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Characterization of Novel Targets for Preventing Fibrosis Evolution in Nonalcoholic Fatty Liver Disease (NAFLD)

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General Abstract

Over the past four decades, non-alcoholic fatty liver disease (NALFD) has become the most prevalent chronic liver disorder worldwide and by now NAFLD progression to nonalcoholic steatohepatitis (NASH) is a leading cause of liver fibrosis/cirrhosis in Western countries. In recent years, the progress in understanding the complexity of the pathogenetic mechanisms responsible for the NAFLD evolution has led to the development of a variety of drugs that are ongoing testing in clinical trials. Nonetheless, in spite that chronic inflammation is seen as the driving force in promoting NASH evolution, therapies targeting liver inflammation and fibrosis development are still very limited. In this setting, this doctoral project has investigated:

a) The capacity of the anti-inflammatory mediator Annexin A1 of influencing the profibrogenic action of macrophages.

b) The anti-inflammatory effects of cholesterol-free ketogenic diet

Annexin A1 (AnxA1) is an important effector in the resolution of inflammation and previous works have shown its involvement in modulating hepatic inflammation in NASH. The first study investigated the effects of the administration of human recombinant (hrAnxA1; 1µg, daily IP) in counteracting the progression of experimental NASH induced in C57BL/6 mice by feeding methionine-choline deficient (MCD) or Western diets. In both experimental models, the treatment with hrAnxA1 improved parenchymal injury and lobular inflammation without interfering with the extension of steatosis. Moreover, hrAnxA1 significantly attenuated hepatic fibrosis as evaluated by the expression of α1-procollagen and TGF-β1 and by collagen Sirius Red staining. Flow cytometry and immunohistochemistry showed that hrAnxA1 did not affect the liver recruitment of macrophages, but strongly reduced the formation of crown-like macrophage aggregates and their capacity of producing pro-fibrogenic mediators like osteopontin and galectin-3. This effect was related to AnxA1 interference with the acquisition of a specific macrophage phenotype characterized by the expression of the triggering receptor expressed on myeloid cells 2 (TREM2), CD9 and CD206, previously associated with NASH evolution to cirrhosis. Collectively, these results indicate that, besides ameliorating hepatic inflammation, AnxA1 is effective in preventing NASH-associated fibrosis by interfering with macrophage pro-fibrogenic features. Such a novel function of AnxA1 gives the rational for the development of AnxA1 analogues for the therapeutic control of NASH evolution.

Lifestyle changes and diet are, so far, the only effective interventions in NAFLD, even though there is no agreement on the most suitable dietary regimen. In recent years, low carbohydrate ketogenic diets (KDs) have been increasingly used for weight loss. However, their efficacy in improving NASH is still controversial. The second study investigated the capacity of a cholesterol-free KD to improve NASH evolution. For the experiments, NASH was induced in C57BL/6 mice by feeding a cholesterol-enriched Western Diet (WD) for up to 16 weeks, followed by switching animals to KD for additional 8 weeks. We observed that KD administration increased by three folds ketone bodies production and significantly reduced liver weights. Moreover, liver proteomic analysis and functional tests evidenced an improved glucose and lipid metabolism along with insulin resistance in KD fed mice. These metabolic effects were associated with an amelioration in transaminase release and in the histological severity of steatosis and necro-inflammation. Mice receiving KD also showed a lowering in the hepatic expression of pro-inflammatory/pro-fibrogenic markers such as CCl2, IL-12, CD11b, α1-procollagen, TGF-β1, osteopontin and galectin-3, which were accompanied by a significant reduction in hepatic monocyte-derived macrophage infiltration and collagen fibres deposition as assessed by the Sirius-red staining. The improvement in liver damage and fibrosis likely relies on the capacity of KD of improving NASH associated dysbiosis leading to a recovery in gut bacterial flora similar to that of healthy mice. Altogether, these results indicate that a cholesterol-free ketogenic diet is effective in improving metabolic derangements and steatohepatitis, and it might represent a potential therapeutic strategy for NAFLD.

1. Introduction

1.1 Non-alcoholic fatty liver disease (NAFLD) epidemiology and histopathological features

Over the past four decades, non-alcoholic fatty liver disease (NALFD) has become the most prevalent chronic liver disorder worldwide, affecting around one-fourth of the adult population (Younossi, et al., 2018). As a result, NAFLD is now the most rapidly increasing cause of end-stage liver disease and liver-related mortality (Younossi, et al., 2018). NAFLD is an umbrella term for a broad spectrum of liver damages, the main feature of which is represented by hepatic steatosis due to an excessive triglyceride deposition within the hepatocytes. NAFLD is defined by the presence of more than 55 mg of triglycerides per gram of liver or, at histology, by the detection of lipid droplets in more than 5% of hepatocytes, in absence of secondary causes of hepatic lipid accumulation (e.g., excessive alcohol consumption) (Brunt, et al., 2015; Kotronen, et al., 2008; Powell, et al., 2021). The spectrum of NAFLD ranges from simple steatosis to non-alcoholic steatohepatitis (NASH), a condition characterized by the concomitant presence of macrovesicular steatosis, parenchymal damage with hepatocyte ballooning and apoptosis, focal necrosis, lobular and portal inflammation, and fibrosis. NASH is a progressive form of NAFLD that can lead to advanced fibrosis and cirrhosis, as well as liverrelated morbidity and mortality (Wree, et al., 2013; Pouwels, et al., 2022) (Fig.1).

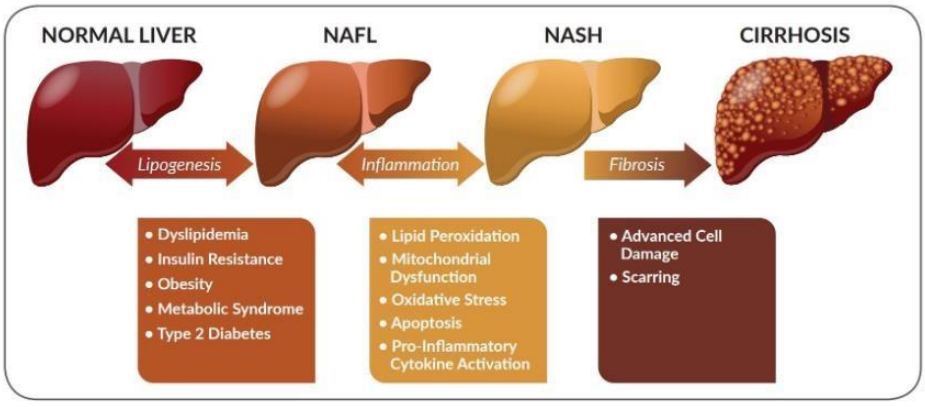

Fig.1: Progression of NAFLD. The evolution and stages of non-alcoholic fatty liver disease (NAFLD). Steatosis (also referred to as "simple steatosis" or "fatty liver") is the initial phase of NAFLD and is characterized by excessive fat accumulation in hepatocytes. Upon developing inflammation, the NAFLD may subsequently transform into non-alcoholic steatohepatitis (NASH) and finally, lead to liver cirrhosis in some individuals. In turn, both NASH and cirrhotic liver has an increased chance to develop hepatocellular carcinoma (HCC). Reproduced from Research Tools for Fatty Liver Diseases | News & Announcements | Cayman Chemical

Although NAFLD patients with simple liver steatosis do not face a significant risk of liverrelated adverse outcomes (Rinella, 2015), about 15% develop NASH (Younossi, et al., 2018; Wree, et al., 2013; Pouwels, et al., 2022; Maurice, et al., 2018; Sutti and Albano, 2019) and among them one-third might progress to advanced fibrosis or cirrhosis with the consequent risk of developing hepatocellular carcinoma (HCC) (Rinella, 2015; Schuster, et al., 2018). As mentioned above, the global prevalence of NAFLD is around 25%, being significantly higher in men than in women (39·7% vs 25·6%; p<0·0001) (Kiarash Riazi, et al., 2022). NAFLD/NASH is more frequent in the Middle East and South America (30%), while its prevalence in Europe and Asia is around 25% and only 13% in Africa. This uneven distribution is likely driven by differences in overall caloric intake, physical activity, body fat distribution, socioeconomic status, and genetic background (Younossi, et al., 2018, Schuster, et al., 2018). The sharp spread of NAFLD in the last decade appears strictly related to the rising incidence of metabolic disorders such as obesity, hypertension, dyslipidemia, and type II diabetes (Anstee and Targher, 2013). In fact, it has become evident that NAFLD is closely associated with the socalled metabolic syndrome (MetS), representing its hepatic manifestation (Brunt, et al., 2015, Yki-Järvinen, 2014). MetS is a complex clinical condition characterized by hyperglycemia, hypertension, hypertriglyceridemia, low high-density lipoprotein (HDL)-cholesterol levels, and obesity. MetS characterizes 70-80% of obese or diabetic subjects affected by NAFLD, while the prevalence of NASH among MetS patients is about 15% (Yki-Järvinen, 2014; Chalasani, et al., 2004). Remarkably, the more components of MetS are present, the higher are the chances of developing NAFLD and eventually advanced fibrosis (Jinjuvadia, et al., 2017). This observation has led to the concept of metabolic dysfunction-associated fatty liver disease (MAFLD). However, this new nomenclature is not currently accepted by the American Association for the Study of Liver Diseases (AASLD) or the European Association for the Study of Liver Diseases (EASL) (Eslam, et al., 2020). The prevalence of NAFLD is also related to body mass index (BMI) and waist circumference. In fact, subjects having a BMI of more than >30 as well as a waist circumference of more than >94 cm for men or >80 cm for women have a greater chance to develop NAFLD (Chaney, 2015; Williams, et al., 2011). However, it is important to note that even people with a healthy BMI can still develop NAFLD, described as lean NAFLD, usually characterized by central obesity or other metabolic risk factors (Powell, et al., 2021). For instance, in Asia, a considerable portion of subjects (8-20%) develops NAFLD at lower BMI

(Fan, et al., 2017). Altogether, these data indicate that NAFLD reflects complex interactions between environmental, lifestyle-related factors and genetic predisposition (Younossi, et al., 2016).

1.2 Pathogenic mechanisms in the NAFLD evolution to fibrosis

The molecular mechanisms underlying the development and progression of NAFLD are complex and multifactorial. Several theories have been proposed to explain the progression of NAFLD to the more advanced stages. Among these, the "two-hit hypothesis" has become very popular and postulates the disease onset through a 'first hit' caused by hepatic lipid accumulation because of obesity and insulin resistance. In its turn, the presence of steatosis enhances liver susceptibility to additional factors representing the 'second hit' activating inflammatory cascade and fibrogenesis (Paschos and Paletas, 2009). However, it has become rapidly evident that such a postulate is too simplistic to recapitulate the complexity of human NAFLD where multiple environmental and genetic factors contribute to making the pathology evolve. Consequently, the theory has been expanded in a "multi-parallel hits" hypothesis, based on the evidence that many processes, including oxidative stress, endoplasmic reticulum (ER) stress, gut dysbiosis, and cytokines production by the adipose tissue can contribute to liver inflammation (Tilg and Mochen, 2010; Wree, et al., 2013). Nonetheless, also in this case the pathogenetic model is still unsuitable to explain the complexity of NASH progression.

i) Mechanisms leading to steatosis and hepatocellular damage

The primary driver of NAFLD is the excessive intake of carbohydrates and lipids causing the expansion of adipose depots as well as ectopic fat deposition. Hepatic steatosis is the main feature of NAFLD, and the mechanisms responsible for the impairment of carbohydrate and lipid metabolism within the liver play a crucial role in the disease's pathogenesis. Physiologically, free fatty acids (FFAs) reaching the liver derive from dietary short-chain fatty acids or from the triglyceride hydrolysis from adipose tissue, and once in the hepatocytes are either partly used as an energy source or re-esterified to triglycerides and exported into the bloodstream as very low-density lipoproteins (VLDL) (Lonardo and Loria, 2002). In addition, hepatocytes synthesize fatty acids from glucose and other non-lipid precursors by *de novo*

lipogenesis (DNL). Thus, any derangement in these processes can lead to the onset of hepatic steatosis. In fact, the liver responds to elevated circulating FFAs as well as to increased lipogenesis by promoting triglyceride synthesis and favouring intrahepatic triglyceride accumulation (Musso, et al., 2009) (Fig.2). Dysregulation of plasma non-esterified fatty acid (NEFA) is one of the consequences of dyslipidemia associated with insulin resistance. Insulin resistance is defined as an impaired response to insulin stimulation of target tissues, primarily the liver, muscle, and adipose tissue, and it plays a pivotal role in causing liver steatosis. Under physiological conditions, insulin binding to insulin receptor leads to its auto-phosphorylation and the activation of insulin receptor substrate (IRS-1/2) proteins. These latter, by interaction with intracellular phosphoinositide 3-kinase (PI3K) and AKT/PKB signalling cascade, mediate the metabolic effects of insulin including glucose uptake, the expression of key lipogenic genes, and the decrease in gluconeogenic genes (Boura-Halfon and Zick, 2009; Peng and He, 2018). Insulin resistance is the result of an impairment of IRS-1/2 signalling leading to a decrease in glucose uptake, glycogen synthase activation, and phosphorylation of forehead box protein O (FOXO) with concomitant stimulation of hepatic gluconeogenesis (Fu, et al., 2014). As a result, insulin resistance induces DNL and dysregulated lipolysis in adipose tissue with the consequent massive release of NEFAs, which are picked up by hepatocytes through fatty acid transport proteins (FATP)-2 and -5 (Tilg and Mochen, 2010; Bugianesi, et al., 2010; Berk, 2008) (Fig. 2). While under physiological conditions saturated FAs (SFAs) are transported to the mitochondria for β-oxidation or esterified for either excretion in VLDLs (Berk, 2008), in NAFLD the overwhelming excess of SFAs coming from adipocyte lipolysis, diet, and free cholesterol from DNL, activates in the hepatocytes a variety of intracellular responses resulting in lipotoxic stress in the ER and mitochondria, respectively, promoting organelle dysfunction, cell injury, and death (Shimada, et al., 2014; 33). In turn, the impairment of the insulin signalling pathway results in the production of adipokines and inflammatory cytokines like tumour necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β contributing to the recruitment and activation of hepatic resident macrophages (Kupffer cells; KCs) mediating inflammation in NASH (Guilherme, et al., 2008; Anderson and Borlak, 2008).

Oxidative stress is known to be of major importance in the progression of NAFLD to NASH. In fact, the disease progression associates with an increase in oxidative stress markers and an

impaired activity of antioxidant enzymes like superoxide dismutase, catalase, and glutathione (GSH) peroxidase along with a reduction in vitamin E and GSH (Sutti and Albano, 2022).

Notably, such variations correlate with the clinical severity of NASH, suggesting that oxidative damages can contribute to the evolution from NAFLD to NASH impacting the inflammatory state either directly through inflammasome activation or indirectly through the action of oxidized proteins and lipid peroxidation products, acting as the damage-associated molecular pattern (DAMPs) in triggering Toll-like receptors (TLR) signalling and the secretion of proinflammatory cytokines (such as TNF-α, IL-1, IL-6, and Fas ligand) playing a key role in cell death, inflammation, and fibrosis (Sutti and Albano, 2022).

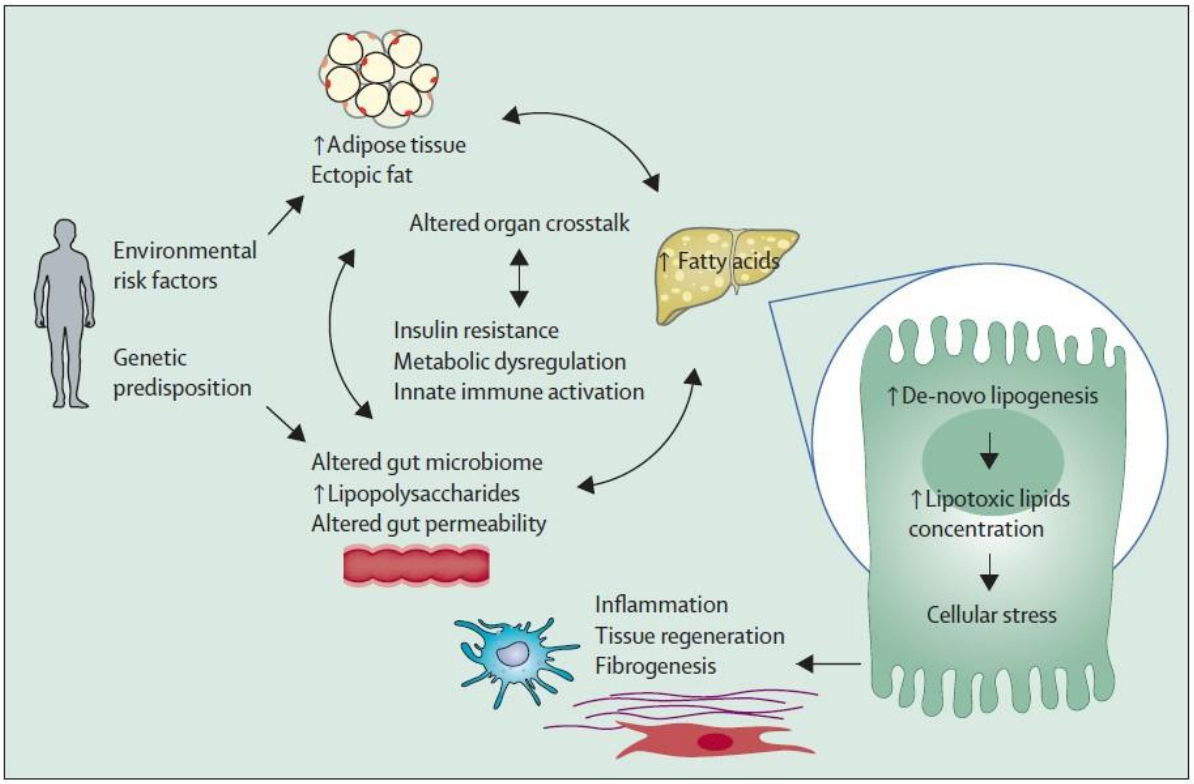

Fig.2 Multiple pathways and interactions between different organs, affect the pathogenesis of non-alcoholic fatty liver disease. In the setting of environmental risk factors and heritable factors, crosstalk between the liver, adipose tissue, and gastrointestinal tract leads to systemic inflammation and insulin resistance, resulting in increased hepatic delivery of fatty acids and de-novo lipogenesis. This metabolic milieu leads to the formation of lipotoxic lipids that contribute to cellular stress with subsequent stimulation of inflammation, tissue regeneration, and fibrogenesis. Reproduced from Powell, et al., 2021.

ii) Inflammatory mechanisms in the progression of NAFLD

Hepatic lobular Inflammation is a hallmark of the progression from simple steatosis to steatohepatitis that characterizes NASH. Growing evidence from clinical and experimental studies indicates that liver inflammation represents the driving force for NAFLD evolution to cirrhosis as well as a specific contributor to extrahepatic injury associated with NAFLD such as type 2 diabetes mellitus, cardiovascular diseases, chronic kidney diseases, osteoporosis, and sarcopenia (Amstrong, et al., 2014). Indeed, chronic inflammation exacerbates tissue injury and might result in an abnormal wound-healing response contributing to the development of NASH and liver fibrosis. In this setting, both innate and adaptive immunity are activated in response to metabolic-related stresses and understanding the interplay between these two systems represents the challenge for unravelling the disease pathogenesis (Peiseler, et al., 2022).

Inflammation in NAFLD is triggered by multiple intrahepatic and extrahepatic factors (Fig.3). The activation of resident KCs is currently seen as a key element in the onset of hepatic inflammation in NASH. In this setting, metabolic derangements consequent to insulin resistance are responsible for causing an excess of circulating FFAs and cholesterol, which directly stimulate Kupffer cells (Kazankov, et al., 2019). Within the hepatocytes, FFAs also cause ER stress and lipotoxicity (Lebeaupin, et al., 2018), which favor the hepatocyte production of pro-inflammatory cytokines and microvesicles capable of stimulating KC responses (Srinivas, et al., 2021). Furthermore, as mentioned above, hepatocyte FFA overload causes lipotoxicity and oxidative stress inducing cell death along with the release of intracellular DAMPs in the hepatic circulation. These molecules are recognized by the innate immune system through pattern recognition receptors (PRRs), responsible for triggering a local inflammatory activity producing inflammatory cytokines such as TNF-α and IL-6 (Feldstein, 2010; Seki and Brenner, 2008). Among PRRs, Toll-like receptors (TLRs) are highly conserved receptors expressed on hepatocytes, Kupffer cells, hepatic stellate cells, and biliary epithelial cells (Kesar and Odin, 2014; Petrasek, et al., 2013; Strowig, et al., 2012). Additional triggers of macrophage response involve the stimulation of inflammasome. In particular, the nucleotide oligomerization domain (NOD)-like receptors (NLRs) appear to play an important role in NAFLD/NASH and by recognizing DAMPs and PAMPs trigger the secretion of IL-1 and IL-18 (Martinon, et al., 2002; Szabo and Csak, 2012). Both these cytokines elicit inflammatory

signals in the liver as well as in the adipose tissue and intestine, triggering steatosis, insulin resistance, inflammation, and cell death (Dixon, et al., 2013).

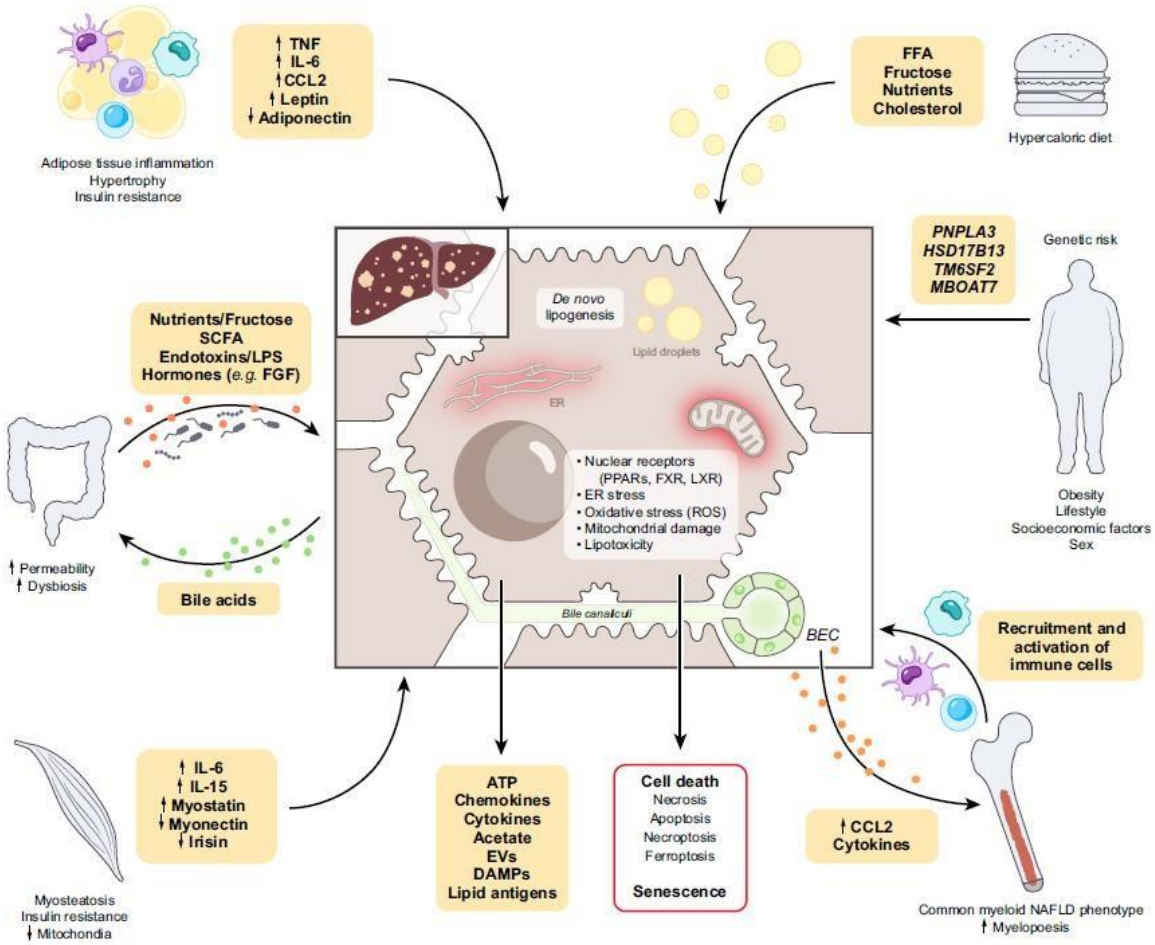

Fig. 3: Triggers of inflammation. Intra- and extrahepatic factors trigger inflammation in NAFLD. The combination of hypercaloric diet, obesity, lifestyle, and genetic risk predispose individuals to NAFLD onset. Hepatocytes overloading with FFAs and increased *de novo* lipogenesis leads to lipid accumulation in hepatocytes. Fat overload in the liver induces lipotoxicity resulting in endoplasmic reticulum (ER) stress, oxidative stress, reactive oxygen species (ROS) production and mitochondrial damage. In turn, stressed hepatocytes release proinflammatory mediators and damage-associated molecular patterns (DAMPs) resulting in robust immune cell activation and infiltration, further damaging hepatocytes. Different forms of cell death occur as well as hepatocyte senescence, triggering a more pronounced immune response. Liver inflammation is also propagated by multiple extrahepatic systems including the adipose tissue, gut, skeletal muscle, and bone marrow. Reproduced from Peiseler, et al., 2022

iii) The role of innate immune cells in NAFLD evolution

Lobular inflammation in NASH involves hepatic infiltration of a variety of immune cells belonging to both innate and adaptive immunity. Innate immune responses are currently seen

as a key element in supporting hepatic inflammation in NASH leading to the activation of resident KCs as well as to the liver recruitment of leukocytes such as neutrophils, monocytes, natural killer (NK) and natural killer T (NKT) cells.

