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# Protective role of liver X receptor alpha (LXRα) in development of Non-Alcoholic Steatohepatitis (NASH)

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# SUMMARY

Non Alcoholic Fatty Liver Disease (NAFLD) is a pathological condition of the liver which includes a spectrum of histological/clinical pictures with an evolutionary course from simple hepatic steatosis (accumulation of triglycerides in hepatocytes) through an inflammatory phase (Non-Alcoholic Steatohepatitis, NASH), up to the development of fibrosis which, in the advanced stages, can lead to cirrhosis with liver failure and an increased risk of the onset of hepatocellular carcinoma (HCC). Furthermore, the presence of hepatic steatosis with minimal fibrosis and therefore in the absence of overt cirrhosis constitutes a risk of neoplastic development, which indicates the need to identify suitable therapeutic strategies even in the early stages of the disease.

NAFLD is rapidly becoming the most common cause of chronic liver disease and cirrhosis, with an estimated prevalence of 30.05% of the world's adult population. The development of NAFLD depends on several partly genetic but mainly acquired factors. There are known racial differences where Hispanics have been identified as being more at risk, while African-Americans have shown a lower degree of steatosis than the general population. These differences may at least in part be explained by the fact that Hispanics more frequently possess a condition of the patatin-like phospholipase domain-containing protein 3 (PNPLA3) rs738409 polymorphism, the major genetic factor currently identified as predisposing to the development and progression of NAFLD.

Among the environmental factors, the presence of the Metabolic Syndrome represents the most important one. By Metabolic Syndrome we mean the association of factors that predispose to the risk of cardiovascular disease, and which include type II diabetes/insulin resistance, an altered lipid profile, arterial hypertension and obesity. Insulin resistance, in particular, is responsible for the alteration of the normal regulation mechanisms of lipid metabolism, the major cause of fat accumulation in the liver. In fact, insulin resistance induces increased lipolysis in the adipose tissue with an increase in free fatty acids which therefore accumulate in the liver. The other sources of lipids that cause hepatic steatosis also derive from the increased synthesis of triglycerides in the liver (de novo lipogenesis), again induced by a condition of insulin resistance and by the amount of fat taken in the diet.

The pathophysiological mechanisms responsible for the progression of NAFLD towards NASH have not yet been completely clarified, which prevents the identification of therapeutic targets suitable for slowing down/inhibiting the progression of the disease.

A factor certainly involved in the progression of liver damage is the development of lipotoxicity, due to the accumulation of free fatty acids in the liver which induces oxidative stress and consequently determines the activation of a chronic inflammatory process and a progressive deposition of collagen fibers at liver level.

Liver X receptors (LXRs) belong to the nuclear receptor family and consist of two isoforms, LXRα and LXRβ, which share most of the targets, including some related to fatty acid metabolism. LXRs are important regulators of lipid metabolism, but are expressed differently in the different organs. Unlike LXRβ, which is ubiquitously expressed, LXRα is expressed at high concentrations in the liver, intestine, adipose tissue, and macrophages.

In the liver, LXR's are the main receptors involved in the regulation of cholesterol metabolism and also have anti-inflammatory and antiproliferative functions. LXRs show pleiotropic actions that, through modification of metabolic conditions associated with tumor growth, can lead to reduced cell proliferation. LXR agonists could prevent lipotoxicity and tumor formation in in NASH-HCC by reducing cell proliferation and toxic lipids formation in NASH. Furthermore, the activation of LXRs in the intestine could block hepatic pro-inflammatory pathways mediated by bacterial translocation (activation of Toll-like receptors, TLRs), known to predispose to HCC development, via the increased concentration of plasmatic High Density Lipoprotein (HDL).

However, the use of LXR agonists for therapeutic purposes is contraindicated by the presence of important side effects such as hypertriglyceridemia and hepatic steatosis, due to the activation of the α isoform in hepatocytes and the consequent increased hepatic lipogenesis.

# INTRODUCTION

## 1. Non-alcoholic fatty liver disease (NAFLD)

Nonalcoholic fatty liver disease (NAFLD) is the term for a number of conditions caused by an accumulation of fat in the liver, due to hepatocyte injury, inflammatory processes, and fibrosis (1, 2). To be defined as such, at least 5% of hepatocytes must be affected by fatty liver disease, in individuals who do not consume or consume small quantities of alcohol and who do not have secondary causes such as viral infections, hereditary diseases or long-term use of drugs (3).

Nowadays NAFLD, along with obesity has become the most common cause of chronic liver disease globally (4). Currently, NAFLD ranks first among liver diseases in Western countries and is typically associated with other comorbidities such as diabetes, in obese patients, and metabolic syndrome in lean individuals (5-7). A sedentary lifestyle, combined with a caloric excess due to a Westernized diet, contribute significantly to the development of simple NAFLD (8, 9), a condition that affects, according to the most recent estimates, 30.05% of the world population (10, 11) (Figure 1).

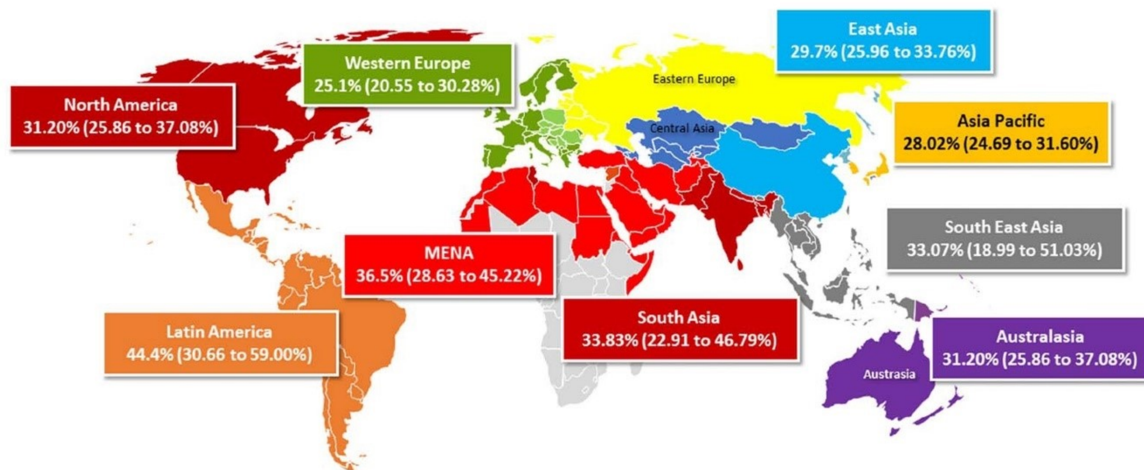


**Figure 1**: Global Prevalence of Nonalcoholic Fatty Liver Disease (NAFLD) (11).

In the United States, it is estimated that by 2030, NAFLD will become the leading cause of liver transplantation (12). Between 10-20% of people with NAFLD have nonalcoholic steatohepatitis (NASH) characterized by chronic liver inflammation, hepatocyte damage, and fibrosis (13). Furthermore, given the high prevalence of NAFLD worldwide, but

particularly in more developed countries, there is growing epidemiological evidence that NAFLD is an underlying etiology for many cases of hepatocellular carcinoma (HCC) (9).
The metabolic syndrome, due to the excessive production by the fatty liver of two metabolites such as glucose and triglycerides, is at the basis of the onset of NAFLD, also resulting in a key factor in the subsequent evolution into NASH (14). Normally triglycerides, together with sterol esters, are stored in the form of reserve organelles called lipid droplets (LDs), they function as an energy reserve, but they are also important regulators of lipid homeostasis as well as buffers of toxic lipid species (15). In case of dysregulated lipid homeostasis, some of the toxic lipids are released inducing cell dysfunction and death, playing an important role in the progression from NAFLD to NASH (16).
The pathogenesis of NAFLD is still unknown, resulting in an obstacle for the treatment of fatty liver disease (17).
Over the past twenty years many treatments have been developed and tested, but none have yet been approved. Therefore, it is of primary importance to focus research on understanding the pathogenesis of NAFLD in order to develop safe and effective drug therapies for the treatment of NAFLD.

## 1.1. From NAFLD to MAFLD

The first to coin the term NAFLD was Schaffner in 1986 (18). Since then, knowledge about it has greatly increased and we know that NAFLD is closely related to metabolic syndrome (3, 19). In fact, in most patients, NAFLD is associated with other metabolic pathologies such as obesity, type 2 diabetes and dyslipidemia (7).
For this reason, a new, more appropriate term has been proposed to define liver disease associated with metabolic dysfunction, namely fatty liver disease associated with metabolic dysfunction (MAFLD) (20-22).
The high prevalence of this pathology is linked to a series of incorrect behaviors, such as a sedentary lifestyle, lack of physical activity, excessive caloric intake and nutritionally unbalanced diets. For better identification, criteria have been proposed that go beyond the criteria used so far, which included the exclusion of other chronic liver diseases, but based on a positive diagnosis, histological tests (biopsy), imaging or blood biomarkers of accumulation of fat in the liver (fatty liver disease) as well as the presence of one of the following criteria, i.e. obesity, diabetes mellitus or metabolic dysregulation (20, 23).

It is evident that the diagnostic criteria are different from those previously used for the diagnosis of NAFLD. In particular, the main differences concern 1) the inclusion of metabolic abnormalities (20) and 2) the diagnosis is no longer made due to the exclusion of liver diseases of other aetiology (24, 25). The transition from NAFLD to MAFLD goes far beyond the change of nomenclature, but offers a number of expedients to facilitate diagnosis and improve clinical care (26).

The heterogeneous characteristics of the disease are the first cause of the attenuated efficacy of compounds under development, representing a major obstacle for the discovery of highly effective drug treatments (23). For the realization of an effective treatment it is necessary that it is targeted on the basis of the phenotype and the specific background of the patient (27, 28).

## 1.2. Gut microbiota in NAFLD

The intestinal microbiota is the set of microorganisms hosted in the intestine by each individual, able to interact with it to regulate essential functions. It is characterized by a pool of genes, called the microbiome (29).

Its composition is not constant over time, but is very dynamic, varying from individual to individual (30). The different intestinal microbiota can give the host unique metabolic characteristics, making it capable of adapting to environmental changes and substrate availability (31). A normal composition of the intestinal microbial population confers numerous benefits to the host such as development of the immune system, protection from pathogens, regulation of intestinal homeostasis and metabolic functions (32). Liver diseases are closely associated with alterations in the composition of the intestinal microbiota. These changes, both quantitative and qualitative, are called dysbiosis. Dysbiosis is characterized by reduction or loss of certain microbial species, associated with disorders of the immune response and metabolism (29). In addition, it can be characterized by an out-of-control growth of potentially pathogenic species. A dysbiotic microbiota compromises mucosal integrity, allowing translocation of bacteria or their products into the circulatory system, causing progression of liver disease (33, 34). The mechanism underlying this progression is a remodulation of the immune system, which involves the inflammasome via the metabolites produced by microorganisms and subsequent activation of the pro-inflammatory pathways (33). In this phase, specific liver receptors intervene, the so-called Toll-Like Receptor (TLR), multiprotein complexes capable of recognizing and binding the antigens derived from pathogens such as lipopolysaccharides (LPS) (35). This

interaction leads to a cascade of events with increased production of inflammatory cytokines and chemokines, including Il-6, Il-1 and TNFα and fibrogenic, such as TGFβ, as well as oxidative stress and endoplasmic reticulum stress ultimately culminating in cell death (32). However, the role of the inflammasome in the development of liver injury still remains to be elucidated.

Furthermore, the presence of products of bacterial derivation indirectly influences the metabolism, in particular that of glucose, by regulating the release of glucagon-like peptide-1 (GLP-1) with a sensitizing effect towards insulin (36).

The identification of the intestinal microbiota and the metabolites deriving from it could represent the right approach for the identification of diagnostic markers and therapeutic targets (32). Several studies in recent years have identified NAFLD-associated microorganisms useful as markers of liver health (37-40). The complete knowledge of the microbiota and how it intervenes in the intestine-liver axis will allow us to introduce new diagnostic methods and develop targeted therapies.

## 2. Non-alcoholic steatohepatitis (NASH)

Nonalcoholic Steatohepatitis, or NASH, is the severe form of NAFLD. It is defined as the presence of steatosis, inflammation, degeneration of liver cells, with or without fibrosis, and can progress to cirrhosis, with a risk of developing HCC (14, 41). In particular, high degrees of steatosis, balloon degeneration, and inflammation are required in the pathogenesis of cirrhosis and HCC, associated with high levels of cellular morbidity and mortality (42) (Figure 2).

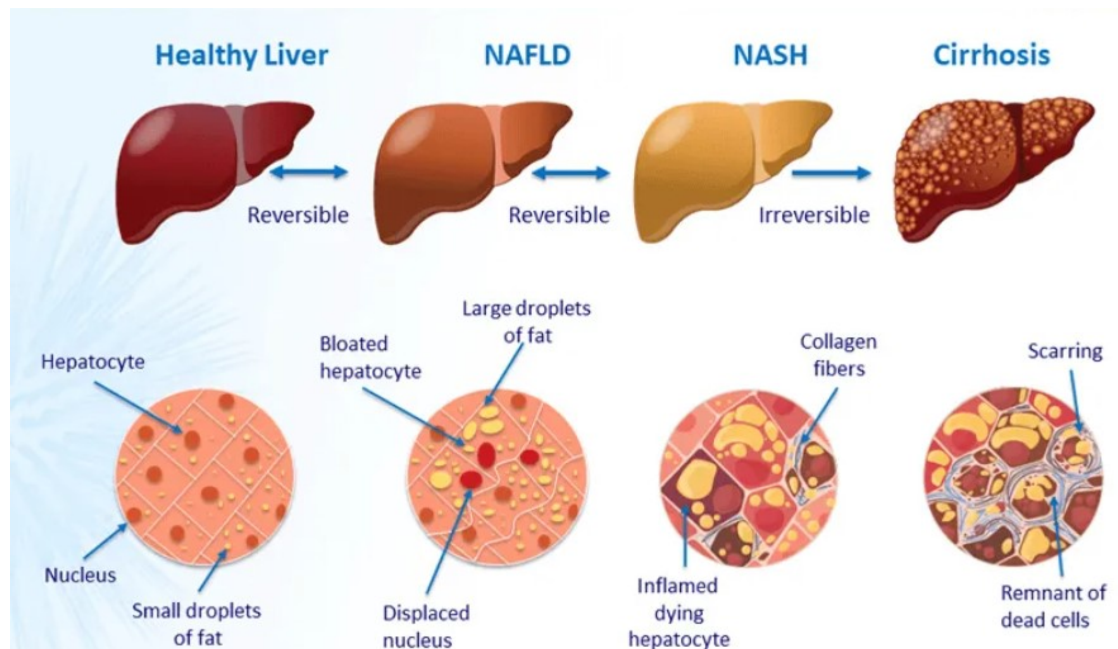


Figure 2: Progression of disease (HCV-triols.com)

Histologically, the most important feature associated with NASH mortality is the presence of significant levels of fibrosis (10). In fact, it has been amply demonstrated that a worse prognosis, with an increase in mortality, occurs in subjects with severe fibrosis (fibrosis stage 3-4) (43).

The global prevalence of NAFLD has increased over the years in parallel with obesity rates (44). It went from 15% in 2005 to 25% in 2010, up to about 40% in 2016 (45, 46). Similarly, in the same period, the NASH rate also almost doubled, from 33% to 59% (10). If we consider the obese population, the data is even more alarming. In fact, obesity was found in 51% of NAFLD patients, reaching up to 82% in individuals with NASH (47).

This is even more worrying considering that these data are constantly growing, making NASH among the leading causes of liver transplantation in the United States.

Among patients with NAFLD, about 30% of cases progress to NASH, and of these, about 20% progress to cirrhosis (8, 48). A common condition of metabolic diseases such as alcoholic liver disease (ALD) and NAFLD is that following exposure to harmful agents, such as a diet high in simple sugars and/or alcohol, there is accumulation of lipids in the liver, which in addition to leading to steatosis, is accompanied by an increase in inflammatory indices, which leads to fibrosis up to cirrhosis and liver cancer (49).

However, the pathophysiological mechanism underlying the progression of NAFLD in NASH remains unclear, although it is known that the basis is an accumulation of lipids in the liver, an increase in inflammation, as well as damage to hepatocytes and the deposition

of collagen fibers. The "two-hit hypothesis" (50), explaining how steatosis evolves towards NASH and fibrosis, is limited and does not illustrate the multifactorial complexity of the disease. Therefore the "multiple impact model" has been proposed and is widely used (51). This model identifies several key factors in the pathogenesis of NAFLD, such as lipid accumulation, insulin resistance, oxidative stress, gut microbiome, apoptosis and inflammation (52, 53). These factors act in a coordinated and cooperative manner, driving the development of NASH.

Lipotoxicity occurs when hepatic lipid homeostasis is compromised, i.e. when the liver is no longer able to use, store or transport free fatty acids (FFA) as triglycerides (TG), as it is saturated by an abundant supply of peripheral FFA mainly from adipose tissue and de novo lipogenesis (29, 51). A part of FFA is first metabolized by mitochondrial β-oxidation or re-esterification into triglycerides in hepatocytes and then stored in the liver in the form of lipid droplets. While, another part of FFA is exported as very low density lipoprotein (VLDL) (54). Toxic lipid species are derived from the lysis of lipid droplets, which cause ER inflammation, oxidative stress, cytokine release, and activation of inflammatory mediators within liver cells (55).

Several immune cells, such as Kupffer cells (KC), are also involved in the progression of fatty liver disease. The KCs, macrophages present in the liver, once activated, in addition to contributing to lipid peroxidation and consequent production of ROS, in collaboration with the proinflammatory macrophages deriving from circulating leukocytes and monocytes, shift the balance towards a proinflammatory state at the expense of the macrophages anti-inflammatories (17). This is due to the increased expression of proinflammatory cytokines such as interferon-γ (IFN), tumor necrosis factor (TNF) and lipopolysaccharide (LPS), activating the signaling pathways cascade of apoptosis and cell death (56).

