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

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INTRODUCTION

The Non-Alcoholic Fatty Liver Disease (NAFLD) is an umbrella term used to described an histological spectrum ranging from simple steatosis, defined by a concentration of hepatic triglycerides exceeding 5% of liver weight, to nonalcoholic steatohepatitis (NASH) characterized by hepatocellular damage, lobular necroinflammation and fibrogenesis. Liver fat accumulation, causes oxidative stress and transdifferentiation of hepatic stellate cells into activated myofibroblasts increasing the production of collagen matrix. The "Two Hits Hypothesis" explains the development of NAFLD with a double hit, the first producing steatosis (leads to insulin resistant and accumulated lipid in the liver) and the second a source of oxidative stress driving a significant lipid peroxidation that facilitates inflammation, progressive steatosis and fibrosis. This hypothesis provides a strong rationale for the prevention and the control of disease progression. Recent results, from the Framingham Heart study, revealed that there was a significant association between NAFLD and subclinical cardiovascular diseases (CVD) outcomes, 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 cytokines.
In recent years, the effects of nutraceuticals on NAFLD and on cardiovascular disease received increasing attention as possible treatment. L-carnitine (LCARN) is an essential nutrient that converts fat into energy into mitochondria and plays an important role in lipid metabolism; it acts as an essential cofactor for the β-oxidation of fatty acids by facilitating the transport of long-chain fatty acids. It is accepted that LCARN administration can ameliorate or prevent liver damage of various etiologies. It is currently used as an adjunctive therapy in various heart conditions with promising results and recognized as a nutritional supplement in cardiovascular disease.
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 and as reported in literature, the nutrient deficient model used to probe changes in liver injury, increased inflammation and pathways involving the elimination of fat is the methionine and choline deficient diet (MCDD).

MATERIALS AND METHODS

Male C57BL/6 mice (n=30), were used for the study at 10 weeks of age and were divided into three 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 3 weeks. After the first 3 weeks, one of the MCDD food group was enriched with 200mg/kg/die oral LCARN (MCDD+LCARN) for other 3 weeks and all animals were sacrificed at the end of the experiments. As reported in literature, male C57BL/6 mice developed the histological features that most closely resemble those seen in human NASH.
To study LCARN role on NAFLD histopathologic progression, liver and heart 8 µm frozen cryosections were evaluated with Hematoxylin and Eosin staining and Masson Goldner Trichrome staining kit. Furthermore, lipid accumulation in liver sections was evaluated by Oil Red O staining.
Oxidative stress and fibrosis development were analyzed by Western Blot and Immunofluorescence assays. Automated quantification on the liver lipid accumulation and immunofluorescence signals were performed using Image J program (http://imagej.nih.gov/ij/). Quantitative measurement of western blot 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.
All experiments were performed three times.
Statistical analysis were performed with GraphPad Prism software (GraphPad). Data are presented as the mean ±SD or SEM. Statistical significances were assessed by two-way Anova tests and Tukey's multiple comparisons test. Results were considered significant when $p \leq 0.05$.
A linear model with random effects (random intercept) was used to model the glycemic level according to time for the 4 different treatments under study (CONTR, MCDD group 3 week, MCDD+LCARN 3 week, MCDD group 6 week, MCDD+LCARN 6 week). Time was modeled using a B-spline with 3 interior knots. The free R software was used for the computations [R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/].

AIM OF THE STUDY

The aim of the study was to investigate the effects of L-Carnitine supplementation on liver fat deposition, oxidative stress and fibrosis development mechanism in a mouse model of steatohepatitis induced by a methionine-choline deficient diet. In the same model we also analyzed the role of L-Carnitine on cardiac tissue, considering the highest rates of mortality in NAFLD due to cardiovascular events.

