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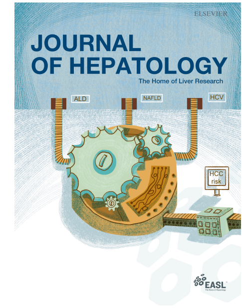

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LANIFIBRANOR

ADVANCED CIRRHOSIS

↓ portal pressure

↑ liver µvascular function

↓ hepatic fibrosis

HSC
Deactivation

↓ collagen deposition

↓ proliferation

↓ contractility

Hepatocytes

Improved function

LSEC
Re-differentiation

↑ fenestrae

↓ pro-inflammatory phenotype

KC

Improved cytokine expression pattern

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Pan-PPAR agonist lanifibranor improves portal hypertension and hepatic fibrosis in experimental advanced chronic liver disease

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Abstract (274 words)

Background & Aims. In advanced chronic liver disease (ACLD), de-regulated hepatic necroinflammatory processes play a key role in the development of liver microvascular dysfunction, fibrogenesis, and increased hepatic vascular tone, resulting in progression of ACLD and portal hypertension. Given the current lack of an effective treatment, we aimed at characterizing the effects of the pan-peroxisome proliferator-activated receptors (pan-PPAR) agonist lanifibranor in two pre-clinical models of ACLD, as well as in liver cells from patients with ACLD.

Methods. Cirrhotic rats (thioacetamide or common bile duct ligation; TAA or cBDL) randomly received lanifibranor (100mg/kg/day, po) or vehicle for 14 days (n=12/group). PPAR expression, systemic and hepatic hemodynamics, presence of ascites, liver sinusoidal endothelial cells (LSEC) phenotype, hepatic stellate cells (HSC) activation, serum transaminases and albumin, hepatic macrophage infiltration, cytokine expression, and liver fibrosis were determined. Hepatic cells were isolated from livers from cirrhotic patients and their phenotype was evaluated after treatment with lanifibranor or vehicle.

Results. TAA-cirrhotic rats receiving lanifibranor showed significantly lower portal pressure than vehicle-treated animals (-15%) without decreasing portal blood flow, indicating improved hepatic vascular resistance. Moreover, lanifibranor-treated TAA-rats showed decreased ascites, improved phenotype of LSEC and HSC, ameliorated hepatic microvascular function, reduced hepatic inflammation, and significant fibrosis regression (-32%). These findings were confirmed in the cBDL rat model, as well as in human liver cells from cirrhotic

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patients, which exhibited a phenotypic improvement upon treatment with lanifibranor.

Conclusions. This study demonstrates that lanifibranor exerts clear beneficial effects in pre-clinical models of decompensated cirrhosis, which lead to amelioration in fibrosis and portal hypertension. Our results in human hepatic cells isolated from cirrhotic patients further encourage its clinical evaluation for the treatment of ACLD.

Lay summary: Advanced chronic liver disease (ACLD) constitutes a serious public health issue still lacking a safe and effective enough treatment at the clinical scenario. This study shows that lanifibranor improves portal hypertension and liver fibrosis, two key elements of ACLD's pathophysiology, in pre-clinical models of the disease. Evaluation of lanifibranor in liver cells from patients suffering from ACLD further supports its beneficial effects.

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1. Introduction

Chronic liver disease (CLD) originates from a sustained hepatic injury that can vary in nature, being excessive alcohol consumption, unhealthy dietary habits and hepatitis B or C virus infection the most common causes. As a consequence of long-term liver injury, tissue wound healing mechanisms may become deregulated, leading to hepatic fibrosis, which can ultimately progress to cirrhosis and, in some cases, hepatocellular carcinoma.[1]

One of the most frequent and severe clinical syndromes associated to advanced CLD (ACLD) is portal hypertension (PH), which is characterized by a pathological increase in the hepatic venous pressure gradient (HVPG) and is considered clinically significant when it reaches values of at least 10 mmHg.[2] An increment in the hepatic vascular resistance (HVR) represents the primary factor in the development of PH. This stems from a generalized dysfunction of all hepatic cell types, and includes hepatocyte necroapoptosis, hepatic stellate cell (HSC) activation, liver sinusoidal endothelial cell (LSEC) capillarization and macrophage activation and recruitment. These phenotypic alterations constitute the main factors responsible for the development of fibrosis, parenchymal remodeling and formation of regenerative nodules, which compose the so-called architectural component of the increased HVR. In addition, deregulated synthesis of vasoconstrictory and vasodilatory factors and increased contractile response at the hepatic microcirculation leads to an increase in hepatic vascular tone, the dynamic component of the HVR, further aggravating PH.[3]

The clinical consequences of PH represent the first non-neoplastic cause of death and liver transplantation in patients with ACLD.[4] Despite extensive

preclinical and clinical research in the last decades, there is still no effective enough treatment for PH at the clinical scenario.[5]

A therapeutic target that has gained relevance over the recent years is the peroxisome proliferator-activated receptors family (PPARs), a group of nuclear receptor transcription factors involved in a wide range of functions. In mammals, there are three PPAR isoforms: PPAR-α, PPAR-δ (also known as PPAR-β) and PPAR-ɣ, which are differentially expressed among various tissues and cell types. Among those, PPAR-α expression is most abundant in the liver, as compared to PPAR-ɣ and PPAR-δ. However, all three PPAR isoforms play a role in maintaining a normal liver function.[6] During CLD, PPAR deregulation contributes to the progression of the disease, affecting lipid metabolism, inflammation, insulin resistance, and fibrogenesis.[7,8] In fact, previous studies have shown the beneficial effects deriving from the activation of one or several PPAR isoforms in pre-clinical models of liver injury. More specifically, PPAR activation can reverse mild liver fibrosis, reduce inflammation and steatosis, and even improve the extrahepatic complications of CLD.[9–15] However, none of these studies investigated the consequences of activating the three PPAR isoforms concomitantly in the clinically-relevant scenario of ACLD, overlooking the fact that each PPAR isoform could follow different expression patterns and have distinctive and complementary roles in different cell types, as some authors have pointed out.[16,17] In this regard, recent studies demonstrated the efficacy of the pan-PPAR agonist of lanifibranor, an indole sulfonamide derivative, in pre-clinical models of mild liver injury and non-alcoholic fatty liver disease (NAFLD).[18–20]

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The objective of the present study was to evaluate the therapeutic potential of pan-PPAR activation for the treatment of advanced cirrhosis. We characterized the effects of lanifibranor on systemic and hepatic hemodynamics, liver sinusoidal cells phenotype, microvascular function, inflammation and fibrosis in pre-clinical models of ACLD. We further evaluated the effects of lanifibranor in human primary cells from cirrhotic patients to verify if the data obtained in rodent models could be translated to cells from human cirrhotic liver, thus approaching our findings to the bedside.

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2. Methods

A detailed description of the methods can be found in the Supplementary Material.

2.1 Animal models of advanced chronic liver disease

Advanced chronic liver disease (ACLD) was induced in male Sprague-Dawley rats (150g, Janvier) by administration of thioacetamide (TAA, Sigma) twice per week during a total of 12 weeks. TAA was previously dissolved in saline solution at a concentration of 125 mg/ml and administered intraperitoneally at a dose of 250 mg/kg body weight.[21,22] Following the last TAA administration, animals with ACLD underwent a detoxification period of 2 to 5 days before beginning pharmacological treatment.

As a second model of ACLD, secondary biliary cirrhosis was induced in male Sprague-Dawley rats (250g, Janvier) by ligation of the common bile duct (cBDL) for a period of 28 days, as previously described. [21,23] Treatments started on the 14th day after surgery.

Healthy Sprague-Dawley rats (350g, Janvier) were used for sample harvesting and subsequential molecular analysis.

Animals were caged in pairs on a 12:12-hour light-dark cycle, in environmentally controlled animal facilities at the Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS). Mortality rate intrinsic to model generation was of 7% and 15% for TAA and cBDL, respectively. All procedures were approved by the Laboratory Animal Care and Use Committee of the University of Barcelona and were conducted in accordance with the European Community guidelines for the

protection of animals used for experimental and other scientific purposes (EEC Directive 86/609).