Cells, undergoing apoptosis and necrosis, release damage-associated molecular patterns (DAMPs), capable of binding particular receptors such as pattern recognition receptors (PRRs) such as tool-like receptors (TLRs) and NOD-like receptors ( NLR) further enhancing the inflammatory response by activating immunoprotein complexes called inflammasomes, such as NLRP (57). The inflammasome induces the activation of caspase-1 which is capable of inducing an inflammatory cell death called pyroptosis (58). Furthermore, the inflammasome is important for the progression of NASH as it causes fibrosis and cirrhosis.

To date, there are no licensed drugs for the treatment of NASH. Since bariatric surgery is effective in a small percentage of cases (59), diet and exercise are the only weapons in dealing with the disease (17). Vilar-Gomez et al. (60) have observed that a 10% weight loss allows a reversal of steatosis, inflammation and fibrosis. However, the long-term beneficial effects of weight loss are still unknown (61).
The realization of a pharmacological treatment capable of blocking the progression of NAFLD in NASH requires the complete knowledge of the mechanism that drives this passage.

## 2.1. Liver fibrosis

NASH is the leading cause of liver fibrosis to date, surpassing chronic HCV and HBV infections as the leading cause. Fibrosis is the result of the wound healing response during chronic liver injury, not dependent on etiology (62). Characterized by the deposition and accumulation of extracellular matrix (ECM) proteins and by the subsequent alteration of the liver parenchyma, over time it can evolve into cirrhosis and HCC (63). It is therefore evident that hepatic fibrosis is a key factor in the development of the disease and the related mortality (64). Fibrosis levels are not only responsible for the progression of fatty liver disease, but are responsible for other clinical manifestations associated with NAFLD. In particular, the increase in the degree of fibrosis is associated with an increase in insulin resistance, with an increased risk of developing type 2 diabetes in subjects with fatty liver disease (65).
Among the cell populations involved in fibrogenesis processes are hepatic stellate cells (HSC) (62). These, located between hepatocytes and endothelial cells, are activated in the presence of chronic liver damage and undergo a process of differentiation into myofibroblasts, capable of abundantly secreting ECM proteins and simultaneously releasing pro-inflammatory and fibrogenic factors , such as transforming growth factor β (62, 66). Furthermore, the increased inflammatory response following chronic insults of the liver has the further effect of activating HSCs, which when activated further secrete pro-inflammatory cytokines. Free cholesterol has also been indicated as a signal capable of sensitizing and activating HSCs, contributing to the progression of fibrosis and steatosis (67).
However, although research has focused on hepatic signals in the development of fibrosis, it must be remembered that it is influenced by extra-hepatic events. The interaction between different organs, in particular fat and intestine, but also vascular abnormalities,

can contribute to the development of liver fibrosis. For example, adipose tissue produces adipokines and molecules involved in pro-inflammatory signalling, such as TNFα and IL-6 with pro-fibrogenic effects (68).

The complete reversibility of fibrosis, especially in an advanced stage, is not yet fully understood. In a stage including cirrhosis, when the scar tissue has thickened, irreversibility is very likely (69). However, based on clinical experience, a regression of fibrosis is possible thanks to interventions aimed at the remodulation of inflammatory cytokines, of the inflammatory microenvironment, but above all of the behavior of the HSCs. The reduction or reprogramming of activated HSCs is the key event for the reversibility of the fibrosis process (70, 71). Macrophages resident in the liver are also an important cellular component for the reduction of fibrosis, thanks to their antifibrotic activity (72), they are also capable of remodulating the anti-inflammatory activity (73).

A new therapeutic approach capable of inhibiting fibrogenesis and promoting the resolution of fibrosis may be the right way to reduce the risks associated with the progression of liver disease.

## 3. Hepatocellular Carcinoma (HCC)

Progression of NASH may lead to cirrhosis or liver failure, representing a risk for the development of hepatocellular carcinoma (HCC), and is expected to outnumber viral hepatitis as the common etiology for HCC (74, 75). This is due to the rapidly growing epidemic of metabolic syndrome. Metabolic syndrome, together with obesity, insulin resistance and type 2 diabetes aggravate the course of NASH, increasing the risk of HCC (75, 76). Approximately 75-85% of liver cancers are represented by HCC, making it the most common primary liver cancer, as well as the sixth most common cancer in the world and third in terms of deaths (77). In most developed countries this proportion has nearly tripled in recent years, increasing incidence associated primarily with the obesity and NAFLD/NASH pandemics (78).

Its pathogenesis is highly complex, as it is linked to both genetic and environmental factors. Most of the proposed molecular mechanisms for tumor development are present in the etiology of NASH (79), such as alterations in metabolism, oxidative stress, endoplasmic reticulum stress and mitochondrial dysfunction (80).

Many genetic factors influencing the development of NASH and HCC are now known, these influence the heterogeneous prevalence of fatty liver disease among different races and ethnicities (81-83). Single nucleotide polymorphisms (SNPs) in several genes, including PNPLA3 (84), TM6SF2 (85), and MBOAT (86), are associated with an increased risk of HCC. However, variants have been identified that reduce the risk of HCC. This indicates that the genetic component plays a fundamental role in the risk of developing liver damage and subsequent NASH/HCC, regulating several signaling pathways crucial in the carcinogenesis process (80). Micro RNAs (miRNAs), involved in the regulation of the expression of several genes involved in a broad spectrum of processes such as lipid and fatty acid synthesis, glucose metabolism, inflammation, proliferation and apoptosis are dysregulated in many types of cancer, including HCC (87).

Other crucial aspects to consider in the development of cancer are lipotoxicity and glucotoxicity. Insulin resistance stimulates the release of insulin-like growth factor 1 (IGF-1) and insulin receptor substrate 1 (IRS-1), important for cell proliferation and apoptosis, promoting the development of HCC (88). The onset and progression of NASH is closely associated with liver inflammation (89). Sterile inflammation, in the absence of pathogens and due to DAMP release causes IL-1β and IL-8 maturation and release, supporting chronic inflammation and cancer progression (9). Furthermore, pathogen-associated molecular patterns (PAMPs), such as LPS, also involved in NASH progression, together

with DAMPs, bind to pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), inducing a response inflammation mediated by cytokines such as TNF-α and IL-6. These, by activating other factors, further drive the oncogenic processes (90). Furthermore, TNF-α and IL-6 promote hepatocyte cell growth as a response to excessive apoptosis through NF-κB, mTOR and STAT3 and the release of chemokines (CCL2 and CCL7) and cytokines (TNF-α, IL-1β, IL-6) (91). Cytokines and chemokines produced by hepatocytes and KC, activate hepatic stellate cells (HSC), stimulating collagen production, fibrotic scar formation and stimulating Fas and TNFR1 receptors inducing apoptosis (91, 92).
Finally, a key role for the transition from NASH to HCC is played by mitochondria. Fundamental for cellular homeostasis, they supply chemical energy in the form of ATP, regulate redox balance, β-oxidation and glucose and lipid metabolism (91). Underlying mitochondrial dysfunction is an excessive accumulation of FA, which increases the levels of β-oxidation resulting in the production of ROS. High concentrations of ROS cause mutations in nuclear and mitochondrial DNA (mtDNA), promoting the activation of oncogenes and inhibition of tumor suppressors (91). Therefore, several biological processes intervene in the transition from NASH to HCC, resulting in determining the severity of HCC disease.

## 4. Nuclear Receptors (NRs)

Nuclear receptors (NRs) belong to a superfamily of transcription factors capable of responding to a variety of ligands, which include both endogenous ligands, such as steroid hormones, cholesterol, fatty acids (FA), bile acids (BA), and vitamins , both exogenous compounds such as drugs and toxins, both natural and synthetic (93).
Since their discovery in the mid-1980s, numerous ligands, both natural and synthetic, have been identified that can cause both activation and suppression of gene transcription. Although different, the NRs exhibit a highly conserved structure (94, 95).
The first class of receptors are the endogenous receptors. They have high affinity for fat-soluble hormones and vitamins. In this class we find the receptors for steroid hormones, Thyroid hormone Receptor (TR), and for vitamins A and D, the Retinoic Acid Receptors (RAR) and the Vitamin D Receptor (VDR), respectively, necessary for the homeostasis of the endocrine system. From a strictly mechanical point of view, these receptors function as homodimers, forming heterodimers with the Retinoic X Receptor (RXR)(94).

The ligands and natural functions of many NRs were initially unknown, thus this class of NRs was termed 'orphan'. For some orphan NRs the receptors have been identified and the ligands defined and therefore renamed "adopted orphans" (96).
Adopted orphan receptors include Farnesoid X Receptor (FXR), Liver X Receptor (LXR), Pregnane X Receptor (PXR), Peroxisome Proliferator-Activated Receptor (PPAR), and Retinoid X Receptor ( RXR) (96, 97). Also in the category of adopted orphan receptors are "enigmatic" adopted orphans, for which a ligand has been identified, but the nature of the ligand-dependent regulation is not known. This group includes receptors whose activity can be modulated by both endogenous molecules and synthetic estrogens (94, 96).
The last class is made up of true orphan receptors, characterized by the fact that the ligand, whether natural or synthetic, has not yet been identified. However, these receptors are most likely thought to be regulated by coactivator availability, receptor expression, or covalent modification (94).
It is the largest group of transcriptional regulators, consisting of 48 members in humans (95) and 49 in mice (94). They play different roles in cell differentiation, development, proliferation and metabolism, often resulting in various liver diseases ranging from simple steatosis and inflammation to fibrosis and cancer (97). Several members of the NR family have a multidomain structure, exhibiting different regions capable of binding the ligand, allowing its activation. The most common NR structure (Figure 3) provides an activation domain independent of the $NH_3$-terminal ligand, which takes the name of AF-1, indispensable for interactions with the cofactors, a central DNA binding domain, with a particular conformation called a zinc finger, a hinge region, and a C-terminal ligand binding domain (LBD). Given the uniqueness of LBD, it allows NRs to distinctly bind the ligand, subsequent polymerization, and interactions with the coregulator (94, 96).

Figure 3: Schematic structure of NRs (nuclear receptors) (98).

The NRs, in the absence of ligands, will be localized in the cytoplasm or in the nucleus, bound to a hormonal response element of the DNA but repressed by a corepressor. Ligand binding results in a conformational change in the FA, allows release of the corepressor and recruitment of a coactivator, ultimately leading to activation of gene transcription (97).
The role of NRs is of fundamental importance, as they regulate a wide variety of metabolic pathways, intervening in the metabolism of hepatic lipids and lipoproteins, in the

maintenance of glucose and bile acid homeostasis, but also in other activities such as embryonic development, reproduction, inflammation, fibrosis as well as some aspects of tissue repair such as liver regeneration and tumor formation (93, 95, 97).
Therefore, NRs play a key role in the control of liver functions, studying them could allow us to better understand liver physiology and explain the pathophysiology, also representing valid targets for the development of new therapies for the treatment of different liver diseases (93).

## 5. Liver X Receptor

Liver X receptors, referred to as LXR, belong to the NRs supergene family of ligand-activated transcription factors. The whole family includes 48 members involved in different physiological and pathological mechanisms. Initially considered as orphan receptors and subsequently reclassified as adopted orphan receptors, since cholesterol-derived oxysterols were identified as natural ligands (99, 100).

Two isoforms of LXR have been extensively described, respectively LXRα (NR1H3) and LXRβ (NR1H2) (101). Both isoforms share 78% of the amino acid sequence in their DNA and ligand binding domain, but differ in their localization (102). While LXRβ is ubiquitously expressed in almost all tissues, in fact it is widely expressed in the immune system, glial cells of the central nervous system, pancreatic islets, prostate epithelium and gallbladder, LXRα expression is mainly limited to organs that control lipid metabolism, such as liver, intestine, adipose tissue, kidneys and macrophages (102). Therefore, expression levels in various tissues are crucial to determine their role in transcription.

Both LXR isoforms consist of four domains: 1) domain with activation function (AF-1) which can stimulate transcription even in the absence of a ligand; 2) DNA binding domain (BDB); 3) a ligand binding domain (LBD) required to bind the ligand and finally 4) an activation domain (AF-2), which stimulates transcription in response to ligand binding (103).

LXR receptors function as heterodimers with the retinoid X receptor (RXR), which is also common to other NRs (104). The LXR/RXR heterodimer (Figure 4) is considered a permissive heterodimer that can be activated either by the LXR agonist or by specific RXR ligands. The RXR/LXR complex, once activated, binds to a liver X receptor (LXRE) response element at the promoter of the target gene. LXRE consists of a fragment of directly repeating DNA (DR-4) comprising two AGGTCA sequences separated by 4 spacer nucleotides.

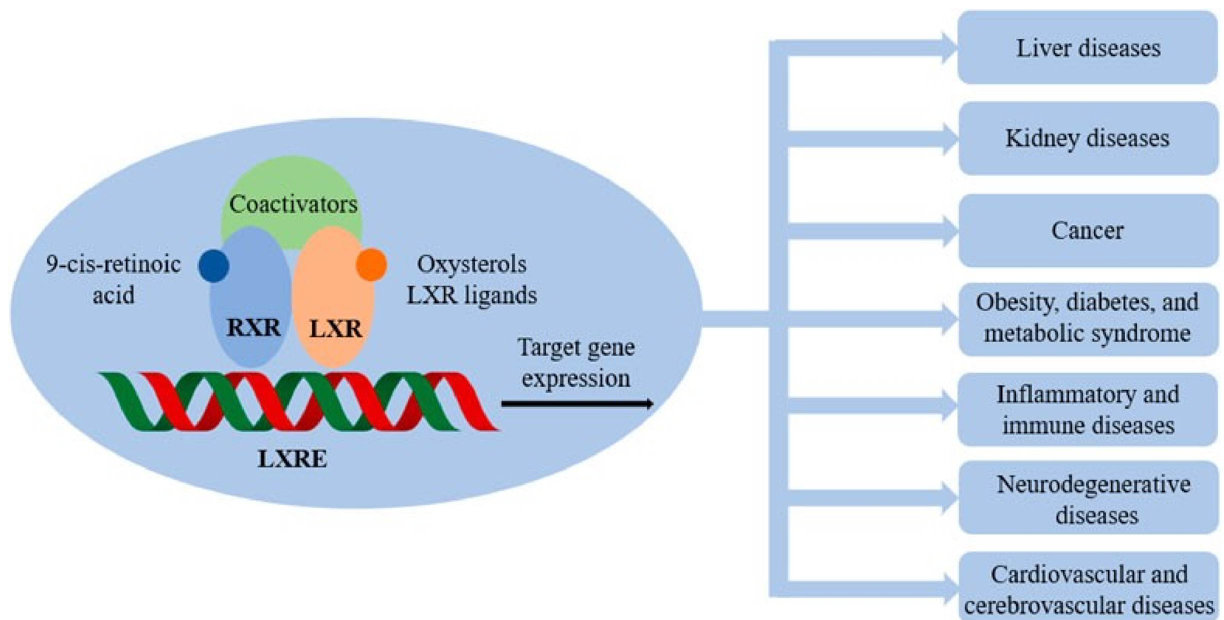


Figure 4: Liver X receptors (LXRs) form a heterodimer with retinoid x-receptors (RXRs) capable of mediating many pathological conditions (105).

Like other receptors, such as PPAR and FXR, LXRs can stimulate transcription even in the absence of ligands, however they can also repress the expression of some genes in the unbound state (106). A three-phase model has been proposed to describe the activation mechanism of LXR. Initially, in the absence of ligands, the RXR/LXR heterodimer inhibits transcription by recruiting co-repressors. After ligand binding, there is initially a removal of the co-repressor, due to a change in the conformation of the receptor, and subsequently activation of transcription, at moderate levels, and recruitment of co-activators. Recruitment of co-activators results in maximal stimulation of transcription (103). Some LXR ligands can also inhibit transcription by several mechanisms, including: 1) by binding to other transcription factors and inhibiting their activity; 2) competition between LXR and other transcription factors for RXR binding; 3) competition between RXR/LXR heterodimers and other transcription factors for co-activators.

LXRs regulate specific gene networks involved in the metabolism of lipids, glucose, and especially cholesterol. They can mediate inflammatory activity and modulate the immune response.

Excess amounts of cholesterol can be detected by LXRs, by different mechanisms, playing a protective role against cells. Reverse cholesterol transport (RTC) regulates the trafficking of cholesterol from peripheral tissues to the liver, where it is excreted in the bile or converted into bile acids. Free cholesterol and its esters are transported to the liver in high-density lipoprotein (HDL) or low-density lipoprotein (LDL) and very low-density

lipoprotein (VLDL). LXRs regulate the expression of cholesterol transporters, complexes that drive cholesterol from the plasma membrane to extracellular acceptors, called ATP-binding cassette (ABC) transporters. ABCA1 and ABCG1 are the most abundant forms, present both in macrophages and in hepatocytes, enterocytes and adipocytes. ABCA1 is defined as a complete transporter, as it is able to transport both cholesterol and phospholipids from the plasma membrane to pre-HDL or free apolipoproteins, forming HDL, while ABCG1 is a half transporter as it only transfers cholesterol to HDL. At the hepatic level, through the type 1 scavenger receptor (SR-B1), the uptake of HDL takes place in the hepatocytes (107, 108).

In addition to efflux, LXRs are also responsible for the regulation of intracellular trafficking of cholesterol, i.e. the passage from the endosomal compartment to the plasma membrane. This step occurs thanks to two vectors, the Niemann-Pick C1 (NPC1) and C2 (NPC2) proteins, which increase their expression following activation mediated by LXR agonists (103).