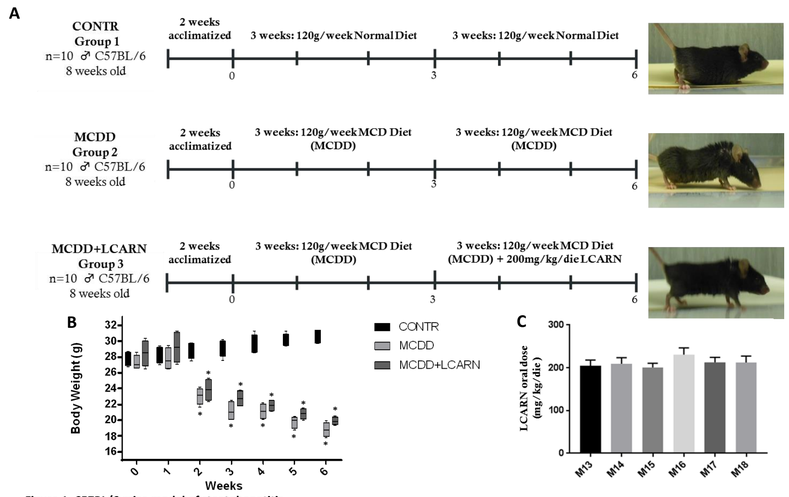

Figure 1. C57BL/6 mice model of steatohepatitis.
A) Experimental protocol;
B) Body weight of the three experimental groups. The MCDD mice loss of weight reflected the experimental model described in literature. Statistical significances: Mice body weight by Anova p<0.0001. Tukey's multiple comparisons test *p<0.0001 vs CONTR;
C) Water consumption and oral LCARN supplementation in MCDD mice supplemented with LCARN (M13-M18) group were recorded every day. Data are presented as mean ± SD.

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

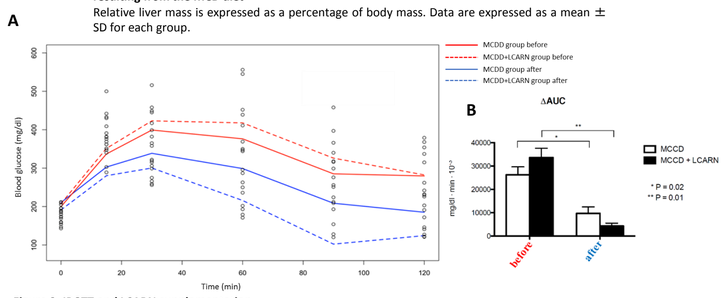

Figure 2. IPGTT and LCARN supplementation.
A) Linear model with random effects was used to model the glycemic level according to time for the 2 different treatments before (week 3) and after (week 6) oral LCARN somministration. There were evidence of interaction between time, treatment type and before/after the treatment itself (p<0.001). This results is confirmed both considering and excluding the CNT group.
B) Area Under the Curve (AUC) before (week 3) and after (week 6) oral LCARN somministration. Data are expressed as ±SD. Statistical significance: t-test *p=0.02, **p=0.01.

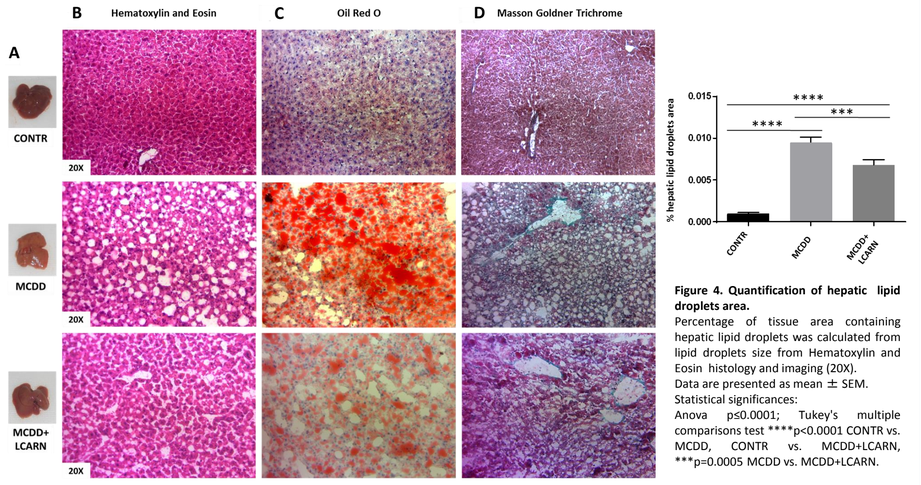

Figure 4. Quantification of hepatic lipid droplets area.
Percentage of tissue area containing hepatic lipid droplets was calculated from lipid droplets size from Hematoxylin and Eosin histology and imaging (20X).
Data are presented as mean ± SEM.
Statistical significances:
Anova p≤0.0001; Tukey's multiple comparisons test ****p<0.0001 CONTR vs. MCDD, CONTR vs. MCDD+LCARN, ***p=0.0005 MCDD vs. MCDD+LCARN.