2.2 Lanifibranor administration

A total of 22 cirrhotic rats were enrolled per group and disease model, and randomized to receive blindly lanifibranor (100 mg/kg body weight/day) or vehicle (1% methylcellulose + 0.05% poloxamer). Both treatments were administered by gavage, daily, for a total of two weeks. The pharmacological treatment was administered by a third person, and therefore, the investigators performing the experiments were not aware of the treatment received by the rats. This blinding was maintained until the final analysis of results. No treatment-related mortality was observed. Of the total number of animals per group, 12 were used for *in vivo* hemodynamic studies and molecular determinations, and 10 for *ex vivo* hepatic microvascular function assessment.

2.3 Human samples

Human liver tissue remnants from liver resections and explants were used for total mRNA extraction and hepatic cells isolation and culture. Non-tumorous liver tissue from hepatic resections was used as a healthy control and compared to liver explants from ethanol and non-alcoholic steatohepatitis (NASH) etiologies. Additional donor information is provided in Suppl. Table 4. The ethics committee of the Hospital Clinic de Barcelona approved the study protocol (HCB/2018/0028), and sample manipulation and isolation procedures were carried out following good laboratory practices. In all cases, patients had signed an informed consent before resection or transplantation.

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2.4 Statistical analysis

A sample size of n=12/group for *in vivo* hemodynamics evaluation was calculated based on a predicted 20% reduction of portal pressure, with a standard deviation of 1.5, 95% power and a p value of 0.05. Results were analyzed with GraphPad Prism v7.04 (GraphPad Software). Data in the figures are expressed as box plots with Tukey-style whiskers, where the central line indicates the median. For line plots, data are expressed as mean ± standard error of the mean (S.E.M.). Differences among groups were tested for statistical significance by Student's t test, when comparing two groups, by one-way ANOVA followed by *post hoc* Tukey's test, when comparing multiple conditions, or by general lineal models (GLM) with repeated measures for microvascular function analysis and gel contraction assay. Differences were considered significant at $p < 0.05$.

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3. Results

3.1 Differential expression of PPAR isoforms in hepatic cells & effects of lanifibranor

The expression of PPAR isoforms was assessed in human and rat liver tissue and rat primary hepatic cells. As shown in Figure 1A, PPAR-α was the most expressed isoform in human liver tissue, both in healthy and cirrhotic individuals from alcohol and non-alcoholic steatohepatitis (NASH) etiologies. Despite an intrinsic variability among patients, especially in healthy samples presumably due to differences in clinical diagnostic or treatment received, PPAR-α was significantly down-regulated in patients with alcohol-derived cirrhosis (-77%, $p=0.046$), and showed a similar trend in NASH-cirrhotic patients (-65%, $p=0.222$). PPAR-ɣ and PPAR-δ followed a similar expression pattern although no statistical significance was reached.

Similarly, both in healthy and TAA-cirrhotic rats, PPAR-α was the most abundant isoform in total liver tissue, followed by PPAR-δ and PPAR-ɣ (Fig. 1B). All three isoforms were significantly down-regulated in livers from cirrhotic animals compared to healthy (-68%, -47% and –86%, for PPAR-α, PPAR-ɣ and PPAR-δ, respectively, all $p<0.001$), but this was not always the case when looking at specific cell types (Suppl. Fig. 1A). Indeed, analysis of each hepatic cell type revealed that hepatocytes, which are the most abundant cell type of the liver, were the main cells expressing PPAR-α (Fig. 1C). Liver sinusoidal endothelial cells (LSEC) were the main cell type expressing PPAR-δ, and notably, LSEC exhibited increased PPAR-α expression in cirrhosis. PPAR-ɣ was predominantly expressed in Kupffer Cells and, importantly, showed up-regulation in cirrhotic HSC. Altogether, these results suggest that the TAA rat

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model closely reflects the marked de-regulation of PPARs that takes place in human cirrhotic livers, and that PPARs expression is cell type-distinct.

Analysis of PPARs expression in liver tissue of cirrhotic animals treated with lanifibranor revealed no differences when compared to those treated with vehicle (Suppl. Fig. 1B), therefore suggesting that the below-detailed effects of lanifibranor would be mRNA expression independent.

3.2 Effects of lanifibranor on portal hypertension in rats with ACLD

Rats with ACLD treated for two weeks with lanifibranor exhibited a significant improvement in portal hypertension (-15% in portal pressure; PP, p=0.003) compared to those that received vehicle (Table 1). This amelioration was not accompanied by any decrease in portal blood flow (PBF) that actually showed a trend for increasing, thus indicating a reduction of HVR (-29% p=0.022) in treated animals. No changes in systemic hemodynamic parameters, such as mean arterial pressure (MAP) and heart rate (HR), were observed. Body and liver weight were equally unaltered. However, lanifibranor-treated animals tended to present less splenomegaly (-19% in spleen weight, p=0.084). Lastly, the number of animals presenting ascites at the time of hemodynamic analysis was significantly lower in the lanifibranor group than in the vehicle group (-76% p=0.020), indicating a favorable effect of lanifibranor in decompensated cirrhosis.

3.3 Effects of lanifibranor on liver sinusoidal endothelial cells phenotype and hepatic microvascular function

To assess whether the improvement in hepatic hemodynamics observed in rats was accompanied by an amelioration at the cellular level, liver sinusoidal endothelial cells (LSEC) phenotype was evaluated. The analysis of liver

sinusoids by scanning electron microscopy revealed that LSEC from lanifibranor-treated rats presented significantly more fenestrae (+25% in porosity p=0.050) than those treated with vehicle, indicating a regression in capillarization (Fig. 2A). Moreover, lanifibranor treated rats had a reduced liver expression of von Willebrand factor, a marker of endothelial dysfunction (-25% p=0.004; Fig. 2B). Lastly, LSEC from treated rats expressed lower mRNA levels of the cell adhesion molecules vascular cell adhesion molecule 1 (vcam1), intercellular adhesion molecule 1 (icam1) and e-selectin (e-sel) (-44% p=0.097, -52% p=0.038 and -85% p=0.020, respectively) than those receiving vehicle (Fig. 2C), suggesting an amelioration of the dysfunctional and pro-inflammatory LSEC phenotype found in ACLD.

In addition, hepatic microvascular function was characterized in *ex vivo* liver perfusion experiments to assess the hepatic vasodilatory capacity. Cirrhotic animals that received two-week lanifibranor exhibited significantly improved hepatic microvascular function, indicated by greater vasodilation in response to incremental doses of acetylcholine (p=0.028; Fig 2D). These observations show an improved phenotype of both LSEC and hepatic stellate cells (HSC) after lanifibranor treatment as compared to the vehicle-treated animals.

Lastly, the effects of lanifibranor on hepatic angiogenesis were evaluated. As shown in Figure 2E, cirrhotic rats receiving lanifibranor showed a trend to reduce VE-cadherin expression (-32%, p=0.189), while other angiogenic and angiocrine markers remained unchanged (Suppl. Fig. 2).

3.4 Effects of lanifibranor on hepatic stellate cell phenotype

HSC phenotype was evaluated through several well-defined markers. Firstly, the expression of α-smooth muscle actin (α-SMA), a protein playing a key role

in cell contractility and considered an activation marker of HSC, was determined in total liver tissue. As shown in figures 3A and 3B, animals with ACLD treated with lanifibranor exhibited a markedly reduced α-SMA expression compared to those treated with vehicle, both at the mRNA and protein levels (-47% p=0.002 and -75% p=0.014, respectively). Moreover, determination of α-SMA by immunofluorescence, confirmed a decrease in expression with a staining reduction of -61% (p=0.047) in livers from lanifibranor-treated rats (Fig. 3C). In accordance to these results, p-moesin expression also tended to be lower in the lanifibranor group (-19%; p=0.098; data not shown), suggesting a decrease in rho kinase activity and, consequently, in HSC contractility. Lastly, hepatic expression of desmin, a marker for both quiescent and activated HSC, was evaluated by immunohistochemistry, revealing that lanifibranor-treated rats presented 36% less desmin-positive cells than those that were given vehicle (p=0.018; Fig. 3D). Altogether, these results show that lanifibranor promotes HSC deactivation, particularly inhibiting its abnormal proliferation and contractility in the context of ACLD.