HDL can exert anti-inflammatory and antioxidant effects in macrophages. This is related to the direct link between lipid metabolism and inflammation (62). LXRs regulate cholesterol levels through the expression of genes associated with the reverse transport of cholesterol, the conversion of cholesterol into bile acids, but also the intestinal absorption of cholesterol (100, 109). They also modulate circulating lipopolysaccharide (LPS)-induced inflammatory response by inhibition of NF-κB signaling (110).

Therefore, given the direct involvement of LXR in numerous pathways related to the development of NAFLD and its progression in NASH/HCC, the possibility of modulating its expression with pharmacological treatment opens the door to the discovery of new drugs useful for the therapy of these pathologies.

# AIM OF THE PROJECT

NAFLD is currently the most common chronic liver disease in Western countries where it is the leading cause of death among liver diseases. The pathophysiological mechanisms underlying the development and progression of the disease have not been fully elucidated, which is associated with the lack of effective drug therapies.

For the development of this project, we used a mouse model which involved the administration of low doses of $CCl_4$ in combination with WD feeding to obtain the histopathological manifestations of human NAFLD (111). The use of this model is essential to investigate the mechanisms underlying liver damage and to investigate inflammatory responses.

LXR belongs to the family of nuclear receptors and is recognized as the main regulator of cholesterol homeostasis; indeed, the accumulation of cholesterol products induces a complex response which, through the activation of LXR target genes, regulates hepatic synthesis of cholesterol, its hepatic and intestinal excretion, intestinal absorption and increases the RCT. However, the use of LXR agonists for therapeutic purposes is contraindicated by the presence of important side effects such as hypertriglyceridemia and hepatic steatosis, due to the activation of the α isoform in hepatocytes and the consequent increase in hepatic lipogenesis.

However, it has been amply demonstrated that a specific activation of LXRα in the intestine determines an upregulation of the genes target of LXR in the intestine and involved in the processes of cholesterol homeostasis, both at the luminal and plasma levels, consequently reducing the hepatic accumulation of triglycerides and hepatic fatty acid synthesis (112).

Based on the results previously obtained by our group, the aim of the present study was to evaluate whether a selective intestinal activation of LXRα could reduce the features of the metabolic syndrome, especially lipid accumulation and liver inflammation, by limiting the intestinal dysbiosis and acting as an antagonist of bacterial components, especially LPS.

The use of an "intestinal" approach would also be fundamental as it is devoid of systemic side effects. In our opinion, this study could pave the way for new targeted and therefore effective therapeutic strategies.

# MATERIALS AND METHODS

## 1. In vivo model

Transgenic mice, with a constitutively active form of LXRα in erythrocytes, called iVP16-LXRα (kindly provided by Prof. Moschetta, University of Bari) and their respective non-transgenic controls, FVB/N, were kept in the breeding facility until between 8 and 12 months of age, at a temperature of 20-24°C with a 12-hour light/12-hour dark cycle with water and a standard diet *ad libitum*.

The iVP16-LXRα animals present the transactivation domain of the *Herpes Simplex Virus* (VP16) coding sequence cloned downstream of the villin promoter. This allows for specific intestinal expression of VP16. VP16 is a potent transcriptional activator which, when expressed as a chimeric protein, results in a constitutive induction of LXRα target genes.

Male mice aged between 8 and 12 weeks, belonging to the two genotypes, were fed a normal standard chow diet (CD, Mucedola s.r.l., 4RF21-PF1610) and normal tap water or, alternatively, a Western diet (WD) enriched with saturated fats (21,1%), sucrose (41%) and cholesterol (1,25%) (Teklad diets, TD. 120528) and a high sugar solution containing d-fructose (23,1 g/L) and glucose (18,9 g/L). Administration of WD or standard diet was combined with Carbon Tetrachloride ($CCl_4$) injection (Sigma-Aldrich, 289116-100ML), at a dose of 0,2 µl (0,32 µg)/g body weight or its control vehicle, corn oil, respectively, intraperitoneally once a week, starting simultaneously with the administration of the diet. The experimental groups, as shown in Figure 6, were as follows: CD/Oil controls (9 mice per group) and WD/$CCl_4$ treated (12 mice per group), for each genotype, for 12 weeks of treatment (Figure 6). At the end of the treatment the animals were anesthetized and sacrificed following the institutional guidelines. Serum, liver and intestinal samples were collected for each animal.

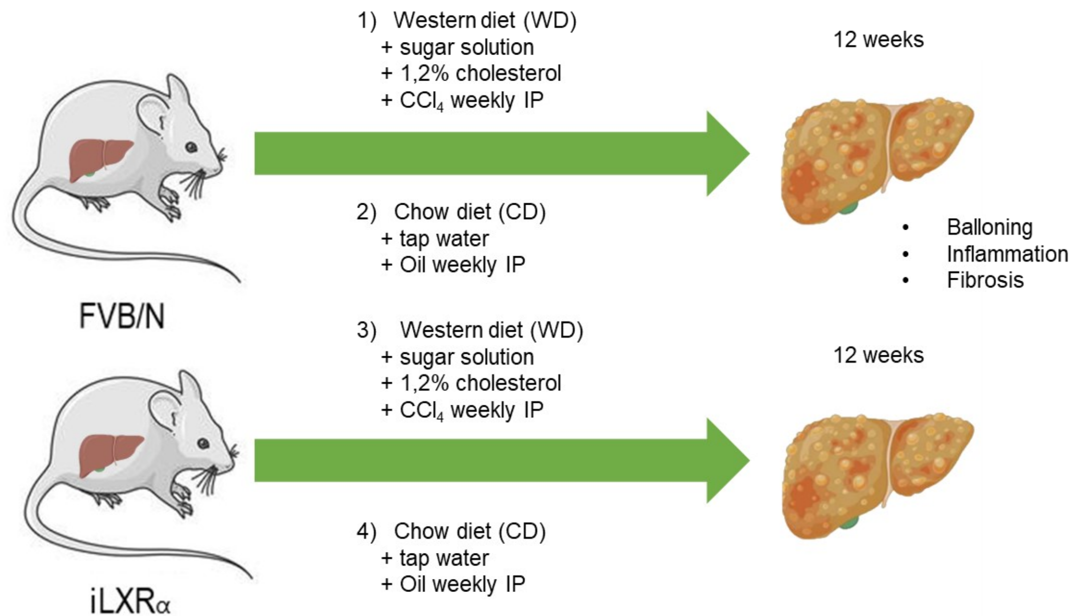


Figure 6: Representative scheme of the treatment on animal models. A total of 4 experimental groups were made as follows: FVB/N mice on a chow/corn oil diet; FVB/N mice on WD/$CCl_4$ diet; iVP16-LXRα mice on chow/corn oil diet and iVP16-LXRα mice on WD/$CCl_4$ diet.

## 2. Histological analysis

Liver samples from VP16-LXRα and FVB/N mice were fixed in 4% paraformaldehyde for 12-24h, dehydrated, and paraffin embedded. Histological sections, 5µm-thick, were stained with hematoxylin and eosin (H&E) for evaluation of liver histology and with Masson's trichrome stain for the identification and quantification of fibrosis and strictures. Steatosis was evaluated by a scale range 0-3 according to Brunt et al. (113). The analysis of fibrosis was quantified calculating the percentage of positive area for Masson's trichrome by Image J software (National Institutes of Health, Bethesda, MA, USA). Ten different fields were counted for each sample and presented as fold change compared with the control (114).

### 2.1. Immunofluorescence

Immunofluorescence was performed on 2 µm-thick sections obtained from formalin-fixed mouse liver tissue embedded in paraffin. Antigen retrieval was performed with ethylenediaminetetraacetic acid (EDTA) (pH 9) (Dako, Glostrup, Denmark). Liver specimens were stained after overnight incubation at +4˚C with: mouse monoclonal anti-CD68 (Abcam, Cambridge, UK) used as a pan-macrophage marker, rabbit monoclonal anti-CD206 used as a marker of M2 macrophages (Abcam, Cambridge, UK. After incubation with primary antibodies, specimens were washed) and treated for 1 h with

labeled isotype-specific secondary antibodies goat- anti-mouse Alexa Fluor 488 (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) and mouse anti-rabbit Alexa Fluor 555 (Life Technologies, Carlsbad, CA, USA). Nuclei were counterstained with Hoechst.

### 3. Quantification of hepatic hydroxyproline content

To quantify collagen content in the liver, hydroxyproline concentration was measured. Livers were homogenized using TissueLyser (Qiagen), precipitated by trichloroacetic acid and incubated for 24 hours at 110°C in 6N HCl. After hydrolysis, samples were processed with Hydroxyproline Assay Kit (#MAK008, Sigma Aldrich, St. Louis, MO, USA) according to manufacturer's instructions. Absorbance was measured at a wavelength of 560 nm (Tecan Group Ltd., Männedorf, Switzerland).

### 4. Quantification of cholesterol and lipoproteins

Total liver cholesterol was assayed using the Cholesterol Fast kit (Diacron Labs, Grosseto, Italy, ref. 10.501). For each sample, 10 mg of liver was homogenized with 200 µl of Chloroform:Isopropanol:NP-40 (7:11:0.1). The obtained product was centrifuged at 15,000xg. The supernatant was recovered and transferred to a new test tube, where it was evaporated at 50°C to remove the chloroform. The product obtained was then subjected to a series of enzymatic reactions which made it possible to obtain a colored compound whose intensity is proportional to the concentration of total cholesterol present in the sample. The reading was carried out with a spectrophotometer (OD=505nm).
Circulating lipoprotein levels (HDL, LDL and VLDL) were determined in serum samples using the HDL and LDL/VLDL Cholesterol Assay kit (Abcam®, Cambridge, UK, ab65390). To separate HDL and LDL/VLDL, 100µl of Assay Buffer supplied by the manufacturer was added to 10mg of tissue. After centrifugation at 13000 xg for 10 minutes, the supernatant was transferred to a new tube to separate HDL from LDL and VLDL by addition of 2X Precipitation Buffer and centrifuged at 2000 xg for 5 minutes. In the supernatant we will have the HDL fraction, while in the precipitate the LDL/VLDL fraction, which will be resuspended in PBS before proceeding to the subsequent steps of the procedure. Finally, we add Total Cholesterol Reaction Mix Free Cholesterol Reaction Mix for each sample in reaction, we incubate for 60 minutes at 37°C away from light sources. Absorbance was measured at a wavelength of 560 nm (Tecan Group Ltd., Männedorf, Switzerland).

## 5. Quantification of triglycerides

The triglyceride content in liver was evaluated by colorimetric determination using the Triglyceride quantification Kit (Abcam®, Cambridge, UK, ab65336), following the protocol provided by the manufacturer. 100 mg of tissue were homogenized in 5% (v/v) of Nonidet-P40 (NP-40) in $H_2O$, incubated at 85°C for 5' and then cooled to room temperature. The samples thus obtained were centrifuged for 2' in order to remove traces of insoluble material. The supernatant was then used for quantification. Briefly, triglycerides were converted to free fatty acids and glycerol upon incubation with lipase. The glycerol is then oxidized to form a product which, by reacting with the probe present in the kit, generates a colored compound measurable with a spectrophotometer at a wavelength of 570nm.

Serum triglyceride levels were measured by using an enzymatic assay (Diacron Labs®, Italy, ref. 10.508) and performed according to the manufacturer's protocol. Triglycerides are hydrolysed in the presence of lipoprotein lipase (LPL) to fatty acids and glycerol; the latter is phosphorylated and transformed forming a purple colored compound, the intensity of which is proportional to the concentration of triglycerides present in the sample, and measured with a spectrophotometer at a wavelength of 570 nm.

## 6. Analysis of lipopolysaccharide (LPS)

LPS concentration was measured in mice serum by a commercially available kit, Limulus amebocyte lysate (LAL) Assay (Hycult Biotech, Uden, Netherlands). Briefly, the samples were incubated with the LAL reagent, causing an enzymatic reaction triggered by the presence of the endotoxin which will cause the color change of the solution, becoming yellow. Subsequently the enzymatic reaction was stopped by adding acetic acid. The absorbance was measured at a wavelength of 405 nm (Tecan Group Ltd., Männedorf, Switzerland).

## 7. *In vitro* experiments

Two cell lines, HepG2 and LX-2, were used. Hepatocellular carcinoma cells of the liver (HepG2 cells, LGC Standards S.r.l., Italy) were cultured in Minimal Essential Medium (MEM) at 37°C in a humidified atmosphere with 5% $CO_2$. The medium was supplemented with 10% fetal bovine serum (FBS), 0.01 ml/ml non-essential amino acids (NEAA), 0.01 Na-pyruvate, 2 mM L-glutamine, 1% penicillin-streptomycin, 10 mg/ml gentamicin. Human liver stellate cells (LX-2 cells, Sigma-Aldrich, Saint Louis, MO) were cultured in

Dulbecco's Modified Eagle Medium (DMEM High Glucose) at 37°C in a humidified atmosphere of 5% $CO_2$. The medium was supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 1% glutamine. All products were purchased from Sigma-Aldrich (Sigma-Aldrich, Saint Louis, MO). The cells were plated at 70% confluency and after 24 hours they were subjected to silencing using the INTERFER reagent in Polyplus-transfection S.A, Illkirch, France), according to manufacturer's instruction either with selected predesigned siRNA or nt-RNA (Ambion Life Technologies Corporation, Woburn, MA).

## 8. Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from the liver and intestine samples using the TRIzol® reagent (Life Technologies Corporation, Woburn, MA) according to the methods indicated by the supplier company. Briefly, approximately 50-100 mg of tissue was homogenized in 1mL of TRIzol®. Subsequently chloroform was added and, after centrifugation, the aqueous phase containing RNA was recovered from each sample. The RNA was then precipitated in 100% 2-Propanol and then rehydrated in 75% ethanol. Finally, the RNA was resuspended in RNase-free water.

The concentration of the extracted RNA, together with its purity, was evaluated by Nanodrop analysis. Subsequently, 1μg of RNA from each sample was reverse transcribed into cDNA by MultiScribe™ Reverse Transcriptase (Thermo Fisher Scientific-Invitrogen, Waltham, MA, USA) and using Random Primers (Abcam, Cambridge, UK). Finally, 50ng of cDNA obtained were used for Real Time qPCR analyses. The relative concentration of the target genes was normalized to cyclophilin, as internal control.

The primers used for the Real Time qPCR were designed with the Oligo 6 and Beacon Designer software with reference to the mRNA sequences obtained from the Gene Bank; the specificity of the primers was confirmed by BLAST analysis. The list and sequences of primers used is included in Table 1.

| Gene name | Sense 5’ → 3’ | Antisense 5’ → 3 |
|---|---|---|
| m CYCLOPHILIN | CAGTGCTCAGAGCTCGAAAGT | GTGTTCTTCGACATCACGGC |
| m ABCG5 | TCAATGAGTTTTACGGCCTGAA | GCACATCGGGTGATTTAGCA |
| m ABCG8 | AATGTCATCCTGGATGTCGTCTC | CCAGCTCATAGTACAGCATTGACC |
| m ABCA1 | AGTGATAATCAAAGTCAAAGGCACAC | AGCAACTTGGCACTAGTAACTCTG |
| m APOA1 | TCACCCACACCCTTCAGGATGAAA | ACACATAGTCTCTGCCGCTGTCTT |

| m COL1α1 | CATTGTGTATGCAGCTGACTTC | CGCAAAGAGTCTACATGTCTCTAGG |
| --- | --- | --- |
| m COL3α | GAGGAATGGGTGGCTATCCG | TTGCGTCCATCAAAGCCTCT |
| m α-SMA | GTTCAGTGGTGCCTCTGTCA | ACTGGGACGACATGGAAAAG |
| m TGF-β | CAACCCAGGTCCTTCCTAAA | GGAGAGCCCTGGATACCAAC |
| m SRB1 | ATGATCGTGATGGTGCCGTC | TGTTGCTTTTGTGCCTGAAC |

Table 1: Primers set used for real-time PCR.

Real Time qPCR analysis was performed using Rotor-Gene 6000 (Corbett Research) with SYBR® Green fluorophore (Life technologies, Woburn, MA).

The data obtained were analyzed using the Gene Expression Macro Genex software (BioRad, Milan, Italy), using a quantitative comparison algorithm. Peptidylprolyl isomerase B (Cyclophillin B) was used as the reference gene for normalization.

## 9. QRT-PCR Open Array for inflammatory genes

Quantitative gene expressions of targets associated with immune responses were analyzed by using RNA isolated from mouse livers tissue by TaqMan® OpenArray® Mouse Inflammation (Thermo Fisher Scientific), according to the manufacturer's instructions. In detail, RNA extraction was performed using Norgen's Total RNA isolation plus kit (Norgen Biotek Corp) and concentration was quantified using a NanoDrop Technologies, Wilmington, USA). Two micrograms of total RNA were reverse transcribed using SuperScript™ VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific-Invitrogen, Waltham, MA, USA) with random hexamer primers. All cDNA was used to analyze a Taqman Mouse Inflammation fixed-content pathway panel covering 632 genes that have been studied as targets for a range of inflammatory diseases, plus 16 endogenous control genes. Real-time quantitative PCR was carried out using optimized TaqMan® OpenArray® Real-Time PCR Master mix and QuantStudio™ 12K Flex Real-time PCR system (Thermo Fisher Scientific-Applied Biosystems). Each sample was evaluated in quadruplicate. Relative quantity (RQ) of gene expression values were calculated using Thermo Fisher Cloud Resources. RQ of each gene in the different samples was normalized against to the respective GAPDH, and the fold induction was calculated against the mRNA levels in the respective controls. TaqMan® probes for the array were reported in Supplementary Table S3. Heatmap representing mean of RQ for each sample was plotted by the online tool CIMMiner by quantile binning algorithm.

