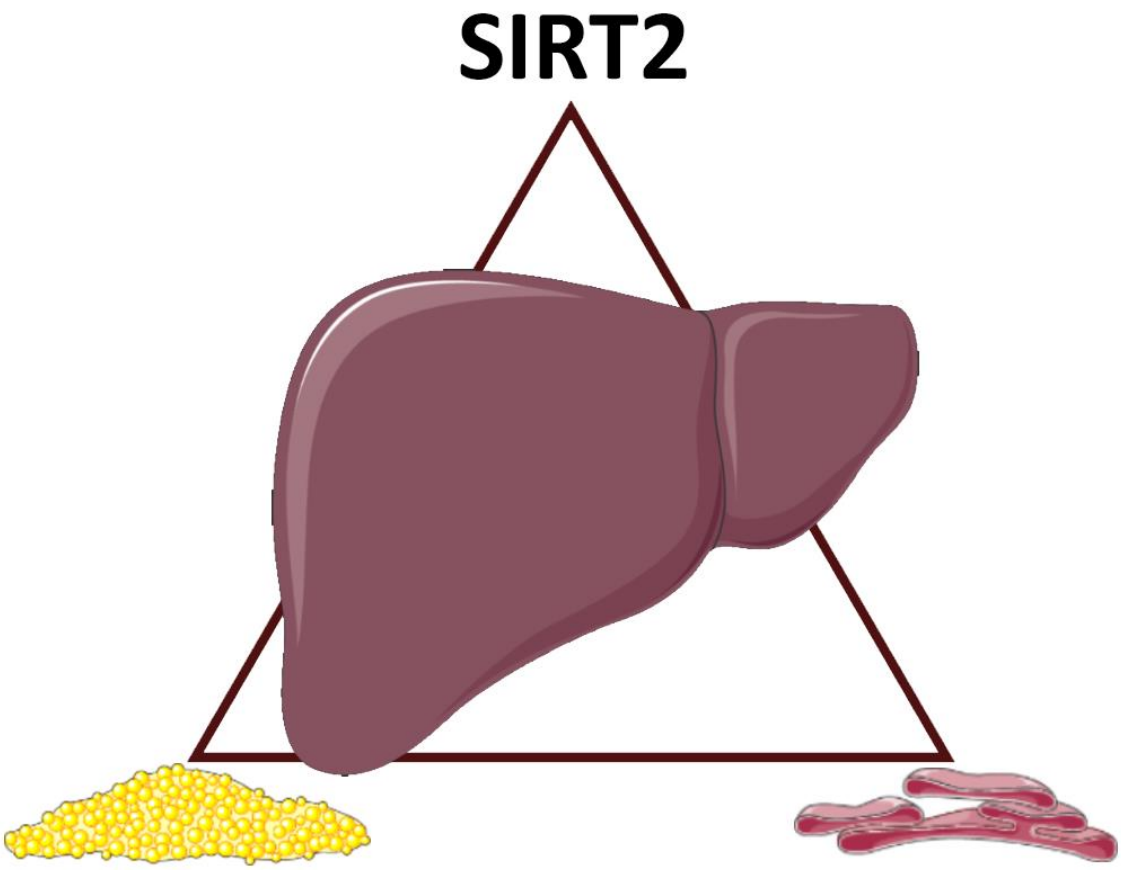

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Dissertação de Mestrado em Biologia Celular e Molecular
orientada pelo Doutor Pedro Gomes e pela Professora Doutora Emília Duarte,
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Junho de 2018

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UNIVERSIDADE DE COIMBRA

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O trabalho aqui apresentado foi realizado no grupo de Neuroendocrinologia e Envelhecimento do Centro de Neurociências e Biologia Celular (Universidade de Coimbra, Coimbra), liderado pela Professora Doutora Cláudia Cavadas, e orientado pelo Doutor Pedro Gomes.

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"*Plus Ultra!*"

Yagi Toshinori

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ACC – Acetyl CoA carboxylase

ACDH – Acyl-CoA dehydrogenase

ACS – Acyl CoA synthase

ACLY – ATP citrate lyase

AMPK – AMP-activated protein kinase

APO - Apolipoprotein

ATF – Activating transcription factor

ATP – Adenosine triphosphate

BCA – Bicinchoninic acid

BIM – Bcl-2-like protein 11

BSA – Bovine serum albumin

CD – Chow diet

CHOP - CCAAT-enhancer-binding protein homologous protein

ChREBP – Carbohydrate response element binding protein

CPT – Carnitine palmitoyl transferase

CR - Chylomicron

DGAT-1 – Diacylglycerol transferase-1

ECH – Enoyl-CoA hydratase

eIF2α – Eukaryotic initiation factor 2α

ER – Endoplasmic reticulum

FAS – Fatty acid synthase

FFA – Free fatty acids

FXR – Farnesoid X Receptor

FOXO – Forkhead box class O

GADD34 - Growth arrest and DNA damage-inducible protein

GK – Glucokinase

GRP78 - 78-kDa glucose-regulated protein (also called BiP)

HACDH – Hydroxy acyl-CoA dehydrogenase

HCC – Hepatocellular carcinoma

HD – Huntington's disease

HDL – High density lipoprotein

HFD – High-fat diet

HSC – Hepatic stellate cells

HIF-1α – Hypoxia-inducible factor-1α

IR – Insulin receptor

IRE1 – Inositol required enzyme 1

IRS – Insulin receptor substrate

KACT - Ketoacyl-CoA thiolase

LDL – Low density lipoprotein

LDLR – Low density lipoprotein receptor

LPL – Lipoprotein lipase

LXR – Liver X receptor

NAFLD – Non-alcoholic fatty liver disease

NAM - Nicotinamide

NASH – Non-alcoholic steatohepatitis

NF-kB – Nuclear factor-kB

ORO – Oil Red O

PC – Phosphatidylcholine

PE - Phosphatidyletanolamine

PGC-1α – Peroxisome proliferator-activated receptor α coactivator-1α

PEPCK – Phosphoenolpyruvate carboxykinase

PERK – Protein kinase RNA-like

PPAR-γ – Peroxisome proliferator-activated receptor –γ

PARP – Poly ADP-ribose parylation

ROS – Reactive oxygen species

SCD-1 – Steroyl CoA desaturase-1

Sir – Silent information regulator

SIRT - Sirtuin

SREBP – Sterol regulatory element binding protein

TG - Triglycerides

TNF-α – Tumor necrosis factor-α

UPR – Unfolded protein response

VLDL – Very low density lipoprotein

Abstract

Non-alcoholic fatty liver disease (NAFLD) is becoming the most common liver disease worldwide, increasing in parallel with obesity and type 2 diabetes. NAFLD is characterized by dysregulated hepatic lipid metabolism, resulting in liver triglyceride accumulation, leading to insulin resistance and activation of ER stress and inflammatory pathways. NAFLD is one of the leading causes of liver transplantations, mostly due to low number or non-efficacy of current pharmacological approaches. Moreover, the hepatic molecular mechanisms that underlie the disease have not been fully elucidated. Sirtuins are recognized as important players in metabolic regulation, as the role of these NAD^+-dependent deacetylases have been implicated in both glucose and lipid metabolism. Recent studies suggest an important role for sirtuin 2 (SIRT2) in metabolic regulation. Our preliminary data using SIRT2-KO mice showed elevated lipid accumulation in the liver, under both chow and high-fat diet feeding, thus suggesting SIRT2 as a potential player in hepatic lipid metabolism. Nonetheless, the role of this protein on lipid homeostasis has not been elucidated. Taking this into account, this study was designed to investigate the role of SIRT2 in hepatic lipid metabolism, by establishing an *in vitro* model for hepatic steatosis using HepG2 cells and modulating SIRT2 levels in this model to establish a relation between SIRT2 expression and lipid deposition. We found that palmitate-induced lipid overload caused ER stress activation and concomitantly reduced SIRT2 expression. Importantly, SIRT2-silenced cells exhibit higher lipid accumulation and ER stress activation under basal conditions, whereas SIRT2 overexpression led to attenuated lipid deposition and ER stress activation upon palmitate exposure. Taken together, our findings demonstrate a role for SIRT2 in ER

stress response and lipid homeostasis, and could have a paramount role as a novel pharmacological approach towards pathologies that comprise lipid metabolic dysregulation such as NAFLD.

Keywords: Sirtuin 2, Hepatic steatosis, Endoplasmic reticulum stress, metabolic dysfunction

Resumo

O fígado gordo não-alcoólico (NAFLD) esta a tornar-se a doença metabólica hepática de maior prevalência no mundo, aumentando em paralelo com doenças metabólicas como a obesidade e diabetes tipo 2. O NAFLD caracteriza-se inicialmente por desregulação metabólica no metabolismo lipídico, causando acumulação de triglicerídeos hepática, seguindo-se por resistência à insulina e desencadear respostas inflamatórias e de ativação do stress do retículo endoplasmático (ER). Esta patologia tem sido insurgente em transplantes hepáticos, principalmente pela baixa existência de terapias farmacológicas atuais. Os principais mecanismos relativos ao metabolismo lipídico na doença ainda não foram totalmente identificados. As sirtuínas, uma família de desacetilases dependentes de NAD^+, têm sido implicadas como reguladores do metabolismo de glicose e lipídico. A sirtuína 2 (SIRT2) tem sido emergente em estudos de regulação metabólica, e dados preliminares do nosso grupo de murganhos knock-out para a SIRT2 (SIRT2-KO), demonstraram uma predisposição para a elevada acumulação lipídica hepática, tanto em dieta controlo, como em dieta hipercalórica, sugerindo um potencial papel da SIRT2 na regulação do metabolismo hepático lipídico. Deste modo, o papel desta proteína na regulação da homeostasia lipídica tem de ser elucidado. Tendo esta informação em conta, este estudo visou a investigação do papel da SIRT2 no metabolismo hepático lipídico, através do estabelecimento de um modelo *in vitro* para esteatose hepática utilizando a linha celular hepática HepG2 incubadas com palmitato, e consequente modulação dos níveis de SIRT2 para estabelecer uma relação entre a expressão de SIRT2 e deposição lipídica. Observámos que a sobre acumulação lipídica tanto ativou as vias do stress do ER como diminuiu a expressão de SIRT2. Deste modo, os nossos resultados relativos a células silenciadas para SIRT2 demonstraram uma

predisposição para acumulação lipídica e ativação do stress do ER, em contraste com células que sobreexpressaram SIRT2, que após exposição a palmitato tiveram uma modulação da ativação do stress do ER, assim como uma prevenção de sobre acumulação lipídica. Os nossos resultados demonstram um papel para SIRT2 na modulação da ativação do stress do ER e consequente regulação no metabolismo hepático lipídico, e em suma, poderá e ser uma nova abordagem terapêutica para patologias que compreendem desregulação lipídica como o NAFLD.

Palavras-chave: Sirtuína 2, esteatose hepática, stress do retículo endoplasmático, disfunção metabólica

Chapter I – Introduction

1.1 Pathophysiology of obesity and related metabolic disorders

1.1.1 Obesity and Type II Diabetes

It is undeniable that obesity and related pathologies are among the main epidemics of the 21st century. High dietary fat intake coupled with a sedentary lifestyle are major risk factors for the onset of most cases worldwide, and the numbers keep increasing over time. The number of deaths due this group of diseases is estimated to increase in the next decades.

Obesity is characterized by excessive accumulation of fat in the adipose tissues, as well as in other key organs, such as the liver, heart and skeletal muscle, also designated by adiposity. This will lead to ectopic fat accumulation in both peripheral and central organs, triggering lipotoxic events, such as metabolic dysfunction and activation of inflammatory responses (Figure 1)[2]. Also associated with this phenotype is insulin resistance. Insulin is an hormone produced by the pancreatic β cells and, in physiological conditions, insulin is necessary to trigger a metabolic signaling cascade by activating its receptors (IR) , that will allow the insertion of glucose transporters (GLUT) in the cell membrane in key tissues, such as the skeletal muscle and adipose tissue, to take up glucose [3]. Insulin also plays a role in the stimulation of fatty acid synthesis, as well as inhibition of adipose tissue lipolysis [1].

Under insulin resistance conditions, insulin receptors become impaired, leading to compromised activation of the signaling cascade, as well as the insulin receptor substrate 1 (IRS-1) that promotes the downstream target activation, for example

through Akt, and ultimately transcription and translocation of GLUT4, leading to diminished glucose uptake [4]. Impaired insulin signaling is most commonly observed in peripheral tissues. Coupled to the impairment of the activation of the receptors, there is less stimulated glucose production in the liver, as well as a deficiency in the hormone synthesis by the pancreatic β cells, leading to hyperglycemia, the hallmark of type II diabetes [5]. This pathology is closely associated with obesity, since dietary habits are a major risk factor, and overall it can lead to cardiovascular complications as well as blindness [6]. The resulting hyperinsulinemia is also a major causal factor of dyslipidemia.

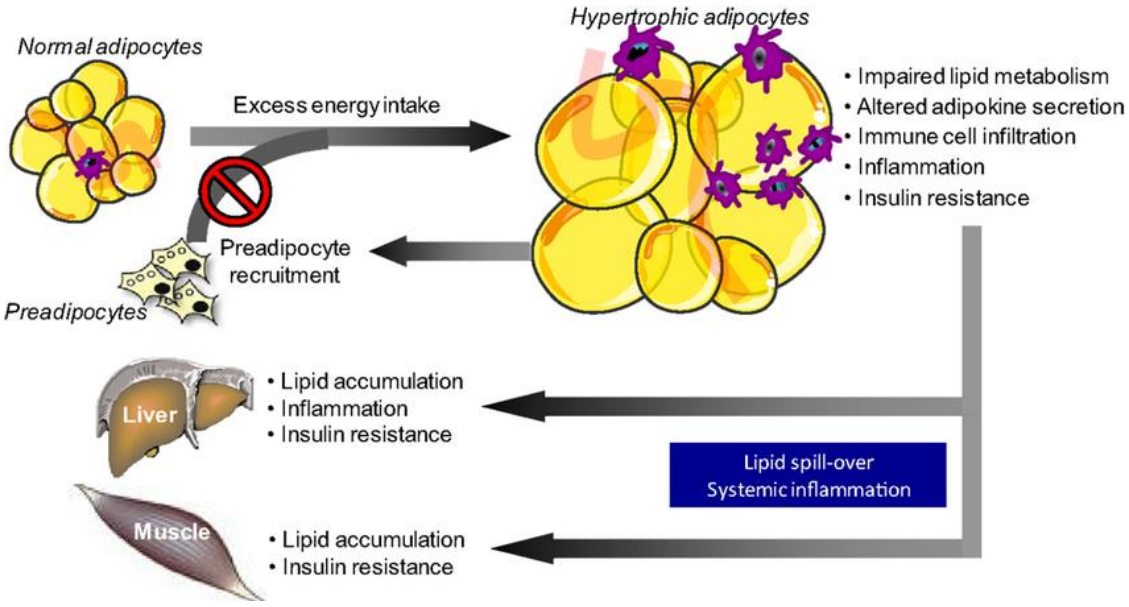

Figure 1. Pathophysiology of obesity. Excess energy intake leads to adipocyte hypertrophy. This will lead to both inflammation and insulin resistance in adipose cells, as well as ectopic fat accumulation on other tissues, triggering metabolic dysfunctions [7].

Since metabolic disorders are growing at an alarming rate, the complications that result from these pathologies are increasing as well. They pose a major economic burden, as the numbers regarding the health care for these diseases are increasing

exponentially, and are expected to grow even more during the next decades [8]. Therefore, there is an urgent need for pharmacological approaches to prevent and treat these syndromes, more efficient than nutritional and exercise programs.

