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Department of Internal Medicine and Therapeutics

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Role of fatty acids and oxidative stress in non-alcoholic fatty liver disease: identification of innovative molecular target

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List of abbreviation

AC: adenylate cyclase

ACC: acetyl-coA carboxylase

ACPD: (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid

ADA: adrenic acid

ADP: adenosine di-phosphate

ALA: α-linoleic acid

ALT: alanine aminotransferase

AP: alkaline phosphatase

APS: ammonium persulphate

ARA: arachidonic acid

AST: aspartate aminotransferase

ATP: adenosine triphosphate

BSA: bovine serum albumin

cAMP: cyclic adenosine monophosphate

CDPPB: 3-cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl)benzamide

CHPG: *(R,S)*-2-chloro-5-ydroxyphenylglycine

ChREBP: carbohydrate response element binding protein

CNS: central nervous system

COX-2: cyclooxygenase-2

CPPHA: N-[5-chloro-2-[(-1,3-dioxoisoindolin-2-yl)methyl]phenyl]-2-hydroxybenzamide

CRD: cysteine-rich domain

CTRL: control

CYP: cytochrome P450

Cys: cysteine

D4D: delta 4 desaturase

D5D: delta 5 desaturase

D6D: delta 6 desaturase

D12D: delta 12 desaturase

D15D: delta 15 desaturase

D9-16D: delta 9-16 desaturase

D9-18D: delta 9-18 desaturase

DAG: diacylglycerol

DAMP: damage-associated molecular pattern

DCD: donation after cardiac death

DCF: dichlorofluorescein

DDAH: dimethylarginine dimethylaminohydrolase

DFB: 3,3′-difluorobenzaldazine

DGAT: diacylglycerol acyltransferase

DGLA: dihomo-gamma-linoleic acid

DHA: docosahexaenoic acid

DHPG: *(R,S)*-3,5-dihydroxyphenylglycine

DMA: Dimethylamine

DMSO: dimethyl sulfoxide

DNL: *de novo* lipogenesis

DPA: docosapentaenoic acid

ECD: extended criteria donor

ECL: enhanced chemiluminescence substrate

ECM: extracellular matrix

EDTA: ethylenediaminetetraacetic acid

eNOS: endothelial nitric oxide synthase

EPA: eicosapentaenoic acid

ETA: eicosatetraenoic acid

FA: fatty acid
FAD: fatty acid desaturase
FAO: fatty acid oxidation
FAS: fatty acid synthase
FBS: fetal bovine serum
FFA: free fatty acid
FXR: farnesoid X receptor
G6P: glucose-6-phosphate
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
GERD: gastroesophageal reflux disease
Glu: glutamate
DHPG: *(R,S)*-3,5-dihydroxyphenylglycine
GLA: gamma linoleic acid
GRM5: glutamate metabotropic receptor 5 gene
GSH: glutathione
HBV: hepatitis B virus
HCC: hepatocellular carcinoma
H_2DCFDA: 2',7'-dichlorofluorescein
HF: high fat
HIF: Hypoxia-inducible factors
HMP: hypothermic machine perfusion
HOPE: hypothermic oxygenated perfusion
HTK: histidine tryptophane ketoglutarate
ICAM: intercellular adhesion molecule
IGL: institues Georges Lopez
IHTG: Intrahepatic Triglyceride
IL: interleukine
iNOS: inducible nitric oxide synthase
IP3: inositol 1,4,5-trisphosphate
IR: ischemia/reperfusion
IRi: ischemia/reperfusion injury
KO: knockout
KRH: krebs/henseleit buffer
LA: linoleic acid
LDH: lactate dehydrogenase
LDL: low dendity lipoprotein
LL: left lobe
LPL: lipoprotein lipase
LXR: liver X receptor
MAPK: mitogen-activated protein kinase
MCPG: (*S*)-α-methyl-4-carboxyphenylglycine
MCP: macrophage chemotactic protein
MDA: malondialdehyde
mGluR: metabotropic glutamate receptor
MHC: major histocompatibily complex
ML: median lobe
MP: machine perfusion
MPEP: 2-Methyl-6-(phenylethynyl)pyridine
MPPA: N-methyl-5-(phenylethyl-2-amine)
MTEP: 3-((2-Methyl-4-thiazolyl)ethynyl)pyridine
MUFA: monounsaturated fatty acid
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
NAFLD: non-alcoholic fattly liver disease
NAM: negative allosteric modulator
NASH: non-alcoholic steatohepatitis
NCBI: national center for biotechnology information
NHBD: non heart beating donor
NMDA: N-methyl-D-aspartate
NF-κB: nuclear factor κ-light-chain-

enhancer of activated B cells

NMP: normothermic machine perfusion

NO: nitric oxide

NOS: nitric oxide synthase

OCA: obeticholic acid

PAM: positive allosteric modulator

PBS: phosphate buffered saline

PEG: polyethylene glycol

PK: protein kinase

PLCβ: phospholipase Cβ

PNF: primary non function

PPARα: peroxisome proliferator-activated receptor α

PRR: pattern recognition receptor

PUFA: polyunsaturated fatty acid

PVDF: polyvinylidene difluoride

Quis: quisqualic acid

RL: right lobe

ROS: reactive oxygen species

RT: room temperature

RT-PCR: real time-polymerase chain reaction

SAM: S-Adenosyl methionine

SCD: stearoyl-CoA desaturase

SCS: simple cold storage

SDS-PAGE: sodium dodecyl sulphate - polyacrylamide gel electrophoresis

SFA: Saturated fatty acid

SOD: superoxide dismutase

SREBP: sterol regulatory element binding proteins

SA: stearidonic acid

SNMP: subnormothermic machine perfusion

TAG: triacylglycerol

TBARS: thiobarbituric acid reactive substances

TEMED: N,N,N,N'-tetra-methyl-ethylendiamine

TG: triglycerides

TGF: transforming growth factor

TLR: toll-like receptor

TNF-α: tumor necrosis factor-α

TZD: thiazolidineodiones

UW: university of Wisconsin

VCAM: vascular cell adhesion molecule

VFD: venus flytrap domain

VLDL: very low dendity lipoprotein

ABSTRACT

Non-Alcoholic Fatty Liver Disease (NAFLD) is a common disease condition characterized by a relevant intracellular lipid deposition, in liver parenchyma hepatocytes. It is a chronic condition that affects about a third of the United States population and it is often associated with a broad spectrum of metabolic abnormalities, including dyslipidemia, hypertension, insulin resistance and obesity; symptoms that are collectively known as "metabolic syndrome" (Barshop et al. 2008). About 10% of patients suffering from NAFLD progresses to Non-Alcoholic Steatohepatitis (NASH), a more serious pathological condition, less common than simple steatosis, that is a risk factor for the development of cirrhosis and development of Hepatocellular Carcinoma (HCC) which occurs, according to recent studies, in 4-27% of cases (Starley et al. 2010).

The development and progression of NAFLD to NASH represent a complex pathophysiological process. Currently, the most accredited hypothesis is that a sequence of insults, called “hits”, are involved in the onset and progression of liver damage (Day & James 1998). The first hit is the process that causes fat accumulation, in particular triglycerides, in hepatocytes. Instead, the second hit is a multifactorial phenomenon in which lipotoxicity, oxidative stress, inflammation and environmental factors participate (Paschos & Paletas 2009). The number of triglycerides depends principally on a complex interaction among four elements: hepatic fatty acid uptake, *de novo* fatty acid synthesis, Fatty Acid Oxidation (FAO) and fatty acid export within Very Low Density Lipoprotein-Triglycerides (VLDL-TG). Another crucial factor that characterizes NAFLD is the excessive formation of Reactive Oxygen Species (ROS) (Sanyal et al. 2001). Following hepatic steatosis, the liver cells become sensitive to the action of oxidative stress, which is usually considered the second hit and so responsible for the progression of NAFLD. This occurs in patients suffering from non-alcoholic hepatic steatosis because the excess of Free Fatty Acids (FFAs) undergoes oxidation within hepatocytes.

FFAs can be subdivided into three categories: saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA). SFAs have negative effect because they reduce receptor-mediated uptake of LDL by the liver and this is the predominant mechanism whereby such saturated fatty increased plasma LDL cholesterol concentrations (Woollett et al. 1992; Spady

et al. 1993). Instead, MUFAs may balance the pro-inflammatory effects of SFAs. Finally, can be distinguished two main types of PUFAs: omega-3, such as α-Linolenic Acid (ALA, 18:3n-3), or Docosahexaenoic Acid (DHA, 22:6n-3), and omega-6 fatty acids, as linoleic acid (LA, 18:2n-6) and Arachidonic Acid (ARA, 20:4n-6). An important feature of the PUFAs is that they are the major component of cell membranes. In particular, omega-3 are considered to be pivotal nutrients for preventing NAFLD (Gormaz et al. 2010); they alleviate lipid accumulation, pro-inflammatory actions, ROS production and promote the FAO. PUFAs are formed by the activity of the desaturases, enzymes that insert double bonds in existing polyunsaturated fatty acids or make the PUFA *de novo* in mammals, such as delta-5 (D5D), and delta-6 desaturases (D6D).

Nowadays, in order to compensate for the growing number of patients on waiting lists for liver transplantation, transplantation centers adopted extended criteria donors for graft selection, moving the limit of acceptance for marginal organs, such as steatotic livers. Since steatotic livers, as well as all marginal organs, are more susceptible to Ischemia Reperfusion (IR) injury and Primary Non Function (PNF) once transplanted into the recipient, there is an evident need to improve liver preservation methods. At the moment, there are two different approaches of preservation: static and dynamic. Simple Cold Storage (CS) at 4°C is the method for static storage, in which the low temperature reduces metabolic activities that might lead to cellular degradation. Instead, Machine Perfusion (MP) at 20°C is a dynamic technique employed to preserve organs during transplantation. The mechanisms underlying the early hepatic graft dysfunction and poor recipient outcome are mainly two. Fat droplets have been seen to expand during cold preservation altering the infrastructure of the cell itself by displacing the surrounding organelles (Takeda et al. 1999). With the expansion of these droplets and the increase in the hepatocytes size secondary to swelling, the microcirculation of the liver tends to deteriorate. Also activation of Kupffer cells appears to play an important role in PNF: in fact, they are responsible of free radical generation that, through the inhibition of β-oxidation, mediates lipogenesis (Takeda et al. 1999; Brass & Roberts 1995).

A variety of techniques has been tested to address one or more of the putative mechanisms that predispose steatotic livers to IR injury. These approaches generally consist of using pharmacological agents or preconditioning methods to turn on protective pathways before subjecting the liver to IR stress (Nativ et al. 2012).

Nowadays, to address the lack of knowledge in molecular mechanisms of NAFLD pathogenesis, novel targets to reduce fatty accumulation have been investigated (Feldstein et

al. 2003; Donato et al. 2006). From the literature, it is known that in the brain, metabotropic Glutamate Receptors subtype 5 (mGluR5) has a role in appetite and energy homeostasis. The acute and chronic appetite suppression and reduction in body weight and adiposity in obese rats suggest a novel role for mGluR5 for intervention in the treatment of chronic obesity in humans (Bradbury et al. 2004).

In this study we used three different models of liver steatosis, a Methionine and Choline Deficient (MCD)-diet rat model, a Zucker-rat model and an *in vitro* model of steatosis in hepatic cells, HepG2 cells.

In the first part, we made a comparison between the MCD nutritional model and the Obese Zucker genetic one preserving the livers either by cold preservation at 4°C or by machine perfusion at 20°C. CS preservation induced a marked release in hepatic and biliary enzymes in Obese Zucker rats when compared with the MCD group. The same trend occurred for the bile flow. No difference was found when comparing MP in MCD and obese Zucker rats. Fatty acid analysis demonstrated that the SFAs/PUFAs ratio was lower in MCD group respect to Obese Zucker rats. The data obtained suggest that the lipid composition of the liver affects the susceptibility of the organ following preservation through cold storage at 4°C. Our results not only confirm the data presented by Fukumori and colleagues on the crystallization of fat at low temperatures, but also provide us further information: the quality of lipid constituents might influence hepatic injury during preservation by CS more than the quantity of lipid content and energy status. The livers of MCD rats have a higher PUFA content. This composition makes them less susceptible to cold preservation than Obese Zucker group, which, instead, shows high levels of SFAs. It is precisely the SFAs, therefore, that at low temperatures form crystalline structures causing cell breakage. Through these analyses, we confirm that the lipidomic liver profiling is a sensitive indicator of graft function and that the quality of lipid constituents might influence hepatic injury. According to this hypothesis, the reduced CS injury observed in our MCD rat model, may be justified by the higher PUFAs levels that makes them less susceptible to cold preservation, possibly reducing the deleterious tendency to lipid crystallization.

Secondly, to further clarify the events that occur during the development and progression of NAFLD, we focused our attention on the changes in fatty acid desaturases, enzymes that insert a stereospecific double bond between specific carbons, D5D, D6D, D9-16D and D9-18D, and their relationship with oxidative stress and TNF-alpha in two rat models of NAFLD.

Liver fatty acid profiling was used for the quantification of desaturase activities. D6D was found only in MCD rats. The activity of D5D and D9-16D was higher in Obese Zucker rats, and in MCD rats was found an increase in the D9-18D activity occurred. D5D and D9-16D showed a negative correlation versus TBARS, ROS and TNF-α, but a positive correlation with GSH. A positive correlation between D9-18D versus TBARS, ROS and TNF-alpha and a negative correlation with GSH was also found. Thus, D9-16D and D9-18D seem to be involved in opposite effects in the experimental rat models considered in this study.

Another important factor involved in the development of NAFLD is DHA, a polyunsaturated fatty acid. In 2014, Stanković and colleagues have demonstrated that DHA had antioxidant and anti-inflammatory effects in MCD group (Stanković et al. 2014). We support these results, since we found a significant negative correlation between DHA versus oxidative stress, and a correlation also between oxidative stress versus TNF-α. Although there is still much to clarify regard the pathophysiology of NAFLD, these results reinforce and extend findings on the identification of potential therapeutic targets able to counteract this common disorder.

To identify novel molecular targets, we used *in vitro* hepatic cellular models of steatosis. Bradbury and colleagues, in their animal model of steatosis, demonstrated that at the level of the brain, mGluR5 might be involved in modulating appetite and energy homeostasis. In our study, we identified mGluR5 in a human hepatocyte-derived cell line, HepG2, and we used a mixture of palmitic acid (PA, C16:0), a saturated FA, and oleic acid (OA, C18:1), a monounsaturated FA as a recognized model of *in vitro* steatosis.

HepG2 cells were subjected to the orthosteric agonist of mGluR5, DHPG 100 µM alone or in combination with various concentrations of negative allosteric modulator of mGluR5: MPEP 0.3, 3, 30 µM. The analysis of lipid content shows, for the first time, that the mGluR5 in HepG2 cells has a role in lipid intracellular accumulation. In particular, the mGluR5 activation by DHPG, increases cell lipid uptake; on the contrary, the blockade by MPEP administration, reduces DHPG-induced cell FA accumulation. Furthermore, mGluR5 seems to be involved also in the apoptotic process: in fact, we demonstrated that the blockade of mGluR5 with MPEP reduces the caspase-3 protein expression.

In conclusion, we firstly showed that not only the amount, but also the composition of fatty acids influence hepatic injury during preservation in steatotic liver. In fact, the MCD fatty livers that have a higher PUFA content, are less susceptible to cold preservation than Obese

Zucker group, which, instead, shows high levels of SFA; this latter forms crystalline structures at low temperatures, causing cell breakage. Secondly, to further clarify the events that occur during the development and progression of NAFLD, the changes in fatty acid desaturases, and their relationship with oxidative stress and TNF-α show that the NAFLD is characterized by high levels of oxidative stress and the activation of fatty acid desaturation leads to pro-inflammatory condition. Finally, our data show for the first time that the mGluR5 in HepG2 cells has a role in lipid intracellular accumulation. In particular, the mGluR5 activation by DHPG increases cell lipid uptake; instead, the blockade by MPEP administration reduces DHPG-induced cell FA accumulation. Moreover, differently from what observed in other models of liver damage, such as ischemia/reperfusion injury and acetaminophen hepatotoxicity, hepato-protection from lipid accumulation observed using MPEP, appears to be associated with a ROS-independent mechanism. Our results support the idea that the advancement in the understanding of the pathophysiology of liver steatosis might lead to the development of novel therapeutic alternatives, whose the mGluR5 negative allosteric modulators represent a feasible and truthful example.

Introduction

1. Non-Alcoholic Fatty Liver Disease (NAFLD)

Non-Alcoholic Fatty Liver Disease (NAFLD) is a common disease condition characterized by a relevant intracellular lipid deposition, in the form of triglycerides, in liver parenchyma hepatocytes. It is a chronic condition that affects about a third of the United States population and it is often associated with a broad spectrum of metabolic abnormalities, including dyslipidemia, hypertension, insulin resistance and obesity; symptoms that are collectively known as "metabolic syndrome" (Barshop et al. 2008). Worldwide, NAFLD affects about a third of the population and, in Western countries, its incidence varies between 20-30% (Browning et al. 2004) up to a value of 54-70% in obese subjects (Angulo 2002). The increasing incidence of this pathology in Western society makes NAFLD the leading cause of recovery in liver clinics in the United States. About 10% of patients with NAFLD progress to a more severe disease state, known as Non-Alcoholic Steatohepatitis (NASH).

Steatohepatitis is distinguishable from NAFLD because it involves inflammatory cells, fibrosis and a higher amount of collagen around the veins and cells. NASH, less common than simple steatosis, is a risk factor for the development of cirrhosis and development of Hepatocellular Carcinoma (HCC) which occurs, according to recent studies, in 4-27% of cases (Starley et al. 2010) (Figure 1.1).

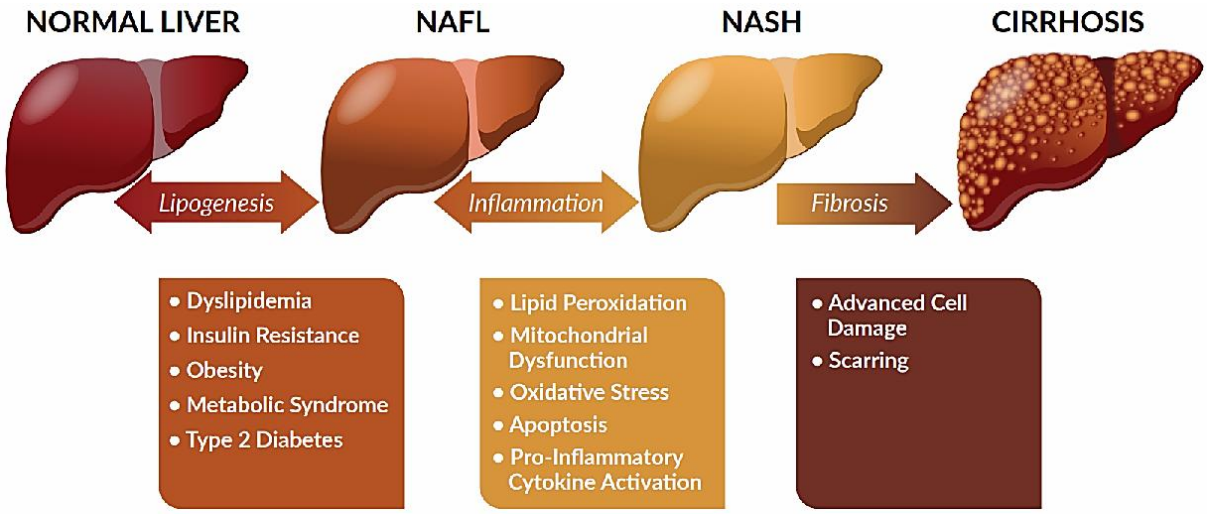

Figure 1.1: Scheme of the progression from a healthy to a cirrhotic human liver. A normal liver can progress to NAFLD due to excessive lipogenesis. NAFLD condition is characterised by inflammation and oxidative stress so can get worse to NASH. NASH is a risk factor for the development of cirrhosis. Both NAFLD and NASH are thought to be reversible (Hebbard & George 2011).

Ludwig and colleagues coined the term Non-Alcoholic Steatohepatitis in the '80s to describe, at morphologic level, the severity of liver injury in 20 patients monitored at the Mayo Clinic over a 10-year period (Ludwig et al. 1980). These patients, despite having never used alcohol, showed the same symptoms of patients with alcoholic hepatitis, such as steatosis and lobular inflammation. However, all patients were obese and showed hyperlipidemia and diabetes mellitus type II. During the years, many other terms have been used to indicate this clinical problem, inclouding, for example, pseudo-alcoholic liver disease, alcohol-like hepatitis, diabetic hepatitis, non-alcoholic Laennec's disease and steatonecrosis (Sheth et al. 1997). However, since the disease counts a wide-range of pathologies, the umbrella term "NAFLD" introduced for the first time in 1986, became the preferred one (Schaffner & Thaler 1986).

Fatty liver disease is a chronic and very often asymptomatic disease. Patients discover that they are affected by routine analysis, since NAFLD causes an abnormal increase in the values of Alanine Aminotransferase (ALT), an hepatic enzyme considered a liver health marker. Since ALT is a general indicator of hepatic suffering, in order to have a more precise diagnosis, the method of election for diagnosis is biopsy. This invasive method allows the evaluation of the degree of steatosis and the presence of fibrosis or necro-inflammatory activity, thus allowing to distinguish steatosis from steatohepatitis (Dyson et al. 2014).

The development and progression of NAFLD to NASH represent a complex pathophysiological process. Currently, the most accredited hypothesis is that a sequence of insults, called "hits", are involved in the onset and progression of liver damage (Day & James 1998). The first hit is the process that causes fat accumulation, in particular triglycerides, in hepatocytes. An example of a first hit can be insulin resistance (Tominaga et al. 1995). It is believed that this occurrence results from decreased catabolism of fatty acids, due to compromised mitochondrial β-oxidation (Reid 2001). Instead the second hit is a multifactorial phenomenon in which lipotoxicity, oxidative stress, inflammation and genetic and environmental factors participate (Paschos & Paletas 2009).

Other putative agents triggering the progression to fibrosis and cancer are the presence of bacterial toxins (Yang et al. 1997), overproduction of inflammatory cytokines as Tumor Necrosis Factor-α (TNF-α), ATP stores depletion and cytochrome P450 Cyp2E1/Cyp4A-enzyme activity dysregulation (Leclercq et al. 2000). Although this scheme represents a useful model to describe the progression of NAFLD, data from animal models have shown that the secondary hits *per se* could induce fatty liver formation. For example, steatosis can result from increased lipid peroxidation-associated oxidative stress (Pan et al. 2004), TNF-α

expression stimulated by free fatty acid (Feldstein et al. 2004a), and inflammation generated by adipocyte-specific Fas receptor, which belongs to the super family of the TNF receptors (Wueest et al. 2010). These studies suggest that fatty liver formation and inflammation often develop simultaneously through multiple inter-related hits. Hebbard and George, in their work, propose that a more appropriate term and model reflecting current knowledge of NAFLD pathogenesis is the "integrated response" model. The "integrated response" hypothesis of NAFLD pathogenesis is based on the concept that tissue crosstalk promotes hepatic steatosis and inflammation. Over nutrition and reduced physical activity in a susceptible person leads to metabolic syndrome and obesity. The increased dietary intake can alter intestinal bacterial flora and increases levels of bacterial products entering the portal circulation and the liver, activating the innate immune system. High levels of circulating free fatty acids are associated with muscle insulin resistance and inhibition of adipocyte insulin receptor function. The retarded response of muscle to insulin causes the deviation of carbohydrates away from muscle glycogen synthesis towards hepatic *de novo* lipogenesis. Increased dietary carbohydrate can magnify pancreatic insulin release. Adipose tissue enlargement and apoptosis and/or necrosis is associated with the recruitment of macrophages to the tissue, culminating in low-grade adipose and systemic inflammation. The net effect of these events on the liver is to inhibit insulin sensitivity and promote hepatocyte apoptosis. Further data suggest that impaired insulin responses in the central nervous system can also affect hepatic function (Hebbard & George 2011) (Figure 1.2).

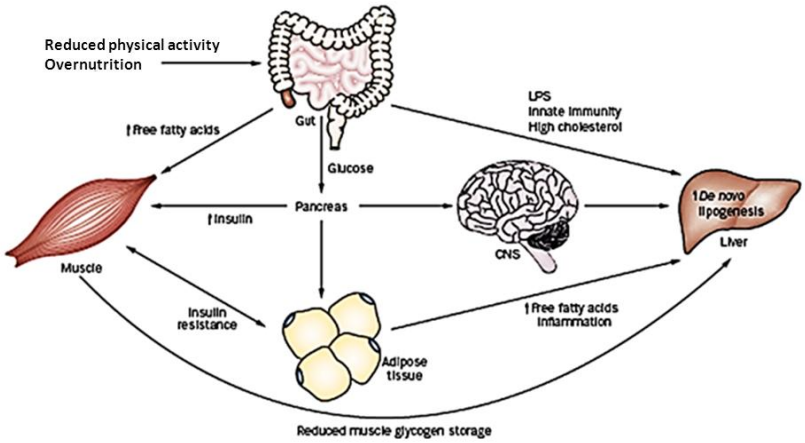

Figure 1.2: Hypothesis of "integrated response". A tissue crosstalk occurs, promoting hepatic steatosis and inflammation (Hebbard & George 2011).

In addition, interactions between the gut and the liver, called the gastro-liver axis, play an essential role in the development and evolution of NAFLD. The portal blood flow connects the intestine to the liver. The blood from the intestine exposes the liver to metabolic products obteined from the intestinal microbiome, including phenols, acetaldehyde and ammonia (Compare et al. 2012), proinflammatory bacterial components such as peptidoglycan and lipopolysaccharides. The liver has a wide variety of immune cells: lymphocytes, macrophages, dendritic cells and natural killer cells (Henao-Mejia et al. 2013). The innate immune system responds to cellular damage or pathogens through Pattern Recognition Receptors (PRRs) that are expressed intracellularly or on the surface of hepatocytes (Bieghs & Trautwein 2014). Damage-associated molecular patterns (DAMPs) released by damaged cells or pathogens produced by bacteria are recognized by PRRs (Pedra et al. 2009). Activated Toll-Like Receptors (TLRs) induce gene transcription that simplifies the responses of the innate immune system (Takeuchi & Akira 2010). Therefore, their activation is important in the development of NAFLD. TLRs are expressed in stellate cells, Kupffer cells and hepatocytes and are able to identify a wide range of pathogens, which can induce the pro-inflammatory response.

Finally, another major cause of non-alcoholic steatosis is choline deficiency. In fact, choline is an essential element for the structural maintenance of the plasma membrane, for the generation of Very Low Density Lipoproteins (VLDLs) and the synthesis of neurotransmitters as acetylcholine (Zeisel & da Costa 2009).

The *de novo* synthesis of the choline does not satisfy the metabolic demands of the human organism; therefore, it is necessary to associate a diet rich in foods that contain in abundance this nutrient such as eggs, beef liver, chicken breast, cauliflower and wheat (Fischer et al. 2007).

Nowadays, since the lack of knowledge in molecular mechanisms of NAFLD pathohenesis, there is no treatment for this pathology but only the symptoms are treated. One of the suggest treatment of patients suffering from NAFLD/NASH is weight loss (Chalasani, Younossi, Joel E. Lavine, et al. 2012). Calories reduction, physical exercise and behavior modification could have beneficial effects on NASH progression. In fact, patients who have started on a diet, with an average weight loss of 9.3% from baseline, display histologic improvement in NASH severity (Promrat et al. 2010). The basis of NAFLD therapy in adults (Hannah & Harrison 2016) and in children (Africa et al. 2016) is the change in lifestyle focused on healthy eating,

weight loss when necessary and regular exercise. When recommending healthy food choices, a Mediterranean diet has proved to be a valid alternative to the Western diet (Abenavoli et al. 2014). Another good option could be bariatric surgery (Corey & Rinella 2016). However, surgery is only possible in a minority of patients and there is clearly a need for drug therapy (Musso et al. 2016). Due to the wide variety of causes and symptoms that characterize NASH, no NASH approved drugs are currently present. As one of the characteristics of NASH is insulin resistance, one of the possible NAFLD/NASH therapies is treatment for diabetes. Biguanides and Thiazolidinediones (TZDs) are insulin sensitizers. In fact, the biguanide *metformin* improves hepatic insulin sensitivity through activation of AMP-activated kinase and following blockade of gluconeogenesis (Zhou et al. 2001). A meta-analysis demonstrated that *metformin* does not give any benefits in NASH patients' treatment, making it useless for treatment of NASH (Vernon et al. 2011). Conversely, TZDs have been considered promising agents as for the NASH treatment. TZD *pioglitazone* is one of the approved and recommended drug for the management of biopsy-proven NASH in both diabetic and non-diabetic patients (Chalasani et al. 2012). Since the encouraging results obtained by TZD administration, it is not difficult to imagine that a category of drugs, able to improve insulin sensitivity and to reduce, at the same time, hepatic inflammation without the PPAR-y-associated side effect of weight gain, has become the principal method to treat NASH.

Previous clinical trial data suggest, for example, that pioglitazone or vitamin E may be useful in non-diabetic patients with NASH (Sanyal et al. 2010) and the benefit of pioglitazone on NASH reversal and improvement of fibrosis has recently been confirmed in diabetic patients (Cusi et al. 2016). In NASH diabetic patients, pioglitazone significantly reduces steatohepatitis (Belfort et al. 2006; Aithal et al. 2008), displaying greater histologic improvement of the disease, compared with lifestyle interventions (Sanyal et al. 2010). However, pioglitazone causes weight gain and other safety drawbacks in long-term administration, which is why its use is limited in patients with NASH. Finally, in NASH patients therapeutic strategies have been used which aim at oxidative stress and inflammation reduction, such as vitamin E and vitamin C. Also this therapy has a limited use because the interaction of vitamin E with cardiac risk (Saremi & Arora 2010) and oncological disease is not yet defined (Lippman et al. 2009).

The approach based on the modulation of metabolism considers hydrophilic Bile Acids (BAs) as the latest candidates for NASH treatment. BA, through the activation of two receptors, Farnesoid X Receptor (FXR) (Makishima et al. 1999; Wang et al. 1999) and TGR5

(Kawamata et al. 2003), control both glucose and lipid homeostasis and show a significant anti-inflammatory activity (Neuschwander-Tetri et al. 2015). Several agonists for both receptors have been produced, although the FXR agonist Obeticholic Acid (OCA) is the first in-class synthetic BA under analysis for NASH treatment (Abenavoli et al. 2018).

Despite all the studies, the molecular mechanisms regulating the onset and the progression of NAFLD to NASH are not yet clear. Several studies are required to investigate and identify better therapies in order to prevent and treat this pathology (Feldstein et al. 2004).

1.2 Steatotic liver

Steatosis is the hallmark feature of NAFLD. The main characteristic of steatotic liver is the accumulation of lipid droplets within hepatocytes. Current quantification and grading of hepatic steatosis utilizes a classification of mild (<33%), moderate (33%-66%), or severe (>66%) steatosis (Adam et al. 1991). The 'gold standard' method to evaluate steatosis remains the microscopic analysis, despite being relatively subjective and can vary between individual observers (Angele et al. 2008). Frozen section examination is the preferred method because of time constraints between graft retrieval and transplantation (McCormack et al. 2007).

There are two forms of steatosis which are classified on the size of hepatocytes' triglycerides (TGs) droplets: microvescicular and macrovesicular steatosis (Figure 1.3).

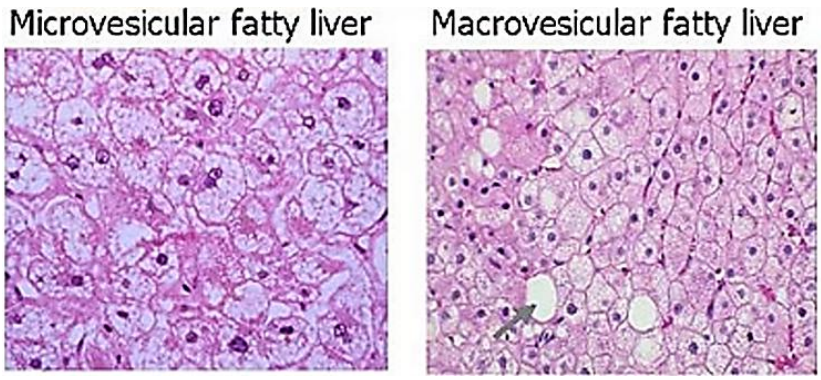

Figure 1.3: Two type of steatosis: microvescicular and macrovescicular. Small lipid droplets characterize microvescicular steatosis, while the macrovescicular one displays large lipid droplets (Hebbard & George 2011)

Microvesicular steatosis is defined as an accumulation of relatively small lipid droplets that do not displace the hepatocyte nuclei and is commonly found in pathological conditions associated with mitochondrial injury, such as acute viral or drug induced liver injury, sepsis and some metabolic disorders.

In contrast, large lipid droplets that occupy most of the hepatocyte cytoplasm and displace the nucleus to the cell periphery characterize macrovesicular steatosis. It is commonly associated with excessive alcohol intake, obesity, diabetes and hyperlipidemia (Angele et al. 2008).

A liver is considered steatotic when the triglyceride content is >5% of liver volume or liver weight, or histologically defined when 5% or more of hepatocytes contain visible intracellular triglycerides (Kleiner et al. 2005).

The liver performs many functions necessary for the body metabolic homeostasis of the organism. These metabolic activities require a rich blood supply for transport and distribution of substrates, hormones and nutrients. During basal conditions, 1.5 L of blood are transported to the liver every minute, which deliver a large load of compounds that require metabolic processing. Excessive accumulation of Intrahepatic Triglyceride (IHTG) is associated with alterations in glucose, Fatty Acid (FA) and lipoprotein metabolism and inflammation, which have adverse consequences on health. However, it is not clear whether NAFLD causes these abnormalities or whether the intrahepatic triglyceride accumulation is a consequence of these metabolic abnormalities (Calmus et al. 1992).

Steatosis develops when the rate of fatty acid input is greater than the rate of FA output. The number of triglycerides depends principally on 4 elements (Figure 1.4):

- hepatic fatty acid uptake
- *de novo* fatty acid synthesis
- Fatty Acid Oxidation (FAO)
- fatty acid export within VLDL-TG

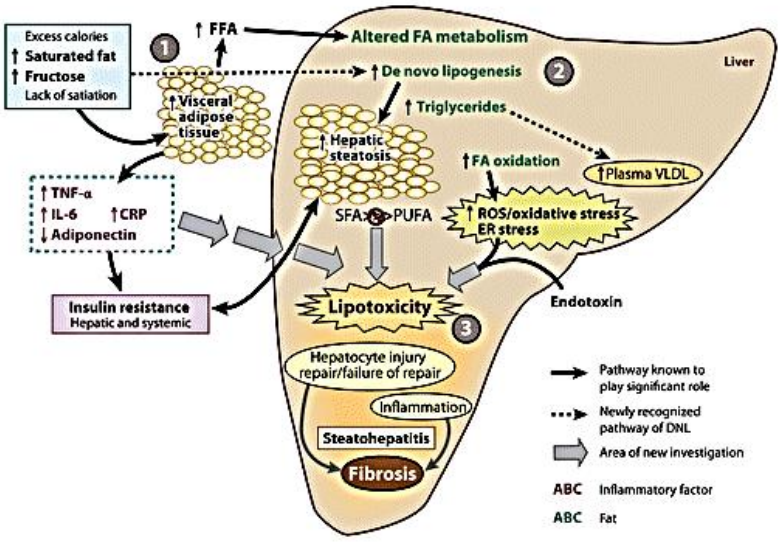

Figure 1.4: Factors that influence the triglyceride content. The number of triglycerides depends on: hepatic fatty acid uptake, *de novo* fatty acid synthesis, fatty acid oxidation and fatty acid export (Tiniakos et al. 2010)

1.2.1 Fatty acid uptake

The rate of hepatic FFA uptake depends on the amount of FFA that reaches the liver and the ability of the liver to transport FFA to other districts. FFA released from subcutaneous adipose tissue enter the systemic circulation. Subsequently, they are transported to the liver from the hepatic artery and from the portal vein, after passing through the splanchnic tissues (Nielsen et al. 2004). In fact, the major rate of FFAs that comes to the liver is released from the adipose tissue. Mittendorfer and colleagues found that the rate of FA release in the systemic circulation is directly proportional to the increase in fat mass; this occurs in both men and women. Thus the rate of release of FFA in relation to fat-free mass is greater in obese subjects than in lean ones (Mittendorfer et al. 2009). Furthermore, lipolysis of TG leads to the formation of FFAs and their accumulation, particularly in obese subjects with NAFLD who have a greater expression of hepatic Lipoprotein Lipase (LPL) compared to subjects without NAFLD (Pardina et al. 2009; Westerbacka et al. 2007) (Figure 1.5).