## 10. Western Blotting

Total protein extraction was performed by homogenizing liver tissue with the TissueLyser (Qiagen) in 1 ml of RIPA lysis buffer (Santa Cruz Biotechnology, Dallas, TX, USA) supplemented with protease inhibitor cocktails, PMSF and sodium orthovanadate (Santa Cruz Biotechnology, Texas, USA). The protein concentration was subsequently determined by means of the Bradford assay (BioRad Laboratories, Hercules CA, USA), by reading the absorbance with a spectrophotometer (Sunrise Tecan, Mannedorf, Switzerland) at a wavelength of 595 nm. Subsequently, a total of 60 µg of protein lysates was analyzed by Western blot (WB). First, the protein lysate was separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel) electrophoretic run on 12% acrylamide gel. After polymerization of the gel, it was placed in the electroporator together with the running buffer (consisting of 25 mM Tris-HCl, 192 mM Glycine, 0.1% SDS (w/v) pH 8.3). Before being placed into gel wells, each protein sample was spiked with 5X loading buffer (0.25 M Tris-HCl pH 6.8, 10% SDS, 50% glycerol, 0.5 M DTT added before loading of the sample) and denatured at 95°C for 5 minutes. To evaluate the molecular weight of each protein sample, a protein ladder marker was loaded. The electrophoretic run was conducted at 100 mV for 2 hours. At the end of the run, the proteins were transferred onto a nitrocellulose membrane using an iBlot™ Dry Blotting System (Invitrogen, Carlsbad CA, USA) at a voltage of 20V for 5 or 7 minutes depends on protein of interest molecular weight. To ensure proper transfer, the membrane was stained with Ponceau Red dye, which binds to proteins nonspecifically.

The obtained membrane was incubated in saturation buffer composed of 10mM Tris-HCl, (pH 7.6), 150mM NaCl and 0.05% v/v Tween-20 (TBS-T) and blocked for 1 h at room temperature in non-fat dry milk 5% (w/v) of TBS-T. Then, the membrane was incubated with the specific primary antibody (Santa Cruz Biotechnology, Dallas, TX) properly diluted, overnight at 4°C. The binding of the primary antibody to the protein of interest was highlighted by incubating the membrane with a specific secondary antibody (dilution 1:2000) conjugated with peroxidase (HRP) (sc-2004, Santa Cruz Biotechnology). The HRP in the presence of the ECL mix (Western Blotting detection reagents, GE healthcare), consisting of two reagents in a 40:1 ratio, catalyzes the oxidation of the chemiluminescent substrate (Pierce, Waltham, MA). The developed chemiluminescence was acquired by using the ChemiDoc™ MP Imaging System (Bio-Rad, Italy) and densitometry of the specific spots was performed with ImageJ v3.91 software. The primary anti-β Actin

antibody (Sigma-Aldrich, Saint Louise, MO) was used for equal loading control and for normalization of results.

## 11. Analysis of inflammatory protein array

The RayBio® C-Series Mouse Inflammation Antibody Array C1 (RayBiotech, Inc., Norcross, GA, USA) was employed to evaluate the expression of 40 different cytokines in serum samples from mice. The assay was carried out according to the manufacturer's protocol. The chemiluminescent signal was acquired by chemiluminescence with iBright Western Blot Imaging Systems (Thermo Fisher Scientific-Thermo Fisher, Waltham, MA, USA). Quantification of each spot was performed by measuring integrated density by Image J v3.91 software.

## 12. Statistics

The results shown below are expressed as mean ± SEM. Student's t test for independent samples was used to determine statistical significance. P values less than 0.05 were considered statistically significant. Comparisons between multiple groups were evaluated using analysis of variance followed by the Tukey test (ANOVA). P values less than 0.05 were considered statistically significant.

# RESULTS

## 1. Intestinal specific LXRα activation

The evaluation of the effect of intestinal LXRα activation on the progression of liver damage, and in particular on the pathogenesis of NASH and its evolution into HCC, was possible thanks to the use of a particular mouse model, called iVP16-LXRα. In this model a constitutive activation of the α isoform of the LXR receptor is induced, thanks to the presence of the VP16 protein deriving from the Herpes Simplex Virus. The VP16 protein and the receptor are specifically expressed in the intestinal epithelium, under the villin promoter, allowing not only a constitutive activation of the receptor at the intestinal level, but also to maintain normal expression in other organs and tissues (115). Non-transgenic mouse models, FVB/N, basic method for the development of the transgenic mouse (108) were used as control groups.

To confirm the functionality of iVP16-LXRα transgene expression, we analyzed both the expression of LXRα and its target genes (Figure 7). In particular, we determined the mRNA expression of the transporters ABCG5 and ABCG8, responsible for the elimination of cholesterol in the intestine.

As we can see from Figure 7, the expression of the α isoform of the receptor remained almost unchanged both in the intestine and in the liver of both genotypes. While the most substantial differences emerged from the analysis of the gene expression of the cassettes.

As expected, we observed an increase in the expression of both transporters in the intestine samples of iVP16-LXRα mice, whereas no statistically significant change in the expression was observed in the liver preparations (Figure 7).

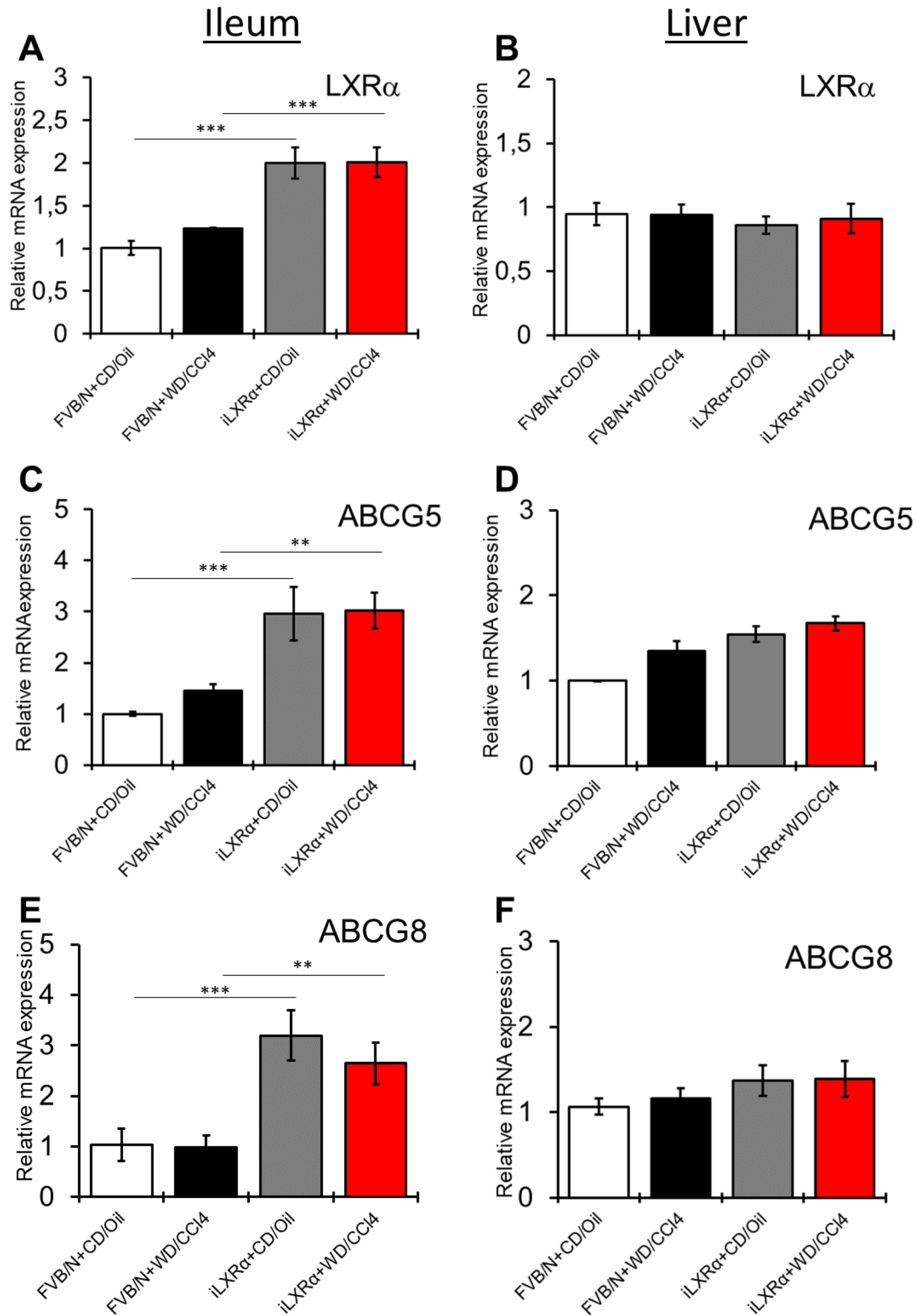


Figure 7: Gene expression of LXRα and the target genes ABCG5 and ABCG8 in the ileum and liver of mouse models. Gene expression was measured by quantitative real-time PCR in ileum (A, C and E) and liver (B, D and F) samples taken from FVB/N and iVP16LXRα mice treated with CD/Oil and WD /$CCl_4$. The endogenous calibrator for calculating expression levels is Cyclophilin B (CypB). Results are expressed as mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

## 2. Treatment with WD/$CCl_4$ induces liver injury in animal models

NAFLD is the most common cause of chronic liver injury in western regions. NAFLD is expected to become the leading indication for liver transplantation by 2030. NAFLD is a metabolic complication associated with obesity induced by the loss of the balance between the lipolytic phenomena (β-oxidation) and those of lipogenesis in the liver.

The iVP16-LXRα mice and their respective controls, FVB/N, were subjected to intraperitoneal injections of carbon tetrachloride ($CCl_4$), simultaneous intake of a Western diet (WD) and a high sugar solution, or in alternative to a standard diet (CD) and intraperitoneal injections of corn oil.

As reported in the literature, the Western diet model associated with the injection of low doses of $CCl_4$ (0.2 of body weight of the animal), much lower than the dose used to induce fibrosis, is used in mouse strains to induce obesity and complications of metabolic syndrome. In particular, the WD/$CCl_4$ model alters the physiological metabolic processes causing both an increase in body weight, associated with hypertrophy and inflammation of the adipose tissue, the onset of hepatic steatosis and inflammation of the liver parenchyma, development of glucose intolerance, and finally insulin resistance (116). During the whole duration of the study, the weight of the animals was measured on a weekly basis and as we can see from Figure 8, both mouse genotypes, FVB/N and iVP16-LXRα treated with WD showed an increase in weight compared to mice treated with chow diet, although $CCl_4$ treatment reduced food intake (Figure 8). However, a reduction in the amount of food is associated with an increase in caloric intake, due to the simultaneous intake of a diet enriched in fats and sugars and a solution with a high sugar content (Figure 8).

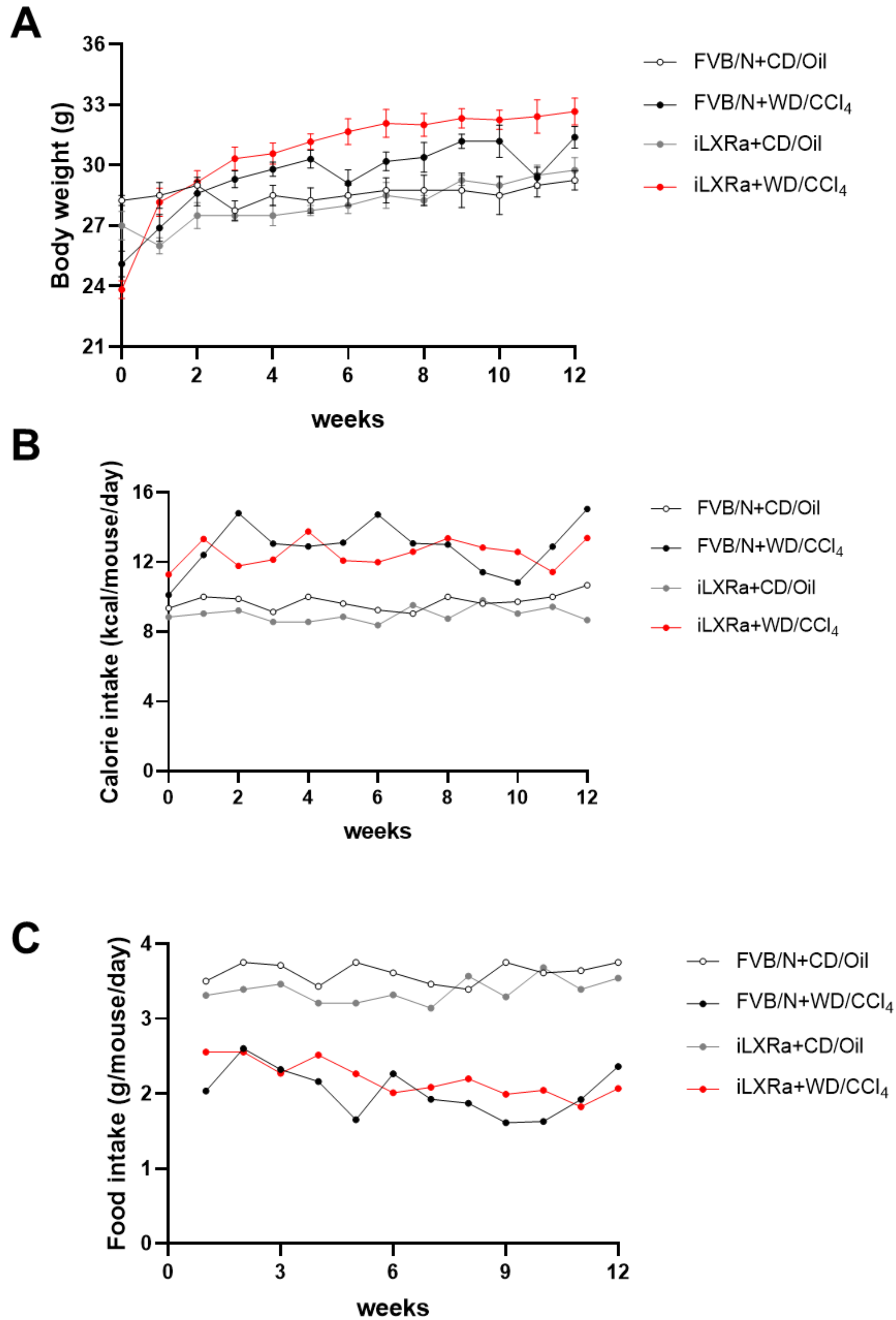


Figure 8: Mice were treated with ND/Oil, WD/Oil, ND/$CCl_4$, and WD/$CCl_4$ for up to 12 weeks. (A) Body weight change for 12 weeks, (B) calorie intake and (C) daily food intake. Results were expressed as mean ± SEM, and were compared by two-way ANOVA test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. ND, normal diet; WD, western diet; $CCl_4$, carbon tetrachloride.

Furthermore, WD/$CCl_4$ retention, in addition to the weight of the mice, increased the liver weight, both in iVP16-LXRα and in controls. The results show that, after 12 weeks of treatment, the FVB/N controls with WD/$CCl_4$ have a significant increase in the ratio of

liver weight (LW) to mouse weight (BW) (LW/BW ratio) (Figure 9), while this effect was not observed in LXRα transgenic mice (Figure 9).

These results are in agreement with the effect on the tissue pattern of steatosis, as shown in the hematoxylin/eosin stain (Figure 9). In fact, the livers of WD/$CCl_4$-treated FVB/N control mice show a change in color saturation, resulting whitish, a hallmark of hepatic lipid accumulation, while the staining of iVP16-LXRα-treated mice did not appear altered.

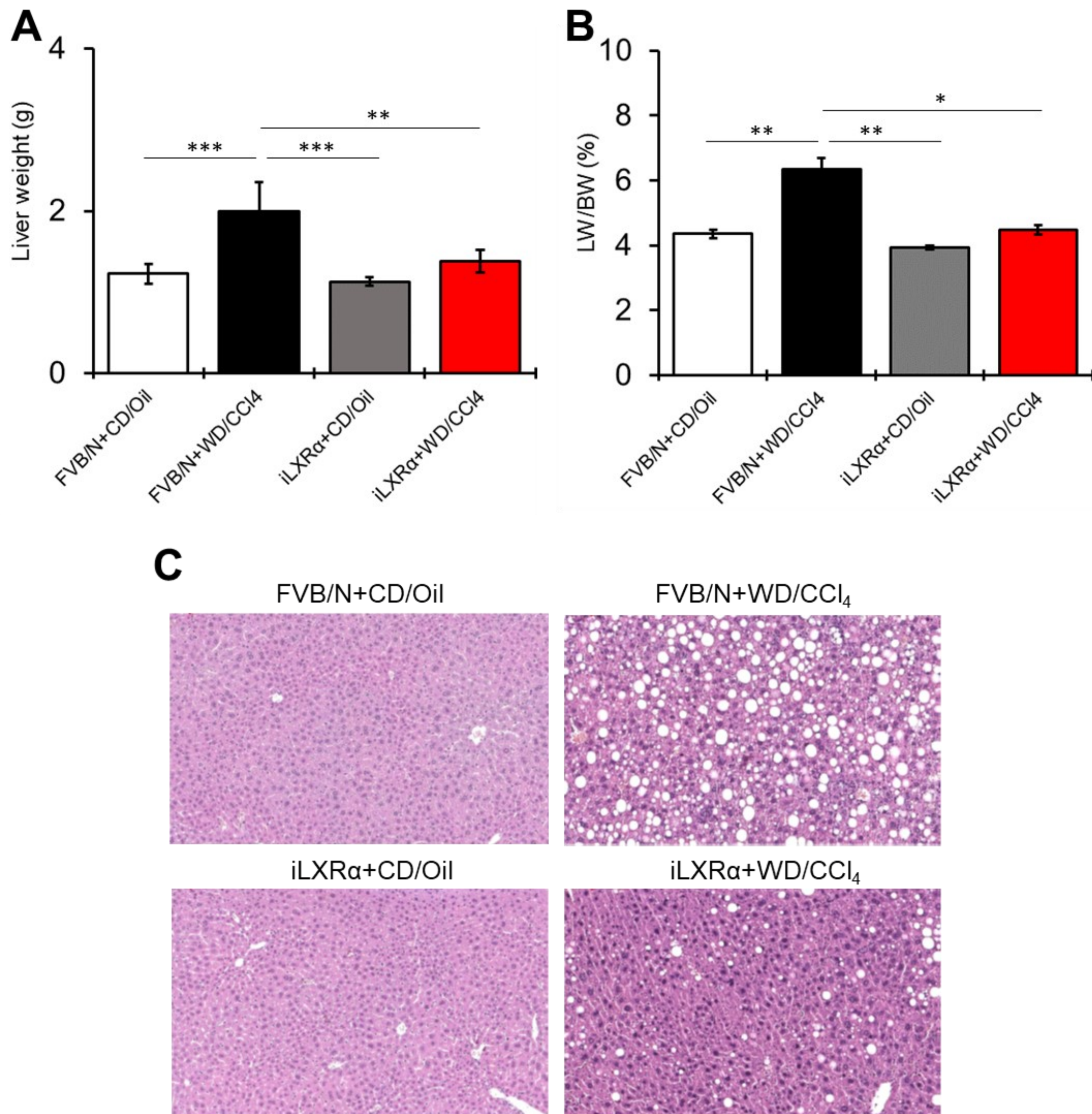


Figure 9: Intestinal activation of LXRα reduces hepatic lipid absorption. (A) Liver weight, (B) liver to body weight ratio and (C), hematoxylin and eosin stain of representative mice treated for 12 weeks. Results were expressed as mean ± SEM, and were compared by two-way ANOVA test. *P < 0.05, **P < 0.01, ***P < 0.001.

