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Title

Enhanced expression of pro-inflammatory mediators and LXR-regulated lipogenic genes in nonalcoholic fatty liver disease and hepatitis C

Short title (running head): Liver X receptor in chronic liver diseases

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Keywords: Liver X receptor; nonalcoholic fatty liver disease; chronic HCV infection; lipogenesis; inflammation.

List of Abbreviations:

NAFLD, Nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; SREBP, sterol regulatory element binding protein; TNF, tumor necrosis factor; IL, interleukin; HCV, hepatitis C virus; LXR, liver X receptor; iNOS, inducible nitric oxide synthase; COX, cyclooxygenase; OPN, osteopontin; ALT, alanine aminotransferase; γ -GT, gamma-glutamyltransferase; NAS, nonalcoholic steatosis; NL, normal liver; PPAR- γ , peroxisome proliferator-activated receptor gamma; FAS, fatty acid synthase; CYP2E1, cytochrome P450 2E1; SOCS, suppressors of cytokine signalling; RXR, retinoid X receptor; LXRE, LXR response element.

ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is one of the most frequent chronic liver diseases worldwide. The metabolic factors associated with NAFLD are also determinants of liver disease progression in chronic hepatitis C virus (HCV) infection. It has been reported that, besides to induce hepatic fatty acid biosynthesis, liver X receptor (LXR) regulates a set of inflammatory genes. We aimed to evaluate the hepatic expression of LXR α and its lipogenic and inflammatory targets in forty-three patients with NAFLD, forty-four with chronic HCV infection, and in twenty-two with histologically normal liver. Real-time PCR and western blot were used to determine hepatic expression levels of LXR α and related lipogenic and inflammatory mediators in the study population. We found that LXR α gene and its lipogenic targets peroxisome proliferator-activated receptor- γ , sterol regulatory element binding protein-1c, sterol regulatory element binding protein-2 and fatty acid synthase were overexpressed in the liver of NAFLD and HCV patients who had steatosis. Moreover, up-regulation of inflammatory genes such as tumor necrosis factor (TNF)- α , interleukin (IL)-6, osteopontin, inducible nitric oxide synthase, cyclooxygenase-2 and suppressors of cytokine signalling-3 was observed in NAFLD and HCV patients. Interestingly, TNF- α , IL-6 and osteopontin gene expression was lower in patients with steatohepatitis than in those with steatosis. In conclusion, hepatic expression of LXR α and its related lipogenic and inflammatory genes is abnormally increased in NAFLD and HCV patients with steatosis, suggesting a potential role of LXR α in the pathogenesis of hepatic steatosis in these chronic liver diseases.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is one of the most frequent causes of abnormal liver function and correlates with central adiposity, obesity, insulin resistance, the metabolic syndrome and type 2 diabetes mellitus [1]. The pathological spectrum of NAFLD ranges from fatty liver (steatosis) to nonalcoholic steatohepatitis (NASH), advanced fibrosis, cirrhosis, and even hepatocellular carcinoma [2]. Accumulation of excess triglyceride in hepatocytes is necessary for the development of NAFLD. Fat either is delivered to hepatocytes in the form of free fatty acids bound to albumin or is synthesized *de novo* within hepatocytes [3]. In this regard, several lines of evidence indicate that hepatic activation of sterol regulatory element binding protein-1c (SREBP-1c) together with other lipogenic or adipogenic transcription factors is crucial for the development of fatty liver [4,5]. While the mechanisms involved in the transition from bland steatosis to NASH have not been fully established so far, there is increasing evidence on the central role of nontriglyceride lipotoxicity in the pathogenesis of NASH [6]. In this hypothesis, the accumulation of triglyceride in the form of lipid droplets within the liver is not needed for the development of NASH and, in fact, it may be protective whereas toxic metabolites derived from free fatty acids could lead to NASH by inducing endoplasmic reticulum stress, inflammation and liver cell death by apoptosis [7,8].

Hepatic steatosis is a common histologic feature in chronic hepatitis C virus (HCV) infection and has been reported to be associated with fibrosis [9]. HCV has been shown to alter host cell cholesterol/lipid metabolism and induce hepatic steatosis [10]. Furthermore, HCV proteins associate with mitochondria and endoplasmic reticulum and promote oxidative stress, increasing gene expression of oxidative stress-mediated inflammatory genes [11-13].

Liver X receptors (LXRs) are members of the nuclear hormone receptor superfamily that work as fatty acid-activated transcription factors [14]. Considerable evidence has emerged indicating that, besides to induce SREBP-1c-mediated hepatic fatty acid biosynthesis [4,14], LXRs repress in macrophages a set of inflammatory genes as IL-6, inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2 and osteopontin (OPN) [15,16]. The latter is considered as a pro-inflammatory cytokine strongly associated with the early stages of development of NASH in animal models [17]. Albeit hepatic mRNA and protein levels of iNOS and COX-2 are increased in HCV patients [18,19], little is known, however, on the role of LXRs in the development of hepatic steatosis and inflammation in chronic HCV infection. Based on these data, it is conceivable that LXR may be a key regulator of hepatic lipogenesis and inflammation in NAFLD and HCV patients. In order to shed light on this hypothesis, we carried out this study aimed to determine hepatic mRNA and protein levels of LXR α and its related lipogenic and inflammatory targets in patients with NAFLD, chronic HCV infection, and histologically normal liver (NL).

PATIENTS AND METHODS

Patients. This study comprised 87 nondiabetic patients with a clinical diagnosis of either NAFLD or chronic HCV infection who underwent liver biopsy with diagnostic purposes. Inclusion criteria for NAFLD patients were based on the absence of alcohol intake, the presence of biopsy-proven steatosis with/without necroinflammation and/or fibrosis, and a negative anti-HCV serum test. Patients with chronic HCV infection were included if they had serum anti-HCV and HCV RNA, persistently abnormal serum alanine aminotransferase (ALT) levels, liver histology findings compatible with chronic hepatitis, and a negative history of alcohol consumption. In order to avoid bias depending of HCV genotype, all HCV patients included were infected by genotype 1. In addition, none of HCV patients studied had ever received antiviral therapy. Patients with other potential causes of chronic liver disease or those receiving drug treatment for any chronic medical disorder were excluded.

We further studied 22 patients with asymptomatic cholelithiasis to whom a liver biopsy was taken, during programmed laparoscopic cholecystectomy, as part of a study protocol and after signing a specific informed consent. All these subjects were otherwise healthy, and had histologically normal liver, normal fasting glucose, cholesterol and triglycerides, normal AST and ALT, and no evidence of viral infections (hepatitis B virus, HCV and human immunodeficiency virus). In addition, none of these individuals drank alcohol.

The study was performed in agreement with the Declaration of Helsinki and with local and national laws. The Institution's Human Ethics Committee approved the study procedures, and written informed consent was obtained from all patients before inclusion in the study.

Clinical and laboratory assessment. After a 12-hour overnight fast, clinical and anthropometric data of each patient were collected at the time of liver biopsy as well as venous blood samples to test hepatic enzymes and metabolic parameters by using a conventional automated analyzer. Serum insulin was determined by a chemiluminescent microparticle immunoassay (ARCHITECT insulin; Abbot Park, IL). Insulin resistance was calculated by the homeostasis model assessment (HOMA-IR) [20].

