic, prior to the angioplasty procedure. Suitable patients were subsequently recruited during their admission in the day care ward in the MHC. All blood samples were processed and stored in Professor Ludwig Neyses' laboratory in the Stockport Building, The University of Manchester. The serum samples were analyzed in Professor

Doug Kell's lab in Manchester Interdisciplinary Biocentre (MIB), The University of Manchester.

2.1.3 Study Population

The study population included elective stable patients with single vessel coronary artery disease.

2.1.3.1 Inclusion criteria

Single vessel coronary artery disease.

2.1.3.2 Exclusion criteria

1. Triple vessel disease and previous CABG: Using the cardiology angiogram web archive system recent coronary angiograms were reviewed and patients with flow limiting triple vessel disease (>70% lesion), chronic total occlusion lesions or history of previous CABG were excluded.
2. LV dysfunction: LV function was assessed with the help of parasternal and apical 2D transthoracic echocardiography and LV angiography performed during previous cardiac catheterisation. Evidence of left ventricular dysfunction with an ejection fraction of less than 60% was an exclusion criterion for the study.
3. Renal failure: All recent blood results were analysed using the hospital clinical laboratory data and patients with renal dysfunction (estimated glomerular filtration rate <60) were excluded.
4. Anaemia: Using the hospital clinical laboratory data, blood results were analysed and patients with anaemia (Hb < 12 for females and Hb <13 for male) were excluded from the trial.

5. Recent acute coronary syndrome/percutaneous coronary intervention: Using patient hospital notes, discharge summary and patient history, any patient with recent acute coronary event and/or intervention (<6 weeks) was excluded.
6. Chronic inflammatory disease & malignancy: With the help of patient hospital notes and detailed history patients with underlying connective tissue disorders or malignancy were excluded from the study.

2.1.4 Definitions of Epicardial Coronary Vessels and Lesions

The epicardial coronary blood vessels consists of left main coronary artery branching into left anterior descending (LAD) and circumflex (LCx) coronary arteries and the right coronary artery (RCA). A coronary atherosclerotic lesion of 70% or more is considered significant requiring coronary revascularization. The American College of Cardiology/American Heart Association (ACC/AHA) Task Force has classified the coronary atherosclerotic lesions as A, B and C. for predicting success and complications during coronary interventions (256). The lesion was defined as type A if it was discrete, non-angulated, or non-calcified lesion with no major branch involvement. Type B lesions were tubular, eccentric, moderately angulated, calcified, with bifurcation involvement or ostial location. Type C lesions were diffuse, extremely tortuous, calcified, and total occlusion for more than three months. Based on the classification, there is an association of slightly decreasing procedural success and increasing procedural risk with lesions staging from A to C.

2.1.5 Procedure and Sample Collection

Blood samples were obtained from 46 patients undergoing elective percutaneous coronary intervention (PCI) to native coronary arteries over a period of 12 months. Peripheral venous samples were collected through a venous sheath inserted into the femoral vein. Coronary sinus sampling was performed in 7 patients following cannulation of the coronary sinus using a 6 Fr Amplatz Left-1 catheter (AL-1) during the procedures. All the PCI procedures were performed via the femoral artery. Most of the participants agreed for the peripheral femoral venous sample collection rather than coronary sinus sampling due to the less-invasive nature of the procedure.

Control samples of venous blood were obtained either from the coronary sinus or via the sheath in the femoral vein once the guide catheter and guide wire were in position before initiation of the PCI procedure (defined as baseline). Myocardial ischaemia was generated during the initial balloon inflation to pre-dilate the target lesion during PCI. Balloon inflations of between 30 seconds and 1 minute were performed until ECG changes, ST segment depression or ST segment elevation and patient symptoms, such as chest tightness or pain consistent with myocardial ischaemia were noted. Individual operators decided upon the type and size of the angioplasty balloon and inflation pressures that were used to pre-dilate the target lesions. Serial venous samples were collected from either the coronary sinus or femoral vein at 1 minute (defined as TP1) and 5 minutes (defined as TP5) after balloon inflation. PCI was then completed as per routine.

Patient demographics and procedural data were collected at the time of intervention. Samples were collected into EDTA Vacutainer tubes (Becton

Dickinson, Franklin Lakes, New Jersey) and transported on ice for immediate centrifugation at 1500g for 15 minutes at 4°C. Plasma was then removed and stored in aliquots at −80°C. Two studies were undertaken, defined as the Discovery Study (DS) and the Validation Study (VS). The process for assignment to the DS or VS groups was based on the time of patient recruitment with the first set of patients recruited being assigned to the DS group (patients 1-25, recruited first 6 months) and the remaining set of patients recruited being assigned to the VS group (patients 26-46, recruited following the second 6 months). Peripheral and coronary sinus blood was collected from 21 and 4 subjects, respectively, in the DS and from 18 and 3 subjects in the VS, respectively. Peripheral blood data for one subject in the DS group was identified as an outlier (the number of metabolite features detected was less than 35% that of the mean of metabolite features detected across all samples) and was removed from the dataset following data acquisition.

2.1.6 Metabolic phenotyping

All reagents used were of HPLC grade purity (Sigma-Aldrich CHROMASOLV, Dorset, United Kingdom). Plasma samples were extracted applying liquid-liquid extraction as follows: 200 µL aliquots of plasma were mixed with 600 µL of methanol using a vortex mixer (15 seconds) followed by centrifugation (13,000 g, 15 minutes). Supernatants were transferred to Eppendorf tubes and lyophilized (HETO VR MAXI vacuum centrifuge attached to a HETO CT/DW 60E cooling trap; Jouan, Gydevang, Denmark). A single pooled quality control (QC) sample (intra-study QC sample) was prepared by pooling 200 µL aliquots of plasma from each sample followed by vortex mixing for 60 seconds. Twenty-

two (DS) and nineteen (VS) 200 µL aliquots of the intra-study QC sample were transferred to separate Eppendorf tubes for extraction as described above for the plasma samples.

All samples were analyzed separately in positive and negative ion modes using Ultra Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS;Waters Acquity UPLC system coupled to a Thermo LTQ-Orbitrap XL) applying a previously described method by Dunn *et al*., 2011 (257). Raw data was processed applying the open-source software XCMS to construct a data matrix for positive ion mode data and a data matrix for negative ion mode data. Signal correction was performed to minimize run-order associated drift in response by applying quality control-based robust LOESS (locally estimated scatterplot smoothing) signal correction (QC-RLSC). Quality assessment and quality-based filtering of the data were performed applying the data acquired for QC samples only. The relative standard deviation (RSD) for QC samples only (from QC sample injection nine onwards) and the percentage of QC samples where a response was reported were calculated for each metabolite feature. Data for all biological samples and QC samples for all metabolites with a relative standard deviation for response >20% in the studied QC samples and after QC-RLSC were removed from the dataset prior to statistical analysis. Data for all biological samples and QC samples for all metabolites which were detected in <60% of the studied QC samples were removed from the dataset prior to statistical analysis. The Wilcoxon signed-rank test was applied to peripheral blood sample data only to identify statistically significant metabolites when comparing baseline to TP1 samples and baseline to TP5 samples. Metabolites were annotated applying the software PUTMEDID_LCMS (258), to

level 2 of the Metabolomics Standards Initiative reporting guidelines for chemical analysis (259). Further work to identify several metabolites based on MS/MS spectra was also performed with matching to an internal retention time and MS/MS library developed by Dunn, Broadhurst, Begley *et a*l., (2011) or to mzCloud (https://www.mzcloud.org/). Metabolites were grouped into 'metabolite classes' based on chemical structure similarity (e.g. fatty acids) or metabolic pathway similarity (e.g. TCA cycle). Box and whisker plots were constructed with MetaboAnalyst using normalized data (260). Pathway enrichment analysis was performed using MetaboAnalyst (pathway analysis, compound name list inputted, visualization method=scatter plot, enrichment method=hypergeometric, topology analysis=relative betweenness centrality, pathway library=homo sapiens (KEGG).

2.2 Methods for the analysis of the concomitant comorbidities and other patient characteristics in the study population.

2.2.1 Preparation of dataset

The purpose of this study was to explore the effects of other factors that may also have an influence on the metabolite profile to support the conclusions made regarding metabolite changes in response to cardiac ischaemia. These factors included co-morbidities such as, high body mass index, diabetes, hypertension, hypercholesterolaemia, and also concomitant medication including those used for angina, ACE inhibitors, proton pump inhibitors, statins, and others.

The analysis plan was divided into 4 separate tasks:

1. Construction of the dataset/s for MVA

2. Exploration of metabolite changes related to diabetes, hypertension, BMI, medication, location of sample collection
    a. Using principal components analysis (PCA)
    b. Using classification and regression models i.e. partial least squares regression (PLS) and its extension to discriminant analysis (PLS-DA)
3. Discriminant analysis to look at groupings of the data according to different cardiac function tests (chest pain, ECG, and clinical chemistry markers Troponin T and cytokines)
4. PLS analysis to explore any correlations between mass spectrometry variables and clinical chemistry measurements.

The data consisted of 4 excel files containing:

- Positive ion data for the Discovery cohort (subjects 1-25)
- Negative ion data for the Discovery cohort (subjects 1-25)
- Positive ion data for the Validation cohort (subjects 26-46)
- Negative ion data for the Validation cohort (subjects 26-46)

The UPLC-MS data had already been peak-picked using XCMS as part of the initial analysis. As each of the cohorts had been peak-picked separately, the location of each peak was not consistent across both sets of data for each ionisation mode (positive and negative). It was therefore not possible to combine the Validation and Discovery sets of data, although it was possible to combine positive and negative ion data for each cohort. It was therefore decided to analyse the Discovery and Validation cohorts separately, focusing on the Discovery cohort. Modelling of the Validation cohort was also carried

although the dataset could not be used as a validation set for the statistical modelling.

Mass spectrometry data were concatenated with the Meta data, which included the co-morbidities, concomitant medication and other subject information. It was also merged with the metabolite identification, the statistical results for the comparison of the different time-points, the unique peak id, m/z and retention time values. The molecular formulae and adduct information were also included. This was done in case it was necessary to track the peak information across different analyses at any time.

2.2.2 Statistical Methods

The MVA approaches used in the analysis were

- PCA
- PLS
- PLS-DA
- The orthogonal signal correction equivalents of PLS and PLS-DA – OPLS and OPLS-DA

Prior to statistical analysis all data were mean centred. Preliminary analysis of the models showed no differential weighting of the positive versus negative ion variables so no block scaling was applied. Mass spectra were pareto-scaled and Meta data continuous variables were unit-variance scaled prior to statistical analysis.

2.2.3 PCA exploratory analysis

PCA was initially used as an exploratory method to look for any natural groupings arising from variation in the data. To aid visualisation of any

groupings, colour coding was used on the scores plots, focusing on the first six principal components. Observations of partial clustering or patterns in the PCA plots helped give confidence to models obtained by supervised methods (PLS and PLS-DA) because the classification/response variables were not used in the PCA calculations.

2.2.4 Model validation

Correlations between all variables with UPLC-MS data were explored using PLS. Group differences were analysed by PLS-DA for binary y-variables – e.g. patients with and without diabetes or hypertension, patients taking anti-angina medication versus those who were not. OPLS and OPLS-DA were then used to remove any variation that was completely uncorrelated with the response or class variable respectively to obtain coefficients for the tables of important variables.

Models were tested for statistical significance using two methods. The first was by cross validation, where each model was generated many times after iteratively leaving out a block of data. For each round of cross validation, values for the excluded data were predicted from the model and these predicted values compared with the values obtained when the samples were included in the model. However, because the dataset included three different mass spectra for each patient (baseline, 1 minute and 5 minutes), but not all time points had different Meta data, there was some replication within the dataset. In order to test the models for statistical significance by cross validation it was therefore necessary to modify the cross-validation protocol so that all samples from a particular subject were excluded simultaneously for each round.

The second method for testing statistical significance was by randomly permuting the y-variable and observing the explained variances R2 and Q2 for the randomly generated model together with their correlation to the original model. Providing there was low correlation between the random and actual model and the R2 and Q2 were lower (preferably negative for Q2), the model was deemed significant.

Chapter 3: Ultraperformance Liquid Chromatography-Mass Spectrometry analysis of the Discovery and Validation cohort in the ischaemia Model.

3.1 Introduction

The transition from reversible to irreversible myocardial injury in ACS is associated with distinct metabolic changes. Identifying metabolomic signatures associated with reversible myocardial injury may provide insight into disease pathophysiology, aid with early diagnosis and help predict prognosis and management. The metabolomic approach systematically evaluates metabolites in biological samples that reflect the state of the system and provide additional insight into disease pathophysiology. Metabolomics have thus emerged as a promising tool and have successfully identified novel metabolite signatures in CVD (261).

This study was performed in an attempt to characterize the metabolic pattern during reversible myocardial ischaemia and evaluate the potential for novel metabolites that may provide information beyond the traditional biomarkers of CHD. This is a prospective case-control study with the patients as their own controls. Plasma samples of the patients were analysed using Ultraperformance Liquid Chromatography-Mass Spectrometry (UPLC-MS), in an untargeted fashion following controlled myocardial ischaemia. The study was conducted independently in DS and VS groups, with matched patient characteristics.

3.2 Results

3.2.1 Population demographics

The study population consisted of a cohort of 46 patients; 25 patients in the DS and 21 patients in the VS. The patient characteristics, lesion data, and procedural data are shown in Table 2. In both groups, the majority of participants were male and Caucasian. There was a significant difference in the vessel diameter (range: 2.50 – 2.99 mm) between the DS and VS. Apart from this, there were no additional significant differences in the target vessels or lesion types between the two study cohorts.

Pre-dilatation of the target lesions was performed with angioplasty balloons inflated between 14 to 22 atmospheres (mean = 15 atmospheres) for a period of between 30 to 60 seconds (mean = 31.1 sec). During balloon inflation, ischemic ECG changes associated with chest discomfort were noted in 26/46 patients (56.5%), with ST-segment elevation in 19/46 patients (41.3%), and ST-segment depression in 7/46 patients (15.2%). In the remaining 20/46 patients (43.4%), there were no ECG changes observed, although 11/20 patients (55.0%) reported transient chest discomfort during this period. A total of 9/46 patients (19.6%) did not have ECG changes or symptoms during the procedure in which balloon inflation was performed up to the maximum period of 60 seconds to create transient ischaemia. The PCI procedure was then completed in all cases, and no major adverse events or complications were documented up to 30 days post-procedure in any of the participants.

Baseline Variables	**DS (n =25)**	**VS (n =21)**	**p value**
Age (years)	63.9 ± 9.0	63.0 ± 9.0	
Male	84%	85.7%	
Caucasians	88%	90.4%	

Risk Factors			0.674
Hypertension	12/25 (48%)	11/21 (52%)	
Diabetes	15/25 (60%)	7/21 (33.3%)	
Hyperlipidemia	22/25 (88%)	19/21 (90.4%)	
Smoking	10/25 (40%)	8/21 (38%)	
BMI (kg/m^2)	27.84±5.35	26.47±1.96	
Drugs			0.992
Antiplatelets	25/25 (100%)	21/21 (100%)	
Beta-Blockers	20/25 (80%)	16/21 (76.2%)	
ACEi	17/25 (68%)	14/21 (66.6%)	
Statins	25/25 (100%)	21/21 (100%)	
Nitrates	6/25 (24%)	4/21 (19%)	
Calcium channel	6/25 (24%)	7/21 (33.3%)	
Lesion			0.777
LAD	16/25 (64%)	12/21 (57.1%)	
RCA	6/25 (24%)	7/21 (33.3%)	
Cx	3/25 (12%)	2/21 (9.5%)	
Vessel Diameter			**0.017**
2.50 – 2.99	11/25 (44%)	1/21 (4.8%)	
3.00 – 3.49	7/25 (28%)	8/21 (38%)	
3.50 – 3.99	6/25 (24%)	8/21 (38%)	
4.50 – 5.00	1/25 (4%)	4/21 (19%)	
Lesion Length (mm)			0.993
10 – 14	4/25 (16%)	4/21 (19%)	
15 – 19	5/25 (20%)	5/21 (23.8%)	
20 – 24	8/25 (32%)	6/21 (28.6%)	
25 – 30	5/25 (20%)	4/21 (19%)	
>30	3/25 (12%)	2/21 (9.5%)	
Lesion Type			0.657
A	3/25 (12%)	1/21 (4.8%)	
B	10/25 (40%)	10/21 (47.6%)	
C	12/25 (48%)	10/21 (47.6%)	
% Stenosis			0.713
50 – 74	3/25 (12%)	2/21 (9.5%)	
75 – 94	15/25 (60%)	15/21 (71.4%)	
>95	7/25 (28%)	4/21 (19%)	
Procedural Data			
Mean Ischemic Period	31.1 ± 1.87	31.6 ± 3.65	
Median ST Elevation	0.5 mm	0.5 mm	
Median ST Depression	0.5 mm	0.5 mm	
Mean Troponin	0.04 ng/ml	0.03 ng/ml	
Mean CK	102.3 U/L	97.3 U/L	

Table 2: Patient characteristics, Lesion and procedural data of the patients in the DS and VS. BMI – Body Mass Index, ACEi – Angiotensin-Converting Enzyme inhibitor, CK – Creatine Kinase, Cx – Circumflex, LAD – Left anterior descending, RCA – Right coronary artery, DS – Discovery Study, VS – Validation Study. Bold defines $p < 0.05$.

3.2.2 Metabolomic phenotyping

The metabolic composition of peripheral plasma samples collected at baseline, and at 1 (TP1) and 5 minutes (TP5) following transient coronary ischaemia, was obtained using UPLC-MS. The significance of the change in the circulating levels of metabolites at 1 and 5 minutes following transient coronary ischaemia was calculated and the results are shown in Figure 28. The changes in circulating metabolites also showed significant variation at 1 and 5 mins when compared to baseline measurements, as shown in the canonical variate analysis (CVA) plot.

There were 2692 and 1012 metabolite features (*m/z*-RT pairs) detected in positive and negative ion modes, respectively, after quality assessment and data filtering in the DS. There were 3163 and 919 metabolite features detected in positive and negative ion modes, respectively, after the same process in the VS. 432 and 480 unique metabolites were annotated in negative ion mode in the DS and VS, respectively, with 179 unique metabolites identified in both the DS and VS studies. 1093 and 1143 unique metabolites were annotated in positive ion mode in the DS and VS, respectively, with 384 unique metabolites identified in both the DS and VS studies. Following statistical analysis, 108 and 203 unique metabolites were identified as significantly differing ($p<0.05$) in the DS and VS, respectively, when comparing baseline and TP1 samples. Of these, 54 unique metabolites were identified as differing ($p<0.05$) in TP1 (Table 3).

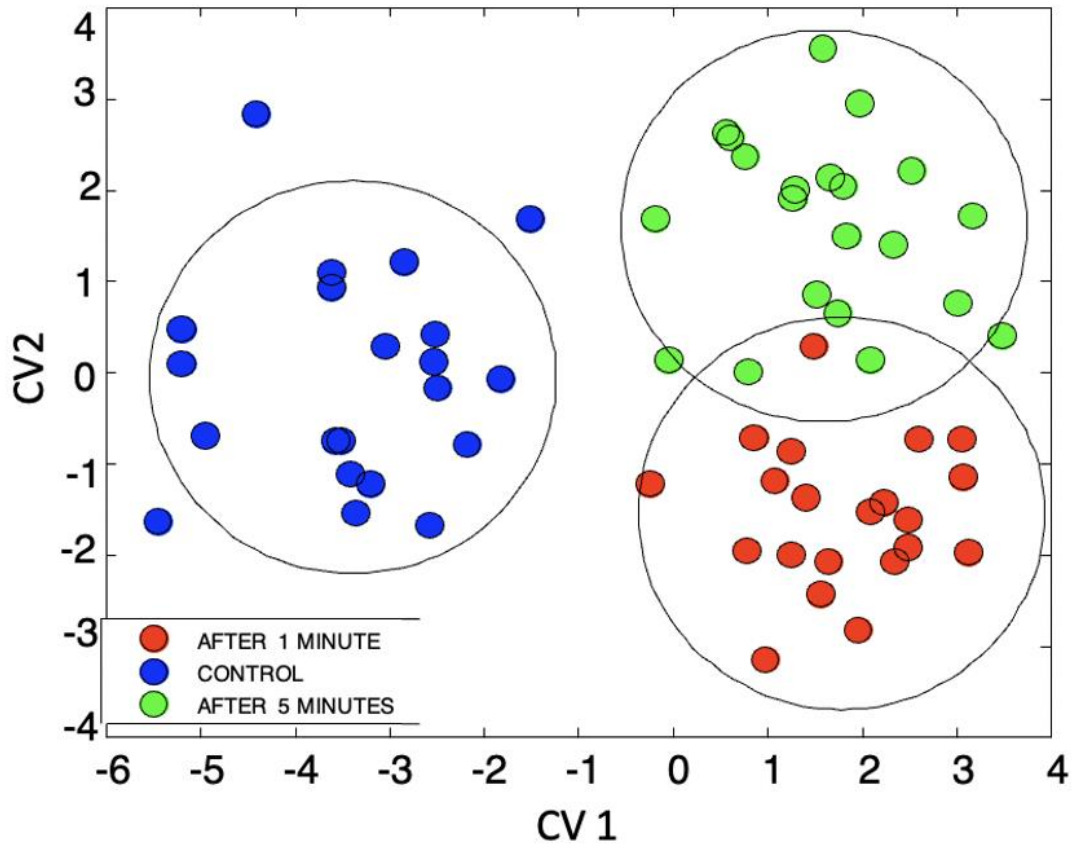

Figure 28: Canonical Variate Analysis (CVA) plot shows statistically significant variance in the metabolic changes observed at 1 and 5 mins when compared to the control group. The position in the x-axis represents first canonical variate axis (CV1) and the position in the y-axis represents second canonical variate axis (CV2). The concentration of metabolites in the control group is shown as blue colored dots, while the red colored dots represent metabolite changes at 1 min and the green colored dots represent the metabolites at 5 mins post transient coronary ischaemia. The plot shows that there is no significant overlap in the metabolites suggesting statistically significant variance at different time intervals. CV1 shows the primary effect implying that the major disruption to metabolism happens before 1 min while the CV2 represents the secondary effect, implying that not all metabolic disruption happens before 1 min and some of them return to normal before 5 mins.

		Discovery study		Validation study	
Metabolite	**Metabolite Class**	**p.value**	**FC**	**p.value**	**FC**
Octanoylcarnitine	Acyl carnitine	0.00628	1.34	0.00005	1.41
Decanoylcarnitine	Acyl carnitine	0.01269	1.23	0.00038	1.43
Tryptophan	Aromatic metabolites	0.00039	2.68	0.00209	1.32
2',6'-Dihydroxy-4'-methoxyacetophenone;"3-(2,3-Dihydroxyphenyl) propanoate";3-(3-hydroxyphenyl)-3-hydroxypropanoic acid;3-(4-Hydroxyphenyl) lactate;3-Methoxy-4-hydroxyphenylglycolaldehyde; Dihydrocaffeic acid; Homovanillate; Isohomovanillic acid; Phenylacetic acid	Aromatic metabolites	0.00048	1.93	0.00158	1.51
Formyl-N-acetyl-5-methoxykynurenamine; Phenylacetylglutamine; N-Acetylserotonin; N-methyltryptophan	Aromatic metabolites	0.00059	1.12	0.01823	1.40
2,5,6-Trihydroxy-5,6-dihydroquinoline;3-succinoylpyridine; adrenochrome; Hippurate; N-Acetylanthranilate	Aromatic metabolites	0.01947	0.78	0.00665	0.75
Hydroxybenzoate; Salicylate	Aromatic metabolites	0.02664	0.96	0.00019	1.20
2-Carboxy-2,3-dihydro-5,6-dihydroxyindole; Hydroxyhippuric acid; Carboxyphenylglycine; Dopaquinone; Salicyluric acid	Aromatic metabolites	0.03623	1.16	0.00281	1.15
Galactose 6-sulfate; Glucose 6-sulfate	Carbohydrate	0.00026	1.17	0.00042	1.41
1,5-Anhydro-D-glucitol; Deoxy-galactose; Deoxy-glucose; Rhamnose; Fucose; Fuculose; Rhamnulose	Carbohydrate	0.03277	1.06	0.02081	1.32
Phenylgalactoside	Carbohydrate	0.03999	1.13	0.03849	0.89
DG (38:6); DG (36:3)	Diacylglycerol	0.02157	1.24	0.03954	1.30
DG (32:2)	Diacylglycerol	0.03277	1.03	0.00896	1.11
Hydroxy-tetradecenoic acid; Oxo-tetradecanoic acid	Fatty acid	0.00102	1.26	0.00769	1.15
Heptenoic acid	Fatty acid	0.00428	2.59	0.03479	1.14

Methyl-octadecanoic acid; Nonadecanoic acid	Fatty acid	0.00730	1.22	0.01743	0.34
Hydroxy-oxo-hexadecanoic acid; Dihydroxycyclo pentaneundecanoic acid; pentadecenoic acid	Fatty acid	0.00944	0.71	0.01823	0.73
Docosahexaenoic acid	Fatty acid	0.01069	0.78	0.00336	0.68
Eicosadienoic acid; Icosatrienoic acid	Fatty acid	0.01362	0.78	0.02685	0.65
Hexadecanoate	Fatty acid	0.02148	0.85	0.00790	0.74
Icosatetraenoic acid; Eicosatetraenoic acid	Fatty acid	0.02664	0.76	0.01593	0.81
Oxo-hexacosenoic acid	Fatty acid	0.02664	1.26	0.00107	0.81
Heptacosanoic acid	Fatty acid	0.04405	1.06	0.01203	1.08
Octadecadienoic acid	Fatty acid	0.04599	0.86	0.01500	1.19
LysoPC (22:6)	Lysoglycerophospholipid	0.00072	0.81	0.00011	0.71
LysoPC (20:2)	Lysoglycerophospholipid	0.00233	0.75	0.01387	0.77
LysoPC (22:5)	Lysoglycerophospholipid	0.00365	0.72	0.00002	0.71
LysoPC (18:2)	Lysoglycerophospholipid	0.00486	0.88	0.00084	0.90
LysoPC (18:1)	Lysoglycerophospholipid	0.01208	0.89	0.03423	0.92
LysoPC (20:4)	Lysoglycerophospholipid	0.01531	0.84	0.00084	0.89
LysoPC (20:5)	Lysoglycerophospholipid	0.01947	0.64	0.00067	0.76
LysoPC (16:0)	Lysoglycerophospholipid	0.02395	0.84	0.02016	1.07
MG (20:0)	Monoacylglyceride	0.03999	1.15	0.00258	1.27
2-Acetamido-2-deoxy-6-O-a-L-fucopyranosyl-D-glucose;3-O-fucopyranosyl-2-acetamido-2-deoxyglucopyranose; N-Acetyl-6-O-L-fucosyl-D-glucosamine	Oligosaccharide	0.02633	0.86	0.00066	0.77
Aminoadenosine; Oxo-Adenosine;Dehydroadenosine ;S-aminomethyl dihydrolipoamide	Other class	0.01531	1.31	0.00042	1.87
1,3-Dimethyl-6,8-isoquinolinediol	Other class	0.01578	1.24	0.00790	1.24
Prolylhydroxyproline	Peptide	0.00628	2.21	0.01743	1.87
10-Deoxymethynolide	Polyketide	0.00286	1.57	0.00209	1.27
Hypoxanthine	Purine metabolite	0.03506	1.80	0.00557	1.95
5-Amino-6-(5'-phosphoribosylamino) uracil	Riboflavin metabolism	0.02664	1.09	0.01593	1.53

2-Methyl-3-ketovaleric acid; Oxohexanoic acid	Short chain organic acids	0.00021	1.29	0.00008	1.20
Ethylhydracrylic acid; Hydroxy-methylbutyric acid; Hydroxyvaleric acid	Short chain organic acids	0.00039	1.56	0.01203	1.15
Hydroxybutanoic acid	Short chain organic acids	0.00048	0.82	0.00665	0.50
Oxohexanoic acid; Methyl-oxopentanoate	Short chain organic acids	0.01578	1.22	0.02016	1.36
Butenoate; Isocrotonic acid	Short chain organic acids	0.02157	0.78	0.00258	0.75
Lactate	Short chain organic acids	0.02958	1.06	0.03479	1.14
Sphingosine 1-phosphate	Sphingolipids	0.04844	1.15	0.01099	1.31
11beta,21-Dihydroxy-3,20-oxo-5beta-pregnan-18-al;16alpha-Hydroxycorticosterone;"17alpha,21-Dihydroxy-5beta-pregnane-3,11,20-trione";18-Hydroxycorticosterone;"1alpha,17alpha,21-trihydroxy-20-oxo-22,23,24,25,26,27-hexanorvitamin D3;"4,5alpha-Dihydrocortisone"; Cortisol	Sterol and steroid metabolism	0.01923	1.12	0.00896	1.38
(20S)-1alpha,20,25-trihydroxy-24a-homovitamin D3;"(20S)-1alpha,25-dihydroxy-20-methoxyvitamin D3;"(23R)-1alpha,23,25-trihydroxy-23-methylvitamin D3;"(24R)-1alpha,24-dihydroxy-26,27-dimethyl-22-oxavitamin D3;"(24S)-1alpha,24-dihydroxy-26,27-dimethyl-22-oxavitamin D3;13'-carboxy-gamma-tocopherol;"1alpha,25-dihydroxy-11alpha-(hydroxymethyl)vitamin D3;"1alpha,25-dihydroxy-11alpha-methoxyvitamin D3;"1alpha,25-dihydroxy-11beta-methoxyvitamin D3;"1alpha,25-dihydroxy-24a,24b-dihomo-22-oxa-20-epivitamin D3;"1alpha,25-dihydroxy-24a,24b-dihomo-22-oxavitamin D3;"1alpha,25-dihydroxy-24a,24b-dihomo-23-oxavitamin D3;"1alpha,25-	Vitamin D metabolism	0.00944	1.16	0.00934	1.12

dihydroxy-26,27-dimethyl-20,21-didehydro-23-oxavitamin D3;"1alpha,25-dihydroxy-26,27-dimethyl-22-oxavitamin D3;"1alpha-hydroxy-18-(4-hydroxy-4-methylpentyloxy)-23,24,25,26,27-pentanorvitamin D3;(22S)-22-hydroxyvitamin D3;(24R)-24-hydroxyvitamin D3;(24S)-24-hydroxyvitamin D3;(5E)-1alpha-hydroxy-3-epivitamin D3;(5E)-1alpha-hydroxyvitamin D3;(5E)-1beta-hydroxy-3-epivitamin D3 ;(5E)-1beta-hydroxyvitamin D3					
(22R)-1alpha,22,25-trihydroxy-26,27-dimethyl-23,24-tetradehydro-24a-homo-20-epivitamin D3;"(22R)-1alpha,25-dihydroxy-22-methoxy-26,27-dimethyl-23,23,24,24-tetradehydrovitamin D3;"(22S)-1alpha,22,25-trihydroxy-26,27-dimethyl-23,23,24,24-tetradehydro-24a-homovitamin D3;"(22S)-1alpha,22,25-trihydroxy-26,27-dimethyl-23,24-tetradehydro-24a-homo-20-epivitamin D3;"(22S)-1alpha,25-dihydroxy-22-methoxy-26,27-dimethyl-23,24-tetradehydro-20-epivitamin D3;"1alpha-hydroxy-18-(4-hydroxy-4-ethyl-2-hexynyloxy)-23,24,25,26,27-pentanorvitamin D3	Vitamin D metabolism	0.01947	0.72	0.01743	1.09
24-Hydroxygeminivitamin D3;1alpha,25-dihydroxy-2beta-(5-hydroxypentoxy) vitamin D3	Vitamin D metabolism	0.02664	1.08	0.00557	1.17

(20S)-1alpha,25-dihydroxy-20-methoxy-26,27-dimethylvitamin D3;"(22R)-1alpha,22,25-trihydroxy-26,27-dimethyl-24a-homo-20-epivitamin D3;"(22S)-1alpha,22,25-trihydroxy-26,27-dimethyl-24a-homovitamin D3;"1alpha,25-dihydroxy-26,27-dimethyl-24a,24b-dihomo-22-oxa-20-epivitamin D3;"1alpha,25-Dihydroxy-2alpha-(3-hydroxypropyl)vitamin D3";"1alpha,25-dihydroxy-2beta-(3-hydroxypropyl)vitamin D3;"1alpha-hydroxy-18-(4-hydroxy-4-ethylhexyloxy)-23,24,25,26,27-pentanorvitamin D3;1alpha-hydroxy-2beta-(3-hydroxypropoxy)vitamin D3;"26,27-diethyl-1alpha,25-dihydroxy-22-oxavitamin D3;"26,27-diethyl-1alpha,25-dihydroxy-23-oxavitamin D3 ;(6R)-6,19-ethano-25-hydroxy-6,19-dihydrovitamin D3;"(6S)-6,19-ethano-25-hydroxy-6,19-dihydrovitamin D3;"1alpha-hydroxy-26,27-dimethylvitamin D3;1-Hydroxyvitamin D5;"25-hydroxy-26,27-dimethylvitamin D3;"25-Hydroxy-6,19-dihydro-6,19-ethanovitamin D3	Vitamin D metabolism	0.03999	1.08	0.00934	1.26

Table 3: Metabolites defined as statistically significant ($p<0.05$) when comparing baseline and TP1 samples in both the discovery and validation studies. FC is fold change, calculated applying the median relative concentrations at baseline/TP1. The metabolites highlighted are the metabolites of interest.

Similarly, 117 and 214 unique metabolites were identified as differing ($p <0.05$) in the DS and VS, respectively, when comparing baseline and TP5 samples. Of these, 55 metabolites were identified as statistically significant in both studies (Table 4). 30 and 77 unique metabolites were identified as differing (p

<0.05) in the DS and VS, respectively, when comparing TP1 and TP5 samples. Of these, nine metabolites were identified as statistically significant in both studies (Table 5).