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3.5 Effects of lanifibranor on liver function and inflammation

In order to evaluate a possible beneficial effect of lanifibranor on liver function, serum samples from lanifibranor and vehicle-treated cirrhotic rats were collected and aspartate aminotransferase (AST), alanine aminotransferase (ALT) and albumin levels were measured. As shown in figure 4A, animals from the lanifibranor group presented lower serum levels of AST (-31%, p=0.006), whereas ALT and albumin remained unchanged.

Effects on inflammation were evaluated as macrophage infiltration (Fig. 4B) and mRNA expression of key pro- and anti-inflammatory cytokines (Fig. 4C and 4D).

While treatment with lanifibranor did not affect the presence of tissue macrophages, measured as CD68 positive cells, it did improve cytokine expression pattern. Animals from the lanifibranor group exhibited a significant down-regulation of interleukin IL-6 (-79%, p=0.025), as well as a tendency to decrease tumor necrosis factor α (tnf-α) and inducible nitric oxide synthase (inos) expression (-50%, p=0.067; and -71%, p=0.121; respectively). Among anti-inflammatory mediators, interleukin IL-10 expression showed a trend to be increased (+73%, p=0.158) in lanifibranor-treated animals, whereas arginase 1 (arg1) and mannose receptor C-type 1 (mrc1) were unchanged.

Taken together, these results suggest that treatment with lanifibranor reduces hepatic pro-inflammatory environment and improves liver function.

3.6 Impact of lanifibranor on liver fibrosis in rats with ACLD

As shown in figure 5A, staining of liver sections with Sirius red revealed that cirrhotic rats receiving lanifibranor exhibited a significant improvement in liver fibrosis compared to those receiving vehicle (-32%, p=0.020) resulting in thinner, less conspicuous and frequently perforated fibrous septa. Accordingly, lanifibranor-treated animals showed a reduced collagen 1α1 mRNA expression in total liver tissue (-50% p=0.003; Fig. 5B).

To investigate if fibrosis amelioration in lanifibranor treated rats was related to enhanced extracellular matrix (ECM) remodeling, mRNA expression of metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) was determined (Fig. 5B). Interestingly, timp1 and 2 were significantly down-regulated (-45% p<0.001 and -31% p=0.018, respectively), suggesting that lanifibranor could favor ECM remodeling through an increase of MMP activity. However, mmp9 and mmp13 expression was also reduced (-56%, p=0.034

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and -32%, p=0.102, respectively) and mmp2 remained unchanged. These results may be explained by the fact that lanifibranor-driven fibrolysis occurred previously to the analyzed timepoint, since MMPs and TIMPs expression obeys to a complex balance that changes during disease progression/regression.

Thus, lanifibranor promotes the deactivation of HSC and could favor ECM remodeling by metalloproteinases, altogether leading to an improvement of liver fibrosis in ACLD.

3.7 Effects of lanifibranor in a rat model of secondary biliary cirrhosis

To confirm lanifibranor's beneficial effects in a second model of ACLD, lanifibranor or vehicle were given to rats with secondary biliary cirrhosis due to common bile duct ligation (cBDL).

PPAR expression, evaluated in healthy and cirrhotic rats, followed an expression pattern similar to that found in humans and rats from the TAA model, with the exception of PPAR-δ, which was upregulated in cBDL-cirrhosis (+655%, p=0.050). Treatment of cBDL-cirrhotic rats with lanifibranor did not alter mRNA levels of either PPAR isoform, which is in agreement with the observed results in the TAA model, suggesting that lanifibranor's effects were independent of mRNA expression.

Hemodynamics evaluation revealed that animals receiving lanifibranor exhibited a small but statistically significant improvement of portal hypertension (-9% in PP, p=0.025), without changes in systemic hemodynamics parameters such as MAP and HR (Fig. 6C). As evidenced by Sirius red staining (Fig. 6D), hepatic fibrosis remained unaltered, albeit new synthesis of collagen 1α1 was significantly reduced upon lanifibranor treatment (-28%, p=0.010). Endothelial characterization, on the other hand, revealed a trend to improve hepatic

microvascular function, measured as sinusoidal vasodilatory capacity (p=0.130; Fig. 6E). This was accompanied by a partial reversal of LSEC pro-inflammatory phenotype, with a significant reduction of icam1 mRNA expression (-54%, p=0.050), as well as a tendency to decrease e-sel expression (-79%, p=0.059). Thus, 2-week lanifibranor-dependent reduction of PP in a rat cBDL model did not obey to an amelioration of the architectural component of the increased HVR but rather to an improvement of the dynamic component.

3.8 Effects of lanifibranor on primary cultured human hepatic cells from cirrhotic patients

To assess the effects of lanifibranor on human liver cells, freshly isolated hepatocytes and HSC from cirrhotic patients were incubated with increasing doses of lanifibranor (1, 3 and 10 μM) or vehicle, both in conventional cell culture and in the advanced co-culture platform ExoLiver.[24]

PPARs mRNA levels in isolated hepatocytes or HSC remained largely unchanged after treatment with lanifibranor (Suppl. Fig 3A and 3B), similarly to what was observed in total liver tissue from treated cirrhotic animals. As shown in figure 7A, HSC exhibited a significant improvement in their phenotype after lanifibranor treatment. This effect was observed as the down-regulation of two key HSC activation markers: α-SMA (-51% p=0.005; -39% p=0.037 and -40% p=0.090, for 1, 3 and 10 μM, respectively) and the platelet-derived growth factor receptor β (PDGFRβ; -44% p=0.045; -47% p=0.029 and -49% p=0.042, for 1, 3 and 10 μM, respectively), whereas collagen levels remained unchanged. Similarly, hepatocyte's phenotype showed a tendency of improvement upon treatment with 10 μM lanifibranor in the ExoLiver platform (Fig. 7B), showing a numerically, albeit not statistically, increased expression of the master regulator

hepatocyte nuclear factor 4 alpha (hnf4α; +284%; p=0.124) and of the transporter solute carrier family 22 member 1 (slc22a1; +181%; p=0.182), while reducing ATP-binding cassette subfamily C member 3 expression (abcc3; -17%; p=0.199).

Treatment with lanifibranor in conventionally-cultured cells had no effects on hepatocytes phenotype markers, possibly due to the spontaneous de-differentiation of cells when mono-cultured in plastic (Suppl. Fig. 3C).[24] It did, however, improve liver cells survival as suggested by a dose-dependent decrease of both AST (-30% p=0.040 and -37% p=0.015 for 3 μM and 10 μM, respectively) and ALT (-28% p=0.050 and -39% p=0.014 for 3 μM and 10 μM, respectively) measured in the culture medium (Fig 7C). In addition, lanifibranor improved HSC's phenotype in spite of the overactivation stimuli inherent to this culture method (Suppl. Fig 3D).

Finally, primary human HSC were used in a cell contraction assay to assess their response to treatment with lanifibranor, vehicle, or 2,3-butanedione monoxime (BDM), a well-known contraction inhibitor, used as a positive control. Interestingly, HSC treated with lanifibranor underwent a much lower contraction than those treated with vehicle, exhibiting a surface reduction of only 21% after 150 hours, whereas in vehicle-treated cells this reduction was of 70% (p<0.001; Figure 7D). In fact, lanifibranor-treated HSC's response was similar to BDM-treated cells, both being significantly different from vehicle-treated HSC but not from one another (data not shown).

These results are in accordance with the phenotypic and functional improvements observed in our *in vivo* studies in TAA-cirrhotic rats, suggesting that lanifibranor's effects could be translated to patients with ACLD.

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4. Discussion

The de-regulation of necroinflammatory processes in the liver is key in the development of hepatic microvascular dysfunction and fibrosis, ultimately resulting in PH, which is considered the most dreadful non-neoplastic complication of ACLD.[4] Advances in the understanding of PH pathophysiology described PPARs as key transcription factors controlling liver cells phenotype, and therefore have been proposed as potential therapeutic targets to improve this syndrome. In this regard, our study shows for the first time the expression pattern of the three PPAR isoforms in healthy and cirrhotic human and rat liver tissue, as well as in liver cells isolated from healthy and cirrhotic rat livers. While PPAR-α has long been known as the predominantly expressed isoform in the liver[25], our results add important novel data revealing the importance of PPAR-ɣ and PPAR-δ in sinusoidal cells such as LSEC, HSC and KC. PPAR-ɣ, in particular, presents two isoforms as a result of alternative splicing (PPAR-ɣ1 and PPAR-ɣ2) that have been shown to play a role in inflammation and fatty liver development, further suggesting its importance in CLD.[26] These novel observations encourage the use of pan-PPAR therapies to improve the microvascular de-regulation of the cirrhotic liver, and depict the pan-PPAR agonist lanifibranor as a promising candidate for the treatment of CLD. Indeed, previous work evidenced the beneficial effects of lanifibranor in pre-clinical models of mild liver injury and NAFLD[18,20], which have been recently confirmed in a phase 2b clinical trial in non-cirrhotic NASH patients.[27] Therefore, we aimed at elucidating its effects on more advanced stages of liver disease.

