UNIVERSITY OF SALERNO

DEPARTMENT OF CHEMISTRY AND BIOLOGY

Ph. D. Course in "Chemistry" - XXXIII Cycle

Ph. D. Thesis in Chemistry

# OLIVE OIL AND OLEATE AFFECT HEPATIC PROTEOME AND MITOCHONDRIAL DYNAMICS: IN VIVO AND IN VITRO APPROACHES

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2017 - 2020

1

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# OLIVE OIL AND OLEATE AFFECT HEPATIC PROTEOME AND MITOCHONDRIAL DYNAMICS: IN VIVO AND IN VITRO APPROACHES

A Thesis presented to the Federal University of São Paulo (Brazil) and to the Department of Chemistry and Biology of University of Salerno (Italy) for the degrees of Doctor in Science and Doctor of Philosophy

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2021

# ACKNOWLEDGEMENTS

My friends, Tania Yuka Yuba, Adriana Sabino, Gabriela Pereira, Camilla Naves Pascoal, Juliana Tamashiro, Denise Eugênia Pereira Coelho, Fernanda Nascimento, Michelle Castro, Duane del Giudice

# ABSTRACT

The Mediterranean diet (MD) has been pointed out as a nutritional approach to prevent metabolic disturbances leading to obesity, diabetes and liver steatosis. These properties have been largely attributed to oleic acid and polyphenol compounds present in olive oil (OO), the main fat source of MD. However, there are controversies regarding the consequences of excessive extra virgin olive oil (EVOO) intake.

Prolonged high-fat feeding may induce obesity, insulin resistance, and non-alcoholic fatty liver disease. Dysfunction of mitochondrial dynamics along with endoplasmic reticulum (ER) stress, have been suggested to play a role in the establishment of these disturbances. The mechanism seems to involve a prevalence of the fission process over the fusion process, and endoplasmic reticulum stress, with activation of the unfolded protein response (UPR) and consequent stimulation of inflammatory pathways.

The dietary fats differ in their lipotocixity degree, with saturated fatty acids being considered more harmful, while the monounsaturated oleic acid has been suggested to protect against lipotoxicity. However, data on the mechanisms involved as well as on the existence of a dose-dependency are not conclusive. Given that the liver is a key organ for the maintenance of the metabolic homeostasis and is highly susceptible to dietary manipulations, focusing on the consequences of high-fat feeding on this tissue is of relevance. With the aim of evaluating these aspects, we used both *in vivo* and *in vitro* approaches.

The animal study examined body, serum, and hepatic parameters after the prolonged intake of diets containing either normolipidic or hyperlipidic amounts of EVOO. To perform a broad analysis of liver metabolism, a proteomic approach was used.

Two months-old swiss mice were fed for 12 weeks with either normolipidic diets (9.5% energy from fat) containing soy oil (control diet, C) or EVOO (NO diet) or a high fat EVOO diet (HO, 39% energy from fat). Body weight and food intake were measured weekly and serum parameters were analyzed by enzymatic methods. Liver proteome was analyzed by LC-MS/MS and the proteomic data were analyzed by one-way Anova followed by Tukey post-hoc and Benjamini-Hochberg correction. Pathway enrichment analysis was performed with Fisher´s test and corrected by the Bonferroni approach.

The high-fat intake of EVOO diet (HO group) inhibited food and energy intake, decreased serum triglycerides while it preserved normal patterns of body weight gain, body adiposity, and glucose levels . However, it increased total cholesterol levels and liver mass

and tended to increase hepatic fat content. The proteomic analysis of the liver identified 2318 proteins and, after application of the inclusion criteria, 487 proteins were quantified. They were allocated in 27 pathways significantly enriched, of which 7 pathways were altered in the HO group, in comparison to both C and NO (lipid metabolism, fatty acids metabolism, gluconeogenesis, metabolism of amino acids and derivatives, citric acid cycle, electron transport chain, and biological oxidations). The examination of the pathway analysis derived from the proteomic data suggested stimulation of both mitochondrial and peroxissomal β-oxidation of fatty acids, and inhibition of lipid synthesis from LCFA and of gluconeogenesis in the HO group. On the other hand, although the NO group failed to show significant alteration of the liver proteome, it presented reduced body fat, body weight gain, and serum triglycerides and glucose levels, with no evident hypercholesterolemia.

The results allow the hypothesis that the hepatic metabolic adjustments in the HO indicated by the proteomic analysis, were partially successful in avoiding/counteracting the detrimental outcomes of a long term high fat feeding. Contrastingly, since the beneficial effects of the NO diet could not be attributed to overt effects on hepatic metabolism, it is suggested that other tissues may have had a more relevant participation.

Because of the indications of altered mitochondrial metabolism by the high EVOO intake, we performed *in vitro* experiments in HepG2 cells to analyze the consequences of crescent doses of either oleate or palmitate on aspects of mitochondrial dynamics, cell viability, apoptosis, and ER-UPR response. To this end, HEPG-2 cells were treated for 24 hours with 10 μM, 50 μM, 100 μM, 250 μM or 500 μM of either palmitate or oleate. The effects on apoptosis and cell viability were evaluated by the caspase-3 activity and MTT assay, respectively. Western blotting analysis was performed to evaluate the protein content of: a) mitofusin 2 (MFN2) and optic atrophy 1 (OPA1), markers of mitochondrial fusion process; b) dynamin-related protein 1 (DRP1), marker of mitochondrial fission process; and c) 78-kDa glucose-regulated protein (GRP78), marker of early ER stress.

Both fatty acids reduced cell viability at doses of 250μM and 500μM, and the highest dose had more pronounced effects. The two highest doses of either palmitate or oleate caused similar increases of Caspase-3 apoptotic activity. GRP78 levels were increased only by palmitate at the lowest and highest doses while no differences were induced by oleate. The levels of MFN2 were not significantly affected by the treatments, although a trend to increased values was observed with all oleate doses, while only the doses of 100 μM, 250 μM and 500 μM of palmitate promoted such a trend. OPA1 and DRP1 levels were not significantly affected by any treatment or dose.

The results showed that the saturated fatty acid elicited a condition of UPR induction and mitochondrial fission trend starting at low doses, whereas a high toxicity with marked reduction of cell viability were elicited at high doses. Differently, the monounsaturated fatty acid tended to induce mitochondria fusion at low doses associated with low toxicity even at high doses. This kind of study can help to understand cellular pathways activated by nutrients with protective effects towards metabolic diseases.

# RESUMO

A dieta mediterrânea (MD) é considerada uma estratégia nutricional vantajosa na prevenção de distúrbios metabólicos que podem acarretar obesidade, diabetes e esteatose hepática. O consumo de azeite de oliva extravirgem (EVOO), principal fonte de gordura dessa dieta, e dos seus constituintes, o ácido oleico e os polifenóis, é uma das principais condutas associadas aos efeitos benéficos atribuídos à MD. Porém os efeitos do consumo excessivo de EVOO são controversos.

A ingestão prolongada de dietas hiperlipídicas pode ocasionar obesidade, resistência à insulina e doença hepática gordura não-alcoólica (NAFLD). A disfunção da dinâmica mitocondrial associada ao estresse do retículo endoplasmático (ER) estão relacionados ao estabelecimento desses distúrbios. O mecanismo pelo qual eles se relacionam envolve a prevalência do processo de fissão em detrimento do processo de fusão mitocondrial e o acionamento da resposta reticular a proteínas mal enoveladas (UPR) e consequente ativação de vias pró-inflamatórias.

As gorduras dietéticas diferem em seu grau de lipotoxicidade, sendo que as gorduras saturadas são consideradas mais prejudiciais enquanto que o ácido graxo monoinsaturado oleico demonstrou efeitos protetores. No entanto, dados sobre os mecanismos envolvidos são inconclusivos quanto à existência de uma relação dose-dependente. Uma vez que o fígado é um órgão primordial para a manutenção da homeostase metabólica e é igualmente susceptível a modificações nutricionais, explorar as consequências do consumo de diferentes dietas hiperlipídicas neste tecido é relevante. Utilizamos técnicas *in vivo* e *in vitro* no presente estudo para proporcionar uma avaliação ampla de tais efeitos.

O estudo com animais avaliou o efeito do consumo prolongado de dietas normolipídicas ou hiperlipídicas enriquecidas com EVOO sobre parâmetros corporais, séricos e hepáticos. A fim de analisar o metabolismo do fígado de maneira ampla, o proteoma foi escolhido como técnica para caracterização e quantificação de proteínas.

Camundongos suíços de dois meses de idade foram alimentados por 12 semanas com dietas normolipídicas (9,5% de energia lipídica) contendo óleo de soja (dieta controle, C) ou EVOO (dieta NO) ou dieta hiperlipídica contendo EVOO (dieta HO, 39% de energia lipídica). A massa corporal e a ingestão alimentar foram medidas semanalmente e os parâmetros séricos foram analisados através de métodos enzimáticos. O proteoma do fígado foi realizado por meio de cromatografia líquida acoplada à espectrômetro de massas (LC-MS/MS) e os dados obtidos foram estatisticamente analisados por Anova de uma via seguida

por teste de Tukey e correção de Benjamini-Hochberg. A análise de vias enriquecidas foi feita através do teste de Fisher e corrigida por Bonferroni.

O consumo prolongado da dieta HO reduziu a ingestão alimentar e calórica e os níveis séricos de triglicérides, concomitantemente ao ganho de peso corporal, adiposidade e glicemia de jejum semelhantes aos grupos normolipídicos. Porém houve elevação do colesterol plasmático total e do peso do fígado associado à tendência de aumento da gordura hepática. A análise proteômica hepática identificou 2318 proteínas e após a aplicação dos critérios de inclusão, 487 proteínas foram quantificadas. Essas proteínas foram alocadas em 27 vias significativamente enriquecidas, das quais 7 foram afetadas no grupo HO em relação aos demais grupos (metabolismo de lipídios, metabolismo de ácidos graxos, gliconeogênese, metabolismo de aminoácidos e derivados, ciclo do ácido cítrico, cadeia transportadora de elétrons e oxidações biológicas). A análise de vias alteradas sugeriu estimulação da β-oxidação mitocondrial e peroxissomal, inibição da síntese de lipídios a partir de ácidos graxos de cadeia longa (LCFA) e inibição da gliconeogênese no grupo HO. Por outro lado, apesar de o proteoma hepático não ter sido substancialmente afetado, o grupo NO apresentou redução da adiposidade, do ganho de peso corporal e dos níveis séricos de jejum de triglicérides e glicose, sem a hipercolesterolemia observada no grupo HO.

Os resultados permitem hipotetizar que os ajustes metabólicos desencadeados pelo fígado frente ao excesso de lipídios da dieta HO foram parcialmente bem-sucedidos. Além disso, sugere-se que os benefícios proporcionados pelo consumo da dieta NO observados não estariam relacionados a alterações do proteoma hepático, podendo outros órgãos exercerem função mais relevante nesta modulação.

Após indicação do proteoma de alteração mitocondrial decorrente do consumo de EVOO, experimentos *in vitro* foram realizados em células hepáticas HepG2 para analisar as consequências de doses crescentes de ácido oleico e palmítico em parâmetros da dinâmica mitocondrial, viabilidade celular, apoptose e UPR do ER. As células foram tratadas por 24 horas com 10 μM, 50 μM, 100 μM, 250 μM or 500 μM de ácido oleico ou palmítico. Os efeitos na viabilidade celular e na atividade apoptótica foram avaliados por meio da atividade da Caspase-3 e do teste colorimétrico de 3(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio brometo (MTT), respectivamente. A análise da expressão proteica de a) Mitofusina 2 (MFN2) e da Proteína da atrofia óptica 1 (OPA1), marcadores do processo de fusão; b) proteína relacionada à dinamina-1 (DRP1), marcador do processo de fissão; c) Proteína 78 regulada pela glicose (GRP78), marcador do estresse precoce do ER; foi realizada por Western Blotting.

Ambos os ácidos graxos nas doses de 250μM e 500μM reduziram a viabilidade celular, e a maior dose produziu efeitos mais proeminentes. As duas doses mais altas de ácido oleico e palmítico também aumentaram a atividade da enzima apoptótica Caspase-3. Os níveis proteicos de GRP78 aumentaram somente com a menor e a maior dose de ácido palmítico e nenhum efeito foi observado com o ácido oleico. Os níveis de MFN2 não foram significativamente alterados por ambos os tratamentos, porém uma tendência de aumento foi observada com todas as doses de ácido oleico e com as doses de 100 μM, 250 μM e 500 μM de ácido palmítico. Os níveis de OPA1 e DRP1 não foram significativamente alterados por nenhum tratamento ou dose.

Os resultados mostram que o ácido graxo saturado induziu à UPR e houve uma tendência de fissão mitocondrial já com doses menores, enquanto que efeitos tóxicos importantes foram observados com doses altas na viabilidade celular e atividade apoptótica. Diferentemente, doses menores do ácido graxo monoinsaturado produziram uma tendência de aumento do processo de fusão associado a menor toxicidade com doses mais altas. Este tipo de estudo pode auxiliar na elucidação de mecanismos protetores de ativação de vias celulares mediados pelos nutrientes e componentes dietéticos contra doenças metabólicas.

# LIST OF FIGURES

## INTRODUCTION

## ARTICLE 1

## ARTICLE 2

# LIST OF TABLES

## ARTICLE 1

# ABBREVIATIONS

ACC - Acetyl-CoA carboxylase

ACOX1 - Acyl-coenzyme A oxidase 1

ACSF2 - Acyl-CoA synthetase family member 2

ACSL- Long-chain-fatty-acid-CoA ligase/ Long chain fatty acyl-CoA synthetase

AMP – Adenosine monophosphate

AMPK - AMP-activated protein kinase

ATF6 - Activating transcription factor 6

ATP - Adenosine triphosphate

BMI – Body mass index

BAT - Brown adipose tissue

BSA – Bovine serum albumin

C – Control group

CAD - Glutathione peroxidase

CHOP - C/EBP homologous protein

CNS – Central nervous system

CPT1 - Carnitine palmitoyltransferase 1

CPT2 - Carnitine palmitoyltransferase 2

DAG - Diacylglycerol

DECR1 - 2,4-dienoyl-CoA reductase 1

DHA - Docosahexaenoic acid

DIO - Diet induced obesity

DM – Diabetes mellitus

DMSO - Dimethyl sulfoxide

DRP1 – Dynamin-related protein 1

DTT – Dithiothreitol

EDTA - Ethylenediamine tetraacetic acid

EPA - Eicosapentaenoic acid

ER – Endoplasmic reticulum

ETC - Electron transport chain

EVOO - Extra virgin olive oil

FA - Fatty acid

FAAH - Fatty acid amide hydrolase

FAO – Fatty acid oxidation

FFA – Free fatty acids

FGH - S-formylglutathione hydrolase

FIS1 – Fission protein 1

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase

GLUT2- Glucose transporter 2

GLUT4 – Glucose transporter 4

GPx - Glutathione peroxidase

GRP78/BIP1 - 78-kDa glucose-regulated protein/ endoplasmic reticulum chaperone BIP

HepG2 - Human hepatocellular carcinoma cells

HF – High-fat

HO – High-fat olive oil diet

IR- Insulin resistance

IRE1 - Inositol-requiring kinase 1

LC – Long chain

LCFA – Long chain fatty acid

LCFA-CoA – Long chain fatty acyl-CoA

MCFA – Medium chain fatty acid

MD - Mediterranean diet

MFF - mitochondrial fission factor

MFN2 – Mitofusin 2

MiD49/51 - Mitochondrial dynamic proteins of 49 and 51 kDa

MS - Mass spectrometry

MTT - Thiazolyl blue tetrazolium bromide

MUFA - Monounsaturated fatty acid

NAFLD - Non-alcoholic fatty liver disease

NASH - Non-alcoholic steatohepatitis

NEFAs - Non-esterified fatty acids

NO – Normolipidic olive oil diet

OA - Oleic acid

OEA – Oleoylethanolamide

OO - Olive oil

OPA1 – Optic atrophy 1

OXPHOS - Oxidative phosphorylation

PBS - Phosphate buffered saline

PEPCK - Phosphoenolpyruvate carboxykinase

PERK - Protein kinase RNA-like endoplasmic reticulum kinase

PI3K - phosphatidyl-inositol- kinase

PMSF - Phenylmethylsulfonyl fluoride

PPAR-α - Peroxisome proliferator-activated receptor α

PUFA – Polyunsaturated fatty acids

ROS - Reactive oxygen species

SCAD – Short chain acyl-CoA dehydrogenase

SDS - Sodium dodecyl sulphate

SOD - Superoxide dismutase

T2D - Type 2 diabetes

TAG – Triacylglycerol

TCA - Tricarboxylic acid cycle

TLR4 - Toll-like receptor 4

UCP -  Uncoupling protein

UPLC - Ultra Performance Liquid Cromatographer

UPR – Unfolded protein response

VLCFA – Very long chain fatty acids

w/w -  weight for weight

**TABLE OF CONTENTS**

# 1.    INTRODUCTION

The Mediterranean diet (MD) is composed by traditional local foods of Mediterranean countries, as vegetables, fruits, fish, whole grains, nuts, and seeds, and has olive oil (OO) as the main fat source. Metabolic effects of MD have been extensively studied and it is pointed as a nutritional approach to prevent heart diseases and cancer, besides being recognized to promote longevity (Katz & Meller 2014).

There are many subtypes of OO and their composition vary according to local of production (70% of OO world production occurs in Mediterranean countries such as Spain, Turkey, Greece, Italy, Morocco, and Tunisia) and the mechanisms of extraction, which yield the classification of  OO as extra-virgin (EVOO), virgin olive oil, refined, or pomace (Foscolou et al 2018).

Olive oil is composed of two distinct portions. Around 98% is comprised by lipid compounds, including the main fatty acid (FA) in EVOO, the monounsaturated fatty acid (MUFA) oleic acid (OA, 18:1, n-9) which comprises 60% to 84% of total fatty acid content, followed by linoleic acid (3% to 21%). The n-9 monounsaturated fatty acids are not essentials since it can be synthesized by endogenous mechanisms. The smallest portion is composed by minor compounds as tocopherols, sterols, pigments and phenolic compounds (Perona et al 2006).

There are studies that attribute the beneficial properties of EVOO to oleic acid, which is able to act as an antitumor agent by suppressing angiogenesis and has antihypertensive effect in rats (Lamy et al 2014, Terés et al 2008).  However, it has been proposed that is not plausible to state that the beneficial effects provided by EVOO should be attributed only to OA, as the minor compounds, especially phenolic compounds, also have demonstrated actions (Visioli et al 2019). The main phenolic compounds present in olive oil are hydroxytyrosol, tyrosol, oleocanthal and oleuropein, which have at least one aromatic ring with one or more hydroxyl groups and act as antioxidants, exerting anti-inflammatory, hypoglycemic, and antitumor, among other activities (Rigacci & Stefani 2016).