1.1.2 Non-Alcoholic Fatty Liver Disease (NAFLD)

Non-alcoholic fatty liver disease (NAFLD) is another metabolic disorder whose prevalence is growing worldwide. NAFLD can be defined as the hepatic manifestation of the metabolic syndrome, where the aberrant accumulation of fat in the liver is mainly due to high-fat food intake, having no relation with either alcohol intake or other specific cause [9]. Currently, it is the most common chronic liver disease worldwide, and the estimates of global NAFLD prevalence range from 25 to 45% [10]. The major risk factors of this pathology are responsible for the growth in parallel of both obesity and type II diabetes, as these conditions aggravate the NAFLD phenotype [11]. This relation was demonstrated in recent studies, with a link between NAFLD and both hyperglycemia and insulin resistance [12].

Physiologically, the starting point and main hallmark of NAFLD is liver steatosis, which is defined as the excess accumulation of lipids in hepatocytes, by both uptake of circulating free fatty acids and over-synthesis [13]. There is a progressive aspect to NAFLD and the worsening state of the liver. As the disease progresses from simple steatosis, there is an increase of the prevalence of both acute and chronic inflammatory states, leading to non-alcoholic steatohepatitis (NASH), cirrhosis and ultimately, hepatocellular carcinoma (HCC) [14]. NASH is characterized by higher inflammatory responses, tissue ballooning and fibrosis, and cirrhosis by elevated scar tissue-like states

that will be irreversible as the disease advances. The ultimate complication of cirrhosis is hepatocellular carcinoma and, eventually, death (Figure 2).

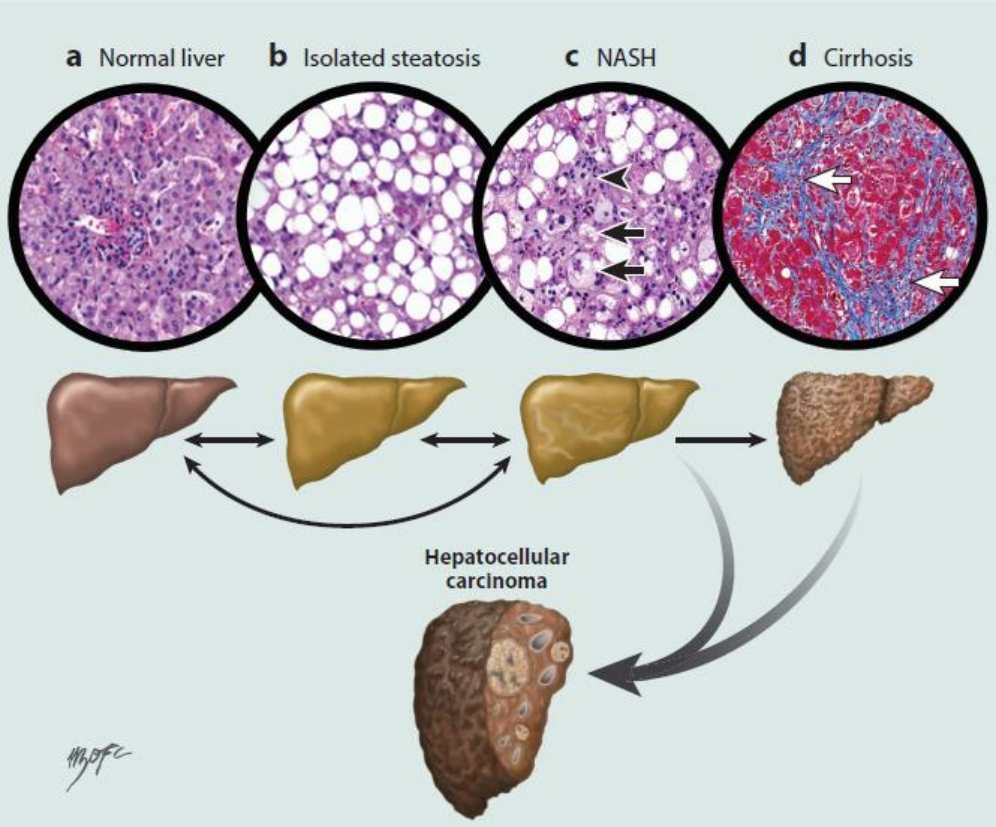

Figure 2. Progressive spectrum of NAFLD. The excess accumulation of fat in the liver causes steatosis, and the aggravation of the phenotype, characterized by high inflammation activation, resulting in NASH, cirrhosis and eventually HCC [15].

To date, pharmacological approaches towards NAFLD are limited and poor in efficacy. The growing incidence of patients with NASH is responsible for the increase of liver transplants, especially in developed countries [16]. The current therapeutic strategies to combat the progression of the disease are mainly targeted towards the correction of its risk factors, as well as liver transaminases levels in the blood. The current diagnostic strategies include liver biopsies, which are invasive, hence increasing

the need for specific biomarkers. Although recent studies have unraveled some targets for pharmacological intervention, it is crucial to develop models that will translate better the metabolic changes in humans with NAFLD [17].

1.2 Hepatic lipid metabolism

The liver plays a central role in lipid metabolism and other metabolic pathways, strongly depending on the nutritional intake and content of both fats and sugar [18]. The main source of the FFA pool in the liver are the circulating FFA, that come from both fat intake and lipolysis in adipocytes during fasting states [19]. Overall, the mechanisms that underlie hepatic lipid accumulation include the processing of lipids obtained from the diet and the result from both adipose and skeletal muscle tissue lipolysis, and both metabolic reactions, *de novo* lipogenesis and fatty acid β-oxidation [1].

1.2.1 Dietary lipids

Lipids derived from diet are converted into bile acids and then converted into chylomicrons (CR) by cells of the small intestine, the enterocytes [20][21]. These CR, enriched in phospholipids, esterified cholesterol and triglycerides, are released into the lymphatic vessels, after acquiring apolipoproteins (APO) B-48, C2 and E [22], reaching key tissues, such as the skeletal muscle or the adipose tissue, where they will suffer an hydrolysis process by lipoprotein lipase (LPL), converting the triglycerides into fatty acids [23]. The CRs will then be reintroduced to the blood circulation and enter the liver,

releasing the lipid content to be further hydrolyzed in order to synthesize very low density lipoproteins (VLDL) [24].

These VLDLs will be responsible for the transport of lipids from the liver to the skeletal muscle and adipose tissues, and subsequent delivery after the action of LPL [23, 25]. Afterwards, the VLDLs will return to the liver, being removed from the circulation, becoming low-density lipoproteins (LDL). This process is mediated by the LDL receptor (LDLR) [26]. This lipid exchange between the liver and the target tissues also include the scavenging of excessive cholesterol, also mediated by lipoproteins, designated as reverse cholesterol transport (RCT) [27]. This clearance mechanism is through high density lipoproteins (HDL) that exchange both cholesterol and triglycerides, with both LDLs and VLDLs, and these lipoproteins are then converted in the liver to bile acids [28].

1.2.2 *De novo* lipogenesis

Hepatic *de novo* lipogenesis comprises the synthesis of fatty acids, using carbohydrates and proteins as precursors, in order to promote the storage of energy in form of these molecules, after the stocks of glycerol are formed. This process is finalized by the esterification into triglycerides (TG), and this activity depends on the nutritional uptake by the organism [29]. Nonetheless, its homeostasis is regulated by a set of enzymes, in order to correctly couple the degradation of carbohydrates (such as glucose), the conversion into fatty acids and the final esterification into TGs [30]. These enzymes include glucokinase (GK), fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), steroyl CoA desaturase 1 (SCD1) and diacylglycerol transferase (DGAT) and are regulated by the transcription factor sterol regulatory element binding protein-1c

(SREBP-1c), which is considered the master regulator of lipogenic gene expression in the liver and was first described as paramount for adipocyte differentiation [31]. This protein is part of the SREBP family, which comprises three transcription factors, which are the main regulators of lipid homeostasis: isoforms SREBP-1a, SREBP-1c and SREBP-2 [32].

SREBP-1a is associated with insulin action and cell proliferation, and SREBP-1c, is abundantly expressed in the mammalian liver, white adipose tissue and skeletal muscle, and is mainly responsible for the transcription of genes involved in lipid synthesis [33] [34]. Nonetheless, the regulation of this transcription factor is crucial in the regulation of both liver and plasma lipids [35]. The induction of SREBP-1c expression may be twofold: on one hand, insulin plays a role in the increased SREBP-1c mRNA, by activation of the IR, which will subsequently phosphorylate intermediates and finally Akt, completing the activation and induction of gene transcription [36, 37]; on the other hand, coordinated with carbohydrate regulatory element binding protein (ChREBP), the liver X receptors (LXR), a family of nuclear receptors involved in the metabolism of cholesterol, display another positive modulatory possibility on the lipogenesis process [30]. Some studies demonstrated that using agonists of the farnesoid X receptor (FXR), in the liver, both *in vitro* and *in vivo*, would lead to a decrease in the activation by the LXRs, and subsequently the level of SREBP-1c and the downstream targets [38].

The other member of the SREBP family is SREBP-2, and its activity has been linked to cholesterol synthesis, when the levels of acetyl-CoA, derived from glycolysis, and this activity is negatively regulated by the levels of cholesterol [39].

1.2.3 Fatty acid β-oxidation

Fatty acid β-oxidation comprises the catabolic reaction that converts FFAs into energy and, in the liver, FFAs are converted into ketone bodies that will generate energy to be used by the entire organism [40]. This pathway is favored in fasting conditions or glucose deprivation, as AMP-activated protein kinase (AMPK) inhibits the anabolic reactions [41]. The gene transcription of key mitochondrial enzymes of β-oxidation is both regulated by PPAR-γ and PGC-1α, and the latter can co-activate PPAR-γ, enhancing the promotion of lipolytic processes that occur under low nutritional conditions, converting esterified lipids (TGs) to FFAs ready to be oxidized in order to produce energy [42].

To be able to oxidize the lipids, these molecules have to be transported into the mitochondria. The first step encompasses the addition of acyl-CoA groups to FFA by acyl CoA synthases (ACS), and need to be shuttled into the mitochondria, since the membrane is impermeable to acyl-CoA groups [43]. This transport is mediated by carnitine palmitoyltransferase (CPT) complex, comprising both isoforms I and II, and inside the mitochondria, a sequence of four β-oxidation spiral genes, such as acyl-CoA dehydrogenase (ACDH), enoyl-CoA hydratase (ECH), hydroxy acyl-CoA dehydrogenase (HACDH), and ketoacyl-CoA thiolase (KACT), will convert the FFA into acetyl-CoA, $FADH_2$ and NADH, which subsequently can be used for ATP production [44] [45].

1.3 Dysregulated hepatic lipid metabolism in NAFLD

1.3.1 Increased hepatic lipid uptake and synthesis

NAFLD patients exhibit high concentrations of both VLDLs and LDLs, in contrast to a reduced number of HDLs [46]. These values are linked to hyperinsulinemia, as there is increased stimulation by insulin of both lipolysis and transport of fatty acids into the liver, mainly from the adipose tissue, combined with loss of VLDL secretion [47]. This first impact of the pathology is also characterized by an excessive activation of LPL in the liver, that will contribute to the over accumulation of fatty acids in the liver by activating lipolysis, converting triglycerides into FA [48]. Hyperinsulinemia is also responsible for the reduction of LDLR expression, causing the increase of circulating LDL, combined with a downregulation of HDL synthesis, decreasing the cleansing effect by these lipoproteins [49]t.

Insulin resistance and the high concentration of hepatic FFAs will also overactivate SREBP-1c and the lipogenic process. Also, the high rates of lipogenesis can be due to the activation by glucose through the ChREBP pathway [50]. Overall, the exposure to high levels of FFAs will dysregulate signaling pathways, hence the insulin resistance in NAFLD due to the compromised signaling cascade (Figure 3).

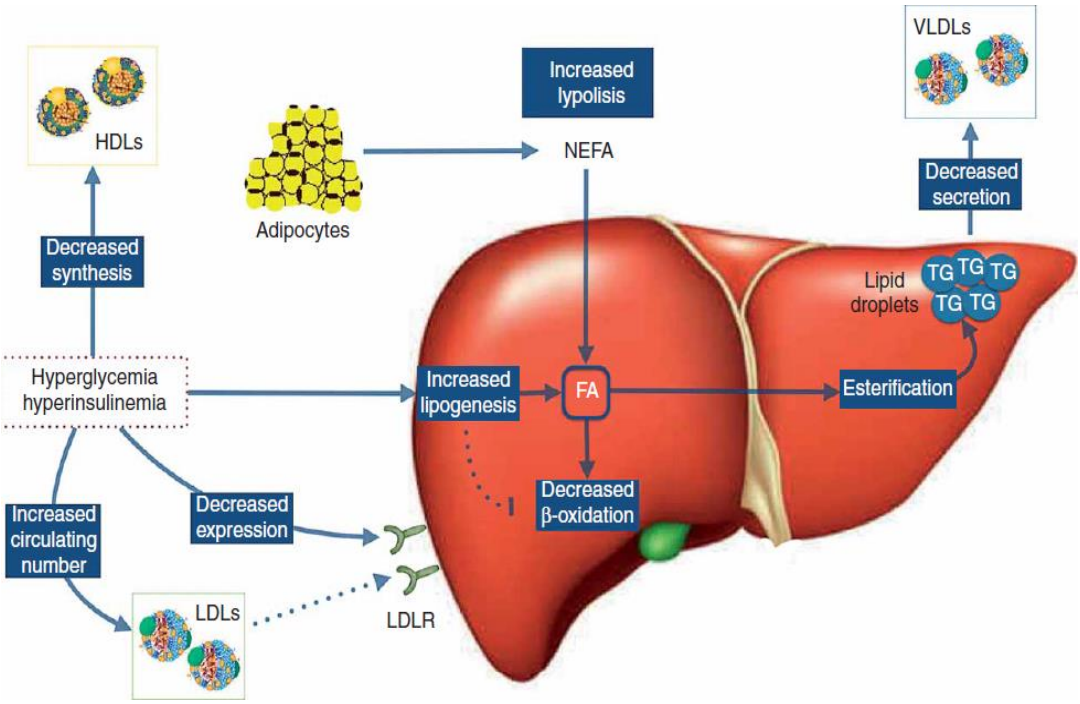

Figure 3. Hepatic lipid metabolic alterations in NAFLD. As the diseases progresses, both the increased uptake of circulating free fatty acids and hyperinsulinemia, will increase the number of circulating LDL and accumulation of VLDL in the liver, coupled to the reduction of circulating HLD. This will ultimately overstimulate lipogenesis and repress fatty acid oxidation, increasing the amount of fatty acids and subsequent esterification into lipid droplets [1].

The excess accumulation of lipids is also explained by the low rates of β-oxidation of FFA. In the fed state, the intermediates of the lipogenic pathway are allosteric inhibitors of CPT-1, preventing the shuttle of FFA into the mitochondria and subsequent oxidation [40] [51]. However, insulin also plays a pivotal role in the inhibition of the process, and moreover, explains why in fed conditions, the process occurs less frequently [52]. Nonetheless, the lipotoxicity that results from FFA also compromises mitochondrial function, especially due to the underexpression of PPAR-γ and other regulators. This is more evident in NASH, as the high production of reactive oxygen species (ROS) will lead to both lipid peroxidation and oxidative stress [53]. This is also

due to the high oxidative rates that are necessary for the removal of extra FFA, but will also generate more ROS consequently, causing oxidative damage to liver cells [54].

This unbalance between the lipid supply to the liver and the rates of both lipid oxidation and export become more evident as the disease progresses, leading to high lipotoxicity and compromised liver function. Nevertheless, the damages due to this dysregulation, as well as others, start to become irreversible in the disease [12].

