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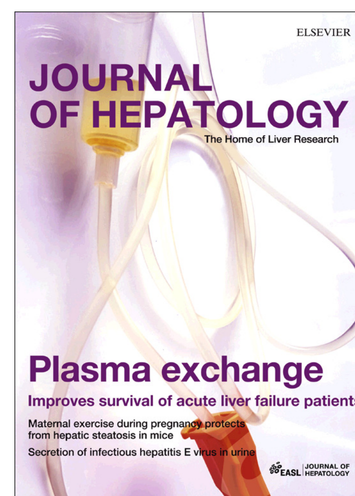

PARP inhibition protects against alcoholic and nonalcoholic steatohepatitis

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PARP inhibition protects against alcoholic and nonalcoholic steatohepatitis

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Abstract

Background & Aims: Mitochondrial dysfunction, oxidative stress, inflammation, and metabolic reprograming are crucial contributors of hepatic injury and subsequent development of liver fibrosis. Poly(ADP-ribose) polymerases (PARP) and their interplay with sirtuins have an important role in the regulation of intermediary metabolism, however, if PARP inhibition is capable to affect alcoholic and nonalcoholic steatohepatitis (ASH/NASH) has not been much studied.

Methods: We investigated the effects of genetic deletion of PARP1 and pharmacological inhibition of PARP with structurally different inhibitors in models of early alcoholic steatohepatitis, as well as on Kupffer cell activation *in vitro* by using biochemical assays, real-time PCR, and histology analyses. The effects of PARP inhibition were also evaluated in high fat or methionine and choline deficient (MCD) diet-induced steatohepatitis models in mice.

Results: PARP activity was increased in livers due to excessive alcohol intake which was associated with decreased NAD^+ content and SIRT1 activity. Pharmacological inhibition of PARP restored the hepatic NAD^+ content, attenuated the decrease in SIRT1 activation and beneficially affected the metabolic-, inflammatory-, and oxidative stress-related alterations due to alcohol feeding in the liver. $PARP1^{-/-}$ animals were protected against alcoholic steatohepatitis and pharmacological inhibition of PARP or genetic deletion of PARP1 also attenuated Kupffer cell activation *in vitro*. Furthermore, PARP inhibition decreased hepatic triglyceride accumulation, metabolic dysregulation, or inflammation and/or fibrosis in models of NASH.

Conclusion: Our results suggests that PARP inhibition is a promising therapeutic strategy in steatohepatitis with high translational potential in light of the availability of PARP inhibitors for clinical treatment of cancer.

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Abbreviations:

ACC1/2: acetyl-CoA carboxylase-1 and -2; AIQ: 5-aminoisoquinoline hydrochloride; ASH/NASH: alcoholic and nonalcoholic steatohepatitis; GK: glucokinase; G6Pase: glucose-6-phosphatase; gp91phox, and p47phox: NADPH oxidase isoforms; HE: hematoxylin and eosin; 4-HNE: 4-hydroxynonenal; iNOS: inducible nitric oxide synthase; MCD: methionine and choline deficient; MIP1-α/2 macrophage inflammatory protein 1 alpha/2; M30: early marker of apoptosis (detects caspase-cleaved cytokeratin 18); MPO: myeloperoxidase; NAD^+: nicotinamide adenine dinucleotide; NAFLD: non-alcoholic fatty liver disease; 3-NT: 3-Nitrotyrosine; PARP**:** poly(ADP-ribose) polymerase; PJ34: *N*-(5,6-Dihydro-6-oxo-2-phenanthridinyl)-2-(dimethylamino)acetamide hydrochloride; ROS/RNS: reactive oxygen/nitrogen species; SIRT1: NAD-dependent deacetylase sirtuin-1; SREBP-1: sterol regulatory element-binding protein 1; TNF-α: tumor necrosis factor alpha.

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Introduction

Chronic alcoholism is a leading cause of liver disease worldwide. The development of alcoholic steatohepatitis involves alcohol and acetaldehyde induced direct hepatocyte injury and death by increasing reactive oxygen and nitrogen species (ROS/RNS) production, lipid peroxidation and oxidative DNA injury, coupled with overactivation of hepatic inflammatory processes (both innate and adaptive immunity) and reprograming of lipid metabolism by promoting hepatic lipid accumulation[1-4]. Non-alcoholic fatty liver disease (NAFLD) may also progress to steatohepatitis (non-alcoholic steatohepatitis; NASH). Oxidative stress, inflammation and hepatocyte injury are also often characteristic features of NASH. More chronic and sustained liver injury, inflammation, and fat accumulation promote scarring, and in susceptible subjects eventually development of cirrhosis.

Poly(ADP-ribose) polymerases are key enzymes involved in DNA repair processes. PARP1 is the most abundant nuclear enzyme and the predominant isoform of the PARP enzyme family. Upon DNA damage (for example induced by ROS/RNS or ionizing radiation), PARP1 activity increases and the ADP-ribose part of NAD^+ is transferred to proteins, giving rise to formation of poly(ADP-ribose) polymers[5]. This posttranslational modification alters the function of many enzymes and structural proteins and may initiate caspase-independent cell death[5]. PARP1 is also a co-activator of key pro-inflammatory transcription factors[6], including NFκB[7]. In the recent years, several additional PARP functions have been discovered, involving the regulation of oxidative stress, mitochondrial function and intermediary metabolism[6, 8]. In experimental models of tissue injury and inflammation PARP inhibition exerts marked protective effects in cells/tissues[5, 9]. In contrast, in cancers with selective defects in homologous recombination repair (cancer cells frequently harbor defects in DNA repair pathways leading to genomic instability), inactivation of PARPs directly causes cell demise[10]. This principle resulted in the development and FDA approval of PARP-inhibitor Olaparib (AZD-2281; Lynparza) for the treatment of BRCA-1 and 2 (tumor suppressor genes) negative ovarian cancer.

Recently, we described PARP activation in livers of subjects with advanced cirrhosis induced by hepatitis B or excessive alcohol consumption, and demonstrated anti-inflammatory and anti-fibrotic effects of PARP inhibition using experimental models of liver fibrosis, as well as utilizing isolated hepatic stellate cells[11].

In this study, we investigated the effects of pharmacological inhibition of PARP with structurally different inhibitors or genetic deletion of PARP1 on hepatocellular injury, mitochondrial function,

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inflammation, metabolic reprograming and lipid accumulation in the liver, utilizing *in vivo* models of early alcoholic steatohepatitis, as well as isolated Kupffer cells stimulated with bacterial lipopolysaccharide *in vitro*. To enhance the translational potential of our study, the effects of PARP inhibition on hepatic lipid accumulation, metabolic reprograming and/or inflammation were also evaluated on mouse models of high fat (HF) or methionine and choline deficient (MCD) diet-induced NAFLD/NASH.

Materials and methods

Detailed description of methods can be found in ***Supplemental Experimental Procedures.***

Human Liver Clinical Samples

Normal human liver samples, and alcoholic fatty livers were obtained from the Liver Tissue Procurement and Distribution System (LTPDS), University of Minnesota. Alcoholic fatty liver samples were collected from donor livers that were not used for transplantation due to excessive steatosis from the Medical College of Virginia through the LTPDS. Liver histology showed significant steatosis and mild inflammation without significant fibrosis. Alcoholic fatty liver and alcoholic cirrhosis (the latter was not used in this study) were diagnosed based on the history of alcohol drinking and liver histology. Normal healthy liver samples were also provided by the LTPDS, and were collected from the part of donor livers that was not used for transplantation. All of samples were collected by snap-freezing in liquid nitrogen (see also supplemental methods).

Animal studies

All the animal protocols conformed to the National Institutes of Health (NIH) guidelines and were approved by the Institutional Animal Care and use Committee of the National Institute on Alcohol Abuse and Alcoholism (Bethesda, MD). C57BL/6 male mice with body weighing more than 20 g were used for ad libitum ethanol feeding, as described in the NIAAA alcohol model[12]. PARP Inhibitors AIQ or PJ34 (10 mg/kg dose) or vehicle were administered once a day intraperitoneally for 10 days. Mice were sacrificed, and then immediately serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were immediately determined. Liver injury was assessed on 5 µm hematoxylin and eosin (HE) FFPE sections, while hepatic lipid content was quantified on fresh frozen sections using Oil Red-O staining. The effect of PARP inhibition (with AIQ, PJ34 or Olaparib) was also studied in mouse models of diet-induced obesity (high fat diet), high fat diet plus a single alcohol binge, and MCD diet-induced NAFLD/NASH, which are described in supplements.