The long term intake of a diet rich in EVOO improved endothelial function in diabetic patients with heart disease (Torres-Peña et al, 2018), and the adoption of a MD pattern for three months was able to reduce body weight, glycosylated hemoglobin, arterial blood pressure and improve serum lipid profile in obese, diabetic and hypertensive patients (Grimaldi et al 2018).

MD has been considered as a valid nutritional approach to prevent several metabolic disturbances and diseases caused by the typical hypercaloric and highly palatable western diet pattern, which contains elevated amounts of saturated and trans fatty acids, salt and refined carbohydrates (Bortolin et al 2018).

Although MD is considered a healthy dietary pattern, especially regarding it antioxidant potential, its effects on body composition and food intake are still controversial. A systematic review found that, although MD improved body weight in overweight and obese individuals, such effect was similar to those obtained with other low-fat or low-carbohydrate diets (Mancini et al 2016). Furthermore, overweight subjects receiving, for 16 weeks, a diet with 12% of total daily energy requirements as olive oil, did not present reduced body weight in comparison to subjects consuming a diet containing the same amount of saturated medium-chain fatty acids (St-Onge & Bosarge 2008).

In rodent studies, the findings are equally discrepant. A long-term consumption of either a high-fat (HF)-EVOO or a HF-saturated diet promoted increased food intake, body and fat mass and insulin resistance, compared with a normolipidic balanced diet. However, cholesterol serum levels and endothelial function were preserved only in the HF-EVOO diet fed rats (Keita et al. 2013). Contrarily, a short-term HF-EVOO diet, either rich or deficient in polyphenols, reduced body weight gain, fat depots, and triglycerides serum levels in comparison to a control balanced diet. Interestingly, only the polyphenols-rich diet was able to increase thermogenesis in brown adipose tissue (BAT) through increased uncoupling protein 1 (UCP1) expression (Oi-Kano et al 2007).

Uncoupling proteins (UCPs) are largely found in BAT mitochondria. These proteins are very important to thermogenesis since they disconnect the electron transport chain from the oxidative phosphorylation, avoiding the complete ATP synthesis from substrate oxidation. UCPs promote heat dissipation, which in turn increases energy expenditure (Klingenberg 1990). It has been reported that HF-EVOO diet induced UCP1, UCP2, and UCP3 mRNA expression in the interscapular brown adipose tissue and UCP3 mRNA in gastrocnemius muscle, in comparison with sunflower oil, palm oil, and beef tallow HF diet. In addition, the HF-EVOO diet increased total-body oxygen consumption (Rodríguez et al 2002).

Body weight improvements induced by olive oil intake can also be due to increased rates of oxidation of oleic acid in both fasting and postprandial states, leading to reduced adiposity (DiNicolantonio & O'Keefe 2017; Galvão Cândido et al 2018). A study in men, with body mass index (BMI) ranging from normal to moderate obesity, compared the

effect of a meal high in EVOO or saturated fat, reporting increased in energy expenditure in the EVOO-fed subjects (Piers et al 2002). However, whether those effects are restricted to overweight or obese subjects remains uncertain.

Excessive intake of fat-dense foods is an important factor in the obesity epidemic and its comorbidities. The consumption of high-fat diets may affect the central mechanisms of feeding regulation as well as peripheral metabolism and the predominant fat type is a relevant aspect conditioning the diet effects (Ravussin & Bogardus, 2000).

Aiming at better understanding the importance and impact of different types and quantities of fat on metabolism and body/serum profile, diet-induced obesity (DIO) animal models have been extensively studied, each mimicking the different characteristics and causes of obesity in humans. We have previously shown that rats fed a high-fat lard-based diet for a long period (8-12 weeks), presented increased body mass and body fat, abolition of central insulin-induced hypophagia, associated with impairment of hypothalamic insulin signaling, and abolition of central glucose-induced hypophagia, associated with reduced hypothalamic glucose transporter 2 (GLUT2) levels and AMP-activated protein kinase (AMPK) activation (Albuquerque et al 2006; de Andrade et al 2015). We have also shown that rats fed a high-fat diet enriched with n-6 fatty acids (soybean oil) presented increased c-fos protein activation in the lateral hypothalamic area, which stimulates food intake (Watanabe et al 2009; 2010), increased body and fat mass (Gaiva et al 2001; 2003), and impaired central insulin-induced hypophagia (Pimentel et al 2012). Additionally, we have found that rats fed a high-fat diet enriched with n-3 fatty acids (fish oil) had reduced body and fat mass and reduced serum levels of leptin and corticosterone, and improvement of serum lipid profile (Pimentel et al 2012). However, impairment of the hypothalamic serotonergic system (Watanabe et al 2009; 2010), hyperglycemia and impaired central glucose-induced hypophagia were also observed (de Sousa et al 2013). These findings indicate that a high-fat intake may have deleterious effects, regardless the type of fat.

The liver plays a crucial role in glucose and lipids metabolism. In the hepatocyte, the postprandial surplus of glucose can either be stored as glycogen or undergo *de novo* lipogenesis. Conversely, hepatic glycogenolysis and gluconeogenesis yield glucose during short-term and prolonged fasting (Rui 2014). Importantly, increased hepatic glucose production due to insulin resistance contributes to diabetic hyperglycemia (Petersen et al 2017).

The central nervous system (CNS) also participates in glucose homeostasis. Although the brain does not need insulin for glucose uptake, insulin receptors are well

distributed in many brain sites and exert many different functions. Insulin levels are proportional to the body fat store and it acts in the CNS stimulating anorexigenic and inhibiting orexigenic neurons (Schwartz et al. 1999 e 2000; Wynne et al. 2005; Ribeiro et al. 2006). In the hypothalamus, insulin acts to suppress hepatic glucose output, an effect mediated by phosphatidyl-inositol- kinase (PI3K) (Obici et al 2002a; Inuoe 2018). Moreover, central administration of oleic acid inhibited hepatic glucose production and food intake, providing relevant evidence that fatty acids can signal body energy status to the CNS and trigger regulatory responses (Obici et al 2002b).

Regarding the central effects of olive oil constituents, oleoylethanolamide (OEA), a fatty acid derived from OA, has been reported to exert an anorexigenic effect after intraperitoneal or oral administration in rats (Nielsen et al 2004, Oveisi et al 2004). As reviewed by Bowen et al (2017), many previous studies showed that the intake of oils rich in OA could rise serum and hepatic oleoylethanolamide (OEA) levels. One study with eutrophic humans showed that, after the ingestion of a meal rich in olive oil or sunflower oil supplemented with OA, serum levels of OEA were increased and that was associated with reduced caloric intake in the subsequent meal. However, only the individuals that consumed the EVOO meal presented reduced hunger and increased fullness and satiety sensations (Mennella et al 2015). The mechanisms involved on OEA anorexigenic activity was related to its high affinity with peroxisome proliferator-activated receptor α (PPAR-α), which in turns modulates lipid metabolism pathways (Fu et al 2003).

The hepatic metabolism of lipids includes biosynthesis, *de novo* lipogenesis, and fatty acid β-oxidation. Hepatic fatty acid oxidation (FAO) occurs in both mitochondria and peroxisomes. Short and medium-chain fatty acids can cross the mitochondrial membrane by diffusion while long-chain fatty acids (LCFAs) need to be transported to undergo β-oxidation. These fatty acids are first activated by the binding of acyl-CoA, catalyzed by long-chain acyl-CoA synthetase (ACSL). Long-chain fatty acyl-CoA (LCFA-CoA) enters mitochondria through the binding to carnitine palmitoyltransferase 1 (CPT1) in the outer mitochondrial membrane and carnitine palmitoyltransferase 2 (CPT2) in the inner mitochondrial membrane (Qu et al 2016).

The process of β-oxidation is highly regulated and AMPK is an important regulator. It acts as a very accurate intracellular metabolic sensor, as it is activated by the reduction of ATP/AMP ratio, indicating a deficit of available energy, such as in prolonged food deprivation (Sandoval et al. 2012; Hardie et al. 2002; Shackelford & Shaw 2009).

Activated AMPK stimulates catabolic pathways, such as fatty acid oxidation, in order to rise ATP production, concomitantly with inhibition of anabolic pathways, such as fatty acid synthesis and gluconeogenesis (McFadden et al. 2014). The modulatory mechanism by which AMPK stimulates β-oxidation consists of its suppressive effect on acetyl-CoA carboxylase (ACC), which catalyzes the conversion of acetyl-CoA to malonyl-CoA. Since malonyl-CoA inhibits CPT1, the reduction of malonyl-CoA stimulates fatty acyl-CoA transport and oxidation. Importantly, this reduces the availability of activated fatty acids for for triacylglycerols (TAG) synthesis in the cytosol (Ruderman & Prentki 2004).

Increased influx of free fatty acids (FFA) to the liver favors triglycerides synthesis and accumulation and is considered a relevant process in the induction of non-alcoholic fatty liver disease (NAFLD) which may progress to non-alcoholic steatohepatitis (NASH) (Tessari et al 2009). The physiopathology also involves impaired mitochondrial β-oxidation and the consequent recruitment of peroxisomal oxidation of fatty acids, elevating of reactive oxygen species (ROS) production, impairing mitochondrial oxidative phosphorylation (Cherkaoui-Malki et al 2012).

Peroxisomes have relevant functions in lipid and hydrogen peroxide metabolism and, along with mitochondria, contribute to cellular ROS homeostasis. Alterations in both peroxisomal and mitochondrial metabolism, such as biogenesis and dynamics, affect both organelles in an interconnected manner (Camões et al 2009). Peroxisomes metabolize only LCFA and very-long chain fatty acids (VLCFA), and the first step is their internalization by ATP-binding cassette transporters of subfamily D (ABCD) (Baker et al 2015). The peroxisomal β-oxidation is incomplete, as these organelles only shorten the fatty acids chain length to 8-6 carbons. This reduces the efficiency of ATP synthesis, constituting an inefficient FA oxidation, since it may disconnect the β-oxidation from energy yielding or storage, what can contribute to decrease body weight and adiposity. However, as $H_2O_2$ is produced, its metabolism is crucial, and any impairment in the anti-oxidant efficiency of peroxisomes may be deleterious (Fiamoncini et al 2013; Schrader et al 2015). Enhanced peroxisomal β oxidation and energy expenditure have been associated with improvement of hepatic lipid accumulation after consumption of high-fat diet enriched with n-3 PUFA, in comparison to saturated fat (Worsch et al 2018). However, chronic stimulation of peroxisomal fatty acid oxidation has been shown to impair hepatic mitochondrial fatty acid metabolism and favor hepatic fat accumulation and dyslipidemia during high-fat intake (Zeng et al 2017).

To our knowledge, there are very few studies which have analyzed the effects of dietary components on peroxisomal β-oxidation. One study reported increased peroxisomal

β-oxidation and reduced hepatic levels of triacylglycerols (TAG) and diacylglycerols (DAG) in mice fed a fish-oil diet, with either normal or high lipid density (Fiamoncini et al 2013). Another study in mice reported that the consumption of a high-fat diet supplemented with walnut polyphenols extract increased the expression of acyl-coenzyme A oxidase 1 (ACOX1), the key enzyme in peroxisomal β-oxidation, while CPT1, the rate-limiting enzyme of mitochondrial β-oxidation, was decreased (Shimoda et al 2009).

Mitochondria are highly dynamic organelles and the mitochondrial bioenergetics is highly dependent on their morphology, which is controlled by the balance between the fusion and fission processes (Figure 1.1). When this balance is disrupted, mitochondria lose the elongated shape, assuming a fragmented shape when the fission process prevails, or an stretched shape along with attachment to each other, when the fusion process is increased (Zemirli et al 2018).

Physiologically, fusion allows mitochondrial communication by the transferring of matrix components and damaged DNA, which is important to regenerate impaired mitochondria. The fusion process is under the control of the mitofusins 1 and 2 (outer mitochondrial membrane) and optic atrophy protein 1 (OPA-1; inner mitochondrial membrane) (Sheridan & Martin 2010).

The fission process is controlled by dynamin related protein 1 (DRP1) and fission 1 protein (FIS1) (Mansouri et al 2018). When fission occurs, cytosolic DRP1 is recruited to the outer mitochondrial membrane in order to promote its constriction and division. This process is important both for mitotic redistribution of mitochondria, which guarantees satisfactory mitochondria content in daughter cells, and for mitochondrial degradation, which allows the separation of damaged and dysfunctional organelles (El-Hattab et al 2018). Mitophagy is responsible for removing damaged mitochondria.

Mitochondrial dynamics, with its strict connection to mitochondrial bioenergetics, is involved in energy balance and diet impact on metabolism. In obese rats, impaired mitochondrial function and reduced MFN2 levels were reported in muscle (Bach et al 2003) and liver (Lionetti et al 2014). A shift towards mitochondrial fission with reduction of MFN2 has been associated with mitochondrial dysfunction, reduced insulin sensitivity and inflammation in obesity. However, different dietary fat sources during chronic overfeeding may affect differently mitochondrial function and dynamics. Saturated fatty acids induced liver and skeletal muscle insulin resistance associated with fission phenotype, whereas ω-3 polyunsaturated fatty acids enhanced insulin sensitivity associated with a shift toward mitochondrial fusion phenotype (Lionetti et al, 2014; Putti et al, 2016).

Obesity presents a low-grade inflammation state and the inflammatory pathways involved may contribute to increased production of reactive oxygen species (ROS). This condition, combined with reduced intracellular antioxidant activity, either by enzymatic (superoxide dismutase – SOD, catalase – CAD, glutathione peroxidase – GPx) or non-enzymatic (antioxidant compounds such as vitamin C and polyphenols) mechanisms, is called oxidative stress and may lead to mitochondrial dysfunction, through the oxidation of DNA, lipids, and proteins (De Mello et al 2018). Since mitochondria participate on lipid β-oxidation and produces ROS during ATP production by electron transport chain (ETC), nutrient oversupply states may overload mitochondria and lead to mitochondrial dysfunction (Lepretti et al 2018). The endoplasmic reticulum (ER) is structural and functionally connected with mitochondria and may also be affected by imbalances in energy homeostasis, triggering unfolding protein response (UPR). Once activated, UPR can modify cellular pathways and protein translation, in order to restore ER functions or, as a last resort, may lead to cell death. UPR in obesity is a regulatory response against increased energy supply and protein synthetic requirements, which are not efficiently sustained by ER (Yilmaz 2017). Insulin resistance, diabetes mellitus (DM), and NAFLD, have been associated with mitochondrial dysfunction, oxidative stress and endoplasmic reticulum (ER) stress (Butterfield et al 2014).
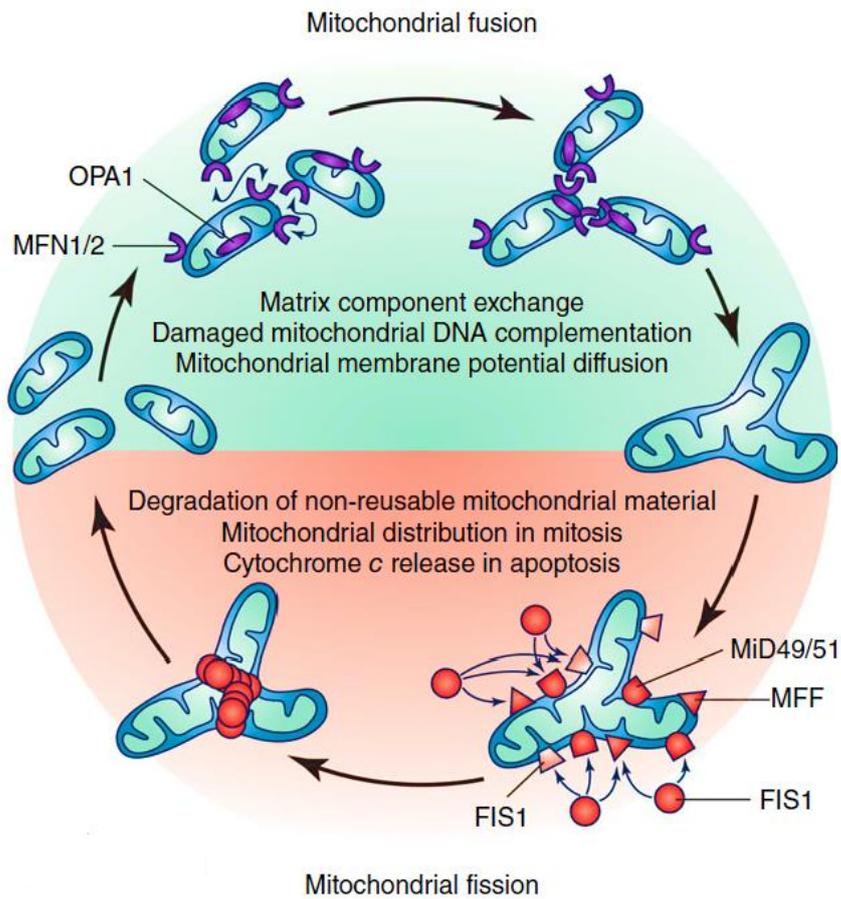
Figure 1.1. Mitochondrial dynamics. Adapted from Vásquez-Trincado et al (2016).

FIS1 - fission 1 protein; MFF - mitochondrial fission factor; MFN1/2 – Mitofusin 1 and 2; MiD49/51 - Mitochondrial dynamic proteins of 49 and 51 kDa; OPA1 - Optic atrophy 1

The Mediterranean diet has been shown to improve NAFLD due to its intrinsic nutritional characteristics that can mitigate steatosis and improve insulin sensitivity. In subjects with NAFLD, the intake of a diet rich in olive oil for 6 weeks reduced liver fat content, in comparison to a low-fat/high-carbohydrate diet. This improvement was associated with increased insulin sensitivity and reduced serum insulin levels (Ryan et al 2013). Mice fed an HF diet supplemented with EVOO presented reduced hepatic expression and activity of lipogenic factors and diminished body fat, serum triacylglycerols and liver FFA levels, effects dependent on the antioxidant capacity of EVOO (Rincón-Cervera et al 2016). Contrastingly, mice fed a HF-EVOO diet have shown increased liver mass and triglycerides content, and increased hepatic expression of lipogenic enzymes, in comparison to high-fat saturated or n-6 polyunsaturated fatty acids (PUFA) (Portillo et al 2001). Additionally, the intake of HF diet rich in either EVOO or palm oil for 8 weeks had similar deleterious metabolic effects in mice, inducing liver steatosis, hyperglycemia, insulin resistance, and exacerbation of the hepatic inflammatory response to an acute dose of lipopolysaccharide, in comparison to the low-fat diet (Meidan et al 2018).

Two studies utilizing proteomic approaches examined the liver after the high-fat intake of EVOO, having found up-regulation of antioxidant proteins enzymes (Arbones-Mainar et al 2007; Wang et al 2014). The same approach in mice fed a normolipidic diet enriched with hydroxytyrosol showed altered hepatic proteins related to oxidative stress (Tomé-Carneiro et al 2017). At the best of our knowledge, no available studies using proteomics performed a broad examination of the effects of different amounts of dietary olive oil on the liver.