## 3. Intestinal LXRα does not induce hepatic steatosis

One of the problems that has prevented the use of LXR agonists for therapeutic purposes is due to the presence of important side effects, such as hyperglyceridemia and hepatic steatosis, and the consequent de novo lipogenesis. An increase in lipogenesis, accompanied by accumulation of hepatic lipids causes lipotoxicity which causes oxidative stress, leading to chronic inflammation and progressive deposition of collagen fibers in the liver.

Histopathologic images (Figure 9) show typical NAFLD/NASH pathology with a pattern of steatosis, inflammation, and fibrosis reminiscent of human NASH. In particular, in FVB/N mice treated with WD/$CCl_4$, we evidenced significantly increased macrovesicular fat accumulation and hepatocyte swelling.

Histological features of NAFLD were evaluated with the use of the scoring system developed by the National Institutes of Health NASH Clinical Research Network. In detail, this system provides for the attribution of a NAFLD activity score, called NAS. We can note that, after 12 weeks of treatment, the NAS score was significantly higher in controls treated with WD/$CCl_4$, while the NAS score of mice with intestinal LXRα activation was significantly reduced (Figure 10).

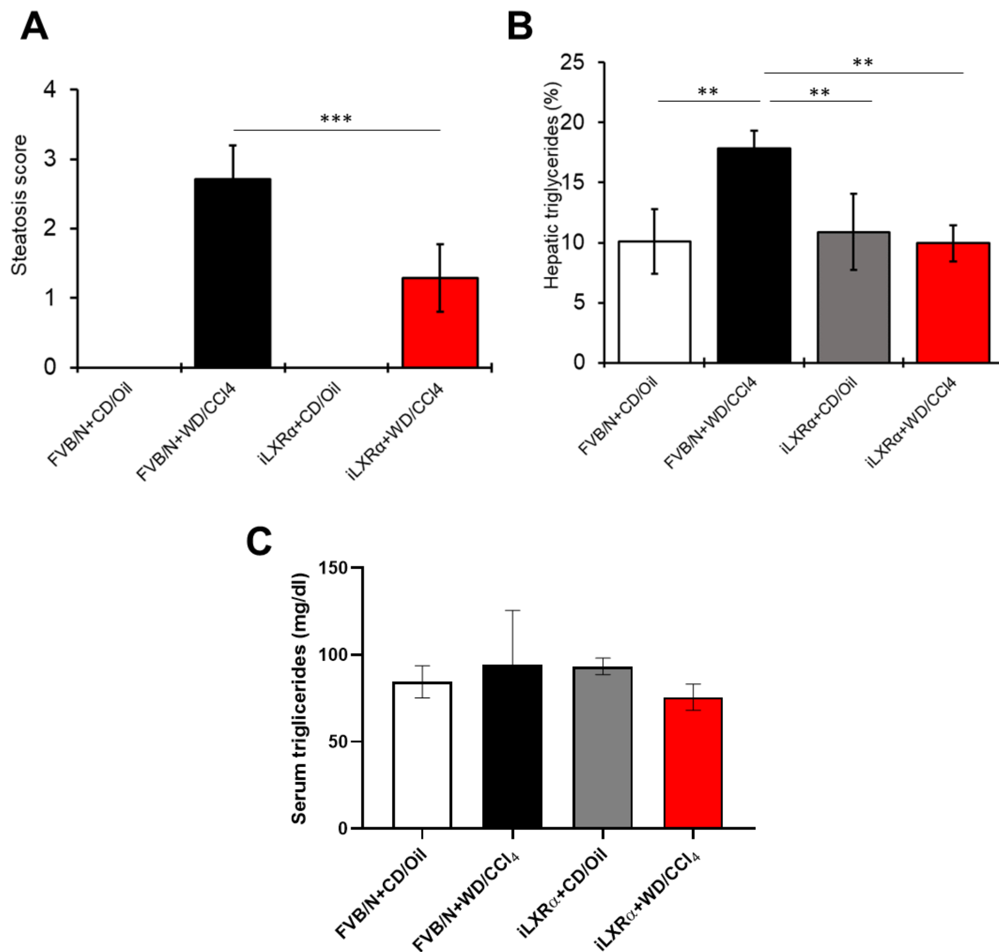


Figure 10: Histology by NAS and triglyceride content. (A) Steatosis grade; (B) hepatic triglyceride content and (C) serum triglyceride content. Results were expressed as mean ± SEM, and were compared by two-way ANOVA test. *P < 0.05, **P < 0.01, ***P < 0.001.

Next, we measured both hepatic and serum triglyceride content to confirm that intestinal LXRα activation does not result in hepatic triglyceride accumulation. Liver TG content is significantly reduced in WD/$CCl_4$-treated iVP16-LXRα mice compared to FVB/N-treated mice, while no change in circulating TG was found (Figure 10).

Finally, we measured the gene expression of the Fatty Acid Synthase (FAS), which encodes the FASN enzyme system, responsible for the de novo synthesis of fatty acids. The results show an increase in gene expression in WD/$CCl_4$-treated controls, whereas a significant reduction occurred in WD/$CCl_4$-treated iVP16-LXRα mice (Figure 11). We also measured the expression of Fatty Acid-Binding Protein (FABP) and Cluster of Differentiation 36 (CD36), also known as scavenger receptor class B member 3 (SCARB3), important regulators of lipid absorption and storage. Their expression was also increased after 12 weeks of WD/$CCl_4$ treatment in FVB/N, while it was unchanged in LXRα transgenic mice, as shown in Figure 11. Moreover, even though diet had no effect

per se on Carnitine Palmitoyltransferase 1A (CPT1A) gene expression, gene responsible for mitochondrial beta-oxidation of long-chain fatty acids, in transgenic mice we observed significant upregulation of this gene (Figure 11).

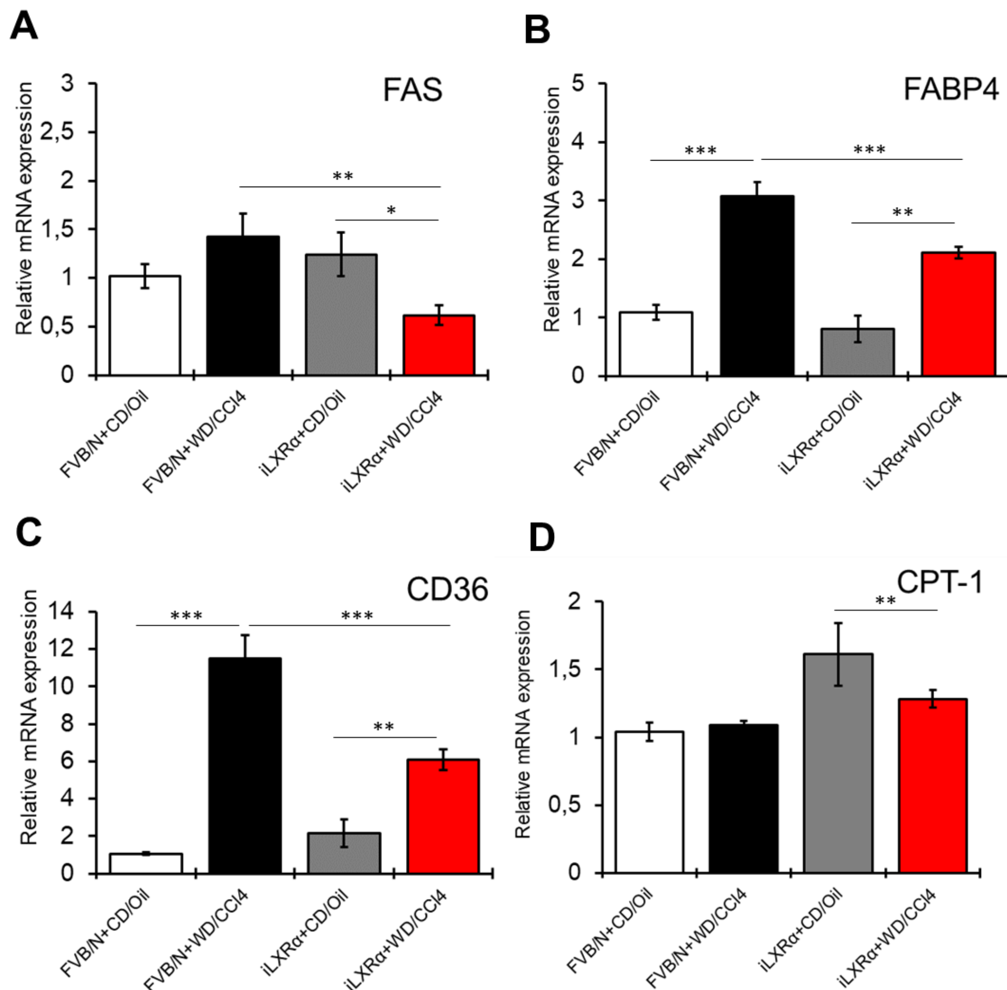


Figure 11: Intestinal activation of LXRα induces changes in lipid metabolism in WD and $CCl_4$-treated mice. (A) Genetic expression of FAS, which catalyzes fatty acid synthesis; (B) gene expression of FABP4, important for the uptake and transport of fatty acids; (C) gene expression of CD36, which is involved in the absorption and storage of lipids and (D) gene expression of CPT1A, responsible for the beta oxidation of fatty acids. Results were expressed as mean ± SEM, and were compared by two-way ANOVA test. *P < 0.05, **P < 0.01, ***P < 0.001.

Next, we measured the expression of peroxisome proliferator-activated receptor alpha (PPARα), acetyl-CoA carboxylase alpha (ACCα), and acyl-CoA oxidase 1 (ACOX1). The first, PPARα is an important regulator of lipid metabolism in the liver, promoting the uptake, utilization and catabolism of fatty acids and is capable of inhibiting pro-inflammatory cytokines. As we can see from Figure 12, its levels increased in the iVP16-LXRα group treated with WD/Oil

While ACCα is involved in fatty acid synthesis, it is not impaired in the four experimental groups.

Finally ACOX1, involved in the beta-oxidation of fatty acids, does not undergo significant changes in the groups treated with WD/$CCl_4$. Overall, these data excluded the regulation of beta-oxidation by intestinal LXRα in the maintenance of lipid homeostasis in the liver.

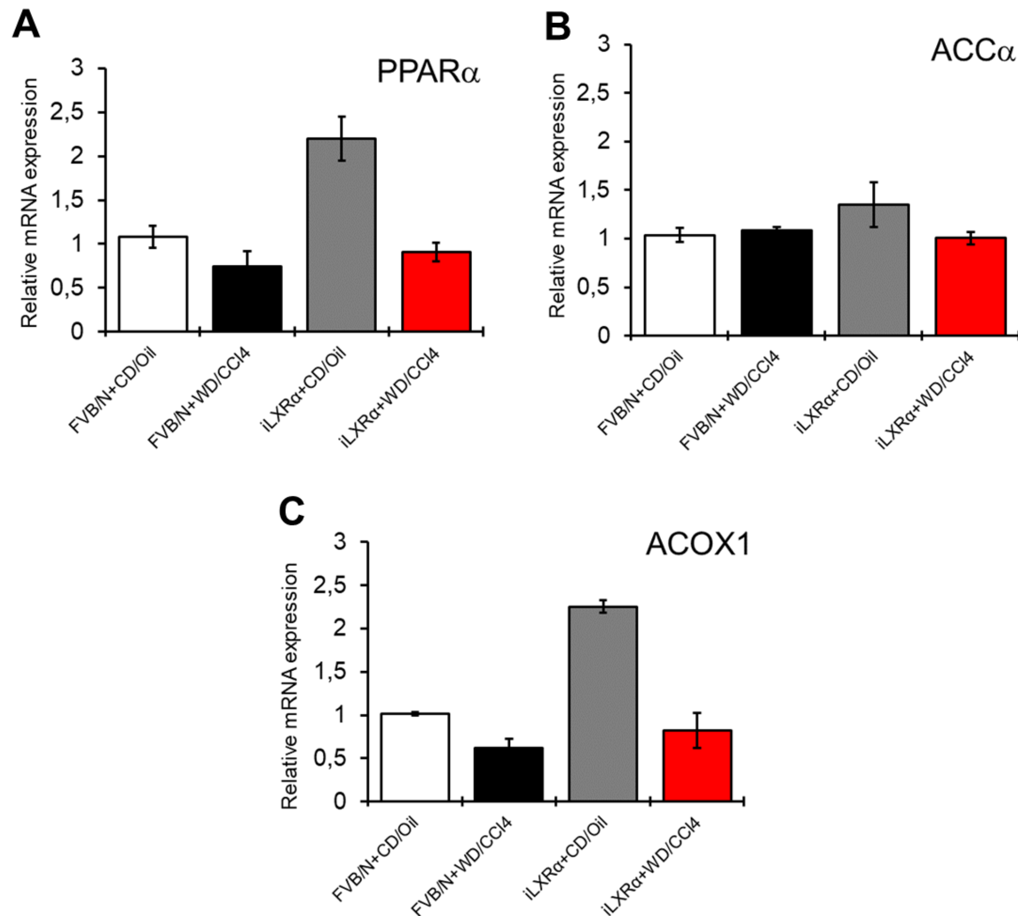


Figure 12: Intestinal activation of LXRα does not induce changes in beta-oxidation. (A) Gene expression of PPARα; (B) ACCα gene expression and (C) ACOX1 gene expression. Results were expressed as mean ± SEM, and were compared by two-way ANOVA test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

## 4. Intestinal LXRα decreases cholesterol absorption and induces RCT

Activation of hepatic lipogenesis is one of the side effects due to LXR activation in the liver. To overcome this problem, alternative strategies have been evaluated in recent years that target a specific tissue activation of LXR without involving the liver and thus overcoming the resulting side effects. Selective activation of LXR in the intestine reduces cholesterol absorption by acting on reverse cholesterol transport (RCT), without changing plasma cholesterol concentration. The main responsible for the reverse transport of cholesterol from tissues to pre-HDL is ABCA1 (belonging to the ATP-Binding Cassette transporters), a membrane transporter whose expression is linked to LXR. As we can observe from Figure 13, constitutive activation of LXR in iVP16-LXRα mice increases

ABCA1 expression in both gut and liver, compared with FVB/N controls. Similar results were obtained from the analysis of apolipoprotein A1 (APOA1), which represents the main component of HDL. Indeed, APOA1 is highly expressed in iVP16-LXRα mice, both in the gut and in the liver (Figure 13).

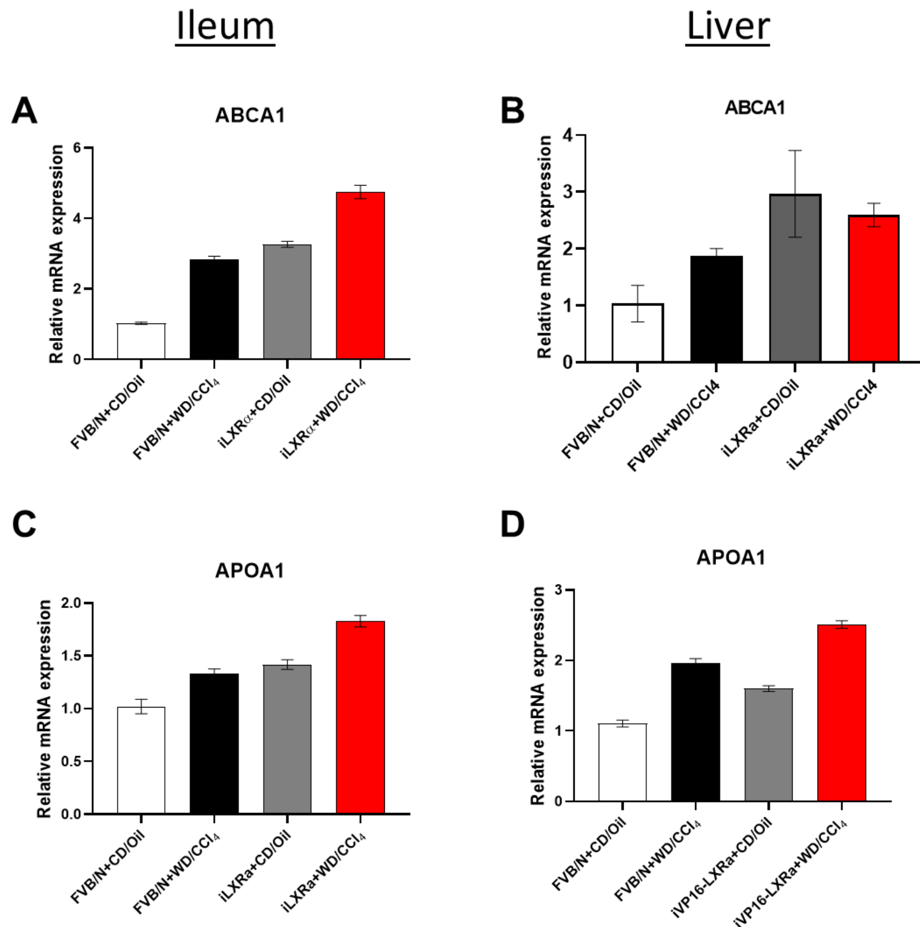


Figure 13: Intestinal activation of LXRα induces reverse cholesterol transport (RCT). (A) ABCA 1 gene expression in gut samples; (B) ABCA1 gene expression in liver samples; (C) APOA1 gene expression in gut samples and (D) APOA1 gene expression in liver samples. Results were expressed as mean ± SEM, and were compared by two-way ANOVA test. *P < 0.05, **P < 0.01, ***P < 0.001.