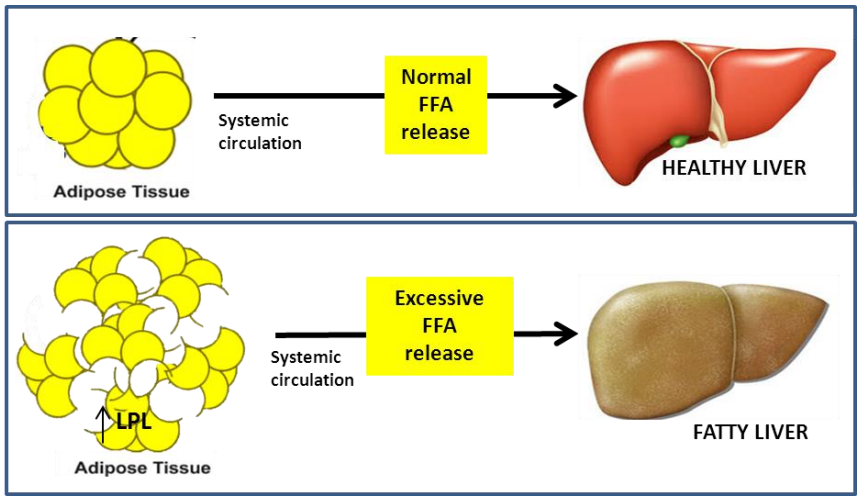

Figure 1.5: Expression of LPL. Subjects with little adipose tissue display a normal release of FFAs and therefore a healthy liver; obese people with a lot of fat present a high expression of LPL and therefore high release of FFAs, which causes fatty liver.

Another factor involved in the hepatic FFA uptake is the direct traffic through membrane proteins, which mediate the movement of FFAs from plasma into tissues. An example of a membrane protein is the FAT/CD36 complex that plays an important role in regulating tissue absorption of FFAs from plasma. In obese subjects with NAFLD, gene expression and FAT/CD36 content increase in liver and skeletal muscles, but decrease in fatty tissue compared to obese subjects with normal intrahepatic triglyceride content (Greco et al. 2008;

Fabbrini et al. 2009). The proteins transporting fatty acids throught membranes, therefore, redirect the absorption of plasma FFA from adipose tissue to other tissues. Hence, these data suggest that the pathogenesis of steatosis and ectopic fat accumulation could be caused by the alterations in adipose tissue lipolytic activity, regional hepatic lipolysis of circulating TG and tissue FFA transport proteins (Figure 1.6).

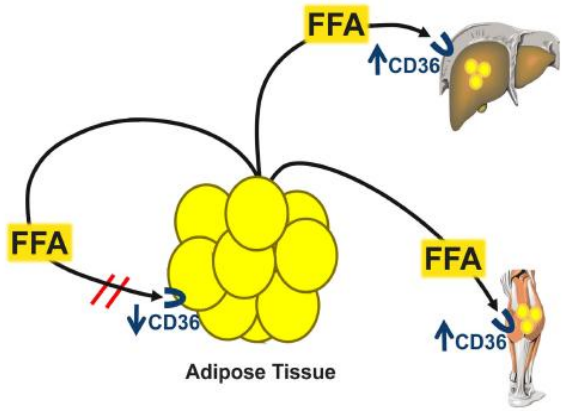

Figure 1.6: Alteration in cellular fatty acid uptake. In the adipose tissue a decrease in the expression of CD36 which regulates the absorption of tissue FFA, occurs. In the liver and skeletal muscle, instead, an increase in the content of intrahepatic and intracellular triglycerides takes place (Fabbrini et al. 2010).

1.2.2 *De novo* lipogenesis

De Novo Lipogenesis (DNL) is the metabolic pathway which converts the excess of carbohydrates in fatty acids . The synthesis *de novo* takes place through a complex cytosolic polymerization in which Acetyl-CoA Carboxylase (ACC) converts acetyl-CoA to malonyl-CoA, to form, at the end, one molecule of palmitate. The rate of *de novo* synthesized fatty acids depends on the Fatty Acid Synthase (FAS) complex, the activity of Acetyl-Coa Carboxylase, Diacylglycerol Acyltransferase (DGAT), Stearoyl-Coa Desaturase 1 (SCD1) and several nuclear transcription factors as SREBPs, ChREBP, Liver X Receptor α (LXRα), Farnesoid X Receptor (FXR), and Peroxisome Proliferator-Activated Receptors (PPARs) (Musso et al. 2009). In particular, the nuclear transcription factors SREBP and ChREBP are activated by insulin and glucose.

Studies conducted on mouse models show that other factors that may stimulate lipogenesis are hepatic overexpression of SREBP-1c or hyperinsulinemia and this in turn causes hepatic steatosis (Shimano et al. 1997; Shimomura et al. 1999). In humans, NAFLD is directly

associated with increased hepatic expression of several genes involved in *de novo* lipogenesis (Mitsuyoshi et al. 2009; Kohjima et al. 2007).

In subjects with NAFLD, 15%-23% of intrahepatic triglycerides derive from the *de novo* synthesis and are secreted in Very Low Density Lipoprotein-Triglycerides (VLDL-TG) (Diraison et al. 2003; Donnelly et al. 2005). Moreover, a study conducted by Petersen and colleagues suggests that the increase in DNL is the decisive step in the development of NAFLD (Petersen et al. 2007). Unlike insulin-sensitive subjects, in insulin-resistant subjects, who have a normal IHTG content, the consumption of a high-carbohydrate meal is associated with a much lower rate of muscle glycogen synthesis and an alteration of the glucose ingested towards hepatic synthesis. The accumulation of IHTG and the orientation of the carbohydrates ingested towards the *de novo* synthesis of fatty acids away from storage, as muscle glycogen, can promote insulin resistance in the muscle.

Although from a quantitative point of view the hepatic DNL is a minor pathway for TG synthesis, the DNL rate could have important metabolic regulatory functions (Chakravarthy et al. 2005).

1.2.3 Fatty acid oxidation

The liver is one of the organs that requires the greatest amount of energy because of all the metabolic processes it carries out. It is estimated that fatty acid and amino acid oxidation provide ~90% of the fuel for basal hepatic energy requirements.

Most of the hepatocellular fatty acid is oxidized mainly in the mitochondria and in much smaller quantities in peroxisomes and microsomes. The FAs can enter the mitochondrial matrix by carnitine-dependent enzyme shuttle (CPT1) and carnitine translocase (CPT2). Mitochondrial β-oxidation consists in shortening of fatty acyl-CoA by two carbon units at each cycle, through a series of dehydrogenation, hydration, and cleavage reactions that involve membrane-bound and soluble enzymes, which are transcriptionally regulated by PPAR-α (Desvergne & Wahli 1999). Fatty Acids Oxidation (FAO) leads to the formation of acetyl-CoA which can continue in two ways: enter the tricarboxylic acid cycle for complete oxidation and proceeds with useful energy for the liver, or can be condensed to form ketone bodies (acetoacetate and beta-hydroxybutyrate) which will subsequently be exported to other tissues as energy endurance (McGarry & Foster 1980).

The inhibition or activation of intrahepatic fatty acid oxidation can influence intrahepatic triglyceride content. Hepatic steatosis can be caused by a lack of enzymes involved in FAO (Zhang et al. 2007; Ibdah et al. 2005), but if their activity and expression are increased, the accumulation of intrahepatic triglycerides can be reduced (Xu et al. 2003; Savage et al. 2006). Today, since there are no methods to measure hepatic FAO *in vivo*, it cannot be determined whether FAO is defective in human subjects with NAFLD.

In addition, in subjects with NAFLD, although CPT1 expression is decreased, gene expression of other hepatic fatty acid oxidative enzymes is generally greater than in those with normal IHTG content (Greco et al. 2008; Kohjima et al. 2007). In contrast, all the subjects with NAFLD display hepatic mitochondrial structural and functional abnormalities.

Typical examples are the followings: loss of mitochondrial cristae and paracrystalline inclusions (Sanyal et al. 2001; Caldwell et al. 1999), a decrease in mitochondrial respiratory chain activity (Pérez-Carreras et al. 2003). Moreover, it could be possible to have a reduced ability to resynthesize ATP after a fructose challenge (Cortez-Pinto et al. 1999) and increasing of hepatic uncoupled proteins (Adams et al. 2005; Kohjima et al. 2007) which only influence energy production but not FAO.

All these anomalies could represent a decoupling between FAO and ATP production; this allows the liver to oxidize excess FA substrates without generating unnecessary ATP.

1.2.4 VLDL kinetics

The liver can produce Very Low-Density Lipoproteins (VLDLs), particles of complex lipoprotein, and secrete them into the systemic circulation. VLDLs play a very important role in the conversion of water-insoluble TG into a water-soluble form. Therefore, VLDLs can export it from the liver and deliver it to peripheral tissues.

VLDLs are generated by the action of the Microsomal Triglyceride transfer Protein (MTP), which mediate the fusion of a newly synthesized apolipoprotein B-100 molecule (apoB-100) with a TG glycerin; each VLDL particle contains a single molecule of apoB-100. Fatty acids esterified in triglycerides and secretions like VLDL are derived from different sources. About 70% of FAs incorporated into VLDL-TG come from systemic plasma or non-systemic sources, such as *de novo* hepatic synthesis, intrahepatic triglyceride lipolysis and lipolysis of visceral adipose tissue (Lewis 1997).

When fatty acids are not oxidized, they are esterified to triglycerides. Therefore, triglycerides may face two different possibilities: be incorporated into VLDL or secreted into the circulation within the liver. Therefore, the secretion of VLDL is very important because it represents a method to reduce the content of intrahepatic triglycerides. Genetic defects, such as familial hypobetalipoproteinemia (Schonfeld et al. 2003), or an inhibition of MTP activity caused by pharmacological agents (Cuchel et al. 2007), or an intrahepatic increase in triglyceride content, may cause the impairment of hepatic VLDL secretion. However, many studies demonstrate that subjects with NAFLD present a higher secretion rate of VLDL-TG with respect to subjects with a normal intrahepatic triglyceride content. Fabbrini and colleagues proved that the rate of VLDL-TG secretion was twice as greater in non-diabetic obese subjects with NAFLD than in those with normal IHTG content. The increase in the contribution of non-systemic FA, derived from lipolysis of intrahepatic and visceral fat, is mainly responsible for the secretion of VLDL-TG (Fabbrini et al. 2008). Furthermore, a difference in the relationship between VLDL-TG secretion and IHTG content was found among the group of subjects with normal IHTG content and those with NAFLD. In fact, in subjects with normal IHTG, the content of intrahepatic triglycerides also increases with increasing secretion of VLDL-TG, but this phenomenon seems to reach a *plateau* in subjects affected by NAFLD, regardless of the IHTG content.

Hence, in NAFLD subjects, the increase in VLDL-TG secretion rate is not able to compensate adequately for the increased rate of IHTG production, for this reason steatosis is maintained.

1.2.5 Fatty acid metabolism

Excessive release of fatty acids from the adipose tissue into the circulation increases the uptake of FFA in the liver and skeletal muscles. This accumulation can lead first of all to the intrahepatic triglyceride but also causes insulin resistance in the liver and skeletal muscles (Boden & Shulman 2002).

The cellular mechanism responsible for fatty acid induced-insulin resistance in muscle and liver is not completely clear, despite several studies demonstrated an association between these lipid intermediates and impaired insulin action.

1.2.6 Oxidative stress: second hit

The principal hit proposed to be important in the progression of NAFLD to NASH is oxidative stress. In fact, following hepatic steatosis, the liver cells become sensitive to the action of oxidative stress, which is usually considered the second hit and so responsible for the progression of NAFLD. This occurs in non-alcoholic hepatic steatosis patients because the excess of FFAs undergoes oxidation within hepatocytes. Mitochondrial β-oxidation is normally the main lipid deposition mechanism, but in NAFLD this process is increased and leads to excessive formation of Reactive Oxygen Species (ROS) (Sanyal et al. 2001).

Oxidative stress refers to an unbalanced cellular state in which the production of reactive oxygen species overcomes the normal mechanisms of elimination of free radicals, such as Superoxide Dismutase (SOD), reduced glutathione (GSH), vitamins A, E, C, and other molecules involved in the counterbalancing ROS production and inactivation (Sauer et al. 2001). Oxidative stress is due to a combination of factors, such as mitochondrial dysfunction, ER stress and inflammation mediated by gut flora. Mitochondrial abnormalities that could elevate levels of oxidative stress are: mutations in mitochondrial DNA, loss of cristae, paracrystalline inclusion bodies within the mitochondrial matrix and nucleic genes encoding mitochondrial proteins (Ricci et al. 2008). Moreover, in a lipid-rich environment, the ER initiates a stress response that involves a myriad of signaling cascades to ensure that misfolded proteins do not accumulate in the cell. It was therefore hypothesized that the ER, overloaded with these reports, could contribute to the production of ROS (Gentile et al. 2011). Finally, a crucial role of gut flora in mediating the progression from steatosis to NASH is beginning to emerge with evidence from animal and human studies (Imajo et al. 2012). However, most of the mechanistic understanding is based on an increase in pro-oxidant species and the potential for decreasing functionality of anti-oxidant machinery remains to be explored.

1.3 Saturated fatty acids, Monounsaturated fatty acids, Polyunsaturated fatty acids

Nowadays, the human nutrition and dietary habits show important changes, with increased consumption of intensively produced- and processed-food. The latter are usually rich in fats and refined carbohydrates and poor in essential nutrients such as anti-oxidants, fibers and n-3 Polyunsaturated Fatty Acids (PUFAs). In particular, FAs consumption is increased significantly in the last decades, and today it represents 28-42% of total energy consumed by European populations (Linseisen et al. 2009). In addition, qualitative changes in dietary FAs have occurred over the past 50 years with increased consumption of saturated fat especially from meat and dairy products. Diet also evolved towards higher intakes in vegetables oil rich in n-6 PUFAs and relative decrease in n-3 PUFA consumption present in fatty fish, nuts, seeds, whole-grain cereals (Simopoulos 1994; Ruidavets et al. 2007). As a result, ω-6 PUFA consumption has become progressively much higher than that of ω-3 PUFA (Anderson & Ma 2009).

In 2007, Zelber-Sagi and colleagues, in their study, revealed that patients with NAFLD consume twice quantity of soft drinks and eat more meat (27%) compared with the "normal liver" group; these dietary differences are associated with an increased risk of NAFLD independently of traditional risk factors. This is the first convincing evidence of an association between nutritional pattern and NAFLD (Zelber-Sagi et al. 2007).

It has been well established now that, through a variety of pathways, including Peroxisome Proliferator Activated Receptors (PPARs), Sterol Regulatory Element Binding Proteins (SREBPs) and Liver X Receptors (LXRs), fatty acids can regulate the expression of a range of genes involved in lipid and lipoprotein metabolism within the liver (Jump 2008; Sampath & Ntambi 2005). However, much of the existing evidence relates specifically to the role of PUFAs in regulating gene expression, but little it is known about the potential roles of Saturated Fatty Acids (SFAs). Moreover, according to the type of dietary fatty acids, immune cells display phenotypic changes. The consumption of SFAs activate M1 genes that stimulate Interleukin-6 and TNF-α production, whereas Monounsaturated Fatty Acids (MUFAs) activate the M2 genes related to the expression of Arginase-1 and Interleukin-10, which are cytokines with anti-inflammatory action (Chan et al. 2015). The consumption of MUFAs and PUFAs has positive effects on glucose metabolism, with a reduction in different parameters related to type II diabetes mellitus. PUFA intake is linked to increased expression of

adiponectin, an anti-inflammatory cytokine, which promotes hepatic metabolic enhancement, and reduces the risk of atherosclerosis, such as increased High Density Lipoprotein (HDL) and decreased Triacyclglycerols (TAG).

1.3.1 Saturated fatty acids (SFAs)

SFAs are fatty acids that have no double bonds between carbon atoms in the chain. Their general formula is R-COOH, in which the R- group is a straight-chain hydrocarbon of the form $CH_3(CH_2)n$ with variable length. The most common and important fatty acids, present in many vegetable and animal fats, contain between 12 and 22 carbon atoms (Rosenthal & Glew 2009). Saturated fatty acids can be divided into 3 groups:

- Short chain: up to 6 carbon atoms, that are volatile molecules, have high water solubility and a small molecular size;
- Medium chain: from 8 to 12 carbon atoms, characterized by a good water solubility;
- Long and very long chain: from 14 atoms onwards, which have low or absent water solubility. Palmitic acid (16:0) and stearic acid (18:0) belong to this class of saturated fatty acids (Figure 1.7).

Palmitic acid is the most abundant saturated fatty acid found in animal lipids (20%–30%), and in all plant seed oils (5%–50%). Useful amounts of palmitic acid (upwards of 50%) are extracted from palm oil and from cottonseed oil, lard and tallow in approximately 20%–30% (Gunstone, 1996). Together with oleic acid and linoleic acid, it is one of the most abundant fatty acids in triacylglycerols of adipose tissue and plasma lipoproteins.

Although well known, stearic acid is found in much lower amounts than palmitic acid. Lard and tallow are useful sources of stearic acid. Cocoa and shea butter contain approximately 30%–45% stearic acid.

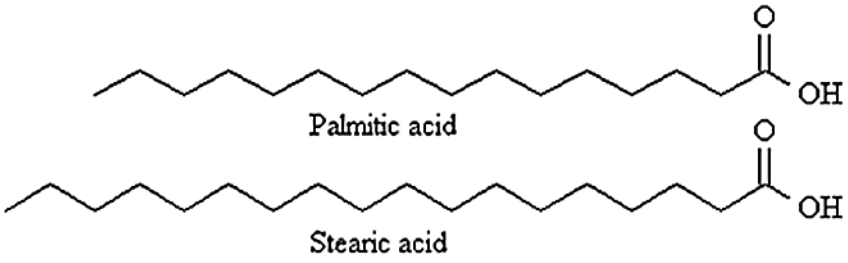

Figure 1.7: Palmitic and stearic acids

Of course, many of the saturated fatty acids can be prepared from the corresponding unsaturated fatty acids through hydrogenation. Because stearic acid has a melting point higher than body temperature, triglycerides containing it in high amounts are poorly absorbed in humans (Rosenthal & Glew 2009).

Major sources of dietary SFAs are meat and dairy products, as well as certain plant fats such as palm oil (Schönfeld & Wojtczak 2016). This type of fatty acids is dangerous: in fact during the diet the recommendations for a healthier cardiovascular system is to reduce SFA intake (Joris & Mensink 2016). According to the American Heart Association Diet and Lifestyle Recommendations, SFA intake should be less than 7% of total energy (Jacobson et al. 2015).

In general, SFA in membranes are confined to the sn-1 position of phospholipids and have 16 to 18 carbon atoms, while in sn-2 there are 18 to 20 carbon atom unsaturated fatty acids. In triacylglycerols saturated fatty acids, often long chain ones or monounsaturated fatty acids, tend to be in sn-1 and sn-3 positions, while in sn-2 position there are polyunsaturated fatty acids are found.

The β-oxidation of the linear chain in saturated fatty acids occurs in the mitochondria. In this catabolic pathway, several enzymes participate and produce acetyl-CoA, $NADH^+$ and $FADH_2$ from fatty acyl-CoA esters. In order to be metabolized, and thus enter β-oxidation, the fatty acid trioesterification of coenzyme A is necessary to form acyl ester-A. Acyl-CoA synthetase catalyzes this reaction at the entry of fatty acids into the cells. Very long-chain fatty acyl-CoA esters (> 20 carbon atoms) are initially shortened by peroxisomal enzymes and subsequently proceed into the mitochondrial network for β-oxidation. L-Carnitine system enables the transportation of long-chain and medium-chain fatty acyl-CoA esters across the mitochondrial membrane. The mitochondrial β-oxidation of the acyl-CoA saturated fatty esters consists in the repetition of four biochemical reactions: oxidation, hydration, second oxidation, and thiolysis (Adeva-Andany et al. 2018) (Figure 1.8).

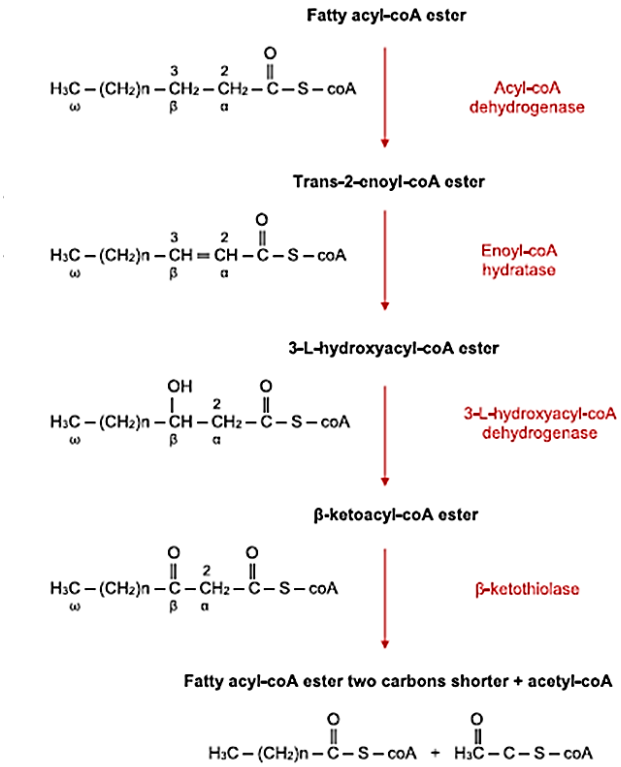

Figure 1.8: Fatty acyl-CoA β-oxidation. Reactions involved in the β-oxidation of fatty acyl-CoA esters.

During each cycle, two carbon atoms shorten the acyl-CoA ester chain. At the end of this process, only acetyl-CoA molecules are obtained. For instance, palmitoyl-coA (C16:0) generates eight molecules of acetyl-coA. In the first step acyl-CoA dehydrogenases catalyze fatty acyl-coA esters, introducing a double bond in the saturated chain to generate a 2-trans-enoyl-coA derivative (α/β-trans-enoyl-CoA thioester) of the same chain length. Then acyl-CoA dehydrogenases catalyzes the hydration of the 2-trans-enoyl thioesters into the 3-L-hydroxyacyl-CoA derivatives. The latter is oxidized by L-3-hydroxyacyl-CoA dehydrogenase into 3-ketoacyl-CoA species. In the fourth and last step, 3-ketoacyl-CoA thiolase catalyses the removal of a thiolytic group of the 3-ketoacyl-CoA chain by the thiol group of a second molecule of coenzyme A, which yields acetyl-CoA and an acyl-CoA ester shortened by two carbon atoms.

B-oxidation of acyl-CoA esters of long and medium chain fats occurs by the trifunctional protein that is located on the inner aspect of the inner mitochondrial membrane. This protein presents three activities: enoyl-coA hydratase, 3-L-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase (Adeva-Andany et al. 2018) (Figure 1.9).

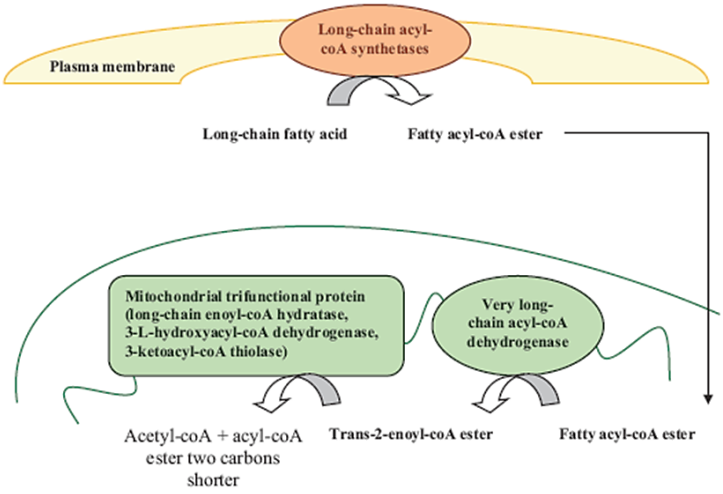

Figure 1.9: Mitochondrial β-oxidation of long and medium chain fatty acids (Adeva-Andany et al. 2018).

Damage of mitochondrial β-oxidation leads to accumulation of non-oxidized acyl-CoA esters, which undergo alternative pathways of oxidation. In alternative, fatty acyl groups are transferred from coenzyme A to L-Carnitine, glycine or taurine to form the corresponding acyl-derivatives (acyl-carnitines, acyl-glycines, acyl-taurines), which are unable to enter biochemical pathways. These acyl-conjugates reach the blood and some of them are excreted in the urine (Adeva-Andany et al. 2018).

1.3.1.1 SFAs in NAFLD

Epidemiological data highlights that high consumption of SFAs has adverse effects on lipid and glucose homeostasis and evolution towards the metabolic syndrome and, hence, NAFLD (Ebbesson et al. 2007).

Dietschy and colleagues demonstrated that saturated fatty acids with between 12 and 16 carbon atoms reduced receptor-mediated uptake of LDL by the liver. The reduced hepatic clearance of LDL was the predominant mechanism whereby such saturated fatty acid increased plasma LDL cholesterol concentrations (Woollett et al. 1992; Spady et al. 1993).

More recently, Vallim and colleagues investigated the impact of a diet rich in saturated fat on the expression of a wide range of hepatic genes. They have formulated a diet able to mimic the fatty acid composition of a typical Western diet. They have shown that in the absence of a cholesterol supplement, this diet modestly increases the concentration of cholesterol VLDL and LDL. There are also evidences that, in the absence of cholesterol, the high fat diet had

little impact on hepatic lipid concentration. On the contrary, by the addition of cholesterol to the diet, the effect is potentiated. In fact, there was a modest increase in hepatic TAG concentration and a dramatic increase in storage of hepatic cholesterol ester (Vallim & Salter 2010).

Major effects of a diet rich in SFAs were observed in the mRNA concentrations for the potential target genes: Acetyl-Coa Carboxylase (ACC), Fatty Acid Synthase (FAS) and Stearoyl-CoA Desaturase (SCD1). Both cholesterol and fat reduced ACC and FAS mRNA concentration. By contrast, while cholesterol on its own had very little effect on SCD1 expression, dietary fat dramatically reduced SCD1 mRNA. Adding cholesterol to the diet appeared to attenuate markedly the effect of fat on SCD1 expression.

Recent experiments conducted in mice demonstrated that the deterioration of hepatic insulin sensitivity partially depends on the presence of stearic acid (van den Berg et al. 2010). However, basically, it is difficult to obtain a rigorous management both of the total and specific SFA dietary reduction. Further data on the therapeutic effects of such reduction on NAFLD needs still to be met.

1.3.2 Monounsaturated fatty acids (MUFAs)

MUFAs are characterized by one double bond in the carbon chain, with the remaining carbon atoms being single-bonded. Oleic acid (C18:1n-9) is the predominant dietary MUFA, accounting for up to 92% of dietary MUFAs (Joris & Mensink 2016). MUFA-rich food include avocados, nuts, olive oil and olives.

As normal saturated fatty acids, also the metabolism of Oleoyl-CoA consists in three cycles of β-oxidation to obtain *cis*-Δ^3-Dodecenoyl-CoA, a 12-carbon fatty acyl-CoA with a *cis* double bond between carbon 3 and 4. Since this product has a cis double bond, it cannot participate directly in β-oxidation. In fact, in order to entry into β-oxidation it needed to be converted from *cis*-Δ3-Dodecenoyl-CoA to *trans*-Δ2-Dodecenoyl-CoA by Δ3,Δ2-enoyl-CoA isomerase. *Trans*-Δ2-Dodecenoyl-CoA now is able acted upon by the enzymes of β-oxidation pathway in five continuous cycles to yield six molecules of acetyl CoA. Then the acetyl-CoA molecules enter the Krebs' cycle (Figure 1.10).

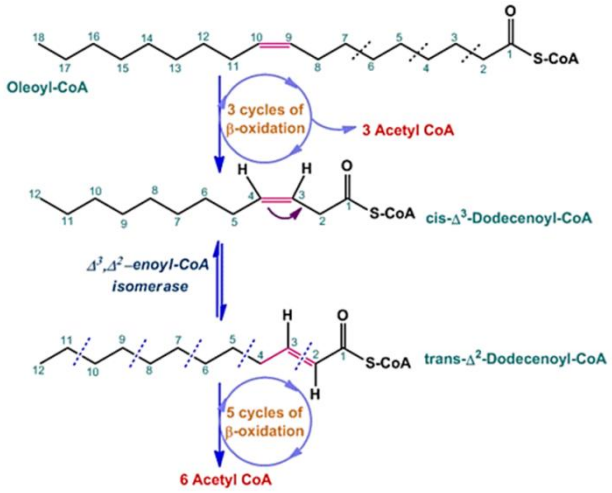

Figure 1.10: Oxidation of monounsaturated fatty acids. https://pharmaxchange.info/2013/10/oxidation-of-unsaturated-fatty-acids/

1.3.2.1 MUFAs in NAFLD

Mediterranean diet is rich in monounsaturated fatty acids (Basu et al. 2006).

In 2007, experiments on rodents demonstrated that olive oil alone, constituted mostly of 73% MUFAs, reduced steatosis by 30% in a model of MCD diet, probably due to inhibition of hepatic triacylglycerol synthesis and stimulation of hepatic peroxisomal β-oxidation (Hussein et al. 2007). In clinical trials, increased MUFAs led to a reduction in glucose, blood pressure, VLDL, and chylomicron remnants as well as to an increase in HDL in patients with type 2 diabetes mellitus (Julius 2003; Rodríguez-Villar et al. 2004). Therefore, an increase in MUFA intakes, particularly as a replacement for SFA, may balance the pro-inflammatory effects of SFAs.

1.3.3 Polyunsaturated Fatty Acids (PUFAs)

PUFAs are characterized by the presence of two or more double bonds. They were considered to be critical nutrients to regulate human health and could modulate brain development and cognition and some symptoms of cardiovascular disease, cancers and diabetes (Lee & Park 2014).

Based on the position of the first double bond from the methyl-end in a chain of fatty acids, two main types of PUFAs can be distinguished: ω-3 and ω-6 fatty acids.

Ω-3 are fatty acids present the first double bond on the third carbon, such as α-Linolenic Acid (ALA, 18:3n-3), Eicosapentaenoic Acid (EPA, 20:5n-3) and Docosahexaenoic Acid (DHA, 22:6n-3). Instead, omega-6 fatty acids present the first double bond on the sixth carbon and include Linoleic Acid (LA, 18:2n-6), Gamma-Linolenic-Acid (GLA, 18:3n-6), Dihomo-Gamma-Linolenic Acid (DGLA, 20:3n-6) and Arachidonic Acid (ARA, 20:4n-6). Linoleic acid and linolenic acid are generally found in plant-derived oils from corn, safflower, sunflower, canola and walnut (Ruiz-Lopez et al. 2012). As humans can not synthesize either LA or ALA, these molecules are considered essential dietary fatty acids. However, only a small portion of fatty acids with more than 20 carbon atoms can be converted by humans; the conversion rates of 18-carbon fatty acids to EPA and from ALA to DHA were reported to be 5%–10% and less than 1%, respectively (Huang et al. 2004). For this reason, fish and their oils, which contain high levels of PUFA over 20 carbon atoms like EPA and DHA, must be assumed with the diet. Another important feature of the PUFAs is that they are the major component of cell membranes. In particular, omega-3 are considered to be pivotal nutrients for preventing NAFLD (Gormaz et al. 2010), autoimmune responses (Simopoulos 2008) and other chronic diseases such as cardiovascular disease (Superko et al. 2014), cancers (Fasano et al. 2017) and diabetes (Zheng et al. 2012). Thanks to their ability to change the cellular membrane composition and to regulate transcription and cellular signaling, these fatty acids provide a variety of biological effects.

Moreover, twenty-carbon PUFAs are precursors of eicosanoids that regulate inflammatory and immune responses through pro- and anti-inflammatory activities; for example DHA is a precursor of anti-inflammatory docosanoids (Wolfe 1982; Calder 2013).

1.3.3.1 PUFAs in NAFLD

Over the years, many studies have been carried out to show that n-3 PUFAs might have beneficial effects in NAFLD. In fact, n-3 PUFAs seem to be involved in the regulation of the metabolic switch from anabolism (lipogenesis) to catabolism (FAO) by inhibiting Sterol Regulatory Element-Binding Protein 1 c (SREBP1c) and activating PPARα, a positive regulator of FAO (Zúñiga et al. 2011). However, there are corcerns about EPA and DHA that are known to improve hypertriglyceridemia (Kris-Etherton et al. 2002). Araya and colleagues, by lipidomic studies, have demonstrated an association between a high hepatic ω-6:ω-3 ratio with the severity of NAFLD (Araya et al. 2004). Some *in vitro* and *in vivo* studies, conducted

by Moreno and collaborators, have challenged these considerations. Indeed, they demonstrated that n-3 PUFA may alleviate lipid accumulation, pro-inflammatory actions, ROS production, and promote the FAO in NAFLD, in part by modulating the production of bioactive adipokines (leptin and adiponectin) that control the crosstalk between adipose tissue and key metabolic organs such as the liver and muscle (Moreno-Aliaga et al. 2010).

1.3.4 Fatty Acid Desaturases

In mammals, Fatty Acid Desaturases (FADSs) have an important role in the synthesis of polyunsaturated fatty acids (Zhang et al. 2016). FADSs are codified by three FADS genes including *FADS1*, *FADS2* and *FADS3*, located on chromosome 11q12.2-13.1 (Nakamura & Nara 2004). FADS1 is also known as Delta-5 Desaturase (D5D) and FADS2 is generally named Delta-6 Desaturase (D6D). The desaturases insert double bonds between the carboxylic-end of a molecule and a pre-existing double bond to interfere with further unsaturation in the existing PUFAs or make the PUFA *de novo* in mammals deprived of dietary PUFAs. Two types of desaturases can produce PUFAs: front-end desaturases and methyl-end desaturases (Meesapyodsuk & Qiu 2012). The first type, such as Delta-4 (D4D), Delta-5, and Delta-6 Desaturases help to introduce double bonds between the carboxylic-end of a molecule and a pre-existing double bond to generate PUFA (Zhang et al. 2016). Unlike front-end desaturases, methyl-end desaturases such as Delta-12 (D12D) and Delta-15 desaturases (D15D), or ω-3 desaturases, assist in adding a double bond between pre-existing double bond and methyl end in fatty acids (Nakamura & Nara 2004). However, mammals, including humans, do not contain these methyl-end desaturases for producing essential fatty acids. Therefore, they must acquire essential fatty acids such as LA and ALA from foods or nutritional supplements.

As has already been said, among front-end desaturases, there are Delta-4 (D4D), delta-5, and delta-6 desaturases.

