

Investigating *S*-acylation in the metabolic dysfunction of the T2D heart



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Abstract

Cardiovascular disease is the leading cause of mortality in type 2 diabetes (T2D) patients. Metabolic dysfunction is apparent within the T2D heart, presenting with an increased reliance on lipid metabolism and decreased glucose metabolism. Emerging work indicates that the main fatty acid (FA) transport protein, CD36, is enhanced on the sarcolemma in T2D, driving an increase in FA uptake. The lipid post translational modification *S*-acylation has been linked to regulating CD36 trafficking. However, it remains unknown whether CD36 is *S*-acylated in the heart and whether CD36 *S*-acylation levels are altered in T2D.

This work began through investigating the *S*-acylation status of substrate membrane transport proteins. CD36 was shown to be *S*-acylated in the heart on four cysteine residues, while the glucose transporters (i.e GLUT1 and GLUT4) and fatty acid transporter FABPpm were not *S*-acylated. Furthermore, in hearts from T2D rats, CD36 *S*-acylation was increased 2.5-fold and associated with a 2.4-fold increase in CD36 localisation at the sarcolemma. The changes in CD36 were accompanied by a 25% increase in fatty acid oxidation (FAO) rates and 65% increase in triglyceride (TG) concentrations in diabetes. The regulatory *S*-acylating enzyme, DHHC4, was increased 2.5-fold in diabetes compared with controls. Furthermore, DHHC4 was shown to have a binding motif for the transcription factor FoxO1, and blunting of FoxO1 decreased DHHC4 mRNA levels in T2D, indicating that enhanced DHHC4 expression in diabetes is driven by increased FoxO1 transcription.

This thesis then went on to explore the manipulation of *S*-acylation using increased palmitate concentrations and pharmacological compounds that inhibit the *S*-acylating DHHCs and de-*S*-acylating acyl-protein thioesterases (APTs). Acute increases in palmitate concentrations decreased CD36 *S*-acylation in Langendorff perfused control hearts, indicating that the abundance of palmitate does not actively drive increases in CD36 *S*-acylation. In T2D rat hearts, the broad spectrum DHHC inhibitor CMA decreased CD36 *S*-acylation by 34% with a 24% decrease in CD36 localised on the sarcolemma. Treatment with CMA also reduced FAO rates by 21% with a 48% decrease myocardial TG concentrations compared to untreated T2D hearts. The decrease in lipid metabolism was accompanied by a 43% increase in cardiac function in T2D hearts perfused with CMA. In contrast, in control hearts, the inhibitor of the de-*S*-acylating enzyme APT1 (ML348) increased CD36 *S*-acylation by 32% with a 30% increase in FAO rates.

The last part of this thesis explored alterations in CD36 in human heart failure (HF) biopsies and myocardial ischemia in rodents. In HF tissue, sarcolemmal CD36 was decreased 29% and were positively correlated with left ventricle ejection fraction (LVEF). In ischemic rat hearts, CD36 *S*-acylation was not altered compared to non-ischemic controls, whereas the metabolic regulators AMPK and ACC were shown to have enhanced *S*-acylation in ischemia, suggesting *S*-acylation as a potential novel regulator of the metabolic response in ischemia.

In conclusion, this work provides compelling evidence that there is an increase in lipid metabolism in diabetes due to enhanced CD36 *S*-acylation driven by FoxO1 mediated increases in DHHC4 expression. Pharmacologically manipulating CD36 *S*-acylation levels has profound effects on downstream metabolism. Lastly, CD36 is altered in human HF tissue and ischemia is a stimulus for enhanced *S*-acylation of AMPK and ACC in rat hearts.

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List of Abbreviations

ACC	Acetyl-CoA carboxylase
ACS	Acyl-CoA synthetase
Acyl-RAC ...	Acyl-resin assisted capture
Acyl-PEG	Acyl-polyethylene glycol exchange
ADP	Adenosine diphosphate
AMP	AMP-activated protein kinase
Akt	Serine/threonine kinase
APT	Acyl-protein thioesterase
ATP	Adenosine triphosphate
CAD	Coronary artery disease
CD36	Fatty acid translocase
CMA	<i>N</i> -cyanomethyl- <i>N</i> -myracrylamine
CBF	Cleaved bound fraction
CUF	Cleaved unbound fraction
CPT	Carnitine palmitoyl transferase
DAG	Diacylglycerols

DbCM		Diabetic cardiomyopathy
DHHC		Asp-His-His-Cys containing palmitoyltransferase
ETC		Electron transport chain
ER		Endoplasmic reticulum
FA		Fatty acid
FABP_{pm}		Fatty acid binding protein (plasma membrane)
FADH₂		Reduced flavin adenine dinucleotide
FAO		Fatty acid oxidation
FASN		Fatty acid synthase
FATP		Fatty acid transport protein
F-2,6-BP		Fructose-2,6-bisphosphate
GAP		Rab-GTPase-activating protein
GLUT		Glucose transporter
GSV		GLUT4-storage vesicles
HF		Heart failure
HK		Hexokinase
IF		Input fraction
IFM		interfibrillar mitochondria
IRS-1		Insulin receptor substrate-1
PI3K		phosphatidylinositol-3 kinase
LCFA		Long chain fatty acid

LCFA-CoA	..	Long chain fatty acyl coenzyme A
LVEF		Left ventricular ejection fraction
MI		Myocardial infarction
NADH⁺		Reduced nicotinamide adenine dinucleotide
PAT		Palmitoyl acyltransferases
PBF		Preserved bound fraction
PCKθ		Protein kinase C theta
PCr		Phosphocreatine
PDH		Pyruvate dehydrogenase
PDK		Pyruvate dehydrogenase kinase
PFK		Phosphofructokinase
PTM		Post-translational modifications
PPAR		Peroxisome proliferator-activated receptor
PI3K		Phosphatidylinositol-3 kinase
PUF		Preserved unbound fraction
SSM		Subsarcolemmal mitochondria
TBC1D1		TBC1 Domain Family Member 1
TBC1D4		TBC1 Domain Family Member 4
TG		Triglyceride
t-SNARES	...	Target-membrane associated SNARES
T2D		Type 2 diabetes

v-ATPase Vacuolar-type H⁺-ATPase

v-SNARES .. *N*-ethylmaleimide-sensitive factor attachment protein receptors

1

General Introduction

1.1 Type 2 Diabetes

In 2023, Diabetes UK reported that over 5 million people were living with diabetes in the UK, an increase of 28% since 2019¹. Type 2 diabetes (T2D) accounts for over 90% of all cases of diabetes, and these patients are at a high risk of vascular complications including nephropathy, neuropathy, retinopathy and cardiovascular disease². Cardiovascular disease is the leading cause of mortality in diabetes patients, associated with increased incidence of myocardial infarction (MI) and heart failure (HF)³. In T2D patients, alterations in cardiac diastolic function can occur in the absence of traditional risk factors such as hypertension and coronary artery disease (CAD), which is known as diabetic cardiomyopathy (DbCM). DbCM is characterised by diastolic dysfunction, fibrosis, inflammation and metabolic dysfunction. There is a growing body of research that has linked altered cardiac metabolism as a key mechanism driving cardiac dysfunction in T2D patients.

1.2 Cardiac metabolism in the healthy heart

It has been well established that optimal cardiac metabolism is crucial for the maintenance of cardiac function. The metabolically healthy heart relies on a constant supply of oxygen and a wide range of fuels to sustain contractile function, utilizing roughly 6kg of ATP per day⁴. Roughly 95% of cellular adenosine triphosphate (ATP) generated by the heart occurs through oxygen-dependent oxidative phosphorylation of substrates in the mitochondria. Substrate breakdown converges on the formation of (NADH⁺) and FADH₂ within the mitochondria, which enters the electron transport chain (ETC), resulting in ATP generation. The remaining 5% of ATP production is from oxygen independent substrate-level phosphorylation in the cytoplasm.

The heart can metabolise a range of substrates to match its high energy needs as illustrated in Figure 1.1. Fatty acids (FAs) are the carbon substrate of choice for the heart for energy extraction under steady state conditions, producing roughly 60% of ATP. Glucose is the second main fuel of the heart, contributing approximately 30% to cardiac ATP production. In addition to FAs and glucose, the remaining 10% of energy can be from amino acids, ketone bodies and lactate to generate ATP⁵. The heart is able to rapidly swap between fuels depending on oxygen availability, hormone levels, workload and substrate concentrations. This metabolic flexibility allows the heart to match ATP production to demand in response to natural fluctuations in substrate supply that occur with fasting, feeding, and exercise.

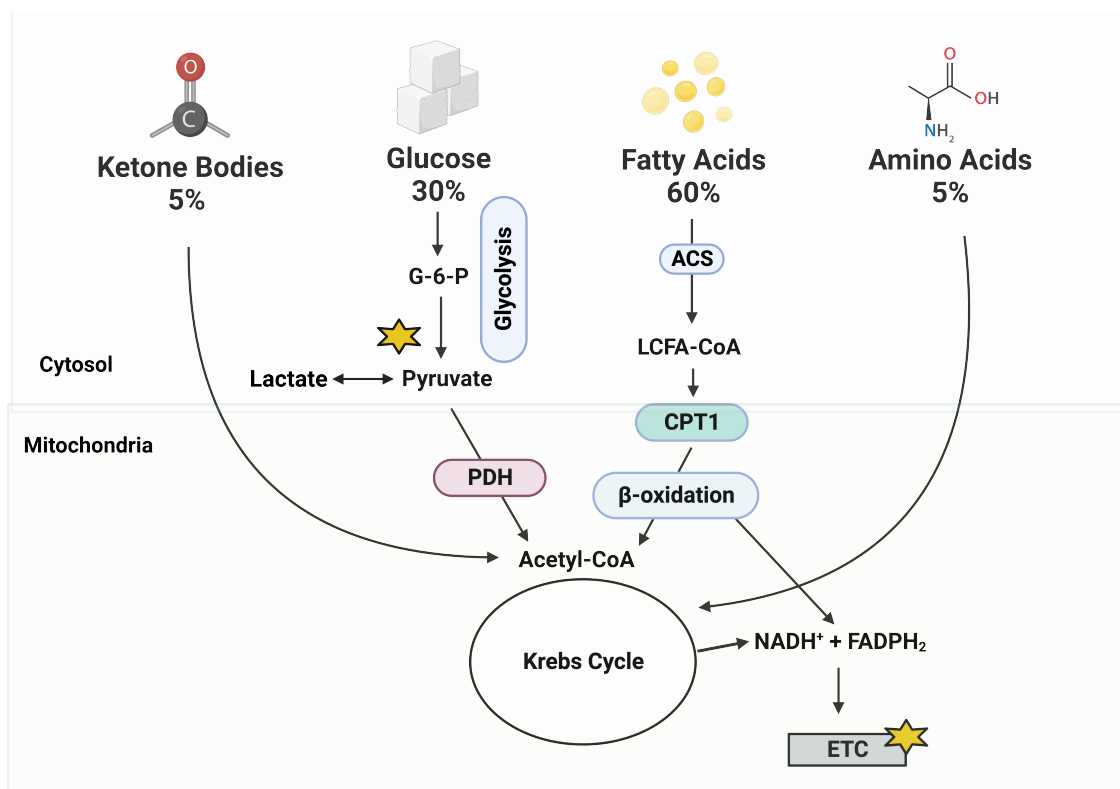


Figure 1.1: Fatty acids, glucose, ketone bodies and amino acids are all substrates used by the heart to generate ATP. Long Chain Fatty Acid (LCFA), Pyruvate Dehydrogenase (PDH), Carnitine palmitoyltransferase I (CPT1), Beta-Oxidation (β -oxidation), Fatty acyl-CoA synthetase (ACS), Glucose-6-Phosphate (G-6-P).

The heart has limited ability to synthesize FA *de novo* and therefore has a large dependency on long-chain fatty acid (LCFA) transported in the blood. Circulating LCFA that are delivered to the heart are transported as non-esterified fatty acids (NEFAs) attached to albumin, as dietary triglycerides in chylomicrons (CM-TG) or as endogenously synthesized triglycerides (TG) in very low-density lipoproteins (VLDL-TG). In the lipoprotein-bound form (i.e. VLDL-TG or CM-TG), the cell must first hydrolyze the TG molecules, which is mediated by the endothelial enzyme lipoprotein lipase (LPL). In contrast, glucose is freely transported in the circulation. In order to be metabolised by the heart to generate ATP, substrates must enter cardiomyocytes through facilitated diffusion across the sarcolemma regulated by membrane substrate transport proteins.

1.3 Membrane substrate transporters

Rapid fluctuations in energy needs by the heart rely on adaptations in substrate uptake across the sarcolemma. The myocardial energy content, ATP and creatine phosphate, can only maintain contractile activity for short periods of time and endogenous myocardial glycogen and TG stores are limited. Therefore, continuous substrate uptake is required by the heart to meet energy demands. The first step of cellular metabolism begins with substrate uptake across the sarcolemma. The sarcolemma encases the cell and is part of an extensive endomembrane system including the endoplasmic reticulum (ER), the nuclear membrane and the Golgi apparatus. Substrate uptake across the sarcolemma is highly regulated and often involves concerted efforts between specific membrane receptors and transport proteins. Substrates are transported across the sarcolemma by transport proteins which confers the physiological benefit of tightly regulating the amount of substrate uptake into the cell in response to metabolic demands, allowing for greater homeostatic control. The amount of glucose and fatty acid transporters

present at the sarcolemma is a major factor determining cardiac glucose and FA utilization⁶.

1.4 Glucose Transporters

While FAs are the main fuel used by the heart, glucose is critical for supporting cardiac metabolism. Intramyocardial glucose can be sourced by either from hydrolysis of intracellular glycogen or uptake of plasma glucose. However, intracellular glycogen levels in the heart are low and therefore to meet the high energy needs of the heart, there is rapid glycogen turnover facilitating 40% of glucose generated⁷, while the remaining 60% of glucose utilisation comes directly from plasma glucose uptake. The rate limiting step for glucose metabolism is uptake across the sarcolemma and glucose uptake has been well established to be mediated by transport proteins of the GLUT family. The glucose transporter 1 (GLUT1) and glucose transporter 4 (GLUT4) are the two main isoforms in the heart. The initial discovery of the GLUT topology was delineated in GLUT1⁸, and GLUT4 is predicted to have a similar structure. The GLUTs are hydrophobic proteins and are comprised of 492 amino acids and have amphipathic alpha helices, 12 transmembrane domains, with one *N*-linked glycosylation site on the exofacial loops and intracellular NH₂ and COOH cytoplasmic tails^{9,10}. The amphipathic helices form an aqueous pore that allows GLUT4 and GLUT1 to transport glucose as well as other hexose sugars down a concentration gradient into the cell^{9,10}.

An important distinction between the GLUT isoforms is that GLUT1 is primarily responsible for basal glucose uptake, while GLUT4 is located in intracellular compartments and translocates to the sarcolemma to enhance glucose uptake in response to extracellular stimuli. The translocation of GLUT4 to the sarcolemma in cardiomyocytes has been well established to be stimulated by circulating plasma

insulin and increased cardiac work¹¹. In the un-stimulated state, GLUT4 is sequestered in endosomes and packaged into small GLUT4-storage vesicles (GSVs). Once stimulated, the GSVs move along the cytoskeletal network aided by motor proteins to the sarcolemma where the vesicles then fuse with the membrane and the GLUT4 transporters are inserted into the sarcolemma. Comprehensive proteomic studies on isolated GLUT4 vesicles in adipocytes have identified 50 proteins that could potentially regulate GSV transport and fusion with the sarcolemma¹². However, it has been well established that Rab proteins are essential for the formation of the GSVs and movement through the cytoskeletal network, while vesicles fusion to the sarcolemma is mediated through vesicle-associated soluble N-ethylmaleimide-sensitive factor attachment protein receptors (v-SNAREs) and interactions with specific target-membrane associated SNAREs (t-SNAREs).

Both insulin and contraction stimulate GLUT4 translocation through distinct signalling pathways, however they converge at the level of the GTPase-activating proteins, AS160 (also known as TBC1D4) and TBC1D1. Once insulin binds to the insulin receptor (IR), it results in the activation of insulin receptor substrate-1 (IRS-1), phosphatidylinositol-3 kinase (PI3K) and Akt2 in sequential order. Contraction stimulation results in the activation of AMP-activated protein kinase (AMPK), either through increased AMP/ATP ratio or elevated intracellular Ca^{2+} . The activation of Akt2 and AMPK lead to an inhibitory Ser/Thr phosphorylation of AS160 and TBC1D1, enhancing their binding with the molecular adaptor protein 14-3-3, which inhibits their Rab-GTPase-activating protein (GAP) activity towards Rabs. Inhibition of the GAP promotes the conversion of a less active GDP-loaded Rab to a more active GTP-loaded Rab¹³. The more active GTP-loaded Rab then facilitates GLUT4 storage vesicle trafficking to the sarcolemma from intracellular endosomal stores¹⁴. It has been proposed that there exists two distinct GSV pools, an insulin sensitive pool and a contraction sensitive pool; with one responding to Akt

signalling and the other responsive to AMPK mediating signalling¹⁵. Additionally, endosomal pH has been shown to play a role in governing GLUT4 translocation to the sarcolemma. As such, insulin stimulated GLUT4 translocation was blunted when the vacuolar-type H⁺-ATPase (v-ATPase), which maintains endosome at pH 5, was inhibited¹⁶. Therefore, GLUT4 trafficking to the sarcolemma is a complicated process regulated by various stimuli and trafficking proteins.

1.5 Fatty Acid Transporters

Similar to glucose transport, the first and rate limiting step of lipid metabolism is FA uptake across the sarcolemma. Following their uptake into cardiomyocytes, FAs are then activated into acyl-CoA esters that can either be incorporated into the dynamic TG pool for storage, undergo mitochondrial β -oxidation to produce ATP, or be used for phospholipid synthesis. It was initially thought that given the lipophilic nature of the sarcolemma that the majority of FA uptake occurred as a result of passive diffusion. However, not having a point of control, such as a transporter, could prove undesirable as the heart would have no control over the rate of FA entry into the cardiomyocyte. While current research has demonstrated that passive diffusion of FAs across the sarcolemma cannot be fully excluded, it has been found to only contribute marginally to the overall rate of FA uptake^{17,18}.

Three types of fatty acid transport proteins have been identified in the heart and have been shown to be largely responsible for FA uptake; a 40 kDa peripheral fatty acid binding protein (FABPpm) located on the outer leaflet of the sarcolemma¹⁹, a family of 63 kDa fatty acid transport proteins (FATP1-6)²⁰ and an 88 kDa fatty acid translocase (CD36)²¹. As such, seminal experiments in giant sarcolemmal vesicles and cardiac myocytes discovered that FA uptake was dramatically suppressed when the FA transport proteins were chemically inhibited, therefore demonstrating the

importance of transporters for FA uptake^{18,22,23}. Decades of research on the FA transport proteins have established that CD36 drives the majority of fat uptake, and is responsible for driving approximately 70% of myocardial FA uptake^{18,24}.

After initially being identified as a membrane glycoprotein on human platelets in 1977²⁵, CD36 was then shown to play a role in various cellular processes including being a leukocyte differentiation antigen²⁶, a cellular receptor for thrombospondin²⁷, a macrophage scavenger receptor for oxidized LDL²⁸ and was shown to be involved in tethering malaria infected erythrocytes to endothelial receptors²⁹. Therefore, CD36 is classified as scavenger receptor SR-B2 and is a member of a superfamily of scavenger receptor proteins (Class B), sharing topology with SR-B1 and lysosomal integral membrane protein 2 (LIMP-2). However, in 1992, a study investigating the inhibitory effects of sulpho-N-succinimidyl oleate (SSO) on FA uptake in rat adipocytes identified an integral membrane protein that was identical to the leukocyte cluster-of-differentiation antigen CD36²¹. When inhibited by SSO, decreased FA uptake was observed²¹ and further work from the group delineated that CD36 also acted as a FA transport protein³⁰.

Recently, human CD36 was crystallized to investigate the binding of the adhesion protein PfEMP1 found in the malaria parasite *Plasmodium falciparum* to CD36²⁹. CD36 is characterized by a hairpin topology consisting of two large transmembrane domain spanning regions and small cytoplasmic tails containing its NH₂ and COOH termini as outlined in Figure 1.2. The large extracellular domain has been shown to contain nine N-glycosylation sites, as well as two phosphorylation sites, which result in the observed 88 kDa molecular mass of CD36. Furthermore, CD36 has three disulphide bonds on the extracellular amino acids between 243-311, 271-333 and 313-323 and two ubiquitination sites on the COOH terminus.

In order to facilitate FA uptake through CD36, the lipid tails of FAs have been proposed to interact with the large hydrophobic cavity running through the entire

length of the CD36 protein, while the FA carboxyl head group interacts with amino acid residues at the opening of the cavity in the extracellular domain^{29,31}. Although not fully delineated, current insight into the structure and topology of CD36 suggests that this protein does not act as a traditional transporter. As such, CD36 has been proposed to facilitate FA uptake across the sarcolemma through three key steps: absorption into the membrane, flip-flop translocation and desorption from the membrane³². More specifically, CD36 possibly promotes FA partitioning for delivery to the outer leaflet of the lipid bilayer³², where the carboxyl group of the FA is then able to move through the bilayer interior and reposition at the inner side of the membrane. Once through to the aqueous intracellular side, CD36 provides a docking site for FAs to bind with cytoplasmic heart-type fatty acid binding protein (H-FABP)^{32,33}, and is able to interact with acyl-CoA synthetase³⁴ for onward metabolism. The desorption step, where FA moves into the aqueous cytosolic phase appears to be the rate limiting step for the transmembrane transport³⁵ and therefore, the overall rate of FA uptake in the cardiomyocyte is determined by the trans-sarcolemmal gradient.

Similar to GLUT4, CD36 has further been classified as a trafficking membrane protein that translocates from intracellular endosomes to the sarcolemma in response to stimuli. Seminal studies using confocal immunofluorescence microscopy³⁷ and sub-cellular fractionation³⁸ demonstrated that CD36 was present at the sarcolemma and in microsomal membranes. Immunoabsorption studies have further characterized that CD36 is located in a subcellular pool that does not contain any GLUT4, indicating that GLUT4 and CD36 are stored separately intracellularly and translocate to the sarcolemma independently¹⁵. CD36 is enhanced on the sarcolemma following insulin stimulation and contraction to facilitate FA uptake, similar to GLUT4. In cardiomyocytes that were treated with the CD36 inhibitor SSO, FA uptake was severely reduced during both insulin stimulation and contraction, demonstrating an

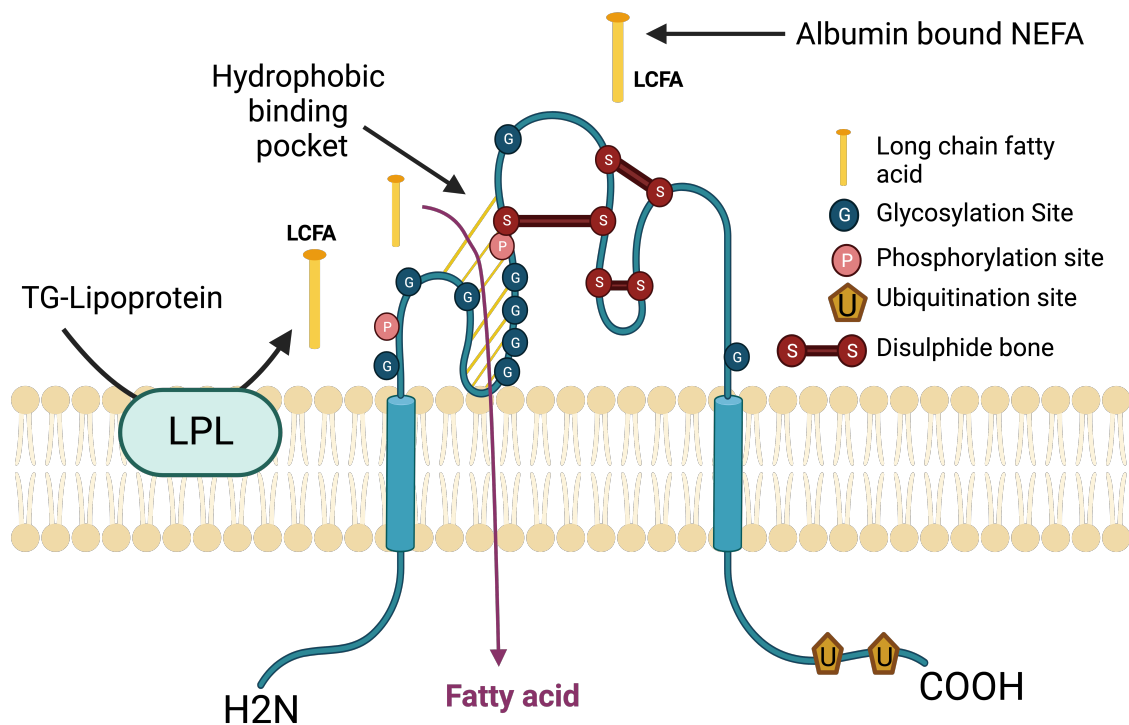


Figure 1.2: Structure and topology of CD36. Figure modified from Glatz et al. 2018³⁶.

irreplaceable role for CD36 in stimulus mediated FA uptake³⁹.

In contrast to GLUT4, the specific trafficking machinery involved in transport and fusion to the membrane dedicated to CD36 is still being unravelled, however, assumptions have been largely made based on GLUT4 trafficking as demonstrated in Figure 1.3; including the role for Rabs in vesicle fission and transport through the cytoskeletal network, and v-SNAREs and t-SNAREs in fusion with the membrane. Furthermore, insulin and contraction mediated AS160 and TBC1D1 phosphorylation has also been implicated in mediating CD36 trafficking to the sarcolemma. However, reports have demonstrated some distinction in translocation responses of GLUT4 and CD36 in response to changes in endosomal pH¹⁶. As such, when endosomal pH is increased under basal conditions by inhibition of the v-ATPase using the pharmacological compounds monensin and bafilomycin-A, CD36 is rapidly translocated to the sarcolemma, whereas GLUT4 remains intracellularly bound⁴⁰.

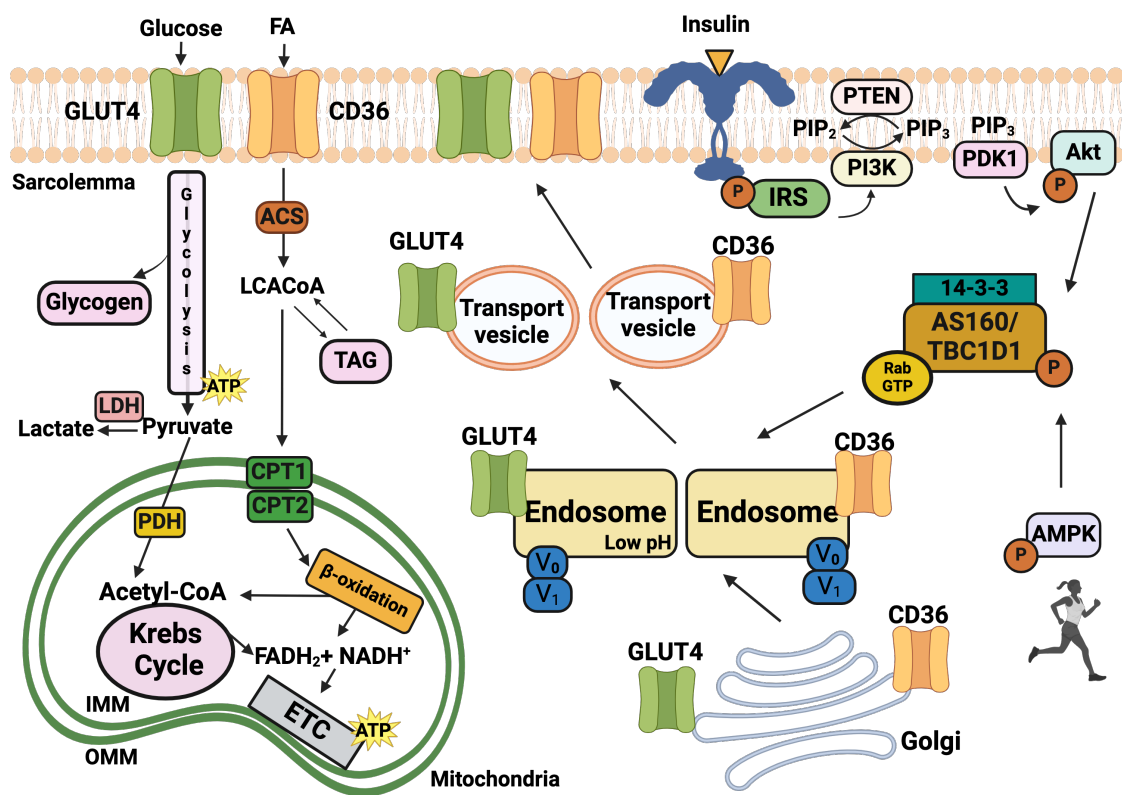


Figure 1.3: In response to insulin and contraction, GLUT4 and CD36 traffic to the sarcolemma and facilitate substrate uptake, thereby regulating downstream metabolism. Figure modified from Glatz and colleagues³⁶.

The other FA transporters, FATP1, FATP6 and FABPpm were found to not traffic between intracellular storage compartments and the sarcolemma in cardiomyocytes and therefore do not contribute to inducible FA uptake. FATP1, FATP6 and FABPpm were also shown to only play a minor role in FA uptake under basal conditions⁴¹. However, the minority of FAs that are thought to be transported by the FATPs are very long chain (VLC) fatty acids (>C22:0) and are rapidly converted to VLC-acyl-CoA thioesters by the synthetase activity of the FATPs. Furthermore, although CD36 has a greater FA transport efficiency compared to FABPpm, it has been suggested that these transporters work in concert as acceptors of for FAs at the cell surface⁴². Therefore, both CD36 and GLUT4 are the major metabolic gatekeepers of substrate metabolism in response to varying stimuli, which puts these

transporters at key regulatory points between cardiac health and disease.

1.6 Altered cardiac metabolism in T2D

As metabolism is imperative for cardiac output, impairments in cardiac metabolism can have dire consequences on cardiac function. Peripheral insulin resistance is a key hallmark of T2D and plays a role in the metabolic dysregulation in the pathophysiology of the disease⁴³. Insulin, which is a hormone secreted by the β -cells of the pancreas, is a master regulator of systemic FA and glucose metabolism. However, T2D is a progressive disease and early stages of T2D are characterized by insulin resistance (hyperinsulinaemia), prior to later-stage development with β -cell impairment (hyporinsulinaemia)⁴⁴. As such, perturbations in cardiac metabolism have been universally reported in T2D as a result of systemic dysregulation of glucose and FA metabolism. Impaired insulin action in the liver and peripheral tissues results in increased hepatic glucose output and decreased glucose uptake respectively, driving enhanced blood glucose levels (hyperglycemia). Similarly, there are increases in systemic FA delivery to the heart (hyperlipidemia), either as non-esterified fatty acids (NEFAs) bound to albumin or as VLDL/ CM-TGs, due to impaired insulin signalling in liver and adipose tissue. Interestingly, circulating FA concentrations have been shown to be increased to a greater extent compared with glucose levels in T2D⁴⁵. Therefore, in diabetes there are chronic changes in supply of substrates to the heart which have been shown to have profound effects on cardiac metabolism.

Cardiac metabolism is altered in T2D, as there is a shift towards excessive dependence by the heart on FA metabolism to meet its high energy needs, as illustrated in Figure 1.4. The increased rate of FA utilisation has been shown to occur with the degree of insulin resistance, as a seminal report in humans demonstrated

increasing rates of FA utilization were associated with the transition from obesity to T2D⁴⁵. In various rodent models of T2D, increases in cardiac FAO rates have been observed^{46,47} and this finding has been confirmed in T2D patients whereby positron emission tomography showed increased levels of overall fat utilization and FAO⁴⁸. Furthermore, reports in rodents have demonstrated that worsening myocardial FA metabolism precedes the decrease in myocardial glucose uptake^{49,50}, suggesting that the increase in FA uptake potentially drives changes in cardiac substrate fuel selection in the development of T2D.

In addition to the increase in FAO rates observed in T2D, there is an increase in systemic lipid accumulation, including an increase in myocardial TG concentrations, which is observed in humans^{51,52} and rodent models of T2D⁵³. Furthermore, myocardial TG concentrations have been shown to negatively correlate with diastolic function in patients⁵¹. As such, in T2D, the heart is overloaded with lipids⁵⁴, which drives enhanced activation of the transcription factor peroxisome proliferator-activated receptor alpha (PPAR α), which in turn increases target genes involved in FA uptake, metabolism and storage^{55,56}. Therefore, increases in FA uptake in T2D promote the activation of lipid-sensitive transcription factors, which further enhances the transcription of FA metabolism proteins and creates a vicious cycle with upregulated FA metabolism and suppressed glucose use. The increase in cardiac lipid storage has also been implicated in generating toxic lipid intermediates (i.e. ceramides, diacylglycerols) which are considered lipotoxic for the heart and have been associated with impaired insulin signalling.

The increase in FA oxidation and storage has been attributed to increased uptake across the sarcolemma. Enhanced FA uptake capacity in T2D has been shown to be associated with increases in CD36 sarcolemma content and decreases in GLUT4 sarcolemma content in rodent models^{57,58}. In humans, CD36 has been demonstrated to be permanently relocated to the sarcolemma in obese and T2D patients, resulting

in increased FA uptake⁵⁹. Interestingly, more CD36 is localised on the sarcolemma in T2D patients compared with obese patients, suggesting CD36 plays an important role in disease progression⁵⁹. Therefore, it appears that there is dysfunctional CD36 trafficking in T2D. Thus, understanding the mechanisms that underpin abnormal CD36 and GLUT4 trafficking in the diabetic heart is of great interest.

Furthermore, the increased in FA metabolism in T2D is accompanied by a decrease in glucose metabolism as illustrated in Figure 1.4. In diabetes, glycolysis is decreased, mediated by allosteric and transcriptional repression. Enhanced long chain fatty acyl coenzyme A (LCFA-CoA) concentrations are increased in diabetes, causing allosteric inhibition of hexokinase (HK), reducing the phosphorylation of glucose to glucose 6- phosphate (G-6-P)⁶⁰. Additionally, increased FA metabolism leads to enhanced Krebs cycle-derived citrate accumulation, which once exported from the mitochondria to the cytosol, results in increased phosphofructokinase (PFK) inhibition. In conjunction with reduced glycolytic flux, glucose oxidation is impaired in diabetes. It has been well established pyruvate dehydrogenase (PDH) activity is suppressed in diabetes. The decrease in PDH activity, which catalyses the oxidative decarboxylation of pyruvate to acetyl-CoA, is largely driven by the increased generation of FA derived acetyl-CoA and NADH, activating pyruvate dehydrogenase kinase 4 (PDK4). Once activated, PDK4 phosphorylates and inactivates PDH, suppressing glucose oxidation. The decrease in glucose utilisation also results in insufficient glycolysis-derived acetyl-CoA, which forms malonyl-CoA by acetyl-CoA carboxylase (ACC), and there is minimal inhibition of CPT1, thus further driving uncontrolled FAO rates at the expense of glucose oxidation.

Impaired glucose uptake across the sarcolemma also plays a central role in the overall decrease in glucose utilization in diabetes. As demonstrated in Figure 1.4, the occurrence of lipid-induced insulin resistance further down-regulates glucose metabolism by blunting GLUT4 trafficking to the sarcolemma. Elevated FA

storage increases myocardial diacylglycerol (DAG) concentrations, which activate protein kinase C theta (PCK θ). PCK θ activation results in the inhibitory serine phosphorylation of the insulin receptor and its adaptor protein insulin receptor 1/2 (IRS-1/2), blunting the tyrosine phosphorylation that is required for the insulin signalling cascade and subsequent GLUT4 trafficking to the sarcolemma⁶¹. Furthermore, consistent findings in rodent models of T2D show that GLUT4 expression is decreased in the heart, further driving the decline of glucose utilization in the heart^{62,63}.

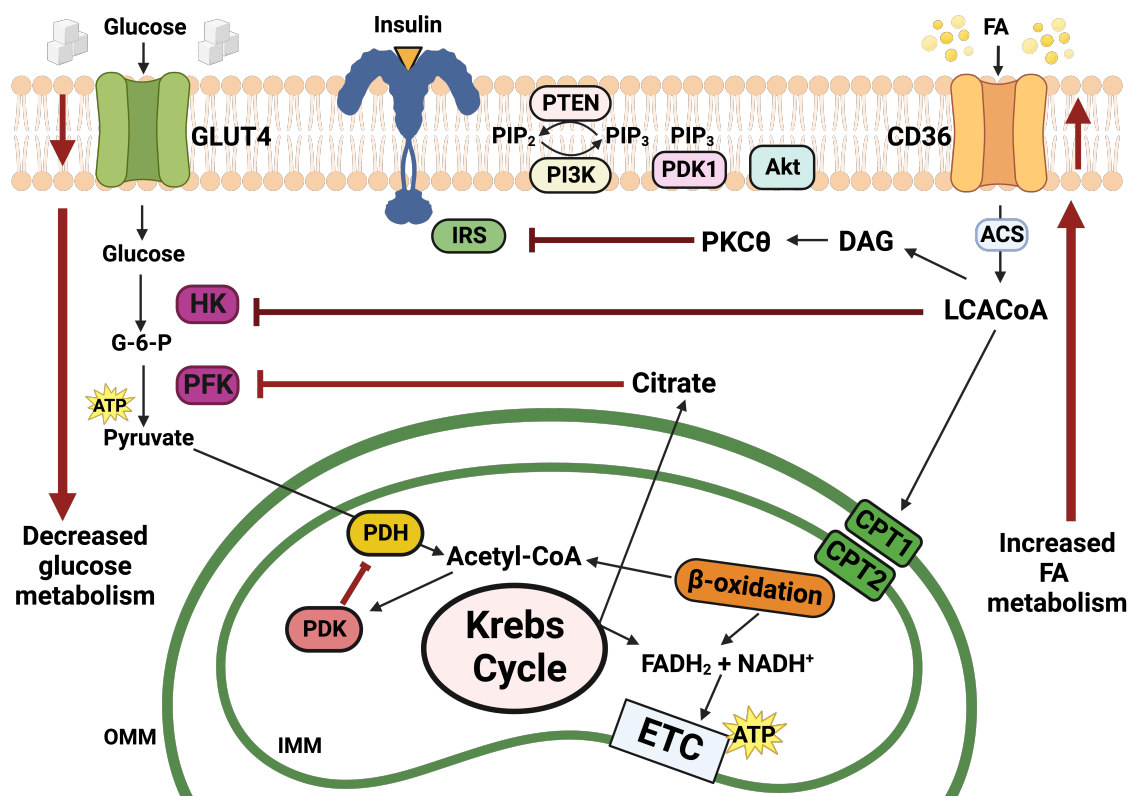


Figure 1.4: Substrate metabolism in Type 2 diabetes. Increased levels of sarcolemmal CD36 drive increased fatty uptake, while decrease levels of GLUT4 result in reduced glucose uptake. Fatty acid oxidation rates are increased, whereas glycolysis and glucose oxidation are decreased. Figure modified from Merewether and colleagues⁶⁴.

Lastly, the dominance of FA utilisation and decreased glucose utilisation in diabetes increases baseline oxygen consumption and mitochondrial uncoupling⁶⁵, and is associated with impaired myocardial energetics. Cardiac energetic dysfunction is observed in patients with T2D, presenting with decreased phosphocreatine (PCr)/ATP ratios⁶⁶. Research in rodent models has shown that elevated levels of cardiac FA metabolism in T2D increase the oxygen requirements of both basal metabolism and excitation-contraction coupling^{67,68}, leading to reduced cardiac efficiency and contractile dysfunction. Both *in silico* modelling⁶⁹ of oxidative phosphorylation coupled with creatine kinase and isolated mitochondria from T2D rodents^{65,70,71} suggest that impaired cardiac energetics in T2D occurs as a result of decreased ATP synthesis and impaired activity of mitochondrial enzymes given the chronic enhanced oxygen demand of FA metabolism. Therefore, alterations in cardiac FA and glucose metabolism as observed in diabetes can have catastrophic consequences on contractile function.

The underlying processes involved in the metabolic shift in T2D has historically been thought to occur largely as a result of Randle cycle mediated mechanisms⁶⁰. However, changes in post-translational modifications (PTMs) of membrane substrate transporters has been proposed as an additional mechanism in the heart. As such, CD36 and GLUT4 have been shown to undergo PTMs. Recently, a lipid PTM, *S*-acylation, which regulates membrane trafficking of various proteins has sparked interest in the field of metabolism. Both CD36⁷² and GLUT4⁷³ have been shown to contain *S*-acylation sites and *S*-acylation has been shown to regulate their trafficking in other tissues.

1.7 Protein *S*-acylation

S-acylation poses a novel mechanism potentially regulating cardiac sarcolemmal substrate transporter levels and metabolism in diabetes. Lipid modifications are one of the most common types of PTMs as roughly 25-40% of the proteome has been found to undergo lipid modifications of some sort⁷⁴. The first published report of the incorporation of lipids into proteins in the brain occurred in 1951⁷⁵. However, the function of this lipid modification wasn't known until a seminal report in the 1990s demonstrated that the observed lipid incorporation into proteins critically influenced trafficking through the secretory pathway⁷⁶. Protein lipidation involves the covalent attachment of lipids, including cholesterol, isoprenoids, phospholipids and fatty acids to target proteins. Of these, the incorporation of fatty acids (usually eight to twenty hydrocarbons (C) in length) to an amino acid residue or *N*-terminal groups of a protein, termed fatty acid acylation, has gained interest due to the high prevalence of dysregulated fatty acid acylation in various diseases. Fatty acid acylation modifications are characterized based on the reactive group being modified; *S*- modifications occur on the thiol of a cysteine (thioester bonds), *N*-modifications occur on an amine group of a lysine, or a protein *N*-terminus (amide bonds) and *O*- modifications on serine and threonine residues (ester bonds)^{77,78}. Fatty acid acylation modifications are further classified by the fatty acid carbon chain length. The main FAs involved in protein acylation are myristoyl (C14:0, e.g. *N*-Myristoylation on the end terminal glycine residue) and palmitoyl (C16:0, e.g. *N*-Palmitoylation and *S*-palmitoylation)^{77,78}.

Compared with the other fatty acid acylation modifications which are irreversible and stable, *S*-palmitoylation is unique as it is the only readily reversible and dynamic lipid PTM as outlined in Figure 1.5. *S*-palmitoylation involves the covalent addition of a long chain FA to a cysteine thiol through a thioester bond^{79,80} and has been shown to target 10% of the proteome^{78,81}. The attachment of palmitate (C16:0)

is the most common FA present on S-palmitoylated proteins, however it has been shown that other long chain fatty acid moieties such as palmitoleate (C16:1), oleate (C18:1) and stearate (C18:0) can also be used in this modification^{82–84}. Given this, there has been a recent shift in the field away from the term to S-palmitoylation to *S*-acylation. Therefore, the term *S*-acylation will be used in this thesis. Due to the labile nature of thioester bonds, *S*-acylated proteins can undergo cycles of *S*-acylation and de-*S*-acylation with timescales ranging from seconds to hours^{85–87}. As such, protein *S*-acylation has been well characterized to regulate numerous cellular processes by mediating protein-membrane affinity by enhancing hydrophobicity, regulating protein activity, stability and protein-protein interactions, as well as influencing other PTMs^{78,88}.

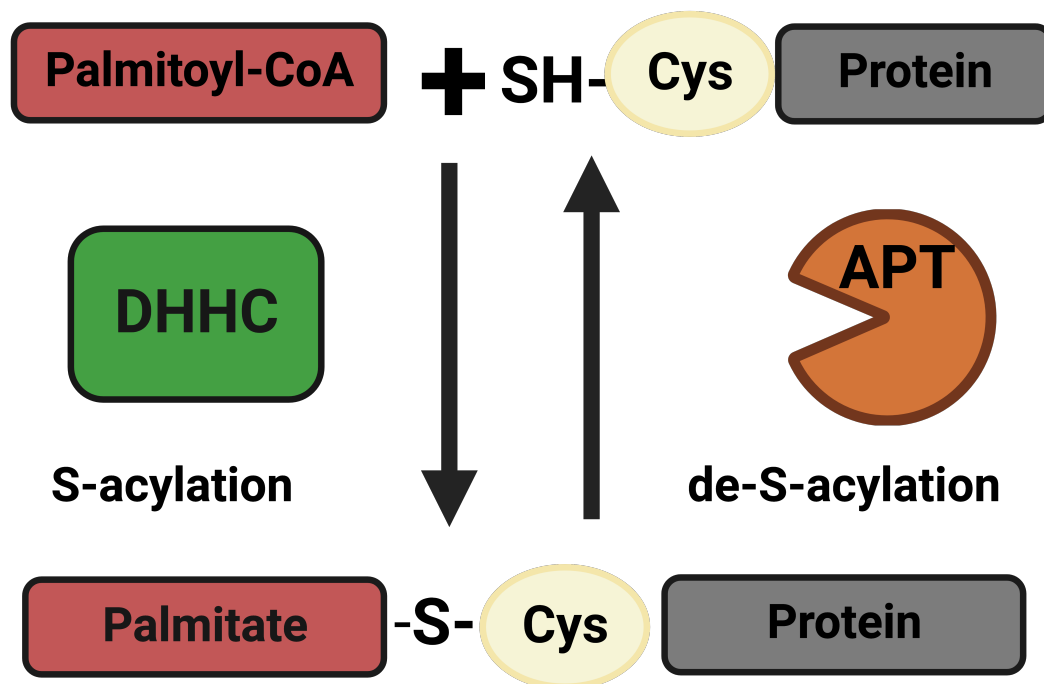


Figure 1.5: *S*-acylation is a reversible lipid PTM whereby a palmitoyl group is covalently linked to a cysteine thiol through a thioester bond on target proteins. Dynamic *S*-acylation cycles are regulated by DHHCs and APTs.

Emerging evidence highlights that *S*-acylation may have a regulatory role in governing cellular metabolism in various tissues. Plasma palmitate is imported into the cell across the plasma membrane, and once in the cytosol, a thioester bond formation is catalyzed by acyl-CoA synthetase (ACS) forming palmitoyl-CoA. In adipose and liver, palmitoyl-CoA can also be synthesized endogenously from *de novo* lipogenesis from non-fatty acid precursors by fatty acid synthase (FASN). In addition to being a source of energy, palmitoyl-CoA is a biologically active signalling molecule that can regulate many physiological processes⁸⁹. More specifically, through the attachment of palmitoyl-CoA derived from exogenous FA uptake or from *de novo* FA synthesis, *S*-acylation has been linked to substrate uptake into the cell by regulating membrane trafficking of transport proteins^{72,90}. More recently, palmitoyl-CoA has been implicated in regulating various mitochondrial metabolic processes. This is of particular interest given that 95% of the total cellular CoA pool is located within the mitochondria⁹¹. Therefore, *S*-acylation potentially sits at a key cross road linking supply of palmitoyl-CoA to the regulation of substrate uptake and onward metabolism in the mitochondria.