1.3.2 ER Stress, Inflammation and Oxidative Stress

As mentioned previously, hepatic lipid accumulation coupled to insulin resistance is a common feature of NAFLD. The high content of hepatic lipids disrupts the membrane in the endoplasmic reticulum (ER) in hepatocytes, by increasing the ratio of phosphatidylcholine [55] to phosphatidylethanolamine (PE), triggering ER stress [56]. This process derives from the accumulation of cellular insults that lead to the excessive amount of misfolded proteins in the lumen, activating signaling cascades that are collectively known as the unfolded protein response (UPR) [57, 58]. Depending on the insult, the UPR cascade will lead either to attenuation of the ER stress or apoptosis. The UPR has three main branches: protein kinase RNA-like ER kinase (PERK), activation transcription factor-6 (ATF-6) and inositol requiring enzyme-1 (IRE1), that when active during stress conditions, lead to the dissociation of ER chaperone BiP (GRP78) [59]. Downstream targets of this cascade include the phosphorylation of eukaryotic initiation factor 2-alpha subunit (eIF2α), which lead to the inhibition of protein translation and promotion of ATF-4 activity, which could lead to the activation of apoptotic pathways, such as the transcriptional activation of C/EBP homologous protein (CHOP) [59]. The

over activation of ER stress pathways could also induce a negative feedback on the UPR, and decrease SREBP-1c activity and subsequently, through activation of GRP78, decrease lipid synthesis [60]. Overall, the ER homeostasis is deeply altered in NAFLD, and this chronic cell insult promotes hepatocyte dysfunction and has been an emerging potential target of interest in the pathology [56, 61]. Nonetheless, ER stress has been linked to insulin resistance upon fatty acid exposition in HepG2 cells [62] [63].

ER stress is highly prevalent in NAFLD, as it is associated with the high inflammatory responses in NASH progression [64]. It has been demonstrated that activation of the NF-kB pathway may be promoted by both PERK/eIF2α and IRE1 pathways [65]. In NAFLD, expression of stress genes has been linked to both the onset and progression of the disease, as it can contribute to both over activation of lipid synthesis and apoptosis [66]. Because hepatocytes are enriched in ER, the susceptibility for the activation of the inflammatory pathway is higher compared to other tissues [67].

However, the activation of the inflammatory pathway may also be due to high production of ROS [49], as a result of mitochondrial dysfunction. This may activate the NF-kB pathway, which will subsequently promote the production of cytokines, such as TNF-α, that will cause the accumulation of neutrophils [68]. The high rate of lipid peroxidation by ROS will also activate the fibrotic pathway in hepatic stellate cells (HSC), leading to pro-fibrotic events, as well as pro-inflammatory states with the presence and action of TNF-α [17]. Overall, this inflammatory state is deleterious in NAFLD [69].

As NAFLD progresses, there is a growing predisposition to develop hepatic fibrosis. These states are often described to be the main cause of liver transplant, but also death due to the resistance to blood flow, causing both insufficiency in liver function

as well as hepatic portal hypertension [70]. The excessive extracellular matrix, whose proteins include mainly collagen, are mainly due to the effect of activated HSC [71]. This activation is stimulated by hepatocytokines and inflammatory responses, and progressive accumulation of collagen fibers will result in a cirrhosis-like state characterized by high scar tissue, leading to an irreversible state of the disease [71].

Although it is accepted that hepatic metabolic pathways are compromised in NAFLD, especially lipid synthesis and degradation, with concomitant activation of both ER stress and inflammatory pathways [15], further research is needed to improve our understanding of the pathophysiological mechanisms of the disease.

1.4 Mammalian Sirtuins

The mammalian sirtuin family comprises NAD^+-dependent proteins with deacetylase enzymatic function. This family is homolog of the silent information regulator (Sir) proteins of the yeast *Saccharomyces cerevisiae* [72]. The seven members of this family differ in terms of subcellular distribution and targets (Table 1). These proteins have been linked to nutrient sensing, inflammation, metabolism and longevity, as their targets include intermediates of these pathways in the organism. However, the functions of the members of this family have yet to be fully elucidated, being SIRT1 the most studied so far [73].

For instance, SIRT1, whose subcellular localization is preferentially in the nucleus, has been reported to both promote the transcription of genes involved in mitochondrial biogenesis, and demonstrated to regulate adipogenesis, both processes linked to

longevity and metabolism [74, 75] [76] [77]. SIRT3 was also linked to metabolism in several studies, reported to be involved in both fatty acid oxidation mitochondrial biogenesis [78]. Overall, the wide cell distribution of sirtuin members allows different metabolic regulations, at distinct pathways and points [76].

Table 1. Sirtuin family members, respective subcellular location, class, enzymatic activity and targets [76].

Sirtuin	Subcellular location	Class	Enzymatic Activity	Substrates/interactions
SIRT1	Nucleus, cytosol	I	Deacetylation	p53, FOXO, NF-kB-p65, histone H1 and H4, PGC1α, ACS1, IRS2
SIRT2	Cytosol, nucleus	I	Deacetylation, demyristoylation	Tubulin, FOXO, histone H4,
SIRT3	Mitochondria	I	Deacetylation	ACS2
SIRT4	Mitochondria	II	ADP-ribosylation	GDH, ANT, IDE
SIRT5	Mitochondria	III	Deacetylation, demalorylation, desuccinylation	Unknown
SIRT6	Nucleus	IV	Deacetylation, ADP-ribosylation	Histone H3
SIRT7	Nucleus	IV	Deacetylation	RNA polymerase I, p53

1.5 Sirtuin 2

1.5.1 Main functions

Sirtuin 2 was initially described to be mostly present in the cytoplasm where it deacetylates α-tubulin at lysine 40 [79]. This function was linked to the regulation of cell cycle, as SIRT2 overexpression delayed the progression of the cell cycle through the mitosis, suggesting its shuttling into the nucleus, decisive in the G2/M transition [80]. This cell cycle modulation is possible through the deacetylation of a lysine motif of histone H4 during the mitotic process [81]. The regulation of cell cycle has been

associated with the stability of chromosomes during cell division, which could also suggest a tumor suppression function of SIRT2, connected to aging and the onset of carcinogenesis [82]. This latter relationship has been proposed based on the low expression of the protein in both breast cancer and hepatic carcinoma, corroborating the cell cycle modulating function of the enzyme [83]. SIRT2-KO mice also display a higher predisposition to develop both breast and hepatocellular carcinoma, reinforcing the role of SIRT2 in genetic stabilization [83].

Similar to SIRT1, SIRT2 has also been reported to be downregulated during aging [84]. Recent studies have started to elucidate the role of SIRT2 in mitochondrial function. It was shown that SIRT2 was responsible for mitochondrial biogenesis and morphology, therefore, has a crucial role in the function of this organelle [85]. The loss of function of this protein promoted the decrease in ATP production, as well as the increase in the production of ROS [49]. Hence, it adds to another of the paramount function of the deacetylase and demonstrates its importance regarding ROS-related pathologies [86]. A recent study showed decreased SIRT2 expression in both insulin-resistant HepG2 cells and liver tissue, as well as an increase in the generation of ROS production [85]. Mitochondrial function and dynamics were also shown to be regulated by SIRT2, since Mfn2, a fusion protein, and Drp1, a fission protein, were shown to be altered in insulin-resistant HepG2 cells, further linking SIRT2 to metabolic regulation and insulin sensitivity (Figure 4) [87].

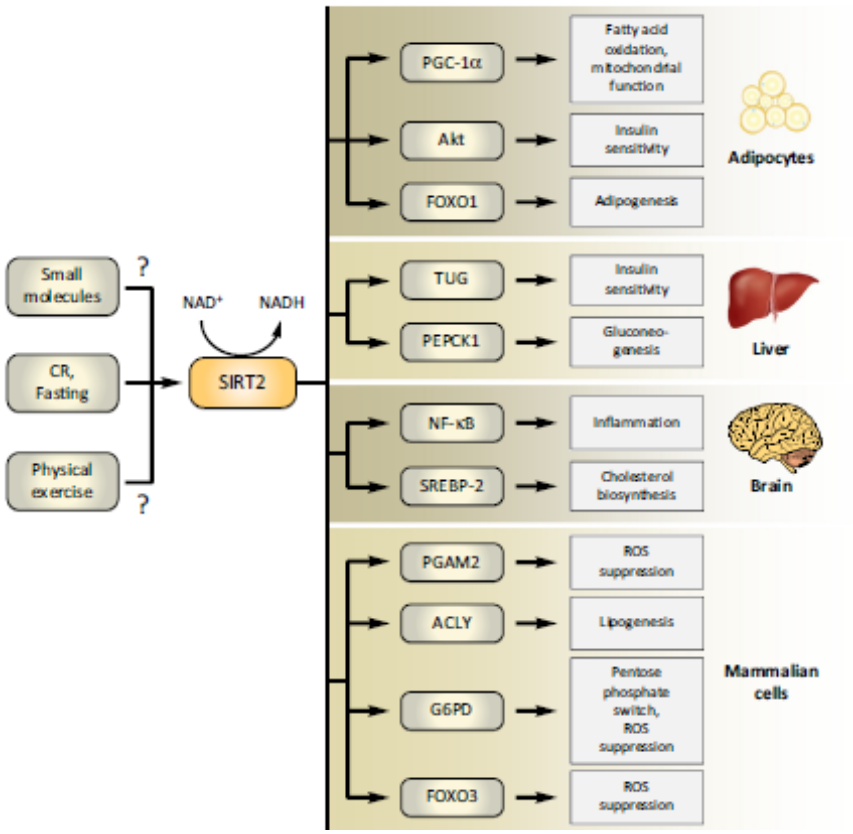

Figure 4. Metabolic targets and processes regulated by SIRT2. Adapted from [72].

SIRT2 seems to play a neuroprotective role through the suppression of inflammation by the deacetylation of NF-kB, considered a key player in inflammation [22]. Another target of SIRT2 is FoxO3a in both adipose and kidney cells, as the deacetylation and further activation of FoxO3a is correlated to the promotion of the transcription of p27 and BIM, under caloric restriction conditions, which will further lead to programmed cell death [88].

1.5.2 Sirtuin 2 and Metabolic Homeostasis

1.5.2.1 Glucose Metabolism

This deacetylase is highly expressed in the brain and adipose tissue. The first study regarding the metabolic function of this enzyme was in the adipogenesis process, in the transition from pre-adipocyte to adipocyte [73].

SIRT2 also contributes to glucose homeostasis by deacetylating phosphoenolpyruvate carboxykinase 1 (PEPCK1), and thus inactivating and stabilizing the kinase which redirects through the gluconeogenesis pathway, therefore modulating the glucose levels in the liver [89]. A recent study demonstrated that liver-specific SIRT2 overexpression improved the hepatic glucose uptake of both obese and diabetic animals, through the deacetylation of glucokinase regulatory protein (GKRP) [90]. Overall, SIRT2 regulates numerous glucose metabolic pathways in several tissues, by modulating the activity of metabolic players [87].

A role for SIRT2 in insulin signaling pathway was suggested based on findings that SIRT2 levels were decreased in livers of rodent models of obesity and insulin resistance, the *ob/ob* mice as well as the high-fat diet (HFD)-fed mice [69]. Other studies regarding SIRT2 and the deacetylation of Akt, an intermediate of the insulin signaling pathway, demonstrated a positive relation between SIRT2 activity and Akt deacetylation status with higher activation and phosphorylation of downstream targets, after insulin stimulation, in adipose cells as well as kidney cells [91]. However, SIRT2 has also been reported to be overexpressed in insulin-resistant conditions in skeletal muscle, meaning that it could affect in a negative manner the uptake of glucose in this tissue [92]. Overall,

this suggests differential roles for SIRT2 in the regulation of the insulin signaling pathway in different tissues and conditions

1.5.2.2 Lipid Metabolism

Several lines of evidence support a role for SIRT2 in the regulation of lipid metabolism. SIRT2 deacetylates ATP-citrate lyase (ACLY), a key enzyme in the beginning of the lipogenic process, by converting citrate derived from glucose into acetyl-CoA, which will be the foundation for lipid biosynthesis. The deacetylation of ACLY by SIRT2 will lead to the ubiquitination and subsequent degradation of the former, hence, decreasing lipogenesis [79]. ACLY is a highly abundant enzyme in certain types of cancer, and the modulation of the levels and activity of this protein via SIRT2 offers additional therapeutic approaches towards those pathologies. SIRT2 has also been described to be part of the sterol biosynthesis in the brain, by promoting the gene expression for enzymes in this process, by targeting SREBP-2, allowing the shuttle of this transcription factor into the nucleus and gene transcription promotion [93]. This same study showed that, in Huntington's disease (HD), the downregulation of the cholesterol biosynthesis by SIRT2 inhibition had a neuroprotective effect. However, a subsequent *in vivo* study demonstrated that the pharmacological inhibition of SIRT2 had no effect on cholesterol biosynthesis or HD progression [94]. This leads to controversial aspects regarding the enzyme function in brain cholesterol synthesis.

As previously mentioned, PGC-1α is crucial for the fatty acid oxidation and a target of SIRT2 in adipocytes. Studies have shown that hypoxia-induced factor 1-α (HIF-1α) is a negative regulator of the fatty acid oxidation by repressing the transcription of

SIRT2, consequently reducing the deacetylation and activation of PGC-1α [55]. Therefore, in obesogenic conditions, where adipocytes are enlarged and the hypoxic conditions are more frequent, there is inhibition of the oxidative process by the repression of SIRT2 action.

1.5.3 Sirtuin 2 and NAFLD

No studies to date have reported an association between SIRT2 and NAFLD. Preliminary data from our group demonstrated that SIRT2-KO mice had a predisposition for metabolic syndrome, and when fed a HFD were hyperglycemic [95] [96]. These changes were mostly observed in HFD-fed SIRT2-KO mice, compared to its WT counterpart, having CD-fed mice displayed slight metabolic alterations (Table 2).

Table 2. Metabolic phenotype of SIRT2-KO mice fed either a CD or a HFD, compared to WT mice. Adapted from [80]

Parameters	Metabolic changes in SIRT2 KO mice compared to WT mice	
	CD	HFD
Body weight gain	=	+
Epididymal WAT weight gain	=	+
Food/energy intake	=	+
Blood glucose	=	+
Glucose tolerance	=	=
Insulin resistance	+	++

= - unaltered; + - mild; ++ - exacerbated

Interestingly, SIRT2-KO fed a CD displayed higher levels of hepatic lipid deposition than WT mice, and this phenotype was further aggravated in response to HFD feeding (Figure 5) [96]. These results were further supported by a trend for a higher expression of lipogenic genes such as *Srebp-1c* and *FasN*, under both CD and HFD, in SIRT2-KO mice.

