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# Non-Alcoholic Fatty Liver Disease and Alcoholic Liver Disease: two sides of the same coin

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## List of abbreviations

**AH**: Alcoholic Hepatitis. **ADH**: Alcohol Dehydrogenase. **ALD**: Alcoholic Liver Disease. **ALDH**: Aldehyde Dehydrogenase. **ASH**: Alcoholic Steatohepatitis. **ATP**: Adenosine Triphosphate. **BMI**: Body Mass Index. **BHT**: Butylated Hydroxytoluene. **CB1/2** Cannabinoid Receptors. **CLP**: Cecal Ligation and Puncture. **CTRL**: chow diet-fed mice or untreated cells. **DAMPs**: Damage-Associated Molecular Patterns. **DIO:** Diet-Induced Obesity. **DTNB:** 5, 5'-Dithiobis-2-Nitrobenzoic acid. **EtOH**: ethanol. **FFA**: Free Fatty Acids. **GSH**: Glutathione. **HFHCD:** High Fat High Carbohydrate Diet. **HNE**: Hydroxynonenal. **IR**: Insulin Resistance. **IRS**: Insulin Resistance Substrate. **LDL**: Low-Density Lipoproteins. **LPS**: Lipopolysaccharide. **LT**: Liver Transplant. **MDA**: Malondialdehyde. **MEOS**: Microsomal Ethanol Oxidation System. **MIF**: Macrophage Migration Inhibitory Factor. **NAFLD**: Non-Alcoholic Fatty Liver Disease. **NASH**: Non-Alcoholic Steatohepatitis. **OCT**: Optimal Cutting Temperature. **O/N**: Overnight. **PAMPs**: Pathogen-Associated Molecular Patterns. **PMA**: Phorbol 12-Myristate 13-Acetate. PUFA: Polyunsaturated Fatty Acids. **RT**: Room Temperature. **RA**: Rheumatoid Arthritis. **ROS**: Reactive Oxygen Species. **SLE**: Systemic Lupus Erythematosus. **T2DM**: Type2 Diabetes Mellitus. **TBS**: Tris-Buffered Saline. **TG**: Triglycerides. **TGF-β1**: Tumor Growth Factor. **TLR**: Toll-Like Receptors. **TUNEL**: Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling. **VLDL**: Very Low-Density Lipoproteins. **WHO**: World Health Organization. **WT**: Wild Type.

**Abstract**

*Background and aims*: The booming prevalence of obesity and diabetes in young age and the increased consumption of alcohol in adolescents always more frequently lead to the development of Non-Alcoholic Fatty Liver Disease (NAFLD) and Alcoholic Liver Disease (ALD). The lack of studies in both fields triggered us in the investigation of these liver damages, separately discussed in task 1 and 2 in the present work. Task 1 is focused on the development and characterization of an *in vivo* model of pediatric NAFLD that, in a second phase, has been used as a platform for the study of the therapeutic properties of Silymarin added to high-fat high carbohydrate diet (HFHCD). The task 2 investigated the source of MIF, a cytokine involved in the pathogenesis of ALD.

*Materials and Methods:* NAFLD *in vivo* study: For the characterization of the model, C57BL/6 male and female mice were exposed to HFHCD or chow diet (CTRL diet) for 16 weeks. The following parameters were screened every 4 weeks: body weight, glycemia, insulinemia, triglycerides (TG), total cholesterol, HDL-C, ALT, AST, liver histology. The biomolecular analysis was performed in order to analyze the pathways involved. Once assessed the pathophysiology of NAFLD, C57BL/6 male and female mice immediately after weaning were fed with HFHCD and CTRL diet for 8 weeks. Then, HFHCD group was divided in 3 subgroups consisting in: 1) HFHCD; 2) HFHCD + Silymarin (33mg/animal/day); 3) HFHCD switched to CTRL diet (lifestyle change). The trial went on for further 12 weeks. Animals exposed only to HFHCD or CTRL diet for the entire trial (20 weeks) were used as controls. Treatment effects were assessed following the same parameters of phase I.
ALD *in vitro* study: MIF expression was measured in HuH7 (hepatocytes) and differentiated THP-1 (macrophages) cells in response to 50mM ethanol. The ethanol-induced liver injury was assessed in C57BL/6 (WT) and *Mif-/-* bone marrow chimeras. MIF was measured in peripheral and suprahepatic serum, as well as visualized by immunohistochemistry in liver biopsies, from patients with alcoholic hepatitis (AH).

*Results*: NAFLD *in vivo* study: Soon after the first week, HFHCD induced a significant body weight gain in both genders. Males, after 4weeks, presented epididymal fat-pads hyperplasia and after week 12, a significant hepatomegaly, with alteration of glycemia, insulinemia, lipid profile, and ALT. Interestingly, comparable body/blood changes were observed in females but at week 16. Liver histology showed in both genders a mixed macro-microvesicular steatosis from week 8 with an increased DGAT2 gene expression. Inflammatory foci were observed only in males, confirmed

also by an increase in MCP-1 and TNF-α mRNA. Conversely, females had no signs of inflammation but rather presented enhanced lipid peroxidation (MDA) and a reduced GSH/GSSG ratio, signs of oxidative stress. Interestingly, from week 8 both genders developed progressive fibrosis (confirmed by increased Col1A1 and αSMA mRNA, and enhanced collagen deposition - Sirius Red staining). HFHCD + Silymarin decreased liver and visceral fat weight and improved ALT and lipid profile. HFHCD switched to CTRL diet reverted all altered parameters under study. Histologically, Silymarin slightly reduced inflammatory foci and fibrosis, a trend confirmed by a decreased α-SMA and Col1A1 mRNA. ALD *in vitro* study: HuH7, but not THP-1 macrophages, released MIF in response to ethanol challenge in cell culture. In chimeric mice expressing MIF in non-myeloid cells (*Mif-/-* →WT), chronic ethanol feeding increased ALT/AST, hepatic steatosis, and cytokine/chemokine mRNA expression. In contrast, chimeric mice not expressing MIF in non-myeloid cells (WT→ *Mif-/-*) were protected from ethanol-induced liver injury. Immunohistochemical staining of liver biopsies from patients with AH revealed a predominant localization of MIF to hepatocytes. Interestingly, the concentration of MIF in suprahepatic serum, but not peripheral serum, was positively correlated with clinical indicators of disease severity and with an increased risk of mortality in patients with AH.

*Conclusions*: Data collected suggested that our juvenile NAFLD model had a faster and more aggressive liver injury progression compared with published adult models, with different molecular mechanisms between males and females. Even if changes of diet habits remain the best choice, Silymarin exerted some beneficial effects without requiring lifestyle improvements, relevant aspect considering the general low compliance of obese subjects in modifying their nutritional behavior. ALD project provided evidence that hepatocyte-derived MIF was critical to the pathogenesis of ALD in mice and likely contributes to liver injury in patients with AH. Altogether these new findings can lead to new therapeutic perspective.

**Riassunto**

*Introduzione e obiettivi*: La rapida diffusione dell'obesità e del diabete nelle fasce giovanili della popolazione e l'aumentato consumo di alcool tra gli adolescenti, contribuiscono ad aumentare lo sviluppo di NAFLD (Non-Alcoholic Fatty Liver Disease) e ALD (Alcoholic Liver Dsease) tra i più giovani. La mancanza di studi specifici in entrambi i settori è stato il fattore che ci ha indotti a investigare queste due forme di malattia epatica, qui discusse separatamente nella Sezione 1 e 2. La task 1 si focalizza nella caratterizzazione di un modello giovanile di NAFLD, utilizzato in una seconda fase del progetto come piattaforma per l'analisi delle potenzialità terapeutiche della Silimarina, aggiunta direttamente nella composizione della dieta ipercalorica (HFHCD). La task 2 invece si basa sulla ricerca dell'origine di MIF, citochina che ha dimostrato avere un ruolo nell'eziologia di ALD.

*Materiali e metodi*: Studio di NAFLD *in vivo*: Per caratterizzare il modello, topi C57BL/6, maschi e femmine, sono stati nutriti per 16 settimane con una dieta ad alto contenuto lipidico e zuccherino (HFHCD), iniziando il trattamento al termine dello svezzamento. Un gruppo di topi sottoposti a dieta bilanciata sono stati usati come controlli sani. Ogni 4 settimane sono stati valutati questi parametri: peso corporeo, glicemia, insulinemia, trigliceridi (TG), colesterolo totale, HDL-C, LDL-D, transaminasi e istologia epatica. Analisi biomolecolari sono state eseguite sui campioni raccolti. Determinata la fisiopatologia della sindrome metabolica in questo modello, nuovi topi appena svezzati sono stati alimentati con HFHCD o dieta controllo per 8 settimane. Dopo questi due mesi il gruppo appartenente alla dieta ipercalorica è stato suddiviso in tre sottogruppi: 1)HFHCD 2)HFHC+Silimarina 3)Sostituzione dieta ipercalorica con dieta bilanciata, continuando il nuovo regime alimentare per ulteriori 12 settimane. Topi che per tutta la sperimentazione (20 settimane) hanno mantenuto dieta ipercalorica e bilanciata sono stati usati come controlli.

*Studio di ALD*: L'espressione proteica di MIF è stata quantificata in un modello *in vitro* di co-cultura, costituito da epatociti (HuH7) e macrofagi (THP1 differenziate), dopo trattamento con 50mM di etanolo. Il danno epatico indotto da alcool è stato valutato anche in un modello *in vivo* di trapianto di midollo osseo (topi C57BL/6 WT e *Mif-/-*). Infine l'espressione di MIF è stata quantificata in campioni di siero periferico e sopraepatico raccolto da pazienti arruolati affetti da epatite alcolica, così come la sua espressione genetica e proteica è stata misurata in biopsie di fegato raccolte dagli stessi soggetti.

*Risultati*: Studio di NAFLD *in vivo*: Già a partire dalla prima settimana, HFHCD ha determinato un aumento significativo del peso corporeo sia nei maschi che nelle femmine. Nei maschi, dopo 4 settimane abbiamo osservato un aumento dell'iperplasia del grasso epididimale, epatomegalia, iperglicemia, insulinemia, iperlipemia e aumentata ALT. Lo stesso profilo patologico è stato rilevato nelle femmine solo dopo 16 settimane. A dispetto delle differenze sieriche, sia i maschi che le femmine hanno riportato a livello istologico una progressiva steatosi epatica a partire dall'ottava settimana, confermata anche dall'aumento genetico dell'espressione di DGAT2. Foci infiammatori sono stati rilevati solo nei maschi, che hanno inoltre riportato un aumento dell'mRNA di MCP-1 e TNF-α. Al contrario, le femmine hanno dimostrato evidenti segni di stress ossidativo, con un incremento dei markers di lipoperossidazione (MDA) e una diminuzione dell'effetto protettivo esplicato dal glutatione (GSH/GSSG). Nonostante queste differenze riscontrate in entrambi i sessi è stata osservata una progressiva fibrosi, associata a un parallelo aumento di Col1A1, αSMA e della deposizione di fibre di collagene nel fegato. L'aggiunta di Silimarina ha dimostrato principalmente un'azione antilipemica, riducendo epatomegalia e adiposità viscerale, con un miglioramento complessivo del profilo lipemico sierico e dei livelli di ALT. Il cambiamento dello stile di vita ha comunque apportato i migliori effetti, normalizzando tutti i parametri alterati analizzati. Istologicamente, la Silimarina ha dimostrato un'azione antifibrotica, confermata del calo dell'espressione genica di α-SMA e Col-1A1. Studio di ALD *in vitro:* Il trattamento con etanolo ha comportato nel modello *in vitro* un aumentato rilascio di MIF solo da parte degli epatociti (HuH7). In linea con questo, in topi che esprimono MIF nelle cellule non-mieloidi (*Mif*-/-→WT) l'esposizione cronica all'etanolo ha comportato un notevole danno epatico, con steatosi e infiammazione. Al contrario, le chimere che non esprimevano MIF nelle cellule non mieloidi (WT→ *Mif*-/-), hanno dimostrato un profilo protettivo non sviluppando danno epatico a seguito del trattamento con etanolo. In linea, l'analisi di biopsie epatiche di pazienti con epatite alcolica ha evidenziato una predominanza di MIF a livello degli epatociti. Infine la quantità di MIF misurata nel siero sopraepatico, ma non in quello periferico, ha correlato positivamente con alcuni parametri di danno severo e con un più alto rischio di mortalità.

*Conclusioni*: I dati raccolti dimostrano che la NAFLD giovanile sembra avere una progressione più rapida rispetto ai modelli adulti pubblicati, con differenti meccanismi molecolari tra maschi e femmine. Sebbene il cambiamento dello stile alimentare rimanga la scelta migliore per la reversione della malattia, l'aggiunta della Silimarina alla dieta ipercalorica ha riportato

interessanti effetti, soprattutto se si tiene in considerazione che nessun miglioramento alimentare è stato imposto in questi animali. Tale aspetto è particolarmente rilevante, perché sebbene la dieta sana sia la scelta ideale nella gestione della NAFLD, la scarsa volontà dei soggetti obesi nel seguirla, spesso rappresenta l'ostacolo più complicato da superare. Per quanto concerne ALD, i risultati ottenuti sono concordanti nel dimostrare che MIF, coinvolto nella patogenesi di danno epatico da alcool, viene rilasciato dagli epatociti, svolgendo un ruolo chiave nella progressione dell'epatite alcolica. Questi nuovi aspetti scoperti possono aprire in entrambe le malattie a nuove prospettive terapeutiche.

# Chapter 1
# Introduction

# 1. Introduction

## 1.1. Fatty liver

*"…the cause of steatosis, and not the fat accumulation by itself, produces cirrhosis" – Heribert Thaler, 1975*[1]

Fatty liver indicates the deposition of intracellular lipid droplets in the hepatocytes[2]. Steatosis per se is benign and completely reversible by removing the factors that caused it. Obesity and alcohol abuse represent the most common risk factors, determining respectively the genesis of Non-Alcoholic Fatty Liver Disease (NAFLD) and Alcoholic Liver Disease (ALD)[3]. Both diseases share the natural history, progressing from simple steatosis to steatohepatitis, until fibrosis/cirrhosis and liver cancer[4].

The term NAFLD was used by Ludwig et al. in 1980[5] to describe a panel of liver injuries similar to alcoholic hepatitis that occurred in absence of relevant alcohol consumption[6] or other known causes of chronic liver disease (HCV, HBV, drugs etc.). It is defined as the hepatic manifestation of metabolic syndrome and it is usually associated with the development of diabetes, insulin resistance, abdominal obesity, and hypertension. The increasing incidence of NAFLD worldwide is in parallel with the booming of obesity and it is expected to become the most frequent indication for liver transplantation by 2030, with high risk for disease recurrence after transplantation[7,8]. Over the last decade, it has been shown that the clinical burden of NAFLD is not only referred to adults, but there is now growing evidence indicating that NAFLD is the most common cause of liver disease also in pediatric population[6]. This worrisome trend is a mirror of the spread of hypercaloric diet and sedentary life habits between young and adolescences. Overweight and obesity are escalating global epidemics in both developed and developing countries. The problem is so diffused that the World Health Organization (WHO) has introduced the term Globesity to define the phenomenon[9].

Another relevant issue that is threatening the modern society is the alcohol consumption and, as obesity, this condition starts to occur in young age groups. Recently, a new drinking behavior has been rapidly spread, the so-called Binge drinking. This definition describes an excessive alcohol intake primarily performed during the weekend by teenagers[10]. It is considered a relevant public health issue, associated with dramatic consequences in terms of violence and traffic accidents. Beyond these social impacts, the generalized binge drinking is also associated with

alcohol dependence, stroke, hepatic disease, sexually transmitted disease[10] due to risky sexual behaviors[11].

Obesity and alcohol are considered two of the most worrisome threats to the public health, with an increasing trend. Many factors determine a strong correlation between these two issues. Alcohol consumption can lead to an excess of calories intake[12], due to the high amount of sugars present in the alcoholic drinks. Furthermore regular alcohol consumers show a higher blood pressure, and excessive alcohol intake has been identified as one of the major causes of hypertension[13]. Considering the worldwide incidence, even more frequent is the coexistence of obesity and alcohol consumption in youth, increasing exponentially the incidence of chronic liver disease.

## 1.2. Epidemiology

### NAFLD

The prevalence of NAFLD is tightly associated with obesity, a global problem issue whose incidence has been nearly doubled since 1980. Data obtained from WHO reveals that in 2008, more than 1.4 billion adults were overweight and more than 500 million were obese. At least 2.8 million people each year die as result of being overweight or obese. Once associated with high-income countries, obesity is now also prevalent in low- and middle-income countries: 65% of the world's population live in countries where obesity kills more people than underweight[14]. This trend interests also the younger age groups: in 2013 the number of overweight children under the age of five was estimated to be over 42 million[15].

In parallel with epidemic obesity, NAFLD has also dramatically increased. It is difficult to establish exactly the incidence because large population studies are not possible due to the necessity of a biopsy-proven diagnosis to certificate the liver damage[3]. For this reason, most studies use other types of non-invasive techniques to certificate the injury, as blood testing, ultrasound, and magnetic resonance spectroscopy and also autopsy samples.

Even fewer studies have been conducted in children and therefore the true NAFLD prevalence in pediatric population remains unknown. Data referred to adult cohorts indicate that the estimated worldwide prevalence of NAFLD ranges from 6% to 35% in the general population[16,17]. In populations with pre-existing metabolic conditions, the prevalence of NAFLD increases to 60–95% in obese persons, 28–55% in persons with type 2 diabetes mellitus and 27–

92% in those with dyslipidaemia[17,18]. Regarding pediatric population, Huang et al. [19] reported in 219 schoolchildren (6-12 years old) that NAFLD was present in 25% of overweight and 76% of obese children.

**ALD**

Estimations of ALD prevalence indicate that 80% of heavy drinkers develops steatosis[3]. This first histological manifestation normally appears in people with an excess of alcohol consumption of 80g/day. The fatty liver is a reversible stage that can be resolved with 2-4 weeks of abstinence. Many studies advice about the alarming increase of alcohol abuse among young. Data referred to 2006[10] indicates that in Europe about 80 million people (15 years and older) reported binge drinking at least once *per* week. Eastern European countries have the highest pattern of this phenomenon, consuming frequently a large amount of alcohol to intoxication[20]. Beer is the most consumed beverage[10]. Bartoli et al.[21] analyzed the prevalence and the correlation of binge drinking among young adults in Italy in 2013 enrolling 654 subjects (mean age 20.6 years). The results reported that more than a third of young adults were binge drinkers. Considering the worldwide fashion for this kind of habit, there is the need of effective and rapid programs to reduce levels of risky drinking in order to reduce the alcohol consumption and consequently to determine a decrease in the attributable burden of disease.

### 1.3. Pathogenesis

**NAFLD**

Non-alcoholic fatty liver disease is the first stage of a wide spectrum of liver disorders. The persistence of the damage determines the progression of the simple steatosis, with the appearance of signs of inflammation that characterizes the presence of Non-alcoholic steatohepatitis (NASH). The development of this condition increases the probability to reach the most advanced stages of the damage such as fibrosis, cirrhosis finally leading to liver cancer. Regarding the causes that can determine the evolution of the disorder some theories have been published in the recent years. The most cited hypothesis was reported by Day and colleagues[22] and is the so-called *Tale of two hits*. In the first hit, a continuous and large nutrient intake can exceed the ability of the liver to metabolize the fats, determining a positive energy balance that triggers the progressive intracellular accumulation of lipid droplets in the hepatocytes, leading to steatosis. The development of fatty liver is strictly associated with obesity and insulin resistance that predispose the liver to the

progression to the second hit, which is characterized by necrosis, inflammation, and fibrosis. One of the mediators that determines the passage from the first to the second hit could be the oxidative stress. Some years later Tilg and Moschen[23] expanded the concept, formulating the *Multiparallel hits hypothesis*. They supported the idea that NASH might be the result of many hits that act in parallel, finally resulting in liver inflammation. According to this model, steatohepatitis is the product of a complex interplay of several factors especially derived from adipose tissue and gut that would have an important role in the evolution of liver inflammation. The most accurate diagnosis of NAFLD relies on the histological analysis of a liver biopsy (considered the gold standard). However, the risk associated with this procedure limits its use. For this reason, the prevalence of NASH is difficult to determine, as large population-based studies are not possible. Epidemiological analysis, based on other non-invasive techniques, indicate that about 30% of patients with NAFLD will progress to NASH. Among subjects with NASH approximately 20% will develop cirrhosis (normally patients with signs of ballooning degeneration and fibrosis) and of those with cirrhosis 30-40% will decompensate and die to liver-related complications over a 10-years period. A lower percentage (0-10%) progresses to liver cancer[3,24] (Fig. 1).

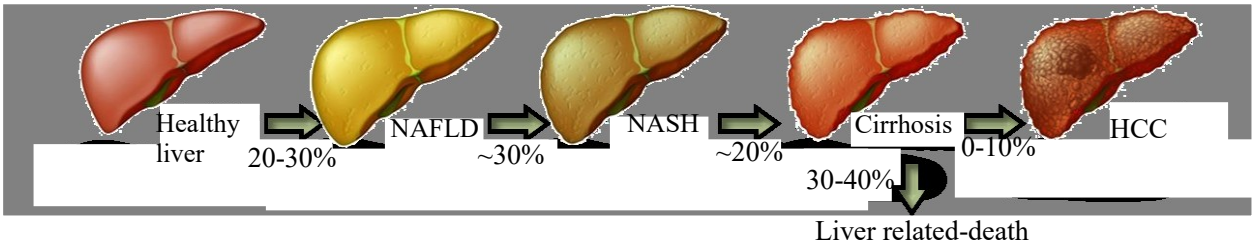


**Fig. 1.1**. The natural history of NAFLD. Percentages from Mc Cullough[24]

Regarding children, the natural history of pediatric NAFLD is not well defined due to the scarce availability of histological information. Despite many case reports describe adult NASH, only a few studies refer to children. Although much remains to be investigated about pediatric NAFLD, it is already evident that children with NASH have a higher risk for cirrhosis[25]. One of the largest pediatric clinical series comprised 36 patients (age 4-16 years old)[26]; liver biopsy was performed in 24 patients and 70% of them had fibrosis. One of them (9 years old) was already cirrhotic. Manton et al.[27] reported a study that enrolled 17 patients (9-15 years old) in which nine

of them had fibrosis and one 11-year-old boy had "evolving cirrhosis". Similar childhood NAFLD has been reported by Roberts in his review[28] (he described a personal communication of Squires RH Jr and Lopez MJ). This communication referred about 39 children, with a histological medical report for 31 of them. Liver biopsy revealed steatohepatitis in all 31, fibrosis in 21 and cirrhosis in 2 children. These findings suggest that pediatric NAFLD/NASH could have a different, and more aggressive, progression than in adults.

Liver biopsy is still necessary to certify the stage of the damage. Histological findings reveal differences between adult and pediatric NASH. For adults, histological features resemble alcoholic hepatitis pattern. It is defined NASH type 1 and is characterized by macrovesicular steatosis, Mallory bodies, and ballooning degeneration and/or perisinusoidal fibrosis in absence of portal features[29]. Inflammatory infiltrate is constituted mainly by polymorphonuclear leukocytes and is usually more severe in the perivenular zone[30,31]. In childhood the histological pattern of NASH defined type 2, is characterized by the presence of steatosis associated with periportal fibrosis and/or inflammation with mononuclear inflammatory infiltrate. Ballooning degeneration is almost absence as well as Mallory bodies[30,31] (Table 1). Some adolescents display an overlapped pattern of lesions, with intermediated features of type 1 and type 2 NASH[30,32].

| | Type 1 | | | Type 2 | | |
|---|---|---|---|---|---|---|
| **Ballooning degeneration** | + | + | - | | - | |
| **Perisinusoidal fibrosis** | - | + | + | | - | |
| **Mallory bodies** | | + | | | - | |
| **Steatosis** | | + | | | + | |
| **Portal inflammation** | | - | | + | + | - |
| **Portal fibrosis** | | - | | - | + | + |

**Table 1.1.** Summary of type 1/type 2 histological pictures. Adapted table from Schwimmer et al.[29]

## ALD

Alcoholic liver disease has the same spectrum of liver disorders observed in NAFLD. ALD ranges from simple steatosis, alcoholic hepatitis (ASH), progressive fibrosis leading to cirrhosis and hepatocellular carcinoma. Approximately 20-40% of steatotic patients with continued alcohol

consumption develop ASH and of those with ASH, 40% progresses to cirrhosis. Usually, severe alcoholic hepatitis is associated with a high mortality rate, instead of mild to moderate ASH can improve with abstinence.

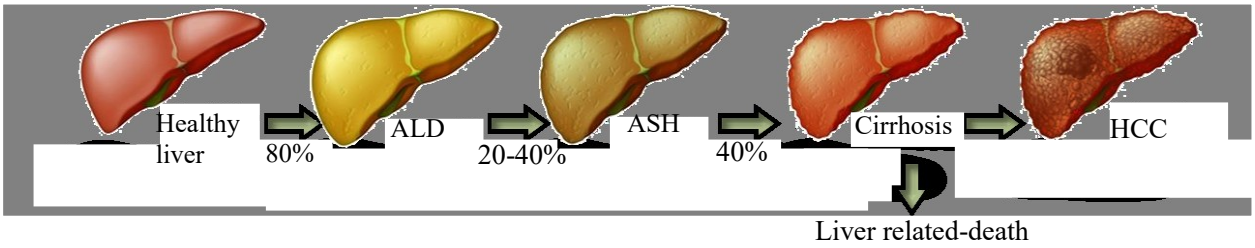


**Fig. 1.2**. The natural history of ALD. Percentages by Grant et al.[33]

The typical histological picture of ASH is characterized by macro and microvesicular steatosis, inflammation, and necrosis. This kind of features is most predominant in the centrilobular region of the hepatic acinus[34]. Ballooning degeneration is usually present. Inflammatory infiltrate normally consists of polymorphonuclear cells and to latter extent mononuclear cells. Mallory bodies can be observed. Fibrosis has not a homogeneous localization, can be pericellular and perisinusoidal. The development of bridging fibrosis is usually the leading event for the subsequent formation of nodules that define the cirrhotic damage.

### 1.4. Risk factors

#### NAFLD

*Age*: In adults, the incidence of NAFLD and liver disorders, such as fibrosis[35], increases with the age. The correlation seems to depend on to the higher prevalence of other risk factors[16], such as hypertension, obesity, and diabetes mellitus that mediate the metabolic syndrome set-up, associated with more severe biochemical, hematological and histological changes[35]. Although the relationship between the elderly and the development of NAFLD is still under analysis, it seems that it could depend on the duration of the disease and the presence of other co-morbidities rather than to age by itself. Regarding the pediatric population, studies have reported that NAFLD can occur in very young children but is more frequent in adolescents. The higher rate in this group of age is correlated to the hormonal changes that happen during puberty. Furthermore, during this period there is an increased control over unhealthy food choices and a trend of a sedentary lifestyle.

It has been reported that puberty is associated with an increased accumulation of lipids in the liver and a reduction in high density lipoprotein production. In addition, adiponectin levels and insulin sensitivity decrease. All these factors may predispose to the genesis of hepatic steatosis.

*Gender*: Population-based studies indicate that NAFLD is more common in men and women in postmenopausal. The same pattern has been identified in the pediatric population in which the higher incidence of NAFLD occurs in boys than in girls. It seems that sex hormones are potentially responsible, mediating protective activity or promoting the pathogenesis of the disorder. Serum level of estrogens can be responsible for the defensive effect mediated in young girls and in premenopausal women, instead, the reduction of the circulating amount of female hormones could explain the burden of NAFLD in postmenopausal. In elder women, several physiological changes occur as result of estrogen reduction. The main alteration is the redistribution of fat in the body that leads to metabolic consequences such as dyslipidemia and glucose intolerance. This condition could be an important factor promoting the pathogenesis of NAFLD. Some animal studies investigated which could be the mechanism for the potential estrogen protective role. Nemoto et al.[36] observed that hepatic steatosis appeared spontaneously in aromatase knockout mice. The deficiency in estrogen production determined the impairment in hepatic fatty acid β-oxidation. The damage was reduced with the replacement of estrogens, leading to the reversion of the steatosis. Furthermore, during steatosis, there is an increase in lipoperoxidation, which can promote the activation of hepatic stellate cells inducing profibrogenic processes. Estradiol exerts an antioxidant activity, contributing to the supposed protective effects attributed to estrogens[37].

*Ethnicity*: The race has been identified as a factor that could influence the development of NAFLD both in adult and pediatric population. The highest incidence was reported in Hispanics, who have the major prevalence of hepatic steatosis and elevated aminotransferases. The lowest occurrence was recognized in African Americans[16]. Ethnic differences seem to be caused by the higher insulin resistance incidence and visceral adiposity at equivalent body mass index (BMI)[38]. It also could be the result of socio-economic factors, linked to the type of diet, exercise attitude, and living location[38].

*Genetic*: An allele in PNPLA3 (rs738409[G], encoding I148M) is the genetic mutation that is recognized to be strongly associated with increased hepatic fat levels and with hepatic inflammation[39]. The physiological function of this gene is unknown. The combination of polymorphism in profibrotic genes such as angiotensinogen and TGF-β1 are correlated with

advanced fibrosis as well as polymorphisms at TNF-α, manganese superoxide dismutase, adrenergic receptors, and interleukin 1β[34].

**ALD**

*Gender*: Women are more vulnerable to alcohol exposure, although ALD is more frequent in men. This condition has been deeply investigated and some hypothesis try to explain the possible reasons. Females have a lower volume distribution[40] and this could explain the major blood ethanol level observed in women than in men, after equal alcohol intake. Furthermore in females has been noted a lower gastric activity of ADH, responsible for the first-step alcohol metabolism and useful to decrease the total ethanol availability and toxicity[41]. Finally seems that estrogens could have a role[42]. These hormones might contribute to increase the gut permeability and consequently the portal endotoxin level, enhancing Kupffer cells activation and liver injury.

*Ethnicity*: Studies regarding the correlation between the ethnicity and the expression of ALD showed that Hispanic patients presented ALD at a significantly earlier age than Caucasians and African Americans, indicating that the race could be a predictor factor[43]. One reason could be that obesity and diabetes are co-morbidities more frequent in Hispanics compared to other ethnic groups. Also in absence of metabolic syndrome, Hispanic patients were more predispose than White/Caucasian groups to develop alcoholic steatosis and cirrhosis[43]. Genetic modification could be another explanation for the higher incidence of ALD in this race. PNPLA3, for example, is strongly associated with alcohol-related diseases and this alteration is more common in Hispanics. Several areas of research are active in the discovery of other potential genetic differences that could be on the base of these race differences.

*Genetic*: The main enzymes implicated in ethanol metabolism are alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH) and CYP2E1. Variants on ADH promote liver damage due to an ethanol accumulation (for the reduction in acetaldehyde formation) or to the activation of other metabolizing pathways, more toxic to the liver[44]. Mutations on ALDH gene can be the cause of individual sensibility to ethanol in women and oriental populations. Studies conducted in Asian cohorts confirm the high rate of this alteration and consequently a major susceptibility to alcohol, even if consumed in low amounts. Polymorphisms in genes encoding inflammatory mediators have been also investigated as possible characters in ALD pathogenesis. Genetic variations located in the promoter region of TNF-α gene determines an increase in cytokine generation, leading to alcoholic hepatitis, as well as polymorphisms in NF-κB, IL-1β receptor

antagonist, IL-2, IL-6, and IL-10. Mutations on important antioxidants, as superoxide dismutase and glutathione-S-transferase, are considered risk factors strongly associated with ALD genesis. As for NAFLD, the PNPLA3 alteration is also in this case associated with higher rate of fibrosis development[34].