1.8 DHHC- palmitoyl acyltransferases (PATs)

S-acylation was initially investigated in the 1990s under the school of thought that proteins were solely auto-*S*-acylated⁹². It wasn't until a seminal study in 2002 employing genetic screening in *Saccharomyces cerevisiae* (yeast) identified a Zinc (Zn^{2+})-finger containing enzyme, denoted ERF2, that *S*-acylated Ras2 and influenced its subcellular localisation⁹³. Others were later identified in yeast as multidomain membrane spanning proteins characterized by a conserved 55 amino acid region containing an aspartate-histidine-histidine-cysteine (DHHC) motif in the enzyme catalytic domain^{80,94} that was identified as being crucial to enzymatic function⁹⁵. These enzymes were then reclassified as DHHC-palmitoyl acyltrans-

ferases (DHHC-PATs), and 23 isoforms of DHHCs have been identified in humans⁸⁸. Recently, the first high resolution structures of two DHHCs, DHHC15 (Zebrafish) and DHHC20 (Human), were determined using X-ray crystallography⁹⁵. DHHCs have four to six transmembrane domains and the conserved DHHC cysteine-rich catalytic domain is located on the cytosolic face (Figure 1.6). Furthermore, DHHCs have been shown to S-acylate proteins across 12% of the human proteome^{81,88,95}.

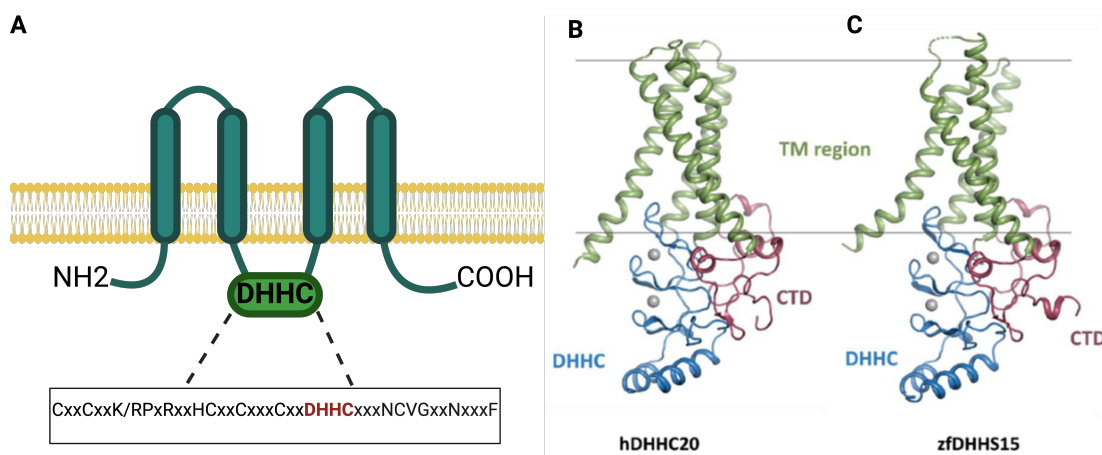


Figure 1.6: Structure of the DHHC family of enzymes (A). Figure modified from Stix and colleagues⁹⁶ (A). Cartoon representation of the crystal DHHC20 (B) and DHHC15 (C) structures taken from Rana and colleagues⁹⁵. The four transmembrane helices are shown in green and the c-terminal domain is in brown (B-C)⁹⁵. The zn^{2+} ions are represented by the two spheres and an extrac helix located in the C-terminal domain is coloured orange (B-C)⁹⁵.

It has been well established that the catalytic activity of DHHC is a two-step process involving palmitoyl-CoA^{98–100} as seen in Figure 1.7. In the first step, DHHC cysteines become auto-*S*-acylated and the long chain acyl-CoA forms an acyl-enzyme

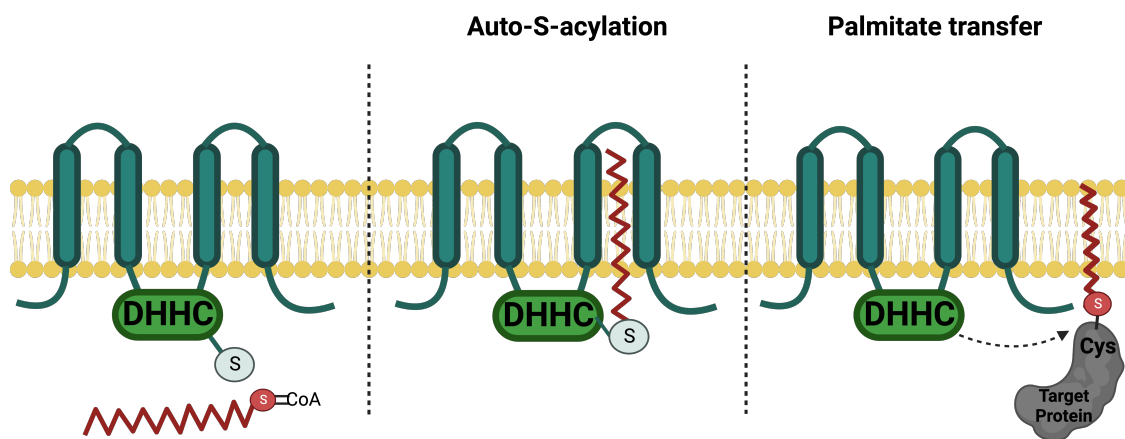


Figure 1.7: Two step ping pong process of DHHC mediated *S*-acylation: auto-*S*-acylation of a conserved cysteine in the DHHC motif by an acyl-CoA with the release of CoA-SH, followed by an *S*-acyl transfer to a target substrate protein cysteine proximal to the DHHC catalytic site. Figure modified from Ocasio and Colleagues⁹⁷

intermediate. The fatty-acyl group is then transferred from the DHHC to the target substrate, resulting in protein *S*-acylation. Interestingly, DHHCs display different auto-*S*-acylating capabilities, catalytic efficiencies¹⁰¹ and display differing acyl-CoA specificities⁹⁹. Another important feature of DHHC is their subcellular locations and tissue specificity. Through studies in HEK293 cells expressing GFP-tagged versions of each DHHC, it was found that DHHCs are located throughout the secretory pathway including the Golgi (e.g. DHHC4, DHHC8), endoplasmic reticulum (e.g. DHHC1, DHHC12) and sarcolemma (e.g. DHHC5, DHHC20), while some can be localised in multiple locations^{74,94,102}. Consequently, the differing subcellular locations of DHHCs have been proposed to allow them to regulate both varying, yet complimentary functions. As such, synergistic efforts between DHHCs in different endomembrane compartments has been implicated in mediating substrate membrane

transporter trafficking to the membrane through the secretory pathway^{72,103}.

While DHHC localisation has been well characterized in endomembrane compartments, there is less known surrounding the presence of DHHCs within or near the mitochondria. Early work employing a bio-orthogonal azido-palmitate identified *S*-acylated substrates within the mitochondria matrix and the mechanism-of-action attributed to *S*-acylation in this organelle was through non-enzymatic processes^{104,105}. As mentioned above, the concentration of total cellular palmitoyl-CoA is highest in the mitochondria and therefore represents a physiological setting where there are high levels of substrate availability. However, DHHC8¹⁰⁶ and DHHC13^{107,108} have been implicated in mediating mitochondrial processes. Although these findings suggest that DHHCs modulate mitochondrial metabolic processes, whether they are localised within the mitochondria remains unclear. Additionally, a recent report has identified DHHC18 within the mitochondria and shown to be the DHHC responsible for malate dehydrogenase (MDH2) *S*-acylation¹⁰⁹.

1.9 Protein acylthioesterases

As *S*-acylation is a reversible process, cleavage of the thioester bond from target proteins is imperative for maintaining the dynamic nature of this lipid PTM. De-*S*-acylation can occur either through slow hydrolysis of the thioester bond between the palmitoyl and cysteine, or through enzymatically driven processes by soluble acyl thioesterases.

The first of these enzymes that were identified are the lysosomal palmitoyl-protein thioesterases (PPTs), which were demonstrated to remove palmitate from [³H] palmitate-labelled H-Ras in the cytosol of bovine brain tissue¹¹⁰. Molecular cloning demonstrated that PPTs contain a S-H-D catalytic triad and a G-X-S-X-G motif, which are common characteristics of lipases¹¹¹ and further work revealed that

PPTs form an α/β -hydrolase fold whereby a hydrophobic pocket accommodates the FA chain and Ser115 acts as the nucleophile residue mediating their de-S-acylating activity¹¹². Although two isoforms have been identified (PPT1, PPT2)¹¹³, only PPT1 has been shown to hydrolyse cysteine thioesters and its ablation has been associated with toxic accumulation of lipidated proteins in neurodegenerative disorders^{113,114}. Interestingly, although originally discovered in the cytosol, further work uncovered that PPTs function mostly in the lysosome to remove FA from proteins prior to their breakdown^{115,116} and therefore were thought to not be the main enzymes responsible for driving protein de-S-acylation¹¹⁷.

In 1998, Duncan and Gilman identified a second class of protein thioesterases, termed Acyl Protein Thioesterases (APTs)¹¹⁸. It was found that the cytosolic localised APT1 was the enzyme responsible for G α de-S-acylation⁹². Interestingly, APT1 was renamed from lysophospholipase I (LYPLA1) as it was previously thought to be a lysophospholipase, however it was found to contain higher enzymatic activity towards acyl thioester-containing proteins over lysophospholipase substrates^{92,119}.

Similar to the PPTs, APT1 is a highly conserved α/β hydrolase that contains both the S-H-D catalytic triad and a G-X-S-X-G motif^{111,112,117} and was found to be highly expressed in the cytosol in yeast and mammalian cells^{112,117,119,120}. Not long after the discovery of APT1, a bioinformatic analysis revealed APT2 (LYPLA2), a homolog sharing 68% sequence similarity with APT1 had a conserved catalytic triad¹²¹. Both enzymes are cytoplasmic, although they can localise to the Golgi apparatus¹²², sarcolemma^{123,124} and more recently were identified in mitochondria^{125,126}. Using a novel mitochondrial targeted de-S-acylation probe (mitoDPPs) in live cells, the de-S-acylating activity of APT1, but not APT2 was observed in mitochondria and was shown to be altered in response to lipid stress, revealing a potential regulatory mechanism of APTs in mitochondrial lipidation¹²⁷. However, the functional relevance of APT1 activity in the mitochondria is unknown.

ATPs have been shown to be *S*-acylated on cysteine (Cys2) near their N-termini^{122,124} and it was found that un-*S*-acylated APTs interact with *S*-acylated proteins in the cytosol, whereas the *S*-acylated APTs are sequestered in the Golgi¹²². Furthermore, in addition to de-*S*-acylating itself, APT1 de-*S*-acylates APT2 to promote dynamic *S*-acylation of both enzymes¹²⁴. While little is known regarding how de-*S*-acylating enzymes including the APTs interact with target proteins, the consensus is that to facilitate de-*S*-acylation, APTs need to come in proximity with the membrane¹²⁸. Recently an interesting three step mechanism-of-action for APT2 binding to the lipid membrane has been uncovered¹²⁸. Long-range electrostatic interactions attract APT2 to the membrane which allows the β -tongue of APT2 to dip into the membrane which holds it in place and becomes stable in the membrane once *S*-acylated by a DHHC¹²⁸. Once APT2 identifies a target protein, it extracts the palmitate from the membrane into its catalytic hydrophobic pocket for hydrolysis¹²⁸ as outlined in Figure 1.8. Unlike DHHCs, substrate recruitment and target selectively by APTs is less known. However, it appears that both enzymes de-*S*-acylate a large number of proteins while showing some selectivity¹²⁹, while some proteins have been shown to not be de-*S*-acylated by PPTs or APTs, indicating that more thioesterases might exist.

As such, a study of the neuronal synaptic protein PSD-95 and N-Ras (which are not regulated by APTs) identified a new class of thioesterases; a family of 19 α/β hydrolase domain (ABHD) containing enzymes that are similar to the PPTs and ATPs and contain a α/β hydrolase fold with a nucleophilic residue mediating their activity¹³⁰. As such, three cytosolic ABHD17 enzymes ($\approx 70\%$ sequence homology) were implicated in mediating N-Ras de-*S*-acylation¹³⁰. Furthermore, ABHD enzymes may localise to other cellular compartments as ABHD10 was discovered to de-*S*-acylate the antioxidant protein peroxiredoxin-5 in the mitochondria, affecting redox homeostasis¹³¹. Interesting, ABHD proteins were found to have

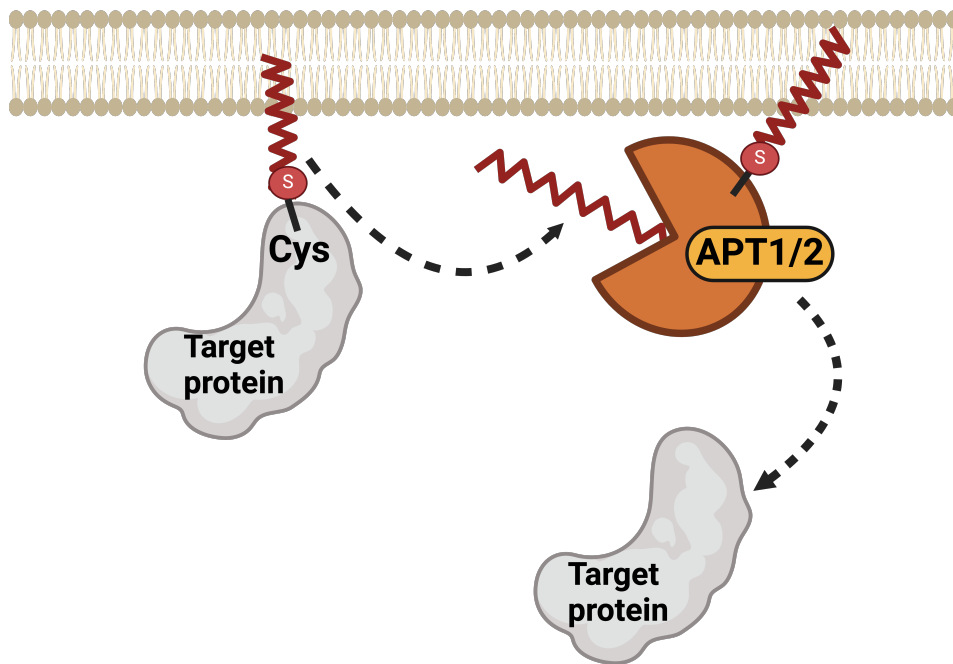


Figure 1.8: APT1 and APT2 mediate the de-*S*-acylating process by extracting palmitate from the membrane into its catalytic hydrophobic pocket for hydrolysis.

acyltransferase motifs in addition to their de-*S*-acylating activity, however it remains unknown whether these enzymes can *S*-acylate target proteins¹³².

S-acylation has been shown to regulate membrane protein trafficking as a result of the dynamic interplay between the DHHCs and APTs as outlined in Figure 1.9. Furthermore, upregulation and downregulation of *S*-acylation has been implicated in promoting pathological conditions including cancer, neuronal defects and inflammatory diseases^{77,88}. Therefore, unpicking whether the cardiac membrane substrate transporters undergo *S*-acylation will provide further insight into the regulatory mechanisms driving cardiac substrate uptake and metabolism. Furthermore, determining if *S*-acylation is altered in diabetes could provide novel therapeutic targets to improve dysregulated cardiac metabolism.

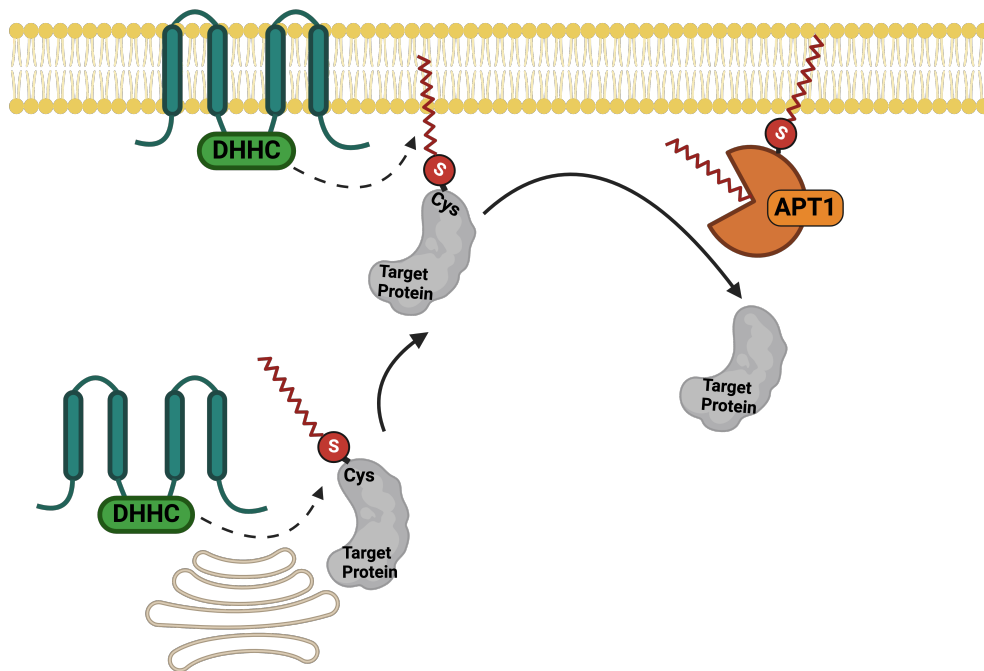


Figure 1.9: *S*-acylation mediates membrane protein cycling through the interplay between the DHHCs and APTs

1.10 Thesis aims

The work described in this thesis intends to characterize CD36 *S*-acylation in a rodent model of T2D and the mechanisms regulating CD36 *S*-acylation. It will further examine the metabolic consequences of pharmacologically altering CD36 *S*-acylation levels by targeting the *S*-acylating and de-*S*-acylating enzymes. The last chapter will investigate CD36 subcellular localisation in human heart failure biopsies and investigate the *S*-acylation status of key metabolic proteins in rat hearts subjected to low-flow ischemia. This will be achieved through four chapters, focusing on:

- 1: Utilizing purification affinity assays to measure the *S*-acylation status of CD36 in rat cardiac tissue and characterizing the expression of the *S*-acylating and de-*S*-acylating enzymes.
- 2: Investigating CD36 *S*-acylation levels and identifying the mechanism driving hyper *S*-acylation of CD36 in diabetes.
- 3: Interrogating whether pharmacological manipulation of *S*-acylation can regulate cardiac metabolism and function.
- 4: Measuring sarcolemmal levels of CD36 and GLUT4 in human heart failure biopsies, and investigate the *S*-acylation status of key metabolic proteins in rat hearts subjected to ischemia.

2

Methods

2.1 Acyl-Resin Assisted Capture (acyl-RAC)

Purification of *S*-acylated proteins using acyl-RAC was carried out by adapting the protocol from Wypijewski and colleagues¹³³ as outlined in Figure 2.1 and contains three broad steps.

2.1.1 Blocking of free thiol groups

Snap frozen rat cardiac tissue (10 mg) was lysed in Blocking Buffer (100 mM HEPES, 1.0 mM EDTA, 2.5% SDS, 1% *N*-ethyl maleimide (NEM) (w/v), pH 7.5) and free cysteines were alkylated by the addition of 1% NEM. Following an incubation for 4 hr at 40°C, excess unreacted NEM was removed by an acetone precipitation (100% acetone) and protein pellets were then washed 5 times with 70% acetone, then left suspended in acetone overnight. Samples were dried at 40°C and then resolubilized in Binding Buffer (100 mM HEPES, 1.0 mM EDTA, 1% SDS, pH 7.5).

2.1.2 Selective cleavage and capture

S-acylated proteins were either captured on pre-equilibrated thiopropyl-sepharose beads (GE Life Sciences) with 200 mM hydroxylamine (HA)(pH 7.4) to cleave the labile palmitate-modified cysteine thioester bond (CBF), or subjected to an identical reaction where HA was replaced with 200 mM NaCl to preserve this bond (PBF) and serve as a negative control. Both HA and NaCl treated samples were then incubated at room temperature for 2.5 hrs.

2.1.3 Elution

Following the incubation, the CBF (HA treated) and PBF (NaCl treated) samples were centrifuged at 15,000g for 1 min and 50 μ L of the supernatant retained representing the cleaved and preserved unbound fractions (CUF and PUF, respectively).

The remaining CBF and PBF resins were then extensively washed (minimum of 5 times) in 1 ml Binding Buffer. Lastly, the captured proteins were eluted in SDS-PAGE loading buffer (30% glycerol, pH 6.8, 0.01% Brilliant blue) supplemented with 100 mM DTT. Equivalent amounts of Laemmli were added to the remaining fractions and all fractions heated for 10 min at 60°C to break tertiary structure prior to resolution by SDS-PAGE.

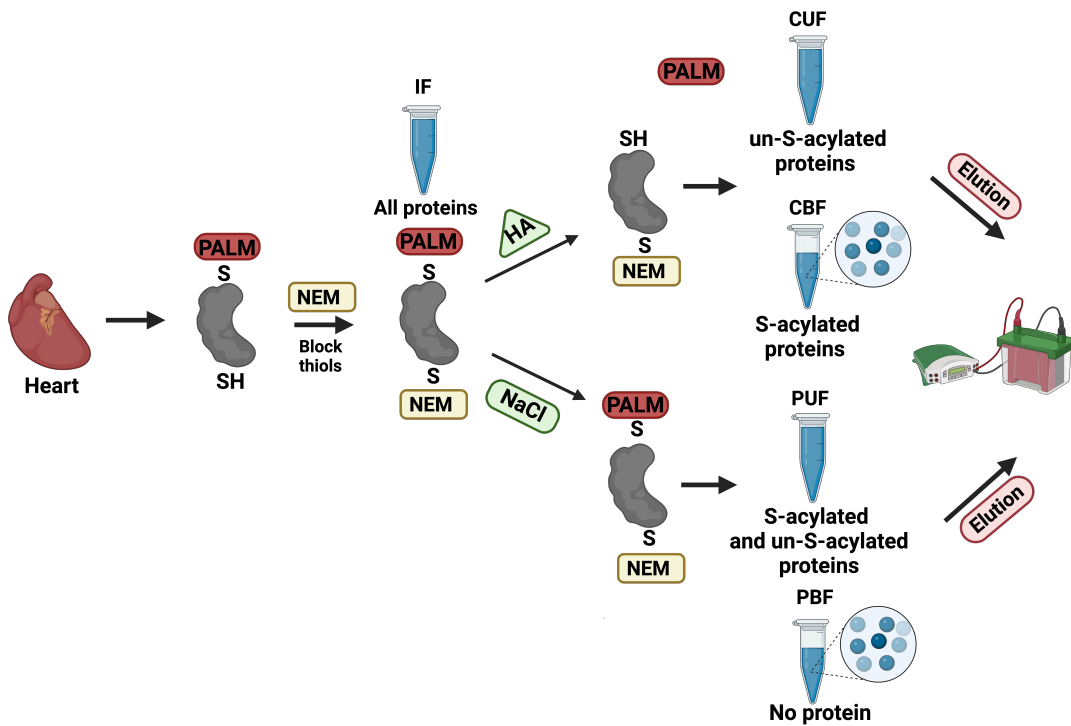


Figure 2.1: Schematic overview of the acyl-RAC assay which produces 5 fractions. Input fraction (IF), Cleaved Unbound Fraction (CUF, un-*S*-acylated proteins), Cleaved Bound Fraction (CBF, *S*-acylated proteins), Preserved Unbound Fraction (PUF, un-*S*-acylated and *S*-acylated proteins) Preserved Bound Fraction (PBF, negative control with no protein).

2.2 Acyl-PEG Exchange

Acyl-PEG exchange was conducted by modifying the protocol from Howie and colleagues¹³⁴ as outlined in Figure 2.2. Similar to acyl-RAC, snap frozen cardiac

rat tissue (10 mg) was incubated with Blocking Buffer for 4 hr at 40°C. Excess unreacted NEM was removed by acetone precipitation (100% acetone) and protein pellets were washed extensively 5 times in 70% acetone, then left suspended in acetone overnight. Samples were dried at 40°C and then resolubilized in Binding Buffer. Using pre-equilibrated Zeba spin desalting columns (Sigma), *S*-acylated proteins were PEGylated using 2 mM 5 kDa PEG maleimide (Sigma) in the presence of 200 mM HA (pH 7.4), or 200 mM NaCl as the negative control, for 2 hr at 37°C. The samples were then heated for 10 min at 60°C in SDS-PAGE loading buffer supplemented with 100 mM DTT.

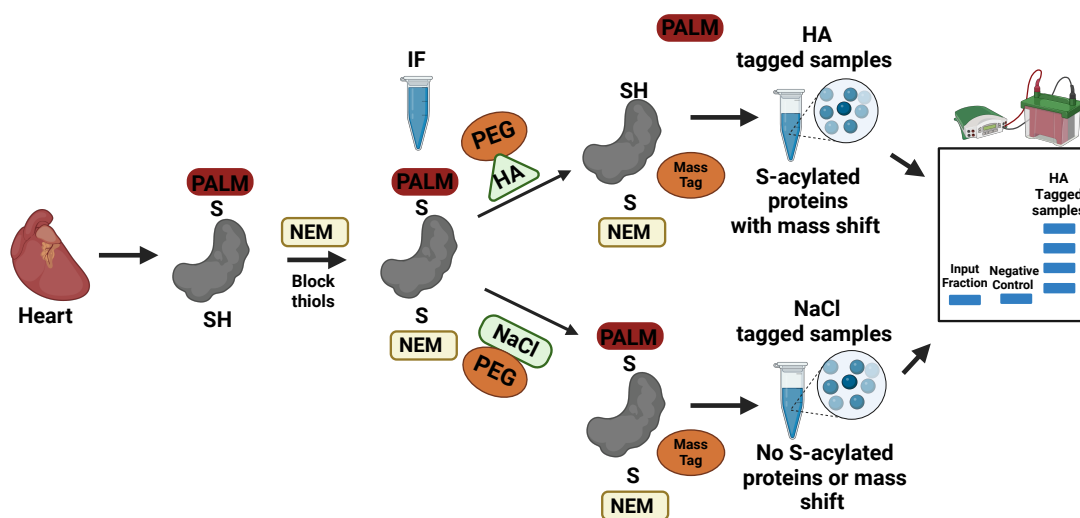


Figure 2.2: Schematic overview of the acyl-PEG exchange assay which generates 3 fractions. The IF total protein fraction, the negative control NaCl PEG treated fraction and the *S*-acylated PEG treated fraction whereby a molecular mass shift occurs reflecting *S*-acylated cysteines.

2.3 Type 2 diabetic rat model

Male Wistar rats 250 g were purchased from Envigo (UK). All investigations conformed to UK Home Office guidelines under The Animal (Scientific Procedures) Act, 1986. Rodents were housed in a dedicated facility (Biomedical Sciences Services, University of Oxford, UK) under a 12 h light-dark cycle and maintained on standard laboratory chow (Harlan Laboratories, Kent, UK) and standard laboratory water, unless specified otherwise. Upon arrival, animals were randomly assigned into the T2D or control cohorts as illustrated in Figure 2.3. The T2D rats were fed a high fat diet (60% kcal from fat, 35% kcal from protein and 5% kcal from carbohydrate, with Atwater fuel energy of 5.3 kcal/g, from Special Diet Services). Control rats were fed chow (with Atwater fuel energy of 3 kcal/g, from Harlan laboratories). Both control and T2D cohorts were fed *ab libitum*. Following 14 days on either diet, rats were subjected to an overnight fast and while control animals were given an intraperitoneal injection of citrate buffer (30 mM citric acid and 20 mM sodium citrate, pH 4), T2D rats were given a low-dose of the β -cell toxin, streptozotocin (STZ) (25mg/kg) (Sigma, UK), dissolved in citrate buffer. STZ is an alkylating agent which has high selectivity for GLUT2 transporters, inducing DNA damage and cytotoxicity to pancreatic β -cells. Following the injections of either citrate or STZ, the animals were maintained on their respective diets for another 5 weeks. This protocol has been previously shown in our group to result in a type 2 diabetes rat model¹³⁵.

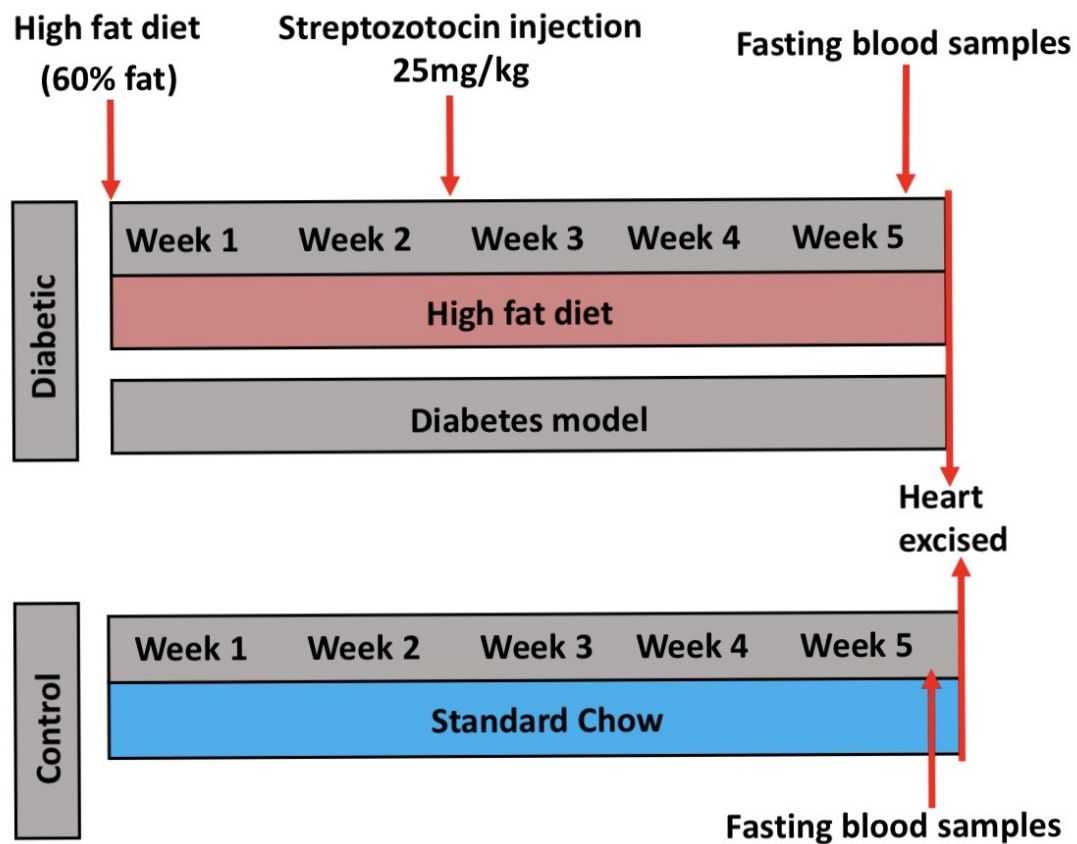


Figure 2.3: T2D rat model timeline.

2.4 *db/db* Mouse Model

Heart tissue from *db/db* and c57BL/6J (control) mice were donated by A/Prof. Dunja Aksentijevic (Queen Mary University of London)

2.5 T2D Mouse Model

Heart tissue from c57BL/6J (control), T2D, T2D FoxO1 KO and T2D AS1842856 treated mice were donated from Dr.Ussher (University of Alberta).

2.6 Porcine Diabetes Model

Heart tissue from diabetic and control pigs were donated by Prof. Dirk Dunker (Erasmus MC)¹³⁶. Briefly, diabetes was induced in the pigs by intravenous injections of streptozotocin (25mg/kg/day) for 3 days. The pigs were then fed a high fat and high sugar diet (25% saturated fats, 1% cholesterol, 10% sucrose and 15% fructose) for 5 months, while control pigs were fed standard chow.

2.7 Insulin Resistant human induced pluripotent Stem cell derived cardiomyocytes (hiPSC-CMs)

Insulin resistant (IR) hiPSC-CM and control hiPSC-CM were generated by Dr. Ujang Purnama⁵⁸. Briefly, IMR90, a healthy human induced pluripotent stem cell (hiPSC) line was cultured and were differentiated into hiPSC-derived cardiomyocytes (hiPSC-CM) TeSR-E8 media. For two days, differentiation was started with RPMI medium supplemented with 1% B27 minus insulin and 6 $\mu\text{mol/L}$ CHIR99201. On day 3, the media was changed for RPMI containing 2.5 $\mu\text{mol/L}$ Wnt-C59. On days 11 and 13, the media was switched to no-glucose RPMI with 1% B27 without insulin. On day 15, the media was changed to RPMI supplemented with 1% B27 complete supplement. On day 16, cells were incubated for 1 week in maturation media (DMEM medium supplemented with 10% horse serum, 5 $\mu\text{g/mL}$ vitamin B12, 0.5 mM ascorbic acid, 0.82 μM biotin, insulin, 100 μM oleic acid:BSA (2:1), 1x MEM non-essential amino acids, and penicillin-streptomycin).

2.8 Isolated mitochondria

Mitochondria isolation was carried out by Dr. Matt Kerr. Hearts were excised from male Wistar rats under terminal isoflurane anaesthesia. Following heart excision, a modified protocol from Palmer and colleagues¹⁶¹ was used to isolate sub-sarcolemmal (SSM) and interfibrillar mitochondria (IFM). Buffers were kept at 4 ° throughout. Hearts were stored in MIS1 (100 mM KCl, 50 mM MOPS, 5 mM MgSO₄, 1 mM EGTA, 1 mM ATP, pH 7.4) and then homogenized in MIS2 (100 mM KCl, 50 mM MOPS, 5 mM MgSO₄, 1 mM EGTA, 1 mM ATP, pH 7.4, 2 mg/mL BSA, 10 mM nicotinamide, 500 mM trichostatin A). SSM were extracted from the heart by polytron homogenization and Potter Elvehjem homogenisation. IFM were extracted following trypsin digestion (5 mg/gww) and further homogenization. Both SSM and IFM mitochondria were purified through centrifugation at 3020 g before re-suspending in 0.4 mL MIS3 (100 mM KCl, 50 mM MOPS, 0.5 mM EGTA, 10 mM nicotinamide, 500 mM Trichostatin A). Frozen isolated SSM and IFM samples were then solubilised with 5% Triton and protein concentrations determined by Pierce BCA protein assay kit. Mitochondria were re-suspended in KME and then extracted by acyl-RAC.

2.9 Measurement of blood plasma metabolites

Following an overnight fast, saphenous vein blood was taken under isoflurane anaesthesia (1.5%) to measure fasted blood samples. Fed state blood samples were collected from the chest cavity immediately after the heart was excised. The Accu-chek compact plus (Accu-chek, Roche, UK) was used to measure blood glucose. Plasma was separated following centrifugation at 16,000 g for 10 mins at 4°C, and stored at -80°C for subsequent analysis. Insulin and non-esterified fatty acids (NEFA) were measured using insulin ELISA (Mercedes) and NEFA colorimetric

assay kit (Randox, UK), respectively.

2.10 Heart Perfusion

2.10.1 Palmitate stock preparation

Palmitate stock (100 mM) was prepared by dissolving 2.56 g of palmitic acid (Sigma Aldrich,UK) in 2 ml of ethanol (Sigma Aldrich,UK) in a water bath adjusted to 70°C. In a very small amount of water, K₂CO₃ was then dissolved and slowly added to the palmitic acid solution. The precipitate was then fully dried, and the resultant salt was dissolved in 100 ml of saline (0.9% NaCl). Prior to the addition to the Krebs-Henseleit (KH)- bovine serum albumin (BSA) buffer, the palmitate stock was warmed to 70°C to ensure binding of the palmitate to the fatty acid free bovine serum albumin (BSA).

2.10.2 Preparation of Krebs-Henseleit buffer

All perfusion studies were performed in modified KH buffer containing 11 mM glucose and 1.5% (w/v) BSA bound to 0.4 mM palmitate. The KH buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.3 mM CaCl₂, 0.5 mM EDTA, 25 mM NaHCO₃, 1.2 mM KH₂PO₄ and 11mM glucose) was prepared with deionised water (ddH₂O). The buffer was gassed with 95% O₂ and 5% CO₂, and filtered with 0.45 µM filters. The BSA was dissolved in KH buffer and placed in dialysis tubing and submerged in the standard KH buffer overnight to dialyse. The next morning the KH-BSA buffer was heated to 30°C and 0.4 mM palmitate was added to the solution using a heated pipette tip to ensure binding of the palmitate to BSA. In studies where fatty acid oxidation rates were measured, the KH-BSA-palmitate buffer was immediately supplemented with [9,10-³H] palmitate (PerkinElmer), as described previously¹³⁷. The calcium and potassium concentrations were adjusted

to a final concentration of 1.75 mM and 4.7 mM respectively.

2.10.3 Langendorff Heart Perfusion

Rats were anaesthetised using isoflurane. Hearts were rapidly excised and placed in ice cold KH buffer following the cessation of the pedal and corneal reflexes. As illustrated in Figure 2.4 the aorta was cannulated on the perfusion apparatus and perfused with standard KH buffer. The pulmonary artery was cut, and an incision was made into the left ventricle to allow a drain and a clingfilm balloon to be inserted. The buffer was then switched to recirculating radiolabelled KH- BSA-palmitate buffer. The hearts were perfused in Langendorff mode at a constant pressure of 100 mmHg and the balloon inflated for an end diastolic pressure between 4-8 mmHg for up to 1 hr. Buffer was gassed with 95% O₂ and 5% CO₂ and maintained at a constant temperature of 37°C.

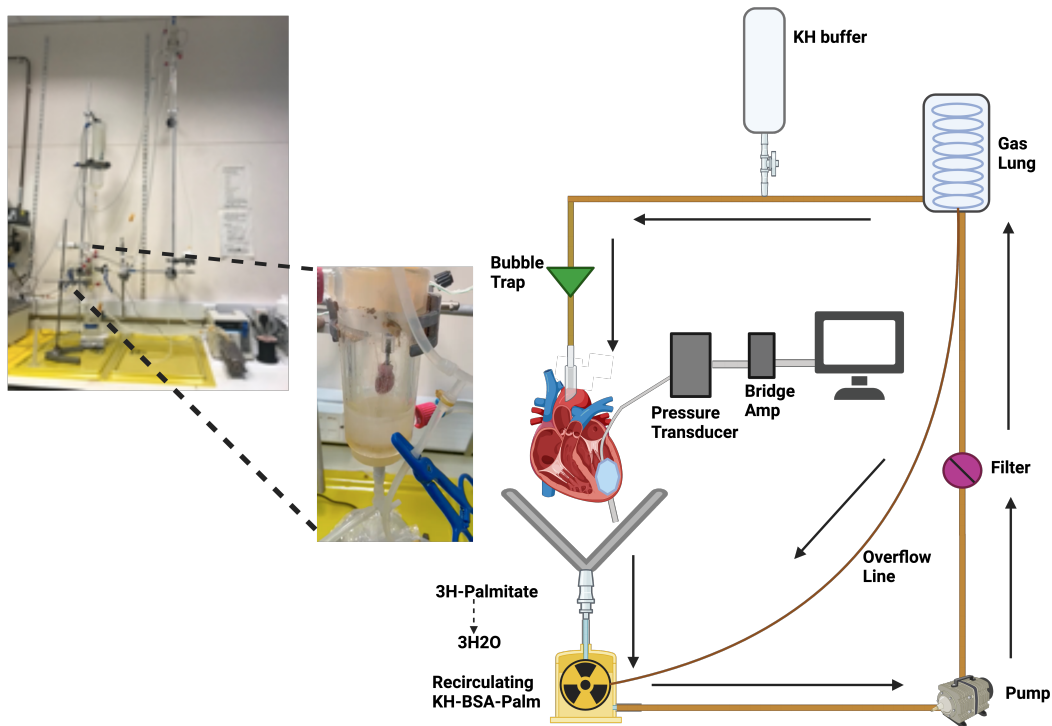


Figure 2.4: Langendorff perfusion apparatus.

In order to record functional data throughout the perfusion protocol as illustrated in Figure 2.5, the fluid-filled clingfilm balloon was attached to a pressure transducer, bridge amplifier, and a PowerLab data acquisition system (ADInstruments, UK). As outlined in Figure 2.5, left ventricular developed pressure (LVDP) was determined as peak systolic minus end-diastolic pressure and the multiple of developed pressure, and heart rate was used to calculate rate pressure product (RPP), which is used as a measure of cardiac function. At the end of the perfusion protocol, which typically lasts between 30 and 60 mins depending on the protocol, hearts were snap frozen in liquid nitrogen for subsequent analysis.

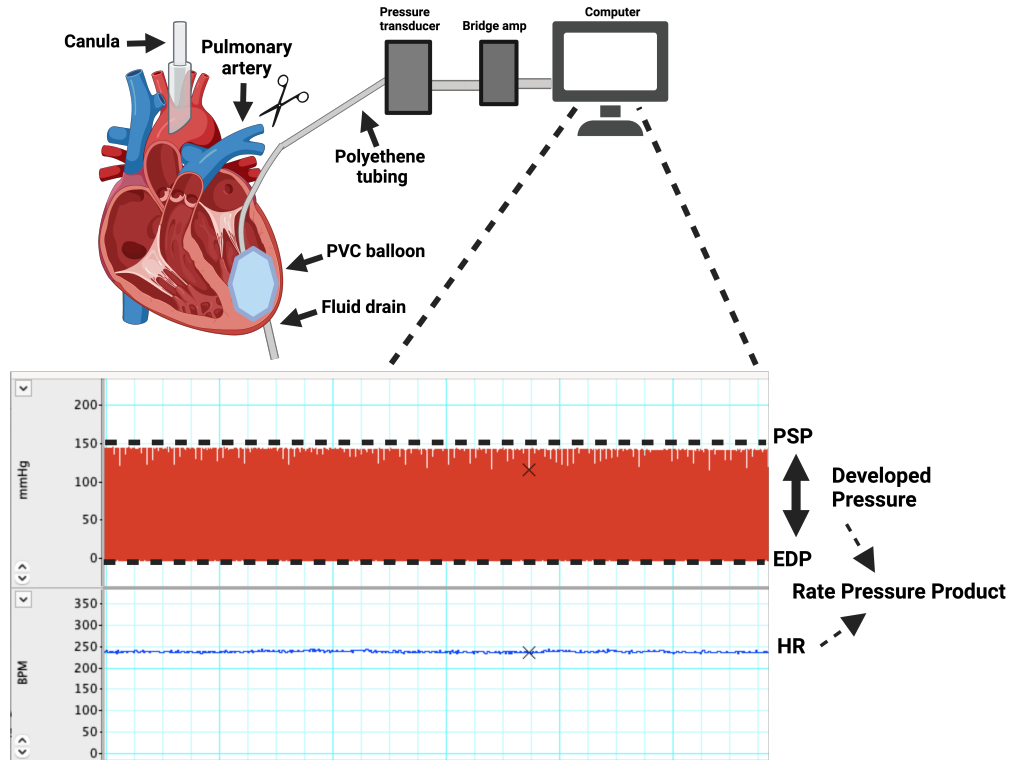


Figure 2.5: Measurement of cardiac function in the Langendorff perfused heart using the PowerLab data acquisition system. The software measures the pressure on the balloon in the left ventricle, and from this, calculates the heart rate. Left ventricular developed pressure is determined as peak systolic (PSP) minus end-diastolic pressure (EDP), and rate pressure product (RPP) is the multiple of developed pressure and heart rate (HR).

2.10.4 Low fat and high fat

Control rat hearts were excised under anesthesia and randomly assigned to be perfused in Langendorff mode for 60 mins with KH buffer containing 11 mM glucose and either 0.2 mM (low fat) or 1.2 mM palmitate (high fat)¹³⁸ supplemented with 0.2 μ Ci/ml [9,10-³H] palmitate as described. Functional data was averaged over the first and last 10 mins.

2.10.5 *N*-cyanomethyl-*N*-myracrylamine (CMA)

CMA was synthesized in Dr. Bryan Dickinson's lab at the University of Chicago by Dr. Ton Lan and Dr. Saara-Anne Azizi¹³⁹. Hearts from T2D rats were excised under anesthesia and perfused in Langendorff mode for 70 mins with KH buffer containing 11 mM glucose and 0.4 mM palmitate supplemented with 0.2 μ Ci/ml [9,10-³H] palmitate. Diabetic hearts were perfused initially for a period of 10 mins to ensure functional stability, then the CMA (5.72 mg dissolved in 100 ml of DMSO) was added to half the hearts (100 μ M in 175ml re-circulating perfusate) for a further 60 mins in the recirculating perfusate. Functional data was averaged over the first and last 10 mins

2.10.6 ML348

Hearts from healthy control rats were excised under anesthesia and perfused for 70 mins in Langendorff mode with KH buffer containing 11 mM glucose and 0.4 mM palmitate supplemented with 0.2 μ Ci/ml [9,10-³H] palmitate. Control rat hearts were perfused initially for a period of 10 mins to ensure functional stability, then the ML348 (5.72 mg dissolved in 100 ml of DMSO) was added to half the hearts (100 μ M in 175ml re-circulating perfusate) for a further 60 mins in the recirculating perfusate.

2.10.7 Acute low flow ischemia

Rat hearts were perfused at a constant pressure of 100 mmHg and an end-diastolic pressure of 4-8 mmHg with KH buffer containing 11 mM glucose and 0.4 mM palmitate at 37 °C. Healthy hearts were randomly assigned to either control or ischemia groups. Control hearts underwent full flow perfusion for 20 mins by gassing the buffer with 95% O₂ and 5% CO₂ and freeze clamped for subsequent analysis. Ischemic hearts underwent the initial 20 mins control perfusion following by 30 mins of low-flow ischemia at a constant flow rate of 0.4mL/min/gww as previously characterised by our group¹⁴⁰. Following 30 mins of low-flow ischemia, hearts were freeze clamped for subsequent analysis. Cardiac function was measured over the last 5 mins for control and pre-ischemia hearts.

2.10.8 Fatty acid oxidation measurements

For the radiolabelled heart perfusions, KH buffer was supplemented with [9,10-³H] palmitate at the time of binding the non-labelled palmitate to 1.5% albumin. A 1.5 ml sample of recirculating buffer was taken at specific time intervals during the heart perfusion protocol, to allow for the measurements of FAO rates. ³H is metabolized to ³H₂O, which is generated by cytochrome c of the electron transport chain and the Krebs cycle, and is quantified in the perfusion buffer collected at set time intervals for a minimum of five linear data points and used to calculate the FAO rates. Using the Folch extraction method, ³H₂O was separated from [9,10-³H] palmitate¹⁴¹. More specifically, 0.5ml of recirculating buffer was added to 1.88 ml Folch solution (2:1 chloroform:methanol), with an additional 0.625 ml chloroform and 0.625 ml of 2 M KCl (prepared in 0.4 M hydrochloric acid (KCl-HCl)). Samples were then mixed for 20 mins and left to stand for an hour to allow separation of the aqueous and organic phases. The aqueous phase was removed and placed in a fresh falcon tube containing 1 ml chloroform, 1 ml methanol and 0.9 ml of KCl-HCl.

The solution was mixed for 20 mins and left to stand for an hour to allow a second separation. The separated aqueous phase was removed, and 0.5 ml added to 10 ml of Ecolite scintillation cocktail (BP Medical, USA) and counted for ^3H using a scintillation counter. The specific activity was measured directly by adding 0.5 ml perfusate taken at $t = 0$ min to 10 ml of scintillation cocktail, and counted on the scintillation counter.

