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L-CARNITINE SUPPLEMENTATION ATTENUATES NAFLD PROGRESSION AND COMPLICATIONS IN A METHIONINE AND CHOLINE DEFICIENT DIET MOUSE MODEL

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of metabolic syndrome and become one of the most common causes of chronic liver disease over the last decade in developed countries. The general prevalence of NAFLD is reported ranging between 20–30 % and 87 % in obese people. It is commonly associated with visceral obesity, type 2 diabetes mellitus, dyslipidemia and hypertension, all components of the metabolic syndrome, so that NAFLD might be considered an additional component of metabolic syndrome itself. As the rate of obesity, diabetes, and metabolic syndrome continue to increase, NAFLD will bring a tremendous impact on health care in the upcoming years. The underlying causes of the disease progression in NAFLD are unclear. Recent evidences suggest the development of lipid droplets (steatosis), subsequent generation of reactive oxygen species (ROS) and fibrosis deposition in the progression to non-alcoholic steatohepatitis (NASH). Moreover, continued elucidation has needed to understand fibrosis progression and regression. The paradigm of hepatic stellate cell (HSCs) activation remains the foundation for defining key translational challenges in order to accelerate the development of new therapies for patients with chronic liver disease.

L-Carnitine (LCARN) is an essential nutrient that converts fat into energy in mitochondria. LCARN plays an important role in lipid metabolism; it acts as an essential cofactor for the β-oxidation of fatty acids. Very recently, LCARN has been proposed for the treatment of various diseases, including liver injury and several studies have shown that LCARN administration can ameliorate or prevent liver damage of various etiologies.

We investigated the potential antioxidant and antifibrotic role of LCARN supplementation on methionine choline deficient (MCD)-diet-induced NAFLD in C57BL/6 mice. Mice were divided into three groups of CONTR (normal diet without any treatment), MCDD (MCD diet only), MCDD+LCARN 200 mg/kg/die group. Liver and heart weight, histological changes and fibrosis progression were assessed after 6 weeks of experiments. The MCD-diet induced severe hepatic fatty accumulation, but the fatty change was reduced in the MCDD+LCARN group. LCARN supplementation showed a role in controlling liver ROS generation and consequently coordinating the HSCs activation. Additionally, the same antioxidant and antifibrotic effect was observed in the myocardium. In conclusion, our findings indicate that LCARN has a potential role in control NAFLD progression to NASH. Therefore, our data suggest that LCARN may acts as a novel and potent supplementation agent against NAFLD cardiac complications.

RIASSUNTO

La steatosi epatica non alcolica (NAFLD), manifestazione epatica della sindrome metabolica, è diventata una delle più comuni cause di malattia epatica cronica negli ultimi dieci anni nei paesi sviluppati. La prevalenza generale della NAFLD è compresa tra il 20-30% e 87% nelle persone obese. È stata comunemente associata all'obesità viscerale, il diabete di tipo 2, la dislipidemia e l'ipertensione, tutti componenti della sindrome metabolica, portando la NAFLD ad essere di conseguenza considerata un'ulteriore componente della sindrome metabolica stessa. Poiché il tasso di obesità, diabete e sindrome metabolica è in continuo aumento, è stato previsto che la NAFLD nei prossimi anni avrà un enorme impatto sulla sanità. Ad oggi, le cause alla base della progressione della malattia non sono ancora state chiarite del tutto. Recenti evidenze hanno suggerito lo sviluppo di depositi lipidici (steatosi), la successiva generazione di specie reattive dell'ossigeno (ROS) e deposizione fibrotica come causa dello sviluppo della steatoepatite non alcolica (NASH). Nonostante questo, il continuo studio di questi meccanismi sarà fondamentale per comprendere l'attivazione della deposizione di collagene, il suo controllo e la regressione della malattia. Il paradigma d'attivazione delle cellule stellate epatiche (HSCs), rimane tuttora la chiave per la definizione e lo sviluppo di nuove terapie per i pazienti affetti da malattia epatica cronica.

L-Carnitina (LCARN) è un nutriente essenziale in grado di trasformare il grasso in energia nei mitocondri. LCARN svolge un ruolo importante nel metabolismo dei lipidi; agisce come un cofattore essenziale per la β-ossidazione degli acidi grassi. Recentemente, LCARN è stata proposta per il trattamento di varie malattie compreso il danno epatico e numerosi studi hanno dimostrato che la somministrazione di LCARN può migliorare o prevenire danni al fegato di varie eziologie.

Lo scopo della tesi è stato di studiare il potenziale antiossidante e antifibrotico di LCARN somministrata come integratore alimentare in un modello murino NAFLD indotto da dieta priva di metionina e colina (MCD). I topi C57BL/6 sono stati divisi in tre gruppi: CONTR (dieta normale senza alcun trattamento), MCDD (dieta MCD), MCDD+LCARN 200 mg/kg/die (MCDD+LCARN). Il peso del fegato e del cuore, le alterazioni istologiche dei due organi e l'attivazione della fibrosi sono state valutate dopo 6 settimane. L'integrazione con LCARN ha ridotto l'accumulo di lipidi epatici, la generazione di ROS e l'attivazione della fibrosi sia epatica che cardiaca. In conclusione, i nostri risultati suggeriscono il potenziale ruolo di LCARN nel controllo non solo della progressione della NAFLD in NASH ma anche dello sviluppo delle complicazioni cardiache ad essa correlate.

1. INTRODUCTION

1.1 Pathology of Non-Alcoholic Fatty Liver Disease

The term nonalcoholic steatohepatitis (NASH), was firstly used in 1980 by Ludwig and colleague to describe the morphologic pattern of liver injury in 20 patients evaluated at the Mayo Clinic over a 10-year period (Ludwig J 1980). The 60% of these patients were female with no history of alcohol abuse, the 90% of these women were obese but incredibly, their liver biopsy had histological evidence of alcoholic hepatitis. In this population, diabetes mellitus and hyperlipidemia were also commonly described. Schaffner and colleague in the 1986 with the term Non-Alcoholic Fatty Liver Disease (NAFLD), included a range of liver injuries and this designation became quickly the preferred one (Schaffner F 1986). The liver injuries included range from hepatic steatosis to NASH, characterized by fatty accumulation exceeding 5% of liver weight, hepatocellular injury, progressive fibrosis and has been defined both histologically and clinically (Brunt EM 2010). This distinction is important, as simple steatosis is unlikely to lead to liver related complications, whereas NASH may lead to increased fibrosis and cirrhosis, and its complications such as hepatocellular carcinoma (HCC) (*Figure 1*) (Sass DA 2005). The accepted scientific consensus is that NAFLD is not a benign condition and a subset of patients develop significant fibrosis or associated morbidity and mortality. Although, some of the pathological characteristic can be associated with hepatic fibrosis and the most important histological feature associated with mortality in NASH is presence of significant fibrosis. NAFLD is progressively being accepted as a clinically important disease, and as with any disease, the clinical importance is relative to its natural history and prevalence. The natural history of NAFLD is dynamic, with interaction and contribution from multiple factors including genetic, environmental and lifestyle factors. NAFLD proves to be a formidable disease entity, with considerable clinical burden, for both the present and the future (Goh GB 2016).

In 1998 Day and James with the "Two Hits Hypothesis" described the development of NAFLD. This disease needs a two-step process dependent from each other. The first hit produce steatosis after a reversible hepatic lipid accumulation that prepare the liver for the second hit represented by a source of oxidative stress determining significant lipid peroxidation that facilitated inflammation, progressing steatosis and fibrosis (Day CP 1998). This hypothesis provides a rationale for the prevention and the control of disease progression in steatosis.
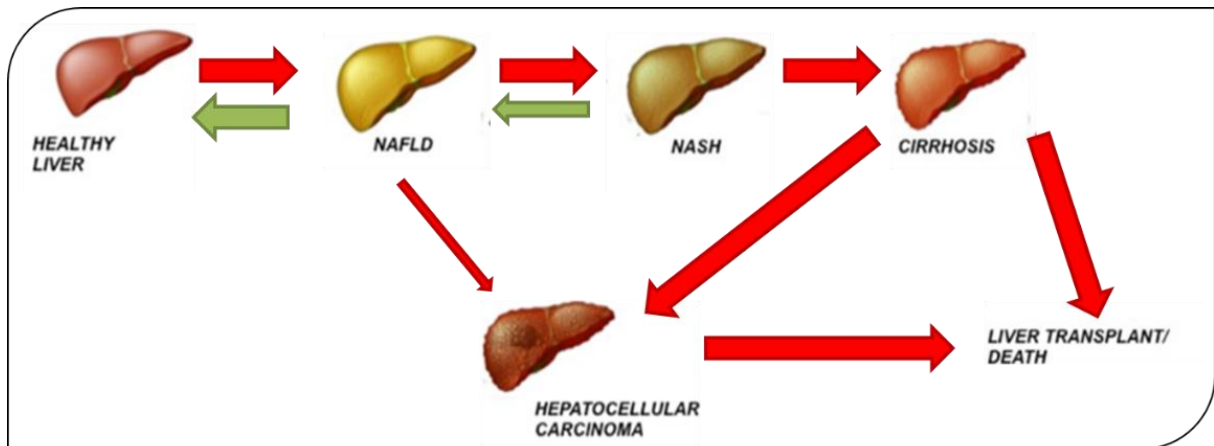
Figure 1. Progression of NAFLD (Adapted from Goh GB, 2016).

1.1.1 First Hit - Lipid accumulation mechanism

Liver plays a central role in the energy homeostasis by storing glucose as glycogen and distributing fuels in the form of glucose and lipids to peripheral organs. Hepatic glucose and free fatty acid uptake occurs and increase linearly with the postprandial rise in plasma concentrations. Dietary lipids in the form of chylomicrons are transported from the gut via the lymphatic system to the liver, where they are incorporated after release from lipoproteins by hepatic lipoprotein lipase (Bradbury MW 2004). Physiologically and during the postprandial phase, dietary lipids are stored in the liver, where they are processed and assembled with apolipoprotein B 100 o form very-low-density lipoprotein (VLDL). The molecular events resulting in intrahepatic lipid accumulation and growth of lipid droplets in hepatic steatosis are poorly understood, but may can be summarized in four different processes (Kallwitz ER 2008):

- Dietary calories in form of triglycerides are transported to the liver as chylomicrons. Moreover, adipose tissue triglycerides can reached the liver in form of free fatty acids (FFAs);
- Increased *De novo* synthesis of triglycerides in the liver;
- Decreased liver β-oxidation;
- Decreased liver export of triglycerides as VLDL.

In developed countries, the common cause of NAFLD is overnutrition that determined an excessive of extra-energy as lipid precursors from adipose tissue into ectopic depots such

as the liver (Valenti L 2016). The initial metabolic stress is represented by the hepatic lipid accumulation and overloading of mitochondrial capacity and is driven by lipotoxicity from FFAs (*Figure 2*). Lipotoxic effects of FFAs have been proposed to be the crucial factor in development and progression of hepatic steatosis.

The modulation of hepatic triglyceride are coordinated by peroxisome proliferators activated receptors (PPARs), a nuclear receptor superfamily. There are three different PPARs isoforms in mammals: PPARα, PPARδ and PPARγ. PPARγ is the most intensively studied and it is also involved in the activation of genes that lead to improved uptake of glucose and lipids, increase glucose oxidation, and decrease FFAs concentration and insulin resistance. Recently, PPARγ is involved in an anti-inflammatory effect interfering with proinflammatory transcription factors as NFĸB, which is inhibited by physical interaction of PPARγ and p65 and p50 NFĸB subunit (Anderson N 2008).
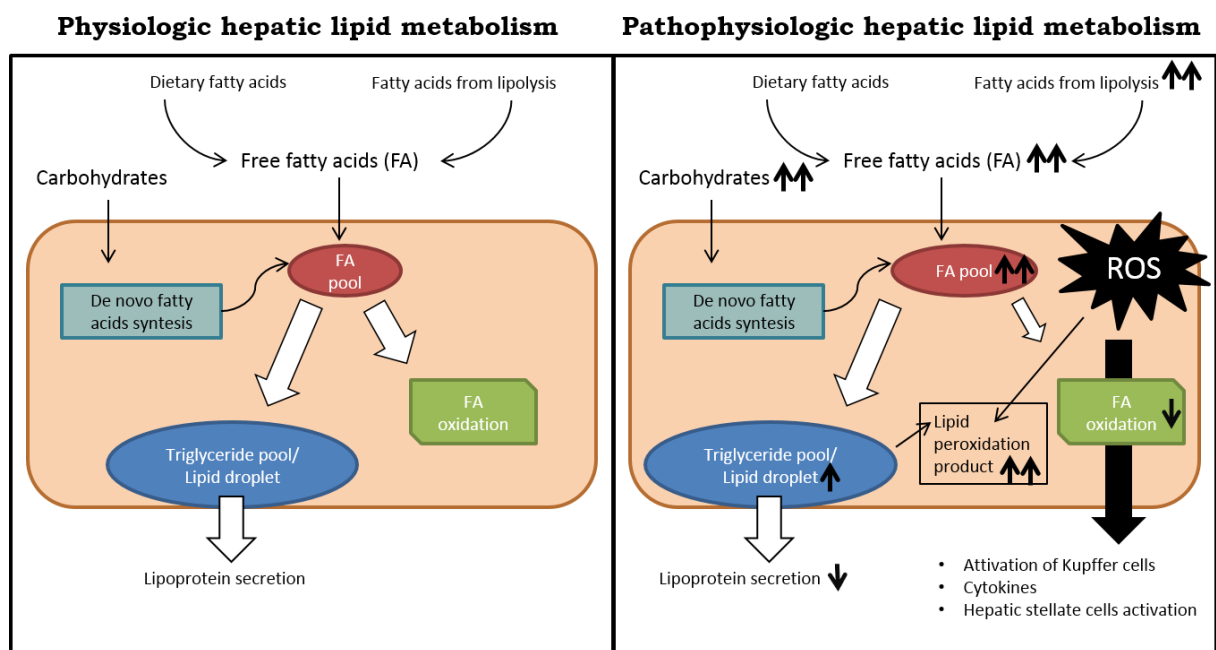

Figure 2. Physiologic hepatic lipid metabolism and pathogenic mechanisms in metabolically induced NASH. The uptake, storage and excretion is balanced in physiological conditions but during the progression to NASH, the molecular events resulting in intrahepatic lipid accumulation and growth of lipid droplets are may arise from: 1) increased uptake of lipids increased, 2) elevated de novo synthesis of fatty acids, 3) impaired lipoprotein synthesis or secretion and/or 4) reduced fatty acid oxidation. (Adapted from Anderson N, 2008).

As described in Figure 2, steatosis and its progression to steatohepatitis may also result from improper fatty acid oxidation (Anderson N 2008). Fatty acids oxidation is activated and

controlled by the AMP-activated protein kinase (AMPK). AMPK. is the downstream component of a protein kinase cascade that plays an important role in maintaining energy balance and plays an important role in whole-body energy metabolism (Kahn BB 2005) (Raney MA 1985). Activation of AMPK requires phosphorylation of threonine 172 (T172) within the T loop segment of the catalytic α subunit (D. M. Hawley SA 1996). The Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), a serine-threonine kinase that is an important mediator of Ca^{2+}, phosphorylates and activates AMPK kinase from rat liver (S. M. Hawley SA 1995). There are four CaMKII isoforms—α, β, γ, and δ—each encoded by a separate gene. The α and β isoforms are mostly neuronal, whereas CaMKIIγ and δ are expressed in a wide variety of tissues. After binding calcium/calmodulin complex, autophosphorylation on Thr287 results in calcium/calmodulin independent activity. CaMKII expression mediates the activation of AMPK primarily in response to Ca^{2+} (Woods A 2005).

Ca^{2+} is the most abundant ion in the body, primarily stored in bones in the form of $CaPO_3$. In bone, Ca^{2+} plays a structural role and also can be dissolved, serving as a source of cations in the blood (Pozzan T 1994). Ca^{2+} is a ubiquitous and versatile signaling molecule controlling a wide variety of cellular processes, including muscle contraction, neuronal transmission, hormone secretion, organelle communication, cellular motility, fertilization, and cell growth (Clapham DE 2007). Given these critical and diverse functions, cellular Ca^{2+} concentration is tightly regulated, and dysfunction of cellular Ca^{2+} homeostasis is associated with several pathological conditions. Importantly, there is a bidirectional relationship between mitochondrial and cytosolic calcium. Changes in $[Ca^{2+}]c$ (Ca^{2+} cytosolic) regulate mitochondrial oxidative metabolism, whilst the uptake of Ca^{2+} into mitochondria is important in buffering and shaping $[Ca^{2+}]c$ signals (Harzheim D 2009) (Gilabert JA 2000). Therefore mitochondrial function is essential not only to match energy supply with the stimulus-strength of glycogenolytic hormones, but also to regulate the amplitude, duration, propagation rates of $[Ca^{2+}]c$ and therefore finely tuned downstream biological responses.

1.1.2 Second Hit - Oxidative stress mechanism

Generation of oxidative stress (OS) is an important factor in lipotoxicity by virtue of its contribution to cellular stress signalling and interference with mitochondrial functions. Reactive oxygen species (ROS) are generated consequentially the hepatic lipid accumulation in NAFLD/NASH conditions. Overload of liver lipids induces the production of ROS and steatohepatitis and elevated production of ROS, contributes to organelle toxicity, suppression

of fatty acid oxidation, and an increase in lipid peroxidation (Figure 3) (Bartlett PJ 2014) (Rolo AP 2012).

OS condition is the key features during different variety of pathophysiological conditions, by oxidizing membrane phospholipids, proteins, and nucleic acids. The duration of the oxidative injury modulates the cellular alterations and leads from plasma membrane changes (i.e. release of cytosolic substances and mitochondrial permeability) to necrosis condition (Ashraf NU 2015). Therefore, OS can be defined as an imbalance between productions of ROS and their elimination by protective mechanisms.