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Biochemical and molecular biology assays

Hepatic triglyceride levels were measured by the Triglyceride Quantification Colorimetric Kit (Biovision, San Francisco,CA). PARP activities in hepatic tissue homogenates were assessed using a colorimetric assay kit (Trevigen Inc.,Gaithersburg,MD). Hepatic total RNA was isolated, reverse-transcribed and the target genes were amplified using the real-time PCR kit from Applied Biosystems (Foster City,CA); for primers see Supplemental Table 1. NAD^+ from liver tissue lysates was determined by colorimetric assay using EnzyChromTM NAD^+/NADH Assay Kit (BioAssay Systems,Hayward,CA). SIRT1 activity was measured by SIRT1 Fluorescent Activity Assay Kit (ENZO Life sciences Farmingdale,NY). The activity of mitochondrial complex I, II, and IV were determined using kits from Abcam. Mitochondrial DNA (mtDNA) determination was performed as previously described[13]. To characterize oxidative and nitrosative stress 4-Hydroxynonenal(4-HNE) and 3-Nitrotyrosine(3-NT) levels were determined using kits from (Cell Biolabs, San Diego, CA, USA and Hycult biotechnology, Cell sciences, Canton, MA, USA, respectively). The levels of hepatic hydroxyproline content from liver lysates were determined by the kit obtained from (BioVision, Mountain View, CA) as described earlier[11]. For detailed descriptions, and also antibodies used for Western blot please see supplements.

Isolation of Kupffer cells

Kupffer cells were isolated by *in situ* collagenase perfusion and differential centrifugation on OptiPrep (Sigma) density gradient. Purity of the cultures was assessed by flowcytometric analysis (anti-F4/80 antibody). More than 90% of cells were Kupffer cells with F4/80 positive staining. Treatment of Kupffer cells with LPS (1 µg/ml) and PARP inhibitors were carried out at the concentrations indicated on Figure 2. for 4 hours; TNF-α, MIP1-α secretion were determined by ELISA (R&D Systems, Minneapolis, MN, USA).

Histology and immunohistochemistry

Hematoxylin and eosin (HE), Oil Red O, M30, myeloperoxidase (MPO; neutrophil marker), Ly6G (marker of monocytes, granulocytes and neutrophils), and Picro Sirius Red staining were processed as described previously[11]; see also supplemental materials.

Statistical Analysis

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All the values are represented as mean ± SEM. Statistical analysis of the data was performed by analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons. The analysis was conducted using GraphPad-Prism4 software. $P < 0.05$ was considered statistically significant.

Results

Increased hepatic PARP and decreased SIRT1 activity and NAD^+ content in steatohepatitis. Pharmacological inhibition of PARP restores attenuated NAD^+ content and SIRT1 activity.

Using a mouse model of chronic and binge ethanol feeding (model of early steatohepatitis)[12, 14], alcohol-fed mice showed significantly increased hepatic PARP activation (Fig 1A) that was associated with depleted NAD^+ levels and decreased SIRT1 activity (Fig 1A,C) and mRNA expression (Fig 1B). Treatment of ethanol-fed mice with PARP inhibitors AIQ and PJ34 significantly decreased alcohol feeding-induced PARP activation, and prevented both the alcohol-induced drop in hepatic NAD^+ content, and the subsequent decrease in SIRT1 activity/expression (Fig 1A-C). There was also increased PARP activity coupled with attenuated SIRT1 activity/expression and hepatic NAD^+ content in liver specimens with alcoholic steatohepatitis from donor livers that were not used for transplantation (Fig S1).

Pharmacologic and genetic inhibition of PARP attenuates hepatocellular injury

The alcohol-induced histopathological injury, steatosis and increase in liver enzymes (indicating hepatocyte injury) were significantly attenuated by either pharmacological or genetic PARP1 inhibition (Fig 1D,E). Various markers of hepatocyte apoptosis (M30, DNA fragmentation, caspase 3/7 activity) and necrosis (transaminase release) were also increased in ethanol-fed mice (Fig 1E-F; Fig S2), and were attenuated by either pharmacological PARP inhibition or genetic PARP1 ablation (Fig 1E-F; Fig S2). Notably, this model is not associated with increased hepatic fibrosis (Fig S3).

PARP inhibition or genetic deletion attenuates alcohol-induced hepatic inflammation *in vivo* and Kupffer cell activation *in vitro*

Ethanol exposure induces activation of inflammatory cells, including Kupffer cells, that further aggravates hepatocellular injury. Indeed, in livers of alcohol exposed animals we found markedly increased expression of the pro-inflammatory cytokines/chemokines TNFα, IFNγ, MIP1α, MIP2, CCR2, IL1β (Fig 2A) and the adhesion molecule ICAM-1 (Fig 2B), as well as

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increased MPO activity, indicative of secondary neutrophil infiltration (Fig 2B) in livers of alcohol-exposed animals. Up-regulation of these pro-inflammatory mediators and neutrophil infiltration were significantly attenuated in ethanol-fed mice by PARP inhibitor treatment or in PARP1$^{-/-}$ mice (Fig 2A,B).

Because Kupffer cell activation (most likely by endotoxin translocated through the damaged gut mucosa or damage-associated molecular patterns released from necrotic hepatocytes) is a key event in alcohol-induced liver injury, we also studied the effect of PARP inhibition on activation of these cells *in vitro*. In isolated Kupffer cells, the LPS-induced increase of pro-inflammatory cytokines TNFα, and MIP1α was concentration-dependently reversed by pharmacological inhibition of PARP or deletion of PARP1 (Fig 2C), showing the crucial role of PARP activation in the initiation of events in steatohepatitis.

PARP inhibition or genetic deletion of PARP1 attenuates alcohol-induced hepatic oxidative/nitrative stress and mitochondrial dysfunction

PARP inhibition also exerted beneficial effect on increased oxidative stress induced by alcohol feeding. The alcohol-induced enhanced hepatic expression of reactive oxygen species generating NADPH oxidase isoforms gp91phox, and p47phox, as well as inducible nitric oxide synthase (iNOS) were largely decreased by both pharmacological PARP inhibitors and in PARP1$^{-/-}$ mice (Fig 3A). Accordingly, hepatic levels of 4-hydroxy-nonenal(HNE), and 3-nitrotyrosine(NT) (markers of lipid peroxidation, oxidative and nitrative stress) decreased by both PARP inhibitor treatment and in the PARP1$^{-/-}$ mice (Fig 3B) upon alcohol feeding, indicating a significant decrease in lipid peroxidation and protein nitration.

Since mitochondrial dysfunction and reactive oxygen species generation are implicated in alcohol-induced liver injury we also tested whether PARP inhibition affects markers of mitochondrial injury, i.e. mitochondrial DNA content and enzyme activities of the electron transport chain. We found that hepatic mitochondrial DNA content (Fig 3C) and activities of complex I and IV, but not II (Fig 3D), were significantly decreased by alcohol feeding. These changes were ameliorated by either pharmacological PARP inhibition or deletion of PARP1 (Fig 3C,D).

PARP inhibition attenuates alcohol-induced steatosis and metabolic disturbances

Liver histology and Oil Red O staining showed obvious hepatic steatosis in ethanol-fed mice (Fig 4A), which were attenuated by PARP inhibition or genetic deletion of PARP1 (Fig 4A). Reprogramming of hepatic lipid metabolism due to ethanol exposure, contributes to the

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development of steatosis in the liver. We found a robust induction of sterol regulatory element-binding protein 1 (SREBP-1), after alcohol feeding, indicating reprogramming of several SREBP-1 target genes involving glucose metabolism and fatty acid and lipid production (Fig 4B). The alcohol-induced up-regulation of SREBP-1 was markedly attenuated by both pharmacological and genetic PARP1 inhibition (Fig 4B). Accordingly, the expression of acetyl-CoA carboxylase-1 and -2 (ACC1 and ACC2), and glucokinase (GK) and glucose-6-phosphatase (G6Pase) were markedly up-regulated, while PARP inhibition or genetic deletion of PARP1 significantly attenuated the alcohol-induced expression alterations (Fig 4B,C). Consistently with Oil Red O staining (Fig 4A), PARP inhibition or deletion of PARP1 attenuated the alcohol-feeding induced increased hepatic triglyceride content (Fig 4D). These results indicated a key role of PARP in the regulation of hepatic intermediary metabolism during alcoholic liver injury.