		Discovery study		Validation study	
Metabolite	**Metabolite Class**	**p.value**	**FC**	**p.value**	**FC**
Octanoylcarnitine	Acyl carnitine	0.00628	1.27	0.00029	1.51
Decanoylcarnitine	Acyl carnitine	0.00902	1.28	0.00021	1.53
Tryptophan	Aromatic metabolites	0.00013	1.60	0.00011	2.59
2',6'-Dihydroxy-4'-methoxyacetophenone;"3-(2,3-Dihydroxyphenyl) propanoate";3-(3-hydroxyphenyl)-3-hydroxypropanoic acid;3-(4-Hydroxyphenyl) lactate; Dihydrocaffeic acid; Homovanillate; Isohomovanillic acid; Phenylacetic acid	Aromatic metabolites	0.00121	2.12	0.00033	1.57
2,5,6-Trihydroxy-5,6-dihydroquinoline;3-succinoylpyridine; adrenochrome; Hippurate; N-Acetylanthranilate	Aromatic metabolites	0.00488	0.76	0.00790	0.75
2-Amino-5-phosphopentanoic acid;2-Methylquinoline-3,4-diol;3-Hydroxy-2-methyl-1H-quinolin-4-one;3-Indoleglycolaldehyde;5-Hydroxyindoleacetaldehyde; Indole-3-acetate; Indoleacetic acid	Aromatic metabolites	0.00710	1.18	0.00557	1.29
Methylquinoline-3,4-diol;3-Hydroxy-2-methyl-1H-quinolin-4-one;3-Indoleglycolaldehyde;"3-Methyl-quinolin-2,8-diol";5-Hydroxyindoleacetaldehyde; Gentianine; Indole-3-acetate;Indoleacetic acid; Naphthoquinone	Aromatic metabolites	0.00801	1.15	0.00934	1.30
1,3-Dimethyl-6,8-isoquinolinediol	Aromatic metabolites	0.01578	1.17	0.00258	1.37

Mandelate;2-(Hydroxymethyl) benzoic acid; 2Hydroxyphenylacetate; "3,4-Dihydroxyphenylacetaldehyde";3-Hydroxyphenylacetate;3-Methoxytropolone;3-Methylsalicylate;4-Hydroxy-3-methoxy-benzaldehyde;4-Hydroxy-3-methylbenzoic acid;4-Hydroxymethylsalicylaldehyde;4-Hydroxyphenacyl alcohol;4-Hydroxyphenyl acetate;4-Hydroxyphenylacetate;4-Methoxybenzoate;4-Methylsalicylate;6-Methylsalicylate; isovanillin; Phenoxyacetate	Aromatic metabolites	0.02148	2.72	0.00336	11.28
2-Carboxy-2,3-dihydro-5,6-dihydroxyindole; Hydroxyhippuric acid; Carboxyphenylglycine; Dopaquinone; Salicyluric acid	Aromatic metabolites	0.02385	1.20	0.00233	1.13
Galactose 6-sulfate; Glucose 6-sulfate	Carbohydrate	0.00730	1.16	0.00002	1.50
DG (38:6); DG (36:3)	Diacylglyceride	0.00186	1.39	0.00066	1.37
DG (38:5); DG (36:2)	Diacylglyceride	0.04208	1.19	0.00029	1.28
Hydroxydecanedioic acid	Fatty acid	0.00013	2.91	0.03954	1.44
hydroxy-oxo-hexadecanoic acid; Dihydroxycyclopentaneundecanoic acid; pentadecenoic acid	Fatty acid	0.00026	0.62	0.00002	0.61
Docosahexaenoic acid	Fatty acid	0.00071	0.72	0.02322	0.83
Icosatetraenoic acid; **Eicosatetraenoic acid**	Fatty acid	0.00121	0.74	0.03423	0.76
Hydroxyhexanoic acid	Fatty acid	0.00639	1.28	0.02685	1.09
Hydroxystearate	Fatty acid	0.00944	0.69	0.02367	0.71
Hexadecanoate	Fatty acid	0.01069	0.81	0.04828	0.74
Eicosadienoic acid; Icosatrienoic acid	Fatty acid	0.01208	0.73	0.03037	0.64
hydroxy-hexadecanoic acid	Fatty acid	0.01718	0.79	0.03423	0.89
hydroxy-tetradecenoic acid; Oxo-tetradecanoic acid	Fatty acid	0.03192	1.48	0.01041	1.18
Dodecanedioic acid; hendecenoic acid; undecenoic acid	Fatty acid	0.03623	0.85	0.04317	0.81
Eicosapentaenoic acid; Icosapentaenoic acid	Fatty acid	0.04844	0.73	0.02081	0.62

LysoPC (20:2)	Lysoglycerophospholipid	0.00000	0.73	0.00896	0.77
LysoPC (20:4)	Lysoglycerophospholipid	0.00004	0.78	0.00665	0.87
LysoPC (18:2)	Lysoglycerophospholipid	0.00013	0.84	0.00233	0.91
LysoPC (22:5)	Lysoglycerophospholipid	0.00026	0.77	0.00029	0.78
LysoPC (22:6)	Lysoglycerophospholipid	0.00048	0.76	0.00008	0.75
LysoPC (18:1)	Lysoglycerophospholipid	0.00059	0.85	0.00233	0.86
LysoPC (16:0)	Lysoglycerophospholipid	0.00121	0.77	0.00557	1.11
LysoPC (20:5)	Lysoglycerophospholipid	0.00143	0.77	0.00042	0.76
LysoPC(O-16:0/0:0)	Lysoglycerophospholipid	0.00639	0.88	0.03479	1.14
LysoPC (17:1)	Lysoglycerophospholipid	0.00831	0.78	0.04317	0.92
LysoPC (16:1)	Lysoglycerophospholipid	0.01208	0.86	0.00042	0.88
LysoPC(dm16:0); LysoPC(O-16:1/0:0)	Lysoglycerophospholipid	0.01718	0.90	0.01286	1.14
1-O-alpha-D-glucopyranosyl-(2-hexadecanoyloxy)-eicosan-1-ol	Other class	0.04208	1.63	0.01743	1.47
16-Hydroxyestrone; estradiol-2,3-quinone; estrone-2,3-semiquinone	Other class	0.04208	1.25	0.01743	1.52
beta-Alanine; Alanine; Sarcosine; Methylglyoxal	Other class	0.04599	1.11	0.00464	1.16
Prolylhydroxyproline	Peptide	0.00375	1.74	0.00790	2.07
gamma-glutamyl-L-isoleucine; gamma-glutamyl-L-leucine	Peptide	0.02958	1.39	0.01823	1.34
10-Deoxymethynolide	Polyketide	0.00801	1.52	0.01286	1.24
Hypoxanthine	Purine	0.00100	1.82	0.00385	2.17
Dimethylguanosine	Purine	0.00554	1.31	0.02322	1.15
Dimethylxanthine; Theobromine; Theophylline	Purine	0.01755	1.09	0.02667	1.20
Hydroxybutanoic acid	Short chain organic acid	0.00001	0.76	0.00658	0.81
Ethylhydracrylic acid; Hydroxy-methylbutyric acid; Hydroxyvaleric acid	Short chain organic acid	0.00005	1.65	0.01387	1.19
2-Methyl-3-ketovaleric acid; Oxohexanoic acid; Methyl-oxopentanoate	Short chain organic acid	0.00186	1.34	0.00001	1.23
dimethyl-butenoic acid; methyl-pentenoic acid; hexenoic acid	Short chain organic acid	0.00248	0.63	0.03052	0.79

Butenoate; Isocrotonic acid	Short chain organic acid	0.01135	0.77	0.00168	0.71
Oxohexanoic acid; Methyl-oxopentanoate	Short chain organic acid	0.01417	1.21	0.01500	1.34
4-Sulfobenzaldehyde	Sulphur metabolism	0.00944	0.82	0.00067	0.40
(22R)-1alpha,22,25-trihydroxy-26,27-dimethyl-23,24-tetradehydro-24a-homo-20-epivitamin D3;"(22R)-1alpha,25-dihydroxy-22-methoxy-26,27-dimethyl-23,23,24,24-tetradehydrovitamin D3;"(22S)-1alpha,22,25-trihydroxy-26,27-dimethyl-23,23,24,24-tetradehydro-24a-homovitamin D3;"(22S)-1alpha,22,25-trihydroxy-26,27-dimethyl-23,24-tetradehydro-24a-homo-20-epivitamin D3;"(22S)-1alpha,25-dihydroxy-22-methoxy-26,27-dimethyl-23,24-tetradehydro-20-epivitamin D3;"1alpha-hydroxy-18-(4-hydroxy-4-ethyl-2-hexynyloxy)-23,24,25,26,27-pentanorvitamin D3	Vitamin D metabolism	0.00375	0.62	0.04477	1.06

Table 4: Metabolites defined as statistically significant ($p<0.05$) when comparing baseline and TP5 samples in both the discovery and validation studies. FC is fold change, calculated applying the median relative concentrations at baseline/TP5. The metabolites highlighted are the metabolites of interest.

		Discovery study		Validation study	
Metabolite	**Metabolite Class**	**p.value**	**FC**	**p.value**	**FC**
1alpha-hydroxy-26,27-dinorvitamin D3 25-carboxylic acid	Vitamin D metabolism	0.0192	1.3	0.0067	1.23
24-Nor-5beta-chol-22-ene-3alpha,7alpha,12alpha-triol	Bile acid metabolism	0.0459	1.26	0.0001	1.18
DG (36:4); DG (34:1)	Diacylglyceride	0.0459	1.11	0.0267	1.14
Heneicosanedioic acid; MG (18:1)	Other class	0.0121	1.14	0.0267	1.28
LysoPC (18:1)	Lysoglycerophospholipid	0.0083	0.95	0.009	0.92
LysoPC (18:2)	Lysoglycerophospholipid	0.0172	0.95	0.015	1.07
methyl-hexadecanedioic acid; Heptadecanedioic acid	Fatty acid	0.0484	0.89	0.00007	1.2
MG (14:0)	Monoacylglyceride	0.0025	1.34	0.00001	1.22
MG (16:0)	Monoacylglyceride	0.001	2.01	0.0046	1.14

Table 5: Metabolites defined as statistically significant ($p<0.05$) when comparing TP1 and TP5 samples in both the discovery and validation studies. FC is fold change, calculated applying the median relative concentrations at TP1/TP5.

During hypoxia there is a mismatch between oxygen demand and supply, hence oxidative phosphorylation cannot continue resulting in decreased ATP production. This causes accelerated glycolysis and a switch to lactate production. This initial myocardial response to acute ischaemia is evident in this study findings. An increase in lactate was observed at baseline and TP1 (Table 3) but this was not observed at baseline and TP5. This suggest that the increase in lactate was a short-term response, in keeping with transient acute ischaemic insult. In addition, it was interesting to find a significant alteration in hypoxanthine, a purine metabolite at TP1 and TP5 when compared to baseline. The treatment of ACS is primarily focused on restoration of blood flow to the ischaemic myocardium. Restoration of blood flow to the ischaemic zone leads

to production of reactive oxygen species (ROS), which causes damage to the cardiomyocytes, in a phenomenon called myocardial reperfusion injury (262). Increased ROS production during ischaemia-reperfusion is thought to be due to the activation of hypoxanthine in the mitochondrial ETC reactions in the cardiomyocytes (263). The method adopted to create ischaemia in this study mimics the similar phenomenon of ischaemia-reperfusion and therefore the observed alteration of hypoxanthine is relevant in this context.

3.2.3 Assessment of specific metabolite changes

3.2.3.1 Acyl carnitines

Metabolites predominantly involved in the lipid metabolism were altered. Two acyl carnitines (octanoylcarnitine and decanoylcarnitine) showed higher concentrations in baseline samples compared to both TP1 and TP5 samples. Box plots for octanoylcarnitine for DS and VS are shown in Figures 29 and 30 respectively**.** There is ample evidence that myocardial carnitine levels quickly diminish during an ischaemic event (264). This study findings similarly demonstrates that the octanoylcarnitine and decanoylcarnitine levels were lower at both time points of ischaemia when compared to the baseline suggesting that the subjects had a controlled ischaemic event as intended. In addition, it is well known that carnitine plays an important role in the energy production in the myocardium and has been shown to transport FFA into the mitochondria for oxidative metabolism in the heart (265). Hence, diminished carnitine during ischaemia interferes with the FA metabolic pathway resulting in accumulation of toxic intermediates that are known to be associated with fatal ventricular arrhythmias. Studies have shown that exogenous supplementation

of L-carnitine replenish depleted myocardial carnitine levels and improve cardiac metabolic and left ventricular function (266).

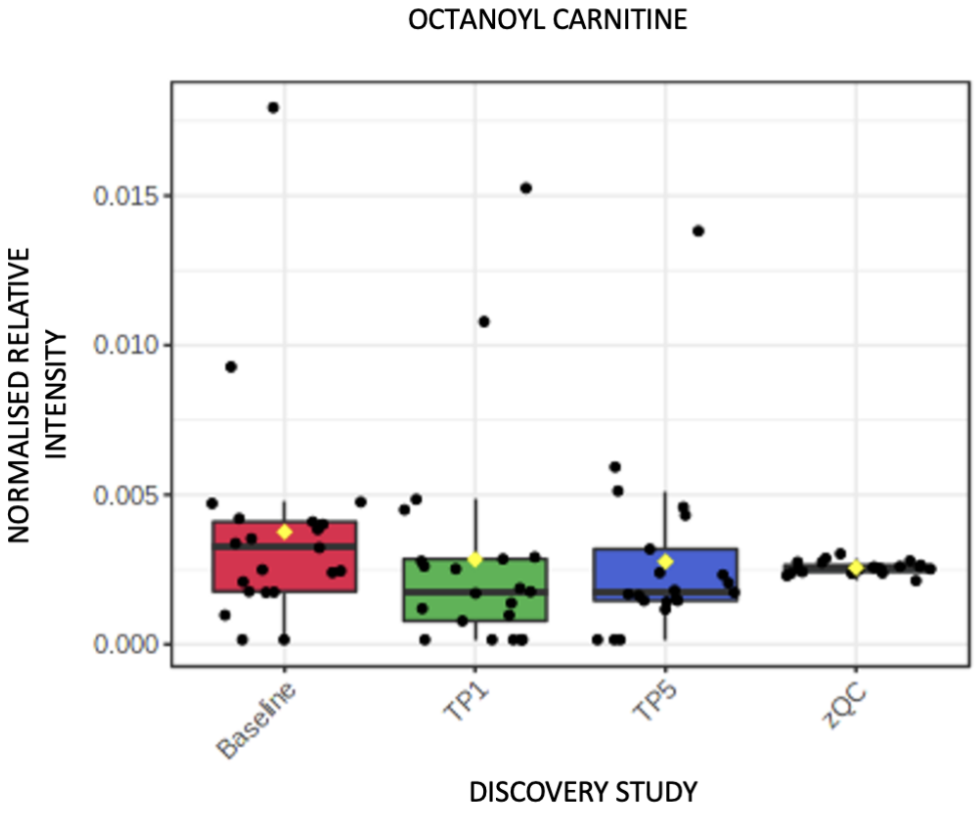

Figure 29: Box and whisker plot describing normalized relative intensity values in the concentration of octanoylcarnitine at three timepoints (0=baseline, 1=TP1 and 5=TP5) for plasma samples ($p<0.05$). QC sample data are included (QC). Data for the discovery study.

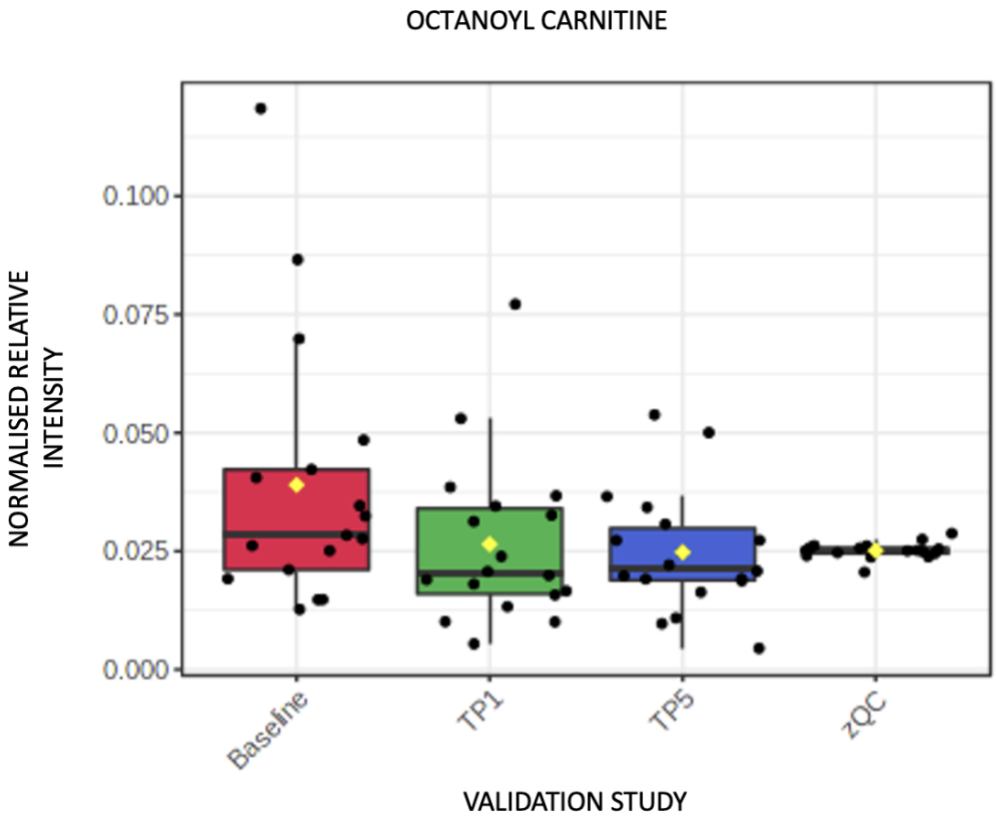

Figure 30: Box and whisker plot describing normalized relative intensity values in the concentration of octanoylcarnitine at three timepoints (0=baseline, 1=TP1 and 5=TP5) for plasma samples ($p<0.05$). QC sample data are included (QC). Data for the validation study.

3.2.3.2 Docosahexaenoic Acid

The levels of docosahexaenoic acid (DHA) were noted to be elevated at 1 minute and 5 minutes in DS and VS when compared to the baseline (Figures 31 & 32). DHA levels showed a consistent elevation both at TP1 and continued to increase at TP5 when compared to baseline in the DS cohort. In the VS cohort, it was observed that although the DHA levels were higher at TP5 when compared to the baseline, the levels were lower than at TP1. This suggests an early spike in concentration of the DHA, which is not consistently elevated following the ischaemic insult. However, the data looks more variable at TP5

which might indicate differential time courses in different patients, with some patients having a transient spike, while the others have a more prolonged increase. Although all the study subjects had controlled ischaemic insult without significant myocardial necrosis, the time duration of balloon dilation varied between patients, depending on the observed ischaemic response either by means of ECG findings or symptoms. Therefore, the degree of ischaemic insult may explain the variation observed.

The observed upregulation of DHA in this study is an important finding as DHA has received considerable attention in the recent decades in its protective role in ACS (267, 268). The elevated levels of DHA during the early phases of myocardial ischaemia utilized in this study have not previously been described. This finding therefore has high clinical significance, as it not only reflects a natural protective effect in response to ischaemia, but also opens up new areas of research. In ACS, plaque rupture is associated with vasoconstriction, platelet aggregation, and thrombus formation mediated by the eicosanoids through the cyclooxygenase (COX) pathway. An unopposed upregulation of the COX pathway during ACS would therefore be detrimental to cardiac function. Although DHA share a common pathway with FA's, the selectivity for the metabolic pathway is determined by its concentration gradient. Thereby, an increased concentration of DHA can shift the pathway from COX to cytochrome p-450 (CYP) causing a vasodilatory and anti-inflammatory effect. Several epidemiological studies have originated and supported this hypothesis (269, 270). Although DHA is not known to have a direct antithrombotic effect, its mechanism of potentially modulating the effect of eicosanoids may thus favorably influence thrombus burden in ACS. Although this is an important

finding in terms of understanding the biological beneficial effect of DHA and its potential therapeutic applications, it would be crucial to know the time duration of elevation of DHA to assess its feasibility as a measurable biomarker. Therefore, future targeted metabolomic studies should focus on assessing blood samples at a time course of 30 mins to one hour in patients presenting with ACS.

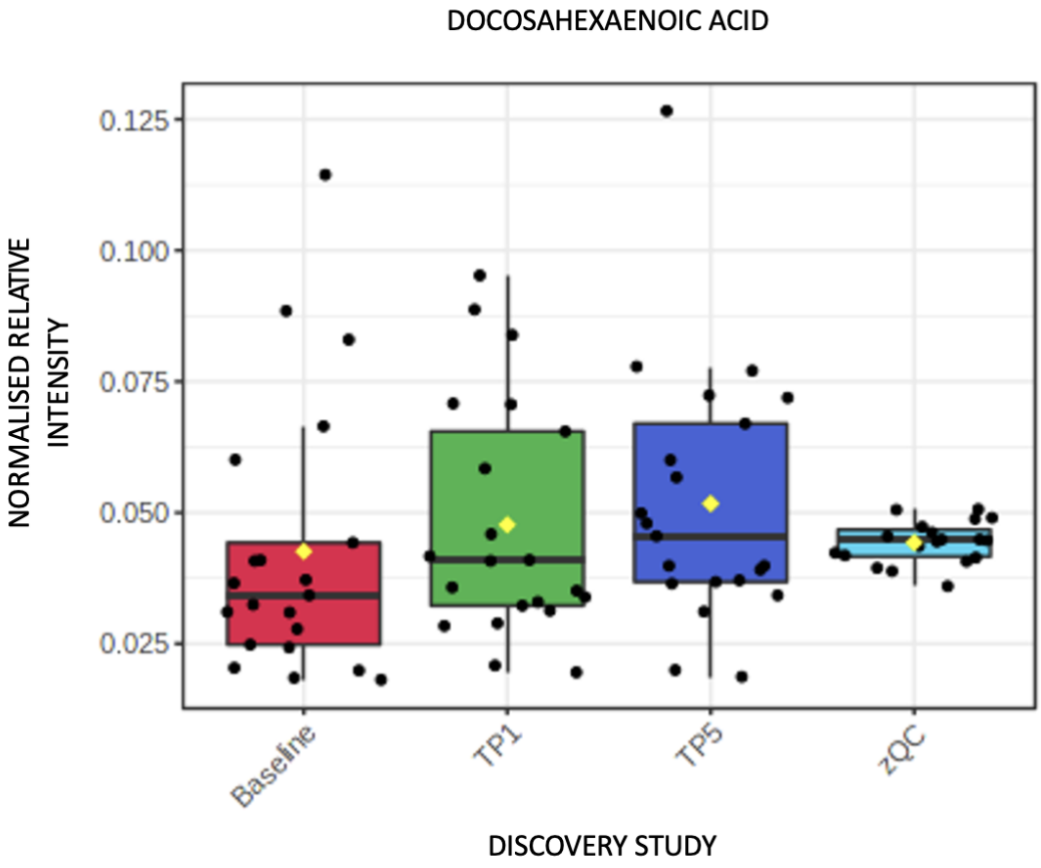

Figure 31: Box and whisker plot describing normalized relative intensity values in the concentration of Docosahexaenoic acid at three timepoints (0=baseline, 1=TP1 and 5=TP5) for peripheral (P) and coronary sinus (CS) samples. QC sample data are included (QC). Data for the discovery study.

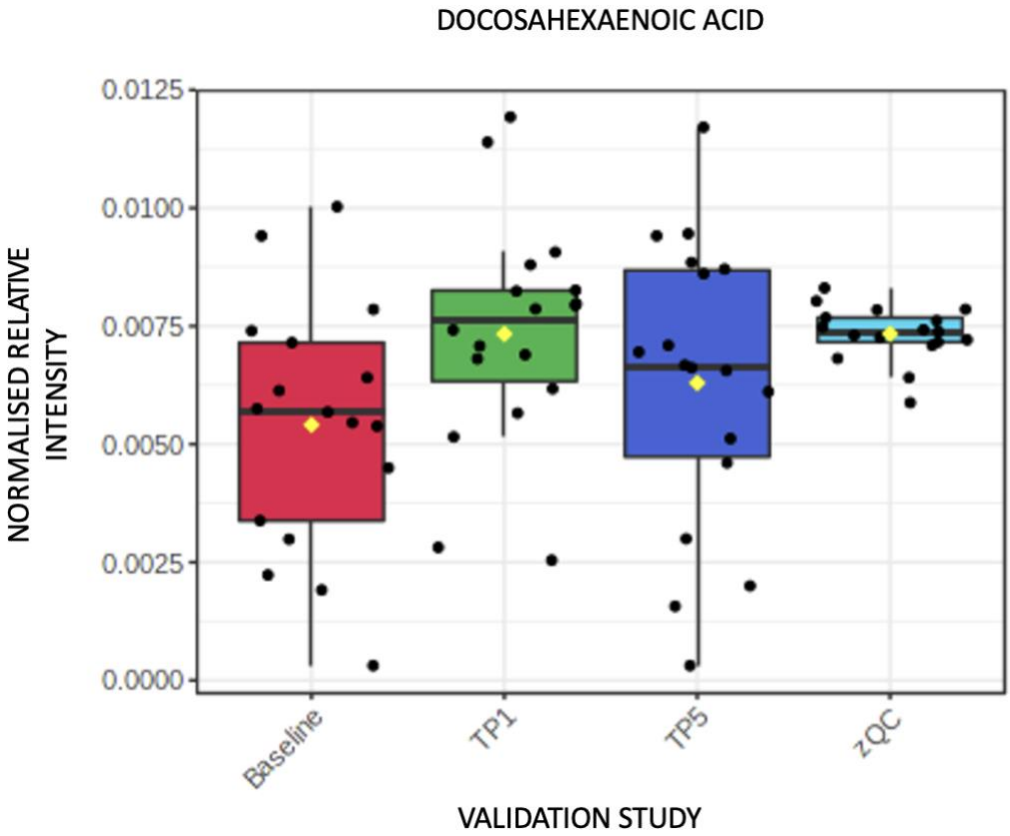

Figure 32: Box and whisker plot describing normalized relative intensity values in the concentration of Docosahexaenoic acid at three timepoints (0=baseline, 1=TP1 and 5=TP5) for peripheral (P) and coronary sinus (CS) samples. QC sample data are included (QC). Data for the validation study.

3.2.3.3 Fatty Acids

C_{16} - C_{22} fatty acids also showed a higher concentration at TP1 and TP5 when compared to baseline levels. Lysophosphatidylcholine (LysoPC) showed higher concentrations in TP1 and TP5 samples when compared to baseline samples in the DS and VS, respectively (Figures 33 & 34). The development of hypoxia during acute ischaemia, is associated with disruption of the cardiomyocyte cell membrane and is characterized by reduced resting membrane potentials, local release of catecholamines and increased intracellular calcium causing delayed afterdepolarization, triggering

arrhythmias (83). Early studies have shown that accumulation of LPC in the ischaemic myocardium and in the venous effluents from the ischaemic regions may enhance these effects causing profound electrophysiological derangements and potentially induce fatal arrhythmias (271).

LysoPC, a principal component of the cell membrane is released as a result of abrupt decrease in cytosolic pH in the ischaemic myocardium due a shift to anaerobic metabolism and lactate production. In addition, under normal conditions cardiac muscle has a relatively higher concentration of LCAC due to its preference for FA metabolism. However, during acute hypoxia, the LCAC concentration is known to increase severalfold which in turn is known to inhibit enzyme that catalyze LysoPC resulting in significant intracellular accumulation (86). Oxfenicine, a known CPT1 inhibitor has been shown to inhibit this pathway thus preventing the accumulation of LysoPC and ventricular arrhythmias (89). Ventricular arrhythmia is a feared complication of ACS, particularly in patients with STEMI and is the commonest cause of sudden cardiac death. Therefore, in addition to restoring blood flow as quickly as possible by PCI, pharmacological measures to minimize the occurrence of arrhythmias are considered. In this regard, this study findings are important and provides insight into future research prospects. The observation of a sustained rise of LysoPC as evidenced by consistent rise in levels from baseline and with significant changes between TP1 and TP5 as shown in Table 5, suggest that there is a potential to evaluate this biomarker at later time points in patients with ACS.

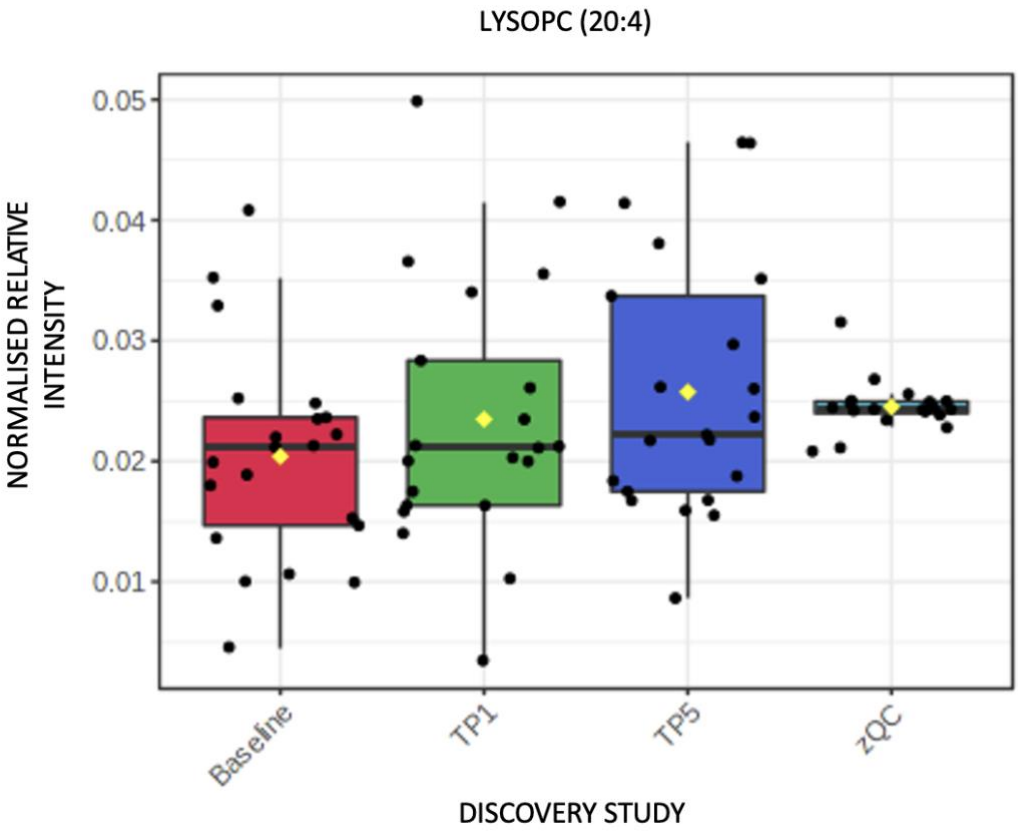

Figure 33: Box and whisker plot describing normalized relative intensity values in the concentration of LYSOPC (20:4) at three timepoints (0=baseline, 1=TP1 and 5=TP5) for peripheral (P) and coronary sinus (CS) samples. QC sample data are included (QC). Data for the discovery study.

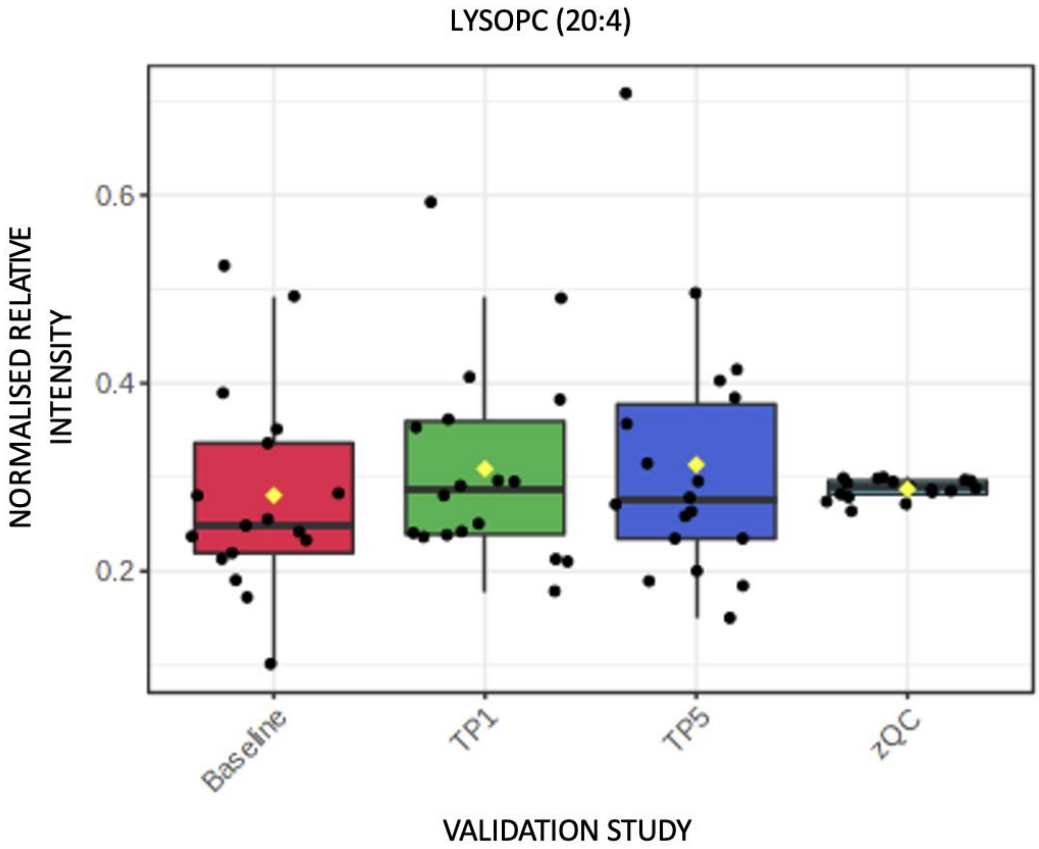

Figure 34: Box and whisker plot describing normalized relative intensity values in the concentration of LYSOPC (20:4) at three timepoints (0=baseline, 1=TP1 and 5=TP5) for peripheral (P) and coronary sinus (CS) samples. QC sample data are included (QC). Data for the validation study.

3.2.3.4 Tryptophan

Significant changes were also observed in aromatic metabolites. Figure 35 & 36 below shows the changes observed for tryptophan in the DS and VS, respectively.