Although periodic increases in mitochondrial matrix $[Ca^{2+}]m$ (Ca^{2+} mitochondrial) are essential for normal cell function, sustained elevations in $[Ca^{2+}]m$ have deleterious effects on mitochondria and whole cell function and are associated with a number of disease states including metabolic syndrome and cardiac disease. Elevated $[Ca^{2+}]m$ results in enhanced flux through the electron transport chain which generates ROS (Rooney TA 1996). The effects of mitochondrially generated ROS are largely mitigated by the cellular antioxidant system via superoxide dismutase and glutathione. However, $[Ca^{2+}]m$ overload can lead to high levels of ROS formation leading to oxidative stress and ultimately cellular dysfunction (Hu F 2011).
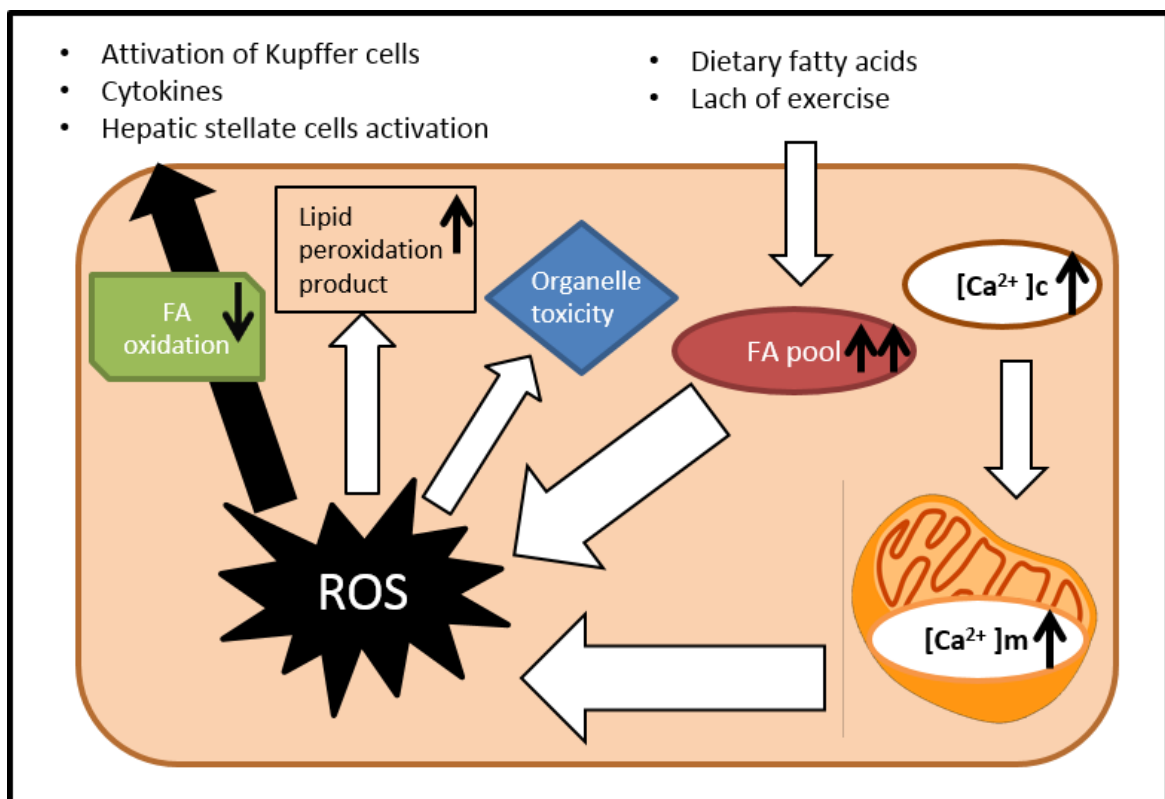

Figure 3. Schematic representation of oxidative stress role in disease progression (Adapted from Rolo AP, 2012).

Another important factor involved in this mechanism is the nuclear factor kappa B (NFκB). NFκB is a ubiquitous nuclear transcription factor in almost all cell types and is involved in numerous biological processes such as OS, inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis. The NFκB has been described as a primary regulator and mediator of OS. It is widely accepted that OS plays crucial roles in the progression of liver injury and, consequently, in the activation of the fibrosis mechanism (Xu J 2003).

It has been documented, that another important molecule that plays a critical roles in NASH, is the extracellular signal-regulated kinases-1/2 (ERK1/2), members of the mitogen-activated protein kinase (MAPK) family with a crucial roles in cellular proliferation, survival, differentiation and homeostasis in normal conditions (Tormos AM 2013). The regulation of cell proliferation and differentiation mediated by growth factors and mitogens during development is mainly regulated by the ERK1/2 pathway (Turjanski AG 2007). In primary hepatocytes reduced survival during ROS exposure was observed when ERK activation was inhibited (Rosseland CM 2005) (Conde de la Rosa L 2006). Comparing primary hepatocytes from young rats (4–6 months) with primary hepatocytes from old rats (24–26 months), the old ones are more sensitive to H_2O_2-induced apoptosis. This decreased survival was associated with reduced activation of ERK, which protect against oxidant injury.

Wang and colleague have been demonstrated that dysregulation of the hepatic ERK1/2 signalling pathway was potential implicated in early-stage liver injury in NASH animal model fed a methionine and choline deficient diet (MCD diet), suggesting that might be represents a novel therapeutic choice for this disease (Wang Z. 2010) (Leclercq IA 2004).

1.1.3 Inflammation

In recent years, the role of the innate immune response in NAFLD has been the focus of intense research. During lipotoxicity, stressed and/or hepatocytes necrosis, are able to release the damage-associated molecular patterns (DAMPs), which can act on various immune cells in the liver starting a homeostatic response to repair tissue injury. Even so, the persistence of these signals can induce an excessive reaction resulting in a full inflammatory response with tissue inflammation and excessive scarring and leading to advanced fibrosis and finally to cirrhosis (Arrese M 2016).

There are different type of cells involved in the inflammatory response during NAFLD progression (Arrese M 2016):

- The Kupffer cells (KCs), resident macrophages of the liver, located in the hepatic sinusoids, the portal tract and hepatic lymph nodes. This cell type derives from circulating monocytes and represents around 15 % of the liver cells, being the largest tissue-specific reservoir of macrophages in the body. The activations of KC, resulting in the production of proinflammatory cytokines and chemokines such as TNF-a, IL-1b, IL-12, IL-13, IL-10, IL-4. This is a key step to trigger local inflammation and to promote additional hepatocyte cell injury, leading to the release of DAMPs. Moreover, some of these cytokines have overlapping proinflammatory and profibrotic properties, contributing to the chemotaxis of inflammatory cells and activation of HSC.

- Dendritic cells (DCs) are tolerogenic immune cells located around the central veins and portal tracts that collectively represent a small fraction of non-parenchymal liver cells and mainly originate in the bone marrow. DC may act as antigen presenting cells as well as in apoptotic cell clearance and removal of necrotic debris. Recent evidence showed that DC rapidly infiltrate the liver, exhibiting an activated immune phenotype expressing increased levels of IL-6, TNF-α.

- Neutrophil accumulation is one of the main features of NASH, and it is thought that this cell type critically contributes to hepatocellular damage in this setting as it can exacerbate the ongoing inflammatory state by contributing to macrophage recruitment and through interaction with antigen-presenting cells.

- Natural Killer cells (NKs) are lymphoid cells that play a role in linking the innate and adaptive immune responses within the liver. Different studies have been shown that NK cells may be activated in NASH in connection with elevated levels of several NK cell-activating cytokines (e.g., IL-12, interferon-c and IL-18) and ligands. Natural Killer T cells (NKTs) comprise a unique immune cell subtype that expresses specific NK cell surface receptors as well as an antigen receptor (TCR) characteristic of conventional T cells. They mainly reside in the sinusoids providing intravascular immune surveillance.

1.1.4 Fibrosis mechanism

In NAFLD, fibrosis takes on a characteristic pattern. All cellular responses during and after these double hits, are converged to activate the hepatic stellate cells (HSCs) and this activation is the responsible of collagen deposition resulting in increased fibrosis.

The pathways of HSC stimulation include signals that promote activation and those that play a part in perpetuation:

- Activation is stimulated by soluble stimuli such as ROS, apoptotic bodies, lipopolysaccharides (LPS) and paracrine stimuli from neighboring cell types including hepatic macrophages (Kupffer cells), sinusoidal endothelium and hepatocytes;

- Perpetuation is stimulated by fibrogenesis itself, proliferation, altered matrix degradation, chemotaxis and contribution of the innate immunity (natural killer cells, dendritic cells, Toll-like receptor).

Resolution of hepatic fibrosis, which occurs following clearance of the primary liver disease, leads to loss of activated HSCs, either through apoptosis, senescence or reversion of activated cells to a more quiescent phenotype (Friedman SL, Evolving challenges in hepatic fibrosis 2010).

The analysis of fibrogenesis are important complements to tests the disease progression. One of the best markers of HSC activation in liver sections is the α-smooth muscle actin (αSMA), the more sensitive indicator of the rate of fibrogenesis and risk of clinical complications. αSMA is an actin isoform that is normally confided to vascular smooth muscle cells, but is also expressed in healing wounds and fibro-contractile lesions (Wang J 2005). Additional previous studies demonstrated that activation and survival of HSCs were closely associated with activation of NFκB and inhibition of its activities is controlled by the activation of PPARγ. These studies suggested that inhibition of NFκB activation might be a potential strategy for prevention and/or treatment of hepatic fibrogenesis (Hellerbrand C 1998) (Lee KS 1995).
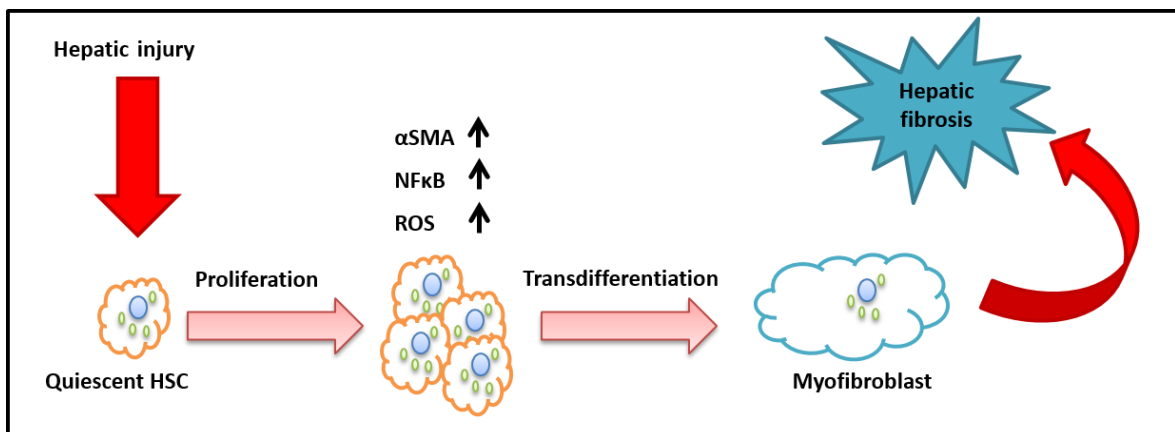

Figure 4. HSC transdifferentiation during hepatic fibrosis progression.

1.2 Histological features and disease diagnosis

The principal histological features of NAFLD (Figure 5) is hepatocellular steatosis and steatosis in more than 5% of hepatocytes is required for the diagnosis of NAFLD (Takahashi Y 2014). Liver steatosis is divided into:

- macrovesicular steatosis: a single large or smaller well-defined fat droplets in hepatocytes cytoplasm, moving the nucleus to the periphery. Steatosis in NAFLD is usually macrovesicular;

- microvesicular steatosis: tiny lipid droplets in the hepatocytes cytoplasm and the nucleus centrally in the cell.

Hepatocellular injury during the disease progression is characterized by ballooning, enlarged hepatocytes characterized as swollen hepatocytes with rarefied cytoplasm. Fat droplets may be observed in ballooned hepatocytes in Haematoxylin and Eosin staining. Hepatocellular ballooning is believed to result from alteration of the intermediate filament cytoskeleton. In ballooned hepatocytes, the two hepatocyte keratins cytokeratins 8 and 18 are disrupted and no longer present throughout the cytoplasm; instead, they are dispersed to the periphery.
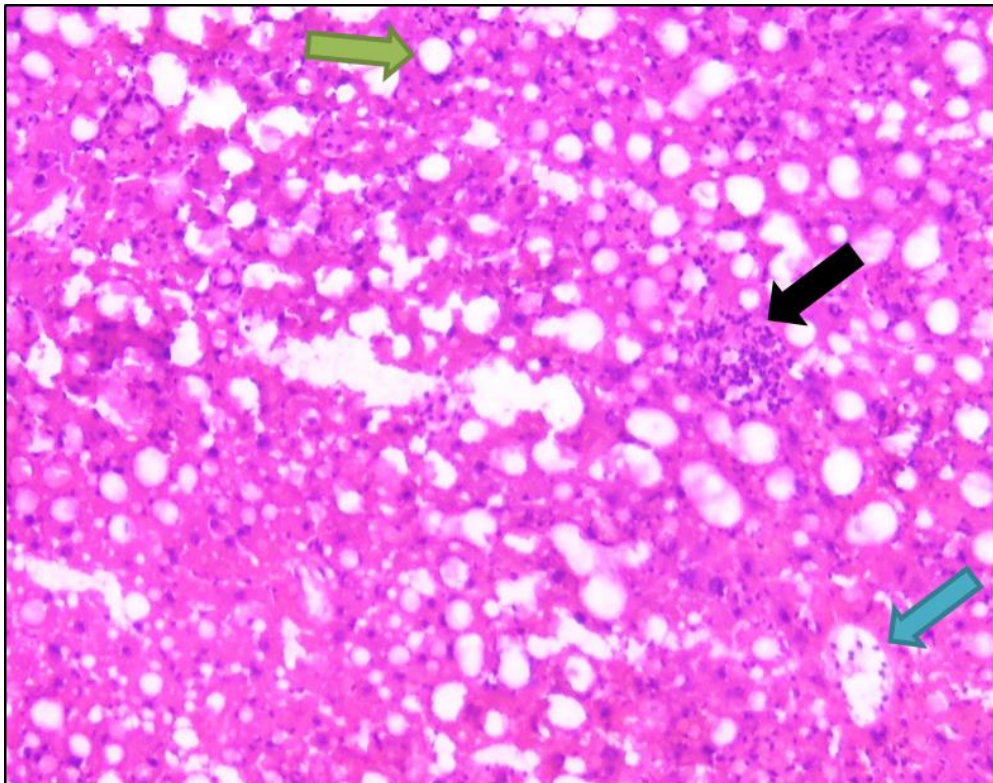

Figure 5. Haematoxylin and Eosin staining (20X) in a 8μm cryosections liver of NAFLD mouse model. Necroinflammatory foci (black arrow), macrovesicular (green arrow) and microvesicular (blue arrow) are distributed in the hepatic lobule.

Intralobular inflammation is also present and it is usually mild, and consists of a mixed inflammatory cell infiltrate (lymphocytes, neutrophils, and Kupffer cells). Scattered lobular microgranulomas (sinusoidal Kupffer cell aggregates) and lipogranulomas (consisting of fat droplets as well as admixtures of inflammatory cells and collagen) are also often observed in NASH. Portal inflammation in NAFLD/NASH is usually absent or mild, and consists mainly of lymphocytes. Chronic portal inflammation (greater than mild) has been associated with the amount and location of steatosis, ballooning, and advanced fibrosis. Therefore, greater than mild chronic portal inflammation in untreated NAFLD could be considered a marker of advanced disease.

Another important histological feature is the fibrosis. The characteristic pattern of fibrosis in NASH is perisinusoidal/pericellular fibrosis and Masson trichrome staining can be useful to evaluate fibrosis (Figure 6). In early lesion, fibrosis begins as a delicate perisinusoidal deposition of collagen fibers. In more advanced lesions, collagen fibers may encircle hepatocytes. Pericellular fibrosis can progress without the development of any appreciable periportal fibrosis for a long time, but commonly, periportal fibrosis may develop. Fibrosis in NAFLD is usually observed with an active necroinflammatory reaction; however, fibrosis without active lesions can also occur, and prior episodes of steatohepatitis are suggested in such cases.
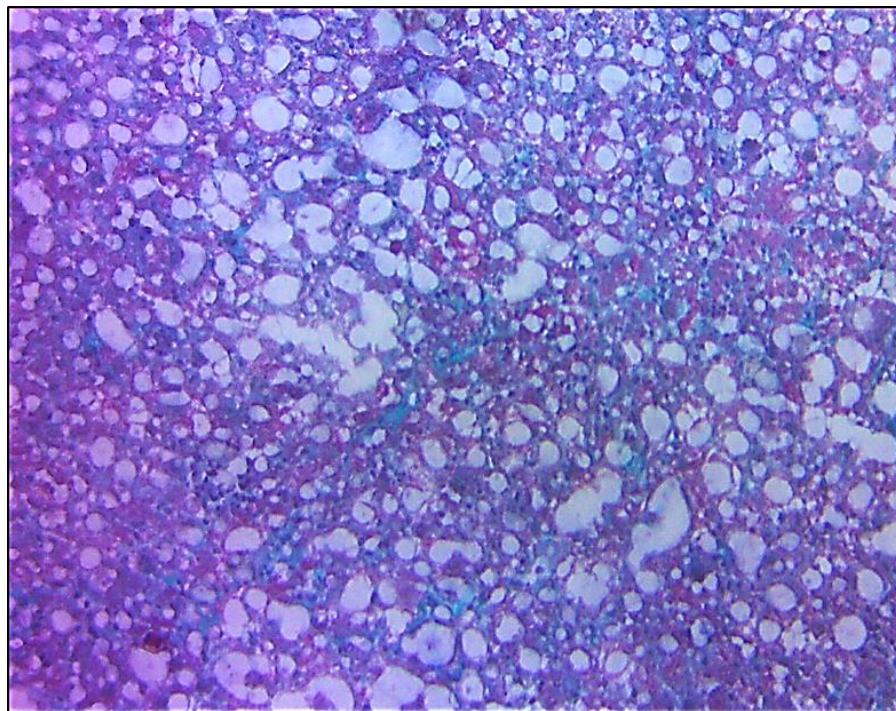

Figure 6. Masson Goldner trichrome staining (20X) in a 8μm cryosections liver of NAFLD mouse model.

In the 1999, Brunt and colleague classified all of these histological features (J. C. Brunt EM 1999). They proposed to classify the necroinflammatory grades of NASH as: grade 1 (mild), grade 2 (moderate), and grade 3 (severe) based on the degree of hepatocellular steatosis, ballooning and disarray, and inflammation (intralobular and portal). Simultaneously, they proposed a scoring system for staging based on the location and extent of fibrosis: stage 1, zone 3 perisinusoidal fibrosis; stage 2, portal fibrosis with the abovementioned stage 1; stage 3, bridging fibrosis in addition to stage 2; and stage 4, cirrhosis. Then, the NASH Clinical Research Network (NASH CRN) subclassified stage 1 into 3 categories: stage 1A, mild perisinusoidal fibrosis in zone 3; stage 1B, moderate perisinusoidal fibrosis in zone 3; and stage 1C, only portal/periportal fibrosis (Kleiner DE 2005). The NASH CRN designed the NAFLD activity score (NAS) for use in clinical research. This score can also be used for the full spectrum of NAFLD, including simple steatosis. The score is calculated as the unweighted sum of the scores for steatosis (0-3), lobular inflammation (0-3), and ballooning (0-2), and ranges from 0 to 8 (Table 1).

ITEM	DEFINITION	SCORE
Steatosis	< 5%	0
	5 % - 33 %	1
	> 33 % - 66 %	2
	> 66 %	3
Lobular inflammation	No foci	0
	< 2 foci per 200X field	1
	2-4 foci per 200X field	2
	> 4 foci per 200X field	3
Ballooning	None	0
	Few balloon cells	1
	Prominent ballooning	2

Table 1. Score for histological evaluation of nonalcoholic fatty liver disease components (Adapted from Takahashi Y, 2014).