## 2.    OBJECTIVES

### 2.1.    *General Objective*

Using in vivo and in vitro approaches, evaluate whether different amounts of olive oil or oleate affect the hepatic metabolism and the mitochondrial dynamics of hepatic cells.

### 2.2.    *Specific objetives*

- Evaluate the effects of the prolonged intake of diets containing either normolipidic or hyperlipidic amounts of EVOO on body, serum, and hepatic parameters.

- To perform a broad analysis of liver metabolism, using a proteomic approach.

- To evaluate the effects of treatments of human hepatocellular carcinoma (HepG2) cells with different concentrations of saturated (palmitate) or monounsaturated fatty acid (oleate) on:

- Mitochondrial fusion/fission processes, analyzing protein levels of MFN2 and OPA1, as markers of mitochondrial fusion, and of DRP1, as marker of mitochondrial fission;

- Early ER stress, by analyzing protein levels of 78-kDa glucose-regulated protein (GRP78), as a marker of UPR;

- Apoptosis and cell viability, by analyzing the enzymatic activity of caspase-3 and thiazolyl blue tetrazolium bromide (MTT) assay, respectively.

# 3.	ARTICLE 1

## Effect of intake of different amounts of extra-virgin olive on the liver proteome

## 3.1.	ABSTRACT

The Mediterranean diet (MD) has been pointed out as a nutritional approach to prevent metabolic disturbances leading to obesity, diabetes and liver steatosis. These propertied have been largely attributed to oleic acid and polyphenol compounds present in olive oil (OO), the main fat source of MD. However, there are controversies regarding the consequences of excessive EVOO intake.

The aim of the present study was to evaluate body, serum, and hepatic parameters after the prolonged intake of diets containing either normolipidic or hyperlipidic amounts of EVOO. To perform a broad analysis of liver metabolism, a proteomic approach was used.

Two months-old swiss mice were fed for 12 weeks with either normolipidic diets (9.5% energy from fat) containing soy oil (control diet, C) or EVOO (NO diet) or a high fat EVOO diet (HO, 39% energy from fat). Body weight and food intake were measured weekly and serum parameters were analyzed by enzymatic methods. Liver proteome was analyzed by LC-MS/MS and the proteomic data were analyzed by one-way Anova followed by Tukey post-hoc and Benjamini-Hochberg correction. Pathway enrichment analysis was performed with Fisher´s test and corrected by the Bonferroni approach.

The high-fat intake of EVOO diet (HO group) inhibited food and energy intake, decreased serum triglycerides while it preserved normal patterns of body weight gain, body adiposity, and glucose levels . However, it increased total cholesterol levels and liver mass and tended to increase hepatic fat content. The proteomic analysis of the liver identified 2318 proteins and, after application of the inclusion criteria, 487 proteins were quantified. They were allocated in 27 pathways significantly enriched, of which 7 pathways were altered in the HO group, in comparison to both C and NO (lipid metabolism, fatty acids metabolism, gluconeogenesis, metabolism of amino acids and derivatives, citric acid cycle, electron transport chain, and biological oxidations). The examination of the pathway analysis derived from the proteomic data suggested stimulation of both mitochondrial and peroxisomal β-oxidation of fatty acids, and inhibition of lipid synthesis from LCFA and of gluconeogenesis in the HO group. On the other hand, although the NO group failed to show significant

alteration of the liver proteome, it presented reduced body fat, body weight gain, and serum triglycerides and glucose levels, with no evident hypercholesterolemia.

The results allow the hypothesis that the hepatic metabolic adjustments in the HO indicated by the proteomic analysis were partially successful in avoiding/counteracting the detrimental outcomes of a long term high fat feeding. Contrastingly, since the beneficial effects of the NO diet could not be attributed to overt effects on hepatic metabolism, it is suggested that other tissues may have had a more relevant participation.

## 3.2.   ABBREVIATIONS

ACOX1 - Acyl-coenzyme A oxidase 1

ACSF2 - Acyl-CoA synthetase family member 2

ACSL- Long-chain-fatty-acid-CoA ligase/ Long chain fatty acyl-CoA synthetase

AMPK - AMP-activated protein kinase

ATP - Adenosine triphosphate

BSA – Bovine serum albumin

C – Control group

DECR1 - 2,4-dienoyl-CoA reductase 1

DHA - Docosahexaenoic acid

DIO - Diet induced obesity

DTT – Dithiothreitol

EPA - Eicosapentaenoic acid

ETC - Electron transport chain

EVOO - Extra virgin olive oil

FA - Fatty acid

FAAH - Fatty acid amide hydrolase

FFA – Free fatty acids

FGH - S-formylglutathione hydrolase

GLUT2- Glucose transporter 2

HF – High-fat

HO – High-fat olive oil diet

LC – Long chain

LCFA – Long chain fatty acid

MCFA – Medium chain fatty acid

MD - Mediterranean diet

MS - Mass spectrometry

MUFA - Monounsaturated fatty acid

NAFLD - Nonalcoholic fatty liver disease

NASH - Nonalcoholic steatohepatitis

NO – Normolipidic olive oil diet

OA - Oleic acid

OEA – Oleoylethanolamide

OO - Olive oil

PEPCK - phosphoenolpyruvate carboxykinase

PPAR-$\alpha$ - Peroxisome proliferator-activated receptor $\alpha$

PUFA – polyunsaturated fatty acids

ROS - Reactive oxygen species

SCAD – Short chain acyl-CoA dehydrogenase

T2D - Type 2 diabetes

TAG – Triacylglycerol

TCA - Tricarboxylic acid cycle

w/w -  weight for weight

## 3.3.    INTRODUCTION

The western diet pattern, characterized by a high intake of energy-dense foods, rich in refined carbohydrates and saturated fat, has an important role in the genesis and progression of obesity and metabolic syndrome (Hariharan et al 2015; Rodríguez‑Monforte et al 2016). Conversely, the typical Mediterranean diet (MD) pattern, characterized by normal lipids and carbohydrates content, has been shown to have several favorable health outcomes including low rates of obesity and metabolic disorders (Davis et al 2015; D'Innocenzo et al 2019).

The main fat source of MD, extra virgin olive oil (EVOO) is rich in oleic acid (OA; C18:1, n-9) and phenolic compounds (Hernáez et al 2016; Martínez-González & Martin-Calvo 2016). A recent systematic review and meta-analysis showed that the intake of 10-20 g/day of olive oil by type-2 diabetic patients reduced glucolysed hemoglobin and fasting glycaemia and, in healthy subjects, decreased the risk of type 2 diabetes (T2D) by 9-13%, with no additional benefits with higher intakes (Schwingshackl et al 2017). A high consumption of EVOO has been set at 15% or more of total energy intake (Martínez-González et al 2014) and the average intake in the MD, of 45 g/day, corresponds to around 20% considering a 2000- Kcal balanced diet (Davis et al 2015). In pre-diabetic patients, the intake of a single balanced meal supplemented with EVOO reduced post-prandial glycaemia and serum triglycerides in comparison with patients receiving a meal without EVOO (Carnevale et al 2017). Moreover, reduced serum triglycerides levels were reported in rats fed a high-fat (HF) diet enriched with EVOO, accompanied by decreased reactive oxygen species (ROS) production, reduced lipid peroxidation and increased fatty acid (FA) oxidation in liver, when compared to a HF diet rich in coconut oil but not to a low-fat standard diet (Lama et al 2017).

Contrastingly, studies with HF-EVOO diets have shown increased liver mass and triglycerides content, and increased hepatic expression lipogenic enzymes, in comparison to high-fat saturated or n-6 polyunsaturated fatty acids (PUFA) (Portillo et al 2001). A recent study in mice reported that the intake of HF diet rich in either EVOO or palm oil for 8 weeks had similar deleterious metabolic effects, inducing liver steatosis, hyperglycemia, insulin resistance, and exacerbation of the hepatic inflammatory response to an acute dose of lipopolysaccharide, in comparison to the low-fat diet (Meidan et al

2018). These findings contrast with the knowledge that EVOO polyphenols have antioxidant and anti-inflammatory properties due to its capacity of curbing free radical species and suppressing lipid peroxidation (Yubero-Serrano et al 2019; Wongwarawipat et al 2018).

The liver plays a crucial role in glucose and lipids metabolism. In the hepatocyte, the postprandial surplus of glucose can either be stored as glycogen or undergo *de novo* lipogenesis. Conversely, hepatic glycogenolysis and gluconeogenesis yield glucose during short-term and prolonged fasting for in short-term fasting (Rui 2014). Importantly, increased hepatic glucose production due to insulin resistance contributes to diabetic hyperglycemia (Petersen et al 2017).

The hepatic metabolism of lipids includes biosynthesis, *de novo* lipogenesis, and fatty acid β-oxidation. In insulin-resistant states, increased influx of free fatty acids (FFA) to the liver favors triglycerides synthesis and accumulation, and is considered a relevant process in the induction of nonalcoholic fatty liver disease (NAFLD) which in turn may progress to nonalcoholic steatohepatitis (NASH) (Tessari et al 2009). The physiopathology involves impaired mitochondrial β-oxidation, leading to recruitment of peroxisomal fatty acid oxidation, elevating ROS production, reducing adenosine triphosphate (ATP) synthesis, and resulting in defective mitochondrial oxidative phosphorylation (Cherkaoui-Malki et al 2012).

The above data indicate that the metabolic and inflammatory aspects of EVOO effects are still controversial, especially when HF diet intake is concerned. The aim of the present study was to evaluate body, serum, and hepatic parameters after the prolonged intake of diets containing either normolipidic or hyperlipidic amounts of EVOO. To perform a broad analysis of liver metabolism, a proteomic approach was used.

## 3.4. MATERIAL AND METHODS

### 3.4.1. Animals, diets and sample collection

All animal experiments were performed in accordance with the Brazilian Council on Animal Research and the Committee on Animal Research Ethics of the Federal University of Sao Paulo. Two months-old male swiss mice were maintained under controlled conditions of light (12:12 h light–dark cycle, lights on at 6 am) and temperature (24 ± 1°C) with free access to food and water. Mice were randomly assigned to receive one of 3 diets *ad libitum* for 12 weeks. The diets were manufactured at the Nutrition Physiology Division of the Federal University of São Paulo and contained either soy or olive oil as the fat source. The control diet (C) was the maintenance AIN-93M diet for adult mice (Reeves 1997), prepared with 4% (w/w) soy oil, the normolipidic olive oil diet (NO) contained 3.4% olive oil and 0.6% soy oil, and the high-fat olive oil diet (HO) contained 17% olive oil and 3% soy oil. The olive oil diets were supplemented with 15% *(w/w)* of soybean oil to achieve the recommended amount of essential fatty acids. The composition of the diets are shown in Table 3.1.

| Macronutrient (% energy) | Diet | | |
|---|---|---|---|
| | Control (C) | Normolipidic olive oil (NO) | High-fat olive oil (HO) |
| Carbohydrate | 75.81% | 75.81% | 46.02% |
| Protein | 14.73% | 14.73% | 14.80% |
| Lipid | 9.47% | 9.47% | 39.2% |
| Calorie content (Kcal/100g) | 380.27 | 380.27 | 460.62 |

**Table 3.1.** Energy and macronutrient composition of the diets

Body weight and food intake were measured weekly. After 12 weeks of diet treatment, the animals were fasted for 14 hours and euthanized for blood and tissue collections. Serum levels of glucose, total cholesterol, HDL cholesterol, and triglycerides were measured by enzymatic methods (Labtest Diagnóstica, Brazil). Fat pads (mesenteric, gonadal, and retroperitoneal) and liver were dissected and weighed. Total hepatic lipid content was measured by Folch´s method (Folch et al 1957).

### 3.4.2. Liver proteomic analysis

### 3.4.2.1 Sample preparation

Aliquots of liver (100 mg) were homogenized in 250 µL of extraction buffer containing 8 M urea, 2 M thiourea, 70 mM dithiothreitol (DTT) and protease/phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL, USA). After centrifugation (19,000 x g for 30 minutes at 4 $^{o}$C), the protein concentration of the supernatants was determined using a Bradford assay (BioAgency, Sao Paulo, SP, Brazil). Aliquots of 200 µg were diluted in 50 mM ammonium bicarbonate to a final volume of 60 µL. RapiGest SF (25 µL, 0.2%, Waters, Milford, MA, USA) was added and the samples were incubated at 80 $^{o}$C for 15 min. Samples were then reduced with 2.5 µL of 100 mM DTT (at 60$^{o}$C for 30 min) and alkylated with 2.5 µL of 300 mM iodoacetamide, at room temperature for 30 min. Protein digestion was performed overnight with trypsin (Promega, Fitchburg, WI, USA). After digestion, the samples were acidified with 5% trifluoroacetic acid, centrifuged and the supernatants transferred to vials for further mass spectrometry (MS) analysis.

### 3.4.2.2. Mass spectrometry and data analysis

Mass spectrometry analysis was performed as described previously (Pedroso et al 2017), with minor modifications. Briefly, six liver samples of each group (C, NO, HO) were analyzed twice on a nanoAcquity UPLC system coupled to a Synapt G2 HDMS Q-TOF mass spectrometer (Waters). Samples (5 µL) were loaded onto a trap column (nanoAquity C18 trap column Symmetry 180 µm x 20 mm, Waters) and transferred by an elution gradient (phase B gradient from 7% to 35% for 92 minutes at a 275 nL/min) through the analytical

column (nanoAcquity C18 BEH 75 µm x 150 mm, 1.7 mm, Waters). The mobile phase A was 0.1% formic acid in water and the mobile phase B was 0.1% formic acid in acetonitrile. Data were acquired in data-independent mode (HDMS$^E$), switching from low (4 eV) to high (ramped from 19 to 45 eV) collision energy. For external calibration, Glu-fibrinopeptide B solution (500 fmol/mL in 50% acetonitrile, 0.1 formic acid, Waters) was infused at 500nL/min every 30 seconds using a nanoLockSpray apparatus.

Data were processed using the ProteinLynx Global Server software version 3.0.1 (Waters) with database search against *Mus musculus* sequences in the UniProtKB/Swiss-Prot database (www.uniprot.org, including 16890 entries). The search parameters included automatic precursor and fragment mass tolerance, 2 missed cleavage sites allowed for trypsin digestion, cysteine carbamidomethylation as fixed modification and methionine oxidation, N-terminal acetylation, glutamine and asparagine deamidation as variable modifications. The protein identification criteria included a minimum of 1 fragment ion per peptide, 5 fragment ions per protein and 2 peptides per protein. The false discovery identification rate was set at 4%. Label-free quantitative assessments based on peptide intensities were performed by integrating the intensities of the three most intense peptides of each identified protein (Silva et al, 2006). Results were exported into Excel files and normalization was performed using the sum of protein intensities. For relative quantitation, only proteins identified in at least 2 technical replicates of at least 4 biological replicates were considered. Additionally, proteins not detected in any of the 12 replicates of one group (indicating that the intensities were below the detection limit), but identified in at least 4 replicates in the other group, were listed and included in the pathway analysis.

### 3.4.3. Western Blotting

To confirm the proteome results, the level of the protein phosphoenolpyruvate carboxykinase (PEPCK) was determined in the liver. The tissue (50 mg) was homogenized in 500 µl of lysis buffer (100 mM Trizma Base, pH 7.5, 10 mM EDTA, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 2mM PMSF, 0.1 mg/ml aprotinin) and the protein content of the samples was determined by the Lowry method (BCA; Bioagency, São Paulo, Brazil). Sample buffer (containing 200 mM of DTT) was added to 50 µg of protein, samples were resolved in 10% SDS–PAGE and transferred to a nitrocellulose blotting membrane. The membranes were blocked with 5% of bovine serum albumin (BSA) and incubated overnight with primary antibody against PEPCK (ab28455,

Abcam, Cambridge, UK). The blots were then incubated for 1 hour at room temperature with peroxidase-conjugated secondary antibody (anti-rabbit, 1: 10,000). The specific bands were revealed by a chemiluminescence (ECL reagent, GE Healthcare Bio-sciences, Pittsburgh, PA, USA), according to the manufacturer's instructions. For evaluation of protein loading, the membranes were striped and re-blotted with anti-beta-actin primary antibody (#8457, Cell Signaling Technology, Inc., Danvers, MA, USA). Band intensities were quantified by optical densitometry (Scion Image software, Scion Corporation, Frederick, MD, USA). The results are expressed in arbitrary units.

### 3.4.4. Statistical analysis

Body weight, white fat depots mass, food intake, liver weight and lipid content, and serum parameters are expressed as mean and standard error. Statistical analysis was performed by one-way analysis of variance followed by Tukey post-hoc test in Statistica 12 Software (StatSoft, Tulsa, OK, USA). Statistical significance was set at $p < 0.05$.

Proteome data were analyzed in IBM SPSS Statistics 21.0 (SPSS Inc, Chicago, IL, USA) by ANOVA with Tukey post-hoc test. Benjamini-Rochberg correction for multiple testing was performed on R software (https://www.r-project.org/). Statistical significance was set at $q < 0.05$.

### 3.4.5. Pathway analysis

The proteins differentially expressed were used to determine enriched pathways. The overrepresentation test was carried out on the online platform Panther, version 14.0, released 2019-04-29 (http://www.pantherdb.org/) (Mi et al, 2017). Whole *Mus musculus* proteome was used as reference list and the annotation dataset was Reactome Pathway. Significantly enriched pathways were based on Fisher's exact test followed by Bonferroni correction for multiple testing and significance was set to FDR < 0.05.

## 3.5. RESULTS

### *3.5.1. Hyperlipidic EVOO intake impaired glucose and cholesterol levels*

As shown in table 3.2, the NO group presented lower body weight gain in comparison to both C and HO groups. The sum of white adipose depots (epididymal, mesenteric and retroperitoneal) was reduced in NO *vs.* C. During the 12 weeks of diet treatment, the cumulative food and caloric intakes of both EVOO groups were lower than those of the C group. Interestingly, the HO diet reduced food and caloric intakes with respect to the NO group.

|  | C | NO | HO |
|---|---|---|---|
| Body weight gain (g) | 23.56 ±0.55 (6) | 15.87 ±1.46 (6)*# | 21.97 ±1.68 (6) |
| Adipose depots (g) | 5.36 ±0.3 (6) | 3.77 ±0.39 (6)* | 4.43 ±0.33 (6) |
| Cumulative food intake (g) | 186.17 ± 6.91 (6) | 151.99 ± 4.00 (6)*# | 89.89 ± 2.29 (6)* |
| Cumulative caloric intake (Kcal) | 687.37 ± 21.64 (6) | 571.91 ± 15.07 (6)*# | 413.50 ± 10.52 (6)* |
| Glucose (mg/dl) | 185.55 ±11.49 (6) | 153.74 ±5.56 (6)# | 199.52 ±12.98 (6) |
| Triglycerides (mg/dl) | 144.34 ±6.71 (6) | 122.99 ±6.94 (6)* | 116.11 ±2.88 (6)**** |
| Total Cholesterol (mg/dl) | 237.38 ±12.19 (6) | 235.48 ±27.69 (6)# | 334.66 ±29.31 (6)* |
| HDL Cholesterol (mg/dl) | 119.20 ±11.71 (6) | 146.43 ±16.40 (6) | 139.30 ±11.93 (6) |
| Liver weight (g/g body weight) | 0.045±0.002 (6) | 0.050±0.002 (6)# | 0.063±0.004 (6)* |
| Hepatic total lipids (g/100 g) | 0.63 ± 0.14 (6) | 0.67 ± 0.08 (6) | 0.82 ± 0.10 (6) |

**Table 3.2:** Body weight gain, adipose depots mass, food mass and calorie intakes, and serum parameters of C, NO and HO groups

Values are expressed as means ± SEM; ( ) = number of animals. Adipose depots: sum of mesenteric, epididymal and retroperitoneal adipose depots. *p<0.05 *vs.* C; #p<0.05 NO *vs.* HO.