Most hepatic macrophages in the healthy state are resident yolk sac-derived KCs located in hepatic sinusoids. These cells are responsible for phagocyting pathogens or bacteria-derived products coming from the portal vein circulation and presenting antigens to cytotoxic and regulatory T-lymphocytes (Lanthier, 2015). As previously mentioned, during NAFLD evolution, quiescent KCs are activated by DAMPs originating from steatotic hepatocytes dying because of lipotoxicity and oxidative stress as well as by the excess of PAMPs consequent to gut dysbiosis with the secretion of pro-inflammatory cytokines like IL-6, TNF-α, IL-1β and chemokine such as CCL2 and CCL5 (Tacke, 2017; Garcia-Martinez, et al., 2016; Mridha, et al., 2017). In their turn, inflammatory cues drive the hepatic recruitment of circulating monocytes, which locally differentiate into monocyte-derived macrophages (MoMFs), characterized by a M1 pro-inflammatory behavior (Szabo and Csak, 2012; Dixon, 2013). During the disease evolution, MoMFs significantly contribute to the inflammatory response and replace KC loss occurring in the early phase of the disease evolution (Peiseler et al. 2022). Furthermore, by surrounding dead/dying-steatotic hepatocytes MoMFs give rise to aggregates known as lipogranulomas or hepatic crown-like structures (hCLSs) (Xiong, et al., 2019; Daemen, et al., 2021; Horn, et al., 2022). Notably, macrophages forming hCLSs display a peculiar phenotype characterized by the expression of triggering receptor expressed on myeloid cells 2 (TREM2), CD63, and the glycoproteins CD9 and NMB (GPNMB) (Xiong, et al., 2019; Seidman, et al., 2020; Daemen, et al., 2021). Because of their association with NASH these cells have been renamed NASH-associated macrophages (NAMs) (Xiong, et al., 2019). Interestingly, from a phenotypical point of view, NAMs resemble scar-associated macrophages described in human fibrotic livers (Ramachandran, et al., 2019). In this respect, NAMs produce pro-fibrogenic mediators such as osteopontin (OPN) and galectin-3 and localize in regions rich in collagen fiber deposition suggesting their possible involvement in NASH-related fibrogenesis (Itoh, et al., 2013). Consistently, recent studies using spatial transcriptomics have shown that in NASH livers $TREM2^{+}$ NAMs localize to sites of hepatocellular damage, inflammation, and fibrosis and that increased circulating levels of soluble TREM2 are an effective marker for the disease activity (Hendrikx, et al., 2022). However, TREM2 deficiency in hematopoietic cells fails to prevent

NASH and causes defective lipid handling and extracellular matrix remodeling, resulting in exacerbated steatohepatitis, cell death, and fibrosis (Hendrikx, et al., 2022). This suggests that NAMs might represent a heterogenous cell population also involved in controlling tissue scarring (Xu, et al., 2012).

Beside macrophages, neutrophils represent the most frequent myeloid cell in hepatic infiltrates in both NASH mouse models and human biopsies. In contrast to their beneficial roles during infection, neutrophils usually have a detrimental effect on chronic inflammatory disorders by releasing ROS, myeloperoxidase (MPO), and proteases, such as neutrophil elastase (NE), proteinase 3, cathepsins, and matrix metalloprotease (MMP)-9 into the extracellular environment. MPO also enhances inflammation by recruiting macrophages and promotes the pro-fibrogenic activation of hepatic stellate cells (HSCs) (Xu, et al., 2012). Accordingly, neutrophil depletion as well as impairment of MPO, NE, or proteinase-3 expression improve liver damage in the experimental model of NASH by limiting inflammation and liver injury. However, such effects are no longer observed as NASH progresses (Cai, et al., 2019). Similarly, dismantling neutrophil extracellular traps (NETs) through deoxyribonuclease1 limits hepatic inflammation, liver injury, and fibrosis in mice. However, recent evidence suggests that neutrophils play also an important role in tissue repair and in the reshaping of extracellular matrix. For instance, neutrophil depletion in the recovery from NASH induced by feeding a methionine, choline-deficient diet (MCD) impairs liver healing along with the phenotype switching of pro-inflammatory macrophages towards reparative macrophages (Peiseler et al. 2022).

Hepatic natural killer (NK) cells are located inside the sinusoidal lumen, adhering to endothelial and KCs. Morphologically they are defined as large granular lymphocytes (LGLs) and functionally as liver-associated NK cells with cytolytic activity toward stressed or apoptotic cells. NK cells are integral modulators of the inflammatory microenvironment and play an important role in NASH evolution, since they secrete a variety of cytokines such as TNF-α, interferon (IFN)-ϒ, and IL-4 recruiting and activating cells involved in adaptive immune response, thus bridging innate and adaptive arms of the immune response (Wallace, et al., 2022; Yoon, et al., 2011). Experimental models of NAFLD have demonstrated the contribution of NK in maintaining M1 polarization of hepatic macrophages by releasing IFN-ϒ

(ToselloTrampont, et al., 2016). Nonetheless, NK cells have also an anti-fibrotic effect favoring the killing of activated mesenchymal cells and myofibroblasts (Vivier, et al., 2008; Stiglund, et al., 2019).

Natural killer T-cells (NKT) comprise a unique immune cell subtype that expresses specific NK cell surface receptors (NK1.1 in mice or CD161/CD56 humans) as well as conventional T-cell antigen receptors (TCRs). Most of the liver NKT cells are represented by type I or invariant NKT (iNKT) and type II or diverse NKT cells (Marrero, et al., 2018). Following activation, type I NKT cells can stimulate dendritic cells (DCs), NK cells, B-cells, and conventional $CD4^+$ and $CD8^+$ Tcells in mediating liver damage. Furthermore, cytokines and chemokines secreted by activated-type I NKT cells (IL-4, IL-10, IFN-γ, and TNF-α) promote the recruitment of neutrophils, myeloid cells, and monocytes to the liver, and can modulate helper T (Th)-1, Th2, and regulatory (Treg) cell activity (Wallace, et al., 2022; Marrero, et al., 2018). Thus, NKT cells can both stimulate or suppress immune/inflammatory responses. During NAFLD evolution the prevalence of liver NKT cells is lowered in steatosis and during the early phases of steatohepatitis (Kremer, et al., 2010), while NKT cell expansion is evident in advanced NASH (Tajiri, et al., 2009; Wolf, et al., 2014). The involvement of NKT cells in the pathogenesis of NASH is demonstrated by several studies showing that interfering with NKT cells effectively improves hepatic parenchymal injury and inflammation preventing NASH progression to fibrosis and hepatocellular carcinoma in different experimental models (Wolf, et al., 2014; Bhattacharjee, et al., 2017; Syn, et al., 2012; Maricic, et al., 2018). In particular, the lack of iNKT cells in $J\alpha18^{-/-}$ mice or NKT cells inhibition by mice treatment with retinoic acid receptorγ agonist tazarotene reduces $CD8^+$ T-cell infiltration in NASH livers (Wallace, et al., 2022; Maricic, et al., 2018) suggesting a strict interplay between cytotoxic T-cells and iNKT cells in the mechanisms supporting steatohepatitis.

iv) The role of the adaptive immune system in NAFLD

Recent studies have evidenced that beyond the key role of innate immunity, adaptive immunity also plays an integral role in orchestrating of inflammation and fibrogenesis during the progression to NASH (Cyster, et al., 2003). Lymphocytic infiltration is frequently observed in liver biopsies of patients with NASH, while experimental NASH models show that the liver

recruitment of $CD4^+$- and $CD8^+$-T- and B-lymphocytes parallels the worsening of steatohepatitis (Peiseler et al. 2022, Sutti, et al., 2014; Bruzzì, et al., 2018).

From the functional point of view, $CD4^+$ T-lymphocytes associated with NASH have been shown to belong to both interferon-γ (IFN-γ)-producing T-helper 1 (Th-1) and IL-17 producing Th-17 cells (Sutti, et al., 2014; Li, et al., 2005; Rau, et al., 2016; Rolla, et al., 2016). These findings are supported by the clinical observations showing that both paediatric and adult NASH is characterized by an increase in liver and circulating Th-1 and Th-17 T-cells (Inzaugarat, et al., 2011; Ferreyra, et al., 2012; Rau, et al., 2016) as well as by data obtained in humanized mice engrafted with a functional human immune system. In these mice, NASH development is accompanied by an expansion of $CD4^+$ T-cells, whereas $CD4^+$ T-cell depletion decreases NASH-associated immune cell infiltration and hepatic injury (Her, et al., 2020). Th-1 lymphocytes contribute to the pro-inflammatory network by promoting macrophage differentiation into the M1 phenotype via IFN-γ, and TNF-α signalling, while Th-17 cells produce chemokines that are chemoattractant for neutrophils and secrete IL-17 that exacerbates steatosis and inflammation (Huby and Gautier, 2022). Consistently, in NASH patients, plasma IFN-γ levels positively correlate with the number and size of hepatic lymphocyte aggregates as well as with the severity of fibrosis (Bruzzì, et al., 2018). The actual role of Th17 cells in NASH pathogenesis has been recently clarified by a single-cell RNAsequencing (scRNA-seq) which identifies a specific subset of hepatic Th17 cells named ihTh17 (Moreno-Fernandez, et al., 2021). These cells are characterized by a high expression of C-X-C Motif Chemokine Receptor 3 (CXCR3) and by the secretion of large amounts of inflammatory mediators (Raza, et al., 2021) and in both experimental and human NASH their hepatic accumulation correlates with the extent of hepatocellular damage (Moreno-Fernandez, et al., 2021).

Along with $CD4^+$ T-cell activation, both human and rodent NAFLD/NASH are characterized by a rise in the liver prevalence of cytotoxic $CD8^+$ T-lymphocytes (Sutti, et al., 2014; Wolf, et al., 2014; Grohmann, et al., 2018; Ghazarian, et al., 2017). $CD8^+$ T-cells are critical effectors of adaptive immunity mainly producing IFN-ϒ, TNF-α, and cytotoxic molecules such as perforins. In early stages of steatosis, $CD8^+$ T-cells do not contribute to liver injury but mediate metabolic dysregulation and insulin resistance. CD8-deficient mice have, in fact, improved metabolic

parameters and the adoptive transfer of $CD8^+$ T-cells isolated from livers of mice with steatosis worsens glucose metabolism (Ghazarian, et al., 2017). Conversely, the selective ablation of $CD8^+$ T-cells ameliorates liver damage in mice with overt NASH (Bhattacharjee, et al., 2017), suggesting an effective role in the pathogenesis of steatohepatitis. In the same vein, singlecell sequencing of intrahepatic T-cells from mice with diet-induced NASH and NASH patients has evinced that the severity of steatohepatitis correlates with the expansion of a $CD8^+$ T-cell population with markers of tissue residency (CXCR6), exhaustion (programmed cell death 1; PD1) and effector function (granzyme B). These $CXCR6^+/PD1^+/CD8^+$ T-cells have autoaggressive behaviour and directly kill hepatocytes via Fas-FasL interactions (Dudek, et al., 2021). Interestingly, mechanistic experiments show that the expansion of $CXCR6^+/PD1^+/ CD8^+$ T-cells depends on IL-15 signalling, while their cytotoxic activity is mediated by metabolic stimuli and extracellular ATP released from dying hepatocytes (Dudek, et al., 2021). Altogether, these data shed new light on the complexity of the contribution of T-cells in the pathogenesis of steatohepatitis suggesting the possibility that the specific metabolic environment of NASH might direct adverse functional responses by immune cells.

Alongside T-cells, B-lymphocytes are also detectable within inflammatory infiltrates in liver biopsies from NASH patients (Bruzzì, et al., 2018; Grohmann, et al., 2018). In mice models of NASH, we have observed that B-cells activate at the onset of steatohepatitis and maturate to plasmablasts and plasma cells. Mice liver B-lymphocytes mainly consist of bone marrowderived mature $B220^+/IgM^+/CD23^+/CD43^-$ B2-cells resembling spleen follicular B-cells. However, a small fraction of $B220^+/IgM^+/CD23^-/CD43^+$ B1-cells is also detectable (Novobrantseva, et al., 2005). The functions of the two B-cell sub-sets are not overlapping, as upon antigen stimulation B1-cells mature in a T-cell-independent manner to plasma cells producing IgM natural antibodies (Tsiantoulas, 2015). Natural antibodies are pre-existing germline-encoded antibodies with broad specificity to pathogens, but also able to cross-react with endogenous antigens, such as oxidized phospholipids and protein adducted by endproducts of lipid peroxidation (Tsiantoulas, 2015). Conversely, the B2 sub-set requires helperT cells to proliferate and to undergo antibody isotype class switching which leads to plasma cells producing highly specific IgA, IgG or IgE (Tsiantoulas, 2015). The expansion of B2-cell in NASH depends upon the up-regulation in the hepatic expression of the B-cell Activating Factor (BAFF) (Bruzzì, et al., 2018), one of the cytokines driving B-cell survival and maturation.

Interestingly, circulating levels of BAFF are higher in patients with NASH than in those with simple steatosis and correlate with the severity of steatohepatitis and fibrosis (Miyake, et al., 2013). Concerning the mechanism triggering B-lymphocyte responses in NASH, recent data indicate a role for gut dysbiosis as fecal microbiota transplantation from NAFLD patients to healthy mice induces histopathologic hallmarks of the disease including an increased number of intrahepatic B-cells displaying an up-regulated expression of antigen-presenting and costimulatory molecules (Barrow, et al., 2021). Beyond gut dysbiosis, oxidative stress can represent another important trigger for B-cell activation, since in NASH patients the prevalence of intrahepatic B/T cell aggregates associates with a higher level of circulating IgGs against oxidative stress-derived epitopes (OSEs) (Bruzzì, et al., 2018). Furthermore, subcutaneous immunization with OSEs worsens experimental NASH by promoting a specific IgG production along with an expansion of IFN-γ-producing $CD4^+$ T helper cells (Sutti, et al., 2014). Indeed, intrahepatic B-cells can directly influence $CD4^+$ T helper (Th) cell functions *in vitro* by promoting Th1 activation (Zhang, et al., 2016). Such an effect is mediated by intrahepatic, but not splenic B-cells, suggesting the presence of a peculiar B-cell phenotype differentiated locally without systemic involvement (Barrow, et al., 2021). A crosstalk between B- and T-cells is further supported by the observation that mice lacking B-cells or harboring functionally defective B-cells show milder hepatic injury and lower liver recruitment of Th1-activated $INF\text{-}\gamma^+CD4^+$ T-cells as compared to control mice (Bruzzì, et al., 2018; Barrow, et al., 2021).

v) Gut dysbiosis is a leading cause in the evolution of steatohepatitis toward fibrosis

One of the still unsolved issues in understanding the mechanisms driving hepatic inflammation in NASH concerns the stimuli maintaining the activation of innate and adaptive immunity. Several factors including lipotoxicity, oxidative stress and DAMPs release by damaged cells represent possible causes. Along with these factors, imbalances in the composition of intestinal microbiota, also known as gut dysbiosis, has progressively attracted attention as a possible mechanism for the induction of chronic inflammation in NASH (Loomba, et al., 2019). Changes in the intestinal microbiota are, in fact, an early event that often precedes the onset of type 2 diabetes and NAFLD (Frost, et al., 2021) and correlates with fat accumulation within the liver (Leung, et al., 2016; Marra, et al., 2018). Conversely, antibiotic treatment, improves

hepatic steatosis in a preclinical model of NAFLD possibly by eliminating dangerous bacterial strains (Bergheim, et al., 2008).

In healthy adults, intestinal bacterial strains belong mostly to two phyla, the Gram-positive *Firmicutes* and the Gram-negative *Bacteroidetes*, whereas *Actinobacteria, Proteobacteria, Fusobacteria*, and *Verrucomicrobia* are less abundant (Vallianou, et al., 2019). Conversely, NAFLD patients, especially those with NASH, show an increased prevalence of Bacteroidetes and a reduction in the relative abundance of *Firmicutes* resulting in a lowered *Firmicutes/Bacteroidetes* ratio (Jadhav, et al., 2020). Furthermore, patients with NAFLD exhibit an increase in species belonging to Clostridium, *Anaerobacter, Streptococcus* and *Lactobacillus* families, while *Oscillibacter, Flavonifaractor, Odoribacter*, and *Alistipes* spp. are less represented (Jiang, et al., 2015). In general, NASH is characterized by an increase in the relative abundance of potential pathogen strains, such as Gram-negative *Proteobacteria*, *Enterobacteriaceae*, and Escherichia spp, whereas are relatively diminished *Faecalibacterium prausnitzii* and *Akkermansia muciniphila* that are endowed with beneficial effects on human health (Zhu, et al., 2013; Gerbes, et al., 2018; Satapathy, et al., 2020). Gut microbiota abnormalities play a crucial role in the pathogenesis and progression of NASH, through multiple mechanisms, including increased intestinal permeability, qualitative and quantitative changes of intestinal bacteria, and impaired intestinal metabolite production (Kessoku, et al., 2021; de Vos, et al., 2022; Marra, et al., 2018). The increase in anaerobic Gram-negative bacterial strains is, in fact, accompanied by a raise in blood levels of lipopolysaccharide (LPS or endotoxin), a component of the outer wall membrane of these microorganisms (Pendyala, et al., 2012; Caricilli, et al., 2013). Endotoxins derived from intestinal Gram-negative bacteria overgrowth translocate through a dysfunctional gut barrier to the portal circulation and reaching the liver promotes hepatic inflammation by the engagement of the toll-like receptor 4 (TLR-4) expressed on inflammatory cells. In line with this, TLR-4 and myeloid-differentiation factor-2 (MD2) deficient mice are protected from diet-induced NASH (Csak, et al., 2011). Besides endotoxin, plasma from mice and patients suffering from NASH contain high levels of mitochondrial DNA, a potent TLR9 activator, thus suggesting that other microbial products might also cause disease progression (Bäckhed, et al., 2004).

Several bacterial metabolites have been regarded as possible contributors to NASH development and progression, among them ethanol is of special interest (Brandl and Schnabl, 2017) since NASH patients exhibit an increased prevalence of ethanol-producing bacteria like Enterobacteriaceae, and Escherichia associated with elevated blood ethanol levels (Zhu, et al., 2013; Engstler, et al., 2016). Gut dysbiosis affects the production of intestinal metabolites displaying essential functions such as bile acids (BAs). BAs are classified into primary BAs, which are synthesized directly from cholesterol within the liver and secondary BAs, which are dehydroxylated by intestinal bacteria once the primary BAs are released into the intestinal lumen (Heuman, 1989). BAs exert a central role in lipid solubilization and digestion, but they have also been implicated in glucose and lipid metabolism along with inflammation (Arab, et al., 2017; Gonzalez, et al., 2016). Because of gut dysbiosis, NASH patients, especially those with severe fibrosis, show significant changes in their bile acid profiles, thus suggesting that an abnormal bile acid metabolism may have possible implications in hepatic fibrogenesis (Kasai, et al., 2022). Aside from secondary BAs, gut microbiota also produces short-chain fatty acids (SFCAs) such as acetate, propionate, and butyrate through the anaerobic fermentation of non-digestible proteins and fibers (Wolever, et al., 1989; McNeil, 1984). SFCAs are mostly produced in the colon and through the portal circulation reach the liver, acting as precursors for gluconeogenesis and lipogenesis (Gao, et al., 2009; den Besten, et al., 2015). Beyond their role as energy substrates, SCFAs can regulate hepatic metabolism by functioning as signaling molecules (Rekha, et al., 2022). In this regard, propionate and butyrate promote lipid metabolism mediating triglyceride hydrolysis, fatty acid release, and fueling β-oxidation (Ge, et al., 2008; Zhao, 2020; Iannucci, et al., 2016; den Besten, et al., 2013; Waldecker, et al., 2008). Recently, it has been demonstrated that SCFAs also mediate anti-inflammatory responses by acting as ligands for G-protein coupled receptors (GPCRs) expressed on immune cells (Huang, et al., 2022; Juanola, et al., 2019; Tan, et al., 2017). It is noteworthy that SCFA plasma concentrations are significantly reduced in patients with NASH or NASH-related cirrhosis compared with those with simple steatosis. Furthermore, a negative correlation is evident between pro-inflammatory molecule and SCFA serum levels. In line with this, SFCA supplementation has shown beneficial effects by reducing the liver and adipose tissue inflammation in mouse models of NAFLD (Zhai, et al., 2019). Altogether these data indicates that the alterations in liver/gut axis caused by dysbiosis can influence in many ways NASH

pathogenesis and suggest that strategies devoted to targeting gut dysbiosis associated with NASH may have beneficial effects by counteracting hepatic inflammation and fibrosis development through multiple mechanisms.