Recently, a new definition based on the SAF score, which does not sub-classify patients with NAFLD based on NASH, has been proposed (Bedossa P 2014). The SAF score assesses three variables: S = steatosis, A = activity, F = fibrosis and steatosis is classified on a scale of 0 to 3 (S0: <5%, S1: 5–33%, S2: 34–66%, S3: >67%). However, this scoring system has been shown to decrease intra-observer variation among pathologists.

The diagnosis of NAFLD requires that there are evidence of steatosis either by imaging or histology and that there are no secondary causes of steatosis, such as increased alcohol consumption, viral hepatitis, use of steroid medications or other causes. Steatosis can be diagnosed either using invasive and/or non-invasive technique. Non-invasive technique includes imaging techniques such as ultrasound or proton magnetic resonance spectroscopy (^{1}H-MRS). Steatosis on ultrasound and/or proton magnetic resonance spectroscopy does not, however, exclude NASH, as they merely quantify steatosis. Invasive technique includes liver biopsy, the gold standard for diagnosis of NAFLD. Histologically, NAFLD encompasses any degree of steatosis alone or steatosis with lobular inflammation but without ballooning. NASH can only be diagnosed by liver biopsy. The presence of ballooning injury is the key to the diagnosis. Ballooning degeneration is a form of hepatocyte cell death where the cells increase in cell size (balloon). Steatosis and inflammation can be observed to any degree. Fibrosis is not required to make the diagnosis of NASH but is often present (Yki-Järvinen H 2016).

1.3 Cardiovascular complications

Liver fat could be an important ectopic fat depot that confers additional risk over other visceral fat depots. One of the most important mortality and morbidity reasons of NAFLD are cardiovascular events. Multiple pathogenetic conditions contribute to the development of cardiovascular diseases (CVD).

The Framingham Heart Study, initiated in 1948 in response to the rapidly increasing incidence of cardiovascular-related death, is the longest running prospective cohort study in the USA. Through over 65 years of discovery, the Framingham Heart Study has contributed to understand obesity, type 2 diabetes mellitus and prediabetes mellitus, the metabolic syndrome and NAFLD, and to how these conditions relate to our overall and cardiovascular-related mortality (Long MT 2016). Recent results, from the Framingham Heart Study (Ma J 2016), revealed that there was a significant association between NAFLD and CVD independently from many other metabolic diseases. NAFLD, not only is a marker of CVD and cardiac function abnormalities but also might be involved in their pathogenesis, possibly through the systemic release of several pathogenic mediators from the steatotic and inflamed liver and other inflammatory cytochines.
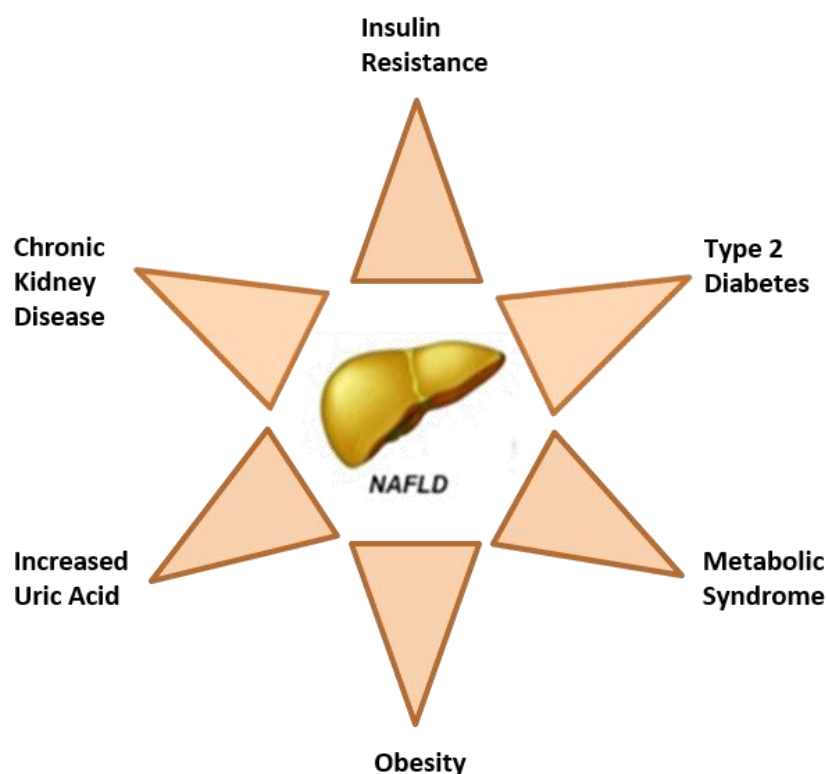
Figure 7. Patients with NAFLD have many CVD risk factors and the typical traits of the metabolic syndrome (Adapted from Lonardo A 2016).

As shown in Figure 7, patients with NAFLD have a many of traditional and non-traditional risk factors for CVD. However, several CVD risk factors associated to the metabolic syndrome are common in patients with NAFLD so it is not clear if this increased CVD risk in patients with NAFLD might be itself a mediator of atherosclerosis (Lonardo A 2016).

In the same way as the liver, cardiac lipotoxicity is associated with increasing ROS production, which leads to damage and death of myocardiocytes. Indeed, in the healthy heart, contractile function depends on the production of intracellular ATP derived primarily from a balance between fatty acids and carbohydrate oxidation. This dynamic use of fatty acids and glucose for energy production is largely a result of fluctuations in plasma fatty acids availability and the resulting changes in fatty acids entry into the cardiac. Immediately following uptake, fatty acids are converted into long-chain acyl-CoA esters that can be used to form acylcarnitines for transport into mitochondria. Once inside the mitochondria, the re-formed acyl-CoA esters undergo β-oxidation to produce acetyl-CoA that, upon entering into

the tricarboxylic acid (TCA) cycle, undergoes further metabolism to provide the energy for ATP production (Brindley DN 2010). Although the healthy heart relies normally on fatty acids oxidation for its predominant energy supply, an overreliance on fatty acids compared with glucose for energy production has been associated with ventricular dysfunction (Brindley DN 2010). Increased fatty acids uptake (for example in diabetes condition) favours the oxidation of fatty acids rather than glucose (especially when combined with insulin resistance). If fatty acids supply exceeds the capacity for fatty acids oxidation, then the excess fatty acids are incorporated into triglycerides, resulting in cardiac steatosis. As in the liver, β-oxidation of long-chain fatty acids can also release electrons to form ROS and may induct of oxidative stress (Mellor KM 2010).

The concomitance of liver and myocardial injury in patients with NAFLD and the probably common molecular pathways of both damages are encouraging therapies to improve liver histology and to control myocardial damage.

1.4 Epidemiology

In the global increases of obesity and diabetes in western countries, NAFLD represents the most common cause of liver dysfunction. However, the lack of sensitivity and specificity of the test used for the diagnosis limited the correct estimates of prevalence *(Bhala N 2013)*. All risk factor of CVD, such as insulin resistance and/or type 2 diabetes, dyslipidemia, hypertriglyceridemia and hypertension characterized the majority of NAFLD patients. In fact, prevalence of NAFLD has been reported in over 76% of type 2 diabetics and over 90% of severely obese patients undergoing bariatric surgery. Given the common risk factors between NAFLD and CVD, cardiac-related death is one of the leading causes of death for NAFLD patients. It is alarming that the prevalence of NAFLD worldwide is thought to be on the rise. The prevalence of NAFLD in the Europe is reported to be between 10% and 30%, with similar rates reported from United States and Asia as reported in Table 2. *(Younossi ZM 2016)*.

REGION	PREVALENCE (%)
Africa	13.48
Asia	27.37
Europe	23.71
Middle East	31.79
North America	24.13
South America	30.45

MEAN AGE	PREVALENCE (%)
30-39	22.43
40-49	26.53
50-59	27.40
60-69	28.90
70-79	33.99

Table 2. NAFLD Prevalence Stratified by Region and Mean Age (Adapted from Younossi ZM, 2016).

Table 2, clearly indicates that the prevalence of NAFLD increases with age. However, very recent evidence suggested that NAFLD in children is becoming a major health concern. Remarkably, the overall prevalence of NAFLD in children has reached approximately 10%, including up to 17% in teenagers and 40%-70% among obese children. While often benign and self-limiting, steatosis can progress with hepatocyte injury into non-alcoholic steatohepatitis (NASH) in 3%-5% of patients. Obesity, hypertension, and other components of metabolic syndrome, including steatosis and severe liver damage has been associated with the progressively increased intake of added sweeteners in the last few decades. When compared with their obese counterparts without NAFLD, the obese children with NAFLD consume more carbohydrates. The epidemics of pediatric obesity and obesity related liver disease (NAFLD and NASH) represents a serious problem. Increasing evidence indicates that affected children are at risk of significant progressive hepatopathy if inflammation and/or advanced fibrosis are already present (Clemente MG 2016).

1.5 Treatments

Adult and pediatric NAFLD does not have a globally efficacious treatment. Lifestyle interventions (i.e. dieting and exercise) represent the pillar treatment, while effective interventions in adults and children are challenging due to a lack of compliance (Clemente MG 2016). In addition to the physical training and diet control, there is no consensus on the most effective pharmacological therapies for NAFLD/NASH caused by the complex pathophysiology. The common approach involves the therapy of the disease complications, such as hepatic fat accumulation, insulin resistance, inflammation, and fibrosis. For example, pioglitazone and metformin, common treatments for glucose intolerance, can enhance insulin sensitivity in patients with NAFLD/NASH; however, other histological features such as fibrosis, are not significantly treated. Pirfenidone, a therapeutic agent used for fibrosis, can reduce the serum alanine aminotransferase and aspartate aminotransferase concentrations and

has anti-fibrotic and anti-inflammatory properties that help to reverse liver injury (Chen G 2016) (P. L. Del Ben M 2014).

NAFLD, a major challenge to healthcare systems worldwide, has no medications approved by the United States Food and Drug Administration. As mentioned above, most people with fatty liver do not develop severe liver disease but have an increased chance of developing cardiovascular diseases. For these reasons, the treatment of NAFLD patients should be based on a global approach, not only addressing the treatment of insulin resistance and metabolic syndrome, but also including strategies focused on reducing oxidative stress, dyslipidaemia and cardiovascular risk. So, next to the many therapeutic strategies approaches, several types of nutraceuticals have been suggested for the treatment of NAFLD and NASH, the most promising of which are those with anti-inflammatory and antioxidant effects (P. L. Del Ben M 2016). In this context, nutraceuticals may have an important role in NAFLD and cardiovascular risk treatment, in combination with the conventional medicament.

1.6 Nutraceutical compounds

1.6.1 History

The term "nutraceutical" was coined from "nutrition" and "pharmaceutical" in 1989 by Stephen L. DeFelice, MD, founder and chairman of the Foundation for Innovation in Medicine (FIM). According to DeFelice, nutraceutical can be defined as "a food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of a disease" (Brover V 1998). In Italy, dietary supplements are defined (Directive 2002/46/EC, implemented by Decreto Legislativo del 21 May 2004, n. 169) as "foodstuffs to supplement the common diet and which are concentrated sources of substances nutrients, such as vitamins and minerals, or other substances with a nutritional or physiological effect, in particular, but not limited to, amino acids, essential fatty acids, fiber and plant-derived extracts, alone or in combination, in unit dose form" (Ministero della Salute s.d.).

Dietary supplements are used by a substantial part of the general population, and the available evidence suggests that the rate of use is even higher among people and patients and the pattern of use varies between type of diet (i.e. vegan) or/and type of disease.

1.6.2 L-Carnitine

L-carnitine (LCARN) was discovered in 1905 as a constituent of muscle tissue (Gulewitsch and Krimberg, 1905; Kutscher, 1905). It owes its name to the high concentration

with which it occurs in meat and its chemical structure was elucidated some 20 years later (Figure 8). After the early 1940s its stereospecificity was clarified and in the 1960s, researcher described the antiomeric forms occurs in the body. The biological activity of LCARN, named vitamin T, was firstly described as essential nutritional factors in mealworms larvae, which are popular with aquaculturists, as fish food. The "T" in this abbreviation stands for the Latin name for the mealworm, *Tenebrio molitor*. Mealworm larvae growing in a state of LCARN deficiency accumulate excessive amounts of fat in their cells and yet seem to die of starvation. This suggested that LCARN might play a role in the oxidation of fat. The function of LCARN in mammals, however, remained a mystery for a long time. In 1955 Fritz and colleague, discovered that adding LCARN to muscle extracts stimulates the oxidation of palmitate. This observation led to the discovery of the mitochondrial carrier function of LCARN and its important role in the burning of free fatty acids in 1962 by Bremer (Wolf G 2006).

Many further biological functions of LCARN have become known since then. The fatty acids with chain lengths of 12 or fewer carbons enter mitochondria without the help of membrane transporters. Those with 14 or more carbons, which constitute the majority of the FFAs obtained in the diet or released from adipose tissue, cannot pass directly through the mitochondrial membranes—they must first undergo the three enzymatic reactions of the carnitine shuttle (Nelson DL Sixth Edition).
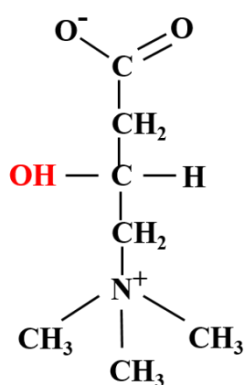

Figure 8. L-carnitine, (β-hydroxy-γ-trimethyl-aminobutyrate). In aqueous solution LCARN, being a zwitterion, is freely soluble in water as its ionisable groups (COO$^-$ and N$^+$(CH$_3$)$_3$) are over 90 % dissociated at a physiological pH (~7.4). In red, the binding site for acyl residues.

The hydroxyl group at the C_2 location of LCARN is virtually undissociated in solution, but is of great physiological significance because it determines one of its functions in the body. The β-hydroxyl group of LCARN reacts with activated fatty acids (acyl-CoA

compounds) and, catalysed by L-carnitine acyltransferases (CAT), forms energy-rich acetyl-L-carnitine in the process. This occurs in principle by the following reaction:

$$\text{acyl-CoA + L-carnitine} \xrightleftharpoons{\text{CAT}} \text{acetyl-L-carnitine + CoA}$$
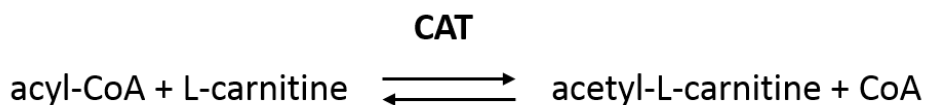
This reaction is reversible, as required, depending on cellular localisation (intra- and extra-mitochondrial) and chain length of the acyl residues. It is catalysed by various L-carnitine acyltransferases.

LCARN is absorbed actively and passively in the intestine. The transport capacity is low when compared with that for glucose and amino acids. LCARN is presumably partially esterified in the intestine before being released into the blood. The liver receives some LCARN from the portal blood and releases it with a time delay back into the blood. The liver with the bile also releases LCARN esters (enterohepatic cycle). LCARN and LCARN esters are readily filterable in the renal glomeruli. In animals with a normal LCARN status, more than 98% of free LCARN is absorbed via the tubules. The absorption of LCARN esters is less efficient. However, tubular absorption can be modified depending on dietary supply, requirement (Wolf G 2006).

1.6.3   L-Carnitine and NAFLD

In recent years, the effects of nutraceuticals on NAFLD have received increasing attention and several types of these agents have been suggested for the treatment of NAFLD/NASH. For some of them, a number of clinical trials highlighted an improvement in liver function tests and a possible positive influence on liver histology (Somi MH 2014) (Malaguarnera M 2010). Very recently *in vivo* studies have been indicated the oxidative mechanism as an important therapeutic intervention strategy for NAFLD (Li W 2016). It has been shown that LCARN administration can ameliorate or prevent liver damage of various aetiologies reinforcing the hepatic mitochondria β-oxidation and the activity of the key ROS-scavenging antioxidant enzymes without an increase in oxidative stress (Ishikawa H 2014).

Several nutraceutical supplements have shown promising results, especially those containing antioxidants and polyphenols. However, present information derives mostly from small trials with considerable heterogeneity with respect to inclusion criteria, sample size, type of experimental interventions and duration. A Cochrane review in 2007 (Lirussi F 2007),

reported that there was insufficient data to either support or refute the use of antioxidant supplements for patients with NAFLD and it may be advisable to carry out large prospective randomised clinical trials on this topic (Lirussi F 2007). In 2016, there are still insufficient data to either support or refute the use of nutraceuticals for subjects with NAFLD. Further randomized controlled studies, with histological changes as an outcome measure, are needed.

1.6.4 *L-Carnitine and cardiovascular disease*

Carnitine deficiency has even been associated with heart failure and supplementation has been recommended in the treatment of CVD and in selected patients with acute myocardial infarction (Gaby AR 2010). In rodent studies, LCARN improves myocardial ischaemia/reperfusion injury via stimulation of glucose oxidation (Broderick TL 1996).

A well-publicized study by Koeth and collaborators. (Koeth RA 2013), indicated that intestinal microbiota can metabolize carnitine to trimethylamine, which is then oxidized in the liver to the proatherogenic trimethylamine-N-oxide (TMAO). In a mouse model, chronic LCARN therapy altered cecal microbial composition, enhanced synthesis of TMAO, and increased atherosclerosis, but this did not occur if antibiotics concurrently suppressed intestinal microbiota. Following this work, nutrition scientists have argued the merits and limitations of the study, resulting in the current state of discord (L. G. Ussher JR 2013). Some investigators have suggested that reducing dietary LCARN and choline is the simplest and safest means to control TMAO levels, and that the development of novel therapeutics targeting the gut microbiome may lead to improved CVD outcomes (Mendelsohn AR 2013). Ussher and collaborators (L. G. Ussher JR 2013) noted that in the Koeth study (Koeth RA 2013), LCARN was delivered concurrently with red meat, confounding the experiment and those experimental doses of LCARN were up to 1000 times higher than a 220gr steak. In addition, LCARN and choline are significant contributors to bile secretions by mass and were likely reabsorbed prior to reaching the cecum/colon for bacterial conversion to TMAO, further confounding biological interpretation

Nevertheless, LCARN has also been recognized as a nutritional supplement in cardiovascular disease and it is currently used as an adjunctive therapy in various heart conditions with promising results (Johri AM 2014) (Strilakou AA 2013).

1.7 Mouse models of NASH

As thoroughly described, the development and progression of NAFLD to NASH represents a complex pathophysiological process. To date, no single animal model has encompassed the full spectrum of human disease progression, but they can imitate particular characteristics of human disease; for these reasons, the appropriate animal selection is very important.