Antibodies against HCV and HIV as well as hepatitis B surface antigen were tested by immunoenzymatic assays (Murex, Dartford, UK). HCV RNA levels were quantified by real-time PCR (COBAS AmpliPrep/COBAS TaqMan HCV test, Roche Diagnostics, Branchburg, NJ). HCV genotyping was performed using a second generation line-probe hybridization assay (HCV genotype 2.0 assay (LiPA), Roche Diagnostics, Branchburg, NJ).

Liver tissue studies. Liver biopsies were divided in at least two pieces of a minimum length of 15 mm each. One of them was fixed in buffered formalin and used for routine histological staining whereas the remaining pieces were immediately snap-frozen and stored in liquid nitrogen.

Histopathology assessment: Paraffin-embedded liver biopsy sections were evaluated under code by a single hepatopathologist blinded to the clinical data. Overall, steatosis was assessed as outlined by Brunt et al [21] and the Kleiner's histological scoring system [22] was used to assess the grade of necroinflammation and the stage of fibrosis in NAFLD patients who were classified into two groups: those with simple steatosis (NAS) and those with definite steatohepatitis (NASH). In the HCV group, the grade of necroinflammation and the stage of fibrosis were scored as proposed by Scheuer [23]. HCV patients were categorized into two groups, depending of the presence of steatosis.

Histologic photomicrographs were acquired by using a high-resolution digital videocamera (Nikon DXM 1200, Tokyo, Japan) connected to a light microscope (Nikon Eclipse E400, Tokyo, Japan) equipped with a planApocromatic 20x objective.

Quantitative real-time PCR. Total RNA of liver biopsy samples from all patients was extracted by using a Trizol reagent (Life Technologies, Carlsbad, CA). First-standard cDNA was synthesized using High-Capacity cDNA Archive Kit (Applied Biosystems, Weiterstadt, Germany). cDNA was amplified using multiplex real-time PCR reactions on a StepOne Plus (Applied Biosystems). TaqMan primers and probes were derived from the commercially available TaqMan® Gene Expression Assays (Applied Biosystems) (Table 1). Relative changes in gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ method. The cycle number at which the transcripts were detectable (CT) was normalized to the cycle number of GAPDH detection, referred to as ΔCt .

Western blot. Protein extraction and western blotting were performed as described [13] with modifications. Briefly, liver biopsy samples from all patients of each group were homogenized in a medium containing: 0.25 mM sucrose, 1 mM EDTA, 10 mM Tris and 1% protease inhibitor cocktail (Roche, Mannheim, Germany). For western blot analysis, total protein were boiled in Laemmli sample buffer and equal amounts of protein (30-50 μ g) were separated by SDS-polyacrylamide gel electrophoresis (12% acrylamide) and transferred to PVDF membranes (Millipore, Massachusetts, United States). Nonspecific binding was blocked by preincubation of the membranes in phosphate-buffered saline containing 5% skimmed milk for 1 h. Membranes were incubated overnight at 4°C with primary antibodies as rabbit polyclonal antibodies against LXR α (Abcam, Cambridge, UK), SREBP-1c (Abcam), PPAR- γ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), TNF- α (Abcam), and iNOS (BIOMOL International L.P., Plymouth Meeting, PA) or mouse monoclonal antibody against OPN (Abcam). Bound primary antibody was detected with HRP-conjugated anti-rabbit or anti-mouse antibodies (DAKO, Glostrup, Denmark) and blots were developed using an enhanced chemiluminescence detection system (ECL kit, Amersham Pharmacia, Uppsala, Sweden). The density of the specific bands was quantified with an imaging densitometer (Scion Image, Maryland, MA).

Statistical analysis. Categorical variables are presented as frequency and percentage. Continuous variables are shown as mean \pm standard deviation. The baseline characteristics of the patients studied were compared by the Pearson χ^2 test for categorical variables and the unpaired t test or Mann-Whitney U test for continuous variables. Data from qPCR and Western blotting were compared by using the Kruskal-Wallis ANOVA test. All statistical analysis were performed using SPSS version 15.0 (SPSS, Cary, NC) software with 2-sided tests, with a p value of < 0.05 considered as statistically significant.

RESULTS

Patient characteristics.

Characteristics of the study population are detailed in Tables 2 and 3. To highlight, all patient groups were well matched in terms of age and sex distribution. NAS patients had significantly higher serum levels of triglycerides ($p = 0.008$) and γ -GT ($p = 0.037$) than NL subjects. In NASH patients, the serum insulin levels and the mean HOMA-IR score were significantly higher than in NL individuals ($p = 0.017$ and $p = 0.004$, respectively), being the higher serum levels of ALT the only significant difference ($p = 0.009$) with respect to NAS patients. No significant differences were observed between HCV patient groups.

At liver biopsy, the degree of steatosis was similar in NAS and NASH patients (Fig. 1, panels A and B). On the other hand, comparing HCV patients with or without steatosis (Fig. 1, panels C and D), no significant differences were found in the grade of necroinflammation and in the stage of fibrosis.

Hepatic expression of LXR α and related lipogenic genes is enhanced in NAFLD patients and in HCV patients with steatosis.

When compared with NL, hepatic mRNA levels of LXR α and PPAR- γ were significantly increased in NAS (+114% and +131%, respectively) and NASH (+131% and +120%, respectively) (Fig. 2, panel A and Fig. 3, panel A). The significant induction in LXR α and PPAR- γ was also shown in HCV patients, being higher in those with steatosis (HCV: +41%, HCV+NAS: +126%; and HCV: +44% and HCV+NAS: +132%, respectively, vs NL) (Fig. 2, panel A and Fig. 3, panel A). Hepatic mRNA levels of SREBP-1c were significantly higher in NAS (+81%) and NASH (+124%) than in NL (Fig. 3, panel B). Regarding HCV patients, hepatic mRNA levels of SREBP-1c were significantly increased with respect to NL only in those with steatosis (+165%) (Fig. 3, panel B). We also found that hepatic mRNA levels of SREBP-2 were significantly higher in NAS (+190%) and NASH (+80%) than in NL (Fig. 3, panel C). Only HCV patients with steatosis had hepatic mRNA levels of SREBP-2 significantly more elevated than NL (+130%) (Fig. 3, panel C). Hepatic gene expression of FAS showed a similar pattern to that observed for LXR α and PPAR- γ (Fig. 3, panel D).

Hepatic content of LXR α , PPAR- γ and SREBP-1c proteins was significantly increased in NAS (+112%, +86%, +126%; respectively) and NASH (+166%, +89%, +176%; respectively) than in NL (Fig. 2, panel B and Fig. 4). Noteworthy, protein amounts of LXR α and SREBP-1c were significantly higher in liver biopsies from patients with NASH than in those with NAS (Fig. 2, panel B and Fig. 4). In addition, a significant increase in LXR α , PPAR- γ and SREBP-1c protein content was shown in HCV patients (+48%, +46%, +43%, respectively, vs NL), being the highest LXR α , PPAR- γ and SREBP-1c levels observed in HCV patients who had steatosis (+155%, +276%, +191%, respectively, vs NL) (Fig. 2, panel B and Fig. 4).

Hepatic expression of oxidative stress and inflammatory genes is upregulated in NAFLD and HCV patients.

Overexpression of cytochrome P450 2E1 (CYP2E1) leads to oxidative stress [24]. Thus, we wanted to determine hepatic CYP2E1 gene expression in our study population. We found that CYP2E1 mRNA levels were significantly increased in the liver of patients with NAS (+74%) and NASH (+72%) with respect to NL (Fig. 5, panel A). To note, only HCV patients with steatosis had hepatic CYP2E1 mRNA levels significantly higher than NL (+105%) (Fig. 5, panel A).