vi) Mechanisms involved in the progression of NAFLD to fibrosis and NASH

A variable degree of collagen deposition within the hepatic matrix, also known as fibrosis, is a common feature in advanced NASH and represent the main risk factor for the disease evolution to liver cirrhosis (Powel, 2021). NASH is now becoming one of the most common causes of end-stage liver disease in western countries with a death rate ascribed to NASHrelated cirrhosis accounting for 12-25% (Younossi, et al., 2022). Hepatic fibrosis is the results of an impairment in the liver repair process characterized by excessive production of extracellular matrix (ECM) that is not adequately balanced by its degradation, thus resulting in net accumulation and the formation of permanent scars (Schwabe, et al., 2020). Liver ECM is a complex network of proteins including fibrillary and nonfibrillar collagen, non-collagenous glycoprotein (elastine, laminin, fibronectin), glycosaminoglycans (hyaluronan), and proteoglycans (aggrecan, fibromodulin, decorin, biglycan, glypicans, and syndecans) (Petrasek, et al., 2013). In the healthy liver, ECM turnover depends on the activity of different mesenchymal cells including hepatic stellate cells (HSCs), portal fibroblasts, and vascular smooth muscle cells. During chronic liver diseases parenchymal damage and unresolved inflammation leads to a continuous stimulation of these cells promoting pathological repair, resulting in substantial ECM. In this setting, HSCs respond to tissue damage, oxidative stress, macrophage production of repair cytokines as transforming growth factor (TGF)-β1, plateletderived growth factor (PDGF), fibroblast-derived growth factors (FGF), by trans-differentiating to collagen-producing myofibroblasts (Schwabe, et al., 2020; Friedman, 2008). In turn, hepatic myofibroblasts are the primary scar-producing cells delivering high amounts of fibrillar collagen, ECM proteins. Furthermore, fibrosis not only involves an increase in ECM but also changes in its composition with an increase in embryonic or wound-healing associated components and extended crosslinks, which render ECM more resistant to degradation, thus contributing to the slow and often incomplete reversibility of advanced fibrosis (Schwabe, et al., 2020). Furthermore, the reduction of hepatic matrix degradation is due to a decreased production of matrix metalloproteases (MMPs) and/or raised production of MMP inhibitors,

in turn, contributing to collagen accumulation (Friedman, 2008). Alongside these factors, adipokine secretion consequent to obesity might have a specific role to induce fibrogenesis. In fact, HSCs selectively express leptin receptors and leptin stimulates HSC survival. The profibrogenic activity of leptin might be also enhanced by the combined lowering of adiponectin that, besides its anti-inflammatory activity, reduces proliferation and increases apoptosis of cultured HSCs (Novo and Parola, 2008). The growing importance of NASH as the cause of liver fibrosis/cirrhosis has led in recent years to a series of studies aiming to characterize the specific mechanisms of NASH evolution to fibrosis. From this research, it emerges that HSCs contribute to approximately 80–95% of the ECM-producing myofibroblasts in NASH. These cells organize in specific structures, named fibrotic niches, in which the interaction networks with liver-resident and non-resident cells such as hepatocytes, macrophages, and recruited immune cells including lymphocytes is critical for supporting the fibrogenic process (Schwabe, et al., 2020). HCS interactions with hepatocytes involve reactive oxygen species (ROS) and DAMP released by damaged cells and more specific signals since ballooned hepatocytes produce the morphogens Sonic Hedgehog (SHH) and Indian Hedgehog which induces metabolic reprograming of HSCs promoting the transition to myofibroblasts (Schwabe, et al., 2020). Furthermore, increased cholesterol accumulation in hepatocytes stabilizes the transcriptional regulator TAZ (WWTR1), which further enhances IHH secretion (Schwabe, et al., 2020). Both TAZ and IHH are increased in human livers with NASH but not with simple steatosis (Schwabe, et al., 2020), suggesting that hepatocyte TAZ could play a specific role in promoting fibrosis progression. Interestingly, hepatocyte stimulation by TLR4 and NOTCH promotes their capacity to secrete OPN that, in its turn enhances HCS response to profibrogenic cytokines and particularly TGFB-1 (Schwabe, et al., 2020). Fibrotic niches in NASH are also characterized by the presence of $CD9^{+}$/$TREM2^{+}$ NAMs that have also been defined as scar-associated macrophages (SAM)(Ramachandran, et al., 2019). As mentioned above, these cells express several fibrosis-promoting genes, including PDGFB, TGFB-1, OPN and Gal-3 along with cytokines and chemokines capable of supporting HSC survival such as IL-1β, TNF-α, and CCL5 and are presently considered the main contributors of NASH-associated fibrogenesis (Peiseler, et al., 2022). In the case of NASH, it has been reported that global or myeloid cell deletion of the efferocytosis receptor c-Met Proto-Oncogene Tyrosine Kinase (MerTK) decreases liver fibrosis in mice fed with a NASH-promoting diet by decreasing HSC

activation (Cai, et al., 2020). Furthermore, a gene polymorphism associated with lower hepatic expression of MerTK is protective against fibrosis in patients with NAFLD. (Petta, et al., 2016) Although we have shown that repairing liver macrophage co-express both TREM2 and MerTK (Ramavath, et al., 2021), it is not clear whether MerTK are a specific sub-set of NAMs with specific pro-fibrogenic activity. The overall picture is further complicated by the fact that liver macrophages can also influence fibrosis by secreting MMP-9, MMP-12, and MMP-13, antiinflammatory mediators (IL-10), and growth factors. The regulation of pro-inflammatory and restorative polarization in liver macrophages is closely associated with the progression of NASH. For instance, the infiltration of Ly-6C^{+} monocytes has been identified as a crucial factor in the progression toward NASH and fibrosis in mice (Baeck, et al., 2012; Lefebvre, et al., 2016), while other chemokine receptors, namely C-X-C motif receptor (CXCR) such as CXCR2 and CXCR3 lead instead to reduced recruitment of infiltration of macrophages, improved hepatic inflammation and fibrosis (Ye, et al., 2017; Zhang, et al., 2016).

An emerging aspect in the understanding the mechanisms responsible for the evolution of NASH to fibrosis concerns the role of immune cells. Recent data indicates that interfering with B and T-lymphocytes as well as NKT cells not only improves steatohepatitis but also prevent fibrosis in experimental NASH (Peiseler, et al., 2022). This latter action can be partially ascribed to the interference with lobular inflammation, but also involve the direct action of lymphocytes on HSCs. In fact, it is now evident that B-cells express pro-fibrogenic genes such as TGF-β1 and tissue inhibitor of metalloprotease 2 (Timp-2) and can as well directly interact with HSCs through the production of the chemokine CXCL4 (Dudek, et al., 2021; Bhattacharjee, et al., 2017; Novobrantseva, et al., 2005). In a similar manner, CD8^{+} T-cells exhibited the ability to directly activate hepatic stellate cells either in vivo or in vitro in mice with NASH (Breuer, et al., 2020). However, the phenotype of these lymphocytes has not been characterized and this strongly limits the full appreciation of CD8^{+} T-cell in liver fibrosis, since in two dietary mouse models of NASH, single-cell transcriptomics has revealed the expansion of a subset of CD69^{+}/CD103^{-}/CD8^{+} tissue-resident memory T-cells during NASH resolution. These cells can kill HSCs through FasL-Fas-mediated apoptosis and their depletion retard collagen degradation (Koda, et al., 2021).

1.3 Development of new therapeutic approaches to NASH

Given the high prevalence and rising incidence of NAFLD/NASH worldwide, finding relevant therapeutic strategies is now more urgent than ever before. At present, weight loss obtained by diet and increased physical activity is the most effective interventions in NAFLD and significantly reduce liver steatosis, although the effects on hepatic inflammation and fibrosis are controversial (Romero-Gómez, et al., 2017; Semmler, et al., 2021). Furthermore, lifestyle changes inducing effective weight loss are hard to maintain, and patient compliance is rather low. The need for pharmacological agents has led in the recent years to development of new classes of drugs, as well as the potential repurposing of currently available agents. Nonetheless, the 2016 clinical practice guidelines suggest that the use of drugs in NAFLD/NASH should be considered for patients with fibrosis stage 2 or higher and with earlystage fibrosis with a high risk of fibrosis progression (older age, diabetes, metabolic syndrome, increased ALT, and high necro-inflammatory activity) (Sumida and Yoneda 2018). In these settings, the endpoints for NASH clinical trials defined by US Food and Drug Administration (FDA) focus on obtaining an improvement of histological features of NASH without worsening of fibrosis or with improvement of at least one fibrosis stage without worsening of steatohepatitis (Dufour, et al., 2022).

Based on the current understanding of NAFLD/NASH pathogenesis the prevalent strategy for pharmacological therapies of NAFLD/NASH has focused on targeting the factors leading to hepatic steatosis and on improving the resultant metabolic stress. Medications in this group include: a) compounds such as pioglitazone, elafibranor, and saroglitazar) acting as agonists of the peroxisome proliferator-activator receptors (PPARs), a family of nuclear receptors including PPARα, β/δ and γ that bind a wide range of fatty acids and fatty acid derivatives and transcriptionally regulate lipid and glucose metabolism; b) agonists of acid-farnesoid X receptor axis (i.e. obeticholic acid, GS-9674) which decrease hepatic lipogenesis and improve peripheral insulin sensitivity; c) thyroid hormone receptor beta (THR-β) agonist (i.e. resmetirom) that improves lipid metabolism; d) inhibitors of de novo lipogenesis (i.e. aramchol, NDI-010976); e) compounds acting on Glucagon-like peptide 1 (GLP-1) signalling (liraglutide) (Rotman and Sanyal, 2017). GLP-1 is a peptide hormone derived from the

proglucagon secreted by intestinal L-cells that acts by stimulating insulin secretion from pancreatic β-cells and by improving peripheral insulin sensitivity (Rotman and Sanyal, 2017). The data so far available indicates that several of the compounds above are effective in controlling steatosis, while phase III clinical trials already concluded does not evidence appreciable effects on hepatic fibrosis (Wiering & Tacke 2022). Nonetheless, more trials are on the way with other compounds with encouraging data (Wiering & Tacke 2022). Another approach consists in targeting oxidative stress with antioxidants such as vitamin E, cell death with the caspase inhibitor such as emricasan or hepatic inflammation (Rotman and Sanyal 2017). On this latter aspect, animal experiments and clinical trials evaluating the effectiveness of antagonists of TNF-α pathway (pentoxifylline) and of blockers of the CCL2/CCL5 receptors (cenicriviroc) have shown improvements in experimental and clinical NASH (Rotman and Sanyal 2017), without a significant reduction in fibrosis (Wiering & Tacke 2022). Furthermore, side effects after prolonged use and/or unknown long-term outcomes has so far limited the employ of compounds interfering with inflammatory mechanisms in NASH patients (Rotman and Sanyal 2017; Sumida and Yoneda, 2018). Therefore, in view of the growing knowledge on the mechanisms involved in the pathogenesis of steatohepatitis, there is the need to explore new approaches for targeting hepatic inflammation in NASH (Wiering & Tacke 2022).

2. Aim of the Project

In recent years, there has been important progress in understanding the complexity of the pathogenetic mechanisms responsible for the evolution of nonalcoholic steatohepatitis (NASH)(Wallace, et al., 2022). These data have led to the development of a variety of drugs that are ongoing testing in clinical trials (Dufours et al. 2022). Nonetheless, in spite that chronic inflammation is seen as the driving force in promoting NASH evolution, therapies targeting liver inflammation and fibrosis development in NASH are still very limited.

From recent studies characterizing the role of cellular interactions involved in modulating hepatic inflammation in my doctoral project, I have investigated some novel therapeutic approaches capable of influencing inflammatory mechanisms in NASH as well as interfering with the disease evolution to fibrosis. In detail, my work focalises on:

a) The capacity of the anti-inflammatory mediator Annexin A1 of influencing the profibrogenic action of macrophages.

b) The anti-inflammatory effects of cholesterol-free ketogenic diets

These issues were addressed using well-established experimental models reproducing the features of human NASH obtained by feeding mice with methionine/choline deficient (MCD) diet or a high fat/carbohydrate diet which allows to reproduce the evolution of chronic liver inflammation to hepatic fibrosis (Lau et al.2017).

3. Materials and Methods

NASH experimental mice models

Wild-type (WT) C57BL/6 male mice at 4th week of age have been bought by Envigo (Bresso, Italy) and employed for the experiments in pathogen-free conditions after four weeks of acclimatizing to their surroundings. Steatohepatitis was induced by feeding eight-week-old male WT mice with either a methionine/choline deficient (MCD) diet for 2 or 8 weeks or a high fat/carbohydrate diet enriched with 1.25% cholesterol Western Diet (WD) for 10-16 weeks. Control animals received either a diet supplemented by choline/methionine or standard chow diet. Mice were treated for 5 days a week by daily intraperitoneal injection of human recombinant AnxA1 (hrAnxA1; 1 μg/daily in saline) according to a previously published protocol that allowed effective pharmacokinetic and avoided production of neutralizing antibodies. Control animals received an injection of saline alone. In some experiments, mice were fed either with a western diet (WD) enriched with 1.25% cholesterol for 16 weeks followed by switching to a cholesterol-free ketogenic diet (KD) for additional 8 weeks. The control littermates were fed with either WD, 1.25% cholesterol or a normal diet (ND) for 16 and 24 weeks. For preliminary experiments mice were fed with the cholesterol-free KD for up to 8 weeks. All the diets used in the experiments have been purchased from Laboratorio Dottori Piccioni (Gessate, Italy).

At the end of the treatments, mice were anaesthetized with sevoflurane, and after checking the anesthesia depth, the blood was collected by retro-orbital bleeding. Afterwards, the mice were euthanized by cervical dislocation. Animal experiments were performed at the animal facility of the Dept of Health Sciences, University of East Piedmont (Novara, Italy) and comply with EU ethical guidelines for animal experimentation. The study protocols received ethical approval by the Italian Ministry of Health (authorizations No. 449/2019-PR and 411/2020-PR) according to the European law requirements.

AnxA1 Recombinant Protein Purification

cDNA of hrAnxA1 carrying a cleavable N-terminal poly-His tag was expressed in Escherichia coli and purified as previously reported (Karlmark et al; 2010). The purity of recombinant AnxA1,

was assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and matrix-assisted laser desorption/ionization dual time-of-flight mass spectrometry and was >95%.

Assessment of liver injury

Livers were rapidly removed, rinsed in ice-cold saline, and cut into five pieces. Aliquots were immediately frozen in liquid nitrogen and kept at −80°C until analysis. The left lobe from each liver was fixed in 10% formalin for 24h and embedded in paraffin. 4 µm thick liver sections were stained with hematoxylin/eosin using a Roche Ventana HE 600 automatic-staining system (Roche Diagnostics International AG, Rotkreuz, Switzerland), while collagen deposition was detected by Picro-Sirius Red staining. Sections were scored blindly for steatosis, lobular inflammation, and the extension of fibrosis by an expert pathologist. Microphotographs were taken using a Nikon Eclips CI microscope fitted with a DSR12 camera (Nikon Europe BV, Amsterdam, Netherlands) using the NIS-Elements F4.60.00 acquisition software. The serum was obtained by centrifugation from whole blood taken from each mouse in the absence of anti-coagulating agents to assay the circulating level of alanine aminotransferase (ALT). The serum concentration of ALT was quantified according to the manufacturer's protocol by using a spectrophotometric assay (Gesan Production S.r.l., Campobello di Mazara, Italy). Total triglyceride liver content was measured by a quantitative enzymatic determination through a commercially available kit (Triglyceride Determination Kit, Sigma-Aldrich, Milano, Italy).

Immunohistochemistry and immunofluorescence

To detect liver macrophages, formalin-fixed paraffin-embedded (FFPE) tissue sections were stained using rabbit polyclonal antibody against F4-80 (Abcam, Cambridge, UK) and goat polyclonal antibodies against galectin-3 (R&D Systems, Minneapolis, USA) and osteopontin (Abcam, Cambridge, UK) in combination with a horseradish peroxidase polymer kit (Biocare Medical, Concord, CA, USA). In some experiments, fluorescence immunostaining on FFPE livers was done using rat monoclonal F4-80 (Biolegend, USA) and rabbit monoclonal TREM2 (Abcam, Cambridge, UK) in combination with secondary antibody cocktail prepared depending on the primary antibodies host species. The image acquisition of fluorescent immunostaining has been performed with Zeiss Observer 7, then large field scanned images were stitched, and a

background subtraction was performed with the default settings using the ZEISS software ZEN 3.1 (blue edition). Single channel grayscale pictures were further processed in FIJI.

Ketone bodies measurement

Ketone bodies were measured weekly on mice urine samples with Keto-Diastix® (Bayer, Basel, Switzerland) according to the manufacturer's indications.

Glucose tolerance test

The glucose tolerance test (GTT) was performed following overnight fasting and the glucose load administration (1.5g/kg) via intraperitoneal injection. Blood sampling has been performed by tail vein incision with sterile needles and the glycemia has been measured at the basal state and 10, 30, 60, 90, and 120 minutes after the glucose load through a glucometer (URight, TD-4279, Munich, Germany).

mRNA extraction and RT-qPCR

Murine liver RNA was extracted from snap-frozen tissue by TRI reagent™ Solution and retrotranscribed with a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a Techne TC-312 thermocycler (TecneInc, Burlington NJ, U.S.A). Real-time PCR was performed in a CFX96™ Real-time PCR System (Bio-Rad, Hercules, California, U.S.A.) using TaqMan Gene Expression Master Mix and TaqMan Gene Expression probes for mouse tumor necrosis factorα (TNF-α; Mm99999068 m1), IL12 (Mm99999067_m1), CCL2 (Mm00441242 m1), CD11b (Mm00434455 m1), CD163 (Mm00474091 m1), TREM-2 (Mm04209422 m1), CD9 (Mm00514275_g1), MerTK (Mm00434920 m1), Gal-3 (Mm00802901 m1), osteopontin (OPN) (Mm01204014 m1), α1-procollagen (Mm00801666 g1), TGF-β1 (Mm00441724 m1) (ThermoFisher Scientific, Milano, Italy) and β-actin (Cat. No. 4352663, Applied Biosystems Italia, Monza, Italy). All samples were run in duplicate and the relative gene expression, calculated as $2^{-\Delta Ct}$ over that of β-actin gene, was expressed as fold increase over the relative control samples.

Flow cytometry analysis of liver leukocytes

Livers were digested by type IV collagenase from Clostridium histolyticum (Sigma-Aldrich, St. Louis, MO, USA), and intrahepatic leukocytes were isolated by multiple differential centrifugation steps. The cell preparations were stained using combinations of the following monoclonal antibodies: CD45 (Clone 30-F11, Cat. 12-0451-82), Ly6C (CloneHK1.4, Cat. 53-5932-80), Ly6G (Clone RB6-8C5, Cat. 47-5931-82,), MHCII (Clone M5/114.15.2, Cat. 56-5321-80), CD11b (Clone M1/70, Cat.56-0112-80), CD206 (Clone MR6F3, Cat. 25-2061-80), CD9 (MZ3FCRUO, Cat. 124815), C-type lectin-like receptor 2 (CLEC-2; 17D9/CLEC-2FCRUO, Cat. 146103), T-cell membrane protein 4 (TIM-4; RMT4-54FCRUO, Cat. 130019) eBioscience, (Thermo Fisher Scientific,Milano, Italy), CD11b (Clone M1/70, Cat. 101212), F4-80 (Clone BM8, Cat. 123113, Biolegend, San Diego, CA, U.S.A.), Triggering Receptor Expressed on Myeloid cells 2 (TREM-2; Clone 78.18, Cat. MA5-28223, Thermo Fisher Scientific, Milano, Italy).

Sample analyses were performed using the Attune NxT flow cytometer (Thermo Fischer Scientific, Waltham, MA, USA) and data were elaborated with FlowJo™ Software (BD Biosciences, San Jose, CA, USA).

Microbiota analysis

Mouse stool samples have been collected into sterile plastic tubes weekly for the whole experimental timeframe (T0-24) and promptly frozen and stored at -80°C before examination for microbiota analyses. The analysis has been conducted on samples collected at the beginning (T0), before (T16), and after (T17) the switching to the cholesterol-free ketogenic diet, and at the end time-point (T24). DNA extraction from 1000 mg of fecal samples was carried out following the SOP 07 guidelines and procedure developed by the International Human Microbiome Standard Consortium (www.microbiome-standards.org, accessed on 1 February 2021). RNAse treatment was then performed on the extracted DNA, quantified by using the QUBIT dsHS kit, and standardized at 5 ng/µL. The V3-V4 region of the 16S rRNA was amplified using the primers 16SF (50-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-30) and 16SR (50-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-30) (Klindworth, et al., 2013), according to the Illumina 16S Metagenomic Se-quencing Library Preparation instructions.

Amplicons were then purified, tagged, normalized, and pooled according to the Illumina protocols. DNA libraries were sequenced on the Illumina MiSeq platform, leadingto 2 × 250 bp paired-end reads. After sequencing, raw reads were imported in QIIME2 software (https://docs.qiime2.org/, accessed on 4 April 2022) for quality, with the chimera-filtering step by the qiime dada2 denoise-paired script (Callahan, et al., 2016). The amplicon-sequence variants (ASVs) obtained were then used for a taxonomic assignment against the SILVA database. QIIME2-diversity script was then used to calculate alpha and beta diversity.

Metabolomics

Extraction of short-chain fatty acids from plasma

Plasma SCFAs were extracted using a liquid–liquid extraction with methyl tert-butyl ether (MTBE). Briefly, 50 uL of plasma was placed in a tube, then 2,5 µL of the internal standard propanoic acid d2 and acetic acid d4 (20.4 ppm) were added, and the sample was vortexed for 20 s, followed by spin centrifugation for 15 s. The sample was then brought to pH 2 using 6 M HCl. 140 µL of MTBE was added, and the tube was placed on a rotator for 15 min, followed by centrifugation for 10 min at 4 °C and 21.1×g. Then, 100 µL of the organic phase containing the SCFAs was analyzed with GCxGC-TOFMS analysis.

For the analysis, a LECO Pegasus BT 4D GCxGC-TOFMS instrument (Leco Corp., St. Josef, MI, USA) equipped with a LECO dual-stage quad jet thermal modulator was used. The GC part of the instrument was an Agilent 7890 gas chromatograph (Agilent Technologies, Palo Alto, CA), equipped with a split/splitless injector. The first dimension column was a 30 m DB-FATWAXUI (Agilent Technologies, Santa Clara, CA) with a diameter of 0.25 mm and a film thickness of 0.25 µm, and the second dimension chromatographic columns was a 2 m Rxi-17Sil MS (Restek Corp., Bellefonte, PA) with a diameter of 0.25 mm and a film thickness of 0.25 µm. High-purity helium (99,9999%) was used as the carrier gas with a flow rate of 1.4 mL/min. 1 µL of sample was injected in split less mode at 250°C. The temperature program was as follows: the initial temperature was 40°C for 2 minutes, then ramped 7°C/min up to 165°C, 25°C/min up to 240°C, maintained for 5 minutes. The secondary column was maintained at +5°C relative to the GC oven temperature of the first column. Electron impact ionization was applied (70 eV). The ion

source temperature was set at 250°C, the mass range was 40 to 300 m/z with an extraction frequency of 32 kHz. The acquisition rates were 200 spectra/s. The modulation periods were 4s for the entire run. The modulator temperature offset was set at +15°C relative to the secondary oven temperature, while the transfer line was set at 280°C.

Data analysis

The chromatograms were acquired in TIC (total ion current) mode. Peaks with signal to- noise (S/N) value lower than 500.0 were rejected. ChromaTOF version 5.31 was used for raw data processing. Mass spectral assignment was performed by matching with NIST MS Search 2.3 libraries adding Fiehn Library. Commercial mix standard of free fatty acids composed by acetic acid, propanoic acid, propanoic acid 2-methyl, butanoic acid, butanoic acid 3-methyl and pentanoic acid was run individually and EI spectra were matched against the NIST library. The calibration curves of the SCFAs were obtained using Excel, while the analytical results were processed and compared with the open-source software MetaboAnalyst 5.0.

Proteomics

Extraction of proteins and digestion

50 mg of liver sample was homogenized in 500 μL of buffer (7 M urea, 2 M thiourea, 4% (w/v) dimethyl [3 propyl] azaniumyl propane- 1-sulfonate (CHAPS), 1% (v/v) immobilized pH gradient (IPG) buffer pH 3–10 NL), 40 mM dithiothreitol (DTT), and Protease Inhibitor Cocktail Complete) (Roche, Mannheim, Germany) and left at room temperature for 1 hour. Then, the sample was centrifuged at 40,000 ×g for 60 min at 4◦C, the supernatant was removed, and protein concentration was measured using the Bradford protein assay. Fifty micrograms (50 µg) of proteins were subjected to reduction with DTT, alkylation with iodoacetamide and tryptic digestion at 37 ◦C overnight. Peptides were then desalted on the Discovery® DSC-18 solid phase extraction (SPE) 96-well plate (25 mg/well) (Sigma-Aldrich Inc., St. Louis, MO, USA) and then analyzed by label-free LC–MS/MS.

Proteomic analysis

Samples were analyzed in two phases: a data-dependent acquisition (DDA) followed by a dataindependent analysis (DIA) on the same sample using the same gradient conditions. All samples were analyzed with a micro-LC Eksigent Technologies (Eksigent, Dublin, USA) system interfaced with a 5600+ TripleTOF system (AB Sciex, Concord, Canada) equipped with DuoSpray Ion Source and CDS (Calibrant Delivery System). Peptides were separated using Halo C18 column (0.5×100 mm, 2.7 µm; Eksigent Technologies Dublin, USA). The reverse phase LC solvents include solvent A (99.9% water +0.1% formic acid) and solvent B (99.9% acetonitrile +0.1% formic acid). A 30 min gradient was used at a flow rate of 15 µL/min with an increasing concentration of solvent B from 2% to 40%. For DDA acquisition, experiments were set to obtain a high-resolution TOF-MS scan over a mass range of 100–1500 m/z, followed by an MS/MS product ion scan from 200 to 1250 Da (accumulation time of 5.0 ms) with the abundance threshold set at 30 cps (35 candidate ions can be monitored during every cycle). The ion source parameters in electrospray positive mode were set as follows: curtain gas (N2) at 25 psig, nebulizer gas GAS1 at 25 psig, and GAS2 at 20 psig, ion spray voltage floating (ISVF) at 5000 V, source temperature at 450 °C and declustering potential at 25 V. Using the same conditions as described above, a SWATH acquisition using DIA was carried out for the labelfree quantification process. The mass spectrometer was operated so that a 50-ms survey scan (TOF-MS) was performed and subsequent MS/MS experiments were performed on all precursors. These MS/MS experiments were carried out in a cyclical manner using an accumulation time of 40 ms per 25-Da swath (36 swaths in total) for a total cycle time of 1.5408 s. The ions were fragmented for each MS/MS experiment in the collision cell using the rolling collision energy. The MS data were acquired with Analyst TF 1.7 (AB SCIEX, Concord, Canada). Peptides (and proteins) were identified using DDA followed by database search, while the quantification was obtained by integrating the area under the chromatographic peak for each ion fragment of identified peptides by using the DIA file.