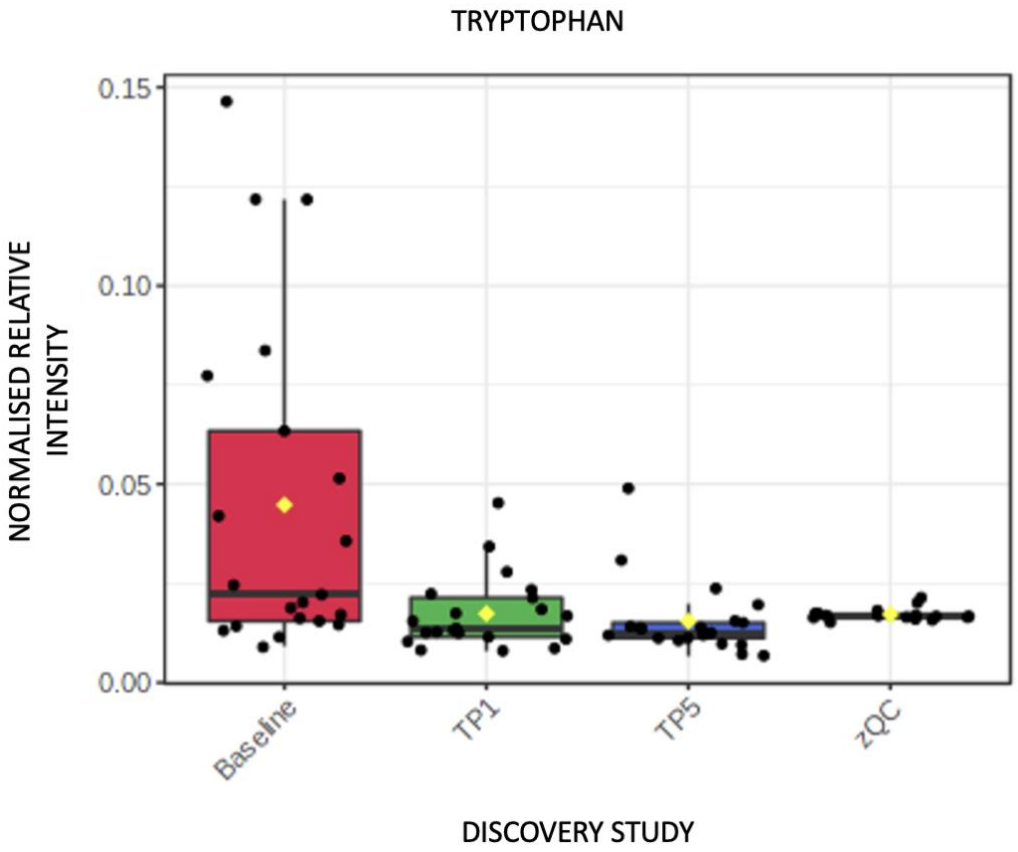

Figure 35: Box and whisker plot describing normalized relative intensity values in the concentration of Tryptophan at three timepoints (0=baseline, 1=TP1 and 5=TP5) for peripheral (P) and coronary sinus (CS) samples. QC sample data are included (QC). Data for the discovery study.

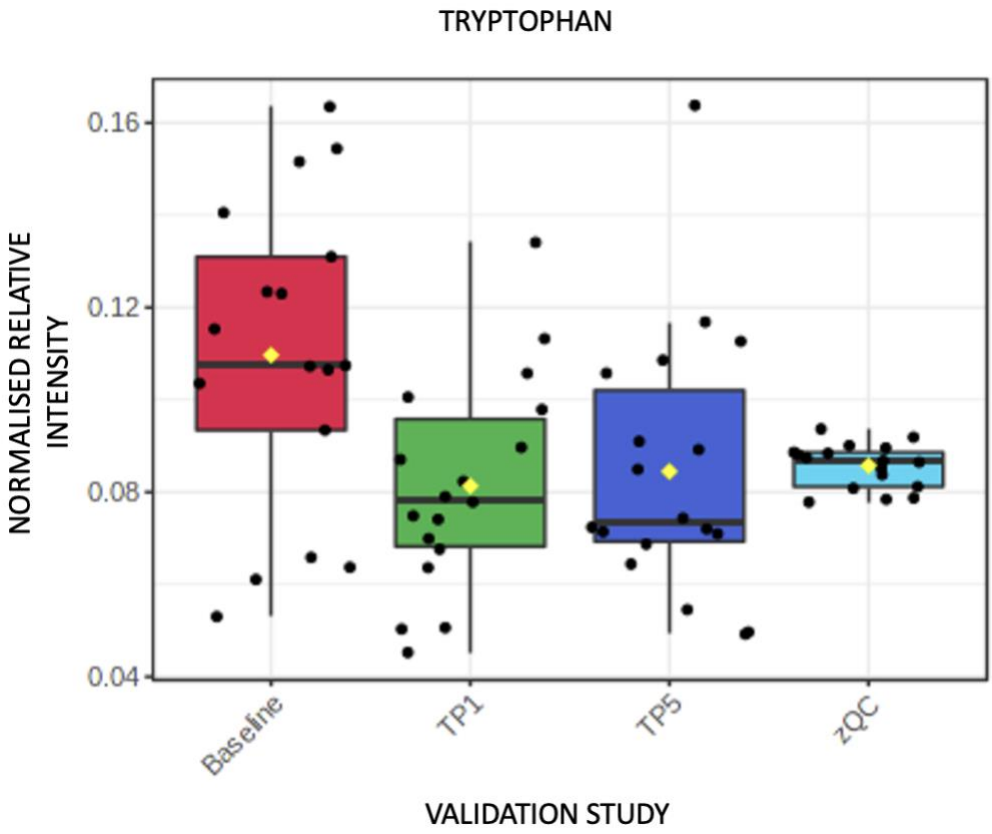

Figure 36: Box and whisker plot describing normalized relative intensity values in the concentration of Tryptophan at three timepoints (0=baseline, 1=TP1 and 5=TP5) for peripheral (P) and coronary sinus (CS) samples. QC sample data are included (QC). Data for the validation study.

In this study population, tryptophan metabolism showed a decrease in concentration both at TP1 and TP5 when compared to baseline, following the ischaemic event, with statistically significant difference in both the DS and VS respectively. Interestingly, the study also identified tryptophan degradation products, kynurenine and N-formyl kynurenine, suggesting enhanced tryptophan catabolism in response to acute myocardial ischaemia. This in turn implies that kynurenine pathway was upregulated as a consequence of acute hypoxia. A decrease in plasma concentration of tryptophan has been observed

in previous studies of patients with CHD and has been attributed to increased catabolism (272). In addition, kynurenine is known to have direct effect in the coronary arteries and recent studies have identified kynurenine as a potent vasodilator (273). This vasodilatory effect is an important determinant in the consequence of ACS and therefore suggest a cardioprotective effect.

3.2.3.5 Arachidonic Acid

Six short-chain fatty acids also showed a statistically significant difference when comparing baseline to TP1 and TP5 samples in both studies with increases and decreases in their concentration when comparing baseline to TP1 and TP5 samples. Arachidonic acid (AA) showed a significant upregulation at TP1 and TP5 in both sample types (peripheral and coronary sinus) after transient coronary ischemia (Figures 37 & 38). Chest pain and distress are a common presentation in ACS, provoking increased sympathetic drive and catecholamine release resulting in increased circulating FFA. This not only suppress the oxidation of glucose but also results in accumulation of FFA intermediates and eicosanoids. Upregulation of AA metabolism and their downstream eicosanoids via COX pathway causes vasoconstriction, platelet aggregation and thrombus burden. An unopposed effect of AA is thus known to have detrimental effect in ACS and modulation of this pathway by shifting from COX to CPY450, would be a feasible and interesting area of further study.

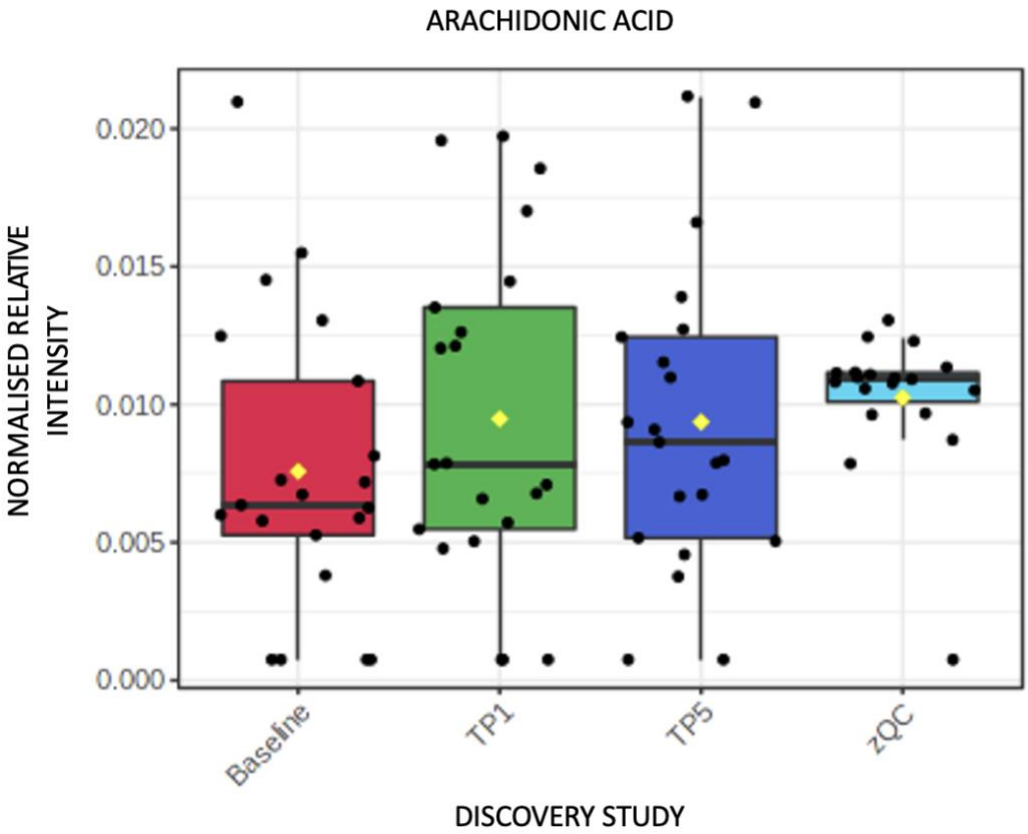

Figure 37: Box and whisker plot describing normalized relative intensity values in the concentration of Arachidonic acid at three timepoints (0=baseline, 1=TP1 and 5=TP5) for peripheral (P) and coronary sinus (CS) samples. QC sample data are included (QC). Data for the discovery study.

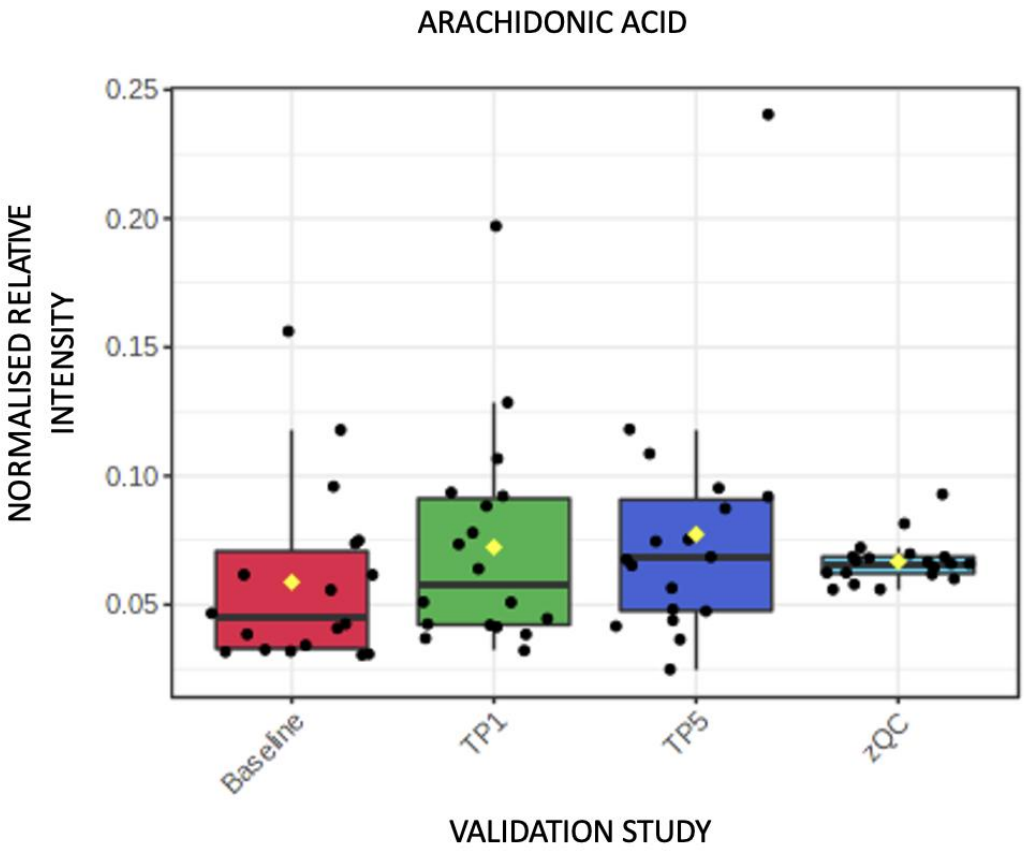

Figure 38: Box and whisker plot describing normalized relative intensity values in the concentration of Arachidonic acid at three timepoints (0=baseline, 1=TP1 and 5=TP5) for peripheral (P) and coronary sinus (CS) samples. QC sample data are included (QC). Data for the validation study.

Robust changes were also observed in other metabolite classes in both the DS and VS. Acyl carnitines (3 and 3 metabolites), aromatic metabolites (15 and 20 metabolites), bile acids (6 and 7 metabolites), carbohydrates (7 and 12 metabolites), diacylglycerides (7 and 6 metabolites), fatty acids (38 and 31 metabolites), lysoglycerophospholipids (25 and 20 metabolites), purine and pyrimidine metabolites (7 and 5 metabolites), short-chain organic acids (4 and 5 metabolites), sterols and steroids (12 and 11 metabolites), vitamin D metabolites (6 and 8 metabolites), TCA metabolites (2 and 1 metabolites) and

branched-chain amino acid metabolism (2 and 1 metabolite) all showed differences in one or both of the DS and VS, respectively.

3.3 Discussion

In a carefully selected cohort of patients with single vessel coronary artery disease, controlled ischaemia was created by transient coronary occlusion. Peripheral and coronary blood samples were analysed using UPLC-MS platform. The study observed statistically significant changes in both DS and VS group with metabolites predominantly involved in the lipid metabolism. Similar changes were observed in coronary sinus samples and in peripheral blood samples for all metabolites, implying that the metabolic changes are myocardial specific. The metabolites of interest were arachidonic acid, lysophosphatidylcholine, docosahexaenoic acid, carnitine and tryptophan.

In addition to the signature metabolites observed during ischaemia, the study also observed interesting phenomenon to demonstrate a valid ischaemia model.

3.3.1 Validity of the ischaemia model

In order to draw a meaningful hypothesis, the model the has to be robust. This study created a myocardial ischaemia model by transient coronary occlusion. From the procedural perspective, balloon inflation of the target coronary vessel was accompanied by contrast injection, confirming total coronary occlusion. Similarly, immediately following deflation of the balloon, coronary angiography confirmed complete reversal of the occlusion. Therefore, undoubtedly there was an objective evidence of coronary occlusion that could generate coronary ischaemia. In addition, although symptoms are quite subjective and ECG

changes does not necessarily represent ischaemia, these observations during the ischaemic insult further supported the model. The study population only involved patients with discreate single vessel coronary artery disease, therefore continued ischaemic insult from elsewhere was impossible, supporting transient reversible ischaemia. In addition, none of the study population had symptoms consistent with acute or chronic decompensated heart failure such as shortness of breath, orthopnea (breathless on lying) or paroxysmal nocturnal dyspnoea, that are known to influence the normal metabolic substrate. Moreover, all participants had an echocardiogram within one month prior to enrolment which confirmed normal left and right ventricular systolic function and a structurally normal heart.

Interestingly, the study results demonstrate specific metabolic perturbation in support of the paradigm of acute myocardial ischaemia. The study observed, homovanillate, a catecholamine metabolite at TP1 and TP5 to be consistently elevated when compared to the baseline, suggesting that there was evidence of metabolic stress. Homovanillate, a major catecholamine metabolite is known to be associated with metabolic stress. Oxidative stress plays an important role in the pathogenesis of ACS and occurs when there is an imbalance between the generation ROS and the antioxidant defense systems in the body so that the latter becomes overwhelmed (274).

Lactate was also observed to increase significantly at TP1 compared to baseline indicating anaerobic metabolism consistent with ischaemia. However, this increase was not observed at TP5 suggesting that this was a short-term response in keeping with transient response. Furthermore, the method used to induce reversible myocardial injury mimics the well-known phenomenon of

ischaemia-reperfusion injury. In support of this phenomenon the study observed significant alterations in the hypoxanthine levels at TP1 and TP5, associated with ATP degradation and ischaemia reperfusion.

3.3.2 Mechanistic relevance of the biomarkers of interest

The study observed myocardial tissue specific metabolic changes related to acute myocardial ischaemia. The study observed significant upregulation of AA, DHA and LysoPC and downregulation of octanoylcarnitine, decanoylcarnitine and tryptophan in a 5-minute period following PCI. This diverse yet specific metabolic pattern identified in response to acute hypoxia involved multiple interlinked pathways that represent several areas of metabolism. Interestingly the pattern had a combination of both detrimental and cardioprotective metabolites. DHA and LysoPC was noted to be consistently elevated at all time points implying its potential for further evaluation at later time points (e.g., 30 mins or 60 mins). This may provide further insight into the feasibility of measuring the metabolite as a potential prognostic biomarker of ACS. Furthermore, tryptophan, its downstream product kynurenine and kynurenine pathway may serve as a potential target for therapeutic intervention in patients with ACS. The study observed significant changes predominantly involving the lipid pathway. Although lipid metabolism involves several complex interlinked pathways such as COX, and CYP-450 epoxygenases pathway that could serve as a target for modulation, the knowledge from this study is not sufficient to support this and requires further targeted studies focusing on patients with ACS.

Chapter 4: subgroup analysis on the impact of co-morbidities and other factors in the study population.

4.1 Introduction

CHD is often present together with an increased prevalence of co-morbidities such as diabetes, hypertension, hyperlipidemia, obesity and metabolic syndrome (275). Excluding these factors are therefore virtually impossible in the study population. However, it is well known that such comorbidities have unique metabolic signatures that are involved in lipid, carbohydrate and amino acid metabolism. A subgroup analysis was therefore performed to assess the impact of the comorbidities, concomitant medications and other factors that may have influenced the study results.

4.2 Aims and objective of the subgroup analysis

The analysis thus far has characterized the effects of myocardial ischaemia on the two separate cohorts. This analysis further explores the effects of other factors that may also have influence on the metabolites profile. The aim was to look for other causes of variance in the data that could elicit variation in the metabolites, as well as offer personalized predictors of myocardial ischaemia and potential outcome of this. The variables analysed included age, gender, co-morbidities (diabetes, hypertension, hypercholesterolemia, body mass index), drugs (beta-blockers, angiotensin converting inhibitors, angiotensin receptor blockers, anti-anginals, statins, proton pump inhibitors, and others),

and procedure related variables (Chest pain, ECG changes, Balloon type and size, and Site of sample collection).

4.3 Results

4.3.1 Inter-individual variability

PCA was initially used as an exploratory method to look for any natural groupings arising from the variation in the data. To aid visualization of any groupings, color coding was used on the scores plots, focusing on the first six principal components.

Metabolite profiles reflect the physiological status of the body. Interestingly, clustering of samples related to each individual was noted in both positive and negative ion modes. This interindividual variability suggest that the metabolic signature of each individual is unique (Figure 39).

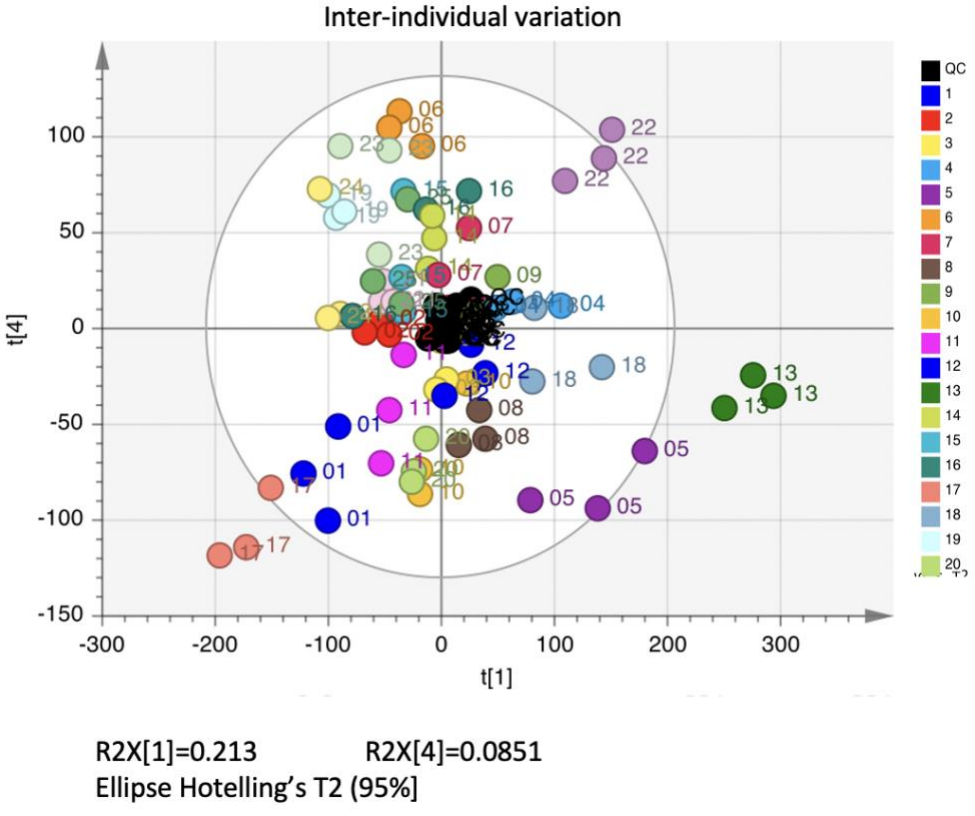

Figure 39: PCA score plot shows interindividual variation. All samples colour coded according to subjects. t[1] shows scores for the samples in the first principal component and t[4] shows scores from the fourth principal component. The plot shows clustering across PC1 and 4 by individual**.** QC samples centred around the mean. R2X[1]: variance in the predictor variable t1=21%; R2X[4]: variance in the predictor variable t4=8.5%.
Ellipse Hotelling's T2: 95% confidence interval of the Hotelling's T-squared distribution**.**

4.3.2 Gender

The study population had a significant male dominance with 21/25 (84%) and 18/21 (85%) in DS and VS respectively. Although CHD remains the leading cause of morbidity and mortality in both men and women, there are substantial gender differences with higher prevalence in men until 75 years of age (276). This could probably explain the gender difference in the study population. The three-dimensional PCA plot across PC 3 to PC5 showed some clustering (Figure 40). However, in view of a significant gender bias, the significance of the observed variance should be interpreted with caution.

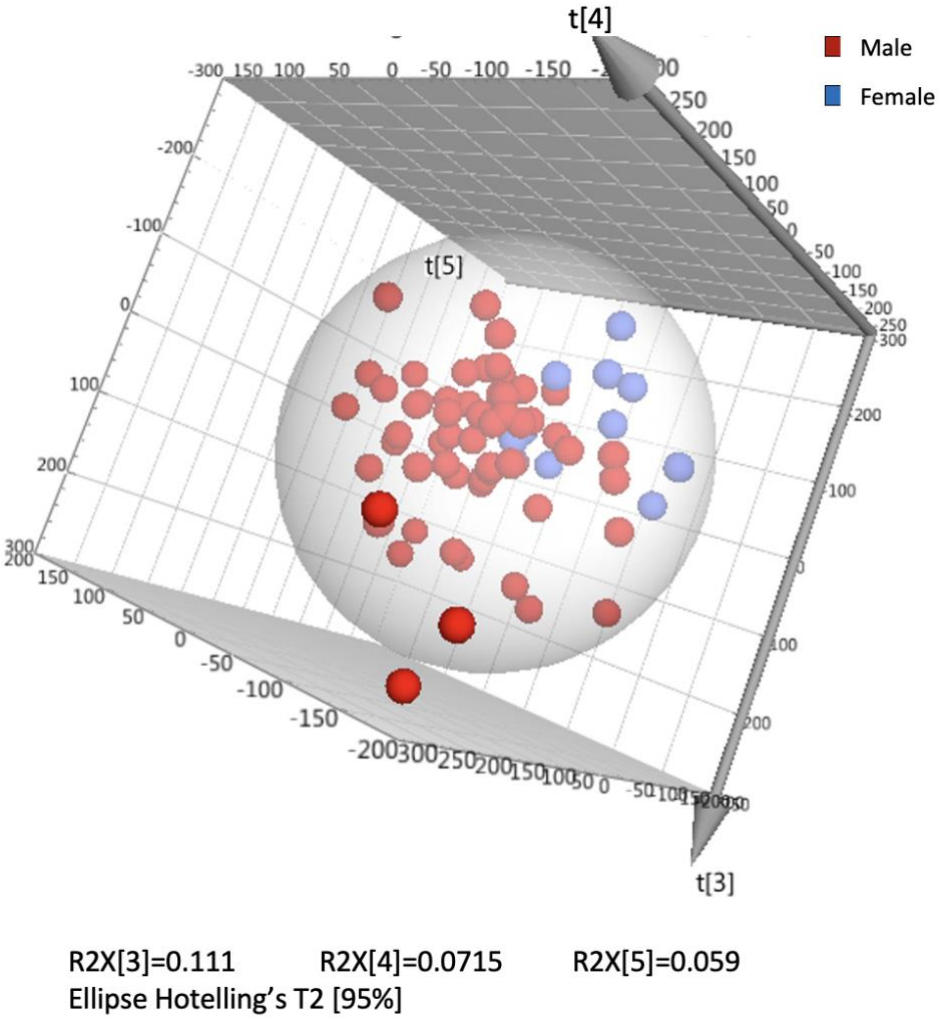

Figure 40: Three dimensional PCA score scatter plot classified by sex in the positive and negative ion combined dataset of the DS group. t[3]: third principal component, t[4]: fourth principal component, t[5]: fifth principal component. Blue dots for females and red dots for males. Score's plot showing the distribution of samples in PCs3-5. R2X[3]: variance explained in the predictor variable t3=11.1%, R2X[4]: variance explained in the predictor variable t4=7.2% and R2X[5]: variance explained in the predictor variable t5=6%.
Ellipse Hotelling's T2: 95% confidence interval of the Hotelling's T-squared distribution.

4.3.3. Comorbidities

4.3.3.1 Diabetes

Diabetes is one of the important comorbidities associated with increased risk of CHD. The 2019 International Diabetes Federation statistics reveal an estimated prevalence of 463 million cases of diabetes among adults between the age of

20 and 79 worldwide (277). In the study population, 15/25 (60%) of the patients in the DS and 7/21 (33%) in the VS had diabetes treated with oral hypoglycemics. The PCA plots of the patients with diabetes in the DS group is shown below. The scores plot show partial clustering across PC1 and PC3 (Figure 41). Diabetes and metabolic syndrome are closely associated and several studies suggest that people with metabolic syndrome are 5-times more likely to develop diabetes (278). It therefore possible that patients with diabetes have unique metabolic signatures. It is also reasonable to speculate that the observed variation could be secondary to enhanced glucose metabolism as an initial response to hypoxia.

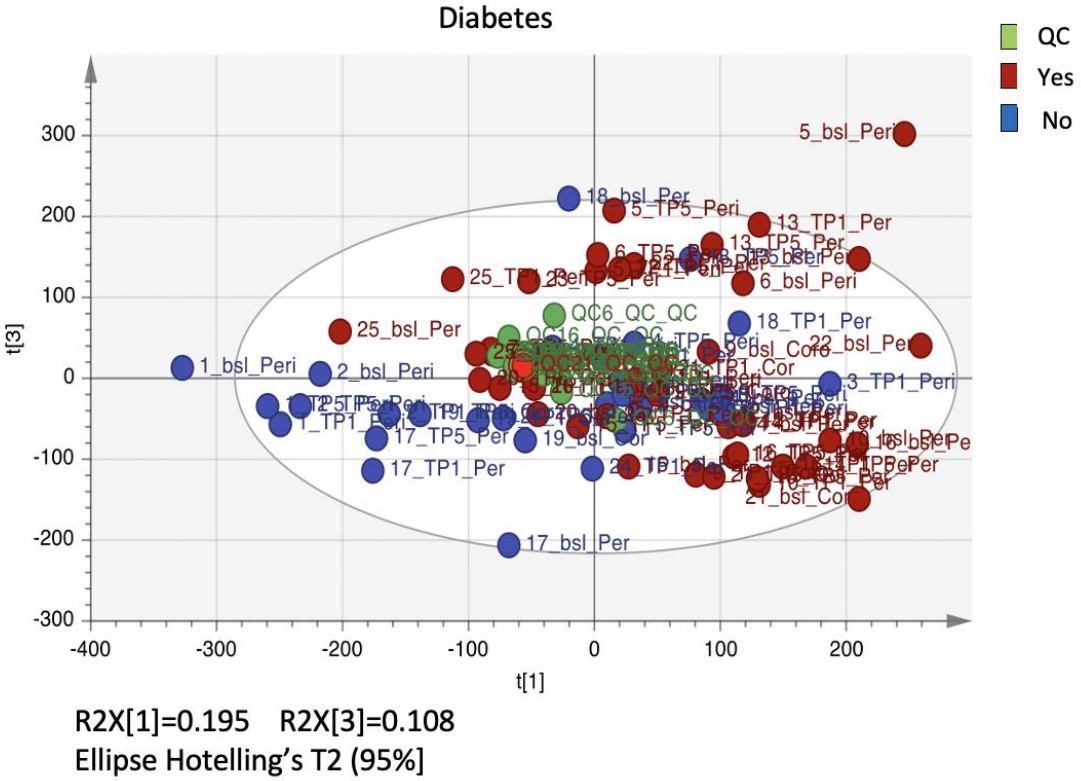

Figure 41: PCA score plot classified by the presence of diabetes. Red dots indicate patients with diabetes, blue dots patients without diabetes and green dots quality controls. t[1] shows scores for the samples in the first principal component and t[3] shows scores from the third principal component. The plot shows partial clustering across PC1 and 3**.** QC samples centred around the

mean. R2X[1]: variance in the predictor variable t1=19.5%; R2X[3]: variance in the predictor variable t3=10.8%.
Ellipse Hotelling's T2: 95% confidence interval of the Hotelling's T-squared distribution

4.3.3.2 Hypertension

About half of the patients were hypertensive with 12/25 (48%) in the DS and 11/21 (52%) in the VS. The PCA plots across PC1 and PC2 did not show any pattern of clustering as shown in Figure 42 below. This may imply that the cardiac work elicited by increased afterload does not markedly alter the metabolic properties of the heart during myocardial ischaemia.

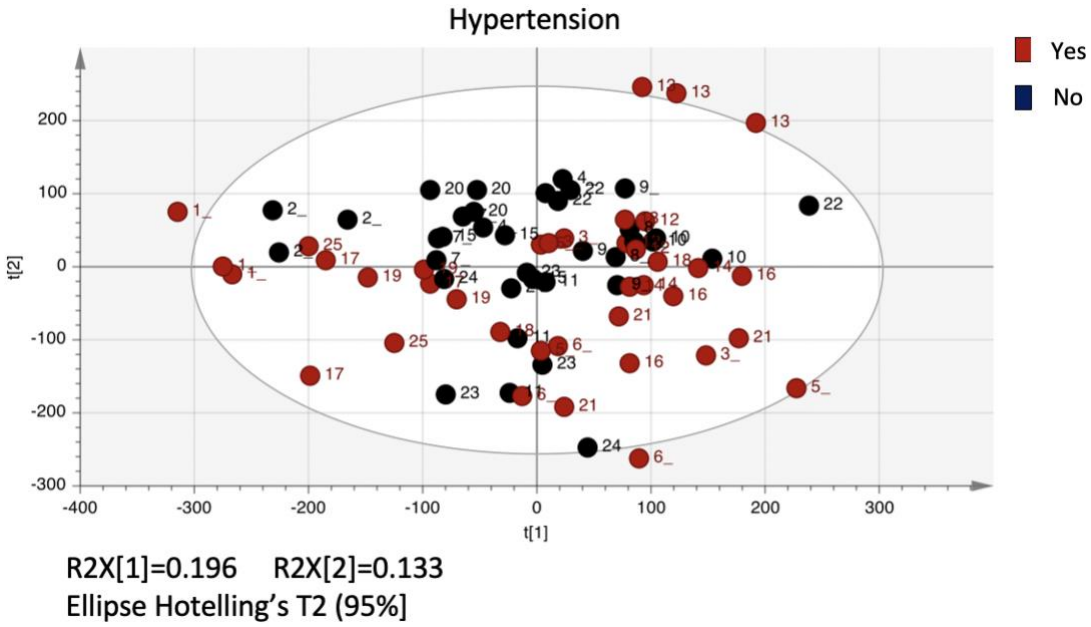

Figure 42: PCA score plot classified by the presence of hypertension. The horizontal ordinate shows the scores for the samples in the first principal component t[1] and the vertical ordinate represents the score for the second principal component t[2]. The red dots indicate patients with hypertension and black dots without hypertension. R2X[1]: variance explained in predictor variable t1=19.6%, R2X[2]: variance in the predictor variable t2-13.3%. The plot shows no clear pattern of clustering across PC1 and 2.
Ellipse Hotelling's T2: 95% confidence interval of the Hotelling's T-squared distribution.

4.3.4 Medications

4.3.4.1 Antianginal medication

The study population had 7/25 (28%) and 4/21 (19%) patients on antianginal nitrates and 4/25 (16%) 0/21 (0%) on nicorandil in the DS and VS group respectively. The PCA plots of patients on antianginal medications in the DS group showed a pattern of clustering across PC1 and PC2 (Figure 43). Similar pattern was also observed in the VS group. The PCA was then performed separately for Nitrates at doses of ISMN 30 mg and ISMN 60 mg and for Nicorandil, which also showed pattern of clustering (Figure 44 & 45). This may be an important finding as the pattern of clustering was seen for nitrates at both doses and also separately for different doses, although majority of them were on the higher dose. It could be postulated that the mechanism of improved blood flow may favor a different metabolic response and hence the observed pattern.

