Cardiac lipids and their role in the diseased heart

Ismena Mardani

Department of Molecular and Clinical Medicine
Institute of Medicine
Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

Cover illustration: Fantasy

Cardiac lipids and their role in the diseased heart
© Ismena Mardani 2019
ismena.mardani@wlab.gu.se

ISBN 978-91-7833-632-6 (PRINT)
ISBN 978-91-7833-633-3 (PDF)

Printed in Gothenburg, Sweden 2019
Printed by BrandFactory

Till Mamma & Pappa♥

Co komu pisane, to go nie minie….

Cardiac lipids and their role in the diseased heart

Ismena Mardani
Department of Molecular and Clinical Medicine, Institute of Medicine
Sahlgrenska Academy, University of Gothenburg
Gothenburg, Sweden

ABSTRACT

Lipids play an essential role within the heart as they are involved in energy storage, membrane stability and signaling. Changes in cardiac lipid composition and utilization may thus have profound effects on cardiac function. Importantly, the diseased heart is associated with intracellular metabolic abnormalities, including accumulation of lipids. In this thesis, I targeted cardiac lipid droplets (LDs) and membrane lipids using genetically modified mice and cultured cardiomyocytes to investigate how myocardial lipid content and composition affect cardiac function.

In ***Paper I*****,** we investigated the LD protein perilipin 2 (Plin2) and its role in myocardial lipid storage. Unexpectedly, Plin2 deficiency in mice result in increased triglyceride levels within the heart as a result of decreased lipophagy. Even though Plin2$^{-/-}$ mice had markedly enhanced lipid levels in the heart, they had normal heart function under baseline conditions and under mild stress. However, after an induced myocardial infarction, cardiac function reduced in Plin2$^{-/-}$ mice compared with Plin2$^{+/+}$ mice.

We have previously shown in both humans and mice that low levels of cardiac Plin5 are unfavorable for heart function. Therefore, in ***Paper II*** we tested the hypothesis that forced overexpression of cardiac Plin5 is beneficial for heart function. We found that Plin5 promotes exercise-like effects, inducing physiological hypertrophy with enhanced left ventricular mass, but with preserved heart function. Furthermore, calmodulin-dependent protein kinase II (CaMKII) and phospholamban activities were increased by Plin5 overexpression, indicating enhanced cardiac contractility and calcium handling.

In ***Paper III***, we found that the sphingolipid glucosylceramide (GlcCer) accumulates in the human heart following injury. We targeted cardiac *Ugcg* (the gene encoding GlcCer synthase) in mice (h*Ugcg*$^{-/-}$ mice) and found that a significant decrease in GlcCer in cardiomyocytes results in Golgi dispersion and defective autophagy regulation, leading to compromised β-adrenergic signaling. h*Ugcg*$^{-/-}$ mice developed dilated cardiomyopathy and died prematurely from heart failure.

In conclusion, our studies show that dysfunctional cardiac lipid storage plays a role in heart function, both in the healthy and diseased heart. Thus, targeting cardiac lipid accumulation may be a future strategy to delay cardiovascular disease progression.

Keywords: Cardiovascular disease, Lipid droplets, Perilipins, Lipid accumulation, Metabolism, GlcCer.

ISBN 978-91-7833-632-6 (PRINT)
ISBN 978-91-7833-633-3 (PDF)

SAMMANFATTNING PÅ SVENSKA

Hjärtsjukdom är den vanligaste orsaken till sjuklighet och dödlighet i världen. Trots förbättrad behandling av hjärtinfarkt, högt blodtryck och klaffsjukdom så utvecklar fortfarande en stor del av de överlevande patienterna hjärtsvikt. Forskning kring nya behandlingsformer för hjärtsjukdom är därför ytterst relevant.

Vid hjärtsjukdom så sker en så kallad remodellering av hjärtat, där vänsterkammaren genomgår form- och volymförändringar. Detta är en omedelbar kompensation för att öka hjärtkontraktiliteten akut men leder i förlängningen till försämrad pumpfunktion och hjärtsvikt. Remodellering av hjärtat är också kopplat till en metabol adaptation där kardiomyocyterna ställer om från att främst använda sig av lipider som substrat till att förlita sig på glukos, vilket i sin tur leder till ökad inlagring av lipid i hjärtat. Lipid lagras i hjärtats celler i så kallade lipiddroppar, främst i form av neutrala fetter (som kolesterolestrar och triglycerider). Lipiddropparnas lagringsförmåga regleras av lipiddroppsproteiner, framförallt perilipiner (Plin). Vid hjärtsjukdom ökar de neutrala lipiderna men också inlagring av lipider som sitter i kardiomyocyternas membran, t.ex. sfingolipider. Lipidinlagring i hjärtat är kopplat till försämrad hjärtfunktion. Dock finns det fortfarande mycket liten kunskap om hur och varför lipidinlagring egentligen skadar hjärtat. I denna avhandling har vi studerat sambandet mellan hjärtats lipidlagring och hjärtfunktion, både i det friska hjärtat och vid sjukdom.

Delarbete I. I denna studie har vi undersökt om Perilipin 2 (Plin2) är involverad i lipidinlagring och påverkar hjärtfunktionen. Avsaknad av Plin2 i möss ledde paradoxalt nog till ökade nivåer av lipid i hjärtat, som ett resultat av minskad lipofagi. Trots att mössen som saknar Plin2 har nästan dubbelt så mycket inlagrad lipid i hjärtat så har de basalt en normal hjärtfunktion.

Delarbete II. Eftersom vi tidigare har visat hos både människor och möss att det är negativt för hjärtfunktionen att ha låga nivåer av Plin5, så undersökte vi i delarbete II hur hjärtat påverkas av ökade nivåer av Plin5. Ökat uttryck av Plin5 leder till fysiologisk hypertrofi i hjärtat, liknande den som induceras av fysisk aktivitet, och resulterar i en skyddande hjärtfunktion.

Delarbete III. I detta arbete studerade vi inlagringen av sfingolipiden glucosylceramid i hjärtat och vilken funktion denna lipid har i kardiomyocyter. Vi fann att kraftigt minskade nivåer av glucosylceramider leder till dilaterad kardiomyopati och prematur död hos möss, vilket visar att denna sfingolipid har en viktig funktion för hjärtats normala fysiologi.

Slutsatsen från våra studier är att en dysfunktionell reglering av lipidlagring och hur lipider används av hjärtat påverkar hjärtfunktionen både i det friska hjärtat och vid hjärtsjukdom.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Mardani I, Dalen KT, Drevinge C, Miljanovic A, Ståhlman M, Klevstig M, Scharin Täng M, Fogelstrand P, Levin M, Ekstrand M, Nair S, Redfors B, Omerovic E, Andersson L, Kimmel A.R, Borén J and Levin M.C. **Plin2-deficiency reduces lipophagy and results in increased lipid accumulation in the heart.** *Scientific reports, 2019 May 6;9(1):6909*

II. Mardani I, Cinato M, Miljanovic A, Drevinge C, Bollano E, Ståhlman M, Levin M, Lindbom M, Scharin Täng M, Klevstig M, Fogelstrand P, Andersson L, Borén J and Levin M.C. **Cardiac-specific overexpression of perilipin 5 promotes physiological hypertrophic remodeling of the heart by fine-tuning calcium-handling regulators.** *Manuscript*

III. Andersson L*, Mardani I*, Miljanovic A, Cinato M, Koh A, Lindbom M, Klevstig M, Ståhlman M, Fogelstrand P, Swärd K, Ekstrand M, Levin M, Tivesten Å, Adiels M, Bergö M, Proia R, Jeppsson A, Borén J and Levin M.C. **Cardiomyocyte-specific deficiency in glucosylceramide synthase results in dilated cardiomyopathy and premature death in mice.** *Authors contributed equally. *Manuscript*

CONTENT

ABBREVIATIONS

Ach	Acetylcholine
ACS	Acetyl-CoA synthetase
ADRP	Adipocyte differentiation-related protein
AMPK	Adenosine monophosphate-activated protein kinase
ANP	Atrial natriuretic peptide
ANS	Autonomic nervous system
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
β-AR	β-adrenergic receptor
BNP	Brain natriuretic peptide
BPM	Beat per minute
CamKII	$Ca^{2+/}$Calmodulin-dependent protein kinase II
cAMP	cyclic AMP
CAT	Carnitine-acylcarnitine translocase
CE	Cholesterol ester
CGI-58	Comparative gene identification-58
CMA	Chaperone-mediated autophagy
CO	Cardiac output

CPT	Carnitine palmitodyltranferase
CVD	Cardiovascular disease
DG	Diglyceride
DGAT	Diglyceride acyltransferase
DHA	Docosahexaenoic acid
ER	Endoplasmic reticulum
FA	Fatty acid
FABP	Fatty acid-binding protein
$FADH_2$	Flavin adenine dinucleotide
FATP	Fatty acid transporter protein
GlcCer	Glucosylceramide
GPCR	G-protein coupled receptor
G6F	Glucose-6-phosphate
HDL	High-density lipoprotein
HEK	Human embryonic kidney
HF	Heart failure
HFD	High fat diet
HIF1-α	Hypoxia-induced factor 1-α
HR	Heart rate

HSL	Hormone-sensitive lipase
HSC70	Heat shock cognate 70 kDa protein
LacCer	Lactosylceramide
LAD	Left anterior descending
Lamp-2a	Lysosomal-associated membrane protein 2a
LCFA	Long-chain fatty acid
LC3B	Light chain 3B
LCS	Lactosylceramide synthase
LD	Lipid droplet
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
LSDP5	Lipid storage droplet protein 5
LV	Left ventricle
MEF	Mouse embryonic fibroblast
MGL	Monoglyceride lipase
MHC	Myosin heavy chain
MI	Myocardial infarction
MLDP	Myocardial LD protein
mTOR	Mammalian target of rapamycin
M6PRBP	Mannose-6-phospate receptor-binding protein

NADH	Nicotine amine dinucleotide
NAFLD	Non-alcoholic fatty liver disease
PA	Phosphatic acid
PDH	Pyruvate dehydrogenase
PGC-1	Proliferator activated-receptor gamma co-activator-1
PLB	Phospholamban
Plin	Perilipin
Plin1	Perilipin 1
Plin2	Perilipin 2
Plin3	Perilipin 3
Plin4	Perilipin 4
Plin5	Perilipin 5
PPAR	Peroxisome proliferator-activated receptor
PKA	Protein kinase A
ROS	Reactive oxygen species
SA	Sinoatrial node
SV	Stroke volume
SEM	Standard error of the mean
SIRT1	Sirtuin 1

TCA	Tricarboxylic acid
TF	Transcription factor
TG	Triglyceride
TIP47	Tail-interacting protein 47
VLDL	Very low-density lipoprotein
WAT	White adipose tissue

INTRODUCTION

Cardiovascular diseases (CVDs) is the main cause of morbidity and mortality in the world. In spite of improved treatment strategies for myocardial infarction (MI), hypertension and valve diseases, a large proportion of surviving patients are still developing heart failure (HF) with time. Research involving new treatment strategies for CVDs are therefore utterly crucial [1, 2].

Remodeling of the heart is occurring in response to pathological injury, that results in a change in shape and volume of the left ventricle (LV). This is an immediate compensation to increased cardiac contractility, but over time it will result in a decreased pump function and HF [3-5]. Remodeling of the heart is also associated with metabolic adaptations, the cardiomyocytes switch from using lipids as the main source for energy to rely on glucose instead. This will result in an increased accumulation of lipids in the heart [6-8]. Lipids are stored in the cardiomyocytes in organelles called lipid droplets (LDs), primarily in the form of neutral lipids (cholesterol esters and triglycerides). The LDs storage ability is regulated by LD proteins, mainly the perilipin (Plin) family [9-12]. In response to cardiac diseases neutral lipids are increasing and lipids that are located in the membranes of the cardiomyocytes are accumulating, e.g. sphingolipids. Lipid accumulation in the heart is associated with a decreased heart function [13, 14]. However, there is still very little knowledge about how and why lipid accumulation in the heart is detrimental. In this thesis, we have studied the correlation between lipid accumulation in the heart and heart function, both in the healthy and diseased heart.

1.1 CARDIOVASCULAR SYSTEM

The heart is the powerhouse of the body that pumps blood through both arteries and veins called the cardiovascular system. The heart is constructed of 4 chambers, left and right atrium and left and right ventricle. With each heartbeat is oxygen-poor blood flowing in from the systemic circulation to the right atrium and then further down to the right ventricle where it is pumped out to the lung through the pulmonary vein to receive oxygen. From the lung, the oxygen-rich blood flows back into the left atrium and then to the left ventricle were it is being pumped out to the systemic circulation that will supply the rest of the body with the oxygenated blood [15]. The heart itself is supplied with blood from the epicardial coronary arteries surrounding the heart. The left coronary artery supplies the left artery, ventricle and interventricular septum (the wall separating left and right ventricle), while the right coronary artery supplies the right atrium, ventricle and the heart's conduction system that regulates contraction of the heart [16, 17].

The left ventricle (LV) ejects approximately 70 ml blood during each heart beat defined as stroke volume (SV), and about 5 litres per minute, known as cardiac output (CO). CO is calculated through SV times heart rate (HR). Cardiac contraction or heart rate is initiated and controlled by electrical impulses that are being produced and directed by specialized cardiac cells in different regions of the heart. The activation begins in the sinoatrial (SA) node, consisting of a band of spontaneous de-polarizing cells in the right atrium. The rate in the SA node is more rapid than in any other region of the heart, and is referred as the pacemaker of the heart. As the impulse spreads to the other regions, it causes the cells to excite.

The heart rate which is normally around 80 beats/min is regulated by the SA node that is further controlled by hormones and nerve impulses. More specifically, it is the autonomic nervous system (ANS) that regulates this machinery and consists of the sympathetic and parasympathetic system that works together [18, 19]. The

parasympathetic system is regulated through the vagus nerve that forms synapses with cells in the SA node. When the neurotransmitter acetylcholine (ACh) is released and binds in to muscarinic acetylcholine receptors, more specifically M_2 receptors on the cardiac cells within the SA node, a decrease in HR referred as a negative chronotropic effect is induced. The sympathetic system regulates through postganglionic fibres that innervate the SA node. The fibres release noradrenaline (NA) that act on β1-adrenergic receptors (β1-AR) on the cells that increase the HR, referred as a positive chronotropic effect. The parasympathetic response on the SA node controls during normal conditions to give a normal HR. An increase in HR is fine-tuned by the sympathetic system that is commonly known as the "fight or flight" response and dominates during exercise, stress and emergency situations [20, 21].

The cardiac cycle consists of two phases, the diastolic phase which is when the heart is relaxing and the heart is filling up with blood, and the systolic phase which is when the heart is contracting and ejecting the blood out to the body [21, 22]. The contracting cells are the cardiac muscle cells, so called cardiomyocytes, that make up 75% of the heart. Additional cell types includes smooth muscle cells, endothelial cells of the coronary vasculature, fibroblasts and other connective tissue cells [23].

1.1.1 B-ADRENERGIC RECEPTOR SIGNALING

The cardiomyocytes which are the contracting cells within the heart are mainly regulated by the β-adrenergic receptors (β-AR) that belongs to the family of G-protein coupled receptors (GPCRs). β-AR activation controls HR and contraction of the heart [24]. The human heart express two classes of adrenergic receptors, the α-adrenergic and β-adrenergic receptors. They are further divided into nine subgroups with β1-ARs being the predominant subgroup of the heart [25, 26]. β-ARs are connected to G proteins and during stimulation with catecholamines (adrenaline and noradrenaline), a conformational change of the G proteins is induced. This results in a dissociation of the G protein into

subunit components that act as signalling units. This starts a cascade causing an accumulation of the second messenger cyclic adenosine monophosphate (cAMP) that binds into protein kinase A (PKA). The kinase then phosphorylates a number of proteins affecting contractility [24, 27]. PKA specifically targets β-ARs themselves, troponin I, phospholamban (PLB) and sarcoplasmic reticular Ca^{+}/ATPase inhibitory proteins, all involved in the regulation of cardiac contraction [28].