Protein Database Search

The DDA files were searched against the mouse UniProt Swiss-Prot reviewed database containing mouse proteins (version 20july2020, containing 23354 sequence entries) using Protein Pilot software v. 4.2 (SCIEX, Concord, Canada) and Mascot v. 2.4 (Matrix Science Inc.,

Boston, USA). Samples were input in the Protein Pilot software with the following parameters: cysteine alkylation, digestion by trypsin, no special factors and False Discovery Rate (FDR) at 1%. For Mascot search, we selected Trypsin as digestion enzyme with 2 missed cleavages, set the instrument to ESI-QUAD-TOF and specified the following modifications for the assay: carbamidomethyl cysteine as fixed modification and oxidized methionine as variable modification. An assay tolerance of 50 ppm was specified for peptide mass tolerance, and 0.1 Da for MS/MS tolerance. The charges of the peptides to search for were set to 2 +, 3 +, and 4 +, and the search was set to monoisotopic. A target-decoy database search was performed, and FDR was fixed at 1%. SwathXtend was employed to build an integrated assay library with the DDA acquisitions to use as the ion library file for all SWATH analysis and quantification.

Protein Quantification

Quantification was performed by integrating the extracted ion chromatogram of all the unique ions for a given peptide. Spectral alignment of the SWATH samples (DIA run) was carried out with PeakView 2.2 (ABSCIEX, Concord, Canada) using the spectral library generated above and the following parameters: 6 peptides per protein, 6 transitions per peptide, XIC extraction window of 5 min and a width of 15 ppm. Shared peptides were excluded as well as peptides with modifications. Peptides with FDR lower than 1.0% were exported in MarkerView 1.2 (ABSCIEX, Concord, Canada) for the t-test.

Data analysis and statistical calculations

Statistical analyses were performed by SPSS statistical software (SPSS Inc. Chicago IL, USA) using a one-way ANOVA test with Tukey's correction for multiple comparisons or the KruskalWallis's test for non-parametric values. Significance was taken at the 5% level. Normality distribution was assessed by the Kolmogorov-Smirnov algorithm.

Statistical analysis of proteomic data was performed using MarkerView software (Sciex, Concord, Canada) and MetaboAnalyst software (www.metaboanalyst.org). Proteins were considered up- and downregulated using fold change >1.3 or <0.769 and p-value < 0.05. The significance of the difference was also analyzed by non-parametric tests using the Prism v.8 software package (GraphPad Software, San Diego, CA, USA), with statistical significance taken

at $p < 0.05$. Bioinformatics analysis of proteomic data was performed using Ingenuity Pathways Analysis (IPA) software (Qiagen, Redwood City, CA, USA).

4. Results Section 1 Annexin A1 treatment prevents the evolution to fibrosis of experimental nonalcoholic steatohepatitis (NASH)

Data published in Clinical Sciences Clin Sci (Lond). 2022;136:643-656. doi: 10.1042/CS20211122.

4.1 Foreword

As previously discussed, targeting hepatic inflammation is still an unmet important objective for the development of new treatments of NASH. Animal experiments and clinical trials have shown that interfering with pro-inflammatory cytokines/chemokines, leukocyte adhesion molecules or gut dysbiosis is effective in ameliorating lobular inflammation and NASH progression to fibrosis (Rotman, et al., 2017; Reimer, et al., 2020). A different approach to control inflammation might rely on the use of physiological modulators that orchestrate the resolution of inflammatory processes and promote tissue healing (Serhan, et al., 2017). Among these pro-resolving factors, Annexin A1 (AnxA1), also known as lipocortin-1, represents a possible candidate.

AnxA1 is a 37-kDa calcium-phospholipid–binding protein highly expressed in myeloid cells and regulated by glucocorticoids (Sugimoto, et al., 2016). AnxA1 interaction with its receptor, formyl peptide receptor 2/lipoxin A4 receptor (FPR2/ALX), down-regulates the production of proinflammatory mediators, such as eicosanoids, nitric oxide and interleukin-6 (IL-6), reduces neutrophil migration to inflammatory sites, and promotes clearance of apoptotic granulocytes (Suigimoto, et al., 2016; Sheikh and Solito, 2018). Furthermore, recent studies showed that endogenous AnxA1 favor epithelial repair and muscle regeneration (Sheikh and Solito, 2018; McArthur, et al., 2020). We have observed that AnxA1 is selectively upregulated in macrophages from mouse and human NASH livers (Locatelli, et al., 2014). Moreover, in NASH patients hepatic AnxA1 transcripts show an inverse correlation with disease progression to fibrosis/cirrhosis (Locatelli, et al., 2014). Furthermore, AnxA1 deficiency enhances insulin resistance and metabolic impairment in mice receiving an obesogenic diet (Purvis, et al., 2019), while it worsens lobular inflammation and hepatic fibrosis in experimental NASH (Locatelli, et al., 2014). These latter effects associate with enhanced macrophage recruitment as well as their pro-inflammatory M1 phenotype and activity (Locatelli I et al. 2014). Consistently, in vitro addition of recombinant AnxA1 to macrophages isolated from NASH

livers reduces M1 polarization by stimulating the production of interleukin-10 (Locatelli, et al., 2014). In the same vein, AnxA1 supplementation improves insulin resistance and type 2 diabetes complications in mice fed with a high fat diet (Purvis, et al., 2019).

Based on these studies and the recent observations that AnxA1 and AnxA1 mimetic peptides are effective in improving inflammation in animal models of diabetic kidney damage and atherosclerosis (Wu, et al., 2021; Kusters, et al., 2015), the present study investigated the possible application of AnxA1 in controlling steatohepatitis in mice with experimental NASH.

4.2 Experimental data

a) Human recombinant Annexin A1 prevents steatohepatitis and fibrosis in mice.

The effects of human recombinant AnxA1 (hrAnxA1) on modifying the severity of NASH were preliminary evaluated in a set of experiments in which steatohepatitis was induced in C57BL/6 mice by feeding a methionine/choline deficient diet (MCD). For these experiments, animals received the MCD diet for 4 weeks to allow the development of extensive steatohepatitis; then, they were injected with hrAnxA1 or saline for further 4 weeks, while maintaining the same diet. At the end of the treatment, liver histology and biochemical analysis showed that administration of hrAnxA1 significantly reduced the severity of liver injury (Fig. 1A) as measured by alanine aminotransferase (ALT) release (Fig. 1B). No changes were evident in the scores for hepatic steatosis (2.2±0.9 *vs.* 1.6±0.6 arbitrary units; n=13; p=0.22) and in liver triglyceride content (Fig. 1C). The same animals also showed a significant lowering in the histological scores for lobular inflammation (2.2±0.8 *vs.* 0.8±0.8 arbitrary units; n=13; $p<0.05$) as well as in the liver expression of pro-inflammatory markers like Tumor Necrosis Factor-α (TNF-α), CCL2, IL-12p40 and the leukocyte marker integrin alpha M (ITGAM; CD11b) (Fig. 1DG). Steatohepatitis in mice receiving the MCD diet for 8 weeks increased liver transcripts for procollagen-1 α and transforming growth factor 1β (TGF-1β) (Fig. 2 A-C) and the onset of liver fibrosis, as evidenced by intra-hepatic collagen staining with Sirius Red (Fig. 2D). Interestingly, mice treated with hrAnxA1 showed significant decrease in the transcripts for procollagen-1α and TGF-1β (Fig.2A-C), along with a lower Sirius Red staining, than those injected with saline alone (Fig. 2D).

Although steatohepatitis induced by the MCD diet reproduces the inflammatory features of human NASH, this experimental model lacks metabolic derangements associated with obesity and insulin resistance that are common features of the human disease (Santhekadur, et al., 2018). Furthermore, the development of fibrosis is usually modest in MCD-fed mice (Santhekadur, et al., 2018). Thus, to better characterize the action of hrAnxA1 on the evolution from NASH to fibrosis, we switched to a nutritional model based on mice feeding with high fat/carbohydrate diet enriched with 1.25% cholesterol known as Western Diet (WD) (Santhekadur, et al., 2018). To this aim, mice were fed with WD for 10 weeks to induce steatohepatitis before being randomized to receive hrAnxA1 supplementation. Preliminary experiments confirmed that 10 weeks feeding of mice with WD significantly increased body weight as compared to chow-fed controls and this associated with an increase in liver weight due to intrahepatic fat accumulation (Fig. 3A). The presence of steatohepatitis was confirmed by histology (Fig. 3b) as well as elevation in the circulating levels of ALT (Fig. 3C) and in the liver transcripts for inflammatory markers (Fig. 3D,E). Although procollagen-1α mRNA was increased in the livers of WD-fed mice (Fig. 3F), histology did not detect changes in collagen deposition (Fig. 3G), suggesting that at this time point, steatohepatitis has not led to marked fibrosis. Thus, 10-week WD-fed animals were a suitable experimental model to investigate whether treatment with hrANXA1 might interfere with NASH evolution to fibrosis.

Six weeks of treatment with hrANXA1 (1 μg/g) of mice receiving WD did not appreciably modify body (33±1.8 g *vs.* 32.5±2.6 g; n=12; p=0.7) and liver weights (2.3±0.32 g *vs.* 2.0±0.32 g; n=12; p=0.12). As expected, 16-week WD feeding promoted the development of insulin resistance, as monitored through the glucose tolerance test (Fig. 4A). Nonetheless, the area under the curve (AUC) did not evidence an appreciable improvement of insulin response following administration of hrANXA1 (Fig. 4B). As observed in the animals receiving the MCD diet, hrANXA1 treatment of mice fed with WD improved liver histology (lobular inflammation score: 1.8±0.4 *vs.* 0.8±0.7 arbitrary units; n=12; $p<0.05$), transaminase release and expression of inflammatory markers (Fig. 5), without affecting the extension of steatosis (2.0±0.9 *vs.* 2.3±0.8 arbitrary units; n=12; p=0.58). According to previous studies (Santhekadur, et al., 2018), WD administration led to diffuse hepatic fibrosis (Fig. 2D-F). In these animals, RT-PCR and Sirius Red collagen staining confirmed that hrANXA1 was effective in preventing the upregulation in procollagen-1α and TGF-1β expression and almost abrogated intra-hepatic collagen

deposition (Fig. 2D-F) as confirmed by morphometric evaluation of collagen Sirius Red staining areas (3.20±0.93% *vs.* 0.35±0.35% n=23 fields; p<0.001).

b) Annexin A1 modulates liver macrophage phenotype

Several reports have pointed on the capacity of ANXA1 to modulate macrophage functions by suppressing pro-inflammatory activities and stimulating pro-resolving functions (Sheikh and Solito, 2018; McArthur, et al., 2020). In our hands, flow cytometry analysis of F4-80$^+$/CD11b$^+$ macrophages infiltrating the liver showed that treatment with hrANXA1 did not interfere with their recruitment to the liver (Fig. 6A). Similarly, the fraction of pro-inflammatory macrophages expressing the lymphocyte antigen 6 (Ly6C), also known as tissue plasminogen activator receptor (Wen, et al., 2021), was not affected (Fig. 6A). On the other hand, hrANXA1 reduced by ~20% the prevalence of cell expressing the mannose-binding protein receptor (MRC1; CD206), a marker of reparative macrophages (Fig. 6A). Macrophages in human and rodent NASH livers are often characterized by the formation of aggregated containing enlarged cells with a foamy appearance due to the accumulation of cytoplasmic lipid droplets and cholesterol crystals reminiscent of crown-like structures detectable in the adipose tissue of obese subjects (Itoh, et al., 2013). Immunohistochemistry for the macrophage marker F480 confirmed the presence of crown-like aggregates in the liver sections from mice fed WD for 16 weeks (Fig. 7A). F4-80 immunostaining also showed that macrophages aggregates were greatly reduced by hrANXA1 treatment (Fig. 7A).

In recent studies, single-cell RNA sequencing has revealed that macrophages expanding in either human and rodent NASH, have a specific phenotype, characterized by the expression of the Triggering Receptor Expressed on Myeloid cells 2 (TREM-2), CD63 and the glycoproteins CD9 and NMB (GPNMB). These cells, also called NASH-associated macrophages (NAM), are the main components of crown-like macrophage aggregates (Remmerie et al., 2020) and their prevalence correlates with the severity of NASH [19], likely in relation to their capability of producing pro-fibrogenic mediators such as osteopontin (OPN) and galectin-3 (Gal-3) (Xiong, et al., 2019; Seidman, et al., 2020; Remmerie et al., 2020; Daemen, et al., 2021). Since the worsening of NASH-associated fibrosis in AnxA1-deficient mice was characterized by an enhanced production of Gal-3 by macrophages in crow-like structures (Locatelli, et al., 2014),

in subsequent experiments we investigated the possibility that the protection against fibrosis observed in hrANXA1-treated mice might be related to action on NAMs. Analysis of TREM-2, OPN and Gal-3 transcripts confirmed a strong up-regulation of these NAM markers in the livers of mice receiving WD that were effectively prevented by the administration of hrANXA1 (Fig. 6 B-D). Moreover, hrAnxA1 promoted the recovery in the expression of hemoglobinhaptoglobin scavenger receptor CD163 (Fig. 6E), a marker of differentiated Kupffer cells (Seidman, et al., 2020). Immunohistochemistry for Gal-3 and OPN confirmed increased staining for both mediators in NASH livers and showed that Gal-3 was selectively produced by macrophages within crown-like aggregates (Fig. 7B), while OPN production was evident in both macrophages and ductular epithelial cells (Fig. 7C). Again, the prevalence of both Gal-3 and OPN expressing cells was greatly reduced by the treatment with hrANXA1 (Fig. 7 B,C).

c) Annexin A1 modulates the differentiation of NASH-associated macrophages.

From these results, and previous observations showing that macrophages in crown-like aggregates in both rodent and human NASH produce AnxA1 (Locatelli, et al., 2014; Jindal, et al., 2015), we postulated that AnxA1 might act in a paracrine manner in modulating the phenotype of hepatic macrophages. To verify such a possibility, we evaluated the effects of hrANXA1 on the differentiation of TREM-2^+/$CD9^+$ NAMs in mice receiving the MCD diet for 2 weeks. Preliminary experiments have shown that the early stages of steatohepatitis in these animals are accompanied by an expansion of F4-80^+/$CD11b^+$ macrophages which included about 40% of cells that were Ly6C^-/TREM-2^+/$CD9^+$/$CD206^{high}$ (Fig. 8). In further experiments, during the second week on the MCD diet, mice were injected with hrANXA1 or saline for 5 days: herein, we observed that administration of hrANXA1 to mice did not affect the liver macrophages pool ($3.9\pm0.7x10^5$ vs $3.8\pm1.3x10^5$ cells/g tissue; p=0.86), but significantly lowered the fraction of TREM-2^+/$CD206^+$ cells (Fig. 9). This effect was accompanied by an increase in the prevalence of F4-80^+/$CD11b^+$ cells expressing the Kupffer cell markers C-type lectin-like type 2 receptor (CLEC-2) and the phosphatidylserine receptor T-cell membrane protein 4 (TIM-4) ($0.45\pm0.05x10^5$ vs $0.55\pm0.06x10^5$ cells/g tissue; p>0.05) and a parallel decrease of CLEC-2^-/TIM-4^- pool ($0.30\pm0.03x10^5$ vs $0.19\pm0.03x10^5$ cells/g tissue; p>0.05) (Fig.

9). No changes were instead observed in the fraction of CLEC-2^{+}/TIM-4^{-} ($1.3\pm0.4x10^{5}$ vs $2.7\pm1.1x10^{5}$ cells/g tissue; p=0.42), supporting the possibility that hrANXA1 prevents NASH evolution to fibrosis by interfering with NAM phenotype in macrophages.

4.3 Figures Section 1

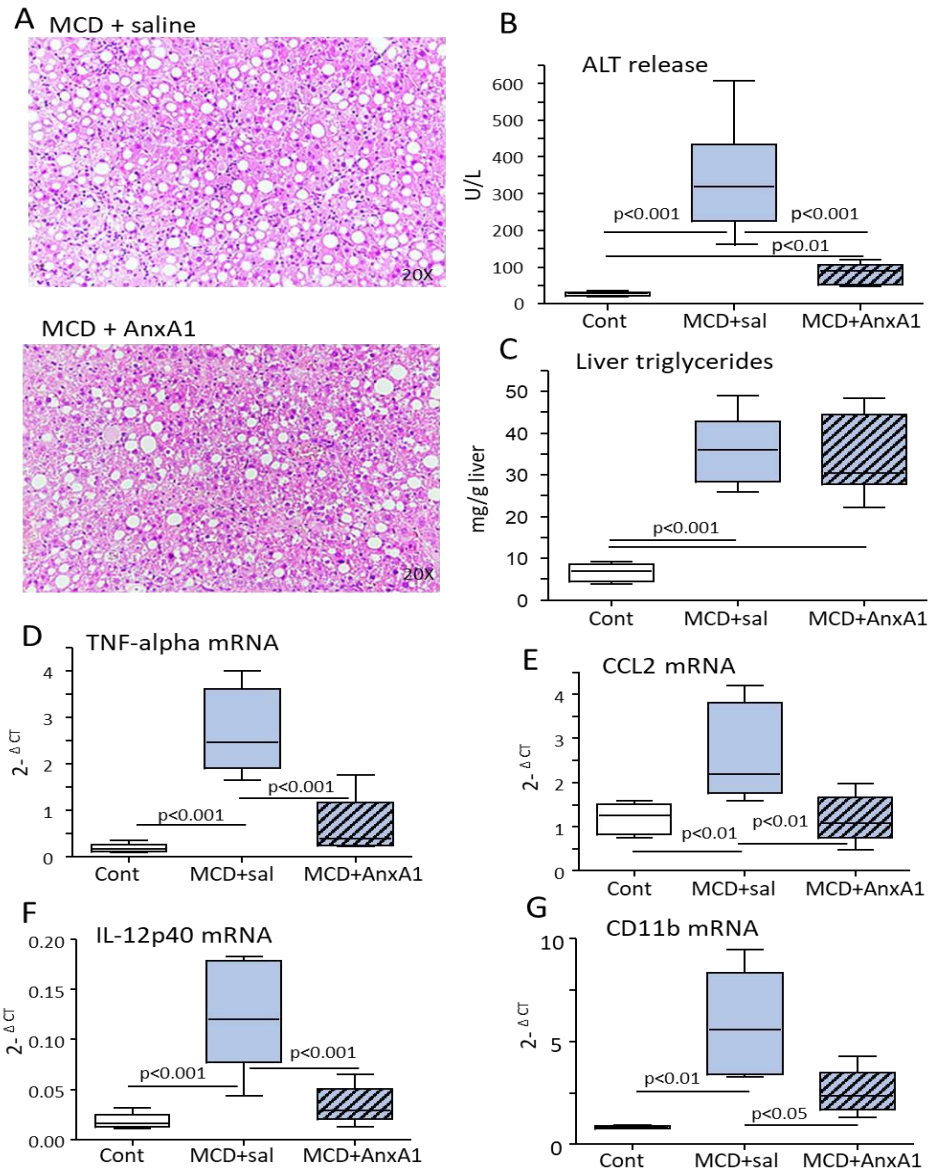

Figure 1: Annexin A1 (AnxA1) supplementation improves NASH-associated hepatic injury and inflammation in mice fed with a methionine/choline deficient (MCD) diet.

NASH was induced in wild type C57BL/6 mice by feeding MCD diet for 4 weeks. The animals were then randomly divided in two groups one receiving AnxA1 (1 µg/daily in saline 5 time a week; MCD+AnxA1) and the other the same volume of saline (MCD+sal) and the administration of the MCD diet was continued for further 4 weeks. (Panel A) Hematoxylin/eosin staining of liver sections (magnification 20x). (Panels B,C) Alanine aminotransferase (ALT) release and hepatic triglyceride content. (Panels D-G) Hepatic transcripts of inflammatory markers TNF-α, CCL2, IL12p40 and CD11b as evaluated by RT-PCR. The values refer to 6-8 animals per group and the boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th-90th percentile) comprise 80% percent of the values.

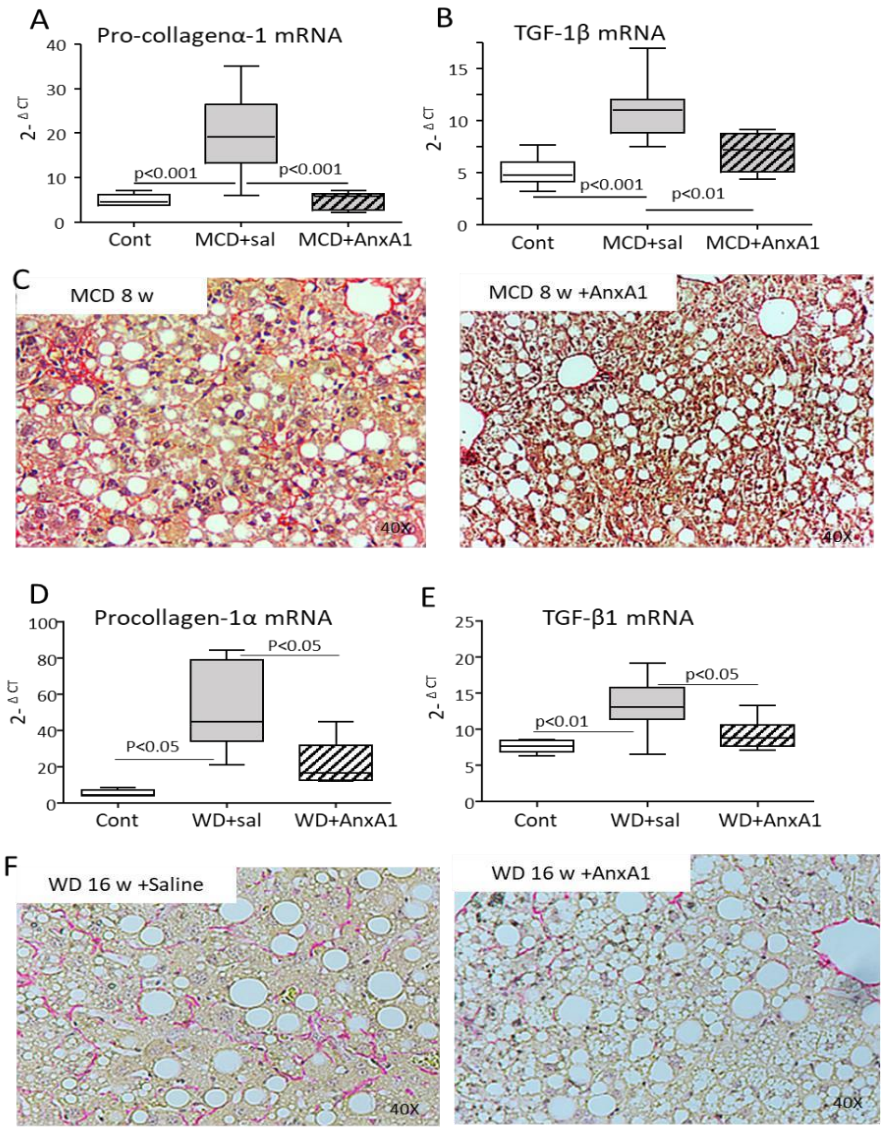

Figure 2: Annexin A1 (AnxA1) supplementation improves hepatic fibrosis in mice with NASH induced by feeding either a methionine/choline deficient (MCD) diet or WD diet.