We further analyzed hepatic mRNA levels of LXR-related inflammatory genes such as TNF- α , IL-6, OPN, iNOS and COX-2. Intrahepatic OPN, TNF- α , and IL-6

mRNA levels were significantly increased in NAS (+271%, +123% and +300%, respectively) and NASH (+116%, +105% and +188%, respectively) as compared with NL (Fig. 5, panels B, C, and D). Interestingly, OPN and IL-6 mRNA levels were significantly decreased in NASH (-57%, and -37%, respectively) with respect to NAS (Fig. 5, panels B and D). However, no differences in the hepatic OPN and IL-6 mRNA levels between HCV patients with or without steatosis were observed (Fig. 5, panels B, and D). In contrast, TNF- α mRNA levels were significantly higher in HCV patients with steatosis (+25%) than in those without steatosis (Fig. 5, panel C). In addition, we found that hepatic suppressors of cytokine signaling (SOCS)-3 mRNA levels were significantly increased in NAS (+92%) and NASH (+107%) with respect to NL, being the highest SOCS-3 mRNA levels observed in HCV patients regardless of the presence of steatosis (Fig. 5, panel E). We also found hepatic iNOS and COX-2 mRNA levels were significantly enhanced in NAS (+87% and +105%, respectively) and NASH (+102% and +130%, respectively) as well as in HCV patients without steatosis (+221% and +524%, respectively) and with steatosis (+250% and +329%, respectively) compared with NL (Fig. 6, panels A and B).

On the other hand, a statistically significant increase in the hepatic abundance of OPN, TNF- α and iNOS proteins was observed in NAS (+137%, +172%, and +98%, respectively) and NASH (+105%, +98% and +104%, respectively) with respect to NL (Fig. 7). Noteworthy, the hepatic content of OPN and TNF- α was significantly lower in NASH patients than in those with NAS (Fig. 7). We also found a significant increase of OPN and iNOS proteins in HCV patients without steatosis (+309% and +137%, respectively) and with steatosis (+317% and +189%, respectively, vs NL). Interestingly, in HCV patients, TNF- α protein content was highest when steatosis was present (HCV: +229% and HCV+NAS: +287%, vs NL) (Fig. 7).

DISCUSSION

There is experimental evidence that LXR α induces the expression of lipogenic genes, such as PPAR γ and SREBP-1c, and downregulates the expression of inflammatory genes, such as TNF α , IL-6, and osteopontin [14,15]. This study demonstrates that LXR α and its related lipogenic and inflammatory gene targets are upregulated in liver biopsies from NAFLD and HCV patients with steatosis. Given the key role of LXR signalling pathways in the cross-talk between lipogenesis and inflammation [15], we propose a direct contribution of LXR α in the development of NAFLD as well as in HCV-associated steatosis.

LXRs belong to the nuclear hormone receptor family and are DNA-binding transcriptional regulators. LXRs form heterodimers with the retinoid X receptor (RXR) α and bind to target DNA sequences known as the LXR response element (LXRE) [25]. There are two isoforms termed LXR α and LXR β , both expressed in the liver among other locations [26]. In response to oxysterols, LXRs activate genes involved in reverse cholesterol transport and hepatic cholesterol metabolism [26]. Hepatic lipogenesis is generally induced by nuclear receptors such as PPAR- γ and LXR α [5, 14, 27]. These transcription factors increase the synthesis of fatty acids by either upregulating SREBP-1c or directly binding to the promoters of some lipogenic genes, including FAS [4,14,28]. LXR α may play a role in enhancing lipogenesis in the liver of NAFLD patients, as previously described [29,30]. In the current study, hepatic LXR α , PPAR- γ , SREBP-1c, SREBP-2 and FAS gene expression was significantly increased in NAFLD patients with respect to NL and, interestingly, the hepatic content of LXR α protein was significantly higher in NASH than in NAS. These findings suggest a steatogenic role for LXR α and its target lipogenic genes PPAR- γ , SREBP-1c, SREBP-2 and FAS. Different factors could induce hepatocyte LXR expression, such as saturated and unsaturated fatty acids, PPAR- γ , LXR agonists and lipid peroxidation products (by stimulating PPAR- γ) [31,32]. In turn, LXR increases the transcriptional activity and gene expression of PPAR- γ through the LXRE contained in its promoter region [32]. In addition, LXR α expression is controlled by an auto-regulatory mechanism, since the human LXR α gene promoter contains three functional LXRE [25]. This mechanism may be responsible for the strong upregulation of LXR α in lipid-loaded hepatocytes.

A novel finding of our study is that hepatic mRNA and protein levels of LXR α and its downstream lipogenic genes were abnormally increased in HCV patients who had steatosis. Mechanisms involved in HCV-induced steatosis seem to be mediated by HCV proteins, whose expression is associated with changes in lipogenic gene expression and/or the activity of lipogenic proteins and effects on mitochondrial oxidative function [10,32]. Thus, HCV core protein indirectly enhances the transcriptional activity of SREBP-1c, increasing the binding of LXR α /RXR α to LXRE as described in an *in vivo* model [33]. Accordingly, HCV core protein may play a crucial role in the development of hepatic steatosis by activating the LXR α signalling pathway in HCV-infected hepatocytes. In addition, HCV NS2 protein is able to induce the transcription of SREBP-1c and FAS, and this could be a contributing factor for HCV-associated steatosis [34].

When an excess of fatty acids occurs in hepatocytes, CYP2E1 gene expression is induced initiating the process of lipid peroxidation [24]. In our study, we found that hepatic mRNA levels of CYP2E1 were increased in NAFLD patients, as previously indicated [35]. Interestingly, CYP2E1 gene expression is also enhanced in HCV patients who had steatosis. The latter is in agreement with previous observations by

Gochee et al. [11], supporting the notion that hepatic CYP2E1 upregulation is not a direct effect of HCV.

In fatty liver, distinct mechanisms such as oxidative stress, endoplasmic reticulum stress and hypoadiponectinemia are able to activate a set of inflammatory pathways including secretion of inflammatory cytokines, such as TNF- α and IL-6 [36]. In particular, TNF- α promotes triglyceride accumulation and hepatic lipotoxicity, at least in part, by inducing hepatocyte lysosomal destabilisation [37]. Our current results show an increased hepatic TNF- α , IL-6 and SOCS-3 gene expression in patients with NAFLD, while TNF- α protein and IL-6 mRNA levels were significantly lower in NASH than in NAS patients. These findings argue against a direct role of TNF- α and IL-6 as pathogenic mediators in NASH. However, many of the biological effects of TNF- α are mediated by IL-6, and experimental evidence exists showing that IL-6 induces hepatic SOCS-3 expression [38]. Since SOCS-3 promotes SREBP-1c expression and subsequent development of hepatic steatosis in mice [39], our findings strongly suggest a key role for SOCS-3 in the development of steatosis in NAFLD patients. On the other hand, we also found an enhanced hepatic TNF- α and IL-6 gene expression in HCV patients, being the highest TNF- α mRNA and protein levels observed in those HCV patients who had steatosis. The latter is in agreement with previous observations [11], suggesting that TNF- α may play an important role in the development of HCV-associated steatosis. Additionally, SOCS-3 gene expression is increased in HCV patients regardless of fat accumulation as previously described in genotype 1 non-responders subjects [40].