2.11 Subcellular Fractionation

For measurement of sarcolemmal CD36, GLUT4 and GLUT1, separation of the sarcolemmal membranes from the intracellular endosomes was carried out by an established protocol of Fuller and colleagues¹⁴² as outlined in Figure 2.6. Frozen tissue (500 mg) was added to a High-Salt (20 mM HEPES (pH 7.4), 2 M NaCl, 5 mM NaN_3) solution which detaches cardiac membranes from myofilaments and the tissue was diced in the High-Salt solution for 4 min on ice, then incubated for 15 min with stirring. The samples were then centrifuged at 1,000 g for 5 min at 4°C. The supernatant was discarded, and the pellet was resuspended in buffer A (20 mM HEPES (pH 7.4), 2 mM EDTA (K^+), 1 mM MgCl_2 , 250 mM sucrose and 5mM NaN_3). The suspension was homogenized 10 times using a glass handheld homogenizer and centrifuged at 1,000 g for 5 min at 4°C. The supernatant was put in a fresh tube, while the pellet was resuspended in 3 ml of buffer A and then homogenized another 10 times before being added back to the supernatant. The mixture was then centrifuged at 100 g for 10 mins and the pellet resuspended in 300 μl western blotting lysis buffer, forming the first subcellular fraction (P1). The supernatant was then centrifuged at 5,000 g for 10 mins and the pellet was resuspended in 300 μl lysis buffer, forming the second subcellular fraction (P2). The third fraction (P3) was formed by the supernatant being centrifuged at 20,000 g for 30 mins and the pellet being resuspended in 300 μl lysis buffer. The

supernatant was then centrifuged for 50,000 g for 30 mins and pellet resuspended in 200 μ l lysis buffer, forming the fourth subcellular fraction (P4). The fifth subcellular fraction (P5) was generate by the supernatant being centrifuged at 190,000 g for 30 mins. The resultant pellet was re-suspended in 100 μ l of lysis buffer (75 mM Tris-HCl (pH 6.8), 3.8% SDS, 4 M urea, 20% glycerol, and 0.1% Triton-X-100) and all samples subjected to a BCA assay to determine protein concentration for subsequent SDS-polyacrylamide gel electrophoresis followed by western blotting.

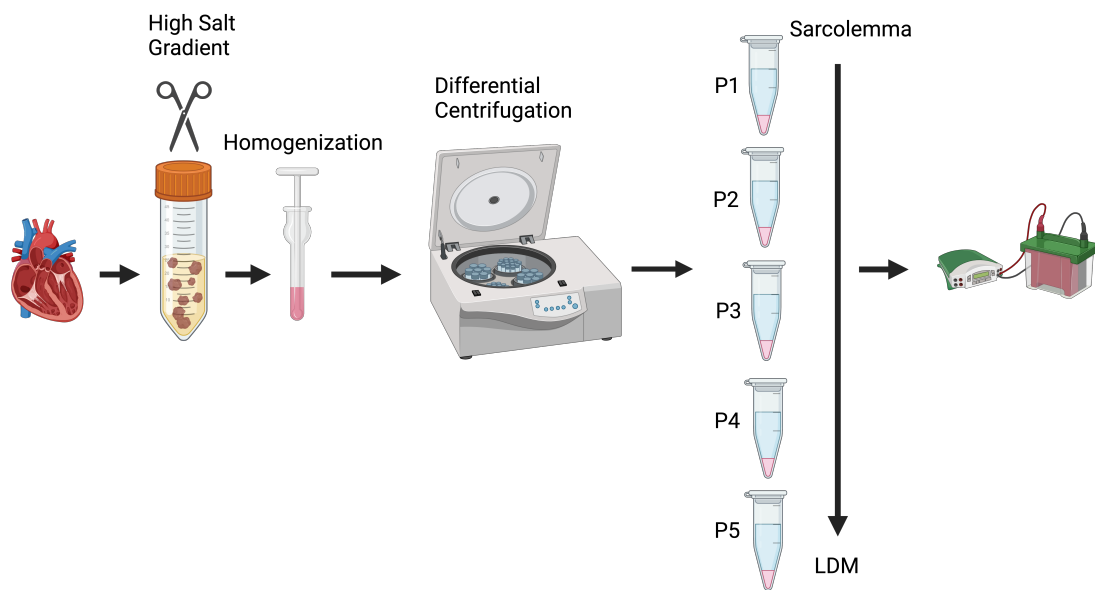


Figure 2.6: Workflow for subcellular fractionation

2.12 Measurement of myocardial triglycerides

520 μl of Folch solution (2:1 chloroform:methanol) was added to approximately 10 mg of tissue, and was homogenized using a Precellys homogeniser for 30 seconds at 5,500 RPM. The homogenate was then added to 100 μl of Folch and 100 μl of ddH₂O and spun at room temperature for 15 mins at 4000 rpm allowing for the top aqueous phase, protein pellet and organic phase to be separated. The organic phase was retained, evaporated for 1.5 hrs and re-suspended in 200 μl of ice-cold ethanol. Samples were then vortexed and left overnight to air dry. The final pellets were re-suspended in 2 μl of ice-cold ethanol and assayed using the Randox Triglyceride colorimetric benchtop assay kit (Randox, UK) following manufacturer's instructions. Total triglyceride (TG) content was normalized to the exact weight of each sample given as gram of wet weight tissue (gww).

2.13 Measurement of myocardial glycogen

As described by Chan and Exton¹⁴³, glycogen concentrations were measured by the conversion of glycogen to glycosyl units following breakdown with amyloglucosidase. Approximately 50 mg of tissue was added to 100 μl of 30% potassium hydroxide (KOH) and heated for 1 hr at 105°C. Samples were then incubated overnight at 4°C with 50 μl of 2% (w/v) sodium sulphate (NaSO₄) solution and 600 μl ethanol. The samples were then centrifuged for 5 mins at 16,000 g and the pellet re-suspended in 500 μl of ethanol. The ethanol was evaporated overnight, and the remaining pellet was resuspended in 375 μl of ddH₂O and 125 μl of amyloglucosidase-containing buffer (5mg of amyloglucosidase (Sigma Aldrich, UK) in 10 ml of 1 M sodium acetate. Following 1 hr incubation at 37°C, 100 μl of ddH₂O was added and incubated for 5 mins. The resultant supernatant was analyzed for glucose using the Abcam glucose benchtop assay kit (Abcam, UK), following manufacturer's

instructions. Total glycogen content was normalized to the exact weight of each sample given as gram of wet weight tissue (gww).

2.14 Lipidomics

Lipidomic analysis was performed by Dr. Christian Cole at the Scripps Research Institutem using GC/MS on 40 mg of snap frozen tissue from control and ischemia perfused hearts.

2.15 Human heart procurement

Human heart samples were provided by Dr. Kenneth Margulies at the University of Pennsylvania. Procurement of human myocardial tissue was performed at the University of Pennsylvania and approved by Institutional Review Boards at the University of Pennsylvania and the Gift-of-Life Donor Program as described^{144,145} and sent to the University of Oxford in accordance with the Central University Research Ethics Committee. HF tissue was obtained at the time of cardiac transplantation and NF tissue was obtained at the time of organ donation from cadaveric donors. Left ventricular ejection fraction as a marker of *in vivo* function was assessed via echocardiography in all hearts at the University of Pennsylvania.

2.16 Gene expression

2.16.1 RNA extraction and cDNA conversion

RNA was extracted from frozen cardiac tissue using the Qiagen RNeasy Mini Kit (Qiagen, Venlo, Netherlands), following the manufacturer's instructions. RNA concentrations were measured using the NanoDropTMUV spectrometer. RNA (1 μ g) was converted into cDNA using the high capacity cDNA reverse transcriptase

kit (Applied Biosystems) according to the manufacturer's protocol.

2.16.2 Quantitative Polymerase Chain Reaction (qPCR)

The resulting cDNA solutions were used for qPCR using SYBR Green technology (SYBR, Thermo Fisher Scientific). Briefly, samples were loaded as 20 μ l reaction volume which contained 12.5 μ g with 10 μ l of SYBR reagent master mix and 1 μ M of primers. Samples were then then loaded onto 96-well qPCR plates (MicroAMP fast, Applied Biosystems) and a standard SYBR cycle was applied using a StepOnePlus Real-Time PCR system (Applied Biosystems). Quantification of qPCR data was carried out using the traditional double delta Ct relative quantification method¹⁴⁶. The Ct of each target gene was normalised to the average Ct value for the house keeping gene (succinate dehydrogenase complex flavoprotein subunit A (SDHA)) and then a second time to the experimental reference sample. Primers were designed using Primer Blast 2 and produced by Sigma Aldrich UK as outlined in Table 2.1.

Table 2.1: Primer Sequences for qPCR Analysis

SDHA	Fp: TCCTTCCCCTGTGCATTACAA Rp: CGTACAGACCAGGCACAATCTG
DHHC4	Fp: CCTAGGGCTTACCCACCGAT Rp: CTGCTGAGGGATTCTCCCC
DHHC5	Fp: GTATCGGCCAGGTTACAGCA Rp: TCTCCACGACTCAACTTGGC
APT1	Fp: ACAGCTCATGTCAGCAGGAAAT Rp: TGTGATGCTGGTGAACGTCT
APT2	Fp: CCGGCTTCCTCATGTCAAGTA Rp: CCCATCAGGTCAAACCAGGAG

2.17 Western Blotting

Approximately 50 mg of frozen tissue was crushed to a fine powder and lysis buffer (75 mM Tris-HCl (pH 6.8), 3.8% SDS, 4 M urea, 20% glycerol, and 0.1% Triton-X-100) was added to the tissue before homogenising using a Polytron (Brinkman Instruments) tissue homogeniser. A Pierce BCA protein assay (Thermo Fisher Scientific, USA) was used to quantify total protein before addition of 5% β -mercaptoethanol. Protein samples were loaded onto pre-cast SDS-PAGE gels and separated by electrophoresis, at a constant voltage of 150 V. Using the wet transfer method, protein bands were transferred onto nitrocellulose at a constant current of 100 V for 1 hr. In order to reduce variability at the transfer stage, gels were cut based on the molecular weight of target proteins and transferred on the same nitrocellulose membrane. If all gels could not fit on one membrane prior to transfer, a control sample was loaded to each gel to allow quantification between membranes.

Membranes were then blocked in TBST containing 5% milk for 1 hr at room temperature before incubating in primary antibody at 4°C overnight. Following the overnight incubation, membranes were incubated for an hour in secondary antibody. Membranes were then exposed with enhanced chemiluminescence (ECL) using Bio-Rad Chemi-Doc MP scanning system (BioRad, USA). Band intensities were quantified using Image Lab software. Equal loading was confirmed using Ponceau staining after transfer. Antibody information is in Table. 2.2.

Table 2.2: Primary antibodies used for western blotting

Protein Target	Antibody	Dilution
CD36	Private donation	1:50,000
GLUT4	Private donation	1:1500
GLUT1	Abcam (ab652)	1:1000
FABPm	private donation	1:1000
UCP3	Abcam (ab3477)	1:2500
MCAD	Abcam (ab92461)	1:5000
DHHC5	Sigma Aldrich (HPA014670)	1:1000
DHHC4	Sigma Aldrich (HPA032124)	1:1000
APT1	Abcam (ab3477)	1:1000
PDK4	Abgent (AP7041b)	1:2500
Phospho-ACC	Cell Signalling (2535)	1:1000
Total-ACC	Cell Signalling (3611)	1:1000
Phospho-AMPK	Cell Signalling (2535)	1:1000
Total-AMPK	Cell Signalling(3611)	1:1000
CAV3	Abcam (ab2912)	1:5000
NCX1	Swant, R3F1	1:1000
Gαi	Pierce antibodies	1:1000
ACAT1	Abcam (ab168342)	1:2000
ANT1	Private donation	1:1000
ANT2	Private donation	1:1000
MLYCD	Abcam (ab234879)	1:1000
HADHA	Abcam (ab300050)	1:3000
PDH E2	Abcam (ab126224)	1:5000

2.18 Statistics

Prism 10 (GraphPad) software was used for the statistical analyses. Results, presented as standard error of the mean (SEM), were considered as significant at $p < 0.05$. Unpaired t-tests with Welch's correction and one-way ANOVA with Bonferroni's multiple comparisons test were used depending on the chapter.

3

Exploring *S*-acylation of membrane
transporters in the heart using affinity
purification assays

3.1 Abstract

S-acylation has been implicated in regulating membrane transport proteins involved in metabolism by modulating trafficking and subsequent membrane affinity in various tissues. However, whether cardiac plasma membrane and mitochondria membrane proteins are *S*-acylated remains unknown. In this chapter, techniques to measure *S*-acylation of cardiac metabolic proteins were optimized, and the *S*-acylation of key regulatory mitochondria and sarcolemmal proteins was investigated.

The known *S*-acylated protein, CAV3, was purified in frozen rat cardiac tissue and isolated mitochondria using acyl-RAC. In agreement with others, CAV3 was found to be 100% *S*-acylated in both whole tissue and in isolated mitochondria as there was no protein in the un-*S*-acylated fraction and all of it recovered in the *S*-acylated fraction. Additional known *S*-acylation targets, NCX1 and G α were found to be *S*-acylated further validating the acyl-RAC protocol. The *S*-acylation status of the membrane fat transporters CD36, FABPpm and the glucose transporters GLUT4 and GLUT1 was subsequently explored. CD36 was found to be *S*-acylated in both whole tissue and isolated mitochondria, while in contrast, no evidence of *S*-acylation was found for FABPpm, GLUT4 or GLUT1 in whole cardiac tissue. Furthermore, the mitochondrial protein ACAT1 was found to be *S*-acylated in isolated mitochondria, while no evidence of *S*-acylation of the mitochondrial proteins PDH, MLYCD, HADHB was found. Using acyl-PEG exchange, CAV3 was found to be *S*-acylated on 6 cysteines and CD36 was shown to be *S*-acylated on 4 cysteines. The *S*-acylating enzymes DHHC4 and DHHC5 and de-*S*-acylating enzymes APT1 and APT2 were also found to be expressed in cardiac tissue.

Collectively, these results demonstrate that CD36 is *S*-acylated in whole cardiac tissue and isolated mitochondria and the regulatory enzymes implicated in mediating its *S*-acylating cycles in other tissues were found to be expressed in the heart.

3.2 Introduction

3.2.1 The importance of *S*-acylation in the heart

Research into the lipid PTM *S*-acylation is a rapidly developing field as both *S*-acylation and its regulatory enzymes mediate a plethora of cellular processes and have been implicated in a number of diseases. In the heart a recent proteomic analysis suggested that over 450 cardiac proteins were *S*-acylated¹⁴⁷. To date, most of the research investigating *S*-acylation in the heart has focused on the role of *S*-acylation in excitation-contraction coupling (E-C coupling), which following electrical excitation of cardiomyocytes, mediates cardiac muscle contraction through a series of intracellular signalling events. As such, Nav1.5 voltage-gated sodium channel, Na⁺/K⁺ATPase, phospholemman (PLM), phospholamban (PLB), Ryanodine receptor (RyR2) and Na⁺/Ca²⁺ Exchanger (NCX1) have been shown to be *S*-acylated, therefore linking *S*-acylation in the modulation of cardiac muscle contraction^{77,134,148–150}. Interestingly, *S*-acylation has been shown to be required for substrate uptake activity of CD36, GLUT4, and GLUT1 by mediating their trafficking and enhancing membrane affinity in adipocytes and brain tissue, respectively^{72,73,151–154}. However, it remains unknown whether these membrane substrate transport proteins are *S*-acylated in the heart. These membrane substrate transporters are heavily expressed in cardiac tissue, and their plasma membrane localisation changes depending on substrate availability in response to varying physiological demands. Therefore, it is plausible that given their role of modulating metabolism in other tissues, that there could be a role for *S*-acylation in governing cardiac metabolism through membrane fat and glucose transporters.

3.2.2 *S*-acylation of mitochondrial proteins

Historically, most of the work in the field of *S*-acylation has identified targets localised to the ER, Golgi and plasma membrane in various tissues, while less had been investigated at the level of the mitochondria¹⁵⁵. In addition to substrate uptake across the plasma membrane, cardiac metabolism is also regulated at the level of the mitochondria. Importantly, the mitochondria contain 95% of the total CoA pool in the cell and consequently contains high concentrations of palmitoyl-CoA⁹¹. Given that palmitoyl-CoA is the primary substrate for *S*-acylation, this lipid PTM may be a relevant mechanism by which mitochondrial metabolism is regulated. Pioneering studies in the field in the late 1980s identified *S*-acylated mitochondrial proteins^{156,157}. Recently, large scale proteomics identified hundreds of potentially *S*-acylated mitochondrial proteins^{104,105}, however, very few of these proteins have had their *S*-acylation status further confirmed and the functional consequences of their *S*-acylation investigated.

Importantly, enzymes that regulate *S*-acylation have been shown to localise near or in the mitochondria. DHHC18 was recently shown to be localised within the mitochondria¹⁰⁹, while DHHC8 and DHHC13 have been shown to modulate mitochondrial metabolic processes^{107,158}. The de-*S*-acylating enzymes APT1^{124,126,127} and ABDH10^{131,159} were also found to be localised within the mitochondria. Therefore, the presence of the *S*-acylation regulatory enzymes indicate that mitochondrial *S*-acylation may not be regulated by levels of palmitoyl-CoA through spontaneous *S*-acylation as originally thought, but may have tighter enzymatic regulation, indicating *S*-acylation may play a role in tightly regulating mitochondrial processes such as metabolism.

3.2.3 Methodology for measuring *S*-acylation

Throughout the years, various techniques have been developed to measure *S*-acylation which have furthered our understanding of how this PTM regulates various cellular processes. While some techniques allow for measuring *S*-acylation dynamics, the majority enable a snapshot of the steady-state *S*-acylation levels in the cell⁷⁷. Widely used and validated techniques include prediction software (i.e. PalmPred), online databases (SwissPalm), radiolabelling, click chemistry and affinity purification assays (i.e. acyl-RAC, acyl-ABE and acyl-PEG exchange), while other techniques such as pan *S*-acylation antibodies in development⁷⁴. These techniques can either be characterized as palmitate-centric (metabolic labelling with radioactive palmitic acid, bio-orthogonal palmitic acid probes) or cysteine-centric based approaches (acyl-RAC, acyl-ABE, acyl-PEG)¹⁶⁰. However, there exists a large amount of variation between sets of *S*-acylated proteins identified depending on which technique is employed¹⁶¹. Therefore, it is important to understand the limitations of each *S*-acylation detection technique and often multiple techniques are combined to increase confidence in the *S*-acylation status of a target protein⁷⁴.

3.2.4 Affinity purification assays (acyl-RAC and acyl-ABE)

Both acyl-RAC and acyl-ABE are easily accessible benchtop affinity purification assays that are widely used methods to allow the assessment of steady-state *S*-acylation levels in a range of protein samples from tissue and cells¹⁶⁰. Acyl-ABE is a biotin switch technique whereby the irreversible alkylation of free cysteines under denaturing conditions is followed by specific cleavage of the thioester bond between palmitate and the *S*-acylated cysteine and finally the capture of the newly revealed cysteines with a sulfhydryl-reactive biotinylation reagent, which is then pulled down on streptavidin beads¹⁶². Acyl-RAC was later introduced to replace the biotinylation step of acyl-ABE. Following the thioester cleavage by hydroxylamine,

the newly exposed free cysteine thiols can be captured by sulfhydryl-reactive solid phase carriers, mainly thiopropyl Sepharose beads^{163–165}. Samples from both acyl-Rac and acyl-ABE are quantified via western blotting or mass spectrometry (MS) based proteomics. Although both acyl-RAC and acyl-ABE are similar, acyl-RAC confers benefits over acyl-ABE as it contains fewer steps, which is beneficial when processing large volumes of samples. Therefore, acyl-ABE and acyl-RAC have enabled research to investigate and quantify *S*-acylation in a range of sample without radiation and complex chemistry probe synthesis.

3.2.5 Acyl-PEG exchange

While acyl-RAC and acyl-ABE are able to detect *S*-acylation of proteins, they are unable to measure the number of *S*-acylation sites on proteins or detect changes between singly and multiply *S*-acylated sites. In contrast, acyl-PEG exchange employs commercially available cysteine reactive PEG maleimides (either 5 or 10 kDa) to induce a molecular mass shift following the cleavage of the thioester bonds resulting in laddering of the target protein, which is detected using western blotting^{134,166}. Thus, the observed laddering represents the number of cysteines *S*-acylated and, therefore acyl-PEG can provide further insight into the *S*-acylation stoichiometry of a protein of interest.

3.2.6 Benefits of the cysteine-centric approaches (acyl-RAC and acyl-PEG)

The cysteine-centric approaches of *S*-acylation detection, acyl-RAC and acyl-PEG confer many benefits as they do not involve radiation or require complex probe synthesis. Furthermore, they are time efficient compared to other methods including Acyl-ABE, which is beneficial when processing large amounts of samples. Additionally, as they are not involved in metabolic labelling, they can be applied

to a range of samples including modified cell lines, frozen animal tissue and human tissue, which is important for investigating alterations of *S*-acylation under pathological conditions. However, a limitation is that they can only report on static *S*-acylation levels and are unable to detect *S*-acylation dynamics. Furthermore, false positives have been reported with both acyl-RAC and acyl-ABE. Therefore, utilizing complementary *S*-acylation techniques, such as acyl-RAC and acyl-PEG exchange together would improve confidence in the detection and investigation of target protein *S*-acylation levels.

3.3 Aims

S-acylation has been shown to regulate the localisation of various membrane proteins involved in metabolism in various tissues, however, it remains unknown whether these proteins are *S*-acylated in cardiac tissue. Therefore, this chapter aimed optimize protocols for acyl-RAC and acyl-PEG exchange for the quantification of protein *S*-acylation in rat cardiac tissue, focusing on sarcolemma membrane substrate transporters and mitochondrial membrane proteins involved in cardiac metabolism. This chapter investigates:

1. The acyl-RAC extraction protocol for measuring *S*-acylation of the well-established *S*-acylated protein CAV3 in frozen rat cardiac tissue and isolated mitochondria
2. The *S*-acylation status of key sarcolemma membrane substrate transport and mitochondrial membrane proteins involved in cardiac metabolism.
3. Acyl-PEG exchange as another method to confirm *S*-acylation of proteins, and further probe the stoichiometry of CD36 and CAV3 in cardiac tissue.
4. The expression of the well characterized DHHCs and APTs in cardiac tissue.

3.4 Methods

3.4.1 Acyl-RAC

Acyl-RAC was carried out following the protocol set out in section 2.1.

3.4.2 Acyl-PEG

Acyl-PEG was carried out following the protocol set out in section 2.2.

3.4.3 Isolated mitochondria

SSM and IFM mitochondria were isolated following the protocol set out in section 2.8.

3.4.4 qPCR

qPCR was carried out following the protocol set out in section 2.16.

3.4.5 Western blots

Western blots were carried out following the protocol set out in section 2.17.

3.5 Results

3.5.1 Validation of Acyl-RAC extraction and selection of positive control protein CAV3

In order to investigate the palmitoylation status of membrane substrate transporter proteins, the acyl-RAC extraction protocol using snap frozen cardiac tissue and isolated mitochondria from rat hearts was optimized. Caveolins are a group of plasma membrane proteins that are localised to subdomains called caveolae¹⁶⁷, with three isoforms of caveolins identified (caveolin-1, caveolin-2 and caveolin-3)¹⁶⁸. Caveolin-3 (CAV3) is a muscle specific isoform that is highly expressed in cardiomyocytes¹⁶⁶. Furthermore, CAV3 is a cysteine rich protein and has been shown to have up to 6 cysteines *S*-acylated in a cluster located in the C-terminal membrane domain¹⁶⁶. Furthermore, as evidenced by its quantitative capture using acyl-RAC and acyl biotin exchange (ABE), CAV-3 was found to be 100% *S*-acylated when at the plasma membrane^{149,166}. Therefore, given its well established *S*-acylation status, abundance in cardiomyocytes, and known number of *S*-acylated cysteines, CAV3 was chosen as the positive control for the acyl-RAC protocol.

Acyl-RAC results contain 5 fractions as detailed in methods section 2.2, including the IF (total protein), CUF (un-*S*-acylated protein), CBF (*S*-acylated protein) and the negative control PUF (*S*-acylated and un-*S*-acylated protein) and PBF (no protein). By using the acyl-RAC protocol, in whole heart tissue (Figure 3.1 A), as well in isolated SSM and IFM mitochondria (Figure 3.1 A-B), CAV3 was confirmed to be *S*-acylated, as protein was devoid in the un-*S*-acylated CUF and the newly freed, previously *S*-acylated cysteines were captured on the thiopropyl sepharose beads in the *S*-acylated CBF. Furthermore, the non-HA treated fractions, which serve as the assay's negative control due to the thioester bond integrity being maintained with NaCl treatment and NEM capping of free cysteines, observed

protein in the PUF that was not recovered on the thiopropyl sepharose beads in the PBF, as expected. We further confirmed our acyl-RAC protocol by observing protein in the *S*-acylated CBF of other well established cardiac *S*-acylated proteins NCX1 and $G\alpha$ (Figure 3.1 C). Taken together, these results demonstrate that the acyl-RAC protocol can be successfully used to identify *S*-acylated proteins in the heart and isolated mitochondria.

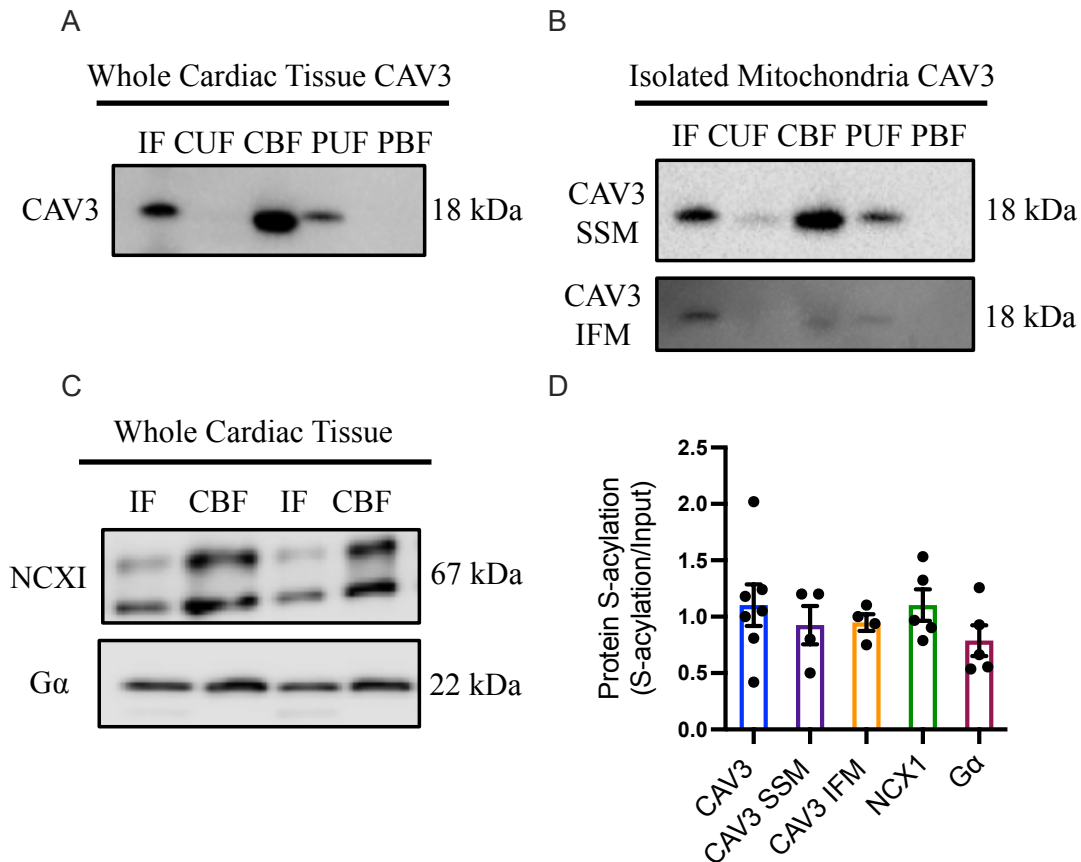


Figure 3.1: Acyl-RAC validation using Caveolin-3 (CAV3). CAV3 is shown to be *S*-acylated in whole tissue (A) and in the subsarcolemmal mitochondria (SSM) and intermyofibrillar mitochondria (IFM) populations (B). Known cardiac *S*-acylation targets $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX1) and G-protein α subunit ($G\alpha$) are used to validate our methodology (C). The fractions are as follows: total protein (input fraction; IF); HA treated un-*S*-acylated protein (cleaved unbound fraction; CUF); HA treated and resin captured *S*-acylated protein (cleaved bound fraction; CBF); NaCl treated and non resin captured (preserved bound fraction; PBF); and NaCl treated uncaptured un-*S*-acylated and *S*-acylated proteins (preserved unbound fraction; PUF). Quantified *S*-acylation blots of CAV3 whole tissue $n=7$, $n=4$ CAV3 SSM and IFM, NCX1 and $G\alpha$ whole tissue $n=5$ (D).

3.5.2 CD36 is *S*-acylated in whole cardiac tissue and isolated mitochondria

Following acyl-RAC optimisation for cardiac tissue and isolated mitochondria, we next investigated whether CD36 *S*-acylation could be quantitatively measured using acyl-RAC. CD36 was first shown to be *S*-acylated in rat adipocytes¹⁶⁹ and further work delineated that it was *S*-acylated on both the NH₂ and COOH [terminal cytoplasmic tails on cysteines 3, 7, 464 and 466 in HEK 293 cells¹⁷⁰. However, it remained unclear whether CD36 was *S*-acylated in cardiac tissue. Therefore, *S*-acylation was probed by immunoblotting against CD36 in whole cardiac tissue and SSM and IFM isolated mitochondria. Similar to CAV3, we observed evidence of CD36 palmitoylation as captured in the CBF fraction in cardiac tissue (Figure 3.2 A) and SSM and IFM mitochondria (Figure 3.2 B). However, in contrast to CAV3, protein was also observed in the CUF fraction which suggests that CD36 is not a 100% *S*-acylated protein. To confirm that the acyl-RAC has worked, protein was also present in the IF and PUF and absent in the PBF, as expected. Therefore, these results show that acyl-RAC is able to demonstrate CD36 *S*-acylation in cardiac whole tissue and isolated mitochondria.

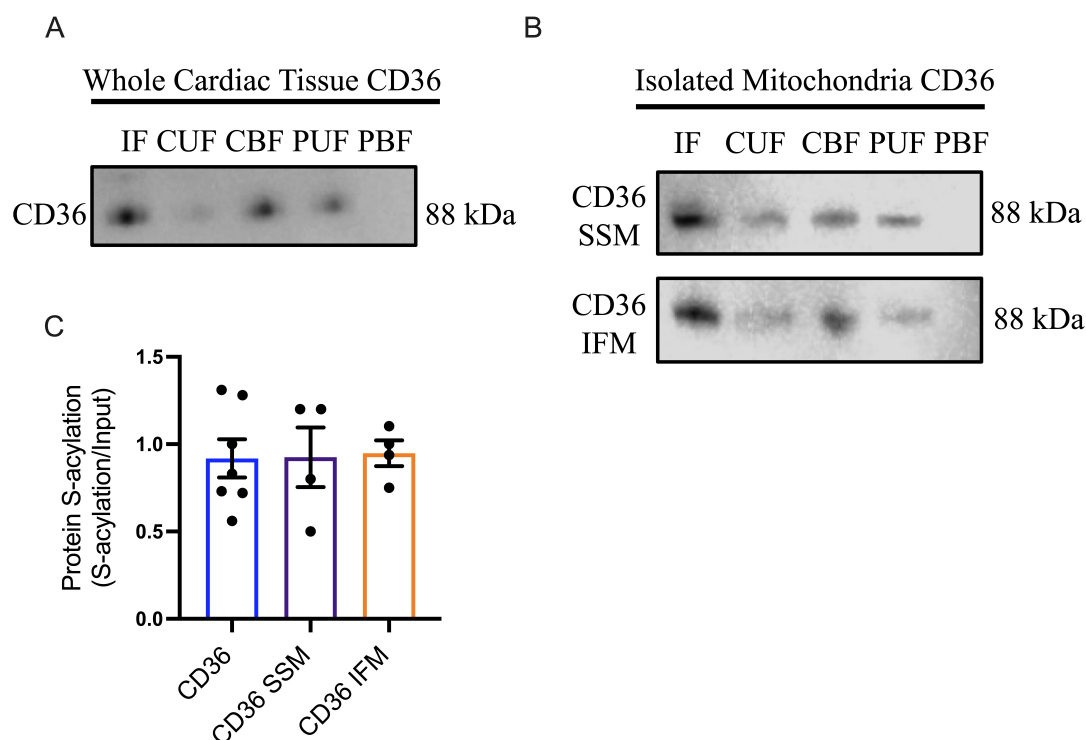


Figure 3.2: CD36 is *S*-acylated. CD36 is shown to be *S*-acylated in whole tissue (A) and SSM and IFM populations (B). Quantified *S*-acylation blots of CD36 whole tissue n=7, CD36 SSM and IFM n=4 (C).

3.5.3 *S*-acylation of membrane substrate transporters and mitochondrial membrane proteins

As both CAV3 and CD36 are *S*-acylated, whether *S*-acylation was ubiquitous to all membrane substrate transporters and mitochondrial membrane proteins was investigated. It has been proposed that in sarcolemmal lipid rafts CD36 facilitates the entry of LCFA into the cell through an interaction with the peripheral membrane protein fatty acid binding protein (FABPpm), however the exact mechanism remains unknown¹⁷¹. Therefore, it was postulated that similar to CD36, FABPpm would be *S*-acylated. However, we observed no evidence of FABPpm *S*-acylation, as demonstrated by no protein in the CBF despite protein in the CUF (Figure 3.3 A). We also observed no evidence of *S*-acylation in the sarcolemmal glucose transporters GLUT4 and GLUT1 (Figure 3.3 A). Therefore, it appears that CD36 is selectively *S*-

acylated membrane substrate transporter and that this lipid PTM is not ubiquitous to all membrane substrate transporters.

Given that mitochondrial CD36, which is located on the outer mitochondrial membrane (OMM) and has been implicated in mediating mitochondrial LCACoA uptake¹⁷² was found to be *S*-acylated, the *S*-acylation status of key mitochondrial membrane proteins were investigated. We investigated the *S*-acylation status of adenine nucleotide transporters (ANT1 and ANT2), which facilitate the exchange of ADP and ATP across the inner mitochondrial membrane (IMM) and are one of the most abundant proteins localised to the IMM¹⁷³. Furthermore, LCACoAs including the main *S*-acylation substrate palmitoyl-CoA, competitively inhibit the activity of ANT1/2⁸⁹. However, we observed no *S*-acylation of ANT1 or ANT2 in SSM and IFM (Figure 3.3 B-C). We also observed no evidence for *S*-acylation of pyruvate dehydrogenase (PDH) E2, malonyl-CoA decarboxylase (MLYCD) or hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit β (HADHB) in SSM (Figure 3.3 B). Interestingly, when we looked at Acetyl-CoA acetyltransferase (ACAT1), which is involved in three major pathways in the mitochondria which produce acetyl-CoA for the TCA cycle, we observed a faint band in the CBF indicating perhaps some *S*-acylation (Figure 3.3 B). As the CBF band was so faint, perhaps ACAT1 is *S*-acylated under certain conditions, but is in a more de-*S*-acylated form under basal conditions. These mitochondrial proteins have been identified via proteomics of *S*-acylated proteins, but had not been validated in the heart or in rats⁸¹.

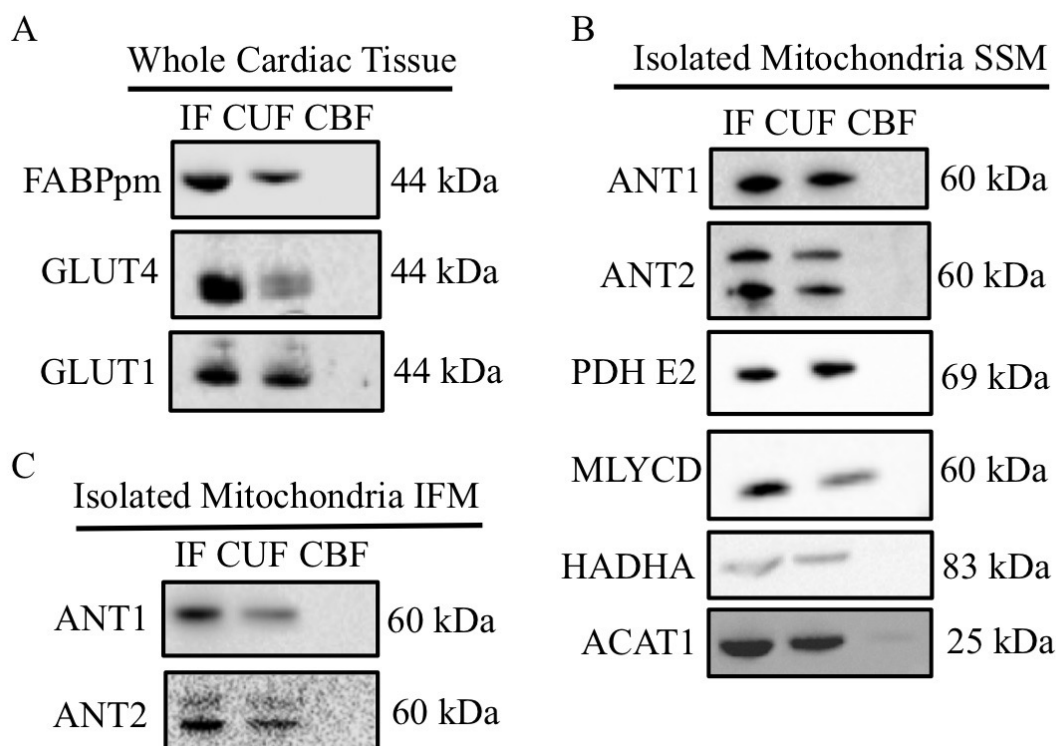


Figure 3.3: Acyl-RAC of membrane substrate transporters and mitochondrial membrane proteins. Fatty acid binding protein (FABPpm), glucose transport protein 4 (GLUT4) and glucose transport protein 1 (GLUT1) (A) had no evidence of *S*-acylation as no protein was recovered in the *S*-acylation CBF. The SSM population adenine nucleotide transporters (ANT1 and ANT), pyruvate dehydrogenase (PDH) E2, malonyl-CoA decarboxylase (MLYCD), hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit β (HADHB) (B) had no evidence of *S*-acylation, however Acetyl-CoA acetyltransferase (ACAT1) had a faint band in the *S*-acylated CBF(B), while the IFM population ANT1 and ANT2 (C) similarly had no evidence of *S*-acylation as no protein was recovered in the CBF. n=7 for each target.

3.5.4 Acyl-PEG exchange for the determination of *S*-acylation stoichiometry

This chapter has shown that CD36 is *S*-acylated in cardiac tissue, however the stoichiometry of CD36 *S*-acylation has not been investigated in the heart. Previous work has identified that CD36 is *S*-acylated on 4 cysteine residues (Cys 3, 7, 464 and 466) in HEK cells¹⁷⁴ and more recently in adipocytes⁷². Exchanging palmitate for a PEG molecule causes a 5kDa band shift for each *S*-acylation site occupied, therefore using the acyl-PEG exchange assay approach allows the stoichiometry of

CAV3 and CD36 to be investigated.

Using CAV3 as a postivie control for acyl-PEG, no molecular weight shift was observed in the non HA treated PEGylation fraction serving as the negative control, while 6 *S*-acylation sites were observed on CAV3 in the HA treated PEGylation sample (Figure 3.4 A) as represented by the 6 band molecular weight shift. These results demonstrate that CAV3 in whole rat cardiac tissue is *S*-acylated up to 6 times and these findings are consistent with previous reports¹⁶⁶. The stoichiometry of CD36 *S*-acylation was then investigated using the the acyl-PEG exchange assay. As expected, CD36 protein was observed in the IF fraction and the non-HA treated PEG fraction with no band shift (Figure 3.4 B). Importantly, a four band shift was observed in the +HA treated PEG fraction for CD36, which is consistent with previous reports demonstrating that CD36 is *S*-acylated on up to four cysteines (Figure 3.4 B). Collectively, these results demonstrate that CAV3 and CD36 are *S*-acylated on up to six and four cysteines, respectively, in rat cardiac tissue.

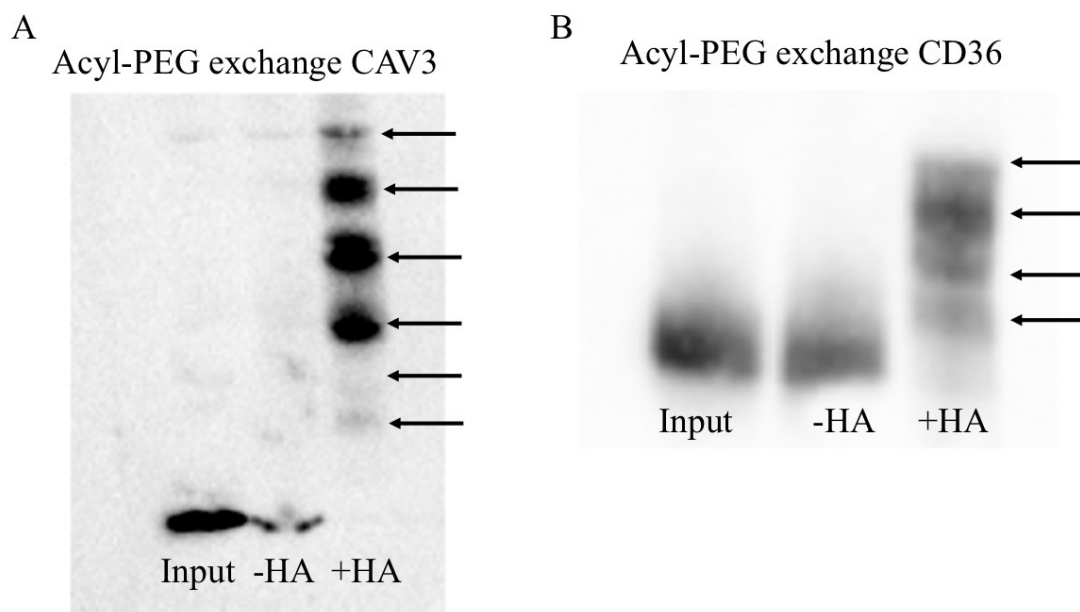


Figure 3.4: Acyl-PEG exchange of CAV3 (A) and CD36 (B). The fractions are as follows: total protein (input fraction; IF); -hydroxylamine (HA) (NaCl treated PEG) ; +HA (HA treated PEG). n=7 for CAV3 and CD36 acyl-PEG.

3.5.5 Expression of DHHCs and APTs in the heart

Given that CD36 was found to be *S*-acylated in the heart, identifying the expression and abundance of the DHHCs (DHHC4 and DHHC5) and APTs (APT1 and APT2) that have been previously reported to regulate CD36 *S*-acylation dynamics in adipocytes^{72,90} was next explored. As such, protein expression of DHHC4, DHHC5 and APT1 were detected in the heart through western blotting using previously published antibodies. Using qPCR, mRNA expression of DHHC4, DHHC5, APT1 and APT2 were also observed. Therefore, these results demonstrate that CD36 is *S*-acylated and the regulatory enzymes determining *S*-acylation dynamics are present in the heart.

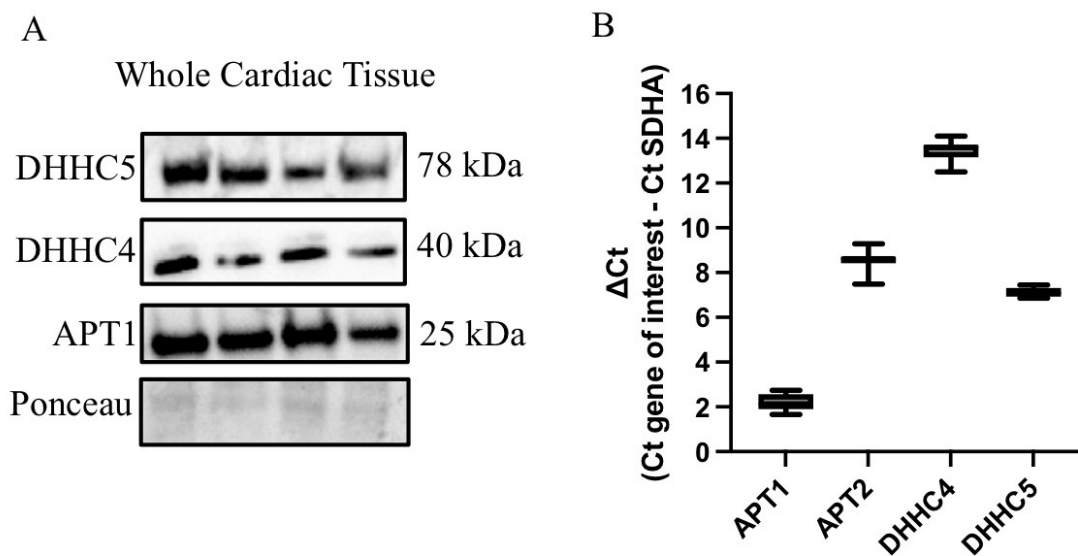


Figure 3.5: Representative western blot images for DHHC5, DHHC4, APT1 (A) $n=4$ for each target. mRNA levels of DHHC5, DHHC4, APT1 and APT2 (B). $n=6$ for total protein and mRNA expression of DHHC5, DHHC4 and APT1.

3.6 Discussion

3.6.1 Acyl-RAC and acyl-PEG demonstrate *S*-acylation of cardiac CD36

Although CD36 has been shown to be *S*-acylated in adipocytes^{72,169}, its *S*-acylation status in the heart remained unknown. These results demonstrate that CD36 is *S*-acylated in whole rat cardiac tissue and in isolated mitochondria. *S*-acylation of CD36 was further confirmed using acyl-PEG which demonstrated that CD36 is *S*-acylated on up to four cysteines, consistent with findings in adipocytes⁷². However, with these methodologies, we are unable to determine exactly which cysteines are *S*-acylated on cardiac CD36. As CD36 is a multifunctional receptor widely expressed in a variety of tissues (i.e. endothelial cells, cardiomyocytes, renal cells, hepatocytes, adipocytes, platelets, macrophages and tubular epithelial cells)¹⁸, it is plausible that cardiac CD36 is *S*-acylated on the same cysteines (i.e. Cys 3, 7, 464 and 466) as has been shown in HEK cells and 3T3-L1 adipocytes^{72,170}. As such, future work using cysteine site mutagenesis in cardiac cell lines is warranted to confirm the *S*-acylated cysteines of CD36 in the heart.

Compared to other known FA transporters, CD36 drives the majority of FA uptake across the plasma membrane in cardiomyocytes¹⁸. In addition, mitochondrial CD36 is located on the outer mitochondrial membrane (OMM) downstream of long-chain acyl CoA synthetase, and has been implicated in FA uptake into the mitochondria¹⁷². Given the prominent role of CD36 in being the gate keeper of lipid metabolism at both the plasma and mitochondrial membranes, understanding mechanisms that regulate its membrane localisation is crucial. Various stimuli have been shown to induce the subcellular trafficking of CD36 including insulin and contraction, however, the exact mechanisms remain unknown. In adipocytes, CD36 *S*-acylation by DHHC4 and DHHC5 working in concert was shown to be required for

its trafficking to the plasma membrane and subsequent FA uptake⁷². Interestingly, the *S*-acylating DHHCs (i.e. DHHC4 and DHHC5) and de-*S*-acylating APTs (i.e. APT1 and APT2) linked with CD36 *S*-acylation dynamics were also found to be expressed in rat cardiac tissue. We were able to confirm DHHC4, DHHC5 and APT1 expression at both the mRNA and protein level. However, APT2 mRNA expression was only shown as an antibody was not commercially available. Importantly, APT1 was been shown to be the main acyl-thioesterase responsible for CD36 de-*S*-acylation⁹⁰. Therefore, as the regulation of CD36 trafficking represents a key mechanism underpinning metabolic control in the heart and *S*-acylation has been shown to regulate membrane affinity and trafficking, CD36 *S*-acylation may therefore represent a novel regulatory mechanism by which cardiac metabolism is governed.