NASH was induced in wild type C57BL/6 mice by feeding MCD diet for 4 weeks or WD diet for 10 weeks. (Panels A-C) The animals were randomly divided in two groups one receiving AnxA1 (1 µg/daily in saline 5 time a week; MCD+AnxA1) and the other the same volume of saline (MCD+sal) and the administration of the MCD diet was continued for further 4 weeks. Panels (D-F) The animals were divided to receive AnxA1 (1 µg/daily in saline 5 time a week; WD+AnxA1) or saline (WD+sal) and the administration of the WD diet was continued for further 6 weeks. The hepatic transcripts for α1-procollagen and TGF-β1 as evaluated by RT-PCR. Collagen deposition was evidenced by staining with Sirius Red (magnification 40x). The values refer to 6-8 animals per group and the boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th-90th percentile) comprise 80% percent of the values.

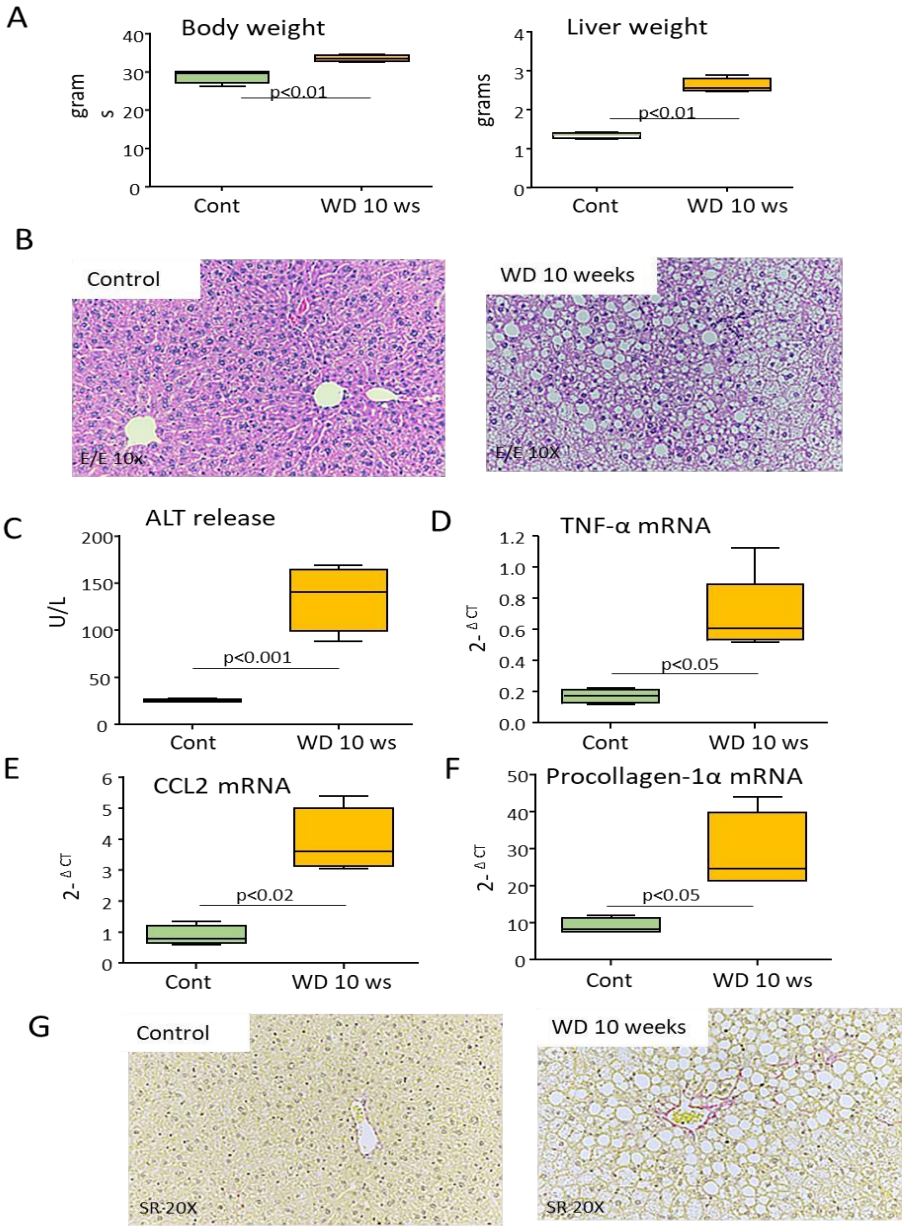

Figure 3: **The development of steatohepatitis in mice fed 10 weeks with Western diet (WT).**

Wild type C57BL/6 mice receiving WD diet or control chow for 10 weeks were evaluated for the development of NASH. (Panel A) liver and body weights. (Panel B) Hematoxylin/eosin (E/E) staining of liver sections (magnification 10x). (Panels C) Alanine aminotransferase (ALT) release. (Panels D-F) The hepatic mRNA levels of inflammatory markers TNF-α and CD11b and procollagen-1 α as evaluated by RT-PCR. (Panel G) Sirius Red (SR) staining for collagen of liver sections (magnification 20x). The values refer to 4-5 animals per group and the boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th-90th percentile) comprise 80% percent of the values.

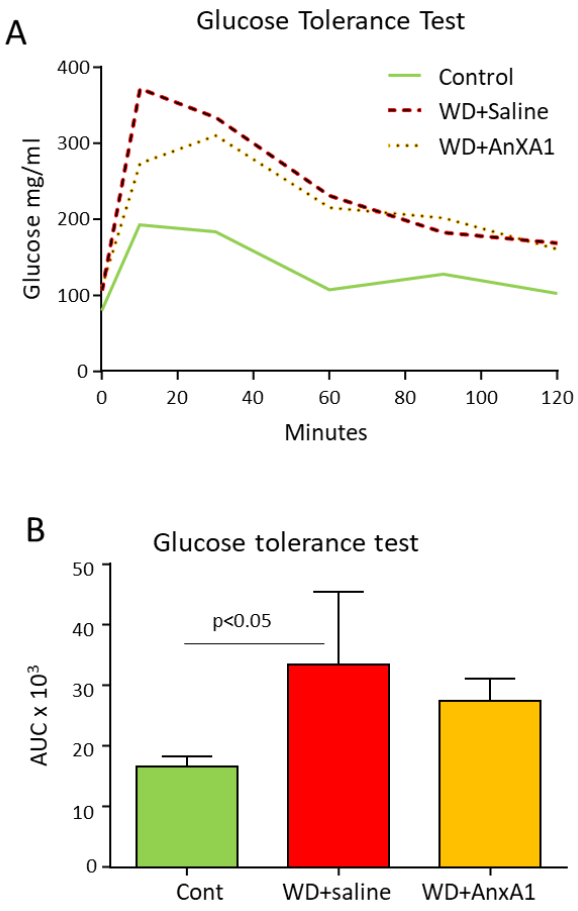

Figure 4: **Annexin A1 (AnxA1) treatment does not improve insulin response in mice receiving Western diet (WT) for 16 weeks.**

NASH was induced in wild type C57BL/6 mice by feeding WD diet for 10 weeks. The animals were then randomly divided in two groups one receiving AnxA1 (1 µg/daily in saline 5 time a week)(WD+AnxA1) and the other the same volume of saline (WD+sal) and the diet was continued for further 6 weeks. (Panel A) Insulin response was evaluated by glucose tolerance test after the intraperitoneal injection of D-glucose (1,5 g/kg body weight). (Panel B) Graphic representation of the area under the curve (AUC) calculated for the glycemic curve. The data refer to 4-5 mice for each experimental group.

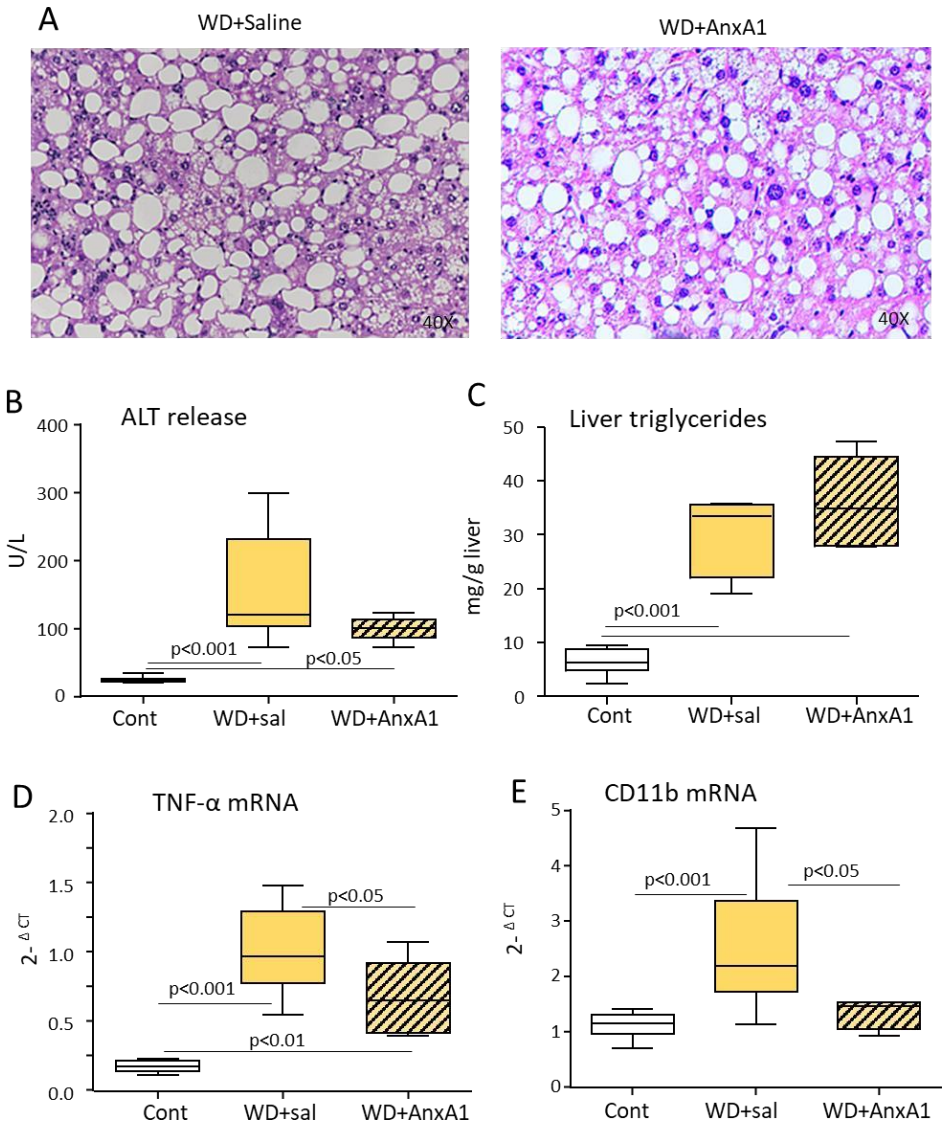

Figure 5: Annexin A1 (AnxA1) supplementation improves steatohepatitis in mice fed with Western diet (WT).

NASH was induced in wild type C57BL/6 mice by feeding WD diet for 10 weeks. The animals were then randomly divided in two groups one receiving AnxA1 (1 µg/daily in saline 5 time a week; WD+AnxA1) and the other the same volume of saline (WD+sal) and the administration of the diet was continued for further 6 weeks. (Panel A) Hematoxylin/eosin staining of liver sections (magnification 20x). (Panels B,C) Alanine aminotransferase (ALT) release and hepatic triglyceride content. (Panels D-E) The hepatic mRNA levels of inflammatory markers TNF-α and CD11b as evaluated by RT-PCR. The values refer to 5-7 animals per group and the boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th-90th percentile) comprise 80% percent of the values.

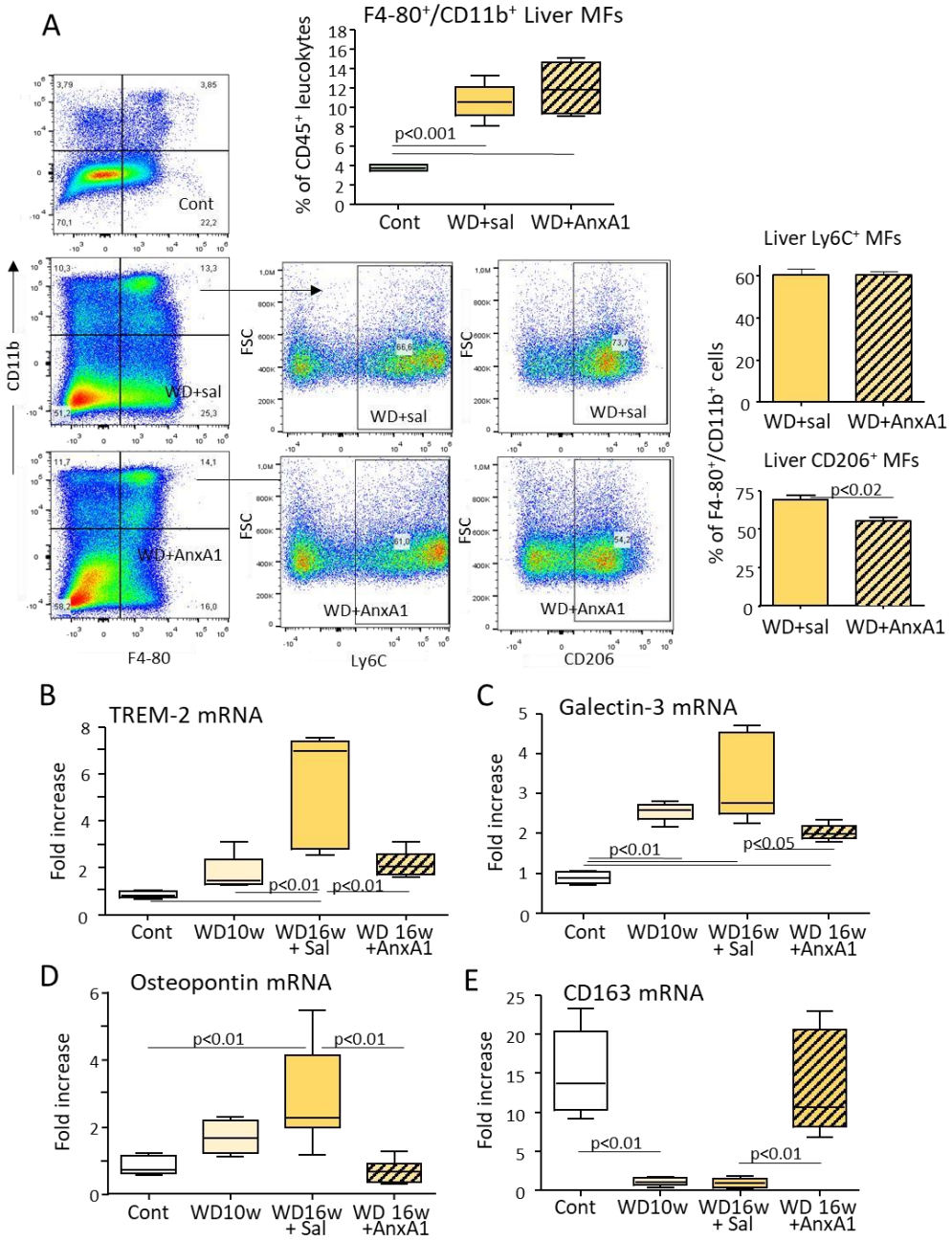

Figure 6: **Effects of Annexin A1 (AnxA1) supplementation on the distribution and features of liver macrophages (MFs) infiltrating the liver of mice with WD-induced steatohepatitis.**

(Panel A) The intrahepatic distribution of F4-80$^+$/CD11b$^+$ MFs and the relative prevalence of cells expressing Ly6C or CD206 was evaluated by flow cytometry in control mice (Cont) or mice receiving WD for 16 weeks in combination with either saline (WD-Sal) or AnxA1 treatment (WD+AnxA1). The values are means ± SD of 3-4 animals for each experimental group. (Panels B-E) Changes in the hepatic transcripts of NASH-associated macrophage (NAM) markers TREM-2, galectin-3 and ostepontin along that of the Kupffer cell marker CD163 in mice receiving WD for 10 or 16 weeks in combination with either saline (WD-Sal) or AnxA1 treatment (WD+AnxA1). The liver mRNA levels were evaluated by RT-PCR in 5-7 animals per group. The boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th-90th percentile) comprise 80% percent of the values.

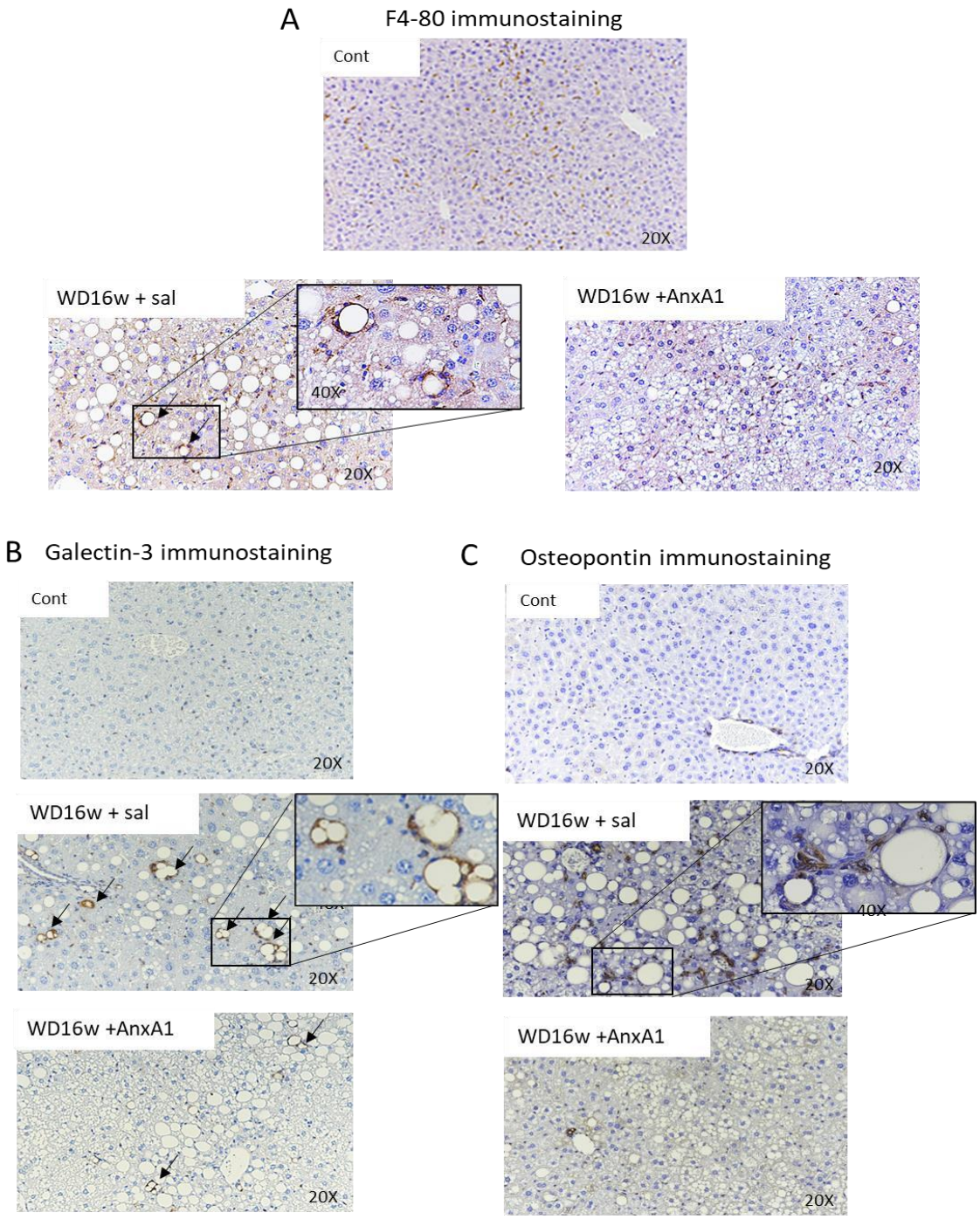

Figure 7: Effects of Annexin A1 (AnxA1) supplementation on the morphology of hepatic macrophages (MFs) and the production of Galectin-3 and Osteopontin.

NASH was induced in wild type C57BL/6 mice by feeding WD diet for 10 weeks. The animals were then randomly divided in two groups one receiving AnxA1 (1 µg/daily in saline 5 time a week; WD+AnxA1) and the other the same volume of saline (WD+sal) and the diet was continued for further 6 weeks. (Panels A-C) Liver macrophages immunostaining for, respectively, F4-80, Galectin-3 and Osteopontin (magnification 20x). The inserts show high magnification of the details of macrophages crown-like structures (arrows).

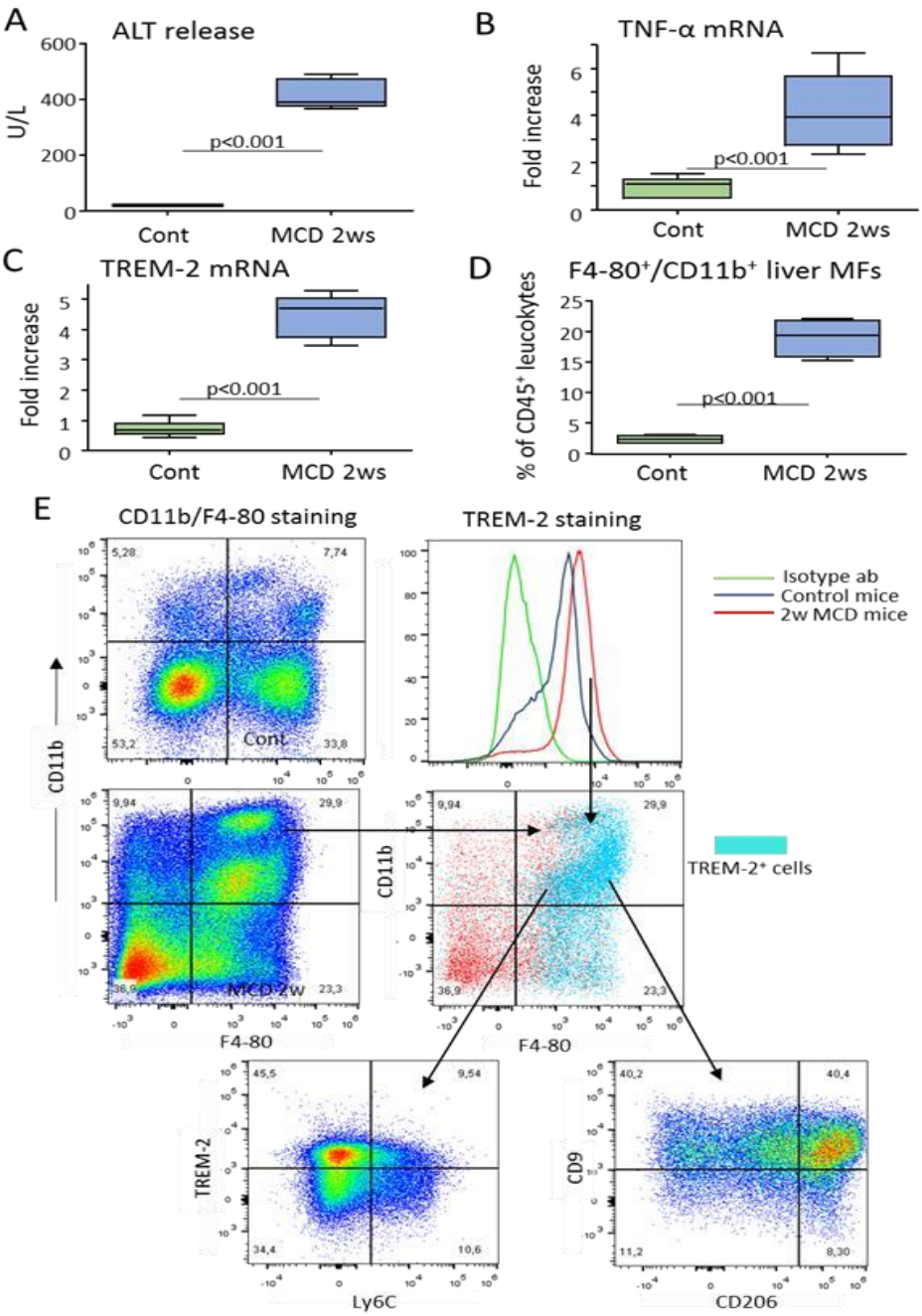

Figure 8: The onset of NASH in mice receiving methionine/choline deficient (MCD) diet parallels with the expansion of TREM-2^{+}/CD9^{+}/CD206^{high} NASH-associated macrophages among liver F4-80^{+}/CD11b^{+} macrophages (MFs).