Figure 3. Liver morphology, histology and lipid accumulation.
A) Macroscopic appearance of livers from mice of the three different group;
B) Hematoxylin and Eosin stained sections from mice showed differences in fatty change (20X). LCARN supplementation controlled fat accumulation and seemed to delay the disease progression;
C) An evaluation with Oil Red O staining technique confirmed lipid accumulation and the action of LCARN supplementation (20X);
D) Masson Goldner Trichrome stained sections from mice used for the detection of fibrotic areas in livers (20X).

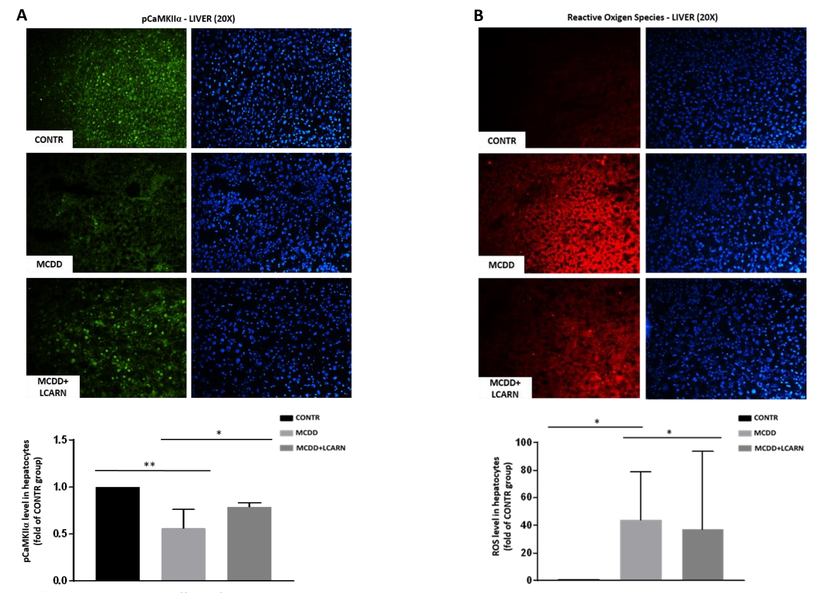

Figure 5. The hepatic antioxidant effects of LCARN supplementation.
A) In green, representative immunofluorescence assay of pCaMKIIα protein content, in mice hepatocytes of the three group and relative quantification. pCaMKIIα Anova p<0.0028; Tukey's multiple comparisons test **p<0.0023 CONTR vs MCDD; *p=0.0308 MCDD vs. MCDD+LCARN. All data shown are means ± SEM.
B) In red, representative immunofluorence 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.
In blue, staining of nuclei with DAPI.