PARP inhibition attenuates high fat diet-induced liver steatosis and fatty acid metabolism dysregulation

To further enhance the translational potential of our observations we also studied the effects of PARP inhibition on hepatic lipid accumulation, metabolic reprograming and/or inflammation/fibrosis, using mouse models of NAFLD/NASH induced by high fat or methionine and choline deficient (MCD) diet, as well as a model of high fat diet and single alcohol binge-induced liver injury.

PARP inhibition for 8 days with 3 structurally distinct inhibitors in mice on high fat diet for 11 weeks attenuated liver steatosis indicated by HE (Fig 5A) and Oil Red O (Fig 5B) staining of liver sections, as well as liver triglyceride content measurements (Fig 5D) in mice following 11 weeks of HF diet. Consistently with these changes, PARP inhibition also beneficially affected dysregulated fatty acid metabolism in livers of high fat-fed animals (Fig 5C). PARP inhibition had no effect on food intake of animals on high fat diet (Fig S4A), but significantly decreased body weight by the end of the observation period compared to vehicle (Fig S4B). To further study these interesting observations we selected the clinically approved PARP inhibitor for evaluation by indirect calorimetry (Fig S4C-F; Fig S5). These results indicated that Olaparib treatment in mice on high fat diet increased O_2 consumption, CO_2 production and total energy expenditure (Fig S4C-E) without affecting ambulatory activity (Fig S4F). This was also associated with significantly increased fat oxidation (Fig S5A; particularly during light periods), and trend in increase in carbohydrate oxidation (Fig S5B), which did not reach statistical significance.

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PARP inhibition attenuates high fat diet and a single alcohol binge induced hepatocellular injury and inflammation

Because nonalcoholic liver steatosis may also increase the sensitivity to acute alcohol-induced liver injury, we also evaluated the effects of PARP inhibition in a model of steatohepatitis induced by high fat diet (3 months) combined with a single alcohol binge[15]. Consistently with previous observations[15] we found marked increase of liver ALT (Fig 6A), neutrophil infiltration (Fig 6B-D) and inflammation (Fig 6D) in the liver, which were attenuated by 3 PARP inhibitors (Fig 6A-D).

PARP inhibition with Olaparib attenuates MCD diet-induced steatohepatitis

We also investigated the effect of PARP inhibition in a model of NASH. MCD diet in mice (described in supplements) was associated with hepatocellular injury (Fig 7A,B), enhanced liver steatosis (Fig 7A,C,D) and dysregulation of fatty acid metabolism (Fig 7E). It also induced marked neutrophil infiltration and inflammation (Fig 8A,B), and promoted pro-fibrotic changes (Fig 8C,D). Chronic Olaparib treatment for 5 weeks markedly attenuated the MCD diet-induced liver injury (Fig 7A,B), dysregulated fatty acid metabolism and steatosis (Fig 7C-E). Olaparib treatment also markedly decreased liver inflammation (Fig 8A,B) and fibrosis (Fig 8C,D).

Discussions

The key findings of our study are demonstration that: a) hepatic PARP activity is increased, coupled with decreased NAD^+ content and SIRT1 activity/expression, in a mouse model of early steatohepatitis induced by chronic and binge ethanol feeding, and likewise in human liver specimens with alcoholic steatohepatitis; b) pharmacological inhibition of PARP restores the hepatic NAD^+ content, attenuates the decrease in SIRT1 activation/expression in mice exposed to ethanol feeding; c) pharmacological inhibition of PARP and genetic deletion of PARP1 protects against ethanol-induced hepatocellular injury by reducing cell death (apoptosis and necrosis) and d) markedly attenuates alcohol-induced liver inflammation *in vivo* (both acute, Kupffer cell driven and delayed, neutrophil-mediated), and endotoxin-induced Kupffer cell activation *in vitro*; as well as e) alcohol feeding-induced hepatic oxidative/nitrative stress, mitochondrial dysfunction, and f) steatosis accompanied by pathological metabolic reprograming; g) PARP inhibition also decreases hepatic steatosis, metabolic dysregulation, and/or inflammation and/or fibrosis in mouse models of NAFLD/NASH induced by HF or MCD diet, as well as in a model of HF diet and single alcohol binge-induced liver injury. These results point towards an important pathological role of PARP in development of alcoholic and

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nonalcoholic steatohepatitis/liver disease not only by controlling oxidative/nitrative stress-induced hepatocellular injury/cell demise and consequent or preceding inflammatory processes, but also by facilitating pathological metabolic reprograming leading to steatosis/steatohepatitis.

Consistently with the important role of PARP activation in liver disease, increased PARP1 activity and PAR accumulation were reported in hepatic tissues from patients with various forms of advanced liver cirrhosis (e.g. induced by hepatitis B or by chronic excessive alcohol consumption) and hepatocellular carcinoma[11, 16, 17]. Increased ADP-ribosylation of proteins was reported after chronic alcohol consumption in livers of rats[18] and in rat/mouse hepatocytes exposed to ethanol or acetaldehyde *in vitro* or ex vivo[19, 20], and most recently in livers from mouse models of NAFLD and human biopsies from patients with various forms of steatohepatitis (including alcoholic), in which CD38 and PARP1 expression were negatively correlated with metabolic function[17, 21]. In line with these observations, we also found increased PARP activation in livers of mice on chronic and binge ethanol feeding, likewise in human liver specimens with alcoholic steatohepatitis.

It is well-established that oxidative/nitrative stress-induced excessive PARP activation in cells leads to energetic collapse and cell death[5]. In pathological conditions such as ischemic-reperfusion injury and various forms of shock, PARP-mediated cell death is primarily necrotic[5]. However, depending on the degree of stress on cells, PARP activation may also mediate or directly/indirectly modulate other cell death pathways, as well as oxidative/nitrative stress and mitochondrial function[5, 8]. We found enhanced oxidative/nitrative stress and cell death (both necrotic and apoptotic) in livers of mice on alcohol diet. PARP inhibition or genetic deletion of PARP1 attenuated the alcohol-induced hepatic cell death and oxidative/nitrative stress.

Activation of pro-inflammatory processes is an important feature of alcoholic liver injury and steatohepatitis. Increased penetration of intestinal bacterial lipopolysaccharide (LPS) is often detected in the circulation of both chronic alcoholics[22] and binge drinking healthy individuals[23], which stimulates resident liver macrophages, the Kupffer cells, to produce reactive oxygen species and cytokines that subsequently trigger hepatocyte injury and activation of hepatic stellate cells [1, 2, 4]. Various damage-associated molecular patterns released from necrotic hepatocytes may also fuel Kupffer cell activation, as well as more delayed neutrophil infiltration, both of which were characteristic features in our model of alcohol-induced liver injury. PARP inhibition or genetic deletion of PARP1 was associated with marked attenuation of alcohol-induced pro-inflammatory response *in vivo*, consistently with our previous results using carbon tetrachloride-induced liver injury models[11]. Our results demonstrating decreased pro-inflammatory activation of LPS-stimulated Kupffer cells by PARP inhibitors or

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genetic deletion of PARP1 *in vitro* suggests that the anti-inflammatory effects of PARP inhibition/PARP1 deletion *in vivo* in alcohol-induced steatohepatitis model involved not only protection of hepatocytes against alcohol-induced cell death, but also direct anti-inflammatory effects on Kupffer cells.