Wild type C57BL/6 mice receiving either the MCD diet or control chow for 2 weeks were evaluated for the development of steatohepatitis. (Panel A) Alanine aminotransferase (ALT) release. (Panels B-D) The hepatic mRNA levels of inflammatory markers TNF-α and CD11b and TREM-2 as evaluated by RT-PCR. (Panel E) The intrahepatic F4-80^{+}/CD11b^{+} MFs and the relative distribution of cells expressing Ly6C, TREM-2, CD9 and CD206 was evaluated by flow cytometry. The values refer to 4-5 animals per group and the boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th-90th percentile) comprise 80% percent of the values.

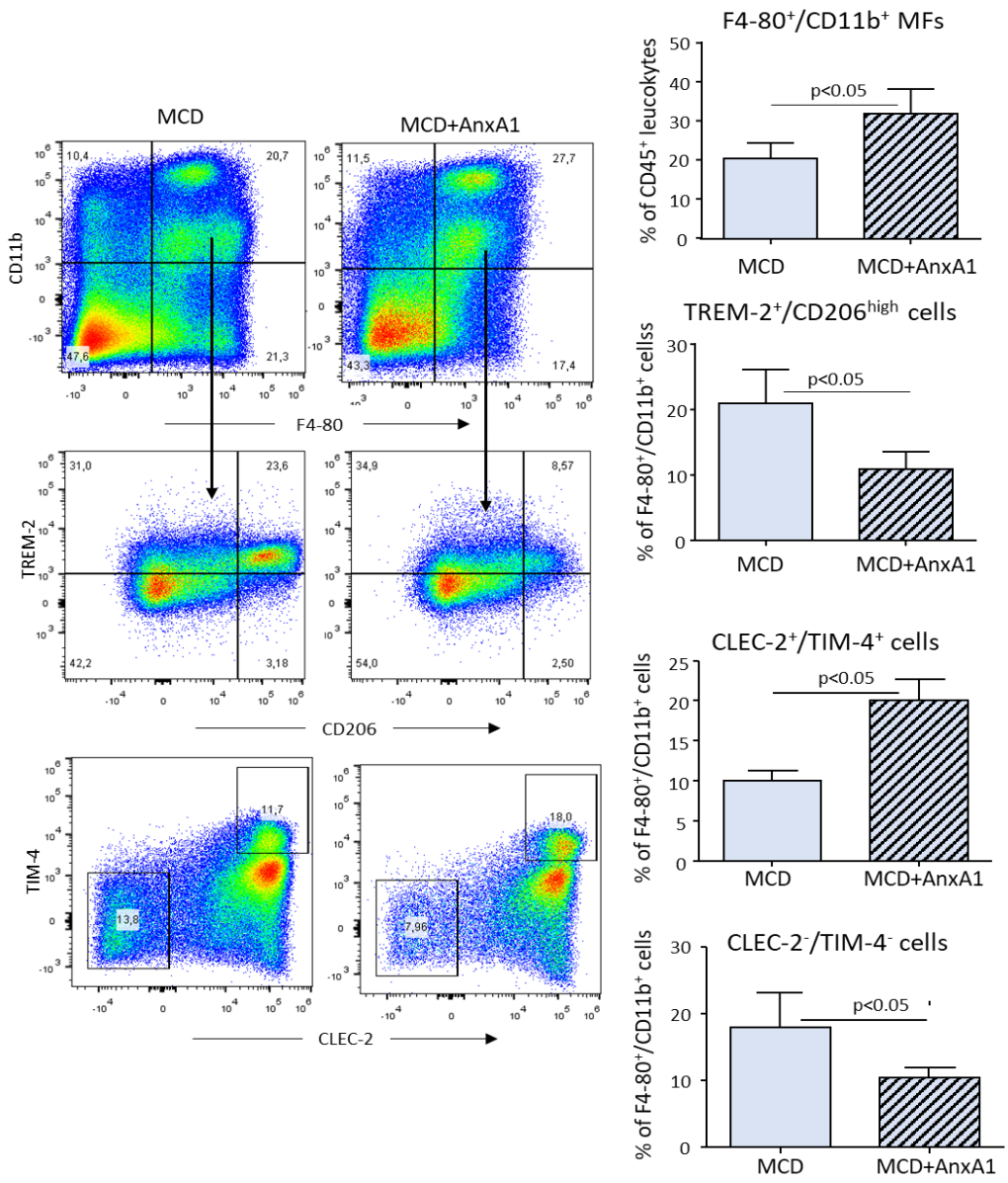

Figure 9: Annexin A1 (AnxA1) supplementation modulates the relative expression of NASHassociated macrophage (NAM) and Kupffer cell markers in liver macrophages (MFs) of mice with NASH.

NASH was induced in wild type C57BL/6 mice by feeding MCD diet for 2 weeks and during the second week the animals received for 5 days AnxA1 (1 µg/daily in saline; MCD+AnxA1) or the same volume of saline (MCD+sal). The intrahepatic distribution of F4-80$^+$/CD11b$^+$ MFs and the relative prevalence of cells expressing NAM markers TREM-2 and CD206 or Kupffer cell markers CLEC-2 and TIM-4 was evaluated by flow cytometry. The values are means ± SD of 3-4 animals for each experimental group.

4.4 Discussion Section 1

As a result of the endemic presence of over-weight and obesity in the western world, nonalcoholic fatty liver disease (NAFLD) is becoming a leading cause of liver cirrhosis, with the prevalence of NAFLD-related end-stage liver diseases expected to further grow over the next decades (Younossi, et al., 2018). On this latter respect, a prospective study in more than 400 patients, with biopsy proven NAFLD with or without steatohepatitis, demonstrated that presence of NASH doubles the rate of disease progression to fibrosis (Singh, et al., 2015). Thus, targeting inflammation represents a key aspect for developing effective treatments for progressive NAFLD. In these settings, the interest for a possible therapeutic use of AnxA1 in NASH steams from the observation that the development of insulin resistance, lipid metabolism derangements, lobular inflammation, and fibrosis are enhanced in AnxA1deficient mice receiving either high-fat or MCD diets (Locatelli, et al., 2014; Purvis, et al., 2019).

By using two different experimental models of NASH, we observe that treating mice with established steatohepatitis with hrAnxA1 not only attenuates liver damage and inflammation but also effectively prevents disease progression to fibrosis. The actions of AnxA1 appear unrelated to effects on metabolic control since, differently from what was reported by Purvis and colleagues (2019), in our experimental settings hrAnxA1 was ineffective in ameliorating liver steatosis and insulin resistance in mice receiving the WD. Such a discrepancy can be explained by the different experimental models. The high-fat diet used in Purvis' experiments causes a lower degree of hepatic inflammation than the cholesterol-enriched WD used in our work (Wiede and Tiganis, 2018) and this might likely influence the severity of the derangements in lipid and glucose metabolism. Furthermore, the time frame between starting the diet and hrAnxA1 administration, was more than two-fold longer in our protocol, thus entailing the possibility of an at least partially effective recovery from liver fat accumulation.

The ability of AnxA1 to ameliorate NASH hepatic damage and inflammation is in line with its recognized action in reducing granulocyte recruitment and macrophage M1 polarization (Sugimoto, et al., 2016; Sheikh and Solito, 2018; Locatelli, et al., 2014). Nonetheless, our results unveil that in NASH AnxA1 is also very effective in preventing disease progression to fibrosis. Such anti-fibrotic action is consistent with previous reports showing that AnxA1 or

AnxA1 mimetic peptides improve lung fibrosis induced by bleomycin or silica particles (Trentin, et al., 2015; Damazo, et al., 2011). In NASH livers, the anti-fibrotic function of hrAnxA1 does not involve changes in the number of hepatic macrophages but it rather associates with the modulation of their phenotype. Treatment with hrAnxA1, in fact, reduces the production of the profibrogenic mediator such galectin-3 (Gal-3) and osteopontin (OPN) that have been previously shown to contribute to liver fibrosis in NASH (Henderson, et al., 2006; Syn, et al., 2011). Moreover, hrAnxA1 affects macrophage capacity of forming crownlike aggregates. The presence of clusters of enlarged and vacuolated macrophages, known as hepatic crown-like structures or lipogranulomas is a feature of both human and rodent NASH (Itoh, et al., 2013). Previous studies have shown that these macrophages derive from liver recruited monocytes and display pro-inflammatory activity (Jindal, et al., 2015; Leroux, et al., 2012; Ioannou, et al., 2013). In addition, these cells can sustain the fibrosis evolution of NASH in view of their colocalization with regions of stellate cell expansion (Remmerie, et al., 2020; Daemen, et al., 2021). So far, the mechanisms leading to the formation of crown-like structures in NASH have not been completely characterized. Studies by Ioannou and coworkers (2013) have shown that macrophage phagocytosis of cholesterol crystals present in dying fat-laden hepatocytes promotes the formation of these crown-like structures. It is noteworthy that these macrophages are also the main producers of AnxA1 in both rodent and human NASH livers (Locatelli, et al., 2014; Jindal, et al., 2015). However, in NASH patients hepatic AnxA1 transcripts inversely correlate with the severity of fibrosis/cirrhosis (Locatelli, et al., 2014), while activated hepatic stellate cells co-localize with Gal-3-positive crown-like structures in the livers of AnxA1-deficient mice with NASH (Locatelli, et al., 2014). This opens the strong possibility that endogenous AnxA1 might be involved in a juxtacrine/paracrine loop that regulates macrophage responses to stimuli, which promote crown-like aggregate formation and fibrogenic mediators' secretion.

Recent reports have outlined the heterogeneity of hepatic macrophages in NASH showing that during disease evolution, resident Kupffer cells are lost, and the liver is enriched by several subsets of monocyte-derived macrophages displaying different phenotypes (Wen, et al., 2021). In more detail, NASH in both rodents and humans is characterized by the abundance of $CD63^+/CD9^+/GPNMB^+/TREM\text{-}2^+$ NASH-associated macrophages (NAM) (Itoh, et al., 2019; Xiong, et al., 2019; Seidman, et al., 2020; Remmerie, et al., 2020), which have similarities with

TREM-2/CD9-expressing lipid-associated macrophages (LAMs) detected in obese adipose tissue (Jaitin, et al., 2019). It is also noteworthy that NAMs contribute to the formation of crown-like aggregates (Remmerie, et al., 2020). The work by Ramachandran and co-workers (Jaitin, et al., 2019) demonstrated that the TREM-2/CD9/OPN/Gal-3 signature also characterizes scar-associated macrophages identified in human fibrotic livers. Lineage tracking indicates that NAMs and scar-associated macrophages derive from liver infiltrating monocytes (Remmerie, et al., 2020; Ramachandran, et al., 2019) and acquire their phenotype in response to specific signals in the interstitial liver niche (Xiong, et al., 2019; Sakai, et al., 2019). We have observed that besides interfering with OPN and Gal-3 gene expression, administration of AnxA1 affects the differentiation of TREM-2^{+}/$CD9^{+}$ macrophage while promoting the acquisition of Kupffer cell markers CLEC-2 and TIM-4. Indeed, recent works have shown that during NASH progression liver infiltrating monocyte-derived macrophages can also acquire Kupffer cell-like features (Remmerie, et al., 2020; Tran, et al., 2020). Although the role of these cells in NASH evolution is presently poorly characterized, our data suggest the possibility that AnxA1 can prevent the development of fibrosis in NASH by skewing liver macrophage-differentiation from pro-fibrogenic NAMs to a phenotype reminiscent that of monocyte-derived Kupffer cells.

In conclusion, our results unveil a novel functional role for AnxA1 in NASH progression by demonstrating its property of interfering with the development of a specific macrophage phenotype associated with the progression of steatohepatitis to fibrosis. Such a novel function of AnxA1 provides a strong rationale for the application of AnxA1, or AnxA1 analogs, to achieve therapeutic control of NASH evolution.

5. Results Section 2 Ketogenic diet improves steatohepatitis and fibrosis in an experimental nonalcoholic steatohepatitis (NASH)

5.1 Foreword

Lifestyle changes, including diet and increased physical activity, are so far the most effective interventions in NAFLD/NASH and significantly reduce liver steatosis, although the effects on hepatic inflammation and fibrosis are controversial (Romero-Gómez, et al., 2017; Brouns, 2018). In recent years low carbohydrates ketogenic diets (KDs) derived from the Atkins diet have been increasingly used for weight loss and anticonvulsant therapy (Kverneland, et al., 2015; Hall and Chung, 2018). KDs are characterized by a very high-fat content that stimulates the liver production of ketone bodies, including acetoacetate (AcA) and β-hydroxybutyrate (βOHB) (Puchalska and Crawford, 2017). Besides the capacity to sustain cell energy production, ketone bodies exert a variety of favourable effects on lipid metabolism, gene expression, and oxidative stress and have specific anti-inflammatory properties exerting beneficial actions on neurodegenerative disease, tumours, and heart failure (Puchalska and Crawford, 2017; Schugar and Crawford, 2012). However, the effectiveness of KDs in reducing systemic and hepatic inflammation has not been investigated in detail. Furthermore, despite epidemiological observations suggest the possibility that alteration in ketones bodies production impact NAFLD/NASH pathogenesis (Goldberg, et al., 2020; Männistö, et al., 2015), the efficacy of KDs in NAFLD remains still controversial (Cotter, et al., 2014; Browning, et al., 2011; Gershuni, et al., 2018). These results are only partially confirmed by a more comprehensive metanalysis (Browning, et al., 2011; Gershuni, et al., 2018). A further limitation in studying the effects of ketosis in NASH derives by the fact that traditional ketogenic diets modelled on Atkins diet cause extensive steatohepatitis when administered to mice despite they achieve substantial weight reduction (Garbow, et al., 2011). Such an effect is likely due to the combination of low protein and choline and high cholesterol content of such diets, all factors known to promote steatohepatitis in mice (Ioannou, 2016; Guerrerio, et al.,2012).

To expand the use of KDs, it would be important to have the possibility of testing new formulations in rodents in order to establish whether these diets are effective in mitigating

systemic and hepatic inflammation and achieving effective control of fibrosis. From more recent data showing that effective ketonemia could be reached also with diets sufficient in protein and choline (Ang, et al., 2020) we devised a choline-sufficient cholesterol-free KD and evaluate its effects in a model of experimental NASH.

5.2 Experimental data

a) Characterization of a new formulation of the ketogenic diet for mice

For assessing the possible beneficial effects deriving from ketosis in rodent NASH, we devised a choline-sufficient cholesterol-free KD (6.7 Kcal/g) containing 0.3% as carbohydrates, 9.2% as proteins, and 90.5% as vegetal fats made up from hydrogenated coconut oil to obtain a consistency suitable for shaping in pellets. a first step we tested the diet palatability for mice to avoid misleading interpretations due to caloric restriction rather than ketogenesis boosting By comparing C57BL/6 mice fed *ad libitum* with the standard or ketogenic diet for up to 8 weeks we did not observe significant differences regarding the amount of food consumed (2,99±0,14 vs 2,98 ±0,19 g/die, $p>0.05$) and body or liver weight (Figure 1A) while, as expected, urine ketone bodies were significantly increased in mice fed with KD as compared with those receiving standard diet ($\sim$ 40 mg/ml vs 0 mg/ml). Since previous studies have shown that a high-fat diet can cause steatohepatitis (Garbow, et al., 2011), histological and biochemical analyses were performed on mice receiving the KD for 8 weeks. Although KD caused a slight but not statistically significant up-modulation in the serological levels of alanine aminotransferase (ALT) (Figure 1A), histological analysis evidenced negligible steatosis without signs of hepatocellular necrosis and portal/lobular inflammation or fibrosis (Figure 1B-C). Furthermore, the analysis of the transcripts for genes commonly up-regulated in NASH showed a modest increase in the mRNAs for TNF-α without appreciable changes in those for the leukocyte marker integrin α M (ITGAM; CD11b) as well as for fibrosis-related markers such as transforming growth factor β (TGF-β) and pro-collagen 1A1 (COL1A1) (Figure 1B-C), confirming the safety of the choline-sufficient/cholesterol-free KD in mice.

b) Effects of the ketogenic diet on metabolic and pathological features of NASH

From these preliminary results, we went on to explore the effects of KD when administered to mice with NASH. To this aim, mice were fed *ad libitum* with a cholesterol-enriched western diet (WD) for up to 16 weeks for inducing extensive steatohepatitis associated with an appreciable collagen fibers deposition (Figure 2B and 7B). After NASH induction, mice were randomly divided into two groups, one switching to KD and the other continuing with the same WD diet for a further eight weeks. The results were analyzed by comparing the switching to KD with the original conditions of mice receiving the WD for 16 weeks as well as with mice maintained with the WD for up 24 weeks. As expected, WD feeding increased body and liver weights and impaired glucose metabolism as assessed by the glucose tolerance test (GTT) (Figure 2A). The switching to KD slightly lowered the body weight without reaching statistical significance (Figure 2A). However, KD administration to NASH mice strongly reduced liver weight and restored the physiological glucose metabolism as also testified by the hepatic upregulation of genes implicated in glucose homeostasis, such as the glucose transporter 2 (GLUT2) (113±13 vs 134±17 $2^{-\Delta Ct}$, $p<0.05$) and insulin receptor substrate-1 (IRS-1) (11,4±3,9 vs 20±5,7 $2^{-\Delta Ct}$, $p<0.01$) (Shearer, et al., 2017; Enooku, et al., 2018). In line with this, Ingenuity Pathway Analysis (IPA) of proteomics data obtained from livers revealed that KD influenced different molecular pathways and in particular stimulated lipid metabolism (Z score ≥ 2) while lowered (Z score ≤ 2) those concerning insulin resistance, glucose metabolism, and inflammatory responses (Figure 3).

These changes impacted NASH features since morphological and biochemical analyses showed that KD administration significantly reduced the extent of hepatic steatosis, lowering liver triglyceride content by 48-61%. (Figure 2B). The regression of steatosis was accompanied by a rise in the gene expression for the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1A) (4±0.9 vs 8±0.6 $2^{-\Delta Ct}$; $p<0.05$). Furthermore, as compared to mice fed with the WD for either 16- or 24-weeks, KD significantly lowered hepatic injury as assessed by hematoxylin and eosin staining and ALT release (Figure 2B).

c) The ketogenic diet modifies gut microbiota and improves hepatic inflammation

Among the beneficial effects of KD, we also observed an improvement of the histological scores for lobular inflammation (2 ± 0.3vs. 0.5±0.2 arbitrary units; n=12; $p<0.01$) as well as in the gene expression of pro-inflammatory markers such as TNF-α, CCL2, IL-12p40 and CD11b (Figure 5A). NASH is often associated with changes in the intestinal microbiota and the loss of gut barrier integrity, which can contribute to hepatic inflammation by increasing the translocation of bacterial products through the portal flow to the liver (Vallianou, et al., 2021). From the knowledge that nutritional behaviors can influence the functional composition of gut microbiota and, in turn, hepatic inflammatory responses (Brandl and Schnabl, 2017), we explored whether changes in gut microbiota could account for the anti-inflammatory properties of KD. To this aim, mouse feces were collected weekly throughout the experimental protocol, the DNA was extracted, and 16S rRNA V3-V4 regions were sequenced. This analysis demonstrated that, according to previous studies, the development of NASH associated with severe dysbiosis characterized by a lowering in the relative abundance of *Lachnospiraceae* and *Ruminococcaceae* and a concomitant raising of *Peptostreptococcaceae*, *Erysipelotrichaceae*, *Bacteroidaceae* and *Sutterellaceae* (Abenavoli, et al., 2022).

KD feeding effectively modified gut microbiota composition with changes that were already appreciable after one week and become more evident at eight weeks from the switching (Figure 4A). Specifically, KD increased the relative abundance of bacterial strains with antiinflammatory properties, such as R-*Ruminococcus spp*, while decreasing that of those displaying a pro-inflammatory behavior like *Sutterella spp* (Figure 4A) (Jiang, et al., 2015; Zhou, et al., 2022). We also characterized the microbiota composition across the gut segments finding that the bacterial genera, colonizing the cecum, ascending, and descending colon, were rather similar, while inside the ileum they appeared more heterogeneous regardless of the type of diet consumed. Therefore, we did not further consider this portion of the intestine. Moreover, since there were no significant differences between the cecum, ascending, and descending colon, we focused our attention on the former. The principal component analysis (PCA) indicated that in this region each dietary regimen was characterized by a welldistinguishable bacterial signature (Figure 4A-B) with KD promoting the enrichment of healthy bacterial strains belonging to specific families such as *Oscillospiraceae*, *Ruminococcaceae,* and *Rikenellaceae*.

Mounting evidence suggests that microbiota plays an essential role in regulating metabolic and immune functions by producing short-chain fatty acids (SCFAs) (Silva, et al., 2020). To explore the possibility that KD's health-beneficial properties might be related to the modulation of SCFA production, we analyzed their serological composition before and after KD switching. In our hands, KD administration modified SCFA abundance by increasing the circulating levels of propionic and acetic acids by 1.2 and 1.4 folds, respectively ($p<0.05$) (Figure 4C). Conversely, no significant changes were appreciable for butanoic and pentanoic acids (0.06±0.012 vs 0.04±0.031 ppm, 0.08±0.001 vs 0.08±0.005 ppm, respectively, $p>0.05$).

d) Ketogenic diet impacts on liver fibrosis

Although proteomic analysis showed that KD feeding affected the hepatic abundance of hundreds of proteins, the production of galectin-3 (Gal-3) appeared significantly downmodulated in 3 out of 5 pathways that emerged as modified (log-fold change = -0.673, p=0.00276). This result caught our attention since Gal-3 plays a fundamental role in driving NASH-associated liver fibrosis being expressed by a subset of liver infiltrating macrophages renamed NASH-associated macrophages (NAMs) (Dou, et al., 2020). Within the livers, NAMs are characterized by the co-expression of the triggering receptor expressed on myeloid cells 2 (TREM-2) and CD9 together with the pro-fibrogenic mediators like osteopontin (OPN) and Gal3 (Peiseler et al. 2022). They also form aggregates surrounding dead/dying hepatocytes, also known as lipogranulomas or hepatic crown-like structures (hCLSs). Of note, NAMs assembly in hCLSs strongly correlates with the disease severity degree and the progression toward fibrosis (Peiseler, et al., 2022).

Multiparametric flow cytometry analysis evidenced that the development of NASH in WD-fed mice increased hepatic recruitment of the pool of infiltrating $CD11b^{high}F4/80^{int}$ macrophages (Figure 5B) that paralleled with up-regulation in the transcripts for TREM-2, CD9, Gal-3 and OPN (Figure 6A) and the progressive accumulation of hCLSs containing $F4/80^{+}$/TREM-2^{+} (Figure 6B-C). As compared to mice kept on the WD the switching to KD almost halved the fraction of infiltrating $CD11b^{high}F4/80^{int}$ macrophages (Figure 5B) reducing the prevalence hCLSs along with the expression of NAM markers (Figure 6A-B). Consistently, we observed that hCLSs were also selectively expressing Gal-3 and that KD strongly reduced the number Gal-3 positive hCLSs (Figure 7A).

From the observation that KD reduced hCLSs and the production of the pro-fibrogenic Gal-3, we investigated whether KD might also have a beneficial impact on liver fibrosis. As expected, steatohepatitis in mice fed WD for 16 or 24 weeks raised the transcripts of the main profibrogenic markers such as COL1A1 and TGF-1β and caused intrahepatic accumulation of collagen fibers as quantified by measuring collagen areas in Sirius Red-stained paraffin sections of liver mice (Figure 8A-B). Noteworthy, mice receiving the KD diet showed a significant down-modulation of the hepatic gene expression for COL1A1 and TGF-1β along with a remarkable reduction in collagen deposition compared to WD-fed animals, thus suggesting the induction of a substantial regression of the fibrosis (Figure 8A-B).