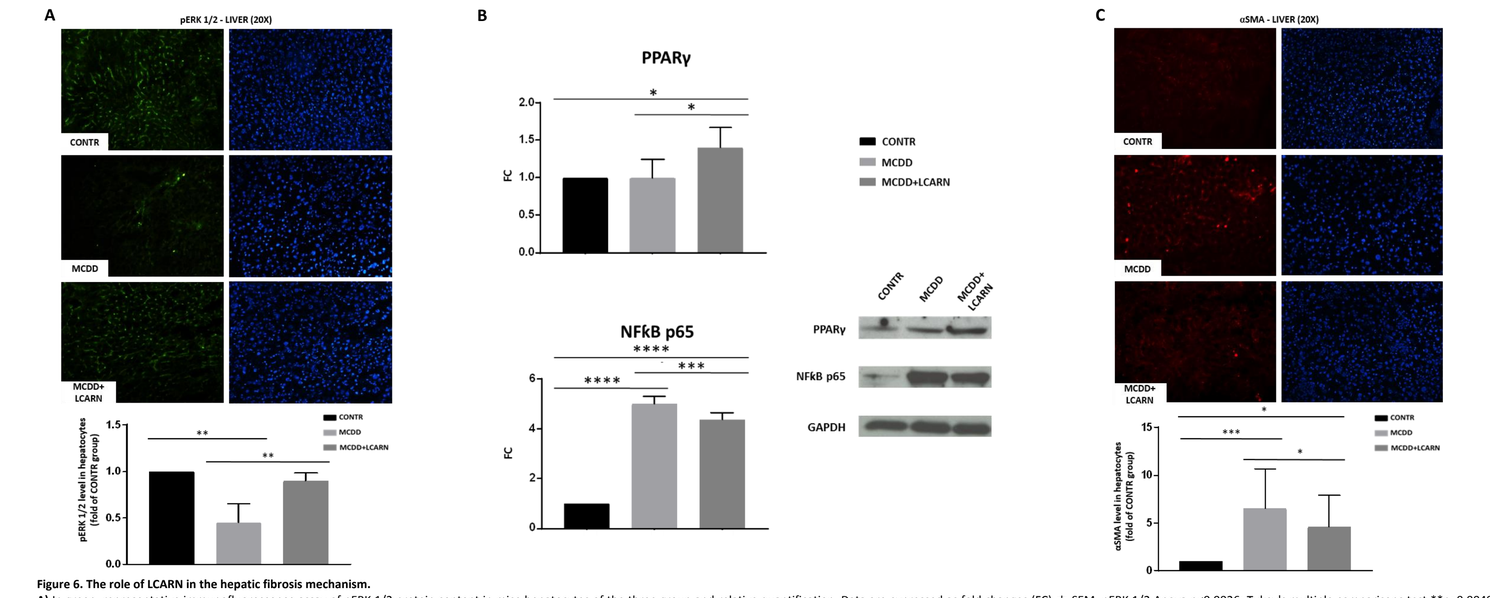

Figure 6. The role of LCARN in the hepatic fibrosis mechanism.
A) In green, representative immunofluorescence assay of pERK 1/2 protein content in mice hepatocytes of the three group and relative quantification. Data are expressed as fold changes (FC) ± SEM. pERK 1/2 Anova p<0.0026; Tukey's multiple comparisons test **p=0.0040 CONTR vs. MCDD, **p=0.0048 MCDD vs. MCDD+LCARN.
B) Western blot data indicated that LCARN supplementation significantly increased the hepatic PPARγ protein content resulting in decreased NfkB 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. NfkB 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.
C) In red, representative immunofluorescence assay of α-SMA protein content 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. In blue, staining of nuclei with DAPI.

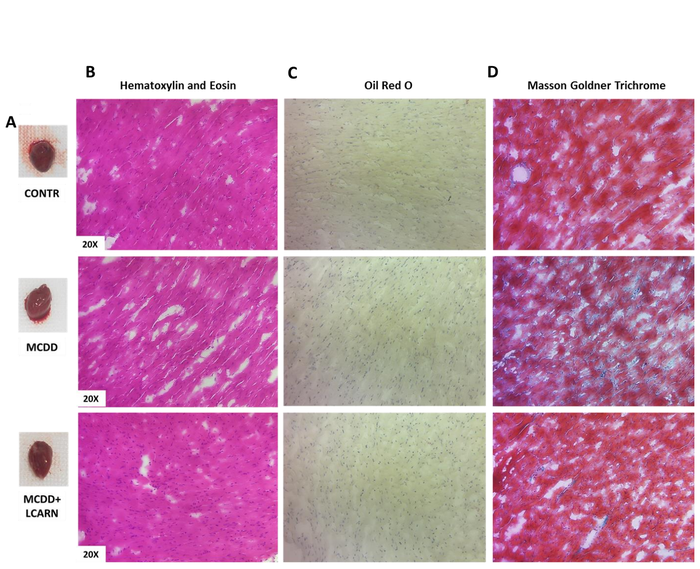

Figure 7. Heart morphology, histology and lipid accumulation.
A) Macroscopic appearance of hearts from mice of the three different group;
B) Cardiac Hematoxylin and Eosin stained tissue did not showed diffuse vacuolar degeneration in the three different groups and myocardiocytes with abnormal size and altered nuclear morphology were not observed (20X);
C) Oil Red O stained sections from mice confirmed no cardiac lipid accumulation (20X);
D) Masson Goldner Trichrome stained sections from mice showed fibrotic areas in MCDD hearts (20X).