Serum glucose levels were significantly reduced in NO only in comparison to HO. Both EVOO groups presented reduced serum triglycerides levels. Total cholesterol levels were increased in HO while the increased HDL-cholesterol levels failed to reach statistical significance. HO presented increased liver weight in comparison to the normolipidic groups, with no significant differences in total hepatic fat content.

### 3.5.2. Hyperlipidic EVOO intake affected hepatic protein expression

Of the 2318 identified proteins, 487 proteins were compared among the groups. The 98 proteins significantly affected by EVOO intake are presented in table 3.3.

The NO group presented only 2 proteins down-regulated in relation to the C group. The HO group presented 43 up-regulated and 38 down-regulated proteins in comparison to the C group and 34 up-regulated and 25 down-regulated proteins in comparison to the NO group. Among this set of affected proteins, 42 proteins wre affected in both comparisons (25 upregulated and 17 downregulated).

With the aim of validating the proteomic results, we performed the analysis of cytosolic phosphoenolpyruvate carboxykinase (PEPCK) levels by Western blot. Figure 3.1 shows that the long term treatment with HO diet promoted a significant decrease of cytosolic hepatic PEPCK protein levels (30% *vs.* C and 34% *vs.* NO), confirming the proteomics data (58% *vs.* C and 55 % *vs.* NO; table 3.3).
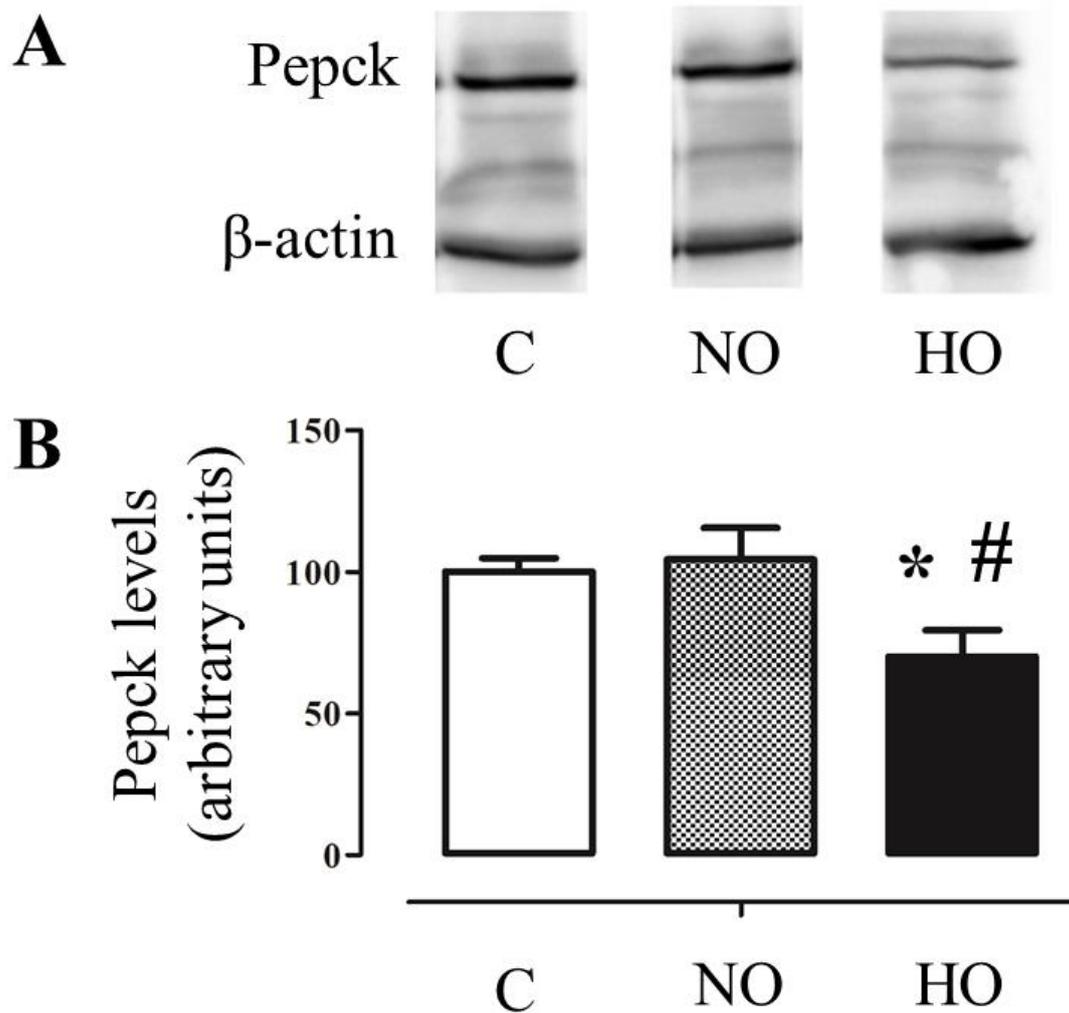
**Figure 3.1:** Cytosolic PEPCK protein levels. **a.** Representative images of Pepck (above) and loading control for protein normalization β actin (below). **b.** Quantifications of expression levels of Pepck protein in mice liver after 12 weeks of treatment with the diets (C/ n=14, NO/ n=10, and HO/ n=8). Data were analysed by one-way anova followed by Tukey´s post hoc test. *p<0.05 vs. C; #p<0.05 vs. NO. Data are expressed as mean ± SEM.

| UniProt ID | Protein | Fold-change (FDR) | | |
|---|---|---|---|---|
| | | NO/C | HO/C | HO/NO |
| Q8K2B3 | Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial | 0.81 (0.0426) | 0.67 (0.0001) | 0.83 (0.0319) |
| P97328 | Ketohexokinase | 0.67 (0.0310) | 0.78 (0.0281) | - |
| Q4LDG0 | Bile acyl-CoA synthetase | - | 0.21 (0.0047) | 0.23 (0.0128) |
| Q64464 | Cytochrome P450 3A13 | - | 0.31 (0.0232) | 0.30 (0.0208) |
| Q8JZR0 | Long-chain-fatty-acid-CoA ligase 5 | - | 0.38 (0.0058) | 0.43 (0.0213) |
| Q9Z2V4 | Phosphoenolpyruvate carboxykinase, cytosolic [GTP] | - | 0.42 (0.0188) | 0.45 (0.0383) |
| Q8VCN5 | Cystathionine gamma-lyase | - | 0.43 (0.0279) | 0.41 (0.0224) |
| Q91YI0 | Argininosuccinate lyase | - | 0.45 (0.0002) | 0.60 (0.0213) |
| P19783 | Cytochrome c oxidase subunit 4 isoform 1, mitochondrial | - | 0.52 (0.0226) | 0.53 (0.0348) |
| O08756 | 3-hydroxyacyl-CoA dehydrogenase type-2 | - | 0.55 (0.0047) | 0.60 (0.0208) |
| Q9DB20 | ATP synthase subunit O, mitochondrial | - | 0.60 (0.0047) | 0.60 (0.0080) |
| P55264 | Adenosine kinase | - | 0.63 (0.0283) | 0.61 (0.0213) |
| Q9QXX4 | Calcium-binding mitochondrial carrier protein Aralar2 | - | 0.64 (0.0079) | 0.67 (0.0213) |
| P00405 | Cytochrome c oxidase subunit 2 | - | 0.64 (0.0045) | 0.67 (0.0083) |
| Q8C196 | Carbamoyl-phosphate synthase [ammonia], mitochondrial | - | 0.67 (0.0188) | 0.64 (0.0116) |
| Q8R0F9 | SEC14-like protein 4 | - | 0.67 (0.0174) | 0.67 (0.0200) |
| Q9DCX2 | ATP synthase subunit d, mitochondrial | - | 0.69 (0.0079) | 0.74 (0.0440) |
| Q9CZU6 | Citrate synthase, mitochondrial | - | 0.72 (0.0347) | 0.72 (0.0440) |
| Q01853 | Transitional endoplasmic reticulum ATPase | - | 1.15 (0.0371) | 1.26 (0.0038) |
| P17742 | Peptidyl-prolyl cis-trans isomerase A | - | 1.22 (0.0290) | 1.25 (0.0213) |
| P05202 | Aspartate aminotransferase, mitochondrial | - | 1.22 (0.0089) | 1.20 (0.0208) |
| Q07417 | Short-chain specific acyl-CoA dehydrogenase, mitochondrial | - | 1.25 (0.0174) | 1.34 (0.0074) |
| Q9R0P3 | S-formylglutathione hydrolase | - | 1.28 (0.0336) | 1.41 (0.0111) |

| | | | | |
|---|---|---|---|---|
| P24549 | Retinal dehydrogenase 1 | - | 1.29 (0.0232) | 1.37 (0.0111) |
| P54869 | Hydroxymethylglutaryl-CoA synthase, mitochondrial | - | 1.29 (0.0232) | 1.31 (0.0213) |
| P14206 | 40S ribosomal protein AS | - | 1.29 (0.0302) | 1.66 (0.0016) |
| P68040 | Receptor of activated protein C kinase 1 | - | 1.31 (0.0371) | 1.34 (0.0335) |
| Q91V76 | Ester hydrolase C11orf54 homolog | - | 1.32 (0.0007) | 1.34 (0.0006) |
| P07724 | Serum albumin | - | 1.34 (0.0238) | 1.35 (0.0283) |
| Q91X34 | Bile acid-CoA:amino acid N-acyltransferase | - | 1.36 (0.0188) | 1.31 (0.0361) |
| Q9JII6 | Alcohol dehydrogenase [NADP(+)] | - | 1.38 (0.0457) | 1.41 (0.0440) |
| P10126 | Elongation factor 1-alpha 1 | - | 1.40 (0.0232) | 1.35 (0.0440) |
| Q9JHW2 | Omega-amidase NIT2 | - | 1.42 (0.0047) | 1.55 (0.0021) |
| Q9R0H0 | Peroxisomal acyl-coenzyme A oxidase 1 | - | 1.44 (0.0065) | 1.45 (0.0083) |
| Q9DBL7 | Bifunctional coenzyme A synthase | - | 1.49 (0.0028) | 1.42 (0.0080) |
| Q9DBF1 | Alpha-aminoadipic semialdehyde dehydrogenase | - | 1.51 (0.0002) | 1.43 (0.0006) |
| Q9Z2I8 | Succinate-CoA ligase [GDP-forming] subunit beta, mitochondrial | - | 1.59 (0.0079) | 1.45 (0.0250) |
| P51660 | Peroxisomal multifunctional enzyme type 2 | - | 1.59 (0.0079) | 1.66 (0.0083) |
| P28474 | Alcohol dehydrogenase class-3 | - | 1.66 (0.0077) | 1.64 (0.0111) |
| Q9D2G2 | Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial | - | 1.75 (0.0047) | 1.45 (0.0213) |
| Q61838 | Alpha-2-macroglobulin | - | 1.79 (0.0002) | 1.90 (0.0004) |
| Q8QZR5 | Alanine aminotransferase 1 | - | 2.15 (0.00005) | 1.73 (0.0005) |
| O70475 | UDP-glucose 6-dehydrogenase | - | 2.22 (0.0019) | 1.59 (0.0208) |
| P51881 | ADP/ATP translocase 2 | - | 0.33 (0.0047) | - |
| P54728 | UV excision repair protein RAD23 homolog B | - | 0.43 (0.0079) | - |
| Q91WN4 | Kynurenine 3-monooxygenase | - | 0.48 (0.0488) | - |

| | | | | |
|---|---|---|---|---|
| Q9DC69 | NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, mitochondrial | - | 0.49 (0.0047) | - |
| Q9D3D9 | ATP synthase subunit delta, mitochondrial | - | 0.52 (0.0080) | - |
| Q01768 | Nucleoside diphosphate kinase B | - | 0.53 (0.0216) | - |
| Q8CHT0 | Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial | - | 0.54 (0.0373) | - |
| Q9JI75 | Ribosyldihydronicotinamide dehydrogenase [quinone] | - | 0.54 (0.0104) | - |
| P56135 | ATP synthase subunit f, mitochondrial | - | 0.58 (0.0188) | - |
| Q9CQN1 | Heat shock protein 75 kDa, mitochondrial | - | 0.62 (0.0300) | - |
| Q99PL5 | Ribosome-binding protein 1 | - | 0.65 (0.0347) | - |
| P51174 | Long-chain specific acyl-CoA dehydrogenase, mitochondrial | - | 0.68 (0.0227) | - |
| P63276 | 40S ribosomal protein S17 | - | 0.70 (0.0188) | - |
| Q9D6Y9 | 1,4-alpha-glucan-branching enzyme | - | 0.70 (0.0232) | - |
| P62897 | Cytochrome c, somatic | - | 0.75 (0.0079) | - |
| Q61176 | Arginase-1 | - | 0.79 (0.0227) | - |
| Q8BMS1 | Trifunctional enzyme subunit alpha, mitochondrial | - | 0.80 (0.0392) | - |
| Q8BH00 | Aldehyde dehydrogenase family 8 member A1 | - | 0.83 (0.0110) | - |
| P50247 | Adenosylhomocysteinase | - | 0.84 (0.0442) | - |
| Q99LC5 | Electron transfer flavoprotein subunit alpha, mitochondrial | - | 0.85 (0.0302) | - |
| P16858 | Glyceraldehyde-3-phosphate dehydrogenase | - | 1.14 (0.0488) | - |
| Q99J08 | SEC14-like protein 2 | - | 1.14 (0.0488) | - |
| Q8VCR7 | Protein ABHD14B | - | 1.23 (0.0047) | - |
| Q91WS4 | S-methylmethionine-homocysteine S-methyltransferase BHMT2 | - | 1.24 (0.0111) | - |
| Q9CQ62 | 2,4-dienoyl-CoA reductase, mitochondrial | - | 1.25 (0.0096) | - |
| O55125 | Protein NipSnap homolog 1 | - | 1.32 (0.0232) | - |
| O08709 | Peroxiredoxin-6 | - | 1.38 (0.0047) | - |

| | | | | |
|---|---|---|---|---|
| P25444 | 40S ribosomal protein S2 | - | 1.39 (0.0232) | - |
| Q9JLJ2 | 4-trimethylaminobutyraldehyde dehydrogenase | - | 1.42 (0.0079) | - |
| P80318 | T-complex protein 1 subunit gamma | - | 1.46 (0.0026) | - |
| P60843 | Eukaryotic initiation factor 4A-I | - | 1.46 (0.0058) | - |
| Q64374 | Regucalcin | - | 1.52 (0.0346) | - |
| Q8VCW8 | Acyl-CoA synthetase family member 2, mitochondrial | - | 1.57 (0.0399) | - |
| Q9CZ13 | Cytochrome b-c1 complex subunit 1, mitochondrial | - | 1.59 (0.0047) | - |
| Q9DBJ1 | Phosphoglycerate mutase 1 | - | 1.83 (0.0365) | - |
| P62259 | 14-3-3 protein epsilon | - | 1.94 (0.0274) | - |
| Q3UEG6 | Alanine-glyoxylate aminotransferase 2, mitochondrial | - | 2.64 (0.0399) | - |
| P47199 | Quinone oxidoreductase | - | 3.76 (0.0020) | - |
| P28666 | Murinoglobulin-2 | - | - | 0.26 (0.0478) |
| P33267 | Cytochrome P450 2F2 | - | - | 0.32 (0.0440) |
| P50172 | Corticosteroid 11-beta-dehydrogenase isozyme 1 | - | - | 0.36 (0.0083) |
| O08914 | Fatty-acid amide hydrolase 1 | - | - | 0.51 (0.0440) |
| Q8VBT2 | L-serine dehydratase/L-threonine deaminase | - | - | 0.53 (0.0208) |
| P11352 | Glutathione peroxidase 1 | - | - | 0.63 (0.0375) |
| P99029 | Peroxiredoxin-5, mitochondrial | - | - | 0.72 (0.0383) |
| Q8CGP6 | Histone H2A type 1-H | - | - | 0.73 (0.0111) |
| P62702 | 40S ribosomal protein S4, X isoform | - | - | 1.36 (0.0440) |
| Q9EQF5 | Dihydropyrimidinase | - | - | 1.43 (0.0208) |
| Q8VCH0 | 3-ketoacyl-CoA thiolase B, peroxisomal | - | - | 1.44 (0.0083) |
| Q8QZT1 | Acetyl-CoA acetyltransferase, mitochondrial | - | - | 1.47 (0.0034) |
| P97823 | Acyl-protein thioesterase 1 | - | - | 1.56 (0.0213) |

| UniProt | Protein | | | FDR |
|---|---|---|---|---|
| Q9QXD6 | Fructose-1,6-bisphosphatase 1 | - | - | 1.57 (0.0083) |
| P0DP27 | Calmodulin-2 | - | - | 1.97 (0.0111) |
| Q9QYR9 | Acyl-coenzyme A thioesterase 2, mitochondrial | - | - | 2.09 (0.0141) |
| P43883 | Perilipin-2 | - | - | 3.12 (0.0171) |

**Table 3.3:** Hepatic proteins significantly altered by EVOO intake

Abbreviations: UniProt, Universal Protein Resource.
FDR: False Discovery Rate (corrected p-values according to Benjamini-Hochberg test).

### 3.5.3. Protein expressions affected by hyperlipidic EVOO intake associated with metabolic pathways

The overrepresentation tests indicated no significant pathway enrichments in the NO group in comparison to the C group.