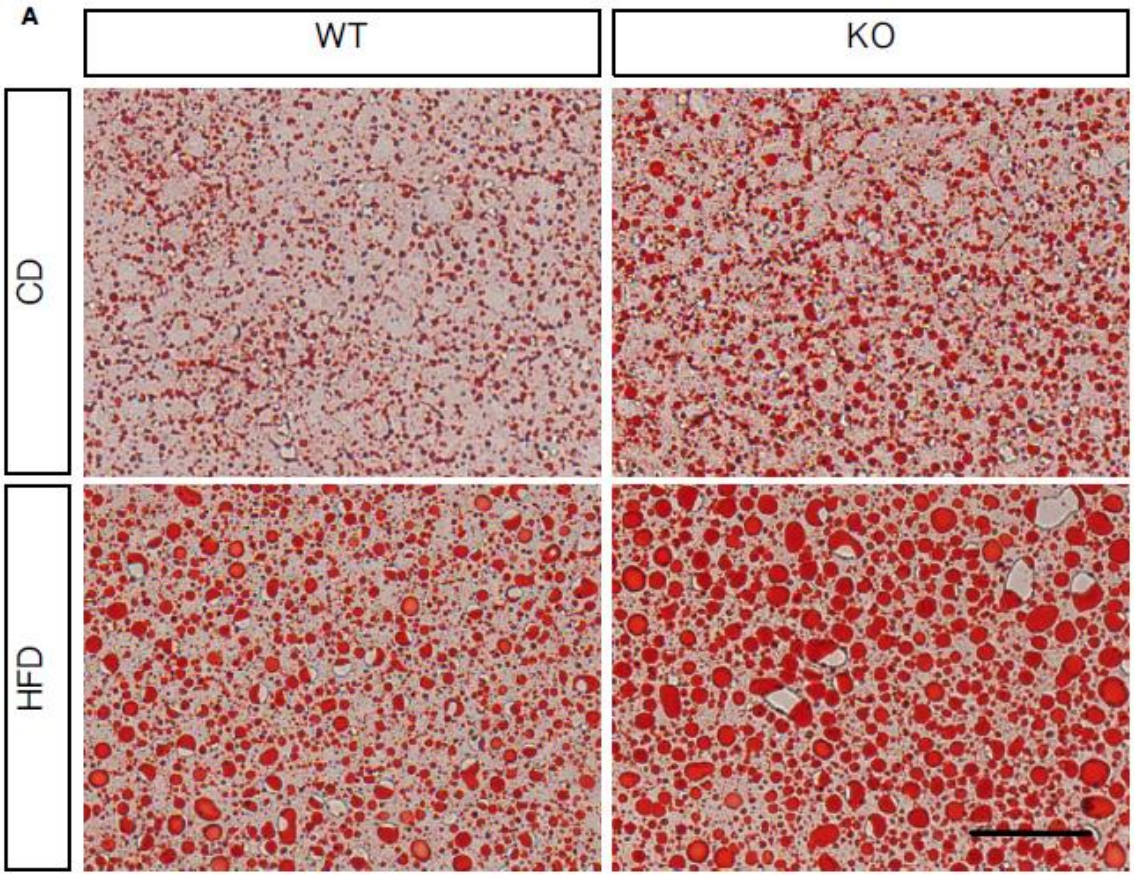

Figure 5. Oil Red O of liver slices of both WT and SIRT2-KO animals, fed both a CD and HFD. As displayed, there is high lipid deposition on both CD-fed SIRT2-KO mice, similar to WT fed a HFD, and this phenotype is exacerbated when fed a HFD [96].

Overall, these results raise the interesting possibility that SIRT2 may exert a regulatory role on hepatic lipid homeostasis.

1.6 General Aims

The prevalence of metabolic disorders has been increasing steadily in the past decades [8]. NAFLD is one of such metabolic disorders, and the rapid progression of the disease is linked to the low availability of therapeutic strategies and high number of liver-related deaths [10]. The high dyslipidemia rates coupled to the exacerbated ER stress and inflammation account for the deleterious phenotype of the pathology [54].

The NAD^+-dependent SIRT2 deacetylase has been implicated in the regulation of various metabolic processes, targeting several intermediates of both signaling and metabolic pathways, including insulin sensitivity, gluconeogenesis, lipogenesis, adipogenesis and fatty acid oxidation [87]. SIRT2 is ubiquitously distributed in the organism, being able to modulate the metabolism in key organs, such as the adipose, hepatic and skeletal muscle tissues, and has been suggested to be a therapeutic target for metabolic disorders [85].

Therefore, the main goal of this study is to establish a link between SIRT2 and the regulation of hepatic lipid metabolism, and to subsequently unravel whether this function has a role in the development of NAFLD. Therefore, this study will attempt to assess if SIRT2 could prove to be a novel pharmacological target towards the treatment of NAFLD.

Chapter II - Methods

2.1 Cell culture

HepG2 cells, a human hepatocyte-derived cell line, were obtained from ATCC (Rockville). Cells were cultured at 37ºC in a humidified 5% CO_2 incubator, in Minimum Eagle's Complete Medium (MEM), supplemented with 1% of Pen/Strep, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2,61 mM sodium bicarbonate ($NaHCO_3$), 2 mM L-Glutamine, 10% heat-inactivated fetal bovine serum (Gibco) and 1 g/L glucose. Cells were cultured in 75 cm^2 flasks (Thermo Fisher Scientific) and plated in either 6 or 12 multi-well plates (Thermo Fisher Scientific), at a density of $1x10^6$ or a $5x10^5$ cells/well, respectively. Cells were subcultured every 6 days (70-80% confluency) using Trypsin-EDTA solution. Cells with less than 20 passages were used.

2.2 Cell treatment

2.2.1 Fatty acid (FA)/bovine serum albumin (BSA) complexation

A 10% stock solution of fatty acid-free bovine serum albumin (BSA) (Calbiochem) was prepared by dissolving BSA powder in 10% FBS containing MEM at room temperature.

A 100 mM stock solution of palmitate (16:0) (Sigma-Aldrich) was prepared by dissolving palmitate in 100% ethanol at 60ºC. This solution was then added to BSA stock solution, and was left stirring for 2 hours at room temperature. Afterwards, the solution was filtered through a 0,45 μm filter in a flow chamber, and stored at -20 ºC in 2 mL-aliquots in a final concentration of 5 mM.

2.2.2 Metabolic stressor treatment

Twelve hours before the treatment, the medium was replaced with FBS-free MEM. The cells were then incubated with either vehicle (BSA) or metabolic stressors (palmitate, high glucose or glucosamine) for 24h.

2.2.3 Insulin stimulation

Following treatment with either BSA or palmitate, HepG2 cells were acutely stimulated with insulin (Humalog). To this end, cells were equilibrated in PBS for 15 minutes and insulin was subsequently added for 15 minutes at different concentrations (10, 100 and 100 nM), or at the concentration of 100 nM for different time points (0, 5, 15 and 30 minutes).

2.2.4 Alamar Blue cell viability assay

Following treatments, cells were incubated with resazurin (0.1 mg/mL), the active ingredient of Alamar Blue, for 1 hour at 37ºC in a CO_2 incubator, in FBS-free MEM. Afterwards, the supernatant was transferred into 96 multi-well plates and absorbance was read at both 570 and 600 nm wavelengths. Viability is determined by the difference between the 570 and 600 nm wavelengths and is presented as percentage of the control group.

2.3 Determination of intracellular lipid content

2.3.1 Oil Red O staining

2.3.1.1 Lipid staining

The intracellular lipid content was evaluated by Oil Red O (ORO), which stains neutral lipids, as previously described [97]. Briefly, control and treated cells were washed twice with PBS and then fixed for 1 hour with buffered 4% paraformaldehyde (PFA). Afterwards, cells were washed twice with distilled water and stained for 1 hour at room temperature with Oil Red O ((Sigma Aldrich, 0,625% in isopropanol) diluted 60:40 distilled water, filtered). Cells were then washed three to five times with distilled water to remove excess dye.

2.3.1.2 Image acquisition

After the Oil Red O staining, cells were counterstained with hematoxylin solution modified according to Gill III (Merck) to stain the nuclei, and then washed with distilled water. Coverslips were then mounted onto slides (Thermo Fisher Scientific) using Mowiol mounting medium (Thermo Fisher Scientific), and were left to air dry for 3 days. Transmitted light images were acquired with an Axio Imager Z2 microscope (Zeiss) with 20x and 40x objectives (Zeiss).

2.3.1.3 Spectrophotometric quantification

In order to quantify the amount of stained lipids, 1 mL of dimethyl sulfoxide (DMSO) was added into the wells and left gently stirring for 10 minutes. The eluted dye

was then transferred into a 96-well plate and the absorbance was read at a wavelength of 520 nm. Absorbance results are presented as percentage of the control group.

2.4 Generation of SIRT2-silenced or overexpressing HepG2 cells

Lentiviral construction, production and cell infection was done as previously described [98]. To accomplish SIRT2 knockdown, HepG2 cells were infected with lentiviral vectors containing either a short hairpin for a random sequence with a sequence for GFP expression (cells referred to as shCTRL), or for SIRT2 (cells referred to as shSIRT2). To achieve SIRT2 constitutive overexpression, HepG2 cells were infected with lentiviral vectors containing a GFP sequence (cells referred to as GFP) or SIRT2 (cells referred to as SIRT2). Infected cells were exposed to either vehicle or palmitate for 24 hours.

2.5 Protein analysis

2.5.1 Protein extraction

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer, containing 50 mM Tris, 150 mM sodium chloride (NaCl), 5 mM EGTA, 1% (v/v) Triton X-100, 0.5% (w/v) deoxycholate (DOC), 0.1% (w/v) sodium dodecyl sulphate (SDS), supplemented with complete mini protease inhibitor cocktail tablet (Roche Diagnostics), 1 mM sodium orthovanadate ($NaVO_4$), 1 mM dithiothreitol (DTT), 10 mM sodium fluoride (NaF), 200

μM phenylmethylsulfonylfluoride (PMSF) and 10 mM nicotinamide (NAM). Cells were then scraped using plastic scrapers, and transferred to 1.5 mL eppendorfs. The eppendorfs were kept on ice for 30 minutes, vortexing each 10 minutes to promote cell lysis, and afterwards were centrifuged at 13.000 rpm for 30 minutes at 4ºC. The supernatant was collected and protein concentration was assessed using the bicinchoninic acid (BCA) method. The samples were denatured with 6x concentrated sample buffer (0.5 M Tris-HCl, pH 6.8; 30% (v/v) glycerol; 10% (w/v) SDS; 0.6 M DTT; 0.01% (w/v) bromophenol blue), and heated at 95ºC for 5 minutes. Samples were then stored at -20ºC until further use.

2.5.2 SDS-Page and Western Blotting

Proteins (30-50 μg) were resolved by SDS-PAGE using 10% polyacrylamide gels. The electrophoresis run was performed in a Tris-Bicine running buffer (25 mM Tris; 25 mM Bicine; 1% (w/v) SDS; pH 8.3), at 70V for the first 10 minutes, and 120-140V for 2 hours, until appropriate separation of the bands of the molecular weight standard. This was followed by an electro-transfer to PVDF membranes, that were pre-activated in methanol (45 seconds), distilled water (5 minutes) and CAPS transfer buffer (5 minutes,) in CAPS transfer buffer (10 mM CAPS; pH 11.0; 10% (v/v) methanol) for 2 hours in a constant current of 1000 mA. Membranes were then blocked for 1 hour with blocking buffer (5% non-fat dry milk or 5% bovine serum albumin in Tris buffered saline (20 mM Tris; 137 mM NaCl; pH 7.6) with 0.1% TWEEN-20), followed by overnight incubation at 4ºC with the corresponding primary antibodies (Table 1). The membranes were subsequently incubated for 1 hour with the corresponding alkaline secondary goat anti-

rabbit or anti-mouse antibody. Proteins were visualized using enhanced chemifluorescence substrate (ECF, GE Healthcare) and scanned using VersaDoc (Bio Rad). Results of the western blot were normalized using the bands of β-tubulin, total Akt, IRE1 or eIF2α. Western blots were analyzed by densitometry using Quantity One software (Version 4.6.6; Bio Rad).

Table 3. List of primary antibodies used in Western blotting analysis.

Antibody	Host/Clonality	Dilution	Reference
Anti-SIRT2	Rabbit polyclonal	1:1000	Sigma S8447
Anti-pAkt (ser473)	Rabbit polyclonal	1:1000	Cell Signaling 9271S
Anti-Akt	Rabbit polyclonal	1:1000	Cell Signaling 9272S
Anti-pIRE1α (ser724)	Rabbit polyclonal	1:1000	Abcam S724
Anti-IRE1α	Rabbit polyclonal	1:1000	Cell Signaling 14C10
Anti-peIF2α (ser51)	Rabbit polyclonal	1:1000	Cell Signaling D968
Anti-eIF2α	Rabbit polyclonal	1:1000	Cell Signaling D7D3
Anti-acetyl-lysine	Rabbit polyclonal	1:1000	Cell Signaling 9441S
Anti-β-tubulin	Mouse monoclonal	1:10000	Sigma T6074

2.6 Flow cytometry

Infected cells were cultured and harvested for flow cytometric quantification of infection efficiency of both vectors encoding SIRT2 silencing and overexpression as previously described [99, 100]. Flow cytometry analysis was assessed by fluorescence-activated cell sorting (FACS) using the Cell Quest software, acquiring 20-25000 events per condition, and sorted by GFP emission. The percentage of GFP-positive cells and

average fluorescent intensities in the control groups were observed in fluorescence histograms, and corresponded to the percentage of both SIRT2 silencing and overexpressing HepG2 cells.

2.7 Statistical analysis

Data are expressed as mean ±SEM, and differences between two groups were analyzed using unpaired non-parametrics Student's t-test, being $P<0.05$ considered significant. All statistical analysis was performed using GraphPad Prism Software (Version 7.0; GraphPad).

Chapter III – Results

3.1 Palmitate induces lipid accumulation in HepG2 cells

To investigate whether the increased hepatic lipid accumulation observed in SIRT2-KO mice was due to the loss of SIRT2 expression, an *in vitro* model of hepatic steatosis was established using human hepatic HepG2 cells [101-103]. Cells were treated with three different metabolic stressors (palmitate, glucosamine and high glucose) and lipid deposition was assessed by Oil Red O staining, using both microscopic analysis and spectrophotometric quantification (Fig. 6A and B). For the control group, we compared both Serum-only treatment with BSA and saw no differences regarding lipid deposition (Data not shown). Therefore, BSA was used as a control reference. We observed increased intracellular lipid deposition after palmitate treatment (~40% increase, $p<0.05$), whereas the other stressors tested did not significantly affect lipid accumulation. To rule out potential cytotoxic effects, cell viability was determined (Fig. 6C). None of the treatments caused a significant decrease in cell viability. Palmitate was therefore selected to induce lipid accumulation.

We then analyzed the effect of two different concentrations of palmitate (0.5 and 1 mM) on lipid accumulation and cell viability. Both palmitate concentrations increased significantly lipid deposition compared to the control group (0.5 mM: ~45% increase, $p<0.05$; 1 mM: ~65% increase, $p<0.01$) (Fig.6 D and E). However, cell viability assays demonstrated that the higher concentration of palmitate caused significant cytotoxicity, whereas the lower concentration did not affect significantly cell viability (Fig.6 F).