Although GLUT4 and GLUT1 have been shown to be *S*-acylated in other tissues, we observed no evidence of their *S*-acylation in the heart. GLUT4 was found to be *S*-acylated in 3T3-L1 and Chinese hamster ovary cells on Cys223 by DHHC7^{73,153}, while GLUT1 was shown to be *S*-acylated on Cys209 by DHHC9 in rat blood brain barrier capillaries, human peripheral blood mononuclear cells and glioblastoma cells^{125,151,154}. Therefore, perhaps in cardiac tissue, GLUT4 and GLUT1 are under different regulatory mechanisms other than *S*-acylation, or require external stimulus such as insulin to become *S*-acylated in the heart.

3.6.2 *S*-acylation of mitochondrial proteins

The understanding of *S*-acylation in mitochondrial proteins is in its infancy. Despite the identification of potentially hundreds of *S*-acylated proteins in the mitochondria, not much work has been done since to investigate these validate these targets further^{104,105}. However, with the recent identification of the DHHCs and APTs localizing in or around the mitochondria^{107,126,158}, identifying their

target proteins within the mitochondria could provide some further insight into the role of this PTM in the mitochondria. This chapter demonstrates that both CD36 and CAV3 are *S*-acylated in isolated mitochondria from rat heart tissue, while other mitochondrial targets that were originally identified as potentially *S*-acylated via acyl-ABE with MS-proteomics were not confirmed using acyl-RAC, suggesting discrepancies between the techniques. Therefore, additional testing using other *S*-acylation techniques on cardiac tissue is warranted to further confirm that these targets are not *S*-acylated. Interestingly, as the CBF *S*-acylation band was so faint in ACAT1, further work is warranted to unpick the extent of ACAT1 *S*-acylation. Recently, *S*-acylation has emerged as playing a crucial role in mediating metabolic aspects of mitochondrial metabolism. Thus, understanding the downstream functional consequences of *S*-acylation of CD36, CAV3 and ACAT1 could identify a novel mitochondrial metabolic regulation mechanism. Although, tools to probe *S*-acylation, such as pharmacological inhibitors and activators, are lacking, a mitochondrial-targeted APT inhibitor was recently developed and demonstrated to de-*S*-acylate Peroxiredoxin-5 (PRDX5) in HEK cells¹³¹. However, this chemical tool to perturb *S*-acylation of mitochondrial APT has not been tested in cardiac specific cells or in *ex vivo* tissue systems.

3.7 Conclusion

In summary, results from this chapter demonstrates that acyl-RAC is able to successfully detect protein *S*-acylation in frozen rat cardiac tissue and in isolated mitochondria using previously confirmed target proteins (i.e.CAV3, NCX1, Gα protein). Novel findings were that CD36 was found to be *S*-acylated in whole tissue and at the level of the mitochondria and using acyl-PEG was found to be *S*-acylated on up to four cysteines. Furthermore, the regulatory enzymes that have been shown to regulate CD36 *S*-acylation were also found in cardiac tissue. This is

the first investigation of CD36 *S*-acylation in the heart and given the major role of CD36 in regulating FA metabolism in cardiomyocytes, these findings potentially link *S*-acylation and cardiac metabolism.

4

S-acylation of CD36 is associated with the
metabolic dysfunction of the type 2
diabetic heart

4.1 Abstract

The dynamic process of *S*-acylation and de-*S*-acylation of target proteins has sparked interest in understanding how this lipid PTM is altered in disease states. While *S*-acylation has been well studied in cancer, neurological disorders, and cardiac physiology, less is known surrounding its role in metabolism. To date, no study has explored CD36 *S*-acylation as a driver of cardiac metabolic dysfunction in T2D. In this work, CD36 *S*-acylation and its regulation is explored in T2D.

Diabetic rats had increased blood glucose, insulin and NEFA concentrations compared with controls, with increased body weight and adiposity, confirmatory of the T2D phenotype. To investigate cardiac function and substrate metabolism, T2D and control rat hearts were perfused in Langendorff mode with ^3H palmitate. T2D rat hearts had decreased cardiac function, increased FA oxidation rates and increased triglyceride storage compared with control hearts. Subcellular fractionation of T2D and control hearts demonstrated a significant increase in CD36 localised to the sarcolemma in diabetes. Acyl-RAC of control and T2D hearts showed an increase in CD36 *S*-acylation in diabetes. Increased CD36 *S*-acylation was also shown in *db/db* mice, diabetic pigs and insulin resistant hiPSC-CM, demonstrating that elevated CD36 *S*-acylation is conserved across species in diabetes. Acyl-PEG demonstrated that CD36 was *S*-acylated on four cysteine residues in both cohorts.

Protein and mRNA expression of the regulatory enzymes, DHHC5 and APT1, implicated in mediating the dynamic *S*-acylation and S-deacylation of CD36 respectively showed no changes, however the S-acylating enzyme DHHC4 was significantly increased at both the total protein and mRNA levels. Lastly, DHHC4 was shown to be a potential target for the transcription factor FoxO1 as following FoxO1 ablation in T2D mice, DHHC4 mRNA expression was significantly reduced.

4.2 Introduction

The FA transporter CD36 is a pivotal protein in regulating LCFA uptake into metabolically important tissues, such as cardiac muscle^{175,176}. Insight into the molecular mechanism by which CD36 is able to act as a dynamic gatekeeper of lipid metabolism occurred when it was discovered that CD36 is able to recycle between intracellular endosomes and the sarcolemma²³. Therefore, the localisation of CD36 regulates the amount of LCFA taken up by the heart. CD36 trafficking to the sarcolemma is induced by insulin and myocyte contraction¹⁷⁷, to allow for the correct coordination of fatty acid uptake rates with myocardial energy needs. Interestingly, chronic lipid overload increases the net translocation of CD36 to the sarcolemma, even before changes in total CD36 protein^{178–180}. Furthermore, altered sarcolemma CD36 expression is associated with pathologies such as T2D, and has been shown to occur prior to the development of insulin resistance^{59,178,180}. As such, studies in patients with T2D and rodents demonstrate a permanent relocation of CD36 to the sarcolemma, which is associated with increased FA transport and downstream metabolic derangement^{59,178–180}. Interestingly, the trafficking machinery dedicated to the recycling of CD36 is still not fully understood in the heart. However, *S*-acylation, which regulates subcellular localisation of a number of proteins, has been proposed as a regulatory mechanism driving CD36 localisation to the sarcolemma.

4.2.1 *S*-acylation of CD36 in metabolic diseases

While the two phosphorylation sites and two ubiquitination sites are well characterized on CD36, the function of *S*-acylation on the four cysteines on CD36 remained largely unexplored (Figure 4.1). The location of the four *S*-acylated cysteine residues on CD36, two on each of the NH2 and COOH cytoplasmic terminals (Cys 3,7,464,466) was discovered in the late 90s¹⁷⁴. However, it was recently shown that

S-acylation of all 4 cysteine residues were required for optimal CD36 trafficking in response to insulin or contraction^{72,181}. Furthermore, *S*-acylation of CD36 was shown to be required for post-transcriptional processing in the ER, and inhibition of *S*-acylation decreases the half-life of CD36 as well as its incorporation into lipid rafts¹⁵². Collectively, these results indicate that *S*-acylation governs CD36 trafficking to the sarcolemma and subsequent lipid uptake. However, not many studies have been conducted to explore the role of CD36 *S*-acylation in metabolic diseases.

Enhanced CD36 *S*-acylation has been implicated the development of non-alcoholic steatohepatitis (NASH) as increased CD36 *S*-acylation was found in the livers of patients and mice with this disease¹⁸². Furthermore, there was increased CD36 localisation on the sarcolemma and increased lipid metabolism observed in mouse NASH hepatocytes, while blunting *S*-acylation through cysteine site mutagenesis of CD36 was protective against the disease development¹⁸². Therefore, increased CD36 *S*-acylation levels are associated with enhanced sarcolemmal localisation and FA uptake, resulting in dysfunction lipid metabolism in liver. However, it remains unknown whether cardiac CD36 *S*-acylation levels are altered in metabolic diseases such as T2D. Therefore, investigating *S*-acylation levels of CD36 in diabetes could provide insight into the effects of this lipid PTM on altered cardiac metabolism, and potentially provide a future therapeutic target to correct dysregulated metabolism in T2D.

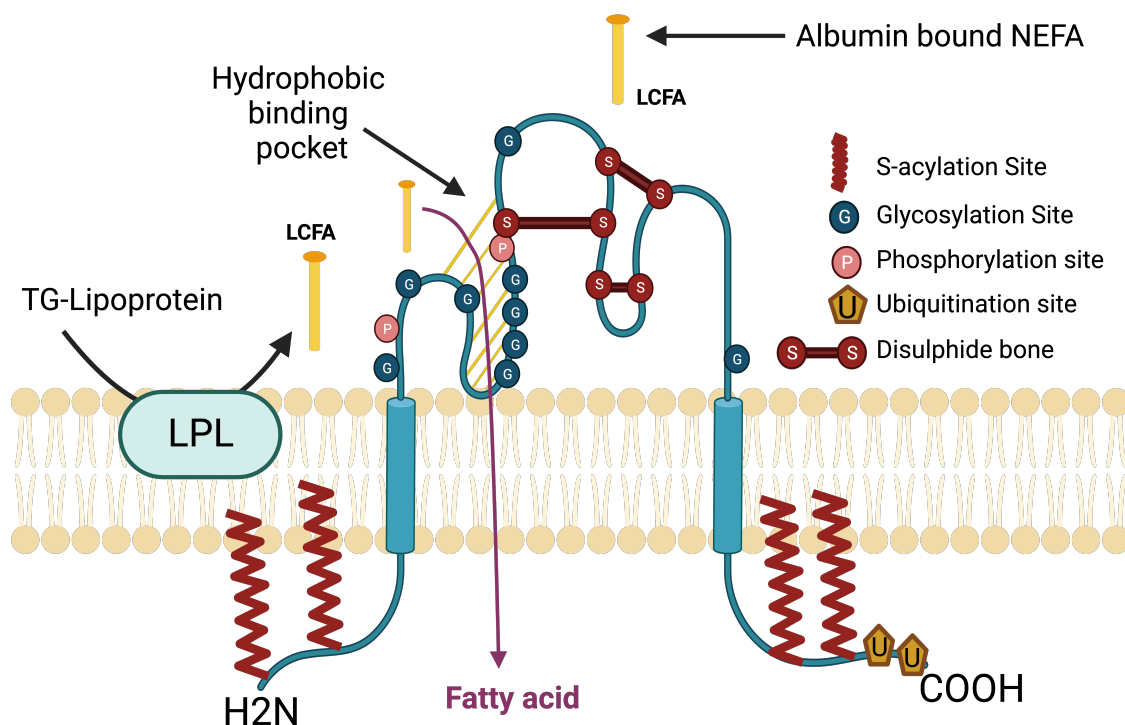


Figure 4.1: CD36 contains four *S*-acylation sites on cysteine residues 3, 7, 464 and 466 on the NH₂ and COOH terminus. Figure modified from Glatz and colleagues³⁶.

4.2.2 FoxO protein transcription factors

The Forkhead Box O (FoxO) transcription factor family is characterized by a wing helix DNA binding motif and the forkhead domain¹⁸³. The FoxO subgroup of forkhead transcription factors consist of FoxO1, 3, 4 and 6¹⁸⁴ and they regulate a plethora of cellular processes including cell growth, differentiation, survival and importantly, metabolism^{185–187}. Furthermore, FoxO proteins have been implicated in regulating glucose and lipid metabolism^{188,189}. The transcriptional activity of FoxO proteins is well established to be regulated as a downstream target of insulin signalling, which results in the inactivation of FoxO-dependent transcription^{190–193}. In the presence of insulin and insulin like growth factor (IGF-1), the P13K-Akt signaling pathway is activated and Akt, as well as the serum and glucocorticoid inducible kinase (SGK) directly phosphorylate FoxO, resulting in its interaction with

14-3-3 binding protein and nuclear exclusion followed by ubiquitylation-dependent proteasomal degradation in the cytosol¹⁹⁴. In the absence of insulin or other similar signalling kinases, FoxO protein binding to 14-3-3 is disrupted and FoxO translocates to the nucleus where it binds to the FoxO promoter region and triggers transcription of a large set of target genes, including those associated with glucose (i.e. PDK4)¹⁹⁵ and lipid metabolism (i.e. CD36, LPL, PGC1- α)¹⁹⁶ in various tissues.

4.2.3 FoxO1 dysregulation in the heart

Although FoxO3, FoxO4, and FoxO6 are also expressed in the heart, FoxO1 is the dominant cardiac member and its expression and function has been linked to the pathophysiology of various cardiovascular diseases. In diabetes, impaired insulin PI3k/Akt signalling drives enhanced FoxO1 nuclear retention and activity as illustrated in Figure 4.2. As such, increases in FoxO1 transcriptional activity have been linked with further impairing the insulin signalling cascade and blunting glucose uptake in cardiomyocytes¹⁹⁷ and rodent models of T2D^{198,199}. Furthermore, FoxO1 was shown to transcriptionally upregulate PDK4, resulting in decreased glucose metabolism in diabetes¹⁹⁹, while FoxO1 deficiency was shown to improve cardiac metabolic dysfunction and protect against diabetic cardiomyopathy by increasing glucose metabolism through PDK4 downregulation in diabetic mice^{198,199}. Furthermore, FoxO1 has been implicated in mediating lipid metabolism, potentially through CD36^{200,201}. In C2C12 myotubes an increase in FoxO1 was accompanied by elevated CD36 on the sarcolemma and increased FA uptake independently of changes in CD36 total protein or mRNA²⁰⁰, and this was further shown in cardiomyocytes supplemented with high palmitate, whereby increases in FoxO1 resulted in increased CD36 on the sarcolemma²⁰¹. However, the mechanism by which FoxO1 drives CD36 to the sarcolemma remains unknown.

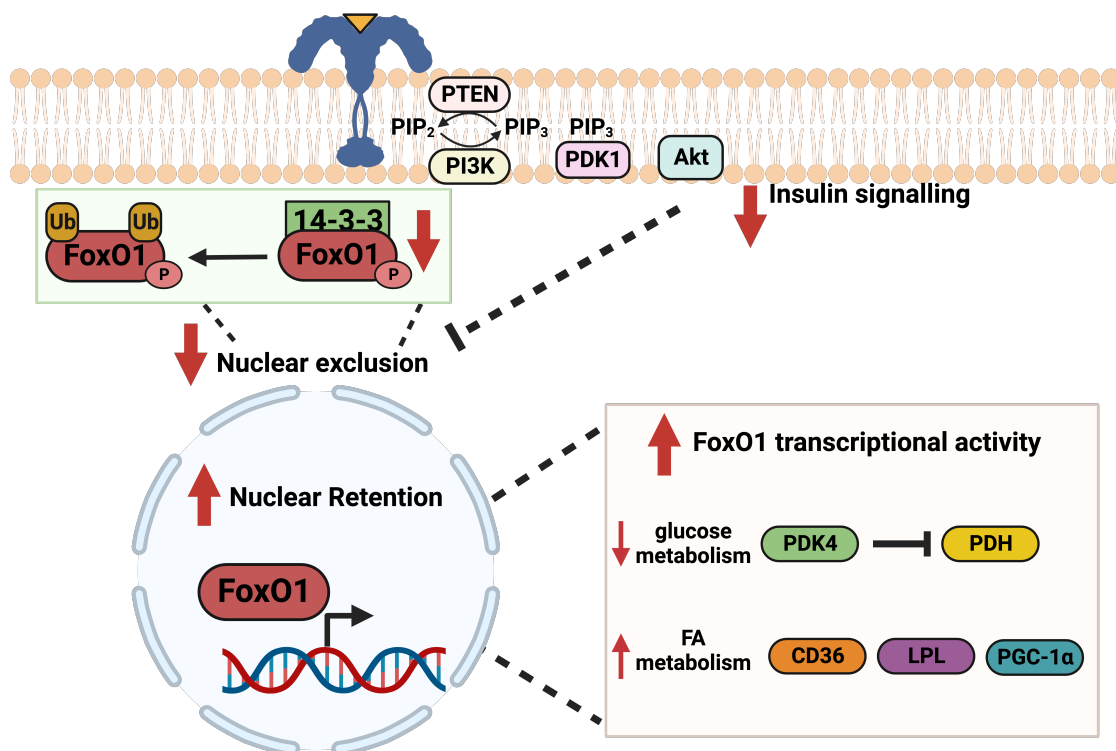


Figure 4.2: In diabetes, FoxO1 has enhanced nuclear retention and increases the transcription of genes involved in increasing FA metabolism and decreasing glucose metabolism. Figure modified from Chistiakov and colleagues²⁰².

4.3 Aims

Elevated *S*-acylation has been shown to be associated with increased sarcolemmal CD36 expression and altered lipid metabolism in liver, however it remains unknown whether there is altered cardiac CD36 *S*-acylation levels in T2D. This chapter therefore aimed to explore *S*-acylation of CD36, sarcolemmal levels of CD36 and regulatory *S*-acylation enzymes (i.e. DHHCs and APTs) in hearts from T2D rats. Furthermore, this chapter aims to unpick the transcriptional regulation of DHHC4 to provide further insight into the enhanced levels of CD36 *S*-acylation in diabetes. This was achieved by:

1. Investigating *S*-acylation levels in control and T2D rat hearts using acyl-RAC and acyl-PEG following Langendorff perfusion. *S*-acylation levels were also investigated in non-perfused hearts from *db/db* mice, T2D pigs and insulin resistant human iPSC-CMs.
2. Determining Sarcolemmal expression of CD36 using subcellular fractionation from control and T2D rat hearts.
3. Quantifying total protein and mRNA expression of the regulatory *S*-acylation enzymes DHHC4, DHHC5 and the de-S-acylating enzyme APT1 between control and T2D rat hearts following Langendorff perfusion.
4. Identifying the transcription factor regulating DHHC4 in diabetes and investigating findings in a T2D mouse model.

4.4 Methods

4.4.1 T2D Rat Model

T2D rat model was generated as carried out following the protocol set out in section 2.3.

4.4.2 Heart perfusions

Diabetic rat hearts underwent Langendorff perfusions were carried out following the protocol set out in section 2.10.

4.4.3 Subcellular fractionation

Subcellular fractionation on control and diabetic rat hearts was carried out following the protocol set out in section 2.11.

4.4.4 Fatty acid oxidation

Fatty acid oxidation measurements was carried out following the protocol set out in section 2.10.8.

4.4.5 Acyl-RAC

Acyl-RAC was carried out on langendorff perfused control and T2D rat hearts and non perfused *db/db* mouse hearts, T2D pig hearts and IR hiPSC-CMs following the protocol set out in section 2.1.

4.4.6 Acyl-PEG

Acyl-PEG was carried out on cyl-RAC was carried out on langendorff perfused control and T2D rat hearts following the protocol set out in section 2.2.

4.4.7 *db/db* Mouse Model

The *db/db* mouse model was carried out following the protocol set out in section 2.4.

4.4.8 Porcine diabetes model

The porcine diabetes model was carried out following the protocol set out in section 2.6.

4.4.9 IR hiPSC-CMs

IR hiPSC-CMs were carried out following the protocol set out in section 2.7.

4.4.10 T2D Mouse model

The T2D mouse model was carried out following the protocol set out in section 2.5.

4.4.11 Measurement of blood plasma metabolites

The measurement of blood plasma metabolites was carried out following the protocol set in section 2.9.

4.4.12 Measurement of myocardial TG and glycogen

Measurement of myocardial triglycerides and glycogen was carried out following protocols set in section 2.12 and 2.13, respectively.

4.5 Results

4.5.1 The T2D Rat Model

The combination of 5 weeks of HFD feed and low dose streptozotocin (STZ) injection resulted in a T2D phenotype in rats. This was evidenced by the development of hyperglycaemia in the diabetic cohort, with a 36% increase in fasting blood glucose concentrations (Figure 4.3 A). T2D rats also exhibited hyperlipidemia as measured by a 2.7-fold increase in fed non-esterified fatty acid concentrations (NEFA) (Figure 4.3 B). This model of T2D is distinct from type 1 diabetes as there was also a 44% increase in fasting insulin concentrations (Figure 4.3 C).

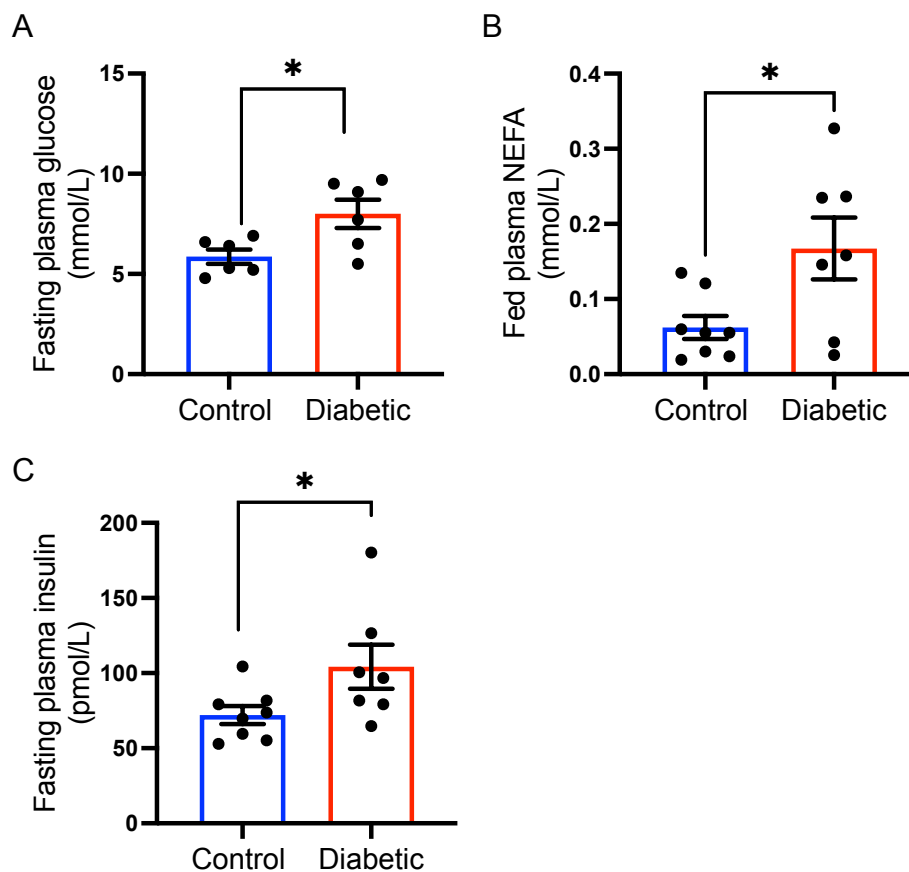


Figure 4.3: T2D rats presented with fasting hyperglycemia (A), hyperlipidemia in the fed state (B) and fasting hyperinsulinemia compared with controls (C). * $p < 0.05$ between control and diabetic.

Furthermore, diabetic animals had a 12% increase in body weight (Figure 4.4 A) and a 67% increase in adiposity (Figure 4.4 B) compared with controls. When normalized to body weight, fat pads were increased 1.5-fold (Figure 4.4 C) in T2D animals compared with controls, demonstrating an increase in adiposity relative to body weight. Finally, although heart weight increased by 13% in T2D rats (Figure 4.4 D), when normalized to the increase in body weight, there was no significant increase in the heart weight to body weight ratio (Figure 4.4 E), which indicates no cardiac hypertrophy in the T2D rat model. Collectively, these results indicate the induction of a T2D phenotype with hyperglycemia, hyperlipdemia, and hyperinsulinemia with increased adiposity following 5 weeks of HFD feeding combined with a low dose STZ injection.

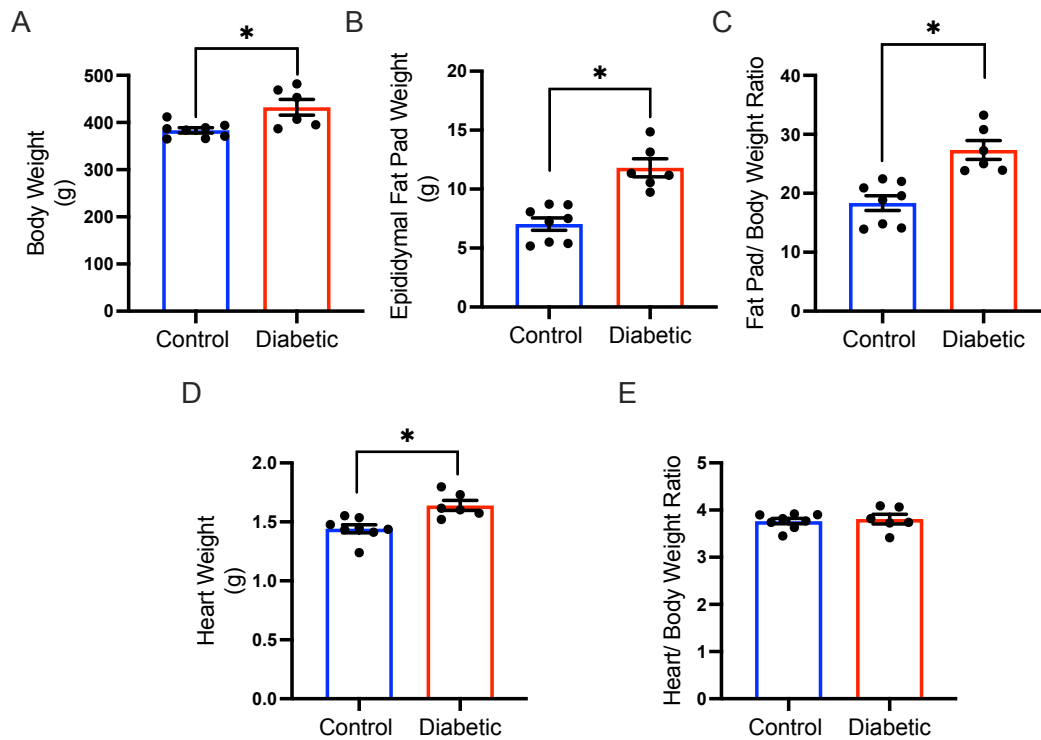


Figure 4.4: Diabetic rats had increased body weight (A), adiposity (B), fat pad to body weight ratio (C), and heart weight (D) with no changes in heart to weight ratio (E) compared with controls.* $p < 0.05$ between control and diabetic.

4.5.2 T2D hearts have normal cardiac function

Hearts were excised under anaesthesia from control and T2D rats, then perfused in retrograde Langendorff mode. Cardiac haemodynamics were measured through a Clingfilm balloon inserted into the left ventricle that was attached to a power transducer. Cardiac function was then measured as rate pressure product (RPP), the multiple of heart rate (HR) and developed pressure (DP). Hearts from control and T2D rats remained stable throughout 20-minute perfusion protocol (Figure 4.5 A) as represented by RPP over time. Consistent with previous findings from the group, no differences in RPP (Figure 4.4 B), DP (Figure 4.5 C) or HR (Figure 4.5 D) were observed¹³⁵.

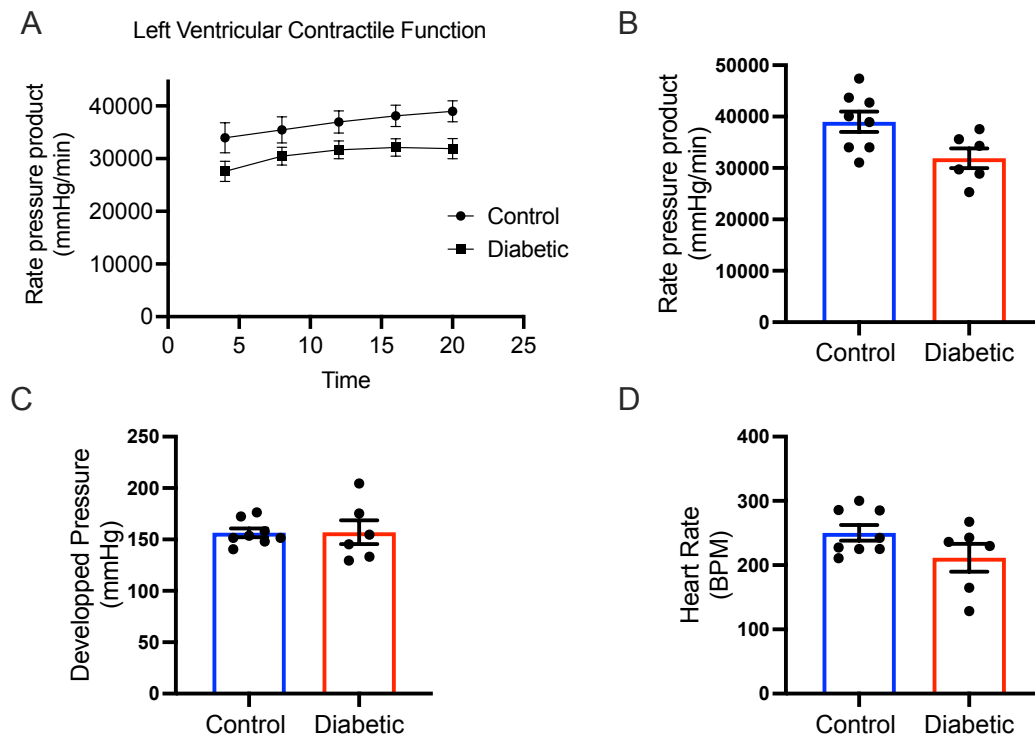


Figure 4.5: Rate-pressure product in retrograde Langendorff hearts from control and diabetic rats was stable throughout the protocol (A). Diabetic rodents presented with no statistically significant changes cardiac function (RPP) (B) DP (C) or HR (D) compared with controls.* $p < 0.05$ between control and diabetic.

4.5.3 T2D hearts have increased fat metabolism

Hearts were perfused with ^3H -palmitate for the measurement of fatty acid oxidation (FAO) rates. In the T2D hearts, FAO rates were increased by 25% (Figure 4.6 A) compared with control hearts resulting in a 66% increase in FAO per unit work (Figure 4.6 B), demonstrating that the diabetic hearts are oxidizing more fat per contraction when compared with controls. Furthermore, myocardial triglyceride concentrations were elevated by 65% in T2D compared with controls (Figure 4.6 C), while no changes in myocardial glycogen levels were observed (Figure 4.6 D).

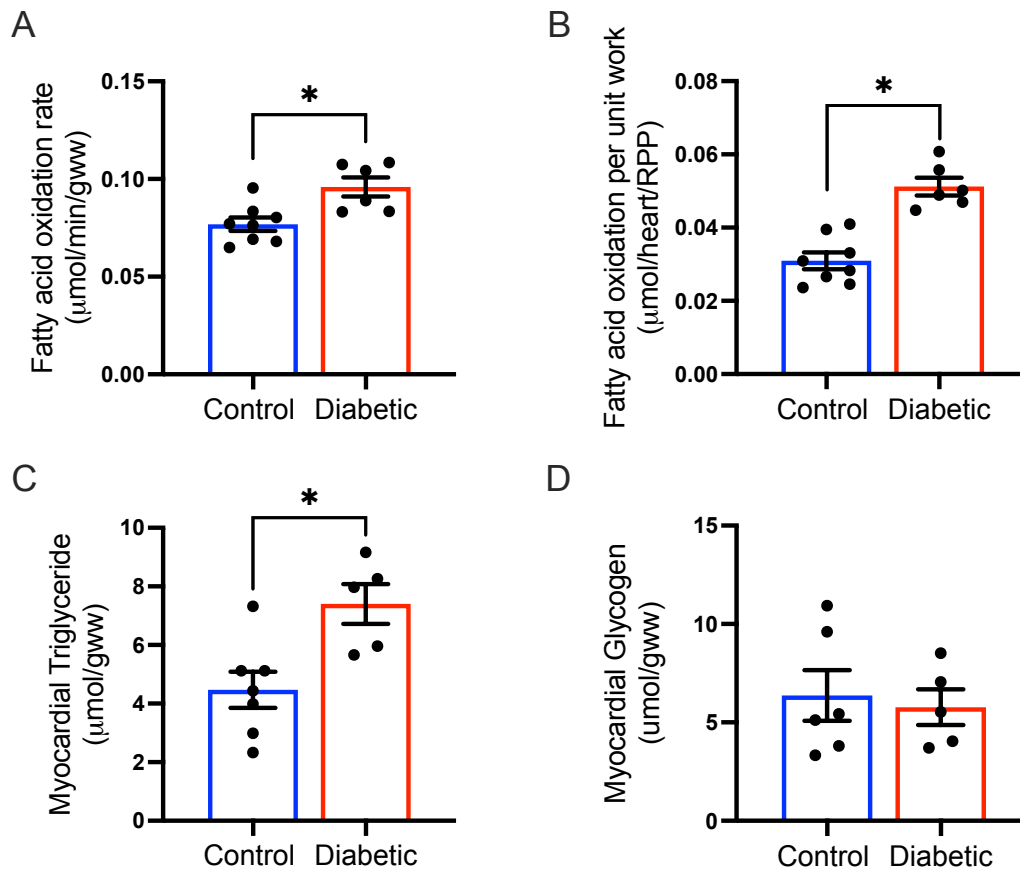


Figure 4.6: Diabetic rats had increased fatty acid oxidation rates (A), fatty acid oxidation rate per unit work (B), myocardial triglyceride concentrations (C), but no change in cardiac glycogen concentrations (D) compared with controls. * $p < 0.05$ between control and diabetic.

In further support of an upregulation in lipid metabolism in hearts from T2D rats, tissue analysis revealed increases in proteins involved in lipid metabolism. As such, the PPAR α targets CD36 and uncoupling protein 3 (UCP3) (Figure 4.7 A-B) were increased 63% and 6-fold, respectively. Protein expression of other PPAR α targets, pyruvate dehydrogenase kinase 4 (PDK4), carnitine palmitoyltransferase-1 (CPT-1), fatty acid binding protein (FABPpm) and medium chain acyl-CoA dehydrogenase (MCAD) did not significantly differ between diabetes and controls (Figure 4.7 F-I). Furthermore, the glucose transporter 4 (GLUT4) was decreased 25% in diabetes (Figure 4.7 C), while the glucose transporter 1 (GLUT1) and lactate dehydrogenase (LDH) protein expression did not change between diabetic and control rat hearts (Figure 4.7 D-G). These results indicate that the T2D hearts are oxidizing more fat, have increased myocardial TG accumulation and have upregulations of key lipid metabolic proteins, indicating a shift towards an increase reliance on lipid metabolism.

4.5.4 Sarcolemmal CD36 is increased in T2D rat hearts

Subcellular fractionation of hearts from control and T2D rats was then performed to look at the subcellular localisation of CD36 and glucose transport proteins. Sarcolemmal content of CD36 had been increased 2.4-fold (Figure 4.8 A) in hearts from T2D rats compared with controls. There was a strong trend towards a decrease in sarcolemmal GLUT4 content ($p=0.0649$ Figure 4.8 B) in diabetes compared with controls. No differences in the sarcolemmal content of the glucose transporter GLUT1 was observed between control and diabetes (Figure 4.8 C). These results indicate that the increase in FA utilization in the T2D may be related to enhanced CD36 at the sarcolemma facilitating an increase in FA uptake into the myocardium.

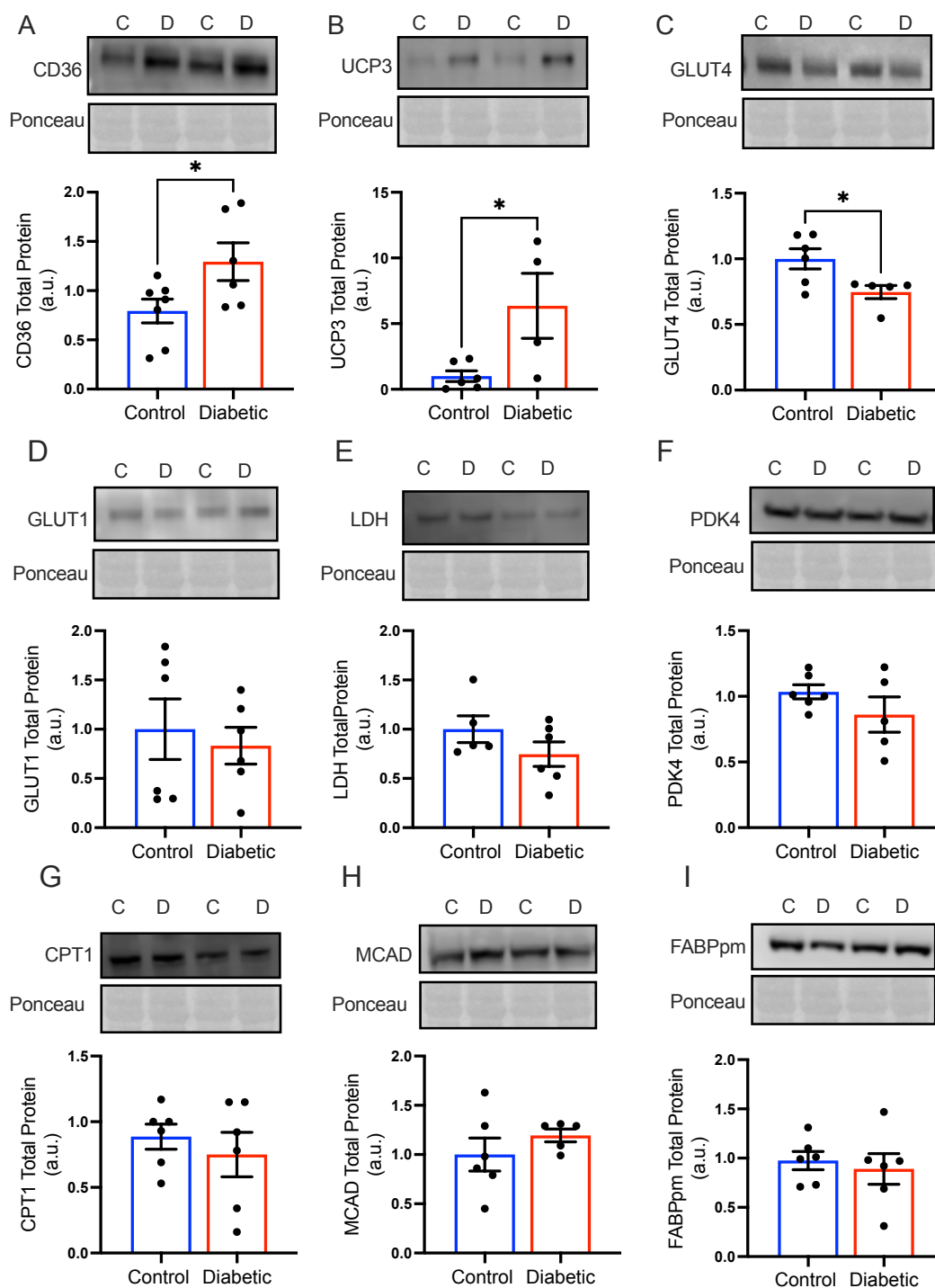


Figure 4.7: Diabetic rats have increased total CD36 (A) UCP3 (B) and decreased GLUT4 (C). There were no changes in FABPpm, CPT1, PDK4, GLUT1 or LDH (D-J) between groups.*p<0.05 between control and diabetic.

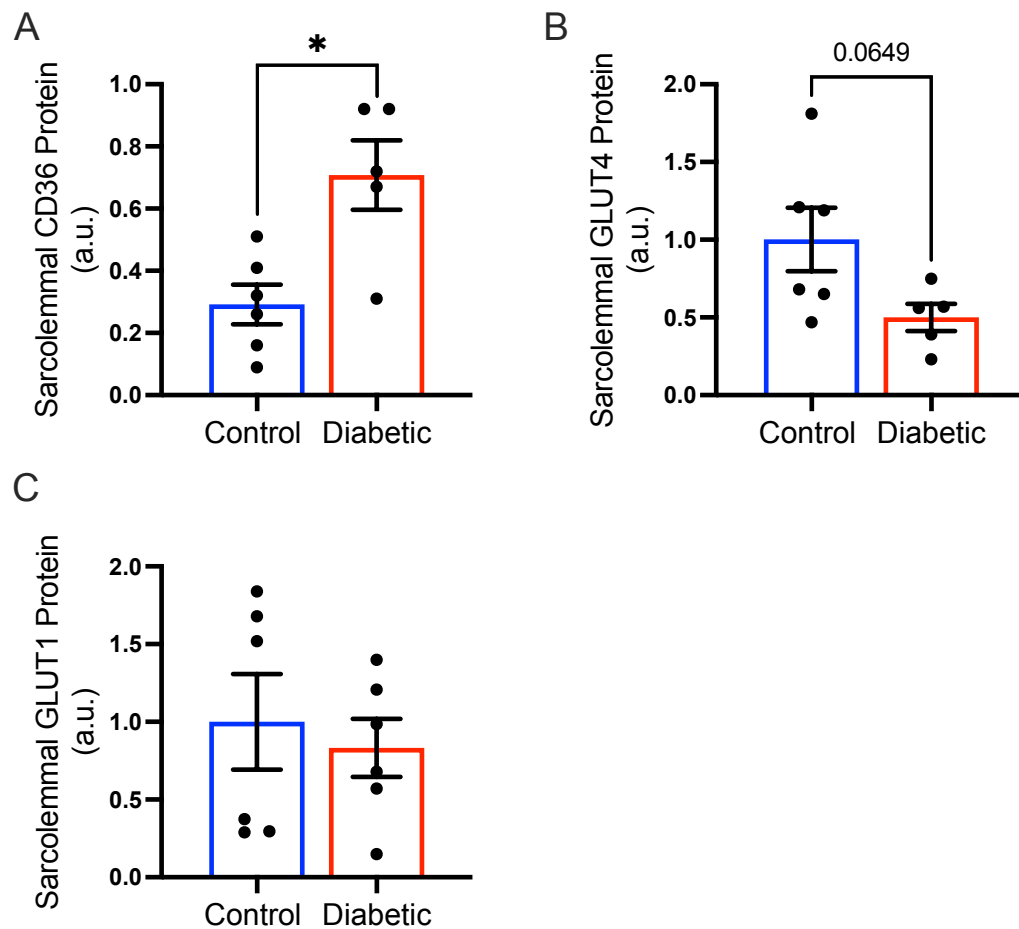


Figure 4.8: Diabetic rats have increased sarcolemma content of CD36 (A), a strong trend towards decreased sarcolemma GLUT4 (B), but no changes in sarcolemma GLUT1 (C). * $p < 0.05$ between control and diabetic. Ponceau staining was used to ensure equal loading.

4.5.5 CD36 is hyper-*S*-acylated in T2D rat hearts

In our T2D rat model in addition to altered cardiac metabolism, there are alterations in the FA transporter CD36; not only is there more CD36 protein content, but more of it is being localised to the sarcolemma. As we have shown that CD36 is *S*-acylated in control rat hearts in Chapter 3, differences in *S*-acylation levels of CD36 between control and diabetes were then explored using acyl-RAC in whole tissue. The reactivity of HA meant that a BCA assay was not feasible to quantify protein loading for western blotting, therefore *S*-acylation levels were normalized to the IF (Figure 4.9 A), which represents the amount of total protein. When

normalized to the amount of CD36 in the IF (which had a 52% increase in diabetes), CD36 *S*-acylation was still increased 2.5-fold in hearts from T2D rats compared to controls (Figure 4.9 B). Therefore, indicating that despite an increase in total CD36, CD36 appears to be *S*-acylated to a greater extent in T2D rat hearts compared with controls. Furthermore, using acyl-PEG, it was determined that CD36 is *S*-acylated on up to four cysteine residues (Figure 4.9 C) in both control and diabetes. These results indicate that CD36 is *S*-acylated to a greater extent in diabetes.

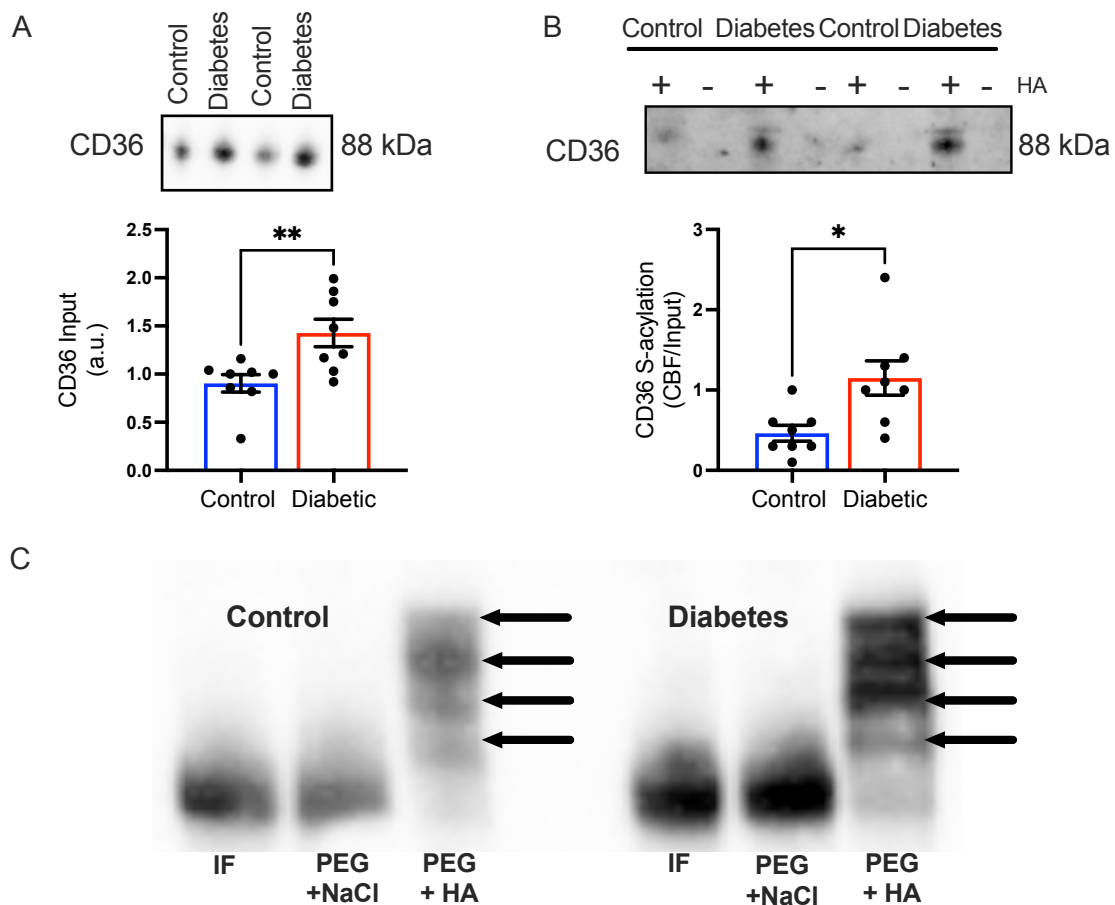


Figure 4.9: Diabetic rats have increased CD36 in the input fraction (A) and greater CD36 *S*-acylation (B) compared with controls. CD36 is *S*-acylated on up to four cysteines in diabetes (C). * $p < 0.05$ between control and diabetic.

4.5.6 Increased CD36 *S*-acylation in T2D mitochondria

Given that CD36 is *S*-acylated to a greater extent in T2D rat hearts at the whole tissue level, mitochondrial CD36 *S*-acylation was investigated in SSM and IFM isolated from T2D hearts. In the T2D SSM population, there was a 2-fold increase in total CD36 protein content (Figure 4.10 A) and 2.2-fold increase in *S*-acylated CD36 when normalized to the input fraction (Figure 4.10 B). In the IFM population, although not statistically significant ($p=0.0870$) total CD36 protein content trended towards an increase in diabetes (Figure 4.10 C), while CD36 *S*-acylation was increased by 70% compared with controls (Figure 4.10 D). Similar to the whole tissue level, despite being normalized to increased total CD36, the amount of CD36 *S*-acylation is still elevated in mitochondria isolated from T2D rat hearts.