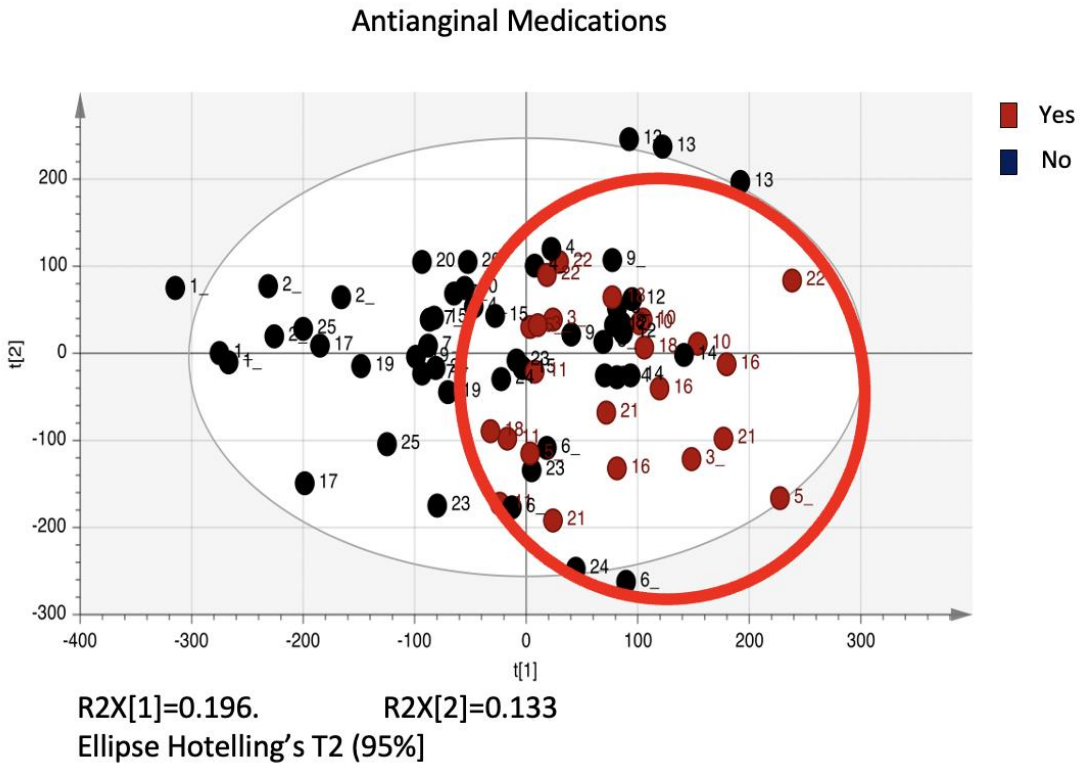

Figure 43: PCA score plots classified by anti-anginal therapy. The horizontal ordinate shows the scores for the samples in the first principal component t[1] and the vertical ordinate represents the score for the second principal component t[2]. The red dots indicate patients on antianginal therapy and black dots without antianginals. R2X[1]: variance explained in predictor variable t1=19.6%, R2X[2]: variance in the predictor variable t2-13.3%. Some partial clustering according to antianginal medications seen indicated by red circle. Ellipse Hotelling's T2: 95% confidence interval of the Hotelling's T-squared distribution.

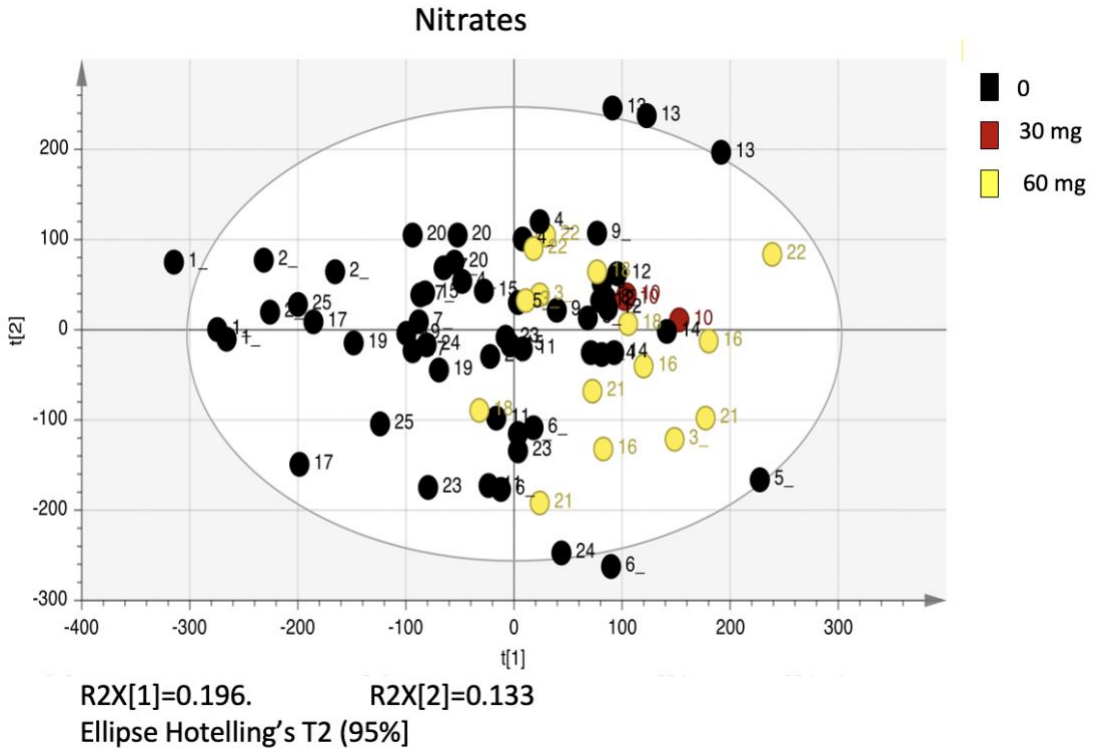

Figure 44: PCA score plot classified by treatment and dose of nitrates. The horizontal ordinate shows the scores for the samples in the first principal component t[1] and the vertical ordinate represents the score for the second principal component t[2]. The red dots indicate patients on Isosorbide mononitrate (ISMN) 30mg, yellow dots for 60mg and black for patients not on ISMN. R2X[1]: variance explained in predictor variable t1=19.6%, R2X[2]: variance in the predictor variable t2-13.3%. Some partial clustering noted across PC1 and 2. Ellipse Hotelling's T2: 95% confidence interval of the Hotelling's T-squared distribution.

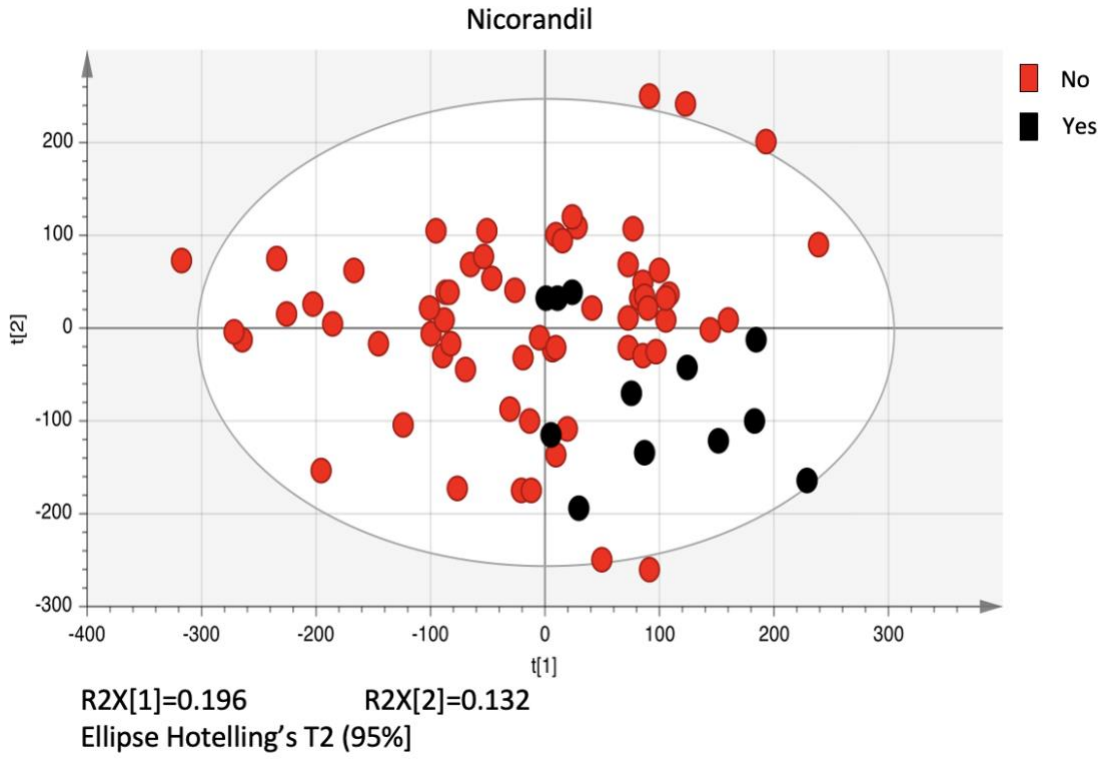

Figure 45: PCA score plot classified by treatment with Nicorandil. The horizontal ordinate shows the scores for the samples in the first principal component t[1] and the vertical ordinate represents the score for the second principal component t[2]. The black dots indicate patients on Nicorandil and red dots for not on Nicorandil. R2X[1]: variance explained in predictor variable t1=19.6%, R2X[2]: variance in the predictor variable t2-13.3%. Some partial clustering noted across PC1 and 2. Ellipse Hotelling's T2: 95% confidence interval of the Hotelling's T-squared distribution.

4.3.4.2 Beta blockers

As expected, majority of the patients in the study population were on beta-blockers with 20/25 (80%) and 16/21 (76%) in the DS and VS respectively. The PCA did not reveal any pattern of clustering in both groups. The score plots of patients on beta-blockers are shown in Figure 46.

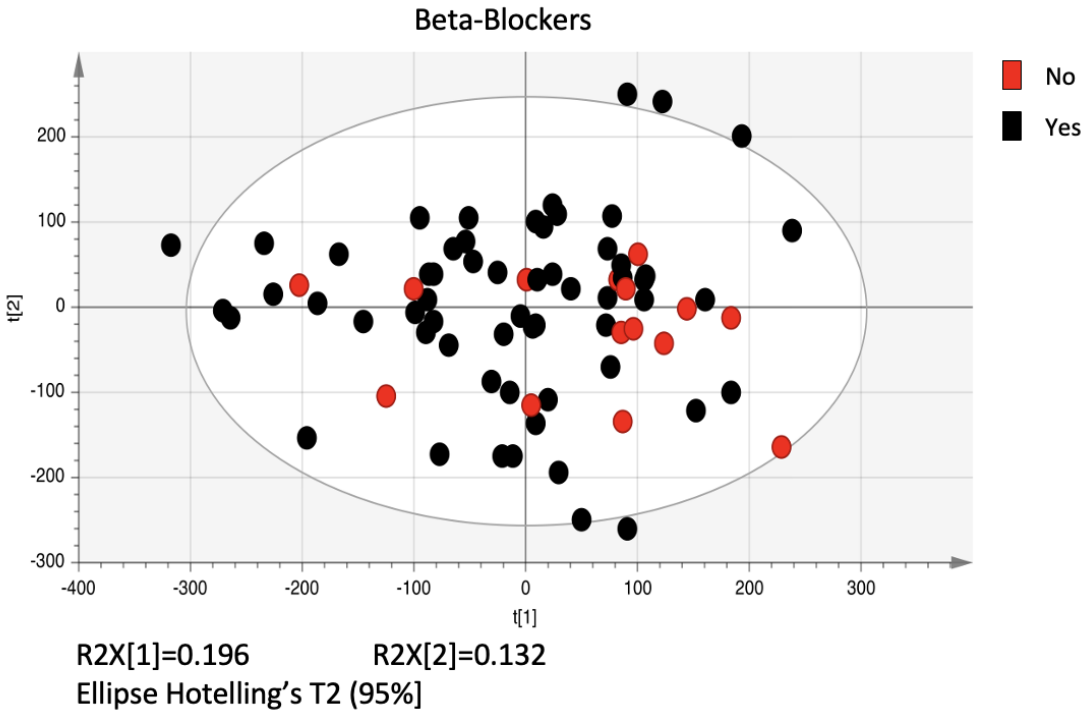

Figure 46: PCA combined positive and negative ion modes. No clustering according to antianginal-Beta-blockers noted. Colour coded as red for not on beta-blockers and black for patients on beta-blockers. R2X[1]: variance explained in predictor variable t1=19.6%, R2X[2]: variance in the predictor variable t2-13.3%. Ellipse Hotelling's T2: 95% confidence interval of the Hotelling's T-squared distribution.

A separate PCA scores plot on patients on a beta blocker (Bisoprolol) at different doses (1.25 mg, 2.5 mg & 5 mg), again did not show any pattern of clustering (Figure 47)**.** The PCA scores plot therefore suggest that there were no influential metabolites that altered based on the use of beta blockers by the patients. This therefore suggest that, the heart rate variability induced by beta blockers and degree of cardiac work does not significantly alter the metabolic responses of the heart during ischaemia.

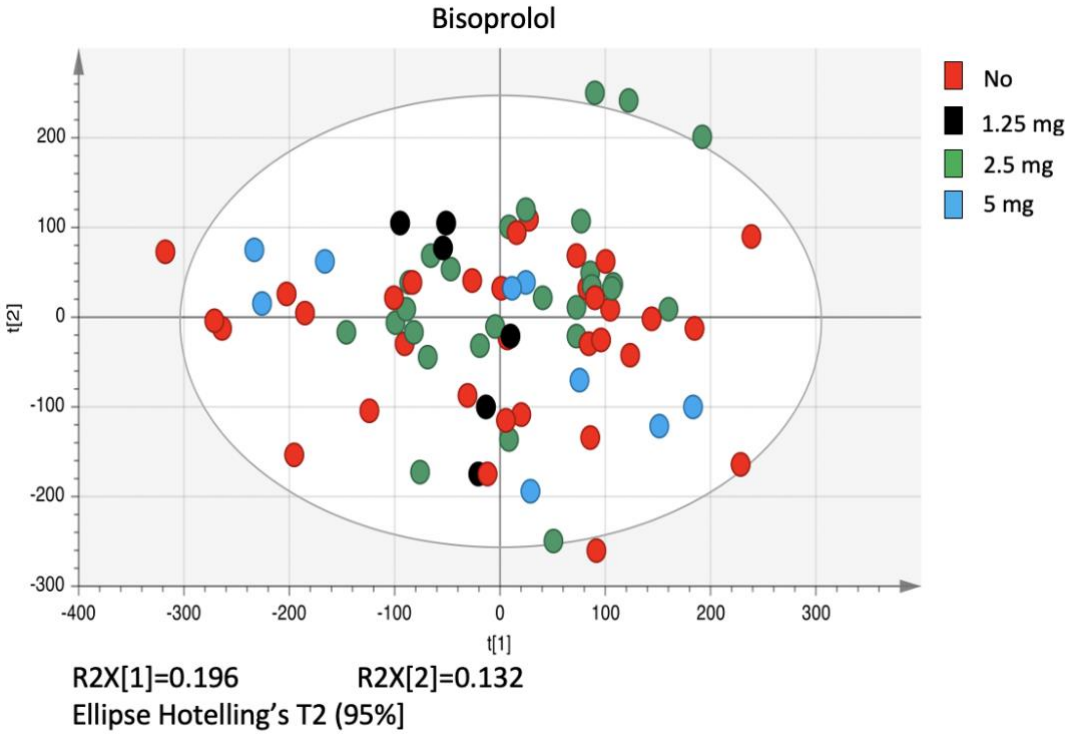

Figure 47: PCA combined positive and negative ion modes. No pattern of clustering according to antianginal-Beta-blockers (Bisoprolol) noted. Colour coded as red for not on bisoprolol, black for 1.25mg, green for 2.5 mg and blue for 5 mg respectively. R2X[1]: variance explained in predictor variable t1=19.6%, R2X[2]: variance in the predictor variable t2-13%. Ellipse Hotelling's T2: 95% confidence interval of the Hotelling's T-squared distribution.

Only one patient was on the angiotensin receptor blocker (ARB), candesartan. As shown in Figure 48, all three samples were grouped at one corner, indicating that this inhibitor may play a role in significantly altering the metabolic signature of this patient. However, as there were only three samples the significance of this observation is currently unclear. For patients on angiotensin converting enzyme inhibitors (ACE-i), lisinopril there was no clear pattern of separation observed (Figure 49).

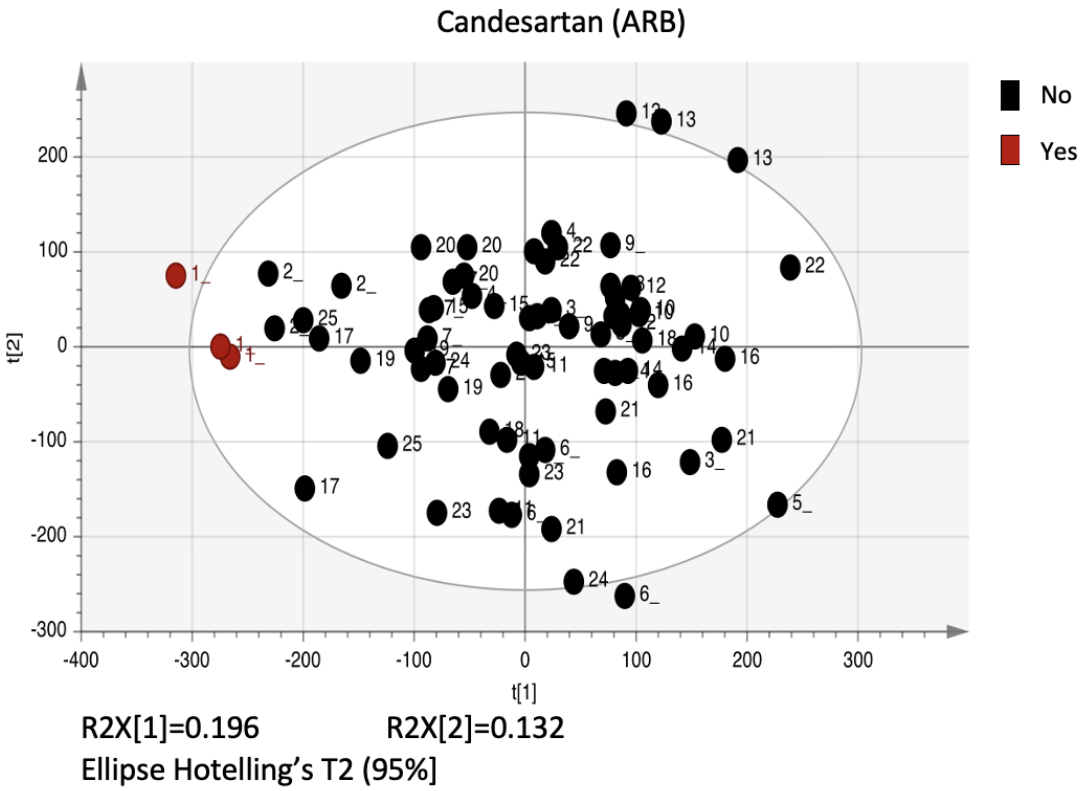

Figure 48 : PCA dataset minus QC samples. Only 3 samples (three timepoints, 1 subject), all grouped on left side of the plot. Colour coded as black for samples not on candesartan and red for samples on candesartan. R2X[1]: variance explained in predictor variable t1=19.6%, R2X[2]: variance in the predictor variable t2-13%. Ellipse Hotelling's T2: 95% confidence interval of the Hotelling's T-squared distribution.

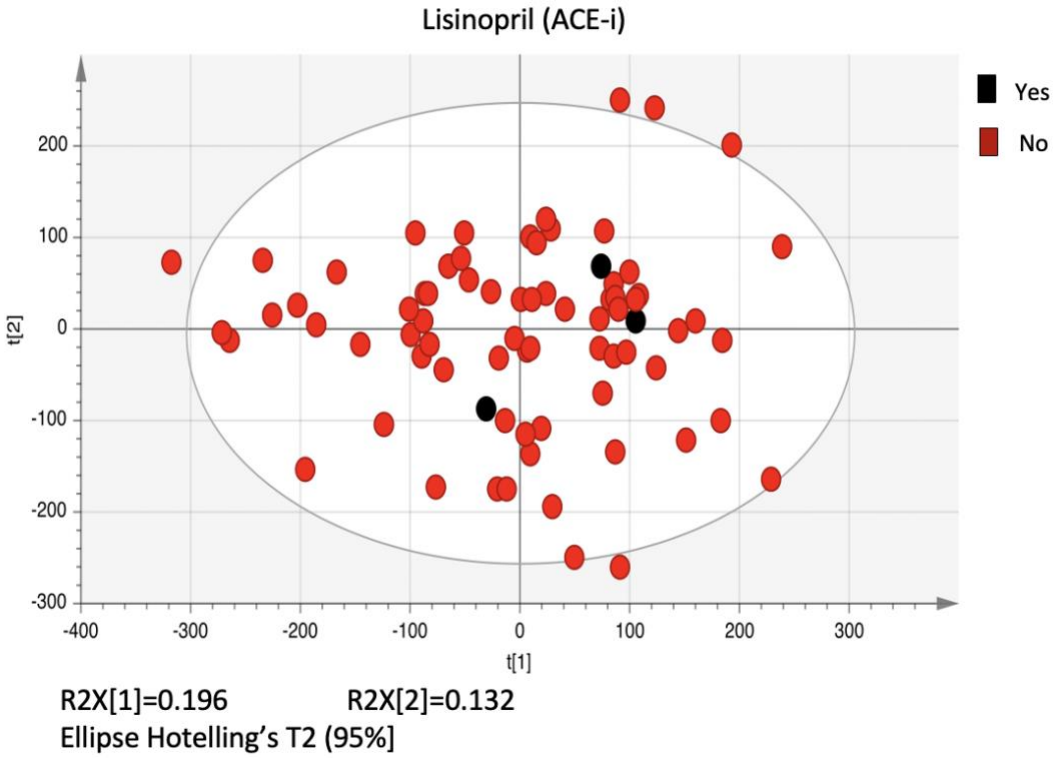

Figure 49: PCA combined positive and negative ion modes. Colour coded as black for patients on lisinopril and red for patients not on lisinopril. No clear pattern of clustering noted. R2X[1]: variance explained in predictor variable t1=19.6%, R2X[2]: variance in the predictor variable t2-13.2%. Ellipse Hotelling's T2: 95% confidence interval of the Hotelling's T-squared distribution.

4.3.4.3 Statins

Hyperlipidemia is an important risk factor for CHD and lipid lower agents have proven clinical benefit in primary and secondary of CHD. Although there are several lipid lowering agents such as hydroxymethylglutaryl (HMG) CoA reductase inhibitors or statins, fibrates, bile acid sequestrants and nicotinic acid, with different mechanism of action, this study population were only on statins. Notably, all participants in both study group DS and VS were on statins (100%) either simvastatin or atorvastatin. No pattern of separation was noted in patients on different statins (Figure 50 & 51). Therefore, it is difficult to assess the impact of statins on the metabolism of patients.

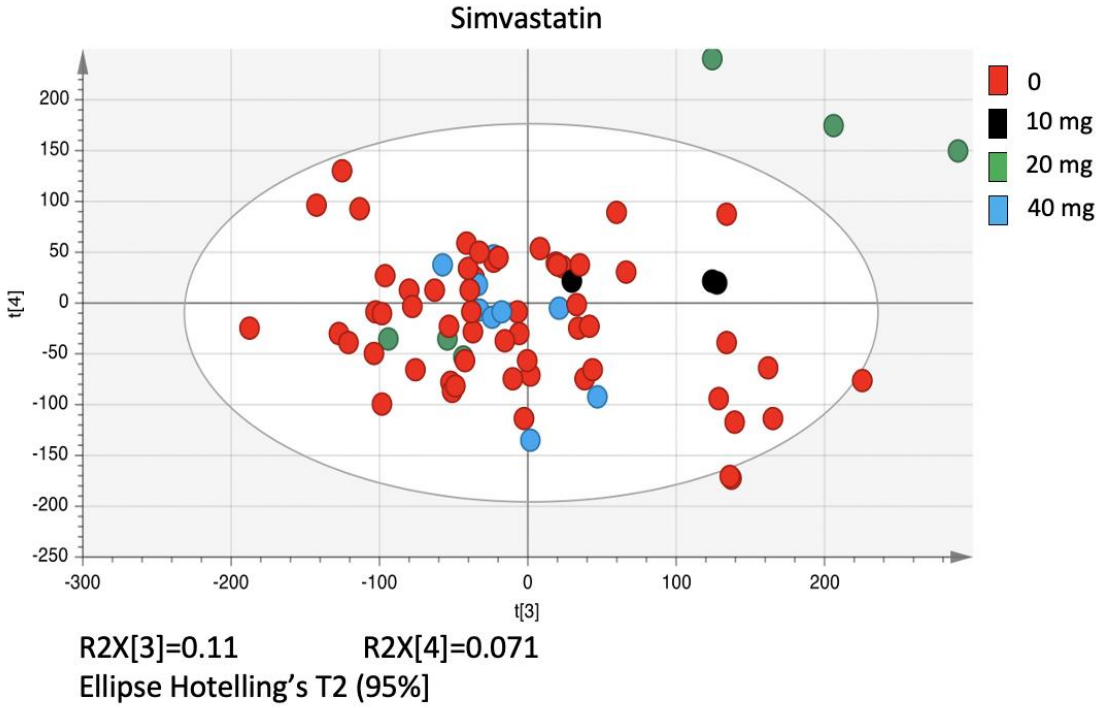

Figure 50: PCA combined positive and negative ion modes. Colour coded as red who are not on simvastatin, black for 10 mg, green for 20 mg and blue for 40mg of simvastatin. No pattern of separation noted. R2X[3]: variance explained in predictor variable t3=11%, R2X[4]: variance in the predictor variable t4=7%. Ellipse Hotelling's T2: 95% confidence interval of the Hotelling's T-squared distribution.

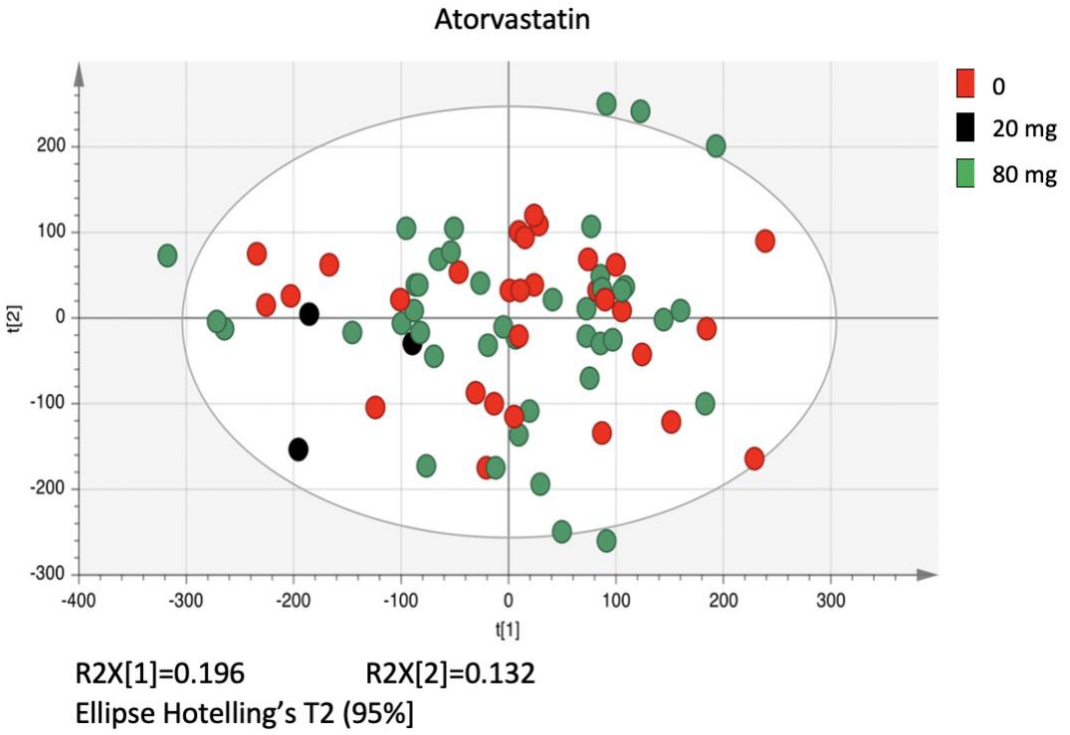

Figure 51: PCA combined positive and negative ion modes. Colour coded as red who are not on Atorvastatin, black for 20 mg, green for 80 mg of atorvastatin. No pattern of separation noted. R2X[1]: variance explained in predictor variable t1=19.6%, R2X[2]: variance in the predictor variable t2-13.2%. Ellipse Hotelling's T2: 95% confidence interval of the Hotelling's T-squared distribution.

4.3.4.4 Other medications

The PCA scores plot for patients on proton pump inhibitors showed no pattern of separation across PC3 and PC4 (Figure 52).

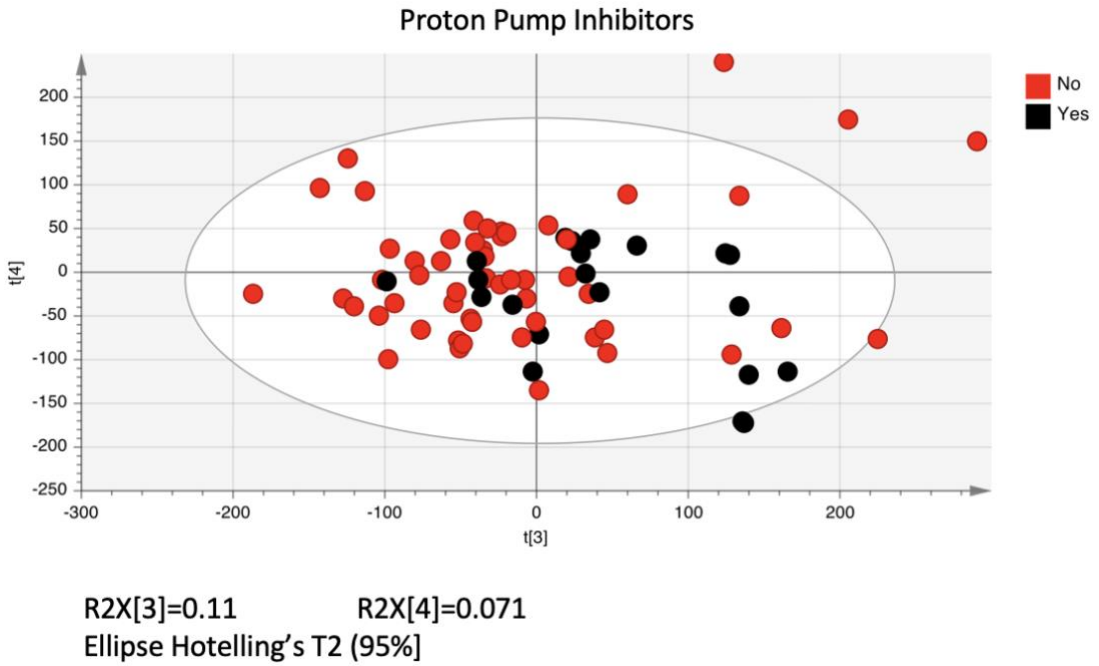

Figure 52: PCA combined positive and negative ion modes. Colour coded as black for patients on Proton pump inhibitors (PPI) and red for patients not on PPI. No pattern of clustering noted. R2X[3]: variance explained in predictor variable t3=11%, R2X[4]: variance in the predictor variable t4=7%. Ellipse Hotelling's T2: 95% confidence interval of the Hotelling's T-squared distribution.

Similarly, PCA was performed on patients on other drugs such as omega 3, fexofenadine (antihistamine) and dosulepin (antidepressant) in the DS group. There was no pattern of separation seen, although the number of subjects were relatively small in all three groups. This is particularly true for patients on dosulepin and fexofenadine, which accounted for just one and three patients respectively (Figure 53).

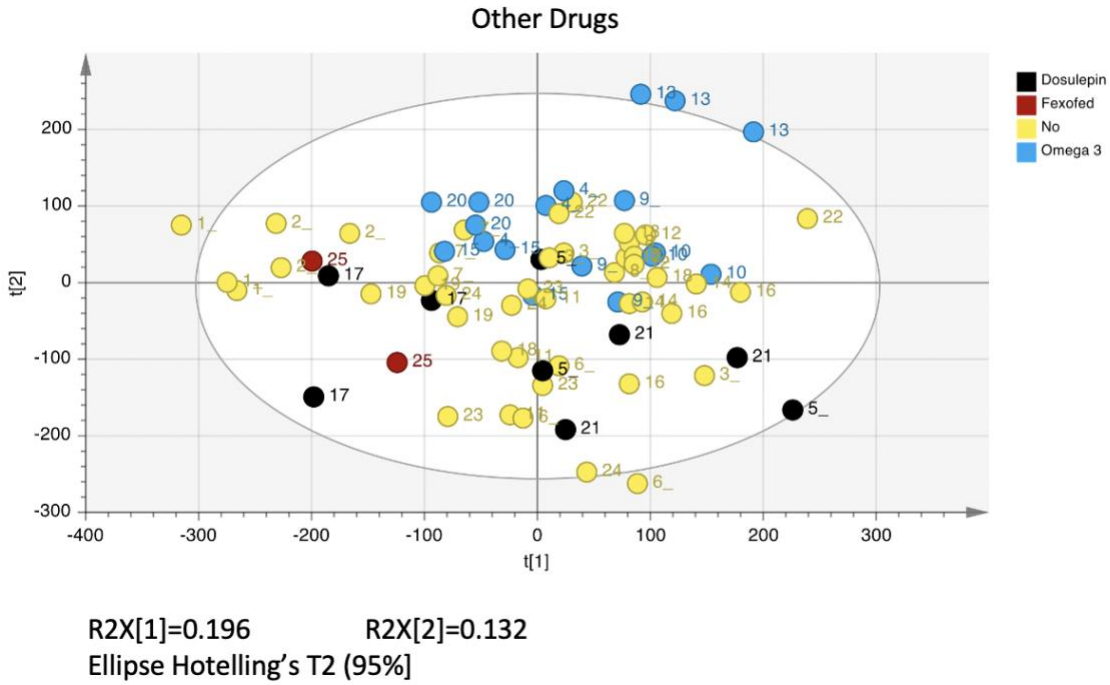

Figure 53: PCA combined positive and negative ion modes. Colour coded as Dosulepin (Black), Fexofed (Red), Omega 3 (Blue), and None (Yellow). No pattern of clustering noted. R2X[1]: variance explained in predictor variable t1=19.6%, R2X[2]: variance in the predictor variable t2-13.2%. Ellipse Hotelling's T2: 95% confidence interval of the Hotelling's T-squared distribution.