5.3 Figures Section 2

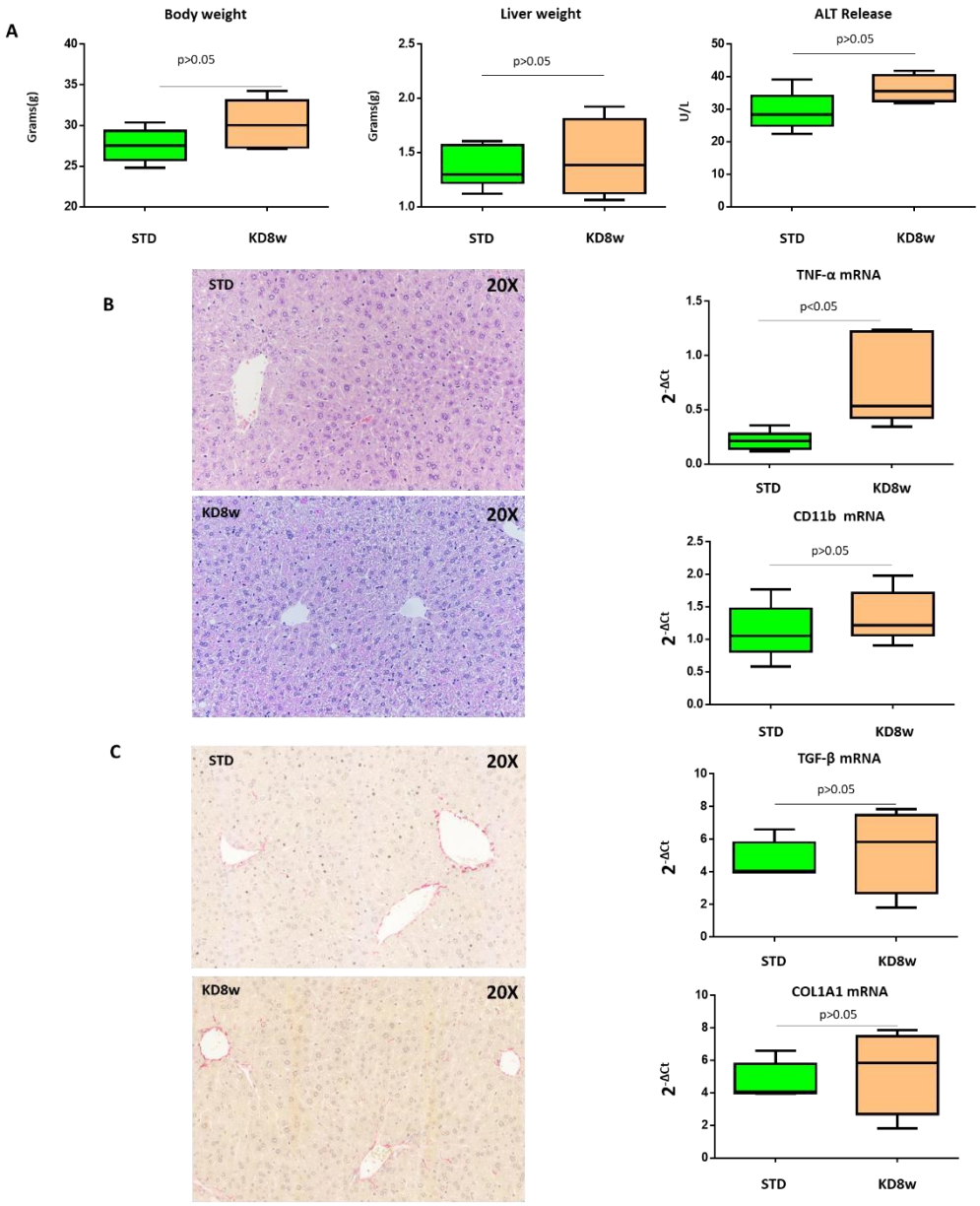

Figure 1. Ketogenic diet (KD) administration does not induce hepatic injury, inflammation, or fibrosis in mice.

Wildtype C57BL/6 mice were fed with standard (STD) or KD diets for up to 8 weeks. (A) Body and liver weights and circulating levels of alanine aminotransferase (ALT). (B) Hematoxylin/Eosin staining of liver sections (magnification 20×) and hepatic transcript levels for the inflammatory markers TNF-α and CD11b evaluated by qPCR. (C) Intrahepatic collagen deposition was evidenced by staining liver sections with Sirius Red (magnification 20×) and hepatic transcript levels for the pro-fibrogenic markers TGF-β and procollagen-1α (COL1A) were evaluated by qPCR. The values refer to six to eight animals per group and the boxes include the values within the 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th–90th percentile) comprise 80% of the values.

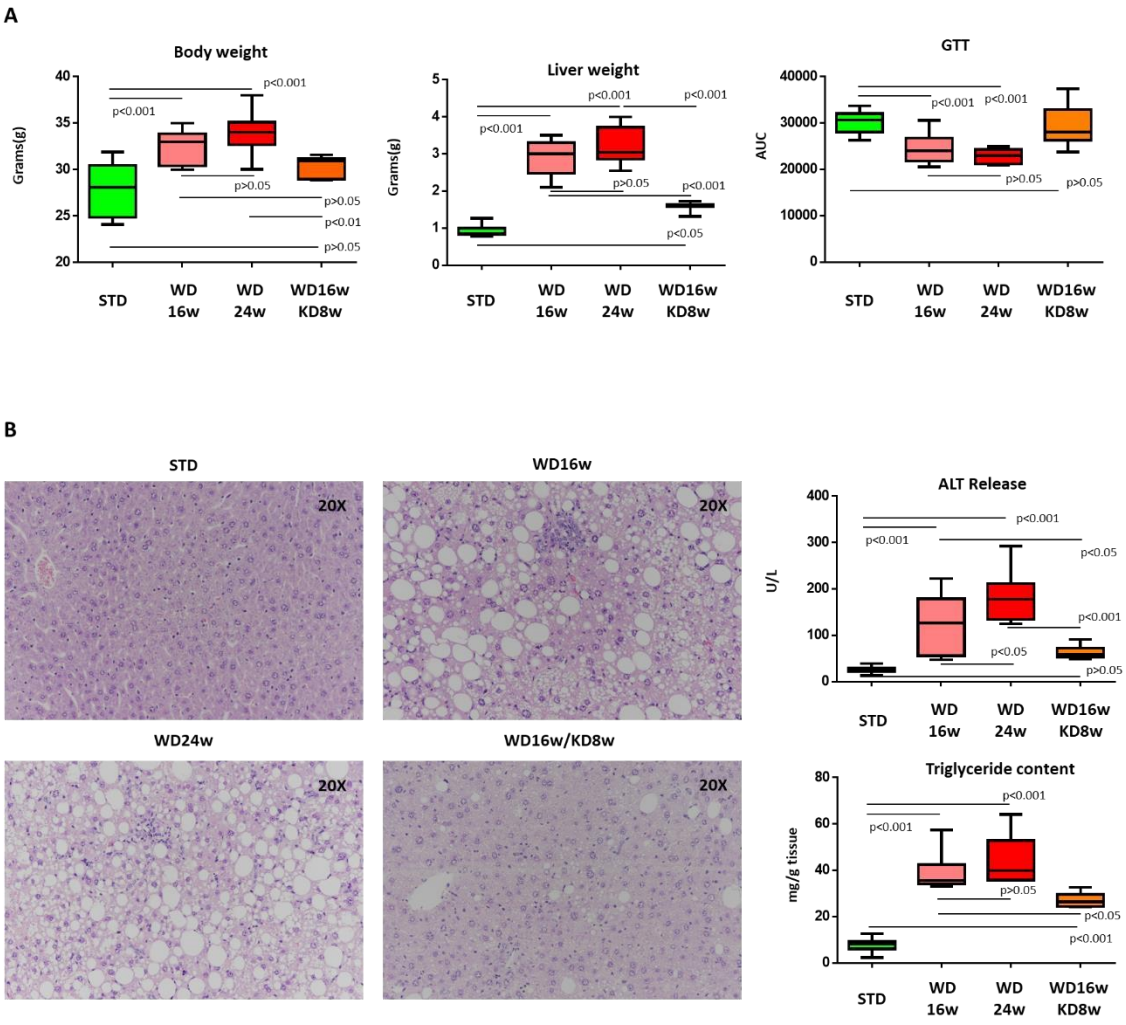

Figure 2. Ketogenic diet (KD) improves NASH-associated hepatic injury and steatosis in mice NASH.

Wildtype C57BL/6 mice were then randomly divided in four groups receiving: standard diet (STD) for 24 weeks; a cholesterol-enriched western diet (WD) for 16 weeks; WD for 16 weeks then switched to KD for further 8 weeks; WD for 24 weeks. (A) Body and liver weights and glucose tolerance test (GTT). (B) Hematoxylin/Eosin staining of liver sections (magnification 20×), circulating levels of alanine aminotransferase (ALT) and liver triglyceride content. The values refer to six to eight animals per group and the boxes include the values within the 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th–90th percentile) comprise 80% of the values.

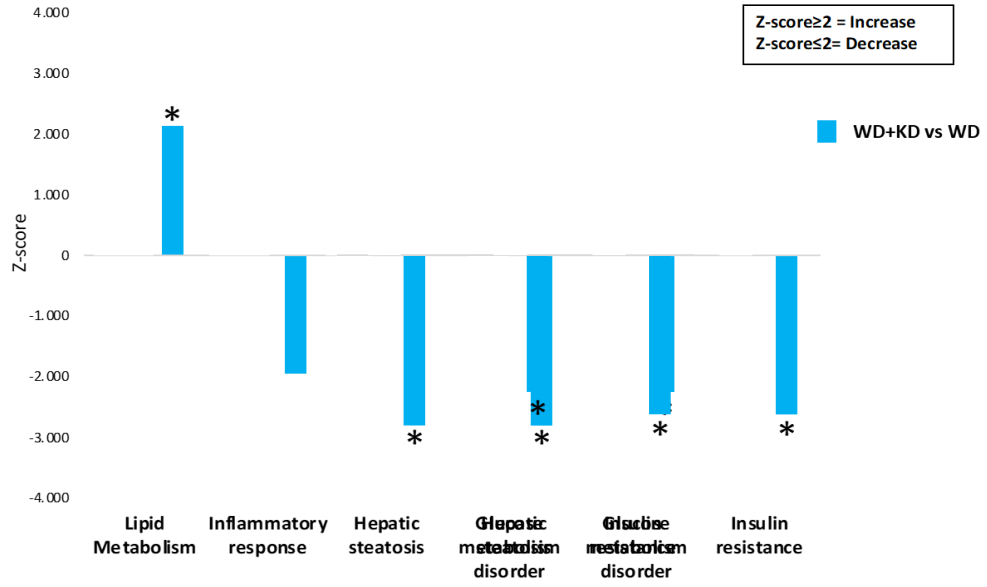

Figure 3. Ketogenic diet (KD) administration modifies multiple hepatic functions in mice with NASH.

NASH was induced in 8-weeks old wildtype C57BL/6 mice by feeding them with WD for 16 weeks before the switching to KD for further 8 weeks. The figure shows the ingenuity pathway analysis (IPA) for lipid metabolism inflammatory response, hepatic steatosis, glucose metabolism and insulin resistance in liver specimens.

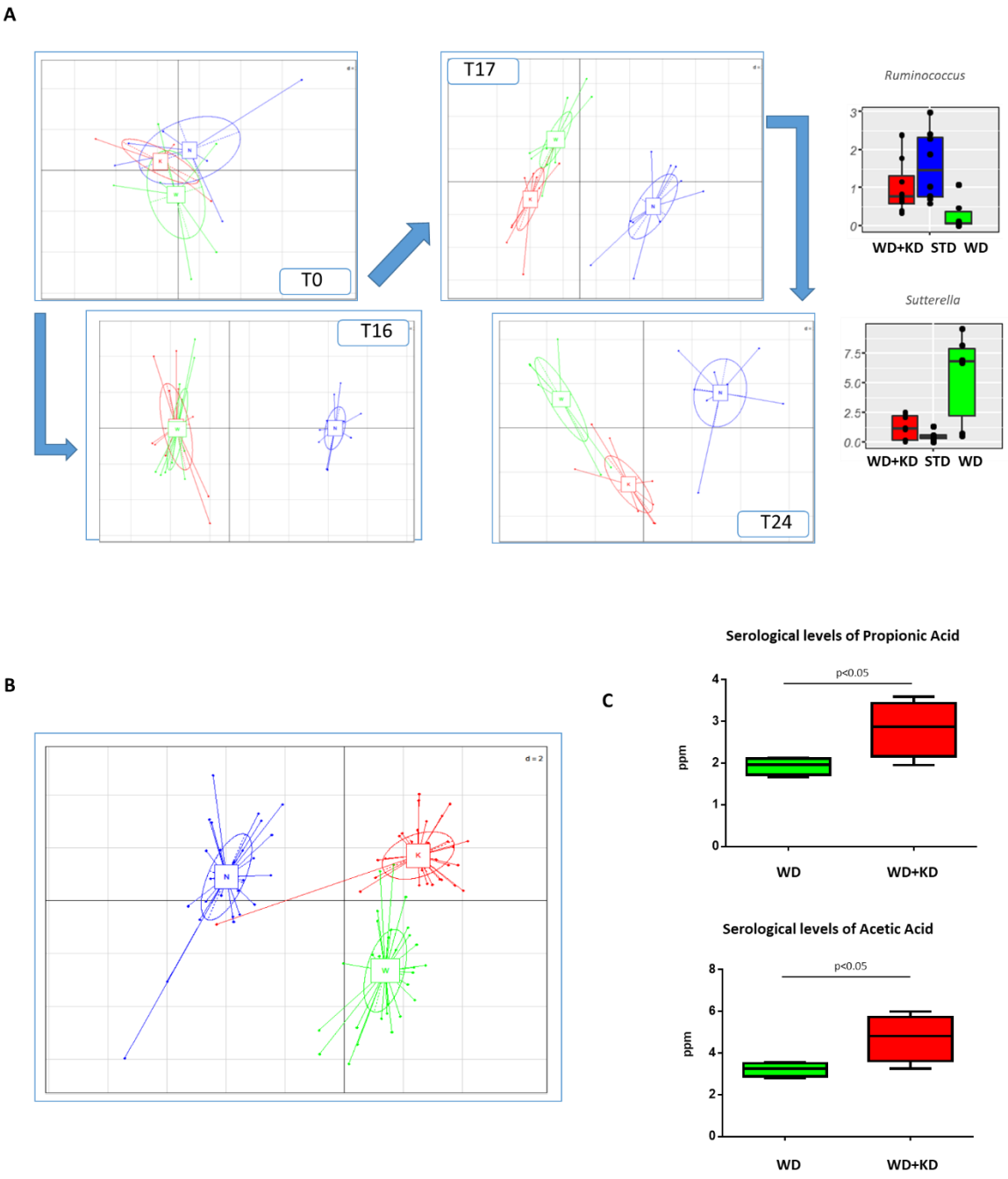

Figure 4. Ketogenic diet (KD) administration reshapes the gut microbiota in mice with NASH.

(A) Principal component analysis (PCA) describing stool microbiota composition in mice receiving standard diet (STD), cholesterol-enriched western diet (WD) or switched from WD to KD over the time. (B) Principal component analysis (PCA) describing cecum microbiota composition in mice receiving standard diet (STD), WD or switched from WD to KD. (C) Serological levels of propionic and acetic acids. The values refer to six to eight animals per group and the boxes include the values within the 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th–90th percentile) comprise 80% of the values.

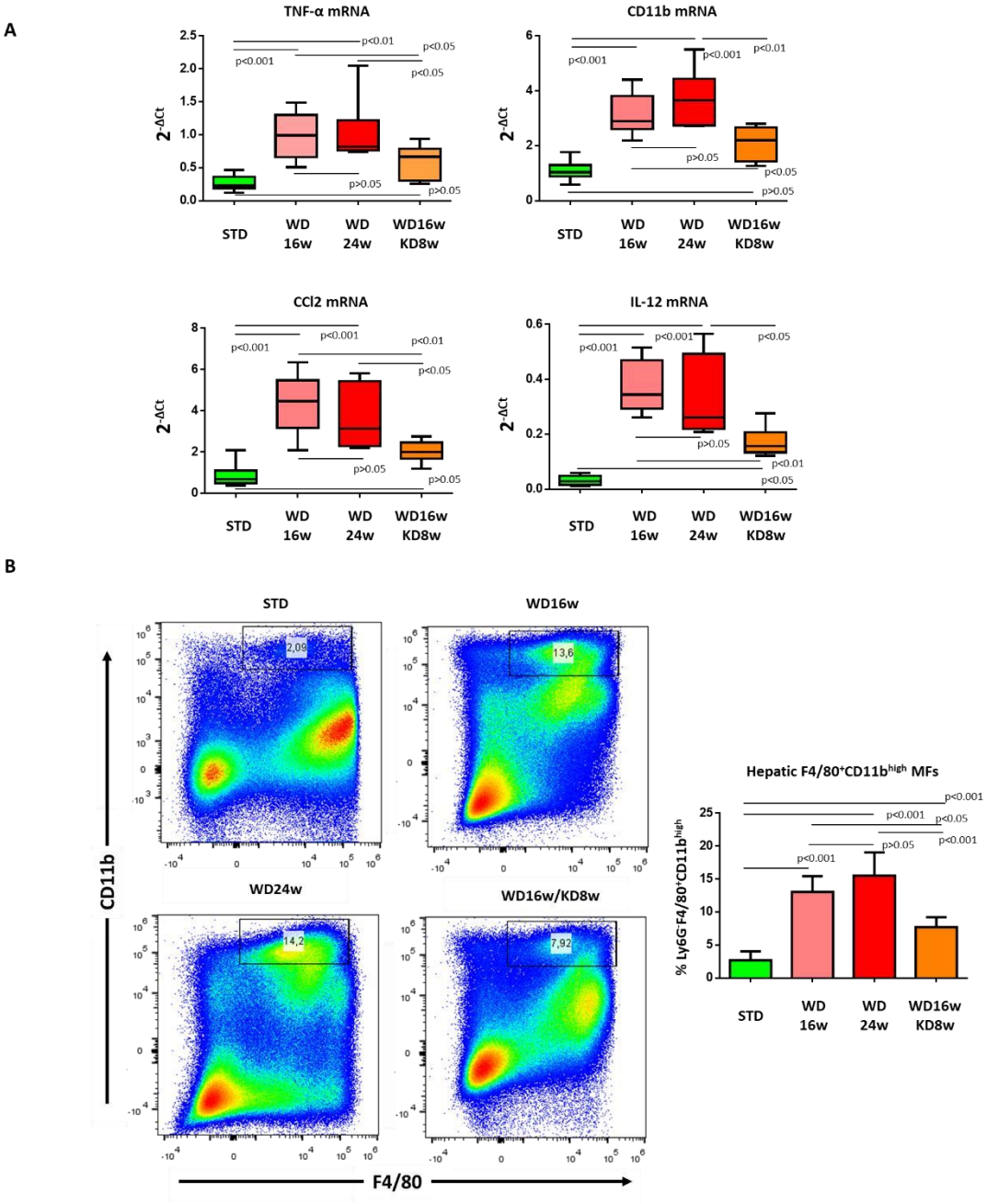

Figure 5. Ketogenic diet (KD) administration ameliorates NASH-associated hepatic inflammation.

Wildtype C57BL/6 mice were then randomly divided in four groups receiving: standard diet (STD) for 24 weeks; a cholesterol-enriched western diet (WD) for 16 weeks; WD for 16 weeks then switched to KD for further 8 weeks; WD for 24 weeks. (A) qPCR analysis of hepatic expression of the pro-inflammatory mediators TNF-α, CD11b, CCL2, and IL12. The values refer to six to eight animals per group and the boxes include the values within the 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th–90th percentile) comprise 80% of the values. (B) The intrahepatic distribution of F4-80+/CD11b+ MFs was evaluated by flow cytometry. The values are means ± SD of three to four animals for each experimental group.

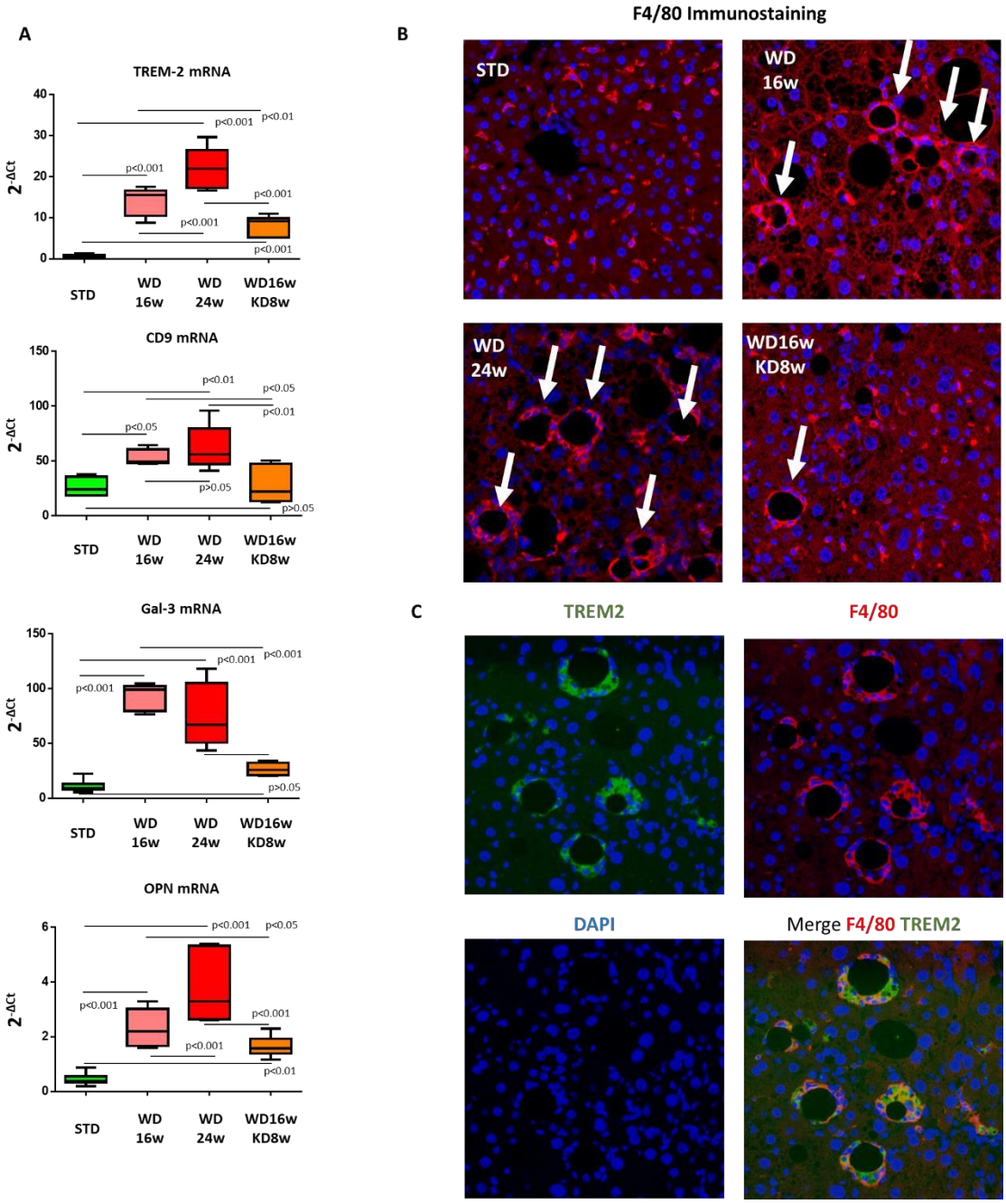

Figure 6. Ketogenic diet (KD) administration reduces the expression of NAM markers and the prevalence of NASH-associated hepatic crown-like structures (hCLSs).

Wildtype C57BL/6 mice were then randomly divided in four groups receiving: standard diet (STD) for 24 weeks; a cholesterol-enriched western diet (WD) for 16 weeks; WD for 16 weeks then switched to KD for further 8 weeks; WD for 24 weeks. (A) Hepatic levels of the transcripts of the NAM markers CD9, TREM-2, galectin-3 (Gal-3) and osteopontin (OPN). The values refer to six to eight animals per group and the boxes include the values within the 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th–90th percentile) comprise 80% of the values. (B) The presence of macrophage aggregates was evidenced by immunofluorescence on FFPE liver sections stained with a fluorochrome-labelled anti-F4/80 antibody. (C) Phenotypic features of liver macrophages forming hCLSs were characterized by double immunofluorescence of FFPE liver sections stained with fluorochrome-labelled antibodies raised against F4/80 and TREM-2.