D4 desaturase: In 2001, D4 desaturase gene was first cloned by RT-PCR approach from *Thraustochytrium*. Despite the study of Sprecher and co-workers in which suggested that DHA biosynthesis in mammals occurs independently of a Δ4 desaturase, subsequent studies have shown the opposite. D4 desaturase catalyze the addition of a double bond at the fourth carbon-carbon bond from the carboxylic acid end in fatty acids.

Mammals, including humans, can not produce omega-3 and omega-6 fatty acid because they do not have delta-12 and delta-15 desaturase. The latter are important enzymes in the synthesis of omega-3 and omega-6 fatty acids beacuase add a double bond at the 12^{th} and 15^{th} carbon- carbon position in fatty acids.

D5 desaturase: The first D5 desaturase gene was cloned in 1998 from the fungus *M. alpine*. The D5 desaturase comprises a cytochrome b_5-like domain fused at the N-terminus of the sequence. D5 desaturase can use two substrates, both ω3 (ETA) and ω6 (DGLA) producing EPA and ARA, respectively. It introduces a double bond at the D5 position (between carbons 5-6). However, besides the two major substrates, some D5 desaturases are active on LA and ALA, producing polymethylene-interrupted D5 desaturated fatty acids such as pinolenic acid (18:3-5,9,12), which was found naturally in seeds of some conifer trees (Haiping Hong et al. 2002). Blond and colleagues in their study demonstrated that in obese rats D5D index is lower compared to normal conditions (Blond et al. 1989).

D6 desaturase: The first D-6 desaturase gene was cloned in 1993 from the cyanobacterium *Synechocystis* using a gain-of-function expression approach (Reddy et al. 1993). The protein sequence shows a similarity to other acyl-lipid desaturases with different regioselectivity from cyanobacteria (Reddy et al. 1993). Like D5 desaturases from eukaryotes, D6 desaturase has a cytochrome b_5-like domain fused to the N-terminus of the main desaturation domain. This modular structure was later observed to be a feature of all the D6 desaturases isolated from eukaryotes including algae (Hoffmann et al. 2008), moss (Girke et al. 1998), plants (García-Maroto et al. 2002), fungi (H. Hong et al. 2002), animals (Zheng et al. 2005) and humans (Cho et al. 1999). These enzymes can introduce a double bond at position Δ6 on the acyl chain of linoleic (C18:2*n*−6) and α-linolenic (C18:3*n*−3) acids. D-6 desaturase is a rate-limiting enzyme in the synthesis of PUFA for generating GLA and SA from LA and ALA, respectively. In 2017, Valenzuela and colleagues have shown that the high fat mice are characterised by a reduction of D6D levels (Valenzuela et al. 2017).

Delta-4 (D4D), delta-5, and delta-6 desaturases take part in the synthesis of 22-carbon fatty acids from oleic acid (Figure 1.11).

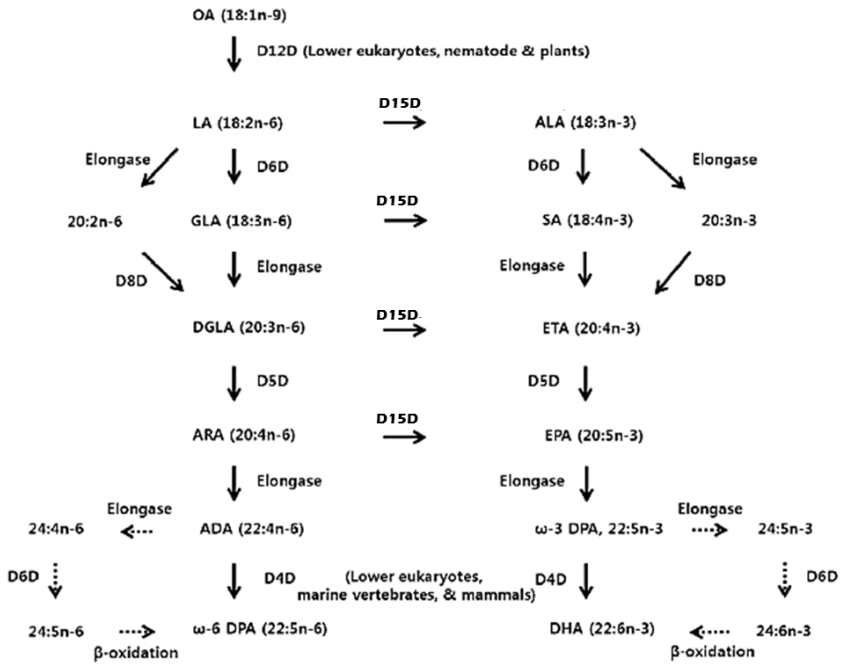

Figure 1.11: PUFA synthetic pathway. PUFA synthetic pathway to 22-carbon fatty acids from oleic acid in eukaryotic system.

Except for mammals, delta-12 desaturase and delta-15 desaturase have been identified in several cellular species: lower in eukaryotes (Sakuradani et al. 1999; Oura & Kajiwara 2004), in plants (Okuley et al. 1994; Vrinten et al. 2005) and in animals (Cripps et al. 1990; Zhou et al. 2011). Thanks to the presence of these two desaturases, these species can produce the ω-3 and ω-6 fatty acids. Delta-12 desaturase can synthesize LA from oleic acid and converted into ALA by delta-15 desaturase (Wang et al. 2013). ω-3 and ω-6 fatty acids compete toward elongases, for the addition of carbons and toward desaturases, for the addition of double bonds in their chains. Delta-6 desaturase converts LA and ALA to GLA and Stearidonic Acid (SA, 18:4n-3), respectively. Successively, GLA and SA are metabolized to DGLA and Eicosatetraenoic Acid (ETA, 20:4n-3), respectively, by elongase activity.

Delta-6 desaturase converts these fatty acids into ARA and EPA, respectively. ARA is elongated to Adrenic Acid (ADA, 22:4n-6). In mammals, it has been thought that ADA is elongated to ω-6 tetracosatetraenoic acid (24:4n-6) and that delta-6 desaturated to ω-6 tetracosapentaenoic acid (24:5n-6). Then ω-6 tetracosapentaenoic acid is β-oxidized to produce ω-6 docosapentaenoic acid (ω-6 DPA, 22:5n-6) (Voss et al. 1991).

Instead, EPA is converted to ω -3 Docosapentaenoic Acid (ω-3 DPA, 22:5n-3), that after the elongation to ω-3 tetracosapentaenoic acid (24:5n-3), the delta-6 desaturated to ω-3 tetracosahexaenoic acid (24:5n-6). In the end, ω-3 tetracosahexaenoic acid is β-oxidized to

DHA (22:6n-3) in peroxisomes (Voss et al. 1991). Another path is also identified for both ADA and ω-3 DPA: they can be desaturated by delta-4 desaturase in primates (Park et al. 2015), marine vertebrate (Li et al. 2010) and lower eukaryotes (Qiu et al. 2001; Tonon et al. 2003).
Moreover, omega-6 fatty acids are converted to omega-3 fatty acids by omega-3 desaturase; this enzyme has been identified in cyanobacteria (Sakamoto et al. 1994), some plants (Okuley et al. 1994; Vrinten et al. 2005), lower eukaryotes (Sakuradani et al. 1999; Oura & Kajiwara 2004) and animals such as nematodes (Spychalla et al. 1997).

For the biosynthesis of monounsaturated fatty acids is required Stearoyl-CoA Desaturase (SCD), a key and highly regulated enzyme which catalyzes the D^9-*cis* desaturation of a range of fatty acyl-CoA substrates. To date four mouse SCD isoforms (1–4) and two human isoforms (1 and 5) have been identified. Human Stearoyl-CoA Desaturase 1 (SCD1) is abundantly expressed in lipogenic tissues, whereas human SCD5 is predominantly expressed in the brain and pancreas (Castro et al. 2011).
SCD1 is involved in the last reaction of *de novo* lipogenesis that catalyzes the biosynthesis of monounsaturated fatty acids from saturated fatty acids that are either synthesized *de novo* or derived from the diet. SCD in conjunction with NADH, the flavoprotein cytochrome b_5 reductase, and the electron acceptor cytochrome b_5 as well as molecular oxygen introduces a single double bond in a spectrum of methylene-interrupted fatty acyl-CoA substrates. The preferred substrates are palmitoyl- and stearoyl-CoA, which are converted into palmitoleoyl- and oleoyl-CoA, respectively. These reactions are catalyze from D9-16D and D9-18D, respectively, by introducing a *cis* double bond between carbon 9^{th} and 10^{th} of the acyl-CoA substrate (ALJohani et al. 2017). Besides changes in D5D and D6D indexes, changes in SCD1 occur in NAFLD condition. In fact, in fatty livers an increase of SCD1 levels was found and in 2010, Buquè and his collaborators attested a significant correlation between SCD1 index and the severity of hepatic steatosis (Buqué et al. 2010).

1.4 Animal models for the study of Non Alcoholic Fatty Liver Disease (NAFLD)

Numerous animal models have been developed to elucidate both the development and progression of NAFLD to NASH and the assessment of the therapeutic effects of different agents. A good animal model of NAFLD and NASH should show the greatest number of histopathological and pathophysiological characteristics found in patients, such as steatosis, insulino-resistence, obesity and hepatocellular ballooning.

Unfortunately, no single animal model has encompassed the full spectrum of human disease progression, but they can mimic particular charateristics of human pathologies (Sanches et al. 2015). Therefore, it is important that researchers choose the appropriate animal model to answer their research question. Currently, two different types of animal models are prevailing: the genetic and the nutritional models (Takahashi et al. 2012).

1.4.1 Nutritional model: Methionine-Choline Deficient (MCD) diet

The most widely nutritional model used to study the pathogenic mechanisms underlying NAFLD/NASH is represented by the Methionine-Choline Deficient (MCD) diet.

The methionine and choline deficient diet has as main components high sucrose and fat composition (40% sucrose, 10% fat), but lacks of methionine and choline.

Methionine and choline are fundamental substances for the β-oxidation of FFAs in the liver and for the synthesis of phosphatidylcholine, an essential phospholipid for the production of Very Low Density Lipoproteins (VLDLs). The deficiency of choline leads, in fact, to a block of VLDLs secretion of and therefore to a failed export of lipids outside the liver with consequent accumulation. Methionine, on the other hand, is an amino acid essential for the synthesis of proteins and two antioxidant molecules: S-Adenosyl Methionine (SAM) and glutathione (GSH), ROS scavenger. The absence of methionine, therefore, causes an ineffective buffering of the oxidizing effect of ROS. The treatment with antioxidants, in fact, attenuates the progression to steatohepatitis and this shows how the oxidative stress is closely correlated with the progress of the disease (Oz et al. 2006).

MCD rats are characterized by body weight loss due to the loss of white adipose tissue (40% in 10 weeks), their liver decreases proportionally in size and develops steatosis, especially in pericentral zone, with consequent onset of necro-inflammation (George et al. 2003). In fact,

MCD fed mice show high levels of inflammation, due to the activation of macrophages and Nuclear Factor-κB (NF-κB), and the infiltration of: Interleukin-6 (IL-6), Transforming Growth Factor β (TGFβ) and Tumour Necrosis Factor-α (Yu et al. 2006; Ip et al. 2004).
Also the increased expression of Intercellular Adhesion Molecule 1 (ICAM1), Vascular Cell Adhesion Molecule 1 (VCAM1) and Macrophage Chemotactic Protein 1 (MCP1), accompanied by raised macrophage phagocytic activity and increased leukocyte adherence contribute to exacerbate liver steatosis (Leclercq et al. 2004; McCuskey et al. 2004). Although MCD animals display severe liver damage, as demonstrated by increased serum ALT levels, there is a reduction of triglyceride and cholesterol levels occurred in plasma. This finding is in contrast with what has been observed in overweight and obese individuals suffering from fatty liver disease (Weltman et al. 1996; George et al. 2003). However, some studies have shown that the MCD diet is responsible for the reduction in insulin sensitivity due to the increase in oxidative stress and the activation of the c-Jun N-terminal Kinase (JNK), in order to suppress the insulin receptor activity (Schattenberg et al. 2006; Schattenberg et al. 2005). 8 weeks MCD diet rats show hepatic steatosis, hepatocyte injury, inflammation and ultimately fibrosis. We assist to a plethora of changes that mimic the hepatic pathology of NASH (Veteläinen et al. 2007).

1.4.2 Other nutritional models

Among nutritional models, rats and mice are subjected to a particular diet, in order to develop metabolic disorders. Examples of diets formulated for this purpose are the High Fat (HF) diet and the cafeteria diet. HF is a Western-style diet, in which most of the caloric intake (45-75%) is given by fats or cholesterol. An example of this food model was developed by Lieber and colleagues and consists in the administration of a liquid consisting of 71% of fats, 18% of proteins and 11% of carbohydrates (Lieber et al. 2004).
These rats show biochemical anomalies similar to those found in human steatohepatitis: lipid deposition, oxidative stress, increase in insulin and TNF-α levels. HF is considered a good model because it is able to represent the majority of the pathophysiological characteristics of the pathology, but it fails to mimic the aetiology, since the administration of the diet is forced (Lieber et al. 2004).

In addition, cafeteria diet imitates a Western-style diet and includes industrially processed foods (biscuits, meat, cereals, cheese) rich in fat, salt and carbohydrates. Represents a better human metabolic syndrome model than HF and gives a phenotype characterized by obesity, glucose intolerance and inflammation (Sampey et al. 2011). Studies conducted on animal models subjected to cafeteria diet have shown that in pro-inflammatory mediators, such as TNF-α, IL-6 and NF-kB, do not increase despite the onset of oxidative stress, insulin resistance, hypertriglyceridemia and obesity (Carillon et al. 2013). Pasarìn and colleagues showed that by treating rats for 15 days simultaneously with a cafeteria diet and a standard diet, they develop above all steatosis, steatohepatitis, chronic inflammation throughout the adipose tissue and also hyperphagia, hyperinsulinemia and glucose intolerance (Pasarín et al. 2012). Therefore, this model adequately mimics the etiopathogenesis of human NAFLD, although it does not progress spontaneously towards fibrosis.

1.4.3 Genetic model: Zucker

Today, Zucker rats represent the most used genetic model for the study of NAFLD. They are characterized by a missense mutation (A→C) at the level of the Leptin receptor gene ($Lepr^{fa}$), localized on chromosome 5, and coding for the leptin receptor (Ob-Rb) (Takaya et al. 1996). Leptin, a hormone produced by white adipose tissue adipocytes, is directed to the brain where it controls appetite, satiety and energy expenditure. It is also involved in thermoregulation, fibrogenesis, modulation of cell death and oncogenesis. Leptin performs its activity through the interaction with its receptor Ob-Rb. Six different isoforms of the alternate splicing Ob-Rb receptor have been detected: the largest isoform is found in the hypothalamus and it is thought to manage the main pathway of leptin-mediated signal transduction; shorter isoforms are found at the level of peripheral tissues (Moran & Phillip 2003). The binding of the receptor ligand leads to its homodimerization and to the activation of the JAK/STAT pathway and other signal transduction cascades (Wang et al. 2000).

However, homodimerization in Zucker rats does not happen because they are homozygous recessive for the mutation (fa/fa). This mutation causes a decrease in the affinity of leptin to its receptor, which results in a marked leptin resistance. Thus, in several studies, heterozygous rats (+/fa) are used as controls (Fellmann et al. 2013). Zucker rats are widely used as models for obesity, diabetes and metabolic syndrome, the principal factors in the pathogenesis of

NAFLD. Zucker rats lose their sense of satiety and become hyperphagic, obese, develop insulin resistance, mild hyperglycemia and hyperlipidemia (Oana et al. 2005). Following leptin resistance, an increased production of SREBP-1c and ChREBP occurs. In particular, SREBP-1c activates the transcription of genes coding for lipogenic enzymes, contributing to the accumulation of triglycerides in the liver (Kakuma et al. 2000).

The lipid deposit in obese rats occurs especially in the periportal area (Carmiel-Haggai et al. 2005). Furthermore the liver of these animals present a low concentration of GSH, vitamin E and a decreased activity of catalase, peroxisomal enzyme responsible for the removal of hydrogen peroxide from cells (Soltys et al. 2001). Although Zucker animals develop macro- or microvesicular steatosis, no spontaneous progression to steatohepatitis occurs. A further stimulus is needed to induce the progression to NASH, such as: exposition to lipopolysaccharides, administration of an MCD diet combined with high saturated fat or a diet rich in disaccharides in association with a low concentration of lipoproteins (Fukunishi et al. 2009; Yang et al. 2012)**.** In addition to liver injury, there are high serum ALT, TNF-α and TGF-β values, an increase of collagen deposition, hyperglycemia and hepatic stellate cells activation, with subsequent expression of oxidation markers such as lipid peroxidation, proteins with carbonyl groups and altered GSH levels (Carmiel-Haggai et al. 2005).

Although Zucker rats faithfully reproduce metabolic dysfunctions, they are not as effective in simulating etiopathogenesis of this pathology. The first discrepancy between experimental group and the human pathological condition is that not all patients suffering from NAFLD have a mutation affecting the gene coding for leptin or for the Ob-Rb receptor (Leclercq et al. 2002).

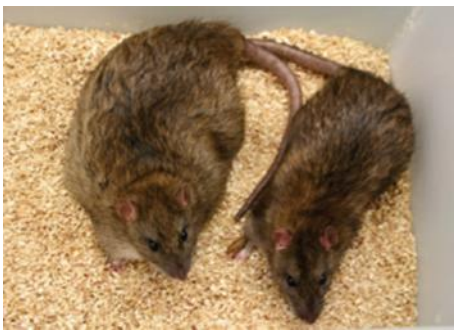

Figure 1.12: **Zucker Rats.** Zucker rats: the recessive (*fa/fa*) genotype develops obesity and liver steatosis (at left side), while the dominant (*lean*) genotype is phenotypically normal (at right side of the picture).

Concluding, genetic models are more suitable for diabetes and NAFLD, while nutritional models, like MCD, are more suitable for studies on lipotoxicity, inflammation and on the pathogenesis of cirrhosis and Hepatocellular Carcinoma (HCC).

1.4.4 Other genetic models

Ob/ob Mice exhibit a spontaneous mutation in the leptin gene, so they are leptin deficient and are not able to produce leptin in their adipose tissue. In physiological conditions, after its secretion by adipocytes, leptin reaches the receptor and regulates the feeding behaviour and energy bursts, promoting a reduced food intake and increasing energy metabolism. In the ob/ob mice, the lack of interaction between leptin and its receptor makes these mice hyperphagic, extremely obese, and inactive (Kucera & Cervinkova 2014). In addition, these animals have an altered metabolic profile and exhibit hyperglycemia, insulin resistance, hyperinsulinemia, and spontaneous development of fatty liver (Diehl 2005).

Db/db Mice present a spontaneous mutation in the leptin receptor gene (Ob-Rb), so, even if they exhibit normal leptin levels, they are resistant to its effects. Thus, the db/db animals are obese, show insulin resistance or diabetes and develop macro-vesicular hepatic steatosis (Wortham et al. 2008).

SREBP-1c Transgenic Mice overexpress Sterol Regulatory Element-Binding Proteins (SREBPs), a family of transcription factors that controls intracellular levels of cholesterol and fatty acids working as a feedback regulatory system. These mice undergo to a deregulation of adipocyte differentiation, which leads to insulin resistance and diabetes. Moreover, whereas the total amount of systemic fat tissue decreases, these animals reveal a significant hepatic lipid accumulation (Shimomura et al. 1998).

1.5 Transplantation

Success in clinical liver transplantation took longer to realize than kidney transplantation. Initial attempts of liver transplantation in 1963 by Starzl in Denver were unsuccessful, but few years later his work showed successful results (Starzl et al. 1963). Calne in Cambridge performed the first transplant in Europe in 1968 (Calne & Williams 1968). In the 1960s, transplants were possible in unrelated people if drugs were taken to suppress the body's immune reaction, but anti-rejection drugs were very poor, until the 1970s with the development of cyclosporine (Borel 2002). In the late 1980s, the field of regenerative medicine emerged as scientists began to apply principles of engineering and cell biology to develop techniques that could restore, maintain or improve body function. Regenerative medicine now includes products that use cells to repair damaged organs and to grow organs outside the body for transplant into the patient. Today, kidney, pancreas, heart, lung, liver, bone marrow and cornea transplantations are performed among non-identical individuals with a large frequency and success increment.

There are many types of transplant: autograft, isograft (syngraft), allograft (homograft) and xenograft (heterograft):

- *Autograft*: it is usually a tissue transplant, typically skin or bone, from one part of the body to another one in the same person. Using the patient's own tissue, the risk of rejection might be reduced.
- *Isograft*: organs or tissues are transplanted between two genetically identical individuals of the same species in a transplant. An isograft transplantation can occur between monozygotic twins; therefore, it is also called syngraft or syngenic graft.
- *Allograft*: it is a transplant of an organ or tissue between two genetically non-identical members of the same species. The allograft transplantations are the most human common transplants.
- *Xenograft*: it is a transplant of organs or tissue from one species to another one.

After transplantation, an immunosuppressive therapy is required to minimize recognition and subsequent rejection of the foreign graft by the recipient's immune system.

There are many types of donor: an organ can be donated by an older person (over 60 to 70 years old), Donation After Cardiac Death (DCD, formerly called Non Heart Beating Donors,

NHBD), and liver with steatosis or exposure to/infection with Hepatitis B or C, in according to the compatibility.
Moreover, other technologies are being investigated, such as the split transplant, in which the organ, usually the liver, is splitted up into two parts, for two recipients, especially an adult and a child, to maximize the benefit of each available donor organ (Broering et al. 2004).

Nowadays, more and more people suffer from hepatic diseases, such as hepatitis, Alcoholic Liver Disease (ALD), Non-Alcoholic Fatty Liver Disease (NAFLD), cirrhosis and cancer. It has been estimated that in 2025 almost 25 million of Americans would be affected by non-alcoholic fatty liver disease (Burke & Lucey 2004). Although the recent improvements afforded by the medicine and by the research in their treatment, the only effective therapy for end-stage liver disease remains the orthotopic liver transplantation (Berlakovich 2002). The growing number of patients on waiting lists for liver transplantation in addition to the shortage of organs have forced many transplantation centers to adopt extended criteria for graft selection, moving the limit of acceptance for marginal livers.
The Expanded Criteria Donors (ECDs), that have proved to be very useful for dealing with this problem, have therefore been drafted. The expanded criteria donors include the use of older donors, the use of donors after cardiac death grafts and the use of livers with high risk of diseases transmission (Vodkin & Kuo 2017). Thanks to these criteria, it is possible to use livers that would have previously been rejected because considered "marginal".
The organs are supposed to be "marginal" when show an increased risk of Primary Non Function (PNF), once transplanted into the recipient, therefore more prone to morbidity or mortality. There are two classes of marginal grafts. The first class is represented by organ with a high risk of technical complications and impaired function, examples of which are steatotic livers, Non Heart Beating Donors (NHBD) also called DCD donors", elderly donors or split livers. Secondly, grafts will be considered marginal if they carry a risk of transmission infection or malignancy to the recipient.
Steatotic livers exhibit intracellular fat droplets. Only the livers with steatosis between 30% and 60% are transplantable because they have only a 25% chance of developing Primary non Function (D'Alessandro et al. 1991). However, livers that show more than 60% of steatosis are discarded because they show increased sensitivity to endotoxins, endothelial damage, decreased ATP deposits, sinusoidal swelling, and congestion following storage and Ischemia Reperfusion (IR) (Chavin et al. 1999; Fukumori et al. 1999). Selzner *et al.*, by comparing

mouse models of microsteatosis and macrosteatosis, showed that macrosteatotic livers are more susceptible to ischemia reperfusion injury than microsteatotic livers, demostrating that it is the presence of macrodroplets that exacerbate the sensitivity to IR (Selzner et al. 2006).

Hepatic steatosis can regress within weeks after liver transplantation, but the immediate post-transplant regenerative capacity of fatty grafts is impaired by more severe ischemia/reperfusion injury (Verran et al. 2003). Liver damage reported in IR injury can be predictive of PNF, acute rejection, biliary complications and is also a good predictor of 1 year graft loss (Ali et al. 2015). The mechanisms underlying the early hepatic graft dysfunction and poor recipient outcome are mainly two. Fat droplets have been seen to expand during cold preservation altering the infrastructure of the cell itself by displacing the surrounding organelles (Takeda et al. 1999). With the expansion of these droplets and the increase in the hepatocytes size secondary to swelling, the microcirculation of the liver tends to deteriorate. Sinusoidal congestion occurs, eventually causing a decrease in blood flow when compared with non-steatotic livers (Teramoto et al. 1994). Also activation of Kupffer cells appears to play an important role in PNF: in fact, they are responsible of free radical generation that, through the inhibition of β-oxidation, mediates lipogenesis (Takeda et al. 1999; Brass & Roberts 1995). During reperfusion, Kupffer cells are further activated by endotoxin insult (Caldwell-Kenkel et al. 1991). Additionally, the fluidity of plasma membrane and the hepatic mitochondrial function are altered during the process of preservation and IR (Fukumori et al. 1999).

A variety of techniques has been tested to address one or more of the putative mechanisms that predispose steatotic livers to IR injury in experimental animals. These approaches generally consist of using pharmacological agents or preconditioning methods to turn on protective pathways before subjecting the liver to IR stress (Nativ et al. 2012).

However, steatotic livers can be transplanted safely with good results for long-term organ survival especially if other contraindications for their use are absent (Angele et al. 2008).

NHBDs represent nowadays a significant source of organs for transplantation. About older donors, during the 1990s, donor liver of about 50 years was usually used, while the elderly pool is currently expanding. In fact, nowadays, a significant number of liver donors over 70 years have the same survival as transplants compared to donors who have fewer than 60 years (Borchert et al. 2005).

Good results were also obtained using liver grafts from donors over 80 year (Zapletal et al. 2005). It should be considered that liver weight and volume as well as blood flow are reduced

with aging (Wynne et al. 1989) and that fibrosis and steatosis incidence are higher in elderly people that younger one. Potential donors with positive serology should not be completely excluded from the donor pool.

Donors with past exposure to hepatitis B infection can be used selectively in some recipients, who were previously administered with vaccines (Hartwig et al. 2005), without carrying Hepatitis B Virus (HBV) infection (Dodson et al. 1999), as it was thought at the beginning.

1.5.1 Liver preservation methods

Currently in the U.S. there are over 100.000 patients awaiting liver transplants with transplant rates of only 38%. This point out an evident need to improve both preservation of the organs and to expand the donor pool. The main goal in organ preservation is to maintain function of the organ and tissue during storage, so that the graft will function at reperfusion. Thus, the maintenance of cellular energy, reached by reducing metabolic demand and ATP hydrolysis, is fundamental (Lee & Mangino 2009). This is pursued by reducing metabolic demand by hypothermia. At the moment, there are two approaches of preservation: static and dynamic (Figure 1.13). Simple Cold Storage (CS) is the method for static storage, and it is the only technique currently employed for livers, lung, pancreas and heart preservation. Among the dynamic approaches, Hypothermic Machine Perfusion (HMP), Normothermic Machine Perfusion (NMP), Subnormothermic Machine Perfusion (SNMP), Hypothermic Oxygenated Perfusion (HOPE) are available.

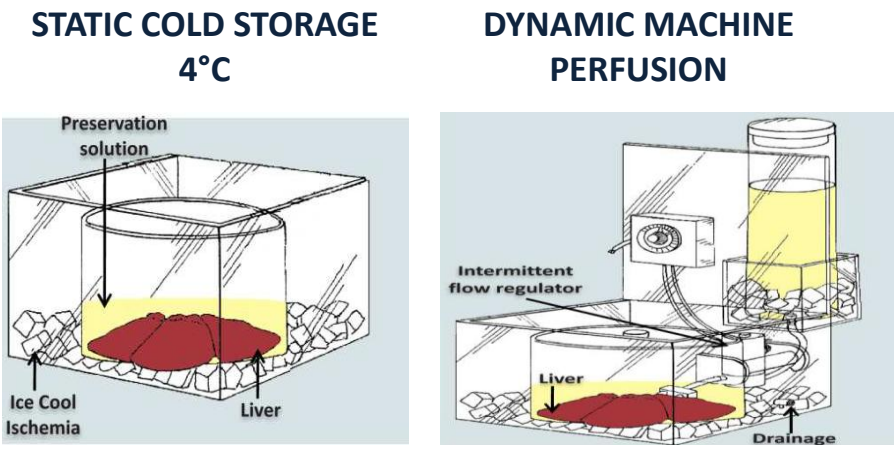

Figure 1.13: Modes of organ preservation. On the left static preservation, on the right dynamic preservation (Jimenez-Castro et al. 2013).

1.5.1.1 Static preservation method

Low temperatures (0-4°C) are applied to organ preservation to reduce metabolic activities that might lead to cellular degradation when oxygen is removed from the donor organ (Lee & Mangino 2009). To enhance storage performances, a cold solution is infused into the portal vein, to wash out the blood and achieve moderate cooling (10-15°C) before final removal from the body (Starzl et al. 1987). Then the organ is stored statically in a plastic bag filled with a preservation solution, and put into an ice-box (Figure 1.14).

Figure 1.14: Simple cold storage. The organ is preserved statically in in a plastic box filled with preservation solution.

To improve organ preservation, several solutions able to mimic intracellular composition and protect intracellular spaces upon the onset of ischemic damage have been developed. The first preservation solution created was the Collins' solution. The development of this buffer increased SCS preservation time to 24 hours for kidneys (Collins et al. 1969).

By using this solution, cellular swelling was avoided thanks a high potassium ion content and a high glucose concentration that acts as a cell impermeant.

Subsequently, modifications of Collins' solution were formulated, such as Euro-Collins solution, in which magnesium phosphate is left out since its precipitation causes crystals (Heinrich 1972), and glucose is replaced with mannitol or sucrose, which provide major protection during prolonged cold ischemia (Andrews & Bates 1985). For the liver, prior to the discovery of the University of Wisconsin (UW) solution, preservation by CS was limited to 6 hours. With the UW solution, preservation improved up to 16 hours and allowed long distance procurement of the donor organ. For this reason, the UW solution has been considered the gold standard for liver preservation since 1989. This formulation includes: impermeants, lactobionate and raffinose, to suppress hypothermia-induced cell swelling and consequent oedema; di-hydrogen phosphate to prevent tissue acidosis; Hydroxyethyl Starch (HES) to

support colloid osmotic pressure, allopurinol and reduced glutathione, as free radical scavengers to prevent oxidative stress; magnesium sulphate as membrane stabilizer, and adenosine and ribose to stimulate ATP synthesis (Belzer & Southard 1988). Successively, to improve mitochondrial function and to prevent early apoptotic events, trophic factors were added to UW solution (Kwon et al. 2007). Experiments on canine kidney preservation confirmed that the employ of an antimicrobial peptide, a neurotrophin, a neuropeptide, and the epidermal and insulin-like growth factors allowed the storage for six days (McAnulty et al. 2002). The UW solution is now used for other donor organs including kidneys, hearts, pancreas, intestine and lungs.

Other solutions have entered the market: Histidine-Tryptophane-Ketoglutarate (HTK) and Celsior. The first solution was initially developed for cardiac surgery by Bretschneider (Bretschneider, 1964), and its effectiveness was later demonstrated also in kidney (Butter et al. 1995) and liver (Rayya et al. 2008). The major components of this solution are histidine, that represents the strong buffer, tryptophan and alpha-ketoglutaric acid, low permeable amino acids, and mannitol, which provides the osmotic barrier. Differently from the previous solutions, the potassium concentration is low as well as sodium and magnesium concentration, and the low viscosity makes the flushing more effective and the cooling of organs more rapid (Guibert et al. 2011). Celsior solution is a "mixture of solutions": in fact, it contained the same buffer of HTK solution, lactobionate and mannitol belonging to UW solution, but with a high content of sodium. At the beginning it was used for heart transplantation, but studies have been performed its efficacy also in lung (Wittwer et al. 1999), kidney (Nunes et al. 2007) and pancreas (Hackl et al. 2010).

In France, a solution very similar to that of UW solution was developed very recently: the Institute Georges Lopez (IGL)-1 solution. Its medium composition differs for high sodium and low potassium concentrations. The peculiarity of this solution is the use of Polyethylene Glycol (PEG) as colloid instead of HES, that has been discovered to be oncogenic (Zaouali et al. 2011). It provided endothelial cell protection and antioxidant defence, in addition to the reduction of immunological responses involving Major Histocompatibility Complex (MHC) type II and the reduction of expression markers of apoptosis (Badet et al. 2005). It has been demonstrated the superiority of IGL-1 to UW solution for rat liver preservation after 24 hour cold storage (Ben Abdennebi et al. 2006; Franco-Gou et al. 2007). More recently, it has been showed that IGL-1 solution more efficiently protected steatotic livers against cold Ischemia Reperfusion Injury (IRI) than UW solution (Ben Mosbah et al. 2006). Despite these beneficial

effects that initially are associated with antioxidant effects, the protective mechanisms of IGL-1 are complex. They may include the involvement of several prosurvival molecules such as Nitric Oxide (NO). NO, in fact, has been demonstrated to activate Hypoxia-Inducible Factor-1α (HIF-1α) in normoxia, by inhibiting prolyl-hydroxylase (Zaouali et al. 2011).

1.5.1.2 Dynamic preservation method

For standard liver graft preservation, SCS with different solutions remains highly successful. Since the need for organ is increased in recent years, the use of novel techniques for optimizing suboptimal graft preservation is arousing increase. Machine Perfusion (MP) is a dynamic technique employed to preserve organs during transplantation. The explanted organ is put into a chamber, continuously perfused, with either an oxygenated or non-oxygenated solution using a pump (Figure 1.15). This continuous perfusion permits better penetration of the preservation solution, a washout of blood and equilibration of the interstitium with the perfusate medium, delivery of oxygen and nutrients, and removal of toxic metabolites. Another advantage of using this technique is to allow real-time monitoring of the functional and biochemical performance of the graft and the provision of metabolic support during preservation (Taylor & Baicu 2010). MP can be performed under different temperatures such as hypothermic, subnormothermic or normothermic conditions, as an innovative approach to reclaiming marginal livers (Kron et al. 2018; Kollmann & Selzner 2017).

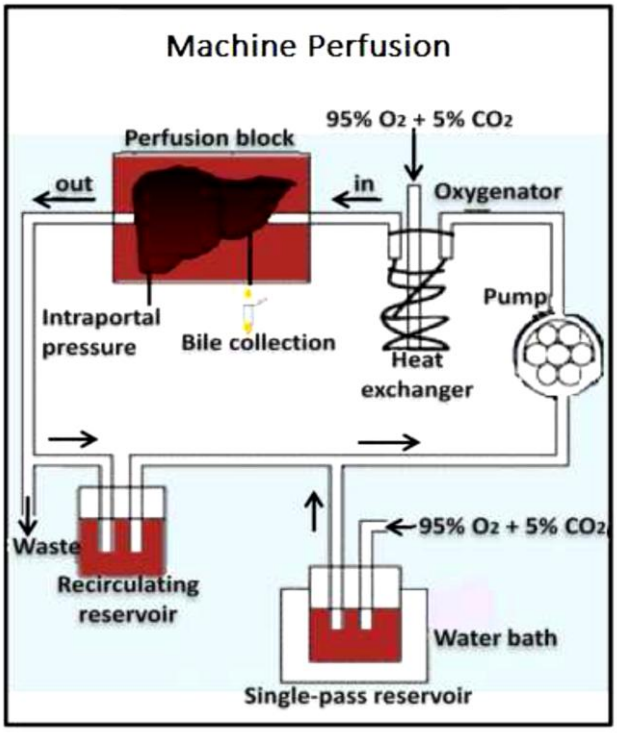

Figure 1.15: Scheme of machine perfusion. The organ is placed in a chamber and continuously perfused, as indicated by the arrows (from Jiménez-Castro et al. 2013).