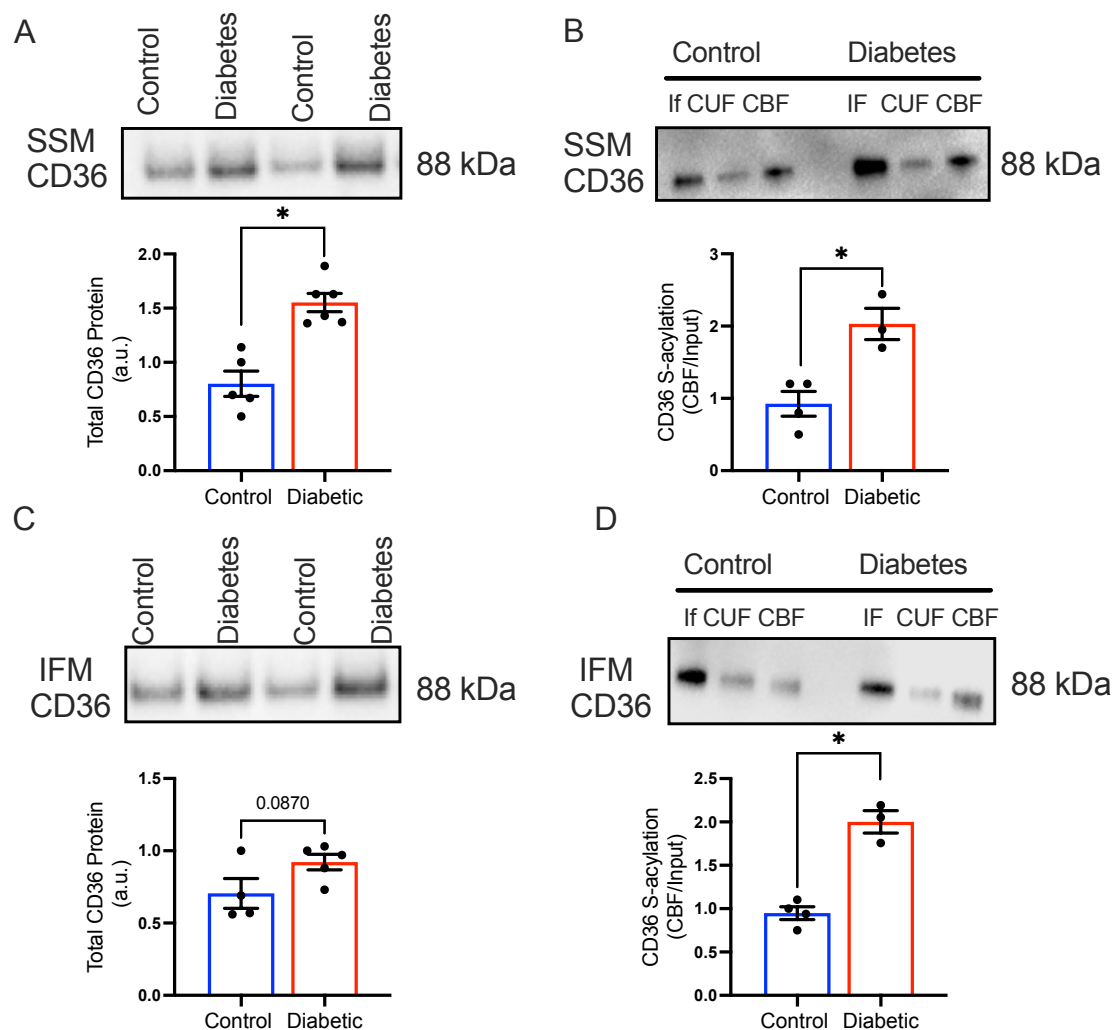


Figure 4.10: T2D rats had increased SSM CD36 total protein (A) and increased SSM *S*-acylated CD36 (B). In diabetes there was a strong trend towards an increase in IFM CD36 total protein (C), and increased IFM *S*-acylated CD36 compared with controls.* $p < 0.05$ between control and diabetic.

4.5.7 Elevated CD36 *S*-acylation in T2D is conserved across species

Whether the increase in CD36 *S*-acylation observed in the T2D rat was conserved across species was next explored by studying diabetic mice, pigs and insulin resistant human cardiomyocytes. In the leptin receptor deficient *db/db* mouse, which models a more severe T2D phenotype, CD36 in the input fraction was increased by 92% (Figure 4.11 A) and CD36 *S*-acylation was found to be increased by 78%, when

normalized to input fraction (Figure 4.11 B). In the diabetic porcine model, no changes were observed in the CD36 input fraction (Figure 4.12 A), with a 46% increase was observed in *S*-acylated CD36 (Figure 4.12 B).

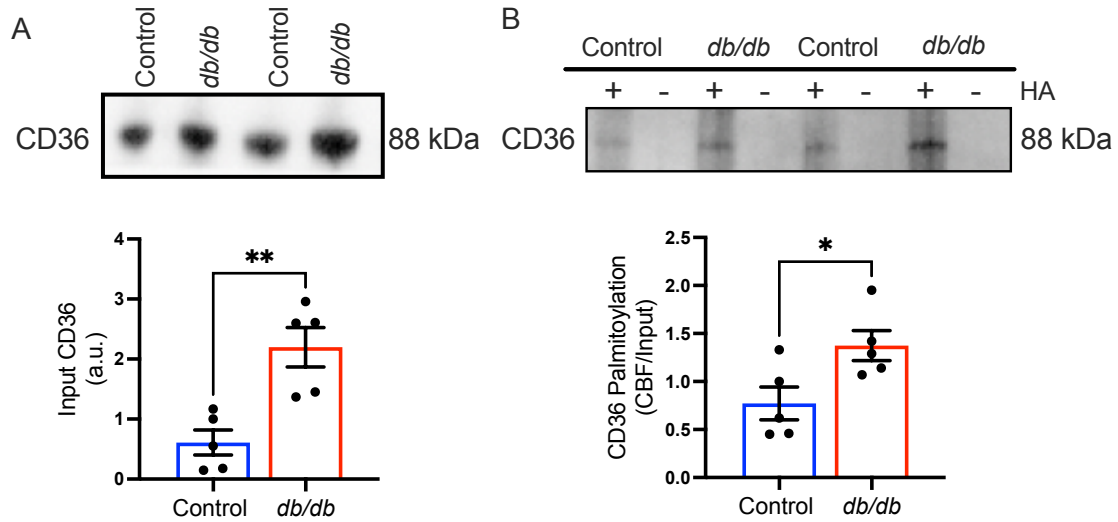


Figure 4.11: Using acyl-RAC, db/db mice had significantly increased CD36 in the input fraction (A) and in CD36 *S*-acylation levels (B). * indicates $p < 0.05$ between control and diabetic.

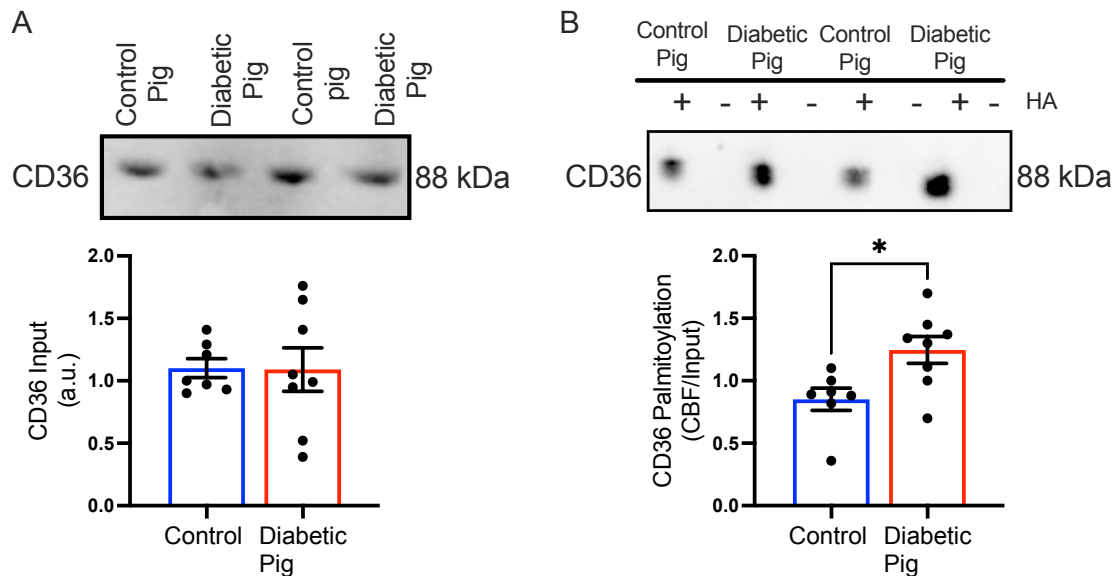


Figure 4.12: Using acyl-RAC, diabetic pigs have a significant increase in CD36 in the input fraction (A) and increased CD36 *S*-acylation levels (B). * $p < 0.05$ between control and diabetic.

Using insulin resistant (IR) human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CM), no changes were observed in the input fraction between control and IR hiPSC-CMs (A), while a 2.5-fold increase CD36 *S*-acylation was observed when compared with controls (B). Collectively, these results therefore suggest an increase in CD36 *S*-acylation in T2D rats, db/db mouse, diabetic porcine and IR hiPSC-CM, suggesting that the increase in CD36 *S*-acylation is conserved between species and often precedes the transcriptional upregulation of CD36 total protein.

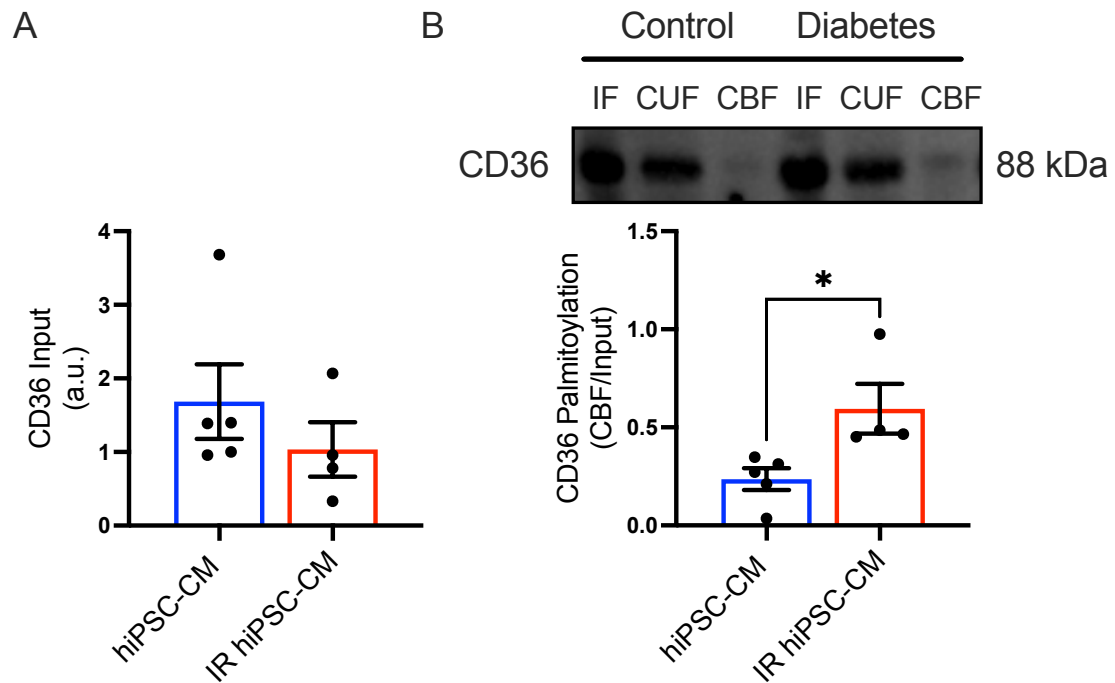


Figure 4.13: Using acyl-RAC, insulin resistant hiPSC-CM had no statistically significant changes in CD36 in the input fraction (A), however CD36 *S*-acylation levels (B) were significantly increased compared with control hiPSC-CM. * $p < 0.05$ between control and IR hiPSC-CM.

4.5.8 CAV3 *S*-acylation is not enhanced in T2D hearts

In contrast to CD36, CAV3 had no alterations in the IF (Figure 4.14 A) or *S*-acylation (Figure 4.14 B) levels between T2D and control rat hearts. Furthermore,

CAV3 was *S*-acylated on six cysteine residues (Figure 4.14 C) in both cohorts.

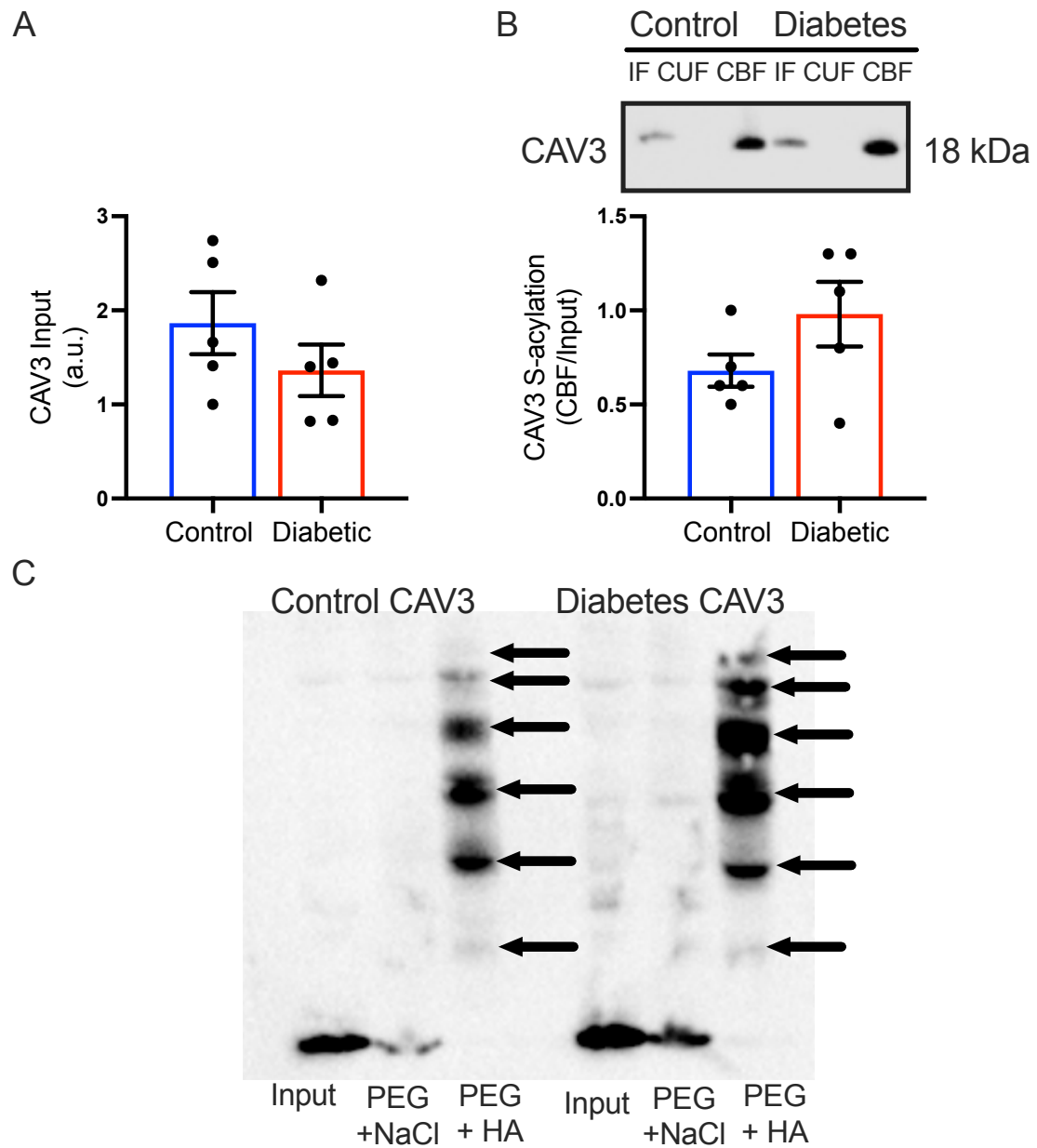


Figure 4.14: Using acyl-RAC, no statistically significant differences in CAV3 in input fraction (A) or CAV3 *S*-acylation (B) in diabetes compared with controls. Acyl-PEG demonstrated that CAV3 is *S*-acylated on up to 6 cysteines in control and diabetic hearts (C).

4.5.9 Other membrane proteins are not *S*-acylated in T2D

In Chapter 3, there was no evidence of *S*-acylation in GLUT4, GLUT1 and FABPpm in control rat hearts, however we questioned whether these proteins would become *S*-acylated in T2D. However, similar to control rat hearts, no *S*-acylation was observed for the glucose transports GLUT4 and GLUT1, as well as the fat transporter FABPpm in diabetes (Figure 4.15 A) as evidenced by no protein in the CBF. Similarly, no evidence of *S*-acylation was observed in T2D hearts for ACAT1, ANT1 or ANT2 in the SSM or IFM populations (Figure 4.15 B-C).

4.5.10 Increased DHHC4 mRNA in T2D rat hearts

The main regulatory enzymes implicated in mediating CD36 *S*-acylation, DHHC4, DHHC5 and de-*S*-acylating APTs are expressed in the heart (Chapter 3). Therefore, changes in the expression of these enzymes were investigated in T2D. Both APT1 and APT2 displayed no statistically significant changes in their mRNA expression between control and diabetes (Figure 4.16 A-B), while DHHC5, which has been implicated in tethering CD36 to the sarcolemma and protecting it from de-*S*-acylation⁹⁰, also had no significant changes in its mRNA expression between T2D and control hearts (Figure 4.16 C). However, DHHC4, which has been implicated in *S*-acylating CD36 at the Golgi to target it for trafficking to the sarcolemma⁷², had a 73% increase in mRNA levels in diabetic hearts compared with controls (Figure 4.16 D).

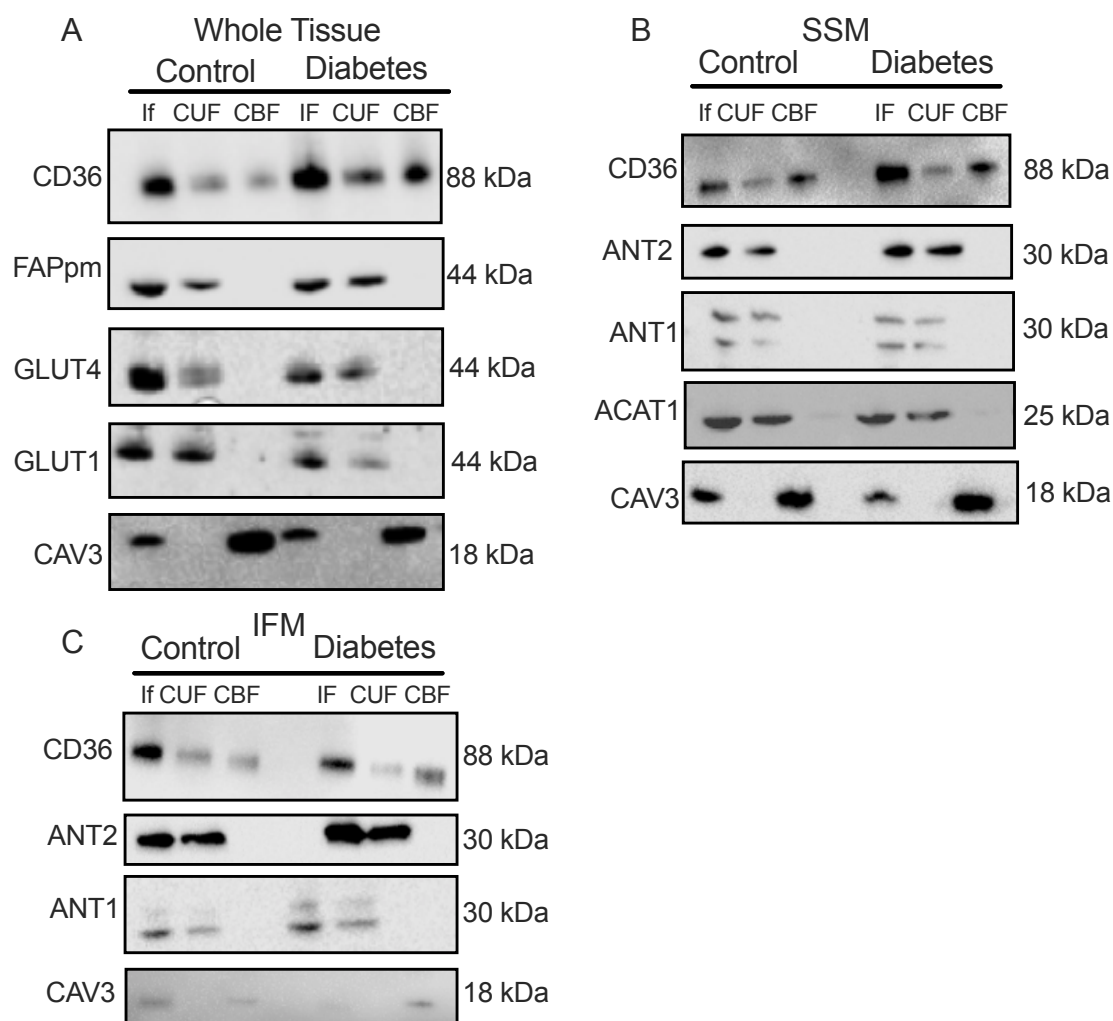


Figure 4.15: T2D rat hearts have increased CD36 *S*-acylation with no evidence of *S*-acylation of FABPpm, GLUT4 or GLUT1 (A) in control or diabetic rat hearts. Similarly, in isolated mitochondria from T2D rats there was no evidence of *S*-acylation of ANT1, ACAT1 or ANT2 in SSM (A) or IFM populations (B) compared with controls. n=7 for control and diabetes.

Total protein levels of the de-*S*-acylating and *S*-acylating enzymes were then explored. Similar to mRNA expression, total protein levels of DHHC5 and APT1 were unchanged in hearts from T2D rat hearts compared with controls (Figure 4.17 B-C). Additionally, mirroring increases in mRNA expression, DHHC4 had a 2.4-fold increase in total protein expression in diabetes compared with controls (Figure 4.17 D). Therefore, DHHC4 is being transcriptionally upregulated in diabetes and is driving an increased capacity for *S*-acylation of its target proteins, such as CD36.

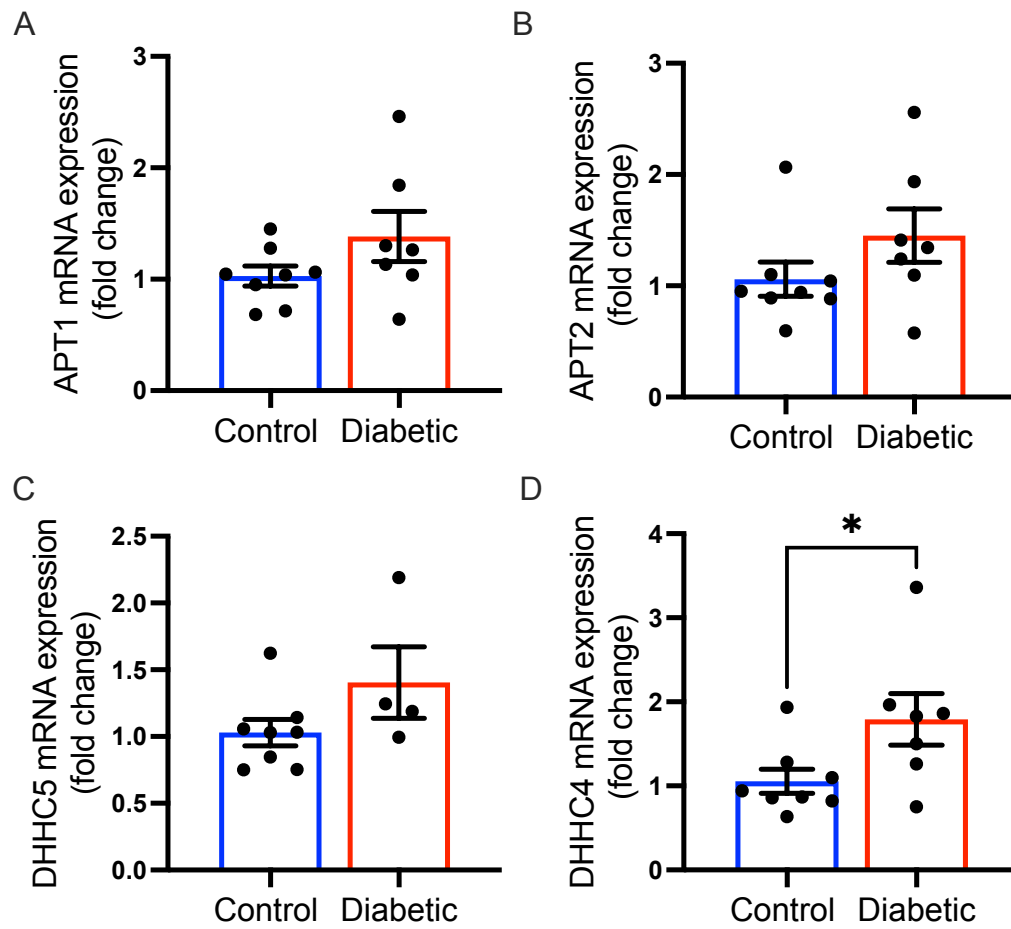


Figure 4.16: No statistically significant changes in APT1 (A), APT2 (B), or DHHC5 mRNA expression (C) between control and diabetic hearts. DHHC4 mRNA (D) is increased in T2D rat hearts compared with controls.* $p < 0.05$ between control and diabetic.

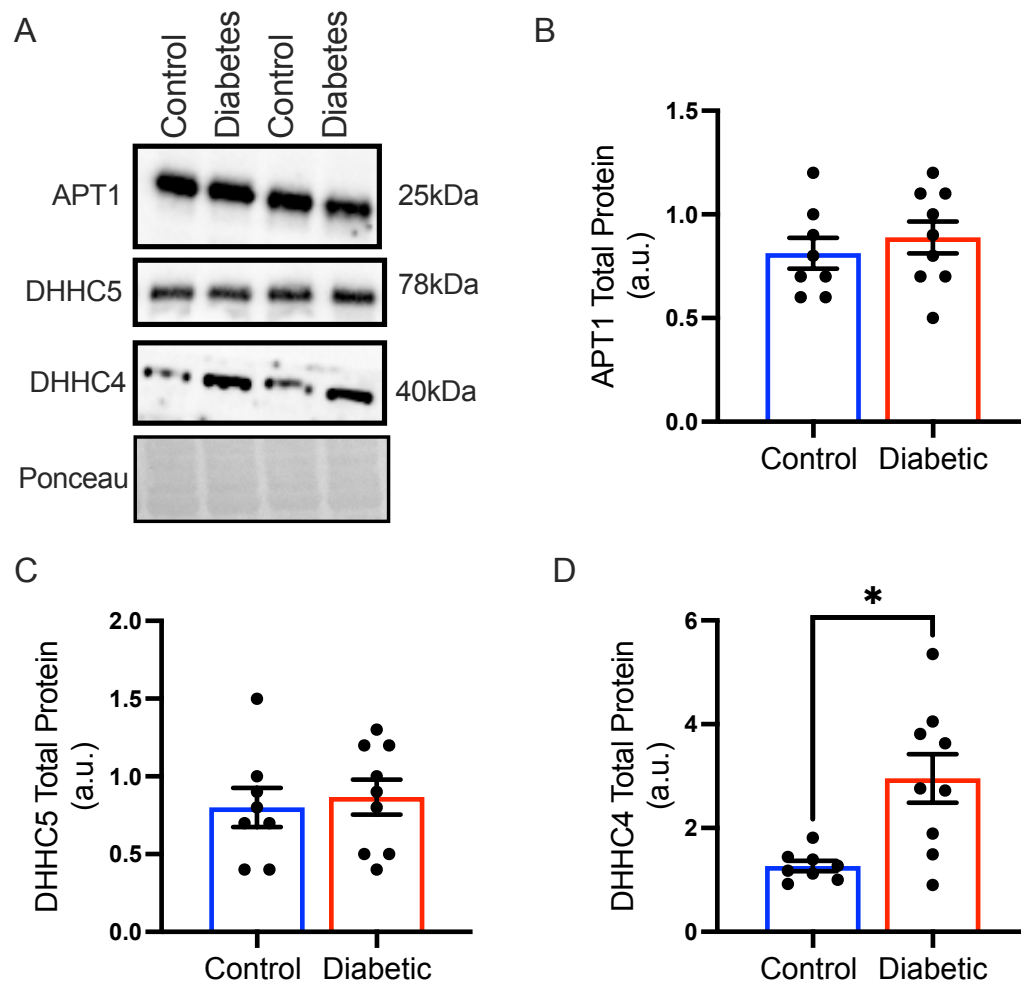


Figure 4.17: Representative western blot images (A). Total protein APT1 (B) and DHHC5 (C) had no statistically differences between cohorts, while DHHC4 total protein had significant increases in diabetes compared with controls (D). * $p < 0.05$ between control and diabetic.

In further support of no changes in DHHC5 total protein or mRNA in diabetes, no statistically significant changes were observed in sarcolemmal DHHC5 (Figure 4.18 A), input fraction (Figure 4.18 B), or *S*-acylation level (Figure 4.18 C).

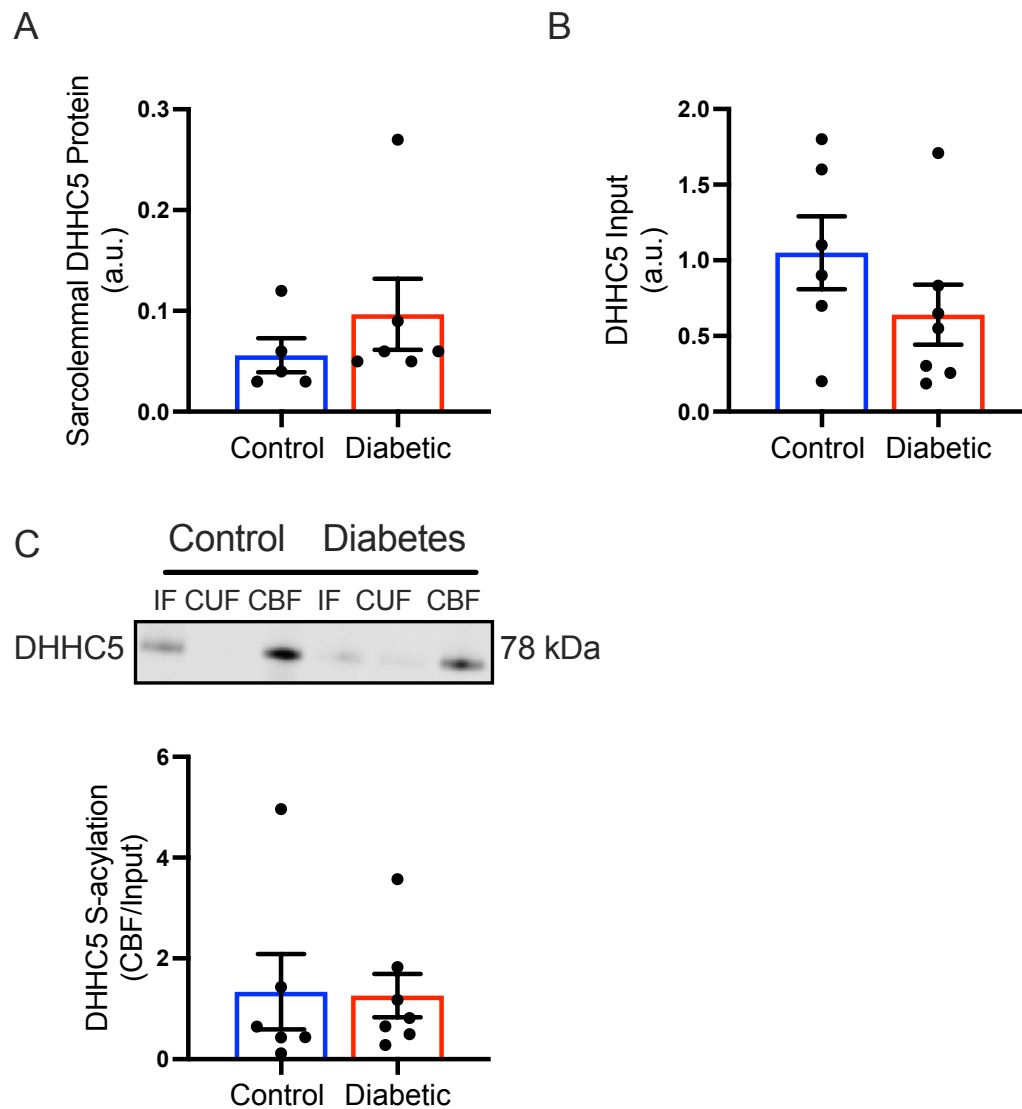


Figure 4.18: Sarcolemma DHHC5 expression (A) as well as DHHC5 IF (B) and *S*-acylation levels (C) had no statistically significant changes between cohorts.

4.6 DHHC4 is a FoxO1 target gene and inhibiting FoxO1 blunts DHHC4 mRNA expression

As we showed that the *S*-acylating enzyme DHHC4 was increased in hearts from T2D rats and mice, the regulatory transcription factors associated with DHHC4 were explored. An *In silico* analysis was conducted by Prof. Sarah De Val first identified the FoxO1 conserved AAACA binding core using JASPAR, an open-access

database of transcription factors binding profiles²⁰³ (Figure 4.19 A).

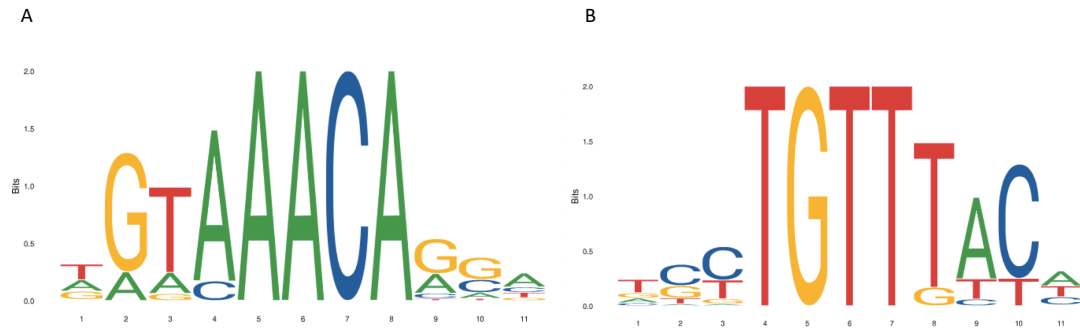


Figure 4.19: JASPAR derived FoxO1 binding motif (A) and the reverse complement (B).

Furthermore, using publicly available mouse heart chromatin immunoprecipitation followed by deep sequencing (ChIP-Seq) data that was deposited into Gene Expression Omnibus (GSE144011)²⁰⁴, demonstrated that DHHC4 contained regions enriched in FoxO1 binding (Figure 4.20 A) as the FoxO1 motif was shown to have a conserved AAACA core within DHHC4. Therefore, these results show that FoxO1 has a binding peak over the promoter region of DHHC4 in mouse heart tissue, indicating that FoxO1 transcriptionally regulates DHHC4.

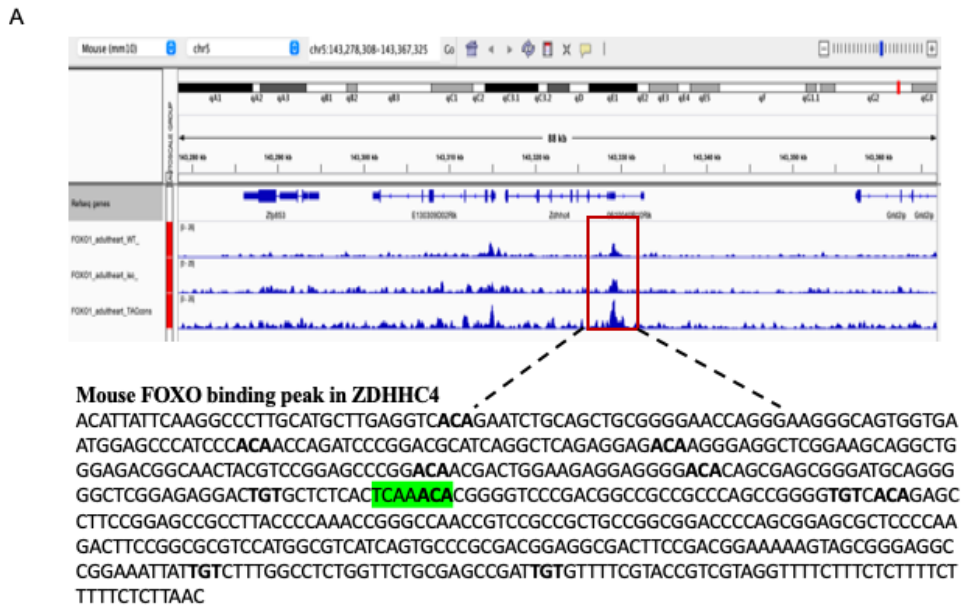


Figure 4.20: Through CHIP-Seq mouse heart data, DHHC4 has a conserved FoxO1 binding site as outlined in green (A).

As FoxO1 has been shown to be increased in T2D^{200,201}, we reached out to collaborators at the University of Alberta who previously characterized cardiac-specific FoxO1 deficient mice (Foxo1 Cardiac -/-) and had used pharmacological agents to inhibit FoxO1 (AS1842856) in experimental T2D¹⁹⁹. Similar to our T2D rat model, DHHC4 mRNA was increased in T2D mice compared to WT controls (Figure 4.21 A). Treating T2D mice with AS1842856, which blocks the transcriptional activity of FoxO1^{195,199}, resulted in DHHC4 mRNA levels decreasing 32% (Figure 4.21 A), with a similar pattern observed in the T2D cardiac-specific FoxO1 deficient mice (Foxo1Cardiac-/-), who displayed a 31% decrease in DHHC4 mRNA (Figure 4.21 A). Therefore, these results indicate that DHHC4 may be a regulatory target of FoxO1, as the increase in DHHC4 mRNA observed in T2D mice is blunted following FoxO1 inhibition.

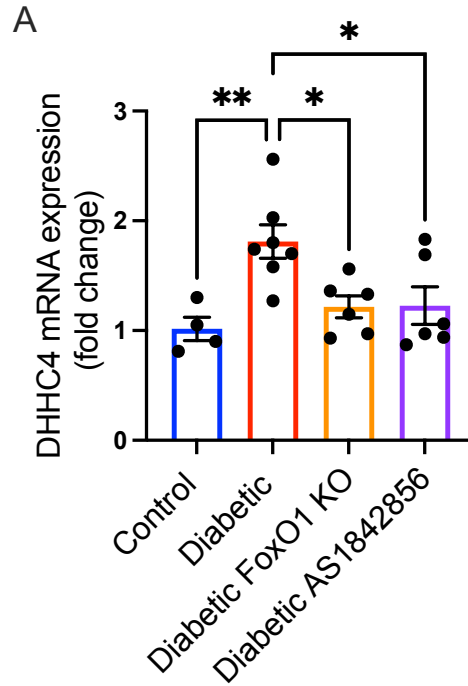


Figure 4.21: DHHC4 mRNA was increased in T2D mice compared with controls, DHHC4 mRNA levels were decreased in cardiac specific FoxO1 KO with experimental T2D. DHHC4 mRNA levels were also decreased compared with control mice in AS1842856 treated T2D mice. * $p < 0.05$ between control vs diabetic. * $p < 0.05$ diabetic vs. diabetic AS1842856 * $p < 0.05$ diabetic vs. diabetic FoxO1 KO.

4.7 Discussion

T2D is a metabolic disorder affecting many organs including the heart and is associated with a pathological systemic overdependence on fatty acid utilization. Our T2D rat model demonstrates hallmarks of the disease after 5 weeks of a HFD combined with a low dose STZ injection, including increase in adiposity as well as elevated insulin, glucose and NEFA concentrations. Furthermore, the metabolic dysregulation extended to cardiac tissue with increases in myocardial FAO rates, myocardial TG accumulation and increases in total protein levels of the PPAR α target UCP3. Our model recapitulates many of the changes in cardiac metabolism identified in patients with T2D⁵². While we demonstrated no systolic cardiac dysfunction in our T2D rat model, a limitation of this current study was not including measurements of dP/dt-min calculated from LVDP in order to investigate

diastolic dysfunction in our model.

4.7.1 Hearts from T2D rats have increased sarcolemmal and total protein CD36

As the main FA transporter in the heart, CD36 is actively involved in governing cardiac lipid metabolism and has been implicated in pathological conditions such as T2D. As such, increases in total CD36 protein have been observed in other rodent T2D models^{24,205,206}. Consistent with these findings, in our T2D rat model, we also observed a significant increase in total CD36 protein content in whole tissue as well as in isolated SSM and IFM populations. Another key perturbation in T2D is the subcellular relocation of CD36 to the sarcolemma, which contributes to an increase in LCFA uptake. Seminal work using skeletal muscle biopsies from obese and T2D patients observed that the increase in LCFA transport rates was associated with an increased retention of CD36 at the sarcolemma⁵⁹. Interestingly, this work also determined that CD36 was the only FA transporter to have enhanced sarcolemmal expression in diabetes and obesity⁵⁹. Further work supported these findings that not only is there more CD36 localised on the sarcolemma, but CD36 was permanently retained on the membrane in skeletal muscle of T2D patients and cultured myotubes from obese patients²⁰⁷. In cardiac tissue, sarcolemmal CD36 was significantly increased in obese Zucker rats^{57,180}, *db/db* mice²⁰⁸ and high fat diet fed rats¹⁷⁹. In line with work in humans and rodents in our T2D rat model, an increase in cardiac CD36 was also observed.

4.7.2 Enhanced CD36 *S*-acylation in diabetes

In addition to an increase in sarcolemma and total CD36 protein content, an increase in CD36 *S*-acylation was found in our T2D rat model at the whole tissue level and in isolated mitochondria. Importantly, this increase in CD36 *S*-acylation was

after normalizing for increases in total CD36 protein content; suggesting that the increase in CD36 *S*-acylation is not as a result of more CD36. Therefore, for each CD36, a greater degree of *S*-acylation is occurring in diabetes, indicating that CD36 is hyper-*S*-acylated. These findings were also replicated in *db/db* mice, a porcine model of diabetes, and IR hiPSC-CMs. These results therefore confirm that the increase in CD36 *S*-acylation is conserved across species with diabetes and insulin resistance. Furthermore, using acyl-PEG, CD36 was shown to be *S*-acylated on up to four cysteines in diabetes and control rat hearts, which has been shown to be critical for CD36 trafficking to the sarcolemma⁷². Interestingly, *S*-acylation not only regulates membrane trafficking of target proteins but enhances membrane affinity through enhanced hydrophobicity⁷⁴. It is plausible that the increase in CD36 *S*-acylation observed in our T2D rat hearts could be the mechanism responsible for driving and maintaining CD36 on the sarcolemma. As such, increases in FAO and myocardial TGs were observed in the hearts from T2D rats, representing an increase in lipid uptake potentially as a result of the increase in CD36 localised to the sarcolemma through *S*-acylation, which has been previously shown in livers of patients and rodents with the metabolic disease NASH¹⁸². However, in those studies it was unclear what was driving the increase in CD36 *S*-acylation.

4.7.3 Elevated DHHC4 in T2D

CD36 *S*-acylation by DHHC4 and DHHC5 has been shown to be required for its trafficking to the sarcolemma and subsequent LCFA uptake in adipocytes⁷². Here we report for the first time that DHHC4 mRNA and total protein levels are increased in diabetes, which suggests a novel mechanism by which *S*-acylation levels are enhanced. As such, in adipocytes, DHHC4 is implicated in *S*-acylating CD36 at the golgi which targets CD36 for trafficking to the sarcolemma⁷². Therefore, it can be postulated that in diabetes, enhanced DHHC4 expression results in enhanced

CD36 *S*-acylation at the golgi and increased sarcolemmal localisation. As our T2D rat model recapitulates early T2D disease progression, it would be of interest to explore alterations in the *S*-acylating and de-*S*-acylating enzymes in a more severe T2D model.

The transcription factor FoxO1 has been implicated in the metabolic dysfunction of the T2D heart^{198,199}. Through work by Prof. Sarah DeVal, DHHC4 was identified as a potential transcriptional gene target of FoxO1. To our knowledge, this is the first study identifying a potential transcriptional regulator of DHHCs. Cardiac FoxO1 is found to be hyperactive in diabetes^{198,199}, and its inhibition through cardiac specific FoxO1 deficient mice (Foxo1Cardiac^{-/-}) was able to correct cardiac metabolism¹⁹⁹. While DHHC4 mRNA was increased in the T2D mice, the cardiac specific FoxO1 deficient mice (Foxo1Cardiac^{-/-}) and AS1842856 treated mice subjected to T2D had blunted DHHC4 mRNA. These results therefore indicate that FoxO1 is driving the increase in DHHC4 in T2D. Interestingly, in FoxO1 cardiac specific FoxO1 deficient mice and following pharmacological Foxo1 inhibition with AS1842856, PDH activity was shown to be increased by decreasing PDK4 expression to increase glucose uptake in T2D^{195,199}. The authors described the concomitant decrease in FAO in the FoxO1 deficient T2D hearts as likely being mediated indirectly via the Randle cycle. However, our data demonstrates that FoxO1 is directly regulating lipid metabolism via DHHC4. In further support of the potential FoxO1-DHHC4-CD36 axis, previous reports have observed that following an increase in FoxO1, there is an increase in CD36 on the sarcolemma despite no changes in total protein or mRNA^{200,201}. Collectively, these results suggest that DHHC4 is a target of FoxO1, and in T2D the observed increases in FoxO1 drives enhanced DHHC4 levels, CD36 *S*-acylation and sarcolemmal localisation.

4.8 Conclusions

In this chapter, we demonstrate that in a T2D rat model there was altered cardiac metabolism with a shift towards an increased reliance on lipid metabolism, which is accompanied by an increase in sarcolemmal and total CD36 protein content. Furthermore, we report an increase in CD36 *S*-acylation levels and enhanced total protein and mRNA levels of the regulatory enzyme DHHC4 in diabetes, mediated by increased FoxO1 activation. This work provides a novel mechanism to explain the metabolic dysfunction in the T2D heart.

5

Pharmacological manipulation of CD36

S-acylation

5.1 Abstract

Increased levels of CD36 *S*-acylation have been implicated in metabolic disease. However, the effect of manipulating CD36 *S*-acylation on downstream metabolism has not been investigated in cardiac tissue. This chapter evaluates the effects of pharmacologically targeting CD36 *S*-acylation on metabolism and function of the *ex vivo* perfused hearts.