In the HO group, 27 pathways were significantly enriched, with 8 pathways being affected in both pairwise comparisons, namely HO *vs.* C and HO *vs.* NO (Figure 3.2, Tables 3.4 and 3.5). These 8 pathways are: *metabolism* (HO/C: q= 2.61E-34; HO/NO: q= 3.07E-29), *metabolism of lipids* (HO/C: q= 1.10E-03; HO/NO: q=1.98E-07), *fatty acid metabolism* (HO/C: q = 1.33E-03; HO/NO:  q= 1.10E-04), *gluconeogenesis* (HO/C: q = 1.87E-02; HO/NO: q = 0,00528), *metabolism of amino acids and derivatives* (HO/C: q = 5.13E-11; HO/NO: q = 0,0000022), *tricarboxylic acid cycle (TCA) cycle and respiratory electron transport* (HO/C: q = 2.04E-07; HO/NO: q = 0,00317), *TCA cycle* (HO/C: q = 3.53E-03; HO/NO: q = 0,000985), and *biological oxidations* (HO/C: q = 8.31E-04; HO/NO: q = 0,00964).

HO vs C          HO vs NO



11
(40.7%)

8
(29.6%)

8
(29.6%)
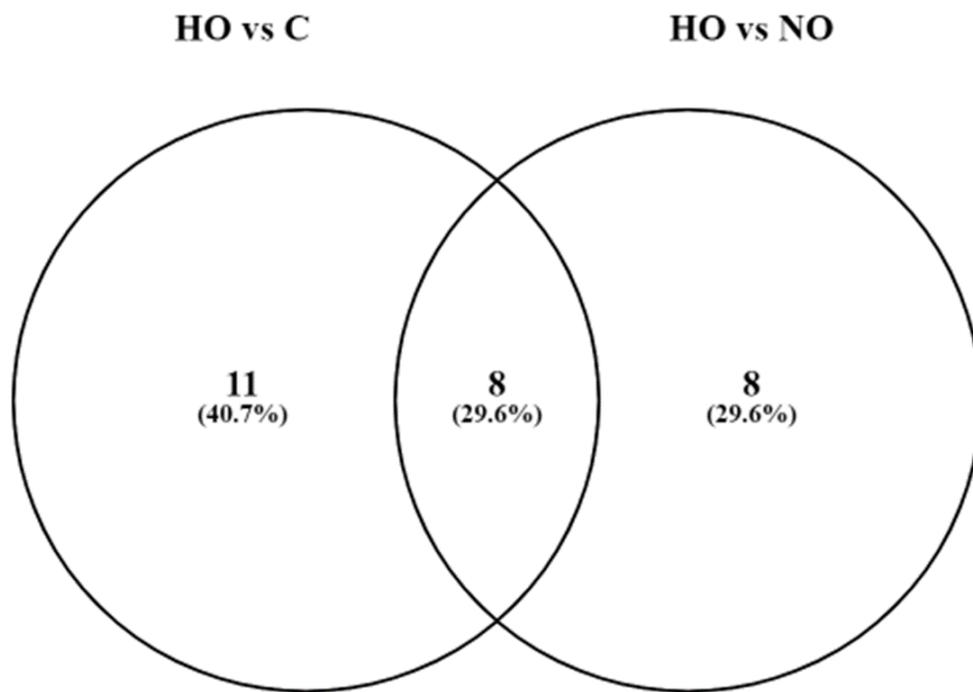
**Figure 3.2:** Number of pathways significantly enriched by the EVOO diets

| Pathways | | | | Protein ID (HO/C) | [b] Overlap ratio |
|---|---|---|---|---|---|
| **M e t a b o l i s m** | | | | ↓P51881, ↓P55264, ↓Q01768, ↑Q9DBL7 | 53/1818 (2.91%) |
| | **Metabolism of lipids** | **FA metabolism** | | ↑P07724, ↑Q91X34,↓ Q64464,↓ Q4LDG0, ↑P54869 | 13/649 (2%) |
| | | | | ↑P51660, ↓Q8JZR0, ↑Q9R0H0 | 8/199 (4%) |
| | | | Mitochondrial FA β-Oxidation | ↑Q8VCW8, ↑Q9CQ62 (Mitochondrial FA β-oxidation of SFA) | 5/39 (12.82%) |
| | | | | ↓Q8BMS1, ↑Q07417, ↓P51174 | 3/9 (33.33%) |
| | Metabolism of carbohydrates | | | ↓P97328, ↑P24549, ↓Q9D6Y9, ↑Q9JII6 | 8/261 (3.065%) |
| | | **Gluconeogenesis** | | ↓Q9QXX4, ↓Q9Z2V4, ↑P05202, ↑Q9DBJ1 | 4/35 (11.43%) |
| | **Metabolism of amino acids and derivatives** | | | ↑Q91WS4, ↓Q8VCN5, ↓O08756, ↓P50247, ↑Q9JLJ2, ↑Q8QZR5 | 15/249 (6.024%)' |
| | | Urea cycle | | ↓Q91YI0,↓Q8C196, ↓Q61176 | 3/10 (30%) |
| | | Glyoxylate metabolism and glycine degradation | | ↑Q9D2G2, ↑P05202, ↓Q8CHT0, ↑Q3UEG6, | 4/30 (13.33%) |
| | | Histidine, lysine, phenylalanine, tyrosine, proline and tryptophan catabolism | | ↓Q91WN4, ↑Q9DBF1,↑Q9D2G2, ↓Q8CHT0 | 4/42 (9.52%) |
| | **TCA cycle and respiratory electron transport** | | | | 10/136 (7.35%) |
| | | Respiratory electron transport, ATP synthesis and heat production | | ↓Q99LC5,↓Q9DC69 | 6/85 (7.06%) |
| | | | Formation of ATP by chemiosmotic coupling | ↓Q9DCX2,↓Q9DB20, ↓P56135,↓Q9D3D9 | 4/19 (21.05%) |
| | | **TCA cycle** | | ↑Q9D2G2,↑Q9Z2I8,↓Q8K2B3,↓Q9CZU6 | 4/22 (18.18%) |
| | **Biological oxidations** | | | ↑Q9R0P3, ↓Q9JI75, ↑P24549, ↑Q8VCR7, ↑Q9JII6, ↓P50247, ↑O70475, ↑P28474, ↓Q64464 | 9/258 (3.59%) |
| Mitochondrial biogenesis | | | | | 4/27 (14.81%) |
| | Cristae formation | | | ↓Q9DCX2, ↓Q9DB20, ↓P56135, ↓Q9D3D9 | 4/19 (21.05%) |
| HSF1 activation | | | | ↑P62259, ↑Q01853, ↑P10126 | 3/8 (37.5%) |

**Table 3.4:** Pathways significantly enriched by the HO diet, in comparison to the C diet, with their allocated proteins

Pathway enrichment analysis: Fisher's exact test followed by Bonferroni correction for multiple testing for FDR < 0.05. [b] Number of proteins with altered expression / total number of proteins in the pathway. Arrows indicate decreased (↓) or increased (↑) on protein expression.

| Pathways | | | | | Gene symbol of associated proteins (HO/NO) | [b]Overlap ratio |
|---|---|---|---|---|---|---|
| **M e t a b o li s m** | **Metabolism of lipids** | | | | ↑P54869, ↓P55264, ↑P97823, ↑Q9DBL7, ↑Q9EQF5 | 42/1818 (2.31%) |
| | | Metabolism of steroids | | | ↑Q8QZT1, ↓ Q64464,  ↑P54869 | 15/649 (2.31%) |
| | | | Bile acid and bile salt metabolism | | ↓P50172 | 5/130 (3.85%) |
| | | | | | ↑P51660 | 4/51 (7.84%) |
| | | | | Recycling of bile acids and salts | ↑Q91X34, ↓Q4LDG0, ↑P07724 | 3/19 (15.79%) |
| | | FA metabolism | | | ↑Q07417, ↓Q8JZR0, ↓P11352, ↓O08914, ↑Q9QYR9 | 8/199 (4.02%) |
| | | | β-oxidation of very long chain FA | | ↑P51660, ↑Q9R0H0, ↑Q8VCH0 | 3/13 (23.08%) |
| | | | Ω-3 and Ω-6 FA metabolism | α-linolenic acid metabolism | ↑P51660, ↑Q9R0H0, ↑Q8VCH0 | 3/14 (21.43%) |
| | **Gluconeogenesis** | | | | ↑Q9QXD6, ↓Q9QXX4, ↓Q9Z2V4, ↑P05202 | 4/35 (11.43%) |
| | **Metabolism of amino acids and derivatives** | | | | ↓Q8VCN5, ↓Q91YI0, ↑Q8QZT1, ↓O08756, ↓Q8VBT2, ↑Q9DBF1, ↑Q9D2G2, ↑P05202, ↑Q8QZR5, ↓Q8C196 | 10/249 (4.02%) |
| | **TCA cycle and respiratory electron transport** | | | | ↓Q9DCX2, ↓Q9DB20 | 6/136 (4.41%) |
| | | **TCA cycle** | | | ↑Q9D2G2, ↑Q9Z2I8, ↓Q8K2B3, ↓Q9CZU6 | 4/22 (18.18%) |
| | | Pyruvate metabolism | | | ↑Q9D2G2, ↑Q9Z2I8, ↓Q8K2B3, ↓Q9CZU6 | 4/51 (7.84%) |
| | **Biological oxidations** | | | | ↑Q9R0P3, ↑P24549, ↓P33267, ↑Q9JII6, ↑O70475, ↑P28474, ↓Q64464 | 7/258 (2.71%) |
| Peroxisomal protein import | | | | | ↑Q8VCH0, ↑P51660, ↑Q91X34, ↑Q9QYR9, ↑Q9R0H0 | 5/68 (7.35%) |

**Table 3.5:** Pathways significantly enriched by the HO diet in comparison to the NO diet, with their allocated proteins

Pathway enrichment analysis: Fisher's exact test followed by Bonferroni correction for multiple testing for FDR < 0.05. [b] Number of proteins with altered expression / total number of proteins in the pathway. Arrows indicate decreased (↓) or increased (↑) on protein expression.

## 3.6. DISCUSSION

In the present work, we evaluated the hepatic proteome of mice fed with diets enriched with different amounts of extra virgin olive oil (EVOO). An important component of the Mediterranean diet, EVOO is well known for its beneficial health properties, attributed to both its oleate and polyphenol contents (Visioli et al 2019). Olive oil comprises 56-80% of OA, a MUFA whose demonstrated effects include suppression of angiogenesis and anti-hypertensive actions (Milinovic et al 2019; Lamy et al 2014; Terés et al 2008). Olive oil also contains phytosterols, tocopherols, and polyphenols, among other microconstituents, whose amount vary as a function of region of production, cultivar type, maturation of olives, oil extraction method, etc (Ghisoni et al 2019, Gorzynik-Debicka et al 2018). Phenolic compounds are phytochemical plant-derived substances, formed by aromatic rings and hydroxyl groups, whose beneficial effects on several metabolic states, such as hyperglycemia and high blood pressure, have been attributed to their antioxidant activity (Rigacci & Stefani 2016). The most prevalent phenolic compounds found in olive oils are tyrosol, hydroxytyrosol, oleuropein and caffeic acid (Perona et al 2006).

It has been reported by us (Dornellas et al 2015, de Andrade et al 2015, Watanabe et al 2010) and reviewed by others (Hariri & Thibault, 2010; Buettner et al 2007), that rats fed chronically with high fat diets enriched with saturated or n-3/n-6 PUFAS, are able to reduce their food mass intake to match their caloric intake to that of control-fed rats. Here, we observed that both EVOO diets reduced both food mass and caloric intakes, with the HO diet having an even more pronounced effect. This finding agrees with a previous report showing that, even when combined with lard in high-fat diets, EVOO had a dose-dependent hypophagic effect (Cintra et al 2012). Based on the oleic acid content of 56-80% in Portuguese olive oil (Milinovic et al 2019) and of around 20% in soy oil (Silva et al 2018), we estimated the average cumulative intakes of oleic acid and found them to be 1.5 g in C, 3.1- 4.3 g in NO and 9.1 - 12 g in HO. These data are compatible with the present finding that the HO group showed the lowest intakes. It is interesting to note that body weight and adiposity were not affected in the HO group, while they even decreased in NO mice.

The hypophagic effect of EVOO diets may be explained by a central effect of oleic acid (Cintra et al. 2012, Obici et al. 2002).

Moreover, stimulation of hypothalamic oxytocin levels by oleate-derived oleoylethanolamide (OEA) has been reported (Sospedra et al 2015; Bowen et al 2017). In the HO group, the present proteomic analysis indicated enrichment of the fatty acid metabolism pathway and reduced expression of the enzyme fatty acid amide hydrolase (FAAH), which metabolizes OEA (Touriño et al 2010). The anorexigenic effect of OEA has been reported after its systemic or oral administration to rats (Nielsen et al 2004, Oveisi et al 2004) and the mechanisms involved have also been related to its high affinity by the peroxisome proliferator-activated receptor α (PPAR-α) (Fu et al 2003). A study in eutrophic humans showed that, after the ingestion of a meal rich in either olive oil or sunflower oil supplemented with OA, the serum levels of OEA were increased and associated with reduced caloric intake in the subsequent meal. However, only the individuals that consumed the EVOO meal presented reduced hunger and increased fullness and satiety sensations (Mennella et al 2015).

Figure 3.3 presents a summary of the main findings of the proteomic analysis. The present proteomic analysis indicated enrichment of the fatty acid metabolism pathway in the HO group. The conversion of long chain fatty acid (LCFA) to long chain (LC)-fatty acyl-CoA, catalyzed by the long-chain-fatty-acid-CoA ligases (ACSL), is the first step of both β oxidation and lipid synthesis. The enzyme ACSL-5 was downregulated in the HO group and previous studies indicated that its main role is to activate fatty acids for lipid synthetic pathways (Bu & Mashek 2010, Mashek et al 2005). Indeed, its hepatic expression was shown to be low in conditions of increased fatty acid oxidation, such as fasting and diabetes while it was enhanced in anabolic states such as insulin treatment and carbohydrate load (Achouri et al 2005). In the presence of oleate, hepatic cells showed reduction of triacylglycerol (TAG) formation and increased FA oxidation after ACSL5 gene-silencing (Bu & Mashek 2010) or increased TAG synthesis and storage after ACSL5 overexpression (Mashek et al 2005).

Differently from ACSL5, the acyl-CoA synthetase family member 2 (ACSF2), also named medium-chain acyl-CoA ligase, activates preferentially medium chain fatty acids (MCFA) (Fujino et al 2001). We found this enzyme to be up-regulated in the HO group. Other 2 enzymes of the β-oxidation of medium- and short-chain fatty acids, 2,4-dienoyl-CoA reductase 1 mitochondrial (DECR1) and SC acyl-CoA dehydrogenase (SCAD, mitochondrial), were up-regulated in the HO group. The enzyme 2,4-dienoyl-CoA reductase (DECR1) is considered an auxiliary enzyme in β-oxidation, since it acts only on unsaturated fatty enoyl-CoA esters. DECR1 null mice presented

hepatic steatosis and increased serum levels of products of incomplete oxidation of unsaturated fatty acids (Miinalainen et al 2009). Conversely, increased mitochondrial DECR1 activity and hepatic fatty acid oxidation were observed in response to a dose of eicosapentaenoic acid (EPA) but not of docosahexaenoic acid (DHA), and these mechanisms were attributed a role in the lowering of serum triglycerides and cholesterol levels promoted by EPA (Willumsen et al 1996).

Additionally, we found that 3 proteins upregulated by the HO diet participate in peroxisomal fatty acid β-oxidation (multifunctional enzyme type 2, acyl-coenzyme A oxidase 1- ACOX1, and 3-ketoacyl-CoA thiolase B). Because incomplete β oxidation of very long chain fatty acids in the peroxisome decreases ATP synthesis efficiency, it favors energy dissipation (Reddy & Hashimoto 2001). Enhanced peroxisomal β oxidation and energy expenditure was associated with improvement of hepatic lipid accumulation after consumption of high-fat diet enriched with n-3 PUFA, in comparison to saturated fat (Worsch et al 2018). However, chronic stimulation of peroxisomal fatty acid oxidation has been shown to impair hepatic mitochondrial fatty acid metabolism and favor hepatic fat accumulation and dyslipidemia during high-fat intake (Zeng et al 2017).

The present results agree with animal and human data showing that polyunsaturated- and monounsaturated-rich diets favored β-oxidation or reduced lipogenesis rate (Sanz et al 2000, Takeuchi et al 1995, Piers et al 2002, Bozzetto et al 2016). In addition, supplementation of mice fed high-saturated diet with EVOO reduced the hepatic expression and activity of lipogenic factors and diminished body fat, serum triacylglycerols and liver FFA levels, effects dependent on the antioxidant capacity of EVOO (Rincón-Cervera et al 2016).

The present findings allow the suggestion that β-oxidation was enhanced while lipid synthesis from LCFA was decreased by the HO diet, what agrees with our finding of reduced serum triglycerides. However, total serum cholesterol presented an increase in the HO group, what could not be totally attributed to increased HDL-cholesterol levels, indicating a deleterious effect of the excess fat intake. It is interesting to note that the NO group also presented beneficial effects of EVOO, namely reduced body fat and weight gain and triglycerides levels, with no evident hypercholesterolemia.

The finding of normoglycemia in the HO group contrasts with the impairment of glucose homeostasis after consumption of high-fat diets rich in saturated or n-6 polyunsaturated fatty acids (Cerf 2007).

The present finding agrees with demonstration of an antidiabetic effect of olive oil polyphenols (Bulotta et al 2014; Alkhatib et al 2018). Importantly, the HO group had reduced hepatic levels of phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting enzyme in hepatic gluconeogenesis, a pathway implicated in increased hepatic glucose production in type 2 diabetes and obesity (Roden & Shulman 2019). It is thus indicated that a reduced gluconeogenic rate contributed to the prevention of hyperglycemia in the HO group.

The present proteome study showed that the electron transport chain pathway (ETC) was significantly affected by the HO diet due to the downregulation of mitochondrial NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9 (Complex I) and ATP synthase (subunits d, O, f and delta). The gluconeogenesis pathway was also significantly altered in the HO group, which showed reduced expression of PEPCK. Although controversial, there are studies supporting the notion of inhibition of ETC Complex I as the mechanism involved in the reduction of hepatic gluconeogenesis induced by the anti-diabetic drug metformin. The consequent reduction of ATP/AMP ratio activates the cellular energy sensor AMP-activated protein kinase (AMPK) which then inhibits anabolic pathways, such as gluconeogenesis, while it stimulates catabolic pathways to yield ATP production (Viollet et al 2012; Hou et al 2018). These data are consistent with the present findings. Hydroxytyrosol, a polyphenol abundant in EVOO, has been shown to stimulate AMPK in the cerebral cortex of diabetic mice. However, this led to stimulation of Complex I (Zheng et al 2015). Polyphenols of green tea and red wine have been shown to inhibit the activity of ATP synthase in rat brain (Zheng & Ramirez 2000).

Liver proteome studies applying diet protocols comparable to the present one are scarce in the literature. A liver proteome and transcriptome study found that a high-fat saturated lard diet affected proteins involved in carbohydrate, lipid, amino acids and energy metabolism and that genes involved in lipogenesis were downregulated while genes of fatty acid oxidation were upregulated. Interestingly, these findings are comparable to the present ones using a high-fat EVOO diet. However, unlike the present results, those lard-fed mice presented increased body mass, NAFLD, increased serum TAG and insulin levels (Kirpich et al 2011). These discrepancies indicate that the presently observed hepatic proteomic alterations, induced by high EVOO intake, were successful in establishing a less detrimental body metabolic status, in comparison to high saturated fat.