*Environment*: Other factors could have a significant correlation with the risk of ALD, linked to the living location and the environment. In the study cited before, Bartoli et al.[21] recognized the presence of binge drinking in more than a third of young adults enrolled in the analysis (mean age 20.6 years). The results indicated that the presence of this liver damage had a significant correlation with the financial availability, the use of cannabis, the interest for discos and parties, the influence of friends and the electronic cigarette smoking. Living with parents was the only protective factor, confirming other previous studies[44] that highlighted that living outside and far from parental control is associated with higher incidence of binge drinking.

## 1.5. Molecular mechanisms in NAFLD

### 1.5.1. Insulin resistance

The occurrence of insulin resistance in NAFLD is a common condition, characterized by a reduction in insulin-suppressing effect on the hepatic glucose production. Many factors can be the cause of IR, including genetic modifications, nutrition, and lifestyle. Normally insulin binds to a tyrosine kinase receptor, inducing the phosphorylation of several substrates including insulin receptor substrates (IRS)-1and IRS-2[46]. The insulin signal propagation finally leads to FFA esterification and triglycerides deposition in adipose tissue, suppressing adipose tissue lipolysis. In insulin resistance (IR), the activation of insulin signaling cascade is impaired and consequently, the insulin-suppressing effect on lipolysis decreases. As result, an increase of FFA efflux from adipose tissue occurs and lipids are accumulated in other tissues. Furthermore, the high level of circulating insulin are associated with an enhanced expression of the SREBP-1c (Sterol Regulatory Element Binding Protein-1c), that is a key transcriptional regulator of the genes involved in the genesis of fats, propagating this fashion[47].

Once obesity leads to liver disease, several mechanisms contribute to the development of NAFLD, but inflammation and oxidative stress have been proposed as the major contributors in the pathogenesis. IR has a strong association with both inflammatory mediators and oxidative markers.

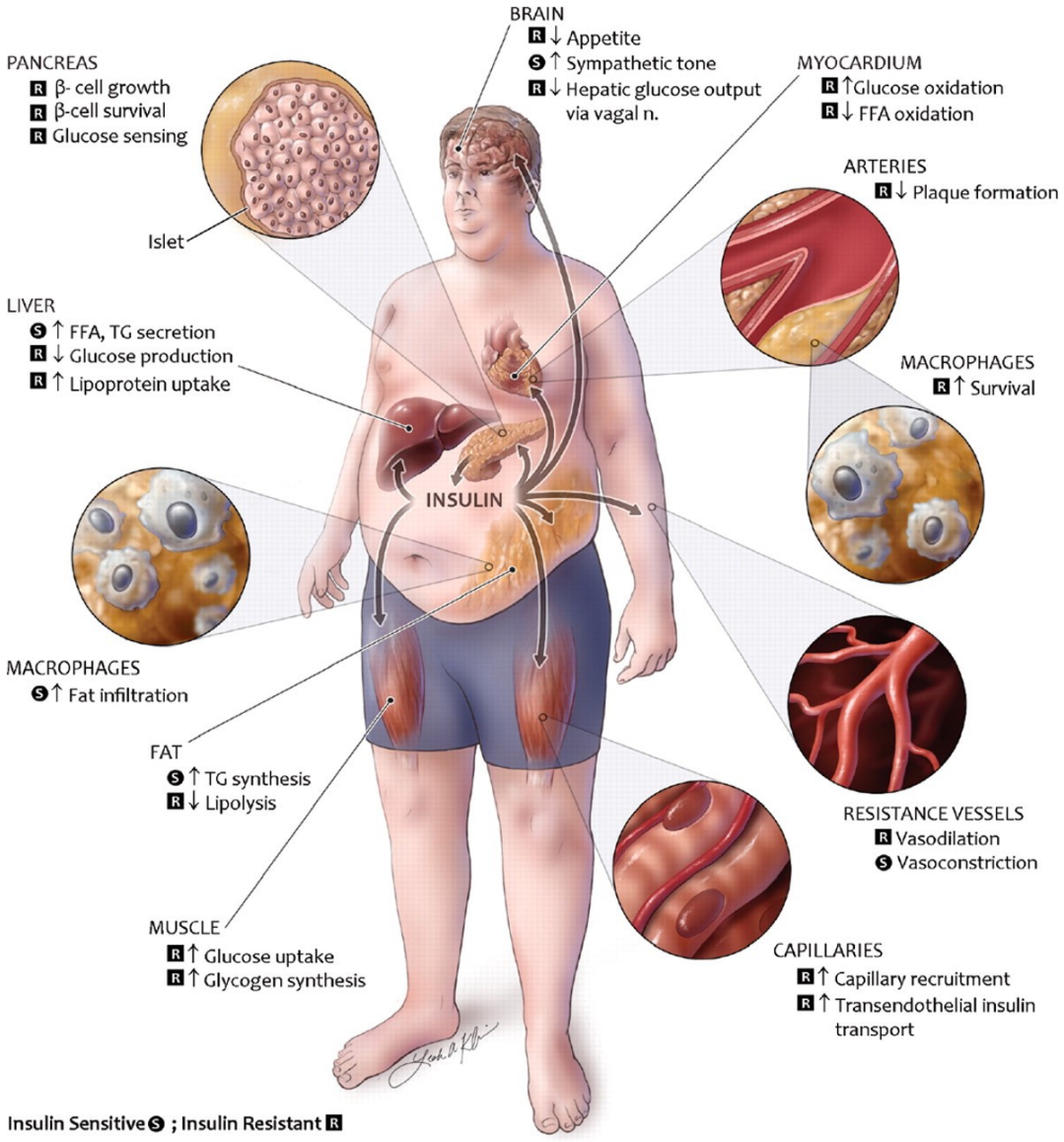


**Fig. 1.3**. **Overview of the physiological insulin signaling and the modifications that happen in metabolic syndrome**. Insulin actions which are insulin resistant and lost during metabolic syndrome are labeled with letter R, whereas insulin actions which remain insulin sensitive in metabolic syndrome and are maintained are labeled with letter S. Image taken from Rask-Madsen et al. review[48]. Original artwork by Leah A. Klein.

### 1.5.2. Inflammation

Nutrient excess and weight gain generate a hypertrophy of adipocytes, increasing the release of FFA into the circulation with a parallel reduction of oxygen delivery to adipose tissue. The association of these factors can be responsible for adipocytes death and for the development of inflammatory response. Macrophages recruitment determines an increase in the release of

cytokines, which induce the activation of inflammatory pathways both in adipose and distal tissues, such as liver and skeletal muscle[47]. The intermuscular fat depots and hepatic lipid droplets generated with obesity and infiltrated with macrophages, contribute to the systemic inflammation, strictly correlated with the development of insulin resistance. In hepatocytes, inflammation holds a key role in the progression of simple steatosis to NASH. The accumulation of lipids as triglycerides may have a protective role in the prevention of hepatic lipotoxicity. This hypothesis is endorsed by animal studies in which the overexpression of DGAT2 (involved in the last step of triglycerides synthesis) determined an increase in steatosis but protected *versus* inflammation and IR[2,49]. On the contrary, the depletion of DGAT2 led only to an improvement of hepatic steatosis, without preventing the lipotoxic injury due to FFA exposure.

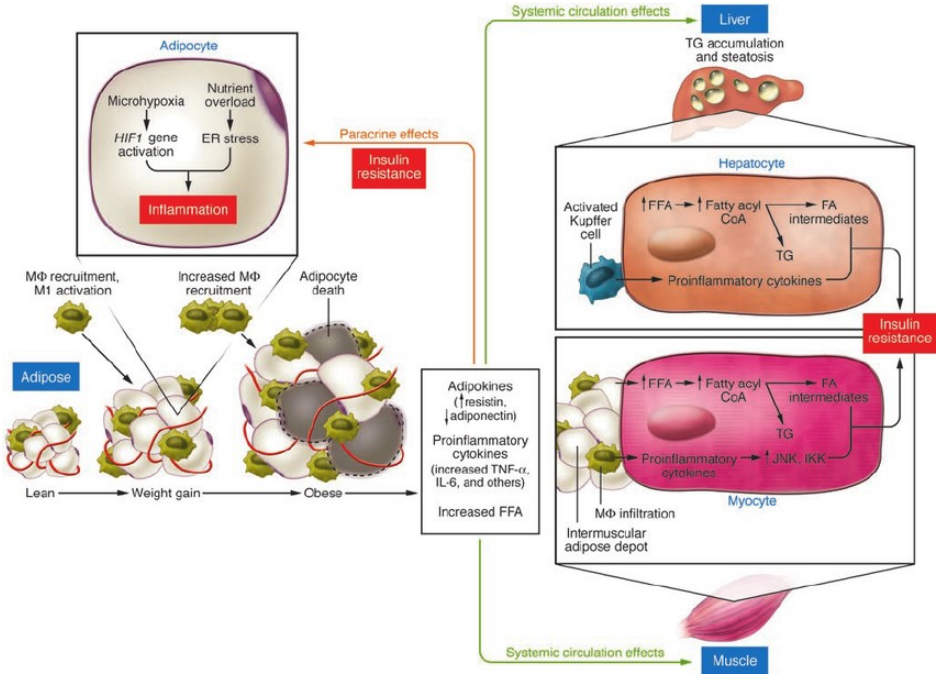


**Fig. 1.4**. Association between obesity, inflammation and insulin resistance in adipose tissue, muscle and liver[47]

### 1.5.3. Oxidative stress

FFA taken by muscles and liver can be β-oxidized in the mitochondria to generate ATP or stored as triglycerides, which, in turn, can be accumulated in the cells or released via VLDL (Very Low-Density Lipoproteins). Mitochondrial β-oxidation is the main pathway for lipid metabolism, but when the concentration of lipids in the cytosol exceeds, other processes are promoted, mediated

by peroxisomes (β-oxidation) and microsomes (ω-oxidation)[50]. Several studies reported that mitochondrial function is impaired in patients with NASH[51], developing lesions similar to those observed in subjects treated with tamoxifen (inhibitor of mitochondrial respiratory chain-MRC). In NASH, mitochondrial dysfunction is usually the result of IR and FFAs excess and it is the main source of reactive oxygen species. The alteration of respiratory chain promotes the interruption of electron flow, with the generation of an intermediate that becomes a donor of electrons to molecular oxygen, producing superoxide anions and hydrogen peroxide[52]. The ROS generation can be amplified by the activation of the alternative pathways in peroxisomes and microsomes that perpetuate the damage. Polyunsaturated fatty acids (PUFA) are susceptible to ROS, leading to lipid peroxidation with the generation of aldehydes such as hydroxynonenal (HNE) and malondialdehyde (MDA)[53]. ROS and aldehydes cause oxidative stress and cell death, inflammatory response and the activation of hepatic stellate cells, finally leading to collagen production and to the perpetuation of inflammation[50]. These effects have the potential to directly induce hepatocyte death and necrosis, steatohepatitis, and liver fibrosis: all of the histologic hallmarks of NASH.

### 1.5.4. Gut microbiota

Many factors including diet, sedentary lifestyle and genetics have been shown to influence the progression from steatosis through NASH to cirrhosis. However not all people who are obese develop NAFLD and neither all patients with NAFLD are obese. The intestinal microbiome is attracting an increasing amount of attention[124]. It is becoming apparent that there is a symbiotic relationship between the intestine and its microbiota and that disturbance in this relationship can be associated with the pathogenesis of metabolic disorders such as NAFLD. The gut represents the main organ of digestion and absorption, but also constitutes the largest site of interaction with the external environment[125]. This symbiotic relationship makes the gut a fundamental barrier that seals the host interior against possible toxic compounds coming from the outside. Maintenance in its integrity is strongly required in order to prevent the absorption of non-safe intruders. The human gut microbiome is characterized by 10-100 trillion microorganisms and encompasses five bacterial phyla (*Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria* and *Verrucomicrobia*) and one Archaea (*Euryarchaeota*). The main component is the bacterial community, with more than 90% of the species belonging to *Firmicutes* and *Bacteroidetes*. Each person has a different and variable microbiota, but a conserved set of gut colonizers (the gut microbiota core) and genes (the core microbiome) are shared among individuals[126]. In a healthy subject the gut microbiota is stable and

a balanced diet and physical activity contribute in maintain the homeostasis. Normal microbes are important to digest fibers and resistant starch and vitamin generation. It is well recognized that NAFLD can lead to dysbiosis, characterized by a reduction of beneficial bacteria and an increased prevalence of potential pathogenic phyla[127]. The relevant role exerted by dysbiosis was well described by animal models. Ley et al.[128] observed in ob/ob mice an increased Firmicutes-to-Bacteroides ratio in comparison with wild-type siblings, although they were fed with the same hypercaloric diet. In line, several human studies reported the same pattern of dysbiosis in human obesity, with high abundance in Firmicutes[128]. On the contrary, Bifidobacteria have been associated with a reduction of the leaky gut by way of increased expression of tight junction proteins[129]. Mice fed with High Fat with a reduction in this phylum displayed an enhancement in body fat mass, inflammation and insulin resistance[129]. Several mechanisms have been proposed to explain how intestinal microbiota can lead NAFLD etiology. An increased energy harvest had been reported in animal model of obesity that displayed an enrichment in carbohydrate metabolizing genes, an increased secretion of SCFAs and a less retain of energy in feces compared with controls[130]. Furthermore, enteric bacteria produces endogenous ethanol that reaches the liver via portal vein where it stimulates Kupffer cells for release nitric oxide and TNF-α[131]. In the liver ethanol leads to the production of two main compounds: acetate and acetaldehyde. The first, generated by ethanol-induced Krebs cycle arrest, acts as substrate for fatty acid synthesis. In parallel, acetaldehyde promotes oxidative stress and lipid peroxidation, responsible for intestinal barrier damage. Intestine membrane is constituted by epithelial cells that are joined to each other thanks to tight junctions, such as zonula occludens and claudins[132]. These proteins seal the intestinal microbial environment and their disruption leads to an increase in leaky gut. Alteration of the normal microbiome can lead to tight junction loss and consequently can increase mucosal permeability, exposing the liver to pathogenic compounds. Translocated bacterial products can activate Toll-like receptors (TLRs) exposed on Kupffer cells and hepatic stellate cells, leading to pro-inflammatory and pro-fibrotic milieu[133,134]. All these findings lead to conclude that dysbiosis might have a central role in NAFLD etiology and could be the target for new therapeutic strategies.

## 1.6. Molecular mechanisms in ALD

### 1.6.1. Alcohol metabolism

Over the 90% of ethanol absorbed from the gut is metabolized in the liver, the remainder is eliminated unchanged with breath, sweat, and urine[54]. The primary pathway involved in the oxidation of ethanol is mediated by alcohol dehydrogenase (ADH), mainly expressed in the liver and in the gastrointestinal tract. This process leads to the production of acetaldehyde, a molecule chemically very reactive that is normally converted to acetate by Acetaldehyde dehydrogenase (ALDH). Two other minor systems that metabolize ethanol are the catalase, whose levels are negligible in the liver and microsomal ethanol oxidation system (MEOS), localized in the smooth endoplasmatic reticulum. The catalase mediates the oxidation of the ethanol coupled with the reduction of hydrogen peroxide to water in the peroxisomes, instead, MEOS activity is based on cytochromes P450 oxidation, particularly CYP2E1 expressed in hepatocytes[55].

### 1.6.2. Oxidative stress

Ethanol metabolism determines the production of reactive oxidative species (ROS), such as superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$). These molecules quickly bind to ethanol and iron atoms, generating other reactive metabolites (ferrous oxide, hydroxyl radical etc.) responsible of lipoperoxidation. Furthermore, mitochondria with respiratory chain abnormalities, endoplasmic reticulum through CYP2E1 and NADPH oxidase of Kupffer cells are also major sources of ROS[56]. Apoptosis and necrosis are evident in ALD, with the generation of damage-associated molecular patterns (DAMPs) that trigger inflammatory response through the activation of macrophages and neutrophils, finally leading to fibrogenesis and hepatic regeneration[55]. In parallel, a long-term ethanol exposure determines a depletion in the antioxidant system (like glutathione and heme oxygenase-1) and hepatocytes become more sensible to oxidative stress.

### 1.6.3. Alcohol-induced steatosis

Chronic alcohol exposure endorses hepatic steatosis, due to the effects of oxidative stress on lipid metabolism. Particularly PPAR-α[57] and AMPK are down-regulated, leading to an impair in the oxidation and export of fats. Furthermore, ethanol promotes the activation of SREBP-1c[58], a transcriptional factor that regulates fatty acid biosynthesis increasing the expression of genes involved in this process. Several studies reported a key role in insulin resistance in the genesis of

steatosis, increasing the speed of the progression toward the most advantage stages in overweight patients.

### 1.6.4. Innate and adaptive immunity

A long-term alcohol exposure determines in the gut an increased serum concentration of lipopolysaccharide (LPS) that arrives at the liver[59], where it is recognized by specific receptors (such as Toll-like receptors- TLRs). LPS promotes a cascade of events that cause the activation of Kupffer cells, with the final induction of inflammatory pathways. LPS translocation depends on the increased leakiness of the gut, consequence of ethanol toxicity. Chronic alcohol exposure is responsible for bacterial overgrowth and dysbiosis that determine oxidative stress and inflammation. It has been reported that also the adaptive immunity could have a role in the pathogenesis of ALD. Alcohol abuse can determine the development of antibodies *versus* lipid peroxidation products, which the activation of adaptive immune response[60]. Ethanol stimulates T cells in the spleen and natural killer T cells in the liver, with a final cytotoxic response against hepatocytes.

## 1.7. Current and emerging therapies: NAFLD management

To date, there is not an approved therapy for NAFLD. The currently available therapeutic alternatives can be divided into two major categories: lifestyle modifications and pharmaceutical approaches.

*Diet and lifestyle interventions.* It is well accepted that obesity is one of the principal risk factors for the development of fatty liver. For this reason, weight reduction, dietary changes, and physical exercises are considered the gold standard for the reversion of NAFLD/NASH. It is generally known that the level of energy intake is significantly higher in patients with NAFLD than in healthy subjects and the calories are mainly consumed in form of carbohydrates (soft drinks represent the main source), saturated fats and cholesterol. An excess of carbohydrates affects glucose and free fatty acids metabolism in the liver, on the contrary, a restriction in the consumption determines weight loss and improvement of lipid profile[61]. Histologically, steatosis, inflammation, and fibrosis get better, confirming the efficacy of carbohydrate limitation in the reversion of NASH[62].

Dietary lipid content has also been identified as an important factor in the development of NASH. It has been reported that specific type of fats has a greater impact on the progression of

simple steatosis or in its improvement. Polyunsaturated fatty acids (PUFA), mainly n-3 and mono-PUFA, exert a protective role in fatty liver, determining adiponectin level increase and a reduction of serum insulin, triglycerides and leptin amount[63]. Furthermore, it has been observed a positive correlation between PUFAs consumption and the upregulation of the gene expression of proteins involved in fatty acid β-oxidation and a decrease of those involved in lipogenesis[64].

Physical activity is the other recommended approach in NAFLD treatment. Regular exercise (more than 3 times/week, at least 30 minutes and for consecutive 3 months) leads to a reduced risk of NAFLD, with a decrease of serum liver enzymes and body weight[65,66].

Over-nutrition, mainly resulting in an excessive intake of saturated fats and simple carbohydrates is strongly associated with obesity. Obesity is a strong risk factor for NAFLD pathogenesis, due to the correlation of overweight with visceral adiposity that has a high lipolytic capability. FFAs released are addressed to the liver through the portal vein, initiating the deposition of lipid content in the hepatocytes. For this reason, weight loss, achieved with dietary modification and physical activity, is the principal key factor in the management of NAFLD.

*Pharmaceutical interventions.* To date, there is not an approved pharmaceutical therapy for NAFLD. Usually, drug interventions are based on the association of several compounds in order to reverse the co-morbidities that characterized the metabolic syndrome. The molecular mechanisms leading to fat accumulation, oxidative imbalance, and liver fibrosis are the targets of the main classes of drugs usually associated with an initial step of lifestyle modification.

*Antioxidant*. The amount of reactive oxygen and nitrogen species, generated by lipoperoxidation, could exceed the capacity of the cellular antioxidant systems, leading to oxidative stress. It has been supposed that this condition is responsible for the progression from NAFLD to NASH, worsening the liver damage. Several antioxidants are normally introduced in NAFLD management to counteract the production of these reactive compounds, in order to prevent the damage progression.

*Insulin sensitizers.* This class of drugs, including metformin and thiazolidinediones (TZDs-such as pioglitazone and rosiglitazone), improves hepatic insulin activity. The improvement in insulin sensitivity by metformin could be ascribed to its positive effects on insulin receptor expression and tyrosine kinase activity[67]. It has also been reported that metformin acutely increases plasma levels of glucagon-like peptide 1 (GLP-1) and induces islet incretin receptor gene expression through a mechanism that is dependent on peroxisome proliferator-activated receptor

(PPAR)-α[68]. TZDs activity could be ascribed to its positive activation on the nuclear receptor PPAR gamma that alters the transcription of several genes involved in glucose and lipid metabolism and energy balance[69]. TZDs reduce insulin resistance in adipose tissue, muscle, and liver. Since insulin resistance is one of the major triggering mechanisms for NAFLD progression, these molecules are often used despite their known side effects[70].

*Lipid lowering drugs*. The main drugs used to reduce the plasma levels of cholesterol are statins[71]. The beneficial effects of these molecules depend on their capacity to reduce cholesterol biosynthesis, mainly in the liver, derived from an inhibitory effect on HMG-CoA reductase. Statins have antiatherosclerotic effects[72], positively correlated with a reduction in low-density lipoprotein (LDL) production.

All these findings emphasize the difficulties to achieve the success in NAFLD clinical management and underline the importance of the discovery of new alternative therapeutic strategies. Recently, the interest has been mainly focused in the study of food bioactive compounds. In the recent years, their promising positive effects in the reversion of the co-morbidities that occur in the metabolic syndrome have been evaluated. In the paragraph 1.7.1, will be discussed specifically Silymarin, obtained by Milk Thistle, since this compound has been used in the project discussed in this thesis. The properties of other bioactive compounds, widely studied for their use in NAFLD regression, are summarized in Table 1.2.

### 1.7.1. Silymarin

Silymarin is a natural extract, composed of three isomers of flavonolignans (silibinin, silydianin, and silychristin) and two flavonoids (taxifolin and quercetin). This mixture is obtained from *Silybum marianum* (commonly known as milk thistle) seeds, a herb studied in hepatology and whose derivatives are used as food supplements worldwide[73]. Silibinin is the major component (50-60%) and is characterized by an equimolar combination of two diastereoisomers, called Silybin A and B[74].

**Fig. 1.5**. Milk thistle (*Silybum marianum*), the seeds and the extract of silymarin obtained from those.

**Pharmacokinetics**

Silibinin is poorly soluble in water and has a low intestinal absorption, leading to a scarce oral bioavailability. For this reason, several approaches, focused on improving silibinin solubility have been used (such as complexation with cyclodextrin and phosphatidylcholine or phospholipids). It has a rapid tissue distribution, with a peak plasma concentration detectable at 30 minutes in the liver, lung, stomach and pancreas and after 60 minutes in the skin and prostate[75]. Silibinin undergoes multiple metabolic reactions, producing sulfate and glucuronic acid conjugates, firstly recognized in human plasma by Hoh et. al.[76]. The two silibinin diastereoisomers are differently metabolized: silibinin B is glucuronidated more efficiently than silibinin A[77,78]. Elimination half-life approximately is estimated at 6h[79]. About 3-8% of an oral dose is excreted in the urine; 20-40% is recovered from the bile and the remaining part via feces in unchanged form[79].

**Pharmacodynamics**

**a. Antioxidant properties**

Oxidative stress is defined as the structural or functional damage, developed when the production of free radicals excesses the anti-oxidant capacity of the defense systems[80]. It is implicated in the pathogenesis of numerous liver disorders, causing cellular membrane alterations and the oxidation of lipids and proteins.

Silibinin exerts radical scavenger activity, reacting with oxidant species, probably due to the phenolic conformation of flavonoids that stabilize reactive molecules, such as hydroxylic and oxygen radicals.

The hepatoprotective effects of silibinin were verified in animal models treated with pro-oxidant agents (ethanol[81], paracetamol[82], $CCl_4$[83]) in which the nutraceutical compound attenuated liver enzyme increase, preserved the integrity and the functionality of cell membrane and influenced positively the defense systems associated with glutathione and superoxide dismutase.

**b. Anti-inflammatory and immunomodulation activity**

Silibinin seems to exert an anti-inflammatory activity, determining the inhibition of neutrophil migration, Kupffer cells[84]activation and a marked inhibition of leukotriene synthesis and the production of prostaglandins[85]. Furthermore, some evidence shows an inhibitory effect on leukocytes, reducing the expression of adhesion molecules, like E-selectin[86] (family of transmembrane components present on leukocytes surface).

It has also been observed a nuclear anti-inflammatory action exerts by silibinin that involved nuclear factor kappa B transcription factor (NF-κB)[87]. Normally, inactive NF-κB is present in the cytoplasm, complexed with the inhibitory protein I- κB. Specific signals are able to cause the dissociation of the inhibitor implicating the translocation of NF-κB to the nucleus and the followed cascade of events associated with this transcriptional factor. Silibinin seems to be able to inhibit this activation process[88]. Silybin exerts anti-inflammatory action interfering with multiple cytokine-induced signaling pathways, downregulating inducible nitric-oxide synthase (iNOS) expression[89], inhibiting the cyclooxygenase (COX)-2 expression[90] and the leukotriene formation in human platelets, endothelial and white blood cells[87].

**c. Anti-apoptotic and anti-fibrotic effects**

Apoptosis is one of the events that participates in the progression of simple steatosis to steatohepatitis. The activation of pro-caspase-3 to the active form of caspase-3 has been defined as one of the major events that characterizes this pathological mechanism. It has been documented that silibinin is able to inhibit this protein activation[91].

Liver fibrosis represents one of the major histological alteration that determines the irreversible stages of the liver damage that finally remodels the liver architecture, causing hepatic insufficiency, portal hypertension, and hepatic encephalopathy. Some promising results reported that silibinin showed antifibrogenic properties, inhibiting collagen type I biosynthesis in animal models of secondary biliary fibrosis. Moreover, it has been reported that this compound inhibits

HSC activation, decreasing the expression of α-SMA and platelet-derived growth factor (PDGF)-induced DNA synthesis and cell proliferation[92,93].

**d. Detoxifying and hepatoprotective effects**

The liver is the organ with the main blood cleaning and detoxifying activity and for this reason is highly exposed to the deleterious effects of toxins and xenobiotics. Silibinin is generally included in detoxification regimens, being considered protective for this organ since ancient times. One of the major examples of the possible hepatoprotective properties of this nutraceutical molecule resides in its activity against *Amanita phalloides*, one of the main worldwide lethal mushroom. Silibinin, in hemisuccinate formulation, is approved as an antidote *versus* this intoxication (at a daily dosage of 20-50 mg/kg/body iv[94] for several days and however until the symptom persistence). It is supposed that its hepatoprotective effect in mushroom poisoning in part depends on to the inhibition of amatoxin uptake in liver cells, through a competitive blocking of the transporter system (OATP1B3), during the toxin metabolism[95]. Furthermore, seems that silibinin is able to stimulate the nucleolar polymerase A, which in turn increases ribosomal protein synthesis and inhibits lipid peroxidation[96]. The drug has to be administrated as soon as possible because the longer time has elapsed after poison exposure the less effective silibinin results.

### 1.7.2. Other natural compounds

Other bioactive molecules have been studied for their hepatoprotective properties. Dongiovanni et al.[97] well-reviewed the main mechanisms of action known for these nutraceutical compounds. The principal information referred are summarized in the following table (Table 2).

| Bioactive nutrient | Origin | Experimental model | Mechanisms |
|---|---|---|---|
| **Omega-3 (PUFAs: EPA and DHA)** | Fish oil, flaxseed, nuts | *In vivo*: HepG2 cells | ↓ FAS, SREBP-1c gene expression (lipogenesis)[98], antioxidant activity (↑SOD, GST, GPX activity) |
| | | *In vivo*: HFD mice, MCD diet rats | ↓steatosis (↓lipogenesis; ↑β-oxidation)[99], ↓fibrosis, antioxidant activity (↑SOD)[100] |
| | | Patients | Adults with NAFLD: 1g/day/12months: ↓liver enzymes, fasting glucose, steatosis[101] Adults with NASH: 2.7g/day/12months: ↓liver enzymes, serum ferritin, and thioredoxin levels; ↓hepatic steatosis, fibrosis, hepatocyte ballooning and lobular inflammation. |

| | | | |
|---|---|---|---|
| | | | Children with NAFLD: 1g/day/12months: ↓liver enzymes, ↓steatosis[102] |
| **Vitamin E** | Vegetable oils and nuts | *In vitro*: human fibroblasts | ↓Collagen1A1, COL1A2 gene expression[103,104] |
| | | *In vivo*: HFD young rats | ↓Oxidative damage, protein nitrotyrosilation, and tissue TNF-α levels; ↓PPAR-γ expression[105] |
| | | Patients | Adults with NASH: 800 IU day/96 weeks; ↓liver enzymes; ↓hepatic steatosis and lobular inflammation[106] |
| **Vitamin D** | Oil fish | *In vitro*: 3T3L1 adipocytes | ↑GLUT4 translocation and glucose utilization[107] |
| | | *In vivo*: VDR mutant mice | Insulin impairment, ↑glycaemia[108] |
| | | Patients | Adults with NAFLD: 50000 IU/every 14 days/4 months: antioxidant activity (↓ MDA, hsCRP)[109] |
| **Polyphenol** (flavonoids and non-flavonoids) | Fruit juices, wine, tea, coffee, chocolate | *In vitro*: HepG2 cells | ↓ACC, FAS, SREBP-1c protein expression (↓lipogenesis), ↑AMPK, PPARα (↑lipolysis)[110]; antioxidant activity (↑SOD, GPX activity)[111] |
| | | *In vivo*: db/db mice, DIO mice, HFM diet mice | ↓lipogenesis, improvement of glucose metabolism, ↓TNF-α, ↓hepatic steatosis and hepatomegaly, ↓serum cholesterol[112–114] |
| **Anthocyanins** (flavonoids subgroup) | Blueberries, red radishes, blackberries, raspberries, strawberries, cranberries, grapes, spinach. | *In vitro*: HepG2 cells | ↓SREBP-1c, FAS, ACC (↓lipogenesis), ↑PPARα, CPT-1 (↑lipolysis), antioxidant activity; ↓cholesterol and triglycerides biosynthesis[115]. |
| | | *In vivo*: MCD diet mice | ↓hepatic lipogenesis; ↓steatosis, inflammation, fibrosis; oxidative stress[116] |
| | | Patients | Adults with NAFLD: 320mg/day/12 weeks: ↓liver enzymes; ↓cytokeratin-18 fragments, ↓myeloperoxidase; IR improvement[117]. |
| **Minerals** | | | |
| **Copper** | Oyster, mushrooms, sesame seeds, nuts | *In vivo*: copper deficient rodent models | ↑hepatic steatosis and insulin resistance, hypercholesterolemia and increased blood pressure[118] |
| | | Patients | Adults with NAFLD: hepatic copper concentrations are lower than in control subjects; ↑hepatic steatosis and fasting glucose[118]. |
| **Iron** | Oyster, liver, nuts, spinach, dark chocolate, tofu | *In vitro*: iron depletion HepG2 cells | ↑GLUT1 and insulin receptor[119] |

| | | | |
|---|---|---|---|
| | | *In vivo*: Sprague-Dawley iron depleted rats | Glucose clearance improvement (↑glucose transport in hepatic tissue); ↑insulin receptor activity[119] |
| | | *In vivo*: iron-enriched diet mice | Insulin resistance development; hypertriglyceridemia and visceral adipose tissue metabolism impairment[120] |
| | | Patients | Adults with NAFLD treated with venesection (350 cc of blood/every 10-15 days until ferritin < 30 ng/mL and/or transferrin saturation < 25%): improvement of hepatic steatosis and ALT value.[121] |
| **Selenium** | Oyster, mushrooms, tuna, beef, lamb and chicken | *In vitro*: C3A cells | ↓TGF-β induced collagen, IL-8 production; antioxidant activity[122]. |
| | | *In vivo*: HFD Sprague-Dawley rats | ↓triglycerides and antioxidant activity[123]. |

**Table 1.2**. **Summary of the properties of other bioactive nutrients studied for NAFLD treatment**. List of abbreviations (alphabetic order): ACC: Acetyl-CoA carboxylase; AMPK: AMP-activated protein kinase; COL1A1/COL1A2: Collagen 1A1/Collagen 1A2; CPT-1: Carnitine palmitoyl transferase I; FAS: Fatty Acid Synthase; GLUT4: Glucose transporter 4; GPX: Glutathione peroxidase; GST: Glutathione S-transferase; HFD: High Fat Diet; HFM diet: High-Fat Milk Diet; hsCRP: high-sensitive C-reactive protein; IL-8: Interleukin-8; IR: Insulin Resistance; MCD: Methionine/choline deficient diet; MDA: Malondialdehyde; NAFLD: Non-Alcoholic Fatty Liver Disease; NASH: Non-Alcoholic Steatohepatitis; PPAR-γ (peroxisome proliferator-activated receptors); PUFA: Polyunsaturated fatty acids; SOD: Superoxide dismutase; SREBP-1c: Sterol regulatory element-binding proteins; TGF- β: Transforming Growth Factor beta; TNF-α: Tumor necrosis factor alpha; VDR: Vitamin D Receptor.