The animal models of NASH could be divided into (Habbard L 2011):

- Dietary-based animal models: MCD diet, high fat, cholesterol and cholate, fructose.
- Genetic models: adipocyte-mediated inflammation, adiponectin, regulation of Wnt signalling, high‑fat‑diet‑promoted adipocyte apoptosis, linking adipose inflammation and steatosis.
- Additional causes of fatty liver: cholesterol, innate immunity, steatotic polymorphisms and mouse models.

The primary driver of NAFLD is over-nutrition and a sedentary lifestyle leading to increased weight and, ultimately, obesity. The majority of animal models focus on providing a diet that cause liver damage. The model used most often is actually nutrient deficient. The methionine and choline deficient (MCD) diet is high in sucrose and fat (40% sucrose, 10% fat), but lacks methionine and choline, which are essential for hepatic β-oxidation and the production of VLDL. This results in the accumulation of intrahepatic lipid and decreased VLDL synthesis. Mice fed the MCD diet lose weight (up to 40% in 10 weeks). With a loss of white adipose tissue, the liver decreases proportionally in size, develops prominent pericentral steatosis, subsequent necroinflammation and fibrosis, resembles that seen in human NASH. MCD fed mice exhibit increased inflammation by way of activated macrophage infiltration into the liver, the activation of NFκB and concomitant increases in interleukin 6, transforming growth factor β and tumor necrosis factor (Habbard L 2011). The main advantages of the MCD diet are that it is widely available and replicates NASH histological phenotype within a relatively shorter feeding time than other dietary models of NASH.

The severity of NASH in mice fed the MCD diet depends on rodent gender and strain. Kirsch and colleague (Kirsch R 2003) analysed male and female C57/BL6 mice fed the MCD diet with respect to liver histology (steatosis and necroinflammation), ultrastructure and lipid biochemistry and compared with Wistar rats, the most susceptible rat strain. In this study, male C57/BL6 mice showed ultrastructural evidence of hepatocyte injury, including profound

mitochondrial enlargement, blebbing of nuclear membranes, and dissociation of rough endoplasmic reticulum from mitochondria. Mitochondrial injury in these animals may be a result of oxidative stress and consequent lipid peroxidation of mitochondrial membranes. Mitochondrial injury activates hepatocyte apoptotic mechanisms and results in hepatocyte necrosis, a prominent feature in male C57/BL6 mice. The increased lipid peroxidation in these mice compared with Wistar rats may, explain differences in necroinflammation in these animals. In conclusion, male C57/BL6 mice develop the histological features that most closely resemble those seen in human NASH.

Another important characteristic in mice fed a MCD diet, is the development of hepatic steatosis characterized by increased free fatty acid (FFA) and triglyceride levels as well as expression of αSMA as marker of activation of HSC and the consequent fibrosis mechanism (Lee SJ 2015).

2.   PRELIMINARY *in vitro* STUDY

As mentioned, NAFLD, a major challenge to healthcare systems worldwide, has no medications approved by the United States Food and Drug Administration. So, next to the many therapeutic strategies approaches, several types of nutraceuticals have been suggested for the treatment of NAFLD and NASH, the most promising of which are those with anti-inflammatory and antioxidant effects (P. L. Del Ben M 2016). In this context, nutraceuticals may have an important role in NAFLD in combination with the conventional medicament.

In our previous work (Montesano A Under Submission), we analyzed the role of LCARN in liver steatosis condition in an *in vitro* model of fructose‑induced lipid accumulation. The effects of LCARN supplementation on HepG2 cells fat deposition, antioxidative response and mitochondrial pathway activation were studied with or without fructose overload (Zhao L 2016). Research results of the last several years, confirmed the initial assumption that HepG2 cells are an excellent tool to detect metabolic properties of biological compounds (Knasmüller S1 2004) and expresses most of the marker proteins of human hepatocytes. Our data showed that LCARN supplementation reduced HepG2 intracellular lipid accumulation induced by fructose. Moreover, LCARN treatment stimulated AMPK activation trough the increase of CaMKII stimulation. Overload of lipids in HepG2 cells, induces the abundant production of ROS that contributes to organelle toxicity and an increase in lipid peroxidation. In our work, LCARN decreased ROS production. Taken together all of these data confirmed the potential protective role of LCARN supplementation.

Our preliminary expertise in the nutraceutical research field and the experimental evidence obtained in an in vitro model of NAFLD, endorses us to demonstrate the role of nutraceutical supplementation in an in vivo mouse model of NAFLD.

3.   AIM OF THE STUDY

Non-Alcoholic Fatty Liver Disease has no approved medications and innovative therapeutic strategies are needed. Nutraceuticals are defined as a new frontier between drugs and food. Due to the complex pathogenesis and complications of the NAFLD, several types of these agents have been suggested for the treatment and the oxidative mechanism is resulted the key target. Malaguernera and collaborators (Malaguarnera M 2010) demonstrated that LCARN supplementation is useful for improving liver function and histological manifestations of NAFLD progression. However, the mechanisms whereby LCARN could mediate its action on liver function and NAFLD complications is still unclear.

The aim of the study was to investigate the nutraceutical effects of LCARN supplementation on liver fat deposition, oxidative stress and fibrosis development mechanism in a mice model of steatohepatitis induced by a methionine-choline deficient diet (MCD diet). Liver fat could be an important ectopic fat depot that confers additional risk over other visceral fat depots. One of the most important mortality and morbidity reasons of NAFLD are cardiovascular events. For this reason, in the same *in vivo* model we also analyzed the shared role of LCARN in cardiac tissue.

4.   EXPERIMENTAL PROTOCOL

As reported in literature (Habbard L 2011) (Kirsch R 2003), animals fed the MCD diet lost weight, led to steatohepatitis and best approximated the histological features of human NASH. After two weeks of acclimation, ten-week-old male C57BL/6 mice were divided into three experimental groups: one group (n=10) were fed with 120g/week of normal diet (CONTR) and two groups (n=10 each group) with 120g/week of MCD diet (MCDD) for nr. 6 weeks. After the first 3 weeks, one of the MCDD food group was enriched with 200mg/kg/die oral LCARN (MCDD+LCARN) until the end of the experiments. All animals were sacrificed at the end of the experiments (nr. 6 weeks of diet) as reported in Figure 9.
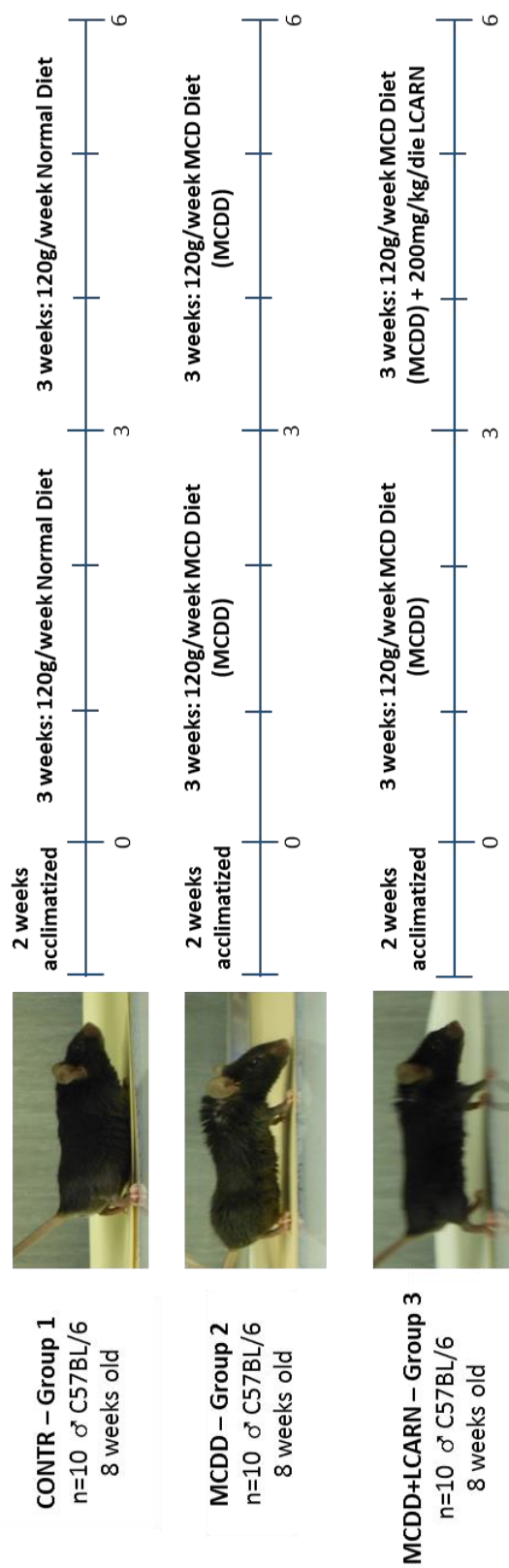
Figure 9. Experimental protocol.

5. MATERIALS AND METHODS

This study was conducted in compliance with approved institutional animal care of the University of Milan.

5.1 Materials

Reagents were purchased from Sigma Chemical Co. (Saint Louis, MO, USA). Primary antibodies against AMPKα 1/2 (sc-25792), CaMKII (sc-9035), pCaMKIIα (sc-12886-R), ERK2 (sc-154), pERK 1/2 (sc-7383), GAPDH (sc-25778), NFκBp65 (sc-109), PPARγ (sc-7196), αSMA (sc-53142), peroxidase-conjugated secondary antibodies for Western blot analysis, and rhodamine-conjugated antibodies for immunofluorescence analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies STAT3 (#9132) and phospo-STAT3 (#9131) were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell ROX® Oxidative Stress Reagents Kit (C10443) from Thermo Fisher Scientific, Life Technologies Italia (Monza-Italy).

Masson-Goldner staining kit (100485), Weigert's iron hematoxylin kit (115973), Entellan®Neo (107961) from Merck Millipore, Merck KGaA (Darmstadt, Germany).

Mayer's Hematoxylin Solution (MHS16-500ML), Eosin Y Solution 0,5% acqueous (HT110216-500ML), Scott's Tap Water Substitute Concentrate (S5134), Oil Red O solution 0.5% in isopropanol (O1391-500ML),Glycerol gelatin aqueous slide mounting (GG1-15ML), Formalin Solution 10% (HT501128-4L) and Bouin's Solution (HT10132-1L) from Sigma-Aldrich® (Sigma-Aldrich Co. LLC.).

5.2 Animal Studies

Animals and experimental protocol

Male C57BL/6 mice (n=30), purchased from Charles River Laboratories (Boston, MA, USA), were used for the study at 10 weeks of age. All animals were kept on a 12 h/12 h light/dark cycle with unlimited access to standard rodent chow food and water. Mice were divided into three paired groups: one group (n=10) were feed with 120g/week of normal diet (CONTR) and two groups (n=10 each group) with 120g/week of MCD diet (ssniff® EF R/M Induction of fatty liver – Ssniff Spezialdiäten GmbH) (MCDD) for nr. 3 weeks. Food of one

of the MCDD group after the first 3 weeks, was enriched with 200mg/kg/die oral L-carnitine (MCDD-LCARN) for other nr. 3 weeks and all animals were sacrificed at the end of the experiment. As reported in literature (Hebbard L 2011) (Kirsch R 2003), mice fed the MCD diet lose weight and promoted liver damage.

LCARN Treatment

LCARN was added to the drinking water at dose of 200 mg/kg body weight per day, for 3 weeks. Control mice received water without LCARN. In our pilot study, water and LCARN were changed daily and the dose adjusted to weight gain each week.

5.3 Experimental Procedures

Protein extracts

Liver and heart protein extracts, were obtained from homogenized mouse tissues by using the following lysis buffer containing: 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM sodium orthovanadate (Na3VO4), 1 mM EDTA, 1 mM PMSF, 1 mg/mL aprotinin, 1 mg/mL leupeptin, and 1 mg/mL pepstatin and shaked for 1 h at 4°C. Detergent-insoluble material was removed from the cell suspension by centrifugation at 12.000 x g for 30 min at 4°C. Protein contents were quantified using Bradford method.

Western Blot Analysis

Aliquots of quantified 30 μg supernatant proteins, were resolved on SDS-PAGE gel and transferred onto nitrocellulose membrane (Protran, Whatman Schleicher & Schuell). The membranes were incubated with specific primary antibodies and then with HRP conjugated anti-species-specific secondary antibodies. To confirm equal protein loading per sample, we used antibody anti-GADPH. Quantitative measurement of immunoreactive bands intensities, visualized by an enhanced chemiluminescence method (Amersham Pharmacia Biotech, Piscataway, NJ, USA), was performed by densitometric analysis using the Scion Image software (Scion Corporation, Frederick, MD, USA). Data were then converted into fold-changes (FC) of the controls.

Histopathologic Analysis

At the end of the study, animals were ether anesthetized, killed, and their livers and heart were removed, weighed and sampled for histological assessment. Additional samples were snap frozen in liquid nitrogen for later analysis of liver lipid levels and lipid peroxidation.

Liver and heart 8 μm frozen cryosections, were examined with different staining technique. Tissue sections were examined with Hematoxilin and Eosin (HE) (Sigma) staining according to the manufacturer's instruction. Images were acquired by phase contrast microscopy and lipid droplets area was calculated with Image J software.

Staining of intracellular lipid droplets was performed with Oil Red O (ORO) (Sigma) technique according to the manufacturer's instruction.

Masson-Goldner (MG) (Merck Millipore) staining method is a trichrome stain that is primarily used for imaging connective-tissue structures in organs. Connective tissue is then counter stained using light green SF solution manufacturer's instruction.

Immunofluorescence Analysis

Liver and heart 8 μm frozen cryosections, were fixed 4% paraformaldehyde for 30 minutes at room temperature. After that, sections were washed with PBS and permabilized with 0.2% Triton X-100. Then were incubated for 30 minutes at room temperature with 10% donkey serum. Finally, were immunostained with specific primary and secondary antibodies, Rhodamine- or FITC-conjugated, and nuclei were revealed with DAPI staining.

Cell ROX® Oxidative Stress Reagents are fluorogenic probes designed to reliably measure reactive oxygen species (ROS) in tissue. The cell-permeable reagents are non-fluorescent or very weakly fluorescent while in a reduced state and upon oxidation exhibit strong fluorogenic signal. Cell ROX® Orange Reagents are localized in the cytoplasm. This staining were performed on CONTR, MCDD and MCDD-LCARN mice liver at the end of the experiments. Slides were mounted with Moviol.

Tissue sections were observed using Nikon Eclipse 50I microscopy and images were captured using Nis-Elements D 4.00 software (Nikon Instruments Europe BV, Netherlands). Data were displayed and analyzed using Adobe Photoshop CS4.

Automated quantification on the immunofluorescence signal was performed using Image J program (http://imagej.nih.gov/ij/), as described (Montesano A Under Submission).

5.4 Statistical Analysis

All experiments were performed three times. Statistical analysis were performed with GraphPad Prism software (GraphPad). Data were presented as the mean ± SD or SEM. Multiple comparisons on data sets were performed using t-test, one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. Results were considered significant when $p \leq 0.05$.

6.   RESULTS

Body weight and LCARN supplementation

There were significant differences in body weight and in food consumption among the three groups (Figure 10A and B). A weight reduction was observed in MCDD and MCDD+LCARN groups, comparing the beginning and the end of the experiment and the MCDD and MCDD+LCARN mice weight were significantly different compared to CONTR mice at each week. At week 4 and 5, the MCDD mice eat/weight compared to the CONTR were significantly different.

Water consumption in the three group recorded every day was not influenced by LCARN oral supplementation (Figure 10C).
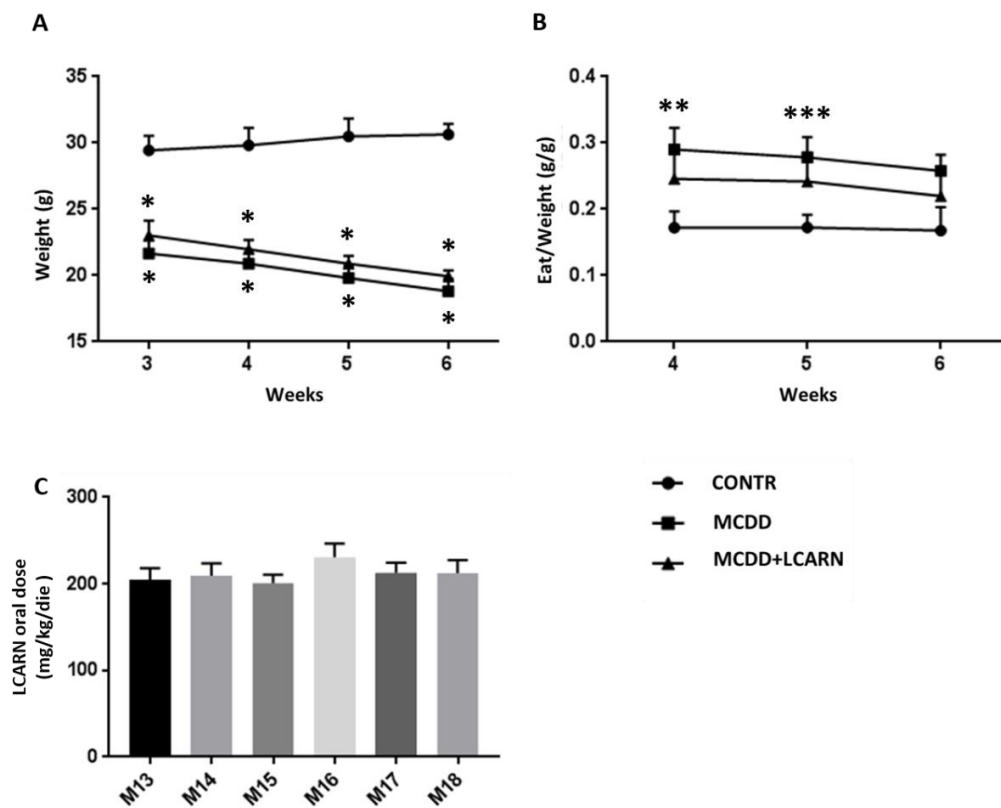
Figure 10. Body weight, food consumption and LCARN supplementation. (**A**) Body weight of the three experimental groups. Every weeks, mice were weighted. (**B**) Food consumption of the three experimental groups. (**C**) Water consumption and oral LCARN supplementation in MCDD mice group were recorder each day. Data are presented as mean ± SEM. Statistical significances: Mice weight by Anova $p \leq 0.0001$; Tukey's multiple comparisons test *$p < 0.0001$ CONTR vs MCDD and CONTR vs. MCDD+LCARN each week. Mice eat/weight by Anova $p \leq 0.0001$; Tukey's multiple comparisons test **$p = 0.0231$ CONTR vs MCDD week 4, ***$p = 0.0169$ CONTR vs MCDD week 5. L-carnitine oral dose by Anova with no significant differences among means.

All of the below results were obtained after the mice sacrificed at the end of the experiment.