4.3.5 Procedural Characteristics

During the balloon inflation, some patients were noted to have symptoms of chest pain, 14/25 (56%) and 12/21 (57%) in the DS and VS, respectively, and 26/46 (56%) patients combined in both groups had ST segment changes. In addition, different types of balloons of varying dimensions were used depending upon the vessel and lesion type. PCA analyses were performed to assess how procedural characteristics alter the metabolic profile of the patients.

4.3.5.1 Experience of Chest pain

Patients reported varying degree of chest pain varying from very mild discomfort to moderate discomfort. None of the patients reported severe chest pain during the procedure. The PCA scores plots across PC1 and PC2 did not reveal any significant pattern (Figure 54). Although the chest pain in the context of coronary occlusion in this study reflects coronary ischaemia, the symptoms are often subjective and do not necessarily reflect the true extent of ischaemic burden, and thus it is not unexpected that there may be no significant difference in the metabolic profiles observed in patients who reported experiencing chest pain compared to those who did not.

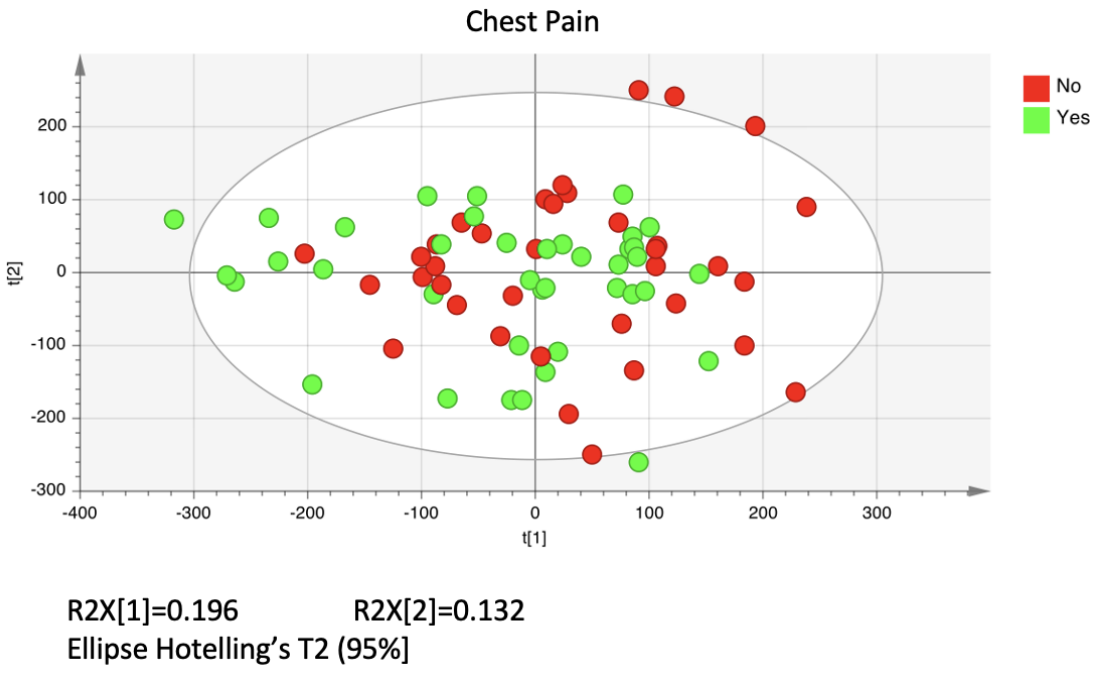

Figure 54: PCA combined positive and negative ion modes. Colour coded as green for chest pain during balloon inflation red for pain free. No pattern of clustering noted across PC1 and 2. R2X[1]: variance explained in predictor variable t1=19.6%, R2X[2]: variance in the predictor variable t2-13.2%. Ellipse Hotelling's T2: 95% confidence interval of the Hotelling's T-squared distribution.

4.3.5.2 ST segment alterations

ST segment changes observed in 26/46 (56%) patients in the study population during the balloon inflation was transient and reversed immediately following deflation of the balloon. The PCA plot showed some pattern of clustering across PC3 and PC4 (Figure 55). The ST segment changes are elicited due to the presence of injury currents triggered by ischaemia-induced alterations in the membrane potential. Therefore, the presence of ST segment elevation or depression is likely to be indicative of more significant myocardial ischaemia compared to those patients in which this was not present – thus the presence of more notable metabolic changes in patients presenting with this change in the ECG validates those metabolic changes are due to the onset of significant myocardial ischaemia in the majority of the patients studied. Further defining of the metabolic properties of those without ST segment changes may also help us identify why some patients don't present with ST segment changes despite the onset of myocardial ischaemia.

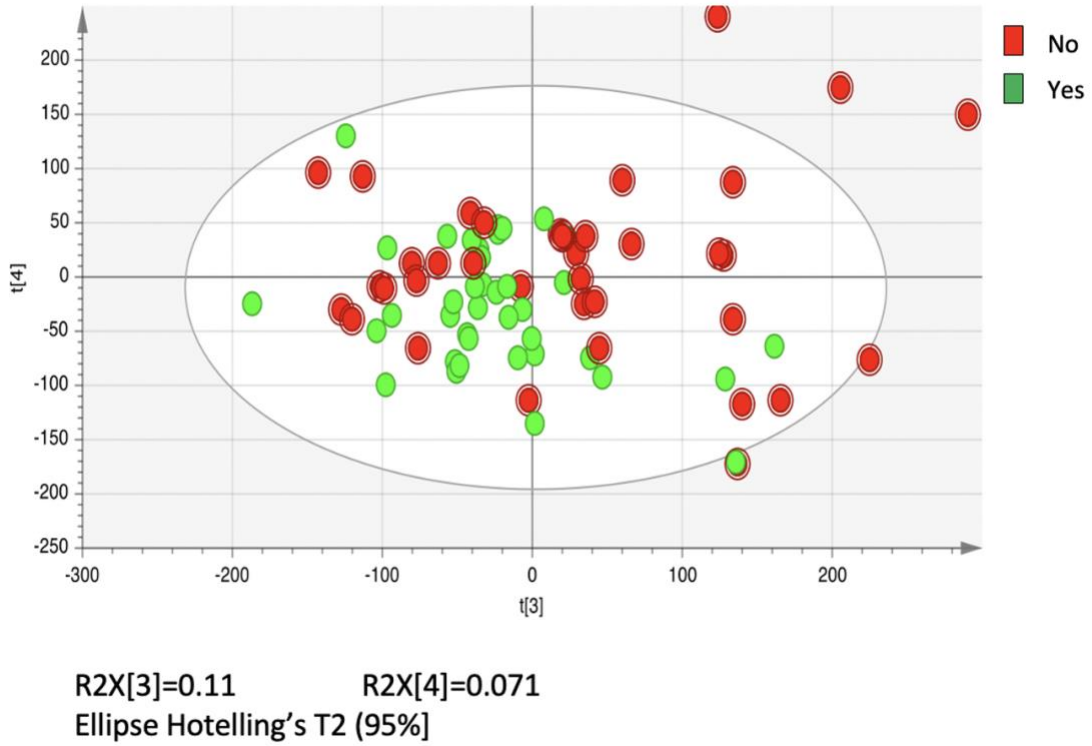

Figure 55: PCA combined positive and negative ion modes. Colour coded as red for ST segment changes during balloon inflation and green for no ECG changes. Pattern of clustering noted across PC1 and PC2. R2X[1]: variance explained in predictor variable t1=19.6%, R2X[2]: variance in the predictor variable t2-13.2%. Ellipse Hotelling's T2: 95% confidence interval of the Hotelling's T-squared distribution.

4.3.5.3 Balloon size

During the procedure the lesions were predilated with a variety of commonly used balloons of different sizes. The PCA plots did not reveal any significant variations suggesting that no significant metabolic alterations were elicited with respect to the choice of the balloon size used in the PCI procedure (Figure 56 & 57). This therefore indicates that the interventionists can utilise the most appropriate balloon size for the patient without concern of exacerbating any adverse metabolic effects of myocardial ischemia either already present in the patient or elicited by angioplasty.

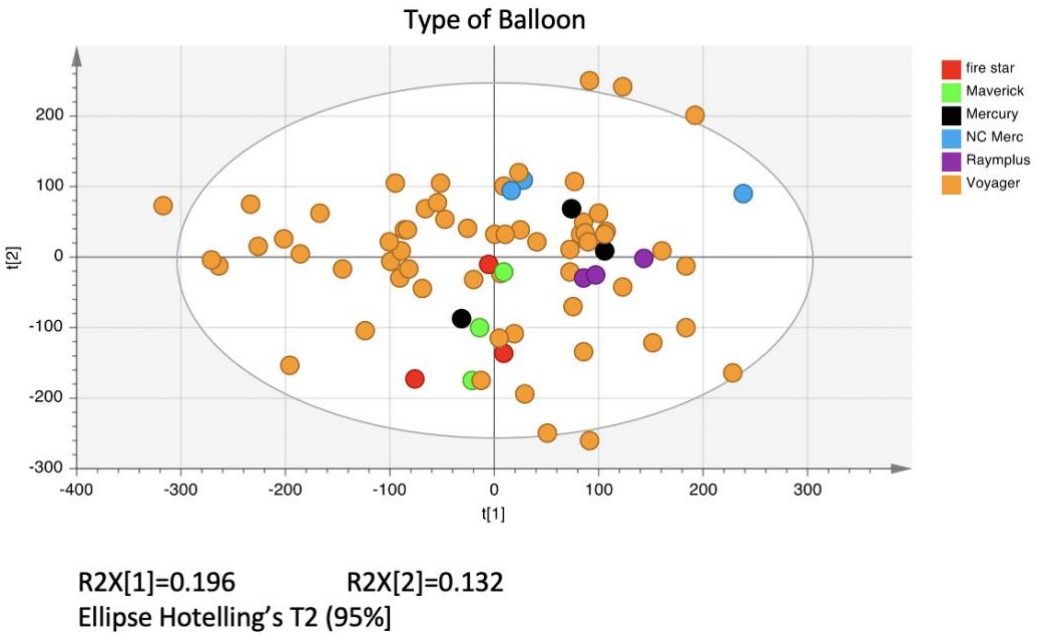

Figure 56: PCA combined positive and negative ion modes. Colour coded according to different type of balloon used for predilatation of the coronary lesions. No pattern of clustering noted. R2X[1]: variance explained in predictor variable t1=19.6%, R2X[2]: variance in the predictor variable t2-13.2%. Ellipse Hotelling's T2: 95% confidence interval of the Hotelling's T-squared distribution.

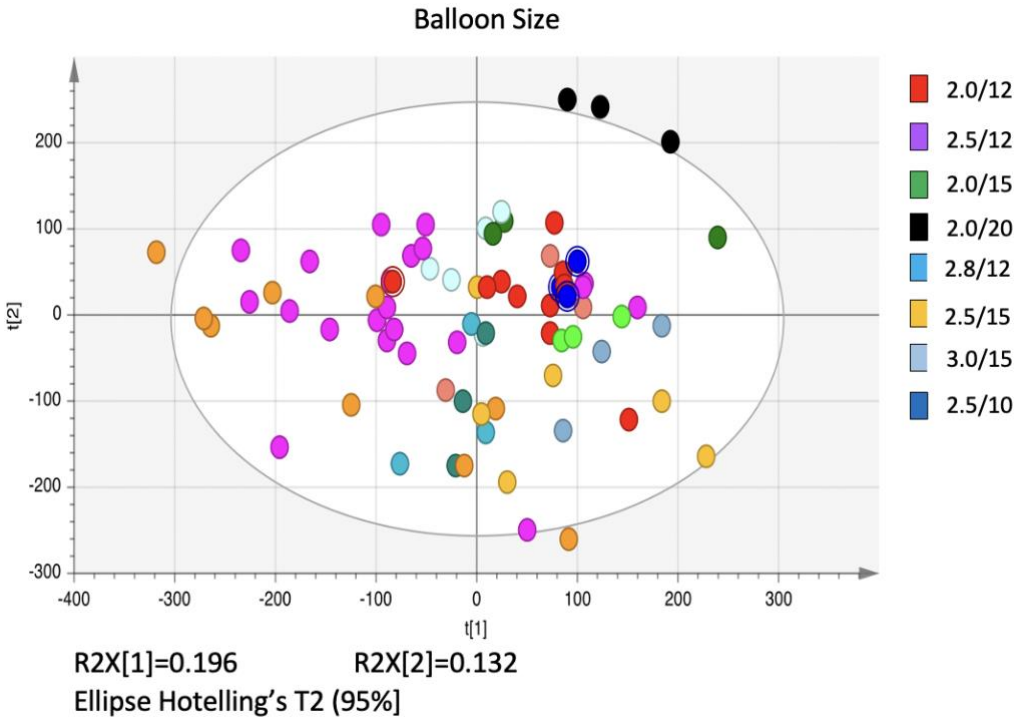

Figure 57: PCA combined positive and negative ion modes. Colour coded according to different balloon size used for predilatation of the coronary lesions. No pattern of clustering noted. R2X[1]: variance explained in predictor variable t1=19.6%, R2X[2]: variance in the predictor variable t2-13.2%. Ellipse Hotelling's T2: 95% confidence interval of the Hotelling's T-squared distribution.

4.3.5.4 Vessel size and location

The baseline characteristics of the patients were largely matched except the vessel dimensions. Majority of the treated coronary artery were LAD with 16/24 (64%) and 12/21 (57%) in DS and VS respectively, followed by RCA with 6/25 (24%) and 7/21 (33%) and the least were Cx with 3/25 (12%) and 2/21 (9.5%) in the DS and VS respectively. The treated vessel diameter ranged from 2.5 to 5 mm. PCA plots of the RCA and LAD coronary vessels are shown in Figures 58 & 59. The analysis showed no pattern of clustering based on the coronary anatomy. This indicate that the coronary anatomy and the corresponding territory of the myocardium that was treated with PCI did

not alter the metabolic pattern. Instead, the ischaemia induced by transient balloon inflation was responsible for the observed metabolic changes.

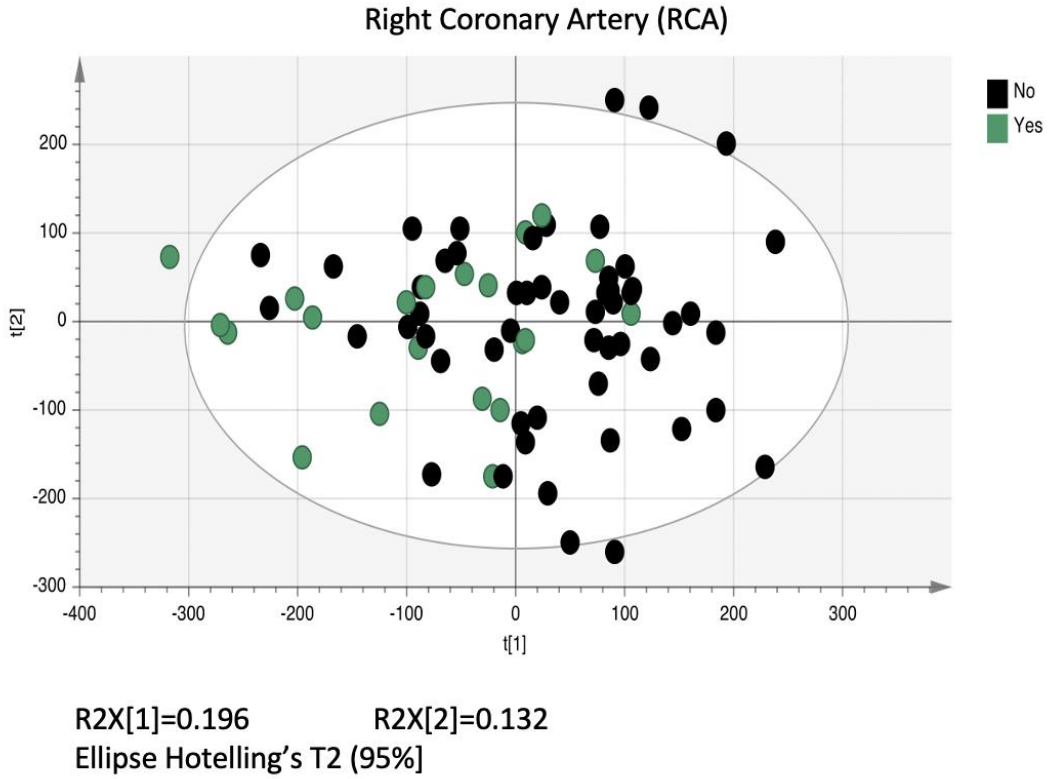

Figure 58: PCA combined positive and negative ion modes. Colour coded according to treated vessel. Green for RCA vessel and black for not RCA. No pattern of clustering noted. R2X[1]: variance explained in predictor variable t1=19.6%, R2X[2]: variance in the predictor variable t2-13.2%. Ellipse Hotelling's T2: 95% confidence interval of the Hotelling's T-squared distribution.

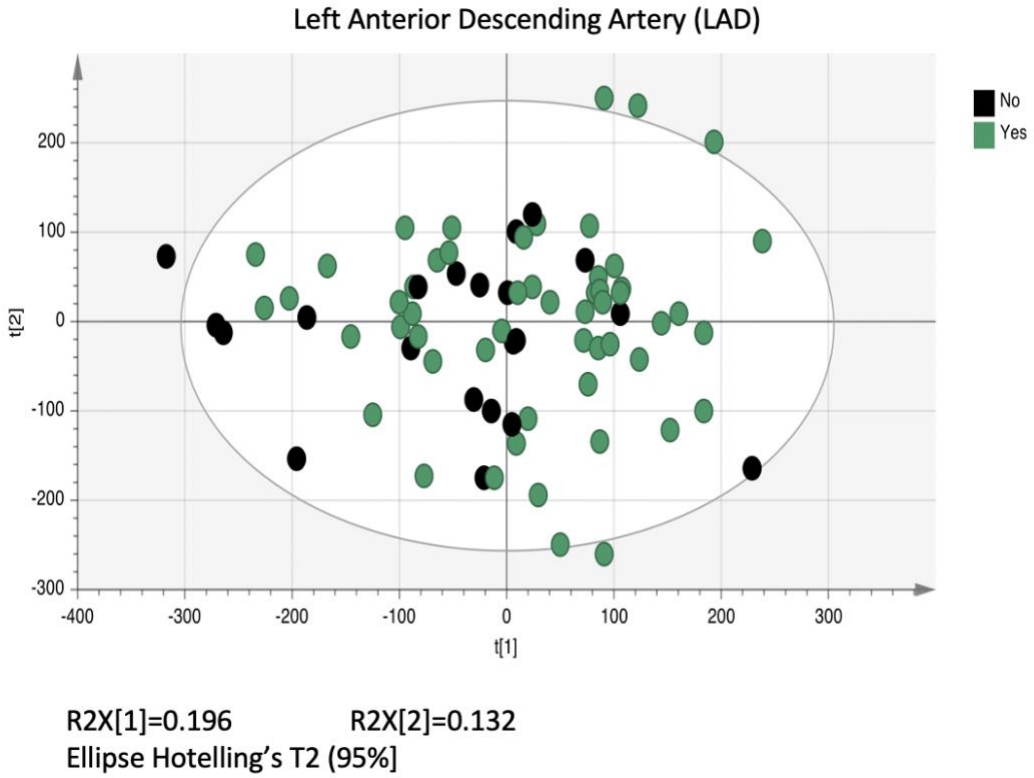

Figure 59: PCA combined positive and negative ion modes. Colour coded according to treated vessel. Green for LAD vessel and black for not LAD. No pattern of clustering noted. R2X[1]: variance explained in predictor variable t1=19.6%, R2X[2]: variance in the predictor variable t2-13.2%. Ellipse Hotelling's T2: 95% confidence interval of the Hotelling's T-squared distribution.

4.3.5.5 Location of venous sampling

The samples were collected from CS and peripheral femoral veins. Due to procedural inconvenience and patient's choice, the CS samples were comparatively low 6/25 (24%) and 1/21 (4.8%) when compared to the peripheral samples19/25 (76%) and 20/21 (95%) in DS and VS respectively. The PCA plots (Figure 60), showed some partial clustering in relation to the site of sample collection. The metabolic effects of ischemia are likely to be better preserved in effluents blood samples taken directly from the heart compared to those in which these substances may have been taken up, metabolized or

excreted from the bloodstream prior to arrival in the peripheral veins. However, the ability to definitively state this is limited by the low number of CS samples collected during this study.

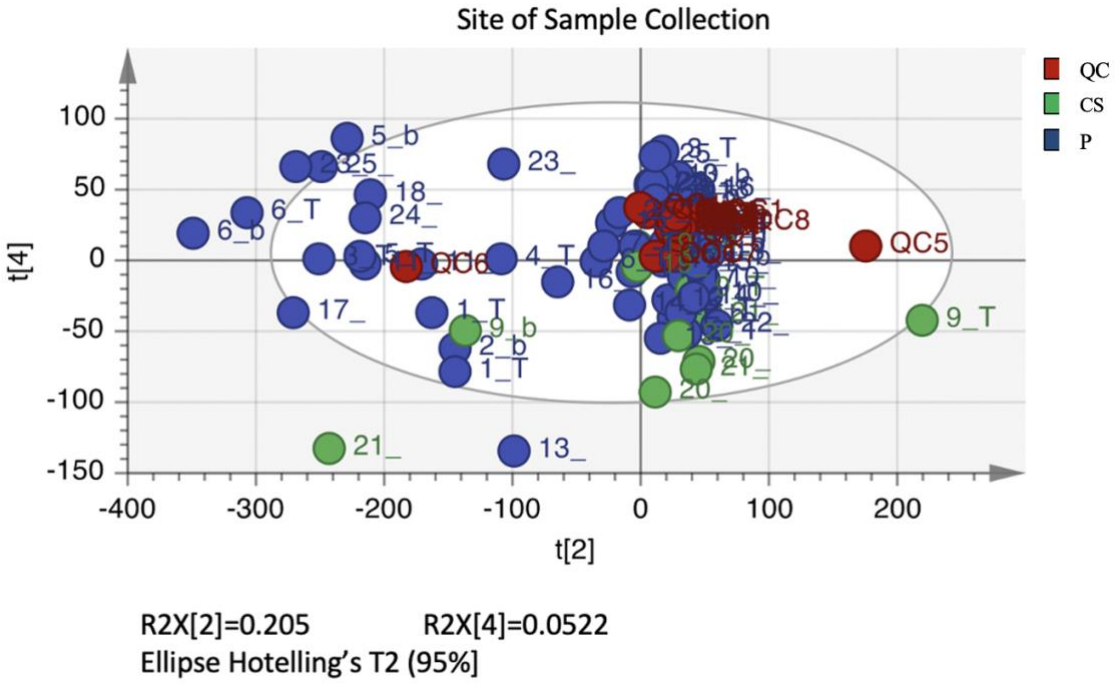

Figure 60: PCA plot according to the site of sample collection, Coronary sinus (CS) and Peripheral (P). Green for CS, Blue for P and Red for Quality control (QC). Partial clustering noted across PC 2 and 4. Two QC samples (QC 5 and QC 6) outliers compared to the other QC samples. R2X[2]: variance explained in predictor variable t2=20%, R2X[4]: variance in the predictor variable t2=5%. Ellipse Hotelling's T2: 95% confidence interval of the Hotelling's T-squared distribution.

4.3.6 Statistical Modelling

Projection to latent structures (PLS) and orthogonal projection to latent structures (OPLS) are the common multivariate statistical analytic strategies used in metabolomics (279). For classification or discrimination these methods are referred as PLS-DA and OPLS-DA were DA stands for discriminant

analysis. The PLS and OPLS models try to find a linear relationship between a *x* predictor matrix (e.g. spectrometric data) and a *y* response matrix (e.g. clinical variable). An appropriate use of statistical package is key to ensure quality of the results and there are several software packages available in the market. This study used *SIMCA* (*Umetrics, Umea Sweden*) to maximise the correlation between the mass spectrometry data and the meta data. To assess the quality of the model, cross-validation was performed, where R^2 measures the goodness of the fit and Q^2 measures the predictive ability of the model. Statistical significance of the model was tested by randomly permuting the y variable and observing the explained variances R^2 and Q^2 for the randomly generated model together with their correlation to the original model. Providing there was low correlation between the random and actual model and the R^2 and Q^2 were lower (preferably negative for Q^2), the model was deemed significant. Following application of these methods, statistically significant models were observed for patients with diabetes, BMI, site of sample collection, antianginals and weak model was obtained for ACE-i

4.3.6.1 Diabetes

Statistically significant models were obtained for patients with diabetes. In the OPLS-DA model for diabetes (Figure 61 & 62), the horizontal ordinate shows the correlation between the m/z and patients with and without diabetes. The vertical ordinate shows the orthogonal PC score values and variation within the group. The model showed that there was a clear variation between the patients with and without diabetes. Similarly, there was a clear separation with lack of overlap between the observed versus the predicted values between patients

with and without diabetes. This shows that there was a distinction between the two groups and the model is a good fit. The permutation test showed Q^2 of –0.3 suggesting a statistically significant model. (Figure 63)

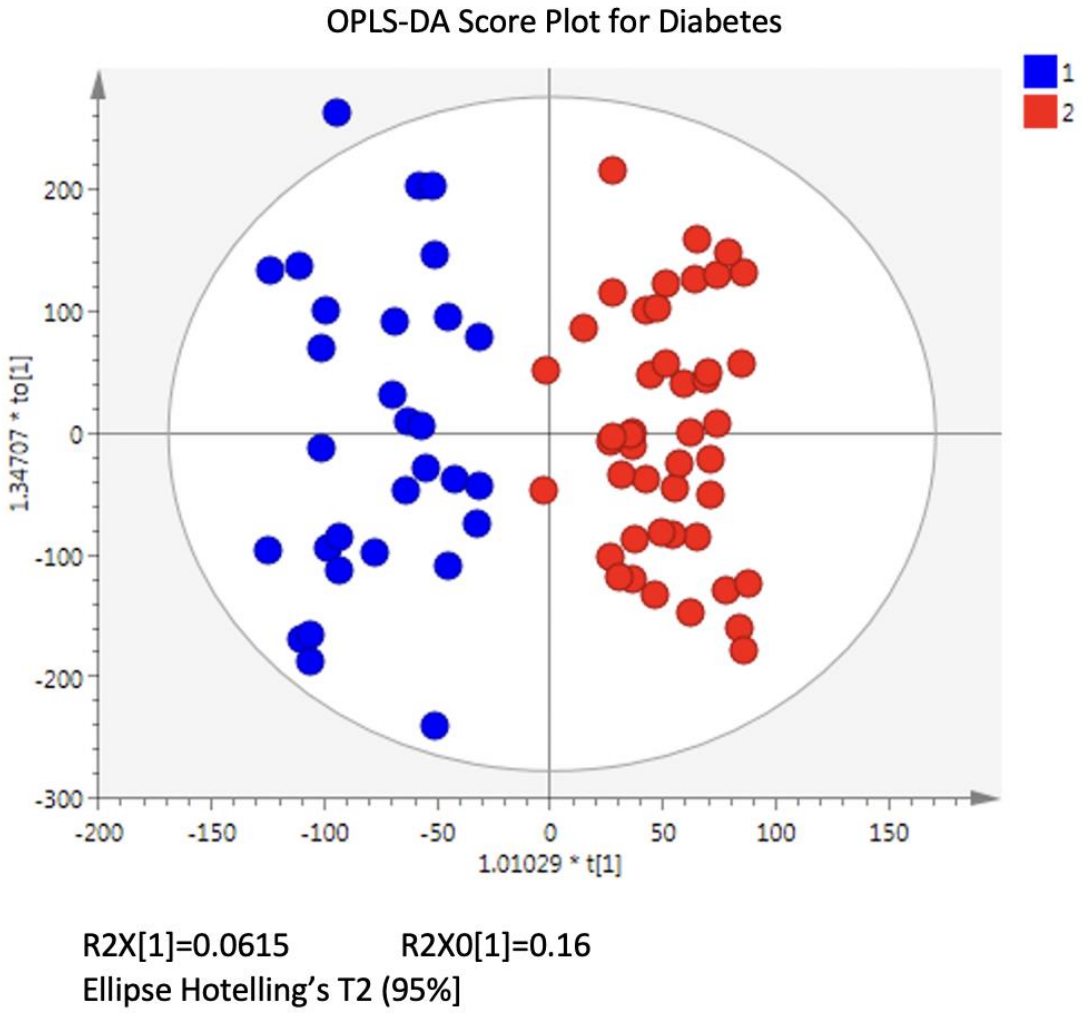

Figure 61: Supervised OPLS-DA models in patients with Diabetes. The horizontal ordinate shows t1: represents correlation with m/z and class (diabetes versus no diabetes) and variation between the groups. The vertical ordinate shows to1: orthogonal principal component-score value and difference in the observation group (within group variation). The plot shows distinction between samples from patients with (red) and without (blue) diabetes. OPLS-DA-Orthogonal partial least squares discriminant analysis.
R2X[1]: variance explained in the predictor variable t1=6.15%, R2X0[1]: variance explained in the predictor variable to1=16%.

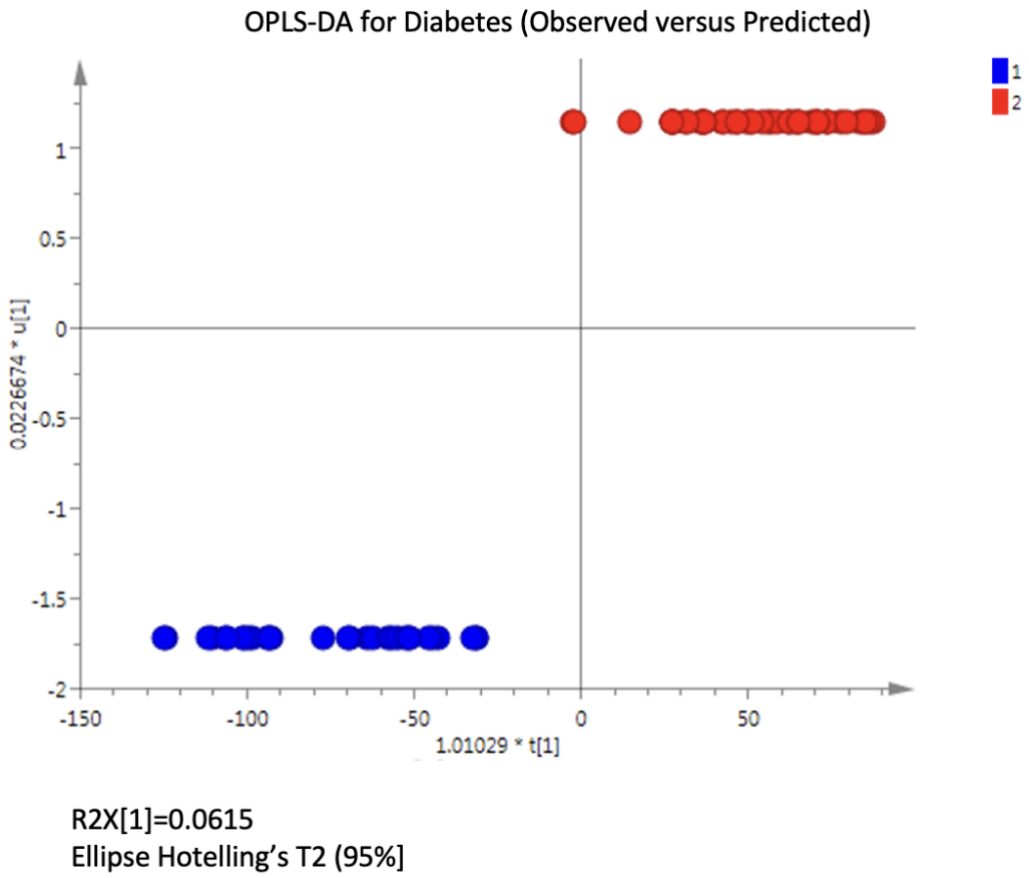

Figure 62: Supervised OPLS-DA model in patients with Diabetes.The plot shows observed versus predicted values and shows distinction between samples from patients with (red) and without (blue) diabetes (6.2% of variation). The separation between class is clearly seen by the lack of overlap vertically between the two groups about x=0 in the observed versus predicted plot. OPLS-DA-Orthogonal partial least squares discriminant analysis.