## 1.8. Current and emerging therapies: ALD management

Scientific studies have made major progress in understanding the pathogenesis of ALD. These advances contributed to set the bases to build a bridge between the bench and the bedside, for the development of new treatments for alcohol-induced damage.

### 1.8.1. Abstinence

The cornerstone of therapy for alcoholic hepatitis is abstinence. It is essential to prevent the progression of the liver injury and it appears to benefit patients at every stage of disease, causing the total resolution of alcoholic steatosis[135] and markedly reducing mortality[136]. Some drugs could be used to facilitate abstinence. One of the first compound used was *disulfiram*, an inhibitor of acetaldehyde dehydrogenase that determines an accumulation of serum acetaldehyde modifying ethanol metabolism. The increased amount of this intermediate produces unpleasant nausea sensation, vomiting, flushing, abdominal pain and tachycardia[137]. This condition should induce

rejection towards further alcohol consumption. Disulfiram could cause hepatotoxicity and this may limit its use. *Naltrexone* is an antagonist of opioid receptors (*versus* mu, kappa and delta receptors) that blocks the central pleasurable ethanol effects, decreasing alcohol craving. The side effects that it can induce encompass nausea, abdominal pain, anorexia, and sedation but it rarely results in hepatotoxic[138]. Another agent that can decrease alcohol dependence is *baclofen*, a gamma-aminobutyric acid-B agonist. Beyond the suppression of alcohol craving, it also improves liver functions, reducing liver enzymes, bilirubin, and albumin levels[139,140]. Besides the pharmacological approach, psychotherapy and family support are fundamental in the management of alcoholic rehabilitation.

### 1.8.2. Nutritional therapy

Malnutrition is common among alcoholics, with a reduction in proteins, minerals and vitamins consumption. These abnormalities are associated with the development of other disorders and the increase in mortality. The causes are different and include poor dietary intake due to anorexia, altered sense of taste and smell, nausea, vomiting, and malabsorption. It has been reported that the severity of malnutrition correlates with the disease outcome[141]. Clinical trials that compared the efficacy of nutritional therapy *versus* untreated patients demonstrated no significant changes in term of mortality reduction but showed an improvement in serum bilirubin level, nitrogen balance, and hepatic encephalopathy[142].

### 1.8.3. Pharmacological approach

*Corticosteroids*. Inflammation is one of the trigger mechanisms and consequently corticosteroids have been proposed as the first choice for the treatment of severe alcoholic hepatitis (AH). The molecular mechanism of this approach consists in the reduction of inflammatory cytokines such as TNF-α, IL-6, and IL-8, with an increase of IL-10. Furthermore, e*x vivo* experiments have shown that corticosteroids reduce the capability of neutrophils and monocytes to produce TNF in presence of LPS[55]. Studies of this class or drugs are based on 13 clinical trials. However, most of them are small, with a limited statistical power and with a wide variety of inclusion and exclusion criteria, dosing, and ethnicities. Five of these studies suggest a decreased short-term mortality *versus* placebo; the others do not highlight any effect[143].

*Anti-TNF antibodies*. Altered levels of TNF were described in AH and FDA-approved anti-TNF antibodies as a treatment in this clinical scenario[135]. However, it has been reported that basal

levels of TNF-alpha are essential for the physiological liver regeneration. Thus, many studies considered the regulation of TNF-alpha rather than a complete abolishment of this cytokine[144]. Results of pilot studies and the first randomized controlled trial published by Tilg et al.[145] indicated an improvement of portal hemodynamics and liver function.

Two of the specific inhibitors of TNF are *infliximab* and *etanercept*. Infliximab is a monoclonal chimeric anti-TNF antibody instead etanercept is a protein that fuses the TNF receptor to the Fc portion of the IgG1[143]. One of the first clinical trials compared the efficacy of the co-therapy of prednisolone with infliximab *versus* the only corticosteroids administration. A reduction in some prognostic markers (cytokine levels, MDF score) was observed, but not significant data has been reported in terms of reduced mortality rate[146]. Another analog trial was stopped due to seven deaths occurred in infliximab group, the most of those related to infectious etiologies[147]. In line with this data, also etanercept did not show an improvement of survival *versus* placebo, but on the contrary, an increase in mortality was observed in a time window of 6 months[148]. This bad outcome suggests that completed inhibition of TNF is not ideal due to its role in liver regeneration and apoptosis.

*Pentoxifylline.* It is an oral phosphodiesterase inhibitor, which demonstrated anti-TNF properties. In cirrhotic animal models[149], this agent prevented the genesis of hepato- pulmonary syndrome and the development of the hyperdynamic circulatory state. In patients with AH, it reduces the risk of hepatorenal syndrome, improving the renal function[150]. However, results about the effect on survival are still controversial.

### 1.8.4. Liver transplantation

Alcoholic cirrhosis is the most common cause of liver transplantation (LT) after viral hepatitis cirrhosis. However, ALD is considered a “self-inflicted” disease and for this reason, the priority for donor organs is given to other end-stage liver diseases[136]. Furthermore, with alcoholic patients, there is a risk of injury recurrence due to a recidivism in alcohol consumption[151]. To prevent the relapse, it has been suggested for LT candidates to undergo assessment by an addictive behavior specialist and at least 6 months of abstinence in order to improve clinical scenario[136]. Besides the medical evaluation, also psychological assessment is mandatory to determine if the patient is suitable for liver transplantation.

### 1.8.5. Innovative approaches

*CXC chemokines.* These mediators, such as interleukin-8 (IL-8), attract polymorphonuclear leukocytes, the main inflammatory cells that infiltrate the liver of ALD patients[152].

*Osteopontin.* It is an extracellular matrix protein that is increased in patients' liver and its expression correlates with the severity of the disorder[153].

*Gut microbiota and LPS*. The modification of the microbiota composition and the increase of the gut permeability lead to an enhanced amount of LPS in the portal circulation. No absorbable antibiotics that modify the gut microbiota, such as rifaximin, demonstrated to be efficacious in the reversion of hepatic encephalopathy and could have a possible application also in ALD[154].

*Endocannabinoids*. The binding to cannabinoid receptors 1 and 2 (CB1 and CB2) is involved in the pathogenesis of ALD and animal models lacking in these target receptors seem to be less responsive to alcohol-induced liver damage[155]. It suggests a possible role of CB1 antagonists and CB2 agonists in the management of ALD.

*Anti-MIF therapy*. MIF is a pleiotropic cytokine with a predominant impact in several autoimmune and inflammatory disorders, such as rheumatoid arthritis, asthma, and systemic sclerosis. Many of these disorders were ameliorated by genetic MIF depletion or neutralization[156,157]. Considering that inflammation is a key factor also in ALD it could be interesting to analyze the role of this cytokine in the pathogenesis of this liver injury. The aim of the second project discussed in this thesis is to investigate the activity of this mediator on an *in vitro* model of alcohol-induced liver damage.

## 1.9. Macrophage migration inhibitory factor (MIF)

MIF is a pleiotropic inflammatory mediator that was initially found to inhibit the random migration of macrophages. MIF is distributed in many tissues and is normally expressed in both immune and non-immune cells. In the liver, it is produced by both hepatocytes and Kupffer cells[158] and it is constitutively stored in performed intracellular pools[159]. Upon stimulation, it does not require *de novo* protein synthesis before secretion and therefore it is rapidly released. It is an upstream regulator of immunity and has a chemokine-like function[160], favoring the recruitment and infiltration of leukocytes in inflammatory and infectious processes. One of the main features is its capability in counter-regulating the pharmacological activities of glucocorticoids[161]. Differently, from other cytokines, MIF's functions are not suppressed by this drug class.

### 1.9.1. Molecular structure

Human MIF consists of 114 amino acids and has a molecular weight of 12.5 kDa[162]. It has two antiparallel alpha-helics and six beta pleated sheets. This protein has been demonstrated to have at least tautomerase and oxidoreductase catalytic activities[163]. Therefore it has been termed "cytokine with enzymatic properties" or *cytozime*. It is also known as secreted enzyme because it is normally expressed and stored in preformed intracellular pools, that are rapidly released after stimulation, differently from other cytokines that are usually produced upon induction[164]. The precise mechanism of secretion of MIF has not yet elucidated.

### 1.9.2. Receptors

MIF is a pleiotropic cytokine and a mediator in numerous inflammatory disorders. Depending on the context, it can trigger a signal through different receptors.

*CD74*. A cluster of differentiation 74 is a single-pass type II transmembrane protein. It lacks in a signal-transducing intracellular domain but it forms heterodimeric complexes with CD44 that once phosphorylated actives Src family non-receptor tyrosine kinase, finally determining ERK1/2 phosphorylation. The binding of this protein mediates the pro-survival and proliferative functions on immune and tumor cells[165].

*CXC chemokine receptors*. MIF promotes the recruitment of monocytes and T cells by interacting with CXCR2 and CXCR4. Through the binding with CXCR2, the chemotactic activity of MIF also extends to neutrophils[166]. However, the atherogenic or inflammatory monocyte recruitment relied not on only in CXCR2 binding but also in the interaction with the co-localized CD74, suggesting the induction of a signal via the formation of a CXCR/CD74 complex. CXCR7 is a transmembrane receptor that can also interact with MIF. This binding can induce the chemotaxis of B cells promoted by MIF and ERK1/2 activation; furthermore, studies of co-immunoprecipitation, fluorescence microscopy, and proximity ligation assay, demonstrated that CXCR7 can also create complexes with CXCR4 and CD74, both after ectopic overexpression and in endogenous conditions[167].

### 1.9.3. Role of MIF in diseases

Numerous studies have been performed to investigate the role of MIF in different disorders. So far, the clearest evidence is linked to the strong impact of this cytokines in conditions of acute inflammatory diseases such as bacterial septic shock in which anti-MIF based therapies have been

already applied. Other studies also clarify the implication of this mediator in chronic inflammatory disorders.

**a. Acute inflammatory diseases**

Microbial toxins stimulate the release of MIF both centrally by the pituitary gland and peripherally by parenchymal and immune cells. The role of MIF in acute inflammation was firstly examined in animal models of endotoxic shock. Mice injected intraperitoneally with LPS developed an endotoxic shock mediated by TNF, IL-1, and MIF as well. Recombinant murine MIF greatly enhanced lethality when co-injected with LPS and anti-MIF antibody conferred full protection against lethal endotoxaemia[168]. In the same way, MIF knock-out mice showed resistance to the LPS-induced damage[169]. The role of this cytokine was investigated also in animal models of bacterial sepsis. Intraperitoneal Escherichia coli injection or cecal ligation and puncture (CLP) models demonstrated a rapid increase of MIF level firstly in the peritoneum and then into the circulation, in parallel with the infection diffusion in the bloodstream. In both systems, the use of anti-MIF antibodies protected the animals from sepsis, even when treatment was started as late as 8h after the infection induction through CLP, demonstrating protection also with delayed administration[170]. This is a very important feature for a compound used in sepsis disorders. Considering that MIF is an essential player in the host responses to infection and a trigger in the induction of innate and adaptive immune systems, another important aspect analyzed was the consequences of a completed MIF deletion. It has been observed that the pre-treatment with anti-MIF antibodies, followed by the injection with LPS does not raise the mortality. In contrast, lethality increased when infection occurred in the setting of the high amount of circulating MIF[170].

**b. Chronic inflammatory diseases**

*Rheumatoid arthritis (RA)*. A pathogenic role of MIF has been reported in several inflammatory disorders and it has also been well established in RA. MIF demonstrated to be the main mediator of the recruitment of synovial leukocytes, the activation of MAPK pathway, alteration of apoptosis, activation of metalloproteinase and cyclooxygenase expression, and the amplification of cytokines such as TNF-α, IL-6, and IL-1[171]. The use of anti-MIF antibodies in animal models of RA demonstrated a marked suppression of the inflammatory response[172]. MIF levels were found enhanced also in patients with osteoarthritis who had 5-to 10-fold higher amount of the protein in the synovial fluid compared with healthy controls[173]. The well-recognized therapy

for RA is based on glucocorticoids. However, a group of patients results no responders towards this therapy. This could be explained thinking to the capability of MIF to counteract the effect of the drug. Santos et al.[174] demonstrated in a murine animal model of antigen-induced arthritis (AIA) that glucocorticoid treatment (dexamethasone) inhibited the injury but the injection of MIF counter-regulated the effect of dexamethasone. On the contrary, the use of anti-MIF antibodies completely reversed AIA and reduced the circulating amount of this cytokine. This suggests that inflammatory processes in specific tissues may respond differently to MIF in the presence of glucocorticoids.

*Systemic lupus erythematosus (SLE)*. It is a chronic autoimmune disease that can affect almost any organ system. Many factors can trigger SLE pathogenesis, but the fundamental element resides on the loss of self-tolerance, generating the release of antibodies by autoreactive lymphocytes. MIF levels are increased in patients with this autoimmune disorder[175] and a positive correlation was detected between MIF levels and SLE-related score and glucocorticoid treatment.

*Lung inflammatory diseases*. Bronchoalveolar lavage fluid obtained from asthmatic patients showed increased levels of MIF, compared to healthy volunteers, suggesting a possible role of this cytokine in the generation of asthma and other lung disorders[176]. Donnelly et al.[177] found a significant amount of MIF in the alveolar space of subjects with acute respiratory distress, inducing an increased release of proinflammatory mediators such as TNF and IL-8. Anti-MIF antibodies reduced this status. These findings suggest that MIF may act as a mediator sustaining the pulmonary inflammatory response and that anti-MIF strategy may represent a novel therapeutic approach.

*Metabolic diseases*. Obesity is associated with a chronic low-grade inflammatory state that leads the genesis of co-morbidities such as insulin resistance, NAFLD, and cardiovascular complications. The primary factor in the metabolic syndrome seems to be the affection of adipose tissue, which is no longer able to respond *versus* an excessive calorie intake, becoming inflame and resistant to insulin[178]. It has been recognized that MIF is increased in this metabolic dysfunction, suggesting a possible release mediated by adipocytes. Different researchers have studied the relationship between circulating MIF and obesity. It has been reported that obese patients have significantly higher MIF plasma levels compared with lean subjects, with a positive correlation between this cytokine and BMI[105,180]. This correlation (higher BMI and an increased waist circumference) was confirmed also in studies conducted in children and adolescents (5-17 years

old)[181,182]. As mentioned before, T2DM is a co-morbidity often associated with NAFLD. Interestingly, a correlation between glucose impairment and MIF level has been also reported. Some studies conducted in Japanese[183], Chinese[184] and Mexican[185] cohorts demonstrates a positive relationship between the circulating amount of MIF and the genesis of this disorder. Moreover, Herder et al.[186] observed an increased MIF serum levels in subjects with impaired glucose tolerance, which was further increased in subjects with T2DM. The role of MIF in obesity and insulin resistance has been investigated also in animal models of diet-induced-obesity (DIO). Saksida et al.[187] analyzed the release of this cytokine in a mouse model of High Fat Diet, which develops an obese phenotype and high levels of insulin. MIF mRNA expression was significantly higher in pancreatic islets, in which palmitic acid-induced the production of this cytokine and apoptosis. The same study demonstrated that MIF-deficient mice were completely protected and resistant to palmitic acid-induced apoptosis. In conclusion, there are some indications that MIF could have a role in obesity and diabetes, and that would be strictly associated with NAFLD. However, this observation must be confirmed in other cohorts of patients from different ethnicities. MIF levels are enhanced in association with expansion of adipose tissue mass and adipocyte size, highlighting a role of this organ as a source of MIF. Cardiovascular disease is strongly associated with obesity and the contribution of MIF to atheroma formation has been demonstrated both in animal and human studies[188,189]. Oxidized LDL uptake increases the release of MIF by macrophages and MIF, in turn, triggers the recruitment of other macrophages, perpetuating the damage.

**c. Hepatocellular carcinoma**

It has been suggested that MIF could have a role in several types of cancer such as in breast, colon, pancreas and lung. It is also overexpressed in hepatocellular carcinoma (HCC) and its level has been correlated with HCC size, intrahepatic metastasis, and vascular invasion[190]. In MIF knock-down models there is an induction of apoptosis-related proteins and a reduction of growth-related proteins[191]. MIF circulating amount is strongly increased in HCC setting, instead, it is markedly reduced after surgical tumor ablation, suggesting that the plasmatic quantity of this mediator should be deeply investigated in the future to understand if it could be considered a potential biomarker for the diagnosis and the follow-up of this liver disorder[192].

**d. Alcoholic liver disease**

As described before, MIF has a recognized role in the etiology of chronic inflammatory disorders. Thus, even more, attention has been drawn to understand the possible role of this cytokine in the pathogenesis of ALD. The first investigations showed a possible contribution of MIF in the immune cell migration and in hepatocellular damage because its expression was localized in the infiltrating cells and in damaged and dying hepatocytes[193]. Increased serum level of MIF was quantified in patients with alcoholic hepatitis and cirrhosis, with a positive correlation with the enhanced levels of hepatic failure markers, such as γ-GTP and AST[193]. A confirmation of the participation of MIF in ALD etiology in given by several animal studies. Rats treated intragastrically with ethanol demonstrated an increased expression of hepatic MIF expression in line with a risen level of TNF and interferon-gamma, as well as with an increased liver damage[134]. In the same way, mice fed with ethanol showed a reduced level of inflammatory cytokines (such as TNF, MCP-1 etc.), adhesion markers (ICAM-1, E-Selectin) and inflammatory infiltrates in MIF-deficient animals, suggesting that this mediator could have a role in the inflammation that in turn leads to the liver injury onset[134]. Furthermore, wild-type mice fed with chronic ethanol liquid diet showed an increase in plasmatic and hepatic MIF expression as well as an enhancement in the recruitment of peripheral monocytes to the liver. In contrast, ethanol feeding to MIF−/− mice decreases F4/80+ cells and does not elicit recruitment of peripheral monocytes. These changes in the hepatic monocyte population are associated with protection in MIF−/− mice from chronic ethanol-induced hepatic steatosis, proinflammatory cytokine production and sensitization to LPS[195]. So far, it is still unclear the main source of this mediator among liver cells in ethanol-induced liver damage and there are some lacks in understanding if once released it exerts autocrine or paracrine effects.

## 1.10. Key conclusive points

NAFLD and ALD share many of the molecular pathological mechanisms that lead the progression from simple steatosis towards more advanced stages. Lifestyle changes introduced even from the young age (such as alcohol abuse and/or hypercaloric diets) are threatening the health of the future generations. This issue is worrisome especially because both liver disorders lack an effective therapy. Thus, lifestyle changes still represent the cornerstone for the injury regression. Unfortunately, low patients' compliance represents the main obstacle to overcome.

The aim of the present study is therefore to elucidate additional aspects involved in alcohol and diet-induced liver injury that are still unclear in an attempt to provide new evidence for the development of future therapeutic strategies to manage NAFLD and ALD.

# Chapter 2
# Aim of the thesis

## 2. Aim of the thesis

Fatty liver is characterized by the deposition of intracellular lipid droplets within the hepatocytes. Obesity and alcohol abuse represent the more common risk factors, determining respectively the genesis of Non-Alcoholic Fatty Liver Disease (NAFLD) and Alcoholic Liver Disease (ALD). Regarding NAFLD, the booming prevalence of obesity and type 2 diabetes mellitus in young age contributes to enhancing the concern towards this liver injury. Besides the spread of obesity, recently another emergent relevant issue among teenagers is the so-called *binge drinking* characterized by an excessive alcohol intake consumed during the weekends. Nowadays, obesity and alcohol are considered two of the most worrisome problems for the public health, with an increased tendency to co-exist in youth, enhancing exponentially the incidence of chronic liver diseases. In literature, there are not many reports that investigate juvenile NAFLD and ALD. The lack of studies in both fields prompted us to analyze these two different sides of the same coin, dividing this thesis into two parts entitled task 1 and 2.

Task 1 is focused in NAFLD study and the principal aims are:

- Development and characterization of a pediatric *in vivo* model of NAFLD, feeding mice with High Fat High Carbohydrate diet (HFHCD) for 16 weeks.
- Evaluation of the therapeutic properties of Silymarin added to HFHC diet. Part of mice followed a change of lifestyle (switching of HFHC to control diet), considered the gold standard for NAFLD management.

Task 2 investigates the role of MIF in the etiology of ALD, a cytokine previously studied in disorders in which inflammation is the central issue. The main targets of this study are:

- Analyze the interaction between hepatocytes and macrophages upon the co-culturing condition and after treatment with 50mM ethanol, in order to analyze the participation of MIF in this cellular cross-talk and more specifically to understand the main source of this mediator in ALD scenario.
- Complete *in vitro* data, using an *in vivo* model of bone marrow transplant between a wild time and MIF KO-mice (WT → WT; WT → *Mif*-/-; *Mif*-/- → WT), feeding animals with chronic ethanol diet in order to analyze the response of chimeras to alcohol exposure.

- Evaluate human serum samples and liver biopsy of alcoholic hepatitis patients to confirm the *in vitro* and *in vivo* findings in order to provide a direct translation from the bench to the bedside.

# Chapter 3
# Materials and Methods

# 3. Materials and methods

## 3.1. TASK 1 ~ NAFLD

### 3.1.1. Project design

Our study was divided into two phases:

**Phase I**: Development and characterization of a juvenile animal model able to reproduce each one of the before mentioned experimental requirements.

**Phase II**: Validation of the model as a platform for therapeutic research. Particularly we assessed the effects of Silymarin in NAFLD treatment.

#### a. Phase I. Characterization of NAFLD juvenile model. Animals and diet.

C57Bl/6 mice pups (37 of each sex) were obtained from Harlan Laboratories S.R.L. (S.Piero al Natisone, Italy). Immediately after weaning, animals were randomly group-housed in cages in a temperature-controlled environment (22±2° C) and on a 12 hours light/dark schedule, and fed *ad-libitum* with control diet (CTRL, D12328, *Research Diets,* New Brunswick, NJ) or HFHC diet (HFHCD: D12331, *Research Diets,* New Brunswick, NJ) (Table 1), plus 42g/L fructose/sucrose in drinking water, for a total of 16 weeks [196]. Four experimental checkpoints, in which part of the animals was sacrificed, were established (4, 8, 12 and 16 weeks of diet). Animal care and procedures were conducted in accordance with the Italian Law (decree 116-92) and by European Community directive 86-609-EEC. All experimental protocol were approved by the local committee of the Medical Research Institute and by the National Authority (Ministero della Salute – Direzione generale della sanità animale e dei farmaci veterinari - Ufficio VI, Rome, Italy, under the Article 7, D.lgs 116/92). The maximal effort was done to reduce the number of animals used and their sufferance in the respect of the 3R rule.

| Control Diet | High Fat High Carbohydrates Diet |
|---|---|
| proteins 16.8 gm% | proteins 23 gm% |
| carbohydrates 74.3 gm% | carbohydrates 35.5 gm% |
| fats 4.8 gm% | fat 35.8 gm% |

**Table 3.1** Control diet and HFHC diet compositions

**b. Phase II. Juvenile model as a therapeutic platform. Study of silymarin properties.**

After 8 weeks of the diet, the group of HFHC mice was divided into the subgroups reported below and the trial was continued for further 12 weeks. Animals exposed to HFHCD for all the experiment (20 weeks) were used as a control to verify treatments effects (silymarin and change of lifestyle). Animals were sacrificed at the end of the trial.

a) CTRL diet (20 weeks)
b) HFHC diet (20 weeks)
c) HFHC diet (8 weeks) + silymarin (33mg/animal/day) (12 weeks)
d) HFHC diet (8 weeks) → Ctrl diet (12 weeks)
e) HFHC diet (8 weeks) → Ctrl diet + silymarin (33mg/animal/day) (12 weeks)

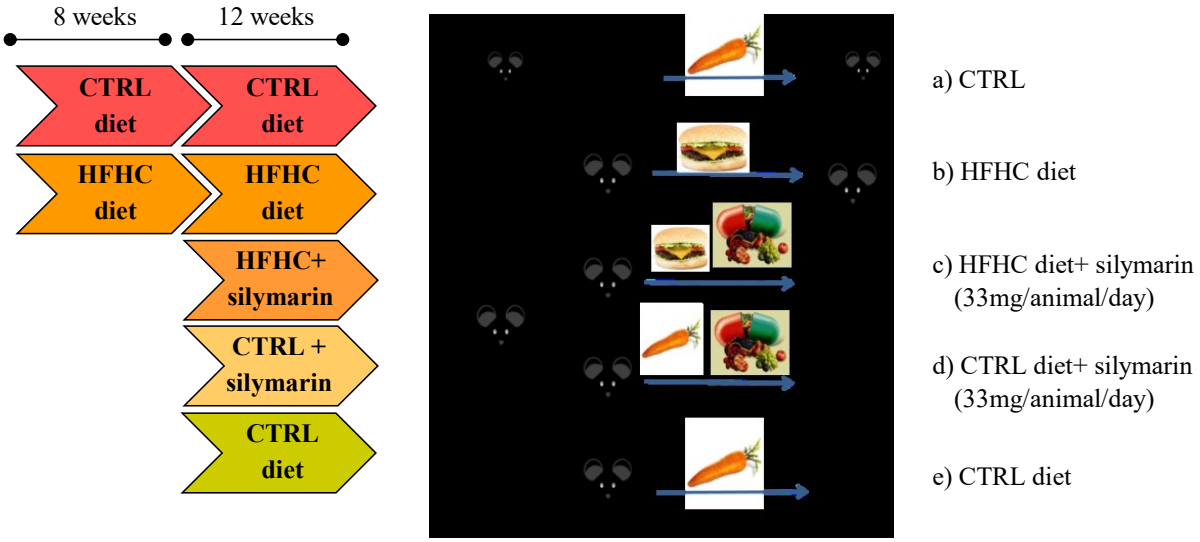


**Fig. 3.1 .**Graphical representation of the project study of phase II

### 3.1.2. Sample collection

*Phase I*: Liver, aorta, adipose tissue, small and large intestine (tissue and content) and blood samples were collected at each experimental checkpoint time (4, 8,12 weeks n=3-5 mice *per* group; 16 weeks n=5-8 mice *per* group).

*Phase II*: Liver and blood samples were collected at the end of the trial.

After O/N fasting, animals were deeply anesthetized (Zoletil 10mg/kg and Xylazine 5mg/kg of body weight, intraperitoneal). Blood was collected by cardiac puncture (exsanguination). Whole

blood was allowed to clot at room temperature (RT) for 20 minutes, followed by centrifugation at 3500g for 5 minutes at RT to separate the serum.

### 3.1.3. Body weight, epididymal fat pads and hepatomegaly

Body weight was recorded weekly, while liver and epididymal fat pads tissues were dissected and weighed only at the animal sacrifice. Moreover, sacrificed animals' body weight and naso-anal length were recorded for indirect computation of body composition via body mass index (BMI), for the confirmation of the development of the obese phenotype [197].

Serum triglyceride, cholesterol, low and high-density lipoprotein quantification

Serum triglycerides (TG), total cholesterol (Chol), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) contents were assessed. For the *Phase I* the analysis was performed in the samples collected at every checkpoint times instead for the *Phase II* only at the end the trial. Enzymatic colorimetric kits (Roche Diagnostics GmbH; Mannheim, Germany) were used to measure these parameters in a Roche HITACHI Cobas e501 instrument (Roche Diagnostics GmbH; Mannheim, Germany) accordingly to manufacturer's instructions.

### 3.1.4. Assessment of glucose homeostasis, serum insulin, and insulin resistance

Glycaemia and insulinemia were measured after 6-hours fasting (two days before sacrifice), collecting some blood drops from a submandibular vein (about 40µL *per* animal) in local lidocaine anesthesia. Glucose was measured in whole blood using One Touch Verio IQ® meter (Life Scan Europe, Zug, Switzerland) accordingly to manufacturer's instructions. Serum insulin content was quantified from the same blood sample, by AlphaLISA Insulin Kit (Perkin Elmer, Waltham, MA, USA) following manufacturer's instructions. The homeostasis model assessment of insulin resistance HOMA-IR was calculated for each animal according to the following formula: blood glucose (mg/dL) x fasting insulin (µU/mL)/405 [198].

### 3.1.5. Quantification of hepatic aminotransferases.

As a measurement of liver damage, serum alanine (ALT) activity was quantified by enzymatic colorimetric assay (Roche Diagnostics GmbH; Mannheim, Germany) accordingly to

manufacturer's instructions in a Roche HITACHI Cobas e501; (Roche Diagnostics GmbH; Mannheim, Germany).