Phosphorylation of β-ARs causes an uncoupling and downregulation of the receptor. The uncoupling of the receptor from the G protein induces internalization of the receptor. The receptor shifts from being on the plasma membrane into cytosolic compartments which is essential to keep a balance between activation and deactivation for normal cellular homeostasis [28, 29]. The diseased heart, such as during HF is characterized by a hyperactivation of the sympatic nervous system leading to an increase of circulating catecholamines as a compensatory response to maintain a normal CO. However, this chronic stimulation is damaging and causing dysfunction in the signalling pathway. β1-ARs undergo downregulation and uncoupling from G proteins making the receptors desensitized. The degree of downregulation and desensitization is linked to the severity of the HF which further can impair heart function causing a vicious outcome [24, 27, 28].

1.2 CARDIOVASCULAR DISEASES

Despite the progress in cardiology, CVDs remain the most common cause of death and morbidity in the industrialized world. The increase of major risk factors such as diabetes, hypertension and obesity combined with a growing population that live longer, additionally increases the burden of CVDs [30-32]. CVDs consists of different types of diseases including myocardial infarction (MI), cardiomyopathy, heart arrhythmias, artery diseases and HF amongst others [2]. More patients are surviving and live longer after a myocardial event today, resulting in a higher incidents of patients developing HF. This phenomenon occurs

due to complex cellular and molecular changes called ventricular remodelling [4, 33]. A major factor contributing to HF is that the human heart has very little regenerative capacity with a renewal rate of 0,5-1 % per year of the cardiomyocytes. The low rate of cardiomyocyte turnover is not enough to compensate for the major loss of cells after an injury such as a MI can induce [34].

1.2.1 CARDIAC REMODELING IN THE DISEASED HEART

In response to a myocardial event such as a MI, the heart is undergoing remodeling to compensate for the loss of contractile function in the injured area. This is shown as molecular and cellular changes identified as alterations in size, mass, geometry and function [33]. Early changes can be seen within hours to days after an insult. Myocardial cell death, so called necrosis results in an infiltration of inflammatory cells such as neutrophils and macrophages [35]. These cells start to break down collagen resulting in a thinning and dilation of the area of injury. Simultaneously fibroblasts are arriving and start to produce new collagen contributing to scar formation. Over the following weeks and months, the cardiomyocytes in the non-injured area are becoming hypertrophic as a response to the increased work-load in the heart resulting in a dilation of the LV. This progression is initially beneficial and aimed to maintain a normal CO. Unfortunately, over time are these changes resulting in a further increased LV size, that causes more wall stress and further dilation and ultimately HF (**Figure 1**) [4, 33, 36]. There are also several molecular changes that are induced that indicates that the remodeling process is ongoing in the diseased heart. In particular, reactivation of the fetal gene program that is silenced in the normal adult healthy heart. This is shown as alterations in the expression of myosin heavy chain (MHC) isoforms, with a decrease in α- and an increase in β-MHC inducing a slower contraction [37]. Increased expression of GLUT-1, reflecting a changed substrate utilization in the diseased heart and increased expression of myocardial stretch markers

atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are detected [3, 4].

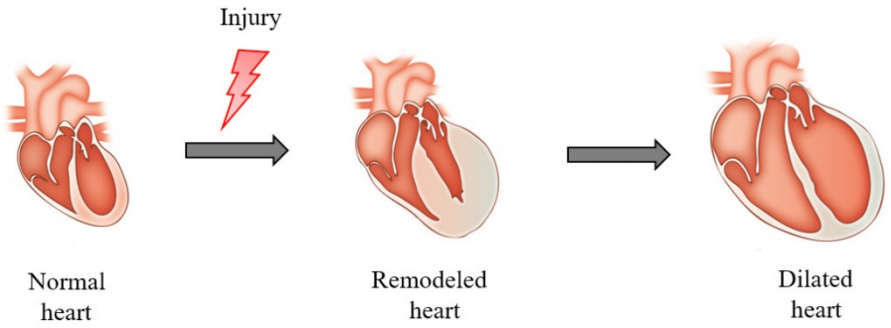

Figure 1. Schematic overview of remodeling in the diseased heart.

Cardiac hypertrophy which is the increase in heart mass is considered to be a bad prognostic sign associated with many forms of HF [38]. Hypertrophy can be divided into two subgroups, pathological hypertrophy and physiological hypertrophy. Pathological hypertrophy is induced in response to heart diseases. It is a condition where the vital cardiomyocytes becomes thicker resulting in a decreased size of the chambers and a reduced capacity to pump blood, this to compensate for the dead cardiomyocytes and the fibrotic remodelling [38, 39] On the contrary, physiological hypertrophy which is seen in athletes, is induced by exercise training and can lead to increased cardiomyocyte size. This is characterized by normal cardiac morphology with normal or enhanced cardiac function completely opposite to pathological hypertrophy [40].

1.3 METABOLIC SUBSTRATE UTILIZATION OF THE HEART

The metabolic demands are the highest for the heart compared to any other organ in the body, and a normal cardiac metabolism is needed to fuel contractility and vitality. The energy for the heart is dependent on sufficient amount of substrates and oxygen to generate adenosine triphosphate (ATP) that fuels the heart [7, 41]. There are 3 kinds of substrates used for the generation of ATP; Fatty acids (FAs),

carbohydrates such as glucose and ketone bodies. FAs are the favoured substrate used in the heart and generates most ATP.

1.3.1 FA UPTAKE AND UTILIZATION

Long-chain fatty acids (LCFA) are important substrates for the heart, but because of their low solubility in water they are provided to the heart attached to plasma albumin or bound in triglyceride (TG) core of circulating lipoproteins, such as chylomicrons and very-low-density lipoproteins (VLDL). These lipoproteins are hydrolysed by lipoprotein lipase (LPL) located on epithelial cells of capillaries within the heart [7, 42]. FAs enter the cardiomyocytes through FA transporters on the cell membrane such as CD36/FAT, fatty acid-binding protein (FABP) and fatty acid transport protein (FATP). Within the cell, the FAs are converted to long-chain acyl-CoA by fatty acyl-CoA synthetase. For the long-chain acyl-CoA to be utilized for the generation of ATP, the enzyme carnitine palmitodyltransferase 1 (CPT1) is converting it to fatty acylcarnitine enabling passage into the mitochondria. Within the mitochondria is the fatty acylcarnitine further transported across to the inner mitochondrial membrane by carnitine-acylcarnitine translocase (CAT). Inside the inner mitochondrial membrane fatty acylcarnitine is converted back to fatty acyl-CoA by carnitine palmitodyltransferase 2 (CPT2) for the utilization during β-oxidation [43]. However, medium-chain FAs do not require these proteins to enter the mitochondria. Each cycle of β-oxidation result in the shortening of the FA by two carbons and the production of acyl-CoA, an acetyl-CoA, flavin adenine dinucleotide ($FADH_2$) and nicotinamine dinucleotide (NADH). The resulting acyl-CoA enters another cycle of β-oxidation, the acetyl-CoA enters the tricarboxylic acid cycle (TCA-cycle), and the electron carries $FADH_2$ and NADH delivers electrons to the electron transport chain [7, 44, 45].

The TCA-cycle, also known as the Krebs cycle and citric acid cycle is located in the mitochondrial matrix [46]. This cycle consists of reactions used to release stored energy through the oxidation of acetyl-CoA and

the generation of NADH and $FADH_2$. The NADH and $FADH_2$ are then further fed into the electron transport chain that contains four complexes that creates a proton gradient. This gradient facilitates the generation of 12 adenosine triphosphates (ATPs) and carbon dioxide (CO_2) through oxidative phosphorylation by ATP synthase, used to fuel the heart [47].

1.3.2 ROLE OF MITOCHONDRIA WITHIN THE HEART

Within the cardiomyocytes are mitochondria occupying one third of the cell volume to cope with the high energy demand of the heart [48]. Mitochondrial fusion and fission is a constant ongoing process which is essential for the maintenance of normal function, and depends on the metabolic needs of the cardiomyocytes [49, 50]. Fusion of mitochondria generates large mitochondrial networks that is beneficial in metabolic active cells and facilitates smoother oxidative phosphorylation capacity. In quiescent cells, mitochondria are instead present as small fragmented spheres or rods in response to fission [51]. However, an optimal function is maintained by having a balanced regulation of fusion and fission and dysfunction in this process is considered as a crucial factor in cardiac pathology [50]. Mitochondrial dysfunction is specifically associated with abnormal electron transport chain activity, reduced ATP production resulting in a shift in metabolic substrate utilization, reactive oxygen species (ROS) overproduction and impaired mitochondrial dynamics that will contribute to the development of different heart diseases [50, 52, 53].

1.3.3 GLUCOSE UPTAKE AND UTILIZATION

The heart is adapted to utilize all kinds of substrates to meet the high-energy demands. Although FAs are known to be the predominant substrate in the heart during resting conditions, the heart is also having a basal glucose uptake and utilization. However, a prominent switch in substrate utilization towards glucose is induced during stressful conditions such as in response to ischemia or pathological hypertrophy [54-56].

Glucose uptake in cardiomyocytes is mediated by the glucose transporter isoforms GLUT1 and GLUT4. GLUT4 is the predominant GLUT isoform in the heart and it is regulated by insulin [55], and by cardiac contraction were its translocation to the plasma membrane in intracellular vesicles dependent on the work-load of the heart [56]. Therefore, GLUT4 transport and uptake is strongly regulated by environmental changes within the heart [54]. GLUT1 is found in the plasma membrane and regulates basal glucose uptake [54]. In addition, GLUT1 has been shown to be regulated by long-term fasting and chronic hypoxia that is mediated by hypoxia-inducible factor 1-α (HIF-1α) involved in the response to low oxygen concentration within the body [57, 58]. When glucose is taken up in the cardiomyocytes it is rapidly phosphorylated into glucose 6-phospate (G6P) that can enter many different pathways. The major pathway G6P enters is glycolysis to generate pyruvate, NADH and ATP. In the cytosol, pyruvate can be converted to lactate, and if transported into the mitochondrial matrix, pyruvate undergoes oxidation to acetyl-CoA for the TCA-cycle [55, 59].

A large number of studies has shown that transcriptional regulation of genes involved in mitochondrial oxidation are changed extensively during the progress towards pathological hypertrophy and HF [55]. Down-regulation of peroxisome proliferator-activated receptor α (PPARα) and proliferator-activated receptor gamma co-activator-1 (PGC-1) which are master regulators of genes involved in FA oxidation has been observed. This impairment facilitates the increased AMP-activated protein kinase (AMPK) activity that further promotes translocation of glucose transporters to the plasma membrane and enhanced glucose uptake and glycolysis. Nevertheless, studies have shown that increased glucose utilization is not harmful for the adult heart, but the reduced ability to utilize glucose might be detrimental in the diseased heart [58].

1.4 LIPIDS IN THE CELL

Lipids are fundamental components of all cells, playing an important role in energy storage, cellular stabilization and signalling. Lipid composition differ in different tissues, cell types and in each organelle suggesting that different lipid compositions are required for different functions [60]. Lipids are components of cellular compartments such as plasma membrane, lipid droplets (LDs), nuclear membrane, endoplasmic reticulum (ER), Golgi apparatus and vesicles like endosomes and lysosomes [60]. The development in mass spectrometry has discovered that living cells consists of thousands of different lipids and through lipidomics we can now address the lipid distribution in different compartments [61].

1.4.1 MEMBRANE LIPIDS

Membrane lipids are amphipathic, meaning that they have a polar hydrophilic end and a non-polar hydrophobic end. In aqueous medium, membrane lipids do organize into bilayers with the polar ends oriented towards, and non-polar ends oriented away from the solution forming a membrane. There are three major classes of membrane lipids, which are; phospholipids, cholesterol and glycolipids, with phospholipids being the predominant membrane lipid [62, 63].

1.4.2 PHOSPHOLIPIDS

The major class of membrane lipids are phospholipids and they are abundant in all biological membranes. The structure of phospholipids consists of two hydrophobic FA tails and a hydrophilic head consisting of a phosphate group. Phospholipids can be built on a backbone of glycerol, carbon alcohol or on a sphingosine. These lipids facilitate the anchoring of proteins in the cell membrane and impact cellular shape. They are also essential for the regulation of exocytosis, endocytosis and chemotaxis [64]. There are four major phospholipids that are abundant in the plasma membrane of mammalian cells and they are; phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and sphingomyelin (SM) [65, 66].

1.4.3 CHOLESTEROL

Cholesterol is structurally different from phospholipids and glycolipids and is built up from four linked hydrocarbon rings. A hydrocarbon tail is at one end of the steroid and a hydroxyl group is attached to the other end. The fused ring structure of cholesterol makes this lipid more robust than the other membrane lipids [66]. Cholesterol do regulate membrane fluidity, supply the body with fat and is involved in synthesis of hormones [66]. Cholesterol is found with varying degree in different membranes. Almost 25% of membrane lipids in certain nerve cells are made up from cholesterol while in some intracellular membranes they are not present [67, 68].

1.4.4 GLYCOLIPIDS

Glycolipids are the sugar containing lipids that have one or more sugar attached to the backbone. These lipids are involved in regulating cell-shape, fuel storage and are receptor components [63]. They do also serve as markers for cellular recognition thus facilitating cell-cell interactions [69].

GLUCOSYLCERAMIDES

Glucosylceramides (GlcCer) is the simplest form of glycolipids consisting of a ceramide with a sugar attached to it [69, 70]. The attachment of the sugar referred as glycosylation occurs by an enzyme called glucosylceramide synthase (GCS) that is encoded by the gene UGCG. GCS is localized to the Golgi apparatus and more specifically to the cis-Golgi site [69]. GlcCer is a precursor for over 300 species of glycosphingolipids, such as lactosylceramides (LacCer) that are important signaling molecules [71]. However, GlcCer must cross the lumen of the Golgi apparatus to be converted to higher order glycosphingolipids such as LacCer. GlcCer are involved in signal transduction, membrane trafficking, cytoskeletal organization and pathogen entry [72]. GlcCer together with cholesterol are further believed to be involved in the formation of lipid rafts and caveolae

structures within the plasma membrane involved in different signaling pathways [63, 73].

Studies have shown that GlcCer is an absolutely essential lipid for the development of mammals, as mice lacking GlcCer do not survive due to embryonic lethality. However, to much GlcCer is also not beneficial reflected in Gauchers disease. This is the most common lysosomal storage disorder caused by a mutation in the gene for β-glucocerebrosidase, which impairs or eliminates the enzyme that normally hydrolyses GlcCer to ceramide and glucose [74]. This results in excessive accumulation of GlcCer in different organs thus facilitating dysfunction in these tissues [75].

1.4.5 LIPID DROPLETS

Lipid droplets (LDs) are evolutionary conserved organelles found in almost all cell types from bacteria to mammals [76]. This dynamic organelle consists of a core of neutral lipids such as cholesterol esters (CE) and triglycerides (TG). The membrane that is surrounding the core consists of phospholipids, cholesterol and proteins with different function with the major protein family being the perilipins (Plins) (**Figure 2**) [10, 76-79]. The formation of LDs is not completely elucidated and there are several theories on how LDs are formed. The most accepted theory is that they are formed *de novo* from ER in eukaryotes. It is believed that a lens of neutral lipids forms on the cytosolic side of the ER bilayer that buds of from the membrane. LDs seem to remain in contact with ER once they are formed, and proteins that associate with both organelles move between them [78, 80].

LDs store lipids that can be used as metabolic fuel, for membrane components, post-translational protein modifications and signaling molecules within the cell [77, 80]. The energy status and nutrient availability within the cell controls whether the LD is expanding, shrinking, fusing or disappearing. They can vary in size from 100 nm up to 100 μm in white adipose tissue (WAT) filling up the entire

adipocyte [10, 80]. Proteomic studies have shown that there is up to 200 proteins that are being associated with LDs, some of which only exist on LDs while others are also found on other organelles such as ER, mitochondria and peroxisomes that are in close interaction with LDs [76, 81]. The proteins coating the LD can vary between droplets within the cell, between metabolic conditions and between cell types, and the limited capacity for proteins to bind on LDs further regulates this [77, 80, 82]. Therefore, macromolecular crowding plays a major role in determining LD protein composition as proteins compete for binding to the surfaces of LDs. As such, LD binding affinity determines protein localization during LD expansion and shrinking [83]. Dysfunctional lipid storage within LDs has been associated with metabolic diseases such as obesity, type 2 diabetes (TD2), nonalcoholic fatty liver disease (NAFLD) and CVDs [82].