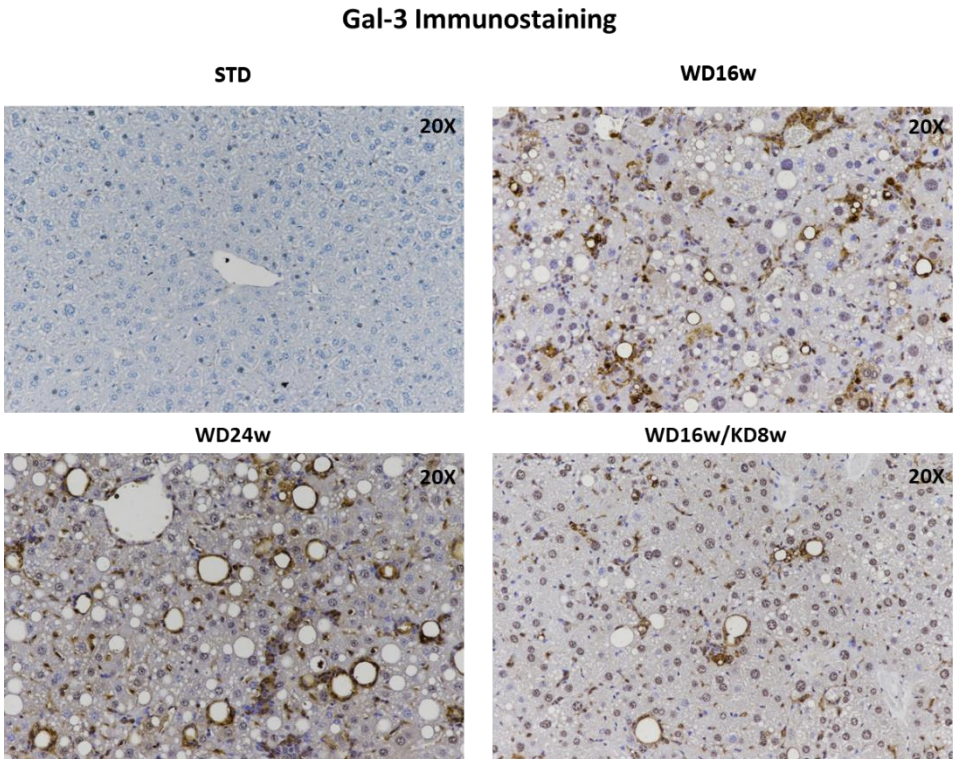

Figure 7. Ketogenic diet (KD) administration reduces the prevalence of NASH-associated hepatic crown-like structures (hCLSs) expressing galectin-3 (Gal-3).

Wildtype C57BL/6 mice were then randomly divided in four groups receiving: standard diet (STD) for 24 weeks; a cholesterol-enriched western diet (WD) for 16 weeks; WD for 16 weeks then switched to KD for further 8 weeks; WD for 24 weeks. (A) The presence of macrophage aggregates expressing Gal-3 was evidenced by immunohistochemistry on FFPE liver sections stained with an HRP-labelled anti-Gal-3 antibody.

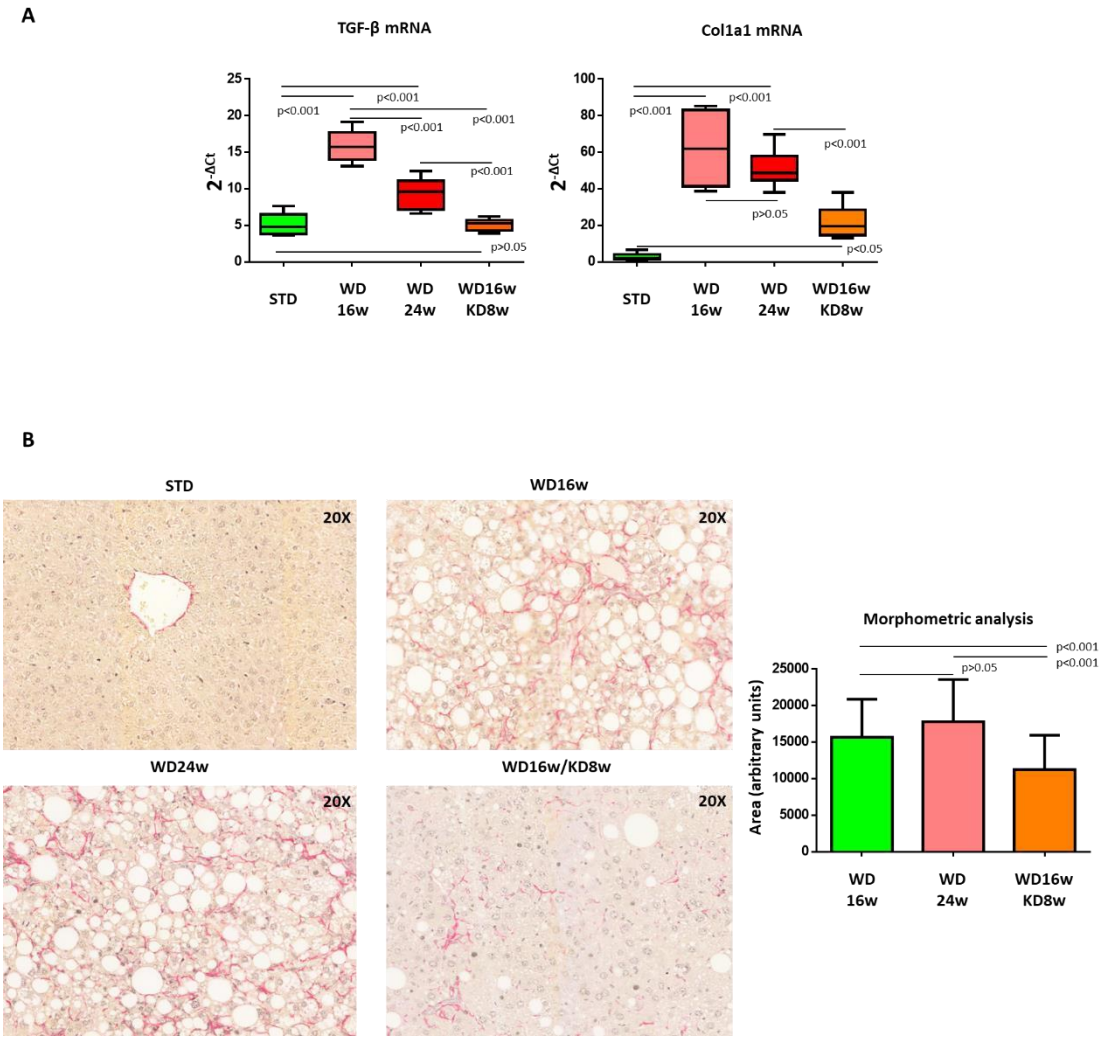

Figure 8. Ketogenic diet (KD) administration improves NASH-associated fibrosis in mice.

Wildtype C57BL/6 mice were then randomly divided in four groups receiving: standard diet (STD) for 24 weeks; a cholesterol-enriched western diet (WD) for 16 weeks; WD for 16 weeks then switched to KD for further 8 weeks; WD for 24 weeks. (A) qPCR analysis of the hepatic transcripts for pro-fibrogenic markers TGF-β and procollagen1α (COL1A). The values refer to six to eight animals per group and the boxes include the values within the 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th– 90th percentile) comprise 80% of the values. (B) Intrahepatic collagen deposition was evidenced by staining FFPE liver sections with Sirius Red (magnification 20×). The values are means ± SD of three to four animals for each experimental group.

5.4 Discussion Section 2

Despite the growing clinical relevance of non-alcoholic fatty liver disease (NAFLD), there are currently no drugs approved for its treatment. According to the European Association for the Study of the Liver (EASL) the guidelines for NAFLD management rely on lifestyle changes related to the dietary regimen and physical activity (Pugliese, et al., 2022). In fact, weight loss achieved through a caloric restriction in combination with physical activity is at present the only treatment that has been proven to ameliorate liver damage in NAFLD patients without severe liver fibrosis (Raza et al., 2021). Among the dietary regimens proposed to ameliorate liver damage, the ketogenic diet (KD), has become popular in the treatment of obese patients due to its forcefulness in inducing satiety and, consequently, weight loss (Martin-McGill, et al., 2020; Moreno, et al., 2016; Moreno, et al., 2014). However, the effectiveness of KDs in NAFLD/NASH has been questioned because of their high content of cholesterol and the lack of an essential nutrient such as choline, two factors well known to promote steatohepatitis *per se* (Garbow, et al., 2011). Considering these limitations, the first aim of this study was fine-tuning a choline-sufficient, cholesterol-free KD to verify whether it could improve ketogenesis in the absence of adverse effects (Schugar and Crawford, 2012; Garbow, et al., 2011; Asrih, *et al.*, 2015).

Hepatocytes are the main responsible for the generation of ketone bodies and, in normal conditions, ketogenesis disposes of up to two-thirds of the lipids entering the liver. Prolonged exposure to high-fat diets, as in NAFLD, progressively decreases ketogenesis likely because of oxidative stress and mitochondrial dysfunction (Mooli and Ramakrishan, 2022). Our newformulated KD *per se* effectively stimulated ketogenesis with a progressive increase in urine ketone bodies, peaking in the last week of treatment. Such an effect can be ascribed to the high content of coconut oil in our formulation. Coconut oil is, in fact, rich in medium-chain triglycerides that are efficiently metabolized in mitochondria to produce β-hydroxybutyrate (β-OHB), acetoacetic acid (AcAc) and acetone (Ac) (Chatterjee, et al., 2020). On the other hand, the presence of vegetal oil as the main calorie source avoids cholesterol overload associated with the use of animal fat.

Ketone bodies have been proposed as direct and indirect epigenetic modifiers of histones involved in regulating chromatin architecture and gene transcription. Among them, β-OHB

seems to be the most effective as, besides increasing global histone acetylation, it has antiinflammatory properties (Puchalska and Crawford, 2017). This latter action involves the inhibition of NF-kB by translocation and degradation of IκB-α (Fu, et al. 2014) and the triggering of G-protein-coupled receptor (GPR)109A, abundantly expressed in many immune cells, including monocytes and macrophages (Watanabe, et al., 2020). In line with the capacity of ketone bodies of regulating gene expression, we observed that the switching of NASH mice to KD modulates multiple metabolic pathways involved in hepatic lipid and glucose metabolism as well as insulin responses. These changes are associated with an improvement in insulin resistance and hepatic steatosis in agreement with previous reports demonstrating the KD capacity of reducing plasma and hepatic triglyceride levels in both rodents and humans (Zhou *et al*. 2022; Lukkonen *et al.*, 2020; Jani, *et al.*, 2022; Mardinoglu, *et al.*, 2018). The anti-steatogenic effect of KD might depend on the empowered energy expenditure and mitochondrial biogenesis as suggested by the increased hepatic expression for the PPARGC1A, corroborating data obtained in other models (Asrih, *et al.*, 2015; Jani, *et al.*, 2022).

Besides improving the fatty liver, we also found that differently from simple dietary restrictions KD effectively counteracts steatohepatitis by lowering hepatic damage and lobular inflammation. In this last respect, hepatic macrophages are the main actors in producing proinflammatory mediators, perpetuating hepatocyte injury and liver inflammation during NASH progression to fibrosis and cirrhosis (Tacke et al. 2017; Wen et al., 2021). Recent studies by single-cell RNA sequencing analysis of liver infiltrating macrophages that expand in both human and rodent NASH have evidenced the phenotype heterogeneity of liver macrophages outlining the importance of the so-called NASH-associated macrophages (NAMs). In fact, the prevalence of NAMs, along with their capacity to form hepatic crown-like structure (hCLS) aggregates surrounding dying hepatocytes correlates with the severity of the disease and its progression toward fibrosis (Peiseler, et al., 2022). In our hands, KD-fed mice show a significant lowering in the fraction of liver infiltrating $CD11b^{high}F4/80^{int}$ macrophages displaying NAM features that parallel with the reduction in the presence of hCLSs. These findings seem to be in contrast with data obtained in a previous report in which KD supplementation fostered hepatic inflammation by promoting macrophage accumulation (Asrih, *et al.*, 2015). However, such a discrepancy can be easily explained considering that the

KD composition employed for those experiments was rich in cholesterol, known to promote hepatic inflammation by stimulating macrophage activation and aggregation in form of hCLSs (Ioannou, et al., 2013).

Growing evidence suggests that gut dysbiosis represents one of the most common features in NAFLD supporting disease progression toward fibrosis by sustaining hepatic inflammatory responses (Lee, et al., 2020). We have observed that KD diet administration reshapes gut microbiota composition associated with NASH promoting the enrichment of bacterial strains belonging to families having anti-inflammatory properties such as *Oscillospiraceae*, *Ruminococcaceae,* and *Rikenellaceae* (Tavella, et al., 2021; Macia, et al., 2015; Khan, et al., 2018). Interestingly, the relative abundance of those bacterial families is decreased in gut microbiota signatures associated with human NAFLD, thus supporting their protective role for liver health (Hrncir, et al., 2021). Gut microbiota contributes to the degradation of nutrients, producing bioactive compounds that can modulate host metabolism. Microbial-derived metabolites include short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate (Vallianou, et al., 2021). SCFAs regulate the immune system and modulate inflammation by influencing activation/differentiation and migration of immune cells, including macrophages (Vallianou, et al., 2021). Furthermore, nutritional behaviors can influence gut microbiota composition and, in its turn, SCFA production along with their metabolic effects. In our hands, KD administration not only reshapes the gut microbiota composition but also significantly modulates SCFA production by raising the serological levels of propionic and acetic acids. Noteworthy, both these metabolites exhibit anti-inflammatory properties by influencing macrophage functional capacities (Al-Lahham, et al., 2012; Yang, et al., 2019). For instance, acetic acid inhibits the activity of the TLR4 signaling pathway in macrophages through the upregulation of the tripartite motif-containing protein 40 (TRIM40), reducing their responsiveness to pro-inflammatory stimuli such as damage/pathogen-associated molecular patterns (D/PAMPs) Yang, et al., 2019). Overall, our results indicate that KD not only is effective in reducing lobular inflammation in NASH but that it is also capable of interfering with several mechanisms involved in promoting steatohepatitis.

A novel finding concerning the beneficial actions of KD in NASH regards the improvement of hepatic fibrosis. We have observed that KD supplementation not only improves

steatohepatitis the main factor responsible for stimulating hepatic collagen deposition but also promotes the regression of already established fibrosis, as evidenced by the reduction of overall intrahepatic collagen content. Such antifibrotic actions of KD likely involve the effect on NAM prevalence, likely in relation to their capability of producing pro-fibrogenic mediators like OPN and Gal-3 (Xiong et al., 2019; Seidman et al. 2020; Remmerie et al., 2020). Indeed, both OPN and Gal-3 liver expression is lowered in mice receiving KD in parallel with those of fibrosis markers. The action of KD on the mechanisms supporting hepatic fibrogenesis is consistent with the data by Moore and colleagues, showing that dietary supplementation with the ketone ester (KE) R, S-1,3-butanediol diacetoacetate (BD-AcAc2) attenuates hepatic stellate cell (HSC) activation and hepatic fibrosis in the context of high-fat diet (HFD)-induced obesity (Moore, et al., 2021). Conversely, our findings differ from those published by Liao *et al.*, which evidence that KD exacerbates carbon tetrachloride (CCl_4)- or thioacetamide (TAA)induced liver fibrosis (Liao, *et al.*, 2021). Such a discrepancy might represent a specific effect of KD in NASH-related fibrosis or depend on the use in Liao's experiments of a traditional KD rich in cholesterol, which is known to promote not only hepatic inflammation but also fibrosis (Teratani, et al., 2012). Although the beneficial effects of ketosis on hepatic fibrogenesis have been proposed some years ago by Puchalska *et al.* (2019) demonstrating its role in preventing tissue scarring, the present study adds new insights regarding the anti-fibrotic action of ketosis, revealing its capability to also mediate fibrosis regression. This is highly relevant because fibrotic livers are fertile ground for HCC development (Yang, et al., 2019; Anstee, et al., 2019) and the severity of fibrosis is the strongest predictor for disease-specific mortality in NAFLD/NASH-affected patients (Ekstedt, et al., 2015). It is now accepted that clinical and experimental liver fibrosis can regress when the causative agent is removed. This effect is associated with the elimination of myofibroblasts derived from activated HSCs and the progressive degradation of collagen in the fibrous scar (Kisseleva and Brenner, 2021). The mechanisms leading to fibrosis regression by KD have not been investigated in detail. It is possible that the anti-inflammatory action related to the improvement of dysbiosis and reduction of NAMs involved in forming hCLSs might play a relevant role. Nonetheless, we cannot exclude that ketosis might favor macrophage phenotypic conversion to a restorative $Ly6C^{low}$ subset, able to secrete matrix metalloproteases (MMPs) like MMP9 and MMP12, and

phagocytosis-associated receptors involved in liver matrix degradation (Kisseleva and Brenner, 2021).

In conclusion, our findings suggest the choline-sufficient, cholesterol-free ketogenic diet is not harmful in the short term and significantly ameliorates steatohepatitis by restoring essential metabolic functions, counteracting dysbiosis, and reducing the hepatic recruitment of NAMs. Strikingly, the new-formulated KD also mediates fibrosis regression by recovering an almost physiological liver morphology. Therefore, such a dietary regimen could represent a novel potential therapeutic tool to be exploited for the treatment of NASH-associated fibrosis.

6. General Discussion

Most of the new therapeutic approaches of NASH that are presently under phase III clinical trials are focused on controlling the factors that promote metabolic alterations at the basis of the disease onset and contribute to its evolution (Dufour et al. 2022). However, the deeper understanding of the complexity in the mechanisms contributing to NAFLD/NASH progression along with the large heterogeneity of NAFLD/NASH patients suggest the possibility that treatments targeting metabolic alterations might be beneficial in early-stages NAFLD, while at later stages anti-inflammatory and antifibrotic therapy should be more appropriated (Dufour et al. 2022). Nonetheless, defining specific ways to control hepatic inflammation and the repair processes in NAFLD/NASH is challenging because of the reciprocal interactions between metabolic dysregulation and the variety of immune cells involved in the process. Such complexity is further increased because extrahepatic disease modifiers including nutrition, gut microbiota, adipose tissue signals, and endocrine dysfunction are influencing the pathogenic mechanisms (Peiseler et al. 2022).

The results obtained during my Ph.D. project indicate that it is possible, at least in an experimental animal setting, to control inflammation in NASH by modulating the phenotype of liver macrophages. It is increasingly evident that the macrophage pool in either rodent and human NASH livers is highly heterogeneous and comprises embryonic and monocyte-derived Kupffer cells as well as monocyte-derived macrophages (MoMFs). It is now well established that in response to C-C motif chemokine receptor 2 (CCR2) signals monocytes are recruited within the liver in the early phases of NASH and MoMF accumulate in the periportal area (Peiseler et al. 2022). These MoMFs are also responsible for the formation of cell aggregates, known as crown-like structure (hCLS) aggregates, the prevalence of which parallel human NASH severity (Peiseler et al. 2022). In our hands, the treatments of NASH mice with both the ketogenic diet or hrAnxA1 are effective in lowering liver injury and hepatic inflammation, and these actions associate with a decrease of hCLSs prevalence Recent data indicate that hCLSs are formed by a sub-set of MoMFs called NASH-associated macrophages (NAMs) that are characterized by the expression of a combination of surface molecules including CD9 and the Triggering Receptor Expressed on Myeloid cells 2 (TREM2). Accordingly, by immunohistochemistry we have observed that TREM2 colocalizes with the macrophage marker F4-80 in hCLSs detected in NASH mice. Both hrAnxA1 and KD also effectively reduce

the hepatic expression of both CD9 and TREM2, suggesting that they might affect NAM differentiation. However, it is likely that different mechanisms can account for such an effect in different experimental settings. In fact, in mice switching to the KD diet the lowering of proinflammatory stimuli associated with dysbiosis and the anti-inflammatory action of ketosis lowers the fraction of $CD11b^{high}/F4\text{-}80^{+}$ MoMFs, which is known to include cells with a proinflammatory behavior likely contributing to hCLSs formation (Peiseler et al. 2022). Conversely, the fraction of $CD11b^{high}/F4\text{-}80^{+}$ MoMFs is unmodified in the liver of mice receiving hrAnxA1, while the lowering of TREM-2^{+} cells is accompanied by an increase in the prevalence of $F4\text{-}80^{+}/CD11b^{+}$ cells expressing the Kupffer cell markers C-type lectin-like type 2 receptor (CLEC-2) and the phosphatidylserine receptor T-cell membrane protein 4 (TIM-4) along with the upregulation in the transcripts for hemoglobin-haptoglobin scavenger receptor CD163, a marker of differentiated Kupffer cells. These observations suggest the possibility that hrAnxA1 might reduce hCLSs by favoring the switching of MoMFs to monocyte derived Kupffer cells. Recent reports by Bonnardel and co-workers (Bonnardel et al. 2019) and Sakai and coworkers (Sakai et al. 2019) have shown that MoMF interactions with liver sinusoidal endothelial cells (LSECs) and hepatic stellate cells (HSCs) are critical for acquiring a Kupffer cell phenotype. Such interactions involve direct cell contact through the Notch 1 receptor with its ligand Delta-like ligand 4 (DLL4) (Bonnardel et al. 2019; Sakai et al. 2019) as well as the production of colony-stimulating factor 1 (CSF1), transforming growth factor-β1 (TGFβ1) and bone morphogenetic protein (BmP) by LSECs and HSCs and of desmosterol by hepatocytes (Bonnardel et al. 2019; Sakai et al. 2019). At present, the mechanisms through which hrAnxA1 drives MoMF phenotypic changes of has not been characterized, but it is possible that the intracellular signals triggered by the AnXA1 receptor FPR2/AXL might enhance MoMF response to the above interactions.

Despite improving hepatic inflammation, we have observed that both KD and hrAnxA1 are also effective in promoting the regression of already established NASH-associated fibrosis. This action is likely related to the interference with hCLSs since we observed that macrophages in these structures strongly express Gal-3. This is consistent with the previous observation by Locatelli and co-workers who reported that the worsening of fibrosis AnxA1-deficient mice associates with a marked upregulation in liver Gal-3 by aggregates containing MoMFs and activated HSCs (Locatelli et al. 2014). Gal-3 is a member of the galectin family, a group of

lectins that participates in the regulation of cell adhesion, proliferation, and survival, as well as in the modulation of tissue inflammation and fibrosis (Henderson and Sethi, 2009). In this latter respect, Gal-3 genetic deletion or inhibition of Gal-3 attenuates HSC activation and hepatic collagen deposition in experimental models of chronic liver diseases (Traber, et al., 2013). However, the reduction in hCLSs can also impact through other mechanisms on the fibrogenic process, considering that hCLSs colocalize with regions of stellate cell expansion (Itoh, et al., 2013). The action of both hrAnxA1 and KD on Gal-3 combines with that on the production of osteopontin (OPN), another pro-fibrogenic cytokine (Song, et al., 2021). The expression of Gal-3 and OPN characterizes TREM-2/CD9 scar-associated macrophages in the fibrotic niches present in the livers of human cirrhotic patients as well as in NASH-related experimental fibrosis (Ramachandran, et al., 2019). Although is presently unclear whether NAMs and scar-associated macrophages represent subsets of phenotypically related cells, it is tempting to speculate that hrAnxA1 and KD might affect the development of scar-associated macrophages.

At present, the interference with macrophage recruitment by blockade of chemokine receptors CCR2 and CCR5 appears a feasible strategy to block cell-cell communication at the level of macrophages. In fact, the CCR2/5 antagonist cenicriviroc is effective in improving macrophage recruitment, steatosis, and fibrosis in mouse models of NASH (Tacke 2018). In clinical trials cenicriviroc ameliorates NAFLD activity score but it does not effectively improve fibrosis (Wiering & Tacke 2022). These inconsistencies along with the recent observation that the impairment of monocyte recruitment in CCR2-knockout mice reduces hCLS formation and the severity of NASH but worsens hepatic fibrosis (Daemen et al. 2021), suggest that blocking NAMs might be also involved in controlling tissue scarring. In these settings, the use of treatments such as AnxA1 supplementation or KD that modulate macrophage phenotype might be more suitable to prevent fibrosis in NASH.

In conclusion, the data obtained during my doctoral training have evidenced that both KD and AnxA1 are effective in controlling hepatic inflammation and fibrosis progression in NASH possibly by regulating macrophage phenotype and functions. These data offer pre-clinical evidence for developing novel therapeutic approaches to personalized therapies in NASH patients.

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