### 3.1.6. Gene Expression analysis by Real Time q-PCR

The mRNA expression of genes listed in Table 3.2 was analyzed by quantitative RT-PCR. Total RNA of liver samples was extracted using Tri-Reagent Kit according to manufacturer's instructions. Briefly, the sample was lysed with the reagent, chloroform was added and cellular RNA was precipitated by isopropyl alcohol. After washing with 75% ethanol, the RNA pellet was dissolved in nuclease-free water and stored at -80°C until further analysis. The total RNA concentration and the purity were assessed by spectrophotometric analysis in a Beckman DU730 spectrophotometer. The integrity of RNA was assessed on standard 1% agarose/formaldehyde gel. Retrotranscription of total RNA (1µg) was performed with High Capacity cDNA Reverse Transcription Kit (Applied biosystems, Monza, Italy) according to the manufacturer's instructions. The reaction was run in a thermal cycler (Gene Amp PCR System 2400, Perkin-Elmer, Boston, MA, USA) at 25°C for 5 min, 37°C for 120 min, and 85°C for 5 min. The final cDNA was conserved at -20°C until used. For the quantitative PCR, primers were designed using the Beacon designer 8.1 software (Premier Biosoft International, Palo Alto, CA, USA) on mice sequences available in GenBank (Table 3.2). The reaction was performed on 25ng of cDNA, with the corresponding gene-specific sense and antisense primers 250nM (with the exception of TNF-α concentration of 400nM), with 1x iQ SYBER Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) in a final volume of 25µL. Reactions were run on an iCycler iQ thermocycler (Bio-Rad Laboratories, Hercules, CA, USA). The thermal cycler conditions consisted of 3 min at 95°C and 40 cycles each at 95°C for 20s, 60°C for 20s, and 72°C for 30s. The PCR was performed in 96-well plates; each sample was analyzed in duplicate. Standard curves were prepared for each target and house-keeping gene. Melting curve analysis was performed to assess product specificity. The relative quantification was made using the iCycler iQ Software, version 3.1 (Bio-Rad Laboratories, Hercules, CA, USA) by the Pfaffl modification of the ΔΔCT equation (CT: cycle number at which the fluorescence passes the threshold level of detection)[199,200], taking into account the efficiencies of the individual genes. The results were normalized to the chosen housekeeping genes and the initial amount of the template of each sample was determined as relative expression vs. one of the

samples chosen as reference which is considered the 1x sample. The data were analyzed using iQ5TM optical system software version 2.0 (Bio-Rad Laboratories, Hercules, CA, USA).

| | Accession number | Forward | Reverse |
|---|---|---|---|
| **GAPDH** | NM_008084 | CCAGTATGACTCCACTCACG | CTCGCTCCTGGAAGATGGTG |
| **β-actin** | NM_007393 | CCTTCTTGGGTATGGAATCCTGTG | CAGCACTGTGTTGGCATAGAGG |
| **DGAT-2** | NM_026384 | CCAAGAAAGGTGGCAGGA | ATGGGAAAGTAGTCTCGGAAG |
| **MCP-1** | NM_011333 | TGTGACTCGGACTGTGAT | CATTGAAAGTGTTGAATCTGGAT |
| **TNF-α** | NM_013693 | AGACCCTCACACTCAGAT | CTTGGTGGTTTGCTACGA |
| **COL-1A1** | NM_007742,3 | AAGAAGACATCCCTGAAG | ATACAGATCAAGCATACCT |

**Table 3.2**. Primer pair sequences.

### 3.1.7. Oxidative stress determination by MDA quantification

Oxidative stress was assessed measuring MDA, one of the aldehydic lipidperoxidation products through the generation of colored products, after the reaction with thiobarbituric acid (TBA) (Sigma Aldrich, Saint Louis, MO, USA). Hepatic tissue was cut by medical scissors and homogenized on ice, using 10mM Sodium Phosphate, 1mM EDTA, 0.2 mM BHT buffer. The homogenate was centrifuged at 10,000 g, 4°C for 10 minutes and supernatant was collected for MDA detection. Briefly, each sample was prepared by adding TBA (0.7% w/v) BHT (2% w/v in ethanol) SDS 8.1% and acetic acid 10% v/v. After 45minutes heat at 100°C pink, pigments were formed. After a quickly cooling process, the samples were centrifuged at 10,000 g for 5 minutes. The supernatant was collected and added to the same volume of TCA 10%, in order to precipitate the proteins. The solution was vortexed and centrifuged at 15,000 g for 3 minutes. The supernatant was collected for absorbance detection at 440nm, 532nm, and 600nm. MDA was calculated using Hodges et al. equation [201].

### 3.1.8. Oxidative stress determination by oxidized/reduced glutathione ratio

Reduced glutathione (GSH) is considered one of the most important scavengers of reactive oxygen species (ROS), and its ratio with oxidized glutathione (GSSG) may be used as a marker of oxidative stress[202].

Total glutathione (GSH) was determined by an enzymatic recycling procedure: the sulphydryl group of the molecule reacts with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent) (Sigma Aldrich, Saint Louis, MO, USA) producing a yellow colored 5-thio-2-nitrobenzoic acid (TNB), and the disulfide is reduced by NADPH in the presence of glutathione reductase.

Hepatic tissue was cut by medical scissors and homogenized on ice, using perchloric acid solution 5%. The homogenate was centrifuged at 10,000 g, 4°C for 10 minutes and the acid-soluble fraction was collected and neutralized with 2M KHCO3. After a centrifugation to remove the formed potassium perchlorate, the supernatant was divided to quantify both GSH and GSSG. For GSH detection, the sample was mixed with 6mM DTNB and 0.3mM NADPH. After incubation at 37°C for 3 minutes, 200 U/mL glutathione reductase was added and immediately the rate of produced TNB was read at 415 nm using the EnSpire® Multimode Plate Reader (Perkin Elmer, Waltham, MA, USA). Glutathione concentrations were calculated using appropriate standards and normalized by µg of proteins.

### 3.1.9. Histopathological analysis

For histopathological analysis, the liver was harvested and a portion of tissue immediately fixed in buffered formalin (formaldehyde 4%, NaH2PO4 4gr/L, Na2HPO4 6.5 gr/L: pH 6.8). Tissue sections were cut at a thickness of 3.5 µm and stained with Hematoxylin & Eosin (H&E) and Gömöri trichrome stain. Histology was read by a single independent pathologist, blinded to experimental design and treatment groups. Steatosis was graded (0-3), portal and lobular inflammation were scored (0-3), and ballooning was rated (0-2). Fibrosis was analyzed separately on a scale (0-4) [203]. Likewise, only for the animals of *Phase I* aortae were also collected at each checkpoint to assess the atheroma plaque formation. The isolated aorta was immersed in OCT (Optimal Cutting Temperature) embedding medium (Leica, Wetziar, GHermany) and frozen at -80°C. Serial sections (7µm thick) were cut on a cryostat and placed on gelatin-coated slides. Sections were stained with Hematoxylin and Eosin (H&E). Photomicrographs and image digitizing were performed using Olympus AX 70 (Olympus, Prague, Czech Republic) with a digital camera Pixelink PL-A642 (Vitana Corporation, Ottawa, Canada).

### 3.1.10. Collagen determination after Sirius Red staining

The collagen content of the liver was assessed by Lopez-De Leon and Rojkind colorimetric method, using Sirius Red for the staining of collagenous proteins and Fast-Green for non-

collagenous proteins. At each sacrifice, the liver was harvested and a portion of tissue immediately fixed in buffered formalin (formaldehyde 4%, NaH2PO4 4gr/L, Na2HPO4 6.5 gr/L: pH 6.8). Tissue sections were cut at a thickness of 3.5 μm and layered on glass slides. Slices were deparaffinized after incubation with xylenes (2x5 min), ethanol 100% (2x 2min), ethanol 95% (2x 2min), ethanol 70% (1x 2min), ethanol 50% (1x 2min) and distilled water (2x2min). The slices were stained with picro-Sirius red stain solution (0.1% direct red 80, 0.1% fast green FCF dissolve in 1.2% aqueous picric acid solution) and incubated for 60min. After the staining, the slides were rapidly dipped in acidified water to destain and finally quickly dehydrate and mount in xylene. The absorbance of stained Sirius red collagen was quantified after dye elution with 1 mL of 0.1% NaOH in absolute methanol (1:1). The eluted color was read immediately in a Beckman DU 640B spectrophotometer (Ramsey, MN, USA) at 540 and 605 nm, the maximal absorbance of Sirius red and Fast green FCF respectively. In order to calculate the concentration of collagen, we followed the procedure reported by Lopez-De Leon and Rojkind [204].

### 3.1.11. Tight junction detection

For immunohistochemical analysis, a portion of tissue fixed in buffered formalin was cut at a thickness of 3.5 μm. Formaldehyde fixing causes cross-linking of proteins within the tissue, maintaining tissue morphology but denaturing the epitopes recognized by the antibody[205]. Therefore, after the hydration of the samples with xylenes and ethanol, antigen retrieval was carried out before staining sections to unmask hidden or denatured target epitopes. The procedure was performed following the heat-induced technique in a microwaveable vessel, using citrate buffer pH 6 solution. The solution was put in the microwave to boil, and immediately after the slices were placed inside. From this point samples were heated for 20 min, maintaining the correct temperature for the antigen retrieval.

When 20 min was elapsed, the samples were brought to room temperature in order to allow the re-formation of the antigenic site after being exposed to high temperature.

After the antigen retrieval, a blocking of endogenous peroxidase with $H_2O_2$ was performed for 20 min in order to reduce background staining due to HRP conjugated antibody used for the detection. Some epitopes could be modified by peroxide, leading to reduce antibody-antigen binding and so the incubation of sections with peroxide could be performed after the use of the primary antibody to avoid this problem. Finally, the slices were washed 2x5 min in TBS plus 0.05% Tween20 (T-TBS) with gentle agitation. 0.05% Tween20 in the TBS reduces surface tension,

allowing reagents to cover the whole tissue section with ease. It is also believed to dissolve Fc receptors and reduce non-specific binding. TBS over PBS is recommended to get a cleaner background. The secondary antibody may cross-react with endogenous immunoglobulins in the tissue. It is necessary to block eventual non-specific sites in order to minimize this risk by pre-treating the tissue with normal serum from the species in which the secondary was raised. Slides were drained for a few seconds after the washes and the glasses were wiped around the sections with tissue paper. A pap-pen was used to draw a barrier on glass slice around the sample to confine the flow of reagents to a defined area. Blocking was performed in 10% normal horse serum in TBS-0.05% Tween20 for 2h at room temperature (RT). At the end, slides were drained for a few seconds and samples were incubated at 4°C overnight with primary antibodies diluted in T-TBS with 2.5% of horse serum. The antibodies used were: ZO-1 Antibody (#PA5-28858; 1:100; Invitrogen, Carlsbad, CA, USA) and Claudin-5 Antibody 4C3C2 (#35-2500; 1:300; ThermoFisher, Waltham, MA, USA) Immunostaining was conducted using the DAB kit. The sections were then followed by hematoxylin staining, dehydrated, and mounted. No specific immunostaining was seen in sections incubated with PBS rather than the primary antibody (data not shown).

### 3.1.12. Statistics

Values are reported as the mean ± standard deviation (SD) of HFHC animals (5-8 mice) *vs*. sex and age-matched controls (3-5 mice). Statistical comparisons were performed using one-way ANOVA and post-hoc Tukey-Kramer's test. A p-value of $<0.05$ was considered statistically significant.

## 3.2. TASK 2 ~ALD

### 3.2.1. Study design and experimental models analyzed

Currently is still unclear the cellular source of MIF, to fill this gap in this regards different experimental models were used. Firstly, the interaction between hepatocytes and macrophages upon co-culturing condition was analyzed in an in vitro co-culture model that recreates the pathophysiology of ALD, in order to confirm the participation of MIF in this cellular cross-talk. Then, and to better explain its source, WT and *Mif-/-* mice have been used in an in vivo model of chronic ethanol feeding. Finally, human serum samples and liver biopsy of alcoholic hepatitis patients were analyzed to confirm the in vitro and in vivo findings, in order to provide a direct translation from the bench to the bedside.

### 3.2.2. *In vitro* model.

#### a. Cell lines and established cell cultures

*HuH7* is a well-differentiated hepatocyte-derived carcinoma cell line, obtained from Japan Health Science Research Resources Bank (HSRRB, JCRB0403). HuH7 cells were maintained in culture in Dulbecco's modified Eagle's high glucose Medium (DMEM) (Euroclone, Pero, MI, Italy) supplemented with 10% fetal bovine serum (FBS), 10,000 U/ml penicillin, 10 mg/ml streptomycin and 2 mM L-glutamine at 37°C, 5% $CO_2$, in a 95% humidified atmosphere.

*THP-1* is a monocyte-derived cellular carcinoma cell line, obtained from Japan Health Science Research Resources Bank (HSRRB, JCRB0403). Cells were grown in suspension in RPMI-1640 Medium (Sigma Aldrich, Saint Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 10,000 U/ml penicillin, 10 mg/ml streptomycin and 2 mM L-glutamine at the same culture conditions described above.

*Co-culture set-up:* THP-1 cells were differentiated to macrophages on 0.4 μm-membrane of transwell inserts (BD Falcon, Bedford, MA, USA) by treatment with 100 nM phorbol 12-myristate 13-acetate (PMA) for 48 hours[206]. After differentiation, inserts with the adherent macrophages were transferred to a 6-well plate containing HuH7 cells. After 24h incubation, cells were used for the experiment.


**Fig. 3.2**. **Co-culture system**. (**A**)Transwell inserts used (BD Falcon, Bedford, MA). (**B**) Graphical representation of co-culture.

### b. Cell treatments

Cells were treated as follows. a) HuH7 monoculture was challenged with LPS and EtOH. Untreated cells were used as control. b) THP1 differentiated to macrophages were treated with LPS and EtOH. c) Cells under co-culturing conditions were exposed to EtOH 50mM. Dosage and time applied for the different treatments are deeply described in the figure legends.

## 3.2.3. *In vivo* model

### a. Animals

Female C57BL/6 (WT) mice were purchased from Jackson Laboratories (Bar Harbor, ME). *Mif* -/- mice on a C57BL/6 background were obtained from Dr. R. Bucala (Yale University, New Haven, Connecticut)[207] and a breeding colony was established at Cleveland Clinic Foundation (CCF).All procedures using animals were approved by the Cleveland Clinic Institutional Animal Care and Use Committee.

### b. Mouse model of chronic ethanol exposure to bone marrow transplanted mice

Bone marrow chimeras were generated as previously described [208] using female WT and *Mif* -/- mice (Fig. 3.3). Four weeks post-transplant, chimeras were dosed with clodronate-containing liposomes (Encapsula NanoSciences, Nashville, TN, USA SKU # 8909) to deplete resident macrophages in the liver as previously described[209]. Seven days post-clodronate, chimeras were placed on a chronic ethanol feeding diet, as previously described[210].Briefly, mice were housed in shoe box cages (two animals per cage) with microisolator lids. Mice were weight-matched, then randomized into ethanol-fed and pair-fed groups. All mice were acclimated to the liquid control diet for two days and then allowed free access to ethanol diet or pair-fed control diets that

isocalorically-substituted maltose dextrins for ethanol. The ethanol content of the diet was increased over time: 1% (vol/ vol) ethanol for 2 days followed by 2% ethanol for 2 days, 4% ethanol for 1 week, 5% ethanol for 1 week, and 6% ethanol for a final week. At the termination of the study, mice were anesthetized, and tissue and blood samples collected as previously described [210]. Mouse body weight and food intakes are shown in Table 3.3.

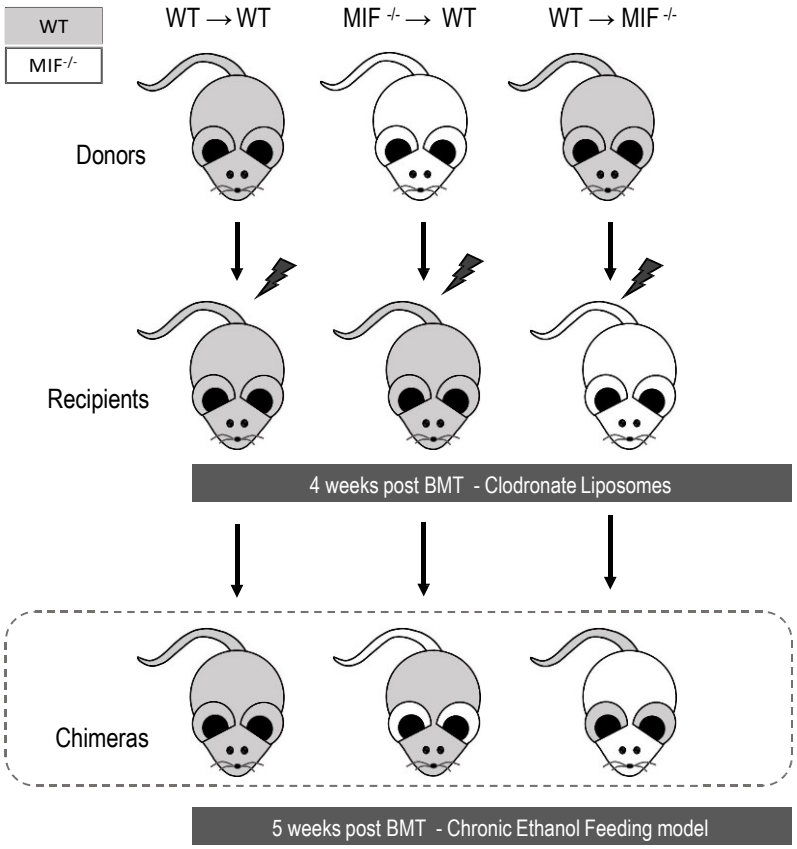


**Fig. 3.3**. **Graphical representation of bone marrow (BMT) model generated.** Bone marrow chimeras were created by lethally irradiating 5-week-old female WT and *Mif* -/- mice. Donor marrow was isolated from 10-week-old mice. 4 weeks post-transplant, chimeras were dosed with clodronate-containing liposomes. Seven days post-clodronate, chimeras started the chronic ethanol feeding paradigm

| Transplant type Donor → Recipient | Pair-Fed | | EtOH-Fed | | EtOH- Fed |
|---|---|---|---|---|---|
| | Initial body mass (g) | Final body mass (g) | Initial body mass (g) | Final body mass (g) | Average daily food intake (mL/cage) |
| **WT → WT** | 17.1 ± 0.4 | 19.8 ± 0.4 | 17.3 ± 0.4 | 19.1 ± 0.4 | 17.6 ± 0.1 |
| **WT → *Mif -/-*** | 16.0 ± 0.5 | 20.7 ± 0.6 | 15.9 ± 0.3 | 17.9 ± 0.1 | 15.0 ± 0.3 |
| ***Mif -/-*→ WT** | 16.6 ± 0.3 | 19.0 ± 0.6 | 16.9 ± 0.2 | 18.5 ± 0.4 | 15.3 ± 0.6 |

**Table 3.3**. Mouse body weight and food intakes

### c. Plasma ALT and AST measurements and liver triglycerides

Plasma alanine aminotransferase (ALT) and plasma aspartate aminotransferase (AST) assay kits were purchased from Sekisui Diagnostics (Framingham, MA, USA). Triglyceride assay kits were purchased from Pointe Scientific Inc. (Lincoln Park, MI, USA).

## 3.2.4. Patients

This study included patients admitted to the Liver Unit of the Hospital Clínic, Barcelona, between January 2000 and September 2007 with clinical, analytic, and histologic features of ALD (patient baseline characteristics are reported in Table 3.5). Inclusion criteria for patients with AH were as follows: patients with active alcohol abuse were defined according to the Diagnostic and Statistical Manual of Mental Disorders IV and excessive ethanol consumption (>60 g/day) for at least 3 months before admission; increased aminotransferase levels (aspartate aminotransferase [AST] > alanine aminotransferase [ALT]), high γ-glutamyl transpeptidase and bilirubin serum levels, and histologic diagnosis of AH characterized by the presence of hepatocellular damage (hepatocellular ballooning and presence of Mallory bodies), inflammatory infiltrate (neutrophils), and pericellular fibrosis. Patients with hepatocellular carcinoma or any other potential cause of liver disease were excluded from the study. A total of 66 patients with AH, 55 patients with decompensated alcoholic cirrhosis without superimposed AH, 36 patients with compensated alcoholic cirrhosis patients without active alcohol intake for at least 6 months. Liver biopsy was obtained using a transjugular approach because most patients with AH have severe coagulation disorders. Serum and plasma were also obtained at the time biopsy. The transjugular approach also allowed for measures of the portal pressure gradient. All patients received nutritional and psychological support for achieving alcohol abstinence for at least 3 months before admission. The

study was approved by the Ethics Committee of the Hospital Clínic of Barcelona and all patients gave informed consent

| **Characteristics** | **Median (25-75 IQR)** |
|---|---|
| Age (years) | 52(46-56) |
| Male n (%) | 53(78) |
| Alcohol Consumption (g/day) | 100(80-160) |
| **Laboratory and hemodynamic parameters** | |
| Hemoglobin (g/dL) | 11.4(9.9-12.8) |
| Leukocyte count $x10^9$ /L | 8.5(6.3-12.5) |
| Platelet count $x10^9$/L | 112(77-199) |
| AST ( U/L) | 117(67-156) |
| ALT (U/L) | 39(24-60) |
| Serum albumin (g/dL) | 2.6(2.3-3.1) |
| Serum creatinine (mg/dL) | 0.9(0.7-1.1) |
| Serum bilirubin(mg/dL) | 6.2(2.7-19.3) |
| International normalized ratio | 1.6(1.4-1.8) |
| **Alcoholic hepatitis severity scores at admission** | |
| MELD score | 19(14-25) |
| ABIC score | 7.83(6.69-8.66) |

**Table 3.4**. Patient baseline characteristics

### 3.2.5. RNA isolation and quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

*Cells/mouse liver:* RNA was isolated from cells or whole liver tissue at the end of the treatment using RNeasy Mini kits per the manufacturer's instructions (Qiagen, Germantown, MD, USA). 2- 4µg of RNA was reverse transcribed and analyzed with Power SYBR qRT-PCR kits (Applied Biosystems, Foster City, CA) on a Mx3000p analyzer (Stratagene, La Jolla, CA, USA). Relative messenger RNA (mRNA) expression was determined using gene-specific primers (Table 3.6). Statistical analyses were performed on the ΔCt values (average Ct of the gene of interest – average Ct of 18S)[199,200].

*Human liver:* RNA was extracted using TRIzol reagent (Invitrogen, Life Technologies). Five hundred nanograms of total RNA were retro-transcribed and 200 ng of cDNA were then amplified (Applied Biosystems Foster City, CA) in a final PCR volume of 10 µl using a StepOnePlus instrument (Applied Biosystems Foster City, CA). TaqMan® Gene Expression Assay primers of MIF (Catalog number: 4331182, ID: Hs00236988_g1) were provided by Applied Biosystems. Results were normalized to 18SrRNA expression and gene expression values were calculated based on the ΔΔCt method.

| | | **Forward** | **Reverse** |
|---|---|---|---|
| **Human** | TNF-α | GTGAGGAGGACGAACATC | GAGCCAGAAGAGGTTGAG |
| | MIF | GGACAGGGTCTACATCAACTA | TCTTAGGCGAAGGTGGAG |
| **Mouse** | MCP-1 | AGGTCCCTGTCATGCTTCTG | TCTGGACCCATTCCTTCTTG |
| | CXCL10 | CCAAGTGCTGCCGTCATTTTC | GGCTCGCAGGGATGATTTCAA |
| | CXCL1 | TGCACCCAAACCGAAGTC | GTCAGAAGCCAGCGTTCACC |
| | CXCL2 | GCGCCCAGACAGAAGTCATAG | AGCCTTGCCTTTGTTCAGTATC |
| | Ly6C | GCAGTGCTACGAGTGCTATGG | ACTGACGGGTCTTTAGTTTCCTT |
| | F4/80 | CCCCAGTGTCCTTACAGAGTG | GTG CCCAGAGTGGATGTCT |

**Table 3.5**. Primer pair sequences.

### 3.2.6. Cytokine release analysis

Cell supernatant: ELISA was performed in the supernatants collected after ethanol treatment, in order to determine the amount of released MIF. The experiment was performed following manufacture's instructions. Quantikine Human MIF ELISA Kit was used (R&D System; Minneapolis, MN).

Human serum: Serum samples were obtained and stored at −80°C. MIF serum levels were measured in peripheral serum of patients with mild AH (n = 46), severe AH (n = 48), compensated alcoholic cirrhosis (n = 23) and decompensated alcoholic cirrhosis (n = 42). MIF serum level was also measured in suprahepatic serum of patients with mild AH (n = 15), severe AH (n = 42), compensated alcoholic cirrhosis (n = 20) and decompensated alcoholic cirrhosis (n = 21). For the analysis, Quantikine Human MIF Immunoassay Kit (R&D Systems, Minneapolis, MN) was used.

Briefly, a monoclonal antibody for human MIF has been pre-coated onto different microplates. Standards and samples were pipetted into the wells and any MIF and TNF- α present were bound by the immobilized antibodies. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human MIF and TNF- α was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of MIF and TNF- α bound in the initial step. The color development was stopped and the intensity of the optical density was measured within 30 minutes, using EnSpire® Multimode Plate Reader (Perkin Elmer) set to 450nm. Wavelength was corrected subtracting readings at 540nm or 570nm, corresponding to plate imperfections.

### 3.2.7. Histological analysis of ethanol-induced liver damage (H&E and TUNEL staining)

Staining for H&E was performed in paraffin-embedded mouse liver sections. Slides were coded and at least 3 images were acquired per tissue section. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) positive staining in liver was analyzed using ApopTag plus In Situ Apoptosis Detection Kit (S7111, Millipore, Billerica, MA). Semiquantification of positive staining was performed using ImagePro Plus software. IHC for MIF was conducted in liver specimens from patients with AH and subjects with healthy livers using the Bond fully-automated slide staining system (Leica Microsystems). Slides are dewaxed in Bond Dewax solution and hydrated in Bond Wash solution. Heat-induced antigen retrieval was performed for 30min at 100°C in Bond-Epitope Retrieval solution. Antigen retrieval was followed with 5min Bond peroxide blocking step and then incubation with primary antibody (MIF, Santa Cruz Biotechnology, Cat# sc-20121) followed with Bond polymer. Chromogenic detection of all antibodies was performed using the Bond Polymer Refine Detection System. Positive and negative controls (no primary antibody or normal serum) were included for each run.

### 3.2.8. Statistics

Values are reported as means ± standard error of the mean (SEM). Data were analyzed by analysis of variance (ANOVA) using general linear models procedure (SAS, Cary, NC). If data were not normally distributed, data were log transformed. Multiple comparisons were analyzed using least square means. For human data, correlations between variables were evaluated using Spearman's rho or Pearson's r, when appropriate.

# Chapter 4
# Results

# 4. Results

## 4.1. NAFLD: juvenile mouse model characterization and possible therapeutic strategies.

### 4.1.1. HFHC diet increases body, liver, and adipose tissue weight in both genders.

HFHC diet induced a significant increment in body weight since the first week of feeding both in males and females ($p<0.001$) (Fig 4.2A). The trend of increase was maintained significantly higher in HFHC groups *versus* controls until the end of the trial. The BMI denoted a marked overweight (ratio ≥ 1.25, based on human data) from week 4 in males (HFHC BMI: 4.0±0.04 *vs*. CTRL BMI: 3.2±0.36, $p=0.06$), and an obese phenotype (ratio ≥ 1.39) at week 12 when the folds of increase of BMI *vs.* control were 1.43 ($p<0.001$) (Fig. 4.2.B). On the other hand, HFHC females showed delayed (after week 12) signs of overweight *vs*. CTRL (HFHC BMI 3.9±0.52 *vs*. CTRL BMI: 3.2±0.23) and a significant obesity at week 16 (1.46-fold of BMI increase *vs*. CTRL ($p<0.001$)). The body weight gain was accompanied also by a progressive increase in the liver weight, which become significantly relevant in males after 12 weeks (1.6±0.2 folds *vs*. CTRL, $p<0.05$) and in females at week 16 (1.5±0.1 folds *vs* CTRL, $p<0.001$) (Fig. 4.2.C). The hypertrophy of epididymal fat (as an indirect measurement of central adiposity) showed a sustained and progressive increase in males with statistic relevant differences from week 8 (4.7±0.6 folds *vs*. CTRL, $p<0.001$). On the contrary, in females, such difference was evident only after 16 weeks (3.2±0.3 folds *vs*. CTRL, $p<0.001$) (Fig. 4.2.D). The representative panel reported below shows the evident difference of adipose tissue accumulation during the time between HFHC males and females (Fig. 3.1).