Liver mass, morphology and histology

Macroscopically livers from MCDD mice appeared pale and fatty compared with controls and mice received LCARN supplementation (Figure 11A).

The livers of CONTR mice in mass were 14.2% heavier than MCDD group and 13% than mice received LCARN supplementation, while the livers of MCDD+LCARN in mass were 1.4% heavier than MCDD mice (Table 3). To control the loss of body mass induced by the MCD diet, liver mass was expressed as a percentage of body mass (relative liver mass).

As shown in Figure 11B, the MCDD group developed hepatocyte steatosis and ballooning at 6 weeks at HE-staining. Hepatocyte steatosis and ballooning was significantly reduced after 3 weeks of LCARN supplementation. Percentage of tissue area containing hepatic lipid droplets was significantly lower in the group supplemented with LCARN (Figure 12). These results were confirmed with ORO-staining technique as shown in Figure 11C.

	CONTR	MCDD	MCDD+ LCARN
Liver mass (g)	1.65 ± 0.21	0.90 ± 0.27	0.94 ± 0.13
Relative liver mass (as % of body mass)	5.46 ± 0.83	4.68 ± 1.19	4.75 ± 0.68
Percentage differences in relative liver mass **MCDD and MCDD+LCARN vs. CONTR** (%)		14.2	13.0
Percentage differences in relative liver mass **MCDD vs. MCDD+LCARN** (%)		1.4	

Table 3. Liver mass absolute and relative and percentage differences in relative liver mass resulting from the MCD diet at the end of the experiment. Relative liver mass are expressed as a percentage of body mass. Data are expressed as a mean ± SD for each group.

Figure 11. Liver macroscopic and histological findings at the end of the experiment. **(A)** Macroscopic appearance of livers from mice of the three different groups. **(B)** HE-stained sections from mice showed differences in fatty change (magnification 20X). **(C)** ORO-stained sections from mice confirmed hepatic lipid accumulation (magnification 20X). **(D)** MG-trichrome stained sections from mice used for the detection of fibrotic areas in livers (magnification 20X).

Figure 12. Quantification of hepatic lipid droplets area at the end of the experiment. Percentage of tissue area containing hepatic lipid droplets was calculated from lipid droplets size from HE-staining sections (magnification 20X). Data are presented as mean ± SEM. Statistical significances: Anova p≤0.0001; Tukey's multiple comparisons test ****p<0.0001 CONTR vs. MCDD, CONTR vs. MCDD+LCARN, ***p=0.0005 MCDD vs. MCDD+LCARN.

As described, fatty acid oxidation is activated and controlled by the AMP-activated protein kinase (AMPK). Ca2+/calmodulin-dependent protein kinase II (CaMKII), is an upstream kinase in the AMPK cascade (Anderson N 2008). In our previous *in vitro* work on HepG2 cells, the treatment with LCARN stimulates AMPK protein synthesis and consequently CaMKII protein levels (Montesano A Under Submission).

CaMKII level was significantly decrease in MCDD and MCDD+LCARN mice in respect of CONTR group. Three-week of LCARN supplementation caused sharp increase of CaMKII compared to the MCDD group in immunofluorescence assay (Figure 13A). Different results were obtained with the phosphorylated active form of CaMKIIα (pCaMKII) (Figure 13 B). LCARN supplementation significantly increased pCaMKIIα compared to the MCDD group.

The AMPK level was detected by immunofluorescence assay and western blot analysis but we did not obtained sufficient data to show.
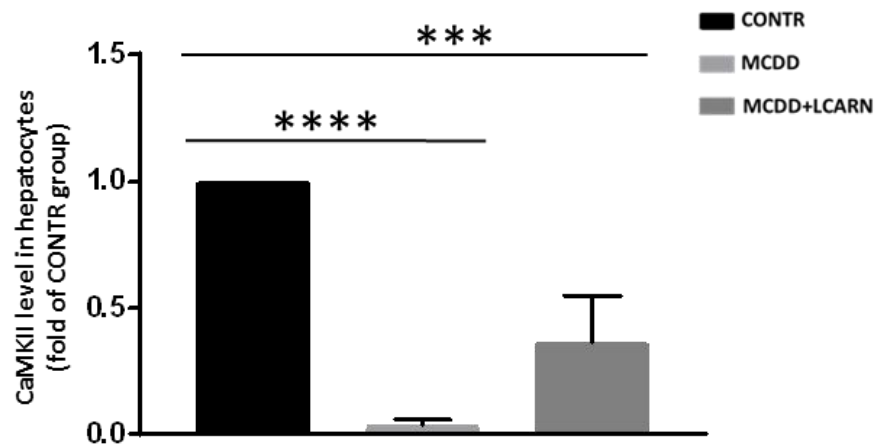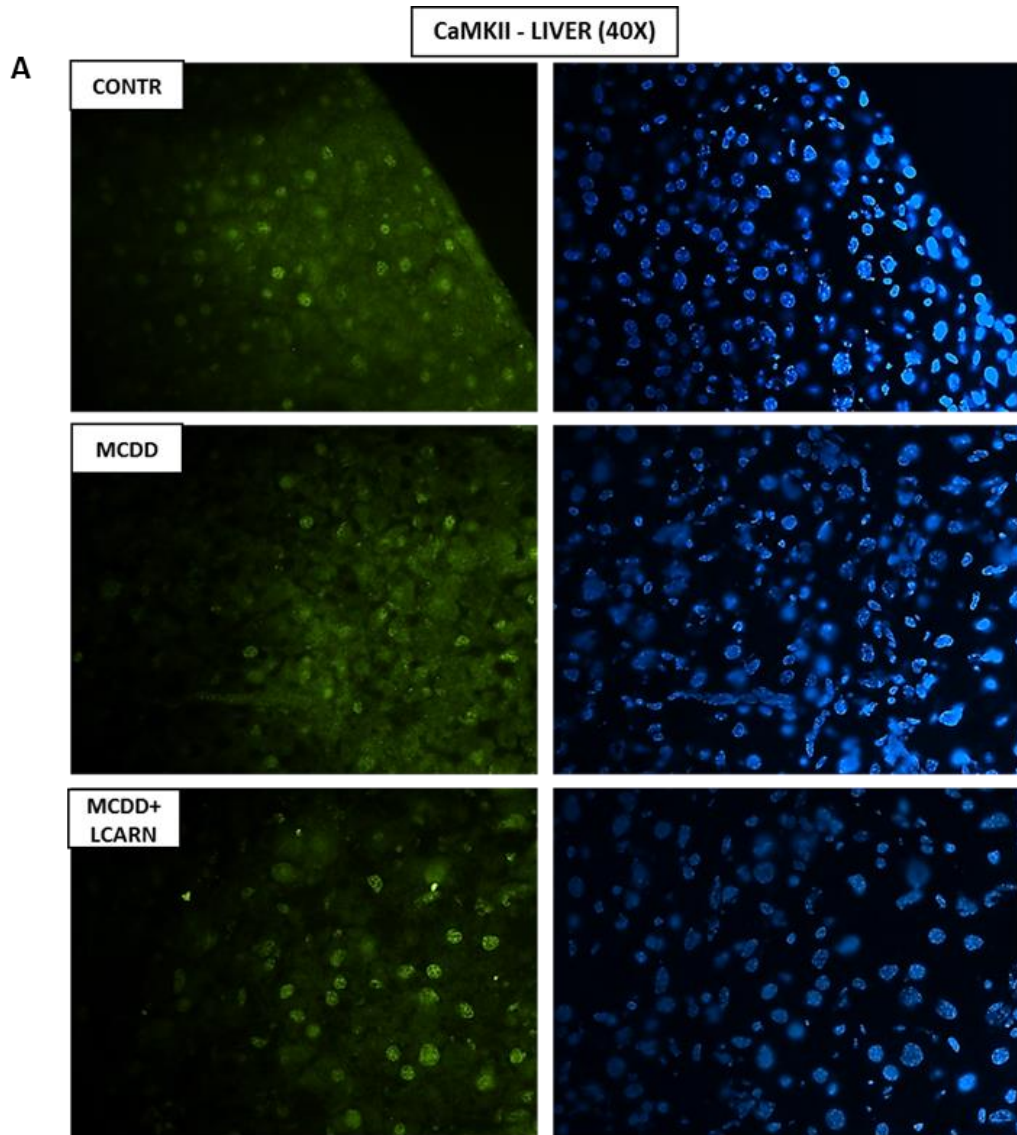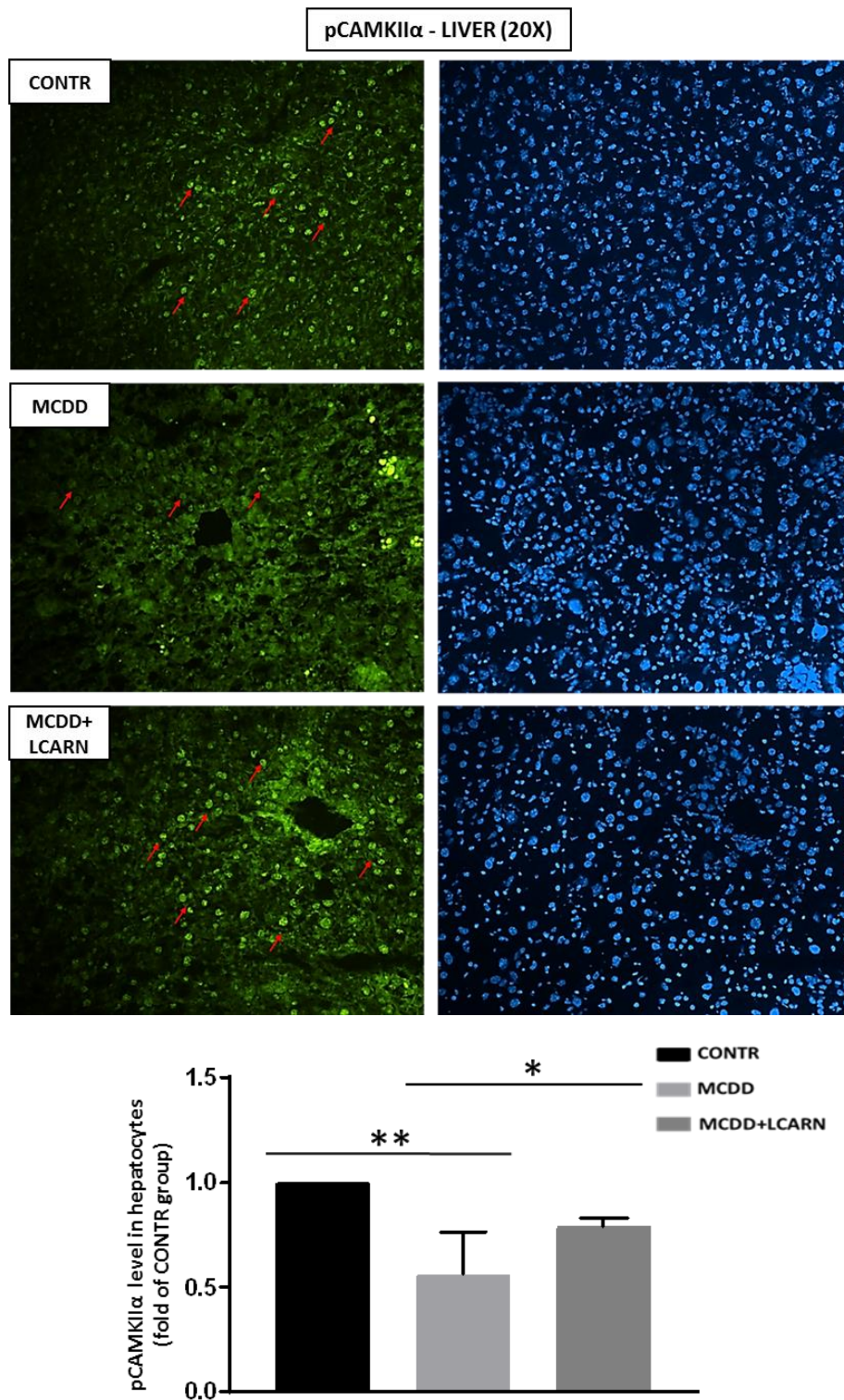
CaMKII - LIVER (40X)

A

Figure 13. Level of CaMKII and phosphorylated CaMKII in hepatocytes. **A)** Representative Immunofluorescence assay of total CaMKII content in mice hepatocytes of the three group and relative quantification. CaMKII Anova p<0.0001; Tukey's multiple comparisons test ****p<0.0001 CONTR vs MCDD; ***p=0.0003 CONTR vs. MCDD+LCARN. **B)** Representative Immunofluorescence assay of phospho CaMKII (pCaMKII) content in mice hepatocytes of the three group and relative quantification. pCaMKII Anova p<0.0028; Tukey's multiple comparisons test **p<0.0023 CONTR vs MCDD; *p=0.0308 MCDD vs. MCDD+LCARN. All data shown are means ± SEM.

The hepatic antioxidant effect of LCARN supplementation

Oxidative stress is induced by reactive oxygen species (ROS) during the progression of NAFLD. Recent evidence have identified potential micronutrient antioxidants that may reduce the accumulation of ROS and finally ameliorate the disease (Chen G 2016). Animal studies have described that mice fed a MCD diet become severely depleted of hepatic anti-oxidants. By decreasing oxidative defense mechanisms, MCD diet increase oxidant stress, a situation that is known to induce inflammation (Li W 2016).

To verify the antioxidative effect of LCARN supplementation, we quantified the ROS level after immunofluorescent assay. As shown in Figure 14, three weeks of MCD diet dramatically increased ROS level, while LCARN significantly reduced ROS level in MCDD+LCARN group compared to the MCDD group.

It is also known that MCD diet feeding for two weeks caused a remarkable reduction in hepatic ERK1/2 phosphorylation and a progression of the liver injury (Wang Z. 2010). In particular, ERK1/2 suppression may be critically involved in the early-stage liver injury and during ROS exposure. To test if LCARN supplementation may prevents ERK1/2 suppression and alleviates liver injury in MCD-fed mice, we analyzed the phosphorylated active form of ERK (pERK) in the liver of the three different mice group. As shown in Figure 14, three-week of LCARN supplementation increased significantly pERK 1/2 level compared to the MCDD group.
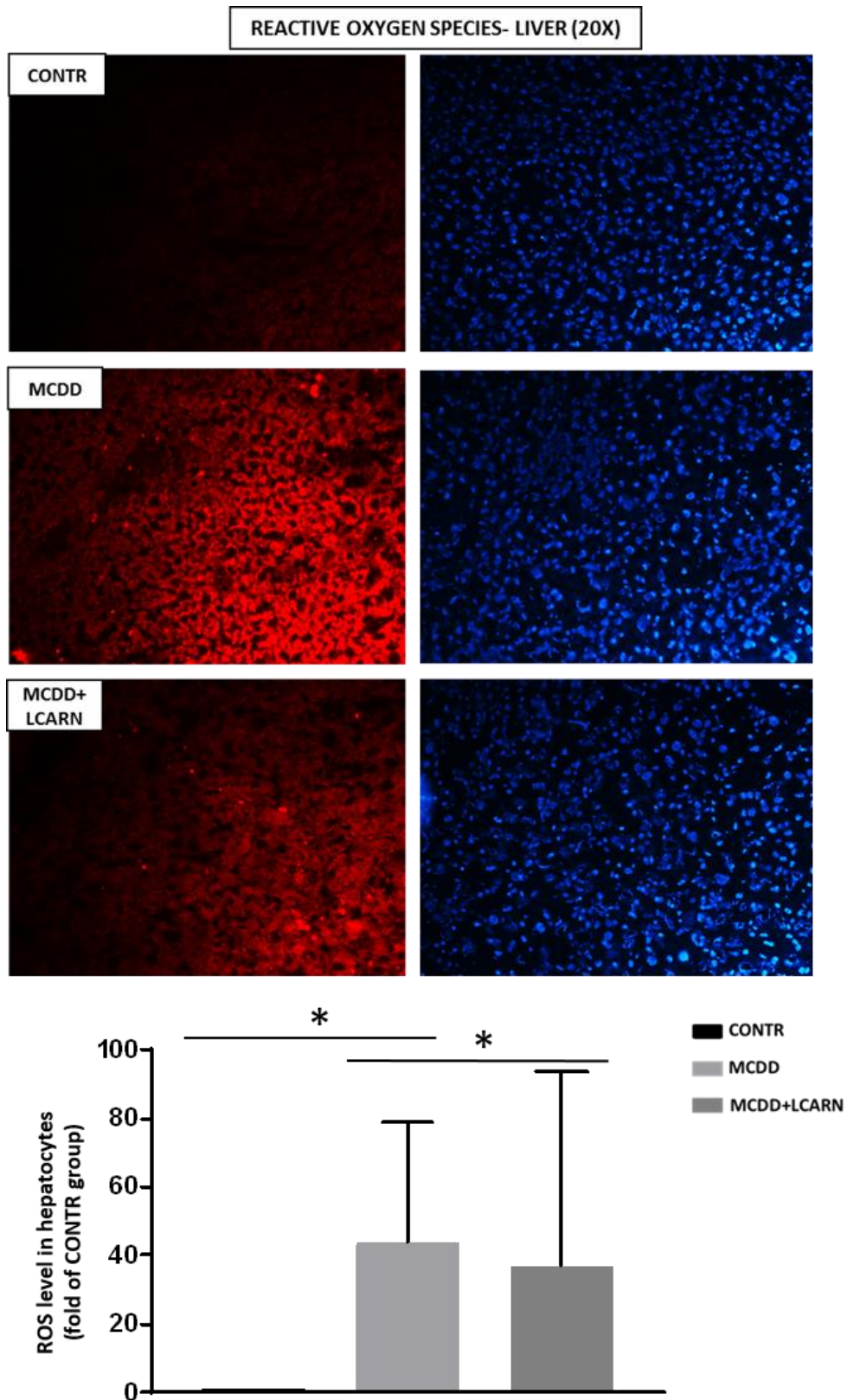
Figure 14. Quantification of hepatic ROS level. The effect of LCARN supplementation on hepatic ROS level in the three mice group. Staining of ROS mice livers and relative quantification. Data shown are means ± SEM; Anova p<0.0161; Tukey's multiple comparisons test *p=0.0430 CONTR vs. MCDD, *p=0.0386 MCDD vs. MCDD+LCARN.
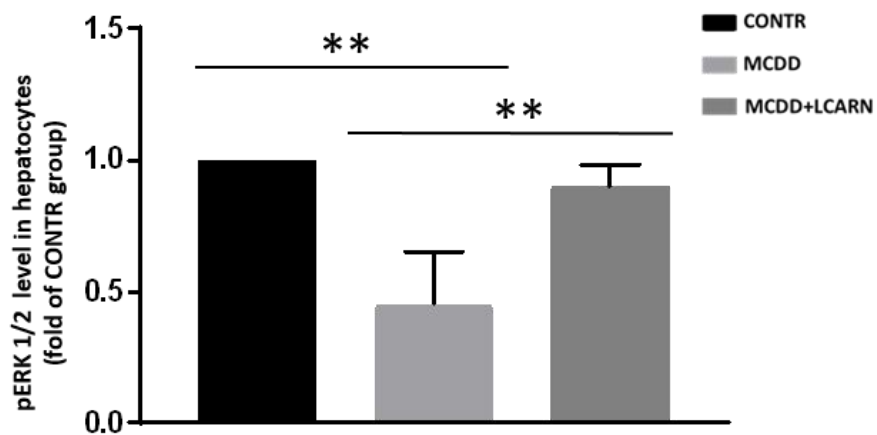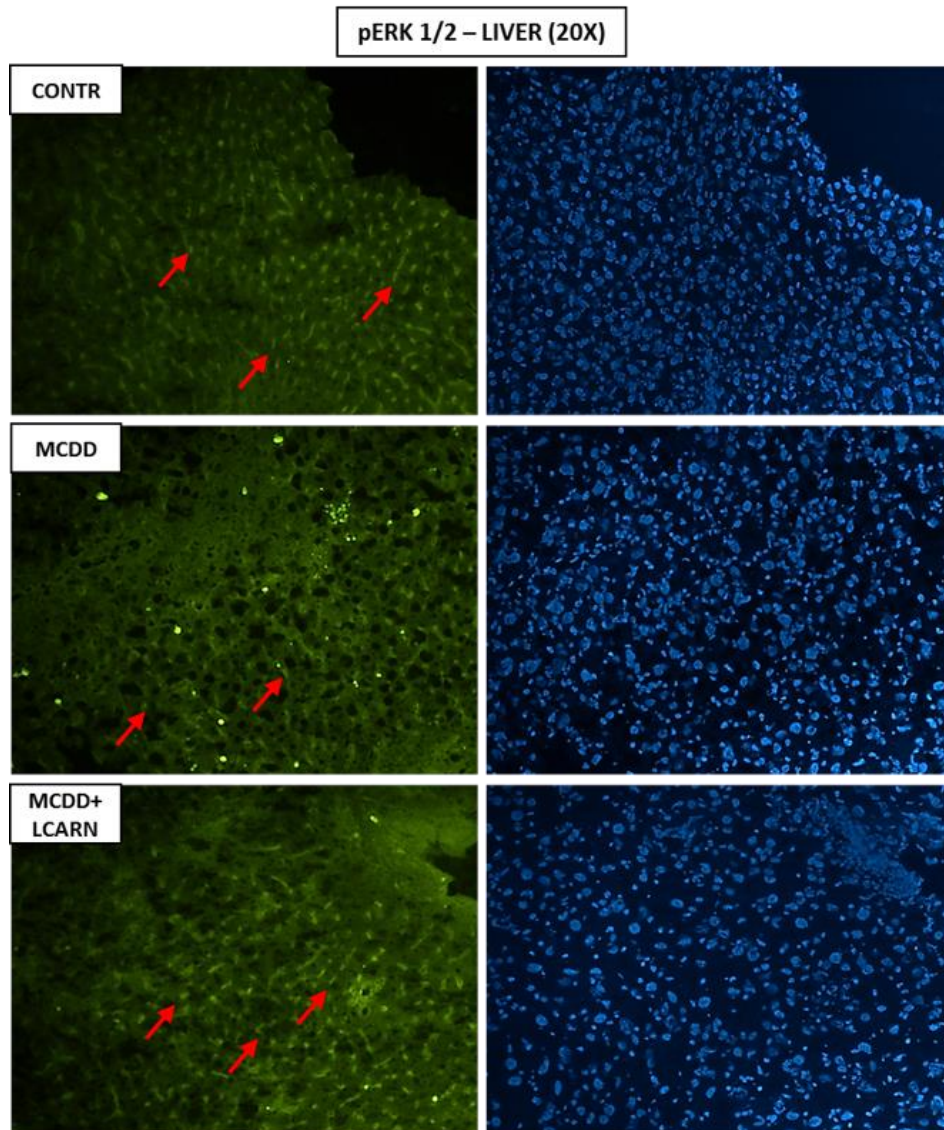
Figure 15. Level of phosphorylated ERK ½ in hepatocytes. Representative Immunofluorescence assay of phospho ERK ½ (pERK 1/2) content in mice hepatocytes of the three group and relative quantification. Data are expressed as fold changes (FC) ± SEM. pERK Anova $p<0.0026$; Tukey's multiple comparisons test **$p=0.0040$ CONTR vs. MCDD, **$p=0.0048$ MCDD vs. MCDD+LCARN.