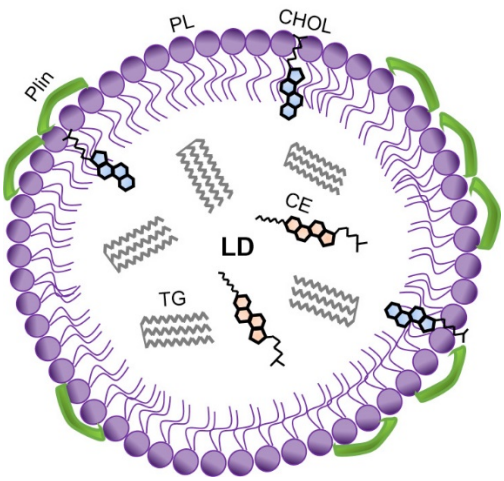

Figure 2. Schematic drawing of LD. LD, Lipid droplet; CHOL, Cholesterol; PL, Phospholipid; Plin, Perilipin; TG, Triglyceride; CE, Cholesterol ester.

1.4.6 TRIGLYCERIDE SYNTHESIS

Triglycerides which is the major storage form of lipids in the LDs is composed of a glycerol backbone and three FAs. The FAs entering the cardiomyocytes can be activated and directly oxidized within mitochondria or they can be esterified and stored as TG. Most of the TG

pool is formed *de novo* within the cell through the glycerol phosphate pathway. *De novo* TG synthesis is initiated by acylation of glycerol-3-phosphate (G3P) with a fatty acyl-CoA to generate lysophosphatidic acid (LPA). This is followed by further acylation and dephosphorylation yielding diglycerides (DGs). The last step to convert DGs to TGs is through acylation by DG acyltransferases (DGATs) within ER generating TGs, which is then stored in LDs. The catabolism of endogenous TG happens likely via the re-esterification of DGs derived from TG breakdown [84, 85].

1.5 PERILIPINS

The most abundant protein family on LDs are the perilipins (Plins) and until now five different Plins are identified in mammals [81]. This protein family differs in size, binding affinity to LDs, stability when not bound to LDs, transcriptional regulation and tissue expression [77].

More specifically, the Plin family, except for Plin4 is characterized by a sequence homology of a 100 amino acid region near the N-terminal end, defined as the PAT-domain. When first grouped as a family these proteins were named the "PAT" family after the first three Plin members identified: perilipin, adipocyte differentiation-related protein (ADRP) and tail-interacting protein of 47 kiloDaltons (TIP47). [82, 86]. This PAT domain is followed by a repeating 11-mer helical motif of varying length. Plin2, 3 and 5 are approximately 300-400 amino acids while Plin4 is 1500 amino acids because of an expanded 11-mer repeat region. Further, Plin1 has 4 splice variants containing the PAT region and 11-mer helical motif but with different C-termini ends in mice [82, 87, 88]. Plin1, 4 and 5 have tissue-specific expression while Plin2 and 3 are ubiquitously expressed. In addition, Plin1 and Plin2 are only stable when bound to LDs while Plin3, 4 and 5 are stable on LDs but also in the cytoplasm (**Table 1**). Studies have shown that the major isoforms of

Plin1, 2 and 5 prefer to bind to LDs enriched in TGs while Plin4 prefer LDs enriched in CE [88-91].

The main group of transcription factors (TF) that regulates Plins are the PPARs. There are three PPAR isoforms (α, β/δ and γ) that are activated by lipids and regulates lipid metabolism in various tissues. The heart has the highest expression of PPARα and all Plins except for Plin3 is regulated by these TFs within the heart [82, 91, 92].

Table 1. Summary box about Plins

Perilipin	Alternative name	Major site of expression	Location
Perilipin 1	Perilipin	WAT	LDs
Perilipin 2	ADRP, Adiphophilin	Liver, ubiquitously	LDs
Perilipin 3	TIP47, M6PRBP1	Ubiquitously	Cytosol, LDs
Perilipin 4	S3-14	WAT, ubiquitously	Cytosol, LDs
Perilipin 5	OXPAT, MLDP, LSDP5	Heart, BAT, skeletal muscle	Cytosol, LDs

Adipocyte differentiation-related protein, ADRP; Tail-interacting protein of 47 kDa, TIP47; Mannose-6-phosphate receptor-binding protein 1, M6PRBP1; Myocardial LD protein, MLDP; Lipid storage droplet protein 5, LSDP5; White adipose tissue, WAT; Brown adipose tissue, BAT; Lipid droplets, LDs.

1.5.1 PERILIPIN 1

Plin1 or perilipin, was the first Plin to be discovered and is mainly expressed in adipocytes of WAT and brown adipose tissue (BAT). It can also be detected in steroidogenic cells of the adrenal cortex, testis and ovaries [82]. Plin1 is essential for the regulation of lipolysis within the adipocytes, and is phosphorylated by protein kinase A (PKA) that is activated by insulin or noradrenaline. This specifically results in the phosphorylation of Plin1 and hormone sensitive lipase (HSL) and the release of comparative gene identification-58 (CGI-58) bound to Plin1, thus facilitating the interaction between the lipase ATGL and CGI-58 and the onset of lipid hydrolysis [91, 93]. Lipolysis will result in the detachment of Plin1 from the LDs resulting in degradation by the proteasomal and lysosomal pathway [82, 94].

Plin1 deficient mice has been generated creating a lean mouse model with an extensive reduction in adipose mass. This as a result of high basal lipolysis because the main Plin coating the LDs is Plin2. Plin2 has been shown to be less protective against lipases than Plin1 in adipose tissue [91, 95-97]. This does however protect the Plin1 deficient mice from weight gain associated with high-fat diet (HFD) [96].

Even if Plin1 is not expressed within the heart, Plin1 deficiency is affecting heart physiology and function. Due to the high lipolysis which generates a high FA flux to the systemic circulation, ectopic lipid accumulation is generated within the heart. This results in increased β-oxidation, ROS production and lipotoxicity that with time injuries the myocardial structure and thus the function. These mice do develop cardiac hypertrophy and ultimately HF with time [98].

In humans, Plin1 mutations are causing lipodystrophy syndrome, associated with hypertriglyceridemia, insulin-resistant and hepatic steatosis [99].

1.5.2 PERILIPIN 2

Plin2, also known as adipocyte differentiation-related protein (ADRP) and adiphophilin is the most abundant LD protein in non-adipose tissue. This Plin is mainly regulated by intracellular lipid accumulation [91, 100]. Plin2 is stable when bound to LDs in presence of high lipid content but becomes degraded by the ubiquitin-proteasomal pathway under lipid poor conditions. Thus Plin2 and LDs provide a reciprocal stabilization [101-104]. Studies were Plin2 has been overexpressed in different cell-lines and tissues results in induced LD accumulation while the opposite holds true for Plin2 deficiency [105-108].

More specifically, Plin2 deficient mice have a 60% reduction of TGs in the liver and they are resistant to diet-induced obesity, fatty liver disease and alcohol induced steatosis [109-112]. Further, in a transgenic mice model with a cardiac-specific overexpression of Plin2 was cardiac

steatosis detected with accumulation of small LDs. This was suggested to be due to reduced activity of HSL suggesting that Plin2 might interfere with HSL [113].

Recent research on Plin2 has shown that overexpression of Plin2 protects LDs against autophagy while Plin2 deficiency enhances autophagy resulting in depleted hepatic TGs levels [111]. This regulation seems to be initiated by the phosphorylation of Plin2 by AMPK enabling the binding of Plin2 with chaperone heat shock 70 kDa 8 (HSPA8/Hsc70) facilitating degradation of Plin2 through chaperone mediated autophagy (CMA) and thus enabling lipophagy and lipolysis of LDs [114, 115].

In humans, a missense polymorphism that has extensive effects on the protein structure and function of Plin2 has been identified. The individuals associated with this variant had lower TG and VLDL concentrations in the systemic circulation [100].

1.5.3 PERILIPIN 3

Plin3 was initially called tail-interacting protein of 47 kDa (TIP47) or mannose-6-phosphate receptor-binding protein 1 (M6PRBP1) and was first discovered to be involved in intracellular trafficking of lysosomal enzymes [91, 116]. Later on, it was discovered that this protein has sequence similarities to Plin2. Plin3 is ubiquitously expressed and in contrast to Plin2 it is stable in the cytosol in addition to LDs [77].

Studies have shown that Plin2 and Plin3 are co-expressed and that Plin3 can compensate for the lack of Plin2. However, in cell culture experiments does Plin3 have the least ability of all Plins to protect the LDs from lipolysis [117]. Recently, it has been shown that Plin3 is an AMPK substrate, and phosphorylation of Plin3 by AMPK promotes LD dispersion that might occur during starvation or AMPK activation [118].

In addition, Plin3 is shown to be targeted for degradation by CMA together with Plin2 [115].

1.5.4 PERILIPIN 4

Plin4 or S3-12 is expressed primarily in fat storing tissues such at WAT and to a lesser degree in skeletal muscle and heart [119]. Plin4 has a unique structure due to the lack of the PAT domain but with a polypeptide length that is three-fold longer than the other Plins [91, 120]. In adipocytes, Plin4 and Plin1 do coat different populations of LDs, and upon lipid loading is Plin4 coating new LDs and permits rapid packing of newly synthesized TGs [119].

A Plin4 null mice has been generated but no major differences were seen in the lipid levels in adipose tissue. However, low TG levels were observed in the heart [120]. These mice did also have reduced Plin5 mRNA and protein levels suggesting that Plin4 might regulate Plin5. The mice maintained a normal heart function and cardiac steatosis was prevented after HFD [120]. Nevertheless, these two genes are located close to each other in the mice genome and it has not been investigated whether the genetic manipulation of Plin4 could have impacted the expression of Plin5 and that these findings are due to alteration of Plin5 and not Plin4.

1.5.5 PERILIPIN 5

First known as OXPAT, myocardial LD protein (MLDP) and lipid storage droplet protein 5 (LSDP5) but now as Plin5 is mainly detected in oxidative tissues such as heart, skeletal muscle and BAT [77, 86, 121]. Plin5 plays an important role for energy balance as it controls TG storage and lipolysis [121, 122]. The expression of Plin5 is increased within the heart upon response to FA exposure, such as during lipid-loading conditions like fasting [82, 86, 123].

We and others have shown that Plin5 whole-body knockout mice have impaired TG storage within the heart. The mice can still maintain a

normal heart function due to increased glucose utilization during normal conditions [122, 124]. However, during dobutamine-induced stress, myocardial ischemia-reperfusion (I/R) injury and after MI, a severely reduced heart function and increased mortality was observed [124, 125]. We have further shown that Plin5 deficiency reduced mitochondrial oxidative capacity and that the lipid composition of the mitochondrial membrane is changed compromising mitochondrial membrane depolarization [126]. In contrary, two other groups have shown that heart-specific Plin5 overexpression within mice increases TG levels within the heart. This increase in TGs result in a decreased mitochondrial function, left ventricular hypertrophy but a maintained heart function [127, 128].

At a molecular level, Plin5 is believed to act as a scaffolding protein against lipases. Cell studies have shown that Plin5 seem to bind to either ATGL or CGI-58 and block their interaction with each other thus inhibiting lipolysis. This blockage is broken when PKA becomes activated and phosphorylates Plin5 that releases itself from the enzymes facilitating lipolysis. However, the precise mechanism by which Plin5 controls LD storage remains to be fully elucidated [86, 123, 129].

Another cell-based study has shown that there is an association between Plin5 and mitochondria. Plin5 facilitates a physiological and metabolic linkage between LDs and mitochondria and directing FAs to LDs thus controlling the availability of FA for β-oxidation [86]. Recently, it has also been detected that Plin5 can induce transcription of genes in the nucleus by forming a complex with PGC-1α and sirtuin-1 (SIRT1) mediating mitochondrial biogenesis and oxidative function [130]. This study opens up for new possibilities that Plin5 might have more roles than just being a LD binding protein.

1.6 AUTOPHAGY

Autophagy is an evolutionary conserved and crucial process within the cells that facilitates the degradation of intracellular compartments such

as proteins, organelles, macromolecular complexes and pathogens [131-133]. The formation of autophagosomes is thought to occur within the cytoplasm and is induced when a cup-shaped double-membrane structure is formed entitled a phagopore. It is not clear where this formation is started but it is suggested that the membrane source may involve contribution from the plasma membrane, ER and mitochondria [132]. When the phagopore is elongating it will start to engulf cellular material, and when it has surrounded its object, it is closing itself forming an autophagsome. The autophagsome is then moving on microtubules until it reaches a lysosome which it fuses with forming a structure called autolysosome. The lysosome consists of enzymes in an acidic environment enabling the degradation of the inner membrane of the autophagsome and the content within [134, 135]. This phenomenon is referred as macro-autophagy and is the most well-known autophagic pathway. There are in addition two other forms of autophagy named Microautophagy and Chaperone-mediated autophagy (CMA) (**Figure 3**) [136, 137].

Microautophagy is the direct interaction of lysosomes with a substrate with an controlled invagination of lysosomal membranes and direct engulfment of a part of the substrate for degradation [76]. CMA is targeting specific proteins for degradation. The proteins contain a specific peptide sequence that signals for lysosomal degradation. This sequence is recognized by Hsc70 that delivers the substrate to lysosomes via lysosomal-associated membrane protein 2a (Lamp-2a) for degradation. [114, 136-140].

Autophagy is divided into being selective and non-selective. Selective autophagy degrades protein aggregates, organelles, bacterial pathogens and signalling complexes that are detrimental and not utilized in the cell [141]. Non-selective autophagy is triggered by starvation and is important for the maintenance of a cellular supply of lipids, amino acids and nucleotides that are needed to provide energy and for the synthesis of new proteins [133, 142, 143]. A basal autophagy is ongoing

constantly and is very essential as a quality control mechanism thus facilitating proper cell physiology [132, 135, 141].

Proteins correlated with autophagy are the autophagy-related (ATG) proteins that are involved in the formation of autophagsomes. One of the proteins that has the strongest association with autophagosomes is microtubule-associated proteins 1A/1B light chain 3B or more commonly known as LC3B, which is a member of the ATG8 protein family. LC3B appears in two different form, LC3B-I and LC3B-II. LC3B-I is free in the cytosol were it becomes lipidated forming LC3B-II, and thereafter translocate to autophagosomes were it seems to be involved in membrane expansion and fusion events and thus works as a good biomarker for autophagy [132, 135].

One of the most known pathways regulating autophagy involves the serine/threonine kinase, mammalian target of rapamycin (mTOR). The induction of autophagy starts by the inhibition of mTOR under starvation conditions [132]. Many additional signals such as growth factors, amino acids, glucose, energy status and different forms of stress further regulates this pathway [133].

1.6.1 LIPOPHAGY

Lipophagy which is the degradation of LDs through autophagy is important for the breakdown of lipids to release free FA (FFA) and to maintain LD homeostasis and prevent lipotoxicity [140]. LDs can be degraded through macro-, microautophagy and CMA, explained in the autophagy section [80, 137, 138]. On LDs are both Plin2 and Plin3 substrates for CMA degradation which enables macro- and microautophagy but also the recruitment of lipases such as ATGL to LDs for lipolysis [114, 138-140]. The induction of either autophagy or lipolysis for the degradation of lipids is tissue-specific. Lipolysis is the main option in adipose tissue that will result in a fast release of FAs as there is a high expression of lipases there. On the opposite, lipophagy is mainly induced in the liver due to low expression of lipases, thus facilitating a slower release of FAs [140].

1.6.2 LIPASES AND LIPOLYSIS

Lipolysis involves the hydrolysis of lipids to break free FAs in cells stored as TGs [144]. This process is regulated by the "lipolytic machinery" including adipose triglyceride lipase (ATGL), the ATGL activator comparative gene identification-58 (CGI-58), hormone sensitive-lipase (HSL) and monoglyceride lipase (MGL). ATGL initiates the rate-limiting step hydrolysing TG to DG and one FFA, while after HSL is performing the second step and hydrolyses DG to monoglyceride (MG) and one FFA. Lastly, MGL bind to MG and generates glycerol and one FFA [78, 80, 86, 144]. The FAs released from TGs in LDs have several fates. They can be used for β-oxidation to generate energy, used as building blocks for membrane lipids, work as co-factors for cell signalling or re-esterified into TGs [80]. Lipolysis is regulated by extracellular signals such as catecholamines and insulin but also by intracellular signals such as the levels of metabolites to balance the hydrolysis of FA with oxidative demands [14, 145]. Interestingly, it has also been proposed that there is an association between ATGL and autophagy/lipophagy. There seem to be a direct interaction between ATGL and LC3B on LDs, and that LC3B might play an important role for the translocation of ATGL to LDs, thus facilitating TG lipolysis within neurons [146]. In addition to this, ATGL is positively regulating SIRT1 within hepatocytes that is an activator of autophagy, thus promoting autophagy/lipophagy [147-149]. However, more thorough studies are needed to elucidate the crosstalk between lipases and the lipophagic machinery.

