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Expression of Type 3 Inositol 1,4,5-Trisphosphate Receptor and Nrf2 Levels in Cholangiocytes in Alcoholic Liver Disease

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Article

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Abstract

Aim

Alcohol-associated liver disease is a major cause of chronic liver disease and cirrhosis. Cholangiocyte dysfunction contributes to disease progression, but the mechanisms underlying the loss of type 3 inositol 1,4,5-trisphosphate receptor, the major intracellular calcium release channel in cholangiocytes, remain unclear. Nuclear factor kappa B, microRNA-506, and nuclear factor erythroid 2-related factor 2 have been implicated in receptor downregulation in other cholangiopathies. This study investigated whether these pathways contribute to receptor loss during alcohol-associated liver disease.

Methods

Liver specimens from 49 patients with cirrhosis secondary to alcohol-associated liver disease, 18 patients with chronic hepatitis C, and 21 liver transplant donors were analyzed by immunohistochemistry, immunofluorescence, and in situ hybridization. Findings were validated in mice exposed to chronic ethanol feeding.

Results

Type 3 inositol 1,4,5-trisphosphate receptor expression was significantly reduced in cholangiocytes from patients with alcohol-associated liver disease and ethanol-fed mice. Serum alanine aminotransferase and aspartate aminotransferase levels were also significantly increased. However, neither activation of nuclear factor kappa B, increased microRNA-506 expression, nor consistent nuclear localization of nuclear factor erythroid 2-related factor 2 was detected. Nuclear localization of nuclear factor erythroid 2-related factor 2 was observed in only 3 of 49 patients.

Conclusion

Downregulation of type 3 inositol 1,4,5-trisphosphate receptor in cholangiocytes during alcohol-associated liver disease occurs independently of nuclear factor kappa B, microRNA-506, and nuclear factor erythroid 2-related factor 2, indicating that alternative regulatory mechanisms underlie cholangiocyte dysfunction.

1 Introduction

Alcohol is the most widely consumed addictive substance worldwide and remains a major cause of preventable morbidity and mortality[1,2]. Alcohol-associated Liver Disease (ALD) encompasses a spectrum of pathological changes ranging from steatosis and steatohepatitis to fibrosis, cirrhosis, and

hepatocellular carcinoma (HCC)[3,4,5,1]. Although hepatocyte injury is the hallmark of ALD, cholestatic features are frequently present and are associated with more severe disease, poorer prognosis, and increased mortality[6,7].

Bile formation depends on the coordinated function of hepatocytes and cholangiocytes. In cholangiocytes, bicarbonate-rich bile secretion is driven by intracellular Ca^{2+} signaling mediated predominantly by the type 3 inositol 1,4,5-trisphosphate receptor (ITPR3), the major intracellular Ca^{2+} release channel expressed in these epithelial cells[8,9]. ITPR3-mediated Ca^{2+} release stimulates ductular bicarbonate secretion and maintains normal bile flow; therefore, ITPR3 loss impairs biliary secretion and promotes cholestasis[9,10,11]. Consistent with these observations, reduced ITPR3 expression has subsequently been reported in multiple cholangiopathies through distinct pathogenic mechanisms, including inflammatory and endotoxin-mediated injury[12], epigenetic regulation by miR-506 in primary biliary cholangitis[13], and oxidative stress-induced repression mediated by Nrf2[14]. Despite growing recognition of cholestatic manifestations in ALD, ITPR3 expression in cholangiocytes during ALD remains underexplored.

Several molecular mechanisms regulate ITPR3 expression under pathological conditions. Our group previously demonstrated that lipopolysaccharide suppresses ITPR3 transcription through Toll-like receptor 4-mediated NF-κB activation, impairing intracellular Ca^{2+} signaling in cholangiocytes[12]. Likewise, miR-506 directly targets ITPR3 mRNA, promoting its post-transcriptional repression in primary biliary cholangitis[13,15], whereas the oxidative stress-responsive transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) represses ITPR3 transcription, thereby linking oxidative stress to impaired Ca^{2+} signaling and biliary secretion[14]. Whether these mechanisms account for the ITPR3 loss in cholangiocytes during ALD remains unknown. Therefore, this study investigated whether ITPR3 expression is reduced in cholangiocytes from patients with ALD and evaluated the contribution of NF-κB, miR-506, and Nrf2 to this process using complementary analyses in a chronic ethanol mouse model.

2 Methods

2.1 Human Liver Biopsies

Formalin-fixed, paraffin-embedded liver biopsy specimens were obtained from the archives of the Department of Pathology and Legal Medicine at the University Hospital, Faculty of Medicine, Federal University of Minas Gerais (UFMG). All research procedures involving human tissue samples were approved by the appropriate Institutional Research Ethics Committee and were performed in accordance with the relevant ethical guidelines and regulations, including Resolution No. 466/2012 of the Brazilian National Health Council (Conselho Nacional de Saúde, CNS). The study cohort included 49 patients with cirrhosis secondary to ALD, 18 patients with chronic hepatitis C virus (HCV) infection, and 21 liver transplant donors as non-alcoholic controls. HCV specimens were collected between 1998 and 2015, whereas donor samples were obtained between 2012 and 2017. The HCV group was included because

previous studies demonstrated preserved cholangiocyte ITPR3 expression despite chronic portal inflammation, allowing discrimination between ethanol-specific and inflammation-related effects[11]. Donors were of comparable age to the ALD and HCV groups.

Liver biopsy samples from patients of both sexes, without age restriction, with a documented cirrhosis secondary to ALD diagnosis for at least five years, were included. The diagnosis was established by a hepatologist at the University Hospital, UFMG. The institution maintains the informed consent forms (Termo de Consentimento Livre e Esclarecido – TCLE), signed by all participants or their legal representatives, authorizing the collection and research use of these archived tissue specimens. Only isolated ALD cases were included. Exclusion criteria comprised viral hepatitis B or C, HCC, primary biliary cholangitis (PBC), primary sclerosing cholangitis, extrahepatic biliary atresia (BA), and malignant or benign biliary obstruction.

2.2 C57BL/6 Mice

The animal study protocol was approved by the Animal Ethics Committee (Comissão de Ética no Uso de Animais - CEUA) da Universidade Federal de Minas Gerais (UFMG), Brazil (protocol No. 280/2018). All experiments were performed in accordance with the relevant guidelines and regulations, including Brazilian Law No. 11,794 of October 8, 2008, Decree No. 6,899 of July 15, 2009, and the regulations issued by the Brazilian National Council for the Control of Animal Experimentation (CONCEA).

To investigate the effects of ethanol exposure on ALD-associated liver injury and ITPR3 expression and to compare the experimental findings with those observed in human liver specimens, 42 male C57BL/6 mice (12 weeks old, isogenic strain) were used. These 21 animals received 5% ethanol (EtOH) for 10 days, as previously described[16–18], whereas 21 untreated animals served as controls. Animals were weighed daily to monitor variations in body weight throughout the experimental period. At the end of the study, deep anesthesia was induced by intraperitoneal administration of ketamine (80 mg/kg; União Química, São Paulo, Brazil) and xylazine (10 mg/kg; Ceva Santé Animale, São Paulo, Brazil). Euthanasia was subsequently performed by rapid decapitation using surgical scissors. At euthanasia, liver weight was measured and analyzed relative to total body weight. Total hepatic lipid content was also determined for each animal, and Western blot analysis was performed on the antioxidant enzymes glutathione peroxidase (GPx), superoxide dismutase 1 (SOD-1), and catalase.

2.3 Histopathological Analysis

Histopathological analysis was performed to characterize inflammatory activity. Formalin-fixed, paraffin-embedded liver biopsy sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope (CX4; Olympus Corporation, Tokyo, Japan). Inflammatory activity was evaluated by the degree of polymorphonuclear and mononuclear leukocyte infiltration and classified as absent, mild, moderate, or severe.

2.4 Immunohistochemical Analysis

IHC analyses were performed to evaluate the expression of ITPR3, Nrf2, NF-κB/RelA (p65), and NF-κB1 (p50). *In situ* hybridization was additionally conducted to detect miRNA-506 in human liver biopsy specimens. Liver samples from patients with sepsis, PBC, and BA were included as positive controls for NF-κB, miR-506, and Nrf2, respectively. IHC was performed using the Novolink™ Polymer Detection System (Leica Biosystems, Newcastle upon Tyne, United Kingdom). Sections were deparaffinized, rehydrated, and subjected to antigen retrieval in 1 mM citrate buffer (pH 6.0), followed by endogenous peroxidase activity, and then incubated with primary antibodies anti-Nrf2 (Abcam, Cambridge, MA, USA) and anti-SOD1 (Santa Cruz Biotechnology, Dallas, TX, USA). Immunoreactivity was visualized with diaminobenzidine (DAB). The ITPR3 expression and Nrf2 in ALD samples were compared with those observed in HCV and liver transplant donors. Images were converted to 8-bit grayscale, and pixel intensity was quantified (0–255 grayscale scale) using ImageJ software (NHI, Bethesda, MD, USA)[18].