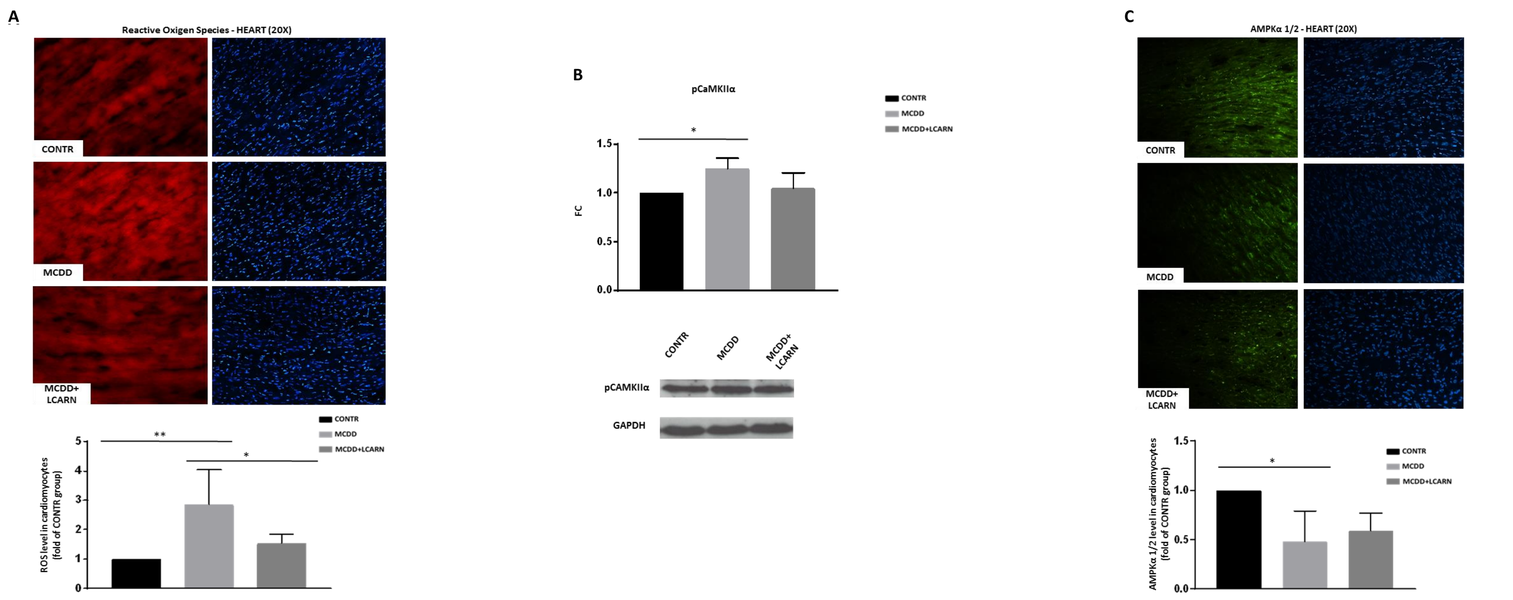

Figure 8. The cardiac antioxidant effects of LCARN supplementation.
A) In red, representative immunofluorescence staining of ROS mice hearts and relative quantification. ROS Anova p=0,0028; Tukey's multiple comparisons test **p=0,0026 CONTR vs. MCDD, *p=0,013 MCDD vs. MCDD+LCARN;
B) Western blot analysis of pCaMKIIα protein level in the three mice groups. Data are expressed as fold changes (FC) ±SD. pCaMKIIα Anova test p≤0.0199; Tukey's multiple comparisons test *p=0.02 CONTR vs. MCDD.
D) In green, representative 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.
In blue, staining of nuclei with DAPI.