In our study, we show that treatment with lanifibranor significantly decreased portal pressure in a TAA rat model of ACLD. This reduction was not

accompanied by any significant changes in portal blood flow, thus indicating an amelioration of hepatic vascular resistance. Such an improved liver resistance was due to the combined effects of lanifibranor decreasing liver fibrosis and reducing the hepatic vascular tone, through the amelioration of HSC and LSEC phenotype (see below). Moreover, the number of animals presenting ascites was strikingly reduced in the treated group, suggesting that lanifibranor favors the regression of ACLD from a decompensated to a compensated stage. It is worth noting that, in patients with liver cirrhosis, lowering portal pressure has been shown to confer protection from variceal bleeding and other complications of ACLD, greatly improving the prognosis of the disease.[28,29] While other studies have described the effects of selective or dual PPAR agonists, such as fenofibrate (PPAR-α) or aleglitazar (PPAR-α/ɣ), on PH[10,13], the role of PPAR-δ in this regard had yet not been investigated. This study shows for the first time the effects of a pan-PPAR agonist, including the improvement of parameters that remained unchanged with other PPAR agonists, such as transaminases levels, and that could be explained by the activation of PPAR-δ.

The underlying mechanisms of the hemodynamic ameliorations involved the phenotypic enhancement of different liver cell types, ultimately resulting in an improvement of the increased hepatic vascular resistance that causes PH. On one hand, HSC showed signs of a marked deactivation upon treatment with lanifibranor, exhibiting a reduced proliferation and contractility. In addition, a shift in the MMP/TIMP expression pattern in lanifibranor-treated animals suggested not only a reduced extracellular matrix deposition by HSC but also an increased remodeling. These results were in accordance with the significant improvement in hepatic fibrosis exhibited by treated animals. It is important to

remark that decreased fibrosis resulted in thinner, less conspicuous and frequently perforated fibrous septa, which have been reported as signs of cirrhosis regression.[30] Our results agree with previous works describing PPAR-ɣ as a key player in HSC phenotype modulation. Although there have been mixed reports, in general, the activation of PPAR-ɣ has been associated with HSC de-activation, likely through the modulation of key pathways such as TGF-β and PDGFR-β.[17,31,32]

On the other hand, animals treated with lanifibranor exhibited a regression of LSEC capillarization. LSEC presented higher number of fenestrae and their pro-inflammatory phenotype was significantly attenuated. Furthermore, lanifibranor-treated rats presented a general improvement of their hepatic microvascular function. These results suggest that lanifibranor ameliorates the endothelial dysfunction that is characteristic of ACLD. This is particularly crucial given the key role of the endothelium in modulating intrahepatic hemodynamics through vasoactive substances, ultimately being of great relevance in the development of PH and in disease progression.[3,33,34] Future work may elucidate whether this endothelial improvement is exerted directly through PPAR-δ activation in LSEC or paracrinally through HSC amelioration.[35]

Finally, lanifibranor-treated animals presented an attenuation of the hepatic pro-inflammatory environment through a shift of its cytokine expression pattern. Our observations agree with previous reports using different PPAR agonists[9,11–14], nevertheless we confirm that anti-inflammatory benefits are also achieved with lanifibranor in pre-clinical ACLD. In this regard, a recent article suggested that lanifibranor combines the beneficial effects of selective PPAR agonists and may counter inflammation and disease progression more potently than single or dual

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PPAR agonists.[20] We are aware that lanifibranor-derived anti-inflammatory effects in the ACLD scenario would require a much deeper characterization, however this is out of the scope of the present manuscript that was mainly focused on depicting its effects on liver fibrosis, PH and microvascular function.

Treatment of cBDL rats (used as a second model of ACLD) with lanifibranor also improved PH without altering systemic hemodynamics. Further characterization of fibrosis and endothelial phenotype revealed that such improvement was related to an amelioration of hepatic microvascular function and LSEC phenotype, rather than a reduction of fibrosis. As cBDL constitutes a more severe model of ACLD in which liver damage persists during the pharmacological treatment phase, these results suggest possible beneficial outcomes of lanifibranor treatment even at very advanced stages of liver disease.

In this regard, and given the current lack of an effective and safe treatment for PH, we further evaluated the effects of lanifibranor on human hepatic cells from cirrhotic patients, to assess the translatability of the findings in pre-clinical models to human disease. It is worth noting that lanifibranor exhibits a similar activity profile in rodents and humans[19], thus strengthening the translatability of its effects on rodent models to the bedside. When cultured in the dynamic co-culture platform ExoLiver[24], hepatocytes exhibited a trend of improvement and HSC's phenotype was clearly ameliorated upon treatment with lanifibranor. Additionally, treatment of primary human HSC with lanifibranor resulted in a striking reduction of their contraction capacity, showing that lanifibranor's effects on human cells are not only phenotypic but also functional. These results were in agreement with what was observed in the rat models of ACLD and suggested

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that the beneficial effects of lanifibranor in regard to PH could also translate to the clinical scenario.

In conclusion, the present study demonstrates for the first time that lanifibranor exerts beneficial effects in a pre-clinical model of decompensated cirrhosis, leading to a marked improvement of fibrosis and portal hypertension. Those beneficial effects may derive from targeting the three PPAR isoforms concomitantly, and therefore impacting on the different hepatic cell types and improving the sinusoidal microenvironment. These results, together with the amelioration of human hepatic cells isolated from cirrhotic patients, encourage the clinical evaluation of lanifibranor for the treatment of ACLD and portal hypertension.

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Abbreviations:

abcc3	ATP-binding cassette subfamily C member 3
ACLD	advanced chronic liver disease
ALT	alanine aminotransferase
arg1	arginase 1
AST	aspartate aminotransferase
BDM	2,3-butanedione monoxime
cBDL	common bile duct ligation
CLD	chronic liver disease
col1α1	collagen 1α1
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
e-sel	e-selectin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
hnf4α	hepatocyte nuclear factor 4 alpha
HR	heart rate
HSC	hepatic stellate cells
HVPG	hepatic venous pressure gradient
HVR	hepatic vascular resistance
icam1	intercellular adhesion molecule 1
IL-10	interleukin 10
IL-1β	interleukin 1β
IL-6	interleukin 6
inos	inducible nitric oxide synthase

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KC	Kupffer cells
LSEC	liver sinusoidal endothelial cells
MAP	mean arterial pressure
MMP	metalloproteinase
mrc1	mannose receptor C-type 1
mRNA	messenger ribonucleic acid
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
PBF	portal blood flow
PCR	polymerase chain reaction
PDGFR-β	platelet derived growth factor receptor β
PP	portal pressure
PPAR	peroxisome proliferator-activated receptors
PPP	perfusion portal pressure
SEM	standard error of the mean
slc22a1	solute carrier family 22 member 1
TAA	thioacetamide
TGF-β	transforming growth factor β
TIMP	tissue inhibitor of metalloproteinase
vcam1	vascular cell adhesion molecule 1
vWF	von Willebrand factor
α-SMA	α-smooth muscle actin

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Author names in bold designate shared co-first authorship.

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TABLES

Table 1. Effects of lanifibranor on hepatic and systemic hemodynamics in a rat model of advanced chronic liver disease (ACLD).