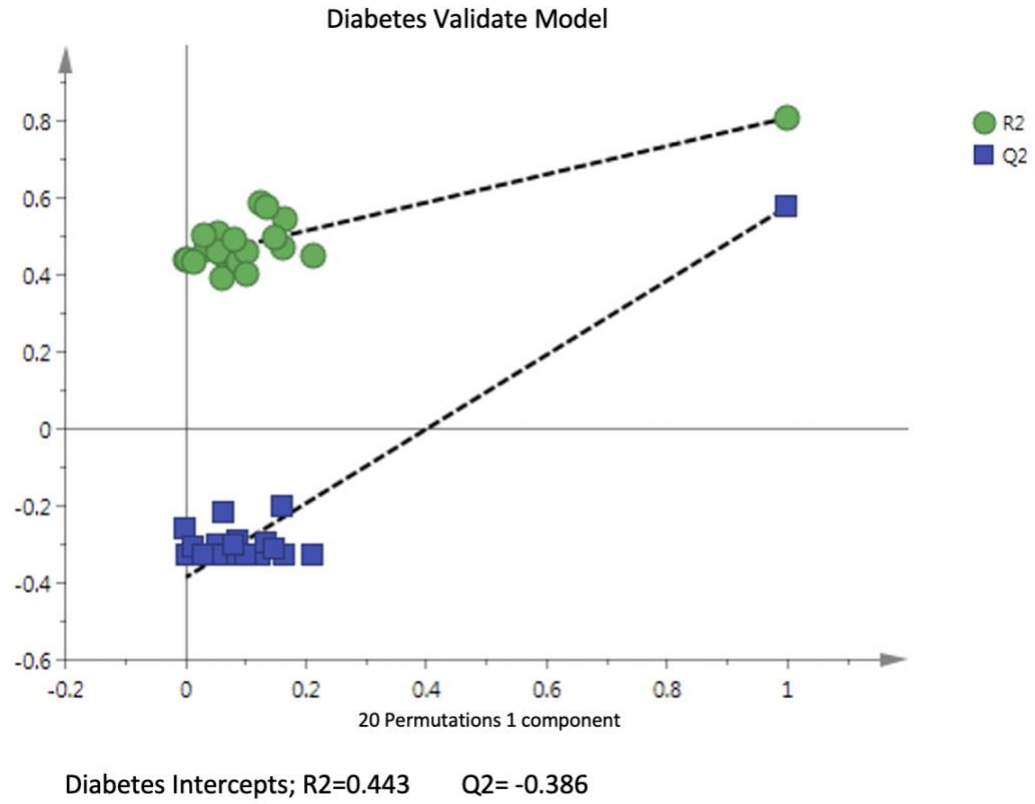

Figure 63: Permutation validation plot for Diabetes. The y-axis intercepts for R2 (Green) and Q2 (Blue) were 0.4 and -0.38 respectively, indicating that the original model was valid. R2 measures the goodness of the fit and Q2 measures the predictive ability of the model.

In patients with diabetes, LysoPC, 2-hydroxybutyric acid, 3-hydroxyisovaleric acid were observed as influential metabolites. This is an interesting finding as hydroxybutyric acid is downstream metabolite produced by FFA oxidation and utilized by the body as an energy source (280). Hydroxybutyric acid is known to be closely associated in patients with diabetes and has been shown to be a sensitive marker in detecting patients with diabetic ketoacidosis (DKA), a life-threatening complication of diabetes as a result of high blood glucose content and ketone bodies in the circulation (281, 282). Hydroxybutyric acid is now available as a serum biomarker in patients with DKA and is thought to be superior to urine acetoacetate testing (282). This evidence supports that the

observed variation in hydroxybutyric acid in this study is likely to be related to diabetes. In addition, hydroxybutyric acid is produced by the β-oxidation of FFAs, this is therefore unlikely to have been elevated as a result of acute ischaemia where the β-oxidation is interfered due to acute hypoxia. It would be interesting to perform further targeted metabolomic analysis focusing on the influence of hydroxybutyric acid as biomarker to determine optimum diabetic control.

The plasma LysoPC concentration is also known to be elevated in diabetes(283). However, a rapid increase in lysoPC observed in this study population at TP1 and TP5 when compared to baseline could only be interpreted as a consequence of acute ischaemia.

4.3.6.2 Body mass index

The average body mass index (BMI) in this study population was high with 27.84±5.35 and 26.47 ±1.96 for the DS and VS respectively. The PLS and OPLS for observed and predicted values for BMI showed a clear association between the observed BMI and the predicted BMI by the mass spectra data. The predicted BMI in the horizontal ordinate and the observed variance in the BMI in vertical ordinate showed a linear correlation (Figures 64 & 65). The permutation test showed a statistically significant model with a Q^2 of – 0.4 for BMI (Figures 66).

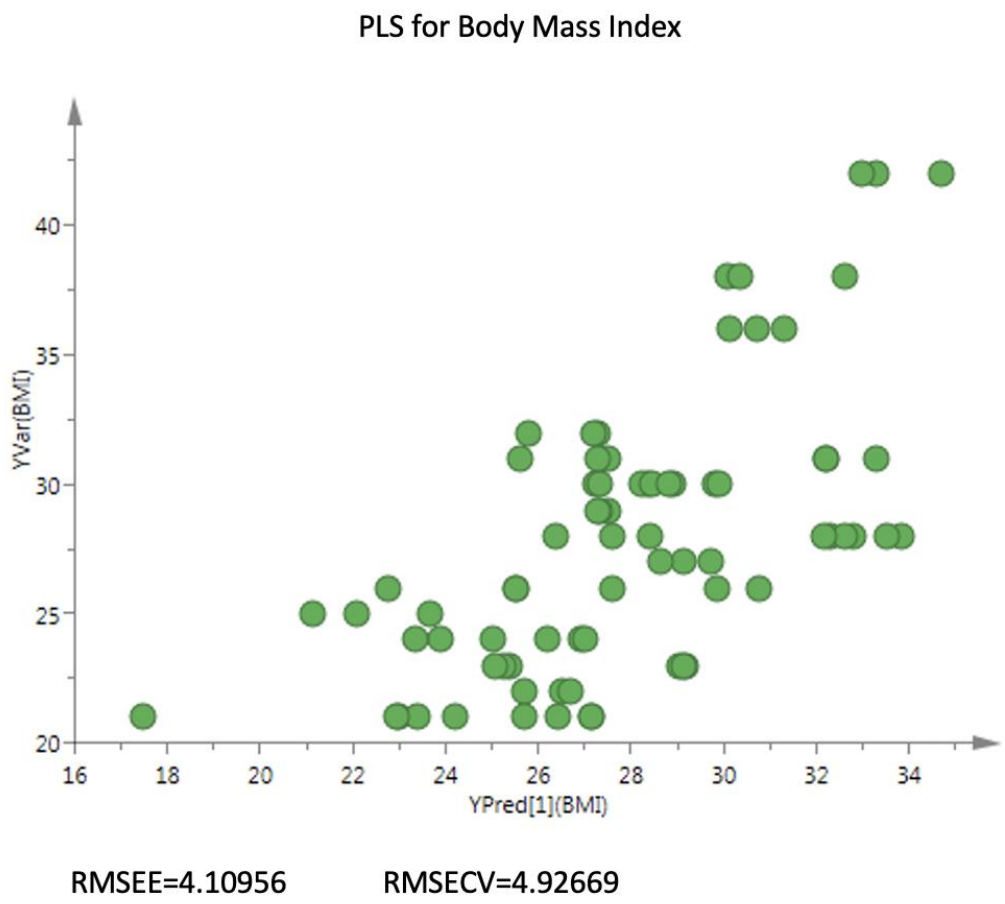

Figure 64: PLS model for observed versus predicted values for Body Mass Index (BMI). Horizontal ordinate YPred(BM) shows the predicted BMI and the vertical ordinate Yvar (BMI) is the observed variance in BMI. RMSEE: root mean square error of estimation, RMSECV: root mean square error of cross-validation. A clear correlation is observed between the observed BMI and the BMI predicted by the mass spectrometry data.

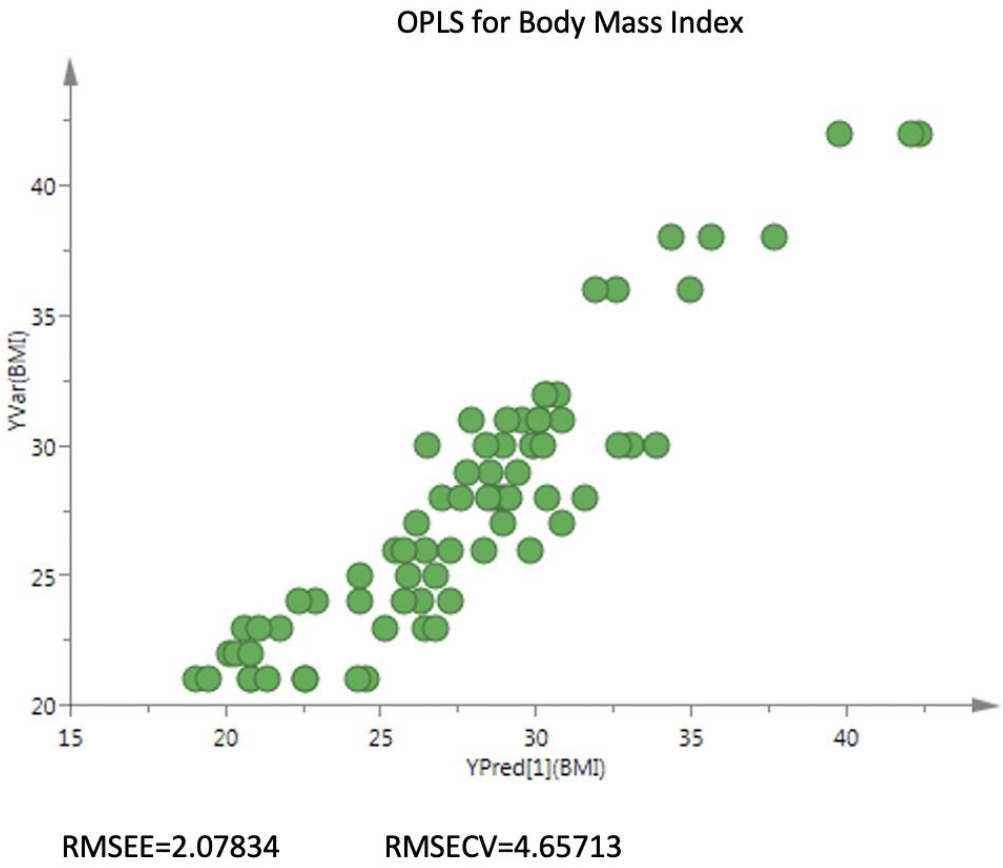

Figure 65: OPLS model for observed versus predicted values for Body Mass Index (BMI). Horizontal ordinate YPred(BM) shows the predicted BMI and the vertical ordinate Yvar (BMI) is the observed variance in BMI. RMSEE: root mean square error of estimation, RMSECV: root mean square error of cross-validation. A clear correlation is observed between the observed BMI and the BMI predicted by the mass spectrometry data.

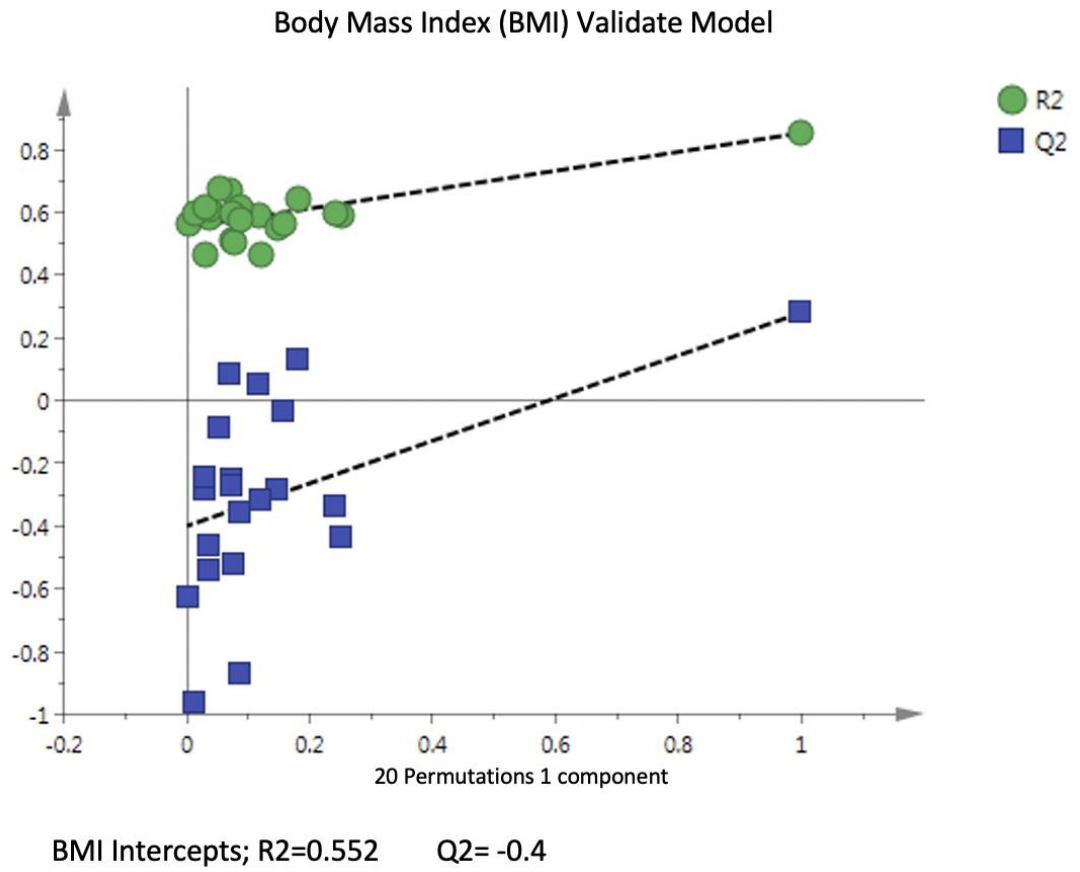

Figure 66: Permutation validation plot for Body mass index (BMI). The y-axis intercepts for R2 (Green) and Q2 (Blue) were 0.5 and -0.4 respectively, indicating that the original model was valid. R2 measures the goodness of the fit and Q2 measures the predictive ability of the model.

Lipid metabolites, Monoacylglycerols, LysoPC and 2-Hydoxybutyric acid were observed to have correlation with the BMI. Obesity and metabolic syndrome is an escalating public health problem that are associated with chronic diseases such as diabetes and hyperlipidemia characterized by increase in plasma FFA and triacylglycerols (284). These findings were similar to the one observed in patients with diabetes and may thus represent that the changes observed could be a consequence of metabolic syndrome rather than myocardial ischaemic insult. However, this observation is vital for further studies particularly to guide life style modification measures in patients with metabolic syndrome and CHD.

In addition, 2-hydroxybuyteric acid could guide as measurable biomarker in patients with morbid obesity in whom aggressive life style modification and bariatric surgery are being increasingly considered.

4.3.6.3 Site of sample collection

Plasma samples were collected from peripheral femoral vein and from the CS in the study population. For patients who underwent CS cannulation a separate femoral venous access was used to advance the catheter into the CS. Understandable, in view of the procedural inconvenience the study had only few patients who agreed on CS cannulation, 6/25 (24%) and 1/21 (4.8%) when compared to the peripheral samples19/25 (76%) and 20/21 (95%) in DS and VS respectively. In the OPLS-DA, the horizontal ordinate shows the variation between the m/z and class (CS versus P). The vertical ordinate shows the orthogonal principal component-score value and the difference within the observation group. The supervised OPLS-DA for site of sample collection showed clear distinction between the two groups (Figures 67 & 68). The permutation test showed a statistically significant model with a Q^2 of – 0.6 (Figure 69).

The effluents form the CS represents close proximity to the heart and therefore the metabolic changes are likely to be better preserved in the effluents from the CS when compared to those from the peripheral blood stream. However, it was interesting to note that the metabolite changes observed in the CS and peripheral venous samples were similar suggesting myocardial specific metabolites in response to reversible coronary ischaemia.

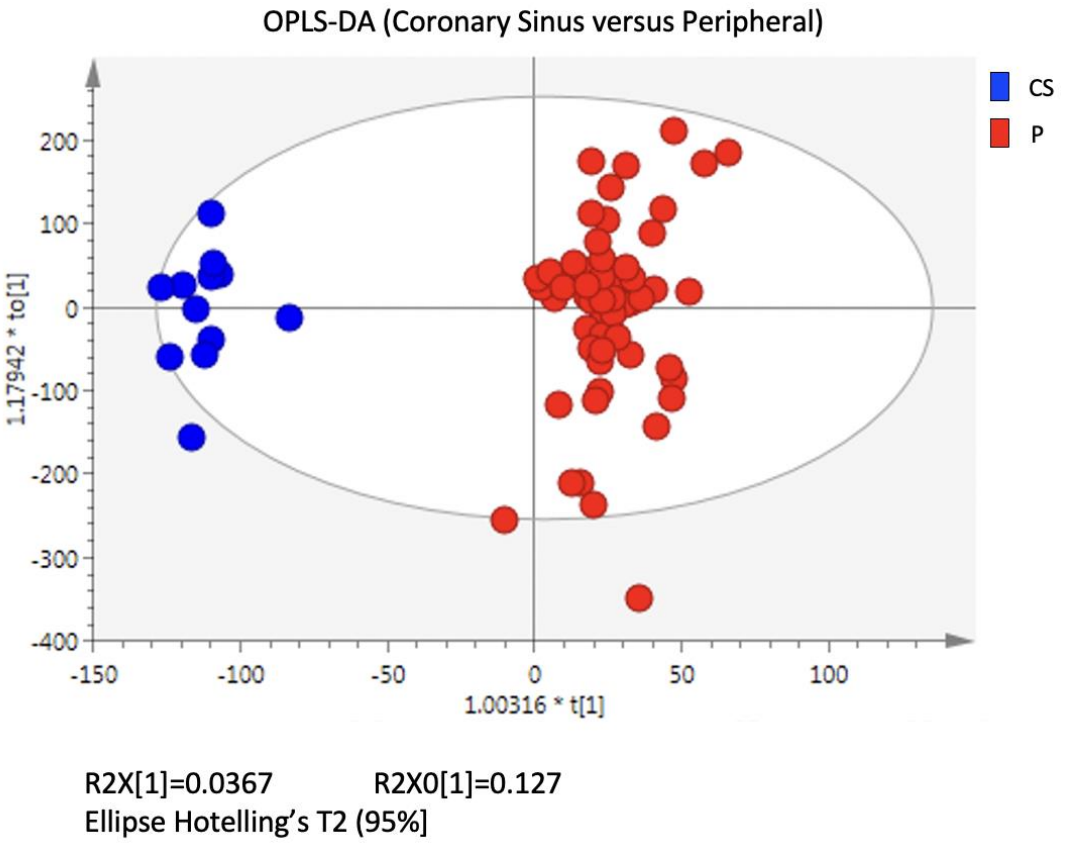

Figure 67: Supervised OPLS-DA models for Coronary sinus (CS) and peripheral (P) site of sample collection. The horizontal ordinate shows t1: represents correlation with m/z and class (CS versus P) and variation between the groups. The vertical ordinate shows to1: orthogonal principal component-score value and difference in the observation group (within group variation). The plot shows distinction between samples from CS (blue) and P (red) accounting for 3.7% of total variation. R2X[1]: variance explained in the predictor variable t1=3.7%, R2X0[1]: variance explained in the predictor variable to1=12.7%. OPLS-DA-Orthogonal partial least squares discriminant analysis.

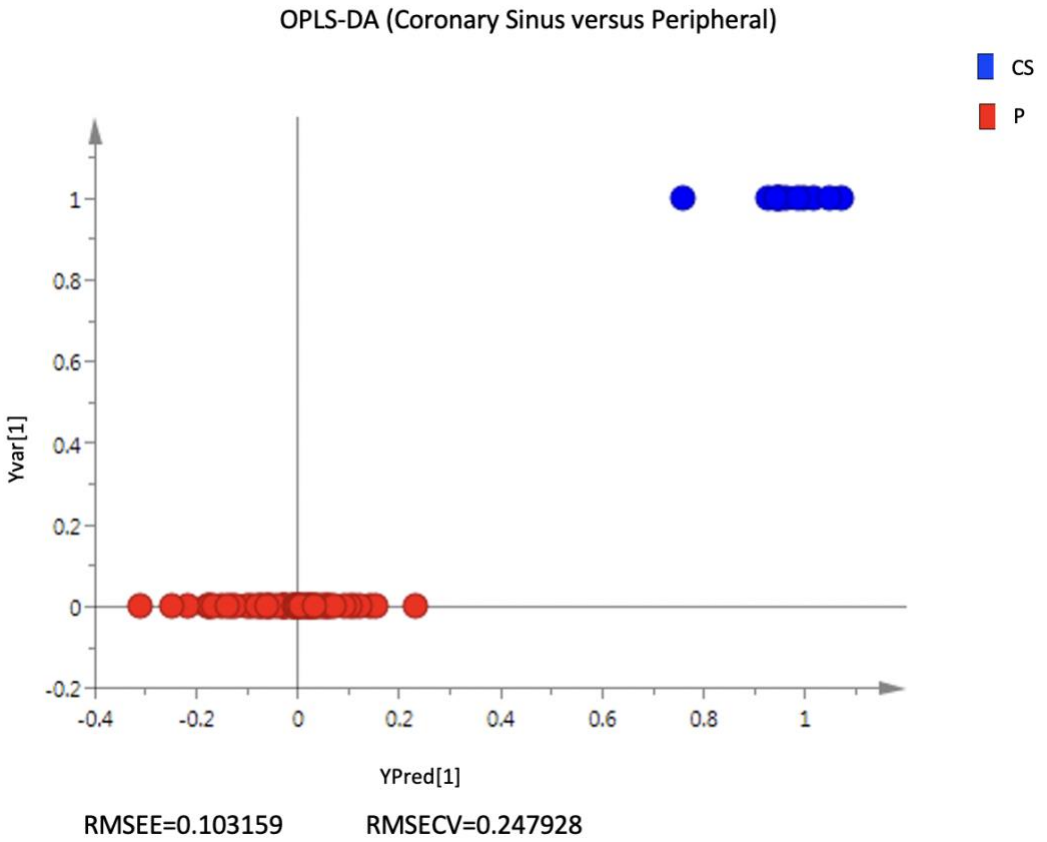

Figure 68: Supervised OPLS-DA model in accordance with the site of sample collection, coronary sinus (CS) and peripheral (P). The plot shows observed versus predicted values and shows distinction between samples from CS (blue) and P(red). The separation between class is clearly seen by the lack of overlap vertically between the two groups about x=0 in the observed versus predicted plot. OPLS-DA-Orthogonal partial least squares discriminant analysis.

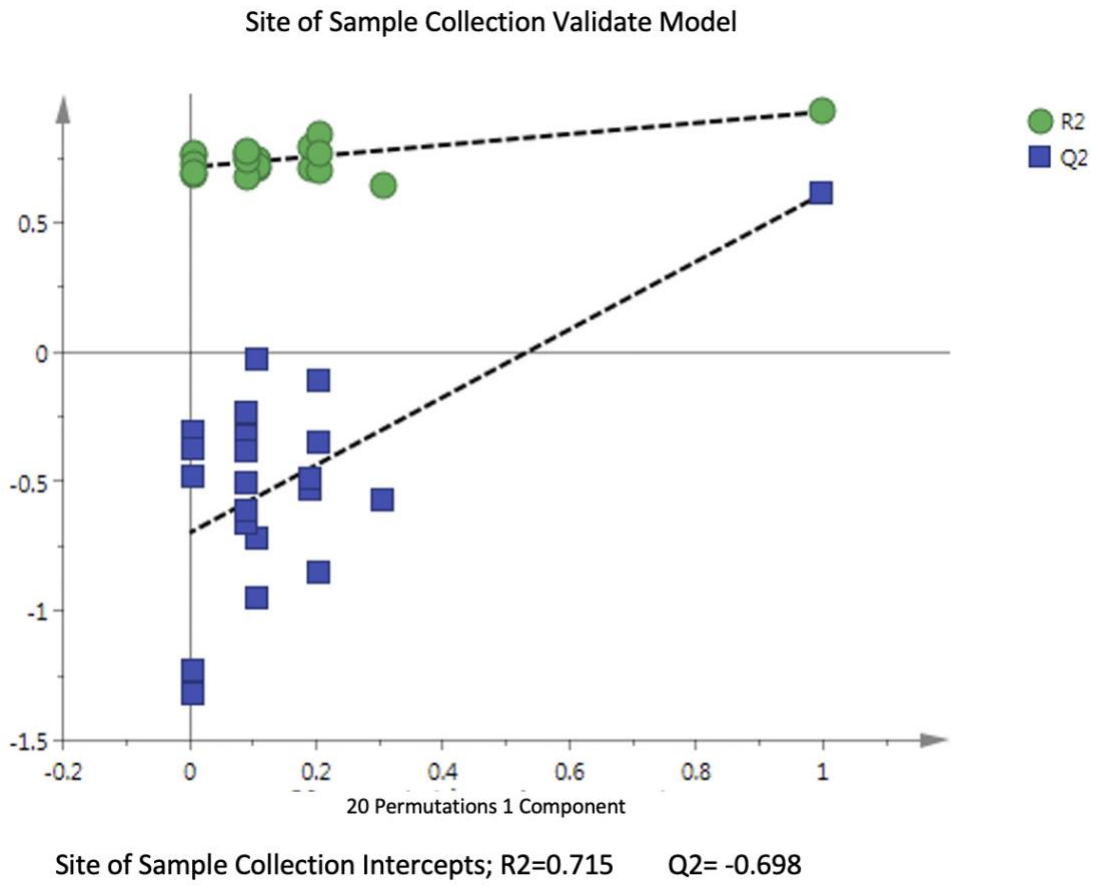

Figure 69: Permutation validation plot for Site of Sample Collection (Coronary sinus versus Peripheral). The y-axis intercepts for R2 (Green) and Q2 (Blue) were 0.715 and -0.69 respectively, indicating that the original model was valid. R2 measures the goodness of the fit and Q2 measures the predictive ability of the model.

4.3.6.4 Antianginal medications

Significant models were also observed in patients on antianginal medications as shown in Figures 70-72. The OPLS-DA plots showed a clear distinction between patients on antianginals and who were not on antianginals. The permutation test showed a statistically significant model with a Q^2 of – 0.6. The mechanism of action of antianginal ISMN is to induce coronary vasodilatation, and thereby attempt to match the metabolic supply of the myocardium to its metabolic demands. The patients in this study population were only included if they were stable, which would mean they would have only been on antianginals

for exertional angina. The results could probably suggest that the alterations in metabolite flux observed here could either be due to the presence of metabolic changes elicited by stable anginal symptoms or the impact of the vasodilatory effect of antianginals resulting in increased recruitment of the glycolytic pathway for energy production. The antianginal benefits of nitrates occur as a result of endogenous reduction to nitric oxide, a potent vasodilator (285). It has been widely accepted for its vasodilatory effect in the cardiovascular system, including reduction in blood pressure, inhibition of platelet aggregation, and protection against ischaemic-reperfusion (286).

Of interest, in this study population, tryptophan was noted to be altered in patients on antianginals. This is an interesting finding because, tryptophan and its degradation product kynurenine that was also observed in the metabolite profiling, share some of the common properties as antianginal drugs in its ability to act as a potent vasodilator (273). However, kynurenine was not observed in this group suggesting that the antianginal medications may not have influenced the kynurenine pathway. This supports the hypothesis that the observed decrease in tryptophan levels at TP1 and TP5 when compared to baseline, associated with its degradation product, kynurenine, suggesting a rapid catabolism of tryptophan through the kynurenine pathway, in response to acute reversible myocardial ischaemia. Nevertheless, future studies may shed more insight into the possible influence of antianginals in the kynurenine pathway.

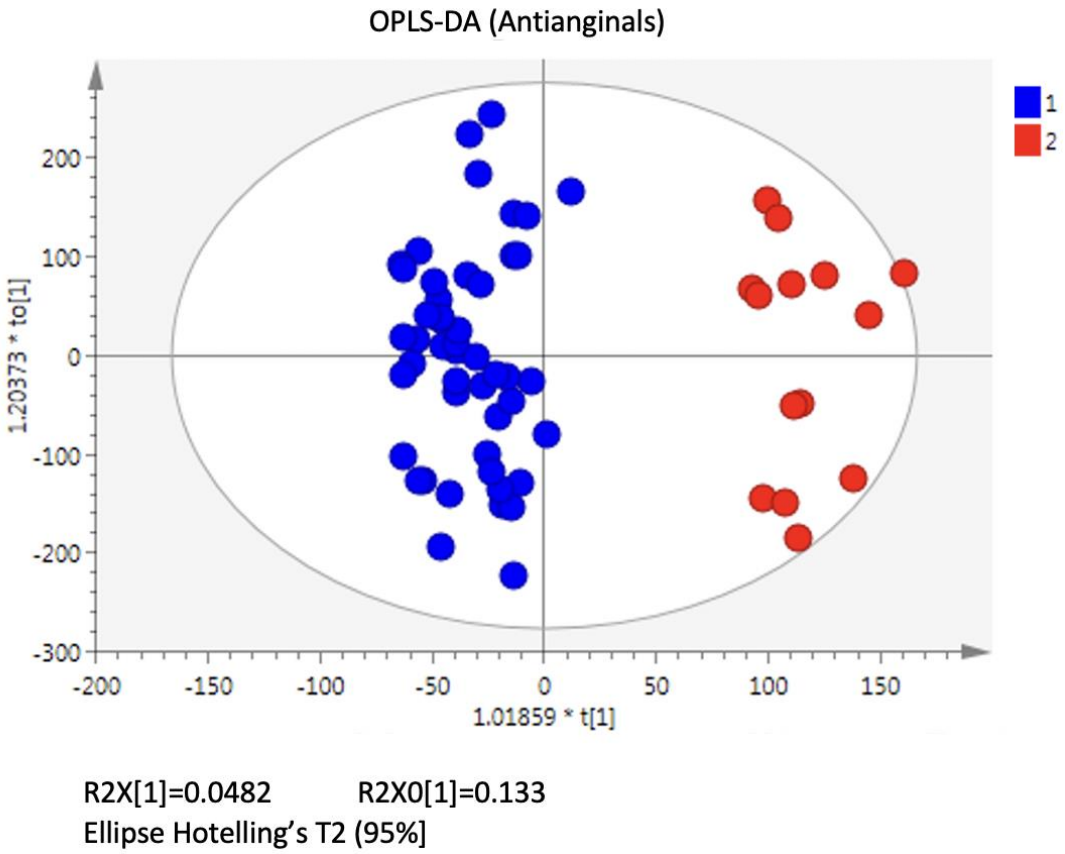

Figure 70: Supervised OPLS-DA models for patients on antianginals. The horizontal ordinate shows t1: represents correlation with m/z and class (CS versus P) and variation between the groups. The vertical ordinate shows to1: orthogonal principal component-score value and difference in the observation group (within group variation). The plot shows clear distinction between patients on antianginals (Red) versus not (Blue). R2X[1]: variance explained in the predictor variable t1=4.8%, R2X0[1]: variance explained in the predictor variable to1=13.3%. OPLS-DA-Orthogonal partial least squares discriminant analysis.

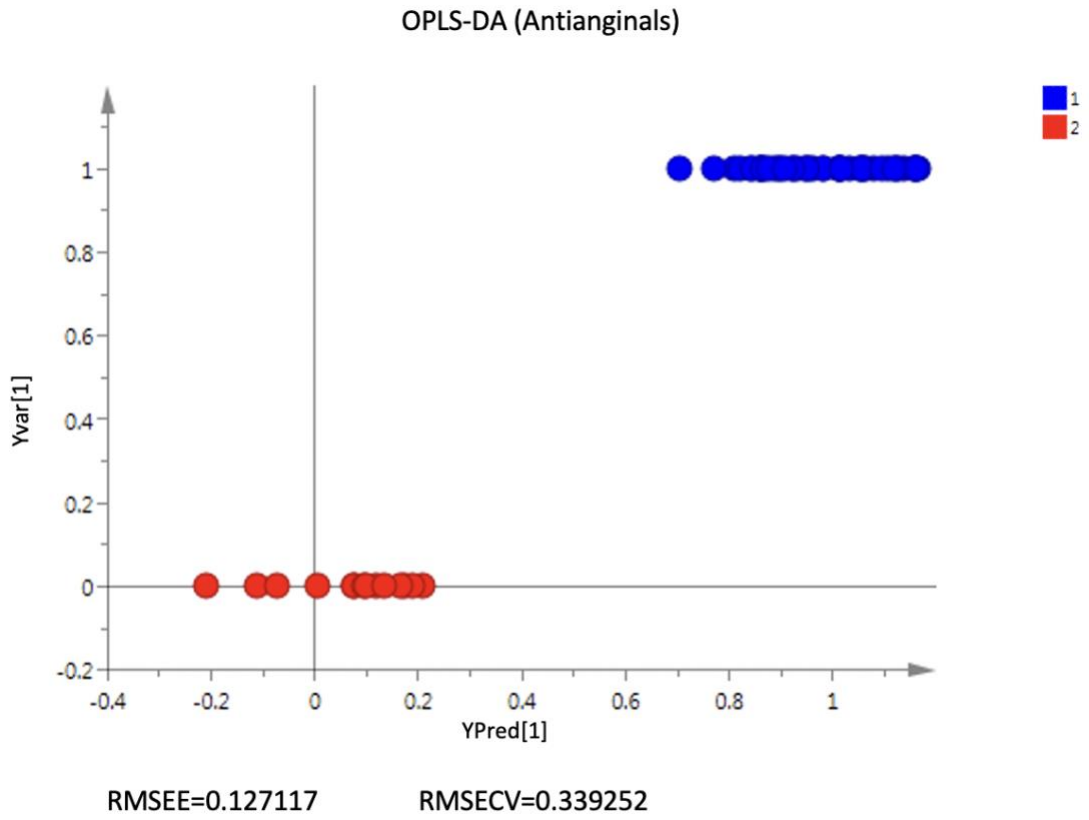

Figure 71: Supervised OPLS-DA model in accordance with the patients on antianginals. The plot shows observed versus predicted values and shows clear distinction between patients on antianginals (Red) from patients not (Blue). The separation between class is clearly seen by the lack of overlap vertically between the two groups about x=0 in the observed versus predicted plot. OPLS-DA-Orthogonal partial least squares discriminant analysis.