Chronic alcohol consumption affects mitochondrial oxidative metabolism in the liver partially by suppressing the synthesis of protein components of the electron transport chain that are encoded on the mitochondrial DNA[24]. These impairments would eventually promote mitochondrial reactive oxygen species formation[25], further aggravating alcoholic liver injury and the deterioration of intermediary metabolism[26]. Furthermore, alcohol consumption also leads to reprograming of lipid metabolism by promoting hepatic lipid accumulation that is, at least in part, due to the blockade of mitochondrial energy production by ethanol (inhibition of SIRT1, PGC-1α, and FOXO1)[1-4, 27].

SIRT1 belongs to the family of sirtuins, essential regulators of metabolism, and whole body energy homeostasis. By the deacetylation of key transcription factors, SIRT1 (using NAD^+ as cofactor for activity) regulates mitochondrial biogenesis (PGC1α), lipid and carbohydrate metabolism (PPARs, SREBPs), and cell proliferation and division (p53). SIRT1 activation protects against metabolic derangements (diabetes, hypercholesterolemia, obesity)[28, 29], while SIRT1 deficiency promotes the development of metabolic syndrome[30]. Recent studies also suggested that SIRT1 plays an important protective role in alcoholic[31] and non-alcoholic hepatosteatosis [32]. A nice recent study demonstrated that hepatocyte specific deletion of SIRT1 enhanced hepatocellular injury, inflammation and metabolic dysregulation in the NIAAA model of ASH [31], whereas boosting cellular NAD+ levels by administration of the NAD+ precursor, nicotinamide riboside (NR), protected against hepatocellular injury in a SIRT1-dependent fashion [17]. Consistently with these results, we also found decreased activity of SIRT1 in livers of mice exposed to alcohol.

Since PARP activation leads to NAD^+ depletion, it can decrease SIRT1 activity by competing for NAD^+ with SIRT1[8, 13]. Indeed, the decreased SIRT1 activity in livers from mice exposed to alcohol was also associated with decreased hepatic NAD^+ content. Pharmacological inhibition of PARP restored the hepatic NAD^+ content, and attenuated the decrease in hepatic SIRT1 activity/expression in mice exposed to ethanol feeding, likewise the mitochondrial dysfunction and lipid accumulation. These results strongly suggest that in addition to attenuating inflammation and cell death, PARP inhibition also exerts important effects on alcohol-induced metabolic dysregulation and lipid accumulation by modulating NAD^+-dependent SIRT1 activity.

To increase the clinical significance of our results we also demonstrate that PARP inhibition decreases hepatic steatosis, metabolic dysregulation, and/or inflammation and/or fibrosis in mouse models of NAFLD/NASH induced by high fat (HF) diet or methionine and choline deficient (MCD) diet, as well as in a model of HF diet and single alcohol binge-induced liver injury.
Furthermore, indirect calorimetry experiments in mice on HF diet treated with Olaparib (the clinically approved PARP inhibitor for the treatment of cancer) also indicated that inhibition of PARP attenuated body weight and liver triglyceride content by enhancing fat burning and normalizing dysregulation of genes involved in fatty acid oxidation, but without influencing caloric intake or ambulatory activity of mice.

In summary, we demonstrated an important role of PARP activation in the sequel of alcoholic liver injury, inflammation and metabolic reprograming, which may involve a molecular link between PARP and SIRT1, providing an additional explanation for the marked protective effect of PARP inhibitors in the present models. We also show that PARP inhibition decreased hepatic triglyceride accumulation, metabolic dysregulation, or inflammation and/or fibrosis in models of NASH. These results, coupled with protective effects of PARP inhibitors in models of ischemic tissue injury and endothelial dysfunction[5], recently described antifibrotic effects in the liver[11], clinical availability of PARP inhibitor Olaparib for the treatment of cancer, and a recent study demonstrating that inhibition of PARP increases fatty acid oxidation and protects against fatty liver disease in mouse models of NAFLD[21] strongly suggest that repurposing PARP inhibitors for the treatment of liver disease may represent a promising new avenue.

Author Contributions

P.M., B.H., M.R., Z.V.V., K.G., D.R., Z.C., E. H., O.P., Z.Z., M.J.X., W.W., G.G., J.P., B.T.N., Y.P., L.L., G. H., P. B., J.A., B.G. and P.P. designed or conducted experiments, analyzed or interpreted data or edited the manuscript. A.H.B. provided knockout mice. P.M., Z.V.V., P.B., P.P. wrote the draft; P.P. supervised experiments and obtained funding.

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Figure Legends

Fig 1. Pharmacological inhibition of PARP restores attenuated NAD^+ content and SIRT1 activity. PARP inhibition or PARP1 deletion attenuates alcohol-induced steatohepatitis.

A) PARP activity, NAD^+ content and SIRT1 activities in control- and alcohol fed mice treated with PARP inhibitors (AIQ and PJ34). n = 6-8/group, $^*P < 0.05$ versus wild type mice treated with vehicle; $^\#P < 0.05$ versus wild type mice+Alcohol (Alc).

B) mRNA and protein level of Sirt1 by Real-time PCR and Western blot respectively. n = 6/group, $^*P < 0.05$ versus wild type mice treated with vehicle for mRNA expression.

C) Representative PGC-1α and FOXO1 acetylation by immunoprecipitation.

D) Representative H&E images from the indicated groups (Scale bar = 100 μm).

E) Left panel: Serum ALT and AST levels, n = 7-11/group, $^*P < 0.05$ versus wild type mice treated with vehicle; $^\#P < 0.05$ versus wild type mice+Alcohol (Alc). Right panel: Serum ALT and AST in the respective groups, n = 6-8/group, $^*P < 0.05$ versus wild type ($PARP1^{+/+}$) mice and $PARP1^{-/-}$ mice treated with vehicle; $^\#P < 0.05$ versus wild type mice+Alcohol (Alc).

F) DNA fragmentation and Caspase 3/7 activity. Left panel n = 6/group, $^*P < 0.05$ versus wild type mice treated with vehicle; $^\#P < 0.05$ versus wild type mice+Alcohol (Alc). Right panel: n = 6/group, $^*P < 0.05$ versus wild type ($PARP1^{+/+}$) mice and $PARP1^{-/-}$ mice treated with vehicle; $^\#P < 0.05$ versus wild type mice+Alcohol (Alc).

Fig 2. Pharmacological inhibition of PARP or genetic ablation of PARP1 attenuates alcohol induced liver inflammation *in vivo* and Kupffer cell activation *in vitro*

A) Real-time PCR analyses of inflammatory cytokines TNFα, IFNγ, MIP1α, MIP2, CCR2 and IL1β. n = 8-20/group, Top panel: $^*P < 0.05$ versus wild type mice treated with vehicle; $^\#P < 0.05$ versus wild type mice+Alcohol (Alc). Bottom panel: $^*P < 0.05$ versus wild type ($PARP1^{+/+}$) mice and $PARP1^{-/-}$ mice treated with vehicle; $^\#P < 0.05$ versus wild type mice+Alcohol (Alc).

B) Real-time PCR analyses of MPO and ICAM, n = 8-20/group, Left panel: $^*P < 0.05$ versus wild type mice treated with vehicle; $^\#P < 0.05$ versus wild type mice+Alcohol (Alc). Right panel: $^*P < 0.05$ versus wild type ($PARP1^{+/+}$) mice and $PARP1^{-/-}$ mice treated with vehicle; $^\#P < 0.05$ versus wild type mice+Alcohol (Alc).

C) Attenuation of inflammatory cytokine expression by pharmacological inhibition of PARP (LPS+AIQ and LPS+PJ34) or genetic ablation of PARP1 in LPS activated Kupffer cells. n = 9/group, $^*P < 0.05$ versus Kupffer cells treated with vehicle; $^\#P < 0.05$ versus Kupffer cells +

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LPS. Or $P < 0.05$ versus PARP1$^{+/+}$ and PARP1$^{-/-}$ Kupffer cells with vehicle $^{\#}P < 0.05$ versus PARP1$^{+/+}$ Kupffer cells+LPS.