2.5 Western Blot Analysis

WB analysis was performed to assess EtOH-induced oxidative stress in mice by measuring the expression of antioxidant proteins GPx, SOD-1, and catalase. Liver tissue (25mg) was homogenized in lysis buffer (100 µL) with protease inhibitor (20 µL). Samples were centrifuged at 13,000 rpm for 10 minutes at 4°C, and the supernatant was collected for protein quantification. Protein concentration was determined by the Bradford assay. Briefly, 1 µL of sample supernatant was added to a microplate containing bovine serum albumin standard solution (1:1000 in Milli-Q water) and 180 µL of Bradford reagent. Absorbance was measured at 595 nm.

For electrophoresis, 15 µL of the sample, mixed with loading buffer and distilled water, was heated at 95°C for 5 minutes and loaded onto a 10% polyacrylamide gel. Electrophoresis was performed at 80–100 V for approximately 1.5 hours. Protein transfer was carried out in a closed transfer system at 20 V for 45 minutes. Membranes were blocked with 25 mL of blocking solution overnight. After washing with Tris-buffered saline (TBS) 1×, membranes were incubated overnight with primary antibodies diluted 1:1000. Subsequently, membranes were incubated with the appropriate secondary antibodies specific to each target protein: SOD-1 (Santa Cruz Biotechnology, C-17, SC-8637; goat), GPx1/2 (Santa Cruz Biotechnology, H-151, SC-30157; rabbit), catalase (Santa Cruz Biotechnology, F-17, SC-34285; goat), and the loading control β-actin (mouse). Incubation was performed at 2–8°C for 1 hour and 30 minutes. After washing with TBS 1x, protein bands were visualized using a chemiluminescent detection solution prepared at a 1:1 ratio according to the manufacturer's instructions.

2.6 Biochemical Analyses

Biochemical assays were performed using serum collected from EtOH-treated and untreated (control) mice to provide complementary and longitudinal assessments of ALD progression. Serum levels of ALT, AST, total bilirubin, and its fractions were measured according to the manufacturer's instructions (Bioclin®, Brazil).

2.7 Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA, USA). Data are presented as the mean ± standard error of the mean (SEM).

Differences between experimental groups were considered statistically significant at $P < 0.05$. Comparisons between two groups were performed using the unpaired Student's *t*-test. For comparisons involving multiple groups, one-way analysis of variance (ANOVA) was conducted, followed by Bonferroni's post hoc test, as appropriate.

3 Results

Among the included samples, there were more males with HCV (81%) and ALD (97%) than females, whereas among liver donors, there were more females (75%). The mean ages were also comparable among the study groups (49 years in liver donors, 55 years in ALD, and 57 years in HCV).

3.1 Inflammatory Activity and Comparative Analysis of Biochemical Parameters in Liver Biopsies

Analysis of inflammatory activity in 49 liver biopsies from patients with cirrhosis secondary to ALD (Fig. 1A) showed mild inflammation in 14 cases, moderate inflammation in 5, severe fibrosis in 2, and no histologically detectable inflammatory activity in 28 cases.

Since previous studies have demonstrated that biochemical alterations, particularly elevated transaminase levels in chronic alcohol users, represent relevant predictors during ALD progression[19–22], we compared biochemical parameters in ALD with liver transplant donors (Fig. 1B). Statistically significant differences ($P < 0.05$) were observed for ALT (41.6 U/L vs. 20.5 U/L), AST (62.8 U/L vs. 39.7 U/L), total bilirubin (3.37 mg/dL vs. 0.57 mg/dL), and indirect bilirubin (1.98 mg/dL vs. 0.32 mg/dL) levels in ALD, but not ALP, GGT or direct bilirubin ($P > 0.05$) (Fig. 1B). Model for End-Stage Liver Disease (MELD) score = 7.7 (donors), 14.3 (HCV), 16.5 (ALD) (Fig. 1C).

3.2 Laboratory Evidence of Ethanol-Induced Effects in C57BL/6 Mice

Serum samples collected at euthanasia demonstrated statistically significant differences ($P < 0.05$) in AST and ALT levels (Fig. 2A1, A2) between EtOH-treated and control groups. In contrast, no significant differences ($P > 0.05$) were observed in total bilirubin or its fractions (direct and indirect bilirubin) between groups (Fig. 2A3 – A5). Furthermore, EtOH-exposed mice exhibited a significant reduction ($P < 0.05$) in the expression of all antioxidant markers analyzed compared with controls (Fig. 2B, C).

In addition, longitudinal monitoring revealed progressive body weight reduction in EtOH-treated group over the 10-day exposure period compared with controls (Fig. 3A). Despite the gradual decrease in overall body weight (Fig. 3B), EtOH-exposed mice exhibited a significant increase ($P < 0.05$) in liver

weight relative to total body weight (Fig. 3C). Histopathological examination of liver tissue sections from EtOH-treated animals demonstrated alterations consistent with early-stage ALD, including hepatic steatosis and hepatocellular ballooning due to marked lipid accumulation. Notably, no neutrophilic infiltration was observed. These findings were absent in control group (Fig. 3D). Consistent with these observations, total hepatic lipid quantification revealed a statistically significant increase ($P < 0.05$) in lipid content adjusted for liver weight in EtOH-treated animals compared with controls (Fig. 3E).

3.3 ITPR3 Expression in Cholangiocytes in ALD and in ethanol-exposed mice.

The IHC analysis showed a significant reduction in ITPR3 expression in cholangiocytes from patients with ALD compared with both liver donors and HCV patients ($P < 0.05$; Fig. 4A–D). No significant difference in ITPR3 expression was observed between the HCV and donor groups. To evaluate ITPR3 expression in the experimental model, cholangiocytes from C57BL/6 mice exposed to 5% EtOH for 10 days were analyzed by immunohistochemistry. EtOH-exposed mice exhibited a significant reduction in ITPR3 expression compared with control mice (Fig. 4E–G). The ITPR3 staining pattern observed in EtOH-exposed mice was similar to that observed in liver samples from patients with ALD.

3.4 Evaluation of candidate regulators of ITPR3 expression in cholangiocytes during ALD

The results demonstrated that the nuclear localization of the NF-κB subunits RelA/p65 and NF-κB1/p50 was not significantly altered in cholangiocytes from patients with cirrhotic-stage ALD compared with donor livers and healthy controls (Fig. 5A–B). Quantitative analysis confirmed the absence of significant differences in the percentage of cholangiocytes exhibiting nuclear p65- or p50-positive nuclei among these groups (Fig. 5B–C). In contrast, cholangiocytes from septic patients, used as a positive control, exhibited significantly higher nuclear localization of both NF-κB subunits than those of all other groups ($P < 0.001$). Likewise, miR-506 expression was not increased in cholangiocytes from ALD patients compared with donor livers and healthy controls (Fig. 5D). These findings indicate that neither activation of the canonical NF-κB pathway nor upregulation of miR-506 is likely to account for the reduction in ITPR3 expression in cholangiocytes during ALD.

Moreover, Nrf2 was not localized in the nucleus in the majority of ALD cases analyzed (46/49). Instead, Nrf2 immunostaining was predominantly cytoplasmic, with a marked perinuclear distribution in cholangiocytes (Fig. 6C). In contrast, cholangiocytes from liver transplant donors and patients with chronic hepatitis C exhibited weak Nrf2 immunoreactivity (Fig. 6A and B), whereas BA, used as a positive cholestatic control, showed intense nuclear Nrf2 staining (Fig. 6D). Nuclear translocation of Nrf2 was observed in only 3 of the 49 ALD biopsy samples, indicating activation of this transcription factor in a small subset of patients (Fig. 6E). Consistent with these observations, semiquantitative analysis of the nuclear histological score demonstrated no significant differences among donors, HCV, and the majority

of ALD samples (ALD-6C). In contrast, BA exhibited significantly higher nuclear Nrf2 expression than donors ($P < 0.001$) and the ALD subgroup with nuclear Nrf2 staining (ALD-6E) ($P < 0.05$), whereas ALD-E displayed an intermediate nuclear histological score (Fig. 6F).

4 Discussion

Dysregulated ITPR3-mediated intracellular Ca^{2+} signaling is a well-established mechanism contributing to cholestasis. Consistent with this concept, we previously demonstrated that NF-κB mediates LPS-induced cholestasis by downregulating ITPR3 expression and impairing Ca^{2+} signaling in cholangiocytes[12]. However, whether NF-κB, miR-506, and Nrf2 regulate ITPR3 expression during ALD remains unclear. In this study, we observed that while ITPR3 expression was downregulated in ALD, there were no significant differences in the activity or expression of NF-κB, miR-506, and Nrf2 compared with liver specimens from HCV patients and transplant donors.