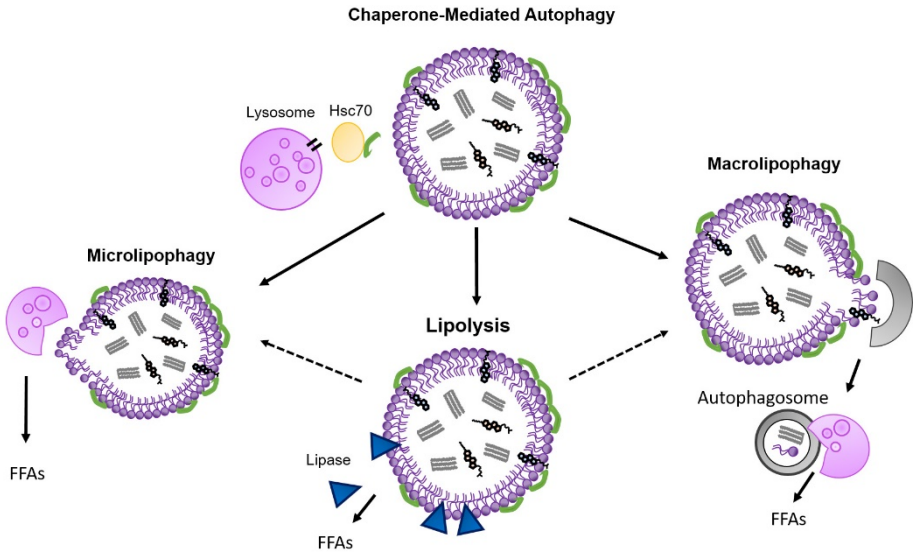

Figure 3. Schematic illustration of chaperone-mediated autophagy, microlipophagy, macrolipophagy and lipolysis. Hsc70, heat shock cognate 70 kDa protein; FFAs, free fatty acids.

1.6.3 METABOLIC ADAPTATION IN CARDIAC DISEASE AND FAILURE

To sum up, the normal heart derives most of its energy for contraction from the oxidation of FAs as previously mentioned. However, the heart adapts quickly to an acute or chronic change in its workload by oxidizing the most efficient fuel. The switch from one substrate to another is regulated by substrate availability, coronary blood flow, oxygen supply, circulating hormones and workload of the heart [150]. During a myocardial insult such as a MI is the heart switching to glucose as the main substrate, because glucose needs less oxygen than FA for the production of ATP [151]. The cardiac dysfunction and remodeling explained previously will promote metabolic abnormalities including reduced β-oxidation of lipids [7, 152], increased glucose uptake and accumulation of myocardial lipids [153-155]. It is well known that neutral lipids (TGs and CEs) accumulate in the failing heart [154, 155]. In addition, we have recently shown that sphingolipids (GlcCer) accumulate in the remodeling heart in response to an ischemic event

[154, 156]. Cardiac lipid accumulation is known to correlate with reduced heart function [157]. However, we do not know if this accumulation of lipids is detrimental or beneficial for the recovery of the heart. Therefore, we have focused on elucidating the role lipids plays within the healthy and diseased heart in this thesis.

2 METHODOLOGICAL CONSIDERATIONS

In this section, considerations regarding selected methods are discussed. A more detailed description of all methods and materials are included in the enclosed papers.

2.1 HUMAN MATERIAL

To study lipid alterations and more specifically GlcCer levels in the remodeled heart, biopsies were taken from patients undergoing coronary artery bypass surgery due to atherosclerosis in the coronary vessels. A patient with an occluded vessel due to atherosclerosis has been exposed to injury as the obstruction of the blood flow is causing limited oxygen and substrate availability in that specific region of the heart, thus facilitates remodeling. Non-ischemic left ventricle control biopsies were obtained from patients that underwent aortic valve replacement, with angiography-verified absence of coronary artery disease in any major myocardial coronary artery branch. These patients have not been exposed to any extensive blockage in the blood flow that could induce ischemia and cell death. This facilitates the use of biopsies from seemingly healthy parts of the heart, without any remodeling. However, we cannot be completely sure that this condition does not affect the whole homeostasis of the heart. Yet, these control biopsies are the closest to physiologically normal as one would obtain, as it would be ethically not justifiable to take biopsies from completely healthy human hearts. It would in addition not be of relevance to take biopsies from a seemingly healthy part of the remodeled heart as it would not be comparable due to lipid level differences in different regions of the heart.

2.2 MOUSE MODELS

The use of animal models has for a long time been debated in the society. Nevertheless, the anatomical and physiological similarities between humans and specifically mice that shares a vast majority of the DNA has allowed us to study different molecular mechanisms and disease

conditions. This has further enabled researchers to test new treatment strategies in animal models before assessing it in humans.

Mice have the advantage over other animal models as they are small and thus need small sized facilities that enables large scale studies cost-efficiently. Mice do have a rapid turn-over rate and goes through birth to death much faster than larger animals, and female mice produce multiple litters per year. All this enables the study of different disease models much easier and faster [158]. The biggest advantage is the ability to produce different transgenic, knock-out and knock-in models resembling a variety of human diseases.

However, we have to keep in mind that there is a considerable difference in size between humans and mice, that is immense and does affect different parameters. Mice have a much faster metabolism with more metabolic tissues such as BAT compared to humans [159]. Mouse cells have a denser mitochondrial organization compared to humans which is associated with a higher metabolic activity [159]. The higher metabolic activity in the mice will generate more reactive oxygen species (ROS) that is damaging for the cells and shortens lifespan [159, 160]. A big difference in the study of CVDs is that mice compared to humans carry much of its plasma cholesterol in high density lipoproteins (HDLs), while humans carry it as LDL. This results in that the non-genetically manipulated mice are resistant to the development of atherosclerosis in their arteries which further makes them less prone to develop CVDs [161].

The human heart is in many ways similar to mice but there are some differences to consider. In addition to the size difference, the human heart beats on average between 60-80 beats per minute (bmp) while the mouse heart beats on average 400-600 bmp, indicating extensive differences in calcium handling and cardiac contraction [162]. The human heart is further resting on the diaphragm which is reflected in more pyramidal heart-shape morphologically. Mice on the other hand

which are four legged has a heart that is moving freely in the pericardial cavity resulting in a more rugby-shaped heart. The human atria are very prominent while in mice this structure is very small. In addition, the location of the coronary arteries is different between mice and humans. This results in that certain aspects are need to be considered when developing and studying different cardiac disease models within mice, and this is further discussed in section Myocardial infarction in mice [163, 164].

2.3 *IN VITRO* MODEL SYSTEMS

2.3.1 HL-1 CELLS

HL-1 cells are a cardiomyocyte-like cell-line, derived from a mouse atrial tumor cell-line and has been extensively used in *Paper III*. These cells contract spontaneously in presence of noradrenaline (NA) in the medium. They have organized sarcomeres and express many genes similar to adult cardiomyocytes. HL-1 cells do maintain their phenotype after several passages and can be genetically manipulated with good efficiency compared to other cardiomyocyte-like cells and primary cardiomyocytes [165]. A disadvantage is that these cells are particular in their needs of a specific growth medium containing adenosine, NA and retinoic acid, in addition to a fibronectin-gelatin matrix to grow on, which limits the development of models of cardiac diseases [166, 167]. In our lab we have used HL-1 cells to elucidate the molecular mechanistic pathways that are affected by Ugcg depletion. This was set-up by transfecting cells with Ugcg siRNA as this gene is expressed in these cells. A downside of using HL-1 cells in our lab is their low expression of Plin5 which has hindered us from studying LD metabolism in relation to Plin5.

2.3.2 PRIMARY CARDIOMYOCYTES

Primary cardiomyocytes isolated from the hearts of different mouse models have been used in all three papers, mainly to confirm that the findings are cardiomyocyte-specific. This has enabled the exclusion of

other cell-types in the heart that might interfere with the results. A disadvantage with this method is that the heart is taken out from its normal environment and the isolation of the cardiomyocytes is occurring in an abnormal setting that could have an impact on the response when performing the experiments. In addition, short-term experiments can only be performed due to the changes in the phenotype over time [168]. Nevertheless, this method is highly applied in our lab and the biological response when performing an experiment will be closer to an *in vivo* situation compared to any cardiomyocyte cell-line. Further, the control over the experimental conditions and settings makes this method very attractive for our studies. The primary cells are maintaining a normal cell morphology, and preserve many of their markers and function seen *in vivo*. Another essential characteristic of primary cells is the possibility to isolate from genetically manipulated mice were factors such as disease conditions but also age and sex can be considered when setting up the experimental model. This is very beneficial as we strive to develop personalized medicine and normal cell-lines cannot contribute with this setting [167].

2.3.3 HEK293 CELLS

In *Paper III*, we are using human embryonic kidney 293 (HEK293) cells as a model system. HEK293 cells are derived as the name states from human embryonic kidney and the cells are believed to be fibroblast-like. However, when characterizing the cells, it was discovered that they express abundantly neuron-specific genes, suggesting that this cell-line might actually be derived from an immature neuronal cell in the kidney. Nevertheless, these cells have been used for years in different contexts due to their stable growth and easy to genetically manipulate in terms of high transfection efficiency [169]. More specifically, HEK293 cells have been extensively used in the study of GPCRs [170].

In our lab, we have created a HEK293 system with stable overexpression of β1-AR for our aims in *Paper III*. We could see in primary cardiomyocytes isolated from the *hUgcg*$^{-/-}$ heart that they had a reduced

responsiveness when stimulating them with isoprenaline (ISO). However, we know that these mice develop dilated cardiomyopathy and die pre-mature, but we do not know when this remodeling is induced. It could be as early as in 8-weeks old mice, which is around the weeks that primary cardiomyocytes are isolated. By generating a HEK293 cell-line with a stable overexpression of β1-AR we are able to study the acute effect of Ugcg deletion through siRNA knockdown. This gives us the possibility to further study the role of GlcCer for receptor signaling.

2.4 MODELS OF MYOCARDIAL DISEASE

2.4.1 MYOCARDIAL INFARCTION IN MICE

In order to evaluate how the heart is responding to pathological stress in the different mice models is MI induced in *Paper I* and *II*. This is done by ligating the left anterior descending (LAD) coronary artery which is the major vessel supplying the LV with blood. This procedure results in an infarction in an anterolateral and apical region of the LV and the injured area affects 40-60% of the heart. The injury is extensive but mice compared to humans can withstand the damage [171]. This disease model enables the study of myocardial changes such as remodeling over time within the heart.

The anatomy of the coronary artery tree may slightly differ between different mouse strains, and one issue is that the LAD coronary artery is hard to detect and therefor a lot of practice is needed to be able to induce similarly sized infarction within the mice. In addition, the coronary artery tree of mice is different compared to humans and not characterized to the same extent as the human coronary vasculature. Mice do have two major coronary arteries, where the left coronary artery generally crosses over the left ventricle and gives off variable branches. A branch has been described as LAD because of its resemblance to the LAD in humans [163, 164, 172]. Further, the right coronary artery commonly divides immediately into two major branches supplying the right ventricle and septal region, thus the septum is saved from injury

during an infarction in mice. In humans, on the other hand is branches of the LAD coronary artery suppling the septum, and the remodeling of the heart will thus be different between the species in response to an injury [172, 173]. Further, the level, width and depth of the suture ligation has prominent effects on the MI size. Ligation higher up of the LAD coronary artery results in larger infarctions, but to high up can results in an increased mortality showing that small differences can have a big impact [174, 175]. In our lab, we are studying heart function and dimensions 24-hours post-MI to evaluate the acute response and around 3-weeks post-MI to study the remodeling of the heart. This is done with ultrasound analysis which is a non-invasive method to evaluate cardiac morphology and function [176]. However, this procedure needs to be optimized due to the small size of the structure, the high HR and the need of sedation of the mice while still remaining a close to normal physiological state.

Another issue to consider when using this disease model is that neovascularization is not observed in the adult mice heart after a MI compared to humans. The lack of new blood vessel formation at the site of injury results in a much larger necrotic area with dead cardiomyocytes contributing to loss of cardiac regenerative capacity [177]. Within humans, the necrotic area is minimized due to newly forming blood vessels that can supply the area of damage with oxygen and substrates contributing to the formation of scar tissue rather than necrotic tissue. Therefore, even if the MI model in the mice is beneficial for the study of cardiac remodeling there are still some differences in the cardiac response between mice and humans. One might also suggest to study the response by inducing ischemia-reperfusion instead of a MI as this resembles more a human situation. However, by introducing reperfusion into the heart, a reperfusion injury is induced. We would thus not know if the reperfusion or the ischemia is causing the remodeling that we want to study, and therefore is the MI-model preferable used for our aims.

2.4.2 DOBUTAMINE- AND ISOPRENALINE-INDUCED STRESS

For the assessment of cardiac response to acute stress is dobutamine used in all three papers. Dobutamine is a β1-adrenergic agonist of the heart that increases contractility and CO with minimal impact on blood pressure. An injection induces a rapid increase in HR and contractility enabling the study of how well the heart work in response to stress similar to an exercise on *e.g.* a treadmill [178].

Isoprenaline (ISO) is a non-selective β-adrenergic agonist that is injected in mice over a time-course to induce cardiac remodeling and used in *Paper III*. This method is non-invasive and induces cardiac dysfunction, and depending on how many injections, dosage and times that are given, different degree of damage can be obtained. An acute model resembles stress-induced cardiomyopathy and a chronic model mimics advanced heart failure similar to humans [179].

2.5 STATISTICAL ANALYSES

The experimental results are presented as mean ± standard error of the mean (SEM) in all papers. All the statistical tests are used to test the null hypothesis. The null hypothesis propose that no statistical significance/difference exist *e.g.* between two experimental groups, and that it is true until statistical analysis proves otherwise [180, 181]. In our papers, statistical significance between groups was evaluated using unpaired two-tailed Student's t test when two unmatched groups were compared. This test can be used when the samples are from a population following a normal distribution and is also referred as a parametric test. When a population cannot be assumed to be normally distributed a non-parametric test is used, *e.g.* Mann-Whitney U-test [182]. Because we work with relatively small sample sizes it is not preferable to perform a normality test to test for normal distribution, as the results would be biased [183, 184]. However, it is assumed that most of the biological or physiological quantitative continuous variables approximately follow

normal distribution, and reach this with increasing sample size, and thus we use parametric tests for our statistical analyses [184, 185].

When comparing three or more independent groups, a one-way ANOVA was used. However, this test only determines if there is a difference between two groups but do not determine between which of the groups. To be able to elucidate between which groups the differences exist, a post hoc test needs to be additionally done. This test should only be done if the one-way ANOVA test shows that indeed there is a significant difference. In *Paper II* we are using *e.g.* Bonferroni's post hoc test, and this test suits when working with small sample sizes. In addition, a one-way ANOVA was used when one factor was considered such as genotype, but when there were more factors to consider like genotype and treatment, a two-way ANOVA was implemented followed by Bonferroni's post hoc test. Further, In *Paper III*, we are additionally using Dunnett's multiple comparisons test as we compare one control group to all the other experimental groups [186]. We are also analyzing the survival in our mouse models and have thus used the log-rank test that compares the survival between two or more groups. This test takes into account the whole survival time and not a specific time-point. In addition, this test does give all the events the same strength independent of the time at which the event happened. In comparison, other survival test can consider earlier events to have more strength [187].

All the statistics were calculated in GraphPad prism, and a p-value <0.05 was considered statistically significant. The p-value is used to determine if there is a significance of the results in correlation to the null-hypothesis. The p-value can be between 0 and 1, and the smaller the value is, the higher probability that the null hypothesis can be rejected. If a p-value is 0.05, there is a 5% possibility that the null-hypothesis is true and that the results are by chance, but it does also mean that that there is a 95% possibility that the null-hypothesis can be rejected and we can accept an alternative hypothesis [180-182].

3 AIMS

The specific aims of the three papers included in this thesis are:

Paper I. To investigate the role of Plin2 deficiency on myocardial lipid storage and utilization and the impact on heart function.

Paper II. To elucidate if cardiac-specific overexpression of Plin5 is beneficial for the heart function and survival post-MI.

Paper III. To define the role of glucosylceramides in heart physiology and function.