Another liver proteome study found that, after induction of liver fibrosis, mice fed a high-fat EVOO diet presented better conditions of hepatic tissue architecture, reduced lipid peroxidation and reduced expression of hepatic fibrosis markers while antioxidant proteins were up-regulated, in comparison to corn oil-fed animals (Wang et al 2014). Interestingly, the protein S-formylglutathione hydrolase (FGH) was up regulated by high EVOO intake in both that and the present study. FGH is a key enzyme in the synthesis of glutathione (Xu & Sun 2017). Hepatic proteomic findings of up-regulation of several anti-oxidant enzymes were also reported by a study comparing high-fat diets rich in palm oil or olive oil. Data compatible with anti-atherogenic and triglyceride-lowering effects of EVOO were also shown (Arbones-Mainar et al 2007).

In summary, the present data showed that the high-fat intake of EVOO diet (HO group) inhibited food and energy intake, decreased serum triglycerides while it preserved normal patterns of body weight gain, body adiposity, and glucose levels. However, it increased total cholesterol levels and liver mass and tended to increase hepatic fat content. The examination of the pathway analysis derived from the proteomic data suggested stimulation of both mitochondrial and peroxissomal β-oxidation of fatty acids, and inhibition of lipid synthesis from LCFA and of gluconeogenesis in the HO group. On the other hand, although the NO group failed to show significant alteration of the liver proteome, it presented reduced body fat, body weight gain, and serum triglycerides and glucose levels, with no evident hypercholesterolemia.

In conclusion, the results allow the hypothesis that the hepatic metabolic adjustments in the HO, indicated by the proteomic analysis, were partially successful in avoiding/counteracting the detrimental outcomes of a long term high fat feeding. Contrastingly, since the beneficial effects of the NO diet could not be attributed to overt effects on hepatic metabolism, it is suggested that other tissues may have had a more relevant participation.

40
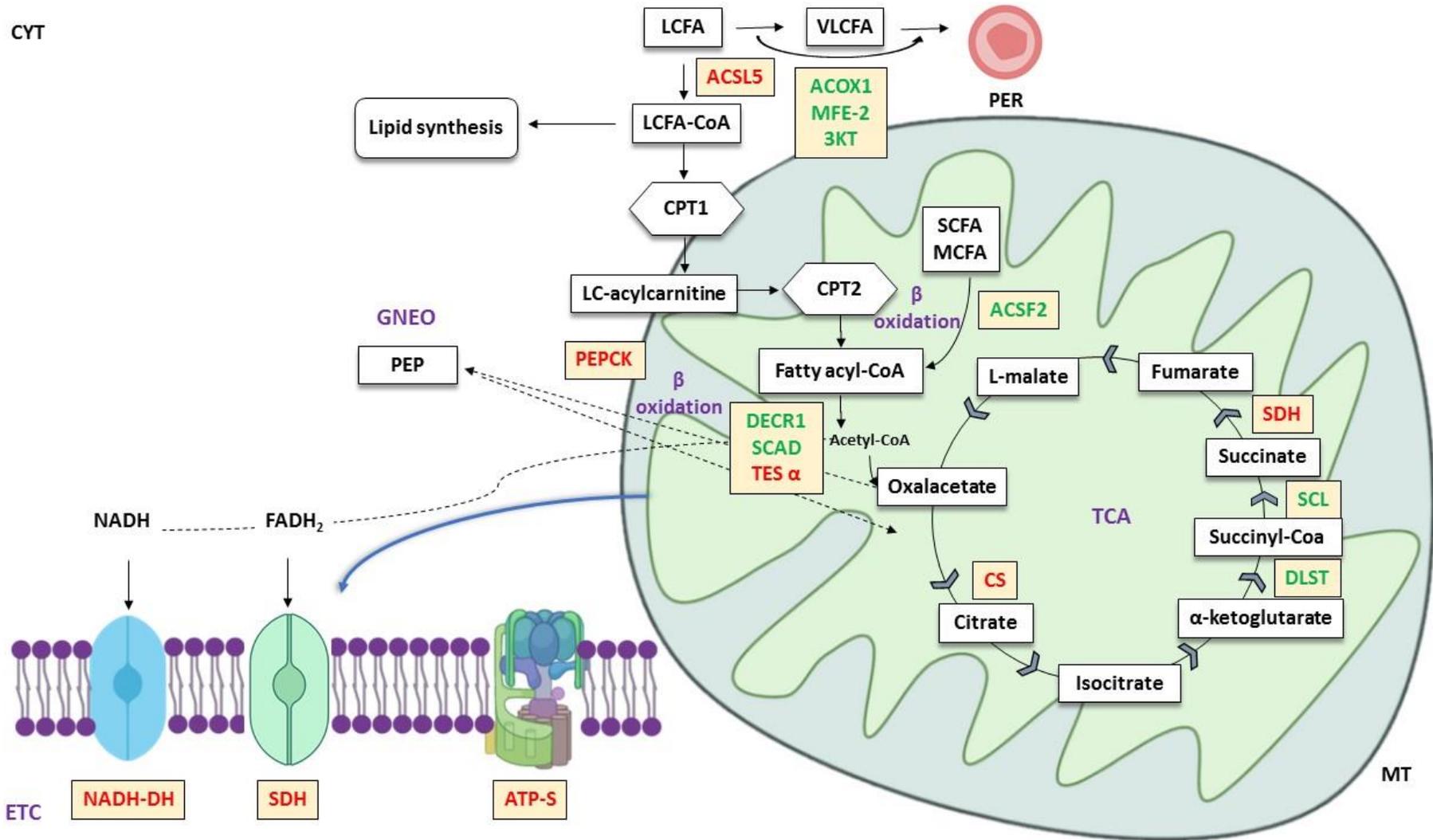
**Figure 3.3**. Summary of the main effects of high-fat EVOO intake on liver metabolic pathways, as indicated by the proteome analysis. Upregulated proteins are shown in green and downregulated proteins are shown in red. The pathways represented are tricarboxylic acid cycle (TCA), β-oxidation in both mitochondria (MT) and peroxisome (PER), gluconeogenesis (GNEO), electron transport chain (ETC).

CYT- cytoplasm; LCFA – long chain fatty acid; LCFA-CoA – Long chain fatty acyl-CoA; VLCFA – very long chain fatty acid; ACSL5- Long-chain-fatty-acid-CoA ligase 5; CPT1- carnitine palmitoyltransferase I; CPT2 - carnitine palmitoyltransferase II; LC – long chain; PEP – phosphoenolpiruvate; PEPCK - phosphoenolpyruvate carboxykinase; SCFA – short chain fatty acid; MCFA – médium chain fatty acid;  ACSF2 - Acyl-CoA synthetase family member 2; DECR1 - 2,4-dienoyl-CoA reductase; SCAD - short-chain specific acyl-CoA dehydrogenase; TESα - Trifunctional enzyme subunit alpha; CS – citrate synthase; DLST - dihydrolipoyllysine  succinyltransferase; SCL - succinate-CoA ligase; SDH - succinate dehydrogenase; ACOX1 - peroxisomal acyl-coenzyme A oxidase 1; MFE-2 - peroxisomal multifunctional enzyme type 2; 3KT - 3-ketoacyl-CoA thiolase B; NADH-DH - NADH dehydrogenase [ubiquinone]; ATP-S – ATP synthase.

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## 4. ARTICLE 2

### Dose dependent effect of palmitate and oleate on mitochondrial fusion and fission proteins and cell viability in hepatic cells (HepG2)

### 4.1. ABSTRACT

Normal mitochondrial dynamics depends on the balance between the fusion and fission processes. Mitochondrial dynamics dysfunction, along with endoplasmic reticulum (ER) stress, have been suggested to play a role in the effects induced by prolonged high fat feeding, including obesity, insulin resistance and non-alcoholic fatty liver disease. The mechanism involves long-term activation of the fission process and the unfolded protein response (UPR), the latter leading to stimulation of inflammatory pathways. Dietary fats differ in their lipotocixity degree, with saturated fatty acids being considered more harmful. The monounsaturated fatty acid oleate, the main constituent of olive oil, has been suggested to protect against lipotoxicity, but studies on the mechanisms involved as well as on the existence of a dose-dependency are not conclusive.

The aim of the present work was to evaluate the dose-dependent effects of saturated and monounsaturated fatty acids on cell viability, apoptosis, mitochondrial dynamics behavior and ER-UPR response *in vitro*. To this end, HEPG-2 cells were treated for 24 hours with 10 μM, 50 μM, 100 μM, 250 μM or 500 μM of either palmitate or oleate. The effects on apoptosis and cell viability were evaluated by the caspase-3 activity and MTT assay, respectively. Western blotting analysis was performed to evaluate the protein content of: a) mitofusin 2 (MFN2) and optic atrophy 1 (OPA1), markers of mitochondrial fusion process; b) dynamin-related protein 1 (DRP1), marker of mitochondrial fission process; and c) 78-kDa glucose-regulated protein (GRP78), marker of early ER stress.

Both fatty acids reduced cell viability at doses of 250μM and 500μM, and the highest dose had more pronounced effects. The two highest doses of either palmitate or oleate caused similar increases of Caspase-3 apoptotic activity. GRP78 levels were increased only by palmitate at the lowest and highest doses while no differences were induced by oleate. The levels of MFN2 were not significantly affected by the treatments, although a trend to increased values was observed with all oleate doses, while only the doses of 100 μM, 250 μM and 500 μM of palmitate promoted such a trend. OPA1 and DRP1 levels were not significantly affected by any treatment or dose.

The results showed that the saturated fatty acid elicited a condition of UPR induction and mitochondrial fission trend starting at low doses, whereas a high toxicity with marked reduction of cell viability were elicited at high doses. Differently, the monounsaturated fatty acid tended to induce mitochondria fusion at low doses associated with low toxicity even at high doses. This kind of study can help to understand cellular pathways activated by nutrients with protective effects towards metabolic diseases.

## 4.2. ABBREVIATIONS

ATF6 - activating transcription factor 6

ATP - adenosine triphosphate

BSA – bovine serum albumin

CHOP - C/EBP homologous protein

DAG - diacylglycerol

DMSO - dimethyl sulfoxide

DRP1 – dynamin-related protein 1

EDTA - ethylenediamine tetraacetic acid

ER – endoplasmic reticulum

FFA - free fatty acids

FIS1 – fission protein 1

GAPDH - glyceraldehyde-3-phosphate dehydrogenase

GLUT4 – glucose transporter 4

GRP78/BIP1 - 78-kDa glucose-regulated protein/ endoplasmic reticulum chaperone BIP

HepG2 - Human hepatocellular carcinoma cells

IR- insulin resistance

IRE1 - inositol-requiring kinase 1

MFN2 – mitofusin 2

MTT - Thiazolyl blue tetrazolium bromide

NAFLD - non-alcoholic fatty liver disease

NASH - non-alcoholic steatohepatitis

NEFAs - non-esterified fatty acids

OPA1 – optic atrophy 1

OXPHOS - oxidative phosphorylation

PBS - phosphate buffered saline

PERK - protein kinase RNA-like endoplasmic reticulum kinase

PMSF - phenylmethylsulfonyl fluoride

PUFA - polyunsaturated fatty acids

ROS - reactive oxygen species

SDS - sodium dodecyl sulphate

TAG – triacylglycerol

TLR4 - Toll-like receptor 4

UPR – unfolded protein response

## 4.3.    INTRODUCTION

Mitochondria are cell organelles responsible for supplying energy to the cell. Impairments in their function are involved in cellular stress and tissue/organ injury and, therefore, in the etiopathogenesis of metabolic diseases linked to imbalanced nutritional supply. At the cellular level, mitochondrial dysfunction, oxidative stress and endoplasmic reticulum (ER) stress have been proposed as mechanisms involved in insulin resistance and hepatic steatosis development (Ozcan et al 2004, Rieusset 2015, Montgomery & Turner 2015, Mollica et al 2011, Putti et al 2015, Putti et al 2016).

Mitochondrial dynamics behavior refers to the balance between two coordinate morphological processes. The fusion process is ruled by Mitofusin 1 and 2 (outer mitochondrial membrane) and by optic atrophy 1 (OPA1), which promote the fusion of the inner mitochondrial membrane and the mitochondrial cristae organization. Fusion is associated with the prevention of metabolic disturbances, being able to protect cells against insulin resistance (Putti et al 2015). The fission process is controlled by dynamin-related protein 1 (DRP1) and fission protein 1 (FIS1) and it is associated with mitochondrial dysfunction development, oxidative stress and apoptosis (Rovira-Llopis et al 2017). When the dynamism of those processes is disturbed, mitochondrial may become dysfunctional.

Mitochondrial dysfunction has been suggested to play a key role in pathology associated to high fat feeding and obesity, such as insulin resistance (IR) and non-alcoholic fatty liver disease (NAFLD). NAFLD has been considered the hepatic manifestation of the metabolic syndrome and is characterized by hepatic steatosis, which may progress to non-alcoholic steatohepatitis (NASH) or even to fibrosis and cirrhosis (Michelotti et al 2013; Mittal et al 2016; Yki-Järvinen 2014).

Mitochondria are functional and structurally linked to ER, and in conditions of chronic overnutrition and positive body energy balance, both mitochondria and ER can experience stress due to the overflow of substrates and metabolic pressure.

The shift of mitochondrial dynamics towards fission process is associate with endoplasmic reticulum (ER) stress. In conditions of lipids oversupply, the homeostasis of the endoplasmic reticulum may be affected and it needs to trigger the unfolded protein response (UPR), which is an intracellular signaling pathway between ER, cytoplasm and nucleus. This response aims to restores the normal function of the cells by interrupting protein translation, degrading misfolded proteins and/or activating pathways to increase

the production of chaperones involved in protein folding, such as glucose regulatory protein 78 (GRP78). However, if none of those mechanisms can effectively counteract the failure of protein-folding process, apoptosis pathways will be recruited (Liu & Kaufman 2003, Ozcan et al 2004).

The impairment of ER function is related to low grade inflammation, lipid accumulation, insulin resistance and cell apoptosis. Moreover, a long-term activation of UPR may lead to metabolic alterations associated to high fat feeding such as NAFLD and diabetes mellitus 2 (Salvadó et al, 2015). It has been shown that UPR sensors are involved in the cellular response to lipotoxic stress and in the regulation of hepatic steatosis. Indeed, ER plays a key role not only in protein folding but also in lipid biosynthesis and trafficking. Toxic lipids can directly activate UPR sensors, independently of the accumulation of misfolded proteins. The mechanism involved in the cross-talk between the UPR sensors, hepatic lipid metabolism, and lipotoxic stress, as well as the possible therapeutic potential of targeting the UPR in NAFLD have been recently reviewed by Song et al. (2019).

A long-term intake of a hypercaloric and high fat diet leads to a positive body energy balance, which induces the accumulation of excess fat in adipose tissue. However, in conditions of energy oversupply, when the fat-storing capacity of adipose tissue is exceeded, increased circulating free fatty acids (FFA), leptin resistance and reduced circulating adiponectin levels lead to decreased lipid oxidation in non-adipose tissues associated with ectopic accumulation of lipids, lipotoxicity, and IR. Hepatic injury occurs when the capacity of the hepatocyte to cope with an increased level of circulating fatty acids is overwhelmed (Sethi & Vidal-Puig 2007, Lionetti et al 2009, Mollica et al 2011). Given that the liver is a key organ for the maintenance of the metabolic homeostasis, any disturbance on its function could affect substantially glucose homeostasis and contribute to insulin resistance onset (Kim et al 2015).

Different types of dietary fatty acids contribute differently to hepatic lipotoxicity in the pathogenesis of NAFLD and insulin resistance (Putti et al 2015). Saturated fatty acids, particularly palmitate, the most abundant saturated fatty acid consumed in a typical western pattern diet, has been pointed out as the major fatty acid responsible for hepatic lipotoxicity and insulin resistance onset (Martins et al 2012, Cao et al 2014, Lee et al 2010, Wei et al 2006, Gustavo Vazquez-Jimenez et al 2016).

Palomer et al (2018) described the main mechanisms by which palmitic acid could lead to insulin resistance. Briefly, the increase on non-esterified fatty acids

(NEFAs) due to visceral obesity, promotes inflammation and insulin resistance by lipotoxicity, impairment of endoplasmic reticulum (ER) and mitochondria function and also by activation of Toll-like receptor 4 (TLR4) which, in turns, increase the activity of the IKK/NF-kB pathway, a proinflammatory pathway. Lipotoxicity is due to the accumulation of toxic lipid-derived substances as diacylglycerol (DAG) and ceramides, which in turn impairs insulin-signaling pathway.

Unsaturated fatty acids have been shown to be less toxic. In particular, omega 3 polyunsaturated fatty acids have been shown to have a protective effect in NAFLD and diabetes (Nobili et al 2011, Tanaka et al 2008, Ostermann & Schebb 2017; Lepretti et al 2018). At the cellular level, dietary omega 3 polyunsaturated fatty acids (PUFA) have been suggested to counteract insulin resistance and hepatic steatosis development by modulating mitochondrial bioenergetics and ER stress (Lepretti et al 2018).

A protective effect against insulin resistance and hepatic steatosis has been also ascribed to monounsaturated fatty acids, namely oleic acid, the predominant component in olive oil (70%-80%), the main fat source in the Mediterranean diet (Oliveira et al 2015, García-Ruiz 2015, Alkhateeb & Qnais, 2017, Palomer et al 2018, Chen et al 2018). In skeletal muscle, in which insulin resistance was induced by the incubation with palmitate, the treatment with 1mM of oleic acid for 12 hours was able to improve insulin signaling by interfering on Akt-2/AS160 phosphorylation and on GLUT4 translocation (Alkhateeb & Qnais, 2017). Moreover, it has been shown that oleic acid is mainly accumulated as triacylglycerols (TAG) whereas palmitic acid is preferably incorporated as DAG, which, unlike TAG, is a lipid-molecule that activates pro-inflammatory and impairs insulin pathways. Interestingly, the coincubation of oleic acid with palmitic acid is also able to revert the impairments caused by the saturated fatty acid (Palomer et al 2018).

In HepG2 cells, saturated fatty acids can reduce the production of ATP on a dose dependent manner, by impairing the activity of oxidative phosphorylation (OXPHOS) complexes. In addition, cells treated with saturated fatty acids present an elevated production of oxidative stress markers. On the contrary, cells incubated with olive oil did not presente any change on ATP production or on TBARS (thiobarbituric-acid-reacting substances) levels (García-Ruiz 2015).