Next, we evaluated hepatic and circulating cholesterol content, to assess the effect of intestinal LXR activation on cholesterol homeostasis. Serum total cholesterol levels (Figure 14) were significantly increased in both groups of mice treated with WD and $CCl_4$, but also in mice with gut activation treated with CD/Oil. We then analyzed the components that make up cholesterol, HDL and LDL/VLDL. As we can see from Figure 14, HDL is significantly increased in iVP16-LXRα mice compared to treated and untreated controls. While, as regards the LDL/VLDL fractions, we found an increase only in control mice treated with WD/$CCl_4$.

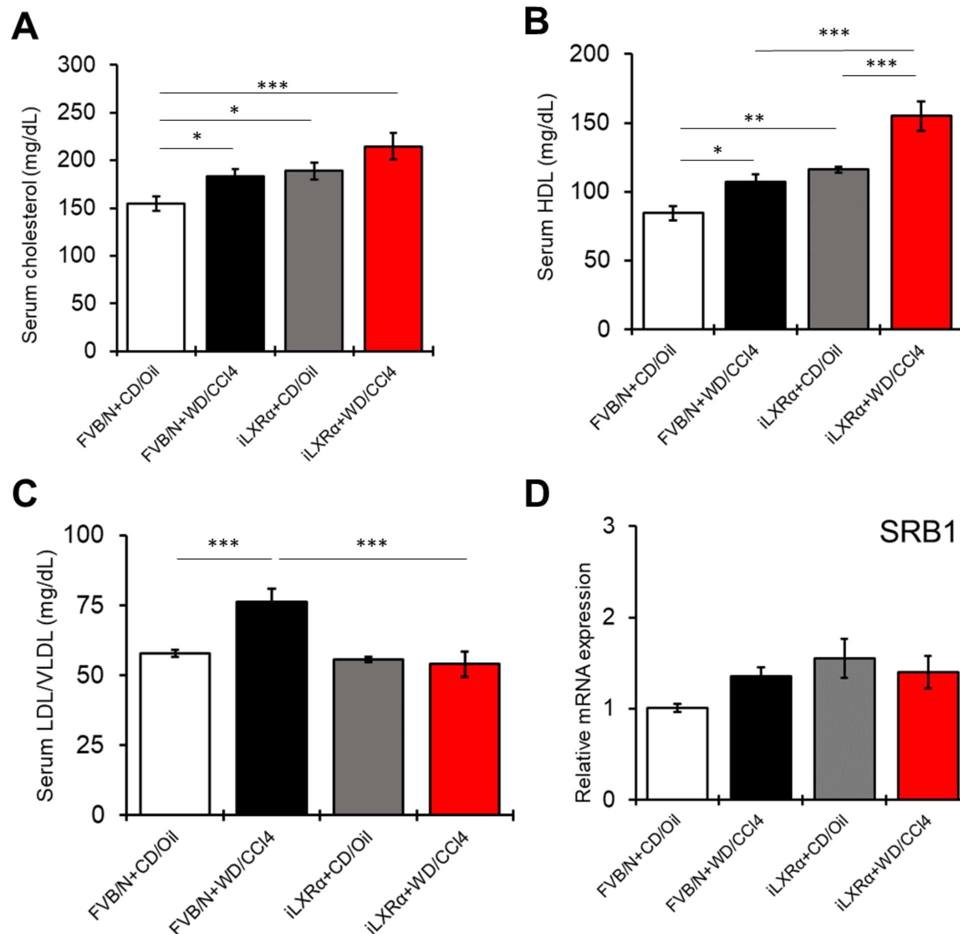


Figure 14: Intestinal activation of LXRα modifies serum cholesterol concentration in WD/$CCl_4$-treated mice. (A) Total serum cholesterol concentration of FVB/N and iVP16-LXRα mice; (B) serum HDL concentration; (C) serum LDL/VLDL concentration and (D) SRB1 gene expression. Results were expressed as mean ± SEM, and were compared by two-way ANOVA test. *P < 0.05, **P < 0.01, ***P < 0.001.

Finally, we analyzed the cholesterol content in the liver. Total cholesterol levels were increased in control mice treated with Western diet and carbon tetrachloride injections compared to both CD/Oil-treated FVB/N controls, but especially compared to WD/$CCl_4$-treated iVP16-LXRα. In particular, as shown in Figure 15, the LDL/VLDL values are altered, increased in the FVB/N treated with WD/$CCl_4$, while in the same experimental group the HDL are reduced after 12 weeks of treatment. Taken together, these data highlight that intestinal activation of LXRα protects against liver injury induced by a high-fat and high-carbohydrate diet.

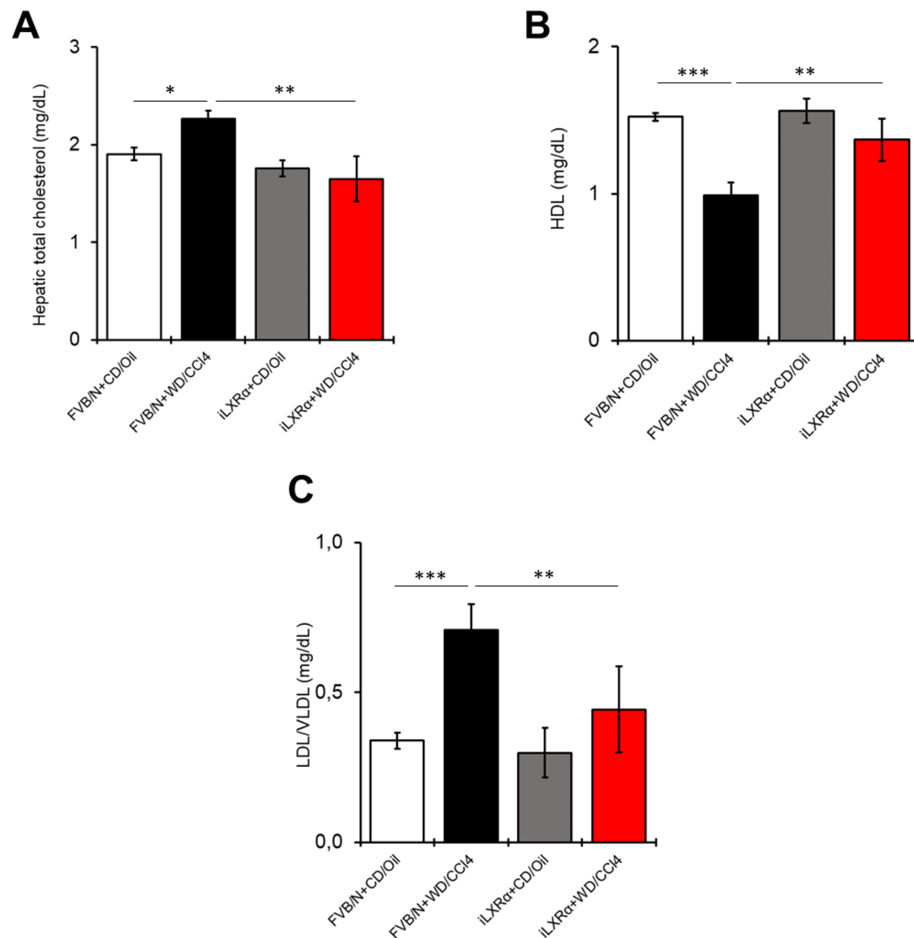


Figure 15: Intestinal activation of LXRα modifies the hepatic concentration of cholesterol subtypes. (A) Total cholesterol concentration in the livers of FVB/N and iVP16-LXRα mice; (B) HDL concentration in the liver and (C) LDL/VLDL concentration in the liver. Results were expressed as mean ± SEM, and were compared by two-way ANOVA test. *P < 0.05, **P < 0.01, ***P < 0.001.

## 5. Intestinal LXRα reduces liver inflammation

In the onset of NAFLD and in general in the progression of liver damage, a fundamental role is played by inflammation. Hepatic macrophage activation represents a major source of inflammatory mediators, including cytokines, chemokines, and eicosanoids. Their presence acts as a support for the activation of HSCs with consequent synthesis and release of collagen. A class of macrophages most represented in the inflammatory infiltrate are the CD68+, able to co-express a huge variety of other markers. Therefore, we performed immunohistochemistry for CD68+ macrophage markers and, as can be seen, in the livers of $CCl_4$/WD-treated FVB/N mice these macrophages are increased compared to Oil/CD controls. Furthermore, we measured the levels of a CD68+/CD206+ double label. The CD206+ marker represents a class of M2 anti-inflammatory macrophages. We can see how the histological number of CD68+/CD206+ macrophages are drastically reduced in

controls treated with $CCl_4$/WD, while iVP16-LXRα mice show a recovery of M2 macrophages compared to the related iVP16-LXRα controls treated with Oil/CD (Figure 16) thus suggesting a reduced inflammation pattern.

Another insult that can induce the progression of liver damage in steatohepatitis is the presence of endotoxins, such as lipopolysaccharide (LPS), capable of stimulating the Kupffer cells in the production of pro-inflammatory cytokines. Therefore, we measured circulating LPS levels and, as expected, observed elevated levels in treated FVB/N mice while decreased values are associated with iVP16-LXRα mice (Figure 16).

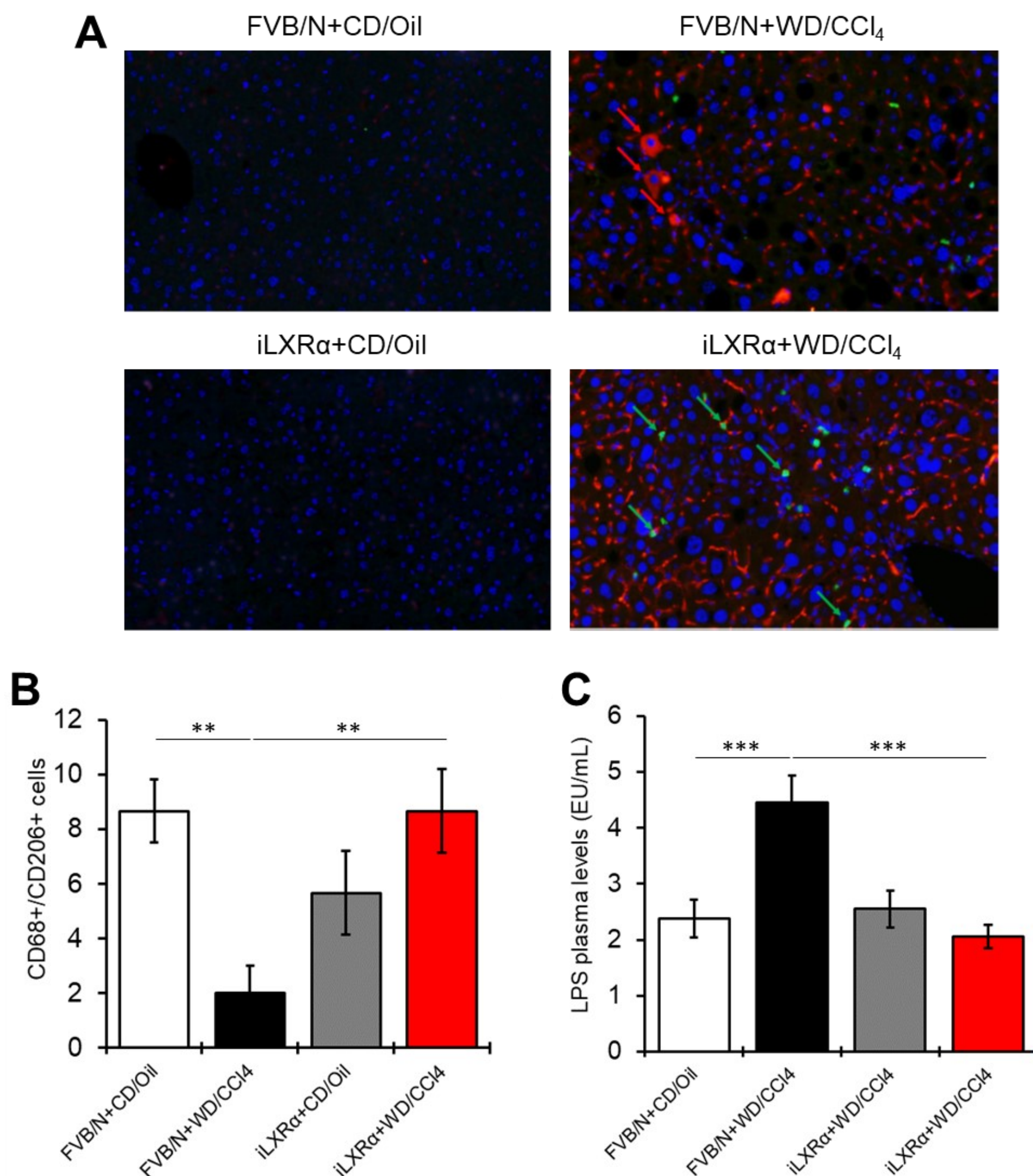


Figure 16: Effects of intestinal LXRα activation on inflammation. (A) confocal microscopy of M1 macrophage marker CD68 (red) and M2 macrophage marker CD206 (green) on FFPE liver tissue double staining, (B) image analysis of hepatic expression of CD68/CD206 ratio and, (C) plasma levels of LPS. Results were expressed as mean ± SEM, and were compared by two-way ANOVA test. *P < 0.05, **P < 0.01, ***P < 0.001.

Finally, to evaluate whether the intestinal activation of LXRα could perform a protective action in the development and progression of liver damage, we evaluated the inflammatory aspect both in the circulation through a protein array that evaluated the expression of 40 inflammatory cytokines on serum (Figure 17), and at the tissue level, by evaluating the gene expression profiles of a profile of 586 inflammatory genes (Figure 18).

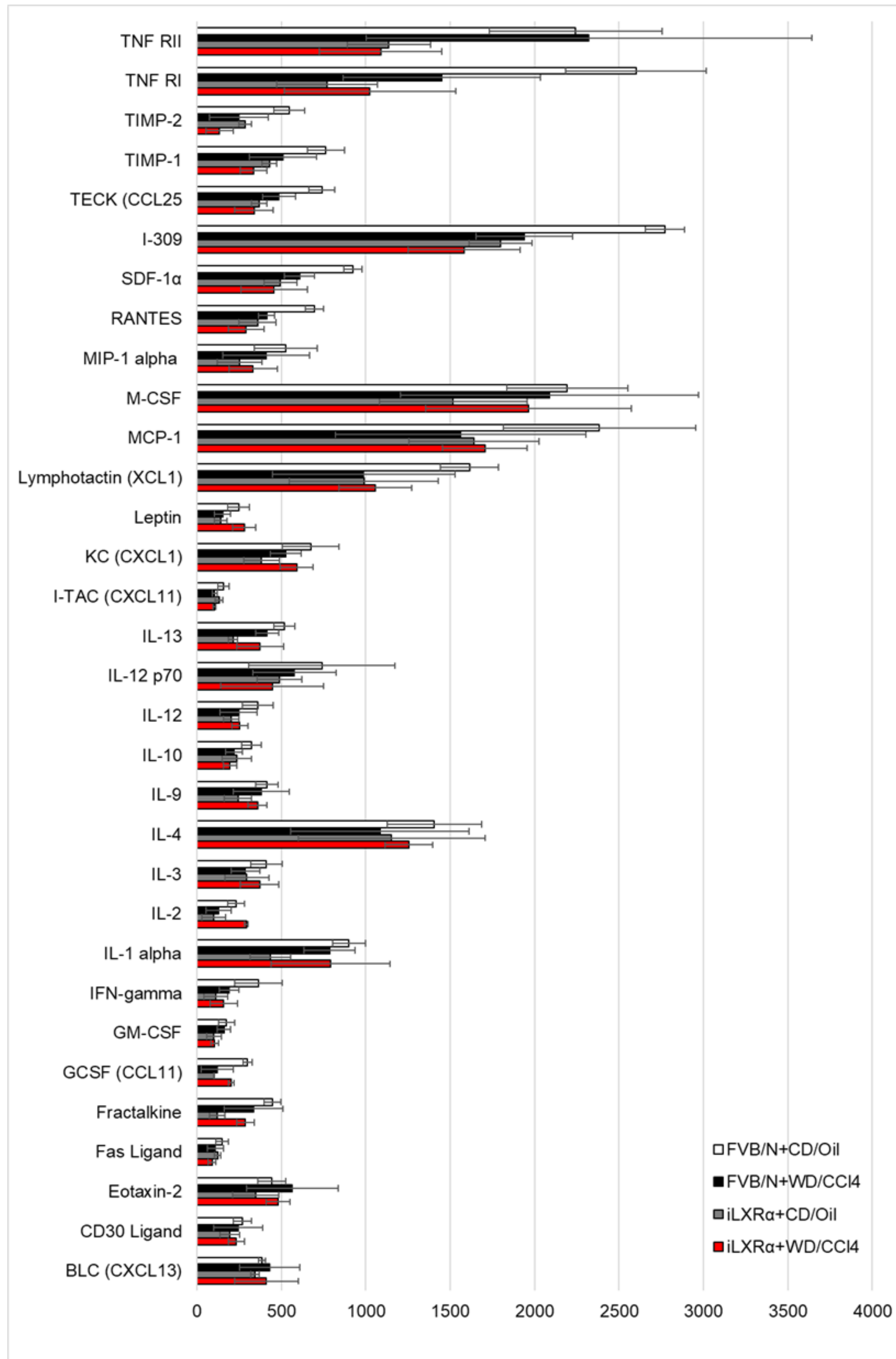


Figure 17: Effects of intestinal LXRα activation on inflammation showed as protein analysis of circulating proinflammatory mediators.