Upregulation of OPN gene expression has been implicated early in the development of NASH in a murine model [17]. In the present study we found that OPN gene expression is markedly enhanced in NAS patients while significantly decreased in those with NASH, in a similar extent to that observed for TNF- α protein and IL-6 mRNA levels. Noteworthy, the highest LXR α protein levels were observed in the same NASH patients. As previously reported, this transcription factor might modulate hepatic inflammation by inhibiting TNF- α , IL-6 and OPN gene expression in Kupffer cells [15,16,41]. The mechanism underlying the repression of inflammatory genes by LXR is poorly understood. LXREs have not been identified in the proximal promoters of the repressed genes [15,16], indicating an indirect mechanism through the regulation of other transcription factors supporting gene transcription. Elucidating whether hepatic upregulation of LXR α occurs in hepatocytes or in Kupffer cells may help to understand the inhibitory action of this transcription factor on inflammatory gene expression in NAFLD patients.

On the other hand, our results also show that hepatic OPN gene expression is markedly increased in HCV patients regardless of the presence of steatosis, as previously indicated regarding SOCS-3 and IL-6 mRNA levels, favouring the hypothesis that inflammatory gene upregulation might be a direct effect of HCV itself. In our study, the highest LXR α protein levels observed in HCV-related steatosis is not accompanied by a repression of OPN, IL-6 and SOCS-3 gene expression. Thus, there is no compelling evidence for a role of LXR α in modulating inflammation in HCV.

iNOS and COX-2 play an important role in development and progression of liver injury in NASH patients [42,43]. Our study results confirm these observations and further show that hepatic iNOS and COX-2 gene expression is similarly increased in NAS and NASH, suggesting that hepatic iNOS and COX-2 upregulation is an early event remaining unchanged in the course of NAFLD. In addition, we also found that hepatic iNOS gene expression is increased in HCV patients, being the highest iNOS

protein content observed in HCV patients who had steatosis. These findings are in agreement with experimental evidence from our group indicating that HCV NS5A and core proteins are able to upregulate iNOS gene expression [13]. An interesting finding of the present study is that hepatic COX-2 mRNA levels were significantly lower in HCV patients with steatosis than in those without fatty liver. There is experimental evidence that HCV core protein can induce COX-2 expression in human hepatocytes [19], but whether fat accumulation may modulate COX-2 expression in HCV-infected hepatocytes, as suggested by our present results, deserves further investigation.

In summary, taking into account all the findings showed in this paper, it is conceivable that an enhanced LXR-induced hepatic lipogenesis may contribute to lipid accumulation in NAFLD and in chronic hepatitis C infection. However, our results do not support an anti-inflammatory effect of LXR and its dependent transcription factors (PPAR γ) in these chronic liver diseases, likely because their potential anti-inflammatory effects are overwhelmed by a number of distinct pro-inflammatory factors, such as lipotoxicity, endoplasmic reticulum stress and mitochondrial dysfunction in NAFLD, and apoptosis and virus-induced production of Th1 cytokines in chronic HCV infection.

In conclusion, this study provides evidence that hepatic expression levels of LXR α and its related lipogenic and inflammatory gene targets are abnormally increased in NAFLD patients and in HCV patients with concomitant fatty liver. These results identify LXR α as a novel factor involved in the pathogenesis of hepatic steatosis in chronic liver diseases. Nevertheless, further experimental studies are needed to establish the specific relationship between LXR and lipogenic and inflammatory pathways in hepatocytes.

Author contribution

All the listed authors have participated actively in the study.

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FIGURE LEGENDS

Figure 1. Liver biopsy features defining the study population. The degree of steatosis, largely macrovesicular, was similar in patients with steatosis (panel A) and in those with steatohepatitis (panel B). Portal and periportal mononuclear cell infiltrates were common findings in HCV patients without steatosis (panel C) and with steatosis (panel D), showing no significant differences between both patient groups. (All photomicrographs: Hematoxylin-eosin staining; original magnification x200). Magnification bar, 100 μ m.

Figure 2. Hepatic overexpression of LXR α in NAFLD and HCV patients. (A). Bar graph shows mRNA levels of LXR α determined by real-time RT-PCR in liver biopsies from different patients groups. Values are expressed as mean \pm SD of the percentage relative to NL (100%). (B) Representative western blotting (two different patients from each group) showing the hepatic protein content of LXR α . Bar graph shows the mean \pm SD of the densitometric analysis of specific bands from all patients studied and are expressed as percentage relative to NL (100%). Equal loading of the gels was demonstrated by probing the membranes with an anti- β -actin polyclonal antibody. LXR, liver X receptor; NL, normal liver; NAS, nonalcoholic steatosis; NASH, nonalcoholic steatohepatitis; HCV, hepatitis C virus. * $p < 0.05$ vs NL. # $p < 0.05$ vs NAS. & $p < 0.05$ vs HCV.

Figure 3. Hepatic mRNA levels of LXR α -related lipogenic genes are increased in NAFLD and HCV patients with steatosis. Bar graph shows mRNA levels of PPAR- γ (A), SREBP-1c (B), SREBP-2 (C), and FAS (D) determined by real-time RT-PCR in liver biopsies from different patient groups. Values are expressed as mean \pm SD of the percentage relative to NL (100%). PPAR- γ , peroxisome proliferator-activated receptor gamma; SREBP, sterol regulatory element binding protein; FAS, fatty acid synthase; NL, normal liver; NAS, nonalcoholic steatosis; NASH, nonalcoholic steatohepatitis; HCV, hepatitis C virus. * $p < 0.05$ vs NL. # $p < 0.05$ vs NAS. & $p < 0.05$ vs HCV.

Figure 4. Hepatic content of PPAR- γ and SREBP-1c proteins is also increased in NAFLD and HCV patients. (A) Representative western blotting (two different patients from each group) showing the hepatic amount of PPAR- γ and SREBP-1c. (B) Bar graph shows the mean \pm SD of the densitometric analysis of specific bands from all patients studied and are expressed as percentage relative to NL (100%). Equal loading of the gels was demonstrated by probing the membranes with an anti- β -actin polyclonal antibody. PPAR- γ , peroxisome proliferator-activated receptor gamma; SREBP, sterol regulatory element binding protein; NL, normal liver; NAS, nonalcoholic steatosis; NASH, nonalcoholic steatohepatitis; HCV: hepatitis C virus. * $p < 0.05$ vs NL. # $p < 0.05$ vs NAS. & $p < 0.05$ vs HCV.

Figure 5. Hepatic expression of oxidative stress and inflammatory genes is up-regulated in NAFLD and HCV patients. Bar graph shows mRNA levels of CYP2E1 (A), OPN (B), TNF- α (C), IL-6 (D) and SOCS-3 (E) determined by real-time RT-PCR in liver biopsies from different patient groups. Values are expressed as mean \pm SD of the percentage relative to NL (100%). CYP2E1, cytochrome P450 2E1; OPN, osteopontin; TNF, tumor necrosis factor; IL, interleukin; SOCS, suppressor of cytokine signalling; NL, normal liver; NAS, nonalcoholic steatosis; NASH, nonalcoholic

steatohepatitis; HCV, hepatitis C virus. * $p < 0.05$ vs NL. # $p < 0.05$ vs NAS. & $p < 0.05$ vs HCV.