4 RESULTS AND DISCUSSION

Paper I: Plin2-deficiency reduces lipophagy and results in increased lipid accumulation in the heart

In this study, we investigated the impact of Plin2 on myocardial lipid storage and cardiac function. Previous publication on Plin2 deficient mice has shown that these mice have lower hepatic TG levels and are protected against fatty liver disease and diet induced obesity, suggesting a beneficial role correlated with Plin2 deficiency [109]. In addition, cardiac-specific overexpression of Plin2 was associated with cardiac steatosis but without any adverse effects on cardiac function [113]. We therefore hypothesized that Plin2 deficiency would result in an opposite effect with a decreased amount of TG within the heart and possibly an improved heart function after MI.

Unexpectedly, we did discover that *Plin2*$^{-/-}$ mice had increased myocardial TG levels within the heart and that this increase was cardiomyocyte-specific. This was not a result of disrupted mitochondrial biogenesis or respiration. Instead, we saw an increased protein expression of Plin3 and Plin5 which could be a compensatory effect, as shown in previous paper [188]. However, the cellular location was intriguing in the *Plin2*$^{-/-}$ cardiomyocytes. Plin3 was mostly coating LDs as expected but Plin5 was more prominent on vesicular structures close to the plasma membrane of the cardiomyocytes and not on LDs, which was not predicted. Previous studies have shown that Plin5 is abundant on cardiac LDs and strongly regulate lipid storage by working as a lipolytic barrier [122, 124, 127, 128, 189]. We therefore hypothesized that the increased amount of Plin5 was bound on the Plin2 deficient LDs and thus contribute to the increased TG levels by shielding the LDs. As we do not see an increased co-localization of Plin5 with LDs in the *Plin2*$^{-/-}$ mice hearts, it is not convincing to expect this. However, in addition to our unexpected finding another paper has detected Plin5 within the nucleus were it works as a transcriptional regulator [130].

Hence, this opens up for new questions of whether Plin5 but also other Plins have additional roles that just being LD-binding proteins, and more studies are needed to clarify this.

To further elucidate why *Plin2*$^{-/-}$ mice have increased accumulation of TG, we investigated markers of lipophagy. Plin2 but also Plin3 has previously been shown to be substrates for CMA and therefore we hypothesized that this pathway might be affected [115]. Indeed, we saw a decrease of LC3BI/II in the Plin2 deficient cardiomyocytes and this was not caused by a higher autophagosomal turnover rate. In addition to this finding, we could confirm that there was a reduced co-localization of lysosomes with LDs suggesting that the lipophagic pathway is somehow affected. An increased accumulation of TG could be a result of a decrease in CMA targets, which Plin2 is. The lack of Plin2 might make it more difficult for the CMA machinery to attach on LDs. This could result in that the LD remains protected and that autophagy/lipophagy is blocked. On the other hand, we do see more Plin3 on the Plin2 deficient LDs which is also believed to be a target for CMA and theoretically would facilitate CMA. However, Plin2 might be more prone for CMA degradation than Plin3, but this needs to be further elucidated.

Interestingly, TG levels were also higher in the cardiomyocyte-specific Plin2 overexpressing mice but as a result of a reduced HSL activity [113]. In our model we have not investigated the activity of lipases but if CMA is blocked it would theoretically also hinder the attachment of lipases. However, within the liver Plin2 overexpression is correlated with an inhibited autophagy and the opposite holds true for Plin2 deficiency, which is opposite to what we see within the heart [111]. Thus, our finding suggests that CMA and autophagy/lipophagy is very much tissue-specifically regulated and that the ratio of the different proteins coating LDs probably strongly influences LD catabolism, and thus heart function during normal condition but also in response to pathological stress.

Paper II: Cardiac-specific overexpression of perilipin 5 promotes physiological hypertrophic remodeling of the heart by fine-tuning calcium-handling regulators

We have previously shown that mice deficient in Plin5 have an altered substrate utilization that affects heart function during stress and myocardial ischemia, leading to increased mortality [124]. Therefore, we hypothesized that overexpression of Plin5 within the heart might be favorable for the outcome after a MI. We generated MHC-*Plin5* mice, with heart-specific overexpression of Plin5. Lipid analysis revealed that MHC-*Plin5* mice have an increased content of TG within the heart, in agreement with previous published papers on models with heart-specific overexpression of Plin5 [127, 128].

We then assessed heart function with echocardiography that showed an increased LV mass in MHC-*Plin5* compared to WT mice hearts, without affecting heart function during baseline conditions similarly to previous publication [127]. Through further characterization we could detect an increase in cardiomyocytes size indicating a form of hypertrophy without any adverse remodelling or fibrosis. Therefore, we decided to identify if it is a pathological or physiological hypertrophic growth induced in the cardiomyocytes. A diseased heart with pathological hypertrophy typically has a re-activation of the fetal gene program with a prominent upregulation of natriuretic peptides [38, 190]. We could detect a downregulation in ANP expression and no difference in BNP expression. In addition to this, we found a downregulation of pathological hypertrophy markers but more interestingly, the PI3K/AKT/mTOR pathway usually upregulated during physiological hypertrophy was not activated [39]. This implies that the mice do have a phenotype similar to physiological hypertrophy without the induction through exercise such as wheel running or swimming [191], nor through the activation of the usual signalling pathway upregulated during

physiological hypertrophy. The MHC-*Plin5* mice thus have a phenotype that is induced through a pathway not identified yet.

In addition to this, physiological hypertrophy is further characterized by a maintained or improved heart function that do not promote the development of dilated cardiomyopathy and HF [38]. As physiological hypertrophy is beneficial for heart function, we decided to induce pathological stress to see how the MHC-*Plin5* hearts persist these conditions. The mice showed to have similar heart function with a maintained SV and CO but with a prominently lower pulse after MI, suggesting that the MHC-*Plin5* hearts might have better cardiac contractility. When investigating markers for cardiac contraction, we could identify an increased CamKII activity that is a prominent regulator of SERCA2a and Ca^{2+} flow implying that this pathway might be changed. However, more thorough studies are needed to evaluate the exact mechanistic behind the increased CamKII activity. This, specifically since CamKII is involved in many other transmembrane electrical processes and its upregulation is in contradiction to our study associated with heart diseases and not with an improved heart function [192, 193].

Comparing the *Plin2*$^{-/-}$ vs. the MHC-*Plin5* mice

We have been elucidating the role of lipid metabolism within the heart for heart physiology and function by using two mouse models with differences in their perilipin expression. Both models result in an increased amount of TGs within the heart and both models do have more Plin5. However, the Plin2 deficient model have a reduced heart function in response to MI, while the MHC-*Plin5* model seem to resist this stress even with a hypertrophic heart. The major difference in relation to LD proteins is that one model lacks Plin2 and the other model express Plin2. We show that Plin2 deficiency seem to affect substrate availability possibly by hindering CMA and consequently autophagy/lipophagy, thus affecting heart function in response to stress. Therefore, we speculate that the major differences we see between these models is because of different perilipin distribution on the LDs but also within the

cell. Even if both mice models have more Plin5, the increased expression does not seem to affect substrate availability. The Plin5 lipolytic barrier is known to be broken during conditions such as fasting within the heart [189]. Hence, if it exists in these mice it is probably also broken during more extensive stress such as MI. Further, we have not completely characterized if Plin5 is the major perilipin coating the LDs in the MHC-*Plin5* mice. Maybe the excessive amount of Plin5 is serving additional functions and is not extensively localized to LDs, such as surprisingly seen in the *Plin2*$^{-/-}$ mice. As our results suggest that overexpression of Plin5 affects cardiac contractility but also the initiation of physiological hypertrophy, it implies once again that Plin5 participates in other signaling pathways also in the MHC-*Plin5* mice.

Paper III: Cardiomyocyte-specific deficiency in glucosylceramide synthase results in dilated cardiomyopathy and premature death in mice

GlcCer are important structural components of cellular membranes, but they are also involved in numerous cellular processes, such as proliferation and differentiation [194, 195]. We have previously shown that ceramides [156] but now also GlcCer levels correlate with heart function in the remodeling human heart following ischemia. In addition, we could translate these findings into mouse models where cardiac remodeling was induced and where we could detect altered GlcCer levels. Therefore, we generated mice with cardiomyocyte-specific deficiency in glucosylceramide synthase (h*Ugcg*$^{-/-}$ mice) to elucidate the role of GlcCer for heart physiology and function. These mice were born and developed normally to adulthood together with a maintained heart morphology, even with reduced GlcCer levels in the cardiomyocytes. Heart function was preserved in young mice during baseline conditions but when exposed to stress, a decrease in systolic function was detected. In addition to this, all the h*Ugcg*$^{-/-}$ mice died premature by the age of 40 weeks due to dilated cardiomyopathy. To clarify what is causing the development of dilated cardiomyopathy, ultrastructure of the cardiomyocytes were investigated. Swollen Golgi

apparatus structures were identified and this is believed to affect the autophagic and endolysosomal pathway [196, 197]. We could confirm an abnormal pattern with autophagic and lysosomal vesicles close to the plasma membrane. In addition, we detected lower levels of LC3B, which was not caused by an increased autophagic flux but instead by a decreased induction of autophagy through the mTOR pathway.

Further, as GlcCer are membrane lipids that are thought to be involved in structures such as lipid rafts and caveolae it is ideal to think that alterations in GlcCer levels can impact receptors on the plasma membrane. In agreement with this, we observed impaired cardiac contraction in response to stress, and we speculated whether the β-AR receptor signaling pathway might be affected. This was confirmed by detecting a reduced responsiveness when stimulating the Ugcg deficient cardiomyocytes with ISO. Thus, our findings show that GlcCer are fundamental for normal heart physiology and function. Absence of this membrane lipid result in mice that die premature due to dilated cardiomyopathy. This is a combined effect of alterations in the Golgi structure, autophagy and endolysosomal regulation that further results in a disrupted β-AR signaling.

However, some aspects to consider regarding this model is that we are additionally using cell systems to confirm that the alterations we see indeed is due to Ugcg deficiency and not that the cardiomyocytes are already diseased, because we do not know when the maladaptation starts. The cell systems are excellent ways to further elucidate the molecular pathways but they cannot completely replicate an *in vivo* situation. It would therefore be valuable to create a mouse model where an acute Ugcg deficiency could be induced that would more precise pinpoint which pathways that are truly affected by the absence of GlcCer. Another limitation is that there is a significant decrease in LacCer levels, which is a lipid species downstream of GlcCer. For now, we do not know whether all the alterations are because of GlcCer depletion or if some could be an effect of LacCer deficiency. To be able

to elucidate which lipid species that gives which specific phenotype, generation of a cardiomyocyte-specific lactosylceramide synthase (LCS) deficient mice model could be a strategy.

Remodeling the cardiac lipid composition

In all three papers we have altered the lipid composition within the heart. It is clearly shown that the absence of the membrane lipid GlcCer seem to give the most detrimental phenotype. This is not surprising as GlcCer are fundamental components of the plasma membrane thus affecting many different cellular pathways within the cardiomyocytes. Thus, our results imply that it is important with GlcCer within the heart and that the increased accumulation of this membrane lipid in mice models of cardiac remodeling in addition to the diseased human heart, do not necessary need to correlate with being detrimental. The accumulation of GlcCer might be beneficial to facilitate a proper remodeling and recovery of the heart, but more studies are needed to clarify this.

In addition, we show that increased TGs levels in the *Plin2*$^{-/-}$ and MHC-*Plin5* mice do not give a harmful phenotype unless exposed to pathological stress, such as seen in the *Plin2*$^{-/-}$ mice. It does further not seem to be the increase in TGs that is damaging for the heart, but rather the inability to utilize the substrate in a sufficient way. Many studies do imply that increased cardiac lipid accumulation is causing heart dysfunction or exacerbates heart diseases specifically in obese or diabetic patients due to increased lipotoxicity [8, 13]. However, from our studies we clearly show that this is not always the case. Hence, an overall insight to consider is that an increased lipid accumulation does not always have to correlate with heart dysfunction.

5 CONCLUSIONS

We conclude that:

Paper I
Plin2 regulates cellular lipid metabolism in a tissue-specific manner. In cardiomyocytes, Plin2 deficiency results in increased lipid accumulation as a result of reduced lipophagy.

Paper II
Overexpression of Plin5 within cardiomyocytes results in physiological hypertrophy, with improved cardiomyocyte contractility and calcium handling.

Paper III
The sphingolipid GlcCer accumulate in human heart following injury. Ugcg deficiency in cardiomyocytes results in Golgi dispersion, abnormal autophagic regulation causing a compromised β-AR signaling, leading to dilated cardiomyopathy and premature death in mice.

6 FUTURE PERSPECTIVES

In this thesis, I have studied and provided insight into the role of cardiac lipid composition for heart physiology and function. However, our studies open up for several unanswered questions.

We still do not know whether it is the elevated levels of Plin3 and/or Plin5, or if it is the deficiency in Plin2, that affects the lipophagic machinery in the *Plin2*$^{-/-}$ mice in *Paper I*. To address this question, we can create additional cell systems/ mouse models. We do have mouse embryonic fibroblasts (MEFs) isolated from *Plin2*$^{-/-}$ and WT mice where we could ideally overexpress Plin3 and/or Plin5 to see if a similar phenotype could be simulated. This would contribute with further information on which perilipins that regulates or are regulated by CMA/lipophagy and clarify substrate availability in relation to perilipins coating the LDs. These findings would increase the knowledge on how lipids are stored within the heart and how lipophagy is regulated within the cardiomyocytes.

In *Paper II*, we show that elevated expression of Plin5 is beneficial for cardiac function. It would be interesting to cross-breed MHC-*Plin5* mice into different pathological heart disease mouse models and see if the heart function is improved or rescued. These results would indicate whether a potential treatment strategy could be to induce increased cardiac expression of Plin5 in patients with a compromised heart function. Because direct delivery of Plin5 to cardiac tissue would be challenging, one way would be to increase transcription of Plin5. To date, Plin5 is known to be regulated by fasting [121] through the transcription factor C/EBPα [198], in addition to PPARα [121]. Thus, a potential way of targeting Plin5 is via C/EBPα or PPARα.

In addition, RNA-sequencing analysis would also give more information on pathways that are affected in the MHC-*Plin5* mice, enabling us to further dissect and clarify how our phenotype is induced.

In *Paper III*, we show that reduced levels of GlcCer and expression of cardiac Ugcg results in severe heart failure. I would like to investigate whether reduced levels of GlcCer and/or gene expression of Ugcg in the heart is a clinically relevant situation. One way to address this question would be to test if genetic variation of genes involved in sphingolipid synthesis may predispose to dilated cardiomyopathy.

ACKNOWLEDGEMENTS

I would like to thank:

Malin Levin, my main supervisor. I am so so grateful to have you as my supervisor. It is a lottery if you get a good supervisor or not, and I hit a jackpot with you!! Thank you for guiding me during these years in research. You have been incredible patient and kind towards me and coming with positive encouragements even when I feel nothing is working. I have always felt very motivated after leaving our weekly Monday meetings and eager to do millions of things in the lab. You are very inspiring and I wish more people in research and in life could be like you.

Jan Borén, my co-supervisor. For the valuable discussions and feedback on my manuscripts to presentations.

The heart group (past and present). Linda, Azra, Mathieu, Martina, Malin, Christina, Meta. Wow, you have always been very supportive and helpful in all the things that I have needed help with in the lab. In accordance to that, thank you for all the nice talks about everything in life from small too big.

Rest of Lab4/5 (past and present), for the nice environment that I have been able to work in. **Sivve**, for all the talks and laughs. **Liliana,** just when I see you, you make me smile ☺. **Elin,** for being so positive and always asking me if I need help. **Mikael,** you are the sunshine of the lab. **Steffi,** for being very funny. **Eva,** you are so sweet and we always have interesting conversations. **Maria,** for all the help with sectioning and stainings. **Kristina,** for being helpful and kind. **Per,** the staining-pro master. **Marcus and Pelle,** for the help with lipidomics. **Annika L,** for learning me how the lab works when I first started as a new and nervous PhD-student.

Marie and Gunilla, for all the administrative help. **Svenne,** for the help with IT related stuff, and the fun cat talks we have had. **Rosie,** for the valuable input on the manuscripts. **Magnus,** for the discussions we have had about everything, you are one of the kindest and most helpful persons I have ever met.