During perfusion, ATP levels recover (Berendsen et al. 2012; Izamis et al. 2013), alleviating some aspects of ischemia/reperfusion injury, and this is well-correlated with enhanced transplant success. MP may also decrease vasospasm and provide additional parameters, such as flow and resistance, to evaluate organ viability. Moreover, machine perfusion is able to maintain the hemodynamic stimulation on the vasculature of the organ, which plays a critical role in vascular function under physiological conditions, despite this potential benefit of machine perfusion remains poorly understood (Yuan et al. 2010).

It was shown that the machine perfusion. In particular hypothermal machine perfusion, can also be used for macrosteatotic-explanted livers. For steatotic liver, the preservation using machine perfusion is better than cold storage preservation. In fact, Bessems and colleagues, demonstrated that after 24 hours of hypothermic storage of steatotic livers using the UW solution or hypothermic MP, bile production, urea production, ammonia clearance, oxygen consumption and the ATP levels during normothermic reperfusion were significantly higher after machine perfusion than after cold storage (Bessems et al. 2007). Subnormothermic machine perfusion on rat liver (Vairetti et al. 2009) as well normothermic machine perfusion

on pig liver (Jamieson et al. 2011) showed a reduced preservation injury in steatotic organs compared to the common cold preservation. *Ex-vivo* normothermic perfusion for 48 hours led to an approximate 50% reduction in lipid droplet size to reach the size found in control lean livers.

This approach represents an alternative to simple cold preservation. Thanks to this technique, it is possible to monitor organ conditions during the entire process and to obtain a time window for a pharmacological intervention. During the years, several variants of machine perfusion have been developed, characterised by different parameters.

Hypothermic machine perfusion

Hypothermic Machine Perfusion (HMP) is a dynamic cold preservation method in which the organ is perfused with a solution oxygenated (Op den Dries et al. 2014) or non-oxygenated (Guarrera et al. 2010) at 4°C. Alexis Carrel first developed this concept in the early 20th century. HMP ensures homogeneous and continuous supply of metabolic substrates to the graft during the *ex vivo* period. HMP is able to supply oxygen to the tissue for ATP synthesis via perfusion of fluids that can carry oxygen.

Thanks to the low temperature, the oxygen need is low, so the demand for oxygen is also low. Moreover, at low temperature also the consumption of energy reserves (glycogen and ATP) is reduced, as well as the production of toxic catabolites. However low temperature can cause vasoconstriction (Jackson 2000). Consequently, this could lead to an increase in flow resistance with disruption of hepatic microcirculation, because of an imbalance in the production of vasoconstrictor and vasodilator substances within the liver (Menger & Vollmar 2000). Livers from DCD rats preserved with HMP showed lower Lactate Dehydrogenase (LDH) and higher bile production, compared to cold storage preserved livers. These findings suggest that HMP protects hepatic function and that worse Sinusoidal Endothelial Cell (SEC) damage during cold storage is which an important pathway graft dysfunction after reperfusion (Lee et al. 2002; Xu et al. 2004). HMP is mainly used for kidney transplantation but despite its advantages, less for the liver. Recently, Guarrera and colleagues conducted a study on 20 adults received HMP-preserved livers. HMP was performed for three-seven hours using perfusion solution at 4–6°C. No cases of primary non-function occur, and serum injury markers were significantly lower in the HMP group. The survival after one year was 90%, and the two death that occurred were caused by pneumonia and cardiovascular diseases, without any correlation with preservation or transplantation techniques (Guarrera et al. 2010).

Currently, this is the only human application of machine perfusion liver preservation. To improving the quality of liver preservation in normal or DCD livers, Oxygenated HMP (HOPE) has been developed. HOPE enables grafts to restore tissue homeostasis and to maintain the functional integrity of hepatocytes during ischemia. Treatment by HOPE results in less oxidative stress, improves liver function and decreases cell death. In the past few years, it has been demonstrated that this technique is more effective than simple cold storage in NHBD liver of rat and pig (Dutkowski et al. 2006).

Normothermic machine perfusion

The principle of Normothermic Machine Perfusion (NMP) is to maintain the liver in a near-physiological environment. So. to perform this technique, a solution containing blood and nutrients is perfused at body temperature (37°C). At this temperature, the amount of oxygen required is great, thus to supply to this lack, the perfusion has to be continuous. NMP is an emerging technology whose potential in liver preservation has been description in several animal studies, which have shown its superiority over CS in the preservation of liver graft (St Peter et al. 2002; Fondevila et al. 2011). Moreover, NMP preservation is logistically more complex than HMP and includes the risk for bacterial contamination during the preservation (Vekemans et al. 2008).

Subnormothermic machine perfusion

In 2009, Vairetti et al. observed that with Subnormothermic (20°C) Machine Perfusion (SNMP) the preservation of steatotic rat livers is better as compared with those preserved by traditional cold storage. The marked damage reduction was evaluated as enzyme and cytokine release, bile excretion, and energy recovery. ATP levels, energy charge, ATP/ADP ratio and bile production were higher, whereas nitrate/nitrite concentration were lower, as well as oxidative stress, TNF-α and biliary Alkaline Phosphatase (AP) release. Fatty livers preserved by subnormothermic machine perfusion showed a better outcome also in terms of morphology, glycogen stores and reactive oxygen species production (Vairetti et al. 2009). This approach represents a compromise between hypothermic and normothermic machine perfusion. In fact, it avoids some of the downsides of hypothermia, such as cell swelling, whilst maintaining mitochondrial function; furthermore, it may circumvent the logistical restraints of NMP.

1.6 Mechanisms of hepatic Ischemia Reperfusion (IR) injury

Liver transplantation is the definitive treatment option for end-stage liver diseases. Unfortunately, liver transplantation is limited by Ischemia Reperfusion (IR) injury. For ischemia we mean an inadequate supply of blood to tissues, causing a shortage of oxygen and glucose needed for cellular metabolism to keep tissues alive. Several factors contribute to the onset of this damage, that could lead to cell death, either for apoptosis or necrosis, while the recipient could suffer from primary non function. The extent of IR injury depends on one hand from preservation period and, on the other, from the organ conditions. In fact, the organ injury is reversible if the organ is subjected to a short ischemia period; on the contrary, when long ischemia period occurs, the injury becomes irreversible. Reperfusion consists into rewarming the organ at 37°C, that is necessary to re-establish the oxygen and nutrients delivery to support cell metabolism and remove potential damaging by-products of cellular metabolism (Kalogeris et al. 2012). According to the temperature at which it is achieved, hepatic IR injury can be subdivided into warm and cold ischemia: the first one takes place at body temperature (37°C), while the latter at 4°C (Baumann et al. 1989).

The warm ischemia reperfusion injury, which is initiated by hepatocellular damage, develops *in situ* during liver transplantation surgery or in some types of toxic liver injury, such as hypovolemic shock, sinusoidal obstruction, Budd-Chiari syndrome and sleep apnea, in which the blood flow is temporarily suspended. It might lead to liver or even multi organ failure.

Cold ischemia, instead, which is initiated by damage to hepatic sinusoidal endothelial cells and disruption of the microcirculation, occurs during *ex vivo* preservation and it is usually coupled with warm IR during liver transplantation surgery (Zhai et al. 2013).

The warm and the cold ischemia reperfusion injury types share common mechanisms in the disease aetiology: morphological changes, ATP depletion, local inflammatory innate immune activation and oxidative stress (Zhai et al. 2011).

During ischemia, hepatocytes show morphological changes, as the formation of protrusions on cell membranes, which are called "blebs". Blebs contain cytosol and endoplasmic reticulum and exclude most organelles, such as mitochondria and lysosomes. All this phenomena lead, in general, to an increase of 30-50% of the total volume of the cell, known as cellular swelling. When blebs break out, the release of enzymes, intracellular catabolites and even the collapse of ionic and electrochemical gradients occur. Moreover, hypoxia causes

mitochondrial impairment, cellular acidosis and Kupffer cells activation (Lemasters 1999).

1.6.1 ATP depletion

Adenosine Tri-Phosphate (ATP) is an essential energy substrate: its hydrolysis, in fact, provides energy for several metabolic and biochemical reactions involved in development, adaptation and cell survival. Oxidative phosphorylation is mediated by an electron transport chain and establishes a transmembrane electrochemical gradient by supporting the accumulation of protons in the intermembrane space of the mitochondria. This gradient is used as an energy source by ATP synthase during the synthesis of an ATP molecule from a molecule of Adenosine Di-Phosphate (ADP) and an inorganic phosphate. Without oxygen, oxidative phosphorylation stops and the proton gradient between the intermembrane space and the inner mitochondria is abolished, thus ATP synthesis is interrupted. The reduction of ATP is a crucial event in ischemic damage.

This rapid fall in intracellular ATP induces a cascade of events leading to reversible cell damage, that, over time, gradually becomes irreversible, causing cell death and destruction of the parenchymal tissue. Thus, to survive, the cell shifts its metabolism from aerobiosis to anaerobiosis, during which glycolytic substrates are able to rescue sinusoidal endothelial cells from lethal cell injury (Nishimura et al. 1998).
Unfortunately, glucose does not protect hepatocytes against anoxic injury since hepatocytes lack hexokinase IV, which catalyses the first glycolysis reaction to obtain Glucose 6-Phosphate (G6-P) from glucose. However, hepatocytes use glycogen as a source of energy and, once it is transformed in glucose 6-phosphate, can enter the glycolytic cycle. When glucose and glycogen are sold out, hepatocytes exploit fructose or, in addition, they can enhance gluconeogenesis (Nieminen et al. 1990). Other consequences of ATP depletion are the release of free radicals and destruction of plasmatic membranes because Na^+/K^+ ATP-ases can not control electrochemical gradient anymore, causing cellular edema (Reddy et al. 2004).

1.6.2 Intracellular acidosis

Another process occurring during IR injury is intracellular acidosis. This event is due to the increased production of protons, caused by metabolic modifications that quickly

saturate the buffering capacity of the cell. Intracellular acidosis alters the physiological functioning of the cell by increasing intracellular Na^+ through the activation of Na^+- and Ca^{2+}-exchangers, increasing the production of free radicals, changing the affinity of proteins and their tertiary structures, inhibiting enzymes and disrupting the function of sarcoplasmic pumps and carriers (Martin & Parton 2006). The production of lactate from pyruvate by Lactate Dehydrogenase (LDH) is the main source of protons during ischemia. The accumulation of extracellular lactate greatly reduces the effectiveness of the lactate/proton co-transporter, preventing the removal of protons. Additionally, the residual metabolic activity also contributes to acidosis, as the hydrolysis of an ATP molecule releases a proton (Gourdin & Dubois 2013).

During ischemia, an increase in intracellular calcium also occur. This event triggers the activation of several enzymes, as proteases which are responsible for cytoskeleton damages and vesicles formation; phospholipases causing deformation of plasmatic membrane; endonucleases determining chromatin condensation; in mitochondria, ATP synthase, pyruvate dehydrogenase, isocitrate dehydrogenase, that contribute to stop ATP depletion. An imbalance of calcium homeostasis leads to cell death of hepatocytes as well as endothelial and Kupffer cells (Schanne et al. 1979).

1.6.3 Kupffer cells activation

Kupffer cells are specialised macrophages within the liver, localised between sinusoid walls that form part of reticulo-endothelial system and blood vessels. Their role is the defence of the liver from bacteria, viruses and other exogenous compounds. As already mentioned, Kupffer cells play a central role in ischemia reperfusion injury. During IR process, Kupffer cells are overproduced, and their products, such as cytokines, are consequently overexpressed. The cytokines produced are TNF-α, Interleukine-1 (IL-1) and Interleukine-6 (IL-6) (Wanner et al. 1996). IL-1 and IL-6 increase the oxidative stress damage and induce apoptosis in hepatocytes; TNF-α stimulate chemokines release that in turn recall other cells of immunity. Moreover, cytokines promote the secretion of selectines and integrines, that occlude sinusoids, prolonging hypoxia and triggering a further activation of Kupffer cells.
During liver IR injury, NO reduction is associated with the worsening of the hepatic damage. In this particular condition, the NO steady state production decreases, due to a reduced eNOS

activity. On the contrary, restoration of NO to more physiological levels diminishes the liver ischemic injury, enhancing hepatic oxygenation and sinusoidal microcirculation (Siriussawakul et al. 2010). There is a still open controversy about the detrimental or beneficial effects of NO in IR injury. In a rat model of hepatic IR, Wang and co-workers observed an increase in iNOS mRNA and protein expression on the first day following hepatic reperfusion. Upregulation of iNOS is directly proportional with increased hepatic injury. Administration of a non-selective nitric oxide synthase inhibitor significantly increased AST and ALT, whereas administration of a selective iNOS inhibitor significantly decreased transaminases levels. These data suggest that the deleterious effects of the non-selective NOS inhibitor were due to inhibition of eNOS, while the protective effects of the selective inhibitor were caused by inhibition of iNOS (Wang et al. 2000).

1.6.4 Oxidative stress

Radicals are molecules or atoms owning an unpaired electron: this electron make the radical extremely reactive and it is able to bind to other radicals or to take away an electron to neighbour molecules, giving rise to new radicals, triggering a cascade mechanism.

Despite oxygen has two unpaired electrons, it is not so reactive per se. However, when it absorbs a certain quantity of energy, it can reach the excited state, being in this way more reactive: Reactive Oxygen Species (ROS) are so formed. Several radicals are formed in the cell: superoxide anion (O_2^-), hydroxyl radical (OH^-), hydrogen peroxide (H_2O_2) and singlet oxygen (O_2). They are generally synthesized in mitochondria by the electron transport chain, in peroxisomes by soluble enzymes, such as xanthine oxidase and aldehyde oxidase, in the endoplasmic reticulum by P450 system or in nuclear membrane (Chalasani et al. 2003). In physiologic conditions, ROS production is not harmful: cells have specific endogenous systems, able to reduce their effect. Superoxide Dismutase (SOD), reduced glutathione, vitamins A, E, C, some elements (selenium, zinc, magnesium) and other molecules, such as melatonin and bilirubin, are the most important antioxidant, involved in the counterbalancing ROS production and inactivation (Sauer et al. 2001) (Figure 1.16).

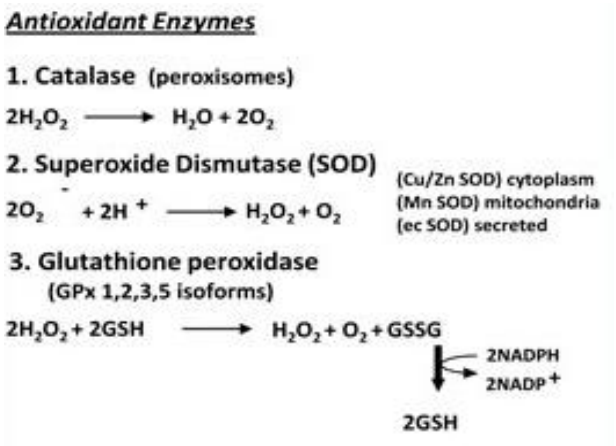

Figure 1.16: Reactions catalyzed by antioxidant enzymes (Novo & Parola 2012).

However, the main factor that determines the establishment of oxidative stress is the depletion of antioxidant defenses that normally protect cells from the action of ROS.

Once producted, ROS can interact with:

- superoxide dismutase type 2 (SOD-2) and other antioxidant enzymes, decreasing its activity;
- the chains of polyunsaturated fatty acids of cardiolipin, phospholipid present in the inner mitochondrial membrane and indispensable for the functioning of the electron transport chain;
- the components of the electron transport chain, inducing the production of further reactive oxygen species;
- mitochondrial DNA, causing mutations and ruptures in the double helix (Paradies et al. 2014; Lismont et al. 2015).

The carbonyl groups found in proteins are considered as initial markers of oxidative stress, since their introduction into protein chains could be due to the reaction between the nucleophilic portion of some amino acid residues (cysteine, histidine, lysine) and the compounds aldehydes produced after lipid peroxidation (Dalle-Donne et al. 2003).

As already mentioned above, ROS can represent one of the factors that determine the development and progression of NAFLD to NASH. NAFLD is a pathological condition characterized by a massive lipid deposit inside the hepatocytes due to an excessive flow of FFAs, or to a consistent lipogenesis. Numerous compensatory mechanisms are activated in response to excess triglycerides in liver, such as increase and acceleration of mitochondrial β-

oxidation and proliferation and enlargement of liver peroxisomes. In fact, peroxisomes are responsible for the metabolism of long chain and branched chain fatty acids that can not enter directly into the mitochondria (Reddy & Mannaerts 1994). Another compensatory mechanism is the expression of cytochrome P-450 reductase (CYP2E1) because it is involved in the oxidative pathway of fats in microsomes (Chalasani et al. 2003). This accumulation causes an alteration in the oxidative capacity of mitochondria, modifying the oxidation-reductive state of the enzymatic complexes that make up the electron transport chain and stimulating the oxidative pathways of fats at the microsomal and peroxisomal level (Rolo et al. 2012). ROS, in addition to being responsible for apoptosis, cause also the release of inflammatory cytokines and lipid peroxidation. The reactive oxygen species, in fact, attack the Polyunsaturated lipids (PUFAs), stimulate the synthesis of Malondialdehyde (MDA) and 4-Hydroxy-Nonenal (HNE), substances that have a higher half-life and mobility than ROS and migrate in different tissues amplifying oxidative stress (Esterbauer et al. 1991). This condition of imbalance between the production of ROS and the presence of antioxidant agents is often considered the main cause of progression from steatosis to steatohepatitis.

The methionine-choline deficient nutritional model has been studied to deepen oxidative stress and to understand the potential role of hepatic lipid composition in the onset of steatosis and in the evolution of the latter in steatohepatitis. In murine specimens subjected to a diet free of methionine and choline, in fact, there is an overproduction of ROS by an equally strong synthesis of antioxidant molecules, a decrease in SFAs and an increase in polyunsaturated fatty acids. As already described, the polyunsaturated fatty acids are preferentially oxidized because of a reduced bond strength between the carbon and the hydrogen of the methyl groups involved in the unsaturations. Following oxidation, the resulting products contribute to the diffusion of stress and oxidative damage (Gardner 1989).

Recently, Freitas and colleagues conducted a study on rats subjected to a MCD diet for four weeks, to evaluate the presence of reactive oxygen species and oxidative markers *in situ*. From the analyses carried out, it appears that this type of feeding induces a consistent production of ROS both in the hepatocytes that are found in the periportal region, where the oxygen tension is higher than the pericentral area, as well as at the perisinusoidal level (Freitas et al. 2016). The main sources of ROS in the periportal region are the NADH coenzyme Q reductase (complex I) and the ubiquinol cytochrome C reductase (complex III); while in the sinusoidal cells the synthesis of such oxidants is due to the activity of NADPH

oxidase (Sauer et al. 2001). It was observed, moreover, that in murine models of NAFLD/NASH, a decrease in the glutathione concentration in the liver occurs. In the case of Ob/Ob mice, glutathione decline is a consequence of the lower transport of GSH from the cytosol to the mitochondria, due to the excessive deposition of cholesterol in the inner mitochondrial membrane (Llacuna et al. 2011). In rats subjected to MCD diet, the decreased hepatic content of glutathione is given by the lack of synthesis of S-adenosylmethionine, the main donor of methyl groups in the liver and precursor of GSH.

The MCD animals represent a suitable model to investigate the correlation between liver steatosis and oxidative stress that occurs in humans. In fact, many studies have demonstrated that, as murine models, also patients with NASH are characterized by an antioxidant status apparently incapable to compensate for oxidative stress and consequent high levels of serum oxidative markers such as thioredoxin, oxidized LDL and TBARS (Sumida et al. 2003; Yesilova et al. 2005).
Moreover, in patients affected by NASH, oxidative stress is associated with a mitochondrial dysfunction characterized by alterations in the number, activity, morphology of the liver mitochondria, which have paracrystalline inclusions and show a loss of ridges (Ricci et al. 2008; Caldwell et al. 1999). Yesilova and colleagues demonstrated in 2005 that a correlation between body fat, lipid metabolism and decreased antioxidants takes place; they contribute to alter oxidative status and lipid peroxidation in NAFLD (Yesilova et al. 2005).

Reactive species are overproduced also throughout IR process. In 1988 Jaeschke and colleagues published a paper describing the two types of damage that characterize the liver reperfusion. The first type of damage occurs during the first few hours following re-oxygenation and it is characterized by a strong oxidative stress induced by Kupffer cells. The activation of Kupffer cells promote the formation of ROS and complement activation (Spencer et al. 2013). ROS act also as signalling molecules that upregulate nuclear transcription factors like NF-κB and subsequently release TNF-α and IL-1 (Cursio et al. 1999). Additionally, ROS can cause damage from oxidant stress, which occurs during the early phase of injury, and activate inflammatory pathways that lead to neutrophil accumulation in the liver in the later phase (Papadopoulos et al. 2013).

1.7 Liver lobe heterogeneity

In recent years, there is an increasing evidence of a functional heterogeneity between the individual liver lobes. The liver parenchyma displays a functional organization known as metabolic zonation: the hepatocytes lined up between the sinusoids along the porto-central axis show structural and functional heterogeneity (Gebhardt & Matz-Soja 2014). This heterogeneity has been found in both healthy and damaged livers. Several studies reported marked lobe variation in the extent and distribution of tissue injury during chemical carcinogenesis (Richardson et al. 1986), acetaminophen hepatotoxicity (Richard et al. 2005), copper distribution (Faa et al. 1995), and cirrhosis (Regev et al. 2002). As well as a heterogeneous distribution of damage within a single lobe or between different lobes, also the presence of a surprising and yet unexplained intra- and intralobular variability in acute liver necrosis has been demonstrated (Malarkey et al. 2005). Palladini and colleagues, in their study, demonstrated that also in IR model a functional lobar heterogeneity of the liver exists, indicating that different events such as modulation of the Extracellular Matrix (ECM) and oxidative stress occur with different intensities in the hepatic lobes (Palladini et al. 2019).

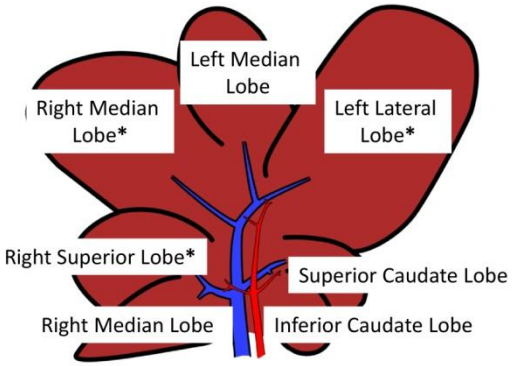

Figure 1.17: Graphic representation of hepatic lobes (Palladini et al. 2019).

One possible explanation may be associated to the distribution of the liver vascular system. Sanger *et al*., described the intrahepatic vascular anatomy in rats and mice liver. Of note, the lobar borders of the liver do not always match vascular territorial borders (Sänger et al. 2015): the Median Lobe (ML) and the Left Lobe (LL) are supplied by the main stem of the portal vein, while the right median portal vein (second order) supplies the right ML. It might be that there is the so-called "Portal Streamlining", that is an incomplete mixing of blood coming from the gastrointestinal tract and spleen, leading to variation in the delivery of nutrients and

toxins to the liver lobes. Another possible mechanism that can play a central role in this hepatic variability is a different lobar gradient of the gene expression profiles (Malarkey et al. 2005), as is the case for paracetamol hepatotoxicity. In this situation the variability of lobar injury correlates with a different regional gene expression (Ruepp et al. 2002).

Finally, as reported by Jacobsonn and colleagues, the difference in the lobe could be due to a phylogenetic difference: the left lobe is older while the Right Lobe (RL) is more recent (Jacobsson et al. 1999). Furthermore, it has been shown that in rat livers a different distribution of vagal afferent neurons correlates with a different functional role (Carobi et al. 1985). Therefore, a possible explanation of the lobes heterogeneity can be attributed to the different microcirculation and different innervations responsible for the specific responses of the different hepatic cell types in which TNF-α and ROS are involved in intercellular communication (Kmieć 2001).

1.8 Liver identification of metabotropic glutamate receptor

Metabotropic Glutamate Receptors (mGluRs) were found in brain in the mid-80 by two different research groups (Sladeczek et al. 1985; Nicoletti et al. 1986). Since then, many articles have been published on this topic. Nowadays, the metabotropic glutamate receptors are among the most studied pharmacological topic, because they are considered as a potential therapeutic targets in the treatment for multiple disease of central nervous system, such as anxiety, depression, pain and Parkinson's disease (Crupi et al. 2019).

However, more recent studies have demonstrated the presence of the mGluRs also in peripheral tissues and have analysed them under physiological and pathological conditions (Julio-Pieper et al. 2011). For example mGluR5 expression has been observed in the gastrointestinal tract at different levels, such as in the mouth and in terminal region of axon innervating human dental pulp (Kim et al. 2009). mGluR5 has been identified also at the gastroesophageal level by Ferrigno and colleagues, which shown its involvement in the Gastroesophageal Reflux Disease (GERD) (Ferrigno et al. 2017). Moreover, the expression of this receptor has been detected also in enteric glia in the ileum and colon of rodents and pigs (Nasser et al. 2007).

In 1997, Sureda and collaborators found metabotropic glutamate receptors in hepatocytes. In particular, they demonstrated that the incubation of hepatocytes with two agonists, quisqualate and (1*S*,3*R*)-1-Aminocyclopentane-1,3-Dicarboxylic acid (ACPD), stimulated polyphosphoinositide hydrolysis (Sureda et al. 1997). Successively, Storto et al. confirmed the presence of mGluR5, and not of mGluR1, in hepatocytes by PCR and immunoblotting analysis (Storto et al. 2000). Storto and colleagues also showed that the activation of this receptor caused cell damage in anoxic hepatocytes. This suggested that the inactivation of mGluR5 protected liver cells from necrosis. The confirmation of the involvement of this receptor in hypoxic conditions was obtained through the use of Knockout (KO) mice for mGluR5 (Storto et al. 2004). The onset of ischemic damage was delayed and viability was improved in cells from KO mice, as well in mouse hepatocytes treated with negative allosteric modulator, compared to wild type control hepatocytes. Furthermore, the blockade of the receptor demonstrated a reduction of ROS production, lipid peroxidation and thiol group oxidation.

As already described, patients with non-alcoholic hepatic steatosis or non-alcoholic steatohepatitis are characterized by lipid accumulation, insulin resistance and obesity. For this reason, the treatment of these pathologies aims to reduce body weight, the intake of calories and promotes physical exercise and behavior modification. In 2004, Bradbury and colleagues conducted a study on rats and mice in which demonstrated that mGluR5 might be involved in modulating appetite and energy homeostasis. In particular, they showed that, by inhibition of mGlu5 receptor with negative allosteric modulators as 3-[(2-Methyl-1,3-Thiazol-4-yl)Ethynyl] Pyridine (MTEP) or 2-Methyl-6-(Phenylethynyl) Pyridine (MPEP), plasma leptin and body weight lowered from decreased adiposity (Bradbury et al. 2004). The acute and chronic appetite suppression and reduction in body weight and adiposity in obese rats suggest a novel role for mGluR5 for intervention in the treatment of chronic obesity in humans (Bradbury et al. 2004).

1.8.1 Classification of mGluR

The molecular cloning of the mGluRs have revealed the presence of eight different isoforms, designated as mGluR 1-8; successively, these subtypes were divided in three groups on the basis of sequence homology and signal transduction mechanisms (Conn & Pin 1997).
Group I include mGluR1 and mGluR5, which are coupled to $G_{\alpha q}$ protein and stimulate the activity of the Phospholipase C (PLC) with formation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Willard & Koochekpour 2013). Group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7 and mGluR8) are both coupled to the $G_{\alpha i}$ protein which inhibits the Adenylate Cyclase (AC), with a consequent decrease in the availability of cyclic Adenosine 3'-5'monophosphate (cAMP) within the cell (O'Connor et al. 2010).
The mGlu5 receptor consists of 3 large domains: the N-terminal domain, the transmembrane domain and the C-terminal domain (Figure 1.18).

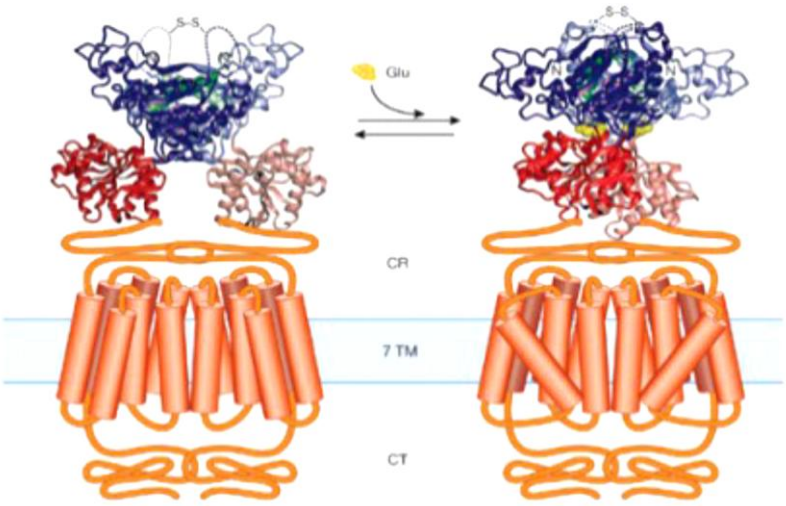

Figure 1.18: Structure of the metabotropic glutamate 5 receptor (mGluR5): The receptor consists of three domains: the extracellular N-terminal that forms a pocket containing the agonist binding site, the seven-transmembrane hydrophobic domain (7TMD) and the Intracellular C-terminal domain that can assume variable dimensions (Lehninger et al. V Edizione).

- The N-terminal is also called Venus Flytrap Domain (VFD). Each VDF is highly preserved (Costantino & Pellicciari 1996) and consist of two lobes separated by a cavity, which represents the orthosteric binding site (Pin et al. 2003). Jingami and colleagues demonstrated that two VFDs dimerize together, back to back, and important conformational changes are induced when agonists bind to one or both venus flytrap domains (Jingami et al. 2003).
- The N-terminal and transmembrane domains are connected by a domain, called "Cystein Rich Domain" (CRD), which contains nine cysteine residues, of which eight are linked by disulfide bridges (Muto et al. 2007). CRD plays an important role because it allows the displacement of the glutamate binding site towards the transmembrane domain (Pin et al. 2003) and it is therefore involved in the transmission of the ligand-induced signal. Indeed, Rondard and colleagues have shown that mutations in the CRD lead to a shutdown of the signal downstream of the receptor (Rondard et al. 2006).
- The domain formed by seven transmembrane hydrophobic segments (7TMD) is involved in signal transduction. The second and third intracellular loops of this receptor form a pocket with which the C-terminal end of the α subunit of the G protein interacts. The longer and less conserved loop 2 represents the recognition site for the G protein. It has been shown, in fact, that the deletion of two specific residues within this loop, Cys694 and Phe781, prevents the activation of the G protein (O'Hara et al. 1993). The loop 3, on the other hand, shorter and highly conserved, plays an important role in the activation of the G protein and, together with the loop 1 and the C-terminal

receptor tail, controls the effectiveness of the interaction with the G protein (De Blasi et al. 2001). Finally, this domain constitutes the binding site of positive and negative allosteric modulators (Conn et al. 2009).

- The C-terminal domain is the least conserved one among the members of the different groups of receptors and can assume variable dimensions. Furthermore, this region is subjected to alternative splicing, with regulation by phosphorylation and protein-protein interactions (Ferraguti & Shigemoto 2006).

1.9 mGluR5 ligands

1.9.1 Orthosteric ligands

The orthosteric ligands bind to the extracellular N-terminal domain and are called "competitive" because they interact with the same binding site of the physiological agonist, glutamate, with the aim of activating the receptor. The glutamate binding site is highly conserved among the different receptor subtypes (Conn et al. 2009).

1.9.1.1 mGluR5 Agonists

The most powerful agonist of group I of mGlu receptors is Quisqualic acid (Quis) which has an efficacy of 50% (EC50) at a concentration of 0.3 μM (Suagstad et al. 1995).
Although it has a high activity on mGlu1 and mGlu5 receptors, it is little used because it is very active also on AMPA receptors.
The first selective agonist for group I of mGlu receptors is (S)-3,5-di-hydroxyphenylglycine (DHPG) which acts exclusively as an S isomer at concentrations between 7 μM and 28 μM.
Another low-potency agonist is 2-chloro-5-hydroxyphenylglycine (CHPG), selective for mGlu5 receptors (Osborne & Krogsgaard-Larsen 1998). A recent publication showed that in rat superior cervical ganglion neurons in which mGlu1 and 5 receptors are expressed, CHPG activates both with the same power and efficacy (Willard & Koochekpour 2013) (Figure 1.19).

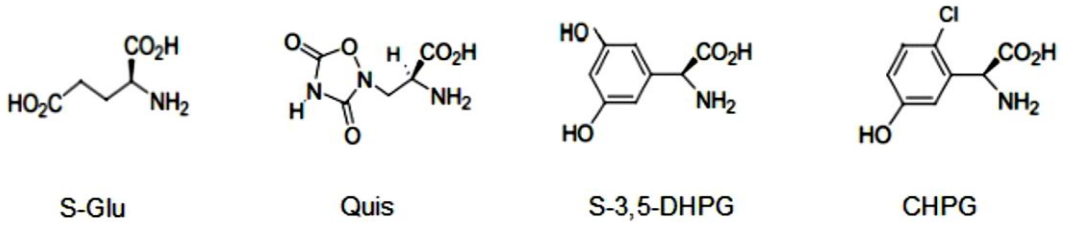

Figure 1.19: Group I mGluR agonists and their chemical structures. From the left, the chemical structure of S-glutamate, Quisqualate, S-3,5-DHPG and CHPG are shown.

1.9.1.2 mGluR5 Antagonists

The first generation of glutamate receptor group I antagonists was composed of 4-carboxyphenylglycine derivatives, such as α-Methyl-4-Carboxyphenylglycine (S)-MCPG. Its affinity was improved when α-methyl-group was substituted with an α-thioxantilmethyl group, as seen in α-thioxantilmethyl-4-carboxyphenylglycine (LY 367366), a molecule that can also antagonize group receptors II and III (Schoepp et al. 1999).

The antagonists that show a maximum selectivity for the mGlu5 receptor are (S)-2-methyl-4-carboxyphenylglycine (LY 367385) and 2-methyl-3-hydroxy-4-carboxyphenylglycine (LY 339840) (Kammermeier 2012) (Figure 1.20).

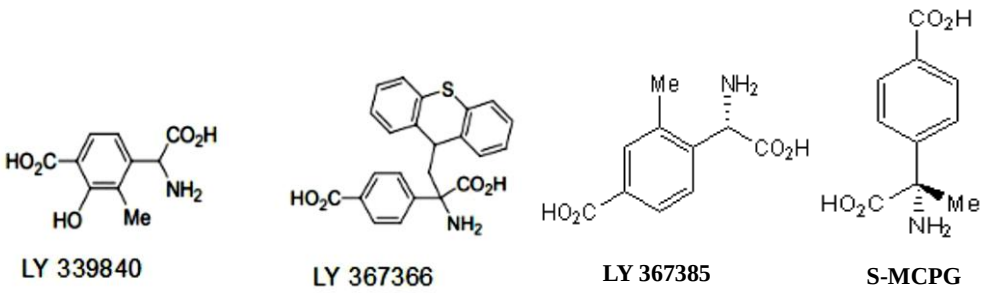

Figure 1.20: Group I mGluR antagonists and their chemical structures. From the left, the chemical structure of LY 339840, LY 367366, LY 367385, S-MCPG are shown.

1.9.2 Allosteric ligands

Allosteric ligands are non-competitive modulators which bind to the allosteric binding site, in the transmembrane heptahelical domain, modulating the response of the receptor (Conn et al. 2009).

Differently from the competitive ligands, these compounds are generally characterized by high potency and subtype selectivity, mainly because their binding site exhibits a great degree of sequence divergence among the different members, a feature that hugely increases the chance to develop potent subtype-selective ligands (Sheffler et al. 2011). Another essential property sharing among the majority of allosteric modulators is their nanomolar potencies, a trait very useful for *in vivo* studies (Niswender & Conn 2010).