Parameter	Vehicle	Lanifibranor	p-value
PP (mmHg)	13.1 ± 0.4	11.2 ± 0.5	0.003
PBF ($mL \cdot min^{-1}$)	19.0 ± 1.7	23.5 ± 2.1	0.1
HVR ($mmHg \cdot min \cdot mL^{-1}$)	0.75 ± 0.1	0.53 ± 0.06	0.02
MAP (mmHg)	81 ± 3	84 ± 2	>0.2
HR ($beats \cdot min^{-1}$)	328 ± 6	334 ± 8	>0.2
Body weight (g)	485 ± 18	484 ± 16	>0.2
Liver weight (g)	14.4 ± 0.3	14.1 ± 0.7	>0.2
Spleen weight (g)	1.6 ± 0.1	1.3 ± 0.1	0.08
Ascites (% of total animals)	67	16	0.02

Results are expressed as mean ± S.E.M. n=12 per group. PP, portal pressure; PBF, portal blood flow; HVR, hepatic vascular resistance; MAP, mean arterial pressure; HR, heart rate.

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FIGURE LEGENDS

Fig. 1. Hepatic PPAR expression in healthy and cirrhotic humans and rats. Relative mRNA expression of PPARs in liver tissue from healthy, alcohol-cirrhotic and NASH-cirrhotic human samples (**A**), liver tissue from healthy and thioacetamide-induced cirrhotic rats (**B**), and primary hepatocytes (Hep), hepatic stellate cells (HSC), liver sinusoidal endothelial cells (LSEC) and Kupffer cells (KC) from healthy and cirrhotic rats (**C**). *p<0.05 and **p<0.01 vs. healthy; †p<0.05 and ††p<0.01 vs. all other PPARs (B) or cell types (C) under the same condition (one-way ANOVA with *post-hoc* test). n=12 in healthy and alcohol groups and n=9 in NASH group (A); n=6 per group (B-C).

Fig. 2. Effects of lanifibranor on LSEC phenotype and function in a rat model of ACLD. (**A**) Scanning electron microscopy representative images of hepatic sinusoidal areas from thioacetamide-induced cirrhotic rats treated with lanifibranor or vehicle (scale-bar 1 μm), and corresponding quantification. (**B**) von Willebrand factor (vWF) immunohistochemistry in liver tissue from rats described in A (scale-bar 100 μm). (**C**) Relative mRNA expression of depicted genes in total liver tissue from rats described in A. (**D**) Hepatic microvascular function from rats described in A. (**E**) VE-cadherin immunofluorescence in liver tissue (scale bar 50 μm; VE-cadherin in red and nuclei in blue). *p<0.05 and **p<0.01 vs. vehicle (Student's t test for A-C and E; repeated measures GLM for D). n=5 per group (A) and n=10 per group (B-E).

Fig. 3. Effects of lanifibranor on hepatic stellate cells phenotype in a rat model of ACLD. (**A**) Relative mRNA and (**B**) protein expression of α-smooth muscle actin (α-SMA) in liver tissue from thioacetamide-induced cirrhotic rats treated with lanifibranor or vehicle. (**C**) α-SMA immunofluorescence in liver

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tissue sections from cirrhotic rats described in A (scale bar 50 μm; α-SMA positive cells in green and nuclei in blue). (**E**) Desmin immunohistochemistry in liver tissue sections from cirrhotic rats described in A (scale-bar 100 μm). *p<0.05 and **p<0.01 vs. vehicle (Student's t test). n=10 per group.

Fig. 4. Effects of lanifibranor on liver function and inflammation in a rat model of ACLD. (**A**) Aspartate aminotransferase (AST), alanine aminotransferase (AST), and albumin levels in serum from cirrhotic rats treated with lanifibranor or vehicle. (**B**) CD68 immunofluorescence in liver tissue sections from cirrhotic rats described in A (scale bar 200 μm; CD68 positive cells in red and nuclei in blue). (**C**) mRNA relative expression of key pro-inflammatory genes and (**D**) anti-inflammatory genes in liver tissue from cirrhotic rats described in A. *p<0.05 and **p<0.01 vs. vehicle (Student's t test). n = 10 per group.

Fig. 5. Effects of lanifibranor on hepatic fibrosis in a rat model of ACLD. (**A**) Sirius red staining of liver tissue sections from cirrhotic rats treated with lanifibranor or vehicle (scale-bar 500 μm), and quantification. (**B**) Relative mRNA expression of depicted genes in total liver tissue from cirrhotic rats described in A. *p<0.05 and **p<0.01 vs. vehicle (Student's t test). n=11 per group (A) and n=10 per group (B).

Fig. 6. Effects of lanifibranor on a rat model of secondary biliary cirrhosis. (**A**) Relative mRNA expression of PPAR isoforms in liver tissue from healthy and cBDL-induced cirrhotic rats, and (**B**) in cBDL-induced cirrhotic rats treated with lanifibranor or vehicle. (**C**) Portal pressure (PP), mean arterial pressure (MAP) and heart rate (HR) of cBDL animals described in B. (**D**) Sirius red staining & collagen 1α1 mRNA expression in livers from cBDL rats described in

B (scale-bar 500μm). (**E**) Hepatic microvascular function (top) and relative hepatic mRNA expression of depicted genes (bottom) from cBDL rats described in B. *$p<0.05$ and **$p<0.01$; †$p<0.05$ and ††$p<0.01$ vs. all other PPARs under the same condition (A). (one-way ANOVA for A; Student's t test for B-D and E; repeated measures GLM for E). n=6 per group (A) and n=12 per group (B-E).

Fig. 7. Effects of lanifibranor on primary human liver cells. (**A**) mRNA relative expression of depicted genes in primary human hepatic stellate cells (HSC) treated with lanifibranor or vehicle. (**B**) mRNA relative expression of depicted genes in primary human hepatocytes treated with lanifibranor or vehicle. (**C**) Aspartate aminotransferase (AST) and alanine aminotransferase (AST) levels in culture medium from primary human hepatocytes treated with lanifibranor or vehicle. (**D**) Primary human HSC contraction over time when treated with lanifibranor or vehicle. *$p<0.05$ and **$p<0.01$ vs. vehicle (one-way ANOVA for A-C; repeated measures GLM for D). n=5 per group (A-H) and n=2-5 per group (G).

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FIGURE 1

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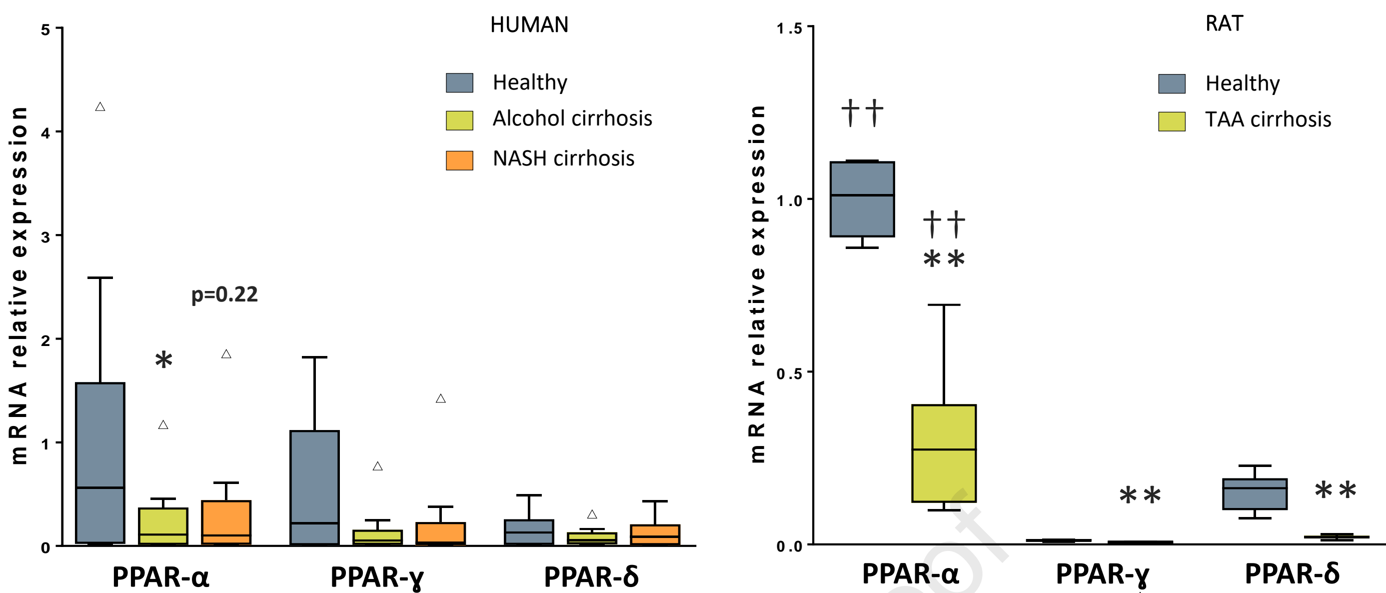