Activation of hepatic PPARγ plays a critical role in inhibition of NFκB activity by LCARN supplementation.

Transcription factor NFκB has been described as a primary regulator and mediator of oxidative stress and it is widely accepted that oxidative stress plays crucial roles in HSCs activation during liver injury. Although the causal relationship remains unknown, previous studies demonstrated that activation and survival of HSCs were closely associated with the activation of NFκB. Furthermore, PPARγ expression was diminished in HSCs as they underwent myofibroblastic activation and it was demonstrated that the level of PPARγ was reduced during HSCs activation in vitro, whereas NFκB activity were increased (Xu J 2003) (Miyahara T 2000).

Liver protein extracts of the three mice group were prepared for western blot analysis. As shown in Figure 16, LCARN supplementation caused a significantly increase of PPARγ level and a consequent NFκB p65 decreased compared with the CONTR group.
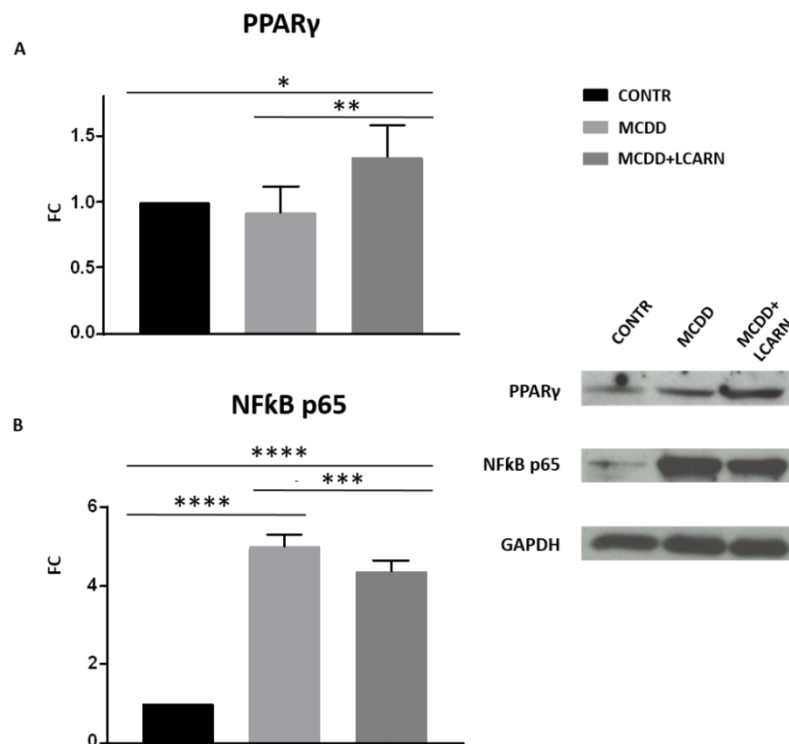
Figure 16. Effect of LCARN supplementation in the control of oxidative stress and fibrosis progression at the end of the experiment. Western blot data indicated that LCARN supplementation significantly increased the **A)** PPARγ level resulting in **B)** decreased NfκB p65 level. Data are expressed as fold changes (FC) ±SD. Statistical significances: PPARγ Anova test p≤0.0077; Tukey's multiple comparisons test *p=0.0297 CONTR vs. MCDD+LCARN and **p=0.0088 MCDD vs. MCDD+LCARN. NfκB Anova test p≤0.0001; Tukey's multiple comparisons test ****p<0.001 CONTR vs. MCDD+LCARN and CONTR vs. MCDD, ***p=0.0009 MCDD vs. MCDD+LCARN.

The role of LCARN in the hepatic fibrosis mechanism

To study if the intracellular fat and oxidative stress were able to induce HSC transactivation and fibrosis development, we first analyzed with MG-staining method the fibrotic area in the liver of the three groups as shown in Figure 11D. LCARN supplementation seemed to delay the fibrosis mechanism progression probably caused by its action on the control of the hepatic lipid accumulation.

In the past few years, several reviews have emphasized the essential role of hepatic stellate cell (HSC) activation into myofibroblasts during the pathogenesis of hepatic fibrosis. The αSMA protein, marker of HSC activation in liver, is a more sensitive indicator of the rate of fibrogenesis and risk of complications (Friedman SL 2010). Recent scientific data indicates that MCD diet-induced NASH mice, induced the expression of αSMA protein (Li YH 2016).

In order to understand the role of LCARN supplementation in liver fibrosis activation and progression, we analyzed the αSMA level after immunofluorescent assay. As shown in Figure 17 and confirmed in Figure 11D, LCARN supplementation seemed to ameliorated HSCs activation and the NASH phenotype of MCD diet-fed mice. Three-week of LCARN supplementation caused a significant decrease of αSMA compared to the MCDD group (Figure 17).
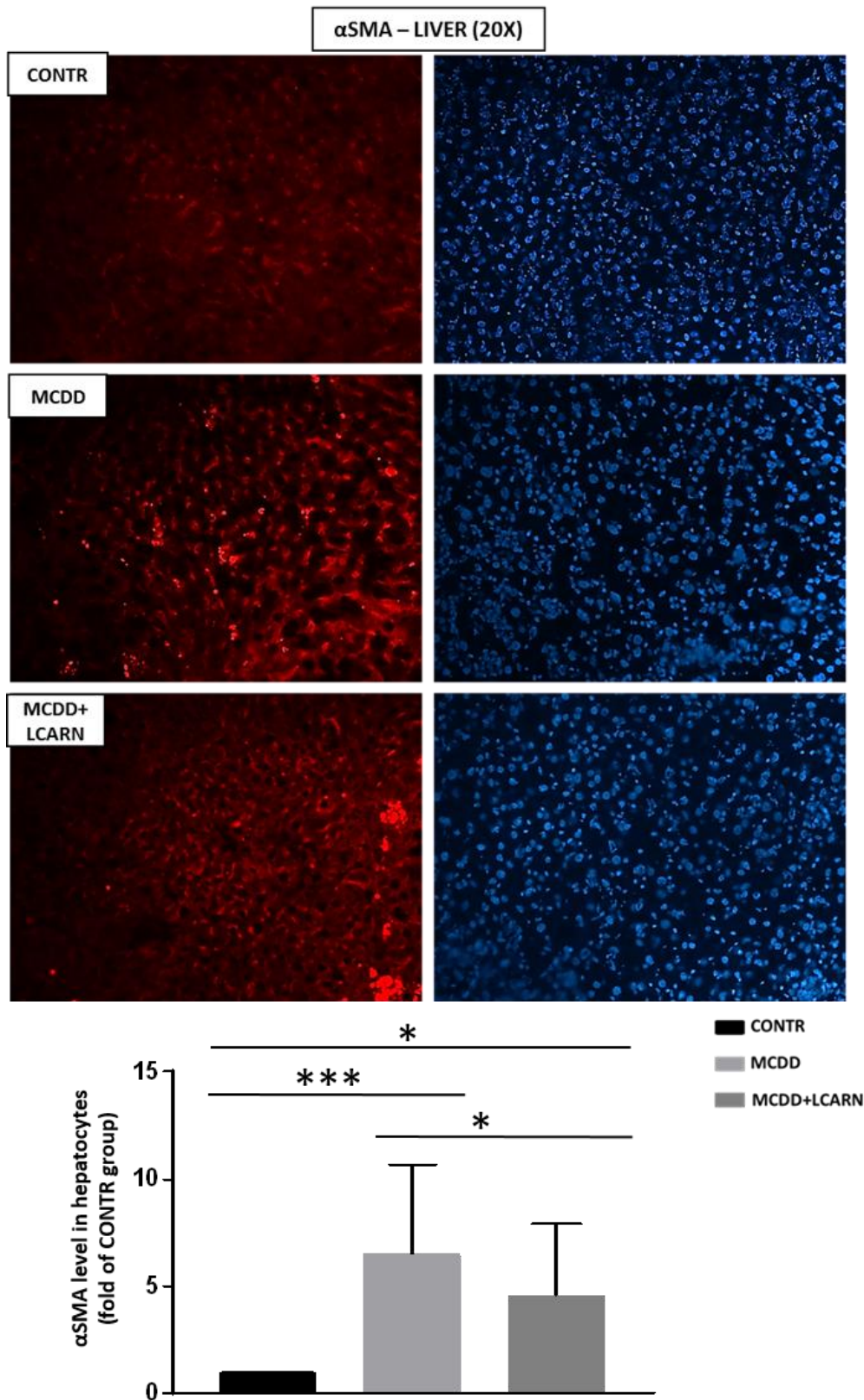
Figure 17. Level of α-SMA in hepatocytes. Representative Immunofluorescence assay of α-SMA content in mice hepatocytes of the three group and relative quantification. Data are expressed as fold changes (FC) ± SEM. αSMA Anova p<0.0012; Tukey's multiple comparisons test ***p=0.0010 CONTR vs. MCDD, *p=0.0206 CONTR vs. MCDD+LCARN, *p=0.0401 MCDD vs. MCDD+LCARN.

Heart mass, morphology and histology

One of the most important mortality and morbidity reasons of NAFLD are CVD events. As reported from the Framingham Heart study, there was a significant association between NAFLD and subclinical CVD outcomes, independently of many metabolic diseases (Fotbolcu H 2016) (Long MT 2016).

Macroscopically hearts from MCDD mice appeared normal compared with controls and mice received LCARN supplementation (Figure 18A).

To control the loss of body mass induced by the MCD diet, heart mass was expressed as a percentage of body mass (relative heart mass). As shown in Table 4, the hearts of CONTR mice in mass were 67.4% less heavy than MCDD group and 34.9% than mice received LCARN supplementation, while hearts of MCDD+LCARN mice were in mass 24.1% less heavy relative to MCDD group (Table 4).

The myocardium of mice fed MCD diet not showed diffuse vacuolar degeneration at HE-staining (Figure 18B), and no intracellular accumulation of lipids with ORO-staining (Figure 18C); myocardiocytes with abnormal size and altered nuclear morphology were not observed.

	CONTR	MCDD	MCDD+ LCARN
Heart mass (g)	0.25 ± 0.01	0.26 ± 0.03	0.22 ± 0.02
Relative heart mass (as % of body mass)	0.83 ± 0.02	1.39 ± 0.17	1.12 ± 0.07
Percentage differences in relative heart mass **MCDD and MCDD+LCARN vs. CONTR** (%)		- 67.4	- 34.9
Percentage differences in relative heart mass **MCDD vs. MCDD+LCARN** (%)		- 24.1	

Table 4. Heart mass absolute and relative and percentage differences in relative heart mass resulting from the MCD diet at the end of the experiment. Relative heart mass are expressed as a percentage of body mass. Data are expressed as a mean ± SD for each group.
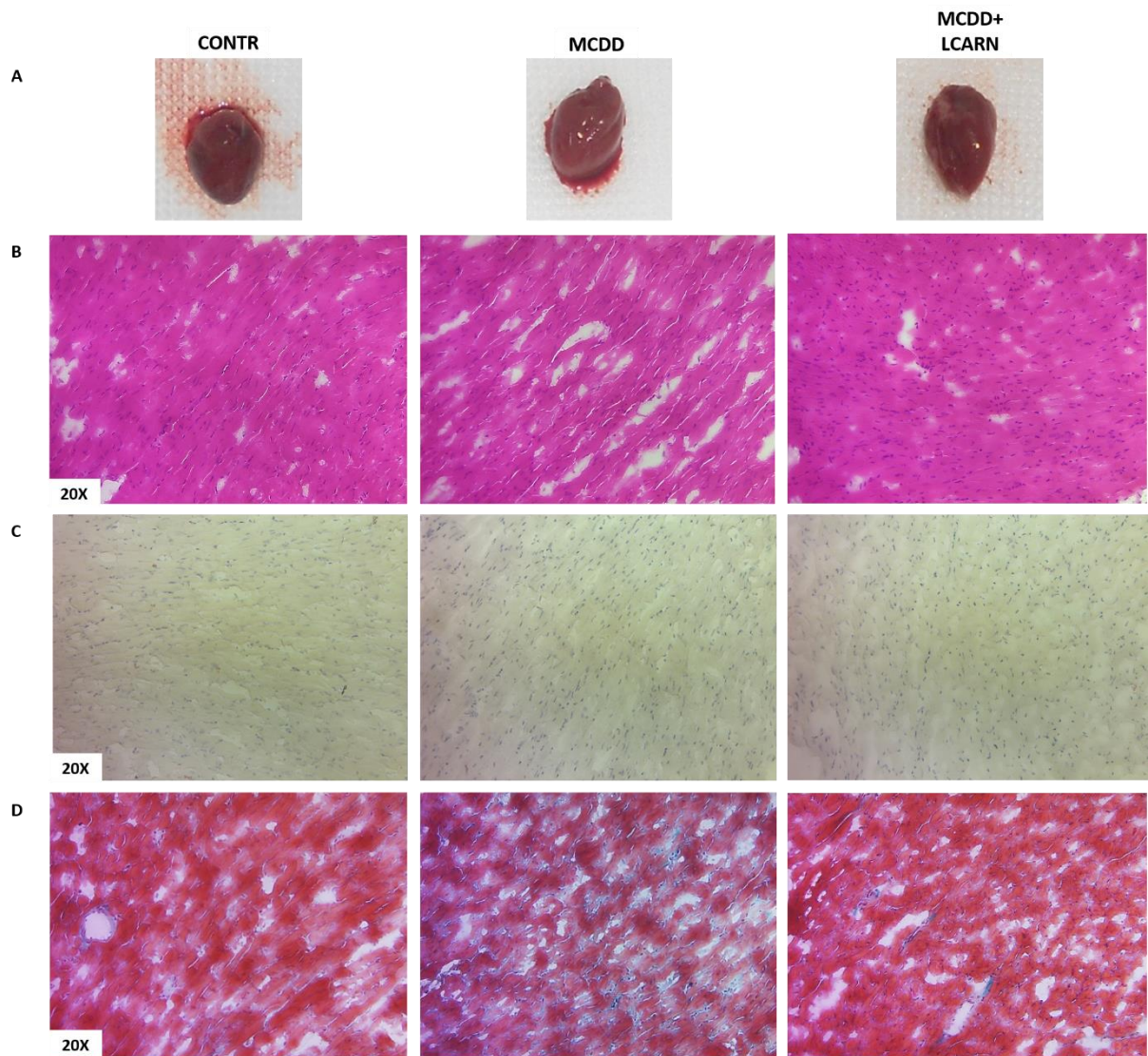
Figure 18. Heart macroscopic and histological findings at the end of the experiment. **(A)** Macroscopic appearance of hearts from mice of the three different groups. **(B)** HE-stained sections from mice showed the heart tissue histology (magnification 20X). **(C)** ORO-stained sections from mice confirmed no lipid accumulation (magnification 20X). **(D)** MG-trichrome stained sections from mice used for the detection of fibrotic areas in heart (magnification 20X).