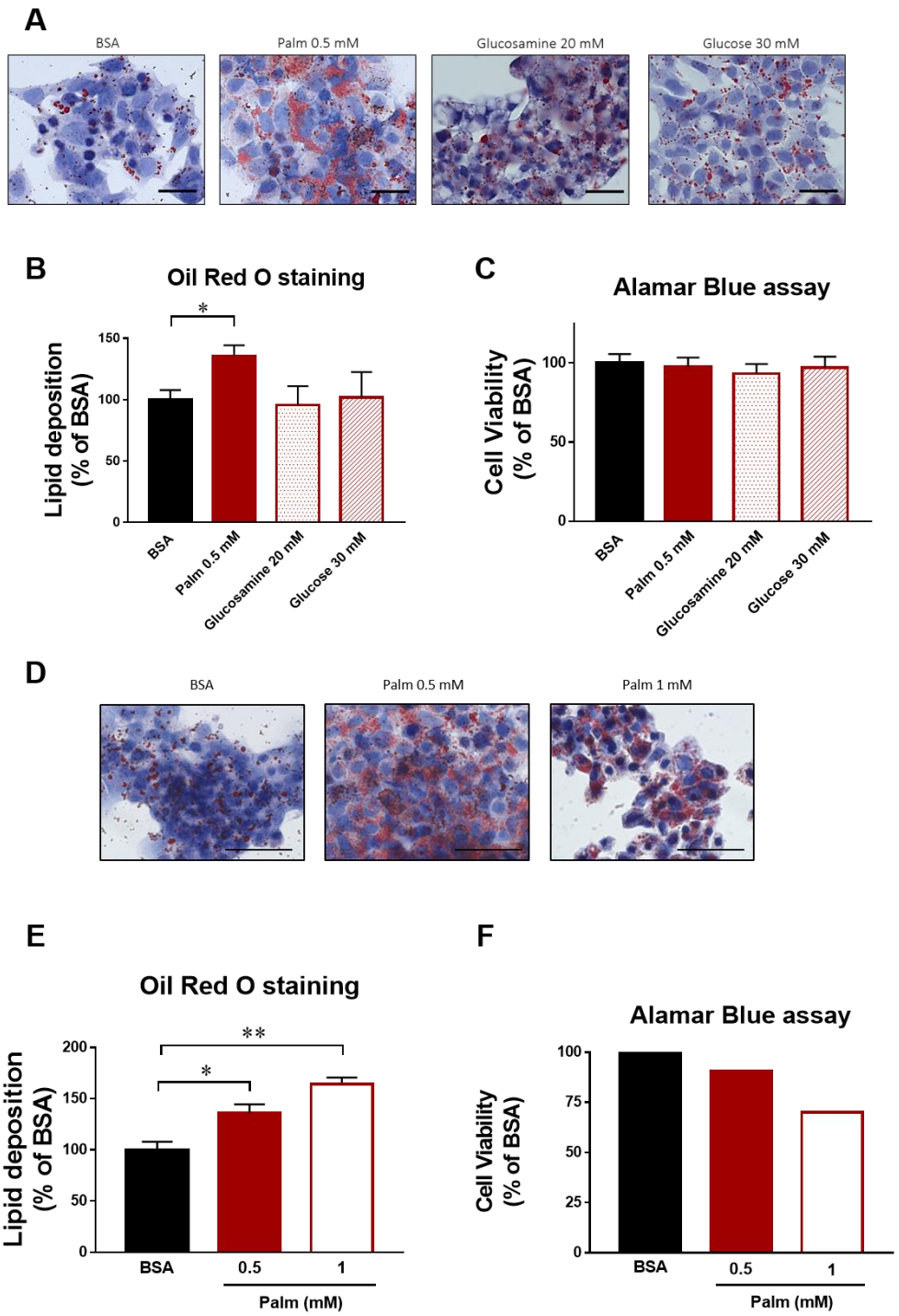

Figure 6. Lipid deposition and cell viability analysis in HepG2 cells exposed to different metabolic stressors. (A) Representative images of Oil Red O (ORO)-stained HepG2 cells, which were incubated with vehicle (BSA), palmitate (0.5 mM), glucosamine (20 mM) or high glucose (30 mM) for 24h. **(B)** Spectrophotometric analysis of stained lipids. **(C)** Alamar Blue assay for cell viability. **(D)** Representative images of ORO-stained HepG2 cells exposed to palmitate (0.5 or 1 mM) for 24h. **(E)** Spectrophotometric evaluation of stained lipids. **(F)** Alamar Blue assay for cell viability. Scale bar represents 25 µm. Results are presented as mean±SEM. Each experiment was performed in triplicate and repeated at least three times (n=3/group). *p<0.05, **p<0.01 compared to control (BSA).

We therefore selected palmitate at a concentration of 0.5 mM to induce hepatic steatosis *in vitro* in subsequent assays.

Insulin resistance is strongly associated with increases in hepatic lipid content [54]. Furthermore, treatment of hepatocytes with palmitate is known to compromise the insulin signaling pathway. To further characterize the hepatic steatosis model, insulin signaling pathway was analyzed by western blotting, by measuring the levels of phosphorylated Akt as a readout. Vehicle (BSA) and palmitate treated HepG2 cells were stimulated with insulin at different time points (Fig.7A and B) and different concentrations (Fig.7C and D).

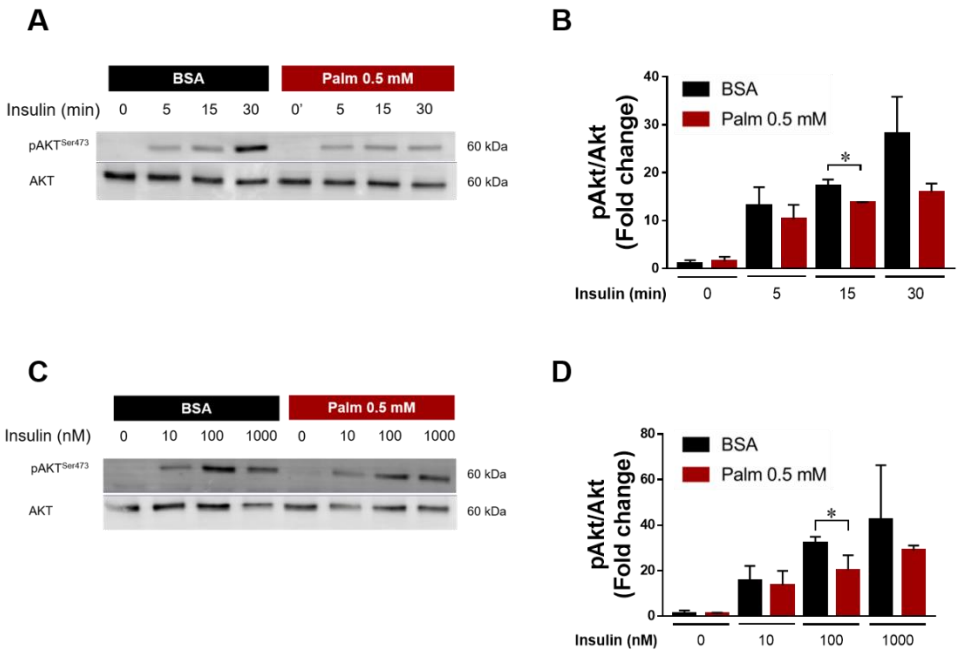

Figure 7. Effect of palmitate on the insulin signaling pathway in HepG2 cells. (A) HepG2 cells were incubated with either BSA or palmitate for 24h, and then acutely stimulated with insulin (100 nM) at multiple time points (0, 5, 15, 30 min), followed by western blot analysis of pAkt Ser473 and total Akt levels. **(B)** p-Akt levels were normalized for total Akt, and the ratios were quantified. **(C)** Representative immunoblot from HepG2 cells incubated with either BSA or palmitate for 24h and then acutely stimulated with insulin (15 min), at different concentrations (0, 10, 100 and 1000 nM). **(D)** p-Akt levels were normalized for total Akt, and the ratios were quantified. Results are presented as mean±SEM. Each experiment was performed in triplicate and repeated at least three times (n=3/group). *p<0.05 compared to control (BSA).

The expression levels of phosphorylated Akt in palmitate-treated cells compared to vehicle was significantly decreased, both after 15 minutes of insulin exposure (5~ fold decrease, $p<0.05$) and at a concentration 100 nM (~15 fold decrease, $p<0.05$). In conclusion, palmitate exposure impaired the insulin signaling pathway, suggesting insulin resistance, characteristic of lipid overload in hepatocytes.

3.2 SIRT2 protein levels are downregulated in steatotic hepatocytes

In order to assess the influence the effects of lipid accumulation on protein acetylation, HepG2 cells were treated with 0.5 mM palmitate for 24 hours. Through western blot analysis, using levels of acetyl-lysine, we observed a significant increase in protein acetylation compared to vehicle (~35%, $p<0.05$) (Fig. 8A and B). A specific band at ~25 kDa was also analyzed, as it seemed to be the most altered region and it was also significantly more acetylated upon palmitate treatment (~30% increase, $p<0.05$) (Fig. 8C). These results showed a significant increase in protein acetylation in cells exposed to palmitate, suggesting downregulation of sirtuin activity and/or expression. Therefore, we analyzed SIRT2 protein expression levels in the same conditions, revealing a significant decrease compared to the vehicle treatment (~40% decrease, $p<0.01$) (Fig.8D and E).

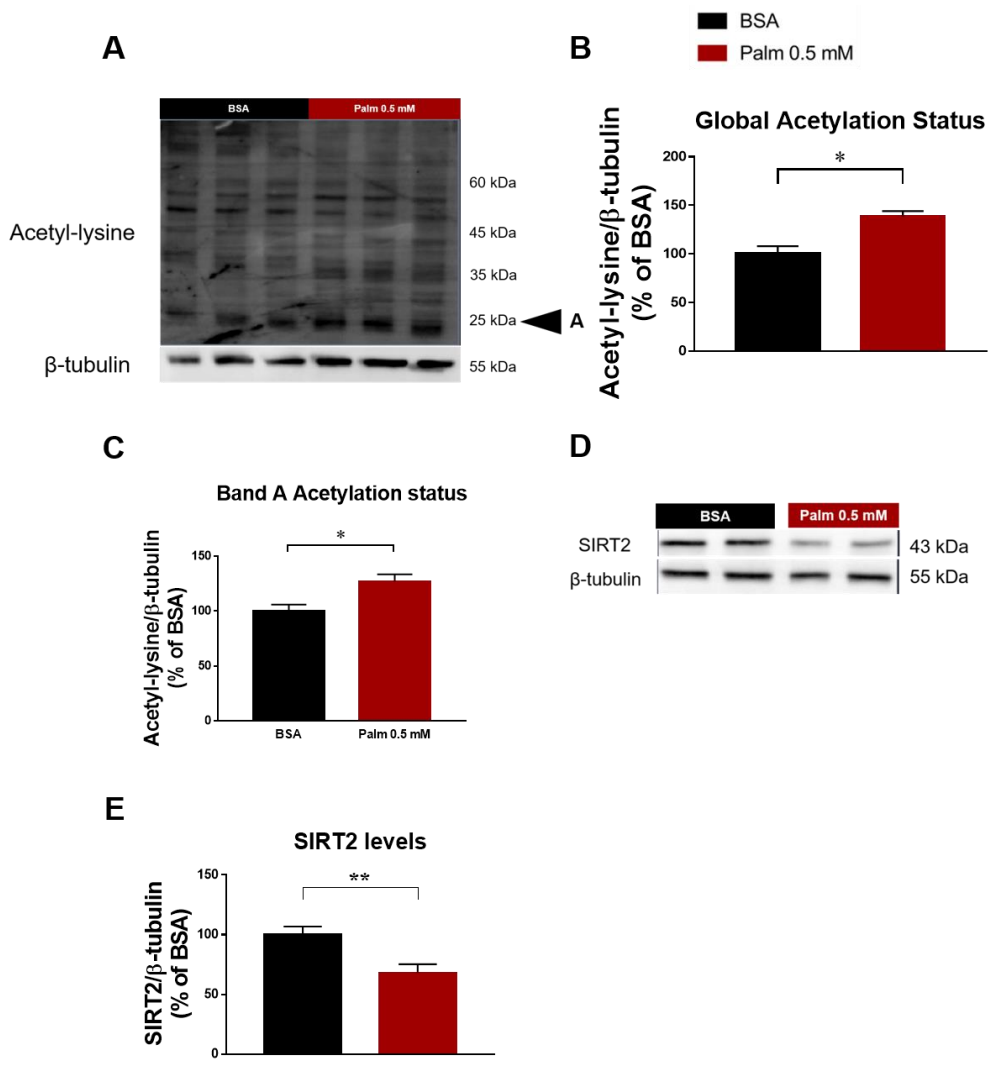

Figure 8. Effect of palmitate on protein acetylation and SIRT2 expression levels. (A) Total protein acetylation was assessed by anti-acetyl-lysine western blotting on HepG2 cells incubated with either BSA or palmitate for 24h. (B) Levels of lysine acetylated proteins were normalized with levels of β-tubulin in the same lane. (C) Levels of lysine acetylated proteins of region A were normalized to the levels of β-tubulin in the same lane. (D) Representative immunoblot showing SIRT2 expression levels. β-tubulin was used as a loading control. (E) SIRT2 levels were normalized to the levels of β-tubulin in the same lane. Results are presented as mean±SEM. Each experiment was performed in triplicate and repeated at least three times (n=3/group). * $p<0.05$, ** $p<0.01$ compared to control (BSA).

Collectively, these results indicate that increased lipid accumulation in hepatocytes is associated with SIRT2 downregulation.

3.3 SIRT2 silencing induces lipid accumulation

To establish a causal relationship between SIRT2 expression levels and the regulation of hepatic lipid accumulation, we produced HepG2 cells in which SIRT2 was silenced or overexpressed by using lentiviral vectors.

To determine which short hairpin sequence for SIRT2 would be more effective in silencing SIRT2 levels, three different human promoters (H1, H2 and H3), with the same short hairpin sequence for SIRT2, were screened by transfecting HEK293 cells and SIRT2 expression analyzed by western blot (Fig. 9A and B). Both H2 and H3 led to ~50% reduction of SIRT2 levels. The selected promoter was H3, which has been previously reported to effectively silence SIRT2 in HEK293 and HCT116 cells [104].

HepG2 cells were infected with either a short hairpin for a random sequence, also encoding for GFP (shCTRL cells), or a short hairpin sequence targeting SIRT2. The efficiency of SIRT2 silencing was assessed through western blotting analysis, showing ~50% decrease in SIRT2 protein expression (Fig. 9C and D).

The percentage of infected cells was evaluated by Fluorescence Applied Cell Sorting (FACS) analysis, where both cells infected with a short hairpin for a random sequence (shCTRL) and with a short hairpin for SIRT2 (shSIRT2) were counted for GFP emission (M2 range) (Fig. 9E and F), as previously described [100]. The use of GFP as reporter of cell infection in shCTRL cells, will correspond to the percentage of cells infected with shSIRT2. The GFP percentage of shCTRL cells was ~61%, and thereby, corresponds to the percentage of cells infected with shSIRT2. To rule out unspecific signal, FACS analysis from both cell types was overlapped, showing a specific GFP signal in shCTRL cells (Fig. 9G).

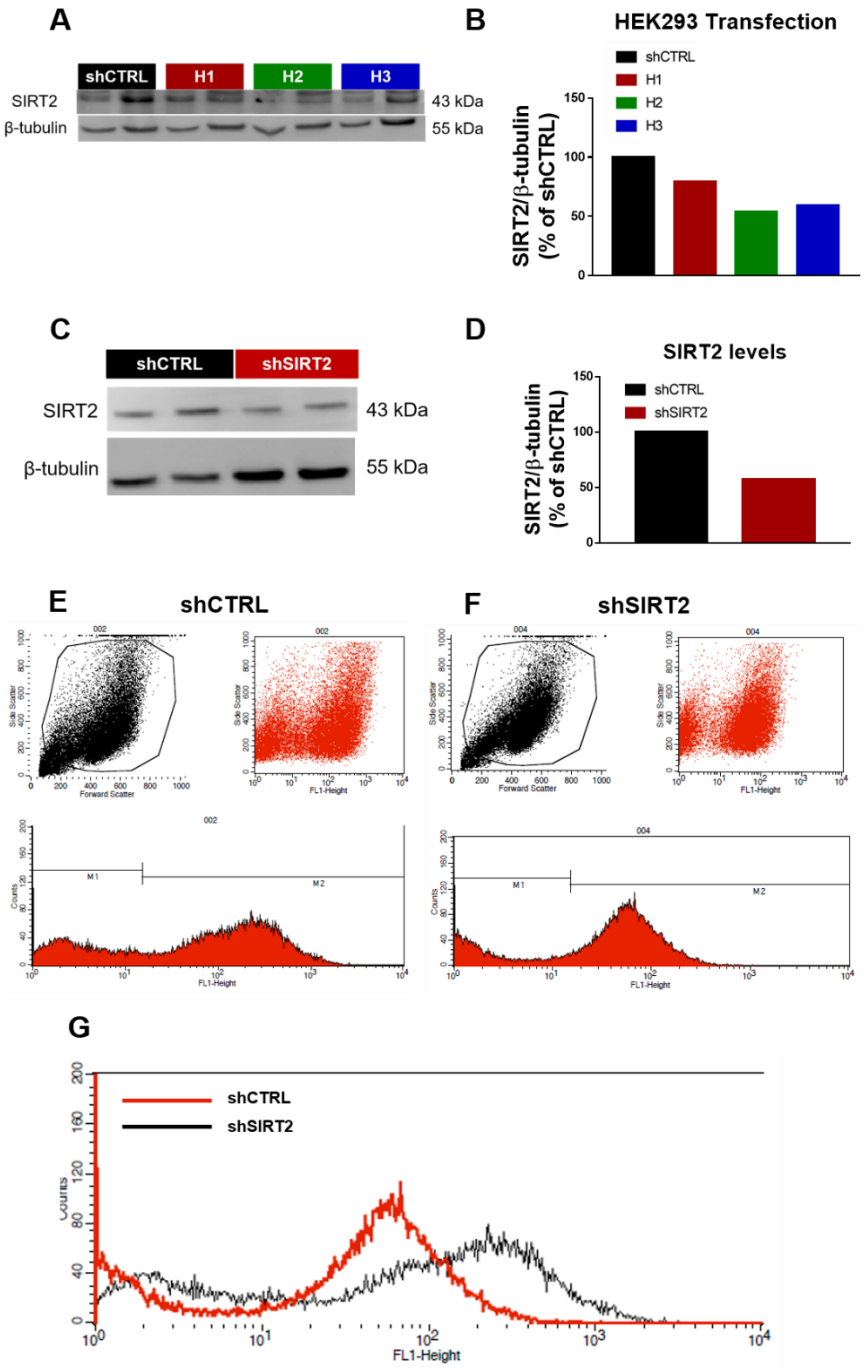

Figure 9. Efficiency of SIRT2-silencing vector in HepG2 cells. (A) HEK293 cells were transfected with three plasmids containing different human promoters to silence SIRT2. **(B)** SIRT2 levels were normalized to the levels of β-tubulin in the same lane. **(C)** Representative immunoblot of HepG2 cells infected with lentiviruses expressing either a shRNA control (shCTRL) or SIRT2 shRNA (shSIRT2). **(D)** SIRT2 levels were normalized to the levels of β-tubulin in the same lane. FACS was used to count shCTRL-infected **(E)** and shSIRT2-infected **(F)** HepG2 cells, by quantifying GFP-positive cells. **(G)** Spectral overlap of both cell types.