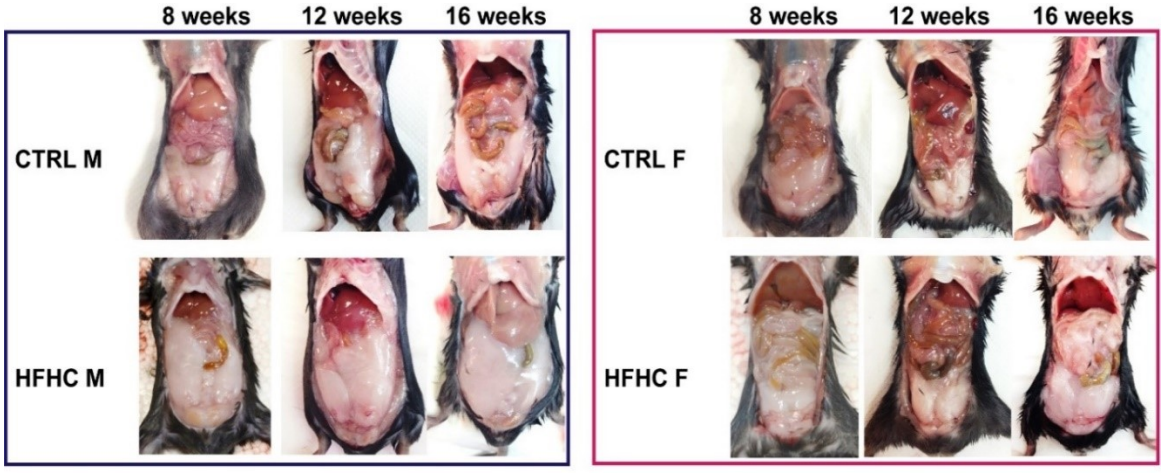


**Fig. 4.1**. **Epidydimal adipose tissue accumulation**.

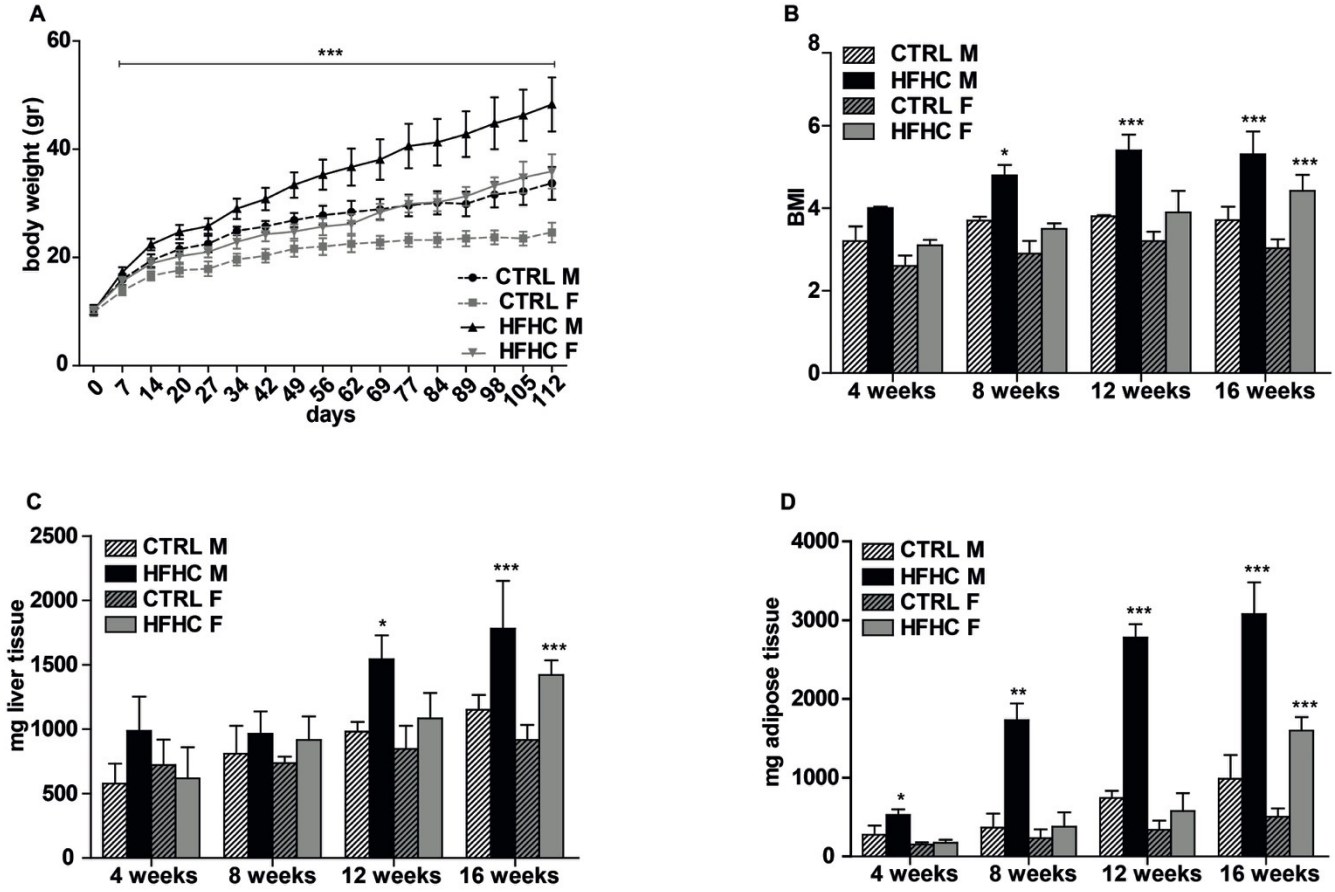


**Fig. 4.2. Representation of the macroscopic parameters.** (**A**) Weekly body weight increase during the experimental time course. (**B**) BMI. (**C**) Liver weight. (**D**) Epididymal fat pads weight. Data are expressed as mean ± S.D. Statistical significance was calculated vs. control. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

### 4.1.2. Changes in life habit strongly improve macroscopical parameters.

Hypercaloric diet determined an increase in the macroscopical parameters under study. It has been observed hepatomegaly and adipose tissue hypertrophy. After 8 weeks of HFHC feeding, a group of animals switched to control-fed diet (HFHC →CTRL mice), followed for further 12 weeks. HFHC→CTRL mice showed immediately a strong reduction in body weight, progressively maintained over the time, with a similar trend in both males and females (Fig. 4.3 A-B). Silymarin administration determined a reduction of body weight in females (Fig. 4.3 B). On the contrary, male mice continued to follow the same behavior reported by HFHC mice (Fig. 4.3 A).

Furthermore, HFHC →CTRL groups showed a reduction in hepatomegaly and fat pad hypertrophy in both genders. Instead, Silymarin did not provide any beneficial effects (Fig. 4.3 C-D).

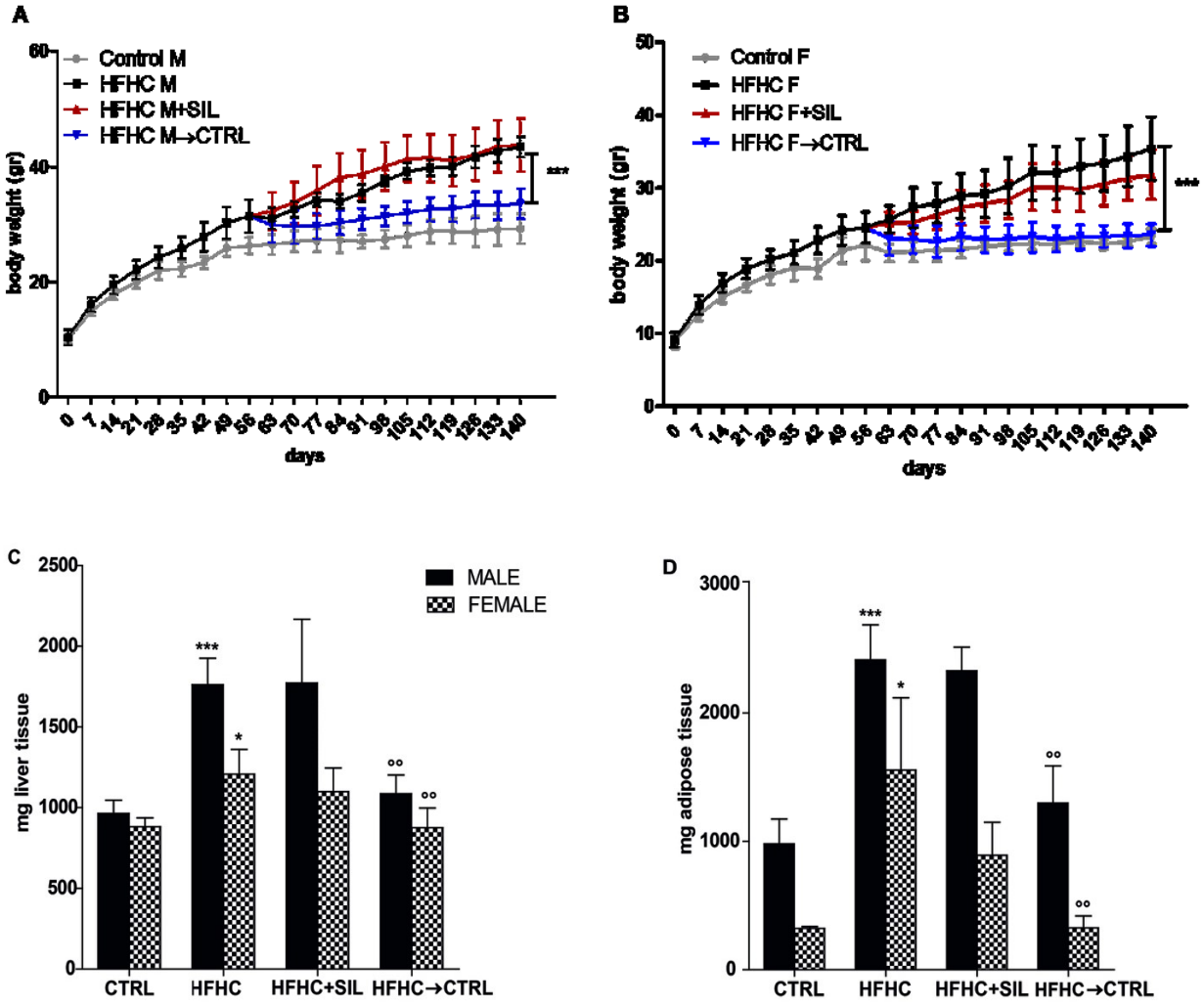


**Fig. 4.3. Effects of healthy diet and Silymarin on macroscopical parameters.** (**A**) Healthy diet reduced body weight in males. (**B**) Both healthy diet and Silymarin administration were able in reducing body weight in females. (**C**) Effects of a healthy diet in hepatomegaly and in (**D**) adipose tissue weight. * p<0.05, ** p<0.01, *** p<0.001vs. CTRL; °° p<0.01vs. HFHC.

### 4.1.3. HFHC diet induces hyperglycemia in both genders but insulin resistance only in males.

HFHC diet-induced hyperglycemia in both genders, starting from week 12 (Table 4.1) and at the same time, increased insulinemia was reported in males. On the contrary, females never displayed any change in this parameter (Table 4.1). HOMA-IR index (Fig. 4.4 A) mirrored this

trend, increasing since week 12 only in HFHC males (2.8±0.5 folds, $p<0.01$), with a further enhanced at week 16 (13.8±7.1 folds, $p<0.001$). Conversely, females never developed insulin resistance (Fig. 4.4 B).

| | HFHC MALE | | | | HFHC FEMALE | | | |
|---|---|---|---|---|---|---|---|---|
| **Parameters** | **4 weeks** | **8 weeks** | **12 weeks** | **16 weeks** | **4 weeks** | **8 weeks** | **12 weeks** | **16 weeks** |
| **Glucose (mg/dL)** | 241.5±16.6 | 203.5± 23.8 | 244.0± 36.0 [c] | 279.7± 49.4 [c] | 180.7± 24.4 | 159.2± 30.2 | 193.8± 20.4 | 201.6± 32.6 [c] |
| **Insulin (µU/mL)** | 3.6±1.3 | 6.0±3.5 | 10.3±2.2 [b] | 56.9±28.9 [c] | 1,4±0.2 | 1.5±0.27 | 2.5±1.3 | 2.3±0.9 |

**Table 4.1.** Glucose and insulin alterations due to hypercaloric diet in males and females over the time. [b] $p<0.01$, [c] $p<0.001$ *vs*. CTRL .

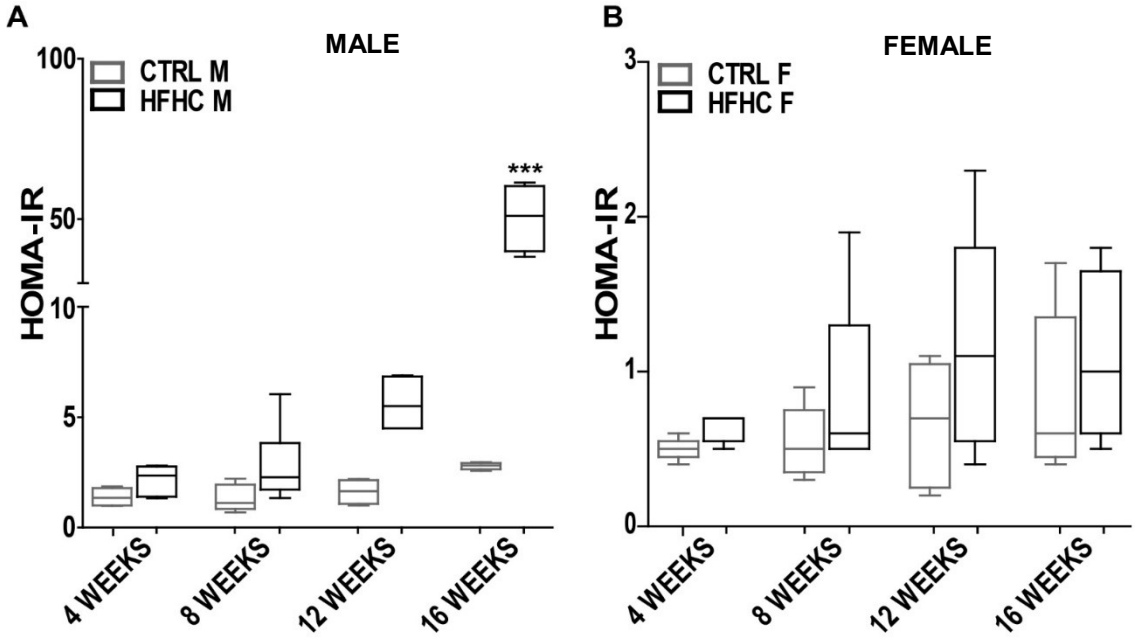


**Fig. 4.4**. HOMA index as a marker of insulin resistance in (**A**) males and (**B**) females over the time. *** $p<0.001$ *vs*. CTRL.

### 4.1.4. Healthy diet restored normal glycemia and insulinemia improving HOMA- IR. Silymarin reduced hyperglycemia in males.

HFHC diet determined an alteration of glycemia in both genders, increasing insulinemia only in males (Table 4.2). Silymarin administration reduced serum glucose levels in males, without improvements of insulinemia and HOMA index (Table 4.2, Fig. 4.5 A). No effects were reported in females (Table 4.2, Fig. 4.5 B). Diet switching had the most effective activity, decreasing all the parameters under study.

| Parameters | MALE | | | | FEMALE | | | |
|---|---|---|---|---|---|---|---|---|
| | CTRL | HFHC | HFHC+Sil | HFHC→CTRL | CTRL | HFHC | HFHC+Sil | HFHC→CTRL |
| Glycaemia (mg/dl) | 164±20 | 260±24*** | 226 ± 17 ° | 167 ±13 °° | 141±10 | 193±25** | 181±31 | 134±6 °°° |
| Insulinemia µU/ml | 1.9±0.4 | 4.1±1.1** | 3.6±1.3 | 2.0±1.2 °° | 0.9±0.1 | 2.5±1.7 | 2.1±0.5 | 1.3±0.3 ° |

**Table 4.2**. Glucose and insulin levels after Silymarin administration (HFHC+Sil) and lifestyle change (HFHC→CTRL) in males and females. ** p<0.01, *** p<0.001 *vs.* CTRL. ° p<0.05, °° p<0.01,°°° p<0.001 *vs.* HFHC.

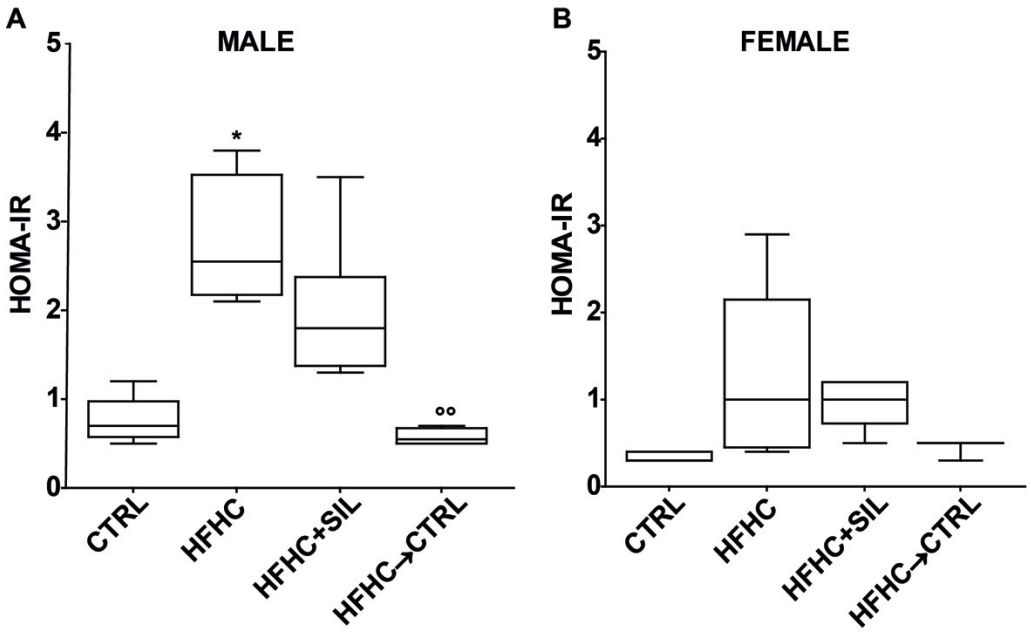


**Fig. 4.5**. **Treatment effects on HOMA-IR**. (**A**) Healthy diet improved insulin resistance in males. (**B**) No alterations were displayed in females. * p<0.05 *vs*. CTRL; °° p<0.01 *vs*. HFHC.

### 4.1.5. Serum lipid profile and transaminases were altered in males since the beginning. Same extent of increase was observed in females after 16 weeks of diet.

The lipid profile showed a different behavior between HFHC males and females. In males, total cholesterol, HDL, and LDL levels rose steadily from week 4 to week 16, whereas in females a significant variation of these parameters was observed only at week 16 (Fig. 4.6.A-B-C). HFHC male reported abnormal levels of serum ALT activity since week 12 ($p<0.05$, Fig.4.6.D) that increased thereafter (16weeks: $p<0.001$). On the contrary, females had a peak in ALT only at week 16 ($p<0.05$).

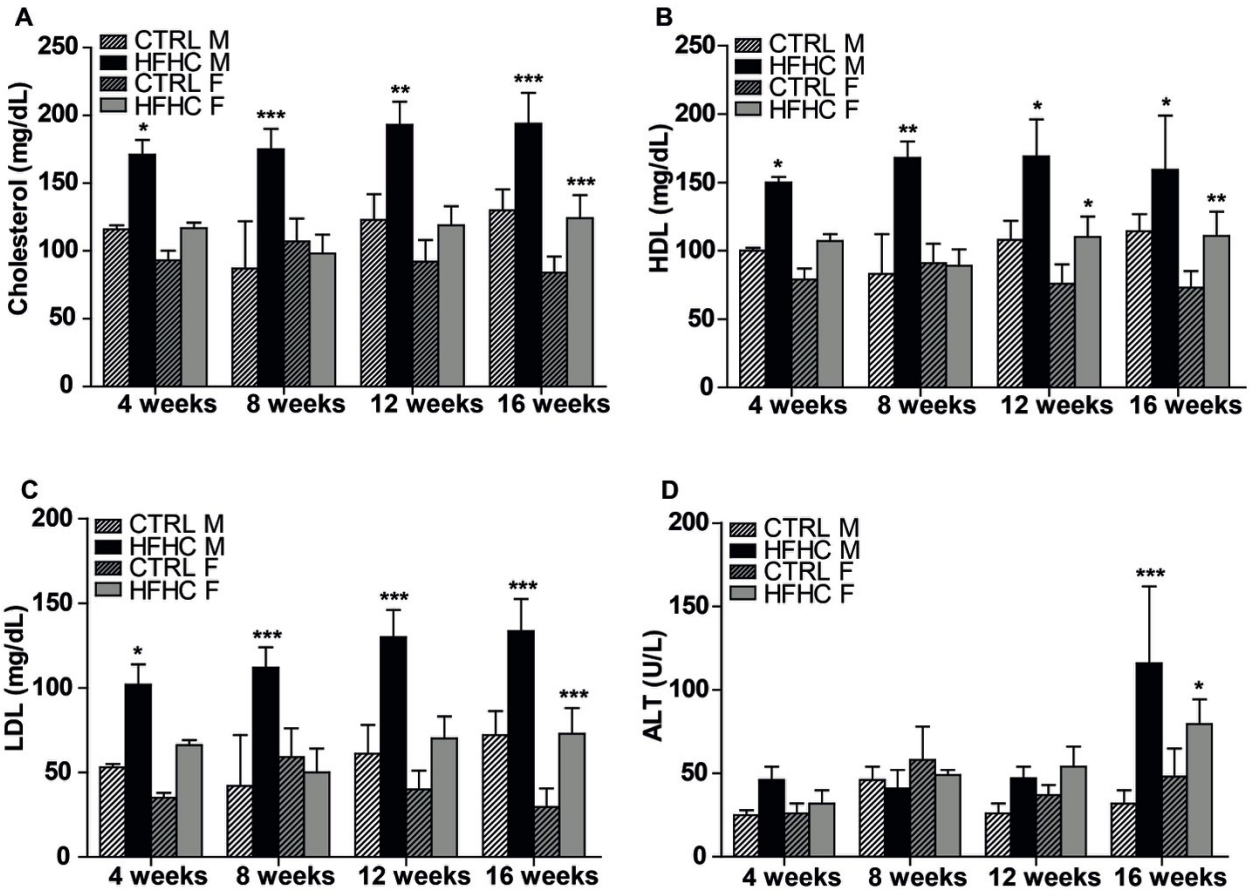


**Fig. 4.6**. **Serum lipid profile and ALT**. HFHC males and females reported a different trend of alteration for (**A**) cholesterol, (**B**) HDL, (**C**) LDL and (**D**) ALT over the time.* $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs*. CTRL.

### 4.1.6. Serum lipidimia and ALT as marker of liver injury were reduced with diet switching and Silymarin administration.

In HFHC groups, total cholesterol, HDL, and LDL levels were increased as well as ALT. Diet switching determined a reduction of all parameters under study, restoring the control profile (Fig. 4.7 A-D). Silymarin demonstrated to be effective in decreasing HDL and ALT in males (Fig. 4.7 B, D) and LDL in both genders (Fig. 4.7 C) No effects were observed in serum total cholesterol level (Fig. 4.7 A).

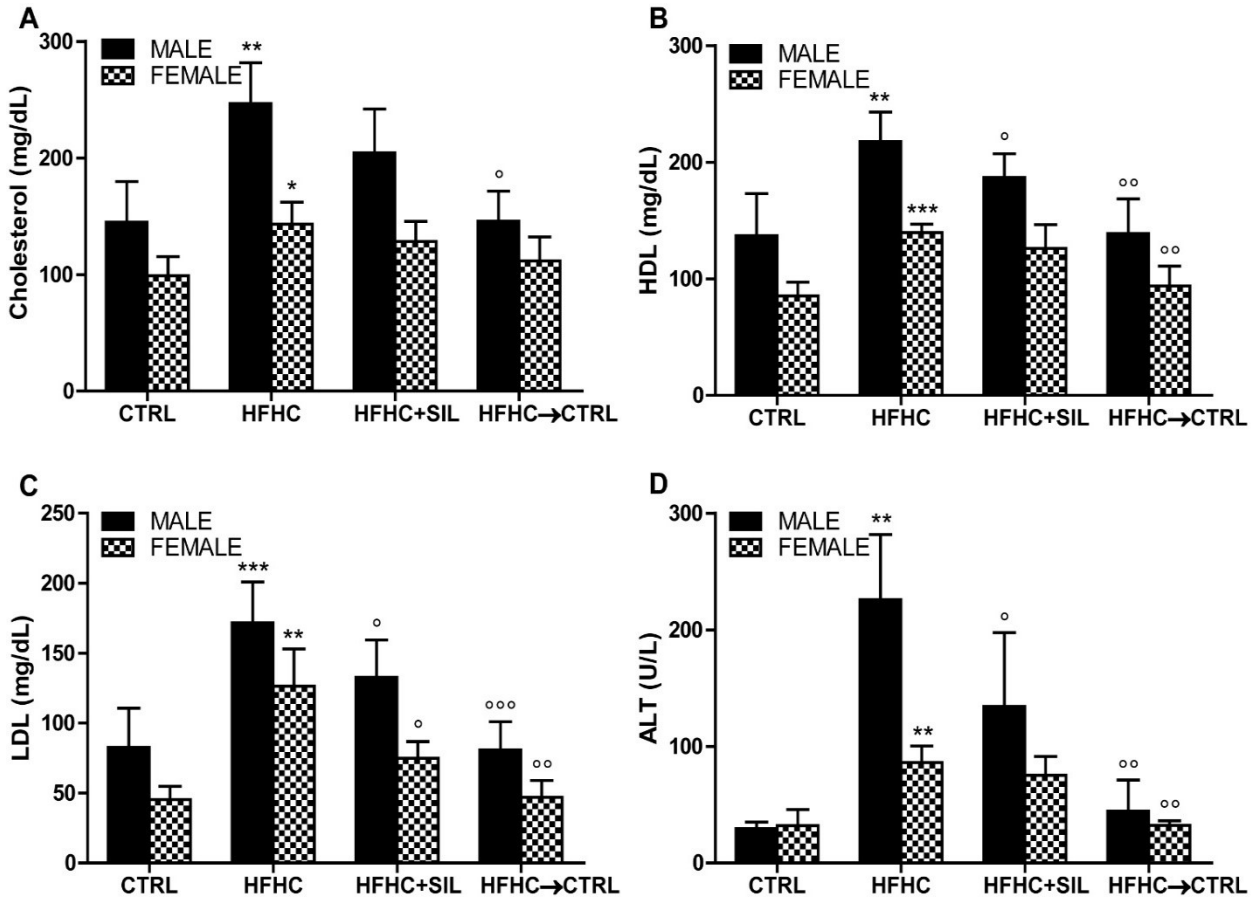


**Fig. 4.7**. **Effects of therapeutic approaches on serum lipid profile and ALT**. * p<0.05, ** p<0.01, *** p<0.001vs. CTRL; ° p<0.05, °° p<0.01, °°° p<0.001 vs. HFHC.

### 4.1.7. HFHC diet-induced steatosis and fibrosis in males and females.

Histological examination of the liver (Fig. 4.8 a-j) showed that HFHC diet induced a progressive macro-microvesicular steatosis in males by week 8 (score 2 for 66% of mice, zone 1)

reaching score 3 in 100% of animals at week 16 (location paracinar). Same trend was reported for HFHC females with a predominant azonal localization. The presence of lobular and portal inflammatory foci was more evident in males with a trend of increase over the time, whereas in females the presence of inflammatory foci was not relevant during all the trial. Interestingly, both genders showed a comparable and progressive induction of portal and periportal fibrosis from week 8$^{th}$ at evolved towards perisinusoidal and portal/ periportal fibrosis at week 16 (86% of males and 71% of females) (Fig. 4.8.k-o). The presence of ballooning injury was rare, observed only in two male mice at week 16. The presence of Mallory-Denk bodies was not found at any experimental checkpoint. The analysis of aorta samples did not reveal any sign of morphological/ atherogenic change (Fig. 4.8p, q).

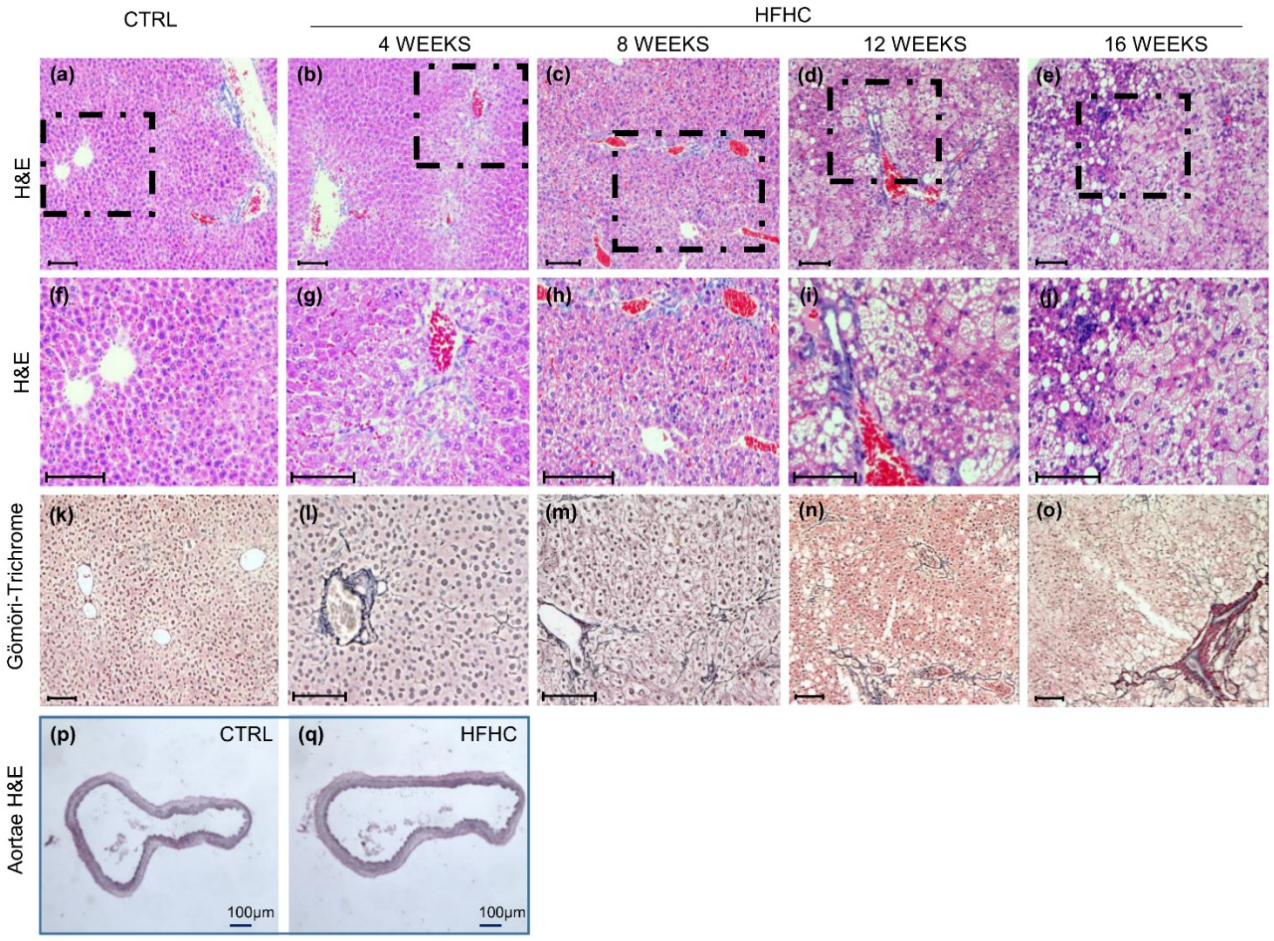


**Fig. 4.8. Hepatic histological analysis.** (**a-j**) Representative H&E staining images of NAFLD progression over the time *vs*. CTRL (**a, f).** (**f-j**) Higher magnification of the dashed area**.** (**k-o**) Gömöri trichrome staining for collagen staining of NAFLD over the time *vs*. CTRL. **(p, q)** Representative picture of Hematoxylin & Eosin staining in aortae of controls **(p)** and HFHC mice **(q).** In all pictures, scale bar is 100 µm.

### 4.1.8. Biomolecular analysis confirmed HFHC diet-induced damage showed by histology

The histological diagnosis was further confirmed through other biomolecular assays. Specifically, liver slices were stained with Sirius Red and Fast Green dyes in order to detect collagen fibers. The representative panel reported in Fig. 4.10 shows the progression of fibrosis, confirmed by staining quantification (Fig. 4.11). In line with this, HFHC-fed mice displayed a significant increase in the expression of Col1A1 (Fig. 4.9 D). Moreover, the microscopical observation of the paraffin-embedded liver samples indicated an evident damage increase highlighted by the presence of altered hepatocytes, the loss of cellular inner integrity and the enhanced accumulation of hepatic lipid droplets. Accordingly, since week 4 both HFHC groups displayed a higher mRNA expression of DGAT2, a gene involved in the last step of triglyceride synthesis (Fig. 4.9 A). Moreover, MCP1 and TNF-α were quantified as markers of inflammation. In line with the histological score, the mRNA expression of these genes was increased in male mice, instead, no changes were observed in females (Fig. 4.9 B, C).