C

PPAR-α
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Healthy
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FIGURE 2

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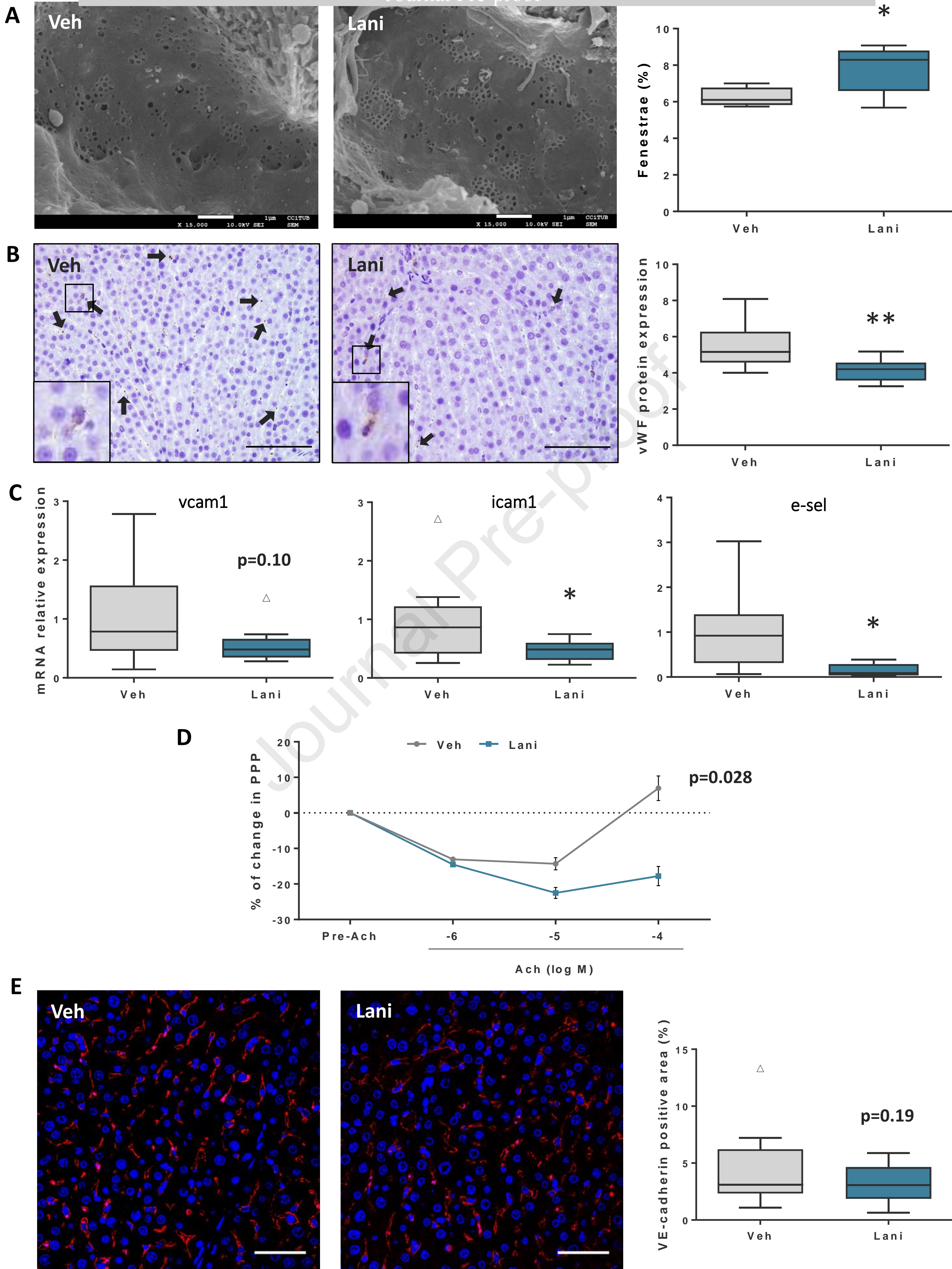

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FIGURE 3

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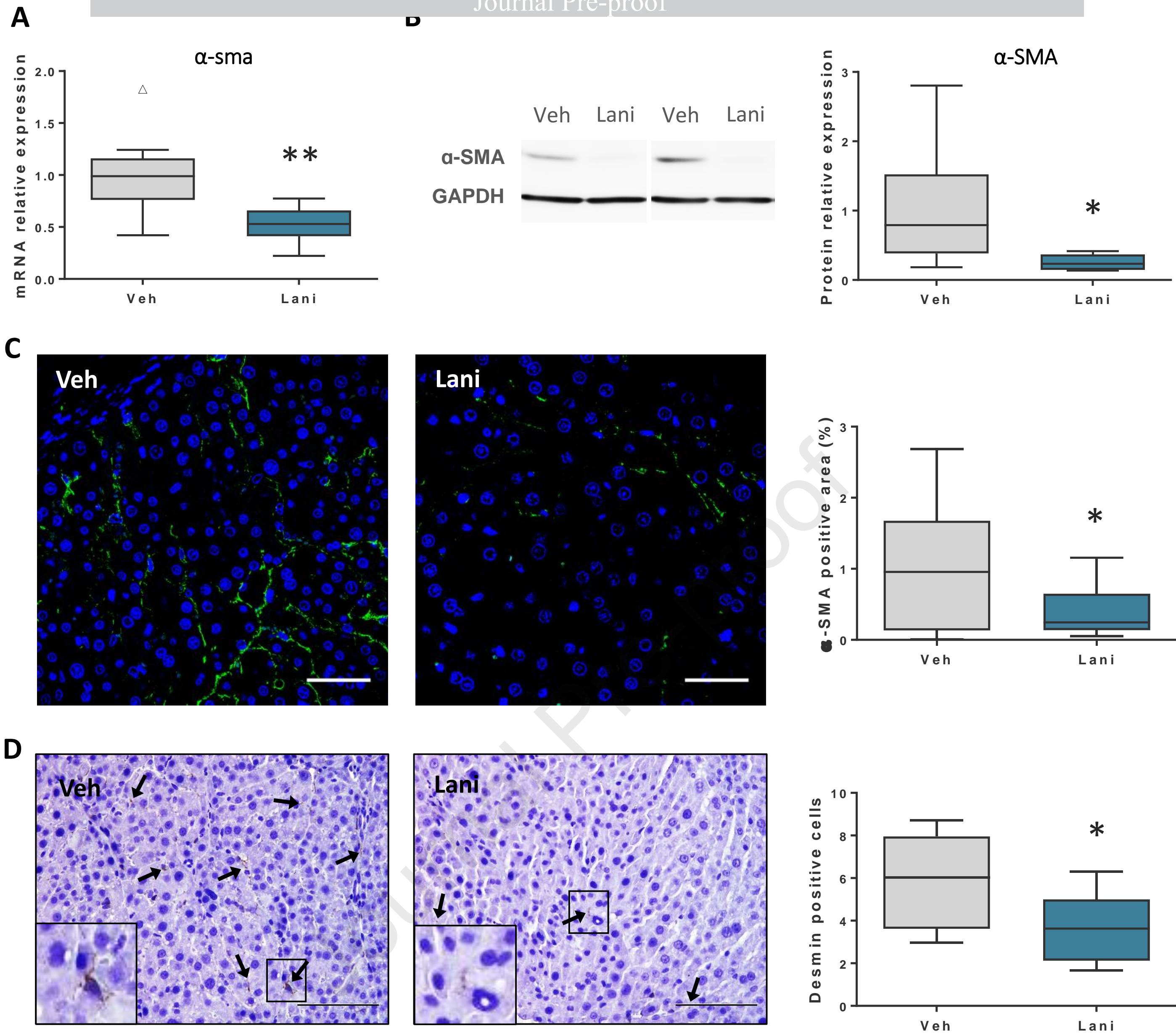