To investigate whether acutely increasing palmitate supply to the heart increases CD36 *S*-acylation, control hearts were subjected to *ex vivo* Langendorff perfusion with either high or low palmitate concentrations in the buffer. Compared to low palmitate, CD36 *S*-acylation was decreased by the high palmitate exposure. To investigate if pharmacologically regulating the *S*-acylation DHHCs affects CD36 *S*-acylation, hearts from T2D rats were treated with the DHHC broad spectrum inhibitor CMA. Perfusion with CMA significantly decreased CD36 *S*-acylation, reduced sarcolemmal CD36 protein, accompanied by decreased FAO rates and myocardial TG concentrations, indicating a metabolic shift away from lipid metabolism in diabetes. CMA treatment also enhanced cardiac function compared with untreated T2D hearts, therefore indicating a functional benefit of reducing CD36 *S*-acylation in diabetes. In contrast, hearts from healthy control rats were perfused with the APT1 inhibitor ML348. In ML348 perfused hearts, CD36 *S*-acylation was found to be increased, accompanied by increases in FAO rates and a strong trend towards increased myocardial TG concentrations, demonstrating that enhanced CD36 *S*-acylation shifts cardiac metabolism to increased reliance on FA metabolism. Collectively, these results demonstrate that CD36 *S*-acylation can be pharmacologically manipulated and that alterations in CD36 *S*-acylation have profound effects on cardiac metabolism, further indicating a role for CD36 *S*-acylation in governing cardiac metabolism.

5.2 Introduction

In Chapter 4, CD36 was shown to be hyper-*S*-acylated in diabetes, with increased CD36 localised to the sarcolemma as a result of increased FoxO1-dependent transcription of DHHC4. As such, CD36 *S*-acylation represents a potential novel target to correct the aberrant lipid metabolism in T2D. However, it remains unknown if *S*-acylation levels can be acutely altered in intact beating hearts. As the regulatory enzymes (i.e. DHHCs and APTs) and abundance of palmitoyl-CoA has been shown to regulate *S*-acylation, Chapter 5 investigates CD36 *S*-acylation in response to increases in substrate availability and pharmacological inhibition of DHHCs and APT1 as outlined in Figure 5.1.

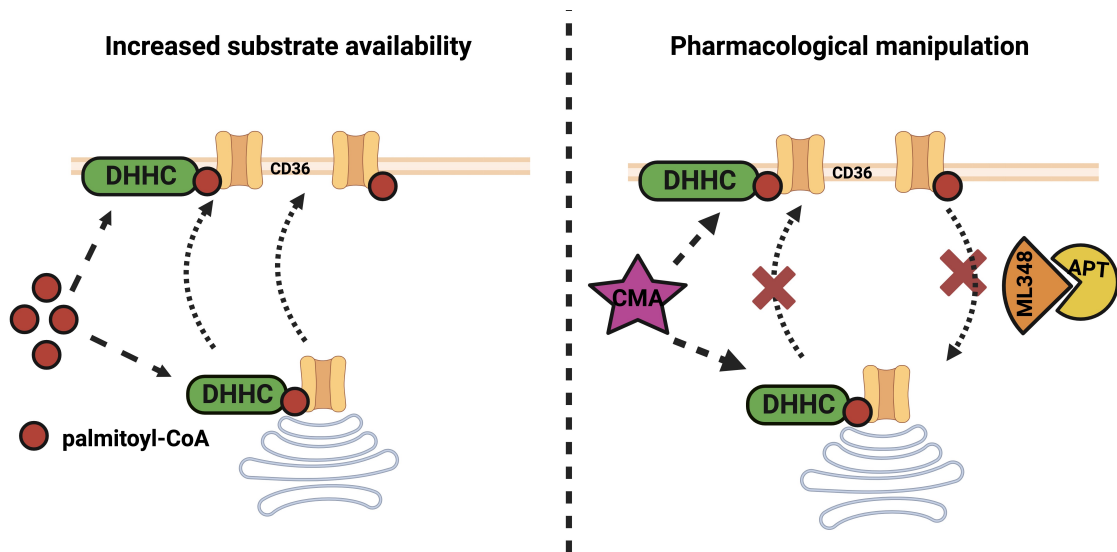


Figure 5.1: Increased substrate availability and pharmacological manipulation as strategies to manipulate CD36 *S*-acylation. Increased palmitoyl-CoA delivery may provide more substrate for the DHHC enzymes and pharmacological inhibition of the DHHCs by CMA and APT1 by ML348.

5.2.1 Regulation of *S*-acylation via non-enzymatic processes

Following the discovery of protein *S*-acylation, unpicking the mechanisms by which palmitoyl-CoA is covalently attached to target proteins has attracted great attention. Initially, it was thought that the majority of *S*-acylation targets were spontaneously

auto-*S*-acylated, therefore relying on non-enzymatic processes regulated by local palmitoyl-CoA concentrations²⁰⁹. As such, an early report in 1996 observed that in the presence of palmitoyl-CoA *in vitro*, the G protein, *Gia1*, was *S*-acylated on Cys3, suggesting a spontaneous auto-*S*-acylation mechanism of action²¹⁰. However, this observation occurred at non-physiological concentrations of palmitoyl-CoA (> 100 μ M)²¹⁰, whereas *in vivo* physiological concentrations of palmitoyl-CoA are tightly regulated. Following the initial discovery of dynamic *S*-acylation cycling²¹¹, pioneering studies in the early 2000s identified families of enzymes that regulate these *S*-acylation cycles^{93,212,213}. The regulation of *S*-acylation has been suggested to potentially occur through both non-enzymatic and enzymatic processes, with the majority of *S*-acylation targets under tight enzymatic regulatory control; DHHCs regulate the *S*-acylation process, while ATPs de-*S*-acylate the target proteins. The mechanism of action for DHHC's is an initial auto-*S*-acylation using palmitoyl-CoA as the substrate, followed by a transfer of the acyl-chain to the target protein. Palmitoyl-CoA levels are known to be increased in pathological conditions such as T2D²¹⁴, which may influence DHHC kinetics to increase *S*-acylation of target proteins. Therefore, understanding whether CD36 *S*-acylation can be enhanced by acute increases in palmitate abundance in intact beating hearts could provide further insight on what is driving the increase in CD36 *S*-acylation observed in diabetes.

5.2.2 Pharmacological manipulation *S*-acylation

Our understanding of the effects of *S*-acylation on target proteins has largely been from genetic DHHC knock-out models, as well as using specific cysteine-site mutagenesis. While beneficial, genetic manipulation can often result in compensatory upregulation of proteins, which can make the interpretation of results difficult, whereas pharmacological manipulation provides a more rapid assessment

with minimal compensatory alterations. However, pharmacological manipulation of *S*-acylation has been far less explored due to difficulties in the development of selective and non-toxic pharmacological inhibitors⁸⁸.

5.2.3 α -Brominated fatty acid 2-bromopalmitate (2BP)

To date, the most common small molecule tool to perturb *S*-acylation events is the lipid based covalent inhibitor 2-BP⁸⁸. 2-BP has been hypothesized to work as an irreversible pan DHHC inhibitor by inactivating the catalytic site through cysteine alkylation and nucleophilic displacement²¹⁵. However, proteomic studies have identified that 2-BP has a high degree of promiscuity and displays a large degree of off-target activity^{216,217}. As such, it was shown that 2-BP has hundreds of targets and 99% of its targets have been shown to not be DHHCs^{95,216–218}. Therefore, following treatment with 2-BP, it is hard to discern if outcomes are as a result of direct loss of *S*-acylation of a specific target or one of the various other targets affected. Additionally, 2-BP has been shown to inhibit both APT1 and APT2, therefore dissecting the specific role for DHHC-mediated *S*-acylation inhibition is not possible²¹⁸. Furthermore, the 2-BP-CoA adduct has been shown to have enhanced reactivity with the proteome²¹⁶ and as such, has been shown to alter lipid metabolism, including inhibiting mitochondrial FAO independently of any DHHC interaction²¹⁹. In conjunction with a high degree of promiscuity, 2-BP also displays poor potency and toxicity as it is frequently used above its IC₅₀⁸⁸. Other *S*-acylation lipid-based inhibitors have been reported (i.e. cerulenin, tunicamycin and compound V), however similar to 2-BP, these chemical tools display high degrees of toxicity, poor selectivity and additionally, haven't been well characterized in the field^{215,217,220}. Therefore, 2-BP represents a poor chemical tool for perturbing *S*-acylation through DHHC inhibition⁸⁸.

5.2.4 *N*-cyanomethyl-*N*-myracrylamine (CMA)

As 2-BP has many drawbacks⁸⁸, a new broad-spectrum DHHC inhibitor CMA, which consists of C₁₄ aliphatic chain and an *N*-cyanomethyl group, was developed¹³⁹. CMA functions both *in vitro* and *in cellulo* and while not perfect, is an alternate chemical tool that has overcome key issues that plague 2-BP¹³⁹. The CMA compound shares a similar inhibiting mechanism as 2-BP, whereby the nucleophilic cysteine is targeted by electrophiles, however the α -halo carbonyl of 2-BP was swapped out for an acrylamide warhead allowing for faster reaction with cysteine thiols compared with serine alcohols¹³⁹. Furthermore, by swapping the carboxylate of 2-BP with an acrylamide warhead, CMA will have limited conversion into reactive and non-specific acyl CoA intermediates^{139,216}. CMA was shown to inhibit DHHC20, a well know 2-BP target, with higher potency and limited toxicity in comparison to 2-BP¹³⁹. Importantly, CMA has been shown to not inhibit APT1 or APT2 *in vitro*¹³⁹, suggesting that CMA has improvements on the crucial limitations of 2-BP. CMA also displays a similar DHHC labelling spectrum as 2-BP and has been shown to engage DHHCs 2,4,5,6,9,11,13,14, 15,16,18,20,23 and 24¹³⁹. Furthermore, CMA treatment decreased CD36 *S*-acylation in 3T3-L1 preadipocytes resulting in reduced FA uptake and lipid droplet storage¹³⁹, consistent with previous findings observed following the knockdown of DHHC4 and DHHC5⁷². Although still with limitations and potential off target effects, CMA confers benefits over 2-BP as it has reduced toxicity and increased potency, which will allow for better monitoring of the dynamics of *S*-acylation on targets such as CD36, without perturbing APT mediated de-*S*-acylation.

5.2.5 ML348

Another method to manipulate *S*-acylation is through inhibiting de-*S*-acylation by APT1. Several inhibitors of APT1 have been described, however they failed to

selectively inhibit APT1 over APT2, and failed to demonstrate APT1 inhibition in animal models^{127,221}. In search of more selective inhibitors, a high-throughput screen using a competitive activity-based fluorescence polarization assay was employed to identify a selective and *in vivo* active APT1 inhibitor²²², which was later optimized to the piperazine amide ML348²²³. ML348 was confirmed to be a potent reversible inhibitor with high selective target engagement *in vitro*, *in situ* and *in vivo* using kinetically tuned Activity-Based Protein Profiling (ABPP)²²³. Furthermore, ML348 was shown to maintain isoform selectivity for APT1 even at concentrations nearing inhibitor solubility ($>10\ \mu\text{M}$)^{222,223}. The orthogonal inhibition of ML348 was determined by a high-resolution structure of APT1 bound to ML348. This demonstrated that the trifluoromethyl group of ML348 was positioned above the catalytic triad of APT in the putative acyl binding region^{223,224} of the hydrophobic channel⁸⁸ whereby the piperazine amide of ML348 forms a hydrogen bond with the G3 Arg149 in APT1⁸⁸. Therefore, ML348 is a validated pharmacological tool to probe the functions of de-*S*-acylation through selective APT1 inhibition. However, despite the *in vivo* bioavailability, very few studies have employed ML348 for *in vivo* research and to date, none have explored the use of ML348 in ex vivo perfused rat hearts.

5.3 Aims

Enhanced CD36 *S*-acylation was observed in hearts from models of T2D and was accompanied by altered lipid metabolism. This chapter proposes that by altering levels of CD36 *S*-acylation, downstream cardiac metabolism will be influenced, demonstrating that CD36 *S*-acylation is a key regulatory step governing cardiac metabolism. Therefore, the aims of this chapter in *ex vivo* Langendorff perfused hearts were:

1. Investigate if CD36 *S*-acylation is enhanced by an acute increase in palmitate supply.
2. Determine if CD36 *S*-acylation can be pharmacologically inhibited in diabetes using the DHHC inhibitor CMA, and investigate the downstream metabolic consequences.
3. Investigate the effect of pharmacological inhibition of APT1 using ML348 on CD36 *S*-acylation and downstream metabolism in healthy control hearts.

5.4 Methods

5.4.1 Low fat and high fat perfusion

Healthy control rat hearts underwent Langendorff perfusions following the protocol set out in section 2.10.4.

5.4.2 N-cyanomethyl-N-myrcylamine (CMA) perfusions

Diabetic rat hearts underwent Langendorff perfusions following the protocol set out in section 2.10.5.

5.4.3 ML348 perfusions

Healthy control rat hearts underwent Langendorff perfusions following the protocol set out in section 2.10.5.

5.5 Results

5.5.1 Acute increase in the abundance of palmitate does not increase CD36 *S*-acylation

In T2D hearts, what is driving the increase in CD36 *S*-acylation remains unknown. As palmitoyl-CoA is the main substrate for *S*-acylation and there are increased levels of palmitoyl-CoA in T2D²¹⁴, whether an acute increase in the availability of palmitate would drive an increase in CD36 *S*-acylation was explored. Healthy rat hearts were perfused with low (0.2mM) and high (1.2mM) palmitate for an 1 hr.

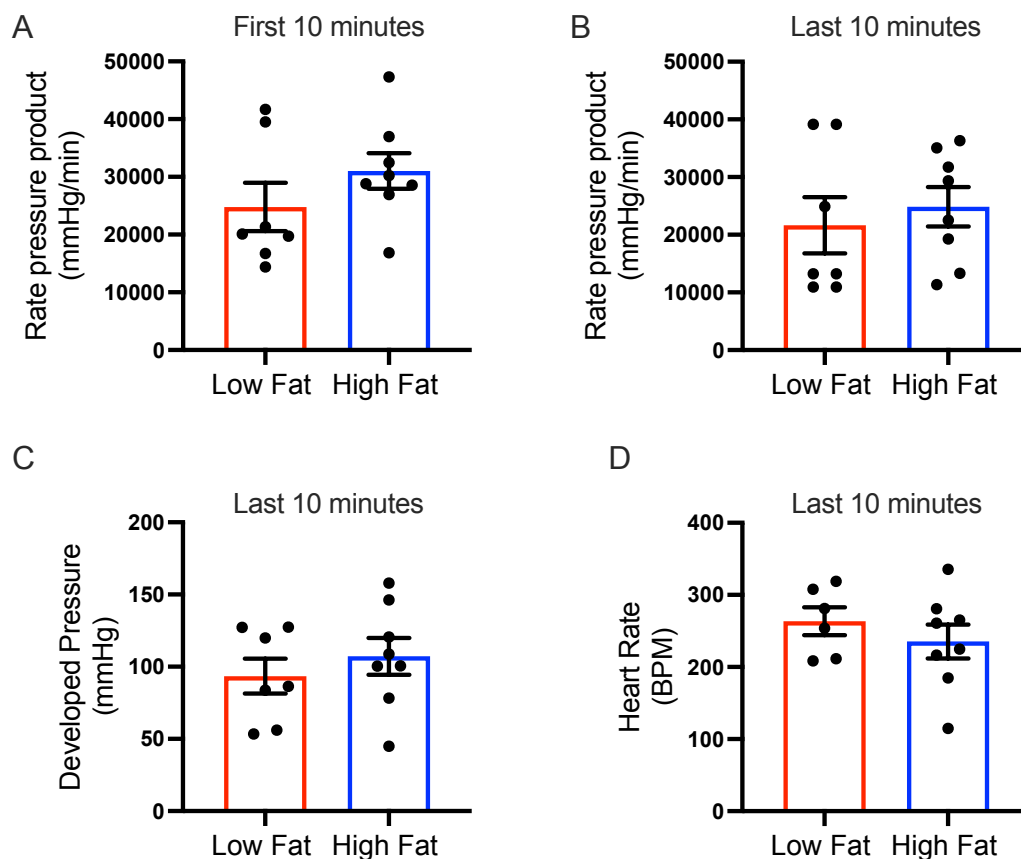


Figure 5.2: RPP in hearts from low fat and high fat perfused groups had no statistically significant changes in function in the first (A) and last ten mins (B), with no changes in DP (C) or HR (D).

Hearts from both low and high fat perfused groups had no changes in RPP in the first 10 mins (Figure 5.2 A), or the last ten mins (Figure 5.2 B). No changes were also observed in DP (Figure 5.2 C) and HR (Figure 5.2 D). Fatty acid oxidation rates were increased 3.8-fold in the high fat perfused group as expected (Figure 5.3 A), but there were no changes in myocardial TG concentration (Figure 5.3 B).

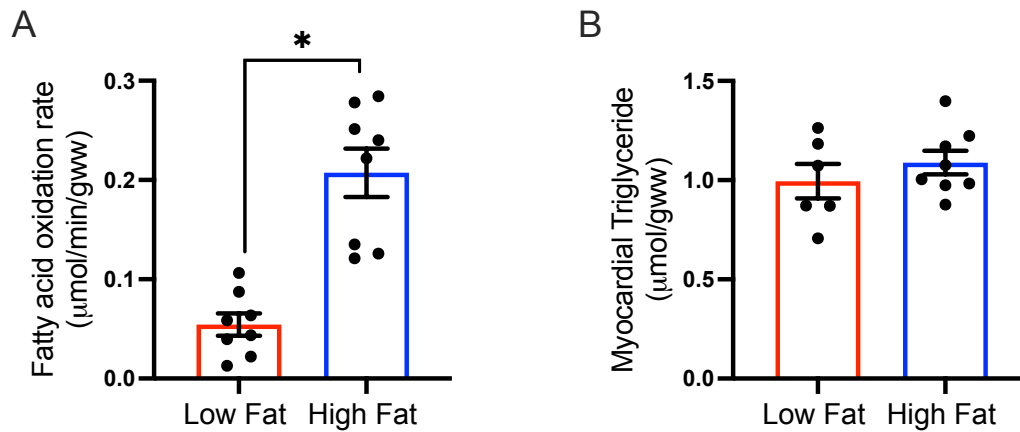


Figure 5.3: Fatty acid oxidation rates were increased in high fat perfused hearts compared with low fat (A). No statistically significant changes in myocardial TG concentrations were observed between groups (B) *p < 0.05 between low fat and high fat groups.

As expected given the one hour duration of fat exposure, no differences between groups was observed in CD36 input (Figure 5.4 A), indicative of total protein. However, CD36 *S*-acylation was decreased 35% in the high fat perfusion group (Figure 5.4B) compared with the low fat perfused group. CAV3 input (Figure 5.4 C) and CAV3 *S*-acylation (Figure 5.4 D) remained unchanged between groups. Collectively, these results indicate that CD36 *S*-acylation is decreased in *ex vivo* perfused hearts following an increase in palmitate exposure, demonstrating that acute abundance of palmitate does not drive increases in CD36 *S*-acylation. This suggests that in our T2D rat model that the alterations in the DHHC4 protein abundance may be responsible for the increase in CD36 *S*-acylation observed, rather than the supply of substrate to the DHHCs.

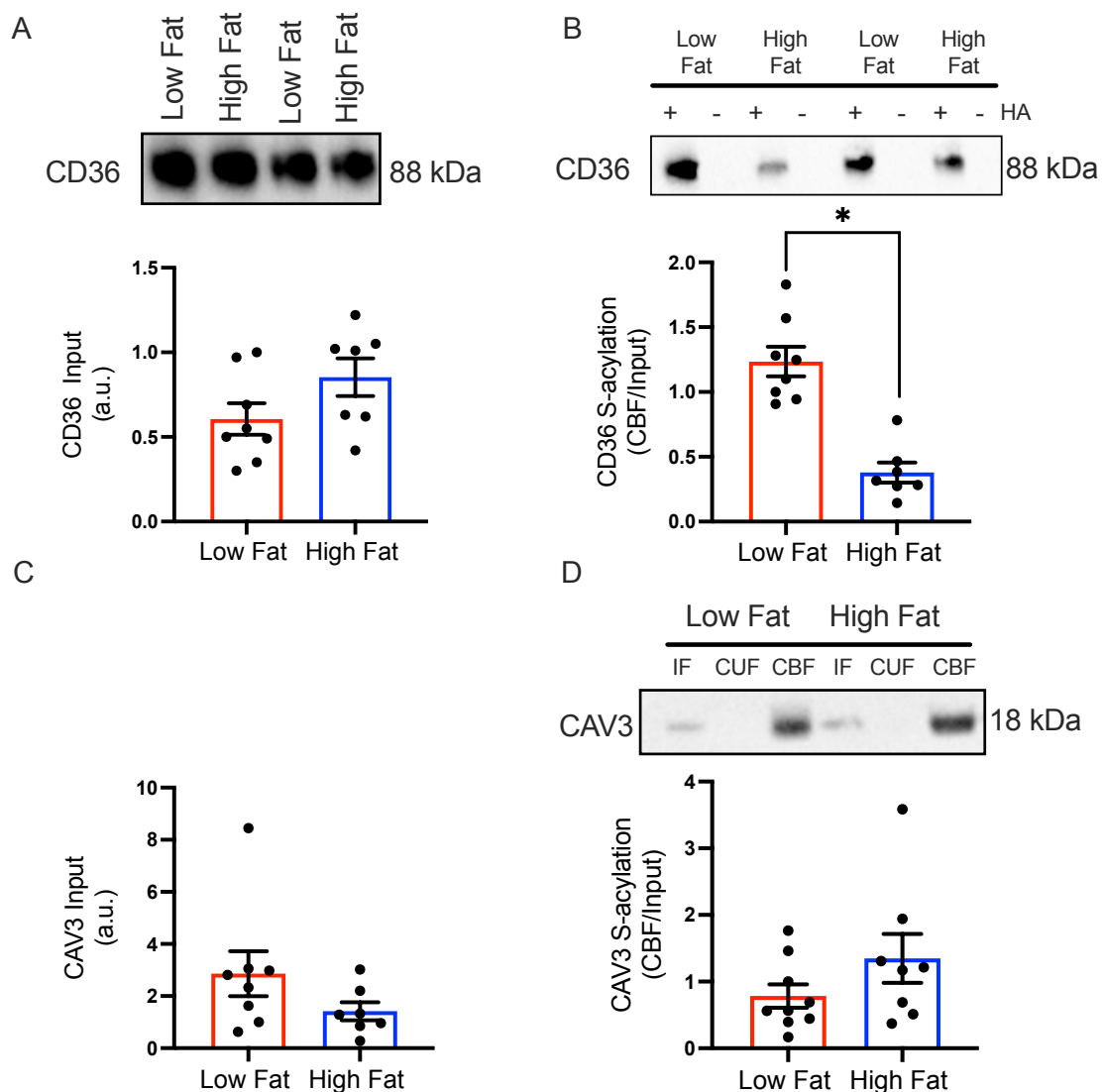


Figure 5.4: Using acyl-RAC, CD36 in the input fraction was unchanged between low fat and high fat perfusions (A) with a decrease in CD36 *S*-acylation in high fat perfused hearts compared with low fat perfused groups(B), with no statistically significant changes in CAV3 input (C) or *S*-acylation (D) between groups.*p<0.05 between low fat and high fat perfused hearts.

5.5.2 CMA decreases CD36 *S*-acylation in control hearts

Given that DHHC4 expression was increased in diabetes, whether pharmacologically inhibiting the DHHCs in *ex vivo* hearts would have an effect on CD36 *S*-acylation was explored as an initial pilot study. Hearts from healthy control rats were perfused with 100 μ M (dissolved in 100 μ L of DMSO) of CMA for 1 hr, to assess the effects on

cardiac function, CD36 *S*-acylation and downstream metabolic markers. Treatment with CMA had no effect on cardiac function in the first (Figure 5.5 A) or last 10 mins (Figure 5.5 B) compared with untreated controls. Furthermore, DP (Figure 5.5 C) and HR (Figure 5.5 D) were unchanged between groups, demonstrating that CMA was not deleterious to the function of *ex vivo* perfused hearts. Following

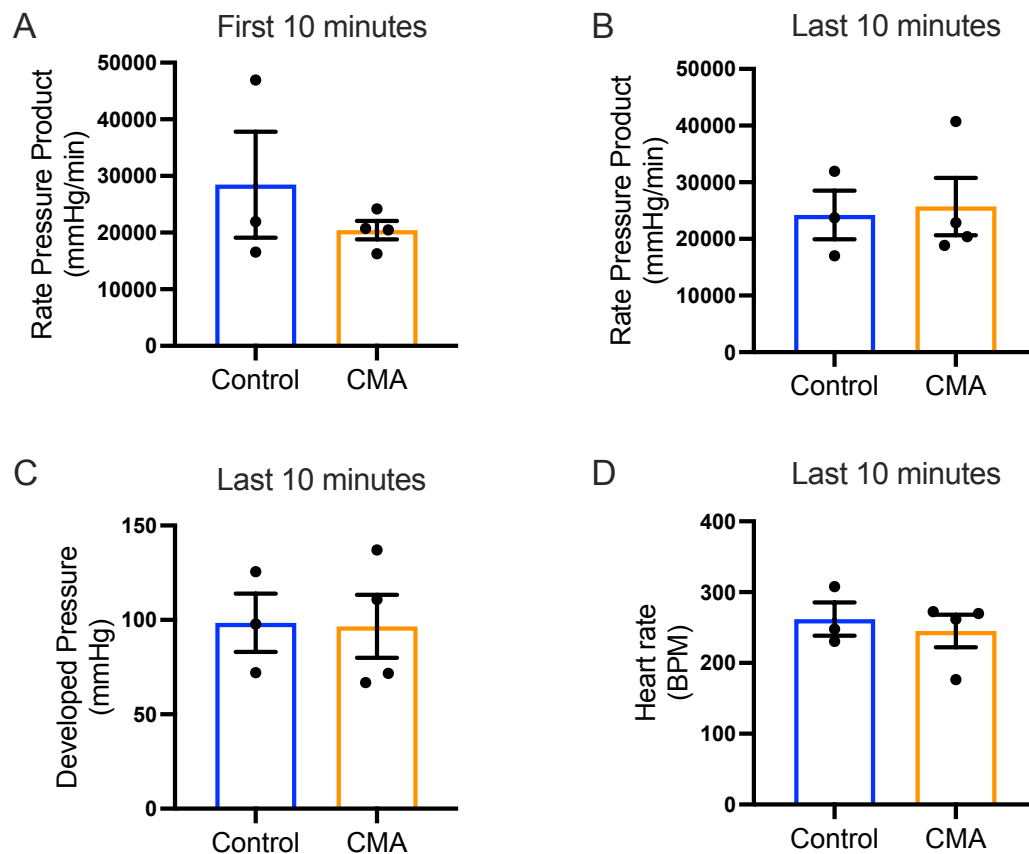


Figure 5.5: CMA Pilot study-hearts from control hearts with CMA or control vehicle had no statistically significant changes in rate pressure products in the first 10 mins (A) or last 10 mins (B). No statistically significant differences between heart rate (C) or developed pressure (D) were found between groups.

treatment with CMA, no changes were observed in the CD36 input fraction (Figure 5.6 A). However, CD36 *S*-acylation was decreased by 44% (Figure 5.6 B) following CMA treatment compared with untreated hearts. Therefore, CMA can effectively decrease CD36 *S*-acylation in *ex vivo* perfused hearts. In contrast, no changes were observed in CAV-3 *S*-acylation or input fraction (Figure 5.6 C-D).

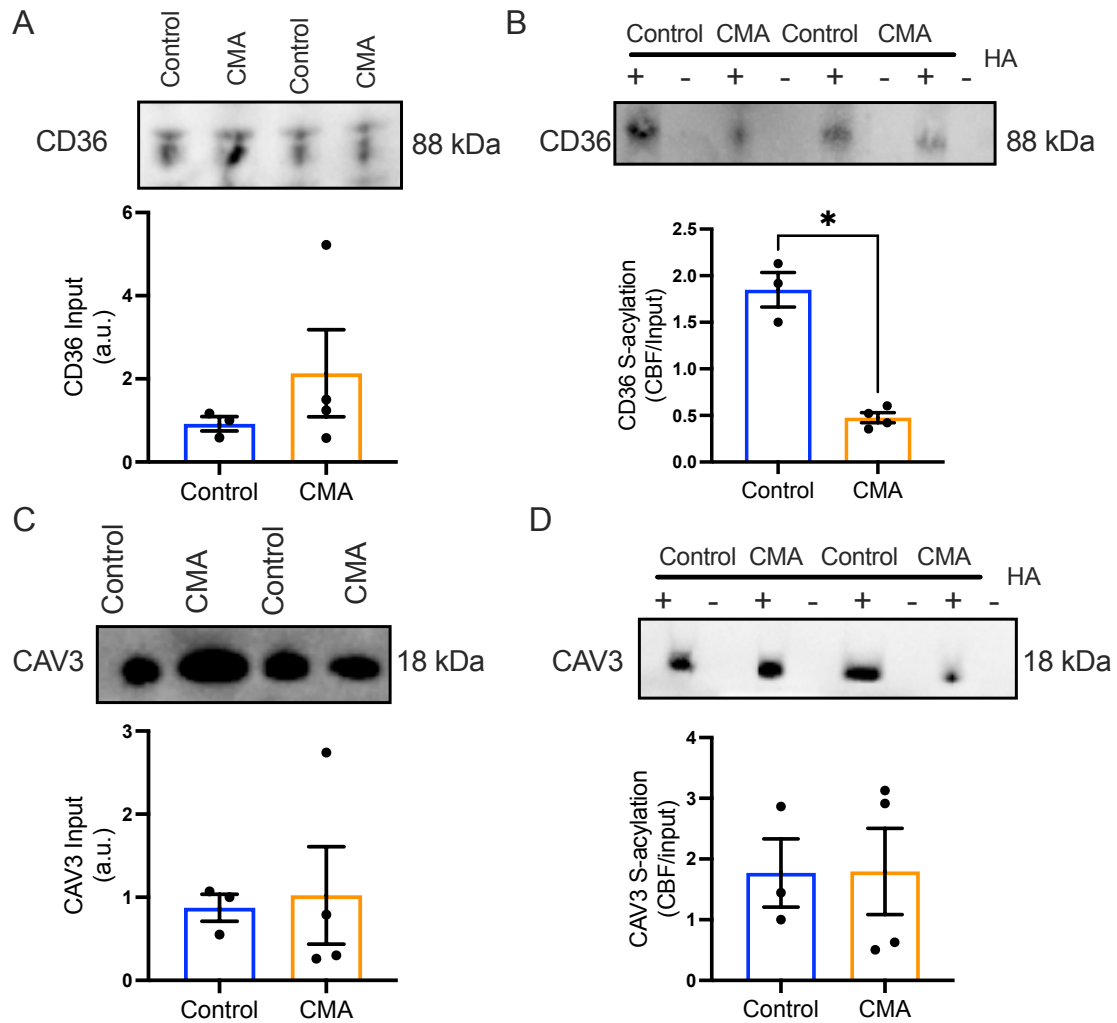


Figure 5.6: Using acyl-RAC, no statistically significant differences were seen between groups for CD36 input fraction (A), however CD36 *S*-acylation was significantly decreased in the CMA treated group compared with the untreated hearts. In contrast, no statistically significant changes were observed in CAV3 input fraction (A) or CAV3 *S*-acylation (B) between groups.* $p < 0.05$ between control and CMA treated groups.

In addition to altering CD36 *S*-acylation, myocardial TG concentrations were decreased 40% (Figure 5.7 A) and lactate efflux rates had a potential trend towards an increase ($p = 0.1490$), however there were low sample numbers ($n = 2$) for the CMA treated group (Figure 5.7 B) as this was a pilot study and we only had a limited amount of CMA. Therefore, perfusing healthy control rat hearts with CMA had no adverse effects on cardiac function, but decreased myocardial TG concentrations and decreased CD36 *S*-acylation. These data suggest that by decreasing *S*-acylation

of CD36, there is a shift away from lipid metabolism, which would be beneficial in T2D where an over-dependence on lipid metabolism is observed.

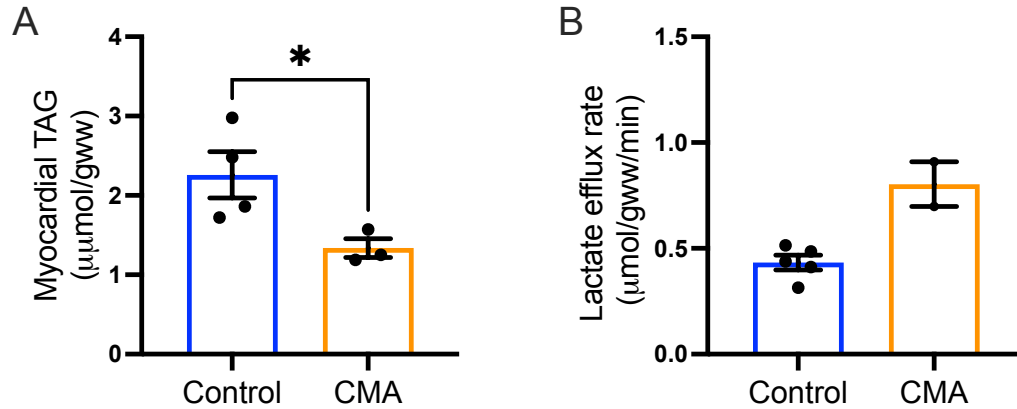


Figure 5.7: Myocardial TG concentrations were decreased in hearts perfused with CMA compared to untreated controls (A) whereas no statistical differences were observed in lactate efflux (B). * $p < 0.05$ between untreated and CMA treated hearts.

5.5.3 CMA decreases CD36 *S*-acylation in T2D rat hearts

Given the increase in CD36 *S*-acylation in hearts from T2D rats and that CD36 *S*-acylation can be reduced using CMA, the effect of CMA on CD36 *S*-acylation was next explored in diabetes. Hearts from T2D rats were excised and perfused in the presence or absence of CMA for 1 hr, following a 10-minute stabilization period. In diabetic hearts, treatment with CMA for 1 hr during the Langendorff perfusion resulted in a 34% decrease in CD36 *S*-acylation levels (Figure 5.8 B), with no changes in the CD36 input fraction compared to untreated diabetic hearts (Figure 5.8 A). Similar to the pilot study, no changes were observed in CAV3 input fraction or *S*-acylation levels (Figure 5.8 C-D) between groups. Therefore, it appears that CMA does not affect CAV3 *S*-acylation in control or in T2D hearts.

In addition to the decrease in CD36 *S*-acylation levels, CD36 sarcolemma localisation was found to be decreased by 24% following CMA treatment of diabetic hearts (Figure 5.9 A), indicating that less of the total CD36 pool was localised to the

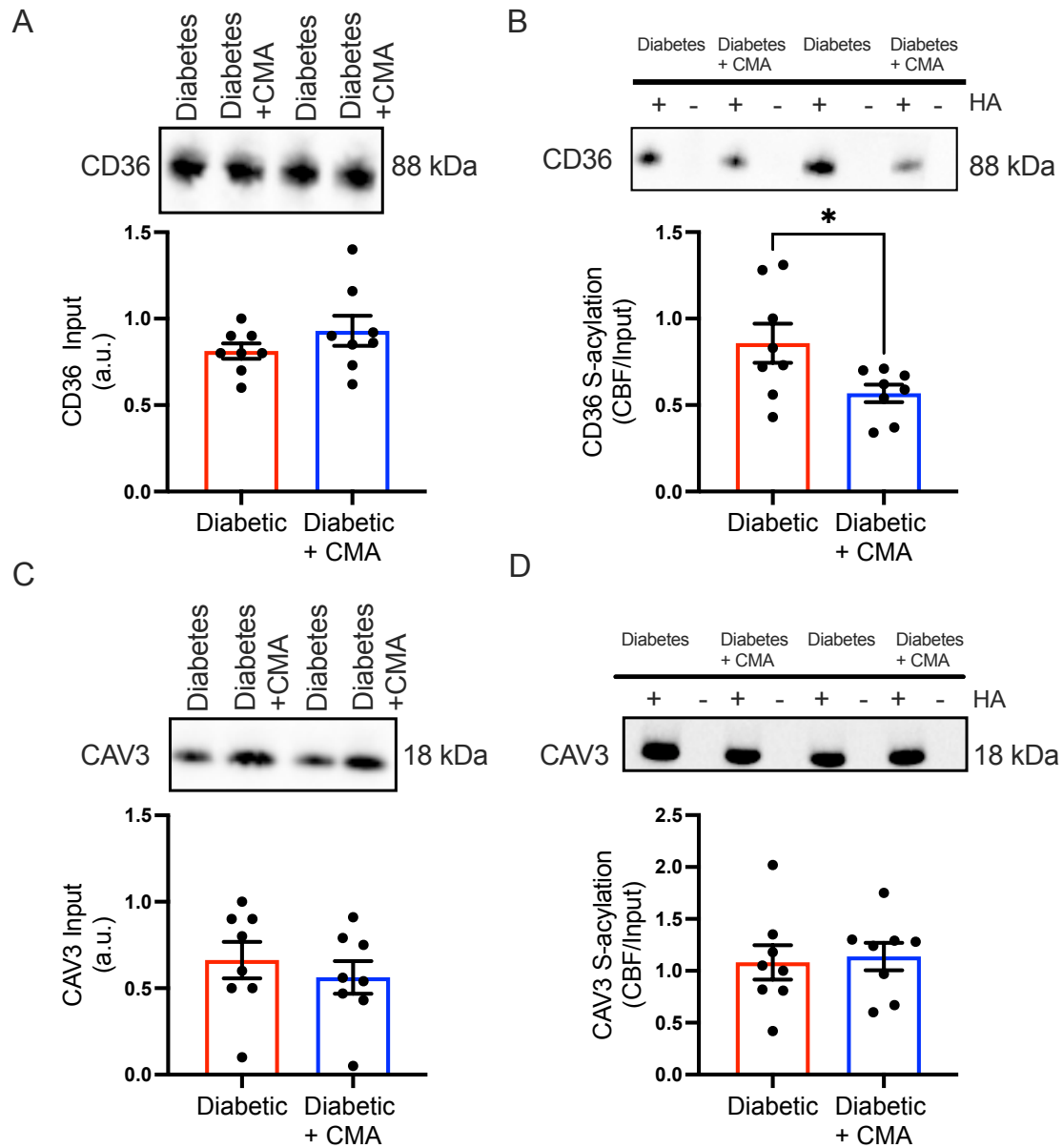


Figure 5.8: Using acyl-RAC, CD36 input was unchanged (A), whereas CD36 *S*-acylation was significantly decreased in CMA treated T2D hearts compared with untreated T2D hearts (B). No statistically significant changes were observed in CAV3 input (C) or CAV3 *S*-acylation between groups.*p<0.05 between CMA treated and untreated T2D hearts.

sarcolemma. With the decrease in CD36 *S*-acylation and sarcolemma expression, myocardial FAO rates were decreased 21% (Figure 5.9 B) and myocardial TAGs were decreased 48% (Figure 5.9 C) following treatment with CMA in T2D rat hearts.

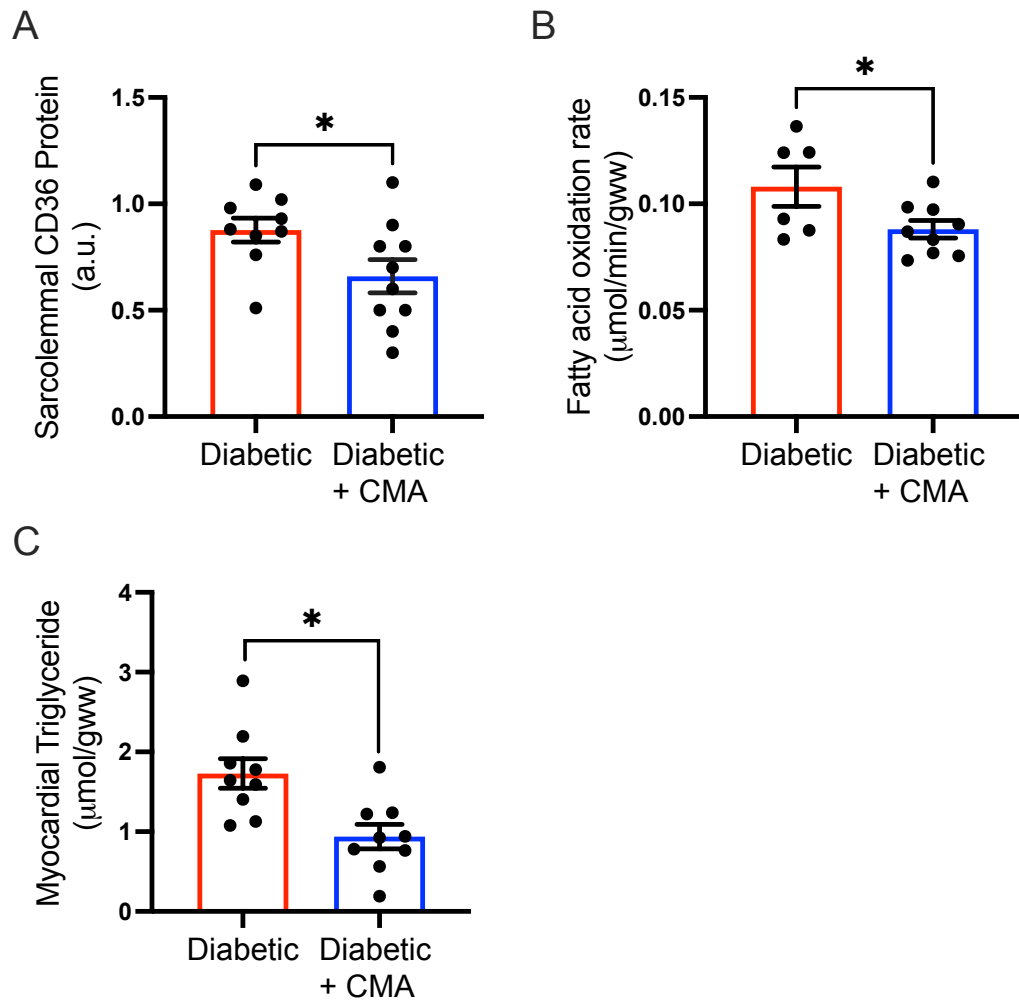


Figure 5.9: Sarcolemmal CD36 (A), FAO rates (B) and myocardial TG concentrations were significantly decreased in CMA treated diabetic hearts compared to untreated diabetic hearts.* $p < 0.05$ between untreated T2D and CMA treated T2D hearts.

No differences were observed in cardiac function (RPP) between diabetic groups prior to CMA being added (Figure 5.10 A). However, following 60 mins of perfusion with CMA, there was a 43% increase in cardiac function (Figure 5.10 B), as a result of 20% increase in developed pressure (Figure 5.10 C) in diabetic hearts. Collectively, these data indicate that CMA treatment in diabetes decreases CD36 *S*-acylation and CD36 localisation to the sarcolemma, which improves dysregulated lipid metabolism and enhances cardiac function.

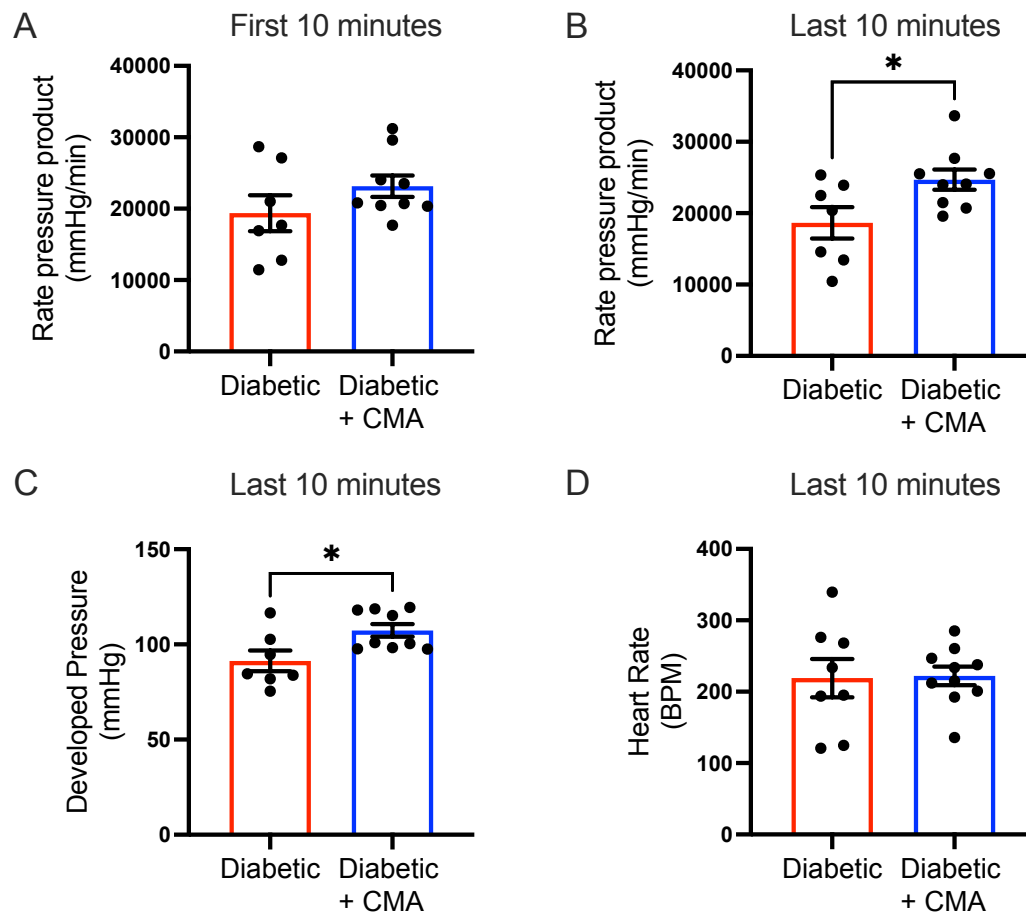


Figure 5.10: T2D hearts in the treated group were perfused for 10 mins without CMA, and had no statistically significant differences in RPP during these first ten mins (A). Following perfusion for 1 hr with CMA, RPP was significantly increased (B), which was a result of increased decreased DP (B) as HR was unchanged (D) between groups.*p<0.05 between CMA treated and untreated groups.

5.5.4 Inhibiting APT1 using ML348 induces a more diabetic profile in hearts from control rats

Given that lipid metabolism was downregulated in diabetes by inhibiting *S*-acylation, we next questioned if lipid metabolism could be upregulated in control hearts by inhibiting de-*S*-acylation. APT1 has been implicated in mediating CD36 de-*S*-acylation⁹⁰ and therefore the well-established APT-inhibitor, ML348, was perfused for 1 hr in healthy rat hearts. Whether APT1 mediated CD36 de-*S*-acylation can be inhibited in *ex vivo* perfused hearts and whether this would result in enhanced CD36

S-acylation was explored. Perfused control hearts had no differences in cardiac function between cohorts prior to the drug being added (Figure 5.11 A). However, following 1 hr of perfusion with ML348, there was a strong trend towards decreased cardiac function ($p=0.0554$) (Figure 5.11 B) as a result of decreased HR (Figure 5.11C) as DP was unchanged (Figure 5.11 D).