Fig 3. **Pharmacological inhibition of PARP or genetic ablation of PARP1 ameliorates alcohol induced oxidative stress and mitochondrial dysfunction**

A) Real-time PCR analyses of ROS generating enzymes: gp91phox, p47phox, and iNOS. n = 8-20/group; top panel: $^{*}P < 0.05$ versus wild type mice treated with vehicle; $^{\#}P < 0.05$ versus wild type mice+Alcohol (Alc), bottom panel: $^{*}P < 0.05$ versus wild type (PARP1$^{+/+}$) mice and PARP1$^{-/-}$ mice treated with vehicle; $^{\#}P < 0.05$ versus wild type mice+Alcohol (Alc). B) Oxidative/nitrative stress markers Nitrotyrosine content and HNE. n = 4-6/group; top panel: $^{*}P < 0.05$ versus wild type mice treated with vehicle; $^{\#}P < 0.05$ versus wild type mice+Alcohol (Alc); bottom panel: $^{*}P < 0.05$ versus wild type (PARP1$^{+/+}$) mice and PARP1$^{-/-}$ mice treated with vehicle; $^{\#}P < 0.05$ versus wild type mice+Alcohol (Alc). C) Mitochondrial DNA content in liver is decreased by alcohol feeding n = 4/group. Top panel: $^{*}P < 0.05$ versus wild type mice treated with vehicle; $^{\#}P < 0.05$ versus wild type mice+Alcohol (Alc). Bottom panel: $^{*}P < 0.05$ versus wild type (PARP1$^{+/+}$) mice and PARP1$^{-/-}$ mice treated with vehicle; $^{\#}P < 0.05$ versus wild type mice+Alcohol (Alc). D) Mitochondrial Complex I and IV activities decreased due to alcohol while there is no significant effect on Complex II activity. n = 4/group. Top panel: $^{*}P < 0.05$ versus wild type mice treated with vehicle; $^{\#}P < 0.05$ versus wild type mice+Alcohol (Alc). Bottom panel: $^{*}P < 0.05$ versus wild type (PARP1$^{+/+}$) mice and PARP1$^{-/-}$ mice treated with vehicle; $^{\#}P < 0.05$ versus wild type mice+Alcohol (Alc).

Fig 4. **Pharmacological inhibition of PARP or genetic ablation of PARP1 attenuates alcohol induced steatosis and metabolic dysregulation in the liver.**

A) Representative Oil-O-Red stained images of liver sections. Magnification: 200x. B) Expression of genes involved in hepatic fatty acid synthesis: sterol regulatory element-binding protein-1c (SREBP-1), acetyl-CoA carboxylase-1 and -2, respectively (ACC1, ACC2). n = 4-7/group. Top panel: $^{*}P < 0.05$ versus wild type mice treated with vehicle; $^{\#}P < 0.05$ versus wild type mice+Alcohol (Alc). Bottom panel: $^{*}P < 0.05$ versus wild type (PARP1$^{+/+}$) mice and PARP1$^{-/-}$ mice treated with vehicle; $^{\#}P < 0.05$ versus wild type mice+Alcohol (Alc). C) Expression of genes involved in hepatic glucose metabolism: glucokinase (GK) and glucose-6-phosphatase (G6Pase). n = 4-7/group, Top panel: $^{*}P < 0.05$ versus wild type mice treated with vehicle; $^{\#}P < 0.05$ versus wild type mice+Alcohol (Alc). Bottom panel: $^{*}P < 0.05$ versus wild type

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(PARP1$^{+/+}$) mice and PARP1$^{-/-}$ mice treated with vehicle; $^{\#}P < 0.05$ versus wild type mice+Alcohol (Alc).

D) Hepatic triglyceride content from liver tissues. n = 6-8/group. Left panel: $^{*}P < 0.05$ versus wild type mice treated with vehicle; $^{\#}P < 0.05$ versus wild type mice + Alcohol (Alc). Right panel: $^{*}P < 0.05$ versus wild type (PARP1$^{+/+}$) mice and PARP1$^{-/-}$ mice treated with vehicle; $^{\#}P < 0.05$ versus wild type mice + Alcohol (Alc).

Fig 5. Pharmacological inhibition of PARP attenuates liver steatosis and fatty acid metabolic dysregulation in diet induced obesity (DIO) model in mice

Mice on high fat diet for 11 weeks were treated with PARP inhibitors or vehicle for 8 days. A) Representative H&E or B) Oil Red O stained images of liver sections from indicated groups. 200x magnification. C) Expression of genes involved in hepatic fatty acid synthesis: SREBP-1, ACC1, ACC2, ACO, FAS and PPARα. n = 6/group. $^{*}P < 0.05$ versus DIO mice. D) Liver triglyceride content n = 6/group $^{*}P < 0.05$ versus DIO mice.

Fig 6. Pharmacological inhibition of PARP attenuates liver injury and inflammation in high fat diet plus binge alcohol model in mice

Mice on high fat diet for 11 weeks were treated with PARP inhibitors or vehicle for 8 days and exposed to a single binge of alcohol on the last day of the treatment. A) ALT level, n=6/group, $^{*}P < 0.05$ versus high fat diet plus binge alcohol (Alc) mice. B) Representative myeloperoxidase (MPO) stained liver sections from indicated groups. 200x magnification. C) Quantification of MPO positive cells expressed as percentage of high fat plus binge alcohol sample, n=16 fields/group from 4 livers in a group. $^{*}P < 0.05$ versus high fat diet plus binge alcohol (Alc) mice. D) Real-time PCR analyses of neutrophil marker Ly6G, adhesion molecule (ICAM1), chemokine (Cxcl2, CCR2) and cytokine (IL1β, TNFα). n=6/group, $^{*}P < 0.05$ versus high fat diet plus binge alcohol (Alc) mice.

Fig 7. Pharmacological inhibition of PARP with Olaparib attenuates methionine-choline deficient (MCD) diet induced steatosis and metabolic dysregulation in the liver.

Mice on MCD diet were treated with PARP Olaparib for 5 weeks. A) Representative H&E and C) Oil Red O stained images of liver sections from the indicated groups, 200x magnification. B) Serum ALT, n=4/group, $^{*}P < 0.05$ versus control mice treated with vehicle; $^{\#}P < 0.05$ versus MCD mice. D) Hepatic triglyceride content, n=4-5/group. P < 0.05 versus control mice treated with vehicle; #P < 0.05 versus MCD mice. E) Expression of genes involved in hepatic fatty acid

synthesis: SREBP-1, SCD1, FAS, ACC1, ACO. n = 5/group, P < 0.05 versus control mice treated with vehicle; #P < 0.05 versus MCD mice.

Fig 8. Pharmacological inhibition of PARP with Olaparib attenuates methionine-choline deficient (MCD) diet induced hepatic inflammation and fibrosis

A) Representative Ly6G stained liver sections from the indicated groups, 200x magnification. B) Quantification of Ly6G positive cells per field taken with 200x magnification, $^{*}P < 0.05$ versus control mice treated with vehicle; n=15-18 fields/group from 3 livers/group. $^{\#}P < 0.05$ versus MCD mice. C) Real-time PCR analyses of inflammatory (TNFα, CXCL2, Ly6G and F4/80) and fibrosis markers (TGFβ, Collagen 1, CTGF and α SMA), n = 5/group, P < 0.05 versus control mice treated with vehicle; #P < 0.05 versus MCD mice. D) Representative Picro Sirius Red staining for fibrosis from indicated groups (200x magnification), and quantification (n=15-27 images/group from 3 livers/group.