Taking into account the deacetylase function of SIRT2, global protein acetylation status was analyzed in shCTRL- and shSIRT2-infected HepG2 cells treated with vehicle or palmitate for 24 hours (Fig. 10A and B). Palmitate treatment induced a significant increase in global protein acetylation of both shCTRL (~30%, $p<0.05$) and shSIRT2 cells (~30%, $p<0.05$), compared to their respective vehicle. We further analyzed the specific region ~25 kDa, and the patterns in both groups were similar (Fig. 10C).

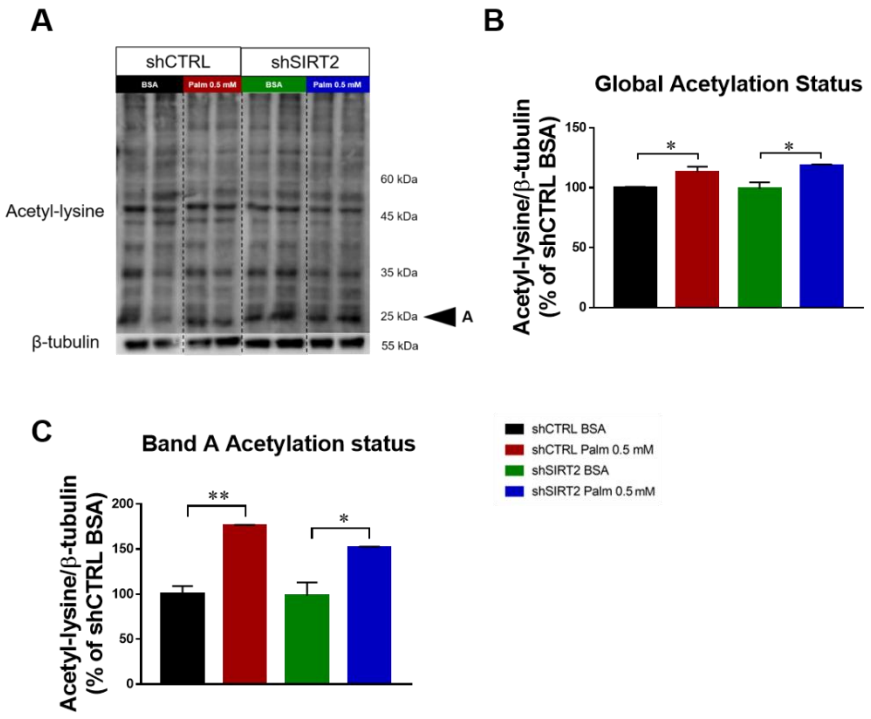

Figure 10. Global acetylation status of SIRT2-silenced HepG2 cells. (A) Total protein acetylation was assessed through western blotting of shCTRL and shSIRT2 HepG2 cells incubated with either BSA or palmitate for 24h. **(B)** Levels of lysine acetylated proteins were normalized with levels of β-tubulin in the same lane. **(C)** Levels of lysine acetylated proteins of region A were normalized to the levels of β-tubulin in the same lane. The black dotted line divides the lanes that were cropped from the same membrane. Results are presented as mean ±SEM. Each experiment was performed in triplicate and repeated at least three times (n=3/group). * $p<0.05$, ** $p<0.01$ compared to control (shCTRL BSA).

To test our hypothesis that reduced SIRT2 levels in HepG2 cells may promote dysregulation in hepatic lipid homeostasis, Oil Red O staining was performed in both shCTRL and shSIRT2 cells treated with vehicle or palmitate. Microscopic analysis demonstrated more lipid droplets in shSIRT2 cells in basal conditions, similar to shCTRL cells exposed to palmitate (Fig. 11A). The spectrophotometric quantification revealed a significant increase in lipid deposition in shSIRT2-infected HepG2 cells in basal conditions (~50% increase, $p<0.05$). However, no significant changes in lipid accumulation were observed after palmitate treatment (shCTRL: 100% increase; shSIRT2: 110% increase) (Fig. 11B). Cell viability was also evaluated to rule out possible cytotoxicity from both palmitate treatment and SIRT2-silencing. No treatment caused significant cytotoxicity (Fig. 11C).

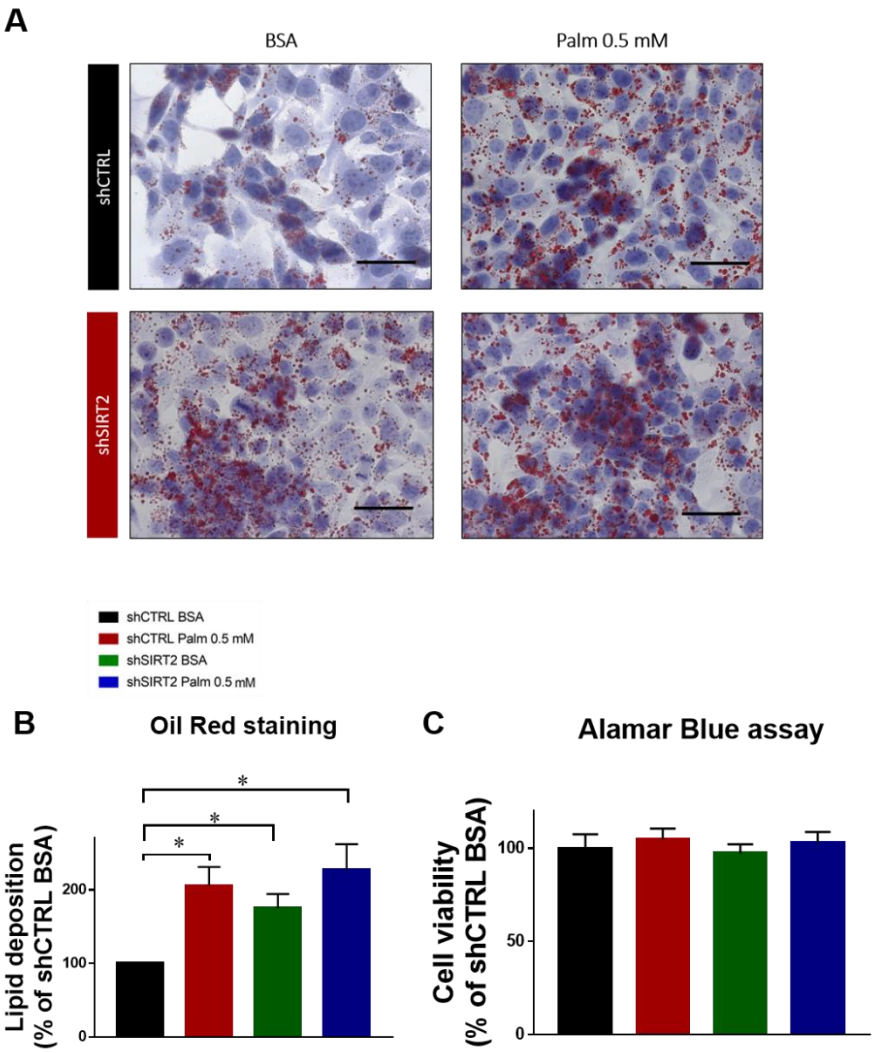

Figure 11. Analysis of lipid deposition and cell viability in SIRT2-silenced HepG2 cells. **(A)** Representative images of Oil Red O (ORO)-stained shCTRL and shSIRT2 HepG2 cells, which were incubated with either BSA or palmitate for 24h. **(B)** Spectrophotometric analysis of stained lipids. **(C)** Alamar Blue assay for cell viability. Scale bar represents 25 µm. Results are presented as mean ±SEM. Each experiment was performed in triplicate and repeated at least three times (n=3/group). * $p<0.05$ compared to control (shCTRL BSA).

SIRT2 silencing promotes increased lipid accumulation in hepatocytes under basal conditions, similar to the *in vivo* findings in SIRT2-KO mice [96]. This finding suggests a crucial role for SIRT2 in the regulation of hepatic lipid content.

3.4 SIRT2 overexpression prevents hepatic lipid accumulation

To assess whether SIRT2 could have a protective role regarding hepatic lipid accumulation, HepG2 cells were infected with either GFP (GFP)- or SIRT2 (SIRT2)-overexpressing lentiviral vectors. Overexpression was confirmed through western blotting analysis, and both GFP and SIRT2 signal demonstrated a clear efficiency of vector overexpression (Fig. 12A).

GFP was again used as an indicator of viral infection. GFP fluorescence was quantified using FACS (Fig. 12B and C) and the measured percentage of ~66% in the control group corresponds to the percentage of SIRT2-overexpressed cells. To rule out unspecific signal, FACS analysis from both cell types was overlapped, showing a specific GFP signal in the control cells (Fig. 12D).

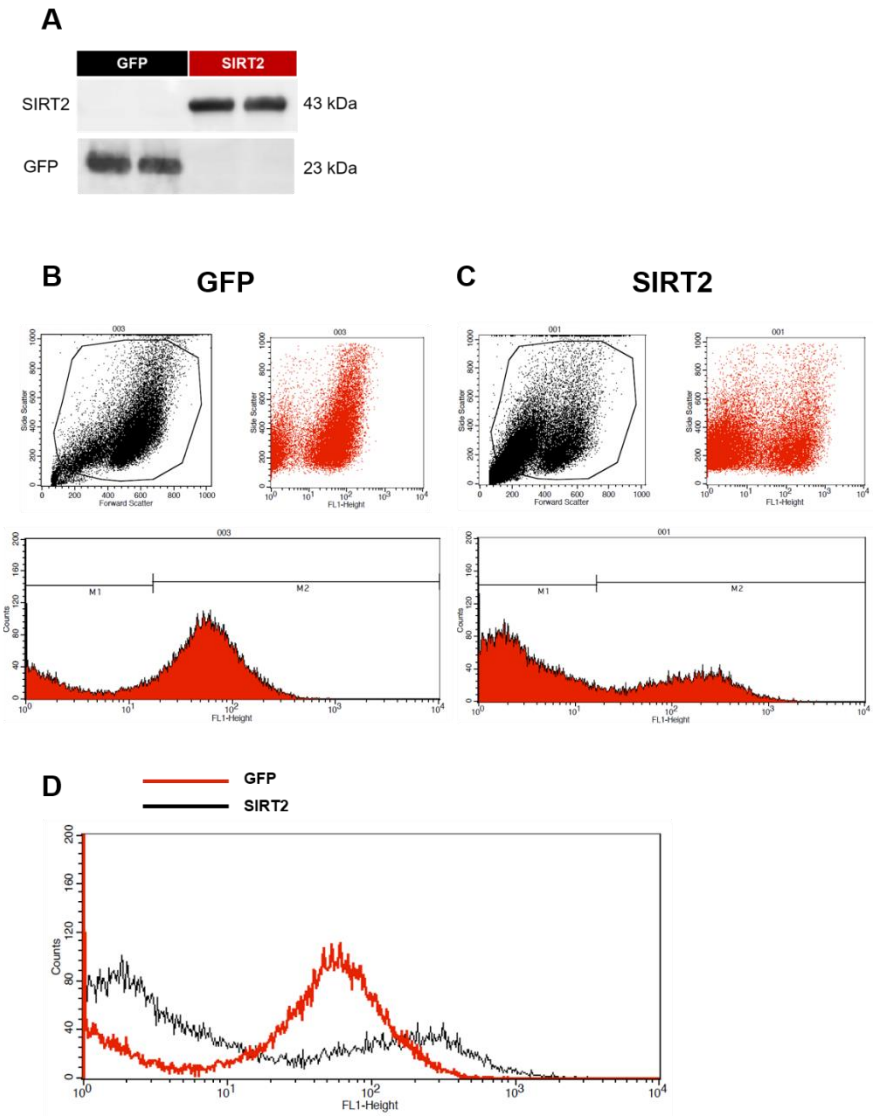

Figure 12. Efficiency of SIRT2-overexpressing vector in HepG2 cells. (A) Representative immunoblot of HepG2 cells infected with lentiviruses expressing either GFP (GFP) or SIRT2 (SIRT2). FACS was used to count GFP-infected **(B)** and SIRT2-infected **(C)** cells, by quantifying GFP-positive cells. **(D)** Spectral overlap of both cell types.

Protein acetylation of both GFP- and SIRT2-overexpressing cells was also analyzed as previously (Fig. 13). Western blot analysis demonstrated that palmitate-induced hyper acetylation is attenuated by SIRT2 overexpression (Fig.13A and B). Moreover, the 25 kDa region was significantly less acetylated in the SIRT2-

overexpression group compared to the GFP group, both under vehicle (~70% decrease, p<0.001) or palmitate (~200% decrease, p<0.001) conditions (Fig. 13C).

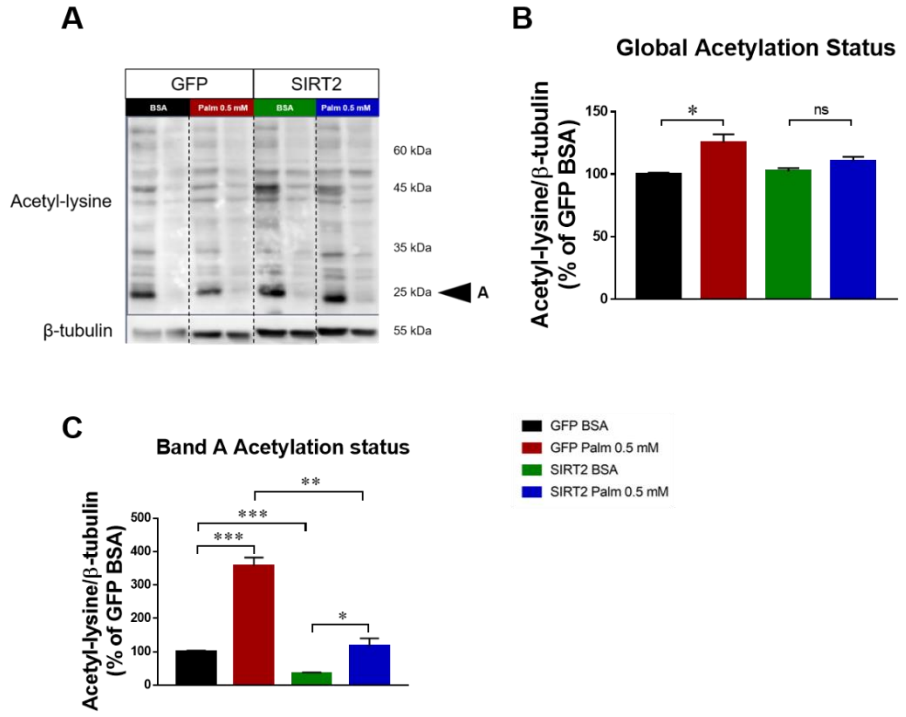

Figure 13. Global acetylation status of SIRT2-overexpressing HepG2 cells. (A) Total protein acetylation was assessed through western blotting on both GFP and SIRT2 HepG2 cells incubated with either BSA or palmitate for 24h. **(B)** Levels of lysine acetylated proteins were normalized with levels of β-tubulin in the same lane. **(C)** Levels of lysine acetylated proteins of region A were normalized to the levels of β-tubulin in the same lane. The black dotted line divides the lanes that were cropped from the same membrane. Results are presented as mean ±SEM. Each experiment was performed in triplicate and repeated at least three times (n=3/group). * p<0.05, ** p<0.01, *** p<0.001 compared to control (GFP BSA).