1.9.2.1 Positive Allosteric Modulators (PAMs) for mGluR5

Positive allosteric modulators of the mGlu5 receptor do not show intrinsic agonist activity, but present the ability to enhance the agonist-induced response, such as glutamate (Glu), Quis and DHPG (Figure 1.21) (O'Brien et al. 2004). The first selective positive allosteric modulators for the mGlu5 receptor to be described are: 3,3'-difluorobenzaldazine (DFB) and N-[5-Chloro-2-[(-1,3-Dioxoisoindolin-2-Y)Methyl] Phenyl]-2-Hydroxybenzamide (CPPHA) (O'Brien et al. 2004). Furthermore, they lack activity on the mGlu1 receptor and receptors of groups II and III, except for the DFB which appears to have weak activity on mGlu4 and 8 receptors (O'Brien et al. 2004). Subsequently, 3-Cyano-N-(1,3-Diphenyl-1H-Pyrazol-5-Yl) Benzamide (CDPPB) was discovered (Kinney et al. 2004) and (S)-(4-Fluorophenyl)[3-[3-(4-Fluorophenyl)-1,2,4-Oxadiazol-5-Yl]Piperidin-1-Yl]Methanone (ADX47273) (Liu et al. 2008), which show intrinsic agonist activity at high concentrations (Engers et al. 2009), antipsychotic effects on animal models (Kinney et al. 2004) and are still being studied as potential drugs for treatment of schizophrenia (Lindsley et al. 2006).

More recently, other PAMs have been described such as N-Methyl-5-(Phenylethyl-2-Amine) (MPPA) (Sharma et al. 2009) and ((4-Hydroxy-Piperidin-1-Il)-(4-Phenylethyl)Phenyl) Methanone (VU0092273). The latter was subjected to an optimization which allowed to obtain the N-Cyclobutyl-6-((3-Fluorophenyl)Ethinyl) Nicotinamide Hydrochloride (VU0360172) which is selective for the mGlu5 receptor (Rodriguez et al. 2010) (Figure 1.21).

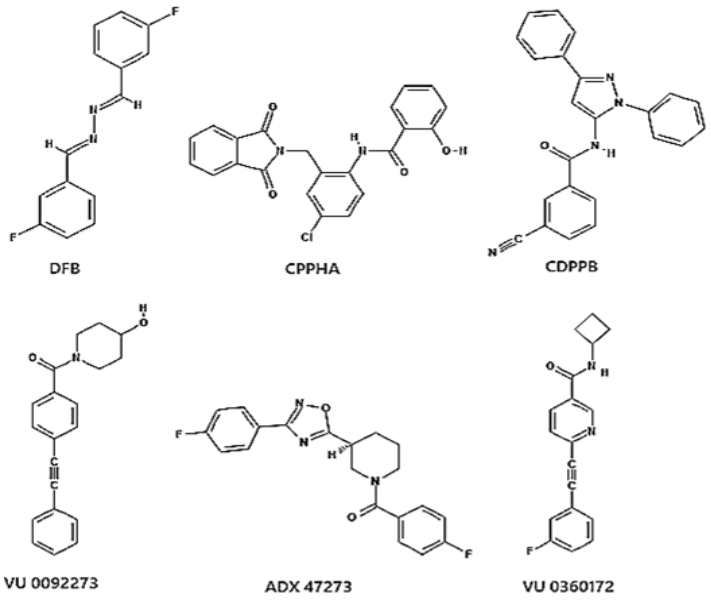

Figure 1.21: Positive Allosteric Modulators for mGluR5 and their structures. From the upper corner, the chemical structures of DFB, CPPHA, CDPPB, VU 0092273, ADX 47273 and VU 0360172 are shown.

1.9.2.2 Negative Allosteric Modulators (NAMs) for mGluR5

Varney and colleagues reported the discovery of highly selective negative allosteric modulators for the mGlu5 receptor: 6-methyl-2-(phenylazo)-3-pyridinol (SIB-1757) and (E)-2-Methyl-6- (2-Phenylethenyl) Pyridine (SIB-1893) (Varney et al. 1999). Another NAM, 2-methyl-6- (phenylethyl) pyridine (MPEP), reduces the activity of the mGlu5 receptor at nanomolar concentrations, decreasing the accumulation of inositol triphosphate, without changing the glutamate EC50 (Schoepp et al. 1999) (Figure 1.22).

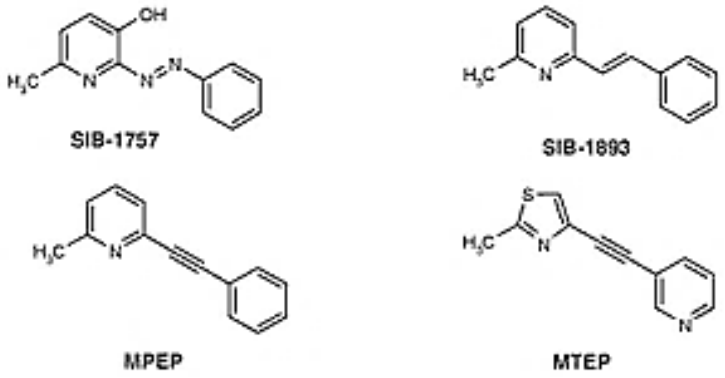

Figure 1.22: Negative Allosteric Modulators for mGluR5 and their structures. From the upper corner, the chemical structures of SIB-1757, SIB-1893, MPEP, MTEP are shown.

Aim of the Study

Non-Alcoholic Fatty Liver Disease (NAFLD) is a spectrum of liver diseases characterized by a relevant intracellular lipid deposition, which cannot be explained by alcohol consumption. It ranges from simple steatosis to Non-Alcoholic Steatohepatitis (NASH) that can have different degrees of fibrosis and progress to liver cirrhosis and end-stage liver disease, including Hepatocellular Carcinoma (HCC) (Machado & Cortez-Pinto 2014). NAFLD is the pandemic liver disease from the twenty-first century. It has been estimated that about one billion individuals worldwide have NAFLD (Loomba & Sanyal 2013). It is currently the leading cause of liver disease in North America, and it is predicted to become the leading cause of liver transplant by 2030 (Doycheva et al. 2017). This could lead to a further aggravation of the existing shortage of donor organs and the demand will be greater than the availability. Nowadays, in fact, in order to compensate for the growing number of patients on waiting lists for liver transplantation, many transplantation centers are forced to adopt extended criteria for graft selection, moving the limit of acceptance for marginal livers. The organs are defined as "marginal" when show an increased risk of Primary Non Function (PNF), once transplanted into the recipient, therefore more prone to morbidity or mortality, such as fatty livers (Koteish & Diehl 2001; Lieber 2004). Nowadays, due to the lack of knowledge in molecular mechanisms of NAFLD pathogenesis, novel targets to reduce fatty accumulation are investigated (Feldstein et al. 2003; Donato et al. 2006). From the literature, it is known that in the brain, metabotropic Glutamate Receptors subtype 5 (mGluR5) has a role in appetite and energy homeostasis. The acute and chronic appetite suppression and reduction in body weight and adiposity in obese rats suggest a novel possible role of mGluR5 for intervention in the treatment of chronic obesity in humans (Bradbury et al. 2004).

Therefore, the first part of this study was aimed to investigate the mechanisms that occur in fatty livers during the transplantation. In particular, since the lipidomic liver profiling is a sensitive indicator of graft function, we analysed the susceptibility of free fatty acid composition following two different types of preservation, static and dynamic, in two models of NAFLD.

Secondly, to further clarify the events that occur during the development and progression of NAFLD, our purpose was to study the changes in fatty acid desaturases, lipogenic enzymes that introduce a stereospecific double bond between specific carbons of fatty acyl chains, and the antioxidant Docosahexaenoic Acid (DHA) and their relationship with oxidative stress.

Finally, we wanted to investigate the role of mGluR5 modulation, using the selective agonist Dihydroxyphenylglycine (DHPG) and the negative allosteric modulator 2-Methyl-6-(Phenylethynyl) Pyridine (MPEP), in a FA-induced lipid accumulation and lipotoxicity model such as the human hepatoma cell line HepG2.

Materials & Methods

3.1 Materials

EAGLE culture medium, antimycotic/antibiotic and pyruvate sodium were purchased by Sigma-Aldrich (Milano, Italy). Trypsin-EDTA was procured by Gibco Life Technologies. Foetal Bovine Serum (FBS) was purchased from Corning, while L-glutamine solution comes from Biological Industries (Milano, Italy).

Bovine Serum Albumin (BSA), sodium oleate, sodium palmitate, Thiazolyl Blue Tetrazolium Bromide (MTT), Hoechst 33342 and Nile Red were provided by Sigma-Aldrich (Milano, Italy).

2'7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) was from Molecular Probes Inc., Oregon, United States.

3.1.1 Antibodies

The mouse antibody anti-Tubulin was purchased from Sigma Aldrich. Santa Cruz Biotechnology, INC. provided the rabbit polyclonal antibody anti-Actin. The rabbit polyclonal anti-mGluR5 was purchased from Millipore, while the rabbit monoclonal anti-Caspase-3 was from Cell Signaling Technology.

Specific peroxidase-conjugated anti-IgG secondary antibodies were from Santa Cruz Biotechnology.

3.1.2 Drugs

6-Methyl-2-(Phenylethynyl)Pyridine (MPEP) was purchased from Sigma Aldrich (Milano, Italy). 3,5-Dihydroxyphenylglycine (DHPG) was provided by Tocris Cookson Ltd (Bristol, UK).

3.2 Animal Models & Operation Procedures

Animal models of hepatic steatosis have improved our understanding of the pathogenesis of Non-Alcoholic Fatty Liver Disease (NAFLD). Two models, normal rats fed Methionine and Choline Deficient (MCD) diet and genetically Obese Zucker rats, have been particularly informative. In fact, rats treated with MCD diet spontaneously progressed to Non-Alcoholic Steatohepatitis (NASH), while in Obese Zucker rats there was no spontaneous progression. For this reason, it is clear that the use of only the genetic or nutritional model is not exhaustive in representing the totality of the factors and mechanisms that contribute to the development of NAFLD/NASH, but, on the contrary, the comparison between the two models can improve understanding and the study of these diseases.

3.2.1 Animals: nutritional Methionine-Choline Deficient-rat model (MCD) and genetic Zucker rats model

Male Wistar rats eight weeks old purchased from Charles River (Italy) were subjected to a methionine-choline deficient diet, obtained from Piccioni Laboratory (Italy), for 2 (n=14), 3 (n=6) or 8 (n=7) weeks or to a control diet. Obese (fa/fa) and Lean (fa/-) male Zucker rats 10-12 weeks old purchased from Charles River (Italy) were used as liver donors. The animal models used were approved by the Italian Ministry of Health and by the Pavia University Animal Care Commission. The animals were allowed free access to water and food until the beginning of all the experiments. Blood samples and hepatic biopsies were collected at the indicated times and snap frozen in liquid nitrogen.

Experimental groups: part I

Static Cold Storage (CS) and dynamic Machine Perfusion (MP) preservation using fatty livers from two weeks-MCD treated rats and Obese Zucker rats were compared.
Animals were divided into two groups:

- **Group 1:** Control (n=14): rats fed with diet supplemented with choline and methionine;
- **Group 2:** MCD (n=14): rats fed with methionine and choline deficient diet;
- **Group 3:** Lean (n=14): heterozygous rats for leptin receptor gene mutation (fa/-);

- **Group 4:** Obese Zucker (n=14): homozygous rats for leptin receptor gene mutation (fa/fa).

Of each group half was subjected to 6 hours of preservation by cold storage or machine perfusion and finally 2 hours of reperfusion as illustrated in the graph (Figure 3.1):

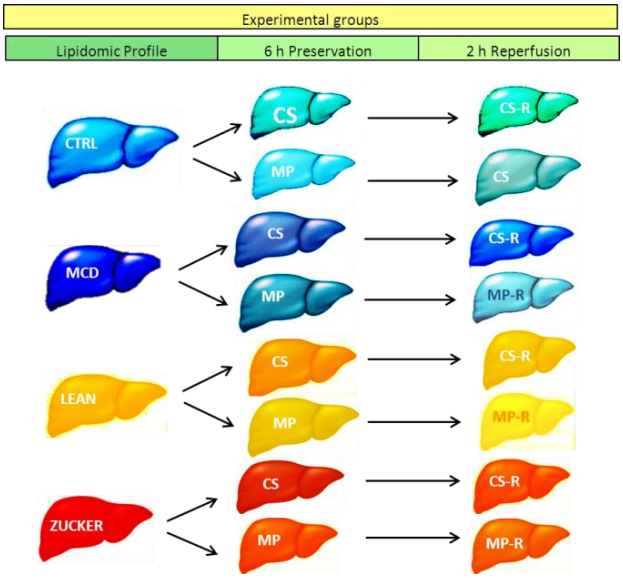

Figure 3.1: Experimental group, part I. CTRL-, MCD-, Lean- and Zucker groups was subjected to 6 hours of preservation by cold storage or machine perfusion and finally 2 hours of reperfusion

Experimental groups: part II

In the second part of this study, again MCD rats fed with a methionine-choline deficient diet for 3 weeks and Zucker rats were used again.

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Animals were divided into two groups:

- **Group 1:** Control (n=6): rats fed with diet supplemented with methionine and choline;
- **Group 2:** MCD (n=6): rats fed with methionine and choline deficient diet;
- **Group 3:** Lean (n=6): heterozygous rats for leptin receptor gene mutation (fa/-);
- **Group 4:** Obese Zucker (n=6): homozygous rats for leptin receptor gene mutation (fa/fa).

Experimental groups: part III

In this part of the work, a cell model of human hepatoma was used, HepG2 cell line, in which fatty acid accumulation was induced.

HepG2 cells were grown in Minimum Essential Medium EAGLE plus 10% Fetal Bovine Serum (FBS), 1% L-glutamine, 1% sodium pyruvate and 1% antibiotic/antimycotic, at 37°C and 5% CO_2.

HepG2 cells were seeded in 96 well-plates at 50.000 cells/well or in a 6-well plates at 500.000 cells/well, 24 hours in their culture medium prior to perform the experiments.

On the day of the treatment, cells were washed once with sterile Phosphate Buffered Saline 1X (PBS 1X) and pre-treated with the mGluR5 orthosteric agonist DHPG 100 µM alone or in combination with the negative allosteric modulator MPEP at 0.3 µM, 3 µM or 30 µM. After 12 hours, culture medium was replaced by medium added by a mixture of oleate:palmitate (2:1) 1.5 mM, associated with pharmacological treatments. After 12 hours, cells were washed once with sterile PBS 1X and incubated with Nile Red 1 µM plus Hoechst 33342 1 µM, to evaluate fatty acid levels, or H_2DCFDA 1 µM plus Hoechst 33342 1 µM for 15 minutes, at 37°C and 5% CO_2, to determine oxidative stress.

3.3 *Ex vivo* studies

3.3.1 Liver isolation

Livers were isolated from Methionine-Choline deficient and genetic Zucker rats, anesthetized with 40 mg/kg of sodium pentobarbital in saline solution, administered intraperitoneally.

After median laparotomy followed by bilateral subcostal incision, the animal received 200 units of heparin per 100 g of body weight via the abdominal inferior caval vein (5000 U/mL, Marvecs Services, Agrate Brianza - MI), to prevent blood clotting and the consequent blood pressure decrease (Cheung et al. 1996). The needle was inserted in a portion of the vessel partially covered of fat, enabling the protection of the hole left by needle extraction. Then the bile duct was cannulated with a 0.61 mm polyethylene tubing (Intramed, Becton-Dickinson, Loveton Circle, MD - USA), to collect bile. The cannulation was made easier by closing the bile duct with a 4/0 silk suture ligation, placed in distal position. In this way the bile duct swelled upstream, becoming more discernible (Figure 3.2 A). Then, after a cut in the bile duct with the spring scissors and polyethylene pipes in the shape of an oblique were inserted

(Figure 3.2 B). When the bile flows in the tube, it was fixed with a 4/0 silk suture ligature (Figure 3.2 C) (Ferrigno et al. 2013).

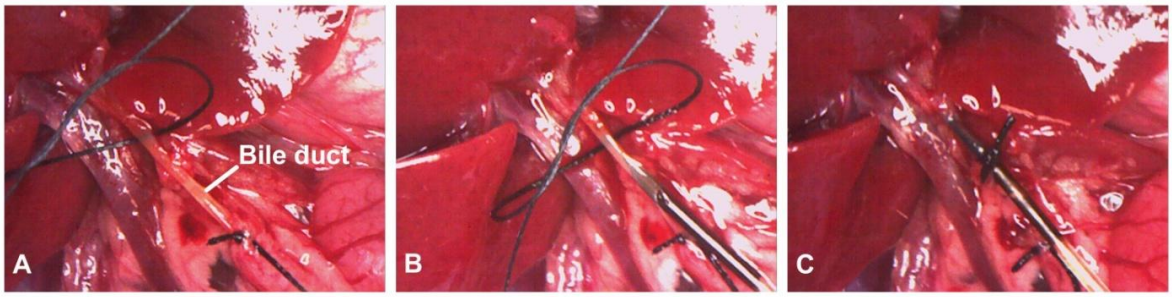


Figure 3.2: Bile duct cannulation. A. The bile duct is ligated with a 4/0 silk suture. **B.** A cut is opened in median position and slant-shaped polyethylene tubing is inserted. **C.** When the tubing completely fills with bile, it is fixed with a 4/0 silk suture ligation (Ferrigno et al. 2013).

Two 4/0 silk sutures were inserted under the portal vein in a proximal position, and two loose double knots were prepared. To avoid leakage through the duodenal vein during perfusion, it was important to place at least one of the sutures between the duodenal branch of the portal vein and the liver (Figure 3.3 A). The fat tissue surrounding the portal vein was carefully scrabbled with two cotton-buds. Then, the portal vein was cannulated with a 22 gauge catheter, stabilized by 6/0 silk suture (Johnson & Johnson, Arlington, UK) (Figures 3.3 B and 3.3 C) to allow the perfusion (Ferrigno et al. 2013).

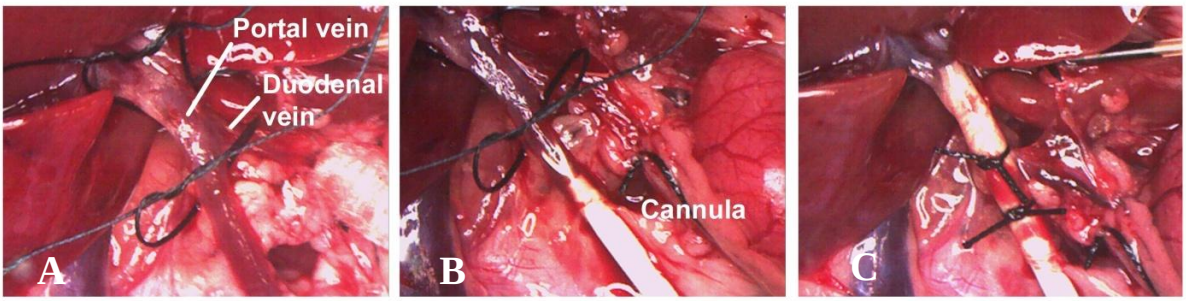


Figure 3.3: Portal vein cannulation. A. Two 4/0 silk sutures are inserted under the portal vein in proximal position. **B.** Portal vein is cannulated with a 22G catheter. **C.** The catheter is ligated with a 6/0 silk suture (Ferrigno et al. 2013).

Afterwards, abdominal inferior caval vein was cut open. Thus, to eliminate all the remaining blood, the liver was washed out *in situ* with 50 mL of modified Krebs/Henseleit buffer (KH) via the portal vein cannula.

3.3.1.1 Liver preservation and reperfusion model

Apparatus

For cold preservation a bag, ice and preservation solution are needed, as illustrated in Figure 3.4.

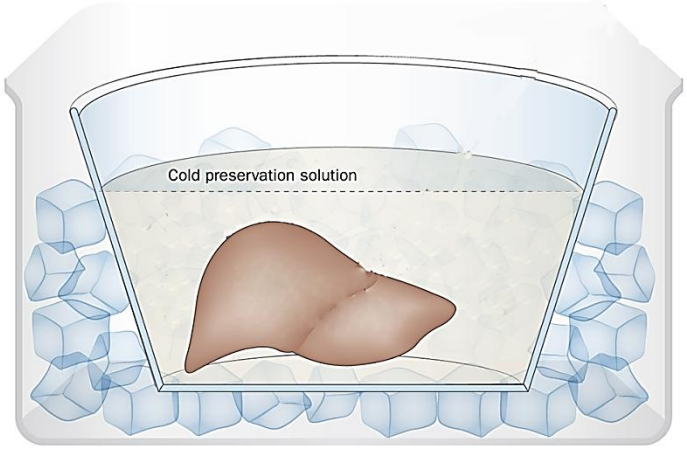


Figure 3.4: Static cold preservation. The organ is usually conserved in a bag filled of preservation solution and kept to 4°C. In our experiments we used a plastic bag.

For machine perfusion preservation a typical apparatus includes several components: peristaltic pump, heat exchanger, bubble trap, reservoir, jacketed perfusion chamber, oxygenator and tubing (Figure 3.5). The perfusion buffer passes through a bubble trap and a Y tube, connected to a manometer and to the portal vein. The perfusate outflowing through the thoracic caval vein was collected in the perfusion chamber, before flowing back to the reservoir by the peristaltic pump.

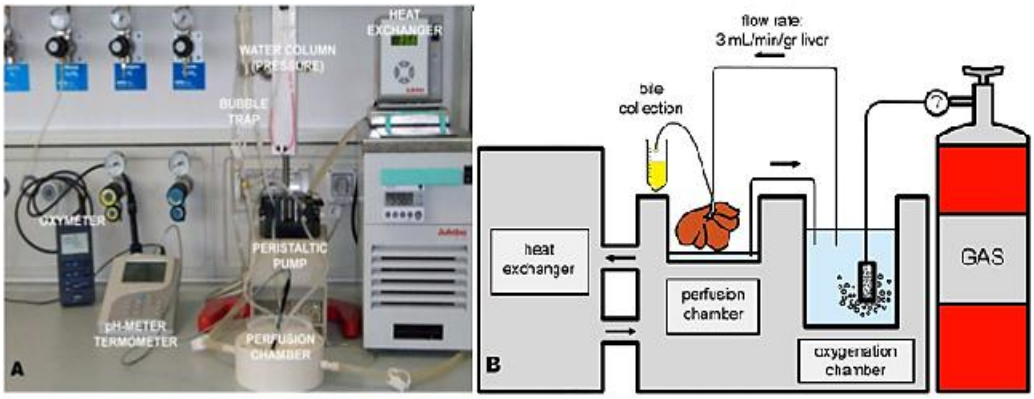


Figure 3.5: Perfusion apparatus. A. The system used into experiments. **B.** The schematic representation of perfusion system. Generally, the apparatus is composed by heat exchanger, perfusion chamber, reservoir, oxygenator, bubble trap and peristaltic pump.

3.3.1.2 Preservation Solutions

Krebs-Henseleit solution: $NaHCO_3$ 25 mM
NaCl 118 mM
KCl 4.7 mM
KH_2PO_4 1.25 mM
$MgSO_4$ * 7 H_2O 1.2 mM
$CaCl_2$ 1.25 mM
HEPES 10 mM

Belzer-UW solution, pH 7.4: Pentafraction 50 g/L
Lactobionic Acid 35.83 g/L
KH_2PO_4 3.4 g/L
$MgSO_4$ * 7 H_2O 1.23 g/L
Raffinone pentahydrate 17.83 g/L
Adenosine 1.34 g/L
Allopurinol 0.136 g/L
Total glutathione 0.922 g/L
KOH 5.61 g/L

Static Cold Storage preservation

The livers, free from ligaments, after washout with Ringer Lactate (50 ml), were submitted to CS: the organs were flushed with the preservation solution Belzer-University of Wisconsin at 4°C (UW, ViaSpanTM, DuPont Pharmaceuticals, Wilmington, DE, USA) for 2 minutes and then placed into plastic bag containing 20 ml UW solution and preserved for 6 hours at 0-4°C.

Dynamic Machine perfusion preservation

The livers preserved by MP were placed in an organ chamber and connected to a standard recirculating perfusion system. The Krebs-Heinseleit medium (KH) solution is collected in a tank (200 mL), oxygenated and maintained at 20°C thanks to a heat exchanger (Julabo-F12). The KH solution is recirculated using a roller pump (Gilson Minipuls-3) maintaining a constant perfusion flow (3.5-4 ml/min/g) throughout the period of liver perfusion. The oxygenation of the perfusion solution takes place through a glass oxygenator

which from a PO2 of about 700 mBar to 20°C. In the liver chamber the perfusate flowed through the suprahepatic vena cava and was recirculated by the roller pump into the reservoir; the risk of emboli were eliminated from the system with a bubble trap.

Reperfusion period

After liver preservation, both by static cold storage or dynamic machine perfusion, the livers were put at room temperature for 10 minutes and successively reperfused with oxygenated (O_2CO_2, 95% and 5%, respectively) KH buffer at pH 7.4, for different times at 37°C. Reperfusion flux was maintained constant during reperfusion. Samples of the reperfusion solution were collected at 0', 30', 60' 90' and 120'.

At the end of reperfusion, liver samples were snap frozen in liquid nitrogen for further analysis.

3.4 *In vivo* studies: two models of NAFLD

Blood samples were collected at indicated times point. Blood was drawn from the vena cava and centrifuged at 3000 x g for 10 minutes at 4°C, to isolate serum.

Bile, secreted by hepatocytes, participates to the digestion process, helping to emulsify the lipids present in food. Thus, bile production requires intact hepatocytes. For this reason it is considered a strong and reliable indicator of overall liver quality and viability. Bile was collected in darkened vials. Bile and serum samples were snap frozen in liquid nitrogen until they were analysed.

The liver was removed, washed in cold (4°C) buffer (30 mM Histidine, 250 mM sucrose, 2 mM EDTA, pH 7.2), dried on paper towels, then a sample of tissue from each lobe was collected. The biopsies have been preserved in liquid nitrogen.

3.5 Biochemical Assays

3.5.1 Transaminases evaluation

Aspartate Transaminase (AST) is an enzyme belonging to the class of transferases detectable in very high concentration in the bloodstream as a result of liver and muscle damage. Alanine Aminotransferase (ALT), also belonging to the class of transferases, differently from AST, is a generic cell necrosis index, present in elevated plasma concentration as result of liver damage of different nature. Therefore it is assessed in combination with more specific indicators. For this reason, the AST/ALT ratio is commonly used as an index of liver damage and can be quantified in serum obtained from all our experimental model rats.

Alkaline Phosphatase (AP) is an hydrolase. There are three different isoforms: at hepatobiliary level, in the skeletal system and in the gut. High levels of AP are associated to hepatic injury.

Liver injury was assessed by serum levels of ALT, AST and AP. Doctor Vittoria Rizzo, Department of Molecular Medicine, IRCCS Policlinico San Matteo Pavia, assessed liver injury markers and glucose, total cholesterol and triglycerides by an automated Hitachi 747 analyser (Roche/Hitachi, Indianapolis, IN, USA).

3.5.2 ATP assay

Adenosine-Triphosphate is a marker for cell viability because it is present in all metabolically active cells and its concentration declines very rapidly when the cells undergo necrosis or apoptosis. ATP is also a marker of the energetic status of the cell, as it rapidly falls in detrimental conditions.

Tissue ATP is measured with the ATPlite monitoring system, based on firefly (*Photinus pyralis*) luciferase. The production of light is caused by the reaction of ATP with added luciferase and D-luciferin. The emitted light is proportional to the ATP concentration and it was measured by using a Victor^2 Multilabel Counter Wallac, Perkin Elmer microplate reader.

Briefly, frozen tissue was homogenized in ice-cold 100 mM phosphate buffer with 3 mM EDTA; the homogenate was immediately precipitated in Tricarboxylic Acid (TCA) 30% and centrifuged at 3000 x g for 15 minutes at 4°C. The supernatant was diluted 50 folds in 100 mM phosphate buffer pH 7.75 and assayed. Tissue ATP was measured with the luciferin-luciferase method using the ATPlite luminescence assay kit (Perkin Elmer Inc., USA), according to manufacturer's instructions with minor changes.

Afterwards, it was performed the ADP evaluation treating the same samples with Phosphoenolpyruvate (PEP) and Pyruvate Kinase enzyme (PK). PK is able to catalyse the pyruvate production, starting from ADP and PEP.

ADP + PEP → ATP + pyruvate

By this step all the ADP present in the sample was converted to ATP and, in this way, it was possible to obtain a total ATP value, given adding ATP and ADP. The values of ATP and ADP were calculated by subtraction (Gorman et al. 2003).

3.5.3 Reactive Oxygen Species (ROS) assay

Reactive Oxygen Species (ROS) include both the so-called "free radicals" (superoxide radical, hydroxyl radical) or molecules containing oxygen, but not radical (hydrogen peroxide). The production takes place during normal metabolic processes involving oxygen, but an excessive release of ROS causes a lot of damages to proteins, lipids and DNA, molecules highly sensitive to oxidative stress.

The hepatic determination of ROS was obtained using the conversion of the 2',7'-Dichlorofluorescein Diacetate (H_2DCFDA) to a fluorescent molecule, the 2',7'-Dichlorofluorescein (DCF). The H_2DCFDA works as a probe: it enters the cell where its two acetyl groups are removed by intracellular esterases and, in the presence of ROS, it is oxidized to DCF.

Tissue samples were homogenized (50 mg/ml) in Locke's buffer (120 mM NaCl, 2.5 mM KCl, 5 mM $NaHCO_3$, 6 mM D-glucose, 1 mM $CaCl_2$, and 10 mM HEPES, pH 7.4) and incubated for 20 minutes at room temperature with 10 mM H_2DCFDA.The production of the fluorescent derivative DCF as a function of time was measured using a Victor^2 Multilabel Counter Wallac, Perkin Elmer microplate reader.

3.5.4 Lipid peroxidation (TBARS) assay

Lipid peroxidation is a process triggered by free radicals. Subsequently, those lipids containing unsaturated fatty acids chains are directly oxidized by molecular oxygen. Then, the oxidative damage spreads with a chain reaction because the electron-deprived lipids try to refill the loss by oxidizing neighbouring molecules. This process grows and involves proteins and also DNA, resulting in the end, in the production of carcinogenic and toxic secondary products, such as aldehydes and ketones. One of these is the Malondialdehyde (MDA) that was used as lipid peroxidation index.

The extent of lipid peroxidation was evaluated in terms of Thiobarbituric Acid Reactive Substances (TBARS) formation, according to Esterbauer and Cheeseman's method (Esterbauer & Cheeseman 1990) and then the samples absorbance was detected spectrophotometrically at the wavelength of 535 nm. The calibration curve was built using MDA as a standard.

3.5.5 Measurement of cell damage: LDH release

Cell viability was monitored by release of Lactate Dehydrogenase (LDH) into the medium, as described respectively by Piccinini et al. and by Wieme and Demeulenaere (Piccinini et al. 2017).

The cytosolic enzyme lactate dehydrogenase catalyses the interconversion of pyruvate to lactate with concomitant interconversion of NADH to NAD^+. When oxygen is absent, or in short supply, LDH converts pyruvate, the final product of glycolysis, to lactate.

$$\text{PYRUVATE + NADH} \xleftrightarrow{\text{LDH}} \text{LACTATE + NAD}^+$$

LDH is considered a marker for the evaluation of cellular damage; in fact, it is released when cells burst out. Hepatocytes viability was assessed through release of lactate dehydrogenase into the perfusate using a spectrophotometer at a wavelength of 340 nm, every 6 seconds within one minute (Wieme & Demeulenaere, 1970). To evaluate LDH concentration, it was necessary to measure its activity by adding to the supernatant saturating concentration of NADH and pyruvate. In this way, the enzyme consumes the substrates at a rate proportional to its concentration. Applying Lambert-Beer's law to the slope of the obtained curve and

knowing the molar extinction of NADH, activity of LDH released from damaged cells was calculated and expressed in mU/min/g.

3.5.6 Glutathione assay

Glutathione is one of the most important antioxidant molecules produced by the human body. Glutathione has an antioxidant action both against free radicals and against molecules such as hydrogen peroxide, nitrites, nitrates, benzoates, and other. From a structural standpoint, glutathione is a tripeptide composed by glycine, glutamate and cysteine. The latter binds to the glycine through a canon peptide bond, whereas it establishes an atypical peptide bond with the glutamate. This kind of bond involves the side chain carboxylic group of glutamate and the amine group of cysteine. Glutathione is produced by the glutathione reductase enzyme, that restores reduced Glutathione (GSH) from oxidized Glutathione (GSSG) by means of NADPH as electron donor.

The hepatic concentration of total GSH was established following the instruction of the enzymatic Glutathione Assay Kit (purchased by Cayman Chemicals) and exploiting the activity of glutathione reductase. GSSG concentration was measured after derivatization of GSH with 2-vinylpyridine.

3.5.7 Lipid assay: Nile Red

Hepatic lipid assay was performed according to Lyn-Cook et al. method (Lyn-Cook et al. 2009). Frozen tissue samples (50–70 mg each) were homogenized in 200 µl of water, and 4 µl aliquots were utilized to determine protein concentration by means of Lowry assay. To extract lipids, 1 ml chloroform–methanol solution (2:1) was added to the homogenized samples and then they were incubated for 1 hour at Room Temperature (RT) with intermittent agitation. After centrifuging of the samples at 3000 x g for 5 minutes RT to separate the lower lipid-containing layer, the latter was transferred to a clean tube and air-dried. Pellets were re-suspended in 100 µl of 100% ethanol, 5 µl aliquots of lipid extract were added to 190 µl of PBS 1X in a 96-well polystyrene black plate, and then, 5 µl of Nile Red solution (1 mg/ml in DMSO) were added to each well. Samples were light-protected with aluminium foil and incubated at room temperature for 10 minutes, under gentle agitation. Fluorescence intensity

(Ex 485/Em 572) was measured using a Victor^2 Multilabel Counter Wallac, Perkin Elmer microplate reader. Results are expressed as total lipid/liver weight (mg/g).

3.5.8 Fatty acid evaluation

Doctor Barbara Mannucci, Centro Grandi Strumenti, University of Pavia performed Fatty Acid (FA) profiling of liver tissue on lipid extracts by Gas Chromatography-Mass Spectrometry (GC-MS). Each identified peak was expressed as relative percentage areas of total methylated fatty acids.

3.5.9 Lowry protein assay

The Lowry assay (Folin-Ciocalteau) is used to quantify the protein content in a specific sample. The Folin reactive (a mixture of tungstate, molybdate and sodium phosphate) and a copper sulphate solution are added to the sample, determining a colour change directly related to the protein amount. The absorbance of the sample is measured spectrophotometrically at 550 nm and the high sensitivity of this method allows to quantify up to 5 μg proteins in a 0.2 ml volume.

Two reagents, A and B (Table 1), were mixed in a ratio of 50 parts of A and 1 part of B, making a final solution C (A+B= C, Table 1). The samples were analysed in duplicate.

Copper ions (Cu^{2+}) in C solution react in a basic environment with CO-NH2 protein groups, causing the violet colour of the mixture. This reaction is specific for polypeptides because almost two $CO\text{-}NH_2$ groups are required.

Subsequently, Folin reactive was added (D solution, Table 1). Tungstic acid and molybdic acid were reduced by the copper protein complex, to tungstate blue and molybdate blue. Folin reactive was added only at the end, because in absence of Cu^{2+} ions, it would bind exclusively to aromatic residues, leaving proteins undetected.