Figure 6. Hepatic expression of iNOS and COX-2 is markedly induced in NAFLD and HCV patients. Bar graph shows mRNA levels of iNOS (A) and COX-2 (B) determined by real-time RT-PCR in liver biopsies from different patient groups. Values are expressed as mean \pm SD of the percentage relative to NL (100%). iNOS, inducible nitric oxide synthase; COX, cyclooxygenase; NL, normal liver; NAS, nonalcoholic steatosis; NASH, nonalcoholic steatohepatitis; HCV, hepatitis C virus. * $p < 0.05$ vs NL. # $p < 0.05$ vs NAS. & $p < 0.05$ vs HCV.

Figure 7. Increased hepatic content of OPN, TNF- α and iNOS proteins in NAFLD and HCV patients. (A) Representative western blotting (two different patients from each group) showing the hepatic amount of OPN, TNF- α and iNOS. (B) Bar graph shows the mean \pm SD of the densitometric analysis of specific bands from all patients studied and are expressed as percentage relative to NL (100%). Equal loading of the gels was demonstrated by probing the membranes with an anti- β -actin polyclonal antibody. OPN, osteopontin; TNF, tumor necrosis factor; iNOS, inducible nitric oxide synthase; NL, normal liver; NAS, nonalcoholic steatosis; NASH, nonalcoholic steatohepatitis; HCV, hepatitis C virus * $p < 0.05$ vs NL. # $p < 0.05$ vs NAS. & $p < 0.05$ vs HCV.

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TABLES

Table 1. Primers and probes used for the multiplex real-time RT-PCR.

Gene	Genbank	Assay ID	Amplicon size	Dye
LXR α	NM_005693.2	Hs00172885_m1	78	FAM TM
PPAR- γ	NM_015869.4	Hs01115510_m1	92	FAM TM
SREBP-1c	NM_004176.3	Hs00231674_m1	84	FAM TM
SREBP-2	NM_004599.2	Hs01081778_m1	50	FAM TM
SOCS-3	NM_003955.3	Hs01000485_g1	111	FAM TM
FAS	NM_004104	Hs01005611_m1	103	FAM TM
CYP2E1	NM_000773.3	Hs00559367_m1	71	FAM TM
OPN	NM_000582.2	Hs00960942_m1	63	FAM TM
TNF- α	NM_000594.2	Hs00174128_m1	80	FAM TM
IL-6	NM_000600.2	Hs00985639_m1	66	FAM TM
iNOS	NM_000625.4	Hs00167248_m1	74	FAM TM
COX-2	NM_000963.1	Hs00153133_m1	75	FAM TM
GAPDH	NM_002046.3	4326317E	122	VIC TM

LXR α , liver X receptor alpha; PPAR- γ , peroxisome proliferator-activated receptor gamma; SREBP, sterol regulatory element binding protein; SOCS-3, suppressors of cytokine signalling 3; FAS, fatty acid synthase; CYP2E1, cytochrome P450 2E1; OPN, osteopontin; TNF- α , tumor necrosis factor alpha; IL-6, interleukin 6; iNOS, inducible nitric oxide synthase; COX, cyclooxygenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Table 2. Demographic, metabolic, biochemical, viral, and histopathological characteristics of patients with normal liver and nonalcoholic fatty liver disease

Characteristics	NL (n=22)	NAS (n=21)	NASH (n=22)
Age (years)	48.7 ± 11.6	49.1 ± 12.5	48.4 ± 12.1
Women (%) / Men (%)	14(63.6%) / 8(36.4%)	12(57.1%) / 9(42.9%)	13(59.1%) / 9(40.9%)
BMI (kg/m ²)	25.3 ± 2.5	26.0 ± 2.4	26.6 ± 2.1
Glucose (mg/dL)	95.2 ± 6.8	96.2 ± 7.4	97.5 ± 6.7
Insulin (μU/L)	8.5 ± 3.1	10.9 ± 6.6	12.3 ± 4.8*
HOMA-IR	1.01 ± 0.5	1.45 ± 1.1	1.77 ± 0.9*
TG (mg/dL)	107.3 ± 34.3	141.6 ± 46.2*	146.9 ± 45.7*
LDL-chol (mg/dL)	137.3 ± 38.3	148.1 ± 47.4	141.2 ± 44.1
HDL-chol (mg/dL)	49.3 ± 8.3	50.7 ± 7.4	48.8 ± 8.7
ALT (IU/L)	16.2 ± 6.2	22.1 ± 15.5	49.6 ± 18.2#
AST (IU/L)	19.2 ± 4.6	20.5 ± 9.3	27.3 ± 9.8
γ-GT (IU/L)	28.8 ± 16.7	61.5 ± 23.6*	73.8 ± 21.5*
Steatosis (%)			
Grade 0	22 (100%)		
Grade 1		10 (47.7%)	11 (50.0%)
Grade 2		9 (42.8%)	8 (36.4%)
Grade 3		2 (9.5%)	3 (13.6%)
Fibrosis (%)			
Stage 0	22 (100%)	21 (100%)	4 (18.3%)
Stage 1			10 (45.4%)
Stage 2			7 (31.8%)
Stage 3			1 (4.5%)
Stage 4			
Necroinflammation (%)			
Grade 0	22 (100%)	21 (100%)	
Grade 1			9 (40.9%)
Grade 2			7 (31.8%)
Grade 3			6 (27.3%)
Grade 4			

Data are shown as mean ± standard deviation or as number of cases (%). NL, normal liver; NAS, nonalcoholic steatosis; NASH, nonalcoholic steatohepatitis; BMI, body mass index; TG, triglycerides; HOMA-IR, homeostatic model assessment-insulin resistant; LDL-chol, low-density lipoprotein cholesterol; HDL-chol, high-density lipoprotein cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GT, gamma-glutamyltransferase. **P* < 0.05 with respect to NL group. #*P* < 0.05 with respect to NAS group.

Table 3. Demographic, metabolic, biochemical, viral, and histopathological characteristics of patients with chronic hepatitis C virus genotype 1 infection

Characteristics	HCV G1 (n=20)	HCV G1+NAS (n=24)
Age (years)	47.9 ± 11.7	47.2 ± 12.1
Women (%)/Men (%)	12(60.0%) / 8(40.0%)	14(58.3%) / 10(41.7%)
BMI (kg/m ²)	25.8 ± 2.2	26.2 ± 2.6
Glucose (mg/dL)	95.1 ± 7.7	96.6 ± 8.2
Insulin (μU/L)	10.1 ± 3.9	10.3 ± 6.9
HOMA-IR	1.47 ± 0.8	1.52 ± 1.0
TG (mg/dL)	105.1 ± 44.7	109.8 ± 49.3
LDL-chol (mg/dL)	139.7 ± 37.4	149.3 ± 48.2
HDL-chol (mg/dL)	51.7 ± 8.1	50.6 ± 9.2
ALT (IU/L)	80.7 ± 26.7	91.6 ± 32.7
AST (IU/L)	42.2 ± 17.6	51.3 ± 23.5
γ-GT (IU/L)	85.4 ± 23.0	83.7 ± 24.2
Viral load (IU/mL)	1.26 ± 0.53 x 10 ⁶	1.43 ± 0.81 x 10 ⁶
Steatosis (%)		
Grade 0	20 (100%)	
Grade 1		12 (50.0%)
Grade 2		10 (41.7%)
Grade 3		2 (8.3%)
Fibrosis (%)		
Stage 0	5 (25.0%)	4 (16.7%)
Stage 1	6 (30.0%)	8 (33.3%)
Stage 2	8 (40.0%)	10 (41.7%)
Stage 3	1 (5.0%)	2 (8.3%)
Stage 4		
Necroinflammation (%)		
Grade 0		
Grade 1	8 (40.0%)	11 (45.8%)
Grade 2	6 (30.0%)	6 (25.0%)
Grade 3	6 (30.0%)	7 (29.2%)
Grade 4		

Data are shown as mean ± standard deviation or as number of cases (%). HCV G1, hepatitis C virus genotype 1; NAS, nonalcoholic steatosis; BMI, body mass index; TG, triglycerides; HOMA-IR, homeostatic model assessment-insulin resistant; LDL-chol, low-density lipoprotein cholesterol; HDL-chol, high-density lipoprotein cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GT, gamma-glutamyltransferase.