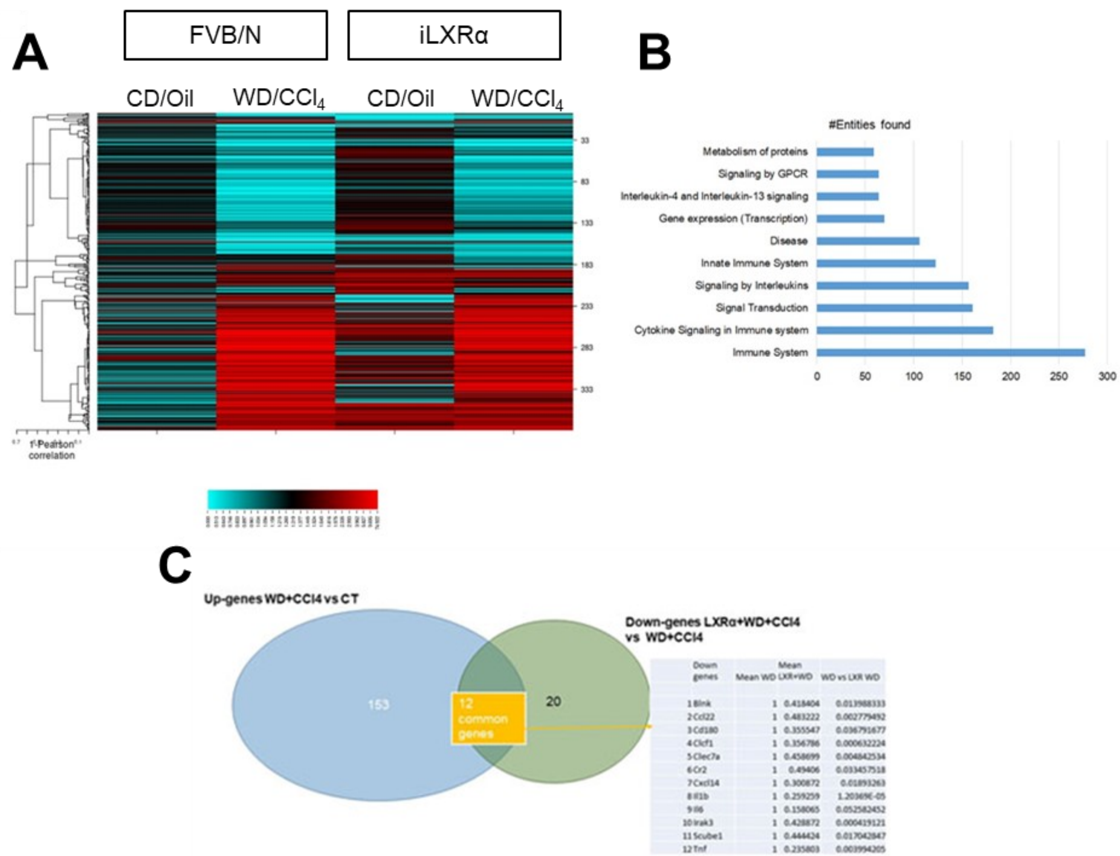


Figure 18: Inflammatory markers in the liver of FVB/N and iVP16-LXRα mice treated with CD/Oil or WD/CCl4. (A) Heatmap of inflammatory gene expression profile performed using the TaqMan OpenArray Mouse Inflammation Panel; (B) pathways of the altered genes and (C) Venn diagram showing the overlapping of upregulated genes by WD+CCl4 treatment with those downregulated upon LXRα constitutive intestinal activation.

As shown in Figure 18, we observed the presence of 153 genes upregulated in the WD/$CCl_4$-treated controls compared to iVP16-LXRα, while 20 genes are downregulated in the LXRα controls compared to FVB/N, of which 12 are in common.

In particular, from the analysis of the inflammatory profile in serum, interleukin 6 (IL-6) and tumor necrosis factor α (TNFα) were significantly altered. In fact, as we can observe from Figure 19, both inflammatory cytokines are increased in the serum of control mice treated with WD/$CCl_4$, while no alteration was found in mice with LXRα activation in the intestine treated with WD/$CCl_4$.

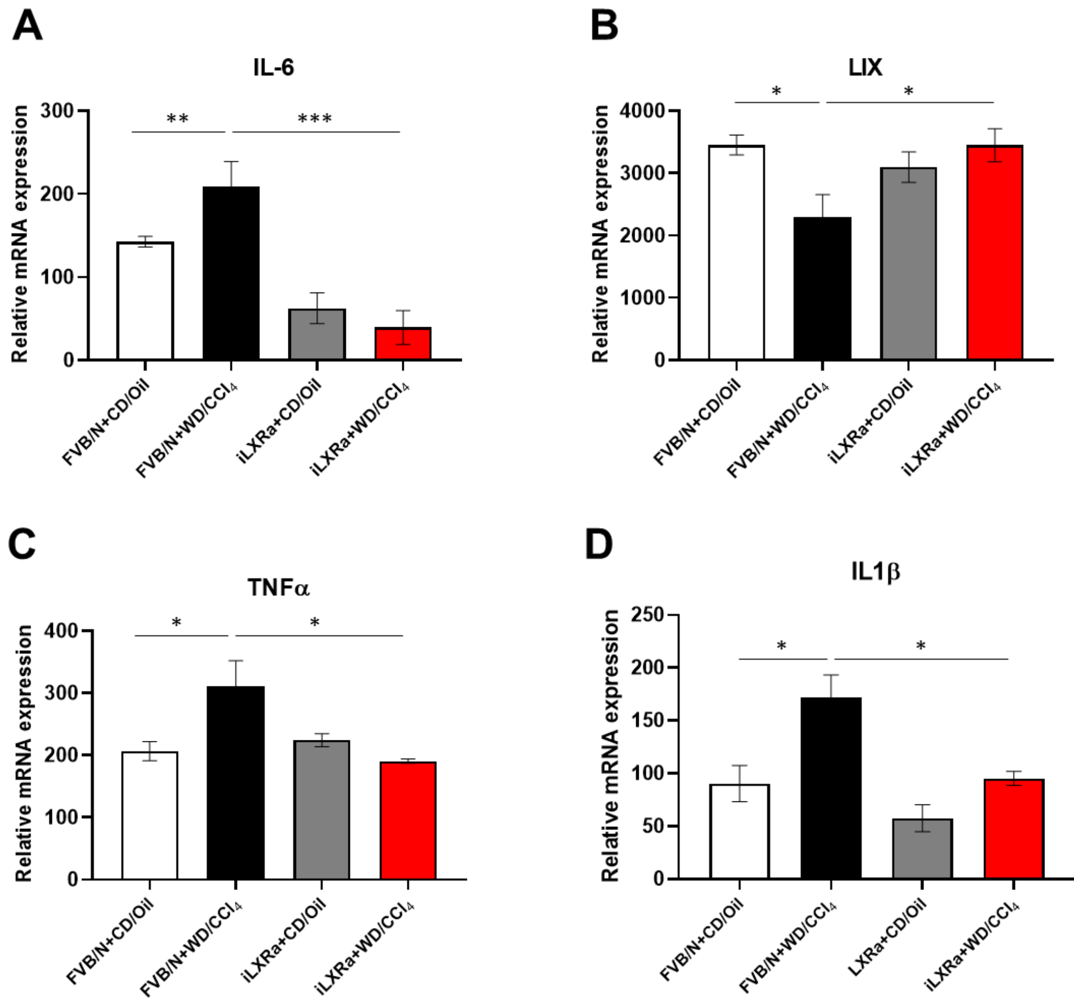


Figure 19: Gene expression of specific circulating inflammatory markers. (A) espressione genica di IL-6; (B) espressione genica di LIX; (C) espressione genica di TNFα, e (D) espressione genica di IL1β. Results were expressed as mean ± SEM, and were compared by two-way ANOVA test. *P < 0.05, **P < 0.01, ***P < 0.001.

## 6. Intestinal LXRα reduces liver fibrosis

Chronic inflammation is associated with a progressive increase in the deposition of collagen fibers, an irreversible process of the disease, which leads to the development of fibrosis.

The degree of liver fibrosis in the modified animals was classified according to the NAS score (113). In the two control genotypes and iVP16-LXRα treated with Oil/CD no trace of fibrosis was highlighted. While the degree of fibrosis was 5,09 ± 1,11 in WD/$CCl_4$ treated FVB/N mice, in contrast to LXRα treated mice, which had scores of 1,24 ± 0,24 (Figure 20). To further evaluate the progression of liver fibrosis, we measured collagen synthesis and its deposition in the liver. We evaluated the expression of collagen 1α (COL1A) and collagen 3α (COL3A) by qPCR, and WD/$CCl_4$-treated iVP16-LXRα mice exhibited

reduced synthesis of both forms of collagen (Figure 20). This was further confirmed by histochemical analysis (Masson's trichrome).

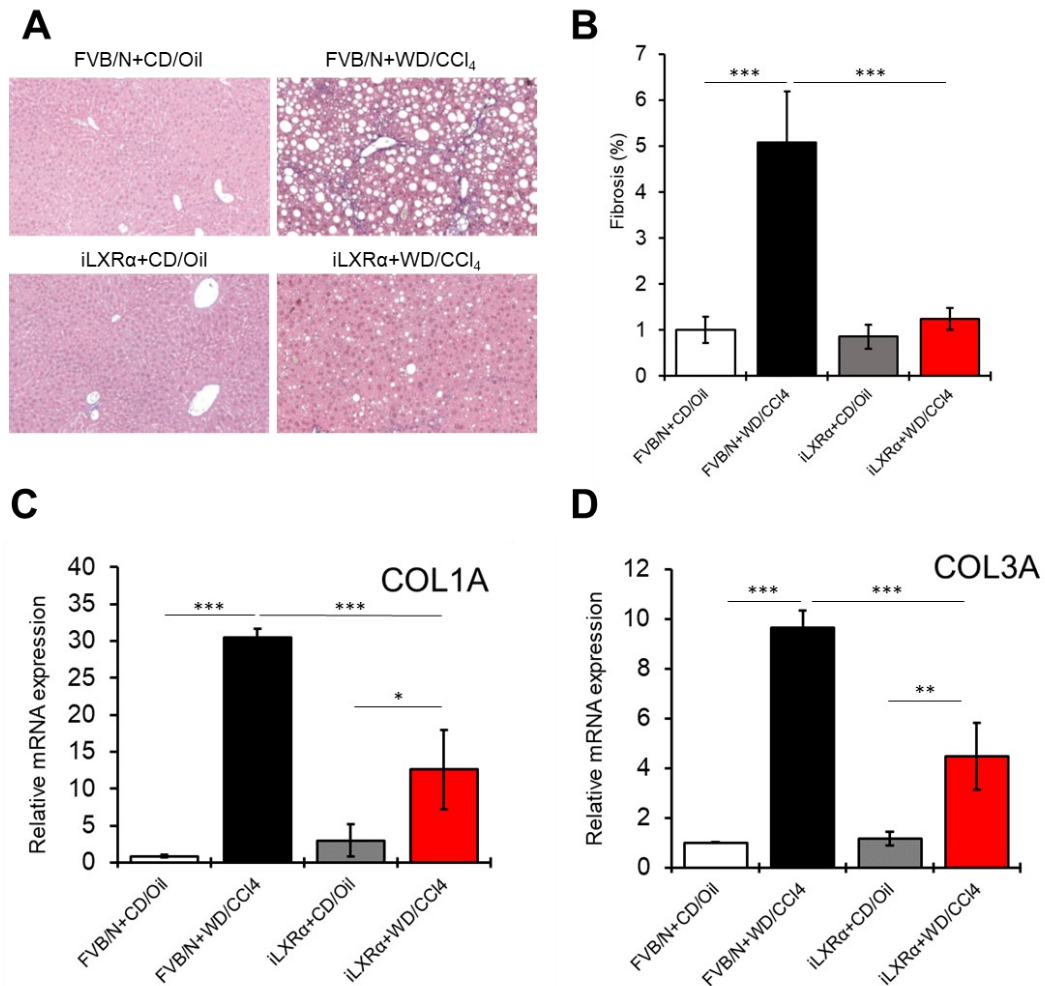


Figure 20: Fibrosis stage of mice treated with diet and $CCl_4$. (A) Masson's trichrome staining of representative FVB/N and iVP16-LXRα mice treated with CD/Oil and WD/$CCl_4$ for 12 weeks; (B) fibrosis score; (C) Collagen 1A gene expression and (D) Collagen 3A gene expression in liver samples taken from FVB/N and iVP16-LXRα mice. The endogenous calibrator for calculating expression levels is Cyclophilin B. Results were expressed as mean ± SEM, and were compared by two-way ANOVA test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

In the pathological cascade leading to the development of NASH, one of the key steps is liver fibrosis, the result of a complex interaction between damaged hepatocytes and non-parenchymal reactive liver cells, including hepatic stellate cells (HSC), Kupffer cells (KC) and lymphocytes. Among these cell populations, HSCs represent the main inducers of hepatic fibrosis, contributing not only to the synthesis and deposition of extracellular matrix (ECM), but also to the inflammatory signaling pathway. In ongoing liver damage, these cells undergo a transformation into myofibroblastic cells, starting to secrete cytokines involved in inflammatory and fibrogenic processes. To evaluate HSC activation, we measured the expression of α-Smooth Muscle Actin (α-SMA), marker of myofibrobastic

activation. We noted lower gene expression in WD/$CCl_4$-treated iVP16-LXRα mice than in controls (Figure 21). To confirm a lower activation of HSCs in mice with LXRα, we measured Transforming Growth Factor β (TGF-β), a cytokine secreted by HSCs and involved in the damage repair mechanism. Again, TGF-β levels were reduced in iVP16-LXRα treated compared with FVB/N controls (Figure 21).

Finally, the lower collagen deposition was further confirmed by the measurement of the hydroxyproline content (Figure 21).

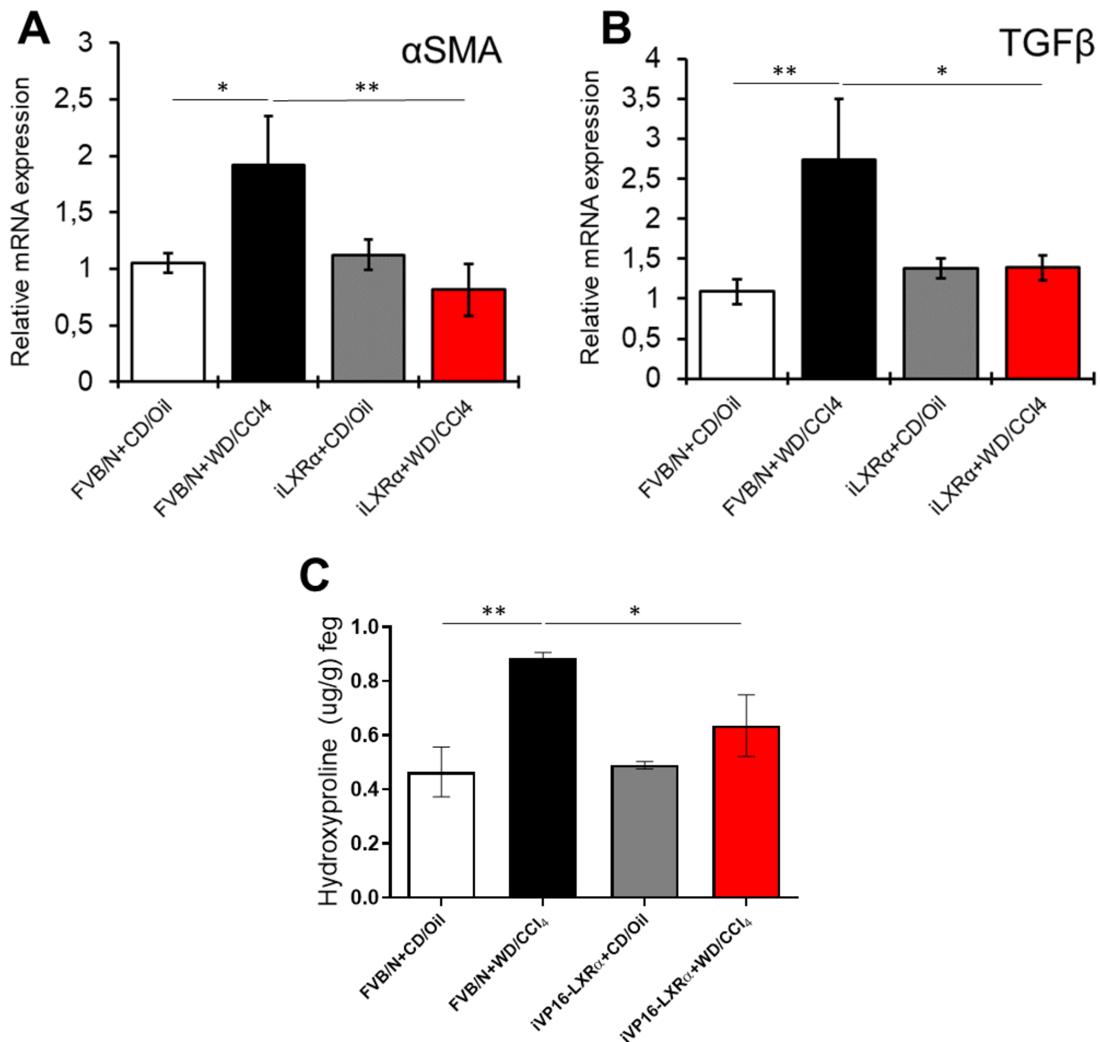


Figure 21: Intestinal activation of LXRα reduces liver fibrosis. (A) gene expression of α-SMA, marker of myofibroblast activation; (B) gene expression of TGF-β cytokine secreted in response to injury; (C) Collagen deposition evaluated by hydroxyproline content. Results were expressed as mean ± SEM, and were compared by two-way ANOVA test. *P < 0.05, **P < 0.01, ***P < 0.001.