To quantify the sample protein content, it was necessary to build a calibration curve, by means of Bovine Serum Albumin (BSA) standard at the concentration of 2 mg/ml. Seven points of the curve were prepared, making serial dilution in bi-distilled water and adjusting the final volume to 200 μl. As the samples, also the curve was analysed in duplicate. To fix the zero point of the curve, a blank sample was prepared and treated like other samples. Both standards and samples were supplemented with 1 ml of C solution (Table 1), stirred and

incubated for 10 minutes at room temperature. Then, 100 µl of Folin reactive (D solution) were added to all tubes. After 30 minutes of incubation, the absorbance was detected spectrophotometrically at 550 nm.

The absorbance value was used to determine the protein concentration according to Lambert-Beer law:

$$\mathbf{A = \varepsilon\lambda \times C \times l}$$

(**A**= absorbance; **ελ**= molar extinction coefficient; **C**= sample concentration; **l**= optical path in cm).

Solution A	NaK 268 mg/l, Na_2CO_3 23.4 g/l, NaOH 4 g/l
Solution B	$CuSO_4$ at 1.56 % in distilled water
Solution C	Solution A + Solution B with a ratio of 50:1
Solution D	Folin + distilled water with a ratio of 1:1

Table 1: Summary of Lowry solutions.

3.5.10 Western Blot procedure

3.5.10.1 Reagents & Antibodies

The CelLytic Buffer and Protease Inhibitor Cocktail were purchased from Sigma-Aldrich (Milan, Italy).

The Enhanced Chemiluminescence substrate (ECL) was from Bio-Rad, as well as the Blotting-Grade Blocker (non-fat dry milk). All reagents were of the highest grade of purity available and were purchased from Sigma-Aldrich (Milan, Italy).

3.5.10.2 Cellular extracts preparation

24 hours before extraction, 2 x 10^6 cells were placed in a petri dish, in their culture medium. After 24 hours of incubation,once the medium was aspirated from the plate and washed with PBS, 500 µl of CelLytic Buffer supplemented with Protease Inhibitor Cocktail (10 µl/mL) was added. The cells, after being scraped, were collected, subjected to agitation and ice alternately and centrifuged at 13000g for 10 minutes at 4°C. Then the supernatant was aspirate and placed in a fresh tube kept on ice, and discard the pellet. The collected supernatant was divided into new tubes and 4 µl of it were used to quantify the protein content

by means of the Lowry method. After that, the supernatant was divided into aliquots containing the same amount of proteins and it was reduced with SDS 2XR, 2% β-mercaptoethanol.

3.5.10.3 Electrophoresis & Immunoblotting

Samples of liver extracts, containing the same amount of proteins, were separated in SDS-PAGE on 7.5% or 10% acrylamide gels, and transferred to Polyvinylidene Difluoride (PVDF) membrane that was blocked for 2 hours with 5% Blotting-Grade Blocker in TBS (20 mM Tris/HCl, 500 mM NaCl, pH 7.5) at 4°C.

After blocking, membranes were washed twice in TBS and incubated with appropriate primary antibodies overnight at 4°C, under gentle agitation. After washing in PBS (Na_2HPO_4 8 mM, NaH_2PO_4-H_2O 2 mM, NaCl 140 mM, pH 7.4, 0.1% Tween 20), membranes were incubated with appropriate peroxidase-conjugated secondary antibodies diluted 1:2000 for all primary antibodies. The membranes were washed again in PBS and, then, reactive proteins were visualized with a chemiluminescence reaction with Bio-Rad Chemidoc XRS+.

Membranes were incubated for 2 hours at room temperature with 1% NaN_3 in PBS to inhibit the peroxidase signal and then they were reprobed with anti-Tubulin or anti-Actin primary antibody as a control for equal loading.

Bands intensity quantification was performed by computer using the Bio-Rad Image Lab software.

3.5.10.4 Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis is a technique consisting in differentiated migration of charged molecules in electric field. Molecules are separated according to their charge, size and according to the applied electric field.

Sodium Dodecyl Sulphate (SDS) is an anionic detergent that stably binds to proteins leading to denaturation. In case of excess of SDS, the proteins acquire a constant amount of negative charge per unit mass: all the protein-SDS complexes will move toward the anode and, according to the gel sorting properties, their mobility (and therefore the distance travelled after a certain period) is inversely proportional to log10 of their molecular weight. Using standards with known molecular weight together with samples, it is possible to establish the molecular weight of the protein in the sample.

Protein sorting in SDS-PAGE was carried out thanks to the use of a chamber for electrophoresis MINI-PROTEAN III, Bio-Rad. The sorting gel dimensions were 88x55 mm and a thickness of 1.5 mm.

Proteins package themselves in the stacking gel, that had a polyacrylamide concentration of 3%, while they separated each other in the linear running gel, that had a polyacrylamide concentration of 7.5% or 10%.

3.5.10.5 Preparing acrylamide gels

Two glass plates, outer and inner, were joined together vertically, using a special support.

The prepared running gel solution (Table 2) was poured between the two glass plates by the use of a syringe. Once the gel polymerized, it formed a texture capable to separate proteins, according to their molecular weight.

Afterwards, we proceeded inserting the stacking gel (Table 2), a gel spacer at the 3% of polyacrylamide, which was poured onto the running gel by means of the syringe. It allows to a special teflon comb to form the sample loading wells.

Solutions	**Running Gel 7.5%**	**Running Gel 10%**	**Stacking Gel 3%**
Acrylamide 30%	2250 µl	3000 µl	250 µl
Tris HCl 1.5 M pH 8.8	2250 µl	2250 µl	-
Tris HCl 1.25 M pH 6.8	-	-	250 ml
SDS 10%	90 µl	90 µl	25 ml
Water	3870 µl	3120 µl	1700 µl
TEMED 1%	450 µl	450 µl	250 µl
APS 10%	90 µl	90 µl	25 µl
Total Volume	**9000 µl**	**9000 µl**	**2500 µl**

Table 2: Polyacrylamide gel solutions.

N,N,N,N'-Tetra-Methyl-Ethylendiamine (TEMED) and Ammonium Persulphate (APS), which are the two cross-linking and polymerizing agents in the reaction, were added just before pouring the solutions between the glass plates.

The samples run simultaneously with a standard mixture of known molecular weight protein. The electrophoresis was carried out for about 2 hours at a constant voltage of 100 Volts, at room temperature. The used buffer for the run was 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3.

3.5.10.6 Western Blotting

This technique consists of moving proteins, previously separated through SDS-PAGE, from within the gel onto a membrane made of Polyvinylidene Difluoride (PVDF). A MINI TRANS-BLOT Bio-Rad chamber was utilized and the transfer took place through the application of an electric field with constant amperage of 200 mA, at 4°C for 2 hours. The procedure consisted in preparing a "sandwich" putting in contact the polyacrylamide gel and the PVDF membrane, the latter was previously activated by soaking it first in methanol for few seconds.

Both the gel and the PVDF were squeezed together by suitable supports. Therefore, to ensure a more closely contact, two layers of sponges and blotting paper were used, soaked in blotting buffer (20 mM Tris, 150 mM glycine, 20% methanol). During the transfer, the gel was turned towards the negative pole, while the membrane was close to the positive one. This is necessary because, since proteins have a negative charge, will tend to migrate toward the positive pole and, therefore, towards the PVDF membrane.

3.5.10.7 Immunoblotting

The PVDF membrane was incubated for two hours at 4°C, under mild stirring, with 5% Blotting-Grade Blocker in TBS (20 mM Tris/HCl, 500 mM NaCl, pH 7.5) to block any non-specific sites. Afterwards, the membrane was washed twice in TBS 1X plus 0.1% Tween 20 and incubated overnight, under mild stirring, with specific primary antibodies depending on the protein of interest: anti-mGluR5, anti-Caspase-3 and anti-Tubulin, diluted 1:1000, except for anti-Actin, that was 1:5000.

After that, the PVDF was washed every 5 minutes for 30 minutes, under stirring and at room temperature with washing buffer PBS 1X, in order to remove the excess of antibody. Then the membrane was incubated for 1 hour at 4°C with appropriate peroxidase-conjugated secondary antibody diluted 1:2000 for all primary antibodies.

Subsequently, the membranes were washed again with PBS 1X. In the presence of the substrate, the peroxidase conjugated secondary antibody, developed a chemiluminescent

reaction which identified the protein of interest, exposing the membrane to the analyser of digital images ChemiDoc XRS+, Bio-Rad.

For the chemiluminescence reaction the Bio-Rad kit, which included two reagents to be mixed in the ratio 1:1, was used. Reagents must be added on the membrane in order to allow the development of the light reaction. At the end, the PVDF was exposed for different times (from a minimum of 30 seconds to a maximum of 10 minutes) to the analyser of digital images ChemiDoc XRS+, Bio-Rad. The intensity quantification of the bands was performed to the computer with the software Image Lab, Bio-Rad. It is possible to inhibit the secondary antibody conjugated with peroxidase, in order to cover again the same PVDF membrane with new antibody, without any interference in the new marking due to the previous one.

In practice, at the end of the immunoblotting development, the PVDF membrane was rehydrated using the washing buffer for few minutes. Subsequently, it was placed in a 1% solution of NaN_3 in PBS 1X and left under stirring at room temperature for about 2 hours. At this point, the excess NaN_3 was removed with several washes, and it was possible to re-probe the membrane with another antibody, without the need of performing again the non-specific sites blocking procedure.

3.6 In vitro studies: HepG2 cells

3.6.1 Cell culture

HepG2 cells were grown in Minimum Essential Medium EAGLE with the addition of 10% Fetal Bovine Serum (FBS), 1% glutamine, 1% sodium pyruvate and 1% antibiotic/antimycotic and were incubated at 37°C and 5% CO_2. HepG2 cells were seeded in 96-well plate at 50.000 cells/well and were used at 75% of confluency.

3.6.1.2 Pharmacological pre-treatment

On the day of the experiment, cells were washed once with PBS and pre-treated for 12 hours with the mGluR5 orthosteric agonist DHPG 100 µM alone or in combination with different concentrations of negative allosteric modulator MPEP: 0.3 µM, 3 µM and 30 µM.

3.6.1.3 Fatty acid preparation and treatment

Palmitic and oleic acids were provided as sodium salt and dissolved in ultrapure water, by means of heating and sonication to a stock solution of 50 mM.

After pharmacological pre-treatment, mixtures of oleate:palmitate (molar ratio 2:1) at final concentration of 4, 2, 1, and 0.5 mM were diluted in serum-free medium supplemented by 1% BSA, with or without the previous pharmacological treatments, and incubated within HepG2 cells for 12 hours. Control cells were incubated with the same medium in which fatty acids were diluted. After 12 hours of incubation, viability, fatty acid uptake and ROS formation were evaluated by MTT assay, Nile Red and H_2DCFDA uptake, respectively.

3.6.1.4 Cell viability assay: MTT

The MTT assay is a colorimetric assay used to evaluate cell viability. This assay is based on the activity of the succinate dehydrogenase enzymes that are only available in living cells. The succinate dehydrogenase is capable of cutting the tetrazolium of a yellow compound, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium (MTT) bromide thus forming crystals of violet formazan, subsequently dissolved in Dimethylsulfoxide (DMSO). The absorbance of the violet solution is measured at 540 nm with a plate reader and from it the percentage of cell viability is obtained.

The MTT assay was performed on HepG2 cells previously subjected pharmacological and fatty acid treatments. After a wash with sterile PBS 1X, 100 µl of serum-free fresh medium with 10 µl of MTT (5 mg/ml in PBS 1X) were added to each well. The negative or "total dead" control was also treated in the same way, to which 4 µl of Tryton X 25% per well were added before treatment with MTT.

After 2 hours of incubation at 37°C at 5% CO_2, the cells were washed with PBS 1X.

To dissolve the formazan crystals, 100 µl of DMSO per well were added, thus obtaining a colored solution, indicating where the cells were vital. The absorbance of the obtained solution was measured at 540 nm from a plate reader. It is important that this dosage is carried out in the dark due to the photosensitivity of the MTT. The percentage of cell viability is obtained through the ratio between the difference in the absorbance values of the dead cells to those of the live cells and the difference between the control averages, all multiplied by 100 (Figure 3.6).

$$\frac{A\ (\text{Campione}) - X\ (\text{Ctrl morte})}{[X(\overline{\text{Ctrl}}\ \text{Vive}) - \overline{X}(\text{Ctrl morte})]} \times 100$$

3.6.1.5 Lipid assay: Nile Red

The lipid content in HepG2 cells was determined using Nile Red, a vital lipophilic dye, calculated in function of labeled dsDNA by means of Hoechst. After incubation with fatty acid and drugs, cells were washed once with PBS and incubated with Nile Red 1 µM plus Hoechst 33342 1 µM for 15 minutes at 37°C and 5% CO_2. After probes incubation, cells were washed once with PBS 1X and observed by means of the fluorescent cell imager ZOE (Biorad). Images were analyzed by ImageJ software, using the subtraction background method.

3.6.1.6 ROS production

Oxidative stress in HepG2 cells was obtained using the conversion of the 2',7'-Dichlorofluorescein Diacetate (H_2DCFDA) to a fluorescent molecule, the 2',7'-Dichlorofluorescein (DCF), compared to stained nuclei as for Nile red assay. HepG2 cells were incubated with H_2DCFDA 1 µM plus Hoechst 33342 1 µM for 15 minutes, at 37°C and 5% CO_2. Then, cells were washed once with PBS 1X and observed by means of the fluorescent cell imager ZOE (Biorad). Images were analyzed by ImageJ software, using the subtraction background method.

Results

4.1 Results part I: Fatty liver preservation: comparison between Cold Storage versus Machine Perfusion

During the first part of this study, static Cold Storage (CS) and dynamic Machine Perfusion (MP) preservation were compared using fatty livers from a nutritional model of fatty livers, Methionine-Choline Deficient (MCD) rats versus a genetic model of fatty livers, Obese Zucker rats. After the preservation period, all livers were subjected to two hours of reperfusion.

Before organ isolation, these two experimental models exhibited similar concentration of hepatic serum enzymes suggesting comparable liver injury (Table 3)

4.2 Model characterization

	MCD	**Obese Zucker**	***p***	**Control MCD**	**Lean Zucker**	***p***
ALT (mU/mL)	124±34	109.3±16.9	ns	23.3±1.6	68±3.5	#; &
AST (mU/mL)	128±16.8	111.5±8.9	ns	59.5±4.8	114.7±18	#
AP (mU/mL)	186.3±25.3	194.2±10.7	ns	134±7.3	191±5.1	ns
Glucose (mg/dL)	151.2±7.5	144.2±8.4	ns	179.5±11.7	128.3±10.7	#
Total Bilirubin (mg/dL)	0.26±0.02	0.13±0.002	$	0.11±0.004	0.11±0.001	#
Direct Bilirubin (mg/dL)	0.14±0.02	0.12±0.002	$	0.12±0.002	0.1±0.001	#
p-Cholinesterase (mU/mL)	886.2±41.4	651.4±28.7	$	272.4±10.9	478.7±39.9	#; &
Cholesterol (mg/dL)	23.8±1.6	180.5±4.8	$	64.7±4.1	100±3.5	#; &
Triglycerides (mg/dL)	16.8±2.3	215.5±38.2	$	72.3±16.8	40±5.1	#; &
TNF-α(pg/dL)	36.9±2.3	9.9±0.4	$	27.2±1.1	9.7±0.5	#

Table 3: Serum hepatic enzymes release. Serum hepatic enzymes before static or dynamic liver preservation were evaluated.
ns: p value not significant; $: p<0.05 MCD vs Obese; #: p<0.05 MCD vs Control; &: p<0.05 Obese vs Lean.

4.3 Perfusate analysis during reperfusion

Liver were exposed to 6 hours of CS preservation at 4°C in UW solution or dynamic MP at 20°C in Krebs-Heinseleit (KH) buffer, followed by 2 hours of reperfusion with KH solution.

4.3.1 AST release

The hepatic damage was assessed through the release of Aspartate Transaminase (AST) during reperfusion in both models, subjecting them to both methods of preservation. After 6 hours CS and 2 hours of reperfusion, a three-fold increase in AST was found in Obese Zucker rats as compared with the MCD group. When the livers were subjected to machine perfusion, the release of AST is comparable in both models (Figure 4.1).

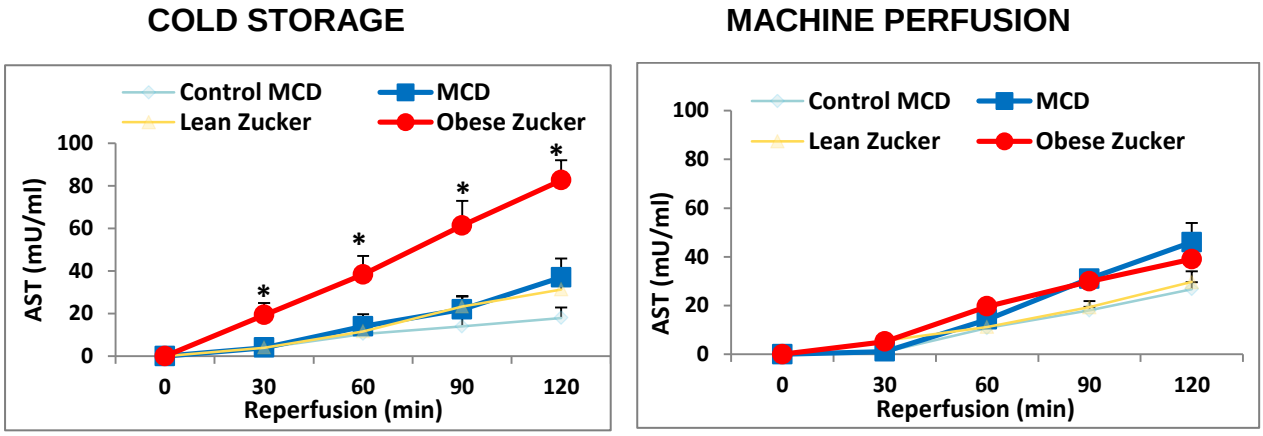


Figure 4.1: AST release. Obese Zucker rats after cold storage preservation showed a significant increase in AST levels when compared with the MCD group. After MP preservation no differences were found between the two steatotic models. The significant *p values, indicated in the chart, are *p<0.05. The results are reported as the mean ± S.E. of 7/groups.

4.3.2 LDH release

Another hepatic damage index is the Lactate Dehydrogenase (LDH) released. After 6 hours CS and 2 hours of reperfusion, in Obese Zucker rats a two-fold increase in LDH levels was observed as compared with the MCD group. No differences in LDH release were revealed when fatty livers of both models were subjected to MP (Figure 4.2).

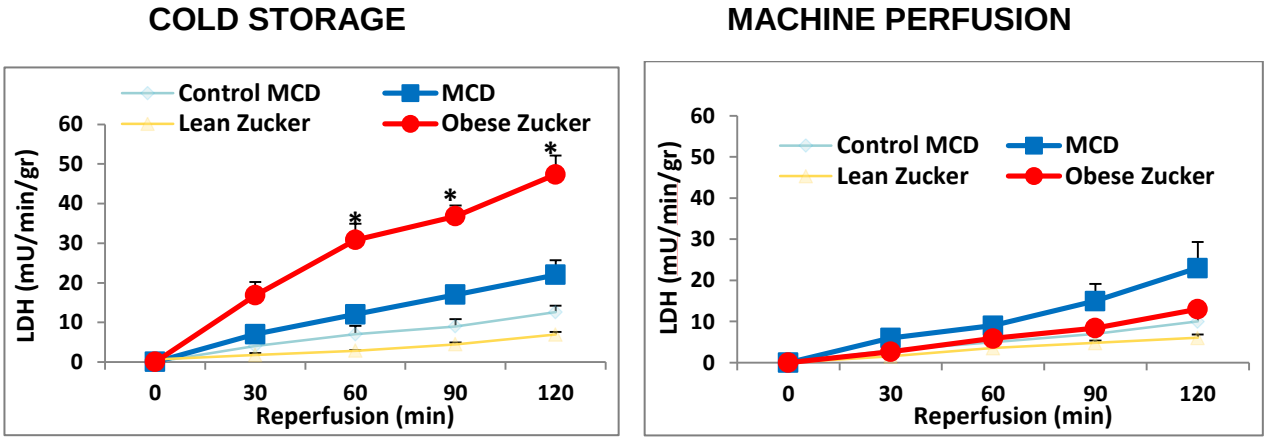


Figure 4.2: LDH release. After cold storage preservation Obese Zucker rats showed a significant increase in LDH levels when compared with the MCD group. No differences were revealed when fatty livers of both models were subjected to MP The significant *p values, indicated in the chart, -are *p<0.05. The results are reported as the mean ± S.E. of 7/groups.

4.3.3 Pressure changes during reperfusion

To assess if an impaired microcirculation occurs in fatty liver, intrahepatic pressure was monitored. During reperfusion, intrahepatic pressure was kept low in MCD throughout the reperfusion period; Obese Zucker rats, instead showed an increase in pressure during the reperfusion time (Figure 4.3).

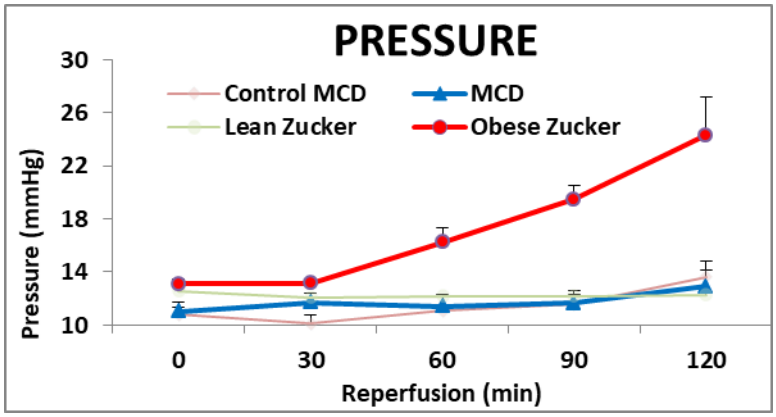


Figure 4.3: Pressure levels. Throughout the reperfusion period, the intrahepatic pressure was maintained constant in MCD rats when compared to Obese Zucker rats. The results are reported as the mean ± S.E. of 7/groups.

4.3.4 Bile flow and biliary enzymes during reperfusion

The bile flow and the release of enzymes in the bile were evaluated. By cold storage preservation, bile flow was significantly greater in MCD rats than in Obese Zucker ones, while the enzyme release within the bile was lower in MCD model than in Obese Zucker rats. On the other hand, by machine perfusion preservation, no differences were found in the biliary flow between the two models, while, the enzyme release into the bile was lower in the MCD rats as compared to the Obese Zucker animals (Figure 4.4).

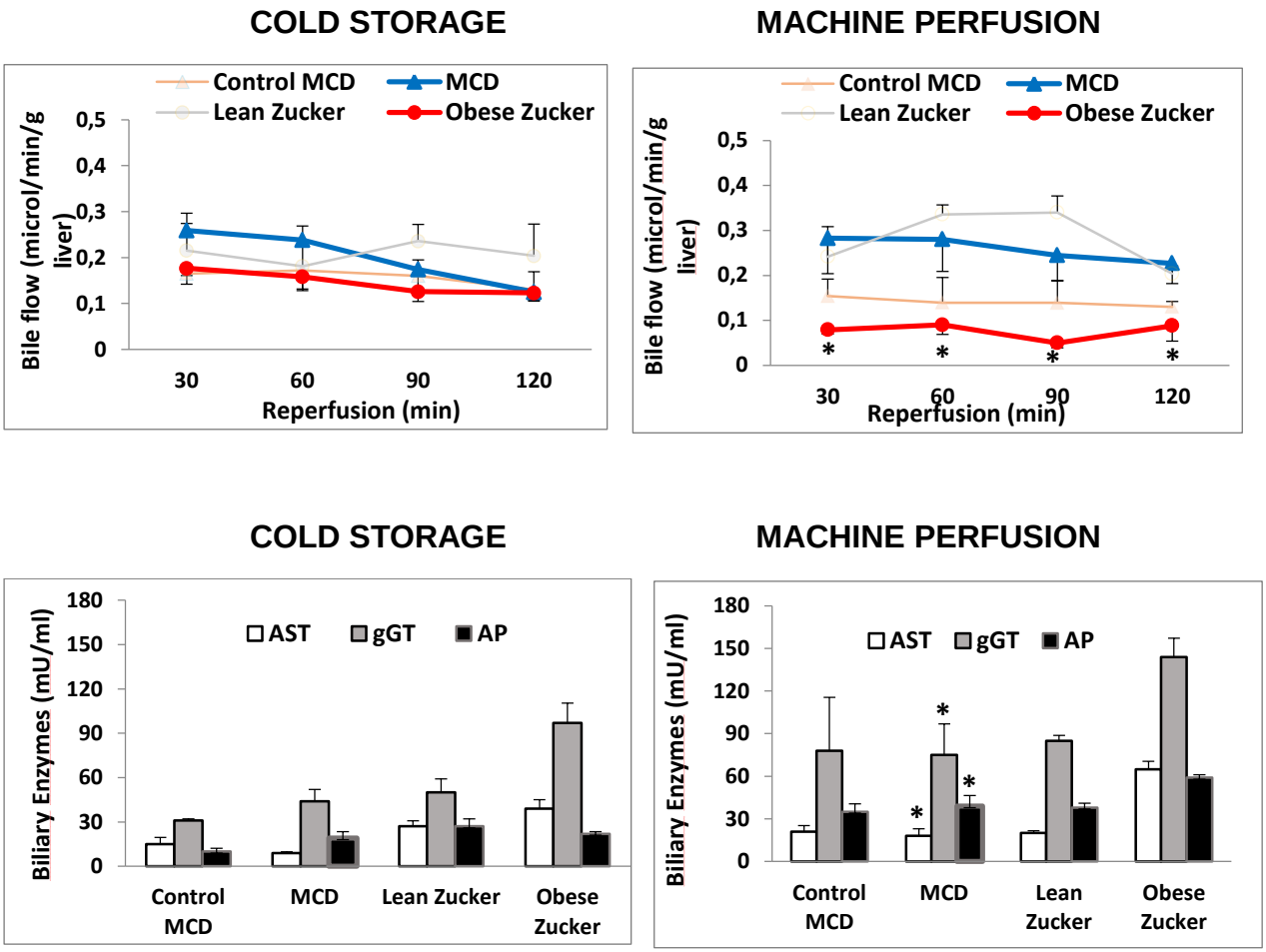


Figure 4.4: Biliary ennzymes. When subjected to CS preservation, MCD group showed a significant decrease in bile flow and biliary enzymes as compared with the Obese Zucker rats, while no differences were found after MP preservation. The significant The results are reported as the mean ± S.E. of 7/groups.

4.4 Mitochondrial function and oxidative stress at the end of fatty livers reperfusion

4.4.1 Mitochondrial function

ATP content is a marker of the energetic status of the cell, as it rapidly falls in detrimental conditions, leading to necrosis or apoptosis. The static cold preservation did not influence the energetic status of the cell of both groups. In fact, at the end of reperfusion, after CS, no difference in tissue ATP/ADP ratio was found in the MCD group compared with the Obese Zucker rats. Significant difference in ATP/ADP ratio were found when compared MCD and Obese Zucker rats with their respective controls (Figure 4.5).

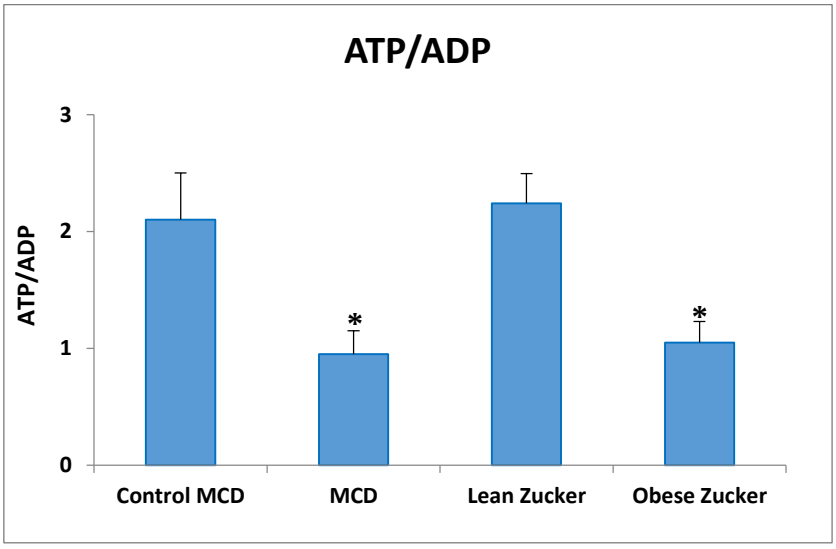


Figure 4.5: Hepatic energy content evaluation. At the end of reperfusion after CS preservation the hepatic ATP/ADP ratio did not show any differences between the two considered groups. Significant differences in ATP/ADP ratio were found between MCD and Obese Zucker rats when compared with their respective controls. The significant *p values, indicated in the chart, are *p<0.05. The results are reported as the mean ± S.E. of 7/groups.

4.4.2 Lipid peroxidation

Lipid peroxidation is a process triggered by free radicals and affecting membrane phospholipids. An uncontrolled increase in free radicals induces the oxidative damage spreads with a chain reaction oxidizing neighboring molecules and worsening the injury. The extent of lipid peroxidation was evaluated in terms of Thiobarbituric Acid Reactive Substances (TBARS) formation, using Malondialdehyde (MDA) as a standard. After 6 hours of CS and of reperfusion, in MCD and Obese Zucker significant differences were revealed as compared with their respective controls. No significant differences were found for the TBARS values between the two models (Figure 4.6).

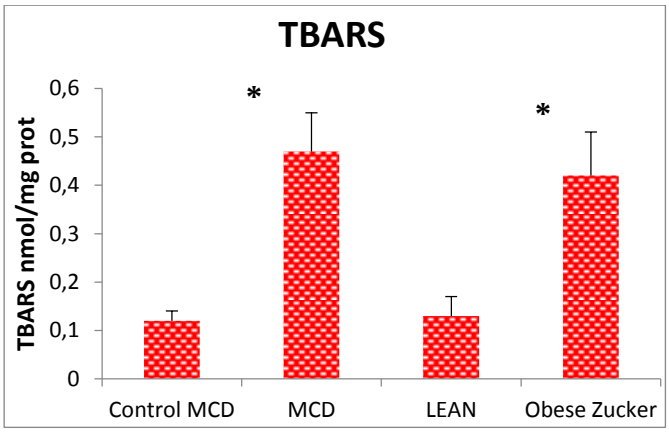


Figure 4.6: Lipid peroxidation evaluation. MCD and Obese Zucker rats showed significant difference as compared with their respective controls. Comparable values of TBARS were found between the two models. The significant *p values, indicated in the chart, are *p<0.05. The results are reported as the mean ± S.E. of 7/groups.

4.4.3 Glutathione content

Glutathione is one of the most important antioxidant molecules, a decrease in reduced Glutathione (GSH) content is considered a marker of oxidative stress-induced injury. As showed in the panel below, MCD and Obese Zucker rats showed significant decrease as compared with their respective controls but no significant differences were found between the two models (Figure 4.7).

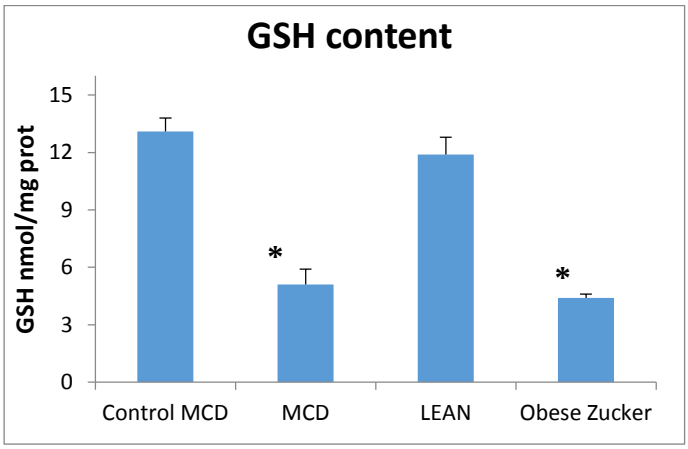


Figure 4.7: Hepatic GSH stores content evaluation. Comparable values of GSH were found between the two models.
A significant difference was found between MCD and Obese Zucker rats with their respective controls. The significant *p values, indicated in the chart, are *p<0.05.The results are reported as the mean ± S.E. of 7/groups.

4.5 Prolonged CS preservation period in liver from MCD rats

Since after 6 hOURS of cold storage preservation no differences were detected in AST, bile flow and pressure in MCD model versus their control, the livers were subjected to cold storage by prolonging the time of preservation to 12 and 24 hours. No increase in AST release, bile flow and pressure variation even after 12 and 24 hours of cold storage were found when compared with 6 hour preservation (Figure 4.8).

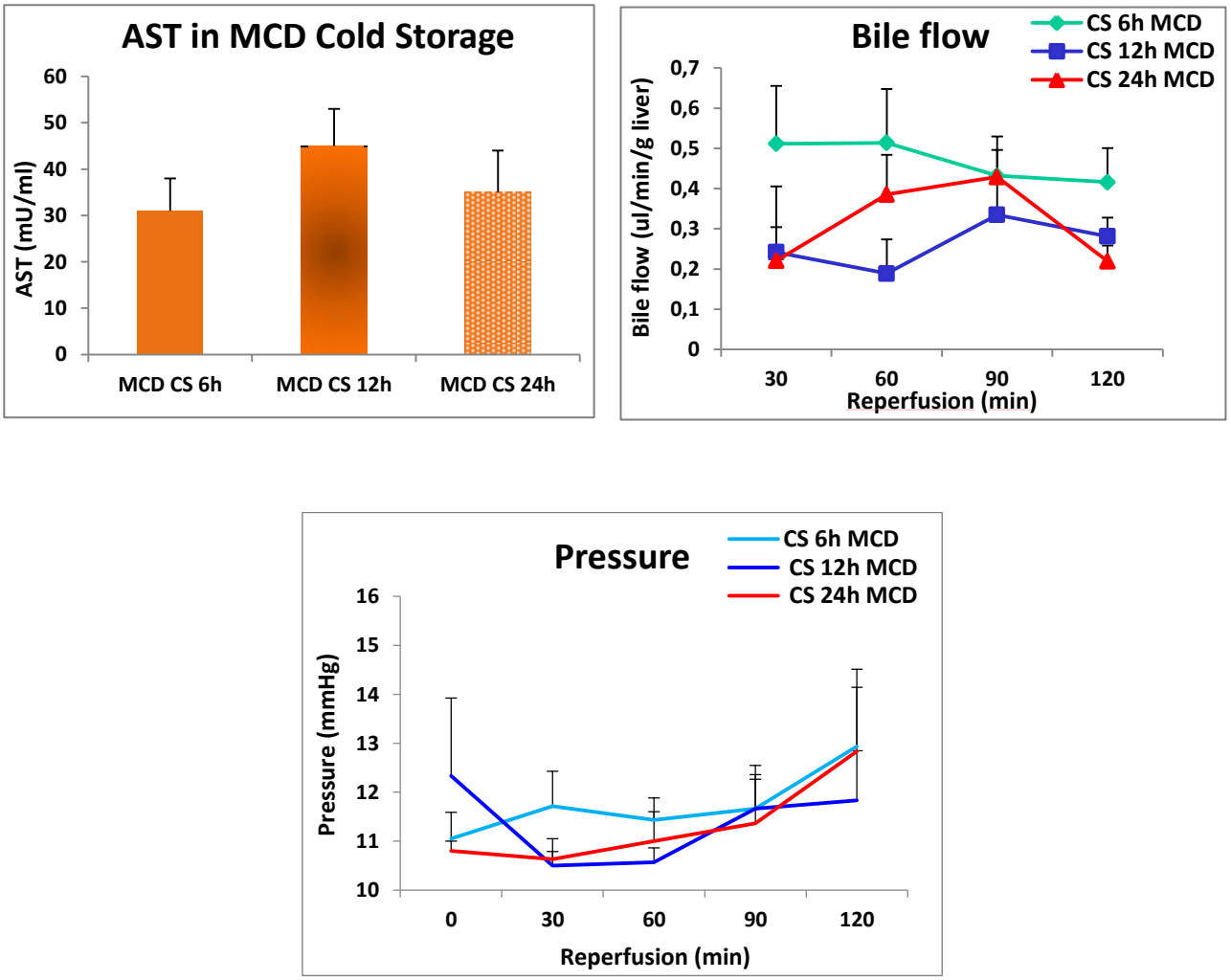


Figure 4.8: Evaluation of AST, Bile Flow and Pressure. AST release, pressure and bile flow remained constant also by prolonging the time of cold preservation to12 and 24 hours for MCD rats. The results are reported as the mean ± S.E. of 7/groups.