Rosellina, Kavitha, Christina, Lisa, Piero, Andrea, Matthias, Oveis, for the nice conversations, laughs, beer drinking, dancing, laserdome and go kart competitions we have had ☺☺

Ara, for buffers, membranes, antibodies, recipes and advices that you kindly share in the lab. It was also so nice to sit in the same writing room as you and talk about life and purple clouds around people ☺

Tony, lab is not the same without you.. just so so soooo serious…

Matias, you are brilliant. I don't know who else I can share my crazy thoughts and ideas with and still being on the same page.

Urszula and Esther, for all the goofiness inside and outside of the lab.

The rest of Wlab, for the friendly environment.

Marta, Ying and Aditi. Girls, you mean tremendously to me, to be able to have you all by my side has been and is priceless! All our crazy and fun adventures we have had together has brighten up my life. No one can get as nice pictures as we can with knifes, face masks, oranges or chop-sticks ☺☺☺☺

Elahe, min syster. För att du alltid finns där. From A to J.

Mardani's, för den persiska touchen i mitt liv.

To my **cousins, aunts and uncle,** all over the world. Third time is a charm and now I will not miss the family reunion.

Menah, my sweet cousin for always keeping in touch with me even when we are superbusy.

Pragnę podziękować mojej **rodzinie w Polsce,** szczególnie babci i dziadziusiowi, oraz moim ciociom Lidii i Marcie, wujkom Stasiowi i Arturowi, za to że zawsze mam w was wsparcie, że pytaliście o moje postępy w pracy naukowej, i że jesteście ze mnie tak bardzo dumni. Teraz gdy moja praca jest gotowa powinniśmy to uczcić toastem....
NA ZDROWIE!!!!!!!!

Samir, min bror, min motsats. Yin och Yang Yosufi´s. Coolaste snubben på radio och på scenen ♫♪♫!!

Mamma & Pappa, vad jag än skriver ner här så kommer det inte ens beskriva en bråkdel av hur mycket ni betyder för mig. Erat stöd är obeskrivligt och när jag tänker på er så vet jag att allt är möjligt.

Amir, för att jag får vakna upp vid din sida varje dag (kanske inte alla dagar nu längre), för att du alltid tar mitt välbefinnande i första hand och för att du får mig att skratta varje dag. Förhoppningsvis blir det mer tid för att resa till Bahamas, Japan, Kuba och kanske Morgendalen.

Alicia, våran älskade diamant. 6:e Februari 2019 kommer för alltid vara den bästa dagen i mitt liv ♥♥♥.

REFERENCES

1. Van Camp, G. (2014) Cardiovascular disease prevention, *Acta Clin Belg.* **69**, 407-11.
2. Stewart, J., Manmathan, G. & Wilkinson, P. (2017) Primary prevention of cardiovascular disease: A review of contemporary guidance and literature, *JRSM Cardiovasc Dis.* **6**, 2048004016687211.
3. Azevedo, P. S., Polegato, B. F., Minicucci, M. F., Paiva, S. A. & Zornoff, L. A. (2016) Cardiac Remodeling: Concepts, Clinical Impact, Pathophysiological Mechanisms and Pharmacologic Treatment, *Arq Bras Cardiol.* **106**, 62-9.
4. Sutton, M. G. & Sharpe, N. (2000) Left ventricular remodeling after myocardial infarction: pathophysiology and therapy, *Circulation.* **101**, 2981-8.
5. Konstam, M. A., Kramer, D. G., Patel, A. R., Maron, M. S. & Udelson, J. E. (2011) Left ventricular remodeling in heart failure: current concepts in clinical significance and assessment, *JACC Cardiovasc Imaging.* **4**, 98-108.
6. Goldberg, I. J., Trent, C. M. & Schulze, P. C. (2012) Lipid metabolism and toxicity in the heart, *Cell Metab.* **15**, 805-12.
7. Lopaschuk, G. D., Ussher, J. R., Folmes, C. D., Jaswal, J. S. & Stanley, W. C. (2010) Myocardial fatty acid metabolism in health and disease, *Physiol Rev.* **90**, 207-58.
8. Schulze, P. C. (2009) Myocardial lipid accumulation and lipotoxicity in heart failure, *J Lipid Res.* **50**, 2137-8.
9. Olzmann, J. A. & Carvalho, P. (2019) Dynamics and functions of lipid droplets, *Nat Rev Mol Cell Biol.* **20**, 137-155.
10. Guo, Y., Cordes, K. R., Farese, R. V., Jr. & Walther, T. C. (2009) Lipid droplets at a glance, *J Cell Sci.* **122**, 749-52.
11. Farese, R. V., Jr. & Walther, T. C. (2009) Lipid droplets finally get a little R-E-S-P-E-C-T, *Cell.* **139**, 855-60.
12. Walther, T. C. & Farese, R. V., Jr. (2009) The life of lipid droplets, *Biochim Biophys Acta.* **1791**, 459-66.
13. Schulze, P. C., Drosatos, K. & Goldberg, I. J. (2016) Lipid Use and Misuse by the Heart, *Circ Res.* **118**, 1736-51.
14. Goldberg, I. J., Reue, K., Abumrad, N. A., Bickel, P. E., Cohen, S., Fisher, E. A., Galis, Z. S., Granneman, J. G., Lewandowski, E. D., Murphy, R., Olive, M., Schaffer, J. E., Schwartz-Longacre, L., Shulman, G. I., Walther, T. C. & Chen, J. (2018) Deciphering the Role of Lipid Droplets in Cardiovascular Disease: A Report From the 2017 National Heart, Lung, and Blood Institute Workshop, *Circulation.* **138**, 305-315.
15. Labrosse, M. R. (2018) Cardiovascular Mechanics.
16. Ogobuiro, I. & Tuma, F. (2019) Anatomy, Thorax, Heart Coronary Arteries in *StatPearls*, Treasure Island (FL).

17. Loukas, M., Sharma, A., Blaak, C., Sorenson, E. & Mian, A. (2013) The clinical anatomy of the coronary arteries, *J Cardiovasc Transl Res.* **6**, 197-207.
18. Kashou, A. H., Basit, H. & Chhabra, L. (2019) Physiology, Sinoatrial Node (SA Node) in *StatPearls*, Treasure Island (FL).
19. Choudhury, M., Boyett, M. R. & Morris, G. M. (2015) Biology of the Sinus Node and its Disease, *Arrhythm Electrophysiol Rev.* **4**, 28-34.
20. McCorry, L. K. (2007) Physiology of the autonomic nervous system, *Am J Pharm Educ.* **71**, 78.
21. Gordan, R., Gwathmey, J. K. & Xie, L. H. (2015) Autonomic and endocrine control of cardiovascular function, *World J Cardiol.* **7**, 204-14.
22. Iaizzo, P. A. (2015) Handbook of cardiac anatomy, physiology, and devices, 817.
23. Tirziu, D., Giordano, F. J. & Simons, M. (2010) Cell communications in the heart, *Circulation.* **122**, 928-37.
24. Wachter, S. B. & Gilbert, E. M. (2012) Beta-adrenergic receptors, from their discovery and characterization through their manipulation to beneficial clinical application, *Cardiology.* **122**, 104-12.
25. O'Connell, T. D., Jensen, B. C., Baker, A. J. & Simpson, P. C. (2014) Cardiac alpha1-adrenergic receptors: novel aspects of expression, signaling mechanisms, physiologic function, and clinical importance, *Pharmacol Rev.* **66**, 308-33.
26. Strosberg, A. D. (1993) Structure, function, and regulation of adrenergic receptors, *Protein Sci.* **2**, 1198-209.
27. Hausdorff, W. P., Caron, M. G. & Lefkowitz, R. J. (1990) Turning off the signal: desensitization of beta-adrenergic receptor function, *FASEB J.* **4**, 2881-9.
28. de Lucia, C., Eguchi, A. & Koch, W. J. (2018) New Insights in Cardiac beta-Adrenergic Signaling During Heart Failure and Aging, *Front Pharmacol.* **9**, 904.
29. Wallukat, G. (2002) The beta-adrenergic receptors, *Herz.* **27**, 683-90.
30. Luepker, R. V. (2011) Cardiovascular disease: rise, fall, and future prospects, *Annu Rev Public Health.* **32**, 1-3.
31. Lavie, C. J., Arena, R., Alpert, M. A., Milani, R. V. & Ventura, H. O. (2018) Management of cardiovascular diseases in patients with obesity, *Nat Rev Cardiol.* **15**, 45-56.
32. Strain, W. D. & Paldanius, P. M. (2018) Diabetes, cardiovascular disease and the microcirculation, *Cardiovasc Diabetol.* **17**, 57.
33. Bhatt, A. S., Ambrosy, A. P. & Velazquez, E. J. (2017) Adverse Remodeling and Reverse Remodeling After Myocardial Infarction, *Curr Cardiol Rep.* **19**, 71.
34. Breckwoldt, K., Weinberger, F. & Eschenhagen, T. (2016) Heart regeneration, *Biochim Biophys Acta.* **1863**, 1749-59.

35. Epelman, S., Liu, P. P. & Mann, D. L. (2015) Role of innate and adaptive immune mechanisms in cardiac injury and repair, *Nat Rev Immunol.* **15**, 117-29.
36. Woodcock, E. A. & Matkovich, S. J. (2005) Cardiomyocytes structure, function and associated pathologies, *Int J Biochem Cell Biol.* **37**, 1746-51.
37. Reiser, P. J., Portman, M. A., Ning, X. H. & Schomisch Moravec, C. (2001) Human cardiac myosin heavy chain isoforms in fetal and failing adult atria and ventricles, *Am J Physiol Heart Circ Physiol.* **280**, H1814-20.
38. McMullen, J. R. & Jennings, G. L. (2007) Differences between pathological and physiological cardiac hypertrophy: novel therapeutic strategies to treat heart failure, *Clin Exp Pharmacol Physiol.* **34**, 255-62.
39. Shimizu, I. & Minamino, T. (2016) Physiological and pathological cardiac hypertrophy, *J Mol Cell Cardiol.* **97**, 245-62.
40. Kavazis, A. N. (2015) Pathological vs. physiological cardiac hypertrophy, *J Physiol.* **593**, 3767.
41. Mubagwa, K., Mullane, K. & Flameng, W. (1996) Role of adenosine in the heart and circulation, *Cardiovasc Res.* **32**, 797-813.
42. van der Vusse, G. J., van Bilsen, M. & Glatz, J. F. (2000) Cardiac fatty acid uptake and transport in health and disease, *Cardiovasc Res.* **45**, 279-93.
43. Fillmore, N., Mori, J. & Lopaschuk, G. D. (2014) Mitochondrial fatty acid oxidation alterations in heart failure, ischaemic heart disease and diabetic cardiomyopathy, *Br J Pharmacol.* **171**, 2080-90.
44. Houten, S. M. & Wanders, R. J. (2010) A general introduction to the biochemistry of mitochondrial fatty acid beta-oxidation, *J Inherit Metab Dis.* **33**, 469-77.
45. Kolwicz, S. C., Jr., Purohit, S. & Tian, R. (2013) Cardiac metabolism and its interactions with contraction, growth, and survival of cardiomyocytes, *Circ Res.* **113**, 603-16.
46. Akram, M. (2014) Citric acid cycle and role of its intermediates in metabolism, *Cell Biochem Biophys.* **68**, 475-8.
47. Czibik, G., Steeples, V., Yavari, A. & Ashrafian, H. (2014) Citric acid cycle intermediates in cardioprotection, *Circ Cardiovasc Genet.* **7**, 711-9.
48. Gustafsson, A. B. & Gottlieb, R. A. (2008) Heart mitochondria: gates of life and death, *Cardiovasc Res.* **77**, 334-43.
49. Scott, I. & Youle, R. J. (2010) Mitochondrial fission and fusion, *Essays Biochem.* **47**, 85-98.
50. Siasos, G., Tsigkou, V., Kosmopoulos, M., Theodosiadis, D., Simantiris, S., Tagkou, N. M., Tsimpiktsioglou, A., Stampouloglou, P. K., Oikonomou, E., Mourouzis, K., Philippou, A., Vavuranakis, M., Stefanadis, C., Tousoulis, D. & Papavassiliou, A. G. (2018) Mitochondria and cardiovascular diseases-from pathophysiology to treatment, *Ann Transl Med.* **6**, 256.
51. Westermann, B. (2010) Mitochondrial fusion and fission in cell life and death, *Nat Rev Mol Cell Biol.* **11**, 872-84.

52. Nguyen, B. Y., Ruiz-Velasco, A., Bui, T., Collins, L., Wang, X. & Liu, W. (2018) Mitochondrial function in the heart: the insight into mechanisms and therapeutic potentials, *Br J Pharmacol*.
53. Knowlton, A. A. & Liu, T. T. (2015) Mitochondrial Dynamics and Heart Failure, *Compr Physiol.* **6**, 507-26.
54. Shao, D. & Tian, R. (2015) Glucose Transporters in Cardiac Metabolism and Hypertrophy, *Compr Physiol.* **6**, 331-51.
55. Kolwicz, S. C., Jr. & Tian, R. (2011) Glucose metabolism and cardiac hypertrophy, *Cardiovasc Res.* **90**, 194-201.
56. Abel, E. D. (2004) Glucose transport in the heart, *Front Biosci.* **9**, 201-15.
57. Ziello, J. E., Jovin, I. S. & Huang, Y. (2007) Hypoxia-Inducible Factor (HIF)-1 regulatory pathway and its potential for therapeutic intervention in malignancy and ischemia, *Yale J Biol Med.* **80**, 51-60.
58. Szablewski, L. (2017) Glucose transporters in healthy heart and in cardiac disease, *Int J Cardiol.* **230**, 70-75.
59. Doenst, T., Nguyen, T. D. & Abel, E. D. (2013) Cardiac metabolism in heart failure: implications beyond ATP production, *Circ Res.* **113**, 709-24.
60. Muro, E., Atilla-Gokcumen, G. E. & Eggert, U. S. (2014) Lipids in cell biology: how can we understand them better?, *Mol Biol Cell.* **25**, 1819-23.
61. van Meer, G. & de Kroon, A. I. (2011) Lipid map of the mammalian cell, *J Cell Sci.* **124**, 5-8.
62. Harayama, T. & Riezman, H. (2018) Understanding the diversity of membrane lipid composition, *Nat Rev Mol Cell Biol.* **19**, 281-296.
63. Malhotra, R. (2012) Membrane Glycolipids: Functional Heterogeneity: A Review, *Biochemistry and analytical biochemistry.* **1**.
64. Bohdanowicz, M. & Grinstein, S. (2013) Role of phospholipids in endocytosis, phagocytosis, and macropinocytosis, *Physiol Rev.* **93**, 69-106.
65. van der Veen, J. N., Kennelly, J. P., Wan, S., Vance, J. E., Vance, D. E. & Jacobs, R. L. (2017) The critical role of phosphatidylcholine and phosphatidylethanolamine metabolism in health and disease, *Biochim Biophys Acta Biomembr.* **1859**, 1558-1572.
66. Alberts B, J. A., Lewis J, et al. (2002) The cell. **4th edition**.
67. Goluszko, P. & Nowicki, B. (2005) Membrane cholesterol: a crucial molecule affecting interactions of microbial pathogens with mammalian cells, *Infect Immun.* **73**, 7791-6.
68. Cooper, R. A. (1978) Influence of increased membrane cholesterol on membrane fluidity and cell function in human red blood cells, *J Supramol Struct.* **8**, 413-30.
69. Ichikawa, S. & Hirabayashi, Y. (1998) Glucosylceramide synthase and glycosphingolipid synthesis, *Trends Cell Biol.* **8**, 198-202.
70. van Meer, G., Wolthoorn, J. & Degroote, S. (2003) The fate and function of glycosphingolipid glucosylceramide, *Philos Trans R Soc Lond B Biol Sci.* **358**, 869-73.