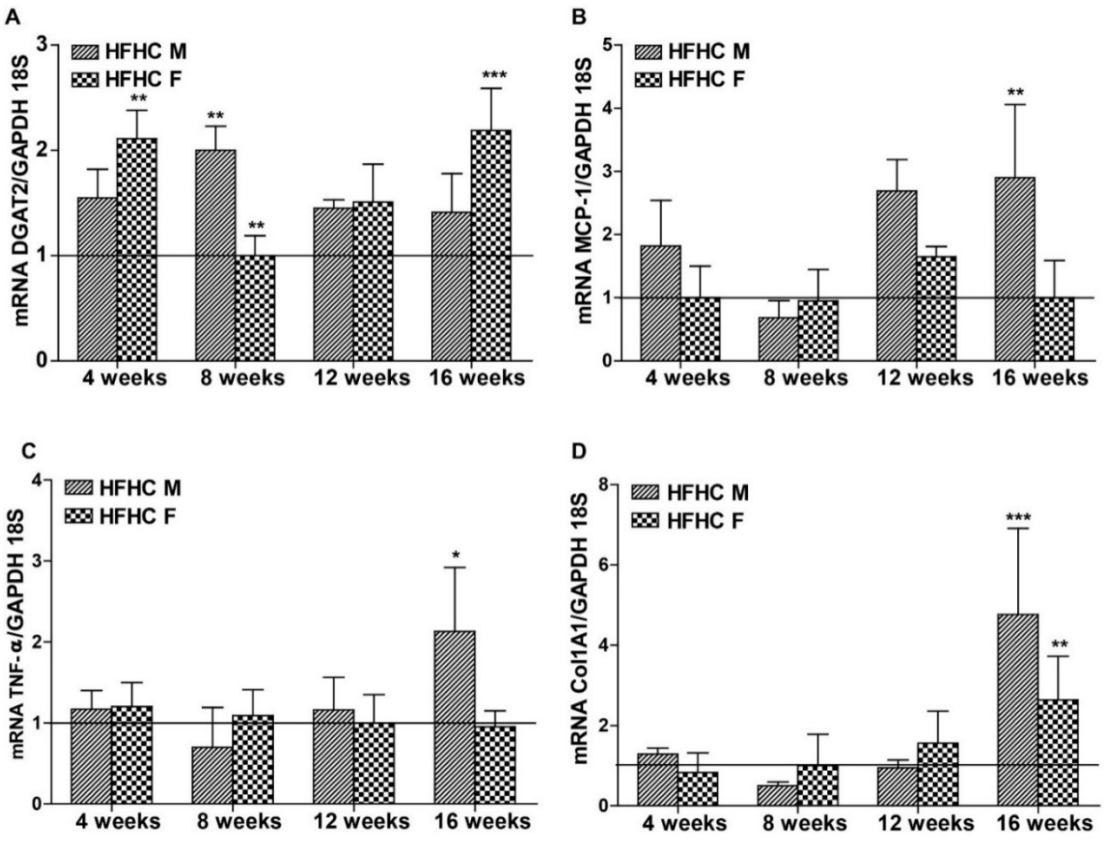


**Fig. 4.9**. Gene expression of (**A**) DGAT2, (**B**) MCP-1, (**C**) TNF-α and (**D**) Col1A1. * p<0.05, ** p<0.01, *** p<0.001 *vs*. CTRL.

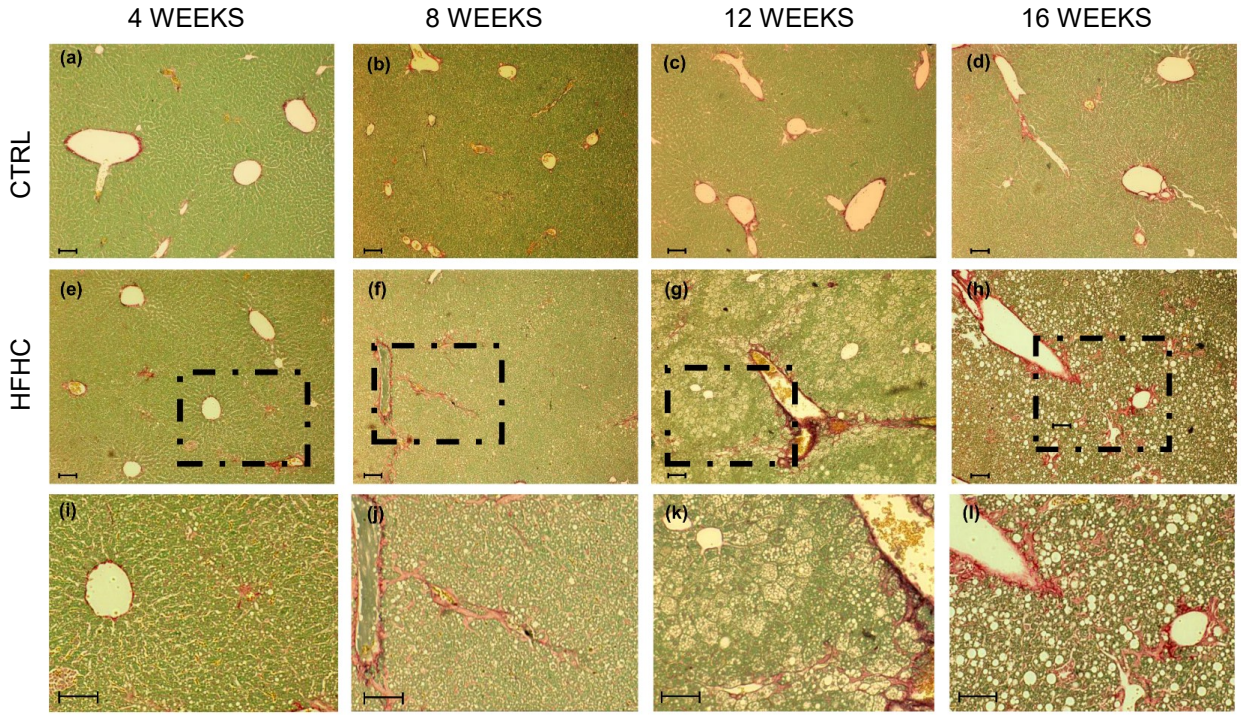


**Fig. 4.10. Sirius red fast green staining.** Representative images of fibrosis progression in HFHC mice (**e-h**) over the time *vs.* CTRL (**a, d**). (**i-l**) Higher magnifications of the dashed area. In red collagen fibers, in green non-collagenous protein. In all pictures, scale bar is 250 μm.

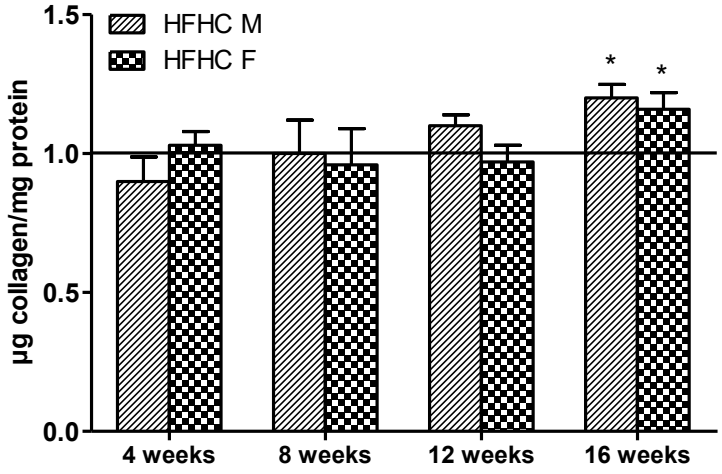


**Fig. 4.11**. Quantification of Sirius red and fast green staining in HFHC males and females. * $p<0.05$ *vs*. CTRL

### 4.1.9. Diet induces an improvement of the histological profile. Silymarin exerts antifibrotic properties

Diet switch determined a general improvement of the histopathological scenario. A reduction of steatosis was observed both in males (50% grade 1; 50% grade 2) and females (75% grade 1; 25% grade 2) (Fig. 4.12 A). Silymarin administration demonstrated a slight improvement of steatosis, reaching score 2 in the majority of HFHC mice. In line with this, both diet switch and Silymarin reverted the DGAT mRNA alteration induced by HFHC feeding in females (Fig. 4.13 A). Inflammation was predominant in males, with lobular localization. Both diet and Silymarin did not change inflammatory infiltration, maintaining the same profile of mice fed with hypercaloric diet. MCP-1 gene expression was enhanced in HFHC males, instead, no increase was observed in females (Fig. 4.13 B). Silymarin reduced this alteration and the healthy diet completely restored a normal condition. On the contrary, TNF-α displayed a different behavior in comparison with the 1$^{st}$ phase, with an increased mRNA expression only in HFHC females and without any significant improvement given by therapeutic approaches (Fig. 4.13 C). Even if serum parameters demonstrated a different trend of alteration in HFHC males and females during the time, at the end, both genders showed the same pathological outcome reaching stage 2 of fibrosis. Lifestyle changes (diet) was able to decrease fibrosis extent that reached stage 1 in males (1a 50%; 1b 25%) and females (1b 75%). It is interesting to underline that a complete resolution of the fibrotic damage was observed in two mice. Silymarin demonstrated antifibrotic properties with a similar pattern in both genders, improving fibrosis score to 1a (50%) and 1c (25%) in male and females (1a 83.3%; 1b 16.6%) (Fig. 4.12 A, B). Interestingly, one male mouse reported a complete damage reversion. In line with these results, Col1A1 mRNA expression was highly increased by hypercaloric diet and effectively reduced by lifestyle change in both genders. In females, Silymarin administration exerted the same effects (Fig. 4.13 D).

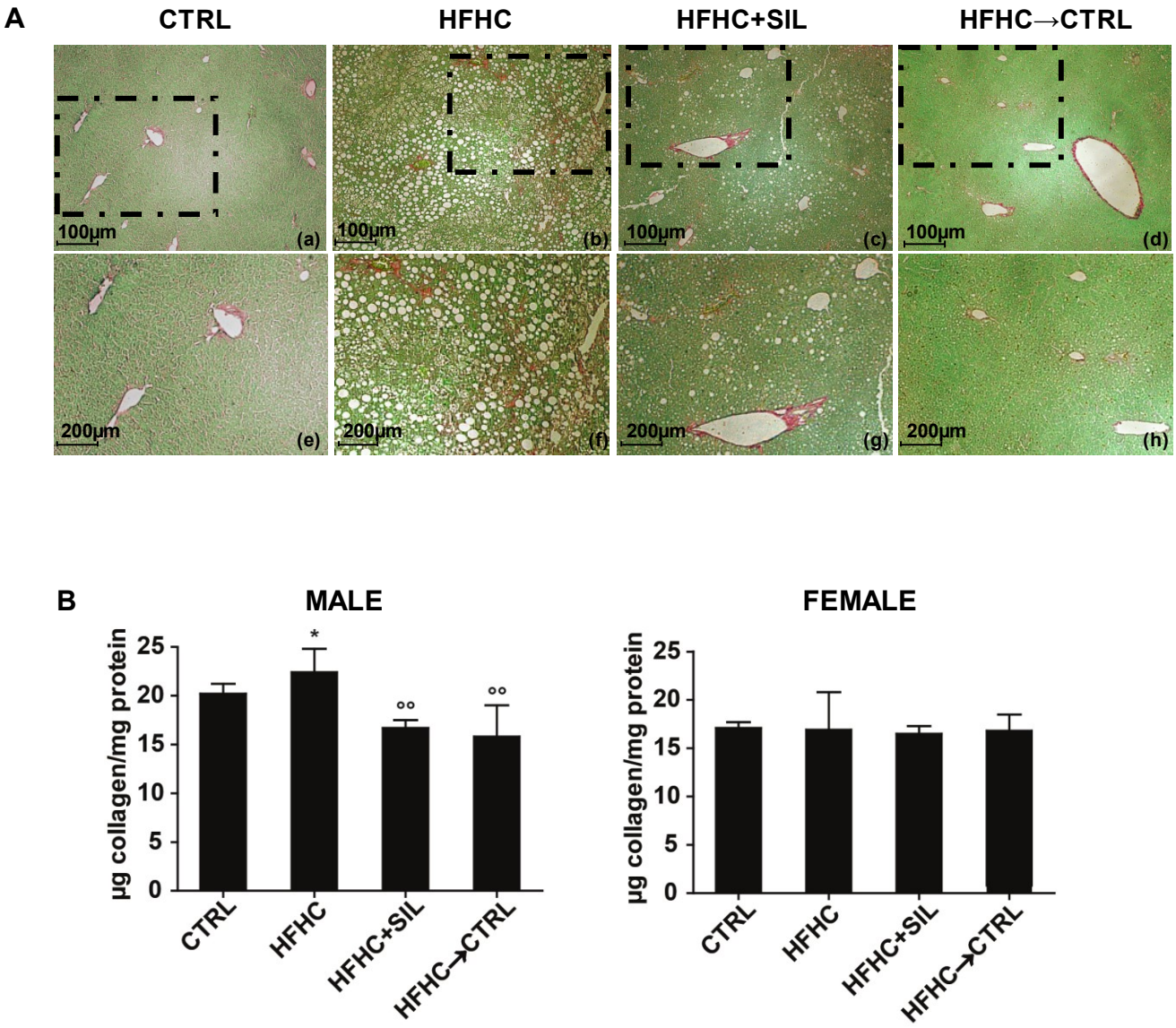


**Fig. 4.12.** (**A**) **Representative panel of Sirius red and fast green staining.** In red collagen fibers, in green non-collagenous protein detected in **(a)** control, (**b**) HFHC, (**c**) HFHC+Sil and (**d**) HFHC→CTRL mice over the time. **(e-h)** Higher magnification of the dashed area. In all pictures scale bar is 250 µm. (**B**) **Staining quantification in male and female mice**. * $p<0.05$, *vs*. CTRL; °° $p<0.01$ *vs*. HFHC.

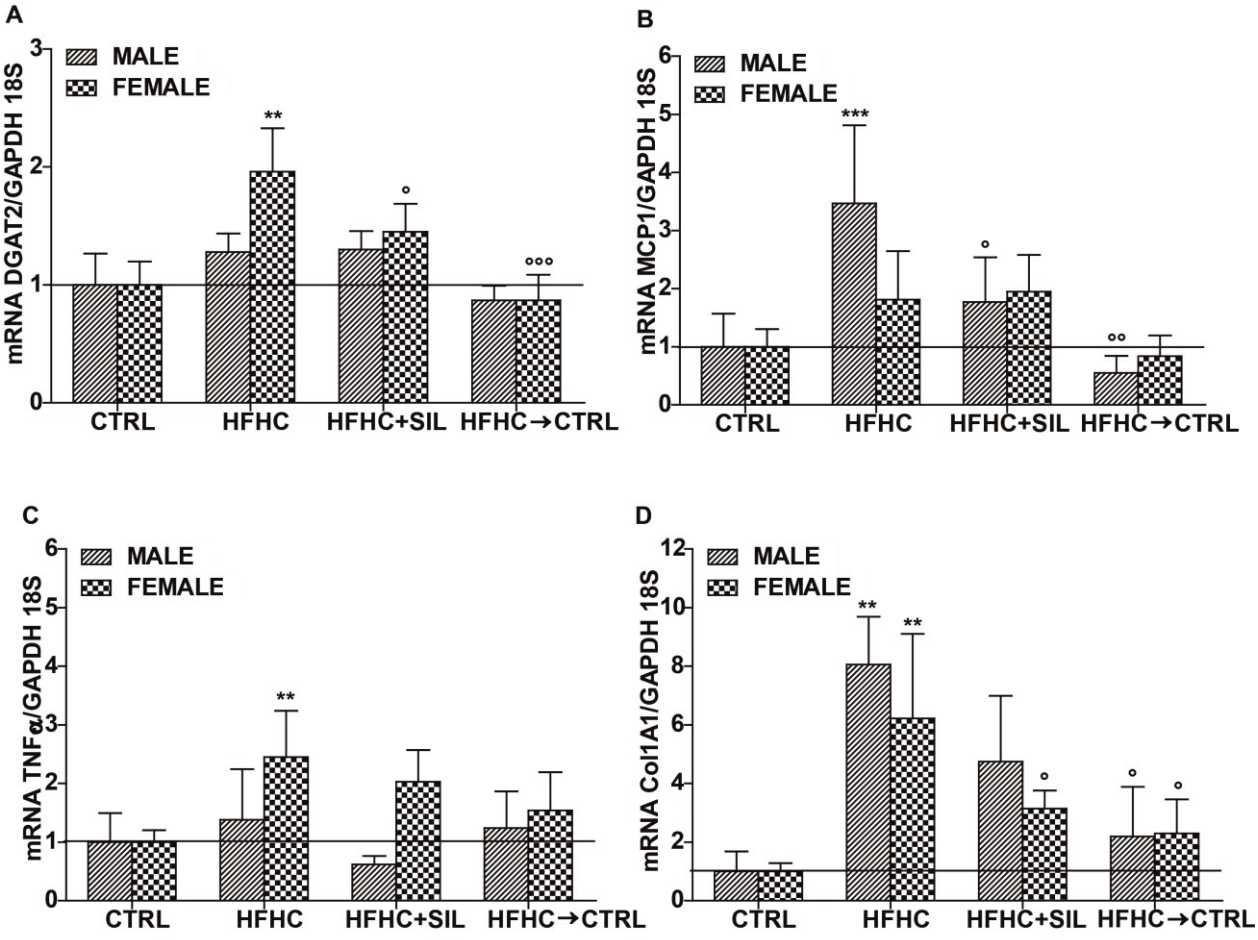


**Fig. 4.13**. Gene expression of (**A**) DGAT2 (**B**) MCP-1 (**C**) TNF-α and (**D**) Col1A1 after Silymarin administration and lifestyle change. ** p<0.01, *** p<0.001 *vs*. CTRL; ° p<0.05, °° p<0.01, °°° p<0.001 *vs*. HFHC.

### 4.1.10. Young female mice generated reactive oxidative species after 16 weeks of HFHC diet

The production of reactive oxidative species is strictly associated with the hepatic manifestation of the metabolic syndrome and is considered one of the main triggering mechanisms that drives the damage progression. Reactive oxidative species were dosed in liver homogenate through the quantification of MDA and GSH/GSSG ratio. A significant MDA increment was observed in females after 16 weeks of diet (3.86 ± 0.37-folds, p<0.001) whereas males did not show any difference (Fig. 4.14 C). This alteration was mirrored by GSH/GSSG ratio that followed the same pattern after 16 weeks of diet (0.53±0.35, p<0.05) (Fig. 4.14 B).Even in this case, HFHC males did not show any difference compared with controls (Fig. 4.14 A).

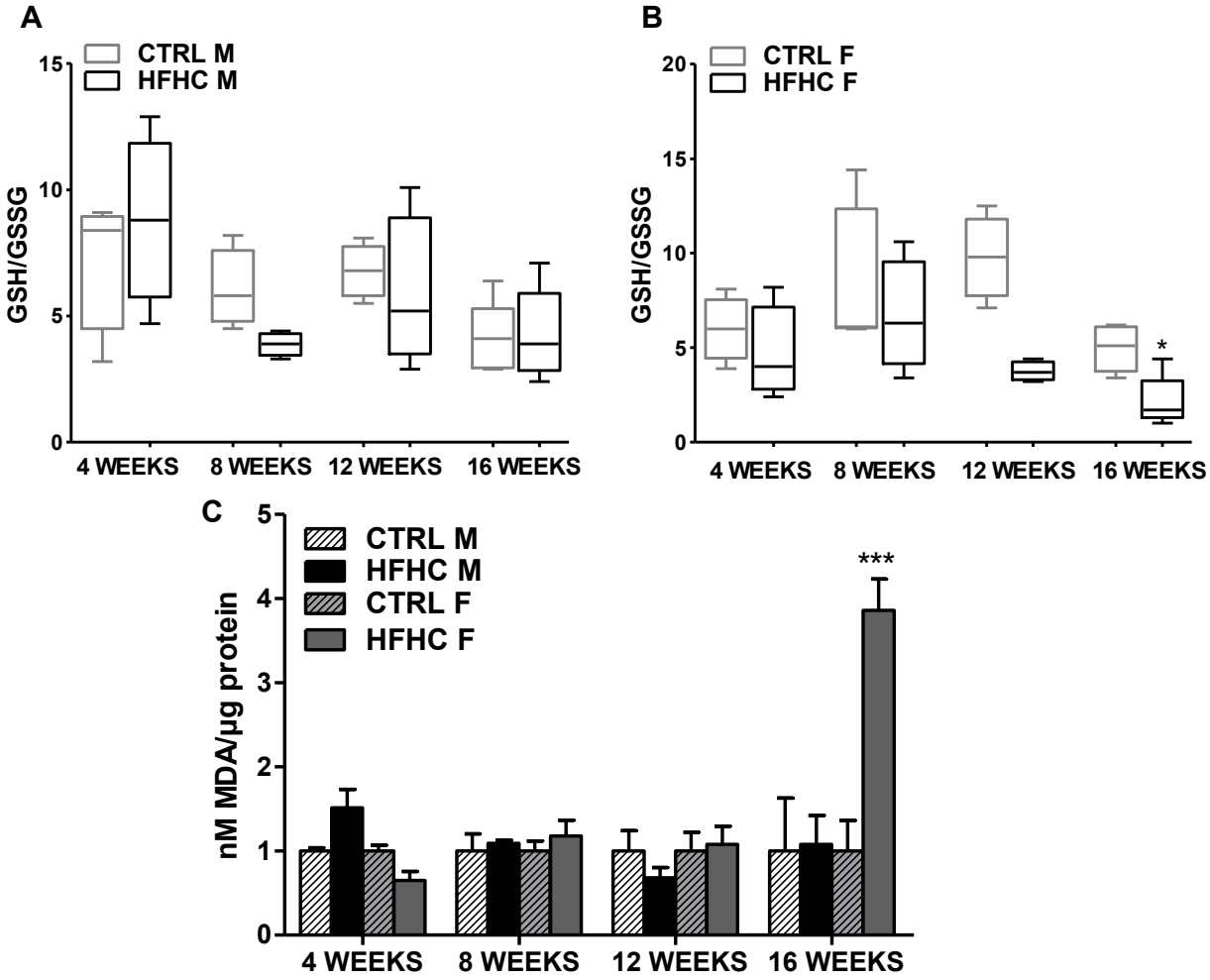


**Fig. 4.14. Oxidative stress assessment**. (**A**,**B**) Determination of GSH/GSSG ratio in males and female mice over the time. (**C**) Quantification of MDA adducts in liver homogenates in mice. * $p<0.05$, *** $p<0.001$ vs. CTRL.

### 4.1.11. Healthy diet decreased MDA production and improved glutathione ratio. Silymarin exerted antioxidant properties in females.

HFHC diet-induced oxidative stress in females was reduced by lifestyle change. Healthy feeding led to a reduction of MDA levels, decreasing the risk of lipid peroxidation. Moreover, an improvement of GSH/GSSG ratio was observed, confirming the antioxidant properties of this therapeutic approach (Fig. 4.15 B, D). Silymarin administration provided similar effects even if the reduction observed was not statistically significant ($p=0.06$). Importantly, among HFHC+SIL females a double response to the treatment was observed and consequently, the data were analyzed

dividing the animals into two subgroups: responsive (HFHC+SIL R) and non-responsive (HFHC+SIL NR) females. In the latter, Silymarin treatment was less effective and those animals had a MDA amount equivalent of HFHC group (Fig. 4.15 B). Regarding GSH/GSSG ratio, an improvement of this redox marker was reported in Silymarin-treated females with a lower extent of success than diet switching. (Fig. 4.15 D). HFHC diet did not exert oxidant effects in males and no differences were observed after Silymarin administration and diet switch (Fig. 4.15 A, C).

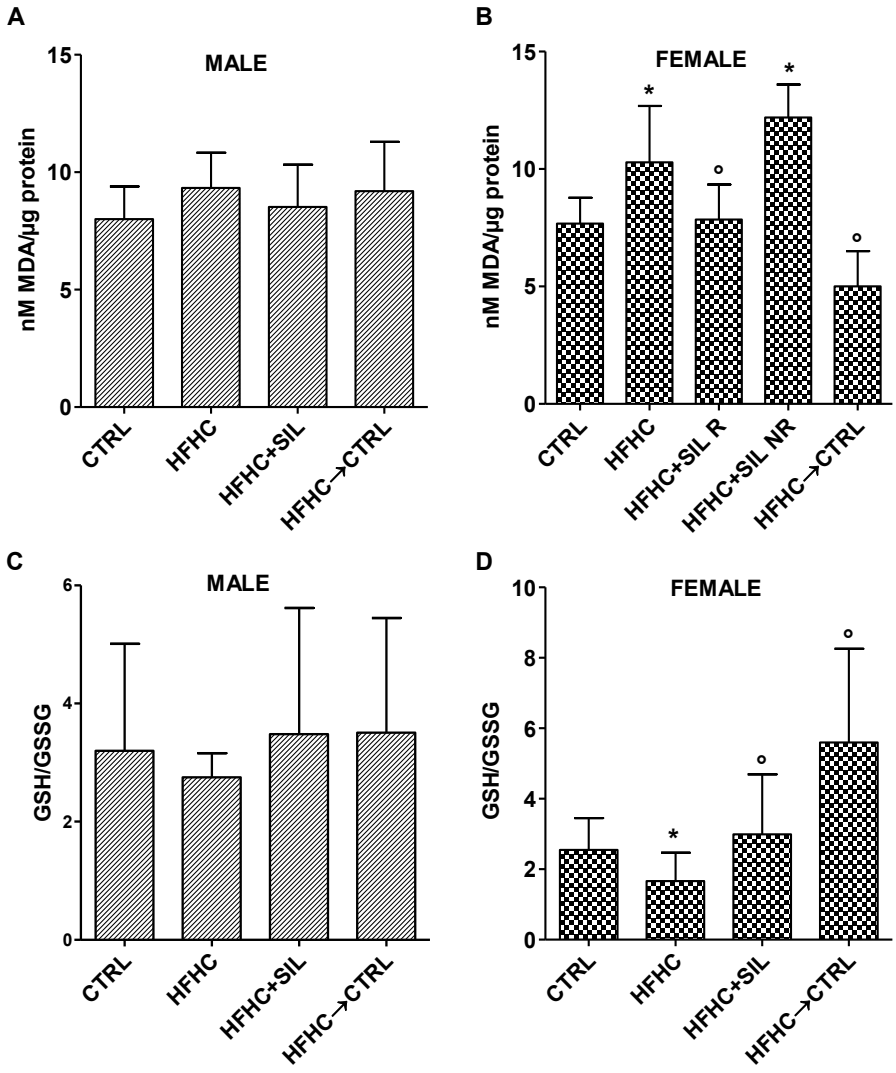


**Fig. 4.15**. **. Oxidative stress level after silymarin administration and lifestyle changes**. (**A**, **B**) Determination of GSH/GSSG ratio in males and female mice. (**C**, **D**) Quantification of MDA adducts in liver homogenates in mice. * $p<0.05$ *vs*. CTRL. ° $p<0.05$ *vs*. HFHC

### 4.1.12. Intestinal tight junction loss and leaky gut

Current evidence suggests that hypercaloric diet induces changes in the gut microbiota and barrier function. The protein expression of zonula occludens-1 (ZO-1) and claudin-5 were detected as molecular markers of tight junction (TJ) integrity in both large and small intestine samples. Large intestine architecture showed the disruption of junctional complex structures and wider gaps between cells. As shown by immunohistochemical staining and positive dye quantification, HFHC diet greatly reduced expressions of ZO-1 and claudin-5 protein in both genders, whereas no differences were detected in the small intestine.

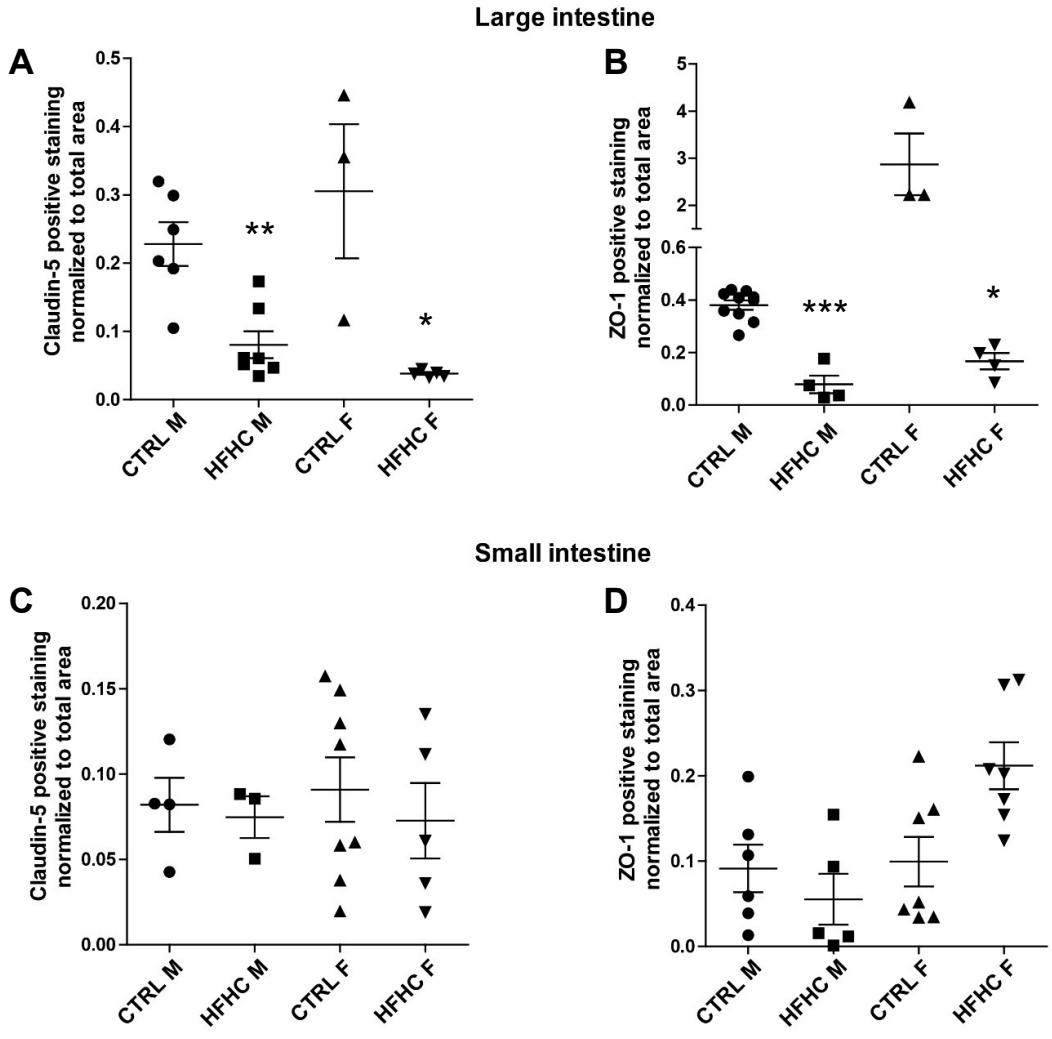


**Fig. 4.16. Quantification of positive staining in intestine samples.** (**A**) Claudin-5 and (**B**) ZO-1 in large intestine and (**C**) claudin-5 and (**D**) ZO-1 in the small intestine. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs*. CTRL.