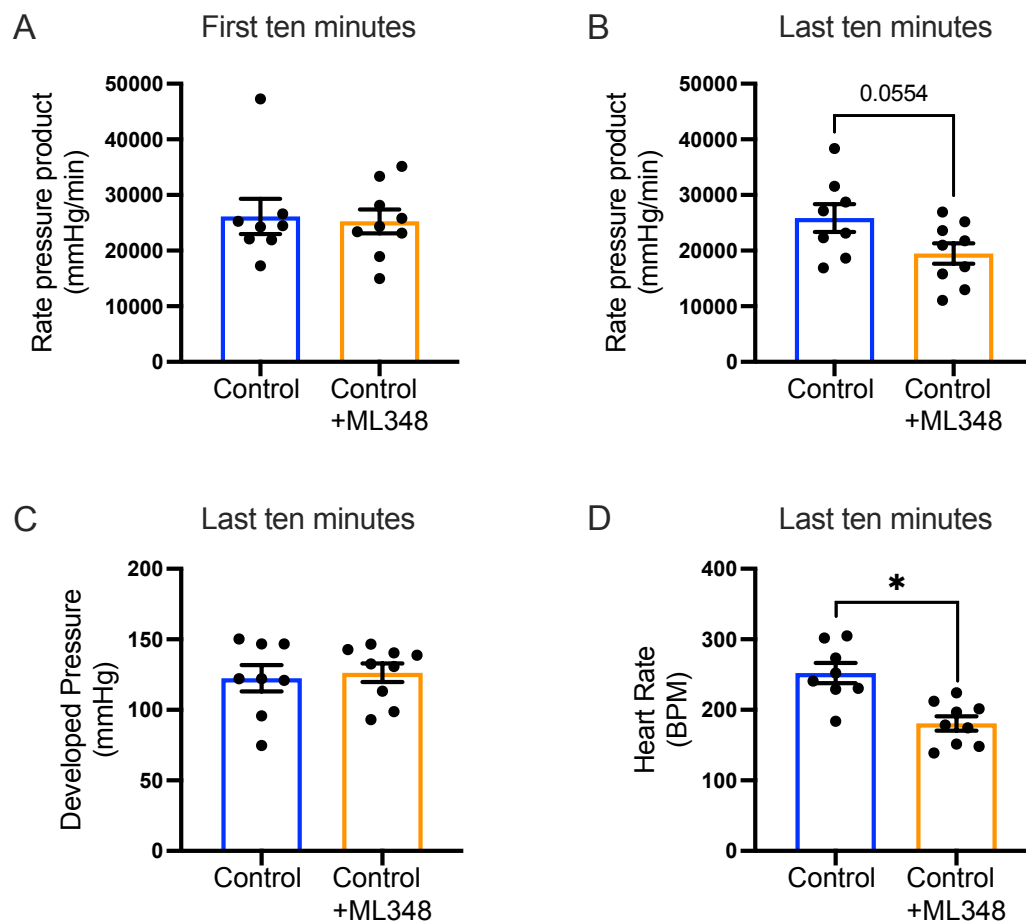


Figure 5.11: Hearts in the treated group were perfused for 10 mins without ML348 and had statistically significant differences in RPP during these first ten mins between the two groups (A). Following 1 hr of perfusion with ML348, RPP had a trend towards a decrease compared to the untreated group (B), which was driven by HR (C) as DP was unchanged (D). * $p < 0.05$ between ML348 treated and untreated groups.

Using acyl-RAC, CD36 *S*-acylation was increased by 32% (Figure 5.12 B), with no changes in CD36 input (Figure 5.12 A) in CMA treated hearts compared with controls. CAV3 input and *S*-acylation levels were unchanged (Figure 5.12 C-D) between cohorts.

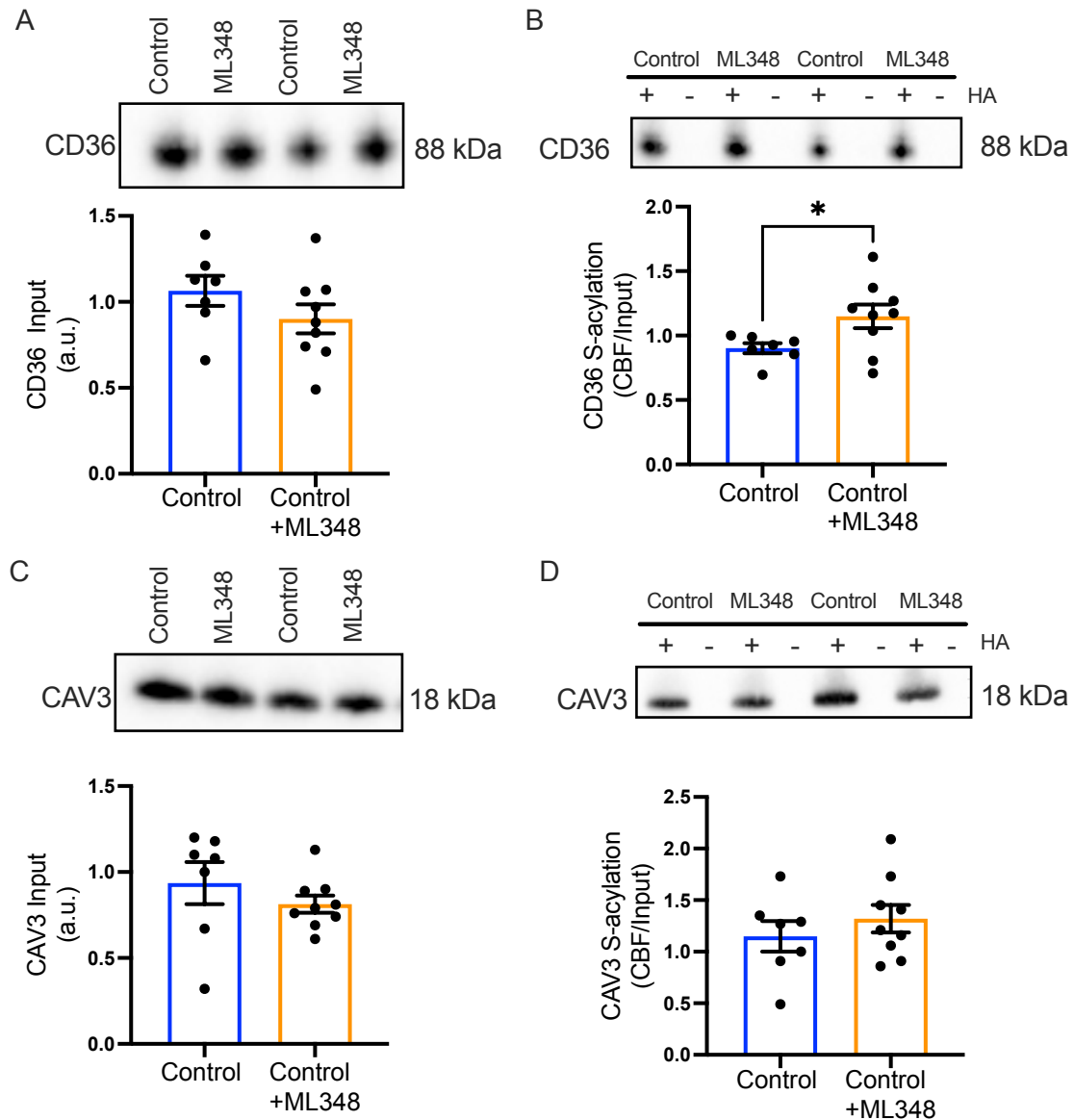


Figure 5.12: Using acyl-RAC CD36 input had no statistically significant changes (A), while CD36 *S*-acylation was significantly increased in ML348 treated hearts compared to untreated hearts (B). There were no statistically significant changes in CAV3 input (A) or CAV3 *S*-acylation between cohorts (B) * indicates $p < 0.05$ between untreated controls and ML348 treated.

Following treatment with ML348, FAO rates were increased 30% (Figure 5.13 A) with a strong trend ($p=0.0835$) towards an increase in myocardial TG concentrations (Figure 5.13 B) compared with untreated controls. Collectively, these results suggest that treatment with ML348 enhances CD36 *S*-acylation in healthy control rat hearts, resulting in decreased cardiac heart rate and elevated FAO rates.

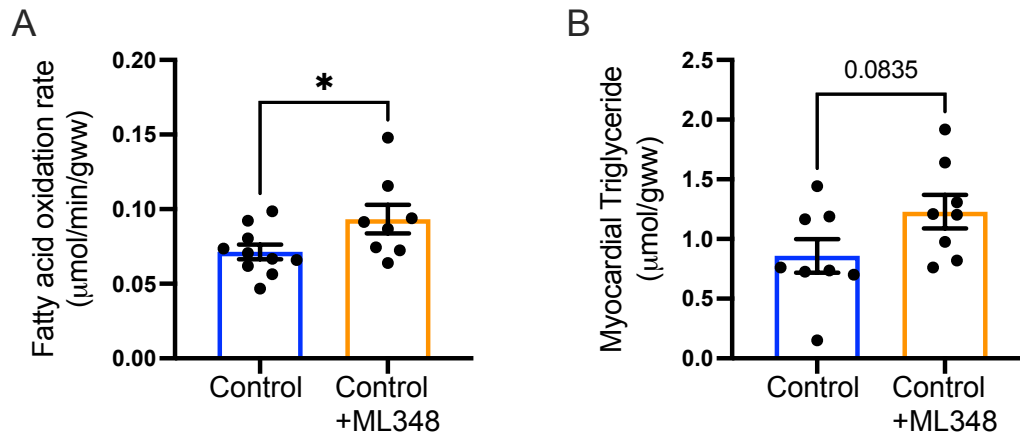


Figure 5.13: ML348 perfused hearts had significantly increased FAO rates (B) and a strong trend towards increased myocardial TG concentrations compared with untreated hearts * $p<0.05$ between untreated and ML348 treated.

5.6 Discussion

5.6.1 Acute increase in the abundance of palmitate decreased CD36 *S*-acylation

This study demonstrated that following an acute increase in palmitate concentrations in intact beating hearts, CD36 *S*-acylation is decreased. This is in contrast to some findings in the literature where an increase in palmitoyl-CoA has increased *S*-acylation of various targets in vitro^{210,225,226}. However, it is possible that this is a target-specific event, as the decrease in *S*-acylation was only evident for CD36 and not CAV3. In support of our findings, when 3T3-L1 cells were treated with oleate for an hour, there was a decrease in CD36 *S*-acylation as early as 5 mins, while no changes were observed in CAV1 *S*-acylation levels⁹⁰. Furthermore, the authors also observed that DHHC5, which has been postulated to maintain CD36 on the plasma membrane⁷², had decreased *S*-acylation levels itself⁹⁰. As sarcolemma CD36 content was shown to be reduced following a decrease in CD36 *S*-acylation and DHHC5 *S*-acylation, this report indicates that *S*-acylation is important for CD36 sarcolemma levels⁹⁰. While the authors concluded that the decrease in *S*-acylation of CD36 and DHHC5 with subsequent movement of CD36 away from the sarcolemma was demonstrating that CD36 facilitates FA uptake through an endocytic mechanism⁹⁰, it could be argued that under acute conditions of high levels of FAs, CD36 internalizes to avoid lipotoxicity.

In ischemia, it is well established that the heart undergoes a metabolic shift away from lipid towards glucose metabolism, mediated in part by CD36 internalisation, to protect against an intramyocardial lipid accumulation¹³⁷. Our results suggest a similar potential protective mechanism in response to acute high palmitate exposure. As such, in addition to a decrease in CD36 *S*-acylation, no changes in myocardial TAG storage was observed despite an increase in FAO, suggesting that the heart

is shifting away from storage to predominantly oxidizing pathways as a protective mechanism. Collectively, these results suggest that CD36 *S*-acylation levels are not regulated by an acute increase in palmitate abundance in ex vivo perfused hearts and further suggest that its *S*-acylation may be largely governed by enzymatic expression of the regulatory DHHCs and APTs.

5.6.2 Pharmacologically decreasing CD36 *S*-acylation in *ex vivo* T2D hearts has profound effects on cardiac metabolism

To date, pharmacological manipulation of *S*-acylation in cardiac tissue has not been explored. Blunting CD36 *S*-acylation was shown to be protective against NASH development in hepatocytes¹⁸², therefore pharmacologically decreasing cardiac CD36 *S*-acylation levels would be of great interest for the diabetic heart. As DHHC4 was shown to be increased in diabetes, with no changes in the de-*S*-acylating enzymes, APT1 and APT2, CMA was chosen as our inhibitor as it has been shown to have greater selectivity for the DHHCs, including DHHC4, while having limited effects on the APTs compared to the well-known inhibitor 2-BP¹³⁹. To our knowledge, this is the first study to show that CMA can decrease CD36 *S*-acylation in hearts, a finding which agrees well with CMA treatment in 3T3-L1 adipocytes¹³⁹. In conjunction with a decrease in CD36 *S*-acylation, treatment with CMA in hearts from T2D rats resulted in CD36 internalization and a subsequent decrease in the elevated cardiac lipid metabolism and lipotoxicity in diabetes; potentially through the detachment of the hydrophobic palmitoyl tails tethering CD36 to the membrane allowing for the internalisation into cytosolic vesicles.

Pharmacological approaches to decrease FA uptake have focused on inhibiting CD36 activity directly. Our group has previously shown that pharmacological inhibiting

CD36 using the inhibitor sulfo-N-succinimidyl oleate (SSO) was beneficial in hearts from T2D rats by decreasing FAO rates, preventing the accumulation of intracellular TGs and improving cardiac function²²⁷. Furthermore, mice treated with the CD36 inhibitor EP-80317 had decreased myocardial FA uptake and improved function during myocardial ischemia and reperfusion²²⁸. Both of these compounds bind to CD36 and prevent FA uptake activity (rather than changing CD36 localisation), which may have negative consequences long term given that FAs are an important fuel source for the heart. However, by pharmacologically altering levels of CD36 on the sarcolemma through manipulating *S*-acylation, this would allow for fine tuning of the amount of FA uptake. It may also allow for greater tissue specificity, as CD36 is widely expressed whereas individual DHHCs display tissue specificity. This makes CD36 *S*-acylating enzymes an attractive therapeutic target for the treatment of metabolic dysfunction in T2D hearts.

5.6.3 Pharmacologically increasing CD36 *S*-acylation in *ex vivo* control hearts results in a metabolic shift towards an increase reliance on lipid metabolism

Pharmacologically inhibiting CD36 de-*S*-acylation in control hearts using ML348 increased lipid metabolism and decreased cardiac function, mimicking the metabolic dysfunction in diabetes. Therefore, by targeting the regulation of fatty acid uptake, control hearts can rapidly resemble a diabetic heart, confirming the critical role of CD36 *S*-acylation in the diabetic pathology. Our results are consistent with other reports where CD36 *S*-acylation was increased following an hour with ML348 treatment *in vitro*⁹⁰. While enhancing CD36 *S*-acylation would be catastrophic in metabolic diseases such as T2D, in other pathologies such as HF, where there is a higher glucose dependency at the expense of fat metabolism, increasing CD36 *S*-acylation may be beneficial. A recent report in non-ischemic HF patients

demonstrated that increasing LCFA uptake and oxidation improved myocardial energetics and contractility²²⁹, suggesting a potential therapeutic role for increased CD36 *S*-acylation to drive enhanced CD36 on the sarcolemma to facilitate an increase in FA uptake into the myocardium.

5.6.4 Conclusions

In conclusion, this chapter provides compelling evidence that altering CD36 *S*-acylation levels by pharmacologically manipulating DHHCs and APT1 has profound effects on cardiac metabolism. The administration of CMA to *ex vivo* perfused hearts from T2D rats had a potent effect on CD36 *S*-acylation, resulting in decreased CD36 on the sarcolemma and a shift away from enhanced lipid metabolism, while facilitating improvements in cardiac function. In contrast, inhibition of APT1 with ML348 in control hearts demonstrated an increase in CD36 *S*-acylation and enhanced lipid metabolism, with negative effects on cardiac function, resembling the diabetic heart. This chapter therefore demonstrates that CD36 *S*-acylation has an important role as a gatekeeper of myocardial lipid metabolism and therefore and that manipulation of CD36 *S*-acylation provides an exciting novel therapeutic target in the heart.

6

Investigating altered cardiac metabolism in
heart failure and myocardial ischemia

6.1 Abstract

A partial metabolic shift away from fat metabolism and increased reliance on glucose metabolism are common features of both heart failure (HF) and acute myocardial ischemia. The mechanisms responsible for the metabolic shifts observed continue to be elucidated. This chapter will firstly investigate the subcellular localisation of CD36 in human HF tissue, and then will evaluate the *S*-acylation status of CD36, ACC and AMPK in acute myocardial ischemia in rats.

As FA import into failing hearts appears to be impaired, a decrease in sarcolemma CD36 represents a good candidate for this link. To investigate this, human HF tissue of varying etiologies was obtained, and a decrease in sarcolemma CD36 was identified and was accompanied by an increase in sarcolemma GLUT4. Furthermore, sarcolemma CD36 was found to positively correlate with left ventricular ejection fraction (LVEF), whereby hearts with the lowest CD36 on the sarcolemma displayed the lowest LVEF. Furthermore, total CD36 protein content negatively correlated with total GLUT4 content, demonstrating that hearts with the lowest CD36 protein had compensatory increases in GLUT4 protein content. This work suggests that reduced sarcolemma CD36 may contribute to the impaired FA metabolism and cardiac dysfunction in HF.

In rat hearts subjected to low-flow ischemia, FAO rates were decreased with an accumulation in individual lipid species in the myocardium, including those involved in *S*-acylation. CD36 was found to have unaltered *S*-acylation levels between ischemic and non-ischemic control rat hearts. However, two key metabolic regulating proteins, AMPK and ACC, were uncovered as being *S*-acylated and had enhanced *S*-acylation in ischemia. Therefore, ischemia is a potent stimulus for *S*-acylation targeting AMPK and ACC, suggesting these key enzymes may have another level of regulation outside of phosphorylation to regulate their metabolic activity.

6.2 Introduction

6.2.1 Heart Failure

Heart failure (HF) is a heterogeneous and complex clinical syndrome with varying etiologies. Decades of research have characterized the failing heart as being in an energy deficit as a result of abnormal cardiac metabolism, resulting in impaired cardiac efficiency and function⁴. This has been attributed to a decrease in mitochondrial oxidative capacity, including a shift away from FAO, which the heart attempts to compensate for by increasing glycolysis²³⁰. As such, in HF, high energy phosphates are reduced as a result of the heart being unable to use energy substrates appropriately to meet its high energetic demands⁴. Therefore, given the role of metabolism in the pathophysiology of HF, identifying metabolic targets that could be targeted therapeutically to reverse the energy deficit and improve cardiac function have received increasing attention.

6.2.2 The role of CD36 in HF

Decreased FA utilization by the myocardium in HF has been linked to cardiac dysfunction. Reduced FA uptake, with compensatory increases in glucose metabolism have been shown to render hearts more susceptible to cardiac dysfunction potentially through energy insufficiency^{231–233}. As such, in chronically infarcted rat hearts, impaired ejection fraction and palmitate oxidation rates were positively correlated with decreased in FA transport proteins (CD36, FATP6, FAPT1 and cFABP)²³⁴. Further work investigating the role of CD36 in HF reported that cardiomyocyte-specific deletion of CD36 accelerates contractile dysfunction in response to pressure overload²³³, while CD36 KO-mice subjected to transverse aortic constriction (TAC) display greater cardiac dysfunction with greater degrees of hypertrophy compared to TAC-WT mice^{235,236}. Alterations in CD36 have been reported in patients.

Biopsies from aortic stenosis patients undergoing valve replacement surgery displayed decreased total CD36 protein content¹³⁷. The decreases in CD36 were accompanied by reduced levels of other FA metabolism proteins; FABPpm, H-FABP, the β -oxidation protein medium chain acyl-coenzyme A dehydrogenase (MCAD), the Krebs cycle protein α -ketoglutarate dehydrogenase (α -KGDH) and the oxidative phosphorylation protein, ATP synthase¹³⁷. Furthermore, hearts with the lowest levels of CD36 had the highest GLUT4 protein content, and this was correlated with increased hypertrophy¹³⁷. These findings suggest a metabolic shift away from FA metabolism to an increase in glucose utilization for ATP generation.

Through metabolomic and genome-wide RNA sequencing approaches, a recent report implicated a defect in FA import into the myocardium in human end stage non-ischemic dilated cardiomyopathy (DCM)¹⁴⁴. Compared to non-failing (NF) hearts, DCM samples displayed energetic defects with reduced myocardial FAs and acyl-carnitines, despite elevated levels in the plasma¹⁴⁴. Furthermore, decreases in short chain hexanoyl-CoA and butyryl-CoA, near end-products of FAO are reduced in DCM with no changes in malonyl-CoA or protein expression of carnitine palmitoyl transport proteins (i.e. CPT1A, CPT1B and CPT2). These results further indicate an impairment in FA import over impaired mitochondrial import¹⁴⁴ driving the decrease in FA metabolism. Although CD36 mRNA was investigated, no changes were observed between DCM and NF hearts¹⁴⁴. Further work in cardiomyocytes isolated from human NF and HF tissue demonstrated that although CD36 mRNA levels were not altered between cohorts, HF cardiomyocytes with higher CD36 mRNA levels had improved contractility, suggesting that mechanisms outside of CD36 protein content may play an important role in cardiac dysfunction¹⁴⁵. Although CD36 levels have been investigated in both rodents and humans, no study has investigated the subcellular localisation of CD36 in HF.

6.2.3 Myocardial Ischemia

HF is caused by multiple different comorbidities, with ischemic heart disease being a prominent contributor. Myocardial ischemia, as a result of decreased blood flow, rapidly alters fuel metabolism by upregulating anaerobic glycolysis and glucose use, while shifting away from FA uptake and oxidation, in an attempt to generate sufficient ATP to maintain cardiac function under oxygen limited conditions. Failure to appropriately respond to acute ischemia may result in contractile dysfunction following reoxygenation^{58,237}.

Under ischemic conditions, the increase in glycolysis and glucose uptake is largely mediated by the intracellular energy sensor AMPK. AMPK is a heterotrimeric protein consisting of a catalytic subunit (α) and two regulatory subunits (β and γ)²³⁸. When oxygen supply is restricted, AMPK is rapidly activated by an increase in intracellular adenosine monophosphate (AMP) concentrations. AMPK is also activated allosterically during oxygen deprivation by a decrease in the phosphocreatine/creatine (PCr/Cr) ratio²³⁹. AMPK activation facilitates an upregulation of glucose metabolism by enhancing GLUT4 trafficking to the sarcolemma²⁴⁰ and by activating 6-phosphofructo-2-kinase (PFK-2), resulting in increased glycolytic flux²⁴¹. Interestingly, AMPK α was shown to be *S*-acylated in a proteomic analysis in adipocytes²⁴², and more recent work demonstrated that it was *S*-acylated on two cysteine residues (Cys209 and Cys543) by DHHC17²⁴³. AMPK α *S*-acylation was demonstrated to be imperative for its localisation and membrane tethering²⁴³. Importantly, following the ablation of *S*-acylation using cysteine site mutagenesis, AMPK α phosphorylation was inhibited and glucose uptake into mouse hepatocytes was impaired²⁴³. This work indicates that *S*-acylation of AMPK is required for its phosphorylation and demonstrates a novel interplay between *S*-acylation and phosphorylation in the governance of metabolism. However, to date, whether AMPK is *S*-acylated in the heart remains unknown and whether AMPK *S*-acylation in

enhanced similar to its phosphorylation under stress conditions such as ischemia has not been studied.

In addition to driving enhanced glucose metabolism, once activated in response to myocardial ischemia, AMPK can phosphorylate and inactivate acetyl-CoA carboxylase (ACC). ACC plays an important role in regulating FAO in the heart by catalyzing the conversion of cytosolic acetyl-CoA to malonyl-CoA, which is an endogenous inhibitor of carnitine palmitoyl CoA transferase 1 (CPT-1)²⁴⁴. Two ACC isoforms exist in the heart, ACC1(250 kDa) and ACC2 (280 kDa), with ACC2 being the dominant isoform. Both isoforms can be phosphorylated by AMPK and subsequently inactivated on serine residues (Ser79 and Ser212), resulting in a decrease in myocardial malonyl-CoA concentrations²⁴⁵. Decreased malonyl-CoA concentrations in the heart release the inhibition on CPT1 and facilitate mitochondrial FA import, resulting in an increase in the relative contribution of FA to oxidative metabolism. All in all, ACC is able to fine tune myocardial lipid metabolism by modulating FAO and, therefore, sits at a key metabolic regulatory axis. However, it remains unknown if ACC is regulated by any other PTMs other than phosphorylation. Given the well-established role of AMPK and ACC in mediating myocardial metabolism, it would be of great interest to investigate if lipid PTMs are involved in the acute response to energetic stress such as ischemia.

6.3 Aims

Cardiac metabolism is altered in heart failure and myocardial ischemia. This chapter proposed that CD36 is decreased on the sarcolemma resulting in the impaired FA uptake observed in HF in patients. Furthermore, this chapter proposed that acute ischemia would drive alterations in *S*-acylation levels of key proteins involved in metabolic adaptations in rodents. These hypotheses were investigated through the interrogation of two key objectives:

1. Determine the subcellular localisation of CD36 and GLUT4 in human HF biopsies.
2. Investigate the levels of CD36, ACC and AMPK *S*-acylation following low-flow ischemia in Langendorff perfused hearts.

6.4 Methods

6.4.1 Human heart procurement

Procurement of human myocardial tissue was carried out following the protocol set out in section 2.15.

6.4.2 Subcellular Fractionation

Subcellular fraction on human tissue was carried out following the protocol set out in section 2.11.

6.4.3 Acute ischemia in isolated Langendorff heart perfusions

Low flow ischemia Langendorff perfusions were carried out following the protocol set out in section 2.10.7.

6.4.4 Lipidomics

Lipidomic analysis was carried out following the protocol set out in section 2.14.

6.4.5 Acyl-RAC

Acyl-RAC was carried out following the protocol set out in section 2.1.

6.5 Results

6.5.1 Sarcolemma CD36 content is decreased in HF tissue and correlates with LVEF

Biopsies from failing human hearts (n=17) displayed a variety of HF etiologies (Table 6.1), while non-failing heart biopsies were classified as NF tissue (n=11).

Table 6.1: Heart Failure Etiology

Non-failing (n=11)	Failing hearts (n=17)
NF	Non-ischemic dilated cardiomyopathy (x8)
NF	Dilated cardiomyopathy (x2)
NF	Restrictive cardiomyopathy
NF	Arrhythmogenic right ventricular cardiomyopathy
NF	Non-ischemic dilated cardiomyopathy/Sarcoidosis
NF	Hypertrophic cardiomyopathy
NF	Familial laminopathy
NF	Pulmonary arterial hypertension Left Heart
NF	Scleroderma/restrictive cardiomyopathy
NF	
NF	

To assess sarcolemmal content of CD36 and GLUT4, subcellular fractionation (SCF) was carried out and fractions assigned as either sarcolemmal or intracellular microsomal. When compared with NF heart samples, there was a 29% decrease in CD36 in the sarcolemma fraction (Figure 6.1 A) and a 28% decrease in the microsomal fraction (Figure 6.1 B) in the failing hearts. Furthermore, HF hearts had a 40% increase in GLUT4 on the sarcolemma (Figure 6.1 C) compared to NF hearts, with no differences in the microsomal fraction between cohorts (Figure 6.1 D). These results therefore suggest that there are alterations in the sarcolemma localisation of the membrane substrate transporters CD36 and GLUT4 in HF.

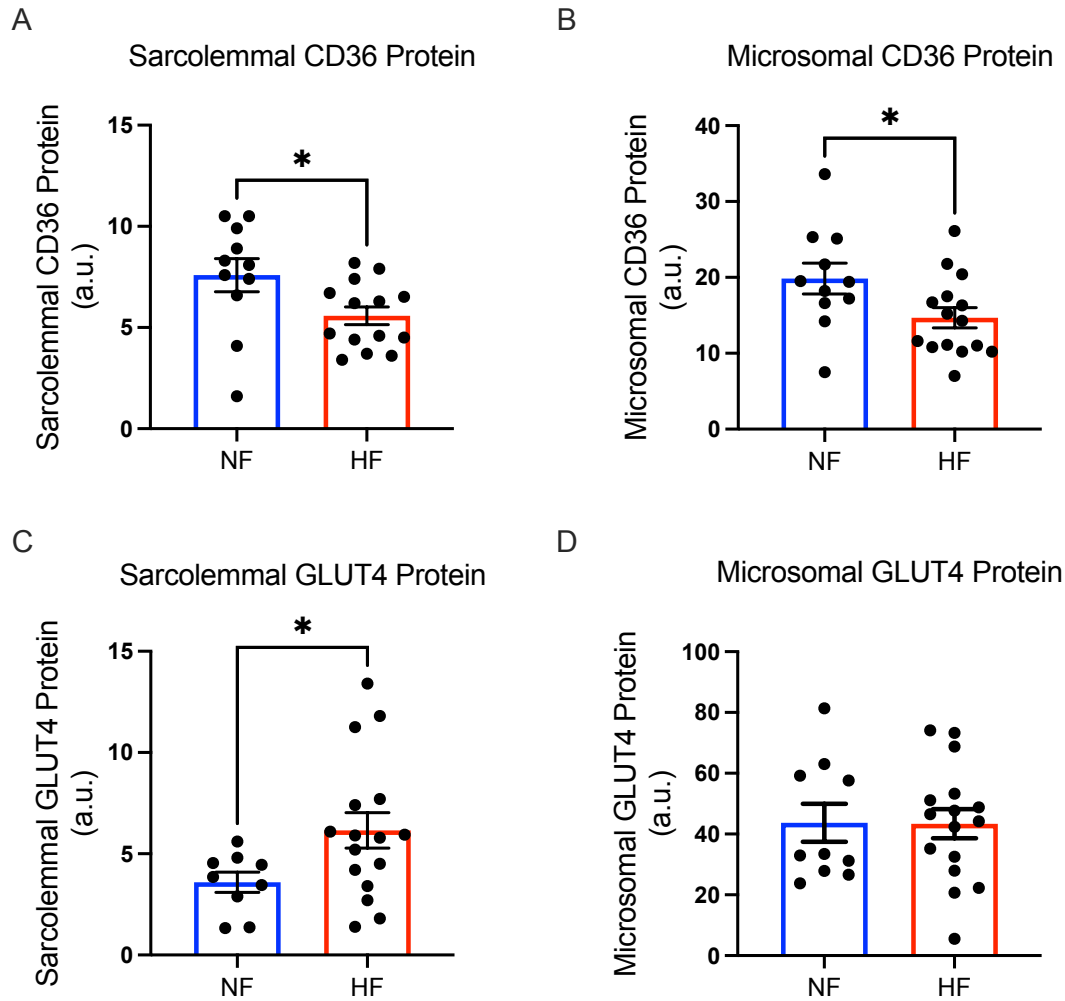


Figure 6.1: In heart failure (HF) tissue CD36 was significantly decreased on the sarcolemma (A) and in the intracellular microsomes (B) compared to non-failing (NF) hearts. GLUT4 was significantly increased on the sarcolemma in HF tissue (C) with statistically significant changes in GLUT4 abundance in the intracellular microsomes (D) compared to NF hearts. * $p < 0.05$ for NF vs HF.

Alterations in total CD36 and GLUT4 protein between HF and NF hearts were then investigated. When using a whole tissue lysate for western blotting, no significant changes in total CD36 protein content were observed (Figure 6.2 A). However, when band intensities of the sarcolemma and microsomal fractions were added together, CD36 was decreased 33% in HF compared to NF hearts (Figure 6.2 B), suggesting a decrease in whole tissue CD36. In contrast to CD36, when the lanes of GLUT4 on the sarcolemma and microsomal fractions were summed, no statistically

significant changes were observed between GLUT4 protein in HF and NF (Figure 6.2 C), however when using a traditional whole tissue lysate for western blotting, total GLUT4 protein was increased 43% in failing hearts compared with NF hearts (Figure 6.2 D).

Using simple linear regression analysis, sarcolemma levels of GLUT4 and CD36 did not significantly correlate (Figure 6.4 A). However, total CD36 was negatively correlated with GLUT4 (Figure 6.4 B) whereby patients with the lowest amount of CD36 protein had the highest GLUT4 protein content. This finding is consistent with previous findings by our group¹³⁷. Furthermore, sarcolemma CD36 levels were found to be positively correlated with LVEF (Figure 6.4 C), demonstrating that patients with the lowest CD36 on the sarcolemma had the lowest LVEF, suggesting that sarcolemma CD36 is associated with cardiac function. In contrast to sarcolemma CD36, no significant relationship between sarcolemma GLUT4 localisation and LVEF was found (Figure 6.4 D). These results therefore suggest that decreased levels of CD36 on the sarcolemma are associated with impaired contractile dysfunction.

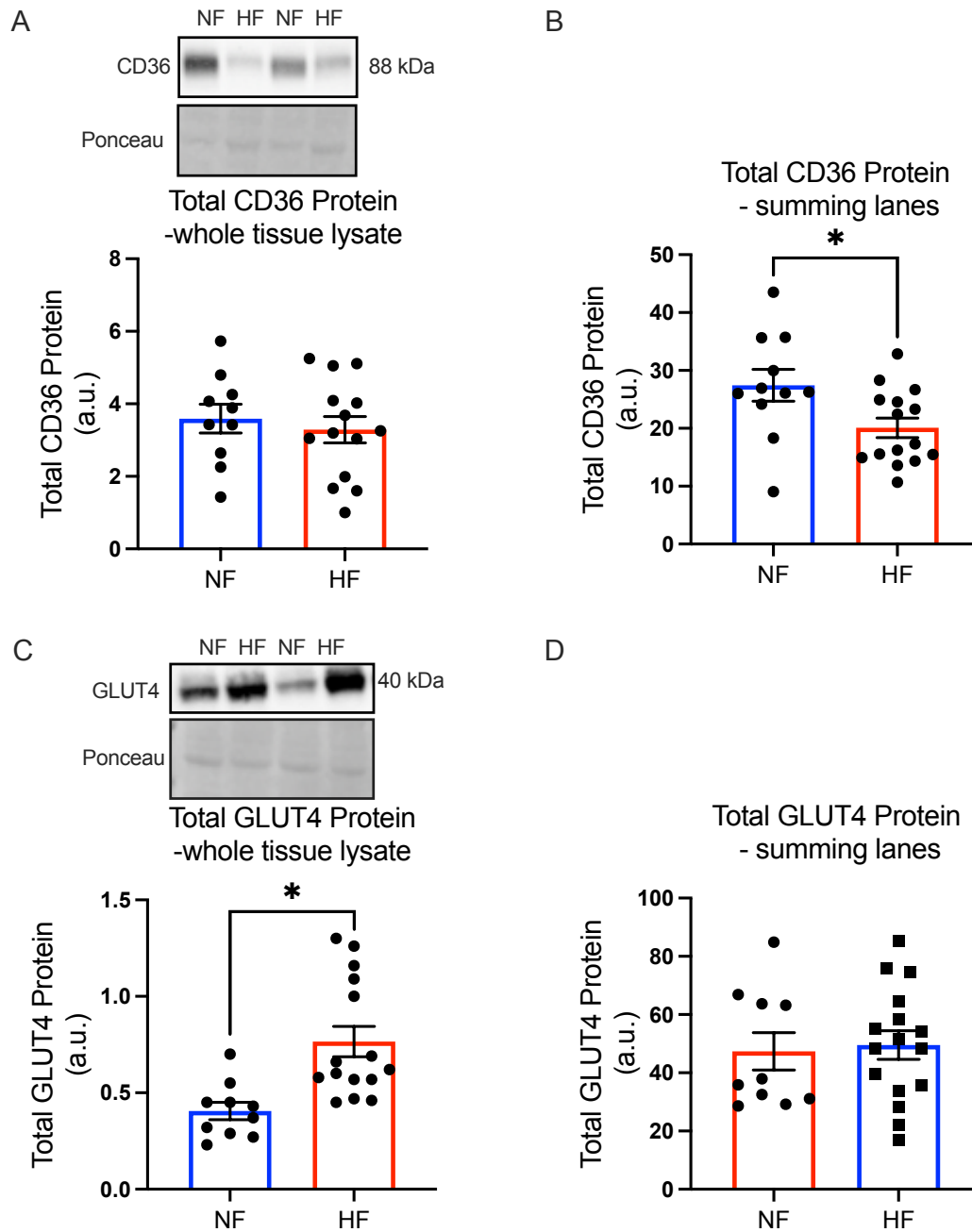


Figure 6.2: No statistically significant changes between NF and HF hearts for total CD36 protein using western blotting (A), but decreases in total CD36 when SCF lanes are summed (B). In contrast, total GLUT4 protein was increased in HF compared with NF tissue using western blotting (C), whereas no changes in GLUT4 protein when SCF lanes were summed (D). * $p < 0.05$ for NF vs HF.

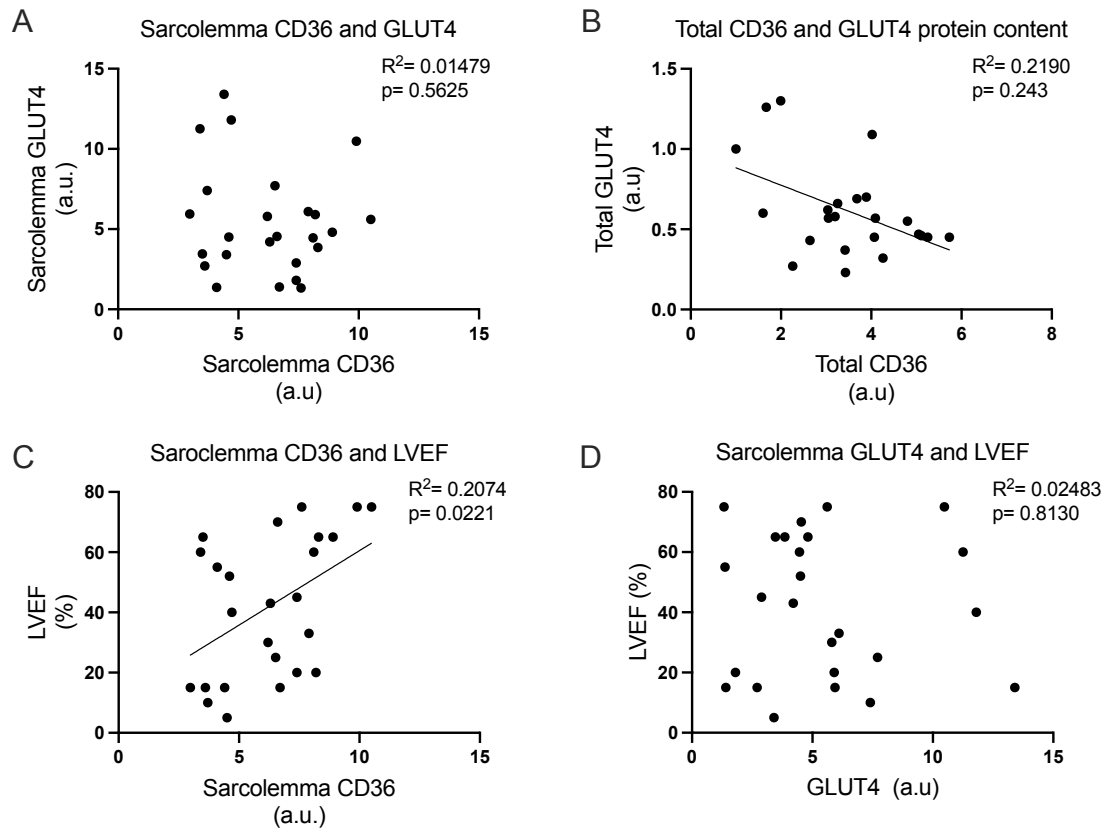


Figure 6.3: Sarcolemma levels of CD36 and GLUT4 had no correlation (A) and total CD36 negatively correlated with GLUT4 (B). Sarcolemma CD36 correlated positively with LVEF (C), whereas sarcolemma GLUT4 and LVEF were not correlated (D).

6.5.2 Potential CD36 Polymorphism

Lastly, one HF patient was shown to be devoid of CD36, both on the sarcolemma and at the whole tissue levels (Figure 6.5 A-B), indicating a CD36 polymorphism that resulted either in no protein being made or reduced protein levels that were no longer detectable by the CD36 antibody. Interestingly, while purely observation, the CD36 deficient sample had one of the highest levels of GLUT4 at the total protein level (Figure 6.4 C), indicating potential compensatory upregulation. This patient was diagnosed with Pulmonary Arterial Hypertension (PAH), was one of the youngest of the HF patients and had the highest left ventricular mass index LVMI, indicative of cardiac hypertrophy (Table 6.2). These results suggest that CD36 deficiency may accelerate HF progression.

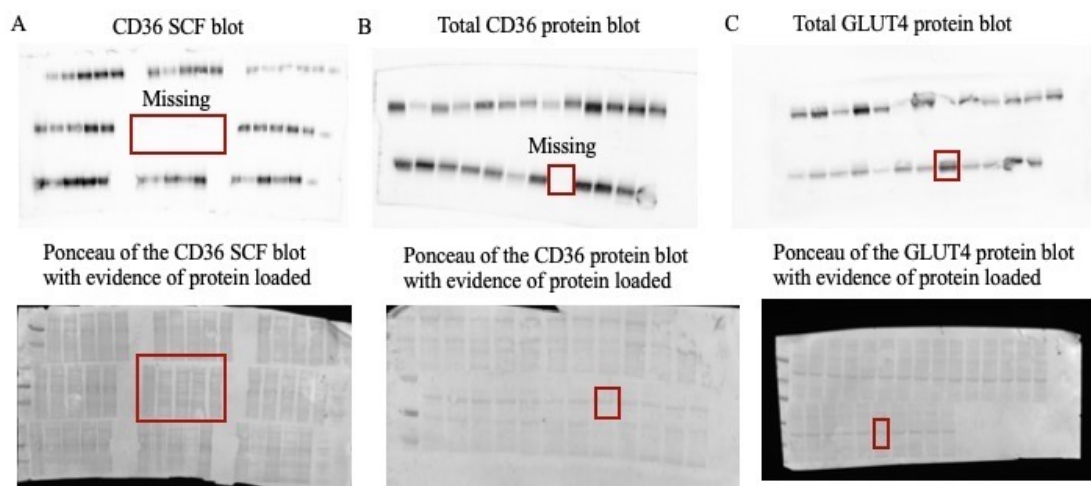


Figure 6.4: A HF patient was devoid of CD36 on the sarcolemma (A) and at the whole tissue level (B), and had increases in whole tissue GLUT4 protein (C)

Table 6.2: Etiology of donor devoid of CD36 and average HF donors

Group	Age	Etiology	LVMI	LVEF
Average HF	49	Mixed	136.54	29
CD36 devoid patient	34	PAH L/V	187.51	75

6.5.3 Decrease in FAO and increased lipid species in the myocardium in ischemia

Using the Langendorff rat heart perfusion protocol, we next investigated *S*-acylation and metabolism in response to ischemia. During low-flow ischemia, cardiac function decreased 98% of pre-ischemic values, consistent with previous findings¹⁴⁰ (Figure 6.5 A-B). Furthermore, FAO rates were measured by A/Prof. Lisa Heather and were found to be decreased 95% in ischemia compared with controls (Figure 6.5 C).

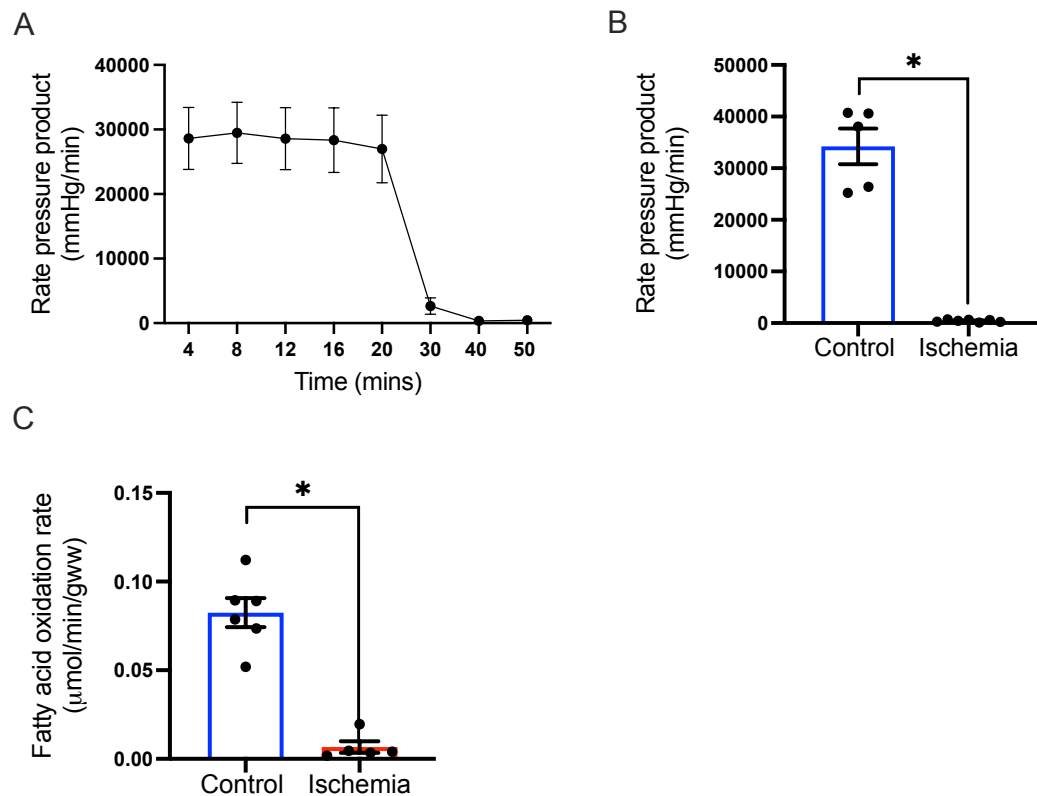


Figure 6.5: Hearts underwent full flow perfusion for 20 mins and were stable (A). As expected, rate pressure product dropped significantly during low-flow ischemia compared to pre-ischemia values (A-B). Cardiac FAO rates also were significantly decreased in ischemia (C) compared to control hearts.* $p < 0.05$ control vs.ischemia.

The total fatty acid pool in control and ischemic hearts was assessed by Dr. Christian Cole at the Scripps Research Institute using Gas chromatography–mass spectrometry (GS/MS). While no changes were observed in the relative abundance of saturated and polyunsaturated FFA between cohorts, monounsaturated FFAs were increased by 33% in ischemia compared with control non-ischemic perfused hearts (Figure 6.6 A). Furthermore, when individual fatty acids were assessed, significant increases were found in individual fatty acids between control and ischemic hearts, including increases in those that have been associated with *S*-acylation, including palmitic acid (C16:0), stearic acid (C18:0), palmitoleic acid (C16:1) and oleic acid (C18:1) (Figure 6.6 B).