4.6 Fatty acid content in steatotic livers

Assessment of Fatty Acid (FA) constituents by Gas Chromatography-Mass Spectrometry (GC/MS) was performed in all the experimental groups used in this study. Estimation of fluorescing FA was performed by analysis of Autofluorescence (AF) spectra with the contribution of Doctor Anna Cleta Croce, Department of Biology and Biotechnology, University of Pavia. FAs evaluated by AF are expressed as relative percentage areas of the total fluorescing fatty acids (Figure 4.9).

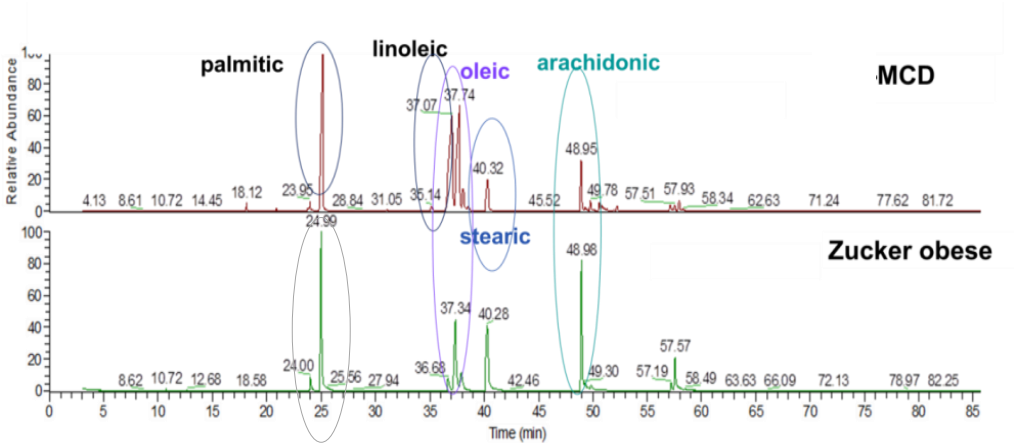


Figure 4.9: Evaluation of fatty acids composition. GC/MS spectra of fatty acids in MCD and Obese Zucker rats.

To get a clearer and more detailed picture of the individual fatty acids composition in the two models, we evaluated the percentage of content of stearate (C18:0), linoleate (C18:2) and arachidonate acid (C20:4). MCD rats showed a significant increase in linoleate levels and low stearic and arachidonic acids when compared to Obese Zucker rats (Figure 4.10).

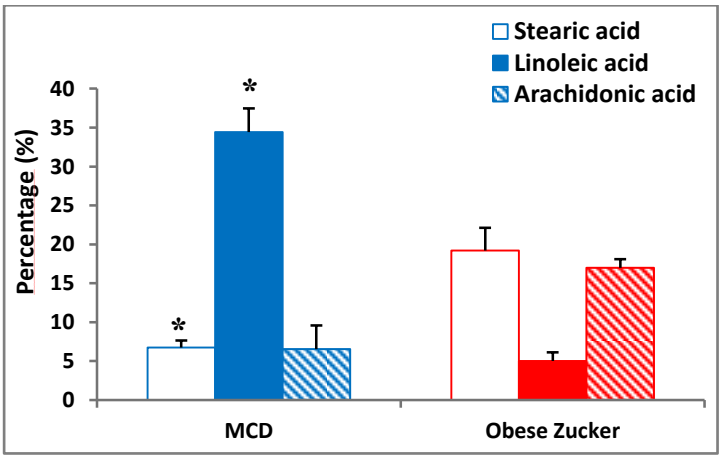


Figure 4.10: Evaluation of stearic, linoleic and arachidonic acid percentage. In MCD group linoleate levels were significantly increased, while stearate levels were significantly decreased, as compared with Obese Zucker rats. The significant *p values, indicated in the chart, are *p<0.05. The results are reported as the mean ± S.E. of 7/groups.

The percentage of Saturated Fatty Acids (SFAs), Monosaturated Fatty Acids (MUFAs), and Polysaturated Fatty Acids (PUFAs) were measured by GS/MS. As shown in the panel, a significant difference was found in SFAs and PUFAs in Obese Zucker and MCD rats, while no difference in MUFAs was found comparing the two models. MCD rats showed a lower percentage of SFAs and a higher percentage of PUFAs as compare with Obese Zucker rats (Figure 4.11).

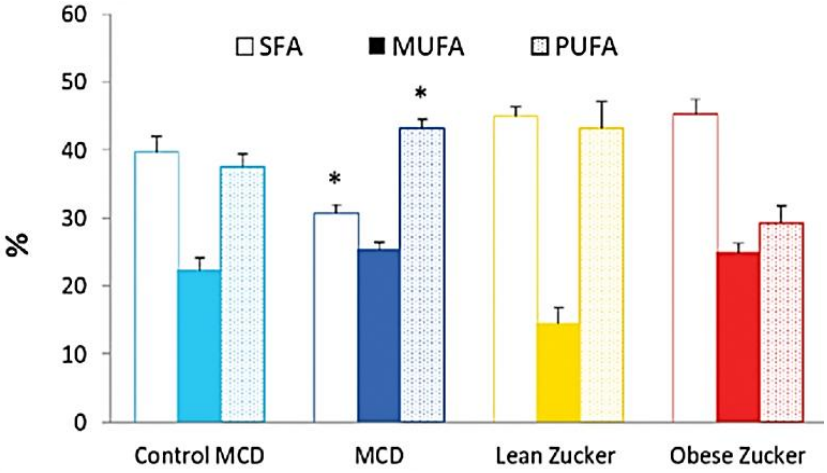


Figure 4.11: Evaluation of fattty acids composition: The percentage of SFAs was lower in MCD group respect to Obese Zucker, while PUFAs were higher in MCD group as compared with Obese Zucker rats. The significant *p values, indicated in the chart, are *p<0.05. The results are reported as the mean ± S.E. of 7/groups.

4.6.1 SFAs/PUFAs ratio

The observed SFAs/PUFAs ratio was significantly decreased in MCD group respect to Obese Zucker rats, in particular it is half in the MCD group compared to Obese Zucker rats (Figure 4.12).

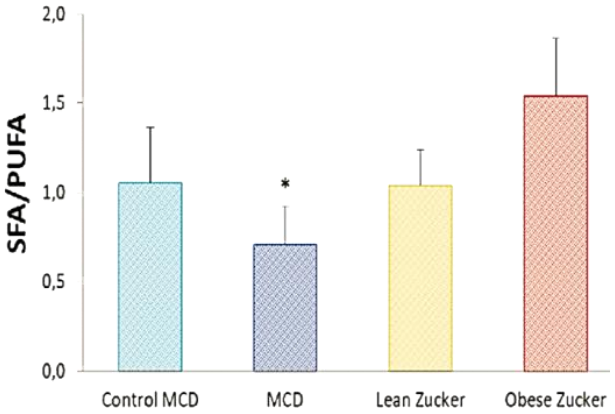


Figure 4.12: Evaluation of SFAs/PUFAs ratio: SFAs/PUFAs ratio was significantly decreased in MCD group respect to Obese Zucker rats. The significant *p values, indicated in the chart, are *p<0.05. The results are reported as the mean ± S.E. of 7/groups.

4.6.2 Total lipid content

The increase in hepatic total lipid amount is considered a marker for severe steatosis. The total lipid content was evaluated in MCD group as well as in Obese Zucker rats. We observed that the lipidomic profile was very different between the two groups: MCD rats showed a significant increased lipid content as compared with Obese Zucker rats (Figure 4.13).

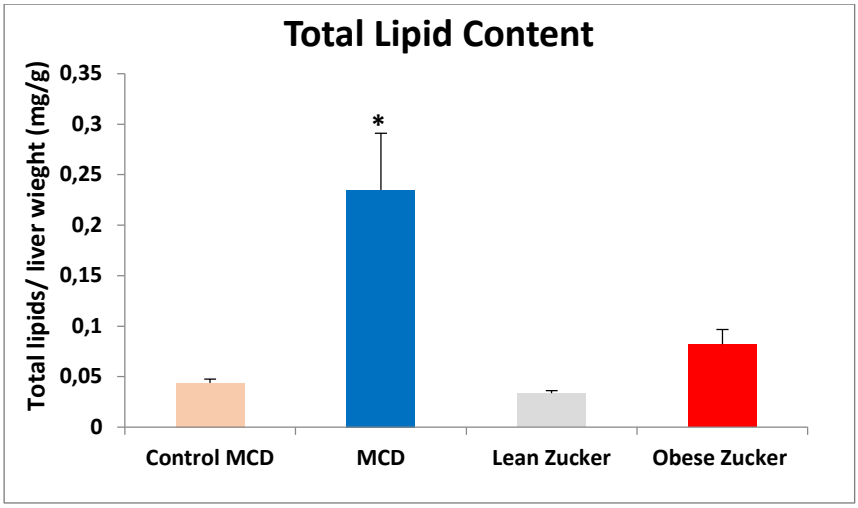


Figure 4.13: Evaluation of hepatic lipid content.
The total lipid content was significantly higher in MCD rats than in Obese Zucker rats. The significant *p values, indicated in the chart, are *p<0.03. The results are reported as the mean ± S.E. of 7/groups.

4.7 Results part II: Oxidative stress and desaturase in NAFLD and NASH rat models

4.7.1 Lobe heterogeneity of oxidative stress in NAFLD

4.7.1.1 TBARS production

Free radicals generate the lipid peroxidation process in living organisms. In particular the overproduction of free radicals and the polyunsaturated fatty acids peroxidation lead to TBARS production. The high formation of TBARS is commonly known as a marker of cell membrane oxidative stress.

In Obese Zucker rats, higher TBARS levels were found in Left Lobe (LL) both in Lean and Obese Zucker rats, when compared with the Right Lobe (RL) and Median Lobe (ML) (Figure 4.14).

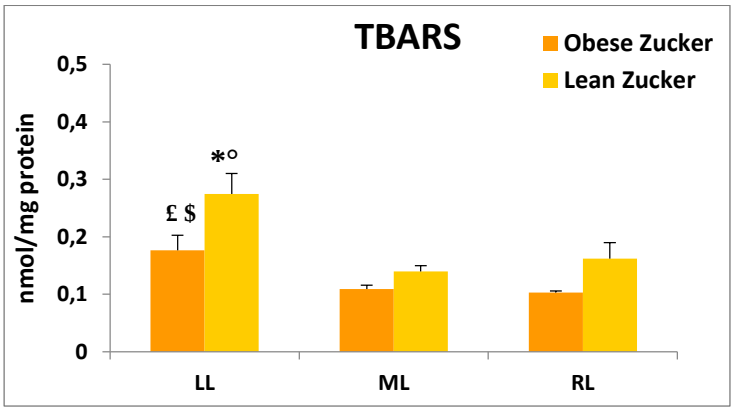


Figure 4.14: Mitochondrial MDA production. Higher MDA levels were found in LL both in Lean and Obese Zucker rats when compared with the RL and ML. The significant *p values, indicated in the chart, are *p<0.05.
* vs ML; ° vs RL; £ vs ML; $ vs RL.

4.7.1.2 ROS formation

The same trend occurred in ROS formation in Obese Zucker rats. In fact, in Zucker model, higher ROS levels were found in LL both in Lean and Obese rats, when compared with the RL and ML (Figure 4.15).

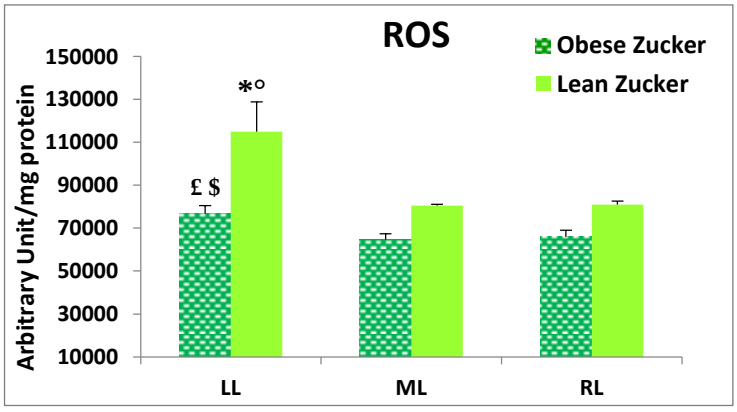


Figure 4.15: Mitochondrial ROS production. In Zucker model, higher ROS levels were found in LL both in Lean and Obese rats, when compared with the RL and ML. The significant *p values, indicated in the chart, are *p<0.05.
* vs ML; ° vs RL; £ vs ML; $ vs RL.

4.7.2 Lobe heterogeneity of oxidative stress in NASH

4.7.2 .1 TBARS production

Non-Alcoholic Steatohepatitis (NASH) is a more serious pathological condition then NAFLD. NASH is distinguishable from NAFLD because it involves inflammatory cells, oxidative stress, fibrosis and a higher amount of collagen around the veins and cells. NASH, less common than simple steatosis, is a risk factor for the development of cirrhosis and development of Hepatocellular Carcinoma (HCC). NASH condition is characterised by oxidative stress and an overproduction of reactive oxygen species.

High TBARS level was observed in the LL as compared with the RL and ML in the MCD animals. In particular at 8 weeks, an increase up to two-fold was detected for LL compared to ML and RL (Figure 4.16).

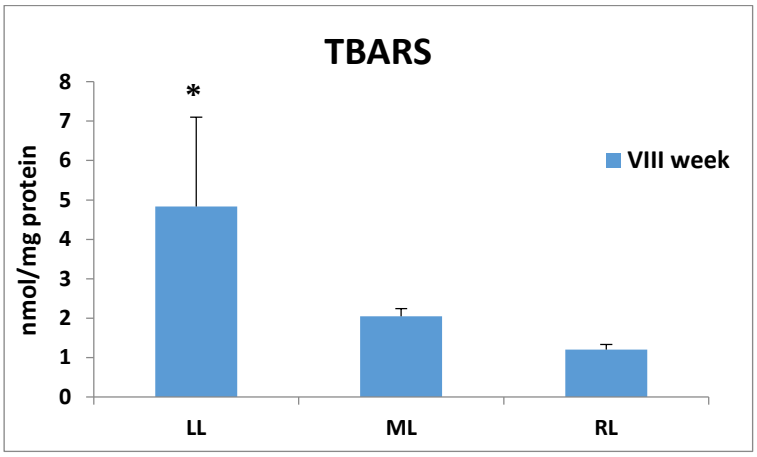


Figure 4.16: Mitochondrial TBARS production. LL of MCD animals showed a significant increase of TBARS level, when compared with the RL and ML. The significant *p values, indicated in the chart, are *p<0.05.
*vs RL; **vs ML

4.7.2 .2 ROS production

A significant increase of ROS levels was observed in LL of Control animals compared with the ML and RL (Figure 4.17).

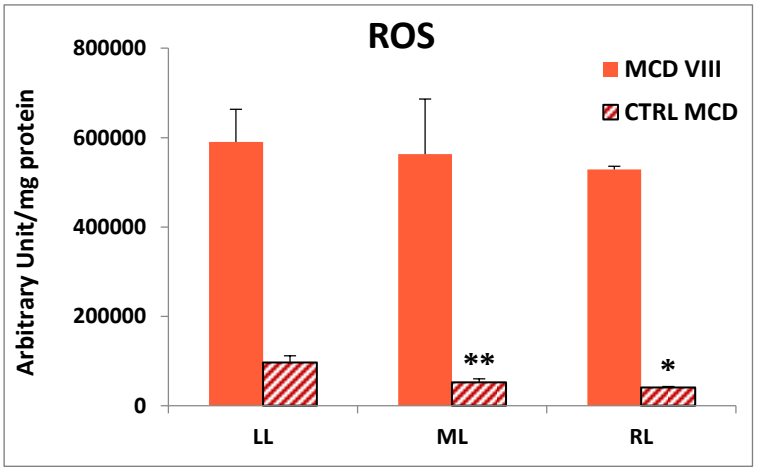


Figure 4.17: Mitochondrial ROS production. LL of Control animals showed a significant increase of ROS level, when compared with the ML and RL. The significant *p values, indicated in the chart, are *p<0.05.
*vs RL; **vs ML

4.8 Desaturase activity and oxidative stress in NAFLD model

4.8.1 Desaturases activity

Desaturases are enzymes able to insert a stereospecific double bond between carbons of fatty acyl chains thus taking part in lipogenesis (Zhang et al. 2016). In mammals, among Fatty Acid Desaturases (FADs) there are Delta-5 Desaturase (D5D) that introduces a double bond between Carbons 5-6 and Delta-6 Desaturase (D6D) that introduces a double bond between Carbons 6 and 7. Stearoyl-CoA Desaturase 1 (SCD1) is the last enzyme of *de novo* lipogenesis and catalyzes the synthesis of MUFAs, mainly oleate and palmitoleate from SFAs, in particular palmitate (D9-16D) and stearate (D9-18D), inserting a double *cis* bond between Carbons 9 and 10 of the acyl-CoA substrate (Aljohani et al. 2017).

The activity of D5D (20:4 n-6/20:3 n-6), D6D (18:3 n-6/18:2 n-6), D9-16D (16:1 n-7/16:0) and D9-18D (18:1 n-9/18:0) were evaluated in fatty livers obtained from the two models of NAFLD, MCD and Zucker rats. When compared with MCD rats, Obese Zucker ones showed higher D5D activity; instead, when compared with their respective controls the D5D activity index was lower in Obese Zucker (Figure 4.18, panel a). D6D was found only in MCD rats and increased when compared with its respective control rats (Figure 4.18, panel b). D9-16D increased in Obese Zucker rats when compared with MCD and Lean Zucker rats (Figure 4.18, panel c). In MCD rats a marked increase in D9-18D occurs as compared with Obese Zucker rats and with their respective controls (Figure 4.18, panel d).

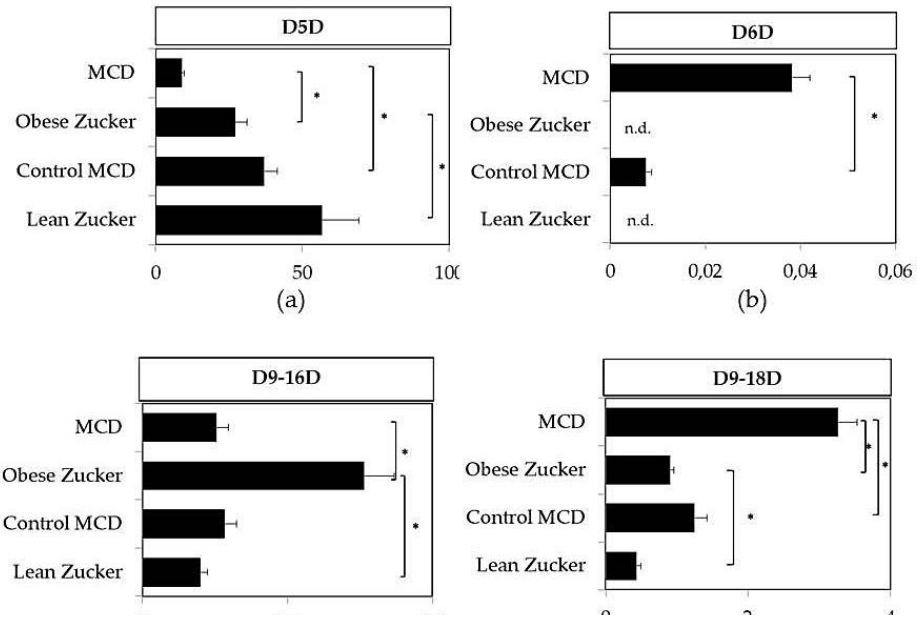


Figure 4.18: Desaturase activity indexes in MCD and Obese Zucker rats. (a) D5D; (b) D6D; (c) D9-16D; (d) D9-18D. p*<0.001; n.d.: not detectable. The results are reported as the mean ± S.E. of 6/groups.

It is known that the excess of hepatic fat lead to lipotoxicity which cause mitochondrial dysfunction with consequent overproduction of Reactive Oxygen Species (ROS).

4.8.2 Oxidative stress: TBARS, ROS and GSH

The two NAFLD models used differed with regard to the levels of oxidative stress. In fact, the MCD rat showed an hepatic increase in ROS and TBARS and a decrease in GSH content (Table 4).

	MCD	Obese Zucker	*p*	Control MCD	Lean Zucker	*p*
TBARS	0.34±0.07	0.11±0.0006	$	0.06±0.003	0.14±0.01	#; &
ROS	3384750±4844.4	111.5±8.9	$	26677.3±7691.3	27671±10713.9	#
GSH	186.3±25.3	194.2±10.7	$	134±7.3	191±5.1	#

Table 4: Oxidative stress indexes. ns: p value not significant; $: p<0.05 MCD vs Obese; #: p<0.05 MCD vs Control; &: p<0.05 Obese vs Lean.

A correlation between the activity index of the desaturases (D5D, D9-16D and D9-18D) versus oxidative stress, ROS, TBARS and GSH was evaluated.

4.8.3 Correlation between D5D versus ROS, TBARS and GSH

D5D showed a negative correlation with both ROS and TBARS and a positive correlation with GSH (Figure 4.19, panel A-C).

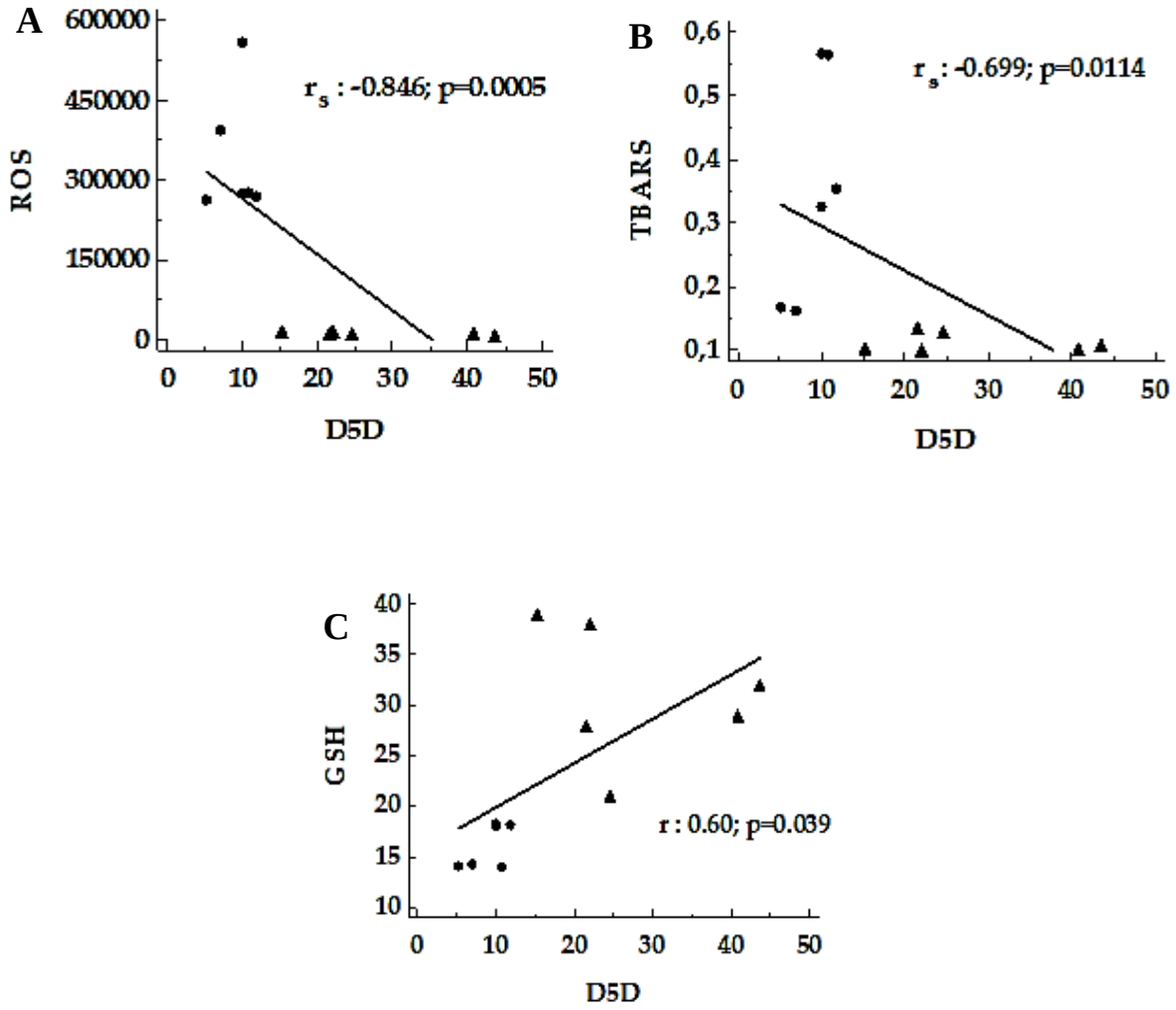


Figure 4.19: Correlation between D5D activity index versus ROS, TBARS and GSH; (A-B) D5D have a negative correlation with ROS and TBARS. (C) A positive correlation between D5D and GSH was found.
●: MCD rats; ▲: Obese Zucker rats. The results are reported as the mean ± S.E. of 6/groups.

4.8.4 Correlation between D9-16D and ROS, TBARS and GSH

In the case of the D9-16D activity related oxidative stress, a negative correlation versus both ROS and TBARS and a positive correlation with GSH were found (Figure 4.20, panel A-C).

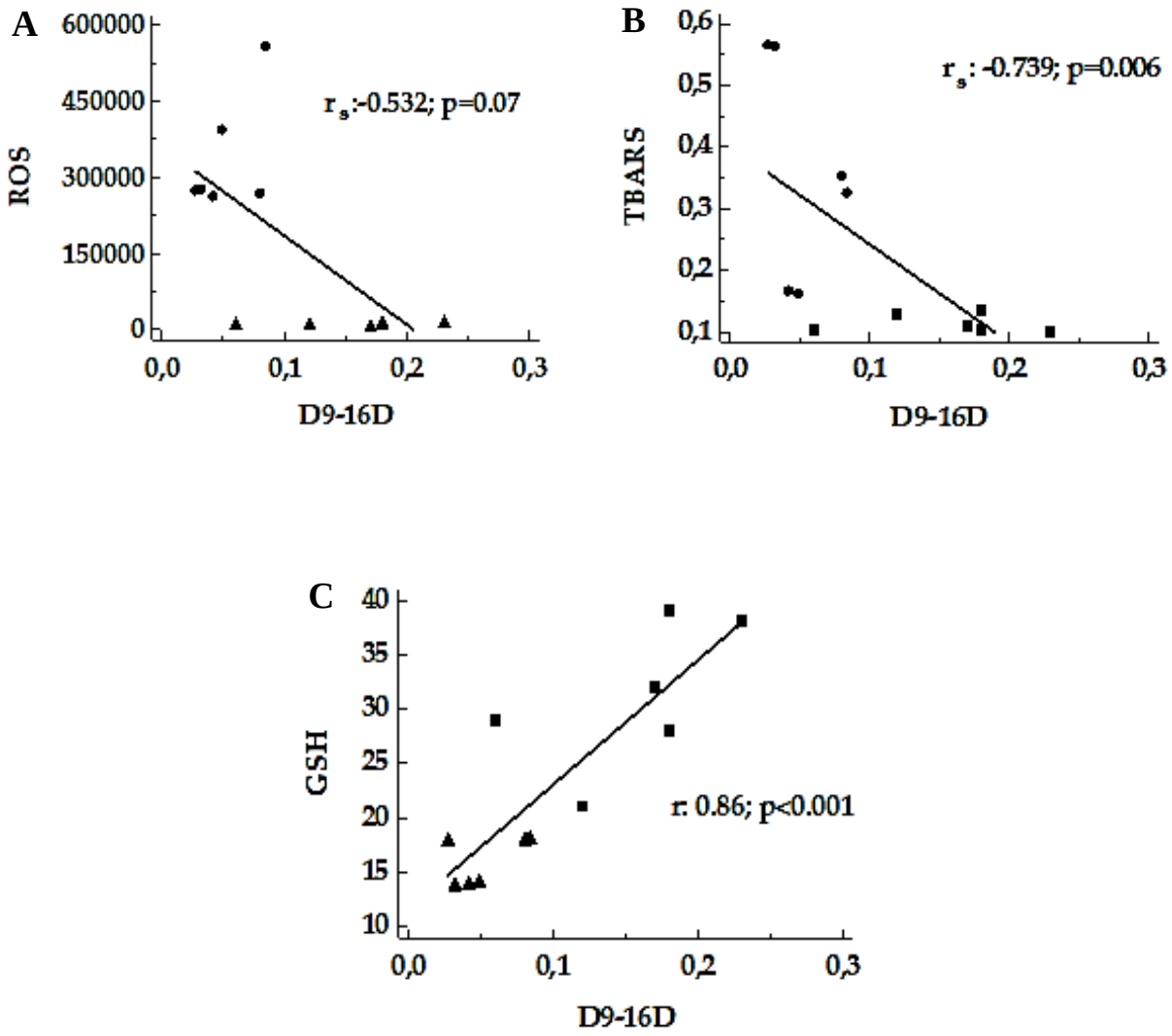


Figure 4.20: Correlation between D9-16D activity index versus ROS, TBARS and GSH; (A-B) A negative correlation between D9-16D with ROS and TBARS was found, while (C) with GSH a positive correlation was observed. ●: MCD rats; ▲: Obese Zucker rats. The results are reported as the mean ± S.E. of 6/groups.

4.8.5 Correlation between D9-18D and ROS, TBARS and GSH

On the contrary respect to D9-16D, a positive correlation between D9-18D versus ROS and TBARS and a negative correlation with GSH were found in MCD rats and Obese Zucker rats (Figure 4.21, panel A-C).

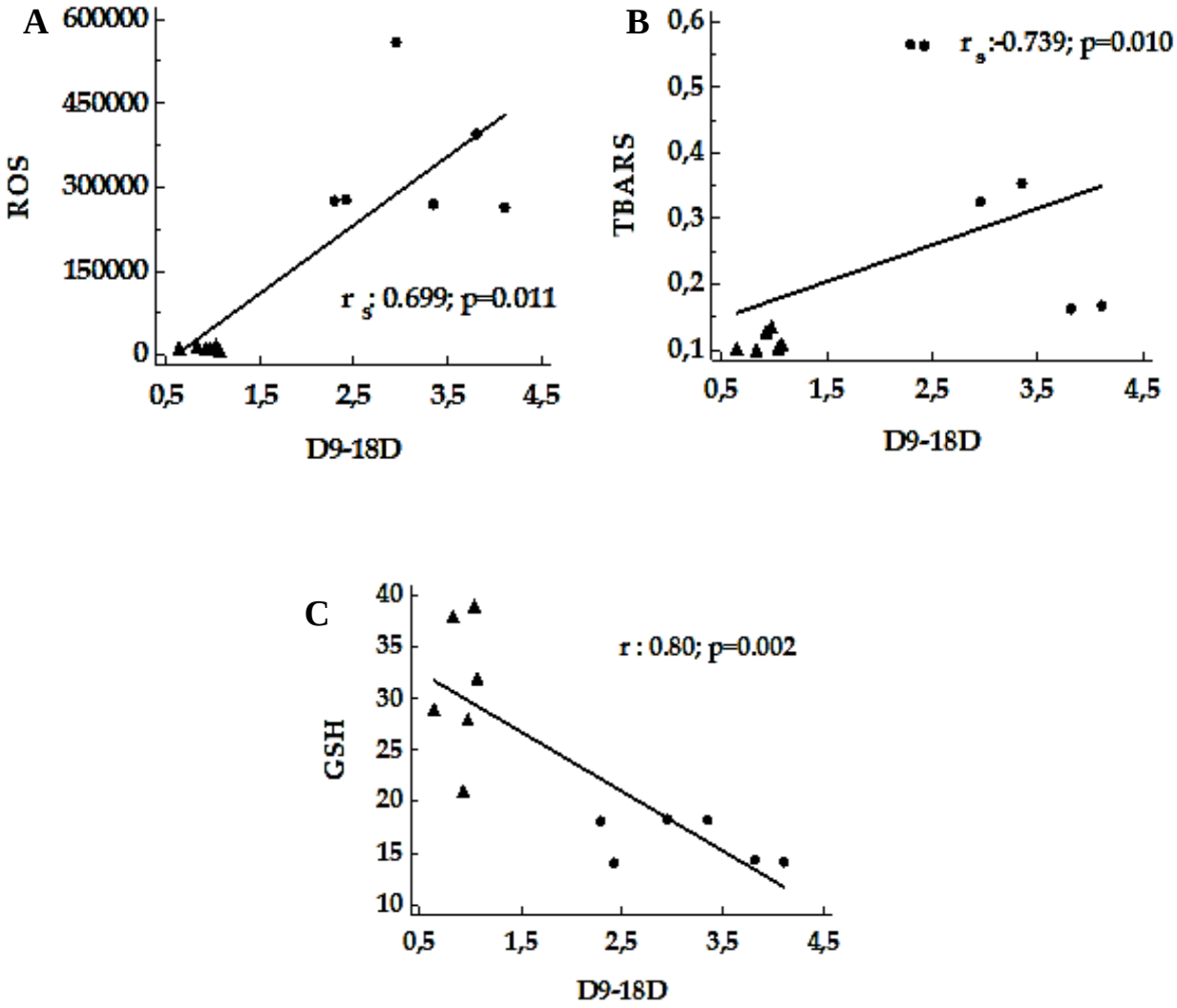


Figure 4.21: Correlation between D9-18D activity index versus ROS, TBARS and GSH; (A-B) Positive correlation of D9-18D with ROS and TBARS was shown. (C) A negative correlation between D9-18D and GSH was found. ●: MCD rats; ▲: Obese Zucker rats. The results are reported as the mean ± S.E. of 6/groups.

4.8.6 Correlation between DHA and ROS, TBARS and GSH

Docosahexaenoic acid (DHA, C22:6), has a strong antioxidant effect, since it tends to bind free radicals, preventing them from destroying living tissue. A recent study demonstrated that, in NAFLD disorders a dietary supplementation with DHA can prevent or alleviate NAFLD (Serviddio et al. 2013). Based on these data, a correlation between DHA and oxidative stress was analyzed. The correlation between DHA versus ROS and TBARS was negative in both cases; on the contrary, the correlation with GSH was positive (Figure 4.22, panel A-C).