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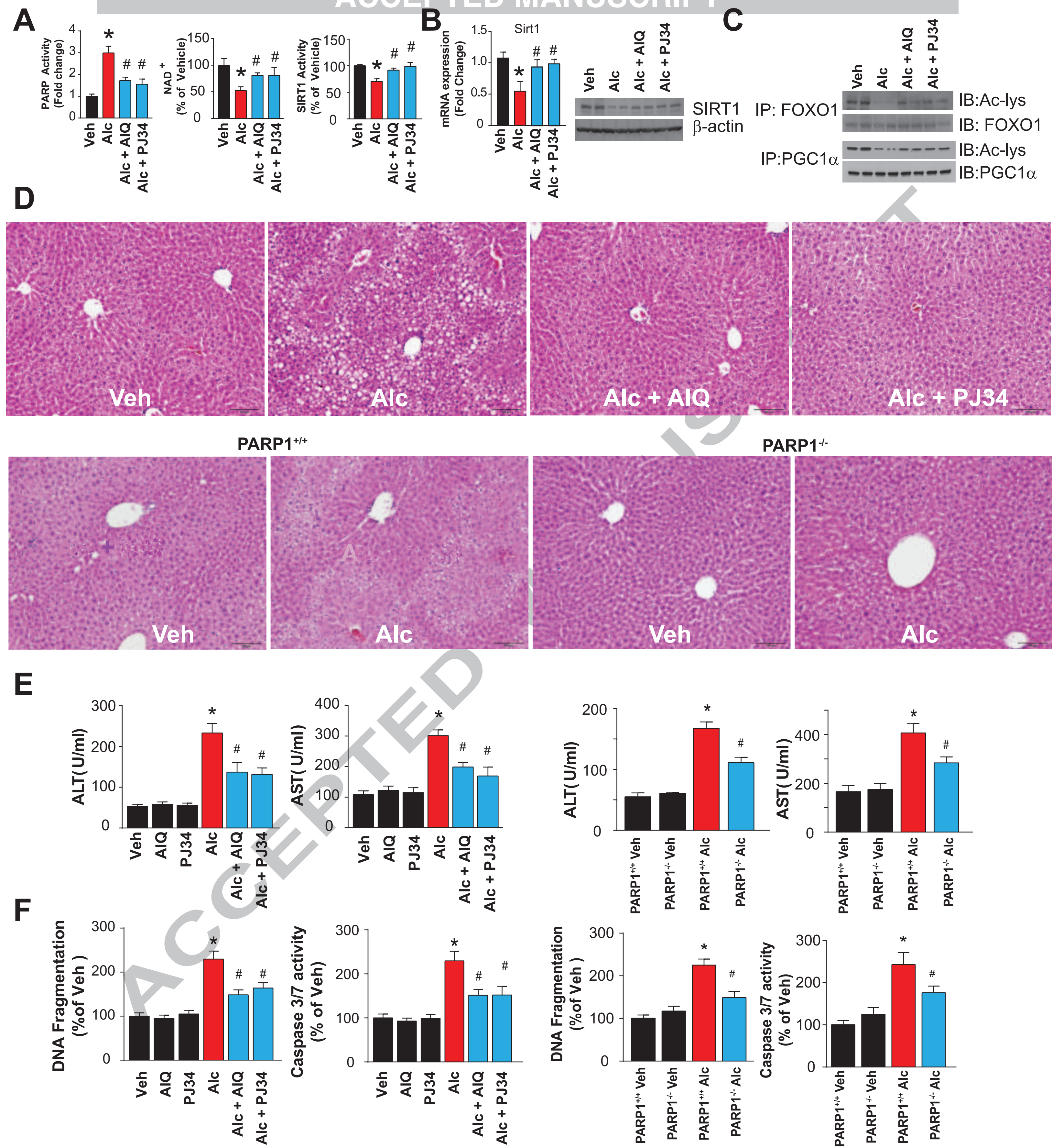

FIG 1

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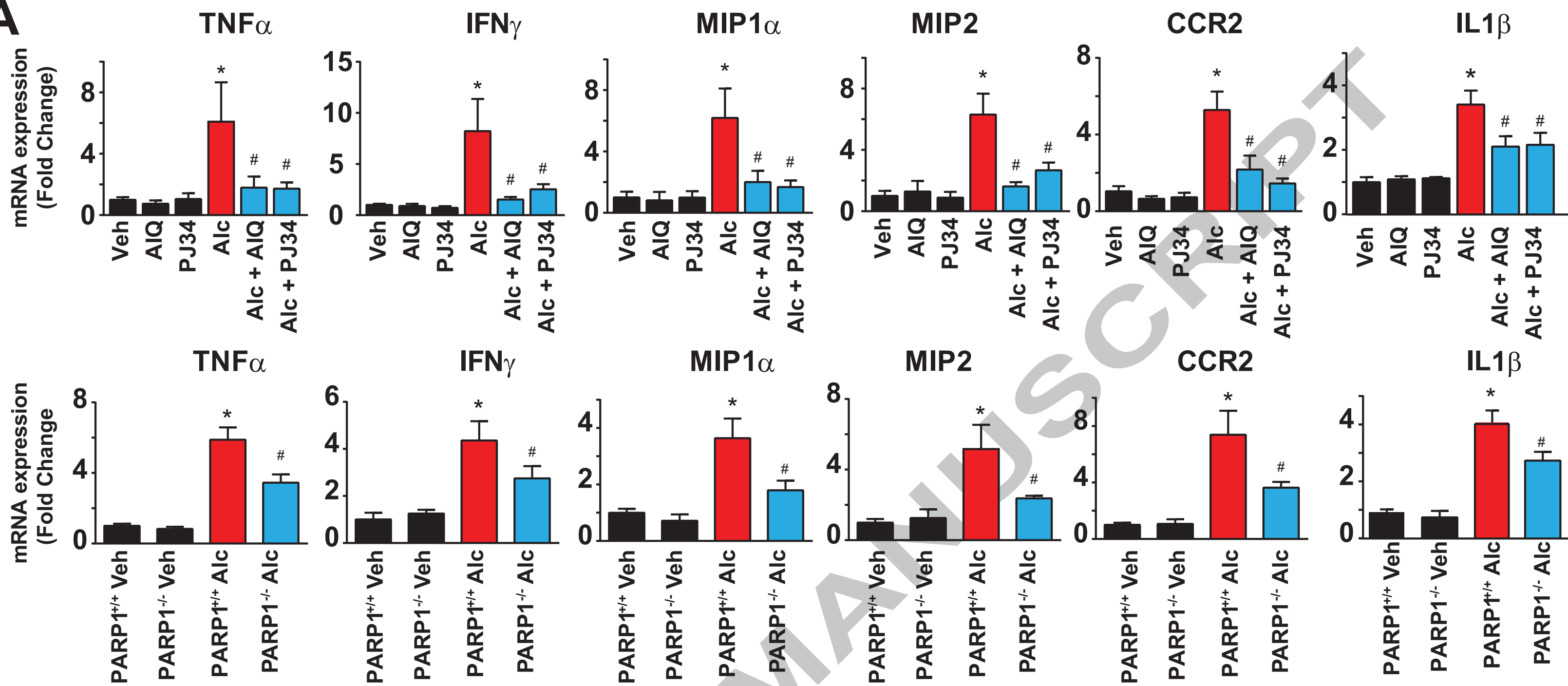