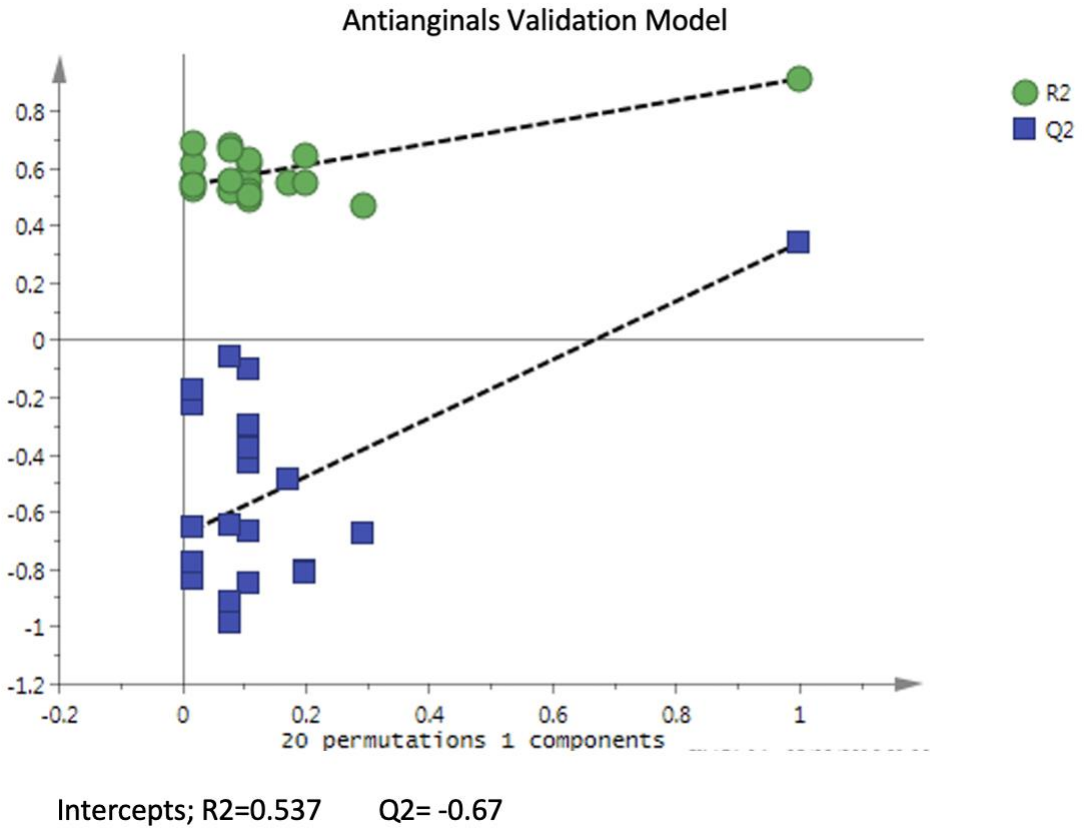

Figure 72: Permutation validation plot for antianginals. The y-axis intercepts for R2 (Green) and Q2 (Blue) were 0.537 and -0.67 respectively, indicating that the original model was valid. R2 measures the goodness of the fit and Q2 measures the predictive ability of the model.

4.3.6.5 Angiotensin converting enzyme inhibitors

Weak models were observed for patients on drug ACE-i. The OPLS-DA plot for ACE-i is shown in the Figures 73 & 74 below. The horizontal ordinate shows the correlation with m/z and the class and the vertical ordinate shows the orthogonal PC score value and difference within the group. The plot showed no clear distinction and overlap between the two groups indicating a weak model. This suggest that ACE-i and any observed variation in the metabolites in patients on this medication may not have necessarily influenced the ischaemia model.

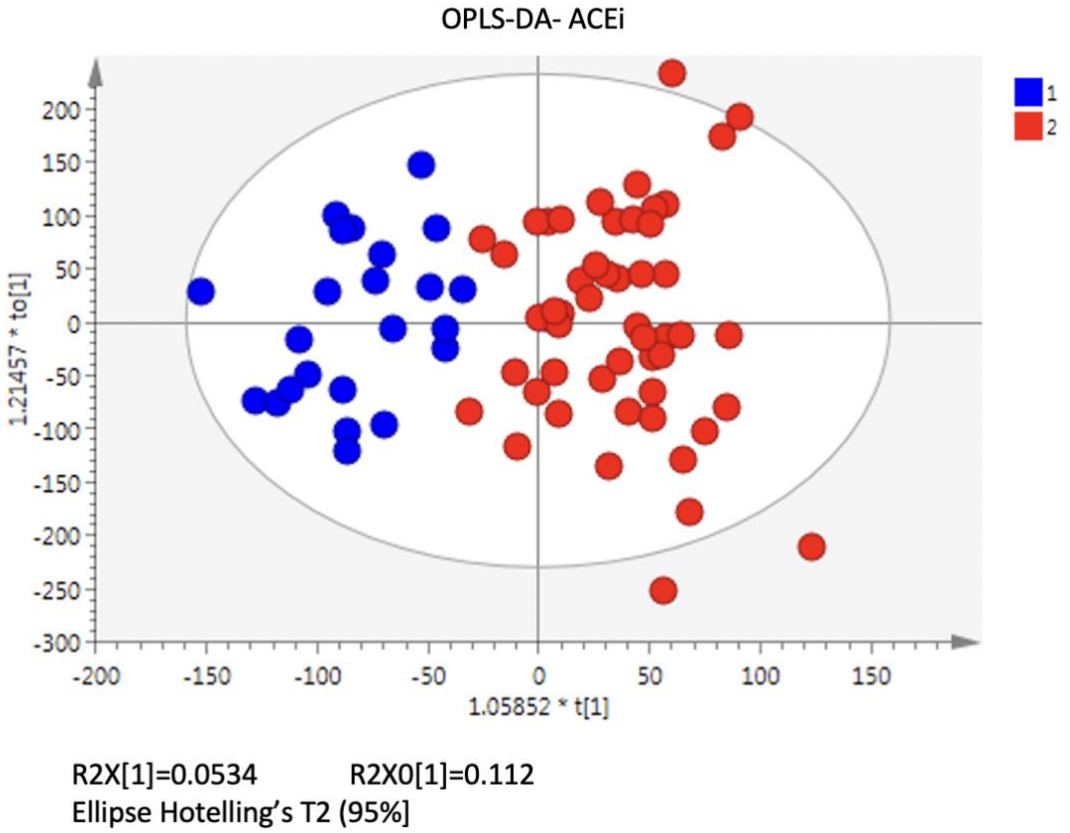

Figure 73: Supervised OPLS-DA models for ACE-i. The horizontal ordinate shows t1: represents correlation with m/z and class (ACE-i versus not on ACE-i) and variation between the groups. The vertical ordinate shows to1: orthogonal principal component-score value and difference in the observation group (within group variation). The plot does not show a clear distinction between samples from patients on ACE-i(blue) and not on ACE-i (red) and doesn't represent a significant model. R2X[1]: variance explained in the predictor variable t1=5.3%, R2X0[1]: variance explained in the predictor variable to1=11.2%. OPLS-DA-Orthogonal partial least squares discriminant analysis, ACE-i- Angiotensin converting enzyme inhibitor.

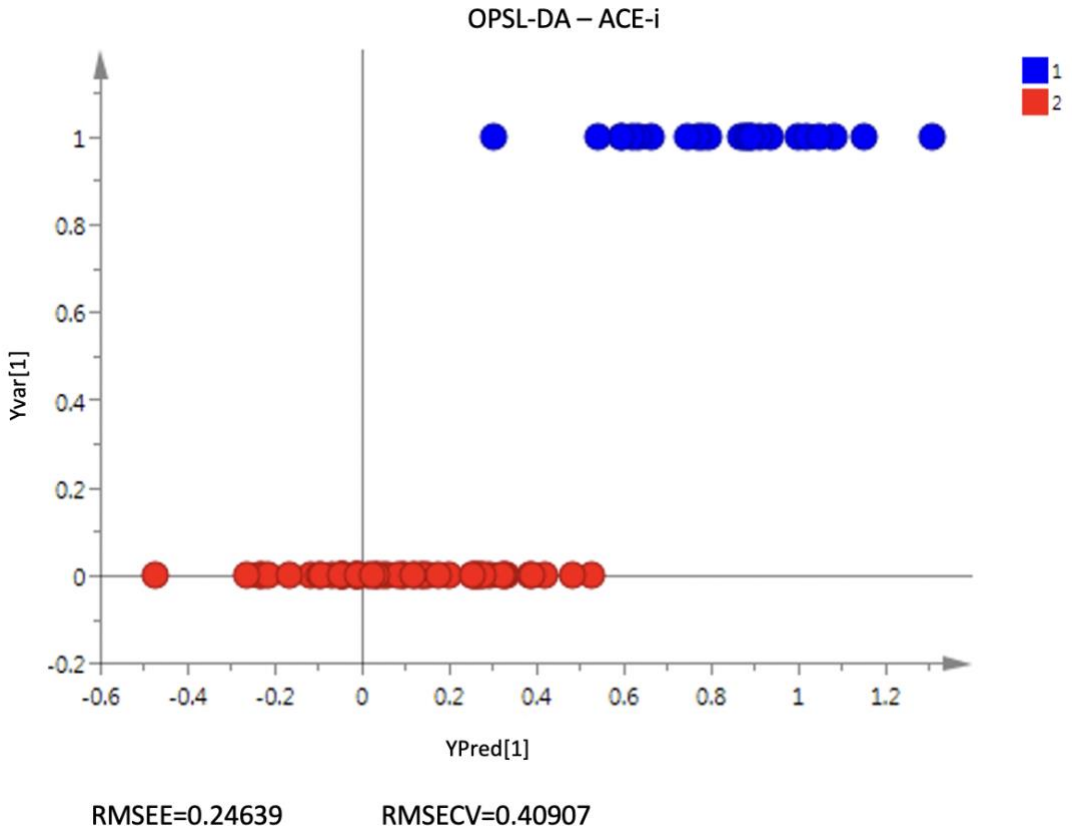

Figure 74: Supervised OPLS-DA model in accordance with the patients on ACE-i. The plot shows observed versus predicted values and shows no clear distinction between samples from patients on ACE-i (blue) and patients not on ACE-I (red). The separation between class is weak as seen by the overlap vertically between the two groups about x=0 in the observed versus predicted plot. OPLS-DA-Orthogonal partial least squares discriminant analysis, ACE-i-Angiotensin converting enzyme inhibitor, RMSEE- Root Mean Squared Error of Estimation.

4.4 Discussion

The subgroup analysis was performed to assess the influence of comorbidities, drugs and procedural factors that may have impact on the metabolites observed. The PCA was initially used as an exploratory method to look for any natural groupings arising from the variation in the data. The observations of clustering or patterns in the PCA plots helped give confidence to models obtained by supervised methods (PLS and PLS-DA). OPLS and OPLS-DA were then used to remove any variation that was completely uncorrelated with the response or class variable respectively to obtain coefficients for important variables. The statistical significance was then tested by permutation test. The analysis showed several interesting findings and statistically significant models were observed for patients with diabetes, BMI, site of sample collection and for patients on antianginals. Weak model was observed for patients on ACE-i drug. None of the other factors were shown to have influential metabolites.

Interesting observation were seen in the metabolite patterns in patients with diabetes and high BMI. The metabolites involved in lipid metabolism, monoacylglycerols, LysoPC and 2-hydoxybutyric acid (HBA) were increased. Of these, 2-hydroxybutyric acid is of particular interest as this has been associated with diabetes and metabolic syndrome (287), and high concentrations of HBA have been observed and used as a reliable marker in patients with DKA, a life-threatening complication of diabetes (288). The observed findings therefore may reflect a baseline altered metabolic state in diabetes and in patients with high BMI. However, from the cardiac perspective, ketone bodies are relevant particularly in the context of cardiac ischaemia.

HBA are small molecules synthesized mainly in the liver and are transported to other organs for oxidation when FA and carbohydrate availability is limited. In this regard, acetoacetate and HBA are the primary ketone bodies utilized by the heart (288). Myocardium is thought to be one of the highest ketone body consumers, especially when the availability of other substrates is limited. Several studies have shown that there is an increased myocardial delivery and oxidation of ketone bodies as an alternative fuel source in patients with advanced heart failure. This is thought to be due to an early adaptive response to maintain adequate ATP production (289, 290). Although ischaemia may be a possible mechanism to the observed upregulation of HBA, it is more likely to represent an underlying hypermetabolic state such as diabetes and obesity. Further studies focusing on HBA as a biomarker of hypermetabolic state would be beneficial. This study model showed good linear correlation with BMI and therefore would serve as a reliable marker in patients with morbid obesity who are being treated with life style modification measures or bariatric surgery.

With the exception of antianginal nitrates, none of the other medications that were used to treat the underlying co-morbidities influenced the metabolic pattern. The observation of low tryptophan levels in the antianginal group was interesting suggesting a synergistic vasodilatory mechanism. However, in the absence of kynurenine, it is reasonable to speculate that the antianginals did not interfere with the kynurenine pathway and hence the observed downregulation of tryptophan is likely to be as a result of acute coronary ischaemia.

Statistically significant model was also observed in the site of sample collection. This is likely due to the close proximity of the CS and therefore the effluents

from the heart preferentially reaches the CS before it could possibly be detected in the peripheral circulation. Although the number of samples were low in the CS group, this finding is interesting and correlates with the proximity of sample collection from the heart. More importantly, the changes observed in the CS and peripheral samples were similar suggesting that metabolite changes were myocardial specific. Hence this model becomes more relevant as a possible surrogate that helps to validate the human myocardial ischaemia model in this study.

The subgroup analysis revealed several interesting metabolites that merits further research. The study unfolds several metabolites and interesting models that may represent underlying chronic hypermetabolic state rather than an acute phenomenon. This study therefore supports that the metabolic perturbation observed in the acute ischaemia model is myocardial specific and occurred as a result of controlled transient reversible ischaemia.

Chapter 5. Discussion

CHD is one of the most common causes of morbidity and mortality worldwide, and thus constitutes an increasing public health burden (291). ACS occurs as a consequence of coronary atherosclerosis, and is associated with significant adverse outcome as a result of irreversible myocardial injury and remodeling. In contrast, myocardial ischaemia is a reversible process, which, when identified in a timely fashion, enables the use of therapeutic options to limit the extent of myocardial injury, providing a significant prognostic benefit for the patient. Due to the complex nature of the molecular and cellular changes occurring in the heart during myocardial ischemia, numerous studies have shown that the use of systems-level, omics approaches, such as genomics, proteomics and metabolomics may provide the optimal strategy for enhancing our knowledge in the diagnosis, management and risk stratification of patients experiencing CHD (292-294). Plasma, a frequently analysed pool of metabolites, is readily available and has been the main source of metabolic profiling (295). Interestingly, myocardial injury is associated with a distinct metabolic pattern compared to a healthy heart. Cellular metabolism is a dynamic process that can be altered by both, exogenous (e.g., diet, drug exposure, exercise, stress, therapeutic interventions) and endogenous factors (e.g., inborn metabolic errors, infections and inflammatory disorders) (296). As metabolites are the final avenue of the cellular process, they can more accurately indicate in a timescale relevant to myocardial ischemia and injury the physiological state of the heart when compared to changes in protein and gene expression, thus allowing us to better understand the earlies events occurring during ACS. Through decades of previous research, metabolomics

has emerged as a promising research tool to enhance our capability to diagnose disease and impact clinical care of patients undergoing CHD. (297). This study has for the first time applied an untargeted metabolomic analysis in a robust human model of controlled early reversible myocardial ischaemia in patients undergoing PCI.

This untargeted metabolomic analysis identified that lipid metabolism was the principal metabolic perturbation observed during early cardiac ischaemia. Interestingly only minor fractions of the metabolome were consistently altered during early cardiac ischaemia suggesting that these are representative of highly specific responses to this pathological state. This analysis has identified changes in plasma concentration of metabolites which have previously been shown to be either detrimental to cardiac function (such as AA, LysoPC and tryptophan), or known to have a cardio-protective effect (like DHA). This combination of both injurious and protective metabolites for cardiac function in response to acute myocardial ischaemia, suggests that metabolic changes provide an interesting mix of pathological changes that further the damage elicited by ischemia, and adaptive responses aiming to mitigate these effect and maintain the integrity of the cardiovascular system.

5.1 Robust human model of myocardial ischaemia

The study design provides a robust human model of myocardial ischemia through recruitment of a carefully selected cohort of patients experiencing single vessel CAD. All significant comorbidities such as HF, CRF, liver failure, chronic inflammatory and infectious disorders and malignancy, which could potentially influence the study findings were excluded. The patient cohort

possess a variety of standard risk factors for CAD such as DM, hypertension, hypercholesterolemia, obesity, and are on concomitant primary prevention medications, making it difficult to exclude these factors as influencing the metabolic responses seen.

The data collected suggests that the induced transient coronary occlusion created by balloon inflations triggers a reversible ischaemic burden, as evidenced by a transient rise of lactate (at TP1 and not TP5), suggesting anaerobic metabolism. In addition, the controlled ischaemia was a reversible injury without myocardial necrosis, as evidenced by lack of significant rise in biomarkers (CK and Troponins), although hs-Tn was not measured in these groups. However, the data presented here represents a true model of myocardial ischaemia. Such a robust model of early myocardial ischemia has not been previously described.

To try to better understand the potential influence that each of these variables have on the metabolic profile observed in the patient cohort, a subgroup analysis of the metabolomic data collected from the initial study was performed. Importantly, most metabolites shown to differ in their concentrations when comparing before and after an acute ischaemic event were not known to be associated with, hypertension, hypercholesterolemia, concomitant medications, ST segment changes during the procedure or procedure characteristics. This indicates that the metabolites discussed above are principally dynamic responders to cardiac ischaemia independent of associated risk factors of CVD. Of the limited number of statistically significant changes, 2-hydroxybutyric acid, monoacylglycerols, 3-hydroxyvalaric acid or 4-sulfobenzyl

alcohol showed changes related to BMI and diabetes. Further studies may be warranted to further investigate the mechanistic basis of this observation.

5.2 Limitations of previous models:

Previous metabolomic studies have used different models representing the continuum of ACS, such as UA, NSTEMI and STEMI (242, 245, 246, 298). While these studies have evaluated the potential clinical usefulness of metabolomics in improving current knowledge about pathophysiological mechanisms and risk stratification of patients with ACS, no conclusive and consistent data regarding the diagnostic and prognostic utility of measuring biomarkers in the early phase of myocardial ischaemia are available in the literature. While UA, in the absence of troponin rise and myocardial necrosis by definition, represents the early phase in the continuum of ACS, they are unstable patients whose cardiac ischaemic symptoms have varying duration, and are at impending risk of atherosclerotic plaque rupture. Although several studies were performed to define changes in metabolite pattern during myocardial ischaemia there were significant limitations in their model as described below.

Although the publication by Sabatine *et al*., (2005) was the cornerstone in the development of the application of metabolomics in IHD, the potential usefulness of their results into understanding the metabolic events in myocardial ischemia was limited by their choice of methodology. The study involved a total of 36 patients who were referred for evaluation of possible myocardial ischaemia. This study used exercise stress test to create reversible ischaemia, which was validated and quantified by myocardial perfusion imaging (18 patients with

reversible ischaemia as cases and 18 without as controls). Using HPLC-MS, targeted metabolomic analysis was performed on blood samples of all participants. Significant changes were detected in circulating levels of amino acids such as γ-aminobutyric acid, citrulline and argininosuccinate and of metabolites belonging to the TCA cycle, such as oxaloacetate and citric acid (245). From the diagnostic point of view, although the exercise stress test is a standard practice which is routinely used to evaluate patients for IHD, this is not without limitations. The use of an ECG recording during the exercise stress test for the detection of CAD is limited by its low sensitivity and specificity, which are 68% and 77% in men, and 61% and 70% in women respectively (299). Therefore, it would not have been possible to accurately determine coronary ischaemia with confidence in all participants. In addition, the exercise stress test involves significant skeletal muscle work load leading to significant oxidative stress in both skeletal and cardiac muscles. Therefore, the metabolite changes observed in this study were likely to not be specific to changes in cardiac metabolism and could have been confounded by the metabolites released from the skeletal muscles. A similar model of ischaemia was used by Barba *et al.,* who performed NMR metabolomics on patients with exertional angina (250). All patients underwent exercise stress myocardial perfusion scan to evaluate for myocardial ischaemia. Analysis of the serum samples identified lactate, glucose, long chain amino acids and lipid that differentiated patients with ischaemia and others. However, similar to the model of Sabatine et al, exercise stress test utilized in this study to evaluate for ischaemia may have influenced the results. Lewis *et al*., (2008), applied LC-MS platform and performed targeted metabolomic analysis in the blood samples of patients who

underwent therapeutic alcohol septal ablation for hypertrophic obstructive cardiomyopathy (HOCM) (246). The study observed changes in TCA pathway, amino acid, purine and pyrimidine metabolism. However, this study was a myocardial infarction model with irreversible myocardial damage rather than a reversible ischaemia model. Therefore, the cardiac metabolites released in this study represent necrotic cardiomyocytes, mimicking the events that might occur in a STEMI rather than those elicited by early myocardial ischemia. With that being said, HOCM by itself is associated with significant metabolic alterations as a result of impaired oxidative phosphorylation. Therefore, in patients with HOCM, there is a constant deprivation of oxygen in the cardiomyocytes and hence does not represent a myocardium with normal baseline metabolic characteristics found in the healthy, uninjured heart. Several investigators have recently applied metabolomics in the context of ACS. In this regard, Surendran *et al*., using LC-MS platform, analysed peripheral venous samples of patients who had suffered STEMI. This work identified significant changes in FA metabolism with perturbations in AA, DHA and LysoPC, consistent with this study findings (242). Similarly, Li *et al*., in their study on a STEMI model, applied UPLC-MS metabolomic platform to compare the metabolic changes in patients with acute MI and unobstructed coronary arteries. The study identified significant variations in plasma concentration of acetylglycine, glutarylglycine and nonanoylcarnitine in samples taken from the peripheral vein. (300) Kohlhauer *et al*., (2018) also performed plasma metabolite profiling in STEMI model and identified succinate as a biomarker of ACS, a substance that was not identified in this study (301). Furthermore, Pouralijan *et al.,* (2020), used NMR spectroscopy and analysed the plasma samples of patients with UA. The

study identified metabolites involved in the steroid hormone biosynthesis and lysine degradation (302) and Wang *et al.,* (2019), applied LC-MS platform to analyze plasma samples of patient with UA. The study identified biomarkers of lipid and amino acid metabolism. Tryptophan metabolism was identified as a key metabolic pathway in UA, consistent with this study findings (298).

While all these studies performed a comprehensive analysis of the metabolites and identified diverse pattern of changes, these occurred in the context of irreversible cardiomyocyte injury, although the extent of severity of ACS varied in the different patient groups studied. Therefore, the previously-described experimental models that are currently present within the literature do not truly represent a model of early myocardial ischaemia. The primary objective of this study was to develop a true human cardiac ischaemia model to allow us to characterize and provide a comprehensive understanding of the metabolic events that may be altered during reversible myocardial damage. The study is unique in this respect and has the potential to open new avenues of targeted research for potential biomarker discovery and develop therapeutic targets to modify cardiac metabolism to favour the production of cardioprotective molecules. Metabolome profiling is typically performed either by targeted or untargeted methods. While targeted metabolomics studies focus on accurate identification of a defined set of metabolites, untargeted metabolomic analysis has the benefit of measuring and comparing as many metabolites as possible across a sample set (303). The untargeted method is therefore particularly suitable for biomarker studies with a potential to identify previously unknown metabolites in the area of interest. Thus, to enhance the capability of extracting the metabolome of myocardial ischaemia without bias, this study used an

untargeted approach. The study analysed multiple different areas of metabolism simultaneously and has identified diverse yet specific areas of metabolism during early myocardial ischaemia.

The heart must contract incessantly, thus, the requirement for energy to fuel optimal function is immense. Consequently, to sustain the high energetic demand in the heart, continuous supply of sufficient oxygen is necessary. Hypoxia is the result of the imbalance between oxygen supply and oxygen demand and heart is challenged to produce similar energy with limited oxygen to maintain normal function resulting in impaired metabolic efficiency. The balance between carbohydrate and fatty acid use as substrates in energy production is well reported in association with cardiac ischaemia (304). The heart is capable of using all classes of energy substrates, including carbohydrates, lipids, amino acids and ketones, for ATP production in the mitochondrion. The heart thus has the capability to adapt and switch the substrate during oxidative stress. In general, during acute hypoxia, accelerated glycolysis becomes the initial response to oxygen deprivation. This is thought to be a natural protective mechanism of the heart to maintain cell function. However, prolonged hypoxia causes accumulation of glycolytic end products such as lactate resulting in intracellular acidosis resulting in inhibition of glycolysis and rapid depletion of the energy store. Thus, FFAs which are readily available becomes the primary source of substrate for the heart. Lipid metabolism thus plays a key role in the continuum of ACS which involves several complex networks of pathways and sequences of events that are known to have both detrimental and cardioprotective effects. This discovery-based mass spectrometry study has defined myocardial tissue-specific

metabolic changes in a human model of acute myocardial ischaemia. The study investigated multiple areas of metabolism simultaneously and have identified several interesting metabolites such as arachidonic acid, docosahexaenoic acid, lysophosphatidylcholine, carnitine derivatives and tryptophan and downstream products. The metabolic pathway analysis, potential mechanisms, interesting interactions and diagnostic and therapeutic targets have been explored and discussed below.

5.3 Metabolites of interest and metabolic pathway analysis

5.3.1 Arachidonic Acid (AA)

In this study, significant increase in the plasma levels of AA were observed at TP1 and TP5 when compared to the baseline following acute myocardial ischaemia. The metabolites, eicosatetraenoic acid, also known as eicosanoids, a free non-esterified AA, was also observed to increase. These findings opened up several questions.

1. Could this observation correlate with comorbidities?
2. Do patients with CHD have high circulating AA?
3. What is the mechanism of increase in AA levels following acute myocardial ischaemia?

Among all FAs, AA is most preferentially incorporated into phospholipids, and as such forms an important constituent of the cell membrane. As a consequence, less AA is partitioned to adipose tissue triglycerides and to oxidation (305). Furthermore, AA esters that are bound to triglycerides are relatively resistant to lipoprotein lipase, and AA is preferentially mobilized from

adipose tissue via hormone-specific lipase (306, 307). Hence, under normal physiological conditions, the circulating concentrations of AA are typically low due to albumin binding and trafficking to cells (308). Therefore, the observed increase in AA concentrations in this study are less likely to be due to a preexisting phenomenon and hence represents a pathological state.

This study population involved patients with DM and high BMI, therefore it is reasonable to speculate that some of the observed findings could be changes associated with metabolic syndrome. However, this speculation was not supported by the subgroup analysis, in which although lipid metabolism was altered in patients with DM and high BMI, AA or its downstream products were not observed as influential metabolites. Furthermore, previous evidence has demonstrated that in patients with high BMI and metabolic syndrome, the AA levels are high in the adipose tissue but lower in the circulation. Additionally, obese subjects have lower concentrations of circulating AA when compared to non-obese individuals (309). These data suggests that the increased plasma level occurs despite the presence of DM and high BMI in our study population, rather than because of it. AA also represents one of the pivotal signaling molecules involved in the initiation and propagation of inflammation, pain and homeostatic function (310). In this regard, previous studies have shown its association in adult patients with inflammatory bowel disease (311) and chronic inflammatory rheumatoid arthritis (312). Importantly, this study excluded patients with preexisting acute or chronic inflammatory disorders. Therefore, considering the prevailing evidence it is possible to confidently conclude that the observed increase in AA concentrations in this study is unlikely to be

secondary to any preexisting comorbidities, and is instead due to the induction of coronary ischaemia in the patients.

Of major importance, the metabolic pattern in this study unfolds a complex network of metabolic pathways that are interlinked and yet have both detrimental and cardioprotective function. This combined effect drives the attention back to the cascade of AA metabolism through the COX, LOX and CYP-450 pathway. AA metabolites derived from different pathways have been long recognized to possess important roles in the inflammatory and immunological process and have shown to mediate a variety of actions including vasodilation, vasoconstriction, and platelet aggregation (313). Under normal circumstances this mechanism is vital for maintaining adequate haemostasis. However, in ACS, increased platelet aggregation and vasoconstriction are associated with an adverse outcome. In this regard, the AA-derived eicosanoids such as prostaglandins (PGE_2, PGF_2, PGD_2), prostacyclin (PGI_2), thromboxane A_2 (TXA_2) and leukotriene B_4, that are involved in vessel tone regulation, platelet aggregation and coagulation, are of particular concern (314, 315). Therefore, the primary goal of treatment in ACS is to target these complex pathways that could favorably influence the outcome. In this regard, COX pathway has been of immense interest for decades. COX1 is usually present in endothelial cells, whilst COX2 is not normally found in abundance in blood vessel or heart tissue, where it only becomes upregulated during pathological stimuli like ischaemia. The anti-thrombotic features of aspirin, the first-line drug used in ACS, are primarily related to the suppression of PG and TXA_2 synthesis, and is the only known non-steroidal anti-inflammatory drug (NSAID) with cardioprotective effects (316). The mechanism

by which aspirin protects the cardiovascular system involves the unique pharmacology of as an irreversible inhibitor of COX which allows this NSAID to target TXA_2 release and inhibit bicarbonate production in the stomach (317). This anti-platelet effect of aspirin is well established and has a central role in the primary and secondary prevention of ACS. In contrast, NSAIDs that are able to inhibit COX2 are known to increase the cardiovascular risk, due to their prevention of production of endogenous anti-platelet compound, prostacyclin, by the endothelium (318). Hence, a subtle alteration in the metabolism of AA by the COX pathway could unbalance the equilibrium from antithrombotic to prothrombotic, thus understanding how AA metabolism changes during myocardial ischemia.

The endogenous AA generation mainly occurs from the cell membrane phospholipids. Liberation of AA from the membrane phospholipid is catalyzed by the enzyme phospholipase A_2 (PLA_2), the activity of which is known to be increased by various cellular activation signals including infection, inflammation or oxidative stress (319). For instance, it is well known that myocardial ischaemia and oxidative stress has dramatic consequences on AA metabolism, as it alters AA release by PLA_2 and induces COX2 over-expression (320). This would probably suggest that cardiomyocyte cell membrane disruption and oxidative stress induced by acute myocardial ischaemia are possible triggers in this study. It is also evident in this study that there were enhanced plasma levels of the downstream products of AA metabolism such as eicosanoids (e.g. eicosatetraenoic acid). These data would be consistent with an enhanced AA generation and subsequent metabolism. It is well known that increased breakdown of myocardial phospholipids to AA is an early feature of myocardial

injury and the enhanced action of PLA_2 is believed to be an important factor in this process (321). The circulating levels of PLA_2 has been regarded as prognostic indicators, and studies revealed that plasma levels of PLA_2 is elevated in patients with coronary artery disease and predicts coronary events in stable patients with CHD (322). Furthermore, increased plasma levels of PLA_2 predicted recurrent coronary events in patients with ACS (323). Several large studies have shown a relationship between PLA_2 and the risk of future cardiovascular events, although the magnitude of the association varied (324, 325). Although, there is not enough data to support PLA_2 as a biomarker of myocardial ischaemia, the pathophysiological influence of PLA_2 in the AA pathway is worth taking into consideration in this study. Interestingly, PLA_2 is already expressed in the normal arterial wall and there is compelling evidence that PLA_2 exist in abundance in atherosclerotic plaques (326-329). Additionally, a high incidence of atherosclerosis and mortality have been associated with patients with high PLA_2 activity (330). PLA_2 is virtually inactive on phospholipids from intact cells and several studies have supported that only those membranes where the differential transverse distribution of phospholipids has been disturbed such as vessel injury offer a convenient surface able to interact with the enzyme (331). This may be an important concept to consider in this study as mechanical disruption of the plaque occurs during balloon inflation. However, this mechanism doesn't necessarily explain the observed rapid flux in the AA metabolites seen and such a rapid change could only have been as a result of acute ischaemia. Therefore, in the absence of any other causal role, it would be reasonable to postulate that, increased levels of AA and

eicosanoids observed in this study is likely to be directly as a consequence of acute myocardial ischaemia.

PLA_2 has not previously been established as a biomarker of ACS and currently there is not enough evidence to support this. In this study, although PLA_2 was not measured, it is tempting to postulate that PLA_2 may have played a major role in the oxidative stress created by acute hypoxia. To determine its feasibility as a biomarker of acute myocardial ischaemia, several factors such as its sensitivity and specificity, its reliability as an early predictor, timing of rise from the onset of symptoms and its dynamic response, need to be established. Generally as a biomarker, PLA_2 is known to be remarkably stable, hence it could remain active for a prolonged period of time in the extracellular matrix of the tissues and circulation (332). Therefore, PLA_2 is a measurable substance and this property makes it an attractive substance for consideration of future research focusing on its feasibility as a biomarker of ACS.

At present there is no firm evidence to support the possible role of eicosatetraenoic acid in modulating the metabolic pathway during myocardial ischaemia. However, it is tempting to hypothesize that eicosatetraenoic acid, as a mitochondrial targeted FA, could improve energy production within the myocardium. Moreover, as a precursor of other non-esterified FAs, it is possible that eicosatetraenoic acid could more accurately reflect PUFA status. Furthermore, eicosatetraenoic acid has been shown to have anti-inflammatory effects by inhibiting the conversion of AA to prostaglandins (333),suggesting a potential beneficial role of eicosatetraenoic acid in CHD – although this will require further study to confirm. This also suggests that the beneficial effects of 3-PUFA in cardiovascular disorders may not be necessarily only be related to

already known PUFA (DHA or EPA), or any other specific PUFA, but rather involve the complex effects of several PUFA's, including eicosatetraenoic acid. Thus, the evidence suggests that in addition to the known detrimental effect of AA on cardiac function, there are several complex networks that may serve as either a diagnostic or therapeutic targets in CHD. It was expected that this study showed an increase in concentration of AA following acute coronary ischaemia, and it is interesting to observe this consequence as an early feature. In addition, this study indicated a potential role of PLA_2 as a viable biomarker for patients with CHD. Moreover, PLA_2 in combination with other inflammatory markers such as CRP or myeloperoxidase, could be useful in the multimarker strategy to identify patients at high risk of future coronary events. Of more interest, the diagnostic utility of PLA_2 as a biomarker of acute myocardial ischaemia has not been established. Targeted metabolomic analysis assessing these metabolites would provide an opportunity to more closely examine this possibility.