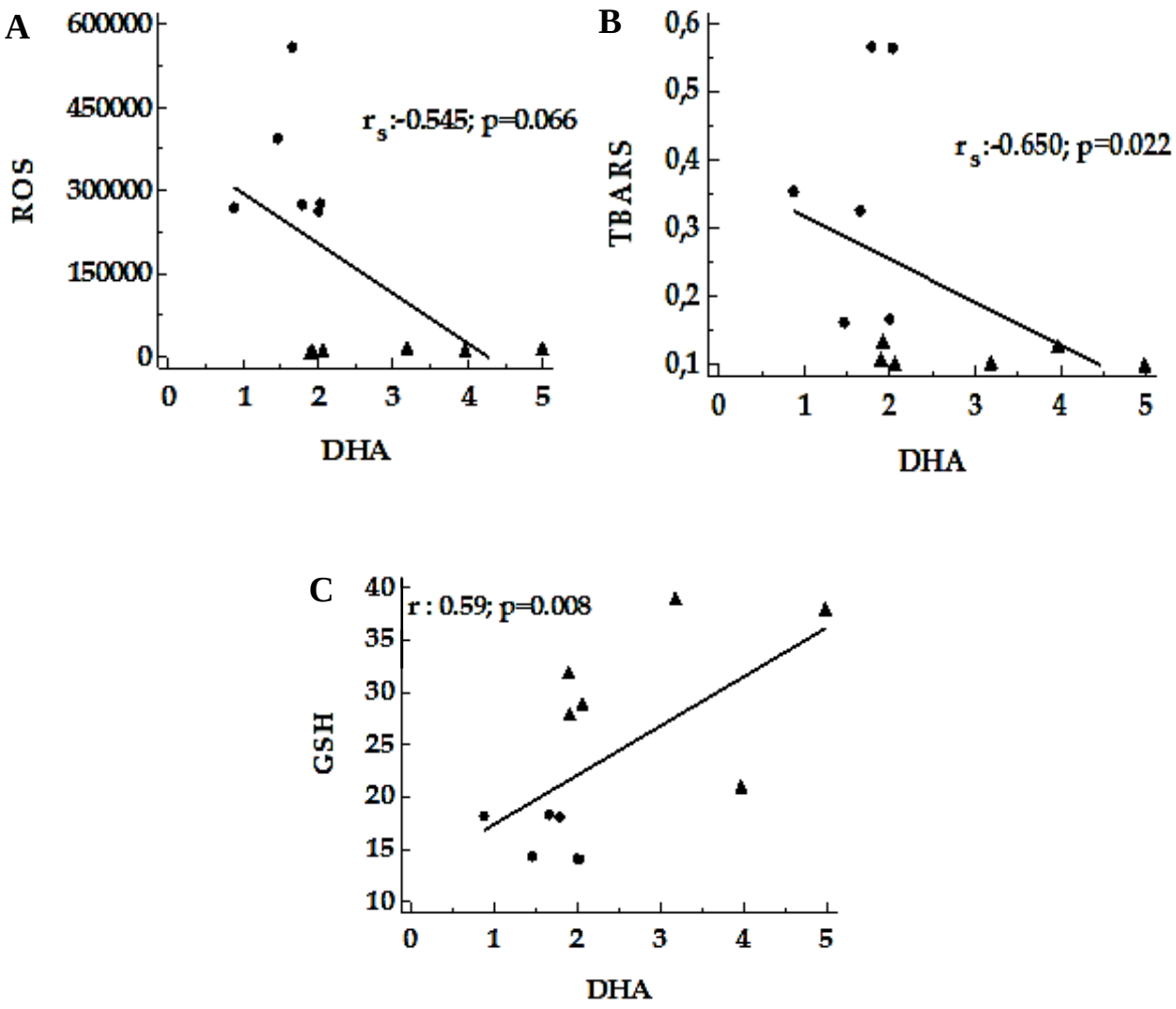


Figure 4.22: Correlation between DHA activity index versus ROS, TBARS and GSH; (A-B) A negative correlation between DHA versus ROS and TBARS was detected. (C) The correlation between DHA and GSH was positive. ●: MCD rats; ▲: Obese Zucker rats. The results are reported as the mean ± S.E. of 6/groups.

4.8.7 Correlation between serum TNF-alpha versus Desaturases and DHA

The following table shows the levels of correlation between serum levels of TNF-α versus hepatic desaturases and DHA. A negative correlation was found between D5D, D9-16 and DHA versus TNF- α. On the contrary, there is a positive correlation between D9-18 versus TNF- α (Table 5).

	Serum TNF- α versus Desaturases and DHA	
	r/r_s	***p***
D5	-0.708	0.010
D9-16	-0.611	0.035
D9-18	0.830	0.0008
DHA	-0.802	0.0017

Table 5: Correlation between serum levels of TNF- α versus liver desaturase and DHA.

4.9 Results part III: Modulation of mGluR5 on *in vitro* models

4.9.1 Cell viability

In the third part of this study, *in vitro* models of steatosis have been used to investigate the possible involvement of metabotropic Glutamate Receptor subtype 5 (mGluR5) during lipid accumulation in hepatic cells of human origin, HepG2.

The dose-dependent effect of oleate:palmitate (2:1) mix (4, 2, 1, 0.5 mM), after 24, 48, 72 hours on cell viability was evaluated by MTT assay.

The cells treated with 4mM of mix died, while with 2 mM cells survived only up to 24 hours respect to Control. Cell viability improved with 1 and 0.5 mM respect to Controls at both 24 and 48 hours (Figure 4.23).

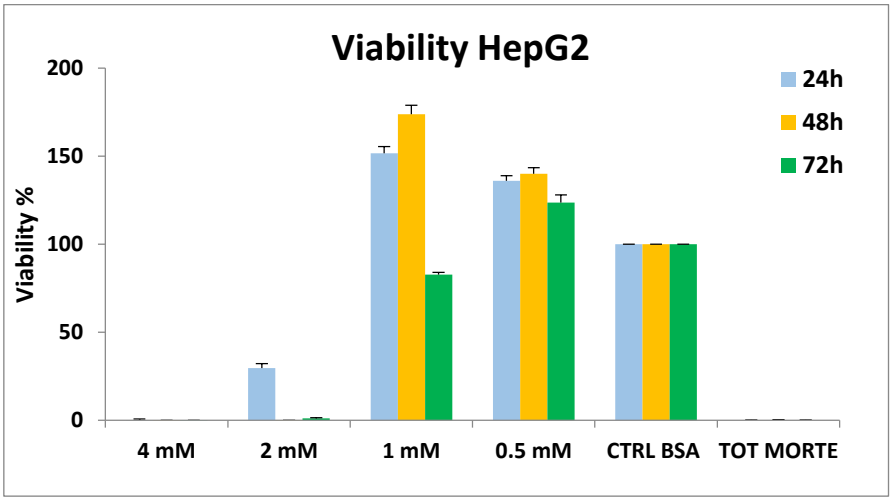


Figure 4.23: Cells viability with mix oleate:palmitate time course. Cells treated with 4 and 2 mM oleate:palmitate (2:1) mix died. At 1 and 0.5 mM cell viability improved.

Based on these results, it was supposed that the most appropriate oleate:palmitate (2:1) mix concentration was in the middle between 2 mM and 1 mM: 1.5 mM. At 1.5 mM oleate:palmitate (2:1) mix in fact, it was observed a damage that did not alter the viability.

4.9.2 Metabotropic Glutamate Receptor protein expression

To assess the modulation of mGluR5 on HepG2 cells treated with oleate:palmitate (2:1) mix, the presence of this receptor was first evaluated comparing cortex, in which it is more expressed, as positive control (Figure 4.24).

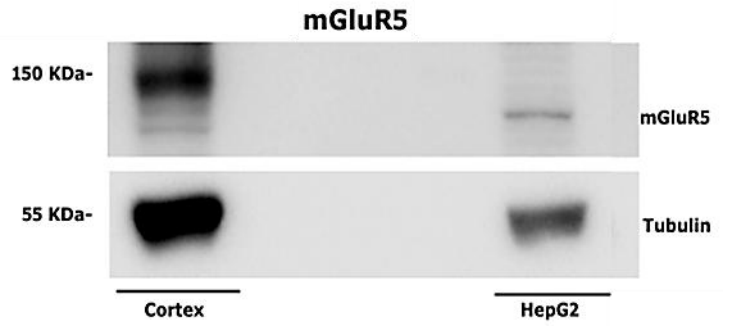
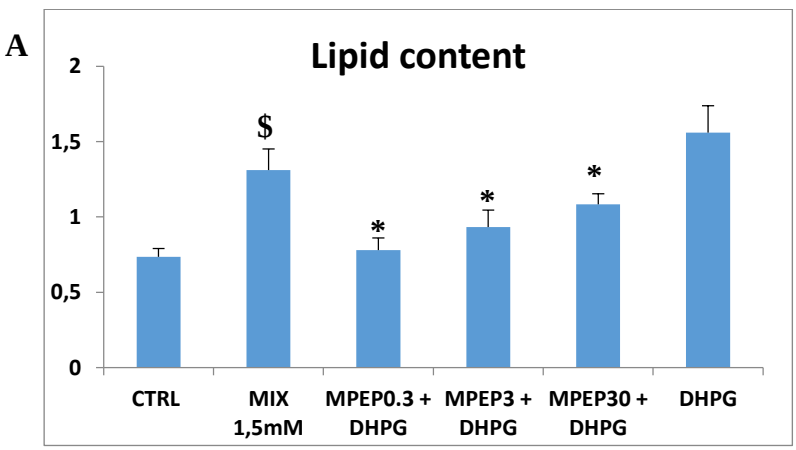


Figure 4.24: Metabotropic Glutamate Receptor 5 expression. HepG2 cells express metabotropic glutamate receptor 5.

4.9.3 Lipid content

HepG2 cells were first subjected to a 12 hour pre-treatment with various concentration of negative allosteric modulator of metabotropic glutamate receptor 5: MPEP 0.3, 3, 30 µM, and the orthosteric agonist DHPG 100 µM. Then, the cells were treated 12 hours with 1.5 µM oleate:palmitate (2:1) mix and DHPF or MPEP+DHPG at different concentrations.

The lipid content was calculated as ratio between fluorescence intensity of Nile Red that marks lipids within the cells and Hoechst, that stains nuclei in blue. FA accumulation increased with the activation of mGluR5 by DHPG, when compared with FA-treated cells. The addiction of MPEP 0.3 µM neutralized the DHPG-induced FAs accumulation (Figure 4.25).



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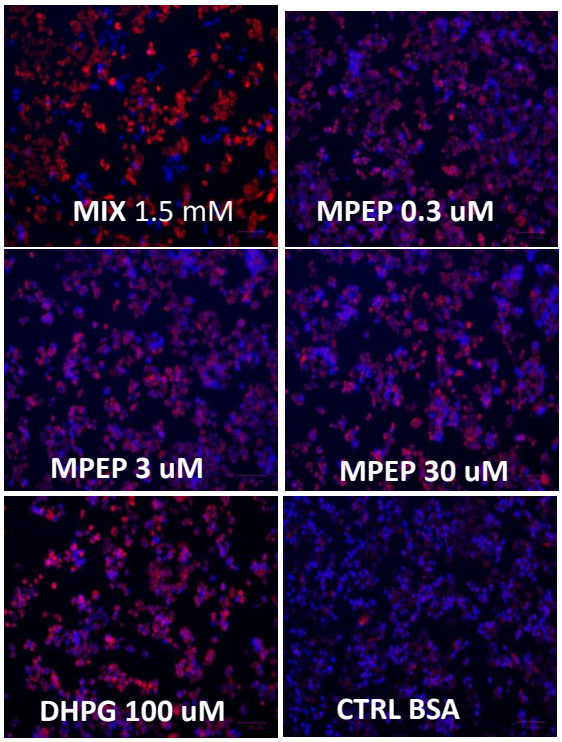

Figure 4.25: (A) Lipid content evaluation. When mGluR5 is activated by DHPG 100 µM, FA accumulation increased. Instead, the blockade of mGluR5 by MPEP 0.3, 3 and 30 µM caused a reduction of FA in HepG2 cells. $: $p<0.05$ MIX vs MPEP 0.3+DHPG; *: $p<0.05$ vs DHPG; **(B)** Respective conditions cell imaging.

4.9.4 ROS formation

ROS formation was calculated as ratio between fluorescence intensity of Dichlorofluorescein (H_2DCFDA) that marks ROS within the cells and Hoechst that stains nuclei in blue.

No differences were observed in ROS formation using MPEP at each concentration and fatty acid treatment, respect to Controls (Figure 4.26).

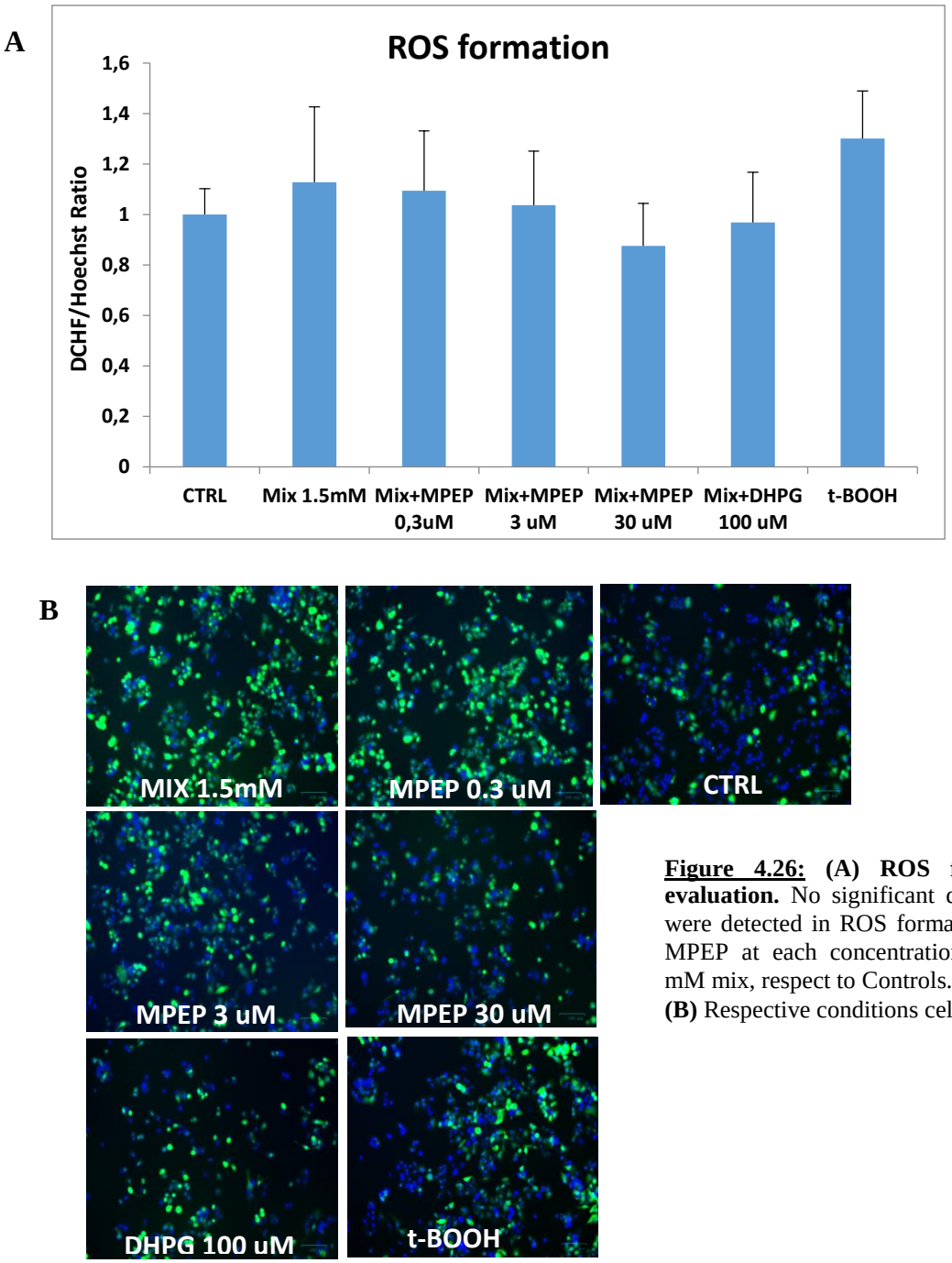

Figure 4.26: (A) ROS formation evaluation. No significant differences were detected in ROS formation using MPEP at each concentration and 1.5 mM mix, respect to Controls.
(B) Respective conditions cell imaging.

4.9.5 Cell viability

After 12 hours of pharmacological and 1.5 mM oleate:palmitate (2:1) mix treatments, cell viability was evaluated with MTT assay.

No changes in cell viability were found in all conditions considered (Figure 4.27).

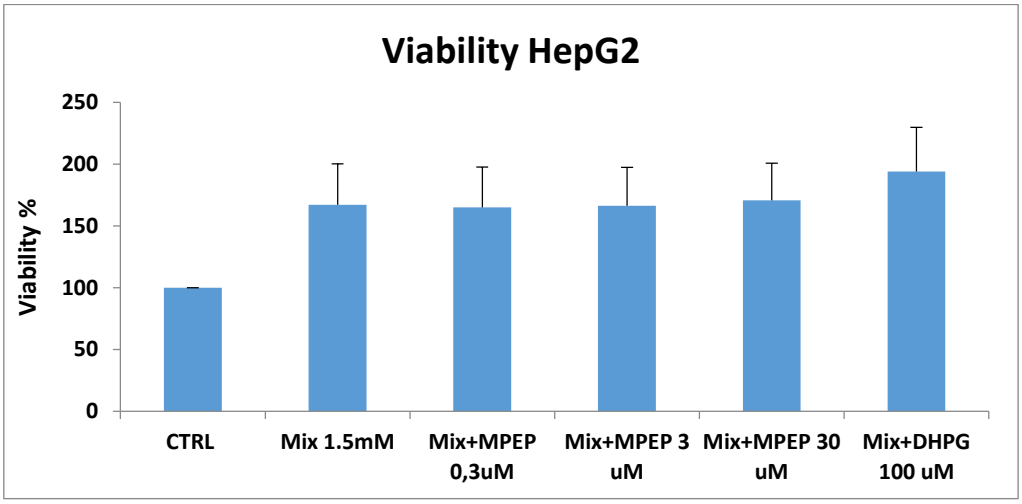

Figure 4.27: Cells viability evaluation. Cell viability did not show difference during the pharmacological and fatty acid treatments respect to Controls.

4.9.6 Apoptosis

We examined the levels of expression of caspase-3, key protein in the apoptosis process. Caspase-3 protein is a member of caspase family, which regulates both the intrinsic and extrinsic apoptosis pathways.

HepG2 cells treated with MPEP 0.3 µM show a significant reduction of the caspase-3 protein expression compared to cells treated only with fatty acids (Figure 4.27).

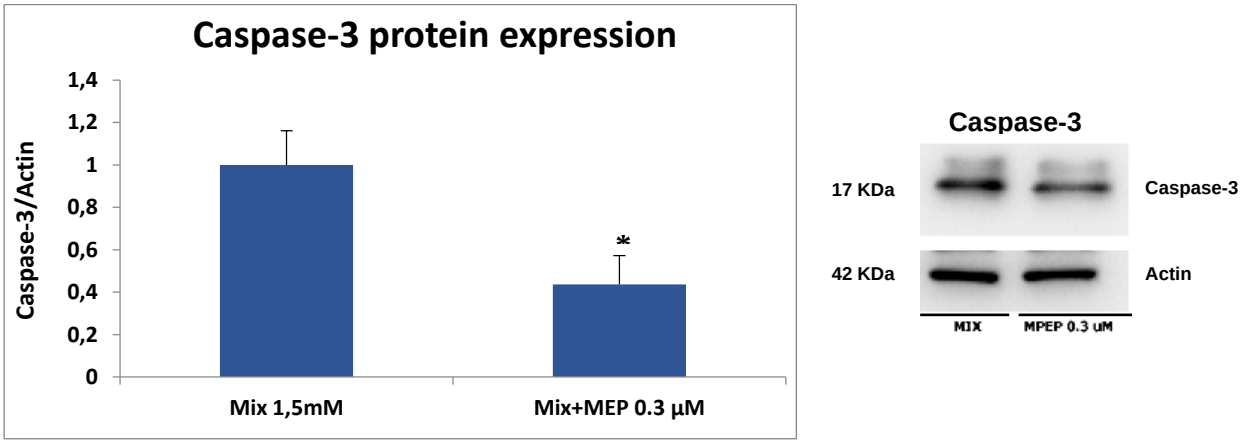

Figure 4.27: Cells apoptosis evaluation. HepG2 cells subjected to pharmacological and fatty acid treatments show a significant reduction of the caspase-3 expression respect to cells treated only with fatty acids.

Discussion

In this work we demonstrated that the lipidomic liver profile is a sensitive indicator of graft function and the choice of preservation strategy varies according to hepatic fatty acid constituent. We showed that a composition rich in PUFAs makes the livers less susceptible to cold preservation, than those with high levels of SFAs. Furthermore, our data revealed that the development and progression of NAFLD is associated with changes in the hepatocyte redox status and oxidative stress. Finally, we investigated mGluR5, as a novel target to reduce fatty acid accumulation. In particular we showed that the blockade of mGluR5 with the negative allosteric modulator MPEP protects hepatocytes from fatty acid accumulation and apoptosis.

5.1 Role of fatty acids composition in fatty liver transplantation

In the first part, we made a comparison between the MCD nutritional model and the Obese Zucker genetic one, preserving the livers either with cold storage or with a perfusion machine for 6 hours and reperfusing the organs for 2 hours at 37°C in the presence of oxygen. The organs of the two models subjected to cold storage have a different release of hepatic enzymes: in fact, the MCD group has a lower release of both AST and LDH compared to that found in the Obese Zucker model. However, when the livers are subjected to machine perfusion, the release of AST and LDH is comparable in both models. Then we evaluated the bile flow and the release of enzymes into the bile. We have observed that by preserving livers with cold storage, bile flow is significantly higher in MCD rats than in Obese Zucker group, whereas the release of enzymes in bile is lower in MCD model, when compared to Obese Zucker rats. On the other hand, by machine perfusion preservation, no differences are found in the biliary flow between the two models, while, as regards the release of enzymes in the bile, it is lower in MCD rats compared to the Obese Zucker animals. Markedly reduced CS damage was found in MCD livers, when compared with organs obtained from Obese Zucker rats. The improved level of CS preservation found in MCD livers is not associated with better energy status because the ATP/ADP ratio is similar in both fatty liver models. The extent of lipid peroxidation was evaluated in term of TBARS formation but no significant differences were found for the TBARS values between the two models. Like for TBARS formation, also for GSH content comparable values were found between MCD and Obese Zucker rats. Then we extended the time of cold storage preservation to 12 and 24 hours. No increase in AST

release, bile flow and pressure variation even after 12 and 24 hours of cold storage were found, when compared with 6 hour preservation. Successively, we examined the total content of lipids and we observed that the total fat content is significantly higher in MCD rats compared to Obese Zucker rats.
Fukumori and colleagues had already reported that the fatty liver is more susceptible to preservation with cold storage because as the temperature decrease, the fats undergo transition, passing from a fluid to a gelled phase to form crystalline structures that cause the breakage of cells (Fukumori et al. 1999). Therefore, based on this result, we could expect that the MCD model is even more susceptible to CS, when compared with Zucker model.

The data obtained regarding MCD rats, however, surprisingly deviates from the expected data. In fact, the lipid content of MCD group suggested a worse outcome with cold storage preservation than compared to Zucker, but this do not happen. Therefore, we hypothesized that the different damage comparing livers from MCD and Zucker rats subjected to CS, was linked to the composition of fatty acids. By examining the fatty acid content within the two groups, we observed that the relationship between SFAs and PUFAs is significantly lower in MCD livers compared to Zucker ones. This suggests that MCD model have more PUFA content than Zucker rats. According to this hypothesis, the reduced CS injury observed in MCD livers could be due to a lower SFA/PUFA ratio, possibly reducing the deleterious tendency to lipid crystallization, probably occurring in Obese Zucker rats (Fukumori et al. 1999). To get a more detailed picture of the individual fatty acids in the two models we evaluated the content of stearate, linoleate and arachidonic acid. MCD rats show very high levels of linoleate and low stearate and arachidonic acid, when compared to Obese Zucker rats. The data obtained suggest a first hypothesis about our conclusions, namely that the lipid composition of the liver affects the susceptibility of the organ following preservation through cold storage at 4°C. Our results not only confirm the data presented by Fukumori and colleagues on the crystallization of fat at low temperatures, but also provide us further information: the quality of lipid constituents might influence hepatic injury during preservation by CS more than the quantity of lipid content and energy status. In fact, the livers of MCD rats present a higher PUFA content, making them less susceptible to cold preservation than Obese Zucker group, which, instead, shows high levels of SFAs. It is precisely the SFAs, therefore, that at low temperatures form crystalline structures causing cell breakage.

A second hypothesis concerns, instead, among PUFAs, the arachidonic acid that is significantly lower in MCD model respect to Obese Zucker one. The arachidonic acid in the liver is 75% metabolized by cytochrome P450, obtaining the metabolite 20-Hydroxyeicosatetraenoic acid (20-HETE). Interestingly 20-HETE was considered to be a potent vasoconstrictor of cerebral microvessels, which contributes to IR injury (Tanaka et al. 2006; Yang et al. 2012). HETE has never been studied in the liver for now, but we know that in kidney transplantation it represent a negative index of functional recovery of the organ following transplantation. Through this analysis, it is possible to affirm that the reduced CS injury observed in MCD livers may be justified by the lower SFA/PUFA ratio, possibly reducing the deleterious tendency to lipid crystallization observed in Zucker obese rats (Fukumori et al. 1999).

5.2 Liver Lobe-Specific Oxidative Stress

In this study, we analyzed the heterogeneity of oxidative stress during NAFLD by the evaluation of TBARS and ROS production each lobe. In Obese Zucker rats, higher TBARS levels were found in Left Lobe (LL), when compared with Right Lobe (RL) and Median Lobe (ML). The same trend occurs also in ROS formation.

Thus, both indexes were evaluated in Non Alcoholic Steatohepatitis (NASH). High vulnerability to oxidative stress is responsible for the "second hit" in the spontaneous progression from simple steatosis to NASH (Guillén et al. 2009). In this case, high TBARS levels were observed in the LL, as compared with the RL and ML in the MCD animals. In particular, at 8 weeks, an increase was detected up to two-fold for LL with respect to ML and RL. Also for ROS production, higher levels were found in LL respect to ML.

Our data suggest that differences in ROS formation in the LL are not only associated with the NAFLD and NASH model as they were also found in control animals and Lean Zucker rats.

These results confirm previous studies in IR and obstructive cholestasis models, which demonstrated a lobar functional heterogeneity of the liver, indicating that different events occur in the different hepatic lobes (Palladini et al. 2019; Ferrigno et al. 2014). One possible explanation may be associated to the distribution of the liver vascular system. It might be an incomplete mixing of blood coming from the gastrointestinal tract and spleen, leading to variation in the delivery of nutrients and toxins to the hepatic lobes. Another possible

difference between the lobes could be due to a phylogenetic origin: the LL is older, while the RL is more recent (Jacobsson et al. 1999).

5.3 NAFLD pathogenesis: correlation between desaturase and oxidative stress

Changes in the hepatocyte redox status and oxidative stress have a critical role in the onset of lipid accumulation (Serviddio et al. 2013). Fatty acid desaturases are enzymes that remove two hydrogen atoms from a fatty acid, creating a carbon/carbon double bond. In particular, the activation of fatty acid desaturation leads to pro-inflammatory condition (Martinelli et al. 2008). In this study, we demonstrated that there are some correlation between desaturase activity and ROS, TBARS and GSH. Among desaturases, we analyzed D5D activity and we have seen that, when compared with MCD, Obese Zucker rats show higher activity. Instead, when compared with their respective controls, the D5D activity is lower in Obese Zucker rats. The correlation with ROS, TBARS and GSH reveals that D5D is privileged under low oxidative stress condition, in fact, D5D shows a negative correlation with both ROS and TBARS and a positive correlation with GSH.

Analyzing D6D, we found that it is detectable only in MCD rats and it is higher compared with their respective control. As demonstrated by Shiri-Sverdlov and successively by Guillén, an association between the expression of SCD-1 (D9-16D and D9-18D) with the degree of steatosis occurs (Guillén et al. 2009) and also a link between SCD1 activity versus inflammation and NASH (Shiri-Sverdlov et al. 2006). Thus, we analysed the activity of D9-16D and D9-18D in MCD and Obese Zucker rats. D9-16D increases in Obese Zucker rats, when compared with MCD and Lean Zucker rats, while, in MCD rats a marked increase in D9-18D occurs as compared with Obese Zucker rats and with their respective controls. We confirm the data of Shiri-Sverdlov and Guillén: in fact the observed high activity of D9-18D is in accord with a more serious steatosis and with the tendency of MCD group to progress from NAFLD to NASH. Therefore, previous studies have shown that SCD1 enzyme plays a key role in lipid partitioning in the liver (Miyazaki et al. 2000), so their inhibition reduces hepatic steatosis and inflammation (Kurikawa et al. 2013). Then, we correlated also D9-16D and D9-18D with oxidative stress. D9-16D negatively correlated with oxidative stress, in particular with TBARS and ROS, but positively associated with GSH. On the contrary, an opposite trend was detected for D9-18D. D9-18D is positively correlated with TBARS and

ROS, and negatively correlated with GSH. Thus, D9-16D and D9-18D seem to be involved in opposite effects in the experimental rat models considered in this study.

In 2014, Stanković and colleagues have demonstrated that DHA has antioxidant and anti-inflammatory effects in MCD group (Stanković et al. 2014). In addition, DHA have been shown to generate a group of lipid mediators called resolvins (E- and D-series) and protectins with potent anti-inflammatory and inflammation resolution properties (Ariel & Serhan 2007).

We support these results, since we found a significant negative correlation between DHA versus TBARS and a key factor in the development of NAFLD, TNF-α (Braunersreuther et al. 2012). On the other hand, in MCD rats that are characterized by a considerable oxidative stress, lower levels of DHA occur. This is another evidence of the antioxidant role of DHA.

5.4 Identification of innovative molecular target: mGluR5

The liver steatosis is characterized by cell dysfunction, lipotoxicity and apoptosis, as well as pathophysiological changes and increased susceptibility to injury (Wang et al. 2006). Animal models are used to investigate the mechanisms involved in both hepatic steatosis and its progression to chronic liver disease. In addition to animal models, *in vitro* hepatic cellular models have demonstrated to be very useful to investigate the pathophysiologic mechanisms and new molecular targets. The metabotropic glutamate receptors are among the most studied pharmacological targets. In fact, Bradbury and colleagues, in their animal model of steatosis, demonstrated that at the brain level, mGluR5 might be involved in modulating appetite and energy homeostasis. In our study, we identified mGluR5 on a human hepatocyte-derived cell line, HepG2, treated with fatty acids. We used a mixture of Palmitic Acid (PA, C16:0), a saturated FA, and Oleic Acid (OA, C18:1), a monounsaturated FA, as recognized model of *in vitro* steatosis.

Once the correct mix oleate:palmitate concentration was determined, HepG2 cells were subjected to various concentration of negative allosteric modulator of metabotropic glutamate receptor 5: MPEP 0.3, 3, 30 µM, and the orthosteric agonist, DHPG 100 µM. The analysis of lipid content shows, for the first time, that the mGluR5 in HepG2 cells has a role in lipid intracellular accumulation. In particular, the mGluR5 activation by DHPG increases cell lipid uptake; on the contrary, the blockade by MPEP administration reduces DHPG-induced cell FA accumulation.

Furthermore, FFAs appear to be important mediators of lipotoxicity, since they are potential cellular toxins and lead to lipid accumulation (Malhi et al. 2006). Several reports show that, individually, palmitic acid and oleic acid have a distinct intrinsic toxic potential (Malhi et al. 2006). Ricchi and colleagues demonstrated that OA is more steatogenic but less damaging than PA in hepatocyte cell cultures and it seems that oleic acid inhibits the oxidative stress caused by PA overloading, contributing to hepatocellular survival (Ricchi et al. 2009). Our results confirm these data: in fact, we did not observe any differences neither in ROS formation nor in cellular viability, treating HepG2 cells with fatty acids and MPEP.

Finally, as demonstrated by Gomez-Lechòn and collaborators, oleic acid is able to reduce caspase-3 activation (Gómez-Lechón et al. 2007). So, we speculated that the receptor blockade might have a further protective effect from the apoptotic process. Therefore, we examined the caspase-3 expression, key protein that regulates both the intrinsic and extrinsic apoptosis pathways. Our data support the hypothesis that an increase of caspase-3 occurred in cell treated with FAs. In addition we found that the blockade of mGluR5 with MPEP 0.3 µM reduces the caspase-3 protein expression and therefore protects against apoptosis. However, further data need to be collected to demonstrate this hypothesis.

Conclusions

Non-Alcoholic Fatty Liver Disease (NAFLD) is a widespread pathological condition, which is characterized by relevant intracellular lipid accumulation in the hepatic parenchyma. About 10% of patients suffering from NAFLD progresses to Non-Alcoholic Steatohepatitis (NASH), a more serious pathological condition, less common than simple steatosis, that is a risk factor for the development of cirrhosis and development of Hepatocellular Carcinoma (HCC) which occurs, according to recent studies, in 4-27% of cases (Starley et al. 2010). It is currently the leading cause of liver disease in North America, and it is predicted to become the leading cause of liver transplant by 2030 (Doycheva et al. 2017). This could lead to a further aggravation of the existing shortage of donor organs so that the demand will be greater than the availability. Nowadays, in fact, in order to compensate for the growing number of patients on waiting lists for liver transplantation, many transplantation centers are forced to adopt extended criteria for graft selection, moving the limit of acceptance for marginal livers, such as the steatotic livers.

In the first part of this project our purposes were to investigate the mechanisms that occur in fatty livers during the transplantation and analyze the role of free fatty acid composition following static or dynamic preservation in two models of NAFLD. We confirm that the lipidomic liver profiling is a sensitive indicator of graft function and that the quality of lipid constituents might influence hepatic injury during preservation by cold storage more than the quantity of lipid content. According to this hypothesis, the reduced CS injury observed in our MCD rat livers, may be justified by the higher PUFAs levels that makes them less susceptible to cold preservation, possibly reducing the deleterious tendency to lipid crystallization.

To understand the NAFLD pathogenesis, we focused our attention on the changes in fatty acid desaturases, enzymes that insert a stereospecific double bond between specific carbons, D5D, D6D, D9-16D and D9-18D, and their relationship with oxidative stress in two rat models of NAFLD. First, we demonstrated that the NAFLD is characterized by high levels of oxidative stress and it plays a key role in the pathogenesis of the NAFLD. In 2008, Martinelli and colleagues showed that the activation of fatty acid desaturation leads to pro-inflammatory condition (Martinelli et al. 2008). In this study we support these data, in fact, we found a correlation between desaturases activity and oxidative stress.

Another important factor involved in the development of NAFLD is the concentration of DHA, a polyunsaturated fatty acid. In 2014, Stanković and colleagues have demonstrated that DHA had antioxidant and anti-inflammatory effects in MCD group (Stanković et al. 2014). We support these results, since we found a significant negative correlation between DHA

versus oxidative stress, and also a correlation between oxidative stress versus TNF-α. Although there is still much to clarify regard the pathophysiology of NAFLD, these results reinforce and extend findings on the identification of potential therapeutic targets able to counteract this common disorder.

Nonetheless, specific pharmacological target and treatment have not been found yet, leaving important medical needs still to be met.

Among the novel targets, the metabotropic glutamate receptor is very studied. In fact, Bradbury and colleagues, in their animal model of steatosis, demonstrated that at brain, mGluR5 might be involved in modulating appetite and energy homeostasis. In our study, we investigated the role of mGluR5 modulation and we found that its activation by DHPG increases cell lipid uptake; on the contrary, the blockade by MPEP administration reduces DHPG-induced cell FA accumulation. Furthermore, mGluR5 seems to be involved also in the apoptotic process, in fact, we demonstrated that the blockade of mGluR5 with MPEP reduces the caspase-3 protein expression. These data allow us to hypothesize that the blockade of mGluR5 could be involved in the control of oxidation or uptake of fatty acids and protection against apoptosis.

Our results support the idea that the advancement in the understanding of the pathophysiology of liver steatosis might lead to the development of novel therapeutic alternatives, whose the mGluR5 negative allosteric modulators represent a feasible and truthful example.

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