Chen et al (2018) evaluated the effects of oleic acid in HepG2 cells and in rats with NASH induced by a high fat diet. The cells were treated with palmitic acid or oleic acid, or with both concomitantly, and the animals received a chow diet or a high fat diet

with partial or total replacement with olive oil. It was observed that oleic acid dose-dependently improved the toxicity framework and the cell apoptosis induced by the palmitic acid treatment. Additionally, it has been seen that oleic acid is able to prevent both oxidative and ER stresses induced by palmitic acid. The addition of oleic acid on cells treated with palmitic acid recovered the cells from the mitochondrial dysfunction caused by the saturated fatty acid.

Analyzing the dose-dependent effects could be useful to understand how cells physiologically adapt their homeostasis to respond to low doses of stressor agents, such as fatty acids, and to clarify the mechanisms by which the cell shifts between a physiological adaptation to a condition of marked stress with induction of cell death/apoptosis at high doses of stressor agents.

The aim of the present study was to investigate, in HepG2 cells, the effect of treatments with different concentrations of saturated (palmitate) or monounsaturated fatty acid (oleate). The effects on apoptosis and cell viability were evaluated by the caspase-3 activity and MTT assay, respectively. Mitochondrial fusion and fission process were analyzed through the protein levels of MFN2 and OPA1 (as markers of mitochondrial fusion) and of DRP1 (as marker of mitochondrial fission), as measured by western blotting. The effect on early ER stress was evaluated by analyzing protein levels of the UPR marker GRP78.

The results of the present study show that the saturated fatty acid elicited a condition of UPR induction and mitochondrial fission trend starting at low doses, whereas a high toxicity with marked reduction of cell viability were elicited at high doses. Adversely, the monounsaturated fatty acid tended to induce mitochondria fusion at low doses associated with low toxicity even at high doses. This kind of study can help to understand cellular pathways activated by nutrients with protective effects towards metabolic diseases.

## 4.4. MATERIALS AND METHODS

### 4.4.1. Cell Culture

Human hepatocellular carcinoma cells (HepG-2) were obtained from Interlab Cell Line Collection (Centro di Biotecnologie Avanzate, Genova, Italy). HepG-2 cells were cultured in 100 x 10 mm Petri dishes in Minimum Essential Medium supplemented with 10% fetal bovine serum (v/v), 1% (v/v) non-essential amino acids, 0.2 mM L-glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin (Invitrogen SRL, Milan, Italy). Cells were maintained at 37 °C in a 5% $CO_2$, 95% air-humidified atmosphere and passaged twice a week. Cells were then treated with saturated (sodium palmitate) or monounsaturated (sodium oleate) fatty acids (Sigma Aldrich) conjugated with bovine serum albumin (BSA). Briefly, cells were plated at 5 x $10^4$ cell/$cm^2$ and then cultured for 24 h in a medium containing palmitate or oleate at five different doses: 10 μM, 50 μM, 100 μM, 250 μM and 500 μM. This dose-range was chosen based on values reported for *in vitro* studies on mammalian cell models (Chen et al, 2018; Moravcová et al, 2015; García-Ruiz et al, 2015). In all cases, cells did not exceed 70% confluence at the time of the analysis.

### 4.4.2. Preparation of BSA-conjugated free fatty acids solutions

Palmitate and oleate 100 mM stock solutions were prepared in NaOH 0.1 M and were dissolved at 75ºC for 15 min under stirring. A 10% BSA fatty acid free (Sigma-Aldrich) solution was dissolved at 55º in NaCl 0.9% and mixed to the 100mM fatty acids solutions and maintained for more than 15 min in a shaking water bath at 55ºC. Then, the stock solutions were diluted to the final concentration of chosen experimental doses (500 μM, 250 μM, 100 μM, 50 μM and 10 μM) and stored at -20ºC. The final concentration of BSA in each experimental dose was 0.05%.

### 4.4.3. Cell viability and Caspase-3 assay

Cell viability was determined by using 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method (M5655, Sigma-Aldrich) which is a colorimetric assay based on the enzymatic conversion of MTT (yellow) into formazan (purple) by metabolically active cells, indicating the quantity of living cells (Grela et al, 2018). After incubation with palmitate or oleate, 0.5 mg/mL of MTT were added to 100µl of cell medium in 96-well plate and incubated for 1 h 30 min at 37 °C and 5% $CO_2$ to allow MTT to be metabolized. The resulting formazan crystals were dissolved in dimethyl sulfoxide (DMSO) and absorbances were measured in a microplate reader at 595 and 655 nm, providing the amount of living cells. The enzymatic Caspase-3 activity assay, for evaluating the apoptosis process, was performed according the manufacturer instructions (Caspase 3 Assay Kit, Colorimetric; CASP-3-C, Sigma-Aldrich).

### 4.4.4. Western blotting analysis

As mentioned above, cells were cultured for 24 h in medium containing palmitate or oleate conjugated with BSA at five different doses: 10 µM, 50 µM, 100 µM, 250 µM and 500 µM. At the end of the treatment, medium was discarded and cells were washed twice with ice-cold phosphate buffered saline (PBS), then were mechanically harvested and lysed with lysis buffer containing 50 mM Tris–HClpH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulphate (SDS), 1% Triton X-100, 1 mM ortovanadate, 2 mM PMSF, 10 mM NaF and the inhibitor cocktail consisting of 104 lM 4-(2-aminoethyl) benzenesulfonyl fluoridehydrochloride, 80 nM aprotinin, 4 lM bestatin, 1.4 lM E-64,2.0 lM leupeptin and 1.5 lM pepstatin A (Sigma–Aldrich).

After 30 min of incubation on ice, cell extracts were centrifuged at 13,000g for 20 min at 4° C, to remove cell debris. The protein concentration on lysates was measured with Bio-Rad Protein Assay Reagent (Bio-Rad, USA) using a BSA curve as a standard and about 30 µg of total proteins were separated by using Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). At the end of separation, protein bands were transferred to a PVDF blotting membrane (GE Healthcare, Life Sciences). The blots were then incubated with 5% skim milk in Tris-buffered saline (TBS) for 60 min, then incubated overnight at 4 °C with the primary antibodies of interest:

Mitofusin 2 (MFN2, 1:1000; Santa Cruz), DRP1 (1:1000; Santa Cruz), GRP78 (1:1000, Santa Cruz), and OPA1 (1: 1000, Novus Biologicals).

The second day, each membrane was washed in three time with TBS-Tween (15 minutes for each one), and incubated for 1 hour at room temperature with the specific secondary antibody: anti-mouse or anti-rabbit peroxidase secondary antibody (1:10,000; Bio-Rad Laboratories); anti goat (santacruz).

Immunocomplexes were revealed using a chemiluminescence detection kit (Immobilon Western, Millipore) according to the manufacturer's instructions. To normalize the amount of each protein in the total cellular extract, anti-GAPDH (1:2000,Abm) was used as loading control guide. Quantification of immunoblot films was performed with Quantify one (Bio-Rad Laboratories, Inc).

### *4.4.5. Statistical analysis*

Statistical analysis was performed using GraphPad prism 5 GraphPad software Inc. San Diego, CA, USA. One-way Anova analysis followed by Bonferroni post-test was used to evaluate the effect of fatty acids (palmitate or oleate) and the dose-effect (10,50,100,250,500μM). P values $\leq 0.05$ were considered statistically significant. Figures show representative blots.

## 4.5. RESULTS

### 4.5.1. Palmitate and oleate effects on cell viability, unfolded protein response (UPR) and apoptosis markers

To evaluate the palmitate and oleate cytotoxicity in hepatocytes, HepG2 cells were treated with five concentrations of both fatty acids (10 μM, 50 μM, 100 μM, 250 μM and 500 μM) for 24 hours. MTT assay showed that palmitate induced an impairment on cell viability in a dose-dependent manner (figure 4.1). No significant difference was found between BSA-treated and non-treated cells (data not shown). The cytotoxic effect of palmitate at the doses of 250 and 500 μM reduced the cell viability to 63% and 34% compared to BSA-treated cells, respectively. The viability of oleate-treated cells was significantly lower (52%) compared to BSA-treated cells only at the highest dose (500 μM). However, compared to palmitate, the viability of oleate-treated cells was significantly higher at 250 and 500 μM.
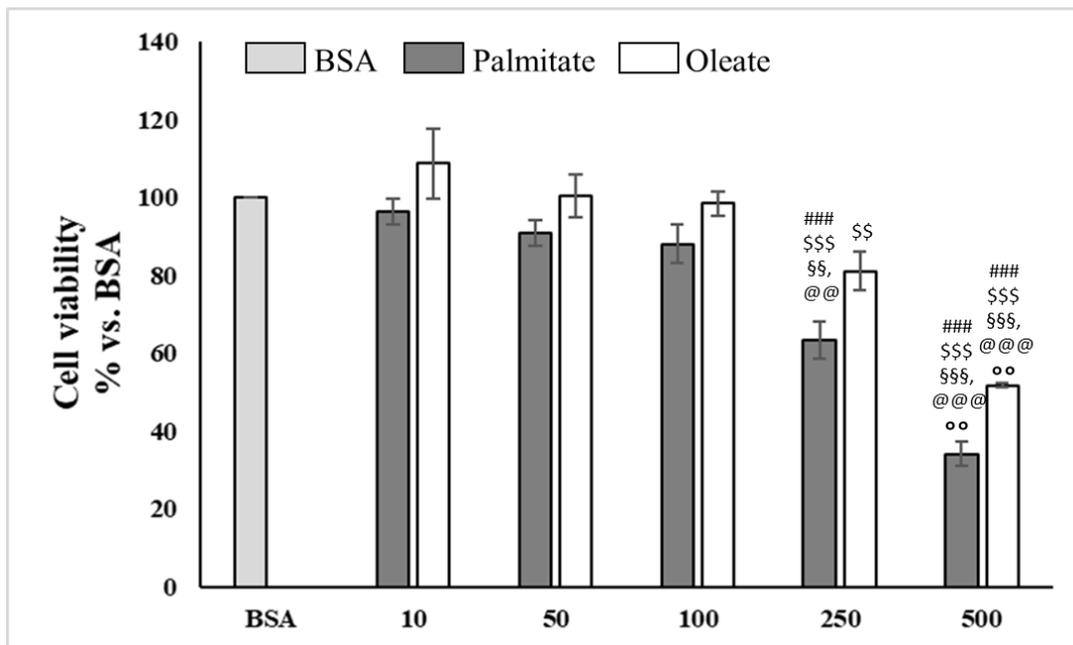
**Figure 4.1**: **Dose dependent effect of palmitate and oleate on HepG2 cells viability**. Cells were treated with five doses of palmitate or oleate (10 µM, 50 µM, 100 µM, 250 µM and 500 µM) for 24 hours. Data are presented as mean ± SEM with 3 biological replicates. One way ANOVA followed by Bonferroni post hoc analysis, was used for statistical analysis. Both palmitate and oleate induced a dose dependent changes in cell viability with the following P values: ### $P<0.0001$ vs. BSA, \$\$\$ $P<0.0001$ vs. 10 µM; §§ $P <0.01$, §§§ $P <0.0001$ vs. 50 µM; @@ $P<0.01$, @@@$<0.0001$ vs. 100 µM; °° $P<0.01$ vs. 250 µM.

To determine the effect on apoptosis, the activity of Caspase 3, a protease that directly participates as effector of programmed cell death by apoptosis, was analyzed. Palmitate induced an increase in caspase 3 activity in a dose-dependent manner (figure 4.2). The doses of 250 µM and 500 µM promoted an increase of around twice of BSA-treated cells levels. Oleate treatment showed a similar trend, but with lower increases (about 70% vs. BSA). Moreover, caspase 3 activity was 25% and 30% lower in oleate-treated compared to palmitate-treated cells at the doses of 250 µM and 500 µM, respectively.
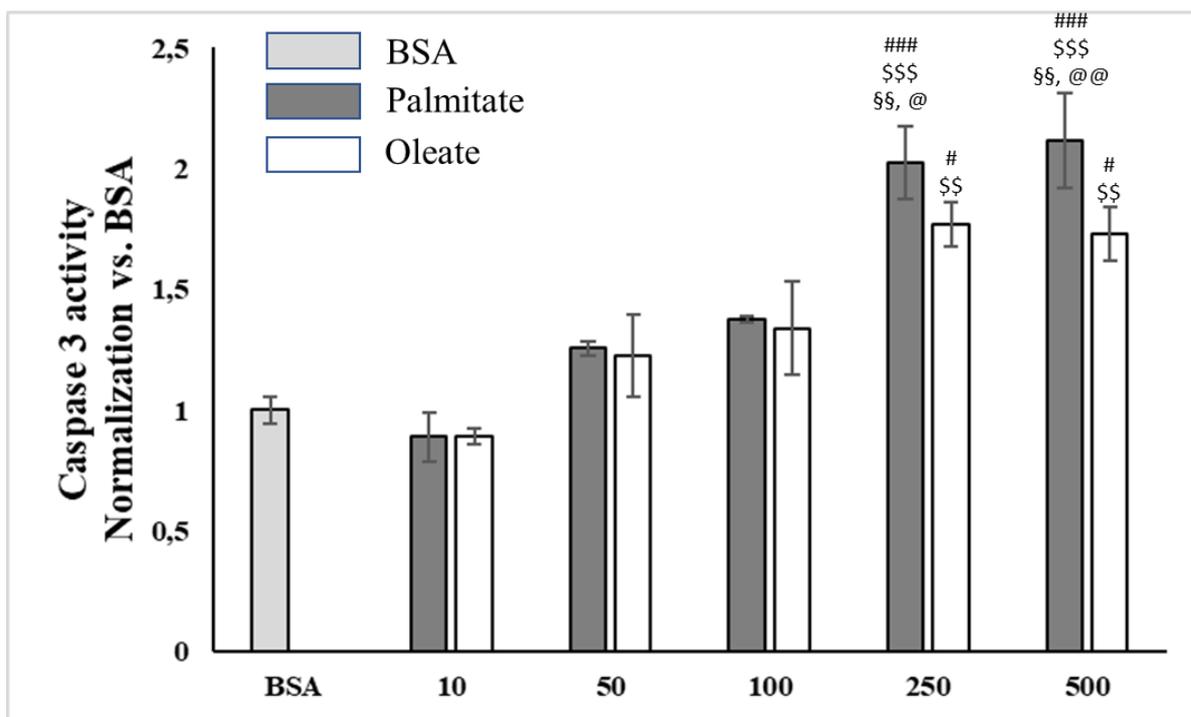
**Figure 4.2:** Effect of palmitate and oleate on caspase 3 activity. Cells were treated with 5 doses of palmitate or oleate (10 μM, 50 μM, 100 μM, 250 μM and 500 μM) for 24 hours. Data are expressed as mean ± SEM for 3 or 4 biological replicates. One way ANOVA followed by Bonferroni post hoc analysis, was used for statistical analysis. Both palmitate and oleate induced changes in caspase 3 activity. # $P<0.05$, ## $P<0.01$; ### $P<0.0001$ compared to BSA, \$ $P<0.05$, \$\$ $P<0.001$, \$\$\$ $P<0.0001$ vs. 10 μM; §§ $P <0.01$ vs. 50 μM  @ $P<0.05$, @@ $<0.01$ vs. 100 μM

GRP78/BIP1 is a marker physiologically ER stress and UPR and its levels were analyzed in the present study. Palmitate treatment induced a significant increase in GRP78 content of around 35% at 10 μM and 500 μM doses compared to BSA-treated cells. Oleate treatment did not induced any significant increment in protein content compared to BSA or palmitate. These findings suggest that palmitate rather than oleate induced the UPR at low and high doses (Figure 4.3).

**A)**
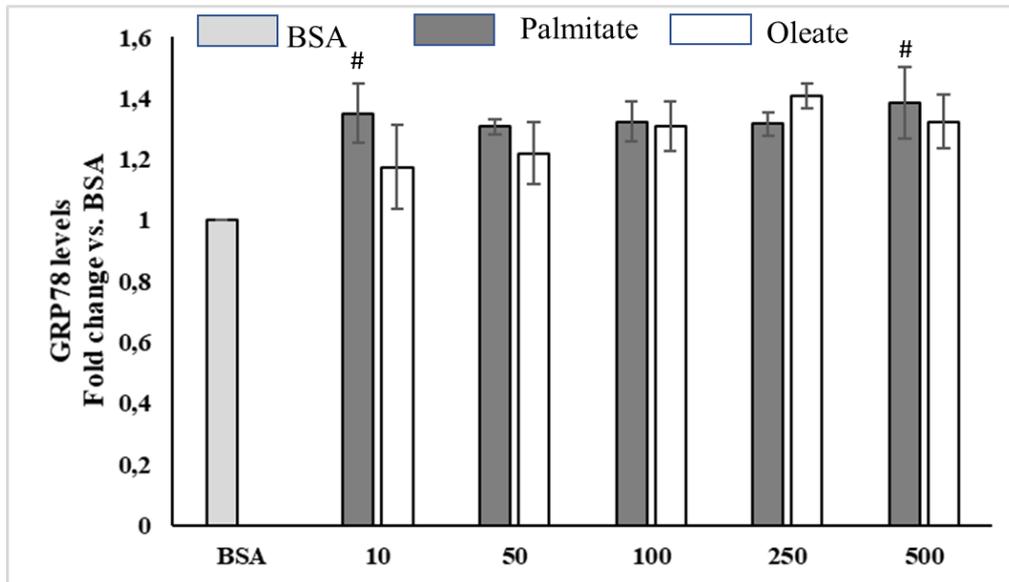


**B)**
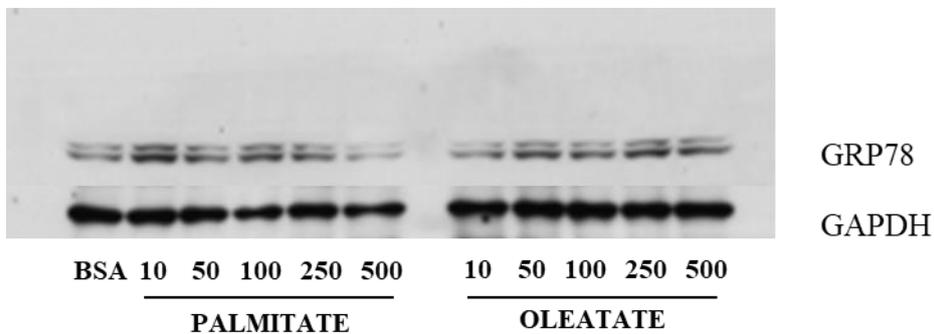


**Figure 4.3: GRP78 protein levels**. **A**. Protein levels of GRP78 in in HepG2 cells treated for 24 hours with palmitate or oleate. Data are presented as mean ± SEM for 4 biological replicates. One way ANOVA followed by Bonferroni post hoc analysis, was used for statistical analysis. Palmitate but not oleate induced increased GRP78 content. #$P<0.05$ compared to BSA. **B**. Representative images of GRP78 (above) and loading control for protein normalization GAPDH (below).