71. Chatterjee, S. & Pandey, A. (2008) The Yin and Yang of lactosylceramide metabolism: implications in cell function, *Biochim Biophys Acta.* **1780**, 370-82.
72. Ishibashi, Y. & Hirabayashi, Y. (2015) AMP-activated Protein Kinase Suppresses Biosynthesis of Glucosylceramide by Reducing Intracellular Sugar Nucleotides, *J Biol Chem.* **290**, 18245-60.
73. Varela, A. R., Goncalves da Silva, A. M., Fedorov, A., Futerman, A. H., Prieto, M. & Silva, L. C. (2013) Effect of glucosylceramide on the biophysical properties of fluid membranes, *Biochim Biophys Acta.* **1828**, 1122-30.
74. Stirnemann, J., Belmatoug, N., Camou, F., Serratrice, C., Froissart, R., Caillaud, C., Levade, T., Astudillo, L., Serratrice, J., Brassier, A., Rose, C., Billette de Villemeur, T. & Berger, M. G. (2017) A Review of Gaucher Disease Pathophysiology, Clinical Presentation and Treatments, *Int J Mol Sci.* **18**.
75. Rosenbloom, B. E. & Weinreb, N. J. (2013) Gaucher disease: a comprehensive review, *Crit Rev Oncog.* **18**, 163-75.
76. Onal, G., Kutlu, O., Gozuacik, D. & Dokmeci Emre, S. (2017) Lipid Droplets in Health and Disease, *Lipids Health Dis.* **16**, 128.
77. Bickel, P. E., Tansey, J. T. & Welte, M. A. (2009) PAT proteins, an ancient family of lipid droplet proteins that regulate cellular lipid stores, *Biochim Biophys Acta.* **1791**, 419-40.
78. Meyers, A., Weiskittel, T. M. & Dalhaimer, P. (2017) Lipid Droplets: Formation to Breakdown, *Lipids.* **52**, 465-475.
79. Walther, T. C., Chung, J. & Farese, R. V., Jr. (2017) Lipid Droplet Biogenesis, *Annu Rev Cell Dev Biol.* **33**, 491-510.
80. Walther, T. C. & Farese, R. V., Jr. (2012) Lipid droplets and cellular lipid metabolism, *Annu Rev Biochem.* **81**, 687-714.
81. Sztalryd, C. & Brasaemle, D. L. (2017) The perilipin family of lipid droplet proteins: Gatekeepers of intracellular lipolysis, *Biochim Biophys Acta.* **1862**, 1221-1232.
82. Kimmel, A. R. & Sztalryd, C. (2016) The Perilipins: Major Cytosolic Lipid Droplet-Associated Proteins and Their Roles in Cellular Lipid Storage, Mobilization, and Systemic Homeostasis, *Annu Rev Nutr.* **36**, 471-509.
83. Kory, N., Thiam, A. R., Farese, R. V., Jr. & Walther, T. C. (2015) Protein Crowding Is a Determinant of Lipid Droplet Protein Composition, *Dev Cell.* **34**, 351-63.
84. Heier, C. & Haemmerle, G. (2016) Fat in the heart: The enzymatic machinery regulating cardiac triacylglycerol metabolism, *Biochim Biophys Acta.* **1861**, 1500-12.
85. Wilfling, F., Haas, J. T., Walther, T. C. & Farese, R. V., Jr. (2014) Lipid droplet biogenesis, *Curr Opin Cell Biol.* **29**, 39-45.
86. Mason, R. R. & Watt, M. J. (2015) Unraveling the roles of PLIN5: linking cell biology to physiology, *Trends Endocrinol Metab.* **26**, 144-52.

87. Lu, X., Gruia-Gray, J., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Londos, C. & Kimmel, A. R. (2001) The murine perilipin gene: the lipid droplet-associated perilipins derive from tissue-specific, mRNA splice variants and define a gene family of ancient origin, *Mamm Genome.* **12**, 741-9.
88. Brasaemle, D. L. (2007) Thematic review series: adipocyte biology. The perilipin family of structural lipid droplet proteins: stabilization of lipid droplets and control of lipolysis, *J Lipid Res.* **48**, 2547-59.
89. Hsieh, K., Lee, Y. K., Londos, C., Raaka, B. M., Dalen, K. T. & Kimmel, A. R. (2012) Perilipin family members preferentially sequester to either triacylglycerol-specific or cholesteryl-ester-specific intracellular lipid storage droplets, *J Cell Sci.* **125**, 4067-76.
90. Sztalryd, C. & Brasaemle, D. L. (2017) The perilipin family of lipid droplet proteins: Gatekeepers of intracellular lipolysis, *Biochim Biophys Acta Mol Cell Biol Lipids.* **1862**, 1221-1232.
91. Itabe, H., Yamaguchi, T., Nimura, S. & Sasabe, N. (2017) Perilipins: a diversity of intracellular lipid droplet proteins, *Lipids Health Dis.* **16**, 83.
92. de la Rosa Rodriguez, M. A. & Kersten, S. (2017) Regulation of lipid droplet-associated proteins by peroxisome proliferator-activated receptors, *Biochim Biophys Acta Mol Cell Biol Lipids.* **1862**, 1212-1220.
93. Granneman, J. G., Moore, H. P., Krishnamoorthy, R. & Rathod, M. (2009) Perilipin controls lipolysis by regulating the interactions of AB-hydrolase containing 5 (Abhd5) and adipose triglyceride lipase (Atgl), *J Biol Chem.* **284**, 34538-44.
94. Brasaemle, D. L., Subramanian, V., Garcia, A., Marcinkiewicz, A. & Rothenberg, A. (2009) Perilipin A and the control of triacylglycerol metabolism, *Mol Cell Biochem.* **326**, 15-21.
95. Tansey, J. T., Sztalryd, C., Hlavin, E. M., Kimmel, A. R. & Londos, C. (2004) The central role of perilipin a in lipid metabolism and adipocyte lipolysis, *IUBMB Life.* **56**, 379-85.
96. Tansey, J. T., Sztalryd, C., Gruia-Gray, J., Roush, D. L., Zee, J. V., Gavrilova, O., Reitman, M. L., Deng, C. X., Li, C., Kimmel, A. R. & Londos, C. (2001) Perilipin ablation results in a lean mouse with aberrant adipocyte lipolysis, enhanced leptin production, and resistance to diet-induced obesity, *Proc Natl Acad Sci U S A.* **98**, 6494-9.
97. Patel, S., Yang, W., Kozusko, K., Saudek, V. & Savage, D. B. (2014) Perilipins 2 and 3 lack a carboxy-terminal domain present in perilipin 1 involved in sequestering ABHD5 and suppressing basal lipolysis, *Proc Natl Acad Sci U S A.* **111**, 9163-8.
98. Liu, S., Geng, B., Zou, L., Wei, S., Wang, W., Deng, J., Xu, C., Zhao, X., Lyu, Y., Su, X. & Xu, G. (2015) Development of hypertrophic cardiomyopathy in perilipin-1 null mice with adipose tissue dysfunction, *Cardiovasc Res.* **105**, 20-30.
99. Gandotra, S., Le Dour, C., Bottomley, W., Cervera, P., Giral, P., Reznik, Y., Charpentier, G., Auclair, M., Delepine, M., Barroso, I., Semple, R. K.,

Lathrop, M., Lascols, O., Capeau, J., O'Rahilly, S., Magre, J., Savage, D. B. & Vigouroux, C. (2011) Perilipin deficiency and autosomal dominant partial lipodystrophy, *N Engl J Med.* **364**, 740-8.
100. Magne, J., Aminoff, A., Perman Sundelin, J., Mannila, M. N., Gustafsson, P., Hultenby, K., Wernerson, A., Bauer, G., Listenberger, L., Neville, M. J., Karpe, F., Boren, J. & Ehrenborg, E. (2013) The minor allele of the missense polymorphism Ser251Pro in perilipin 2 (PLIN2) disrupts an alpha-helix, affects lipolysis, and is associated with reduced plasma triglyceride concentration in humans, *FASEB J.* **27**, 3090-9.
101. Xu, G., Sztalryd, C., Lu, X., Tansey, J. T., Gan, J., Dorward, H., Kimmel, A. R. & Londos, C. (2005) Post-translational regulation of adipose differentiation-related protein by the ubiquitin/proteasome pathway, *J Biol Chem.* **280**, 42841-7.
102. Masuda, Y., Itabe, H., Odaki, M., Hama, K., Fujimoto, Y., Mori, M., Sasabe, N., Aoki, J., Arai, H. & Takano, T. (2006) ADRP/adipophilin is degraded through the proteasome-dependent pathway during regression of lipid-storing cells, *J Lipid Res.* **47**, 87-98.
103. Takahashi, Y., Shinoda, A., Kamada, H., Shimizu, M., Inoue, J. & Sato, R. (2016) Perilipin2 plays a positive role in adipocytes during lipolysis by escaping proteasomal degradation, *Sci Rep.* **6**, 20975.
104. Sletten, A., Seline, A., Rudd, A., Logsdon, M. & Listenberger, L. L. (2014) Surface features of the lipid droplet mediate perilipin 2 localization, *Biochem Biophys Res Commun.* **452**, 422-7.
105. Imamura, M., Inoguchi, T., Ikuyama, S., Taniguchi, S., Kobayashi, K., Nakashima, N. & Nawata, H. (2002) ADRP stimulates lipid accumulation and lipid droplet formation in murine fibroblasts, *Am J Physiol Endocrinol Metab.* **283**, E775-83.
106. Gao, J. & Serrero, G. (1999) Adipose differentiation related protein (ADRP) expressed in transfected COS-7 cells selectively stimulates long chain fatty acid uptake, *J Biol Chem.* **274**, 16825-30.
107. Feng, Y. Z., Lund, J., Li, Y., Knabenes, I. K., Bakke, S. S., Kase, E. T., Lee, Y. K., Kimmel, A. R., Thoresen, G. H., Rustan, A. C. & Dalen, K. T. (2017) Loss of perilipin 2 in cultured myotubes enhances lipolysis and redirects the metabolic energy balance from glucose oxidation towards fatty acid oxidation, *J Lipid Res.* **58**, 2147-2161.
108. Bosma, M., Hesselink, M. K., Sparks, L. M., Timmers, S., Ferraz, M. J., Mattijssen, F., van Beurden, D., Schaart, G., de Baets, M. H., Verheyen, F. K., Kersten, S. & Schrauwen, P. (2012) Perilipin 2 improves insulin sensitivity in skeletal muscle despite elevated intramuscular lipid levels, *Diabetes.* **61**, 2679-90.
109. McManaman, J. L., Bales, E. S., Orlicky, D. J., Jackman, M., MacLean, P. S., Cain, S., Crunk, A. E., Mansur, A., Graham, C. E., Bowman, T. A. & Greenberg, A. S. (2013) Perilipin-2-null mice are protected against diet-induced obesity, adipose inflammation, and fatty liver disease, *J Lipid Res.* **54**, 1346-59.

110. Carr, R. M., Peralta, G., Yin, X. & Ahima, R. S. (2014) Absence of perilipin 2 prevents hepatic steatosis, glucose intolerance and ceramide accumulation in alcohol-fed mice, *PLoS One.* **9**, e97118.
111. Tsai, T. H., Chen, E., Li, L., Saha, P., Lee, H. J., Huang, L. S., Shelness, G. S., Chan, L. & Chang, B. H. (2017) The constitutive lipid droplet protein PLIN2 regulates autophagy in liver, *Autophagy.* **13**, 1130-1144.
112. Chang, B. H., Li, L., Saha, P. & Chan, L. (2010) Absence of adipose differentiation related protein upregulates hepatic VLDL secretion, relieves hepatosteatosis, and improves whole body insulin resistance in leptin-deficient mice, *J Lipid Res.* **51**, 2132-42.
113. Ueno, M., Suzuki, J., Hirose, M., Sato, S., Imagawa, M., Zenimaru, Y., Takahashi, S., Ikuyama, S., Koizumi, T., Konoshita, T., Kraemer, F. B. & Ishizuka, T. (2017) Cardiac overexpression of perilipin 2 induces dynamic steatosis: prevention by hormone-sensitive lipase, *Am J Physiol Endocrinol Metab.* **313**, E699-E709.
114. Kaushik, S. & Cuervo, A. M. (2016) AMPK-dependent phosphorylation of lipid droplet protein PLIN2 triggers its degradation by CMA, *Autophagy.* **12**, 432-8.
115. Kaushik, S. & Cuervo, A. M. (2015) Degradation of lipid droplet-associated proteins by chaperone-mediated autophagy facilitates lipolysis, *Nat Cell Biol.* **17**, 759-70.
116. Diaz, E. & Pfeffer, S. R. (1998) TIP47: a cargo selection device for mannose 6-phosphate receptor trafficking, *Cell.* **93**, 433-43.
117. Sztalryd, C. & Kimmel, A. R. (2014) Perilipins: lipid droplet coat proteins adapted for tissue-specific energy storage and utilization, and lipid cytoprotection, *Biochimie.* **96**, 96-101.
118. Zhu, J., Xu, M., Liu, Y., Zhuang, L., Ying, K., Liu, F., Liu, D., Ma, W. & Songyang, Z. (2018) Phosphorylation of PLIN3 by AMPK promotes dispersion of lipid droplets during starvation, *Protein Cell.*
119. Wolins, N. E., Skinner, J. R., Schoenfish, M. J., Tzekov, A., Bensch, K. G. & Bickel, P. E. (2003) Adipocyte protein S3-12 coats nascent lipid droplets, *J Biol Chem.* **278**, 37713-21.
120. Chen, W., Chang, B., Wu, X., Li, L., Sleeman, M. & Chan, L. (2013) Inactivation of Plin4 downregulates Plin5 and reduces cardiac lipid accumulation in mice, *Am J Physiol Endocrinol Metab.* **304**, E770-9.
121. Dalen, K. T., Dahl, T., Holter, E., Arntsen, B., Londos, C., Sztalryd, C. & Nebb, H. I. (2007) LSDP5 is a PAT protein specifically expressed in fatty acid oxidizing tissues, *Biochim Biophys Acta.* **1771**, 210-27.
122. Kuramoto, K., Okamura, T., Yamaguchi, T., Nakamura, T. Y., Wakabayashi, S., Morinaga, H., Nomura, M., Yanase, T., Otsu, K., Usuda, N., Matsumura, S., Inoue, K., Fushiki, T., Kojima, Y., Hashimoto, T., Sakai, F., Hirose, F. & Osumi, T. (2012) Perilipin 5, a lipid droplet-binding protein, protects heart from oxidative burden by sequestering fatty acid from excessive oxidation, *J Biol Chem.* **287**, 23852-63.

123. Kimmel, A. R. & Sztalryd, C. (2014) Perilipin 5, a lipid droplet protein adapted to mitochondrial energy utilization, *Curr Opin Lipidol.* **25**, 110-7.
124. Drevinge, C., Dalen, K. T., Mannila, M. N., Tang, M. S., Stahlman, M., Klevstig, M., Lundqvist, A., Mardani, I., Haugen, F., Fogelstrand, P., Adiels, M., Asin-Cayuela, J., Ekestam, C., Gadin, J. R., Lee, Y. K., Nebb, H., Svedlund, S., Johansson, B. R., Hulten, L. M., Romeo, S., Redfors, B., Omerovic, E., Levin, M., Gan, L. M., Eriksson, P., Andersson, L., Ehrenborg, E., Kimmel, A. R., Boren, J. & Levin, M. C. (2016) Perilipin 5 is protective in the ischemic heart, *Int J Cardiol.* **219**, 446-54.
125. Zheng, P., Xie, Z., Yuan, Y., Sui, W., Wang, C., Gao, X., Zhao, Y., Zhang, F., Gu, Y., Hu, P., Ye, J., Feng, X. & Zhang, L. (2017) Plin5 alleviates myocardial ischaemia/reperfusion injury by reducing oxidative stress through inhibiting the lipolysis of lipid droplets, *Sci Rep.* **7**, 42574.
126. Andersson, L., Drevinge, C., Mardani, I., Dalen, K. T., Stahlman, M., Klevstig, M., Lundqvist, A., Haugen, F., Adiels, M., Fogelstrand, P., Asin-Cayuela, J., Hulten, L. M., Levin, M., Ehrenborg, E., Lee, Y. K., Kimmel, A. R., Boren, J. & Levin, M. C. (2017) Deficiency in perilipin 5 reduces mitochondrial function and membrane depolarization in mouse hearts, *Int J Biochem Cell Biol.* **91**, 9-13.
127. Wang, H., Sreenivasan, U., Gong, D. W., O'Connell, K. A., Dabkowski, E. R., Hecker, P. A., Ionica, N., Konig, M., Mahurkar, A., Sun, Y., Stanley, W. C. & Sztalryd, C. (2013) Cardiomyocyte-specific perilipin 5 overexpression leads to myocardial steatosis and modest cardiac dysfunction, *J Lipid Res.* **54**, 953-65.
128. Pollak, N. M., Schweiger, M., Jaeger, D., Kolb, D., Kumari, M., Schreiber, R., Kolleritsch, S., Markolin, P., Grabner, G. F., Heier, C., Zierler, K. A., Rulicke, T., Zimmermann, R., Lass, A., Zechner, R. & Haemmerle, G. (2013) Cardiac-specific overexpression of perilipin 5 provokes severe cardiac steatosis via the formation of a lipolytic barrier, *J Lipid Res.* **54**, 1092-102.
129. Wang, H., Bell, M., Sreenivasan, U., Hu, H., Liu, J., Dalen, K., Londos, C., Yamaguchi, T., Rizzo, M. A., Coleman, R., Gong, D., Brasaemle, D. & Sztalryd, C. (2011) Unique regulation of adipose triglyceride lipase (ATGL) by perilipin 5, a lipid droplet-associated protein, *J Biol Chem.* **286**, 15707-15.
130. Gallardo-Montejano, V. I., Saxena, G., Kusminski, C. M., Yang, C., McAfee, J. L., Hahner, L., Hoch, K., Dubinsky, W., Narkar, V. A. & Bickel, P. E. (2016) Nuclear Perilipin 5 integrates lipid droplet lipolysis with PGC-1alpha/SIRT1-dependent transcriptional regulation of mitochondrial function, *Nat Commun.* **7**, 12723.
131. Yu, L., Chen, Y. & Tooze, S. A. (2018) Autophagy pathway: Cellular and molecular mechanisms, *Autophagy.* **14**, 207-215.
132. Ravikumar, B., Sarkar, S., Davies, J. E., Futter, M., Garcia-Arencibia, M., Green-Thompson, Z. W., Jimenez-Sanchez, M., Korolchuk, V. I., Lichtenberg, M., Luo, S., Massey, D. C., Menzies, F. M., Moreau, K., Narayanan, U., Renna, M., Siddiqi, F. H., Underwood, B. R., Winslow, A. R.