Consistent with previous studies[21–25], alterations in serum transaminases and bilirubin have been reported in patients with ALD, underscoring their value as markers of liver injury. In this study, the biochemical results from animal models showed significant differences in AST and ALT but not in total bilirubin or its fractions, which contrasts with findings from liver biopsies in patients with ALD. This may be justified by differences in the degrees and stages of alcoholic liver injury between human ALD and EtOH diet-induced animal models, which rarely reproduce all stages of ALD, particularly the chronic stages of alcohol intake. Thus, certain histopathological alterations in the translational history of ALD progression may not be observed throughout the natural history of ALD progression may not be observed in rodents because they do not exhibit the full spectrum of the disease[26,27,16], even though they represent the preferred animal models for mimicking human ALD as the use of historically preferred primates (especially baboons)[28] is limited by stricter ethical regulations, high costs, and the prolonged time required to conduct research[16].

Reduced ITPR3 expression in cholangiocytes has previously been described in several pathological conditions, including LPS-induced inflammatory injury[12] and AH[9]. In contrast, chronic HCV infection, despite persistent hepatic inflammation, was not associated with comparable loss of cholangiocyte ITPR3 expression, suggesting that inflammation alone is insufficient to account for ITPR3 downregulation[12]. Here, we demonstrate a significant downregulation of ITPR3 in liver of patients with cirrhosis secondary to ALD, whereas its expression was preserved in liver specimens from HCV-infected patients and transplant donors. This disease-specific pattern was corroborated in our experimental model, in which mice exposed to a 5% EtOH diet for 10 days also exhibited a significant reduction in cholangiocyte ITPR3 expression. These findings are consistent with previous reports showing that chronic alcohol exposure impairs biliary function and promotes intrahepatic cholestasis through mechanisms involving cholangiocyte dysfunction and bile duct injury[7,29,30]. Together, these observations indicate that ALD induces a selective loss of cholangiocyte ITPR3 expression.

Beyond transcriptional regulation, evidence suggests that ITPR expression may also be controlled through post-translational mechanisms. A recent study from our group demonstrated that activated neutrophils transfer elastase to hepatocytes, promoting proteolytic degradation of IP_3 receptors and disrupting intracellular Ca^{2+} signaling during alcohol-associated hepatitis[31]. Although the primary analyses focused on hepatocyte IP_3R2, supplementary data demonstrated that all three IP_3 receptor isoforms, including IP_3R3, are susceptible to elastase-mediated degradation in cholangiocytes. These findings suggest that chronic inflammation and neutrophil activation may contribute to the loss of cholangiocyte ITPR3 through enhanced protein degradation, representing a mechanism distinct from transcriptional or post-transcriptional regulation. Therefore, ALD appears to induce cholangiocyte ITPR3 loss through mechanisms that extend beyond the transcriptional pathways investigated in the present study.

To determine whether transcriptional regulators previously implicated in cholangiocyte ITPR3 downregulation could account for this phenotype during cirrhosis secondary to ALD, NF-κB (RelA/NF-κB1), miRNA-506, and Nrf2 were investigated. However, no evidence was found to suggest the involvement of NF-κB or miRNA-506 in the negative regulation of ITPR3 in cholangiocytes during cirrhosis secondary to ALD. NF-κB is a key cytoplasmic transcription factor involved in inflammatory and oxidative stress responses. Upon activation by cytokines, oxidative stress, or bacterial endotoxins, it translocates to the nucleus to regulate target gene expression[32–36]. The discrepancy between the results presented by França et al. (2019) and the present study suggests differences in pathophysiological mechanisms operating across the spectrum of ALD, highlighting our still-incomplete understanding of its heterogeneous manifestations and the role of NF-κB across its stages.

MiRNA-506 has been described as a negative regulator of ITPR3 expression in cholangiopathies[13,15,37,38]. In mice treated with an alcoholic diet, hepatic expression of miRNA-122 is decreased, suggesting a direct or indirect role for miRNA-122 in alcohol-induced fibrosis[15]. Downregulation of miRNA-506 (expressed in bile duct epithelial cells) also increases the activity of the Cl^-/HCO_3^- exchanger system in cultured cholangiocytes from patients with PBC[15]. Transfection of miRNA-506 into cholangiocyte cell lines expressing ITPR3 also leads to loss of ITPR3, resulting in impaired biliary secretion. Likewise, inhibition of miRNA-506 increased ITPR3 levels in cholangiocytes, confirming its regulatory role in ITPR3-mediated Ca^{2+} signaling and secretion[13]. Our group has also shown that ITPR3 expression is regulated by promoter methylation, which varies according to the etiology of liver disease[39]. However, whether these epigenetic mechanisms contribute to the downregulation of ITPR3 observed in cholangiocytes during ALD remains unknown. Despite extensive evidence regarding the regulatory potential of miRNAs in hepatic injury, including during alcohol consumption[40], previous studies have not clearly shown whether miRNAs participate in regulating ITPR3 expression in cirrhosis caused by ALD. Clarifying this mechanism would improve our understanding of the pathways responsible for altered Ca^{2+} signaling and impaired biliary secretion in this disease. In our current study, we found no evidence that miRNA-506 is involved in ITPR3 downregulation expression in

cholangiocytes of patients with liver cirrhosis secondary to ALD. These findings suggest that miRNA expression profiles differ among liver diseases with distinct etiologies.

Since the 1990s, it has already been observed that prolonged alcohol exposure reduces antioxidant defenses and increases ROS production in human cells[41–43]. Consistent with these findings, we observed decreased levels of antioxidant enzymes in EtOH-exposed animal models. Nrf2 is a key regulator of antioxidant and detoxification gene expression, as it is considered a sensor of oxidative stress, with evidence indicating that it negatively regulates ITPR3 expression in cholangiocytes[14,44]. Evidence suggests that Nrf2 promotes cellular survival by preventing increases in reactive oxygen species (ROS) under various oxidative stress conditions, and it is considered the most important endogenous antioxidant signaling pathway in the liver, where it is highly expressed[45–47]. Liu et al. (2013) demonstrated that maximal activation of Nrf2 decreased serum ALT levels in mice, whereas Nrf2-knockout mice exhibited increased ROS and ALT activity[48–50]. Despite the significant attenuation of antioxidant enzymes in EtOH-treated animals, there was no corresponding reduction in the expression of the transcription factor Nrf2. This suggests that EtOH-induced oxidative stress in cholangiocytes of the ALD phenotype is not mediated by the Nrf2 transcription mechanism.

In our study, Nrf2 nuclear localization was absent in 46 of the 49 ALD samples analyzed, whereas only 3 cases exhibited nuclear translocation. Cholestatic diseases encompass a broad spectrum of hepatobiliary etiologies, including impairment of intrahepatic bile production, dysfunction of transmembrane transport, or mechanical obstruction of bile flow, and are characterized by the accumulation of bile components [51,52]. Previous work from our group demonstrated that Nrf2 represses ITPR3 expression in inflammatory cholangiopathies, indicating that distinct signaling pathways may converge to regulate cholangiocyte ITPR3 expression[12]. The heterogeneous pattern of Nrf2 nuclear localization observed in our cohort may reflect differences in disease stage or clinical history. Notably, all patients had undergone at least six months of alcohol abstinence before liver transplantation, and the absence of ongoing alcohol-induced oxidative stress may have contributed to the limited Nrf2 activation observed in most samples. Importantly, the lack of detectable changes in Nrf2 expression or nuclear localization does not exclude functional involvement of this pathway. Indeed, polymorphisms in the promoter region of NFE2L2 have been associated with susceptibility to alcohol-related cirrhosis[53], suggesting that the contribution of Nrf2 to ITPR3 regulation may depend on genetic background and disease context rather than changes in protein abundance or nuclear localization alone.

Collectively, our findings indicate that the loss of cholangiocyte ITPR3 expression in ALD cannot be explained solely by the transcriptional regulators investigated in the present study. Instead, the available evidence supports a multifactorial model in which disease-specific transcriptional responses, genetic variability, and post-translational degradation of IP_3 receptors may all contribute to regulating ITPR3 expression during ALD, although the relative contributions of each mechanism remain to be established.

5 Conclusion

The present study demonstrates that ALD is characterized by a selective loss of cholangiocyte ITPR3 expression, a feature that was not observed in chronic HCV infection despite persistent liver inflammation. In contrast to previous findings in other cholangiopathies, our data indicate that ITPR3 downregulation in cirrhosis secondary to ALD is not associated with activation of the canonical NF-κB pathway, upregulation of miR-506, or Nrf2 nuclear translocation. These findings suggest that distinct molecular mechanisms underlie the loss of cholangiocyte ITPR3 in ALD. Future studies should investigate alternative pathways, including post-translational mechanisms such as inflammation-associated proteolytic degradation of IP_3 receptors, to better define the processes underlying impaired Ca^{2+} signaling and cholestasis in ALD.