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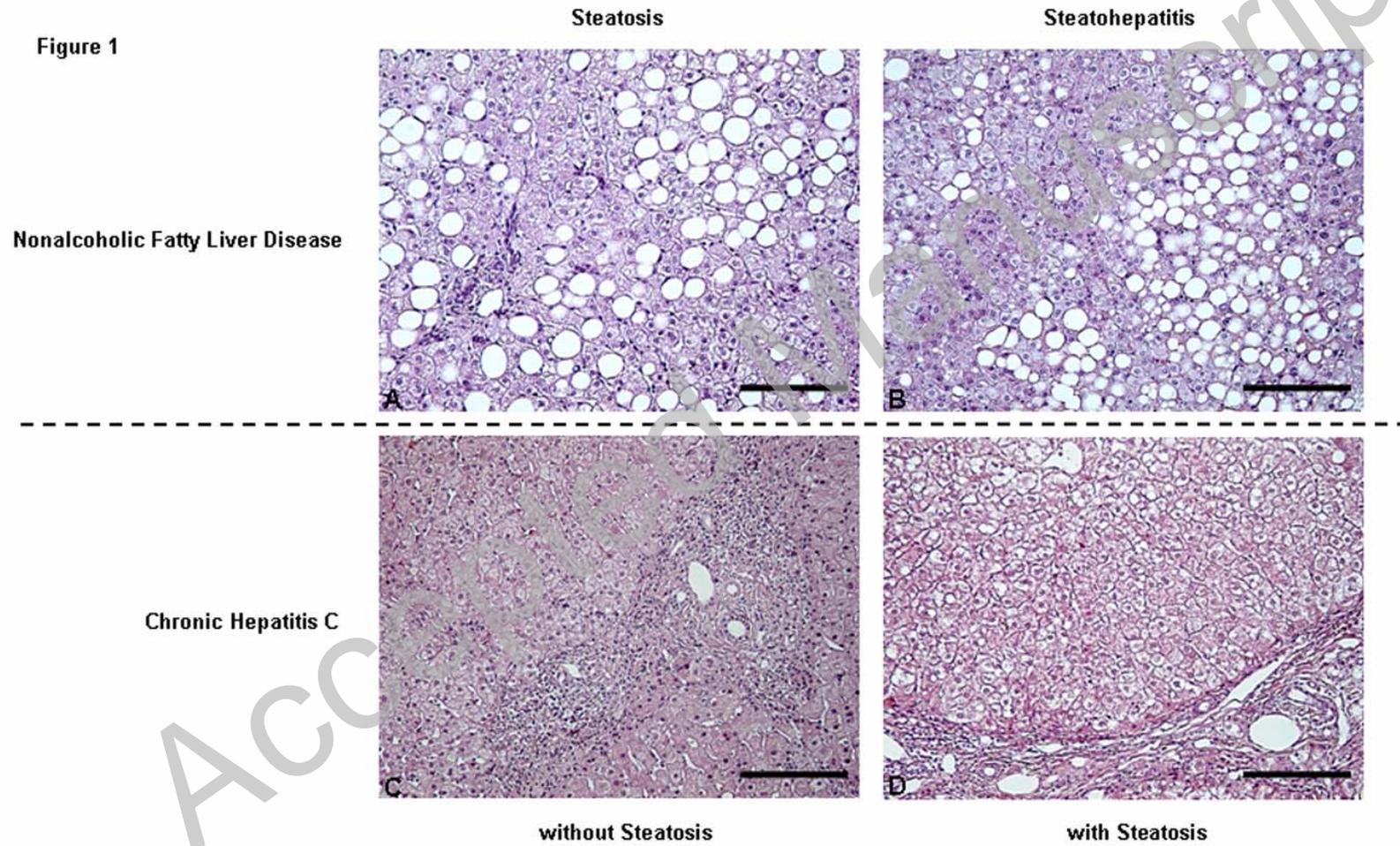