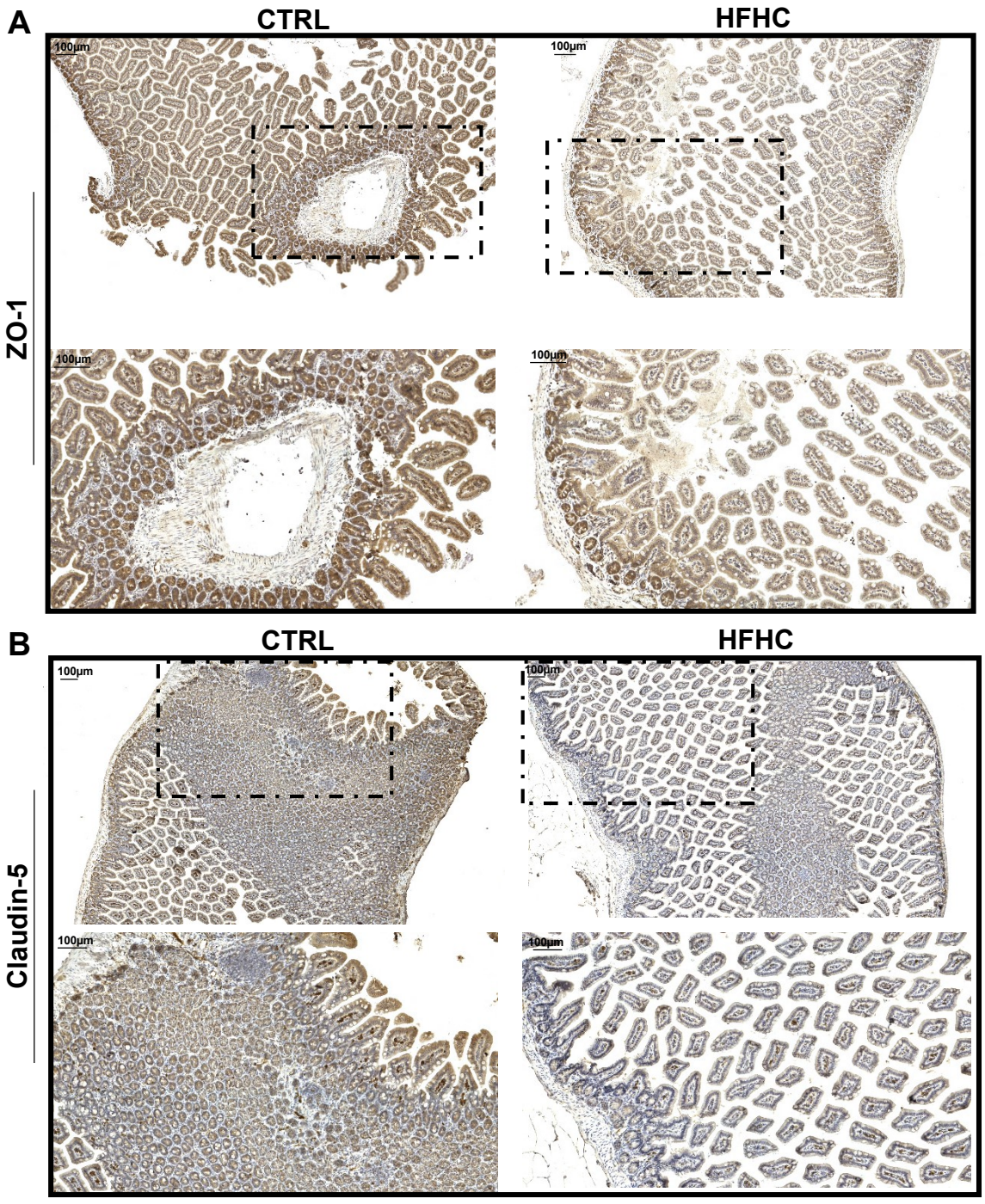


**Fig. 4.17. Positive staining for ZO-1 and Claudin-5.** Representative images of (**A**) ZO-1 and (**B**) Claudin-5 positive staining of CTRL and HFHC mice. The lower images of each panel represent the higher magnifications of the dashed area. In all pictures, scale bar is 100 µm.

## 4.2. ALD: the role of MIF in the pathogenesis of ethanol-induced liver damage

### 4.2.1. Co-culture of activated macrophages and HuH7 increased MIF release

Protein production was assessed both in the monoculture and co-culture systems in terms of MIF and TNF-α release.

Regarding MIF, monoculture HuH7 reported a comparable release of this mediator between untreated and treated cells. Surprisingly, HuH7 under co-culturing conditions displayed a strong increase in MIF release compared with the monoculture, even if ethanol did not contribute to further increase (Fig. 4.17A).Regarding TNF-α, no relevant variation was observed in both HuH7 and macrophages. Cells under co-culturing conditions followed the same trend of the monoculture (data not shown).

In macrophages, we observed a similar pattern. Monoculture cells reported the same MIF release, with no particular changes induced by ethanol. Interestingly, macrophages under co-culturing conditions reported a consistent increase in the release of this mediator, both in untreated and ethanol-treated cells (Fig. 4.17B).

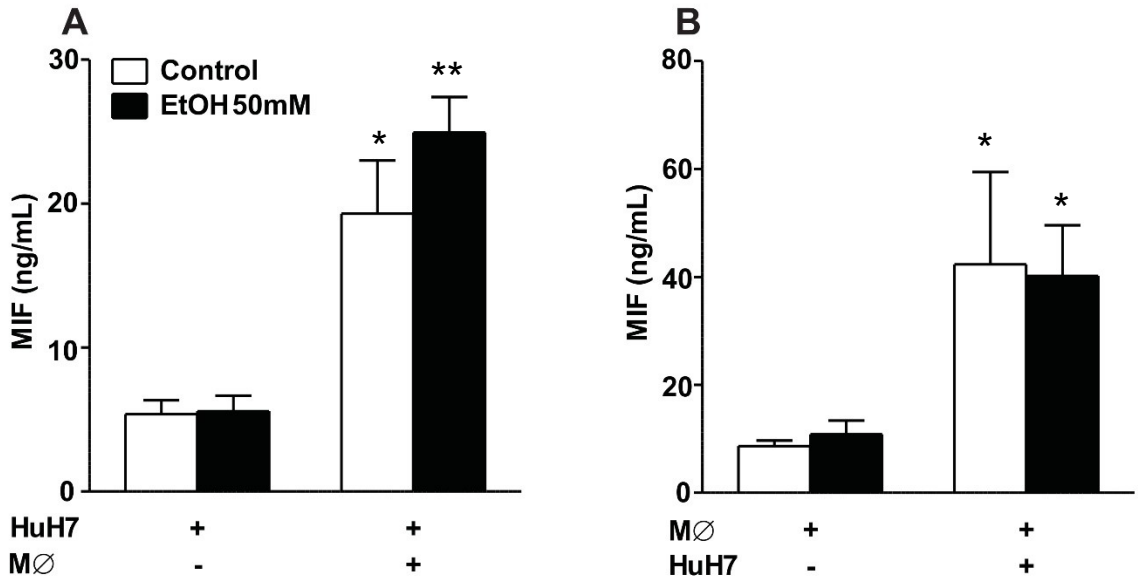


**Fig. 4.18**. **Co-culturing condition determines an increase secretion of MIF by cells.** The supernatant was separately collected from the bottom of the well and the transwell insert of (**A**) monocultured and co-cultured HuH7 and (**B**) monocultured and co-cultured MØ. MIF release was measured after 24hrs of 50mM ethanol -treatment by ELISA. Values represent means ± SD. * $p< 0.05$, ** $p<0.01$ *vs*. matched-control.

### 4.2.2. Activated macrophages increased RNA expression of TNF-α in HuH7 under co-culturing condition

The mRNA expression of MIF and TNF-α were analyzed both in the monoculture and co-culture system.

In monocultured HuH7, 50mM ethanol determined an increase of the mRNA expression of MIF and TNF-α. The regulation of both cytokines was also observed under the co-culturing condition and the analysis was performed by comparison with monoculture. Co-cultured HuH7 cells demonstrated a no significant increase of MIF expression that maintained the same trend in presence of ethanol (Fig.4.18A).Regarding TNF-α, co-culturing condition induced in HuH7 cells a strong up-regulation of the mRNA expression, without any additional effect due to ethanol treatment (Fig. 4.18C).

In macrophage monoculture, 50mM ethanol determined a decrease in MIF mRNA expression. In contrast, the co-culture canceled this effect restoring the condition of the single-cultured cells (Fig. 4.18B). Regarding TNF-α, we observed the same trend in the monoculture, in which ethanol determined a reduction in the mRNA expression of this cytokine. Contrarily, untreated macrophages under co-culturing condition demonstrated an induction of TNF-α, further exacerbated by ethanol treatment (Fig. 4.18D).

The co-culture model did not permit to identify which cell type is mainly involved in MIF release, due to the capability of this small protein to go through the transwell membrane, but rather allow to define the MIF release as the result of cellular cross-talk. To assess the singular cell response to different stimuli (EtOH, LPS, TNF-α), MIF release was quantified in monoculture of each cell type.

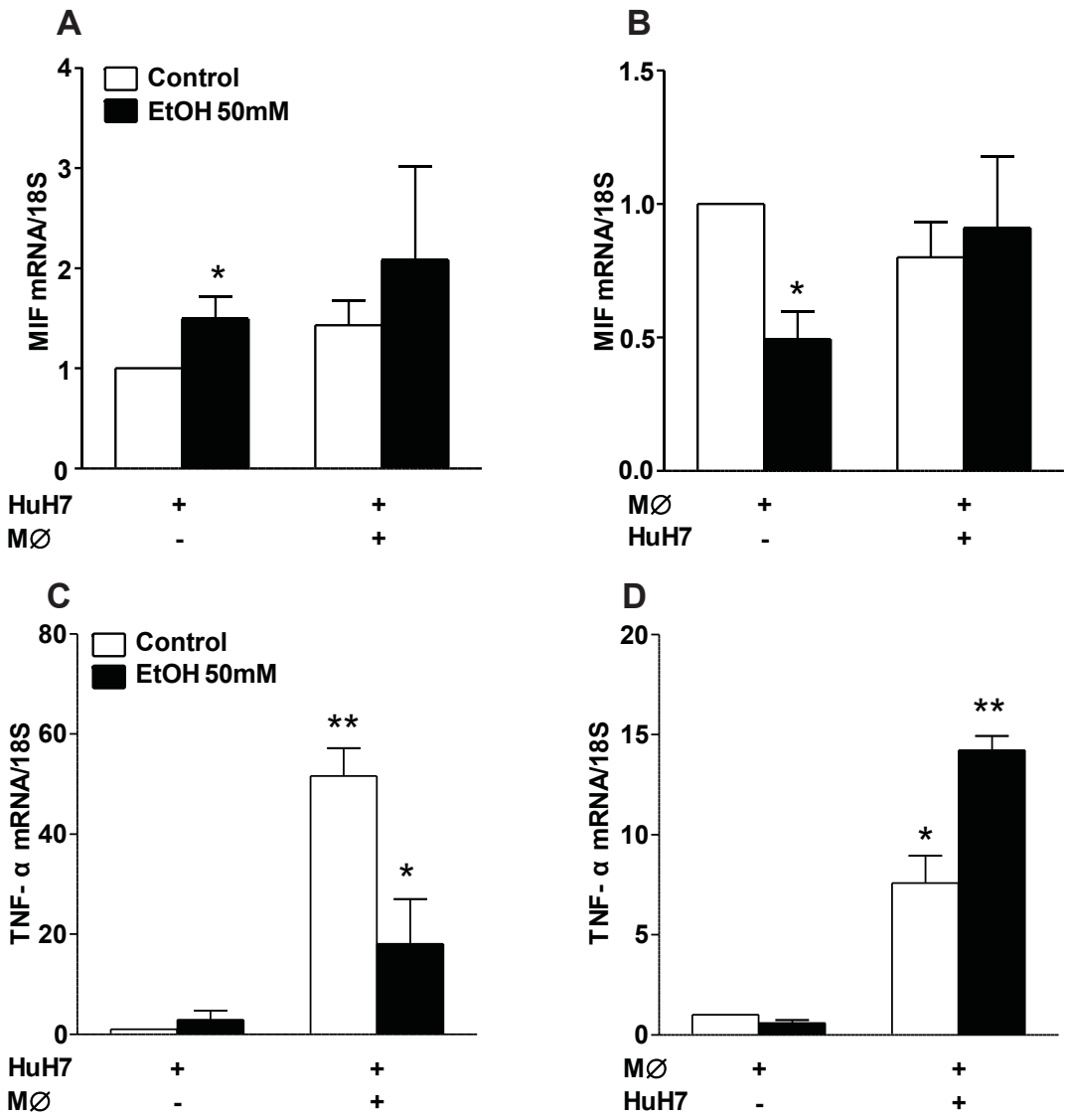


**Fig. 4.19**. **The mRNA expression of MIF and TNF-α were analyzed both in the monoculture and co-culture system**.
In monocultured HuH7, 50mM ethanol determined an increase of the mRNA expression of MIF and TNF-α. The regulation of both cytokines was also observed under co-culturing condition and the analysis was performed by comparison with monoculture. (**A**) Co-cultured HuH7 cells demonstrated a no significant increase of MIF expression that maintained the same trend in presence of ethanol. (**C**) Regarding TNF-α, co-culturing condition induced in HuH7 cells a strong up-regulation of the mRNA expression, without any additional effect due to ethanol treatment. (**B**) In macrophage monoculture, 50mM ethanol determined a decrease in MIF mRNA expression. In contrast, the co-culture canceled this effect restoring the condition of the single-cultured cells. (**D**) Regarding TNF-α, we observed the same trend in the monoculture, in which ethanol determined a reduction in the mRNA expression of this cytokine. Contrarily, untreated macrophages under co-culturing condition demonstrated an induction of TNF-α, further exacerbated by ethanol treatment.

### 4.2.3. Non-myeloid cell lines increased MIF release after inflammatory induction

In order to study the contribution of myeloid and non-myeloid cells to MIF production, HuH7 (non-myeloid) and differentiated THP1 (myeloid) cells were exposed to different noxious stimuli and MIF was quantified in cell culture media.

HuH7 cells were exposed to EtOH50mM and LPS 200ng/mL. MIF release was followed at different times (2, 4, 8 and 24hrs). The maximal response was observed after 8hrs of treatment with both ethanol and LPS (Fig. 4.19A).

Differentiated THP-1were challenged with LPS, TNF-α and EtOH. No changes in the release of MIF were observed in this myeloid cell line, even after 24hrs of exposure in all the conditions under study (Fig. 4.19B).

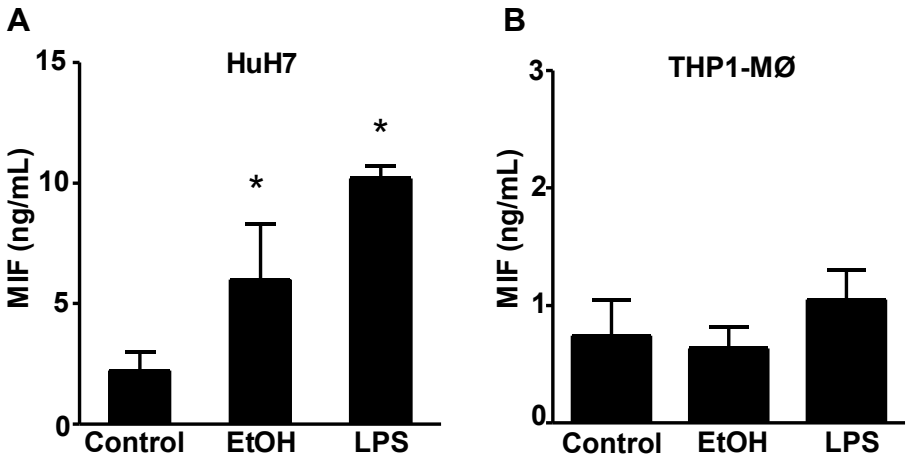


**Fig. 4.20. MIF release in each monoculture.** In order to study the contribution of myeloid and non-myeloid cells to MIF production, HuH7 (non-myeloid) and differentiated THP1 (THP1-MØ) (myeloid) cells were exposed to EtOH 50mM and LPS 200ng/mL. MIF release was followed at different times (2, 4, 8 and 24hrs). (**A**) In HuH7, the maximal response was observed after 8 hrs of treatment with both ethanol and LPS. (**B**) THP1-MØ did not show any change in the release of MIF, even after 24hrs of exposure.

### 4.2.4. MIF expression in non-myeloid cells contributed to ethanol-induced liver injury in chimeric mice.

In order to better understand the different contributor of myeloid and non-myeloid cells in MIF production, bone marrow transplants were carried out to generate the following chimeric mice: WT → WT (expressing MIF in both myeloid and non-myeloid cells), WT → *Mif-/-* (expressing MIF only in myeloid cells) and *Mif-/-*→ WT (expressing MIF only in non-myeloid cells). Bone marrow chimera were fed with chronic ethanol diet. If non-myeloid cells were involved in MIF release, then WT → *Mif-/-* mice would be protected from ethanol-induced liver damage.

Chronic ethanol feeding increased ALT and AST, as well as hepatic triglycerides, in both WT → WT and *Mif-/-*→ WT mice (Fig. 4.20A, B, C). Hepatic steatosis and inflammatory infiltration (Fig. 4.21A) and increased TUNEL positive nuclei (Fig. 4.21B) were also observed in ethanol-fed WT → WT and *Mif-/-* → WT mice. In contrast, WT→ *Mif-*/- mice were resistant to a chronic ethanol-induced increase in ALT/AST, hepatic steatosis, inflammatory infiltrates and apoptosis.

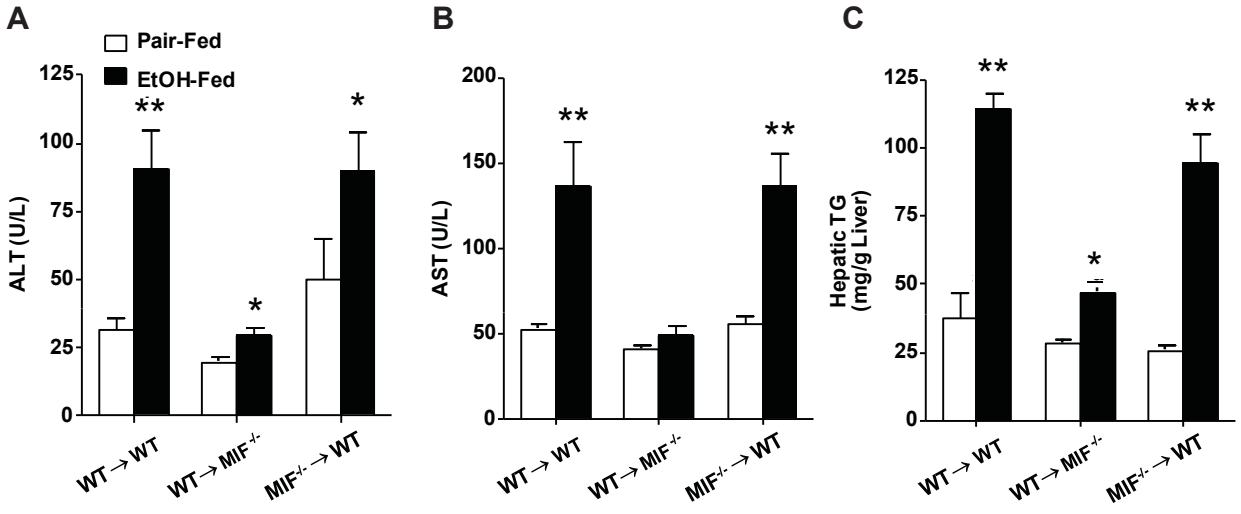


**Fig. 4.21**. **Ethanol-induced liver damage detection.** A group of mice underwent bone marrow transplant at 5 weeks of age (WT → WT; WT → *Mif-/-*; *Mif-/-* → WT) in order to identify the cell types involved in the production of MIF. After the transplant, recipient mice at 10 weeks of age were fed with ethanol. Hepatic transaminases (ALT and AST), as well as liver triglyceride accumulation, were measured. If the myeloid lineage of blood cells is the source of MIF, then chronic ethanol feeding should induce in WT → *Mif* -/- chimeras the same extent of liver injury of WT → WT mice. On the contrary, *Mif-/-* → WT should be protected by ethanol exposure. (**A, B**) In WT → WT and *Mif-/-* → WT mice fed with ethanol, hepatic ALT and AST were elevated compared with pair-fed mice. (**C**) Enhanced triglyceride content was reported in these ethanol-fed chimera groups.

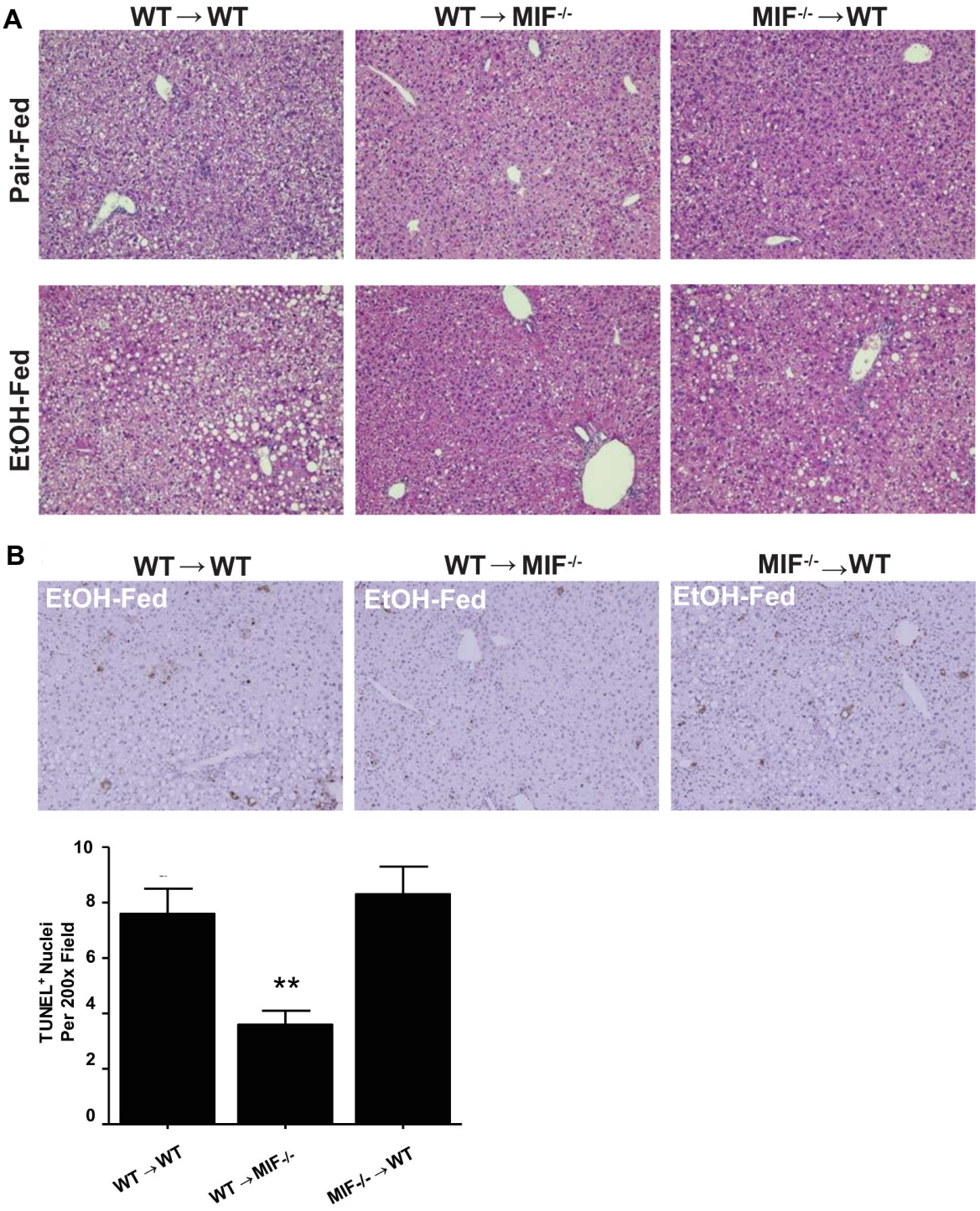


**Fig. 4.22. H&E and TUNEL staining.** Paraffin-embedded liver sections were stained with (**A**) hematoxylin and eosin and (**B**) Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), quantified using ImagePro Plus software. All images were acquired using a 10X objective. Values represent means ± SEM, n = 4 pair-fed and n = 6 ethanol-fed. Values with different alphabetical superscripts were significantly different from each other ($p < 0.05$).

### 4.2.5. MIF-deficiency in non-myeloid cells protected mice from chronic ethanol-induced expression of mRNA for immune cells and cytokines/chemokines

Consistent with the histological evidence for increased inflammatory cells after chronic ethanol feeding to WT → WT and *Mif*-/- → WT mice, expression of F4/80, a marker of resident macrophages, and Ly6C, a marker of infiltrating monocytes, were also higher in chimeric mice expressing MIF in non-myeloid cells after chronic ethanol feeding compared to WT→ *Mif*-/- mice (Fig. 4.22 A,B). Similarly, expression of MCP-1, CXCL10, CXCL1 and CXCL2 mRNA was higher in *Mif*-/- → WT mice compared to WT→ *Mif*-/- mice after chronic ethanol feeding (Fig. 4.22 C-F). Taken together, these data from chimeric mice suggest that bone marrow-derived cells do not contribute to MIF release after ethanol feeding in mice. Instead, non-myeloid cells represent the critical cell type generating MIF in response to chronic ethanol feeding. Importantly, the absence of MIF in non-myeloid cells protected mice from chronic ethanol-induced liver injury to a similar extent as in global MIF-deficient mice[195].

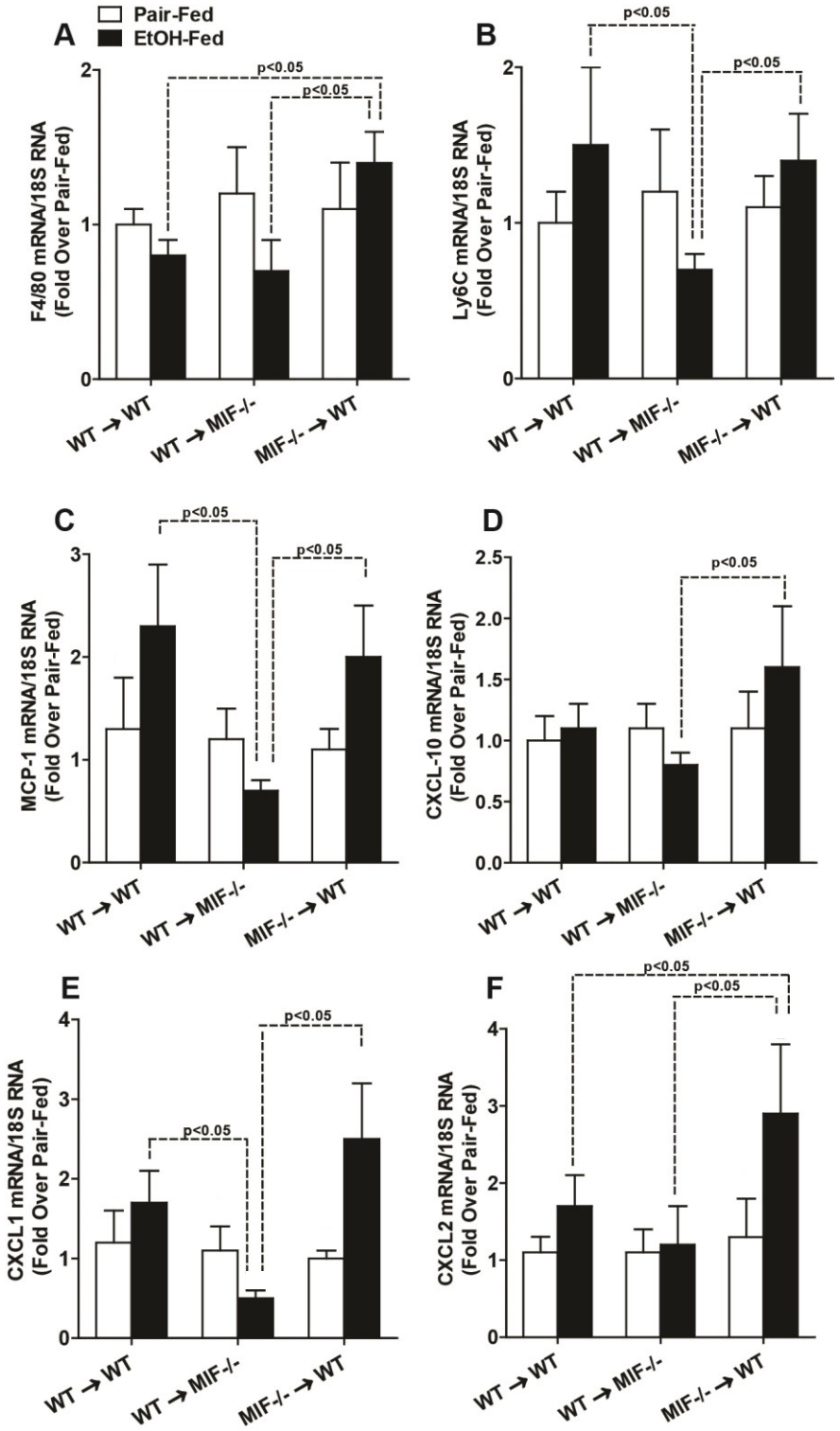


**Fig.4.23**. **(A, B)** Expression of F4/80, Ly6C and (**C-F)** MCP-1, CXCL10, CXCL1, CXCL2 mRNA was detected in mouse livers using qRT-PCR. Gene expression was normalized to 18S. Values represent means ± SEM, n = 4 pair-fed and n = 6 ethanol-fed. Significant differences are reported in the graph.

### 4.2.6. MIF expression in liver of alcoholic hepatitis patients

We next investigated the expression and localization of MIF in liver biopsies from patients with alcoholic hepatitis, autoimmune hepatitis (disease control) and healthy controls. Increased MIF staining was detected in liver biopsies from patients with alcoholic hepatitis, as well as autoimmune hepatitis, compared with controls (Fig. 4.23A). Expression of MIF mRNA was increased 1.4 fold in patients with AH compared to healthy controls (Fig. 4.23B). However, the cellular localization differed between alcoholic hepatitis and autoimmune hepatitis. MIF was predominantly localized to hepatocytes and ductular cells in alcoholic hepatitis cases, while MIF was primarily localized in non-parenchymal cells in autoimmune hepatitis cases. Hepatocytes and non-parenchymal cells were identified according to their cellular and nuclear morphology. Although the majority of MIF staining was localized in the cytosol of hepatocytes, nuclear staining was also detected in a few cells in sections from cases of alcoholic hepatitis.