FIGURE 4

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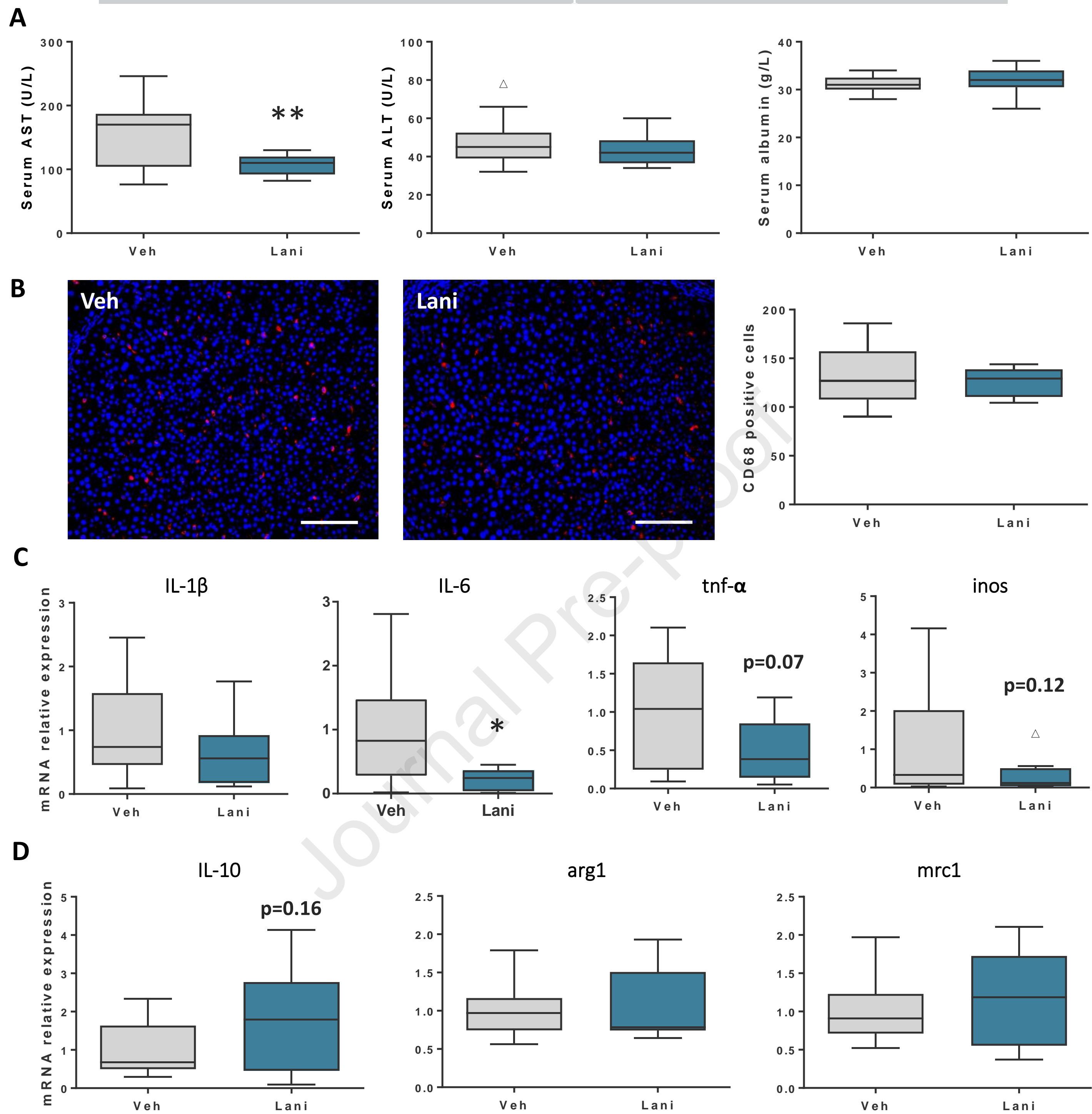

FIGURE 5

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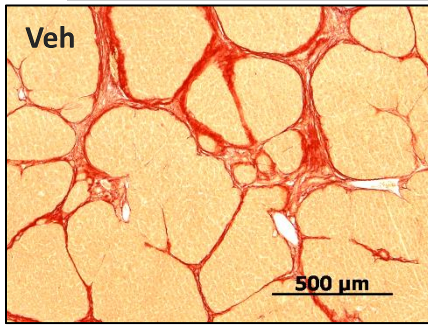
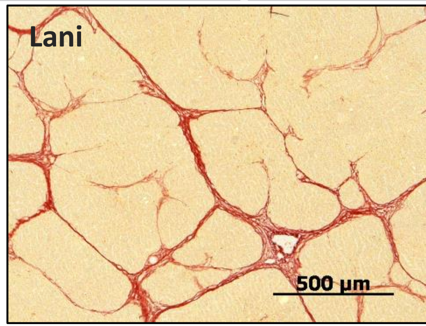
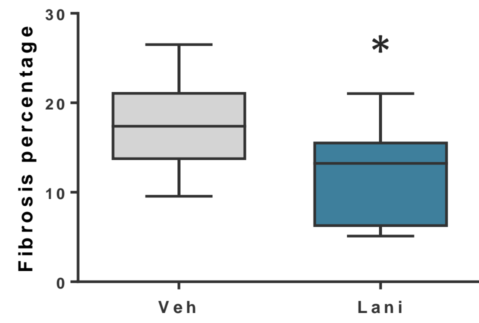
A







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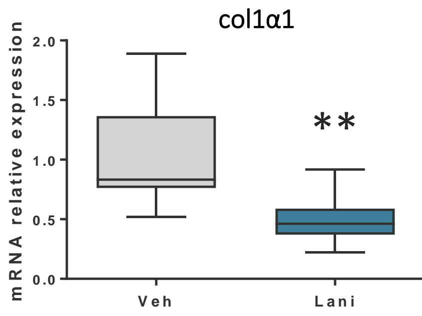