Figure 2

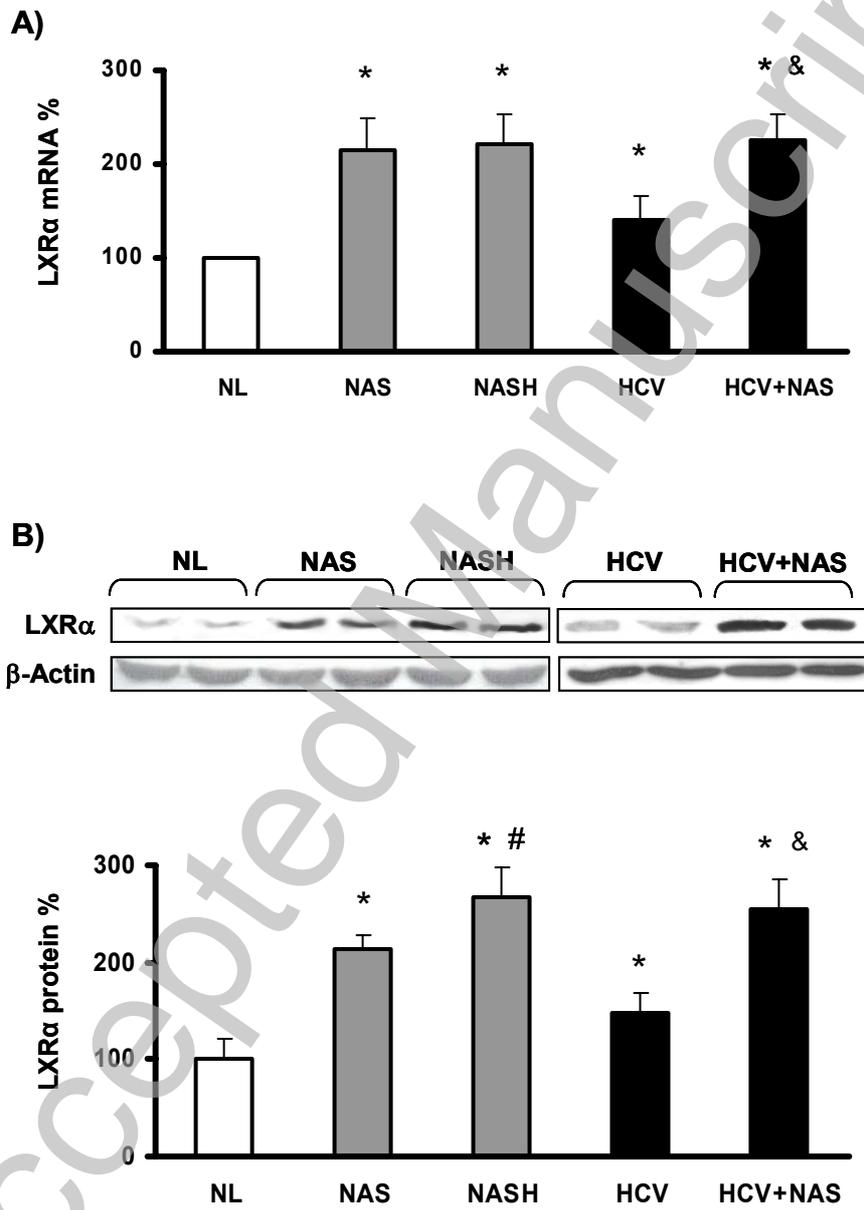


Figure 3

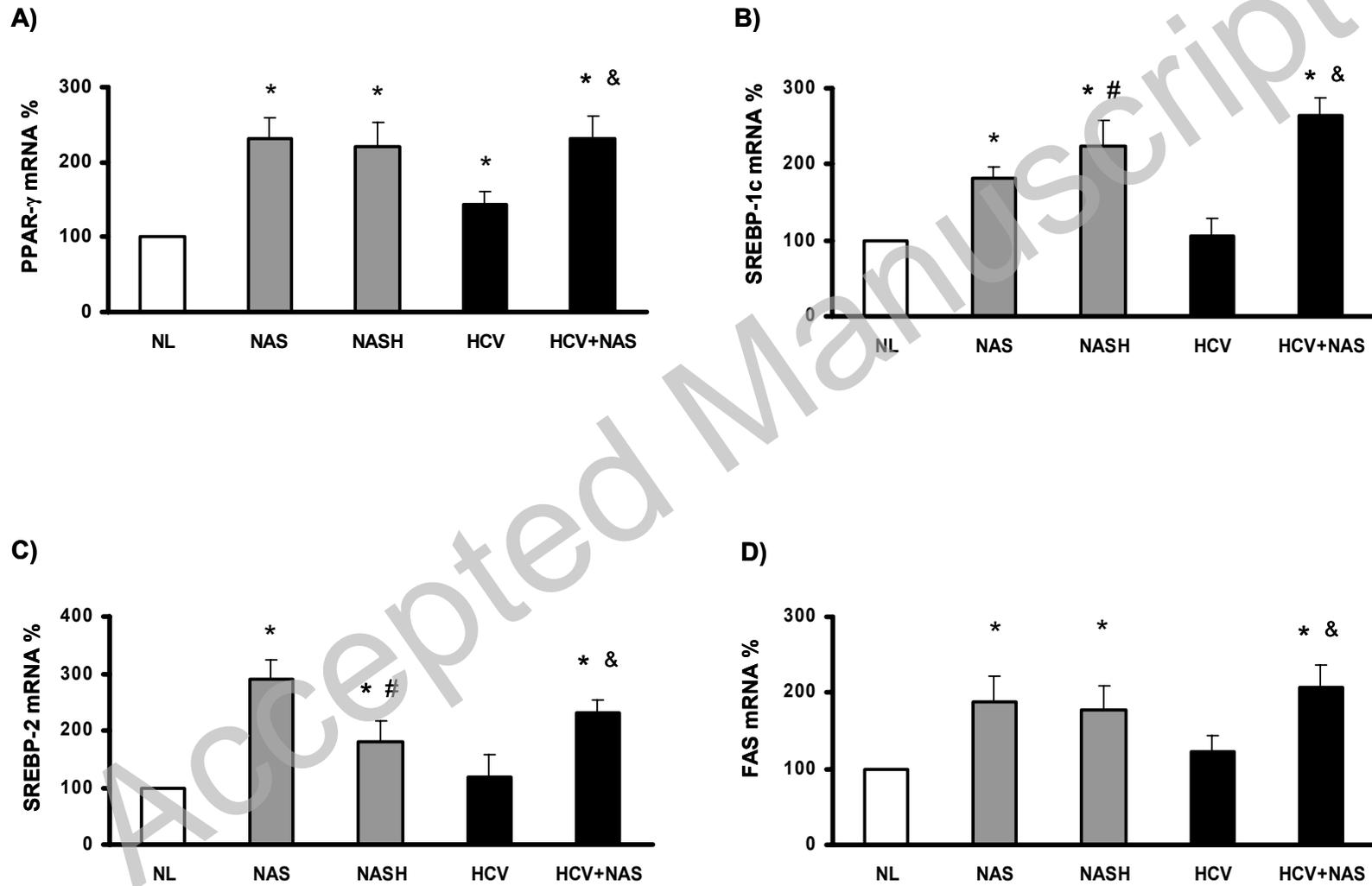
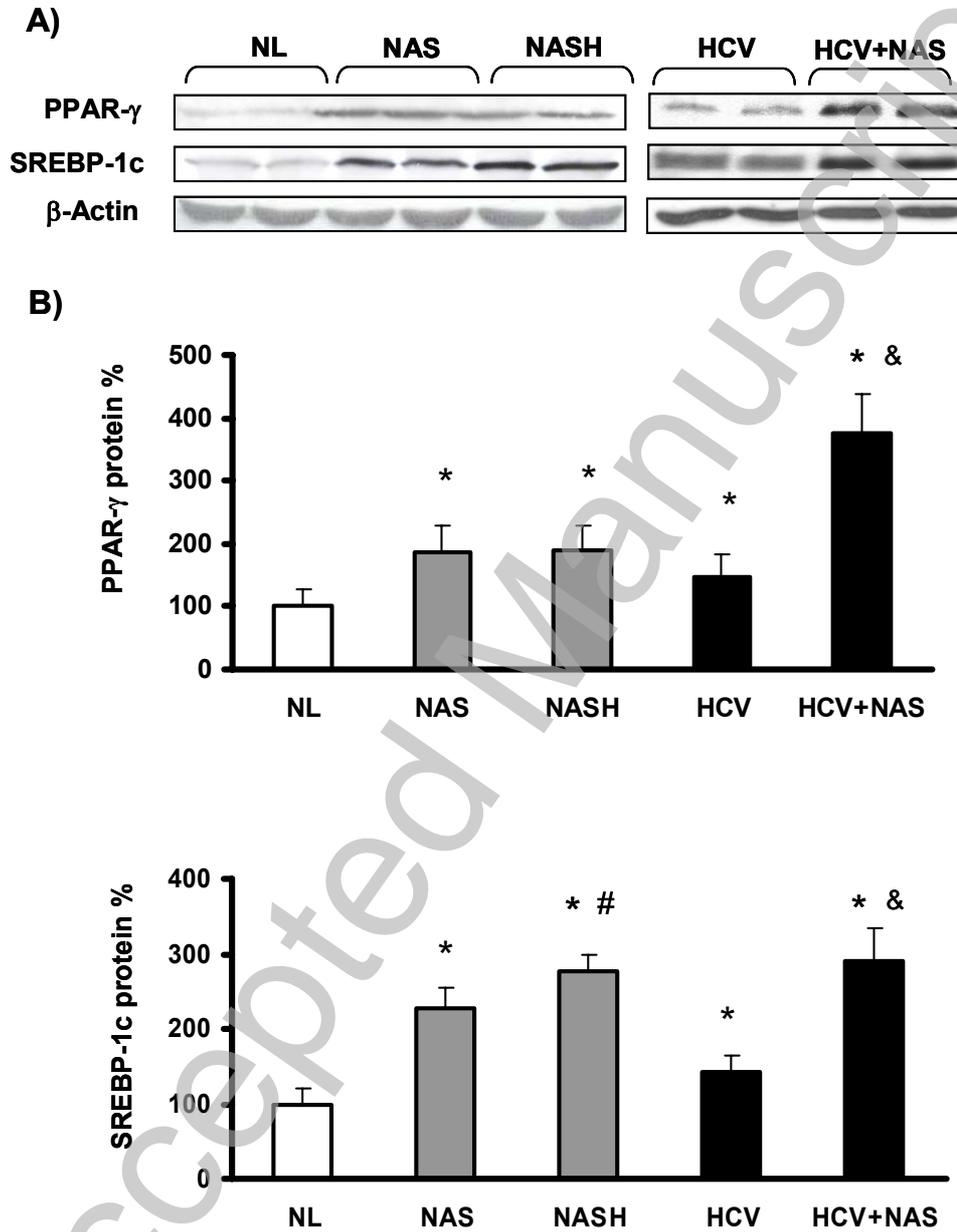