The effect of LCARN supplementation in cardiac stress

Enhanced oxidant stress is an important feature of cardiovascular diseases. Indeed, a working concept for many chronic diseases is that increased oxidant stress is maladaptive and promotes disease progression and severity. Increased oxidative stress may also represent a shared pathophysiological condition between CVD and NAFLD. In fact, abundant studies described increased oxidative stress in a number of chronic diseases, such as metabolic syndrome, hypercholesterolemia, obesity, peripheral artery disease and obstructive sleep apnea syndrome all associated to NAFLD and increased CVD risk (Bhatia LS 2012) (Targher G 2008). General agreement emerging from these studies indicates that patients with NASH are at higher risk of cardiovascular diseases than those with simple steatosis, emphasizing the role of chronic inflammation caused by oxidative stress in these patients. In fact, in different clinical studies in adults and children with NAFLD have been found elevated systemic markers of oxidative stress and lipid peroxidation (Yesilova Z 2005) (Nobili V 2010).

To investigate the shared antioxidant role of LCARN supplementation in heart, we quantified the ROS level after immunofluorescent assay. As showed in Figure 19, the ROS level significantly decrease in MCDD+LCARN group compared with the MCDD group.

Due to the particularly elegant relationship between the structure and function of the kinase, CaMKII is able to translate a diverse set of signaling events into downstream physiological effects. Excessive CaMKII activity promotes cardiomyocyte death, likely by mitochondrial and extramitochondrial pathways. Similarly, elevated ROS contributes to cardiomyocyte death. Based on these hypothesis, Anderson and collaborators demonstrated that CaMKII is a necessary connection between ROS and cardiomyocyte death (Anderson ME 2015). They reported that CaMKII is activated by ROS and proposed a potentially wider role for CaMKII in physiological and pathological responses to cardiac oxidant stress. These observations suggested that oxidation-dependent CaMKII activity plays a critical role in numerous pathological processes in the heart.

As shown in Figure 20, western blot analysis of pCaMKIIα level reveled that this kinase was significantly increased only in MCDD mice group. These results support the hypothesis that cardiac CaMKII might be activated by ROS and LCARN supplementation could ameliorate cardiac stress condition. CaMKII activity is increased in myocardium under stress conditions, such as hypertrophy, where oxidation is also elevated.
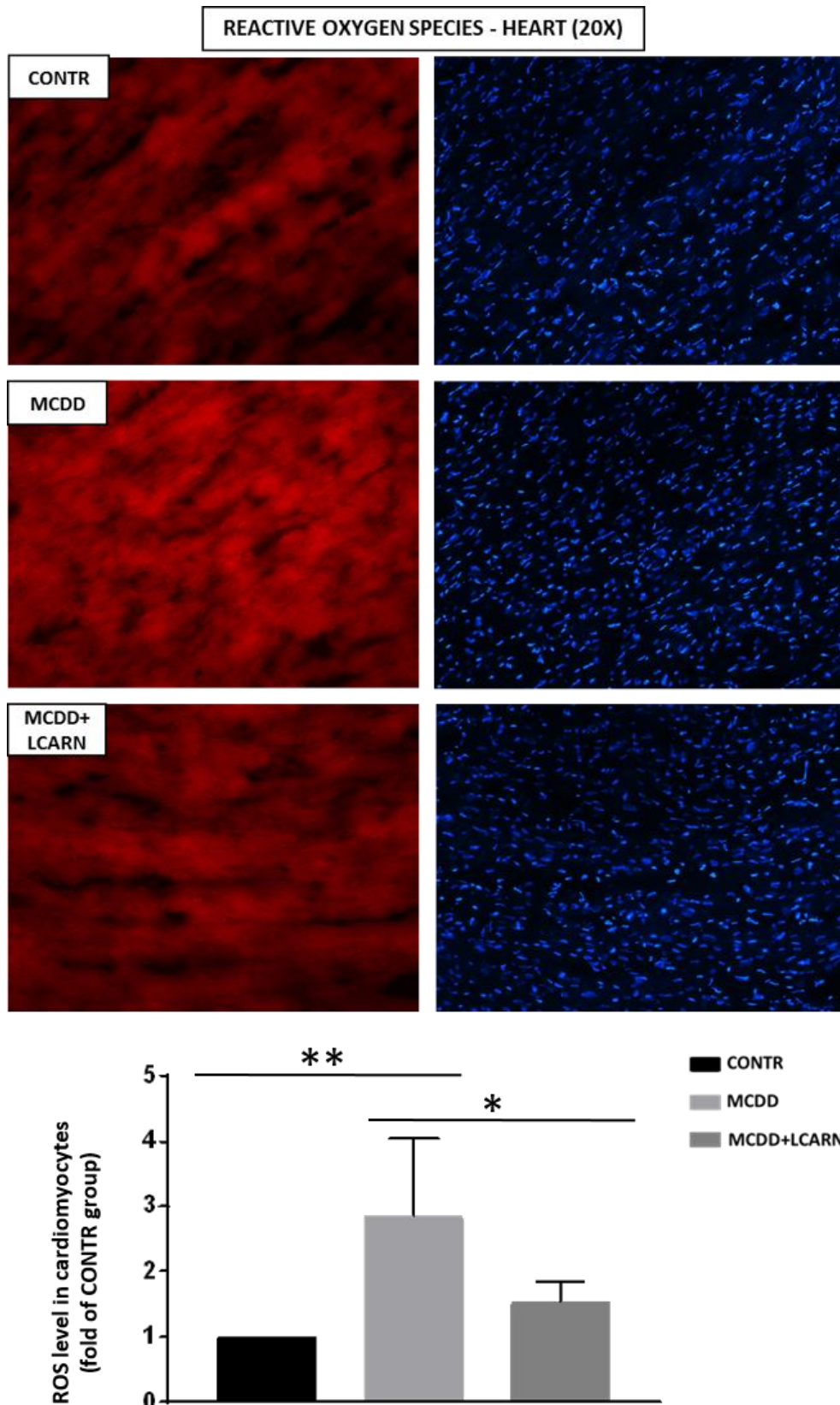
Figure 19. The effect of LCARN supplementation on cardiac ROS level. Staining of ROS mice hearts and relative quantification. ROS Anova p=0,0028; Tukey's multiple comparisons test **p=0,0026 CONTR vs. MCDD, *p=0,013 MCDD vs. MCDD+LCARN.
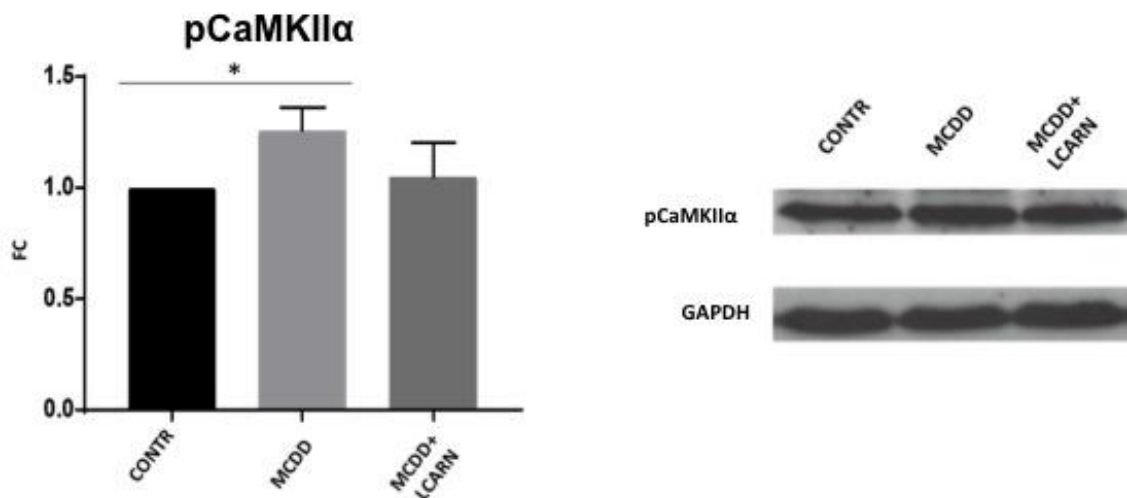
Figure 20. The effect of LCARN supplementation in cardiac stress at the end of the experiment. Western blot analysis of pCaMKIIα protein level in the three mice groups. Data are expressed as fold changes (FC) ±SD. pCaMKIIα Anova test $p \leq 0.0199$; Tukey's multiple comparisons test *$p=0.02$ CONTR vs. MCDD.

An increased CaMKII activity consequent activates the AMP-activated protein kinase (AMPK). One of the many important cellular control systems is AMPK, which is the putative metabolic or energy sensor of the cell. AMPK signaling appears to have broad implications in cardiovascular health and disease (Shirwany NA 2010). AMPK signaling has a specific physiological role in the heart; its importance is accentuated under conditions that place a stress on this organ. For example, AMPK activates the glycolytic pathway, enhances fatty acid β-oxidation and improves ATP availability. Cardiac hypertrophy can develop under physiological conditions as is seen in trained athletes as a compensatory response to intense exercise. It can also occur pathologically in response to different conditions. In recent years, evidence has begun to emerge that AMPK can modulate the development of cardiac hypertrophy and interestingly, reports suggest that pharmacological activation of AMPK can mitigate the hypertrophy (Juric D 2007) (Russell RR 3rd 2004). In conclusion, in the heart signaling by AMPK appears to regulate the bioenergetic status of the cardiomyocyte as well as maintain the heart muscle in optimum condition.

To investigate the AMPK level and the role of LCARN supplementation in heart, we performed an immunofluorescent assay. As showed in Figure 21, LCARN supplementation is seemed to restore the AMPKα 1/2 level compared with the control group and there was no significantly difference of AMPKα level between CONTR group and MCDD+LCARN group.
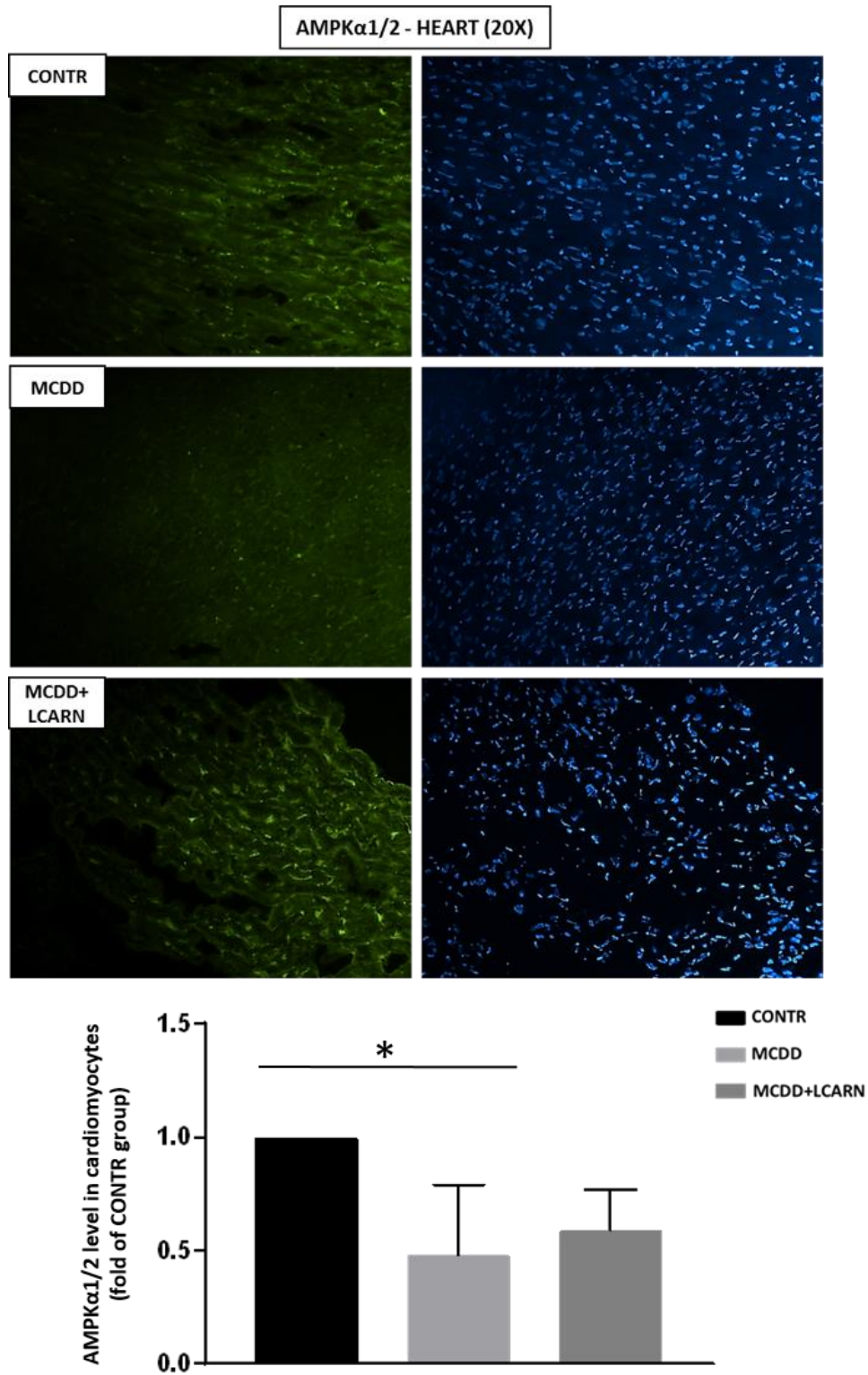
Figure 21. The effect of LCARN supplementation in cardiac stress. Immunofluorescence assay and relative quantification, confirmed a significant decrease of AMPKα 1/2 protein expression in MCDD group mice. AMPKα 1/2 Anova p=0,0473; Tukey's multiple comparisons test *p=0,0399 CONTR vs. MCDD.

The role of LCARN in the cardiac fibrosis mechanism

Consistently with liver findings, we observed fibrotic area also in heart tissue with MG-staining as shown in Figure 18C. To confirm these data, we investigated by western blot technique the pERK2/ERK2 protein level in cardiomyocytes. ERK1/2 pathway is a central downstream signaling pathway that is activated in cardiac muscle cells during hypertrophy (Liang Y 2016). As shown in Figure 22A, LCARN supplementation significantly decreased pERK2/ERK2.

We also investigated the role of the signal transducer and activator of transcription 3 (STAT3). STAT3 mediated intra- and intercellular communication within the heterogeneous cellular network of the myocardium to coordinate complex biological processes. STAT3 modulates proliferation, differentiation, survival, oxidative stress, and/or metabolism in cardiomyocytes, fibroblasts, endothelial cells, progenitor cells, and various inflammatory cells (Haghikia A 2016). In Figure 22B, western blot analysis shows the LCARN supplementation role on pSTAT3/STAT3 protein level. LCARN supplementation significantly decreased pSTAT3/STAT3 protein level that on the contrary is significantly increased in the MCDD mice group.