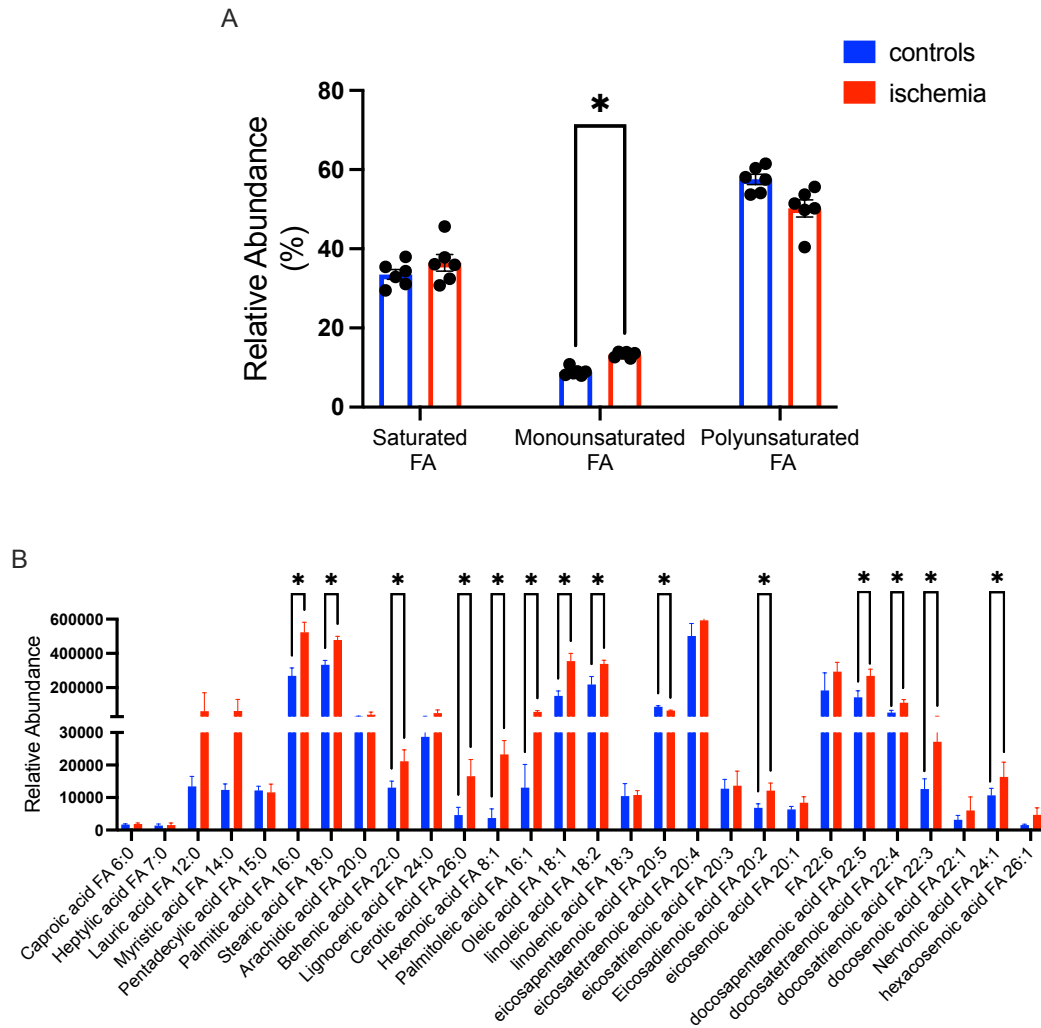


Figure 6.6: Relative abundance of monounsaturated FAs are significantly increased in ischemia compared control non-ischemic hearts (A) and there were significant increases in individual lipid species compared to non-ischemic control hearts (B). * $p < 0.05$ control vs. ischemia.

6.5.4 CD36 *S*-acylation is unchanged in ischemia

As CD36 internalizes during ischemia¹³⁷ and CD36 *S*-acylation has also been shown to be required for its presence on the sarcolemma⁷², we next explored whether CD36 *S*-acylation levels were altered in acute myocardial ischemia. No differences were observed in CD36 input fraction (Figure 6.7 A) or CD36 *S*-acylation levels (Figure 6.7 B) between non-ischemic controls and low-flow ischemia perfused hearts.

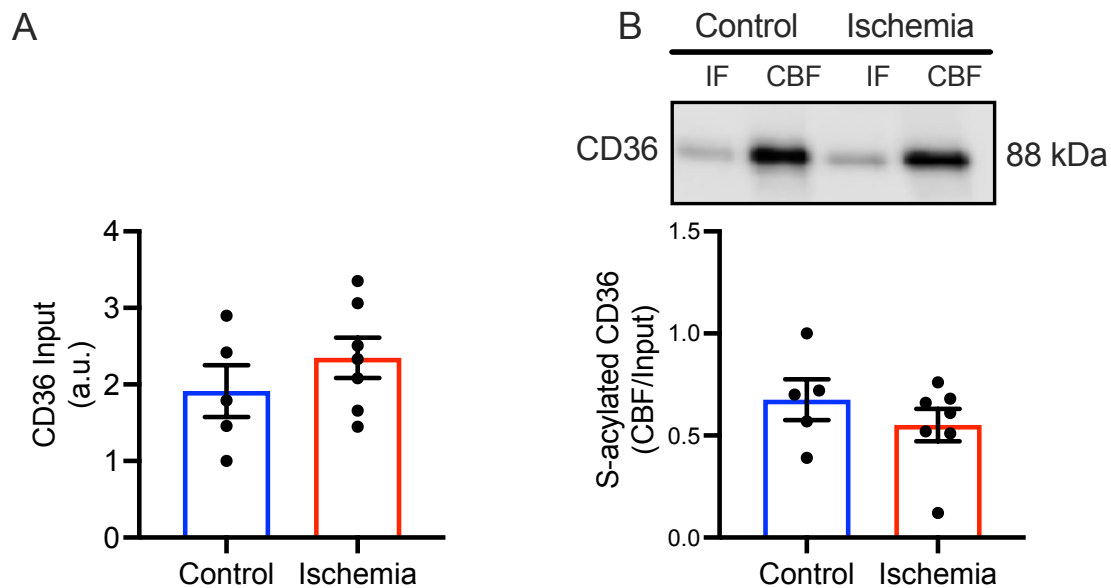


Figure 6.7: In ischemia, no statistically significant differences were found in the input fraction (A) and *S*-acylation levels of CD36 (B), compared with controls.

Similarly, no differences were observed in DHHC5 input fraction (Figure 6.8 A) or *S*-acylation levels (Figure 6.8 B) between control and ischemia perfused hearts. Lastly, no differences were observed in CAV3 input (Figure 6.8 C) and *S*-acylation between cohorts (Figure 6.8 D). Therefore, these results suggest that acute myocardial ischemia does not alter the *S*-acylation levels of CD36, DHHC5 and CAV3.

6.5.5 ACC and AMPK are *S*-acylated in rat hearts and their *S*-acylation is increased by ischemia

Although CD36 *S*-acylation was found to be unaltered in ischemia, we next explored the *S*-acylation status of two key proteins involved in governing cardiac metabolism in ischemia, AMPK and ACC. Total protein levels of AMPK and ACC did not differ between controls and ischemia (Figure 6.9 A-B). However, as expected, there was a 3.8-fold increase in phosphorylated AMPK and a 2-fold increase in phosphorylated ACC in ischemic hearts (Figure 6.9 C-D). These results demonstrate that both ACC and AMPK are phosphorylated in rat hearts subjected to low-flow ischemia.

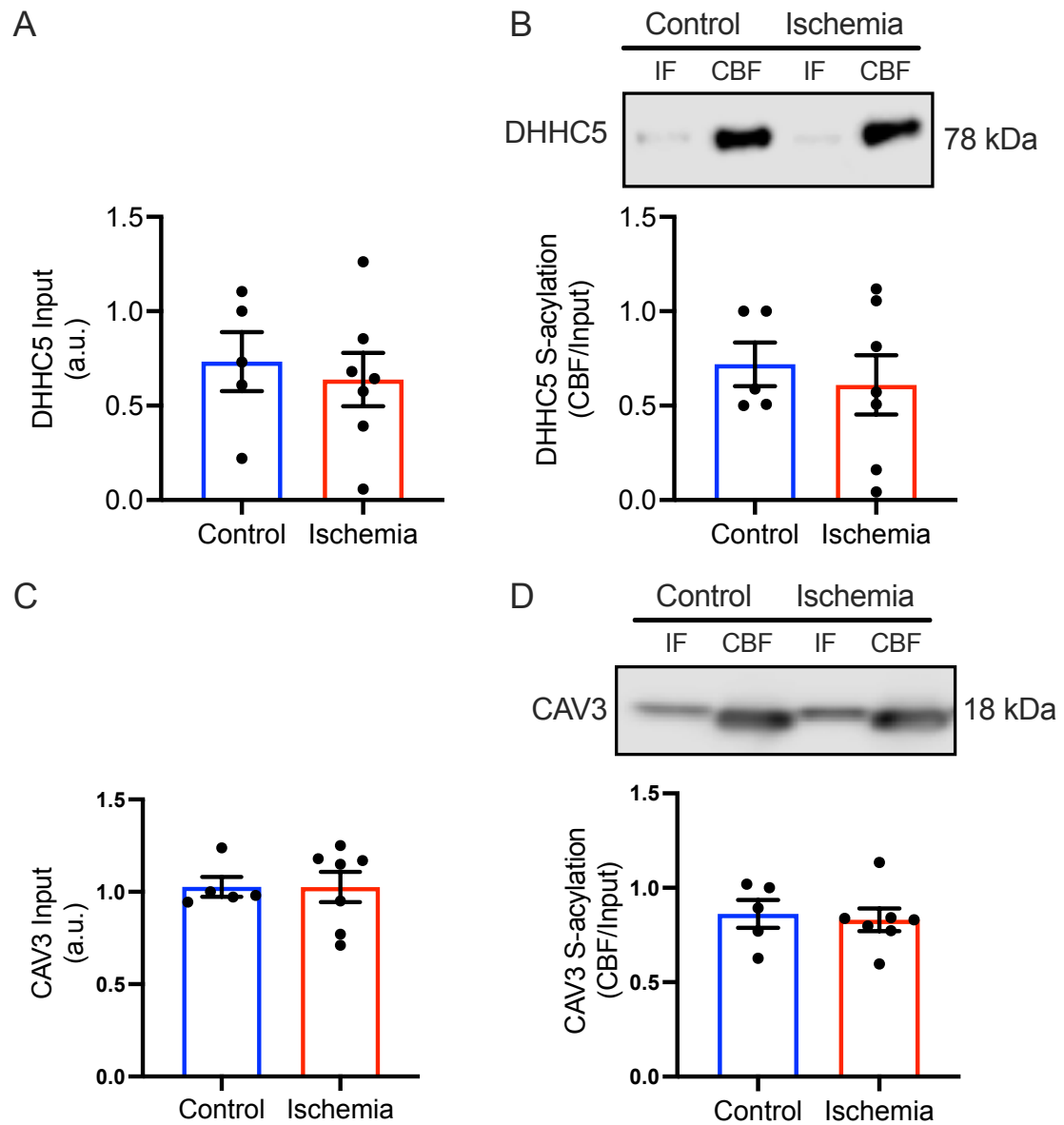


Figure 6.8: In ischemia, no statistically significant differences were found in DHHC5 input fraction (A) or DHHC5 *S*-acylation level (B) compared with control hearts. CAV3 also had no statistically significant alterations in the input fraction (C) or *S*-acylation levels between ischemic and control hearts (D).

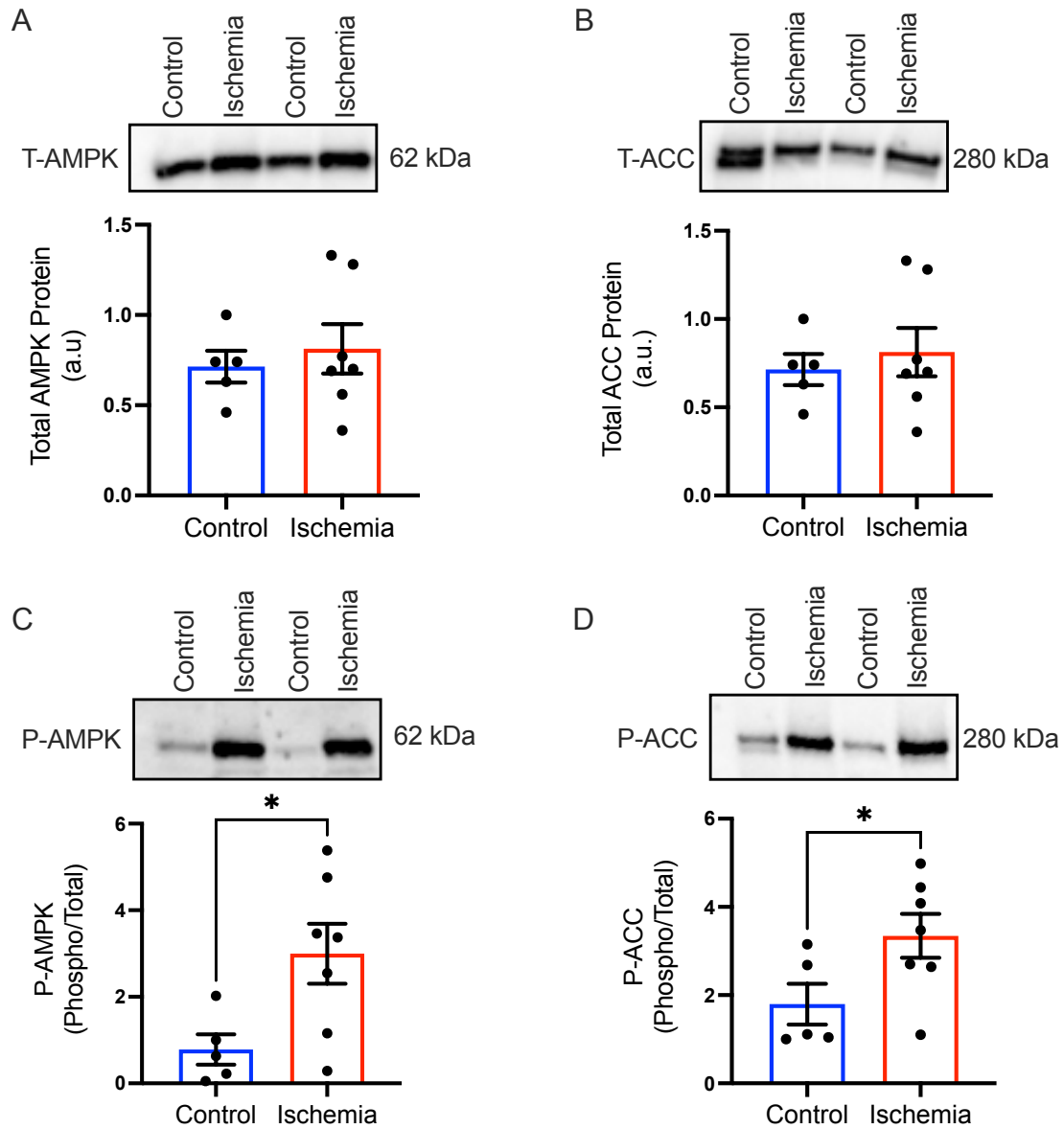


Figure 6.9: Total AMPK (T-AMPK) (A) and Total ACC (T-ACC) were not statistically significantly different between non-ischemic control hearts and ischemic hearts (B). Phosphorylated AMPK (P-AMPK) (C) and Phosphorylated ACC (P-ACC) were significantly increased in ischemia (D) compared with non ischemic controls.* $p < 0.05$ control vs.ischemia.

Interestingly, AMPK has been shown to be *S*-acylated in adipocytes and *S*-acylation was required for AMPK phosphorylation in response to energy stress^{242,243}. Given the metabolic demands of the heart in ischemia, many of which are regulated by AMPK mediated signalling, whether AMPK was *S*-acylated in the heart was investigated. AMPK was shown to be *S*-acylated in control rat hearts (Figure

6.10 B). Following 30 mins of low-flow ischemia, AMPK input had no statistically significant differences (Figure 6.10 A), while AMPK *S*-acylation increased 42% compared to non-ischemia perfused control hearts (Figure 6.10 B). In addition to

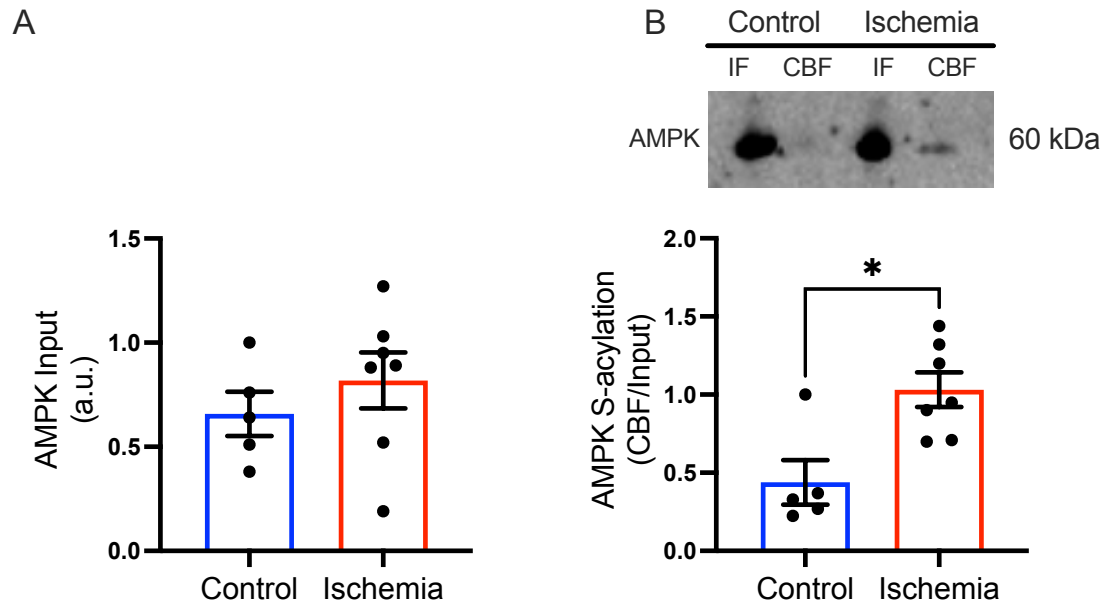


Figure 6.10: In ischemia, no statistically significant differences were observed in AMPK input fraction (A) compared with control hearts (A), while AMPK *S*-acylation was significantly increased (B) in ischemia.* $p < 0.05$ control vs.ischemia.

AMPK, ACC has a crucial role in mediating the metabolic response in ischemia and therefore the *S*-acylation status of ACC was also explored in rat cardiac tissue. Similar to AMPK, ACC input fraction had no statistically significant differences (Figure 6.11 A), however, ACC was found to be *S*-acylated in control rat hearts (Figure 6.11 B) and a 37% increase in ACC *S*-acylation was observed following ischemia compared to non-ischemia perfused controls (Figure 6.11 B). Taken together, these results demonstrate that AMPK and ACC are *S*-acylated in the heart and that their *S*-acylation levels are enhanced in acute myocardial ischemia.

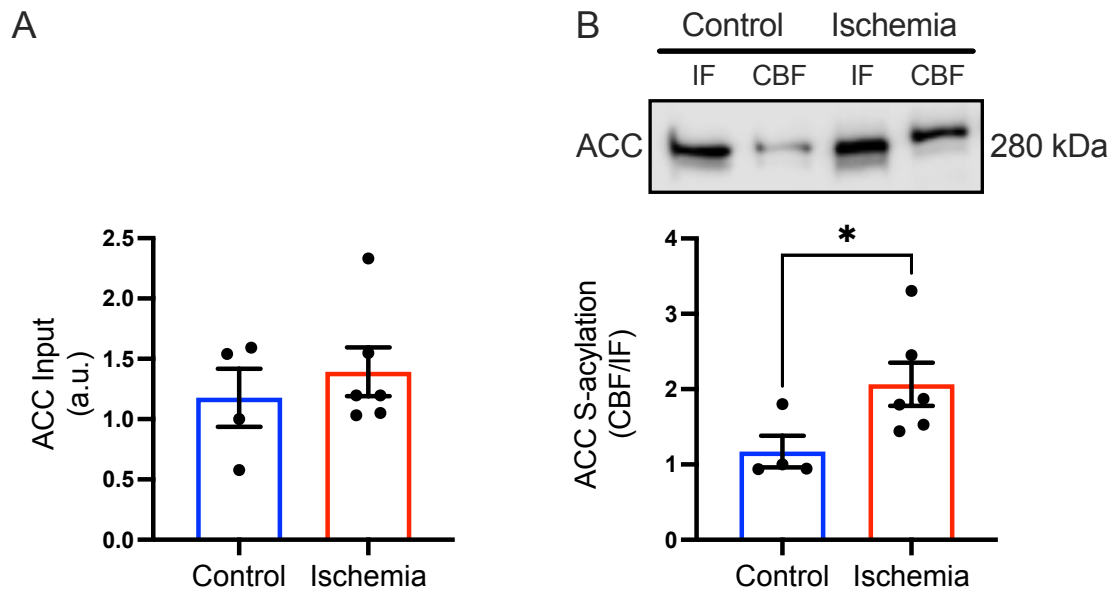


Figure 6.11: Using acyl-RAC no differences were observed in input fraction for ACC (A), whereas ACC *S*-acylation was increased in ischemia compared with non-ischemic controls (B). * $p < 0.05$ control vs. ischemia.

6.6 Discussion

6.6.1 Subcellular localisation of membrane substrate transport proteins in HF

Failing hearts are commonly associated with metabolic reprogramming with a shift away from FA metabolism towards glucose metabolism. Recent work in humans has suggested that increasing lipid metabolism is a novel treatment for HF^{144,145,229}. Our data demonstrates that sarcolemma CD36 is decreased in HF tissue compared to NF tissue, with compensatory increases in GLUT4 on the sarcolemma. While this is the first study to our knowledge to look at the subcellular localisation of CD36 and GLUT4 in human cardiac tissue, these findings agree well with what is seen in human patients and rodent models that report alterations in total protein of CD36 and GLUT4 transporters in HF^{137,234} and therefore indicates that increasing CD36 is potentially an exciting novel target by which to increase FA uptake in the myocardium in HF. Furthermore, we demonstrated that LVEF was positively

correlated with sarcolemma CD36, implicating sarcolemma CD36 in the contractile dysfunction of HF. This finding potentially provides a mechanism by which FA uptake is impaired in HF, resulting the ‘engine out of fuel’ phenotype leading to eventual cardiac dysfunction^{4,144}. However, it is important to note that correlation does not mean causation and further work is warranted to unpick this finding.

Interestingly, reports investigating human HF have largely explored alterations in mRNA and total protein levels, mainly the glucose transporters, while CPT1 is the only marker of lipid metabolism classically investigated. Results have varied depending on the etiology of HF, however, overall there is a decrease in the glucose transporter mRNA with no changes in CPT1^{144,246–249}. Reports investigating both GLUT4 and CD36 mRNA have reported no alterations in their abundance¹⁴⁴, however CD36 and GLUT4 are trafficking membrane substrate proteins and alterations in their subcellular localisation have been linked to altered metabolism in pathological conditions such as diabetes prior to alterations in total protein⁵⁹. Therefore, understanding changes in their localisation would provide further insight into the metabolic shifts observed in HF etiologies. Furthermore, alterations in CD36, including its sarcolemmal abundance, have been shown to occur prior to the development of insulin resistance, therefore unpicking at what stage the alterations of CD36 occur in HF could provide further insight into the development of the pathology. Furthermore, it remains to be elucidated whether the decrease in sarcolemmal CD36 in HF is adaptive or maladaptive.

6.6.2 Limitations of this study

There are two limitations in this study. The first is that we report conflicting findings of total CD36 and GLUT4 protein using traditional western blotting and summation of the subcellular fractions, which should represent total protein. As both of these methods were performed on the same hearts, they should yield similar

results. However, while CD36 demonstrates a decrease in protein content as a result of an overall reduction of CD36 on the sarcolemma and intracellular microsome in HF, this is not seen when the whole tissue lysate is immunoblotted. Similarly, for GLUT4, the summations of subcellular fractionation observed no change in total GLUT4, while whole tissue lysate had significant increases in GLUT4 in HF. The disparity observed between the two methods is unknown and further work is warranted to unpick whether there are alterations in CD36 on the sarcolemma as a result of decreased total amounts of the protein or if there are no changes in total protein and less of the total CD36 is being localised to the sarcolemma in HF.

Another limitation of this study is the variation in the etiologies of HF samples. A recent report by Sayour and colleagues investigated alterations mRNA expression of glucose transporters in various etiologies of end stage HF and observed dramatic differences in mRNA changes depending on the HF etiology²⁴⁸. Therefore, it is important to explore sarcolemma CD36 and total CD36 in within same HF etiology in order to fully delineate the role of CD36 in the pathophysiology of HF.

6.6.3 CD36 deficiency may be a risk factor for the development of HF

Our data also indicates that one of the HF donors was potentially deficient in CD36. This finding is of interest as CD36 deficiency has been reported in humans, largely in individual case studies, and has been suggested to accelerate HF^{250–253}. Flow cytometry has identified two categories of CD36 deficiency. Type 1 CD36 deficient patients are devoid of CD36 on platelets or monocytes, while Type 2 patients lack CD36 on platelets only²⁵⁰. Myocardial LCFA β -methyl-iodophenyl pentadecanoic acid (123-I-BMIPP) scintigraphy studies have identified that Type 1 deficiencies have no LCFA accumulation in the heart, while Type 2 deficiencies have reduced LCFA uptake compared to healthy hearts^{250,254}. As such, Type 1

deficient patients were shown to not express CD36 on capillary endothelial cells, whereas Type 2 deficiencies only have reduced CD36 levels on capillary endothelial cells compared to non-deficient controls²⁵⁰. Furthermore, Type 1 deficiencies are associated with CD36 gene mutations: the base mutation C to T at nucleotide position 478 (C478T), the two base deletion A and C at nucleotide positions 549 and 540 (Del539AC) and the single base insertion of A at position 1159 (Ins1159A)²⁵². Therefore, Type 1 deficiencies are characterized by having a combination of two of the CD36 mutations, impaired 123-I-BMIPP accumulation and are devoid of CD36 on platelet, monocytes and endothelial cells²⁵². Interestingly, Type 1 deficiencies have been largely implicated the development of heart diseases including hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), coronary artery disease (CAD), hypertension, and valvular heart disease^{250–253}. Though purely observational, we report that while total CD36 protein content was devoid in the CD36 deficient patient, there appeared to be a potentially compensatory increase in total GLUT4 protein content. This is in support of a more recent case study where a mitral valve prolapse patient who was diagnosed with a Type 1 CD36 deficiency had decreased FA metabolism and enhanced glucose metabolism, which was implicated in the severity of their HF²⁵⁴. Therefore, similar to findings in rodent models devoid of CD36^{233,236}, it appears that humans with Type 1 CD36 deficiencies are at a greater risk of developing heart diseases and heart failure. The HF donor was devoid of CD36 was diagnosed with pulmonary arterial hypertension caused by left heart disease (PAH L/D), which is a remodelling disease that affects the pulmonary vasculature leading to HF and premature death. As such, this donor was the youngest of the HF donor patients and had the highest left ventricular mass index (LVMI), indicative of cardiac hypertrophy. These results are similar to findings in aortic stenosis patients where it was found that the patients with the least amount of CD36 had greater LVMI¹³⁷.

6.6.4 AMPK and ACC have elevated *S*-acylation levels in ischemia

As myocardial ischemia plays a role in HF, we next aimed to explore the *S*-acylation status of key metabolic targets. Given that CD36 has been shown to internalize during acute ischemia in rat hearts¹⁴⁰ and we have shown a decrease in CD36 on the sarcolemma in human HF tissue, we hypothesized that CD36 *S*-acylation would be decreased during acute ischemia. However, we observed no alterations in CD36 or DHHC5 *S*-acylation between control and ischemic hearts. Therefore, this chapter indicates that following acute ischemia in intact hearts that *S*-acylation is not the mechanism-of-action driving CD36 away from the sarcolemma. Further work is warranted to elucidate the mechanisms behind the decrease in CD36 from the sarcolemma in acute ischemia and in HF.

In the ischemic heart, AMPK and ACC play crucial roles in governing the metabolic response, therefore it is important to investigate the regulatory mechanisms driving their activity. Although the regulation of AMPK and ACC by phosphorylation has been well characterized, less is known surrounding the role of *S*-acylation on these proteins. AMPK has recently been shown to be *S*-acylated by DHHC17 and importantly *S*-acylation was required for AMPK phosphorylation and subsequent metabolic responses²⁴³. This finding demonstrates a potential novel *S*-acylation-phosphorylation regulatory axis of metabolism. Ischemia represents a potent stimuli for AMPK phosphorylation and is a physiological setting where there is an acute increase elevations of long chain fatty acids, including those implicated in *S*-acylation as we demonstrated in this chapter. Furthermore, recently DHHC17, the palmitoylating enzyme responsible for AMPK *S*-acylation in adipocytes has been identified in the heart¹⁴⁷. Therefore, it could be plausible that *S*-acylation plays a regulatory role in mediating signalling kinase activity of AMPK by enhancing membrane affinity and subsequent phosphorylation resulting in the metabolic

response to cardiac ischemia. In further support of the *S*-acylation-phosphorylation regulatory axis, *S*-acylation was shown to regulate the activity of another key signalling kinase, Akt. Akt was shown to be *S*-acylated in adipocytes and Akt *S*-acylation was shown to be required for its cycling to the plasma membrane following insulin stimulation and importantly, Akt was shown to be phosphorylated only if *S*-acylation was present²⁵⁵. Therefore, understanding the functional role of enhanced AMPK *S*-acylation in ischemia could unravel another level of regulation of this key metabolic signalling kinase. Investigating whether AMPK *S*-acylation and DHHC17 are altered under chronic ischemic conditions such as infarcted rat heart models would also be of interest.

In contrast to AMPK, this study is the first to our knowledge where ACC has been shown to be *S*-acylated in any tissue, and that ischemia is a potent stimulator for its *S*-acylation. Given the important role of ACC in mediating FAO in ischemia through modulating malonyl-CoA levels, understanding the function of ACC *S*-acylation could provide further insight into the regulation of cardiac lipid metabolism. Pre-clinical evidence has suggested that inhibiting FAO may be an effective approach to treating ischemic heart disease²⁵⁶. Therefore, if *S*-acylation plays a regulatory role in ACC activity, modulating FAO by manipulating the *S*-acylation of ACC could serve as a method of altering malonyl-CoA levels to fine tune cardiac metabolism and improve cardiac contractile function during situations of metabolic stress, such as myocardial infarction.

6.7 Conclusion

This chapter explored cardiac metabolism in two conditions, HF and acute ischemia. In human HF tissue of varying etiologies, sarcolemma CD36 was shown to correlate with cardiac function, indicating that the subcellular localisation of CD36 plays

an important role in HF. This work also uncovered two novel *S*-acylation targets in the heart, AMPK and ACC and demonstrated that ischemia is a stimulus for *S*-acylation.

7

General Discussion

Work in the field of cardiac metabolism has presented mounting evidence that dysregulated lipid utilization is driven by alterations in FA uptake into the myocardium. The inability to fine tune FA uptake, either too little or too much fat, has been shown to have detrimental effects on cardiac function. Based on this information, the main FA transporter in the heart, CD36, has been proposed as a key metabolic regulator driving concomitant changes in FA utilization that occur in various cardiac pathologies³⁶. Therefore, this thesis was an investigation behind CD36 as a causal factor implicated in dysregulated lipid metabolism in two opposing metabolic pathologies as illustrated in Figure 7.1:

1. T2D: increased lipid metabolism
2. HF and acute myocardial ischemia: decreased lipid metabolism

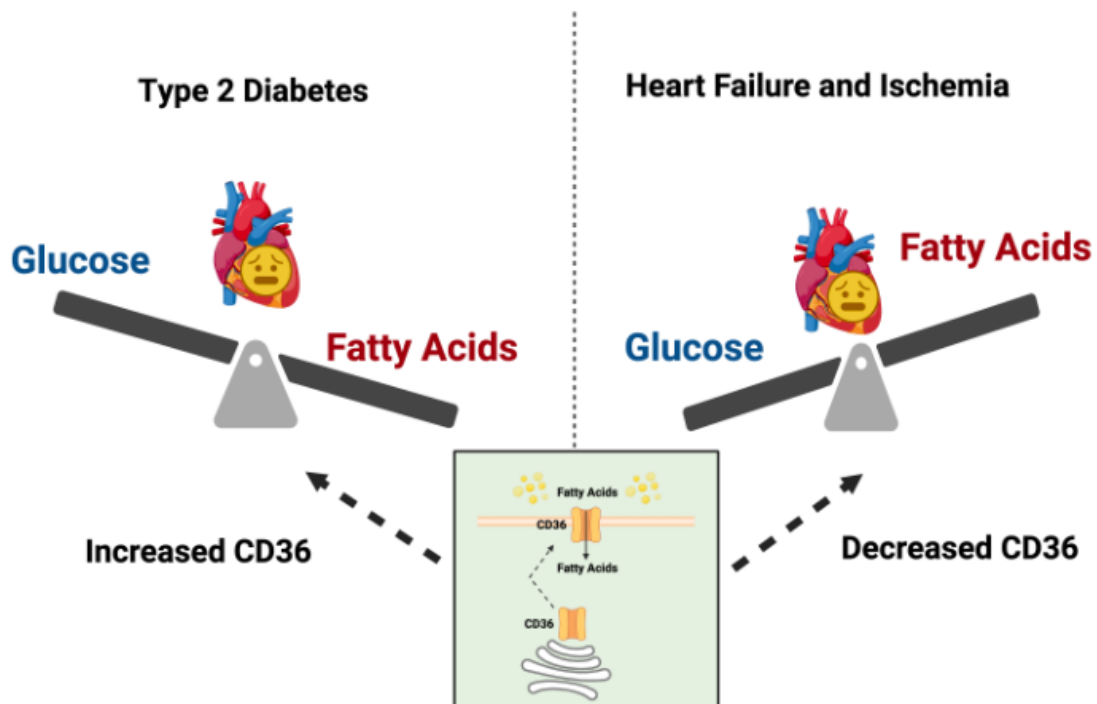


Figure 7.1: This DPhil thesis explores alterations in CD36 in two opposing metabolic pathologies

7.1 Identification of CD36 *S*-acylation as a novel cause of enhanced lipid metabolism in the T2D heart

T2D represents a pathological condition where there is chronically increased cardiac FA uptake at the expense of glucose metabolism. To date, most pharmacological therapies and pre-clinical research has focused on increasing glucose metabolism as a mechanism for improving cardiac function. However, studies have demonstrated that targeting FA metabolism directly, mainly through FAO, has beneficial effects on cardiac outcomes. Although suppressing FAO with CPT1 and β -oxidation enzyme inhibitors has shown beneficial short-term cardiac effects in T2D²⁵⁷, long-term inhibition of FAO has been shown to have deleterious effects on the heart as a result of elevated lipid storage in CPT1 KO mice²⁵⁸. Furthermore, a pharmacological activator of PPAR α resulted in enhanced FAO, which was shown to improve dyslipidemia in T2D patients²⁵⁹ and rodents^{260,261}. However, our group recently demonstrated in a T2D rat model that inhibiting CD36 function using the pharmacological drug SSO reduced both FAO and myocardial TG accumulation back to healthy control levels, and this had beneficial effects on cardiac function in T2D hearts following an ischemic insult²²⁷. In further support, CD36 ablation is cardioprotective in HFD fed mice¹¹. Therefore, targeting CD36 directly in rodents has shown to be effective in re-balancing substrate metabolism in the T2D heart.

Interestingly, the amount of CD36 present at the sarcolemma is the major determinant of FA utilization in the myocardium⁶. Increased sarcolemmal CD36 has been associated with increased palmitate uptake and myocardial TAG accumulation in both rodents⁵⁷ and humans⁵⁹. However, mechanisms behind the increase in CD36 on the sarcolemma in T2D has largely been unexplored. Targeting CD36

sarcolemma trafficking would provide a mechanism by which to fine tune myocardial FA uptake, as opposed to completely abolishing FA uptake, which may be deleterious long term.

7.2 Identification of CD36 *S*-acylation as a novel driver of enhanced lipid metabolism in the T2D heart

The literature surrounding the effects of T2D on the heart indicate that CD36 is increased at the sarcolemma in humans and this translates well into rodent models of T2D. In rodents, chronic activation of the PPAR α receptor has been well established to result in increased CD36 total protein in T2D, however mechanisms to explain the increased sarcolemmal abundance of CD36 are unknown. Therefore, work in Chapters 3-5 investigated a novel mechanism of CD36 trafficking, *S*-acylation. Chapter 3 utilised affinity purification assays, acyl-RAC and acyl-PEG exchange, to demonstrate that CD36 in rat hearts is *S*-acylated on four cysteine residues. Various PTMs have been implicated in the pathology of T2D, including hyper-acetylation²⁶², increased UDP-GlcNacylation²⁶³ and increased glycation²⁶⁴. However, lipidation in T2D has recieved much less attention. Therefore, Chapter 4 demonstrated that *ex vivo* perfused hearts from T2D rats displayed increased sarcolemmal CD36 and hyper-*S*-acylated CD36 levels, with increases in downstream metabolism, including FAO rates and myocardial TG accumulation. By investigating mRNA and total protein levels, Chapter 4 also established that the increase in CD36 *S*-acylation is as a result of increased levels of DHHC4 through enhanced FoxO1 transcription. The data in Chapters 3 and 4 therefore present the first measurements of CD36 hyper-*S*-acylation within the T2D heart and propose a novel FoxO1-DHHC4-CD36 regulatory axis that may be driving the enhanced CD36 sarcolemmal levels observed

in both rodents and humans.

7.3 Targeting CD36 *S*-acylation enables fine tuning of myocardial metabolism

Over the course of chapter 4, a novel adaptation of CD36 within the T2D heart was observed; an increase in CD36 *S*-acylation. This adaptation potentially provides a mechanism by which CD36 has enhanced levels at the sarcolemma. Chapter 5 presented data that CD36 *S*-acylation was decreased following an acute increase in palmitate levels, indicating that perhaps *S*-acylation levels are not driven by substrate availability. However, when the metabolic state of the T2D heart is considered; presenting with chronic increases in FA levels, which increase levels of transcription factors such as FoxO1 that we have linked to DHHC4, it can be concluded that T2D may be a potent driver of enhanced CD36 *S*-acylation. Therefore, more work is required to fully unpick the FoxO1-DHHC4-CD36 axis and whether manipulating levels of FoxO1 is another avenue to decrease CD36 *S*-acylation. The increase in CD36 *S*-acylation appears to be resulting in increased CD36 localisation at the sarcolemma, resulting in further FA uptake and dyslipidemia; suggesting that CD36 *S*-acylation is a key player driving a vicious cycle between FA uptake and enhanced FA metabolism, potentially leading to eventual contractile dysfunction observed in T2D hearts. Therefore, pharmacologically targeting *S*-acylation in *ex vivo* perfused hearts as a method to alter CD36 on the sarcolemma was considered, and which led to uncovering that manipulating CD36 *S*-acylation is an effective approach to alter cardiac metabolism.

Chapter 5 also presented data showing that *S*-acylation levels of CD36 can be pharmacologically manipulated with profound effects on cardiac metabolism. Hearts were perfused with the broad spectrum DHHC inhibitor CMA and the APT1

inhibitor ML348, to better understand how opposing levels of CD36 *S*-acylation affect downstream metabolism. By decreasing CD36 *S*-acylation in hearts from T2D rats, FAO rates were decreased, while increasing CD36 *S*-acylation levels increased FAO rates. Therefore, over the course of Chapters 3-5, CD36 hyper-*S*-acylation was identified as a promising candidate for the source of increased FA uptake within the T2D heart, and targeting CD36 *S*-acylation levels has therapeutic potential alter FA metabolism.

We have demonstrated that CMA and ML348 can be used in *ex vivo* perfused hearts to acutely alter CD36 *S*-acylation levels as illustrated in Figure 7.2. However, there is still a need for more selective small molecule inhibitors⁸⁸. While targeting the regulatory enzymes involved in CD36 *S*-acylation has shown to be effective, these enzymes have been shown to be involved in *S*-acylation cycling of other targets in the cell. Therefore, the development of a specific drug that could directly target and alter CD36 *S*-acylation would be invaluable to fully unpick the metabolic consequences of this lipid PTM on CD36. Furthermore, although ML348 has been shown to have a high degree of specificity and selectivity, CMA is a relatively promiscuous compound. Despite this, given the short time course of the drug perfusion, the degree of off target effects should be minimized. However, future work should aim to conduct proteomics analysis to determine what other targets are being affected by CMA. Lastly, long term *in vivo* work is needed to evaluate both the metabolic and functional consequences of chronically decreased CD36 *S*-acylation in diabetes, however this will be hampered by the lack of chemical tools presently available. Regardless, we have demonstrated for the first time to our knowledge that altering CD36 *S*-acylation levels in an intact beating hearts has downstream metabolic consequences and provides a strong framework for CD36 *S*-acylation as a therapeutic target to improve the metabolic dysfunction of the T2D heart.

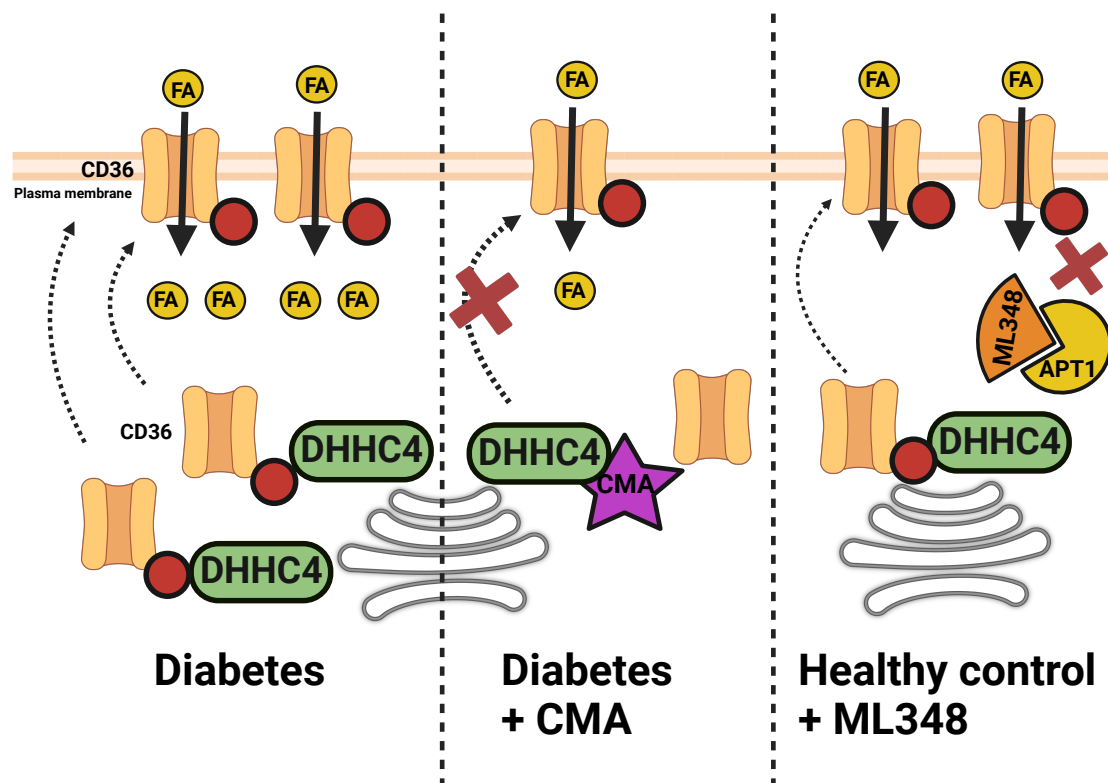


Figure 7.2: In T2D there is an increase in DHHC4 driving CD36 *S*-acylation and trafficking to the sarcolemma to increase FA uptake. Treatment with CMA in diabetic hearts decreases CD36 *S*-acylation and CD36 sarcolemma localisation. Treatment of healthy control hearts with ML348 increases CD36 *S*-acylation, mimicking the diabetic phenotype.

7.4 CD36 in HF, and ischemia *S*-acylation targets

In contrast to T2D, HF and acute myocardial ischemia are characterized by a partial decrease in lipid metabolism. Therefore, a smaller portion of this thesis aimed to investigate alterations in the subcellular localisation of CD36 as potential mechanism resulting in impaired FA uptake in human HF tissue and identified that two key metabolic proteins involved in metabolic response to ischemia are *S*-acylated in rodent hearts.

Within the literature, both glucose and FAO are decreased in HF, however FAO rates are proportionally lower, suggesting metabolic reliance on glucose metabolism

for ATP production²²⁹. Although current therapies have aimed at promoting myocardial glucose metabolism, recent reports have suggested that increases in glucose use alone are not sufficient to increase the energy deficit experienced by the failing heart^{145,229}. Chapter 6 displayed data showing that in failing hearts, CD36 at the sarcolemma is decreased and importantly, the sarcolemma localisation of CD36 correlated with LVEF. However, it remains unknown whether there is reduced CD36 on the sarcolemma as a result of a total decrease or whether more of the total CD36 pool is being internalized. This is the first study to investigate CD36 sarcolemma localisation within the failing heart and identifies CD36 as an exciting potential target for the amelioration of contractile function in HF. In support, recent reports in human HF patients²²⁹ and isolated cardiomyocytes from failing hearts¹⁴⁵ have demonstrated that increasing FA utilization improves contractile function.

In addition to CD36, ACC is a master regulator of lipid metabolism and sits at a key regulatory-axis by governing FAO rates. Identifying mechanisms by which ACC is post-translationally regulated will provide further insight into how FAO is regulated. Chapter 6 demonstrated that ACC is hyper-*S*-acylated in ischemia, although more work needs to be done to understand the functional relevance of this lipid PTM on ACC, and if altered levels of *S*-acylation can modulate ACC activity in the heart. In addition to acute ischemia, determining if ACC is hyper-*S*-acylated in chronic models of ischemia, such as the myocardial infarction rat model, would reveal the time-course of ACC *S*-acylation. Furthermore, future work should identify which regulatory enzymes are involved in ACC *S*-acylation cycling and confirm the *S*-acylated cysteine residues. All in all, ACC *S*-acylation is potentially a novel mechanism by which to fine tune FAO in the myocardium, which would have profound benefits for various cardiac metabolic diseases.

7.5 Conclusion

Therefore, in this thesis, CD36 has been investigated in two opposing metabolic states. Decreasing CD36 on the sarcolemma by targeting *S*-acylation has been shown to be an effective metabolic modulator in T2D, while a decreased level of sarcolemma CD36 are observed in failing hearts; indicating that CD36 plays a critical role in balancing just the right amount of fat the heart needs (Figure 7.3). Collectively, this thesis demonstrates that targeting CD36 and potentially ACC through *S*-acylation is an effective way to re-balance myocardial lipid metabolism, which will have beneficial outcomes for a plethora of metabolic diseases. .

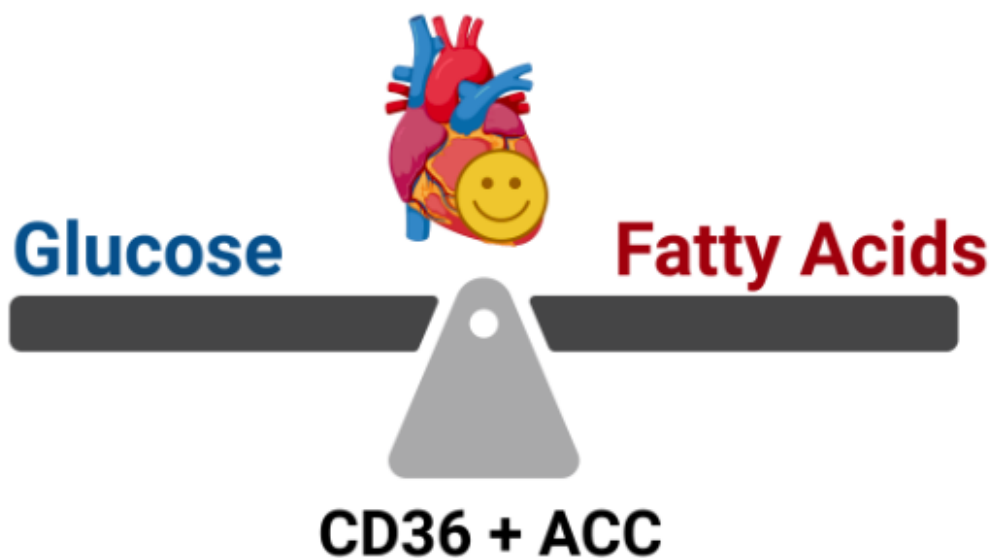


Figure 7.3: CD36 and ACC sit at a key metabolic axis

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