Abbreviations

ALD
alcohol-associated liver disease
ALP
alkaline phosphatase
ALT
alanine aminotransferase
AST
aspartate aminotransferase
Ca^{2+}
calcium
CEUA
Institutional Committee for Ethics in Animal Use
EtOH
ethanol
GGT
gamma-glutamyl transpeptidase
GPx
glutathione peroxidase
HCC
hepatocellular carcinoma
HCV
hepatitis C virus
IHC
immunohistochemistry
ITPR3
type 3 inositol 1,4,5-trisphosphate receptor
MELD
Model for End-Stage Liver Disease

miR
506-microRNA-506
NF
κB-nuclear factor kappa B
Nrf2
nuclear factor erythroid 2-related factor 2
ROS
reactive oxygen species
SOD
1-superoxide dismutase 1
UFMG
Federal University of Minas Gerais.

Declarations

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflict Of Interest Disclosure

The authors declare no conflict of interest.

Ethics Approval Statement

The study protocol was approved by the Research Ethics Committee of the UFMG, Brazil, on May 31, 2017 (CAAE 67814117.6.0000.5149; approval no. 2.093.116). The animal study protocol was approved by the Institutional Committee for Ethics in Animal Use (CEUA) on February 26, 2019 (protocol no. 280/2018).

Author Contributions

KNS and ARRA were equally responsible for methodology, validation, formal analysis, investigation, data curation, writing – original draft, writing – review and editing, and visualization. **ACMLF and PVTV** contributed to resources, methodology, investigation, and writing – review and editing. **AIA** contributed to investigation, and writing – review and editing. **IBC and MFL** was responsible for

conceptualization, methodology, validation, formal analysis, investigation, writing – original draft, writing – review and editing, visualization, project administration, supervision, and funding acquisition.

Declaration Of Generative Ai And Ai-Assisted Technologies In The Manuscript Preparation Process

During the preparation of this manuscript, the authors used ChatGPT (OpenAI) to assist with language editing, improving readability, and refining the organization of the text. The authors critically reviewed, revised, and approved all content and take full responsibility for the accuracy and integrity of the manuscript.

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References

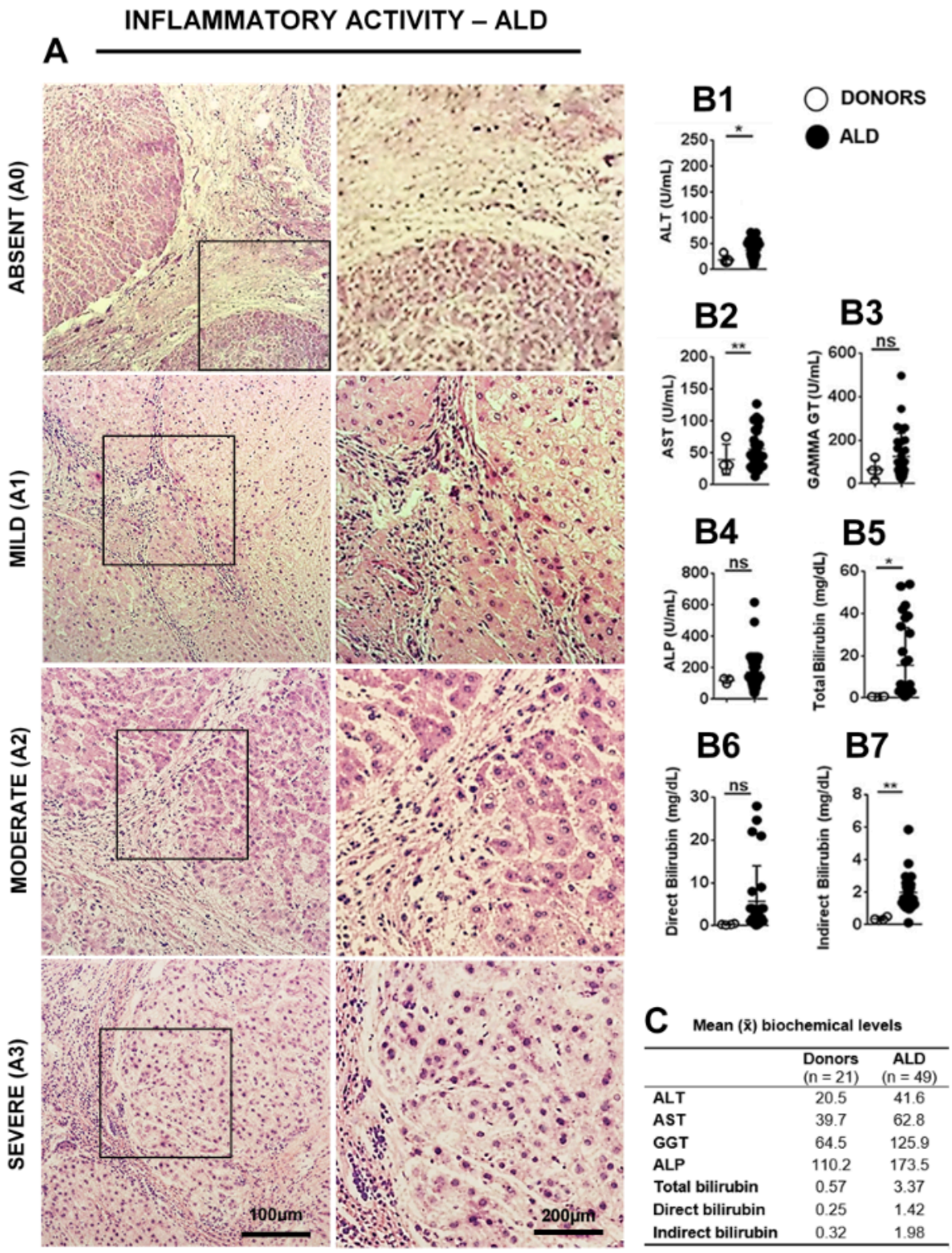
1. Avila, M. A. et al. Recent advances in alcohol-related liver disease (ALD): summary of a Gut round table meeting. Recent advances in clinical practice. **0**: 01–17. (2019).
2. Rehm, J. & Shield, K. D. Global Burden of Alcohol Use Disorders and Alcohol Liver Disease. *Biomedicines* **7** (4), 99 (2019).
3. O'Shea, R. S., Dasarathy, S. & McCullough, A. J. Alcoholic liver disease. *Hepatology* **51** (1), 307–328 (2010).
4. Lucey, M. R., Mathurin, P. & Morgan, T. R. Alcoholic hepatitis. *N. Engl. J. Med.* **360** (26), 2758–2769 (2009).
5. Kong, L. Z. et al. Early Diagnosis, and Therapeutic Management of Alcoholic Liver Disease. *Int. J. Mol. Sci.* **20** (2712), 1–27 (2019).
6. Altamirano, J. et al. R. A histologic scoring system for prognosis of patients with alcoholic hepatitis. *Gastroenterology* **146** (5), 1231–1239 (2014).
7. Kasztelan-Szczerbinska, B., Slomka, M., Celinski, K. & Szczerbinski, M. Alkaline phosphatase: the next independent predictor of the poor 90-day outcome in alcoholic hepatitis. *BioMed Research International*. ; 2013(5): 1–8. (2013).
8. Hirata, K. et al. Regulation of Ca^{2+} signaling in rat bile duct epithelia by inositol 1,4,5-trisphosphate receptor isoforms. *Hepatology* **36** (2), 284–296 (2002).
9. Guerra, M. T. & Nathanson, M. H. Calcium signaling and secretion in cholangiocytes. *Pancreatology* **15** (4), 44–48 (2015).