Collectively, these results may suggest a SIRT2 target involved in hepatic lipid metabolism regulation

The lipid analysis of both GFP and SIRT2-overexpressing cells was performed with ORO staining (Fig.14A). Spectrophotometric quantification demonstrated that SIRT2 overexpression prevented palmitate-induced intracellular lipid accumulation, as

compared to GFP-overexpressing cells (~80% decrease, p<0.05) (Fig. 14B). No significant changes in cell viability were observed for all the conditions tested (Fig. 14C).

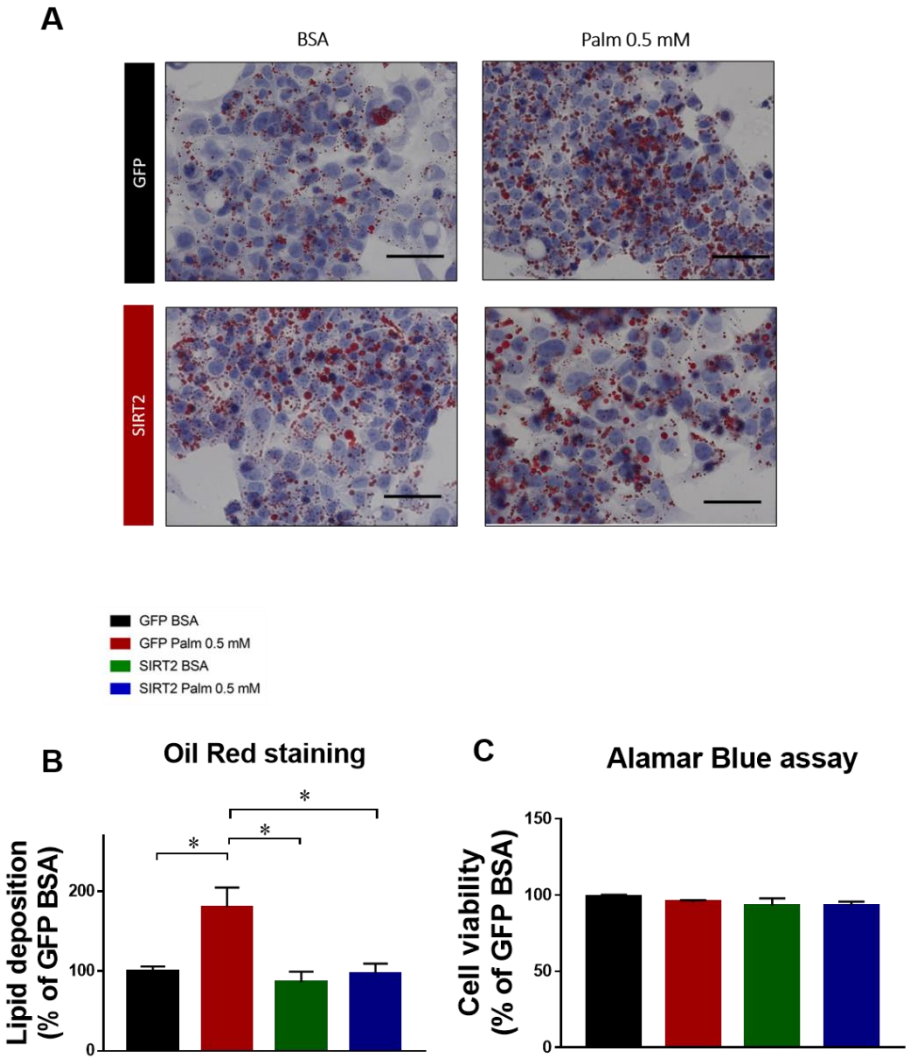

Figure 14. Analysis of lipid deposition and cell viability in SIRT2-overexpressing HepG2 cells. (A) Representative images of ORO staining in both GFP and SIRT2 HepG2 cells, incubated for 24 hours with either BSA or palmitate. **(B)** Spectrophotometric analysis of stained lipids. **(C)** Alamar Blue assay for cell viability. Scale bar represents 25 µm. Results are presented as mean ±SEM. Each experiment was performed in triplicate and repeated at least three times (n=3/group). * p<0.05 compared to control (GFP BSA).

These results further suggest a role for SIRT2 in maintaining hepatic lipid homeostasis, as SIRT2 overexpression protected from hepatic lipid overload.

3.5 Palmitate induces endoplasmic reticulum stress

Endoplasmic reticulum (ER) stress has been described to be upregulated in hepatic steatosis [66]. Moreover, HepG2 cells incubated with palmitate have been demonstrated to activate ER stress, and ER stressors have been shown to promote lipid accumulation [63] [66]. Therefore, the ER stress may be an underlying mechanism of hepatic lipid overload.

We therefore analyzed the levels of phosphorylated and total forms of IRE1 and eIF2α, well-known ER stress markers, in HepG2 cells treated for 24 hours with either vehicle or palmitate. Western blot analysis showed that palmitate induced significantly more phosphorylation of IRE1 (~25% increase, $p<0.01$) comparing to control (Fig.15A and B). Levels of phosphorylated eIF2α were also significantly higher upon palmitate exposure (~30% increase, $p<0.05$) compared to the control (Fig. 15C and D).

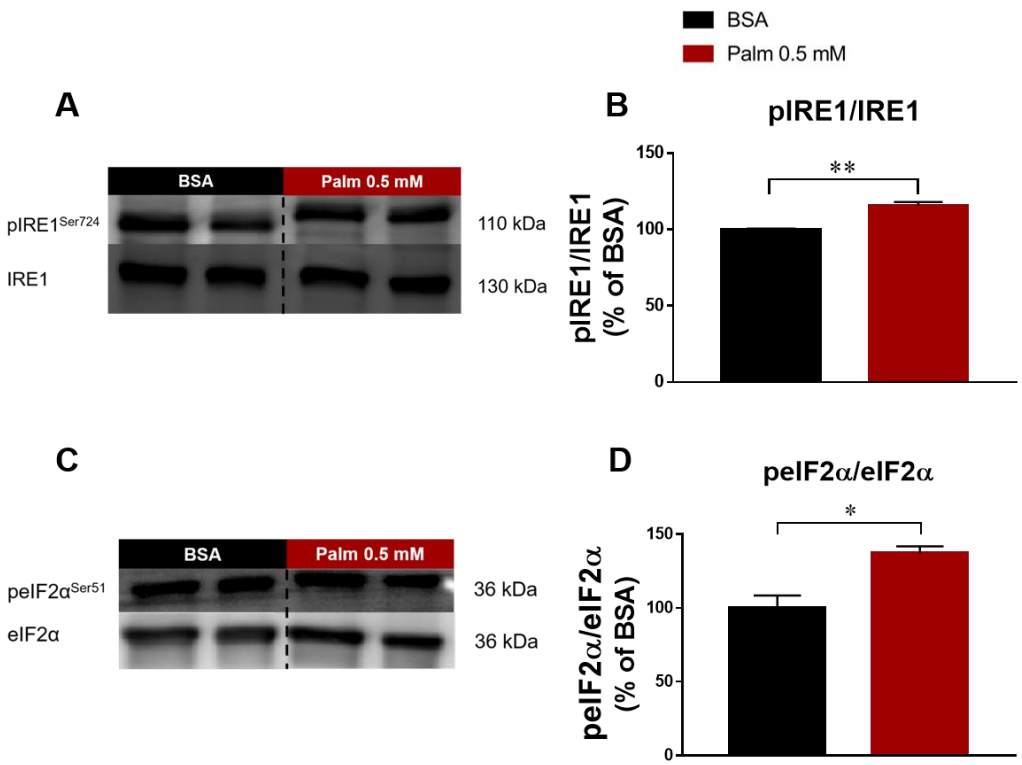

Figure 15. Effect of palmitate on the expression of ER stress markers. (A) HepG2 cells were incubated for 24h with either BSA or palmitate, followed by western blot analysis of pIRE1 Ser724 and total IRE1. **(B)** pIRE1 levels were normalized for total IRE1 in the same lane, and the ratios were quantified. **(C)** Representative immunoblot of peIF2α Ser51 and total eIF2α. **(D)** p-eIF2α levels were normalized for total eIF2α in the same lane, and the ratios were quantified. The black dotted line divides the lanes that were cropped from the same membrane. Results are presented as mean±SEM. Each experiment was performed in triplicate and repeated at least three times (n=3/group). * $p<0.05$, ** $p<0.01$ compared to control (BSA).

Collectively, these results suggest an activation of ER stress upon lipid overload, characteristic of hepatic steatosis.

3.6 SIRT2 silencing induces ER stress

To establish a link between lipid deposition, SIRT2 levels and ER stress, shCTRL and shSIRT2-infected HepG2 cells were incubated for 24 hours with either vehicle palmitate followed by assessment of ER stress markers.

Western blot analysis demonstrated a significant increase in the levels of phosphorylated IRE1 in shSIRT2 cells in basal conditions (~10%, $p<0.05$), when compared to shCTRL cells treated with vehicle. Palmitate exposure in shSIRT2 cells did not cause a significant increase in the phosphorylation of IRE1 when compared to the vehicle treatment (Fig. 16A and B). Phosphorylation of eIF2α followed a similar pattern, as there was a trend for an increased phosphorylation of shSIRT2 cells under basal conditions ($p=0.06$) compared to the control group (Fig. 16C and D).

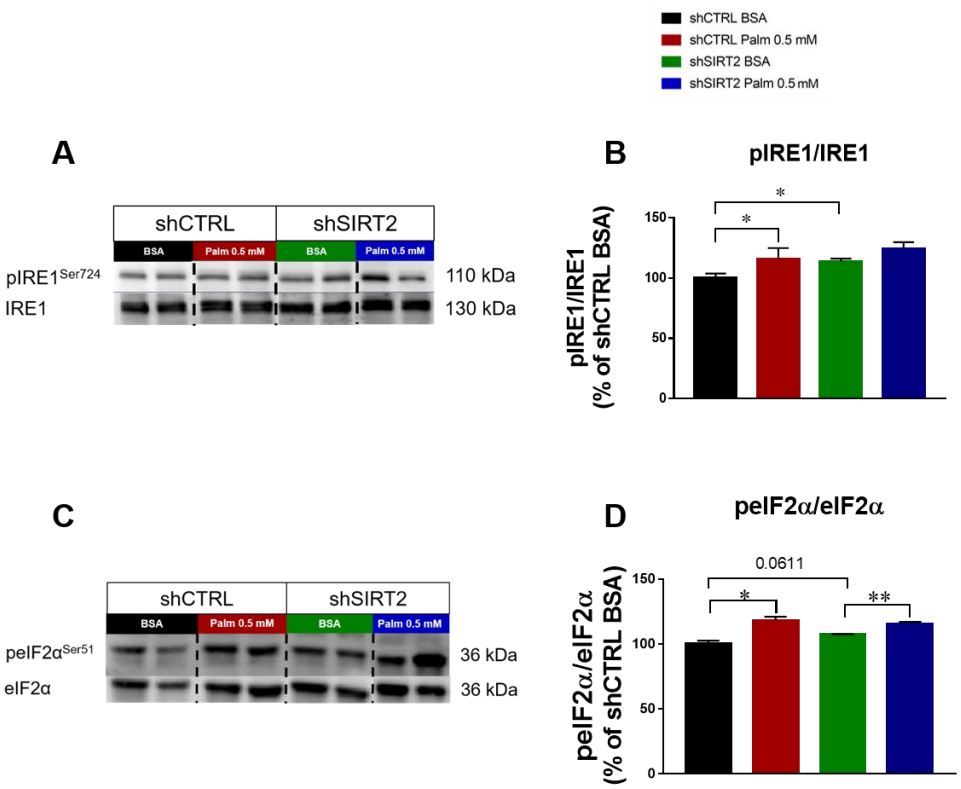

Figure 16. Expression of ER stress markers in SIRT2-silenced HepG2 cells. (A) Both shCTRL and shSIRT2 infected HepG2 cells were incubated with either BSA or palmitate for 24h, followed by western blot analysis of pIRE1 Ser724 and total IRE1. **(B)** pIRE1 levels were normalized for total IRE1 in the same lane, and the ratios were quantified. **(C)** Representative immunoblot of, and peIF2α Ser51 and total eIF2α. **(D)** peIF2α levels were normalized for total eIF2α in the same lane, and the ratios were quantified. The black dotted line divides the lanes that were cropped from the same membrane. Results are presented as mean±SEM. Each experiment was performed in triplicate and repeated at least three times (n=3/group). * $p<0.05$, ** $p<0.01$ compared to control (shCTRL BSA).

Overall, these results demonstrate that SIRT2 silencing in HepG2 cells promotes ER stress, concomitant with the higher lipid deposition displayed in these cells. Therefore, the data suggests a putative role for SIRT2 and ER stress activation.

3.7 SIRT2 overexpression attenuates palmitate-induced ER stress

Having shown that ER stress is activated as a result of SIRT2 silencing, we also wanted to assess if SIRT2 overexpression attenuates ER stress.

Therefore, both GFP and SIRT2-overexpressing HepG2 cells were exposed for 24 hours to either vehicle or 0.5 mM palmitate. SIRT2 overexpression did not promote differences in the levels of phosphorylated IRE1 when compared to the control group, as it prevented palmitate effect on IRE1 phosphorylation (Fig.17A and B). SIRT2 overexpression also prevented eIF2α phosphorylation induced by palmitate (~45%, $p<0.01$) compared to GFP cells (Fig.17C and D).