Finally, cardiac fibrosis is characterized by the excessive deposition of collagens and extracellular matrix proteins (ECM) that lead to impaired organ function. Fibroblasts are the predominant cell type responsible for the homeostatic maintenance of tissue ECM, healing after injury. Myofibroblasts are characterized by increased protein synthesis, including collagens, other ECM proteins, certain cytokines and α-smooth muscle actin (αSMA), a contractile protein and marker of profibrogenic cardiac fibroblasts activation (van Putten S 2016). As studied in the liver with immunofluorescence assay, we analyzed cardiac αSMA level. LCARN supplementation, as showed in Figure 23, significantly decreased αSMA level compared to MCDD group and confirming once again the delay of the fibrosis progression.
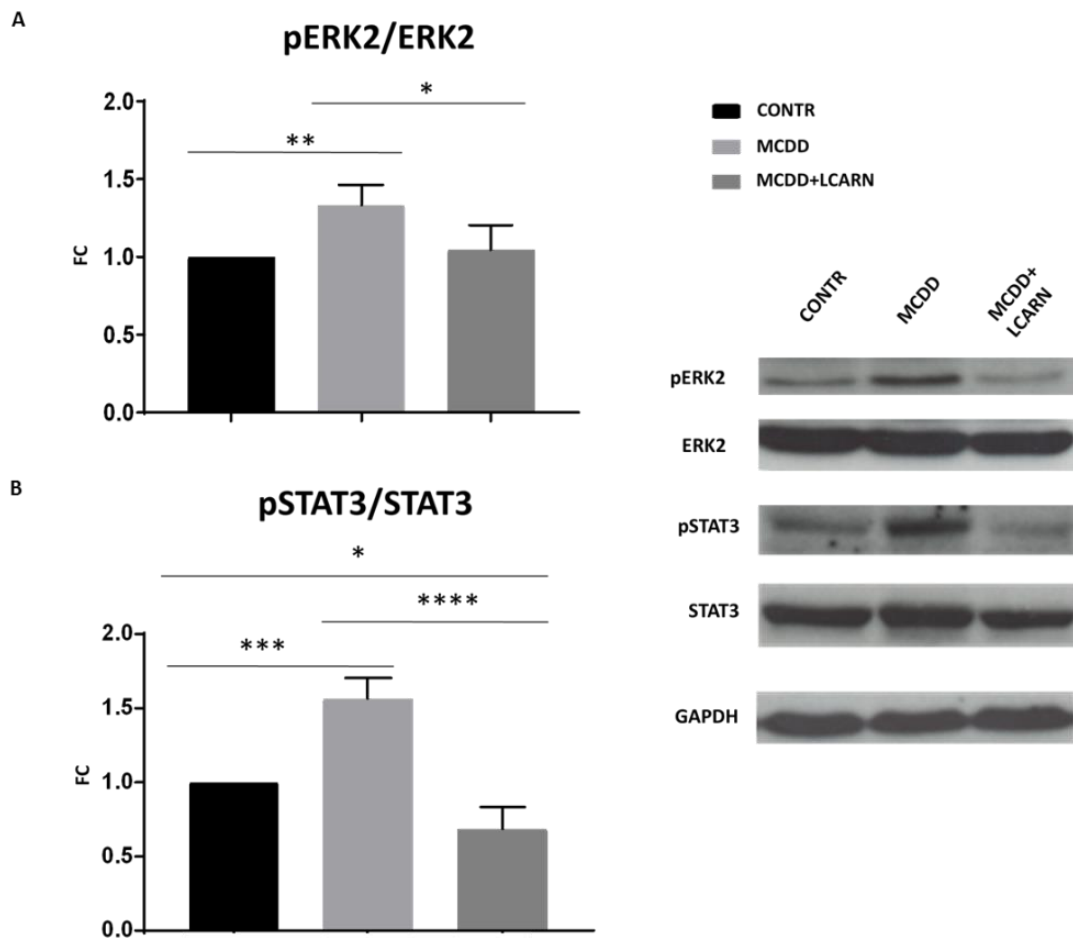
Figure 22. LCARN supplementation role in the cardiac fibrosis mechanism at the end of the experiment. **A)** Western blot analysis of LCARN supplementation effect on pERK2/ERK2 protein level in the three mice group. **B)** Western blot analysis of LCARN supplementation effect on pSTAT3/STAT protein level in the three mice group. Data are expressed as fold changes (FC) ±SD. pERK2/ERK2 Anova test p≤0.0062; Tukey's multiple comparisons test **p=0.001 CONTR vs. MCDD, *p=0.02 MCDD vs. MCDD+LCARN. pSTAT3/STAT3 Anova test p≤0.0001; Tukey's multiple comparisons test ***p=0.0003 CONTR vs. MCDD, *p=0.01 CONTR vs. MCDD+LCARN, ****p<0.0001 MCDD vs. MCDD+LCARN.
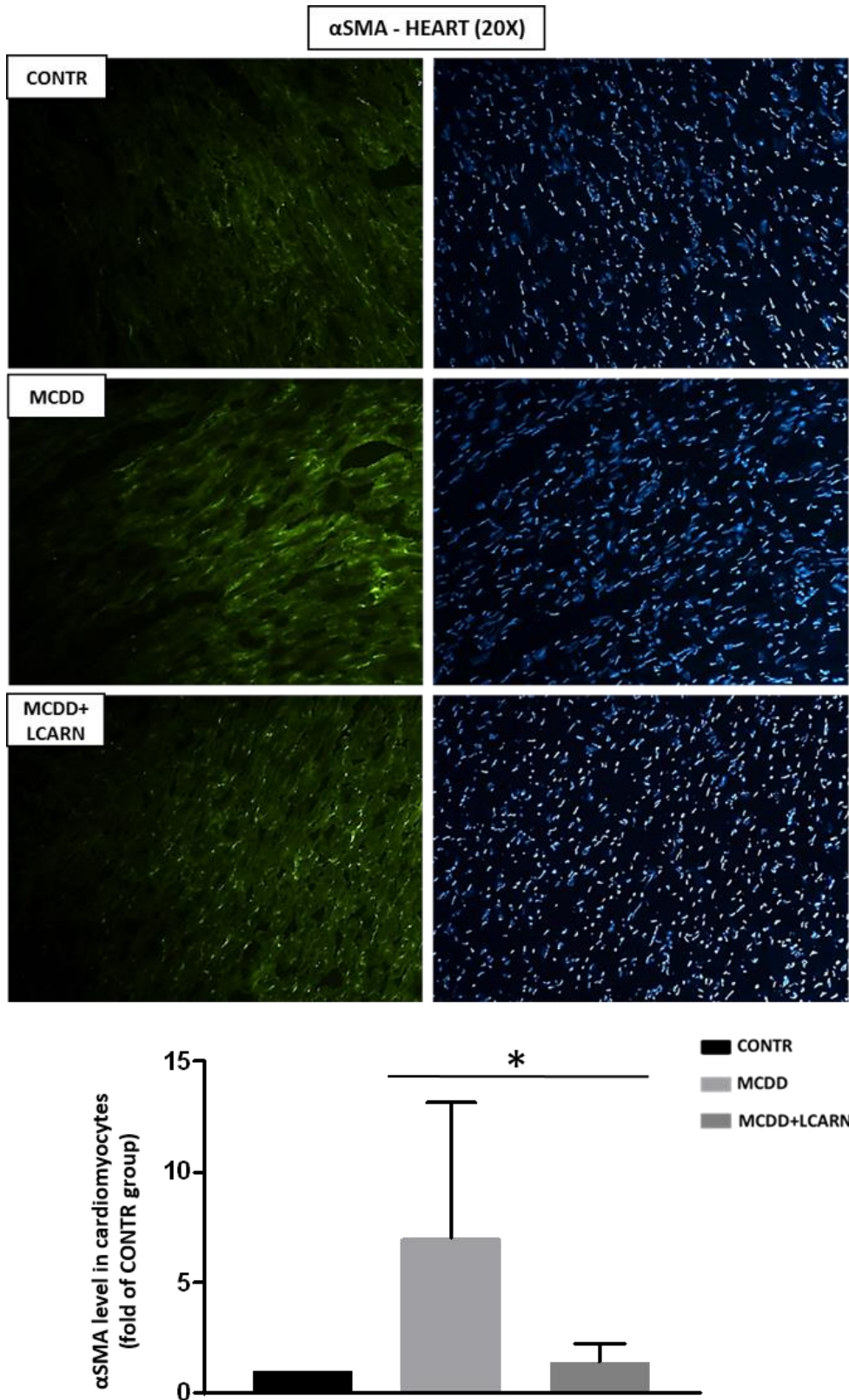
Figure 23. LCARN supplementation role in the development and control of heart fibrosis. Immunofluorescence assay described LCARN supplementation action on αSMA level (20X). αSMA Anova p=0,0437; Tukey's multiple comparisons test *p=0,0385 MCDD vs. MCDD+LCARN.

7. DISCUSSION

In this study, we investigated the effect of LCARN supplementation both in the liver and in the myocardium of C57BL/6 male mice fed a MCD diet for nr. 6 weeks. The MCD diet leads to the development of steatohepatitis with fibrosis and serves as animal model for NAFLD. The MCD diet is essential for hepatic β-oxidation and production of VLDL and methionine/choline deficiency impairs hepatic VLDL secretion. Consequentially, lipids are accumulated in the liver. In addition, cytokines changes, oxidative stress and adipocytokines occur, contributing to the liver injury (Ibrahim SH 2016). Our animal model not presented accumulation of lipid droplets in the myocardium as Salamone and collaborators found in myocardial tissue (Salamone F 2012), probably caused by the different mice group (six-week-old male BKS, Cg-m^{+}/$^{+}$ Lepr^{db}/J - db/db - obese mice and six-week-old male heterozygous db/m lean as control mice) and the dissimilar weeks of MCD diet somministration (all mice were fed a MCD diet for 4 weeks). Despite these differences, in our study, we demonstrated the effects of LCARN supplementation on hepatocyte ballooning, oxidative stress induced by the disease progression and fibrosis development in liver. In addition, LCARN supplementation controlled the induction of cellular stress responses in the myocardium.

ROS production is a highly regulated process that deeply affects cellular function and homoeostasis in all organisms. In eukaryotic cells, ROS are generated in multiple organelles including the ER and mitochondria as by product of oxidative protein folding, mitochondrial respiration and detoxification. Several evidences suggest that oxidative stress and lipid peroxidation has been implicated in the pathogenesis of NAFLD/NASH (Wei Y 2008) (Negre-Salvayre A 2010). The role of oxidative stress in NAFLD pathogenesis has been discussed in relation to metabolic changes and pro-inflammatory transcription factor expression. Inhibition of mitochondrial fatty acid oxidation is thought to be a major cause for intrahepatic lipid accumulation and it has been further suggested that impaired hepatic lipid clearance via VLDL may be a possible cause of hepatic lipid accumulation in NAFLD (Ashraf NU 2015) (Anderson N 2008) (Zhang D 2007). Carnitine plays a vital role in conveying long-chain fatty acids from the cytoplasm to the mitochondria, where they are used as a source of energy via β-oxidation. Several recent studies have suggested a potential role of LCARN in the treatment of lipotoxicity and steatohepatitis. LCARN enhances both lipolysis and fatty acid oxidation in NAFLD (Jun DW 2011). Our recent *in vitro* work on HepG2 cells (Montesano A Under Submission), verified that LCARN supplementation can reduce HepG2

intracellular lipid accumulation and this *in vitro* study confirmed the potential effect of LCARN supplementation on hepatic lipid droplets formation and oxidative stress response.

Recent results, indicate that AMP-activated protein kinase (AMPK) is responsible for the incidence of NAFLD and regulates hepatic lipid metabolism including lipid biogenesis and uptake, fatty acid oxidation, triglyceride and cholesterol metabolism. Furthermore, suppression of AMPK induces the lipid accumulation in the hepatocytes, decreases the ability of mitochondria to oxidize free fatty acids, and subsequently increases production of ROS, which trigger inflammation and ultimately results in the progression from NAFLD to NASH (Qiang X 2016). AMPK is activated by the kinase CaMKII an ubiquitous enzyme with a mitochondrial localization. In liver, LCARN supplementation showed a significantly improvement of pCaMKII level as observed in our previous *in vitro* study (Montesano A Under Submission). In myocardium, LCARN supplementation limited the increase of pCaMKII level that is significantly revealed in MCDD group. The pCaMKII increase in MCDD mice myocardium did not affect AMPK level that was significantly lower compared to the CONTR diet. On the contrary, LCARN supplementation showed a tendency to improve cardiac level of AMPK that represents the key regulator of energy metabolism in the heart.

In the "Two Hits Hypothesis" (Day CP 1998) an oxidative stress condition caused by lipid accumulation in the liver, is capable of inducing enough lipid peroxidation that overcome the normal cellular defense mechanisms and produce inflammation and fibrosis (Tariq Z 2014). During hepatic fibrogenesis, HSCs represent the key fibrogenic elements in response to chronic liver injury (Chen RJ 2015) (Friedman SL 2010) (Gressner AM 1996). HSCs undergo trans-differentiation from a quiescent into an activated phenotype state characterized by an increase expression of αSMA (Barbero-Becerra VJ 2015). αSMA is an actin isoform that is normally confined to vascular smooth muscle cells, but is also expressed in healing wounds and both hepatic and heart fibro-contractive lesions (Wang J 2005). In this work, LCARN supplementation showed a role in limit αSMA level both in liver and heart tissues. Interesting, we reported that this supplementation dose acted also on hepatic ERK levels and counteracted this protein increase during the disease progression. It has been reported the involvement of ERK1/2 in cellular response to oxidative stress and activation of ERK1/2 is also required to prevent the development of NASH. (Martindale JL 2002) (Aghazadeh S 2002). Furthermore, ERK activity and phosphorylation are then critical in altering the cardiac myocyte response to stress-induced hypertrophy and disease progression (Jun DW 2011) (Bueno OF 2000). Our results demonstrated the effect of LCARN

supplementation in decreasing ERK level in cardiomyocytes, confirming once again its effect on the disease progression.

Stimuli initiating HSCs activation derive from injured hepatocytes, neighboring endothelial and Kupffer cells. Hepatocytes and Kupffer cells are a potent source of reactive oxygen intermediates and these compounds exert paracrine stimulation of stellate cells in myofibroblasts. Moreover, their activity is amplified *in vivo* by depletion of antioxidants as typically occurs in diseased liver (Friedman SL 2000). It is known that fibrosis is a reversible process, and fibrotic tissue can be spontaneously resorbed once the injurious stimulus is over (Friedman SL 2012). Central to fibrosis regression is the programmed death of activated HSCs in response to apoptotic stimuli, which limits the number of cells performing scar tissue deposition (Iredale JP 1998). PPARs are a family of nuclear ligand-activated transcription factors regulating metabolism, above all the lipid one, in inflammation, cell growth and differentiation. PPARγ is mainly known for its central role in driving adipogenesis and lipid metabolism, since it promotes the expression of genes involved in lipid uptake and storage and it is poorly expressed in the liver (Panebianco C 2016). Moreover, PPARγ has a protective role against liver fibrosis, since *in vivo* fibrogenesis upon injury turned out to be decreased in PPARγ-depleted and enhanced in PPARγ-overexpressing rat livers (Wang Z 2011) (Yang L 2006). This modulation of liver fibrosis by PPARγ is likely due to its effect on HSCs, since PPARγ agonists, ectopic expression of PPARγ or treatment with the adipocyte differentiation mix in activated HSCs, were shown to inhibit proliferation, induce apoptosis and cell cycle arrest and promote reversal from an activated to a quiescent state (Hazra S 2004) (Yu J 2010). PPARγ also inhibits the expression of the inducible nitric oxide synthase and inhibits gene expression in part by antagonizing the activities of the transcription factors as NFκB (Ricote M 1998). Our results shown that LCARN supplementation in liver controls the fibrosis progression and also significantly increased PPARγ level and consequently reduced NFκB p65.

Finally, in order to investigate the parallel action of LCARN supplementation on cardiac fibroblasts proliferation, we analyzed STAT3 activity. STAT3 has been shown to be an integral part of the responses of the myocardium to various cardiac insults, including myocardial infarction, oxidative damage, myocarditis, hypertrophy and remodeling (Dai B 2013). STAT protein sequences contains highly conserved ERK phosphorylation sites; and therefore STAT protein can be activated by ERK (Ng DC 2001). The cross-talk between these two signaling cascades has been shown to participate in a series of physiological and pathological processes (Fischer P 2007). Based on our pERK2/ERK2 results, LCARN

supplementation showed the ability to significantly reduce the pSTAT3/STAT3 level in cardiomyocytes.

Taken together, these results demonstrated that the supplementation effects of LCARN could act on both liver and cardiac hits. As show in Figure 24, the fundamental mechanism during the progression of NAFLD and the consequent CVD risk is the oxidative stress. In liver, LCARN acted on lipid accumulation and consequently controlled the oxidative stress imbalance. We also demonstrated that LCARN acts in this shared mechanism in both tissue and its central role is to control the oxidative stress resulting to delaying the fibrosis progression.
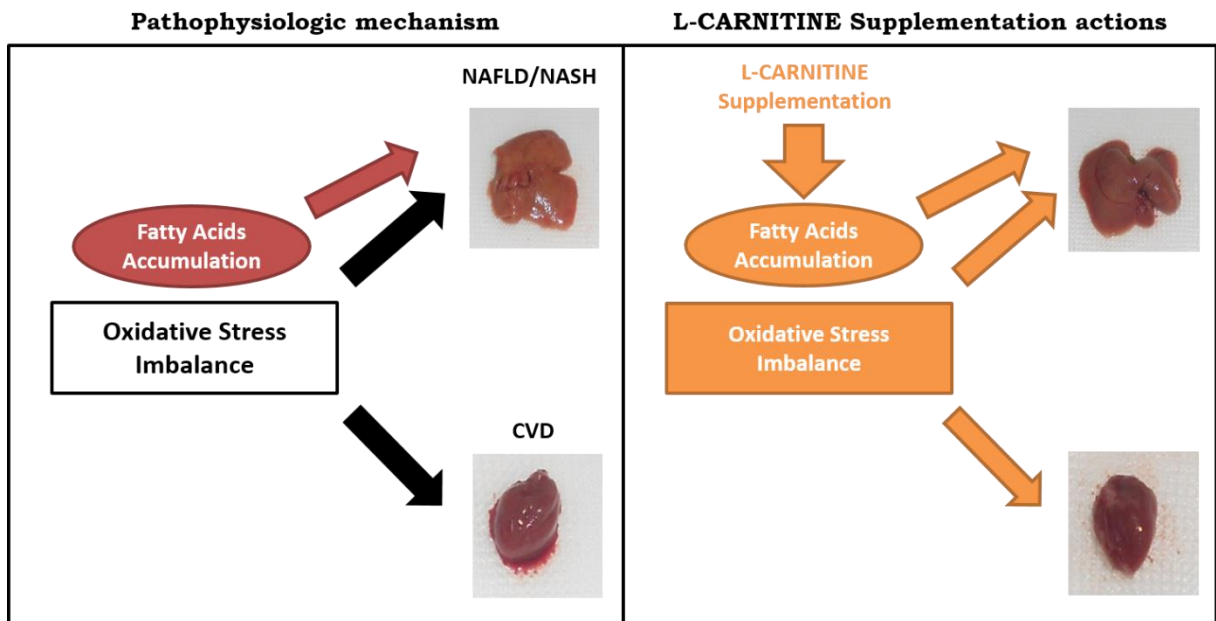
Figure 24. Possible pathophysiological mechanisms linking NAFLD/NASH to CVD and the possible role of LCARN supplementation in the control of disease progression and complications.

8. CONCLUSIONS

In recent years, the effects of nutraceuticals on NAFLD and on cardiovascular disease received increasing attention as possible treatment. We have demonstrated that LCARN supplementation decreased the severity of experimental NAFLD progression via different mechanism likely to involve the lipid accumulation in liver, oxidative stress imbalance and fibrosis accumulation in both liver and cardiac tissues analyzed.

Our data are consistent with the proposition that oxidative stress represents an important stimulus in this model of NAFLD progression. Another important point of our results is the potential role of LCARN in modulating the shared oxidative stress mechanism pathway involved in heart.

Further studies are required to determine whether a long-term LCARN supplementation is able to control the pathophysiologic evolution of the disease and related complications.

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consumption of the three experimental groups. **(C)** Water consumption and oral LCARN supplementation were recorder each day. Data are presented as mean ± SEM. Statistical significances: Mice weight by Anova p≤0.0001; Tukey's multiple comparisons test *p<0.0001 CONTR vs MCDD and CONTR vs. MCDD+LCARN each week. Mice eat/weight by Anova p≤0.0001; Tukey's multiple comparisons test **p=0.0231 CONTR vs MCDD week 4, ***p=0.0169 CONTR vs MCDD week 5. L-carnitine oral dose by Anova with no significant differences among means.   33

Figure 11. Liver macroscopic and histological findings at the end of the experiment. **(A)** Macroscopic appearance of livers from mice of the three different groups. **(B)** HE-stained sections from mice showed differences in fatty change (magnification 20X). **(C)** ORO-stained sections from mice confirmed hepatic lipid accumulation (magnification 20X). **(D)** MG-trichrome stained sections from mice used for the detection of fibrotic areas in livers (magnification 20X).   35

Figure 12. Quantification of hepatic lipid droplets area at the end of the experiment. Percentage of tissue area containing hepatic lipid droplets was calculated from lipid droplets size from HE-staining sections (magnification 20X). Data are presented as mean ± SEM. Statistical significances: Anova p≤0.0001; Tukey's multiple comparisons test ****p<0.0001 CONTR vs. MCDD, CONTR vs. MCDD+LCARN, ***p=0.0005 MCDD vs. MCDD+LCARN.   36

Figure 13. Level of CaMKII and phosphorylated CaMKII in hepatocytes. **A)** Representative Immunofluorescence assay of total CaMKII content in mice hepatocytes of the three group and relative quantification. CaMKII Anova p<0.0001; Tukey's multiple comparisons test ****p<0.0001 CONTR vs MCDD; ***p=0.0003 CONTR vs. MCDD+LCARN. **B)** Representative Immunofluorescence assay of phospho CaMKII (pCaMKIIα) content in mice hepatocytes of the three group and relative quantification. pCaMKIIα Anova p<0.0028; Tukey's multiple comparisons test **p<0.0023 CONTR vs MCDD; *p=0.0308 MCDD vs. MCDD+LCARN. All data shown are means ± SEM.   38

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