10. Minagawa, N. et al. Cyclic AMP regulates bicarbonate secretion in colangiócitos through release of ATP into bile. *Gastroenterology* **133** (5), 1592–1602 (2007).
11. Shibao, K., Hirata, K., Robert, M. E. & Nathanson, M. H. Loss of inositol 1,4,5-trisphosphate receptors from bile duct epithelia is a common event in cholestasis. *Gastroenterology* **125** (4), 1175–1187 (2003).
12. Franca, A. et al. Effects of Endotoxin on Type 3 Inositol 1,4,5-Trisphosphate Receptor in Human Cholangiocytes. *Hepatology* **69** (2), 817–830 (2019).
13. Ananthanarayanan, M. et al. Post-translational Regulation of the Type III Inositol 1,4,5-Trisphosphate Receptor by miRNA-506. *J. Biol. Chem.* **290** (1), 184–196 (2015).
14. Weerachayaphorn, J. et al. Nuclear Factor Erythroid 2-like 2 Regulates Expression of Inositol 1,4,5-trisphosphate Receptor, Type 3 and Calcium Signaling in Cholangiocytes. *Gastroenterology* **149** (1), 211–222 (2015).
15. Banales, J. M. et al. Up-regulation of microRNA 506 leads to decreased Cl-/HCO_3^-anion exchanger 2 expression in biliary epithelium of patients with primary biliary cirrhosis. *Hepatology* **56** (2), 687–697 (2012).
16. Lamas-Paz, A. et al. Alcoholic liver disease: Utility of animal models. *World J. Gastroenteroly*. **24** (45), 5063–5075 (2018).
17. Sueblinvong, V. et al. Nuclear Thioredoxin-1 Overexpression Attenuates Alcohol-Mediated Nrf2 Signaling and Lung Fibrosis. *Alcoholism: Clin. experimental Res.* **40** (9), 1846–1856 (2016).
18. Sueblinvong, V. et al. Chronic Alcohol Ingestion Primes the Lung for Bleomycin-Induced Fibrosis in Mice. *Alcoholism: Clin. experimental Res.* **38** (2), 336–343 (2014).
19. Matos, L., Batista, P., Monteiro, N., Henriques, P. & Carvalho, A. Hepatite alcoólica aguda - Artigo de Revisão. *Jornal Português de Gastrenterologia*. **20** (4), 153–161 (2013).
20. Bruha, R., Dvorak, K. & Petrtyl, J. Alcoholic liver disease. *World J. Hepatololy*. **4** (3), 08–90 (2012).
21. Bang, N. U., Iversen, K., Jagt, T. & Madsen, S. Serum glutamic oxalacetic transaminase activity in acute and chronic alcoholism. *JAMA* **168** (2), 156–160 (1958).
22. Summerskill, W. H. J., Wolfe, S. J. & Davidson, C. S. Response to alcohol in chronic alcoholics with liver disease. *Lancet* **1**, 335–340 (1957).
23. Hed, R. Clinical Studies in Chronic Alcoholism. *J. Intern. Med.* **165** (3), 161–166 (1959).
24. Neame, P. B. & Joubert, S. M. Postalcoholic hypoglycemia and toxic hepatitis. *Lancet* **278** (7208), 893–897 (1961).
25. Collares, M., Valverde, M., Fernández, I. & Ormaechea, G. Colestasis intrahepática: un desafío diagnóstico. *Arch. Intern. Med.* **36** (1), 33–38 (2014).
26. Bertola, A., Mathews, S., Ki, S. H., Wang, H. & Gao, B. Mouse model of chronic and binge ethanol feeding (the NIAAA model). *Nat. Protoc.* **8**, 627–637 (2013).
27. Brandon-Warner, E., Schrum, L. W., Schmidt, C. M. & McKillop, I. H. Rodent models of alcoholic liver disease: of mice and men. *Alcohol* **46** (8), 715–725 (2012).

28. Lieber, C. S., Leo, M. A., Mak, K. M., DeCarli, L. M. & Sato, S. Choline fails to prevent liver fibrosis in ethanol-fed baboons but causes toxicity. *Hepatology* **5** (4), 561–572 (1985).
29. Amini, M. & Runyon, B. A. Alcoholic hepatitis 2010: A clinician's guide to diagnosis and therapy. *World J. Gastroenteroly*. **16** (39), 4905–4912 (2010).
30. Panqueva, R. P. L. Approaches to pathological diagnosis of cholestatic diseases. *Revista Colombianas de Gastroenterología*. **29** (2), 183–192 (2014).
31. Ogino, N. et al. Neutrophils insert elastase into hepatocytes to regulate calcium signaling in alcohol-associated hepatitis. *J. Clin. Invest.* **134** (16), e171691 (2024).
32. Ahmad, S. F. et al. Regulation of TNF-α and NF-kB activation through the JAK/STAT signaling pathway downstream of Histamine 4 receptor in a rat model of LPS-induced joint inflammation. *Immunobiology* **220** (7), 889–898 (2015).
33. Hinz, M., Arslan, S. C. & Scheidereit, C. It takes two to tango: IκBs, the multifunctional partners of NFkB. *Immunol. Rev.* **246** (1), 59–76 (2012).
34. Li, Q. & Verma, I. M. NF-κB regulation in the immune system. *Nature Reviews Immunology*. ; 2(2002): 725–734. (2012).
35. Wang, T., Zhang, X. & Li, J. J. The role of NF-κB in the regulation of cell stress responses. *Int. Immunopharmacol.* **2** (11), 1509–1520 (2002).
36. Dallot, E., Méhats, C., Oger, S. & Leroy Marie-Josèphe; Breuiller Fouché, M. A role for PKC in the LPS-induced translocation NF-κB p65 subunit in cultured myometrial cells. *Biochimie* **87** (6), 513–521 (2002).
37. Jing, N., Yin, L., Sun, J., Cao, Z. & Mao, W. Expression levels of miR-205 and miR-506 in colon cancer tissuesand their relationships with clinicopathological features. *Oncol. Lett.* **16** (4), 4331–4336 (2018).
38. Krawczyk, P. et al. Evaluation of miR-506 and miR-4316 expression in early and non-invasive diagnosis of colorectal câncer. *Int. J. Colorectal Disease*. **32** (7), 1057–1060 (2017).
39. Guerra, M. T. et al. M.F. Expression of the type 3 InsP3 receptor is a final common event in the development of hepatocellular carcinoma. *Gut* **68** (9), 1676–1687 (2019).
40. Marin, J. J. G., Bujanda, B. & Banales, L. MicroRNAs and cholestatic liver diseases. *Curr. Opin. Gastroenteroly*. **30** (3), 303–309 (2014).
41. Toyokuni, S. Reactive oxygen species-induced molecular damage and its application in pathology. *Pathol. Int.* **49** (2), 91–102 (1999).
42. de Groot, H. Reactive oxygen species in tissue injury. *Hepato-gastroenterology* **41** (4), 328–332 (1994).
43. Li, M. et al. Caffeic acid phenethyl ester inhibits liver fibrosis in rats. *World J. Gastroenterol.* **21** (13), 3893–3903 (2015).
44. Cho, H. Y. & Kleeberger, S. R. Association of Nrf2 with airway pathogenesis: lessons learned from genetic mouse models. *Arch. Toxicol.* **89** (11), 1931–1957 (2015).

45. Kang, J. S. et al. Ethanol extract of Prunus mume fruit attenuates hydrogen peroxide-induced oxidative stress and apoptosis involving Nrf2/HO-1 activation in C2C12 myoblasts. *Revista Brasileira de Farmacognosia*. ; 26(2016): 184–190. (2016).
46. Hybertson, B. M., Gao, B., Bose, S. K. & McCord, J. M. Oxidative stress in healthand disease: the therapeutic potential of Nrf2 activation. *Mol. Aspects Med.* **32** (4–6), 234–246 (2011).
47. Niture, S. K., Khatri, R. & Jaiswal, A. K. Regulation of Nrf2-an update. *Free Radic. Biol. Med.* **66**, 36–44 (2014).
48. Liu, J., Wu, K. C., Lu, Y. F., Ekuase, E. & Klaassen, C. D. Nrf2 protection against liver injury produced by various hepatotoxicants. *Oxidative medicine and cellular longevity*. ; 2013: 01–08. (2013).
49. Lamle, J. et al. Nuclear factor-eythroid 2-related factor 2 prevents alcohol-induced fulminant liver injury. *Gastroenterology* **134** (4), 1159–1168 (2008).
50. Wu, K. C., Liu, J. & Klaassen, C. D. Role of Nrf2 in preventing ethanol-induced oxidative stress and lipid accumulation. *Toxicol. Appl. Pharmcol.* **262** (3), 321–329 (2012).
51. Ananth, R. & Neonatal Cholestasis A Primer of Selected Etiologies. *Pediatr. Ann.* **47** (11), 433–439 (2018).
52. Blokker, B. A. et al. Fine-Tuning of Sirtuin 1 Expression Is Essential to Protect the Liver From Cholestatic Liver Disease. *Hepatology* ; (2019). 999 – 716.
53. Santos, K. N. et al. Polymorphism in the Promoter Region of NFE2L2 Gene Is a Genetic Marker of Susceptibility to Cirrhosis Associated with Alcohol Abuse. *Int. J. Mol. Sci.* **20** (14), 3589 (2019).