### 4.5.2. *Palmitate and oleate effects on mitochondrial fusion and fission protein markers*

To evaluate the effect of fatty acids on mitochondrial stress, particularly associated to their morphology and functional network, the main proteins involved in the mitochondrial dynamics machinery (MFN2 and OPA1 for fusion and DRP1 for fission) were evaluated in cell homogenates. Both palmitate and oleate did not induced significant changes in the level of MFN2 compared to control BSA-treated cells (figure 4.4), however it was observed a trend to decrease (about -15%) at the lowest palmitate dose (10 μM), whereas a trend to increase around +20% was observed at the highest doses (250 μM and 500 μM). Contrarily, oleate induced a trend to increase in MFN2 content at all doses studied, compared to BSA. This increase was dose dependent with an increase of around 10% with the lowest dose and 40% at the highest doses (250 μM and 500 μM). Moreover, in comparison with palmitate treatment, oleate induced a trend of increased MFN2 content at all doses (figure 4.4).

**A)**
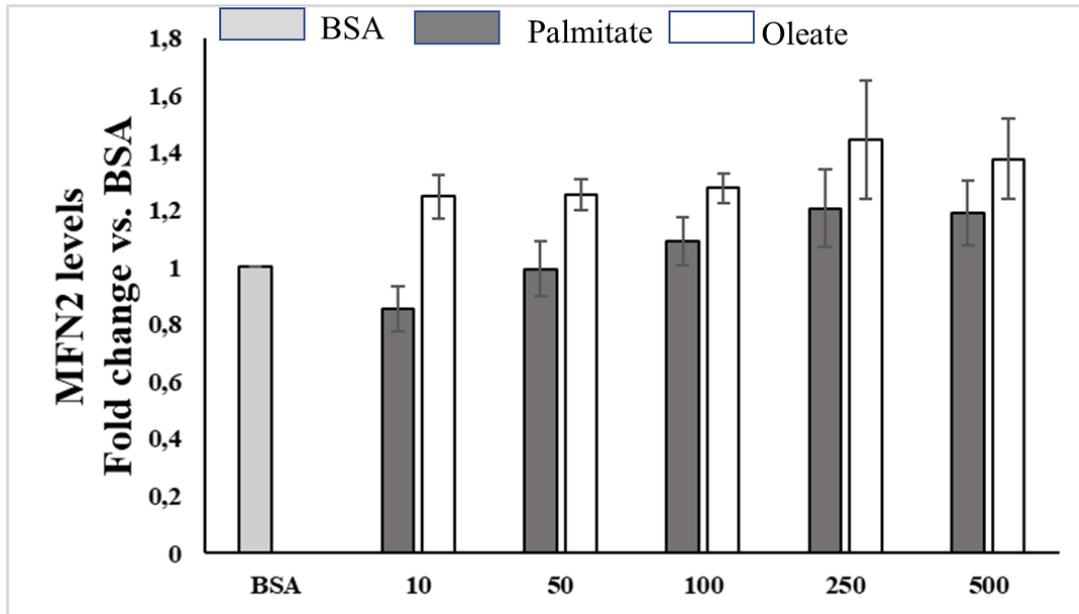


**B)**
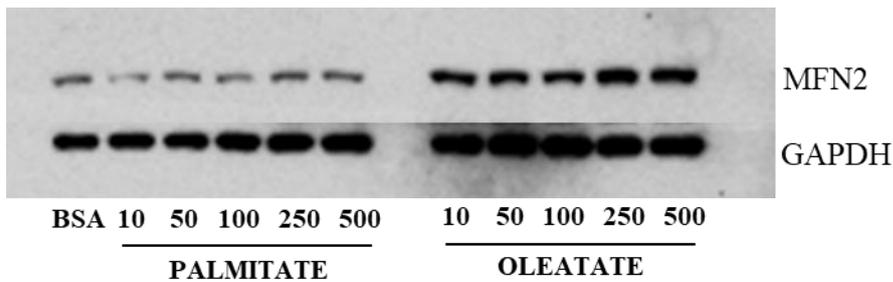


**Figure 4.4: Mitofusin 2 protein levels. A.** Protein levels of Mitofusin 2 in HepG2 cells treated for 24 hours with palmitate or oleate. Data are presented as mean ± SEM for 5 biological replicates. One-way ANOVA followed by Bonferroni post hoc analysis was used for statistical analysis. **B.** Representative images of Mitofusin 2 (above) and loading control for protein normalization GAPDH (below).

As concerns to palmitate and oleate effects on cellular OPA1 content, no significant differences were observed(figure 4.5).

A)
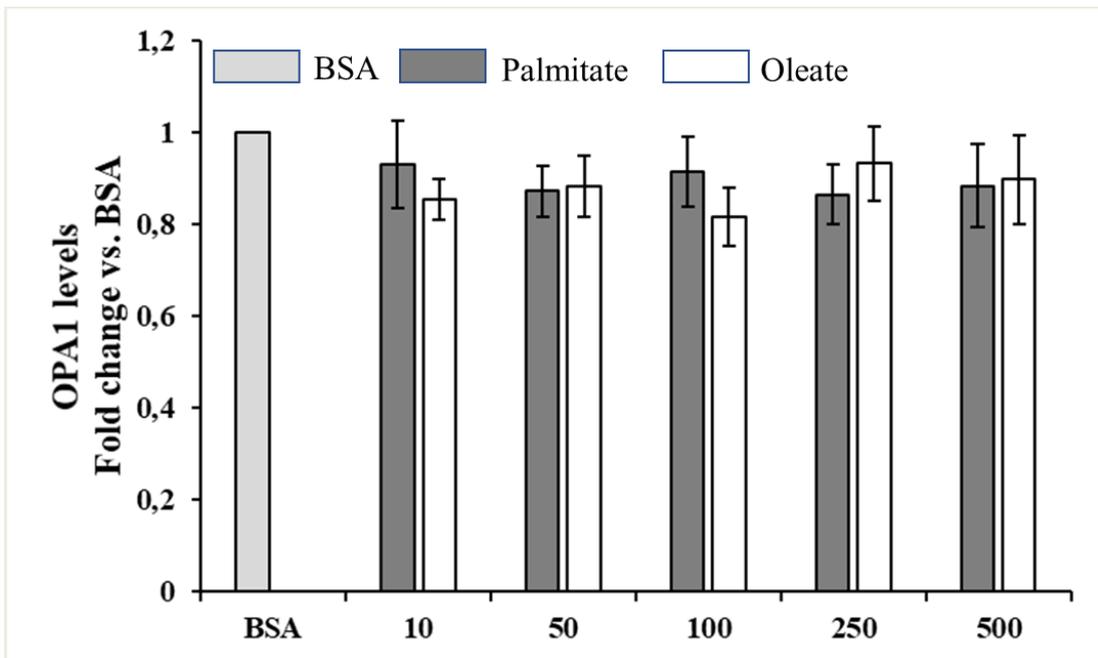


B)
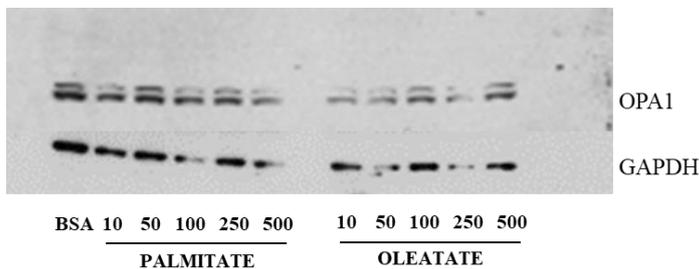


**Figure 4.5: OPA1 protein levels. A.** Protein levels of OPA1 in HepG2 cells treated for 24 hours with palmitate or oleate. Data are presented as mean ± SEM for 4 biological replicates. One-way ANOVA followed by Bonferroni post hoc analysis was used for statistical analysis **B.** Representative images of OPA1 and loading control for protein normalization GAPDH (below).

As regards to fission process, Figure 4.6 shows that palmitate induced a trend to increase in DRP1 protein levels compared to BSA of around 40% at the lower doses (10 μM, 50 μM and 100 μM) and around 20% at the higher doses (250 μM and 500 μM). Oleate induced a trend to decrease in DRP1 content (about 20%) at the lowest dose (10 μM) compared to palmitate, and a trend to increase of 30% at all other doses compared to BSA.

**A)**
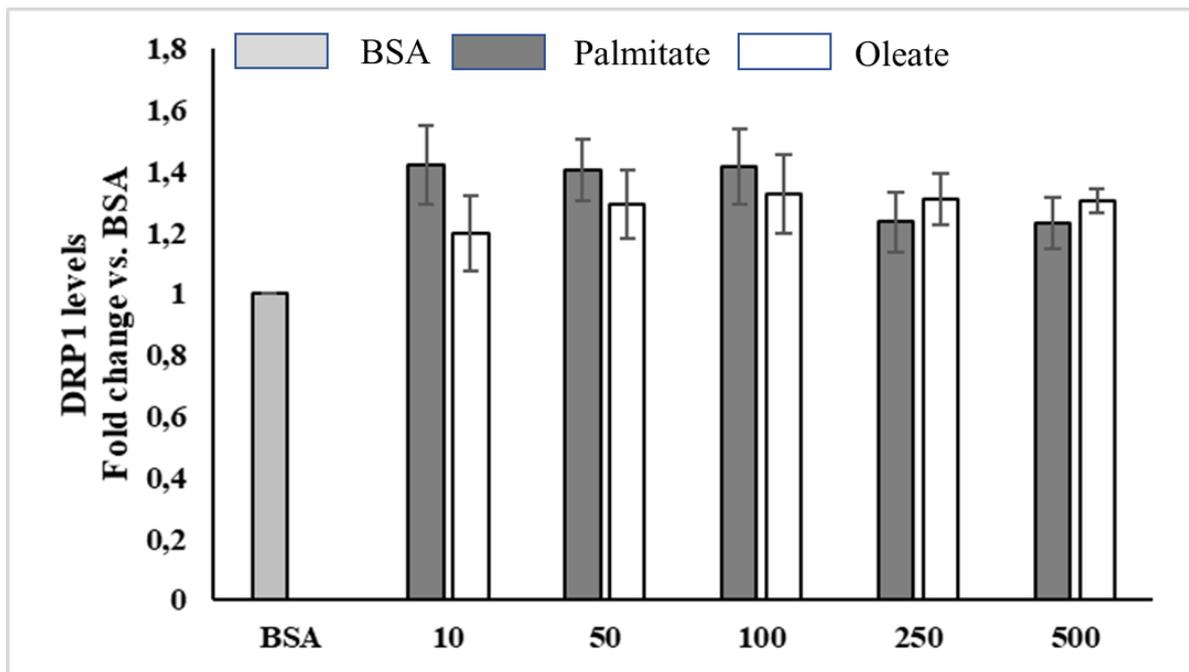


**B)**
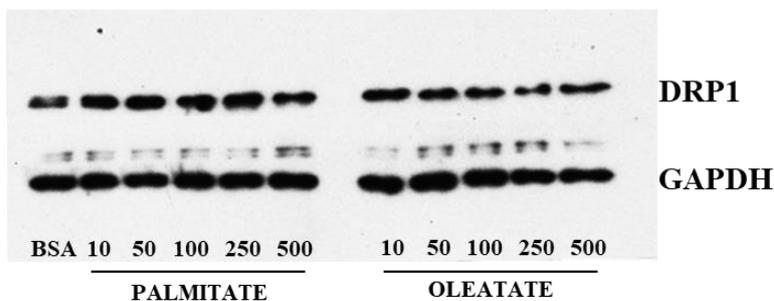


**Figure 4.6: DRP1 protein levels. A.** Protein levels of DRP1 in HepG2 cells treated for 24 hours with palmitate or oleate. All data are presented as mean ± SEM for 6 biological replicates. One-

way ANOVA followed by Bonferroni post hoc analysis was used for statistical analysis. **B.** Representative images of OPA1 and loading control for protein normalization GAPDH (below).

## 4.6. DISCUSSION

In this study, we showed that the type (saturated or monounsaturated) of fatty acids differently affects mitochondrial fusion/fission processes, ER stress onset and cell viability in a dose dependent manner in HepG2 cells. At a low dose (10 μM), cell viability was maintained through cellular adaptive responses against to the oversupply of fatty acids. Palmitate, rather than oleate, induced a trend to increased fission protein DRP1 associated with early ER stress onset, as shown by the increase GRP78 content. At the same dose of 10 μM, oleate trended to induce a shift towards fusion process on mitochondrial dynamics as shown by the trend to increase in MFN2 content. In addition, compared to both BSA and palmitate, GRP78 content was not affected, suggesting that UPR was not triggered. This response contributed to the maintenance of higher viability in oleate than in palmitate treated cells, in particular at the highest doses. Indeed, viability in palmitate treated cells decreased 63% and 34% at the doses of 250 μM and 500 μM respectively, compared to control cells. At the highest dose (500 µM), oleate induced a less cytotoxic effect (decrease to 52%) compared to palmitate (decrease to 34%) compared to control. In line with these findings, the caspase 3 activity was less induced in oleate than palmitate treated cells, at the highest doses (250 μM and 500 μM).

In agreement with our data, Kim et al (2019) observed also a dose-dependent decrease in mesangial cells number with palmitate doses ranging from 12.5 µM to 400 µM. It was also observed that palmitate induced apoptosis and oleate, even when administrated at a high dose as 800 µM, was able to protect the cells against it (Chen et al, 2018). However, in disagreement with this, Liu et al (2019) have observed any impact in the viability of pancreatic cell line with oleate treatment. This could be due to the inherent differences between the cells and their tissue origin. In addition, an increase on apoptotic cells was also described, in accordance with the raise in caspase-3 activity observed in the present study (Kim et al 2015).

These results confirm previous data showing that palmitate is lipotoxic to the cells. Lipotoxicity occurs when the adipose tissue becomes unable to store the excessive amount of circulating fatty acids, distributing the excess to other peripheral tissues (liver, muscles, pancreas), as well as their toxic metabolites, i.e. ceramides, diacylglycerol, and fatty acyl-Coa and also may impair the production of insulin by pancreatic β cells (Ye et al, 2019).

There are studies that correlate lipotoxicity with NAFLD, insulin resistance and, diabetes 2 onset. The possible mechanisms involved could be metabolic and cellular disturbances, as endoplasmic reticulum stress, mitochondrial dysfunction and inflammation (Engin 2017). It is well known that saturated fatty acids are lipotoxic, while unsaturated fatty acids could be less toxic or even reduce apoptosis promoted by saturated fatty acids. One of the mechanism by which palmitate could impairs mitochondrial function could be the increased reactive oxygen species (ROS) production which, in turns, induce apoptosis and alters mitochondria genetic material (Manucha et al 2015). Oleic acid is the most abundant fatty acid present in olive oil.  In vivo and in vitro experiments have demonstrated that the treatment with oleic acid is able to reverse the oxidative stress, apoptosis, ER stress, mitochondrial dysfunction, inflammation and fibrosis caused by palmitic acid (Kwon et al 2014; Chen et al 2018; Rial et al 2010).

When cells are submitted to a stressful stimulus, such as oversupply of nutrients, the unfolding protein response (UPR) can be triggered in ER, inducing inflammatory responses. The UPR is mediated by 78-kDa glucose-regulated protein (GRP78) and the C/EBP homologous protein (CHOP). In an homeostatic state, GRP78 remains associated to other three UPR sensors: activating transcription factor 6(ATF6), inositol-requiring kinase 1 (IRE1) and protein kinase RNA-like endoplasmic reticulum kinase (PERK) and, when misfolded proteins are detected, GRP78 splits from those proteins and triggers the UPR (Ayaub et al 2016).

 ER stress has been associated with NAFLD and with insulin resistance in organs, such as liver, muscles, and adipose tissue (Lionetti et al 2009, Mollica et al 2011, Lepretti et al 2018). It has been reported that diabetic patients present impairment in the communication between mitochondria and endoplasmic reticulum in beta cells (Thivolet et al 2017).

In the present study, we focused only on the content of GRP78 as marker of early ER stress and UPR onset, due to its role in the cellular adaptive response, to restore ER function and cellular homeostasis avoiding cellular death. Our results showed that GRP78 protein content increased only in palmitate treated cells. With the limitation that other markers of ER stress should had been analyzed, we can suggest that ER stress was induced at the low palmitate doses, and that this ER stress had evolved towards apoptosis at the highest doses, as shown by the increase in caspase 3 activity. Adversely, oleate may had induced cellular adaptive responses that attenuated the lipotoxicity and cellular death due to high fatty acid supply.

The present findings suggest that oleate induced a trend to increase in MFN2 with no variation in DRP1 cellular content. It has been shown that obese and diabetic patients presented reduced muscular MFN2 expression and after weight loss this condition was reversed (Lahera et al 2017). Taken together, these results suggested that at the lowest dose used in this study, palmitate induced a shift toward fission processes, whereas oleate induced a trend shift toward fusion processes. No changes we found in the content of OPA1 both in palmitate and in oleate treated cells. This finding is in contrast with the suggestion of increased fusion processes with oleate. However, we measured total OPA1 content in cell homogenates, whereas differences could be found in isolated mitochondria and/or in the content of the different isoforms of OPA1. Further experiments are needed to elucidate OPA1 contribute to oleate and palmitate effects on HepG2 cells.

The results of the present study show a dose-dependent effect of both palmitate and oleate in mitochondrial fusion/fission markers, UPR and cell viability. At low doses, no changes in cell viability were observed with both fatty acids, but a different cellular response was probably activated by either fatty acid to maintain cellular homeostasis and viability at the high doses. In the present study, saturated fatty acid induced a shift towards fission processes on mitochondrial balance associated with induction of UPR, whereas monounsaturated fatty acid induced a shift towards fusion processes. Mitochondrial fission and UPR onset at low doses of palmitate were associated with a lower cell viability and while high doses of palmitate induced higher apoptosis activity. Mitochondrial fusion was associated with higher cell viability and lower apoptosis induction at high doses of oleate. Therefore, it can be suggested that the mechanism of cellular adaptation at low doses can predict the outcome at high dose exposure and that oleate is protective towards cell stress and toxicity.

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## 5.    CONCLUSION

The results of both the *in vivo* and the *in vitro* studies indicated that hepatic metabolic effects of EVOO and oleic acid are influenced by the quantity administered.

The 12 weeks on an EVOO high-fat feeding probably challenged the liver in terms of adaptive responses against the deleterious effects of a long-term lipid oversupply. These responses were triggered and counteracted efficiently the eventual and expected detrimental effects of high-fat feeding on corporal and serum parameters, normally provoked by imbalanced diets, as high-fat saturated diets. However, whether these adaptations will persist after a more prolonged consumption of the HO diet needs to be determined. Moreover, the long-term consumption of the NO diet induced beneficial outcomes in body and serum parameters, without affecting liver metabolism. This may indicate the participation of tissues other than the liver in the effects of normolipidic EVOO effects.

The mitochondrial dynamics alterations seen with oleate in HepG2 cells demonstrated that the high doses were also detrimental and that the type of fatty acid is an important criterion to be considered.

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