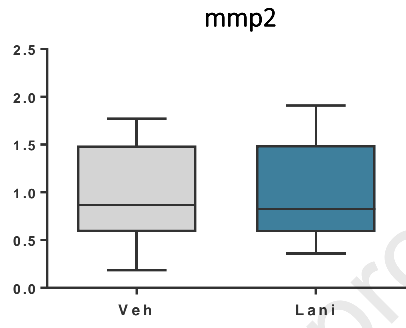

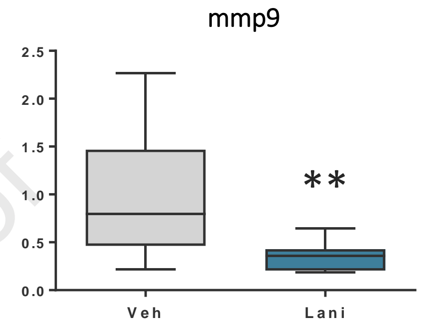

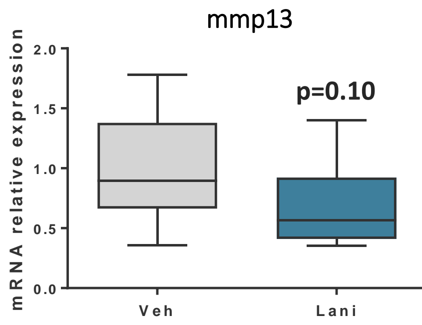

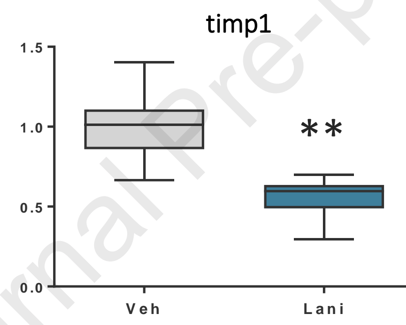

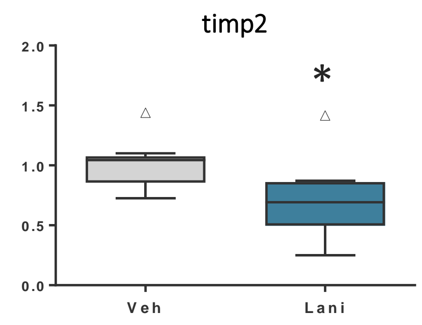

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FIGURE 6

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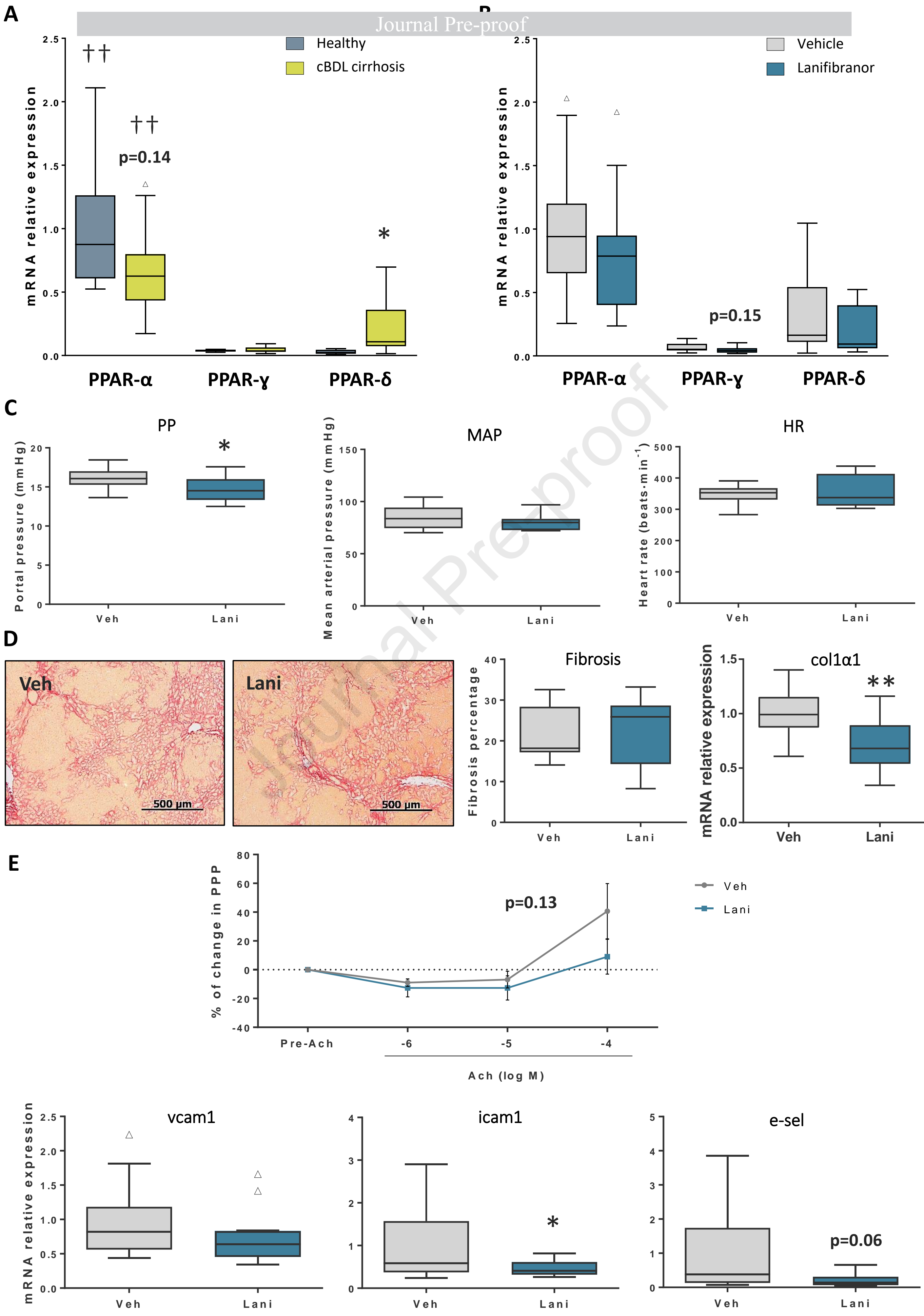

FIGURE 7

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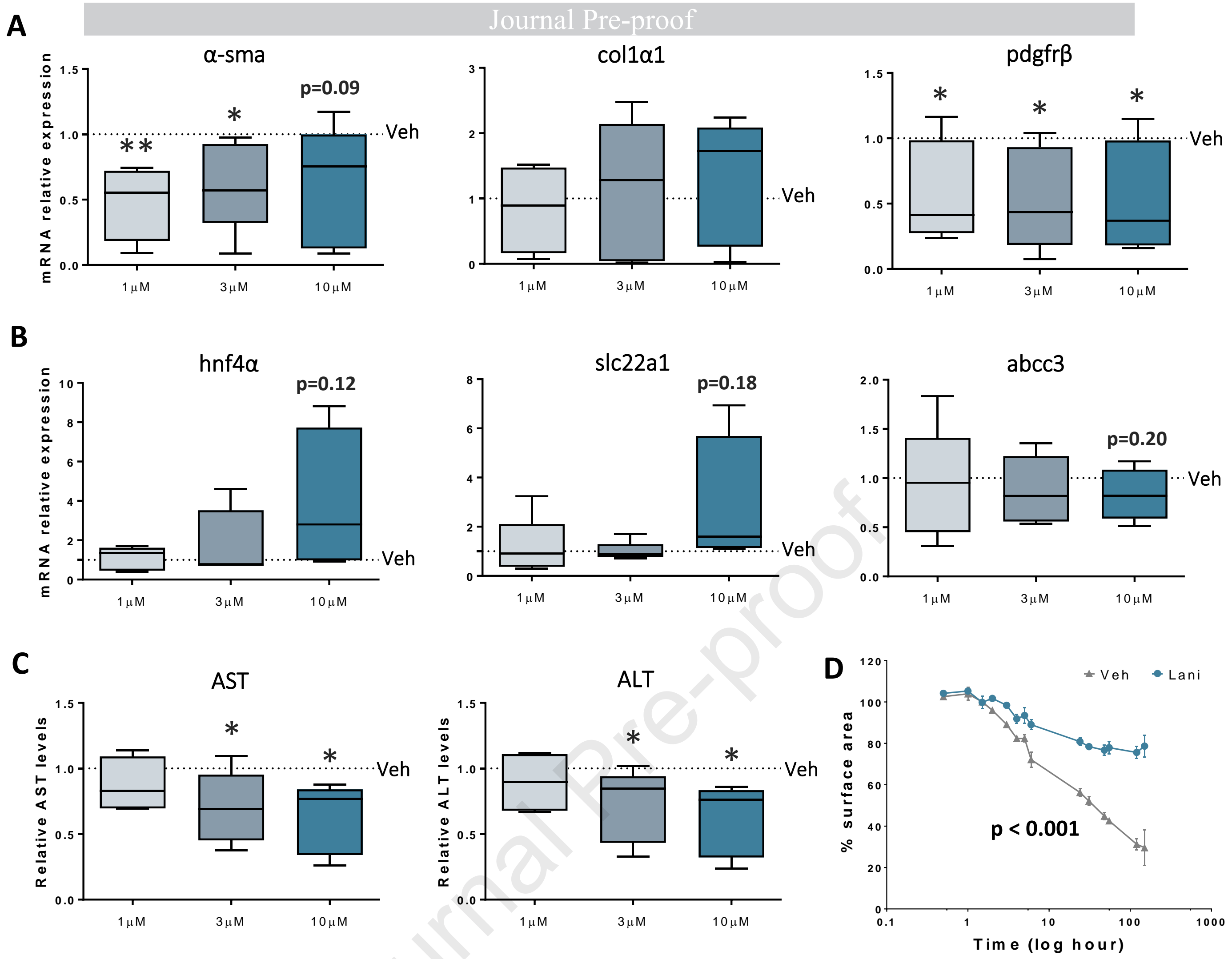

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Highlights:

- PPAR-α, PPAR-ɣ, and PPAR-δ are differentially expressed among hepatic cell types, and are de-regulated in chronic liver disease.
- Treatment with lanifibranor improved portal hypertension in experimental models of ACLD.
- Reduced portal pressure was explained by fibrosis regression and improved hepatic microvascular function.
- *In vitro* lanifibranor treatment phenotypically improved hepatic cells from patients with ACLD.

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