Figures



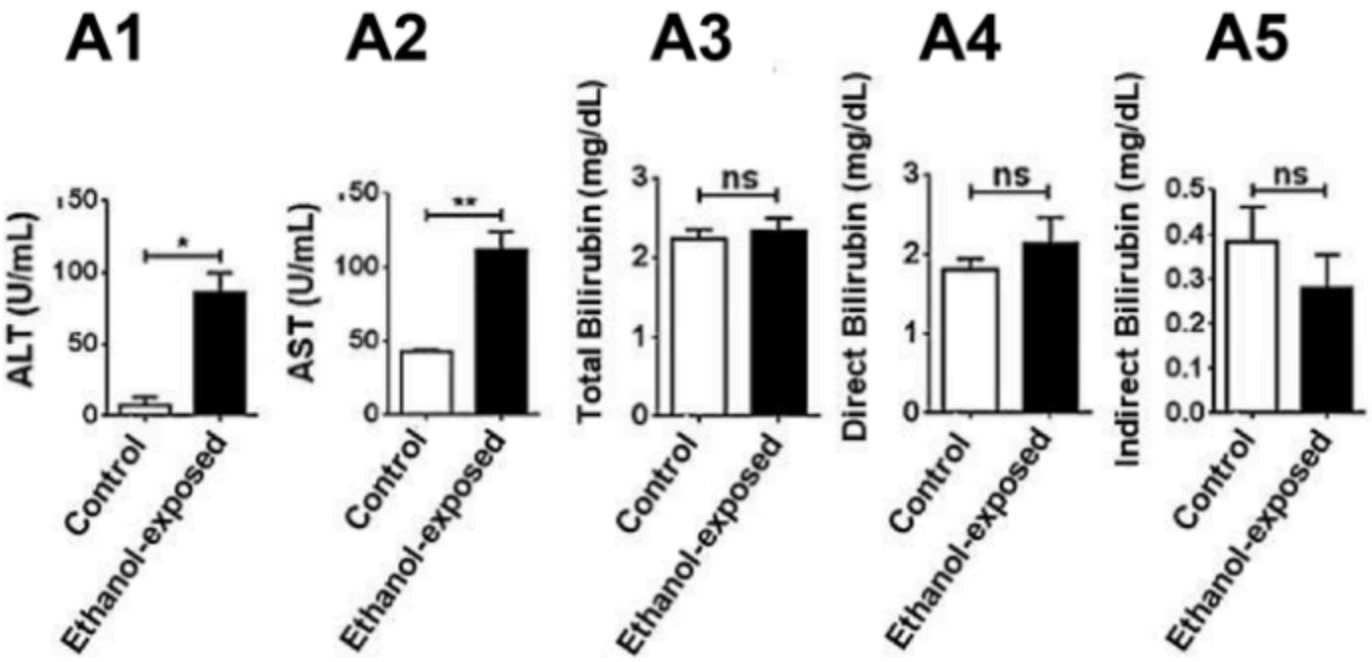
C Mean ($\bar{x}$) biochemical levels

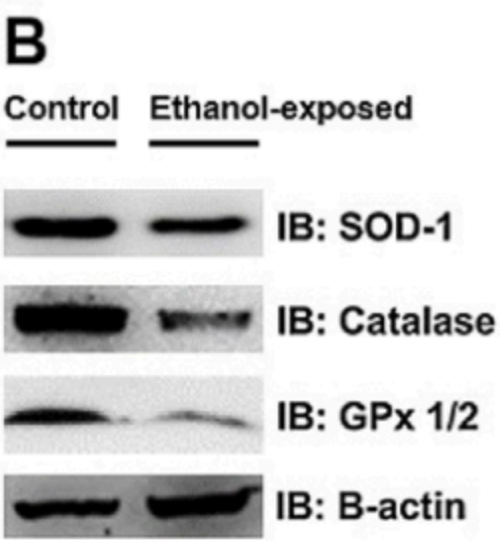
	Donors (n = 21)	ALD (n = 49)
ALT	20.5	41.6
AST	39.7	62.8
GGT	64.5	125.9
ALP	110.2	173.5
Total bilirubin	0.57	3.37
Direct bilirubin	0.25	1.42
Indirect bilirubin	0.32	1.98

Figure 1

Classification of hepatic inflammation and biochemical alterations in patients with ALD-related cirrhosis. (A) Histopathological analysis of 49 human liver tissue samples from patients with ALD identified 14 cases of cirrhosis with mild inflammatory activity (A1), 5 cases with moderate inflammatory activity (A2), 2 cases of cirrhosis and fibrosis with severe inflammatory activity (A3), and 28 cases without histologically detectable inflammatory activity (A0), according to the METAVIR scoring system.

Analysis of clinically relevant biochemical laboratory parameters in ALD demonstrated significantly elevated levels ($p < 0.05$) of ALT (B1), AST (B2), total bilirubin (B5), and indirect bilirubin (B7) in patients with ALD compared with liver donors, with mean values of 41.6 U/L for ALT, 62.8 U/L for AST, 3.37 mg/dL for total bilirubin, and 1.98 mg/dL for indirect bilirubin in ALD patients, whereas liver donors presented mean values of 20.5 U/L for ALT, 39.7 U/L for AST, 0.57 mg/dL for total bilirubin, and 0.32 mg/dL for indirect bilirubin (C). No statistically significant differences ($p > 0.05$) were observed for alkaline phosphatase (ALP) (B4), gamma-glutamyl transferase (GGT) (B3), and direct bilirubin (B6).





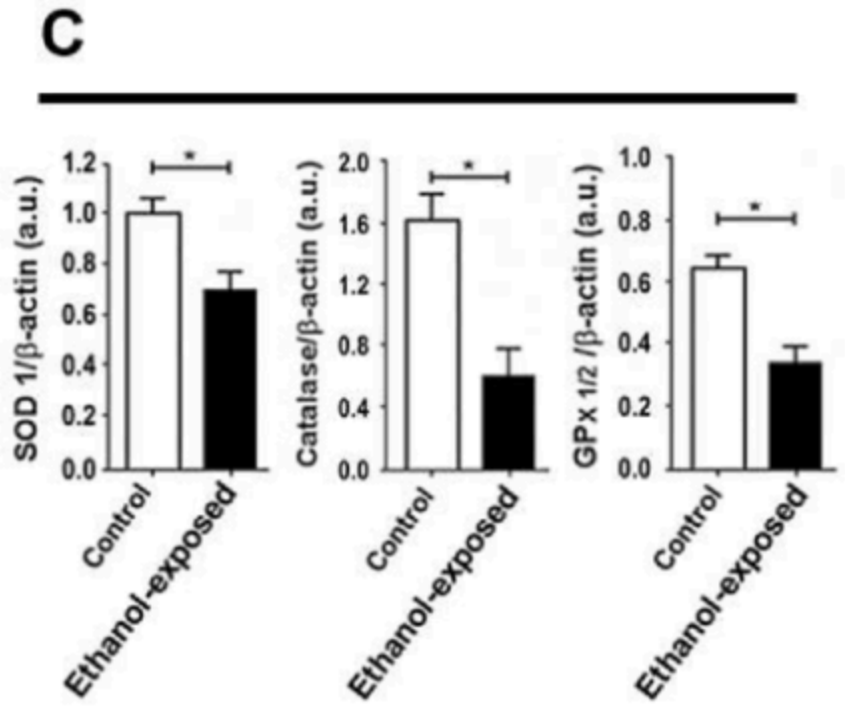


Figure 2

Alterations in biochemical markers expressed during alcohol-induced oxidative stress in mice treated with 5% ethanol. Biochemical laboratory analyses performed in 21 mice exposed to 5% ethanol and 21 control animals demonstrated significant differences ($p < 0.05$) in transaminase levels (ALT and AST) in ethanol-exposed mice compared with the control group (A1, A2). No significant differences ($p > 0.05$) were observed in total bilirubin levels and their fractions between the groups (A3–A5). Western blot analysis (B) demonstrated a significant reduction ($p < 0.05$) in the expression of SOD-1, GPx, and catalase (C) in the 21 ethanol-exposed mice compared with the control group ($n = 21$), which are important oxidative stress markers regulated by the transcription factor Nrf2. The Western blot images presented in panel B were cropped from the original full-length blots, which are provided in Supplementary Information 1.