& Rubinsztein, D. C. (2010) Regulation of mammalian autophagy in physiology and pathophysiology, *Physiol Rev.* **90**, 1383-435.
133. Chen, Y. & Klionsky, D. J. (2011) The regulation of autophagy - unanswered questions, *J Cell Sci.* **124**, 161-70.
134. Hurley, J. H. & Young, L. N. (2017) Mechanisms of Autophagy Initiation, *Annu Rev Biochem.* **86**, 225-244.
135. Tanida, I., Ueno, T. & Kominami, E. (2008) LC3 and Autophagy, *Methods Mol Biol.* **445**, 77-88.
136. Cingolani, F. & Czaja, M. J. (2016) Regulation and Functions of Autophagic Lipolysis, *Trends Endocrinol Metab.* **27**, 696-705.
137. Schulze, R. J., Sathyanarayan, A. & Mashek, D. G. (2017) Breaking fat: The regulation and mechanisms of lipophagy, *Biochim Biophys Acta Mol Cell Biol Lipids.* **1862**, 1178-1187.
138. Dong, H. & Czaja, M. J. (2011) Regulation of lipid droplets by autophagy, *Trends Endocrinol Metab.* **22**, 234-40.
139. Orenstein, S. J. & Cuervo, A. M. (2010) Chaperone-mediated autophagy: molecular mechanisms and physiological relevance, *Semin Cell Dev Biol.* **21**, 719-26.
140. Garcia, E. J., Vevea, J. D. & Pon, L. A. (2018) Lipid droplet autophagy during energy mobilization, lipid homeostasis and protein quality control, *Front Biosci (Landmark Ed).* **23**, 1552-1563.
141. Yin, Z., Pascual, C. & Klionsky, D. J. (2016) Autophagy: machinery and regulation, *Microb Cell.* **3**, 588-596.
142. Bento, C. F., Renna, M., Ghislat, G., Puri, C., Ashkenazi, A., Vicinanza, M., Menzies, F. M. & Rubinsztein, D. C. (2016) Mammalian Autophagy: How Does It Work?, *Annu Rev Biochem.* **85**, 685-713.
143. Feng, Y., He, D., Yao, Z. & Klionsky, D. J. (2014) The machinery of macroautophagy, *Cell Res.* **24**, 24-41.
144. Lass, A., Zimmermann, R., Oberer, M. & Zechner, R. (2011) Lipolysis - a highly regulated multi-enzyme complex mediates the catabolism of cellular fat stores, *Prog Lipid Res.* **50**, 14-27.
145. Knapp, M. & Gorski, J. (2017) The skeletal and heart muscle triacylglycerol lipolysis revisited, *J Physiol Pharmacol.* **68**, 3-11.
146. Martinez-Lopez, N., Garcia-Macia, M., Sahu, S., Athonvarangkul, D., Liebling, E., Merlo, P., Cecconi, F., Schwartz, G. J. & Singh, R. (2016) Autophagy in the CNS and Periphery Coordinate Lipophagy and Lipolysis in the Brown Adipose Tissue and Liver, *Cell Metab.* **23**, 113-27.
147. Lee, I. H., Cao, L., Mostoslavsky, R., Lombard, D. B., Liu, J., Bruns, N. E., Tsokos, M., Alt, F. W. & Finkel, T. (2008) A role for the NAD-dependent deacetylase Sirt1 in the regulation of autophagy, *Proc Natl Acad Sci U S A.* **105**, 3374-9.
148. Khan, S. A., Sathyanarayan, A., Mashek, M. T., Ong, K. T., Wollaston-Hayden, E. E. & Mashek, D. G. (2015) ATGL-catalyzed lipolysis regulates SIRT1 to control PGC-1alpha/PPAR-alpha signaling, *Diabetes.* **64**, 418-26.

149. Sathyanarayan, A., Mashek, M. T. & Mashek, D. G. (2017) ATGL Promotes Autophagy/Lipophagy via SIRT1 to Control Hepatic Lipid Droplet Catabolism, *Cell Rep.* **19**, 1-9.
150. Taegtmeyer, H., Golfman, L., Sharma, S., Razeghi, P. & van Arsdall, M. (2004) Linking gene expression to function: metabolic flexibility in the normal and diseased heart, *Ann N Y Acad Sci.* **1015**, 202-13.
151. Lee, L., Horowitz, J. & Frenneaux, M. (2004) Metabolic manipulation in ischaemic heart disease, a novel approach to treatment, *Eur Heart J.* **25**, 634-41.
152. Hochachka, P. W., Buck, L. T., Doll, C. J. & Land, S. C. (1996) Unifying theory of hypoxia tolerance: molecular/metabolic defense and rescue mechanisms for surviving oxygen lack, *Proc Natl Acad Sci U S A.* **93**, 9493-8.
153. Bilheimer, D. W., Buja, L. M., Parkey, R. W., Bonte, F. J. & Willerson, J. T. (1978) Fatty acid accumulation and abnormal lipid deposition in peripheral and border zones of experimental myocardial infarcts, *Journal of nuclear medicine : official publication, Society of Nuclear Medicine.* **19**, 276-83.
154. Drevinge, C., Karlsson, L. O., Stahlman, M., Larsson, T., Perman Sundelin, J., Grip, L., Andersson, L., Boren, J. & Levin, M. C. (2013) Cholesteryl esters accumulate in the heart in a porcine model of ischemia and reperfusion, *PLoS One.* **8**, e61942.
155. Perman, J. C., Bostrom, P., Lindbom, M., Lidberg, U., StAhlman, M., Hagg, D., Lindskog, H., Scharin Tang, M., Omerovic, E., Mattsson Hulten, L., Jeppsson, A., Petursson, P., Herlitz, J., Olivecrona, G., Strickland, D. K., Ekroos, K., Olofsson, S. O. & Boren, J. (2011) The VLDL receptor promotes lipotoxicity and increases mortality in mice following an acute myocardial infarction, *J Clin Invest.* **121**, 2625-40.
156. Klevstig, M., Stahlman, M., Lundqvist, A., Scharin Tang, M., Fogelstrand, P., Adiels, M., Andersson, L., Kolesnick, R., Jeppsson, A., Boren, J. & Levin, M. C. (2016) Targeting acid sphingomyelinase reduces cardiac ceramide accumulation in the post-ischemic heart, *J Mol Cell Cardiol.* **93**, 69-72.
157. Torffvit, O. & Agardh, C. (2000) The prognosis for type 2 diabetic patients with heart disease. A 10-year observation study of 385 patients, *Journal of diabetes and its complications.* **14**, 301-6.
158. Vandamme, T. F. (2014) Use of rodents as models of human diseases, *J Pharm Bioallied Sci.* **6**, 2-9.
159. Perlman, R. L. (2016) Mouse models of human disease: An evolutionary perspective, *Evol Med Public Health.* **2016**, 170-6.
160. Hulbert, A. J. (2008) The links between membrane composition, metabolic rate and lifespan, *Comp Biochem Physiol A Mol Integr Physiol.* **150**, 196-203.
161. Suckling, K. E. & Jackson, B. (1993) Animal models of human lipid metabolism, *Prog Lipid Res.* **32**, 1-24.

162. Breckenridge, R. (2010) Heart failure and mouse models, *Dis Model Mech.* **3**, 138-43.
163. Wessels, A. & Sedmera, D. (2003) Developmental anatomy of the heart: a tale of mice and man, *Physiol Genomics.* **15**, 165-76.
164. Doevendans, P. A., Daemen, M. J., de Muinck, E. D. & Smits, J. F. (1998) Cardiovascular phenotyping in mice, *Cardiovasc Res.* **39**, 34-49.
165. Claycomb, W. C., Lanson, N. A., Jr., Stallworth, B. S., Egeland, D. B., Delcarpio, J. B., Bahinski, A. & Izzo, N. J., Jr. (1998) HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte, *Proc Natl Acad Sci U S A.* **95**, 2979-84.
166. White, S. M., Constantin, P. E. & Claycomb, W. C. (2004) Cardiac physiology at the cellular level: use of cultured HL-1 cardiomyocytes for studies of cardiac muscle cell structure and function, *Am J Physiol Heart Circ Physiol.* **286**, H823-9.
167. Peter, A. K., Bjerke, M. A. & Leinwand, L. A. (2016) Biology of the cardiac myocyte in heart disease, *Mol Biol Cell.* **27**, 2149-60.
168. O'Connell, T. D., Rodrigo, M. C. & Simpson, P. C. (2007) Isolation and culture of adult mouse cardiac myocytes, *Methods Mol Biol.* **357**, 271-96.
169. Stepanenko, A. A. & Dmitrenko, V. V. (2015) HEK293 in cell biology and cancer research: phenotype, karyotype, tumorigenicity, and stress-induced genome-phenotype evolution, *Gene.* **569**, 182-90.
170. Thomas, P. & Smart, T. G. (2005) HEK293 cell line: a vehicle for the expression of recombinant proteins, *J Pharmacol Toxicol Methods.* **51**, 187-200.
171. Wang, J., Bo, H., Meng, X., Wu, Y., Bao, Y. & Li, Y. (2006) A simple and fast experimental model of myocardial infarction in the mouse, *Tex Heart Inst J.* **33**, 290-3.
172. Michael, L. H., Entman, M. L., Hartley, C. J., Youker, K. A., Zhu, J., Hall, S. R., Hawkins, H. K., Berens, K. & Ballantyne, C. M. (1995) Myocardial ischemia and reperfusion: a murine model, *Am J Physiol.* **269**, H2147-54.
173. Muthuramu, I., Lox, M., Jacobs, F. & De Geest, B. (2014) Permanent ligation of the left anterior descending coronary artery in mice: a model of post-myocardial infarction remodelling and heart failure, *J Vis Exp*.
174. Chen, J., Ceholski, D. K., Liang, L., Fish, K. & Hajjar, R. J. (2017) Variability in coronary artery anatomy affects consistency of cardiac damage after myocardial infarction in mice, *Am J Physiol Heart Circ Physiol.* **313**, H275-H282.
175. Curaj, A., Simsekyilmaz, S., Staudt, M. & Liehn, E. (2015) Minimal invasive surgical procedure of inducing myocardial infarction in mice, *J Vis Exp*, e52197.
176. Pistner, A., Belmonte, S., Coulthard, T. & Blaxall, B. (2010) Murine echocardiography and ultrasound imaging, *J Vis Exp*.
177. Uygur, A. & Lee, R. T. (2016) Mechanisms of Cardiac Regeneration, *Dev Cell.* **36**, 362-74.

178. Puhl, S. L., Weeks, K. L., Ranieri, A. & Avkiran, M. (2016) Assessing structural and functional responses of murine hearts to acute and sustained beta-adrenergic stimulation in vivo, *J Pharmacol Toxicol Methods.* **79**, 60-71.
179. Chang, S. C., Ren, S., Rau, C. D. & Wang, J. J. (2018) Isoproterenol-Induced Heart Failure Mouse Model Using Osmotic Pump Implantation, *Methods Mol Biol.* **1816**, 207-220.
180. Tenny, S. & Abdelgawad, I. (2019) Statistical Significance in *StatPearls*, Treasure Island (FL).
181. Schober, P., Bossers, S. M. & Schwarte, L. A. (2018) Statistical Significance Versus Clinical Importance of Observed Effect Sizes: What Do P Values and Confidence Intervals Really Represent?, *Anesth Analg.* **126**, 1068-1072.
182. Michael J. Campbell, D. M., Stephen J. Walters (2011) Medical statistics, a textbook for the health sciences.
183. Krithikadatta, J. (2014) Normal distribution, *J Conserv Dent.* **17**, 96-7.
184. Kwak, S. G. & Kim, J. H. (2017) Central limit theorem: the cornerstone of modern statistics, *Korean J Anesthesiol.* **70**, 144-156.
185. Kiernan, D. Natural resources
Biometrics.
186. Kim, H. Y. (2015) Statistical notes for clinical researchers: post-hoc multiple comparisons, *Restor Dent Endod.* **40**, 172-6.
187. Bland, J. M. & Altman, D. G. (2004) The logrank test, *BMJ.* **328**, 1073.
188. Sztalryd, C., Bell, M., Lu, X., Mertz, P., Hickenbottom, S., Chang, B. H., Chan, L., Kimmel, A. R. & Londos, C. (2006) Functional compensation for adipose differentiation-related protein (ADFP) by Tip47 in an ADFP null embryonic cell line, *J Biol Chem.* **281**, 34341-8.
189. Pollak, N. M., Jaeger, D., Kolleritsch, S., Zimmermann, R., Zechner, R., Lass, A. & Haemmerle, G. (2015) The interplay of protein kinase A and perilipin 5 regulates cardiac lipolysis, *J Biol Chem.* **290**, 1295-306.
190. Maillet, M., van Berlo, J. H. & Molkentin, J. D. (2013) Molecular basis of physiological heart growth: fundamental concepts and new players, *Nat Rev Mol Cell Biol.* **14**, 38-48.
191. Wang, Y., Wisloff, U. & Kemi, O. J. (2010) Animal models in the study of exercise-induced cardiac hypertrophy, *Physiol Res.* **59**, 633-44.
192. Zhang, P. (2017) CaMKII: The molecular villain that aggravates cardiovascular disease, *Exp Ther Med.* **13**, 815-820.
193. Beckendorf, J., van den Hoogenhof, M. M. G. & Backs, J. (2018) Physiological and unappreciated roles of CaMKII in the heart, *Basic Res Cardiol.* **113**, 29.
194. Hannun, Y. A. & Obeid, L. M. (2008) Principles of bioactive lipid signalling: lessons from sphingolipids, *Nat Rev Mol Cell Biol.* **9**, 139-50.
195. Li, J. F., Zheng, S. J., Wang, L. L., Liu, S., Ren, F., Chen, Y., Bai, L., Liu, M. & Duan, Z. P. (2017) Glucosylceramide synthase regulates the

proliferation and apoptosis of liver cells in vitro by Bcl2/Bax pathway, *Mol Med Rep.* **16**, 7355-7360.
196. Schreij, A. M., Fon, E. A. & McPherson, P. S. (2016) Endocytic membrane trafficking and neurodegenerative disease, *Cell Mol Life Sci.* **73**, 1529-45.
197. Zhang, X. & Wang, Y. (2018) The Golgi stacking protein GORASP2/GRASP55 serves as an energy sensor to promote autophagosome maturation under glucose starvation, *Autophagy.* **14**, 1649-1651.
198. Zhou, L., Zhang, L., Meng, Q., Niu, C., Jin, D., Yu, A., Gan, L. & Yang, Z. (2012) C/EBPalpha promotes transcription of the porcine perilipin5 gene, *Mol Cell Endocrinol.* **364**, 28-35.