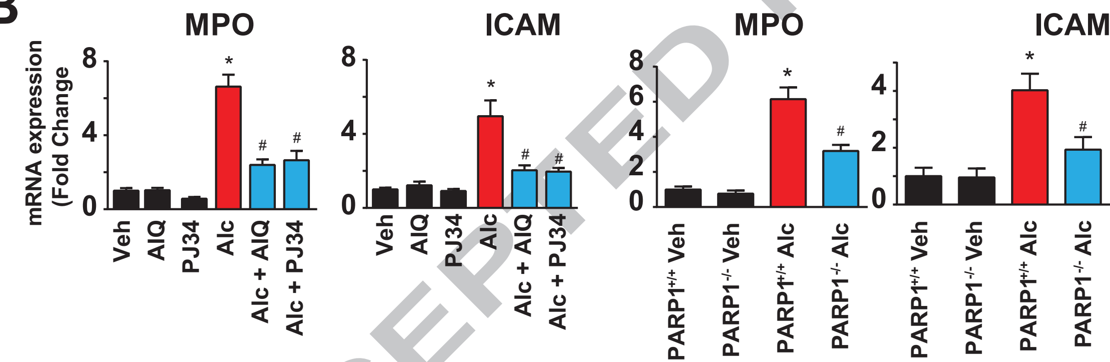

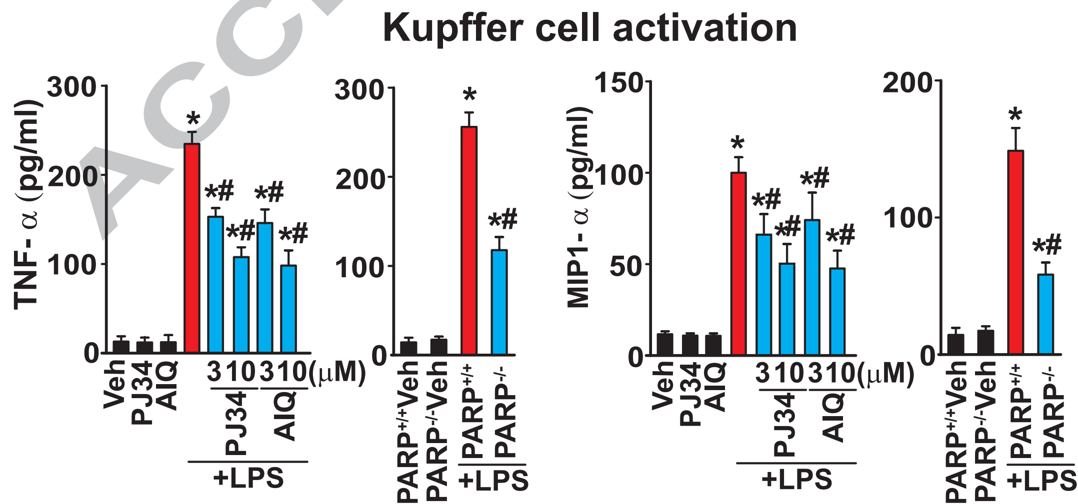

FIG 2

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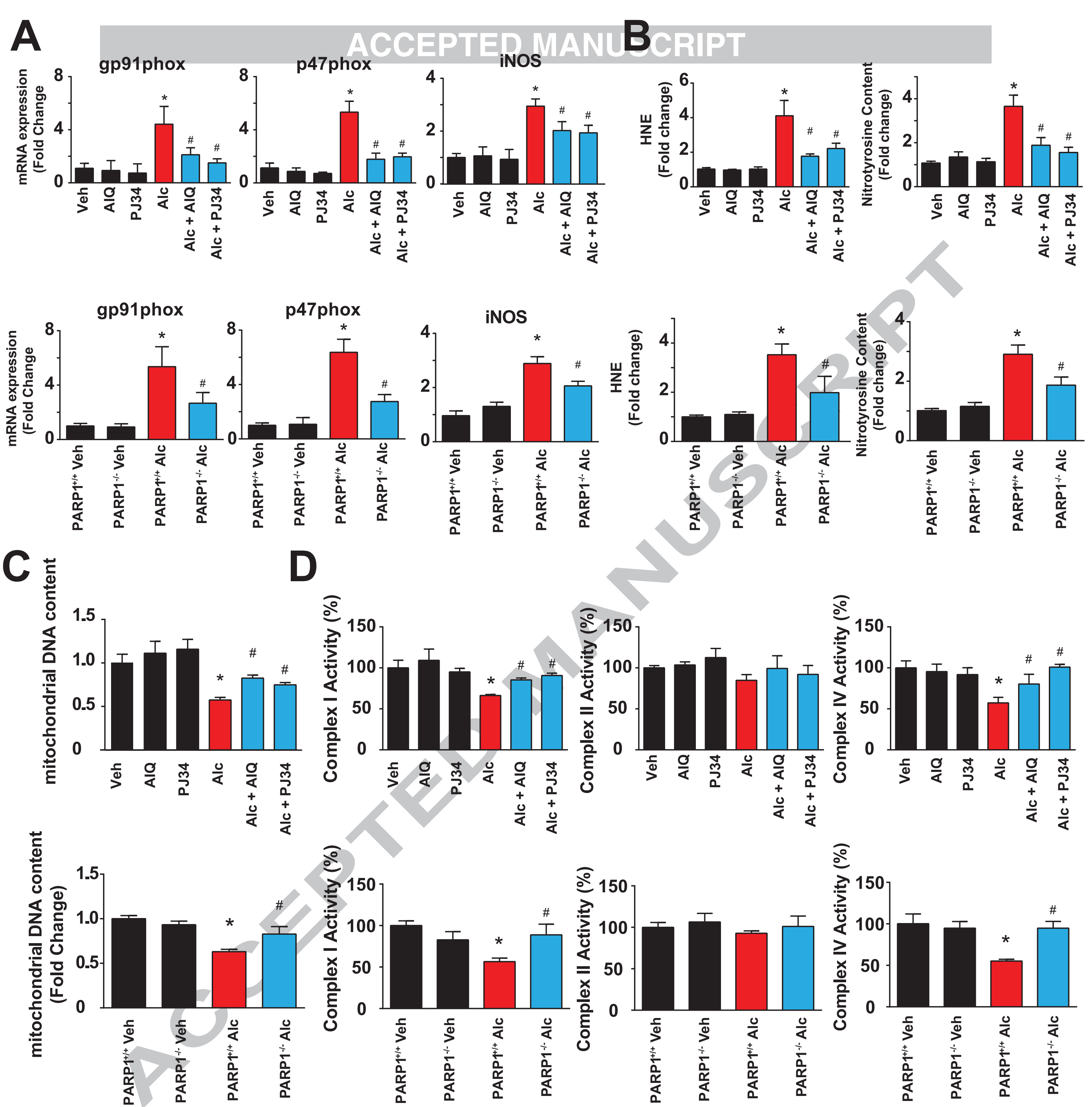

FIG 3

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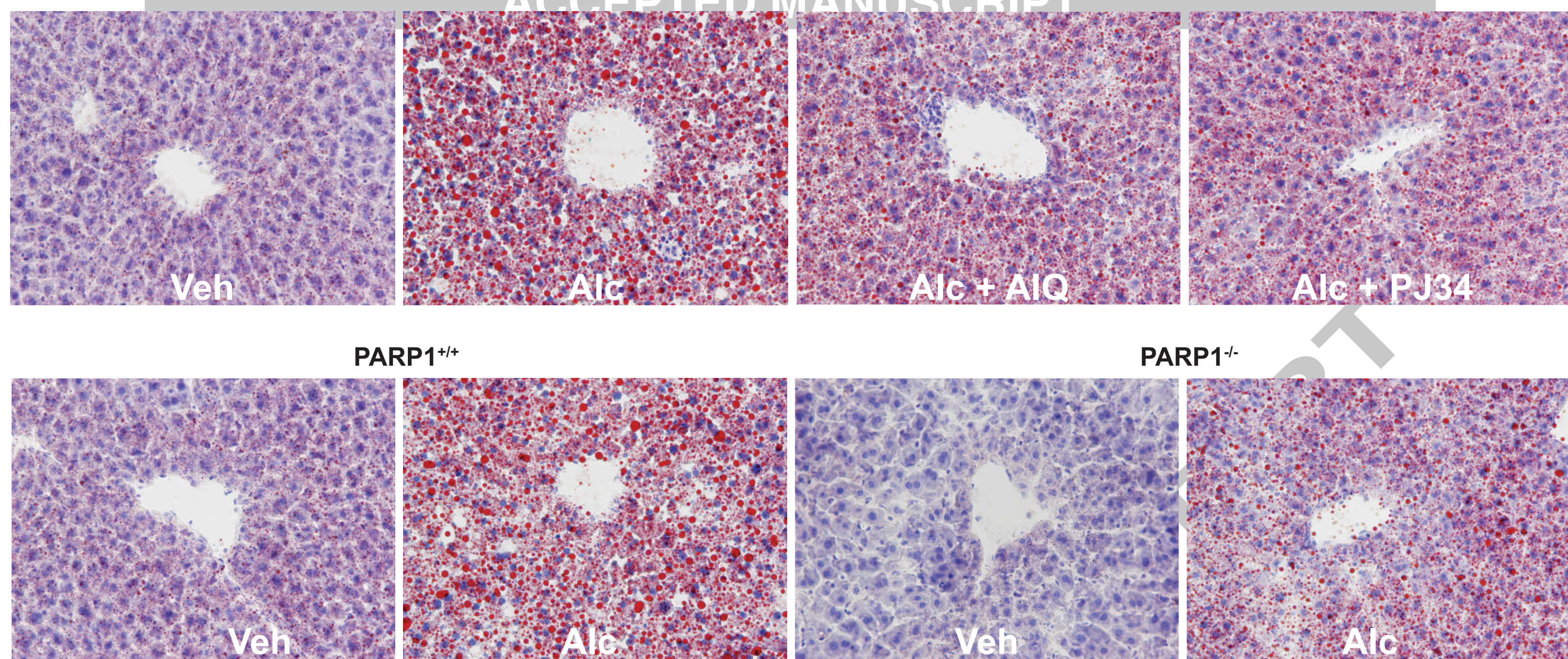