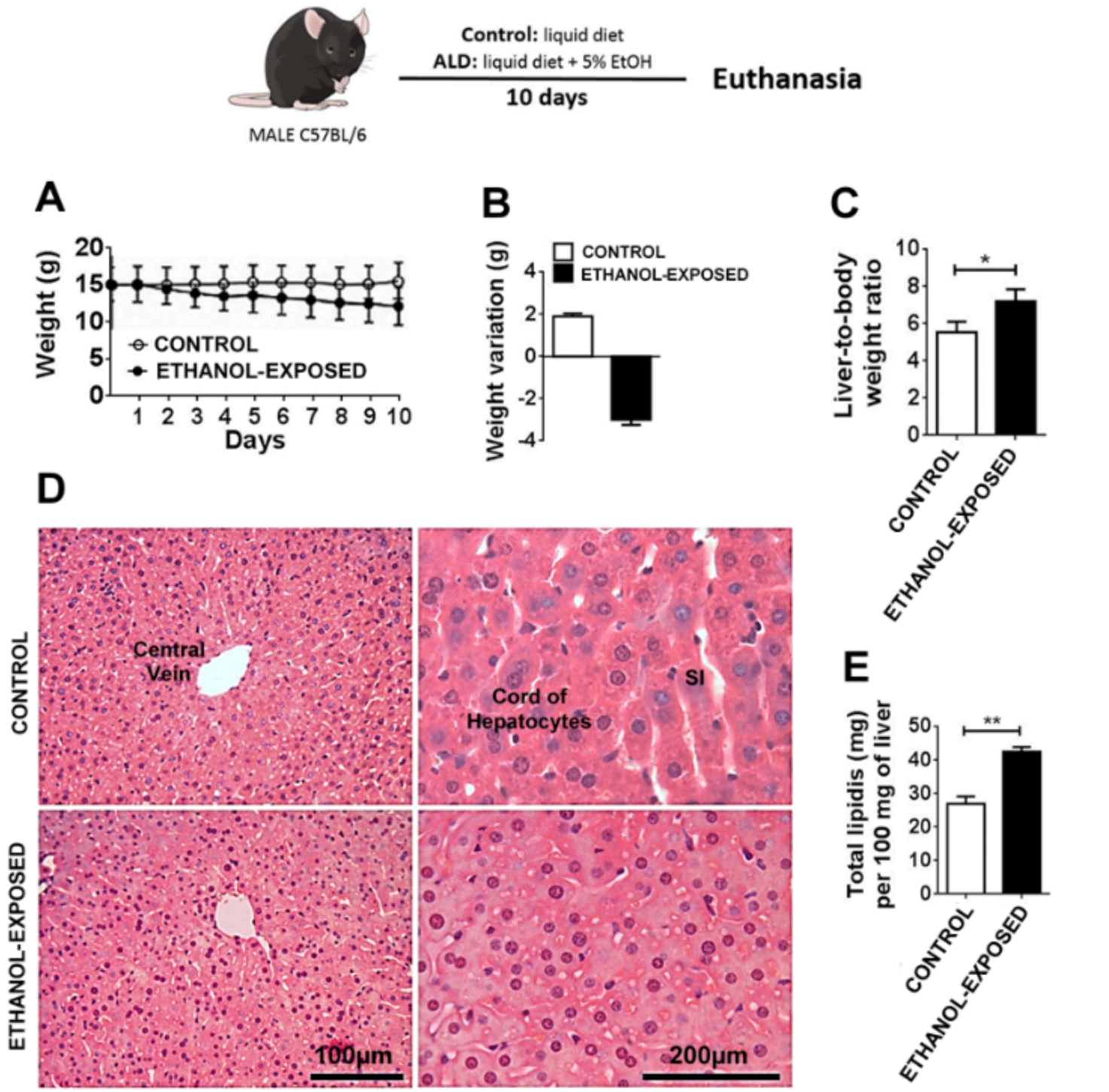


Figure 3

Evaluation of alcohol induction in mice treated with 5% alcohol. A gradual reduction in mean body weight was observed in the 21 ethanol-exposed animals compared with the control group (n = 21) over 10 days of exposure to 5% ethanol (A, B). A significant increase ($p < 0.05$) in liver size/weight was observed in ethanol-exposed animals compared with the control group (C). Histological analysis of hematoxylin and eosin (H&E)-stained liver sections from C57BL/6 mice demonstrated histopathological features characteristic of hepatic steatosis, without inflammatory infiltrate (D), as well as significant hepatic lipid accumulation in the ethanol-exposed group compared with the control group (E).

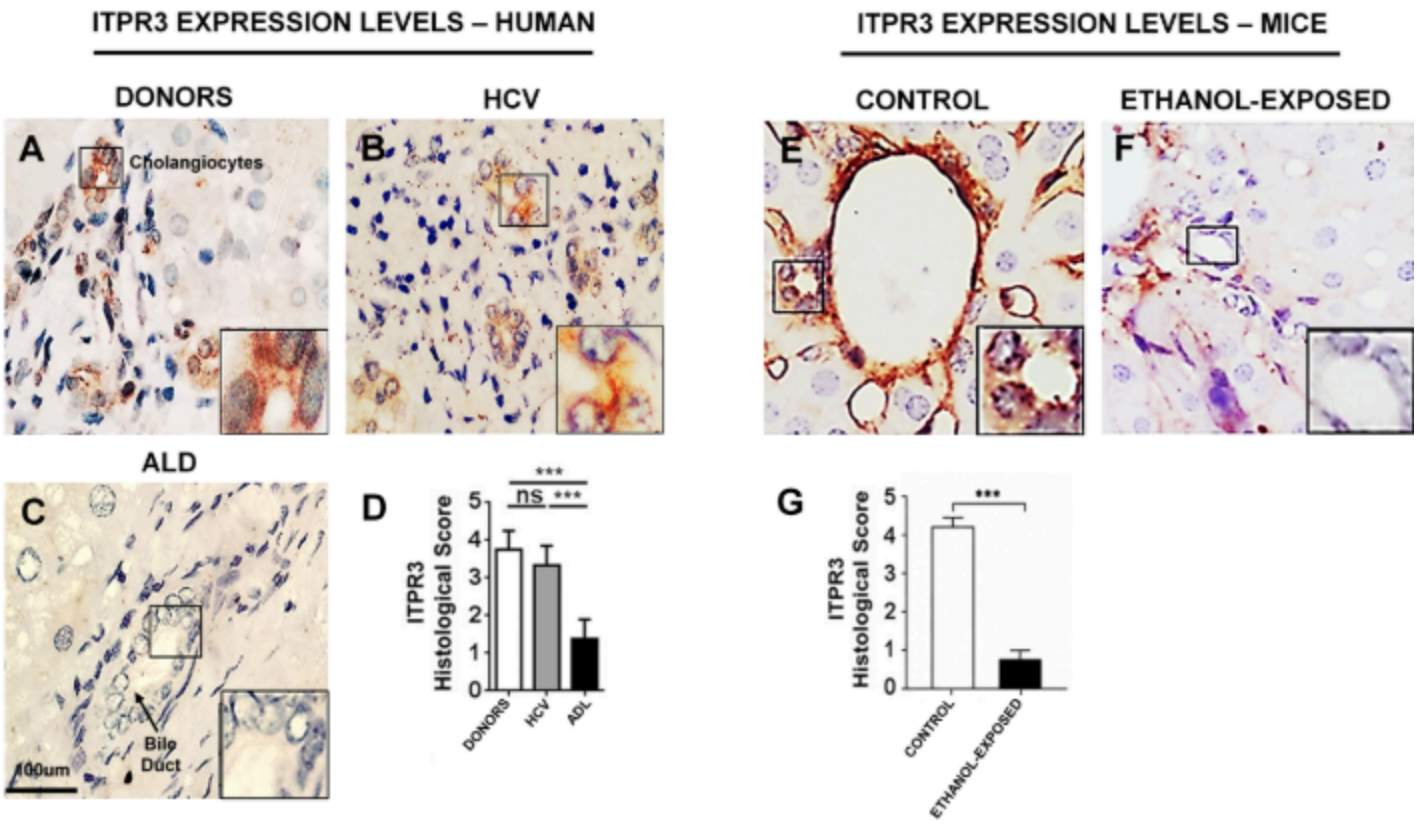


Figure 4

Decreased ITPR3 expression in cholangiocytes in ALD and in ethanol-exposed mice. Immunohistochemical analysis of human liver biopsy samples revealed reduced ITPR3 expression in cholangiocytes among the 49 patients with ALD-related cirrhosis (A) compared with 18 HCV-infected patients (B) and 21 liver donors (C), with significantly lower expression levels ($p < 0.05$) in ALD patients relative to the other groups, as demonstrated by the histopathological scoring graph (D). Histological analysis of liver sections from C57BL/6 mice also demonstrated decreased ITPR3 immunostaining in cholangiocytes among the 21 animals after 10 days of exposure to 5% ethanol compared with the 21 animals in the control group (E, F), with significantly lower expression levels ($p < 0.05$) observed between the groups (G).