The previous data show a marked reduction of inflammation and oxidative stress, accompanied by a reduction of extracellular matrix deposition in the iVP16-LXRα genotype. This is not due to an induction of LXR in the liver, as evidenced by the lack of increase in the expression levels of the target genes, ABCG5 and ABCG8. These results

led us to hypothesize a mechanism leading to the reduction of hepatic damage involving HDL and its cellular receptor SRB1. In particular, we hypothesized a protective role of HDL, and an intervention in the reduction of macrophage-mediated inflammation and oxidative stress. We therefore evaluated the variation of fibrotic genes in hepatic stellate cells (LX-2) via an SRB1 receptor silencing approach and treated in cultures by incubation with Transforming Growth Factor-β (TGF-β) and/or HDL, in order to evaluate the protective role of HDL. We first evaluated the degree of silencing by western blot. As shown in Figure 22 from protein profile analysis, siRNA-treated cells have approximately 50% reduction in SRB1 compared to those treated with control (Figure 22).

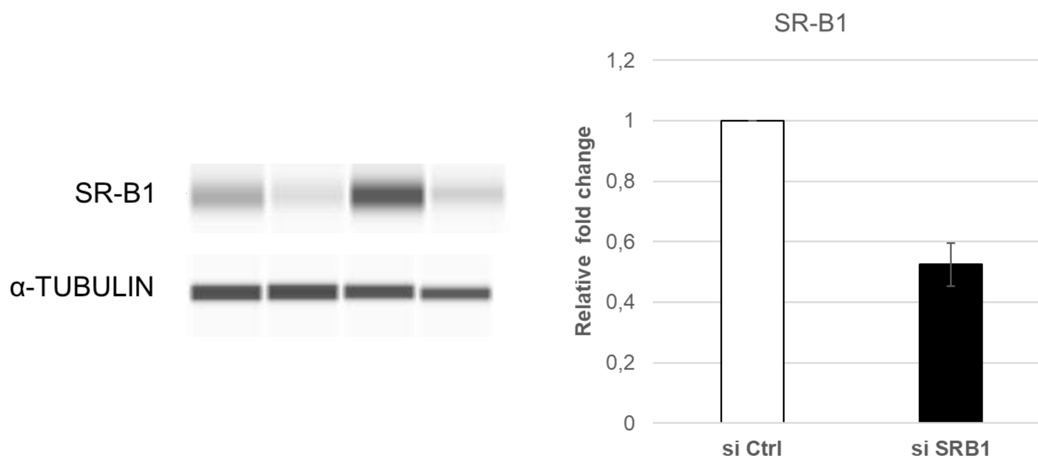


Figure 22: SRB1 silencing in LX-2 cells. The histograms represent relative protein expression a SRB1 after treatment with specific siRNA for this target. Results were expressed as mean ± SD, and were compared by two-way ANOVA test. *P < 0.05, **P < 0.01, ***P < 0.001.

We then investigated the role of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) and protein kinase B (AKT) in profibrotic signaling, hypothesizing a connection with the cholesterol receptor SRB1 (Figure 23).

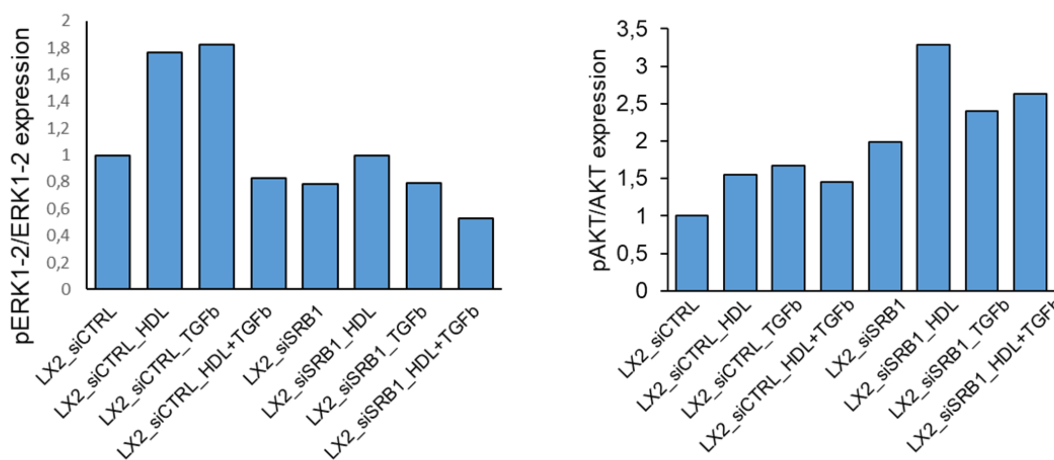


Figure 23: Markers of fibrosis. Western blot analysis of pERK1-2/ERK1-2 e pAKT/AKT.

These data are further confirmed by the analysis of the expression of the genes involved in the fibrosis process, αSMA and COL1. As shown in Figure 24, LX2 cell silencing of SRB1 inevitably reduced the protective role of HDL, increasing gene expression levels following TGF-β treatment.

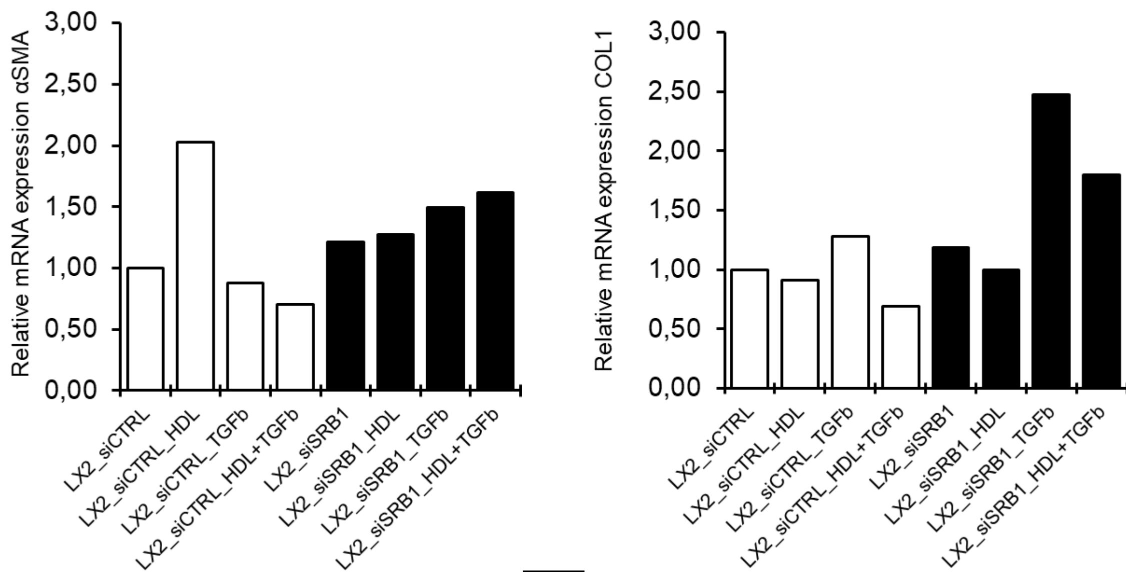


Figure 24: Markers of fibrogenesis. Quantification of αSMA and COL1 expression.

# DISCUSSION

Fatty liver disease has become a major global public health problem in recent years. Despite a relative improvement in living standards, the incidence of fatty liver disease, particularly NAFLD, has increased dramatically (4). The development of NAFLD is closely associated with lipid accumulation, oxidative stress, endoplasmic reticulum stress and lipotoxicity. In addition to these symptoms, patients with NAFLD show associated symptoms such as metabolic syndrome, obesity, type 2 diabetes, and hypertension (5, 7).

To date, unfortunately, the pathogenesis of NAFLD is unknown, and this represents an obstacle for the development of drugs useful in the treatment of fatty liver diseases. Therefore, no drugs have been clinically approved for the treatment of NAFLD, and their treatment is primarily through dietary control in combination with exercise and lifestyle changes.

Liver X receptors (LXRs) include among their functions that of regulating lipid metabolism, therefore they may represent a fundamental control point for the understanding of liver-related pathologies and for the development of targeted therapies (62). Two differently expressed isoforms of the receptor, LXR α/β, are known. The β isoform is ubiquitously expressed throughout the body and the α isoform is expressed at the highest levels in the liver, intestine, adipose tissue and macrophages.

In addition to regulating lipid metabolism and cholesterol homeostasis, LXRs and other receptors belonging to the NR family have important anti-inflammatory activities and are involved in the regulation of glucose homeostasis and have anti-proliferative effects.

Over the years, many LXR agonists have been developed and tested as potential drugs, however, their use has not been authorized as they had various side effects, including important ones, such as hyperglyceridemia and increased lipogenesis, adverse effects due in part to the activation of LXRα in hepatocytes (117).

To circumvent the side effects, alternative strategies were adopted which aimed at an extra-hepatic activation of the LXR receptors. One of these strategies is represented by the intestinal activation of LXRα. It has already been amply demonstrated that the intestinal activation of LXR reduces the absorption of cholesterol and also increases the reverse transport of cholesterol (RCT), increasing the production of HDL and subsequent reduction of circulating cholesterol, thus limiting the effects of hepatic activation of LXR (112).

In this project, therefore, we investigated the mechanism underlying the progression of liver damage in a NAFLD-NASH context and subsequently evaluated the protective

capacity due to intestinal activation of the LXRα receptor. For this purpose, two mouse genotypes were used, the first is a transgenic mouse called iVP16-LXRα, which presented a constitutive activation of the α isoform of the LXR receptor, while the second, an FVB/N, is the base strain from which the transgenic was generated. While, as a model of NASH we used a combination of Western diet (WD) and low doses (compared to those used to induce fibrosis) of carbon tetrachloride ($CCl_4$). With this treatment we have a development of the histological features of NASH, with a progression of fibrosis and transcriptomic features of human NASH (116).

In particular, the WD/$CCL_4$ model induces the alteration of physiological metabolic processes determining, ultimately, the increase in body weight of the mice associated with hypertrophy and inflammation of the adipose tissue, the onset of hepatic steatosis and inflammation of the liver parenchyma , and the development of glucose intolerance and insulin resistance (118).

To clarify the role of intestinal activation of the α isoform of LXR in lipid metabolism, we first monitored, during the 12 weeks of treatment, both the weight of the animals and the amount of food consumed, measuring the weight ratio of the liver/body weight, tissue and blood lipid content. Despite a reduction in food intake, iVP16-LXRα mice and their FVB/N controls treated with WD/$CCl_4$ consumed more calories than those treated with Oil/CD. However, from the analysis of liver weights we noted a significant increase in controls treated with Western diet and $CCl_4$, while this was not observed in LXRα transgenic mice. In fact, after 12 weeks of treatment, the ratio of liver weight to body weight was also higher in FVB/N mice treated with WD/$CCl_4$ than in controls treated with CD/Oil, but above all higher than iVP16-LXRα mice treated. The increase in liver mass in control mice treated with a fatty diet and carbon tetrachloride injections was confirmed by the degree of steatosis, which proved to be higher than in the genetically modified mice. The same trend was observed in the assessment of hepatic triglyceride content.

The reduced accumulation of lipids, as assessed by gene expression analysis of genes involved in lipid synthesis and storage/absorption, led us to hypothesize a key role of LXR in NASH progression.

Furthermore, it is known that the accumulation of fats is the main factor of lipotoxicity and oxidative stress, which leads in the long run to chronic inflammation and progressive fibrosis with deposition of collagen fibers (55).

Following treatment with CCL$_4$ and WD, the expression of circulating inflammatory markers such as TNFα, IL1 and IL6 was reduced in the iVP16-LXRα mouse model compared to control mice.
We found that LXRα activation induces a translation of the macrophage phenotype from pro-inflammatory M1 macrophages, present in WD/CCl$_4$-treated control mice, to an anti-inflammatory M2 phenotype, present in higher numbers in LXRα transgenics, suggesting that this model is also protected from hepatic tissue inflammation. These data are supported by the analysis of 632 genes that could be affected in inflammatory diseases. Among these 382 gene exhibited changes in gene expression among groups of mice. Of note, 153 genes were significantly up-regulated in FVB/N + WD/CCl$_4$ compared to FVB/N + CD/Oil mice, but only 12 of them were rescued in iVP16-LXRα+CCl$_4$/WD mice. Reactome analysis highlighted that these gene are involved in inflammatory pathways but also in signal transduction and in the control of gene expression.
A reduction of inflammation levels, is also linked to the lower activation of HSCs, as we can evidence from the reduced expression of αSMA in the iVP16-LXRα models. This has the effect of a reduction in collagen deposition and a general attenuation of liver damage.
These data justify the lowering of the levels of hepatic fibrogenesis and the deposition of collagen following the treatment.
The improvement of the hepatic picture in the model with intestinal activation of LXRα, both from an inflammatory point of view and of steatosis and fibrosis, correlated with a lower hepatic fat content, not only in terms of triglycerides but also of cholesterol. HDLs not only have the task of eliminating excess plasma cholesterol, but also mediate anti-inflammatory, anti-oxidant and infectious responses (62).
In this work, we demonstrated how intestinal activation of LXRα is able to stimulate RCT. LXR expression increases the levels of the transporters ABCG5 and ABCG8, responsible for the efflux of cholesterol into the intestinal lumen, and of ABCA1, responsible for the regulation of cholesterol and phospholipid homeostasis. The consequence of increased transporter expression is a reduction in cholesterol absorption accompanied by an increase in HDL particles.
At the hepatic levels there are no statistically significant changes in ABCA1 gene expression. While there were significant changes in the scavenger receptor class B type 1 (SRB1) mainly involved in the cholesterol uptake and clearance into the liver. In particular, SRB1 gene expression levels in WD/CCl$_4$ treated FVB/N mice and in not-

treated and treated iVP16-LXRα mice were higher than in WT mice, even if in transgenic mice the hepatic levels of total cholesterol and HDL were significantly down-regulated.
Overall, our data demonstrate that intestinal activation of LXRα results in an increase in RCT, consequently increasing HDL levels, playing an important role in anti-inflammatory processes and a reduction of fibrogenic mechanisms. At the same time, no increase in the intensity of steatosis, one of the main adverse effects of the use of LXR agonists for therapeutic purposes, was observed.
Furthermore, the preliminary data obtained in vitro led us to hypothesize a key role of the SRB1 receptor. In particular, SRB1-mediated removal of cholesterol from caveolae might suppress the activity of high molecular weight phosphatase complex, by inhibiting the PI3K/Akt and ERK1/2 signaling pathway resulting in a reduction of inflammation levels and by blocking the cascade of pro-fibrogenic events. In fact, the silencing of SRB1 inhibited the potential protective role played by HDL, increasing the deposition of collagen in the cells and therefore further worsening the levels of fibrosis. However, whether this mechanism indeed contributes to HDL-induced signaling mediated by SR-B1 remains to be clarified.
The involvement of bacterial flora in the progression of liver damage has been known for years (33). Indeed, elevated blood levels of lipopolysaccharide (LPS) were detected in FVB/N mice treated with WD/CCl4 and decreased in iVP16-LXRα mice. LPS is responsible for the activation of Toll-like receptors (TLRs), resulting in increased inflammation and liver damage. Furthermore, the active role of bacterial translocation during the progression of NASH is known, acting on intestinal permeability (35). The involvement of the gut-liver axis is an interesting aspect that will surely be investigated later.
However, further evaluations will be needed to provide a more complete picture of the role of intestinal LXRα on NASH progression in HCC. Exploiting the previously described combined WD/$CCl_4$ treatment model and pushing it up to 24 weeks, as has been extensively described (111), results in complete HCC development in mouse models. The next objectives will be to investigate the causes that induce this progression and to verify whether the intestinal activation of LXRα can block or slow down the onset of HCC.

# CONCLUSIONS

In conclusion, this work demonstrates that specific activation of intestinal LXRα can exert overall beneficial effects on the process leading to NASH. We have demonstrated how the activation of the RCT causes an increase in HDL levels with a consequent reduction of inflammatory, steatotic and fibrotic processes.

An approach that aims to increase HDL levels may represent a new strategy for the treatment of chronic liver disease. Due to the lack of an effective therapy in NASH, selective intestinal LXRα activation could represent a new specific treatment for the most common form of chronic liver disease, without the side effects due to systemic LXR activation, such as fatty liver disease.

Therefore, the results obtained may open the door to a possible development of a pharmacological therapy based on intestinal-acting LXR agonists: however, some dynamics involved in the pathogenesis process of NASH will need to be further investigated.

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