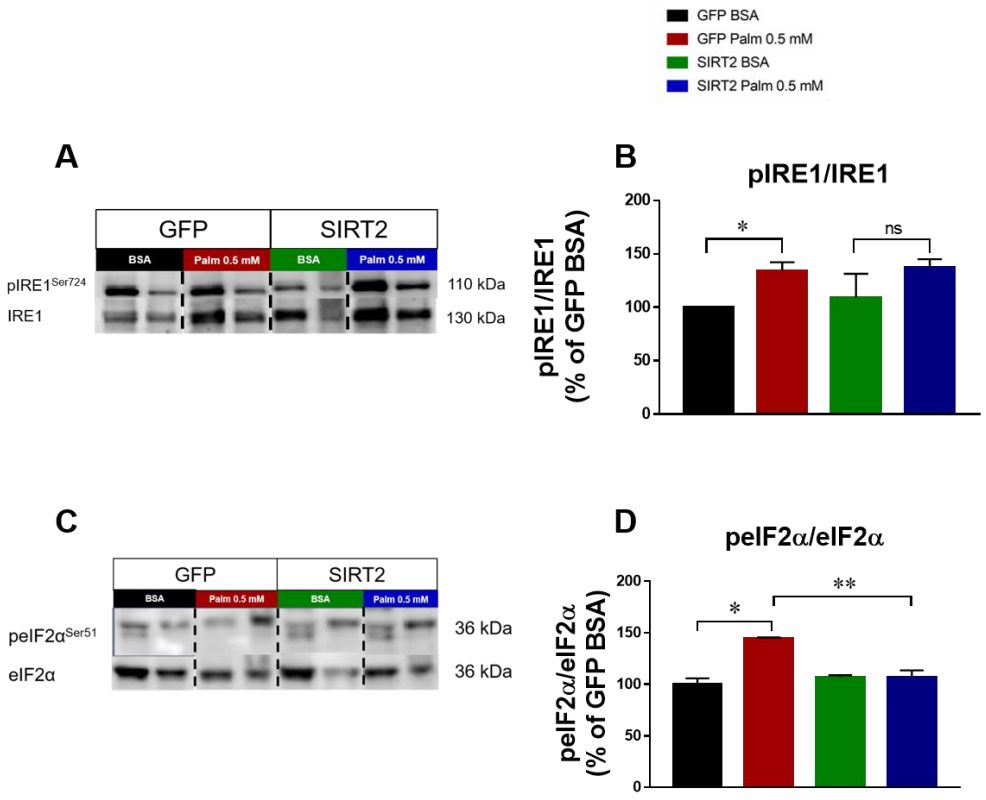

Figure 17. Expression of ER stress markers in SIRT2-overexpressing HepG2 cells. (A) Both GFP and SIRT2 infected HepG2 cells were incubated with either BSA or palmitate for 24h,

followed by western blot analysis of pIRE1 Ser724 and total IRE1. **(B)** pIRE1 levels were normalized for total IRE1 in the same lane, and the ratios were quantified. **(C)** Representative immunoblot of, and peIF2α Ser51 and total eIF2α. **(D)** p-eIF2α levels were normalized for total eIF2α in the same lane, and the ratios were quantified. The black dotted line divides the lanes that were cropped from the same membrane. Results are presented as mean±SEM. Each experiment was performed in triplicate and repeated at least three times (n=3/group). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to control (GFP BSA).

Overall, these set of results demonstrated a link between SIRT2 and hepatic lipid homeostasis. We could observe a decrease in SIRT2 levels upon lipid overload, as SIRT2 silencing promoted lipid over accumulation, in contrast to SIRT2 overexpression, that attenuated hepatocytes steatosis induced by palmitate. Moreover, we observed a novel link between SIRT2 levels and ER stress activation, as this mechanism may be regulated by SIRT2 to promote hepatic lipid homeostasis.

Chapter IV – Discussion

SIRT2, the primarily cytosolic isoform of the sirtuin family of NAD^+-dependent deacetylases, has recently emerged as a key player in the regulation of mammalian metabolism, linked to both glucose and lipid metabolism. However, there is no evidence that SIRT2 may participate in the regulation of hepatic lipid metabolism and no link has been established between the protein and non-alcoholic fatty liver disease (NAFLD)[72]. Preliminary data from our group suggested a connection between SIRT2 and hepatic lipid homeostasis, as SIRT2-KO mice had a predisposition to develop metabolic syndrome and hepatic steatosis [95] [96]. Therefore, the present study was designed to investigate the putative role of SIRT2 as a regulator of hepatic lipid homeostasis.

In the present work, we show that SIRT2 has a regulatory function on hepatic lipid homeostasis by modulating the activation of ER stress. Therefore, in this study, we established an *in vitro* model of hepatic steatosis by using HepG2 cells treated with the saturated fatty acid palmitate to study hepatic lipid overload, as it induced significant lipid deposition (Fig. 6), and compromised insulin signaling pathway, evidenced by the lower phosphorylation levels of Akt (Fig. 7), concomitant with the insulin resistant states of the pathology [46]. SIRT2 levels were shown to be decreased upon lipid overload (Fig. 8), suggesting SIRT2 to be downregulated as a consequence of dysregulated lipid metabolism. Therefore, by modulating SIRT2 levels, we observed that SIRT2 silencing promoted a predisposition for steatosis in basal conditions (Fig. 11), evidenced by the high lipid deposition observed, in contrast to SIRT2 overexpression, which attenuated the lipid overload induced by palmitate exposure (Fig. 14). Overall, the present study demonstrates that SIRT2 exerts a crucial role in maintaining hepatic lipid homeostasis,

and this function is loss by SIRT2 downregulation-derived from lipid overload in hepatocytes.

Based in our findings and previous literature, we suggest that SIRT2 may maintain hepatic lipid homeostasis by regulating both *de novo* lipogenesis and fatty acid β-oxidation. Preliminary *in vivo* data suggested a trend for an increase in the hepatic expression of *SREBP-1c*, the master regulator for lipogenesis, in SIRT2-KO mice, compared to the WT group, on both CD and HFD, concomitant with increased lipid accumulation in the liver of these animals [96]. Similar findings have been previously obtained for SIRT1 in hepatic lipid metabolism, by both deacetylating and inhibiting SREBP-1c activity [105]. Therefore, SREBP-1c deacetylation by SIRT2 may be a mechanism towards hepatic lipid homeostasis. In addition, SIRT2 has been described to regulate mitochondrial function and biogenesis, as PGC-1α, a master regulator of mitochondrial function and fatty acid β-oxidation, has been identified as a SIRT2 target in mammalian cells [79, 104]. Additionally, SIRT2 ablation in mice has been shown to promote an increase in oxidative stress and to compromise mitophagy, as well as mitochondrial function, leading to dysregulation of lipid metabolism [106]. Therefore, the downregulation of SIRT2 in HepG2 cells may have caused dysregulation of hepatic lipid metabolism by overstimulating SREBP-1c and lipogenesis, as well as promoting mitochondrial dysfunction, repressing PGC-1a and fatty acid oxidation, whereas SIRT2 overexpression promoted metabolic homeostasis. Collectively, SIRT2 might regulate hepatic lipid metabolism, by regulating lipogenesis by deacetylating and inactivating SREBP-1c, and fatty acid oxidation by targeting PGC-1a and consequentially maintain mitochondrial function.

Fatty acids in liver cells suffer esterification and are converted into triglycerides (TG), and these molecules in physiological conditions are secreted from hepatocytes in the form of very low density lipoproteins (VLDL) to avoid over accumulation [107]. Palmitate-induced lipid accumulation and insulin resistance have been reported to downregulate VLDL secretion [108]. Therefore, the observed high levels of intracellular lipids may correspond to the excessive accumulation of intracellular VLDL and loss of this secretory function. SIRT1 was also implicated to regulate VLDL secretion in previous studies, as heterozygous mice had a decrease in the liver export of TG [109]. Furthermore, SIRT2 may also regulate lipid homeostasis in the liver by maintaining VLDL secretion, and preventing TG accumulation in hepatocytes. This possibility should be addressed in future studies, by evaluating the content of VLDL in the media in both shSIRT2 and SIRT2-overexpressed cells.

Previous reports showed that palmitate exposure in HepG2 cells induces ER stress activation, as lipid overload depletes the intra-ER calcium pools, and induces UPR-like responses [63]. This was further confirmed in our cellular model of hepatic steatosis, in which IRE1 and eIF2α phosphorylation levels were significantly increased upon lipid overload (Fig. 15). The activation of ER stress has been linked to a dysregulation of hepatic lipid metabolism [110], as recent studies demonstrated that pharmacological activation of ER stress was linked to stimulate *de novo* lipogenesis, by activating SREBP-1c transcription by the IRE1 pathway, coupled to the gene transcription promotion of enzymes in fatty acid synthesis, such as acetyl CoA carboxylase 2 (*Acc2*), diacylglycerol acyltransferase 2 (*Dgat2*) and stearoyl CoA desaturase 1 (*Scd1*) [110] [111]. Phosphorylation and activation of eIF2α was also correlated with the expression of this same set of lipogenic genes, as recent studies described the development of hepatic

steatosis of knock-out mice for a specific eIF2α phosphatase, GADD34 [112] [113] [114]. Other studies demonstrated the development of fatty livers upon pharmacological activation of ER stress in animals, coupled to a downregulation of fatty acid oxidation and mitochondrial dysfunction [115, 116]. Additionally, ER stress activation has been linked to a decrease in VLDL secretion, by inhibiting APOB100 expression, the main component of VLDLs, by activation of the PERK-eIF2α pathway [117].

In comparison with control cells, SIRT2-silenced HepG2 cells had higher basal levels of ER stress, evidenced by the high phosphorylation of the two assessed markers (Fig. 16). This may suggest a novel link between SIRT2 downregulation, ER stress activation, and subsequent dysregulation in lipid accumulation. Importantly, SIRT2 overexpression attenuated palmitate-induced ER stress activation (Fig. 17), suggesting the downregulation of ER stress as the mechanism to prevent lipid overload. SIRT7 was also reported to modulate ER stress activation upon lipid overload and prevent hepatic steatosis by repressing the transcription factor Myc, an activator of ER stress [118]. Myc was reported to activate the PERK/eIF2α pathway [119], and previous studies identified Myc as a SIRT2 target in cancer cells, for subsequent inactivation and degradation [120]. Overall, these findings suggest that SIRT2 hepatic regulatory functions may encompass the modulation of ER stress activation-derived from lipid overload in hepatocytes, and ultimately, our findings reveal that SIRT2 plays a role in the regulation of hepatic lipid homeostasis by downregulating ER stress activation. This hypothesis should be addressed in future studies by evaluating Myc activity upon SIRT2 level modulation.

Finally, to assess if lipid overload would have any influence in sirtuin function, we analyzed the global acetylation patterns, as fatty acid chronic exposure was described

to cause protein hyperacetylation [121]. We observed that palmitate-treated HepG2 cells had a significant increase in protein acetylation (Fig. 8), concomitant with the decrease in SIRT2 expression, suggesting loss of SIRT2 a consequence of dysregulated lipid homeostasis. Nevertheless, shSIRT2 HepG2 cells did not have any differences regarding the global acetylation patterns, and may indicate a compensatory mechanism of other sirtuins (Fig. 10). However, SIRT2 overexpression attenuated the observed hyperacetylated state in cells treated with palmitate (Fig. 13). The cofactor NAD^+ is the main substrate for sirtuins, and is continuously synthesized in the cell in most redox metabolic pathways [122]. NAD^+ was shown to be depleted in NAFLD and, as a consequence, to downregulate sirtuin function in NAFLD [122]. Nicotinamide, a precursor of NAD^+, was recently shown to replenish NAD^+-depleted pools, and upregulate SIRT1 expression and function to prevent ER stress activation and hepatic steatosis [123] [124]. Collectively, by promoting mitochondrial function, SIRT2 also contributes to the continuous NAD^+ biosynthesis by promoting redox metabolism, and both preventing NAD^+ depletion and promoting the deacetylation required for hepatic metabolic regulation.

We additionally analyzed a specific region at ~25 kDa, as it was the most altered in HepG2 cells treated with palmitate. Although SIRT2 silencing did not promote differences in the acetylation of this area, SIRT2 overexpression attenuated the acetylation of this region. Overall, this may indicate a specific region of interest for SIRT2 function, containing targets that may be related to the hepatic lipid regulation role displayed by SIRT2. Further studies require the analysis of the modifications in protein acetylation in this region in different subcellular organelles in order to confirm this hypothesis.

Our ongoing studies are investigating whether pharmacological induction of ER stress modulates SIRT2 expression and function. Moreover, analysis of both gene and protein expression of other ER stress markers (CHOP or GRP78) [125], reported to be altered in NAFLD, and enzymes in the metabolic processes comprised in liver metabolic pathways and regulators such as SREBP-1c and PGC-1α, should be assessed in our models. Additionally, we are also investigating the expression of ER stress markers in liver samples of both WT and SIRT2-KO animals to further corroborate our hypothesis.

The limitations of this study may comprise the SIRT2 silencing model using HepG2 cells. As the validation demonstrated, there was an intermediate infection percentage with the shSIRT2 lentiviral vector (Fig. 9), compared to the SIRT2 overexpressed cells, which had a slightly higher infection percentage, however, displayed a more distinct effect in SIRT2 modulation (Fig. 12). Moreover, this partial silencing of SIRT2 may account for the absence of differences upon global protein acetylation, as well as the specific region near the 25 kDa. Nonetheless, the model demonstrated significance in the analysis of both lipid deposition and ER stress activation between cell groups, as it demonstrated that SIRT2 downregulation promotes higher rates of lipid overload and activation of ER stress at basal conditions.

The prevalence of metabolic disorders such as NAFLD has been increasing at an alarming rate [126] [15]. This condition is mainly characterized by a disruption in the hepatic lipid homeostasis and accounts for high mortality rates and increase in the surge for liver transplants, mainly due to the lack of available efficient therapeutics [11]. This work identifies SIRT2 as a regulator of hepatic lipid metabolism, as our findings demonstrate that SIRT2 overexpression attenuates lipid overload by preventing the

activation of ER stress-induced by the saturated fatty acid palmitate, highly abundant in the modern day diets. Future assessments should focus on the overexpression of SIRT2 in the liver of NAFLD animal models. If successful, these studies may identify SIRT2 as potential novel pharmacological approach to effectively combat NAFLD.

Chapter V – Conclusions

The goal of the present study was to investigate the regulatory functions of SIRT2 on hepatic lipid metabolism. We show that lipid-overloaded hepatic cells exhibit decreased SIRT2 expression and function, associated with ER stress activation. SIRT2 silencing revealed an increase in ER stress activation and lipid overload, whereas SIRT2 overexpression attenuated ER stress activation and subsequently prevented lipid accumulation upon palmitate treatment. Further studies may encompass the effects of pharmacological ER activators on SIRT2 levels, in order to assess the possible modulation of ER stress activation on SIRT2 levels in hepatocytes, as well as the the assessment of ER stress markers in SIRT2-KO animals under both CD and HFD.

Overall, our work demonstrated that the SIRT2-ER stress axis plays a crucial role in maintaining hepatic lipid homeostasis, thus revealing SIRT2 as a putative target to tackle fatty liver diseases, such as NAFLD.

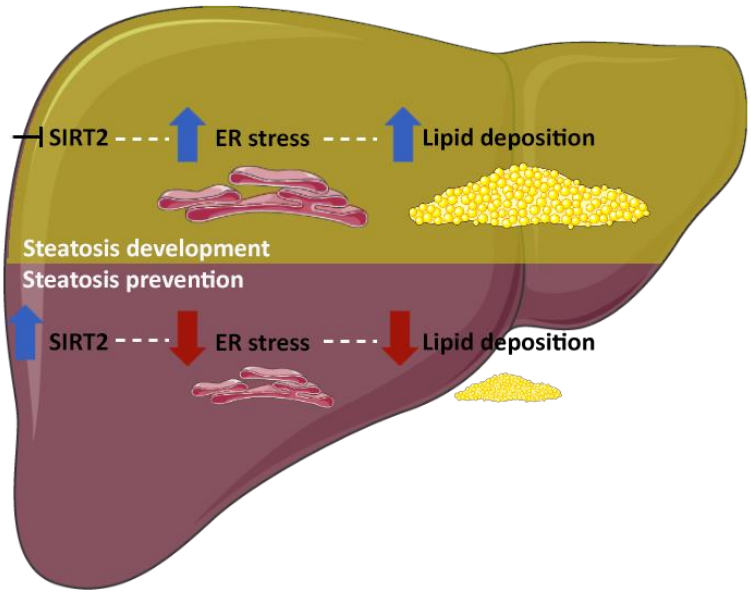

Figure 18. SIRT2 regulatory function on hepatic lipid metabolism. SIRT2 downregulation resulted in higher lipid deposition, possibly through ER stress activation, whereas SIRT2 overexpression prevented palmitate-induced lipid overload by dampening ER stress activation.

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