	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 2. Heart mass absolute and relative and percentage differences in relative heart mass resulting from the MCD diet
Relative heart mass is expressed as a percentage of body mass. Data are expressed as a mean ± SD for each group.

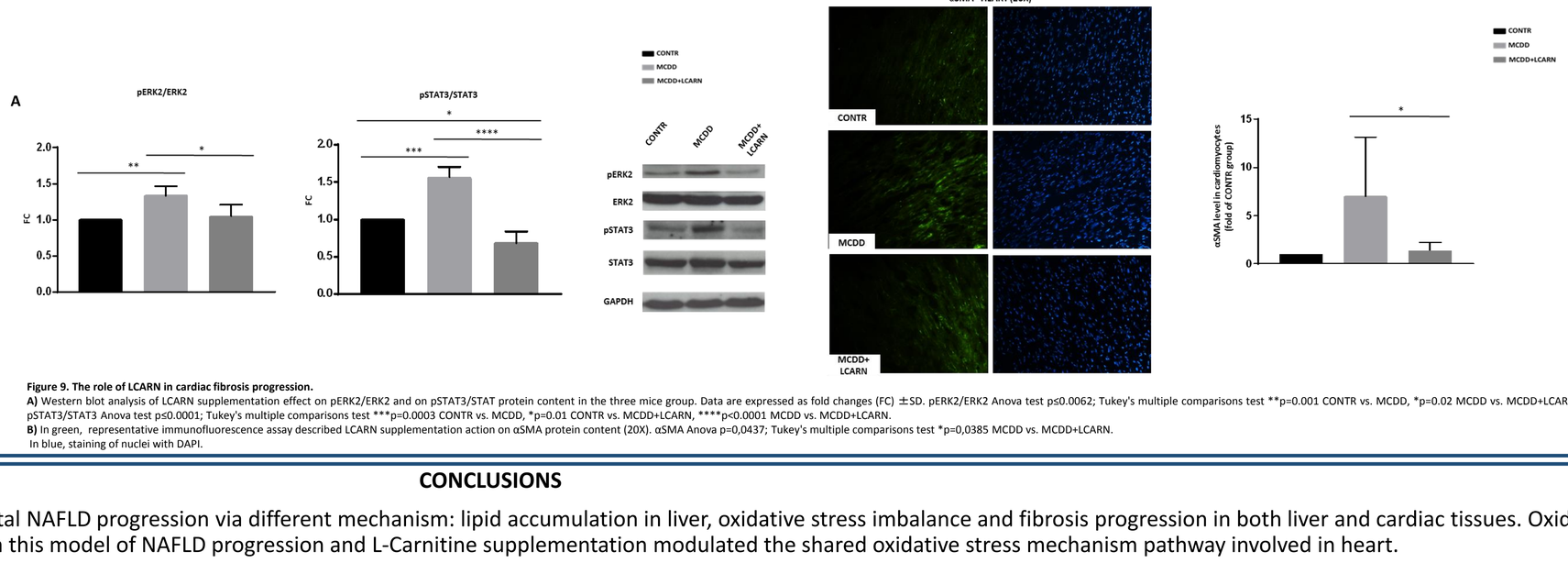

Figure 9. The role of LCARN in cardiac fibrosis progression.
A) Western blot analysis of LCARN supplementation effect on pERK2/ERK2 and on pSTAT3/STAT protein content in the three mice group. Data are expressed as fold changes (FC) ±SD. pERK2/ERK2 Anova test p≤0.0062; Tukey's multiple comparisons test **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.
B) In green, representative immunofluorescence assay described LCARN supplementation action on αSMA protein content (20X). αSMA Anova p=0,0437; Tukey's multiple comparisons test *p=0,0385 MCDD vs. MCDD+LCARN.
In blue, staining of nuclei with DAPI.

CONCLUSIONS

L-Carnitine supplementation decreased the severity of experimental NAFLD progression via different mechanism: lipid accumulation in liver, oxidative stress imbalance and fibrosis progression in both liver and cardiac tissues. Oxidative stress represented an important stimulus in this model of NAFLD progression and L-Carnitine supplementation modulated the shared oxidative stress mechanism pathway involved in heart.