5.3.2 Docosahexaenoic Acid

This study observed a significant increase in DHA at 1 min and 5 mins in both groups. This is an important finding as DHA is known to have beneficial effect for the viability of the myocardium. The observation in this suggests the presence of an endogenous cardioprotective reflex elicited in response to acute ischaemia. This predominant complimentary cardioprotective effects of the cascade is mediated thorough the cytochrome P-450 (CYP) pathway. The molecular mechanism of this action is only partially understood and include changes in membrane structures, direct interaction with ion channels and

alterations in eicosanoid biosynthesis (334). In acute myocardial ischaemia, an increased reliance of cardiomyocytes on the oxidation of FFA is present, but associated with low ATP yield per oxygen and likely decreased energy efficiency (335). In addition, accumulation of FA intermediates in the ischaemic heart promotes ROS production due to inhibition of oxidative phosphorylation. Heart is an omnivore and has the ability to selectively choose the available substrate in response to the need. The oxidation of DHA is more than two times faster than the oxidation of FFA, therefore one could assume that DHA may be more energy efficient, and therefore a preferred substrate during myocardial ischaemia and oxidative stress (336). Thus, during hypoxia, DHA being a preferred substrate for mitochondrial metabolism, inhibition of FA oxidation could lead to comparably higher increase in levels of DHA (337). (338). In addition, DHA may compete with AA for binding and conversion by COX and LOX pathways and thus modulate the production and bioactivity of prostanoids and leukotrienes (339). These mechanisms may possibly explain the observed increase in the DHA in this study.

The metabolic pathways of DHA and AA are known to be interlinked and, in this regard, CYP-450-mediated eicosanoid formation, known as a third branch of the AA cascade, has received considerable attention for its beneficial effect in CHD. Importantly, recent studies have demonstrated that the same CYP isoforms that metabolize AA, also efficiently metabolize DHA and EPA (340). In addition, EPA and DHA can partially replace the AA from cell membrane to alleviate the proinflammatory, vasoconstrictive and prothrombotic effects (341, 342). Therefore, the balance between these two pathways is thus tightly regulated to the cellular signaling mechanisms and biological activities. Hence,

in acute ischaemia, there is an inherent competing mechanism to counter regulate the adverse effects. This fine balance is subject to modulation and intervening on this pathway have shown to alter the downstream metabolites towards a favorable equilibrium (340).

This study population involved patients with stable CAD, in whom a significant increase in DHA would counteract the adverse effects of AA eicosanoids that were also observed during acute ischaemia. Although they share a common pathway, the selectivity for the metabolic pathway and the biological effects of the metabolites produced is determined by its relative concentration (343). Supplementation of EPA or DHA can cause a shift of the CYP-450 as well as COX and LOX mediated metabolic pathways (344). Numerous epidemiological studies have originated based on the concept of competitive inhibition of the AA cascade by exogenous administration of DHA and succeeded on this hypothesis by dietary means. Diet rich in omega-3-PUFA showed significant benefit in CVD by shifting the pathway from the detrimental effects of AA towards a beneficial effect of DHA (340). Dietary and plasma level of eicosapentaenoic acid (EPA) and DHA were inversely associated with incidence of CVD (345). In addition, several trials have shown that DHA and its precursor EPA levels in blood were associated with reduced cardiovascular mortality (346, 347). In this regard, the Diet and Reinfarction (DART) trial examined the relationship of a diet rich in DHA with incidence of IHD and showed a significant reduction (29%) in all-cause mortality at two years following MI (348). Similarly, the GISSI-Prevenzione trial was another large study which supported this view and showed that the risk of sudden cardiac death was reduced by 45% (347). DHA is known to affect the basic properties

of cell membranes including elastic compressibility and ion permeability, owing to its antiarrhythmic properties and potential mortality benefits (349). The common cause of death during the first 24 hours of presentation of ACS is as a result of acute ventricular arrhythmia. (350). DHA has been demonstrated to have an anti-arrhythmic effect in a number of previous studies. In an animal model it was found that omega-3-PUFA can protect against fatal ventricular fibrillation after surgical occlusion of a coronary artery (351). Supplementation of omega 3 FA have shown to prevent post op AF in patients who underwent CABG (352).Similarly, in patients who had implantable cardiac defibrillator, fewer incidence of ventricular tachycardia was noted (353). The postulated mechanism is that the omega 3 FAs directly inhibit the sodium and calcium channels in the cardiomyocytes which can reduce oxidative stress (354). Such effect directly or indirectly prevents the arrhythmias and ischaemia induced ventricular fibrillation (355). A follow-up GISSI-P study that looked at the time course of benefits of omega-3 on mortality documented by the GISSI-P trial showed that there was a 45% reduction in Sudden Cardiac Death from omega-3 supplements in just 4 months.

In patients with stable CAD, DHA has shown to be useful in stabilizing atherosclerotic plaques and hence reducing the occurrence of ACS. Like other fatty acids, DHA forms part of the cell membrane, replacing the unsaturated FAs, and thereby modulating cellular function. A number of changes have been observed upon incorporation of DHA into the cell membrane. Among them the modulation of the eicosanoid system towards vasodilatation and anti-inflammatory responses is key in minimizing the risk of potential atherosclerotic plaque instability in patients with CHD. In support of this notion, Daviglus *et al*,

showed that Americans who consumed 34g or more of fish per day had a 40-50% reduced risk of myocardial infarction and much lower risk of CHD (356). Similarly, the 10-year Multiple Risk Factor Intervention Trial (MRFIT) found that increased consumptions of DHA and EPA up to 664 mg/day were associated with an approximate 40% reduction in CVD, CHD, and a significant reduction in all-cause mortality (357). He *et al.,* on this meta-analysis involving over 220,000 subjects with an average follow-up period of 11.8 years showed that increased fish consumption per week was associated with a 35-45% reduction in CVD mortality (358). In patients with chronic stable angina the impact of DHA in reducing the pro-atherogenic cytokines and stabilizing atherosclerotic plaque has been well recognized. In keeping with these findings, studies have shown more regression and less progression of coronary lesions in patients treated with DHA when compared to control subjects. In a randomized control trial, in patients waiting for carotid endarterectomy, histological analysis found plaque regression and inflammation was less frequent in patients treated with DHA than in the control subjects (359).

While the beneficial effect of DHA is well established in promoting atherosclerotic plaque stability in patients with stable CAD and for secondary prevention in patients following myocardial infarction, the impact of DHA during acute coronary ischaemia is still in its infancy. In ACS, plaque rupture is associated with vasoconstriction, platelet aggregation and subsequent occlusion of the coronary arteries. In such circumstances, the AA eicosanoids derived through the COX and the LOX pathway, such as the prostanoids and leukotrienes, could enhance the effect of platelet aggregation and vasoconstriction aggravating myocardial damage (360). Therefore, an

unopposed effect of the COX and the LOX pathway would be unfavorable because of the potential increased risk of enhanced vasoconstriction and platelet aggregation in the continuum of ACS. In this regard, EPA and DHA has been shown to induce nitric oxide mediated vasorelaxation through endothelium dependent nitric oxide release (361). Hence, by attempting to balance this adverse effect, DHA may promote vasodilatory and platelet inhibitory response and tip the balance towards a more favorable equilibrium. In an acute setting of ACS this effect of DHA is crucial and could perhaps partly explain the reason for different presentations of ACS ranging from UA to NSTEMI to STEMI. The clinical presentations of ACS are closely interlinked and the progression for UA to STEMI is unpredictable. It is well known that early diagnosis and intervention by means of combined pharmacological and interventional strategies have a significant impact in the progression of the events and reduce morbidity and mortality (362). As a first-line management of ACS, in addition to antiplatelet therapy, pharmacological vasodilation is recognised as an important measure to alleviate the distressing symptoms of chest pain and shortness of breath (363). In this respect, DHA by inducing nitric oxide may serve as an effective vasodilator in the immediate management of ACS.

Based on the available knowledge It could be postulated that, in patients with ACS, prognosis could be favorably influenced by increased concentration of DHA within the plasma. This study finding therefore is important in suggesting that production of increased levels of DHA may represent an early protective effect during coronary ischaemia. However, future targeted studies should focus on this hypothesis in models of ACS.

5.3.3 Lysophosphatidylcholine (LysoPC)

In this study, LysoPC plasma concentration was consistently found to be increased at 1 and 5 mins following the onset of acute coronary ischaemia. Rich in oxidized low-density lipoproteins, LysoPC have been identified as an inflammatory lipid that is critically involved in the pathogenesis of atherosclerosis and CHD (364). LysoPC is a class of lipid biomolecule derived by cleavage of PC by PLA_2 (365). Under normal circumstances, circulating LysoPC content is low and has been shown to accumulate rapidly under ischaemic conditions as consequence of enhanced catabolism of PC. Thus, LysoPC is also released into the blood by cardiomyocytes during hypoxia due to disruption of the cell membrane (366). Another potential mechanism of increased lysoPC, would be that a substantial portion of lysoPC that is produced in the circulation is converted back into the parent PC by the action of enzyme lysophosphatidylcholine acyltransferase (LPCAT), but this process requires the presence of acyl-CoA (367). During ischaemia, there is considerable downregulation of acyl-CoA in the plasma, thus inhibiting the conversion of lysoPC back to PC. However, this mechanism may be only relevant in the circumstance of prolonged ischaemia. The patients in this study had transient ischaemia, therefore it is unlikely that the later mechanism played a significant role. It is therefore reasonable to speculate that the observed increase in concentration of LysoPC in this study, is as a consequence of cell membrane disruption due to induced ischaemia.

During hypoxia, the cell membrane disruption and subsequent accumulation of LysoPC impairs the stability and permeability of the cell membrane (368, 369). Thus, LysoPC would dramatically alter the electrophysiological properties of

the membrane by influencing membrane potentials and ion transport functions causing disturbance in the Ca^{2+} homeostasis in the cardiomyocyte. Studies have shown that increased Ca^{2+} permeability correlated with phospholipid breakdown (370). Cultures of the cardiac cells exposed to LysoPC, displayed an accelerated Ca^{2+} flux resulting in Ca^{2+} overload in the cardiomyocyte (371). Exogenous addition of LysoPC to the perfused hearts of hamster and rat produced cardiac arrhythmias (372). Although, the time course of accumulation of LysoPC and the relationship with occurrence of electrophysiological alterations is still controversial, previous studies have shown a positive correlation between the tissue level of LysoPC and the arrhythmia occurrence and found that the severity of ventricular arrhythmias appears to be directly related to the magnitude of LysoPC accumulated during ischaemia (373). This is particularly true in patients with STEMI, in whom the extent and severity of myocardial injury is considerably large, associated with significant cardiomyocyte cell disruption. In such circumstances, ventricular arrhythmias commonly occurs in the early stage of STEMI and are associated with significant mortality (374). Sudden cardiac death (SCD) is a rapid unexpected event that occurs from cardiac dysfunction, often as a consequence of ACS. The most prevalent electrophysiological events leading to SCD are lethal ventricular arrhythmia, particularly ventricular tachycardia and ventricular fibrillation (375). Cardiomyocytes are rich in mitochondria and it is well known that oxidative stress induced by lysoPC, can result in mitochondrial dysfunction which has a close correlation with the onset of lethal arrhythmias (376). Additionally, enhanced oxidative stress is known to inhibit the migration of the endothelial cell to the sites of vascular damage and in turn causes endothelial

dysfunction and downregulation of endothelial nitric oxide resulting in vasoconstriction (377-380). This consequently has a significant adverse impact in the thrombus burden and overall outcome for the patient with ACS.

In the continuum of ACS, the combined mechanism of plaque rupture, platelet aggregation and vasoconstriction occur simultaneously, resulting in significant hemodynamic instability and even death. Therefore, the management of ACS is primarily focused on counteracting these diverse adverse events, collectively by pharmacological therapy and coronary intervention. Therefore, the finding of an increased plasma level of LysoPC during early myocardial ischemia could be significant factor in the onset of ischemia-induced arrhythmias. Better understanding the mechanisms eliciting this increased production of LysoPC and how this may be prevented could prevent new methods to improve the outcome for patients experiences acute myocardial ischemia. Therefore, therapeutic strategies that target the production, metabolism, or signalling pathways may prove to be attractive means to prevent or attenuate the complications of CHD.

5.3.4 Acylcarnitines - Octanoylcarnitine and Decanoylcarnitine

The most important biological function of L-carnitine is in the transport of FAs into mitochondria for subsequent β-oxidation, a process that results in the esterification of L-carnitine to a wide variety of acylcarnitine derivatives ranging from short-chain, medium chain to long-chain acylcarnitines (381). The endogenous carnitine pool is thus comprised of L-carnitine and acylcarnitines.

Interestingly, in this study population, plasma concentrations of medium chain acylcarnitines, Octanoylcarnitine (OC) and Decanoylcarnitine (DC) were noted

to be decreased during early myocardial ischaemia. The study observed that the concentrations of OC and DC were lower at TP1 and TP5 compared to the baseline. To begin with, we know that numerous medical conditions have been associated with lower concentrations of circulating carnitine caused by decreased biosynthesis such as in chronic renal (382) and liver failure (383), as well as malabsorption, bowel disorders and chronic infectious disorders (384). The population in this study were a carefully selected cohort of patients and did not involve patients with renal failure, liver failure or with other chronic illness. Therefore, we could interpret with confidence that the observed results correlate with induced ischaemic burden.

In general, the plasma concentrations of acylcarnitines are maintained within relatively narrow limits for normal biological function in their pivotal roles in FA metabolism and maintenance of energy production (385). Therefore, the observation of a lower concentration of medium chain acylcarnitine in this study could have a number of consequences in the outcome of myocardial ischaemia. Firstly, the study represents an early phase of acute myocardial ischaemia and therefore, observed low levels of acylcarnitine in the early phase of myocardial ischaemia may suggest an increased utilization of acylcarnitine in mitochondrial β-oxidation by the threatened heart as an attempt to enhance cellular ATP production. Mitochondrial FA β-oxidation represents the most efficient method for energy production in the normal human heart, and is thus essential for the maintenance of cardiac contractility. During β-oxidation, L-carnitine is one of the key metabolites that transports cytosolic FAs across the inner mitochondrial membrane, by forming acylcarnitines (386). It is therefore conceivable that in the acute phase of myocardial ischaemia (<5mins), an accelerated response to

utilize the available FA substrate, could result in depletion of the substrate and hence could explain the low observed levels of octanoylcarnitine and decanoylcarnitine in this study.

Secondly, the CPT-II, which is regarded as the key regulator of mitochondrial β-oxidation has previously been shown to be inhibited by OC and DC *in vivo* and *in vitro* models (387, 388). Inhibition of CPT-II, may result in decreased acyl-CoA entering mitochondrial β-oxidation and consequently decreased energy production. In the already threatened hypoxic myocardium, a further interruption of this pathway would accelerate cell death. Therefore, it is possible that the myocardial cells adapt their metabolism to reduce OC and DC levels to disinhibit β-oxidation as part of an adaptive reflex during early ischaemia. These complex interactions between the mitochondria and the regulation of metabolism or cell death has been previously studied and the emphasis on the role of mitochondria as potential therapeutic targets or as candidates for risk-predictor biomarkers has been described (389).

Thirdly, it is well known that FA and glucose pathways compete as a metabolic substrate in the heart and accelerated glycolysis becomes the initial response to ischaemia and oxygen deprivation (55). As upregulation of glucose metabolism is known to occur during ischaemic insult (29), this may result in transient downregulation of the flux through FA metabolic pathways. Although carnitine is typically viewed as a catalyst for FA oxidation, and low myocardial acylcarnitine levels can compromise the efficiency of myocardial FA oxidation, it also has the potential to promote myocardial oxidation of glucose particularly during ischaemia (390). Although the predominant metabolic perturbation in

this study were observed in lipid metabolism alterations in carbohydrate metabolism have also been observed.

Another potential mechanism underlying the observed low plasma levels of OC and DC in this study may be decreased production of myocardial L-carnitine. Normally, carnitine enters the systemic circulation through food intake and its availability primarily depends on dietary conditions, although the concentration found with the heart has also been found to decrease with age (391, 392). Although significant carnitine accumulation occurs in cardiac tissues, the heart fails to produce carnitine, and hence gains this from carnitine generated from the amino acids, lysine and methionine in the liver and kidney (393). Cardiac carnitine concentration also depends upon the capability of these tissues to uptake carnitine using specific transporter proteins in their plasma membrane (394). During hypoxia, in view of the impregnable nature of the cell membrane reduced cellular carnitine uptake may result in low cardiac and plasma concentrations (395). Experimental and clinical studies have shown that myocardial carnitine depletes quickly in the ischaemic, infarcted or failing myocardium (396). It is therefore conceivable that the extent and severity of carnitine depletion would be dependent upon the severity of the ischaemic insult. This would be another possible mechanism of the observed low levels of acylcarnitines in this study. As carnitine is necessary for the transfer of cytosolic FFA to the mitochondria, this process is halted resulting in accumulation of FA intermediates. This carnitine depletion would therefore lead to a significant downregulation of mitochondrial ATP production, which would exacerbate myocardial dysfunction during ischemia. In support of this notion, in a randomized, double blind placebo-controlled, L-Carnitine Ecocardiografia

Digitalizzata Infarto Miocardico (CEDIM) Trial, 472 patients with a first acute myocardial infarction received either placebo (239 patients) or L-carnitine (233 patients) within 24 h of onset of chest pain. Placebo or L-carnitine was given at a dose of 9 g/day intravenously for the first 5 days and then 6 g/day orally for the next 12 months. Patients on carnitine treatment showed significant improvement and recovery of their LV function. Additionally, reports of cardiac failure and demise dropped significantly, as evident from a rate of 4% and 10% in carnitine-treated patients against 10% and 14% in the placebo, respectively (397). Xue *et a*l., (2007), enrolled 96 patients with NSTEMI to assess the benefit in patients undergoing PCI. Patients were randomly assigned to receive either L-carnitine, 5 gm/day as an initial IV bolus and 10 gm/day IV for 3 days or placebo. The values of CK-MB and troponin I were significantly lower in the L-carnitine group. As these cardiac biomarkers represent the extent of myocardial damage the results suggest that L-carnitine supplementation was able to limit the extent of myocardial damage after NSTEMI in patients undergoing PCI (398). Therefore, the observed drop in OC and DC in this study indicates a potential target for pharmacological therapies aimed at improving cardiac function during and following cardiac ischemia through improving carnitine availability in cardiomyocytes.

It is well known that myocardial ischaemia enhances build-up of FA intermediates, potentially noxious to continued subcellular performance and elevated levels of long chain FAs have been linked with increased risk of cardiovascular death among dialysis patients (399). Acylcarnitine, with both polar and lipophilic properties, acts as a detergent and damages the double-layered lipid membrane, triggering the release of phospholipids, glycolipids and

cholesterol (400). Decreased myocardial carnitine content has also been noted in animal models of hypertrophic cardiomyopathy (401) and L-carnitine treatment improved the functional capacity in hypertrophied heart when compared to normal heart (402). Interestingly, a recent untargeted metabolomic analysis of the plasma samples of 70-year-old Swedish population using UPLC and TOF-MS, identified DC as a novel biomarker for atrial fibrillation (403). Similarly, another metabolomic study identified OC and DC as novel biomarkers for cardioembolic stroke and prediction of increased risk of recurrent stroke (404). OC and DC are therefore a measurable biomarker for its suitability for further targeted metabolomic analysis in an ACS model.

Given the fundamental importance of L-carnitine in facilitating mitochondrial and cytosolic FA oxidation, as well as in the conversion of CoA into acyl-CoA, it is not unexpected that endogenous plasma and tissue concentrations are normally maintained within relatively narrow limits in healthy individuals. However, their circulating concentrations are prone to significant alterations during the early stages of myocardial ischaemia, which may have serious implications for myocardial function if hypoxia burden is prolonged, as in ACS. In this regard, the observed results of this study are a novel finding. In the heart muscle, the physiological regulation of OC and DC has not been clearly established, but appears to be maintained at or near its equilibrium position in the intact tissue and appears to decrease significantly following acute reversible myocardial ischaemia. This implies that it could be possible to shift the position to a favorable equilibrium. However, until such measurements are made directly, the question remains open and the proposed mechanism unproven. Moving forward, perhaps a further targeted metabolomic studies in the role of

OC and DC in animal models of ischaemia and infarction would shed more insights into how these metabolic changes come about and may indicate potential methods for modulating carnitine levels to attempt to improve myocardial function during ischemia. From the therapeutic point of view, this study has opened up several possibilities for further research; for example, assess the outcome of carnitine as an adjuvant therapy with cardiac resynchronization therapy-pacemaker (CRT-P) and defibrillator (CRT-D), in patients with end stage heart failure.

5.3.5 Tryptophan

This study observed a decrease in plasma concentration of Tryptophan at TP1 and TP5 compared to baseline following the onset of acute myocardial ischaemia. Interestingly, the study also identified the presence of the tryptophan degradation products, kynurenine and N-formyl kynurenine. These findings are in keeping with the wealth of observational evidence in the literature regarding the inverse relationship of tryptophan with CHD (405-407). Tryptophan, an essential amino acid, constitutes a central component in human protein synthesis. It also serves as an important substrate for the generation of nicotinamide adenine dinucleotide (NAD), nicotinic acid, serotonin and kynurenine, as mediated by the enzymes, tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-deoxygenase (IDO) (408). In this study, it is apparent that there was an enhanced catabolism of tryptophan elicited by ischemia enhancing the generation of kynurenine. The production of kynurenine is only possible through the activation of the kynurenine pathway. Therefore, the observed increases in the downstream products of tryptophan, kynurenine and

N-formyl kynurenine in this study, would also support the conclusion of increased flux of metabolites through the kynurenine pathway.

In the context of acute coronary ischaemia, kynurenine is known to have an important role in endothelium-dependent vasodilatation (409), and oxidative stress (407). Nitric oxide, a potent vasodilator depends on cyclic guanosine monophosphate (cGMP) to exert their vasodilatory effects. Kynurenine, by activating the cGMP pathway has shown to promote blood vessel relaxation (273). This is an interesting concept as vasodilatation is an important therapeutic area of focus in patients with CHD. Chest pain is a frightening symptom during ACS, resulting in a fear of impending death with increased sympathetic drive and vasodilation with nitrates has shown significant symptomatic benefits (410) Nitrates are the most commonly used vasodilators in clinical practice. Although vasodilatation has no proven prognostic benefit in patients with CHD (411, 412), its role in improving ischaemic symptoms and quality of life has been well established in patients with angina (413). In this regard, kynurenine formation within atherosclerotic arteries possibly represents a counterregulatory protective mechanism. It is interesting to observe such a rapid down-regulation and enhanced catabolism of tryptophan during acute ischaemia, in this study. However, the impact of tryptophan or kynurenine as a vasodilator have been poorly investigated in patients with CHD.

The transient ischaemia model in this study mimics the ischaemia-reperfusion (*I-R*) model in clinical practice, in the fact that transient ischaemia was immediately followed by reperfusion of the ischaemic zone, although the intensity and extent of ischaemia was considerably small. A recent

metabolomics analysis in rats reported that *I-R* was associated with increase in plasma kynurenine (414). This was further confirmed by another nontargeted metabolomic study in plasma samples in rats, and furthermore, this study also showed that kynurenine was cardioprotective when injected prior to the coronary occlusion (415). This is an interesting finding, because reperfusion arrhythmias are a common occurrence in clinical practice. Reperfusion arrhythmias are often observed during PCI and tends to occur simultaneously upon revascularization of the ischaemia myocardium. Of major importance, unlike ischaemia ventricular fibrillation, the reperfusion arrhythmias are noted to be a benign phenomenon that resolves spontaneously without sequelae (416). This is because the genesis of reperfusion arrhythmias, unlike ischaemic arrhythmias operate non-reentrant mechanisms, such as enhanced automaticity and triggered activity due to afterdepolarization (416). Although, several factors such as ROS, calcium and other mediators are known to play a key role in the reperfusion arrhythmias, the increased tryptophan catabolism and kynurenine concentration may also have significant cardioprotective effect. These results therefore indicate that these rises in plasma kynurenine levels may have cardioprotective effects, although this will need further assessment. Therefore, from the therapeutic point of view, kynurenine may be an area of immense interest.

As a prognostic biomarker, the utility of kynurenine-tryptophan ratio (KTR) in individuals suffering from CHD have been previously described (417). High KTR have been suggested to predict cardiovascular prognosis, particularly in CHD and after cardiac arrest (418, 419). Furthermore, KTR has been useful in determining all-cause mortality for individuals suffering from CHD (418). Moving

forward, perhaps, further studies focusing on a multimarker strategy using, tryptophan, KTR and CRP, as a prognostic indicator of patients with CHD would be a viable option. In addition, this observation may aid with the management, for example; in patients with documented CHD, in whom the symptoms are atypical or in whom there is a dilemma in terms of the risk-benefits of coronary intervention, it is possible that elevated levels of KTR may identify subjects with vulnerable lesions that may justify the benefit for coronary intervention. The prevailing evidence support the concept that tryptophan and kynurenine may be an important risk predictor of coronary events. Further research into the function of tryptophan and kynurenine pathway as a biomarker of early coronary ischaemia and cardiovascular outcome would shed more comprehensive insight.

Chapter 6: Conclusion

CHD is one of the commonest causes of morbidity and mortality worldwide. No other disease has been so extensively studied. The work of several decades has yielded remarkable advances in our understanding, diagnosis and management of CHD. Although we consider our current understanding and therapy of CHD state-of-the-art, we are still challenged by the unpredictability and adverse outcome of the disease. Despite several advances in the health care system, CHD continues to remain a global burden. This is because acute thrombosis frequently complicates the course of coronary atherosclerosis, causing ACS and sudden cardiac death. The mechanism responsible for the sudden conversion of a stable disease to a life-threatening condition is usually plaque disruption with superimposed thrombosis. Although conventional risk factor modification plays a crucial role in the progression of CHD, alarmingly only few adults achieve favorable results. Today's challenge is to identify and treat the patients in a timely fashion to halt the transition from reversible to irreversible myocardial damage. This could only be possible if biomarkers that aid with the early diagnosis and management of ACS are consistent, reliable, and readily available with high sensitivity and specificity. Cardiac hs-Tn appears to be the most sensitive and specific biomarker among all the diagnostic biomarkers of ACS in clinical use. However, hs-Tn is a marker of myocardial necrosis and therefore reflects irreversible myocardial injury. In addition, the temporal rise in the serum concentration of hs-Tn do not permit an early diagnosis and does not support maximal sensitivity until 6 hours after the onset of symptoms. Therefore, discovery of a novel biomarker have been and area of immense interest.

The transition from reversible to irreversible myocardial injury is a fine balance that involves several interconnected complex metabolic pathways. Changes in the plasma concentration of metabolites in the human body are rapid and reflects the physiological state of the body very closely. The recent advances in metabolomics have revolutionized the field of biomarker discovery, that provide the ability to monitor rapid and dynamic metabolic changes in the human biofluid and tissues. The advances in bioinformatics coupled with cross-disciplinary collaborations have enhanced the ability to retrieve, characterize and analyse large amount of data with precision. High-throughput platforms in metabolomics has evolved as a powerful platform for more global approach to discover the mechanism and molecular fingerprints of ACS.

This study involved a robust human model of acute reversible myocardial ischaemia to define the cardiac metabolic changes in acute hypoxia. In a carefully selected, matched cohort consisting of two study groups; acute transient coronary ischaemia was created and plasma samples were collected at baseline, TP1 and TP5. Using UPLC-MS, the plasma samples were analysed. Interestingly, the study observed specific metabolic perturbation in support of acute myocardial ischaemia. Importantly, interesting phenomena were demonstrated that validated the ischaemia model. In support of transient coronary ischaemia, the study observed homovanillate, a catecholamine metabolite, suggesting metabolic stress. Lactate was observed to increase significantly, but transiently, in keeping with anaerobic metabolism and hypoxanthine levels were observed to increase representing ischaemia-reperfusion. To further support the ischaemia model, a subgroup analysis was

performed to assess the influence of comorbidities, drugs and procedural factors that may have impact on the metabolites observed. The analysis showed several interesting findings and statistically significant models were observed for patients with diabetes, BMI, site of sample collection and for patients on antianginals. However, none of the metabolites observed in the subgroup had influence on the metabolic pattern observed as a consequence of ischaemia.

The study investigated different areas of metabolism simultaneously and identified statistically significant changes with metabolites predominantly involved in the lipid metabolism. Interestingly the pattern had a combination of both detrimental and cardioprotective metabolites. Although it was evident that there was a switch from carbohydrate to FFA metabolism, the observation of increase in AA and DHA implied a tendency of the heart to selectively modulate the pathway, as an innate reflex protective phenomenon. Several other interesting metabolic patterns were observed in respect to the cardioprotective effects. The tryptophan levels were noted to be low with high kynurenine suggesting an upregulation of kynurenine pathway and tryptophan catabolism, a known cardioprotective response. Similarly, OC and DC were noted to be decreased, which could be postulated as a cardioprotective effect, although further studies are required to support this concept in the context of coronary ischaemia. The subgroup analysis revealed several interesting metabolites that merits further research. The subgroup analysis unfolds several metabolites and interesting models more probably representing an underlying chronic hypermetabolic state than an acute phenomenon. This study therefore supports that the metabolic perturbation observed in the acute ischaemia model is

myocardial specific and occurred as a result of controlled transient reversible ischaemia

From a biomarker perspective, the levels of DHA and LysoPC were noted to be consistently elevated at all time points implying its potential for further evaluation at later time points (e.g., 30 mins or 60 mins). This would be an interesting area of further research to create potential novel diagnostic biomarkers in an ACS model. From a therapeutic point of view, tryptophan, its downstream product kynurenine and kynurenine pathway may serve as a potential target for therapeutic intervention in patients with ACS who are at high risk of life-threatening arrhythmias and SCD. In addition, 2-hydroxybutyric acid, identified in the subgroup analysis is known to be associated with metabolic stress, a consequence of DM and obesity. This may serve as a prognostic biomarker in patients with metabolic syndrome in whom aggressive life style modifications and bariatric interventions are performed routinely.

Using an untargeted metabolomic approach, this study has defined metabolic perturbation in a human model of acute reversible myocardial ischaemia. This diverse yet specific metabolic pattern identified in response to acute hypoxia involved multiple interlinked pathways that represent several areas of metabolism. This may provide further insight into the feasibility of measuring the metabolite as a potential diagnostic and prognostic biomarker of ACS. New developments and their implementations are guided by science and there is a great potential for metabolomics to provide insights into the fundamental biological processes for biomarker discovery and provide surrogate targets for therapy.

6.1 Limitations

There are a number of limitations for this study. Firstly, atherosclerosis represents a chronic inflammatory and immunological phenomenon, which involves multiple complex inflammatory processes mediated by macrophages and cytokines. It is therefore possible that some of the observed changes may have been influenced by the inflammatory cascade. However, the observed metabolic pattern revealed a valid ischaemia model and moreover, the metabolic responses observed were rapid with similar changes seen in the CS and peripheral samples suggesting cardiac tissue-specific response to ischaemia.

Secondly, PLA_2 exists abundantly in the atherosclerotic plaque and has an important role in the metabolism of AA. Therefore, it is possible that the enzyme could have influenced the AA cascade. However, observed changes in the plasma was rapid and occurred immediately following ischaemia, suggesting an acute response to myocardial ischaemia.

Thirdly, it is possible that some of the metabolite changes that we observed may relate to vascular trauma.

6.2 Future perspective

This study has identified several important metabolites with both detrimental and cardioprotective effects in a human model of acute myocardial ischaemia and have opened up several avenues for further research.

1. A targeted metabolomic analysis of impact of tryptophan in an animal model of ACS.

Ventricular arrhythmias are the common cause of sudden cardiac death in the acute phase of STEMI. Increased tryptophan catabolism and kynurenine concentration is known to have cardioprotective effect. However, the role of tryptophan and KTR has been poorly studied in patients with ACS. The cardioprotective effect of tryptophan renders it as an attractive biomarker with huge potential. It would be interesting to study the impact of ventricular arrhythmias with tryptophan therapy in an animal model of ACS.

2. A targeted metabolomic analysis on 3-Hydroxybuteric acid as a predictor of response to the management of morbid obesity (bariatric surgery/aggressive life style modification), in patients with cardiovascular disease.

Obesity is a huge public health problem and is associated with significant cardiovascular morbidity and mortality. Bariatric surgery and aggressive life style modification measures have shown to be beneficial in reducing all-cause mortality and these interventions have been increasingly considered in this population. This study showed a linear correlation of metabolites with BMI and hydroxybutyric acid may be of interest in this regard.

3. Assess the outcome of carnitine as an adjuvant therapy with cardiac resynchronization therapy-pacemaker (CRT-P) and defibrillator (CRT-D), in patients with end stage heart failure.

Several studies have previously supported the benefit of exogenous carnitine administration in ACS (398, 420, 421). Hence, from the therapeutic point of view, this study has opened up several possibilities for further research. This

would be a feasible study to perform, as there are a large cohort of patients with end stage heart failure, as a consequence of ACS, who receive treatment with CRT. Therefore, there is a potential to perform a large cohort trial, not only to assess symptomatic benefit but also the impact of arrhythmia burden by device interrogation.

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Ref: 10562-Ltr 2-Neyses

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Dear Professor Neyses

Research Study: **"Identification of novel serum markers of cardiac ischaemia using serum metabolomics"**
PIN: 10562 (Please use this reference number in any future correspondence)

Thank you for submitting a Pan Manchester Research Notification for the above study. I am pleased to be able to confirm that the R&D Office now has all the required information concerning this research and that the Trust's Director of Research and Development has given approval for the project be undertaken.

We acknowledge that the *Central Manchester and Manchester Children's University Hospitals NHS Trust* has accepted the role of Research Sponsor for this study (ref. Research Governance Framework[1] as issued by the Department of Health).

Details of the project have been recorded on the Trust R&D Management Information System and the project has been given a unique identification number (PIN), as shown above. (A copy of the signed Notification Form is enclosed for your records).

Please note, it is a requirement of the approval given by the Trust that the research project is being conducted in line with the guidance given within the Research Governance Framework[1]. Further guidance is available on the R&D web pages (see above), or request a CD from the R&D office.

Please draw your attention to the need to comply with both the Health and Safety at Work Act and the Data Protection Act. If you require further information or advice in any of these latter areas please contact the Trust's Health & Safety Advisor, Mr Ken Wood, on 276 4262 or the Trust's Data Protection Officer, Ms Cara Lally on 276 4878.

In line with this framework I would be grateful if you would inform me of the actual start date of this particular project and any changes that might be made to it during its course. Your help and support would be gratefully received.

I would like to take this opportunity to wish you well with your research

[1] 'Research Governance Framework for Health and Social Care' Version 2 DoH 2005
http://www.doh.gov.uk/research/rd3/nhsrandd/researchgovernance/govhome.htm