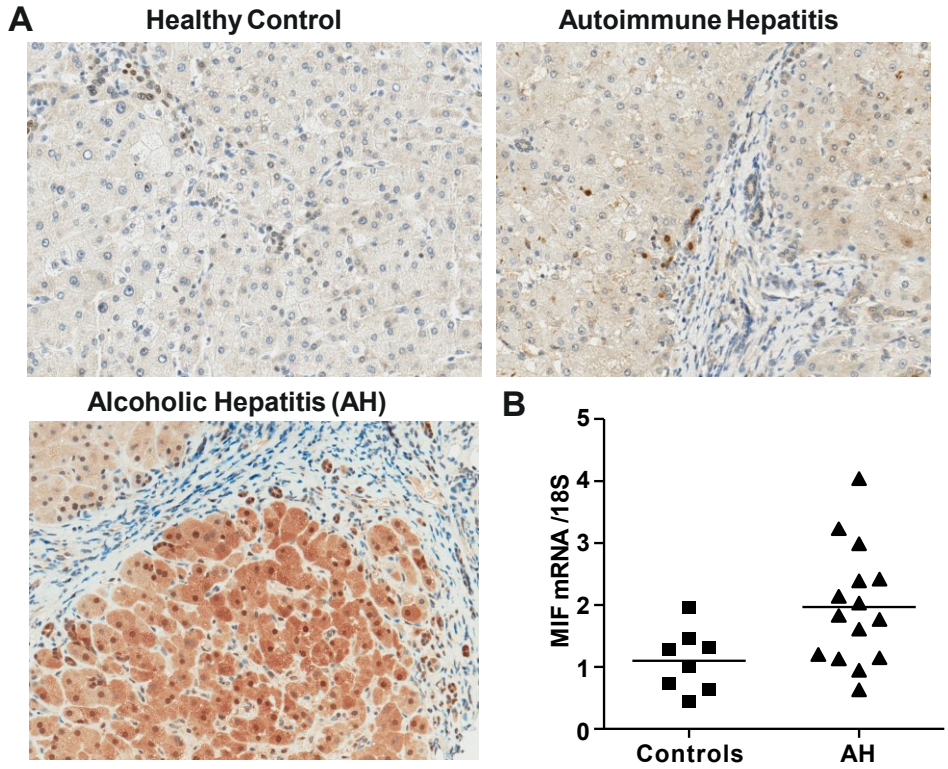


**Fig. 4.24. MIF detection in patient liver slices.** (**A**) Paraffin-embedded liver sections were stained for MIF in samples taken from patients with alcoholic hepatitis, auto-immune hepatitis or healthy controls. All images were acquired using a 40X objective. Images are representative of 5 samples for AH, 5 autoimmune hepatitis and 4 for healthy controls. **(B)** qRT-PCR analysis of MIF mRNA in livers of patients with alcoholic hepatitis and healthy controls. Values represent means ± SEM, n = 17 AH and n = 7 healthy controls**.**

### 4.2.7. Positive correlation between MIF in suprahepatic serum and biochemical markers of liver disease in patients with alcoholic hepatitis

If hepatocytes are an important source of MIF in response to ethanol, then the concentration of MIF in suprahepatic serum should be correlated with the extent of liver disease in patients with alcoholic hepatitis. In order to test this hypothesis, MIF was quantified in the peripheral and suprahepatic serum of patients with alcoholic hepatitis. The concentration of circulating MIF was correlated with clinical measures of the severity of alcoholic hepatitis in this patient cohort. A positive correlation was found between MIF concentration in the suprahepatic, but not peripheral, serum including bilirubin, AST, GGT and circulating triglycerides (Table 4.3). Increased MIF in the suprahepatic serum was also associated with an increased risk of mortality (Fig. 4.24).

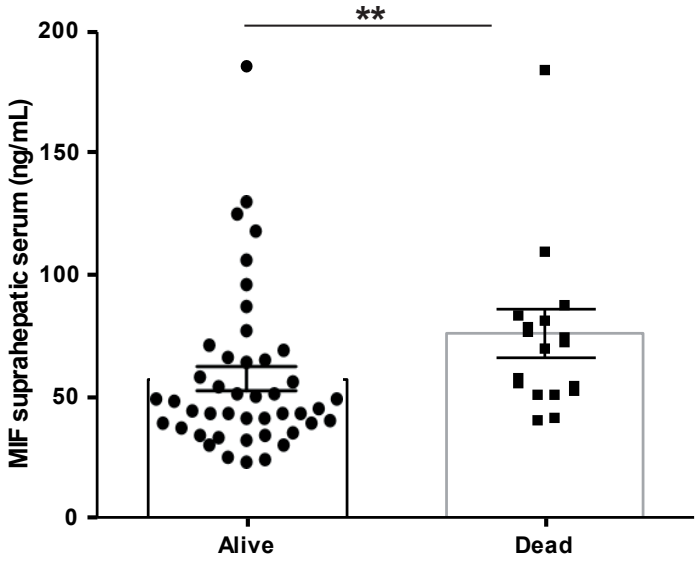


**Fig. 4.25**. MIF concentration in suprahepatic serum was higher in alcoholic hepatitis patients who died within 180 days of diagnosis compared to those who survived.

| | Peripheral Blood MIF (n=65) | | Suprahepatic Blood MIF (n=58) | |
|---|---|---|---|---|
| Biochemical parameters | Pearson's r coefficient | p-value | Pearson's r coefficient | p-value |
| Leucocyte count ($10^9$) | -0.06 | 0.6 | 0.16 | 0.2 |
| Bilirubin serum levels (mg/dl) | 0.008 | 0.9 | 0.30 | **0.02** |
| AST (IU/l) | -0.13 | 0.2 | 0.28 | **0.03** |
| ALT (IU/l) | -0.10 | 0.4 | 0.1 | 0.9 |
| Cholesterol serum levels (mg/dL) | -0.12 | 0.3 | 0.25 | 0.8 |
| Triglycerides serum levels(mg/dl) | 0.1 | 0.9 | 0.27 | **0.04** |
| GGT (IU/l) | -0.13 | 0.3 | 0.40 | **0.002** |
| Albumin (g/dl) | -0.05 | 0.6 | -0.05 | 0.6 |
| Alkaline Phosphatase (IU/l) | -0.08 | 0.5 | 0.05 | 0.7 |
| HVPG (mmHg) | -0.12 | 0.3 | -0.10 | 0.4 |

**Table 4.3**. Correlation between the amount of MIF quantified in peripheral and suprahepatic serum with clinical parameters indicative of disease severity.

# Chapter 5
# Discussion and Conclusions

# 5. Discussion and Conclusions

Obesity and alcohol consumption represent two important public health issues of the 21st century, strongly associated with the rising of NAFLD[211,212] and ALD, respectively in Western Countries. Each liver disorder is characterized by a wide pathological spectrum that includes simple steatosis, steatohepatitis, and fibrosis, until the end stages of cirrhosis and hepatocellular carcinoma. Currently, the most worrisome aspect resides in the fast spread of these liver damages among juvenile population.

Regarding NAFLD, the increasing incidence in adolescents mirrors the parallel spread of hypercaloric diet and sedentary life habits among young. For several aspects, pediatric NAFLD differs to adults. Particularly, histological results highlighted that there is a unique pattern of lesions in children[213,214], and progression to most severe prognosis seems to follow a different trend[214]. Anyway, many aspects regarding the pathogenesis of pediatric NAFLD are still unknown due to the lack of specific experimental models. Taken together, the fast spread of this health issue and the absence of investigations focused on young, make research in this field necessary. Most of the published adult models reproduced NAFLD pathogenesis inducing genetic mutations or feeding mice with hypercaloric diets. The latter approach better resembles human condition, but the lack of progression to advanced stages often represents a limit difficult to overcome. Genetic mutations or more aggressive diets (such as methionine-choline or leptin-deficient diet) reach the most severe outcomes but are far to recreate the conditions encountered bedside. Considering that juvenile NAFLD/NASH models are almost absent in literature, initially, the aim of the first project (Task 1, phase I) was to fill this gap.

Male and female WT mice were fed with high-fat high carbohydrate (HFHC) diet, immediately after weaning. Soon after the first week, both genders showed an increased body weight and BMI, differently from published adult models that required at least 4 weeks[215,196,216]. In addition, since week 4, males presented early dyslipidemia that progressively rose over the time. Increased hepatomegaly and transaminase alterations, as well as enhanced glycemia and insulinemia, were evident after week 12. The biochemical observations were confirmed by histological analysis that highlighted some alterations of the hepatic parenchyma. Specifically, micro-macrosteatosis was reported since week 4, followed by the presence of inflammatory infiltration with the absence of ballooning degeneration. Interestingly, a progressive fibrosis was

also described, reaching stage 2 after 16 weeks of HFHC diet. Female mice developed comparable body and blood alterations but with a slower progression. Specifically, increased adipose fat-pads, hepatomegaly, altered serum lipid profile, as well as ALT, were observed after 16 weeks of the diet. At the same time, enhanced amount of circulating glucose was also detected, even if insulinemia and HOMA-IR remained unchanged. In spite of the later alteration of the serological markers, histological analysis revealed the development of progressive steatosis and fibrosis by week 8. Differently from males, no inflammatory infiltration was reported over the time. Taken together these data showed that even if the underlined mechanisms were different, the final pathological outcome was similar in males and females. Anyway, the absence of early serum alterations (ALT, IR etc.) in young females, suggested a silent profile in the onset and progression of the disorder for this gender. Recent studies[217] indicated that estrogens might exert anti-inflammatory and anti-oxidant functions, with a general hepatoprotective effect[39,217–219]. In line with this, clinical observations described that women after menopause had a burst of hepatic injury[50,220] and a fast visceral fat accumulation[221] caused by estrogen decline[222]. NAFLD diagnosis resides on liver biopsy that is considered the gold standard for liver damage confirmation. Due to the high risk of this technique, a biopsy is performed when there is a real suspicion, based on blood marker alterations and the presence of some metabolic syndrome features (obesity, hypertension, and diabetes for example). These indications may suggest that the absence of serological alterations reported by female mice would be the result of estrogenic masking effect rather than a more protective phenotype, considering the comparable fibrosis developed after 16 weeks of the diet. This trend might be particularly worrisome if confirmed also in humans, increasing the possible number of undiagnosed girls.

NAFLD pathogenesis is strongly associated with cardiovascular risks, but the analysis of aorta morphometry in our mice revealed the absence of atherosclerotic lesions in both genders. A possible explanation might be in the composition of the HFHC diet used, completely lacking in animal-derived fats and for this reason defined “no atherogenic” diet[19]. Anyway, the liver histology demonstrated an early degeneration towards fibrotic complications, differently from adult models that required longer hypercaloric feeding to reach the advanced stages of injury[215]. Normally in adults, simple steatosis represents the first benign phase of NAFLD and is *per se* completely reversible, with a slow pace of progression. Our findings might suggest that this trend

cannot be totally confirmed in adolescents, considering the more aggressive phenotype displayed by our model. A possible explanation might reside on the biological changes associated with puberty, characterized by reduced adiponectin level, decreased insulin sensitivity and increased secretion of sex hormones that lead to a general body fat redistribution[6]. Children with diet-induced fat excess, insulin resistance, and hormonal alterations might be more sensitive to the physiological changes given by puberty and may be more predisposed to liver disease onset[6].

Male mice showed, in the histological analysis, the presence of inflammatory infiltration that was completely absent in females, suggesting that the underlying mechanisms that led to fibrosis were different between the two genders. To better investigate this aspect, mRNA of TNF-α and MCP-1 was quantified in the liver lysate. The results showed an increased expression of both genes in males and no alterations in females, confirming the previously observed histological pattern. Oxidative stress is another reported mechanism implicated in the pathogenesis of NAFLD and NASH[223]. MDA adducts and glutathione ratio were quantified in mice hepatic homogenates in order to evaluate oxidative stress level. Interestingly, our data showed an involvement of this pathological mechanism exclusively in females that after 16 weeks of HFHC diet with an alteration in both markers of oxidative stress. On the contrary, males did not reveal any change, neither in aldehyde amount nor in GSH/GSSG ratio. Taken together these findings revealed a pro-inflammatory profile in males and a pro-oxidant pattern in females, suggesting different biomolecular mechanisms between the two genders.

Overall, our data indicated that different processes in males and females may dictate the development of fibrosis and this can be particularly relevant considering that NAFLD does not have a specific therapeutic approach. Therefore, the present findings might suggest that gender-addressed treatments might be considered to achieve more successful results in reversing the pathological features described for juvenile NAFLD/NASH. Based on the faithful reproduction of the main aspects of the metabolic syndrome, the described model was then used as a platform for a pre-clinical treatment evaluation (Task 1, phase II). Specifically, have been tested the pharmaceutical properties of Silymarin, a nutraceutical compound extracted by milk thistle. Moreover, as gold standard, lifestyle change (switching of HFHC diet to control feeding) was applied for a group of mice.

Silymarin administration decreased hepatomegaly and visceral fat deposition in both genders as well as plasma LDL-C, TG, and ALT.

In males, it also decreased glycemia but without any effect on insulin resistance (HOMA-IR), since insulin level remained unchanged. In line with the improvement of the biochemical scenario, liver histology showed a reduction of steatosis and fibrosis in both males and females. These results were confirmed at the molecular level. Silymarin decreased significantly the gene expression of Col1A1 and Sirius red staining in males, confirming its effects in improving fibrosis. Furthermore, the nutraceutical compound determined DGAT2 and MCP1 mRNA normalization, respective markers of steatosis and inflammation. The anti-inflammatory properties of Silymarin have been deeply investigated. In literature is reported that Silymarin interferes with the transduction cascade controlled by NF-κB that is inducible and ubiquitously expressed DNA-binding protein that acts as transcription factor for genes involved in inflammation, cell survival, differentiation, and growth[87]. During homeostasis, NF-κB interacts with inhibitory protein 1 κ B α (IκBα) and is stored in the cytoplasm. Upon oxidative stress and inflammatory stimuli, IκBα is phosphorylated and degraded, dissociating NF-κB that translocates to the nucleus where triggers the activation of inflammatory genes. Silymarin has been demonstrated to suppress IκBα phosphorylation and degradation, inhibiting NF-κB activation and translocation. The anti-inflammatory activity of Silymarin is also related to its interference with several pathways that control cytokine-induced signaling that normally downregulates inducible nitric-oxide synthase (iNOS) expression. Moreover, Silymarin inhibits cyclooxygenase (COX)-2 expression and activity and leukotriene formation in human platelets, white blood cells, and endothelial cells.

In females, oxidative stress component detected after HFHC feeding was decreased by Silymarin administration, specifically with a reduction of MDA adducts and an improvement of GSH/GSSG ratio, confirming its antioxidant properties.

Silymarin can contribute to the antioxidant defenses in different ways, firstly acting as direct free radicals scavenger[224] that stabilizes free radical species donating a hydrogen atom, given by Silymarin phenolic groups. Secondly, Silymarin demonstrated antioxidant properties exerting a defending mechanism on mitochondrial structure and functions. Indeed, it protects mitochondria from pathological events by triggering pro-survival cell signaling, optimizing electron-transport chain, decreasing electron leakage and ROS formation and directly reducing activities of ROS-

producing enzymes in the mitochondria. In rats subjected to ischemia/reperfusion and to a consequent mitochondrial bioenergetic impairment, Silymarin supplementation prevented ATP level decrease, membrane potential impairment, and the associated cell dysfunction[225]. Moreover, it has been reported that Silymarin can contribute to the antioxidant defenses positively modulating gene expressions which encode for antioxidant enzymes.

Beyond the study of Silymarin, we also managed the metabolic syndrome in our model applying the most important and effective therapy recognized so far for NAFLD: the lifestyle modification. Switching to hypercaloric diet with healthy feeding improved the serological profile and the histological damage, confirming that is the best approach to revert all the co-morbidities previously described. The main challenge of this treatment is to convince patients to maintain healthy behavior and habits in the long term. Due to the lack of strong histological-based data, no firm recommendation are still available for treating NAFLD. Some published reports indicated that 5-10% weight loss would be desirable, even if would be more important an increased nutritional education rather then the only weight reduction. Patients should learn the benefits that healthy diet has, in order to reconsider their food preferences. For example, they should be advised to reduce the consumption of saturated fatty acids and to increase the use of polyunsaturated fatty acids (PUFAs). The intake of n-3 PUFAs elevate adiponectin and decrease insulin, TG and leptin concentration[226]. A decreased amount of sugars intake should be considered, avoiding soft drinks and sodas as well. The correlation between fructose consumption and NAFLD rising has been strongly demonstrated. Mainly because fructose increases free fatty acid hepatic delivery with a consequent enhancement of insulin resistance and oxidative stress[227]. It is important to mention how the family environment influences children weight status, considering that parents are responsible for early food and eating experience in kids. Data available indicated that parents with a wrong diet education reflected their bad nutritional habits in children, leading to a familial pattern of overweight[228]. For this reason, an important goal should be the development of family-based prevention programs, focused on parenting education in preferences and selection of healthy foods, learning also how to promote the acceptance of these new foods in children. Providing information about portion sizes, time and frequency of meals would be essential for the development of a conscious nutritional knowledge, important to the growth of healthy children.

Overall these data showed that the progression of NAFLD is different according to the genders. In males, the injury onset was immediately evident, with early alterations of biochemical markers and an inflammatory profile that drew to liver fibrosis. On the other hand, females showed a silent progression of the disease, with serological manifestations that appeared when liver parenchyma was already compromised and with oxidative stress as a leading mechanism. Furthermore, we demonstrated that addition of Silymarin to HFHC diet helped in reducing the spread of metabolic damage, even if lifestyle modification confirmed to be the best choice in NAFLD management.

Obesity is not the only factor that can induce liver steatosis. Alcohol consumption is considered the major cause of preventable morbidity and mortality worldwide, with a pathophysiological scenario that has the same spectrum of liver disorders observed in NAFLD.

In healthy conditions, the normal architecture of the liver is characterized by a close interaction between parenchymal and non-parenchymal cells. The liver is a crucial organ that performs several functions and receives the main blood supply from the intestine. The gut is the largest site of interaction with the external environment[125], and transports not only nutrients but also bacterial products. Therefore, the liver is permanently exposed to noxious compounds. The detoxification and synthesis activities conducted by the liver contribute to the resilient phenotype of this organ, and hepatocytes are the main players in the regenerative process. These cells are tightly balanced with the activities of non-parenchymal cells, such as Kupffer cells (KC) that are critical components of the robust hepatic inflammatory functions. Prolonged alcohol exposure represents one of the main triggers for liver damage. Alcohol abuse leads to the generation of intracellular oxidative stress that induces lipid peroxidation and apoptosis. During ethanol-induced liver injury, cellular components of the innate immune system, including KC, become activated, secrete pro-inflammatory mediators and contribute to the damage progression[229]. Furthermore, through the portal vein, ethanol can drive the translocation of LPS from the gastrointestinal lumen to the liver that binds KC receptors, perpetrating inflammation.

The induction of inflammatory response is not only the result of an autocrine mechanism but depends also on the recruitment of immune cells to the site of the injury. Among the numerous chemokines and cytokines participating in this process, MIF has a relevant role in maintaining KC number in the liver and in eliciting the infiltration of myeloid cells. It has been demonstrated that

MIF led the infiltration of $CD11^+$ and $Ly6C^+$ monocytes in mice fed with chronic ethanol diet[195]. Furthermore, MIF showed to be directly involved in ALD progression since *Mif-/-* mice did not develop steatosis nor alcoholic hepatitis after chronic ethanol feeding[195]. Both myeloid and non-myeloid cells can contribute to the release of MIF, however, it is still unclear which is the main source of this protein in ALD scenario. Therefore, the main goal of the second project (task 2) was to define the contributors of myeloid and non-myeloid cells in MIF generation during chronic ethanol exposure.

Initially, the interaction between hepatocytes and macrophages was analyzed, using an *in vitro* model that recreated the pathophysiology of ALD. A transwell co-culture system was developed in order to study the effect of the soluble mediators released by the cells in presence of ethanol. Here we described that hepatocytes and Kupffer cells showed significant changes in the inflammatory profile when cultured together, in contrast to the monoculture. An increased TNF-α gene expression was observed in both cell lines, which was further exacerbated by alcohol treatment only in macrophages. However, this up-regulation was not translated in terms of protein response. On the contrary, the presence of released MIF in culture media was strongly enhanced upon co-culturing condition. Conversely, no relevant changes were observed at mRNA level. MIF is constitutively expressed and stored in preformed intracellular pools. Therefore, it does not require *de novo* protein synthesis before secretion and this can explain why no relevant differences have been observed at transcriptional level[230]. The collected data clearly suggested that there is a different cellular response when hepatocytes and macrophages are together, and MIF seems to be one of the first mediators participating in the intercellular cross-talk. This co-culture system recapitulates the key features of known ALD cellular interplay, but does not clarify the main source of MIF. This point was better elucidated treating HuH7 cells and differentiated THP1 separately with ethanol, LPS and TNF-α, in order to trigger the inflammatory response. Hepatocyte cell line reported an increased release of MIF after stimulation, reaching the maximum after 8hrs of treatment. On the contrary, differentiated macrophages did not report any alteration even at longer incubation time with the noxious compounds (24hrs). The *in vitro* model suggested that in the co-culture system, HuH7 cells (non-myeloid) might be mainly responsible for MIF production.

To better understand the different contributors of myeloid and non-myeloid cells in MIF production, occurring during ALD, bone marrow chimeras (WT→ WT, *Mif*-/- → WT, WT→ *Mif* -/-) were fed with chronic ethanol diet. If non-myeloid cells were directly involved in the release of MIF, then WT → *Mif*-/- chimeras would be protected by ethanol-induced liver damage.

In WT → WT mice, alcohol-enriched diet induced a higher amount of hepatic triglycerides (TG) and increased activities of plasmatic ALT and AST that reflect liver injury and cellular death due to sustained inflammation [231]. The histological analysis provided an additional confirmation, showing an enhanced intracellular lipid deposition and inflammatory foci. Moreover, in WT → WT mice ethanol feeding increased MCP-1 mRNA as well as CXCL1 and MIP2. The quantity of F4/80 and Ly6C gene expression was also detected and an indication of increased mononuclear cell recruitment was reported since Ly6C mRNA was enhanced compared with pair-fed animals.

Similarly, ethanol-fed *Mif*-/- → WT reported a behavior comparable with WT → WT. Plasmatic transaminases and hepatic TG were significantly augmented and histologically hepatic steatosis and inflammatory infiltrations were confirmed. Moreover, the biomolecular analysis showed a higher MCP-1 mRNA expression, in line with the transcriptional increase of IP-10, CXCL1, and MIP2. Ethanol-fed *Mif*-/- → WT demonstrated also an altered quantity of F4/80 expression, a marker used to detect hepatic macrophage population. This finding is consistent with published reports that indicated a role of MIF in macrophage/monocyte replenishment and recruitment during ethanol exposure[195,232]. Specifically, Barnes et al.[195] described that ethanol-fed *Mif*-/- mice had a reduction in hepatic F4/80$^{+}$cell number, suggesting the positive correlation between MIF presence and hepatic macrophage population maintenance in ALD. Moreover, the same ethanol-fed *Mif*-/- mice did not develop steatosis and inflammation, as well as cellular injury and hepatocyte apoptosis, confirming that MIF is critical to driving ALD progression. Based on these concepts, if MIF-inducing liver damage in ALD had a myeloid origin, then we would expect a protective profile for *Mif*-/- → WT mice from ethanol toxicity. However, data discussed above revealed a comparable damage extent for *Mif*-/- → WT and WT →WT chimeras, leading to the conclusion that MIF has a non-myeloid origin.

Further confirmation of this hypothesis was obtained by analyzing ethanol-fed WT → *Mif* -/- mice. Interestingly, hepatic triglycerides, as well as transaminase activities were reduced. Histological analysis revealed the absence of hepatic steatosis and inflammatory infiltration. In line with that, cytokine and chemokine mRNA expression did not change *versus* pair-fed mice and it

was significantly lower in comparison with *Mif* -/- → WT and WT →WT transplants. Importantly, the gene expression of MCP1 was strongly reduced in ethanol-fed WT → *Mif*-/-. Several published animal models describe a direct correlation between MCP1 increment and the higher recruitment of myeloid-derived Ly6C$^{+}$ monocytes to the liver [233–235].

Human serum samples and liver biopsies of subjects with alcoholic hepatitis were also analyzed to confirm the *in vitro* and *in vivo* findings. MIF was quantified both in peripheral and suprahepatic serum and was next correlated with the biochemical parameters screened in the same cohort of patients. A positive correlation was obtained only between suprahepatic MIF and bilirubin, AST, GGT and TG. On the contrary, no relevant results were obtained between the peripheral serum and the clinical markers analyzed. The suprahepatic serum was collected through vena cava and it is supposed to be enriched in hepatic products. The transjugular approach was used also to collect liver biopsy. Since suprahepatic MIF concentration was positively associated with several indicators of liver injury, the expression and localization of MIF was then investigated in liver biopsies collected. Autoimmune hepatitis samples were used as positive disease control. Increased MIF staining was detected in both alcoholic hepatitis and autoimmune hepatitis biopsies, compared with healthy controls. However, the cellular localization differed between alcoholic hepatitis and autoimmune hepatitis. In alcoholic hepatitis cases, MIF was predominantly localized in hepatocytes and ductular cells, while in autoimmune hepatitis MIF was mostly localized in non-parenchymal cells.

Overall, our present findings revealed a complex interplay between myeloid and non-myeloid cells during ethanol-induced liver injury in mice. Utilizing BMT model, in conjunction with *in vitro* co-culture system and human sample studies, we identified that hepatocytes are responsible for MIF production in ALD. Thus, our data support that MIF is essential for the progression of alcoholic hepatic damage. This last point is particularly important if we consider that ALD remains one of the frequent causes of death worldwide, without a specific therapeutic choice. Because of the key role of MIF in the pathophysiology of ALD, inhibition of its activity with molecules directly addressed to hepatocytes may be an important therapeutic approach to investigate.

# Chapter 6
# References

## 6. References


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# Chapter 7

# List of Publications

## 7. List of Publications

### Original manuscripts, reviews, book chapters

**Marin, V**; Poulsen, K; Odena, G; McMullen, M; Altamirano, J, Sancho-Bru, P, caballeria, J, Tiribelli, C; Rosso, N, Bataller, R Nagy, L. Hepatocyte-derived macrophage migration inhibitory factor mediates alcohol alcohol-induced liver injury in both mice and patients. *Submitted to J. Hep*

**Marin, V**; Rosso, N; Dal Ben, M; Raseni, Boschelle, M; A; Degrassi, C; Nemeckova, I; Nachtigal, P; Avellini, C; Tiribelli, C; Gazzin, S. An animal model for the juvenile Non-Alcoholic Fatty Liver Disease and Non-Alcoholic Steatohepatitis. PlosOne, 2016, Vol. 11 No.7.

**Marin, V**., Odena, G., Poulsen, K., Tiribelli, C., Bellentani, S., Barchetti, A., Sancho Bru, P., Rosso, N., Bataller, R., and L.E. Nagy. Role of MIF in Hepatic Inflammatory Diseases and Fibrosis, in MIF Family Cytokines in Innate Immunity and Homeostasis, ed. R. Bucala, Springer Press, in press for 2016

Rosso N, **Marin V**, Giordani A, Persiani S, Sala F, Cavicchioli L, Rovati LC and Tiribelli C. The Pros and the Cons for the Use of Silybin-Rich Oral Formulations in Treatment of Liver Damage (NAFLD in Particular). Current Medicinal Chemistry, 2015, Vol. 22, No. 1

Giraudi PJ, Barbero Becerra VJ, **Marin V**, Chavez-Tapia NC, Tiribelli C, Rosso N. The importance of the interaction between hepatocyte and hepatic stellate cells in fibrogenesis induced by fatty accumulation. Exp Mol Pathol 2014; Vol 98.

### Posters

Poster presentation at Liver Meeting 2015 (AASLD). *A novel mouse model for the study of pediatric Non-Alcoholic Fatty Liver Disease (NAFLD)* **Marin, V**; Rosso, N; Dal Ben, M; Raseni, A; Degrassi, C; Tiribelli, C; Gazzin, S. HEPATOLOGY October 2015 -Volume 62, Issue Supplement S1, 1378A. IF: 11.05

Poster presentation at Liver Meeting 2015 (AASLD). *Cross talk between hepatocytes and macrophages in Alcoholic Liver Disease: Which language do they use?* **Marin, V**; Poulsen, K; Nagy, L; Tiribelli, C; Rosso, N. HEPATOLOGY October 2015 -Volume 62, Issue Supplement S1, 868A. IF: 11.05

Poster presentation at Liver Meeting 2015 (AASLD). *Macrophage Migration Inhibitory Factor is Protective in a Model of Chronic-Binge Ethanol Feeding in Mice.* Poulsen, K; Rosso, N; **Marin, V**; McMullen, M. R; Morris, A. R; Tiribelli, C; Nagy, L. HEPATOLOGY October 2015 -Volume 62, Issue Supplement S1, 871A. IF: 11.05

Poster presentation at AISF, Modena October, 8-10 2015. *Effect of age and gender in the progression of NAFLD towards NASH in a juvenile mice model.* **Marin, V**; Rosso, N; Dal Ben, M; Raseni, A; Degrassi, C; Tiribelli, C; Gazzin, S. Digestive and liver disease SUPPLEMENT N°3 VOL. 47 2015 (IF 2.96)

Poster presentation at AISF, Modena October, 8-10 2015. *Therapeutic approach to NAFLD/NASH: is Silymarin able to ameliorate liver injury?* **Marin, V**; Gambaro, SE; Dal

Ben, M; Raseni, A; Degrassi, C; Tiribelli, C; Gazzin, S, Rosso, N. Digestive and liver disease SUPPLEMENT N°3 VOL. 47 2015 (IF 2.96)

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Poster presentation at ILC EASL, Wien April, 22-26 2015. *Alcoholic Liver Disease: effect of hepatocytes and macrophages co-culture in the inflammatory response.* **Marin, V**; Giraudi, P; Tiribelli, C; Rosso, N. Journal of Hepatology SUPPLEMENT N°2 VOL. 62 2015, p.S774 (IF:10.4)

Poster presentation at ILC EASL, Wien April, 22-26 2015. *The importance of the interplay between hepatocytes and hepatic stellate cells during fibrogenesis in a NASH in vitro model.* Giraudi, P; Barbero Becerra, V; **Marin, V**; Chavez-Tapia, N; Tiribelli, C; Rosso, N. Journal of Hepatology SUPPLEMENT N°2 VOL. 62 2015, p.S696 (IF:10.4)

Poster presentation at ILC EASL, Wien April, 22-26 2015. *Characterization of an in vivo model of juvenile Non-Alcoholic Fatty Liver Disease.* **Marin, V**; Rosso, N; Dal Ben, M; Raseni, A; Degrassi, C; Tiribelli, C; Gazzin, S. Journal of Hepatology SUPPLEMENT N°2 VOL. 62 2015, p.S711-S712 (IF:10.4)

Poster presentation at ILC EASL, Wien April, 22-26 2015. *Progression from NAFLD to NASH: Gender does really matter?* **Marin, V**; Gazzin, S; Dal Ben, M; Raseni, A; Degrassi, C; Tiribelli, C; Rosso, N. Journal of Hepatology SUPPLEMENT N°2 VOL. 62 2015, p.S707 (IF:10.4)

Poster presentation at 24$^{th}$ Conference of APASL, Istanbul March, 12-15 2015. *Interaction between hepatocytes and hepatic stellate cells as a crucial factor during fibrogenesis in a NASH in vitro model.* Giraudi, P; Barbero Becerra, V; **Marin, V**; Chavez-Tapia, N; Tiribelli, C; Rosso, N. Hepatol Int (2015) 9 (Suppl 1):S1–S391 (IF:2.46)

Poster presentation at 24$^{th}$ Conference of APASL, Istanbul March, 12-15 2015. *Hepatic inflammatory response to ethanol: the effect of hepatocytes and macrophages co-culture.* **Marin, V**; Giraudi, P; Tiribelli, C; Rosso, N. Hepatol Int (2015) 9 (Suppl 1):S1–S391 (IF:2.46)

Poster synopsis in Treatment Strategies- Cambridge Research Centre. *Protective effects of Silybin In an in vitro model of Non Alcoholic Fatty Liver Disease.* **Marin, V**; Tiribelli,C; Sava,G; Rosso,N May,2014 EASL Poster Synopsis Treatment Strategies Hepatology Volume 1 Issue1 P(35)

Poster presentation at ILC EASL, April 9-13 2014, London. *Protective effects of Silybin In an in vitro model of Non Alcoholic Fatty Liver Disease.* **Marin, V**; Tiribelli,C; Sava,G; Rosso,N. April, 2014 J .Hepatology, Vol. 60, Issue 1, S147

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