Figure 4



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Figure 5

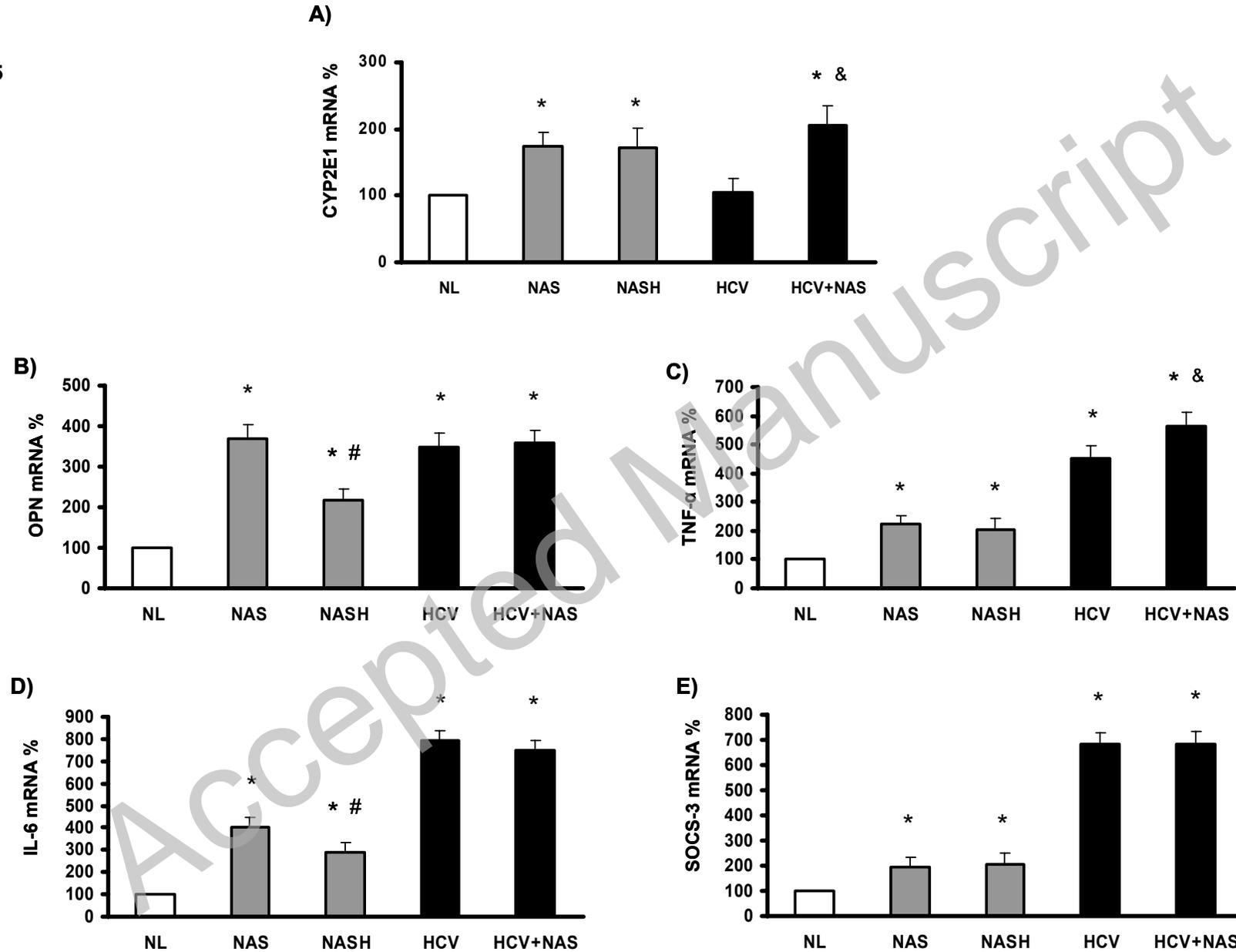
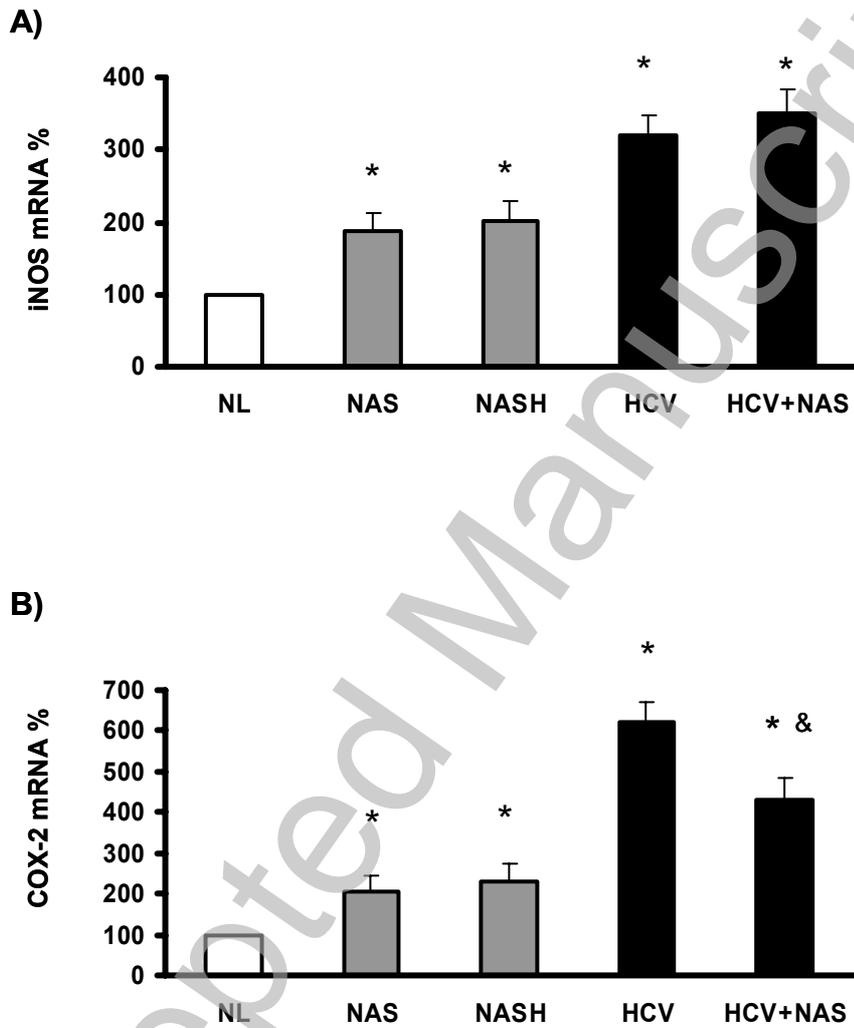


Figure 6



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Figure 7