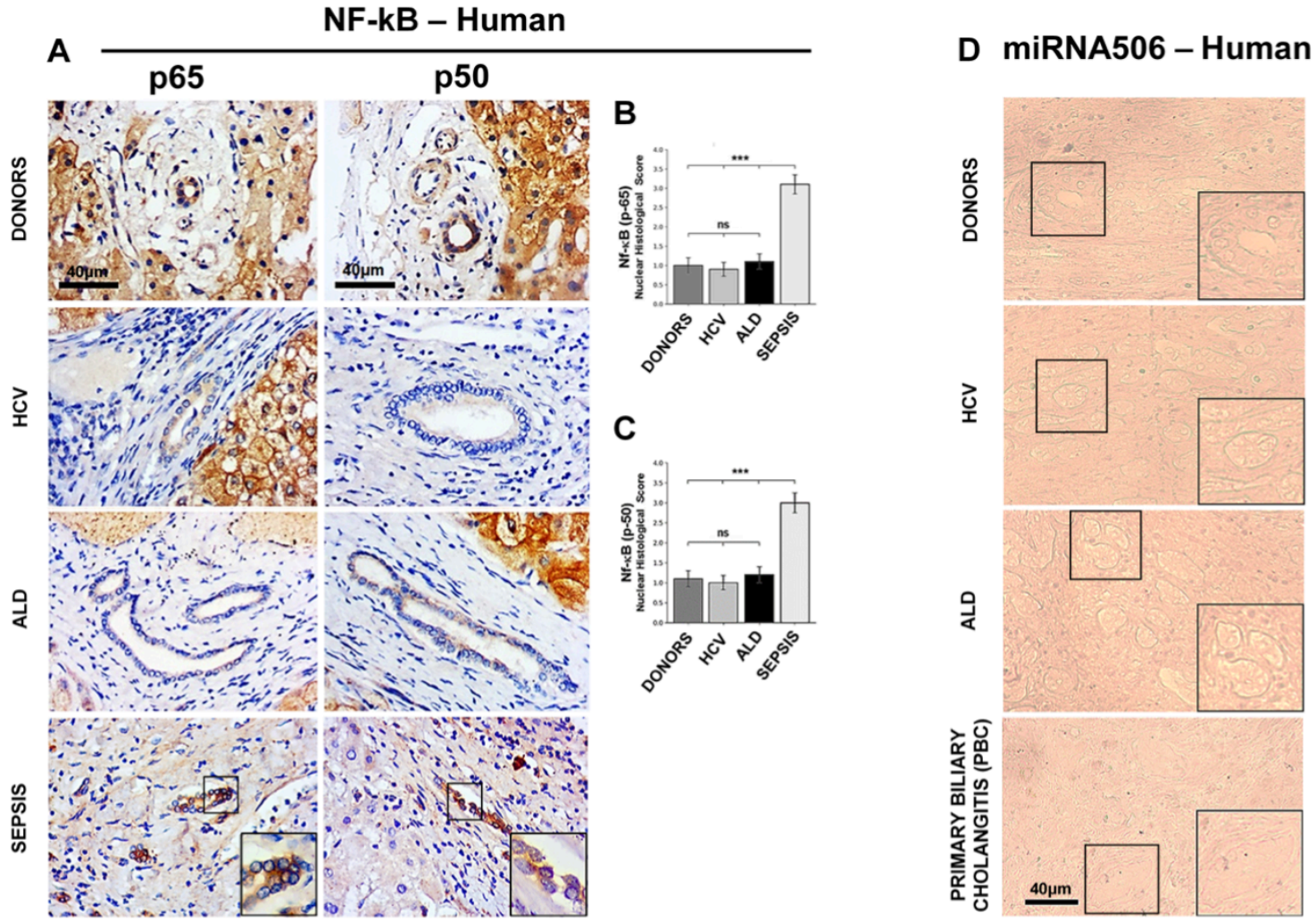


Figure 5

Classical transcription factors are not involved in the negative regulation of ITPR3 expression in cholangiocytes in ALD. Immunohistochemical analysis of human liver tissue demonstrated the absence of nuclear translocation of the transcription factors NF-κB p65 (RelA) and NF-κB1 (p50) among the study groups (A – C), suggesting that NF-κB is not the transcription factor involved in the negative regulation of ITPR3 expression in cholangiocytes in ALD. Cases of sepsis were used as positive controls for NF-κB (p65 and p50) nuclear translocation. In situ hybridization was performed on liver tissue sections from the 49 ALD cases and compared with the HCV-infected group (n = 18) and liver donors (n = 21). No differences in microRNA-506 staining were observed among the evaluated cases (D), further suggesting that microRNA-506 is not associated with the negative regulation of ITPR3 expression in cholangiocytes during ALD. Cases of primary biliary cholangitis (PBC) were used as the positive control group.

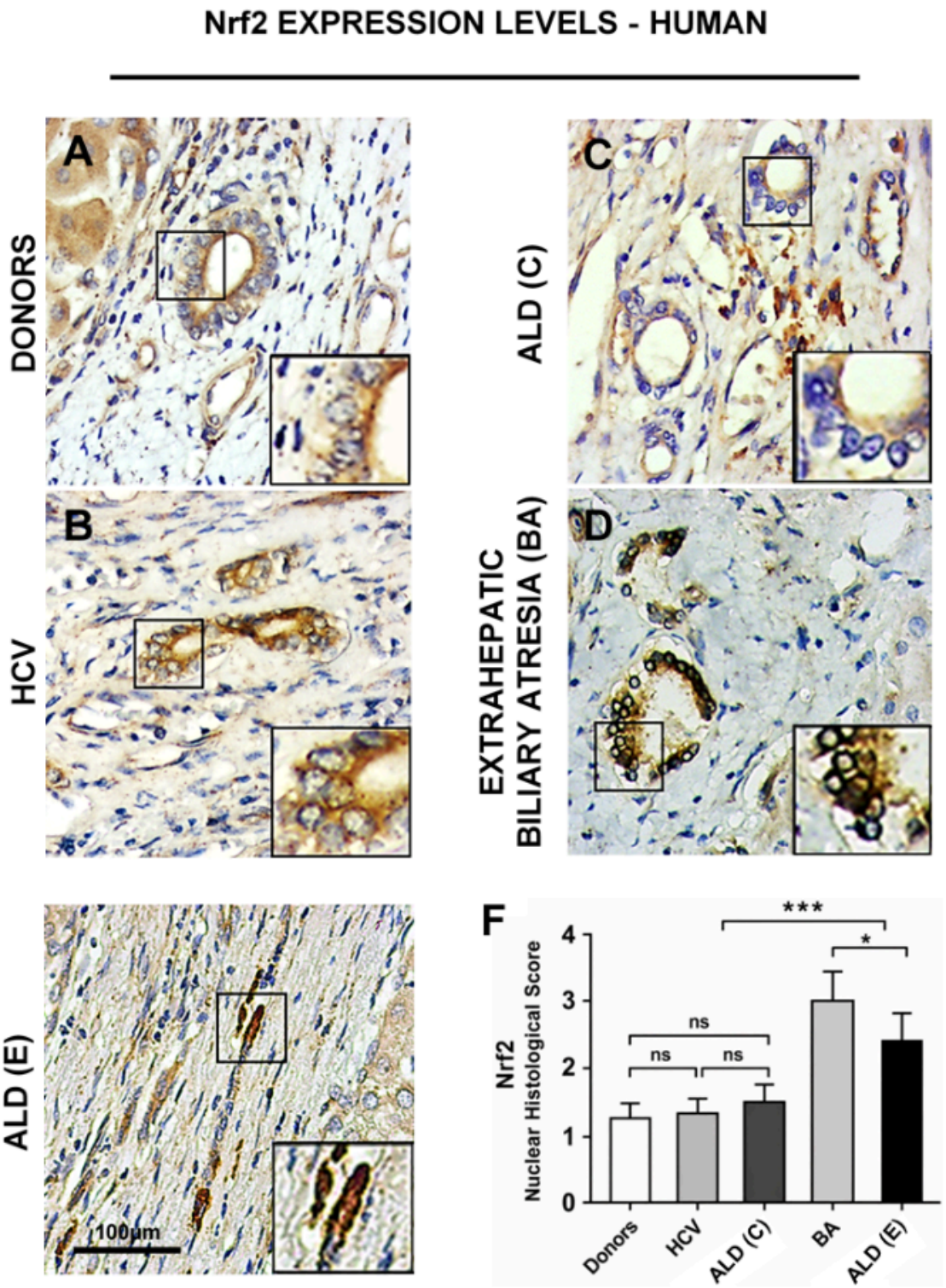


Figure 6

Nuclear translocation of the transcription factor Nrf2 in cholangiocytes in ALD. Immunohistochemical analysis of histological sections from human liver biopsies obtained from patients with cirrhotic-stage ALD (n = 49) demonstrated the absence of nuclear translocation of the transcription factor Nrf2 in cholangiocytes in 46 cases (C), with no significant differences compared with the 21 liver donors (A) and the 18 HCV-infected patients (B). However, nuclear translocation of Nrf2 in cholangiocytes was observed

in 3 of the 49 liver tissue samples from patients with ALD (E), similar to cases of extrahepatic biliary atresia used as positive controls (F), demonstrating a significant association between the groups (F).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryInformation1.docx