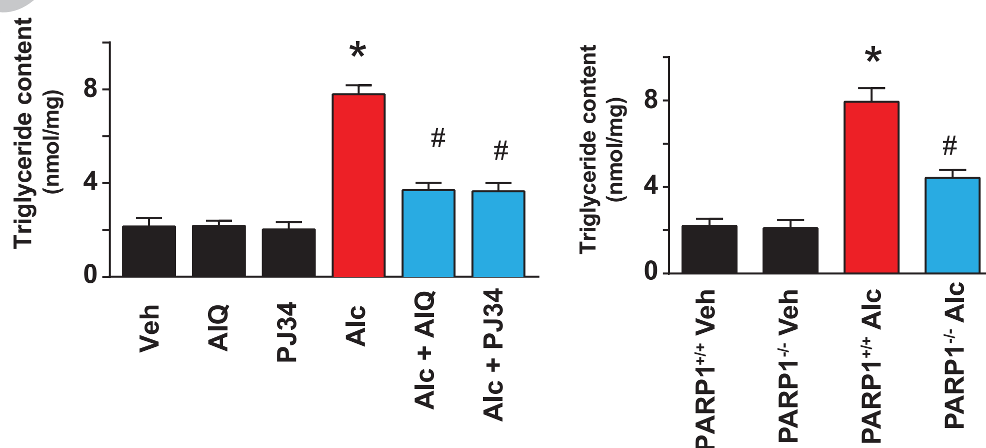

FIG 4

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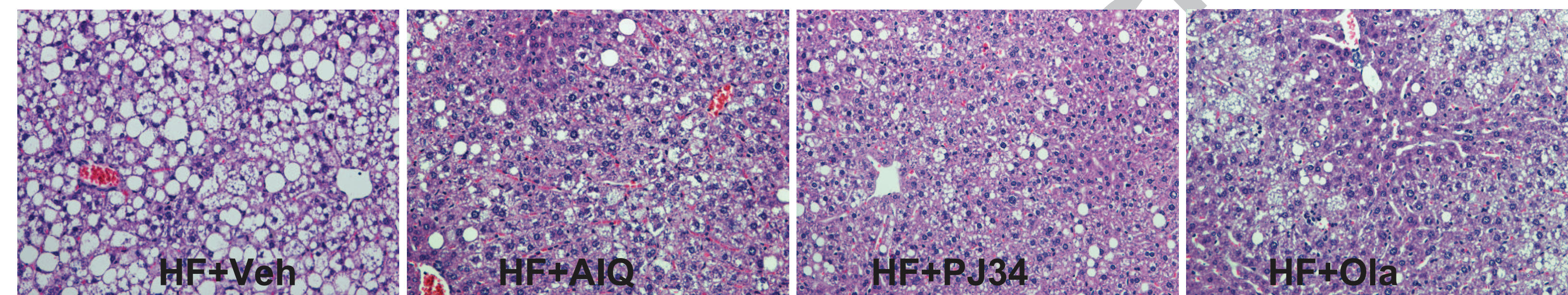
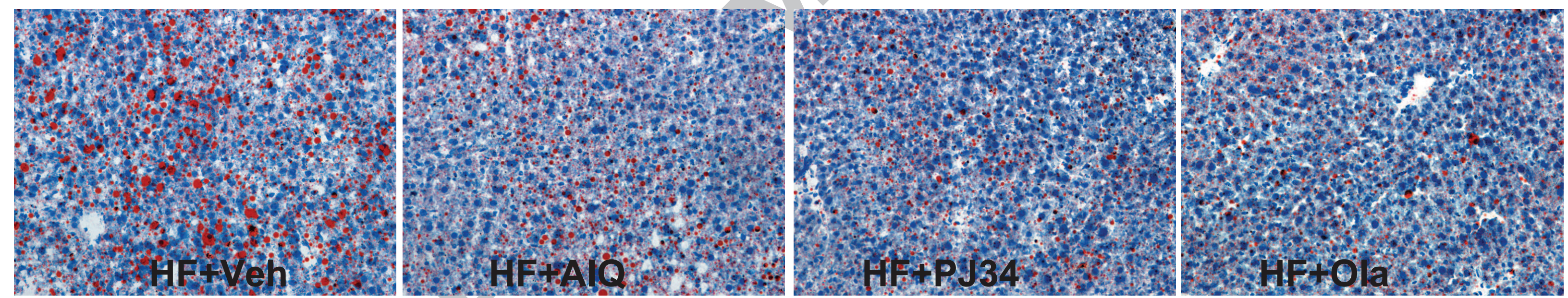
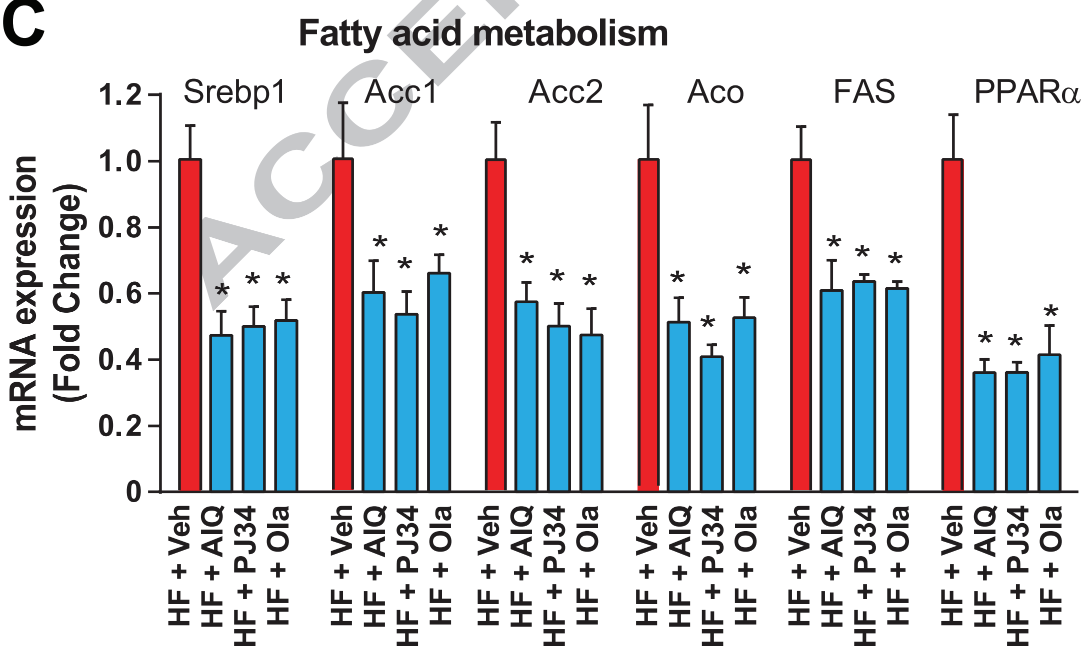
A Haematoxylin and Eosin Staining



B Oil Red O Staining





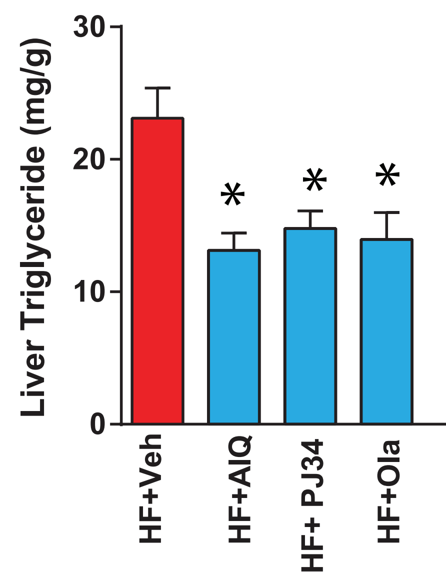


FIG 5

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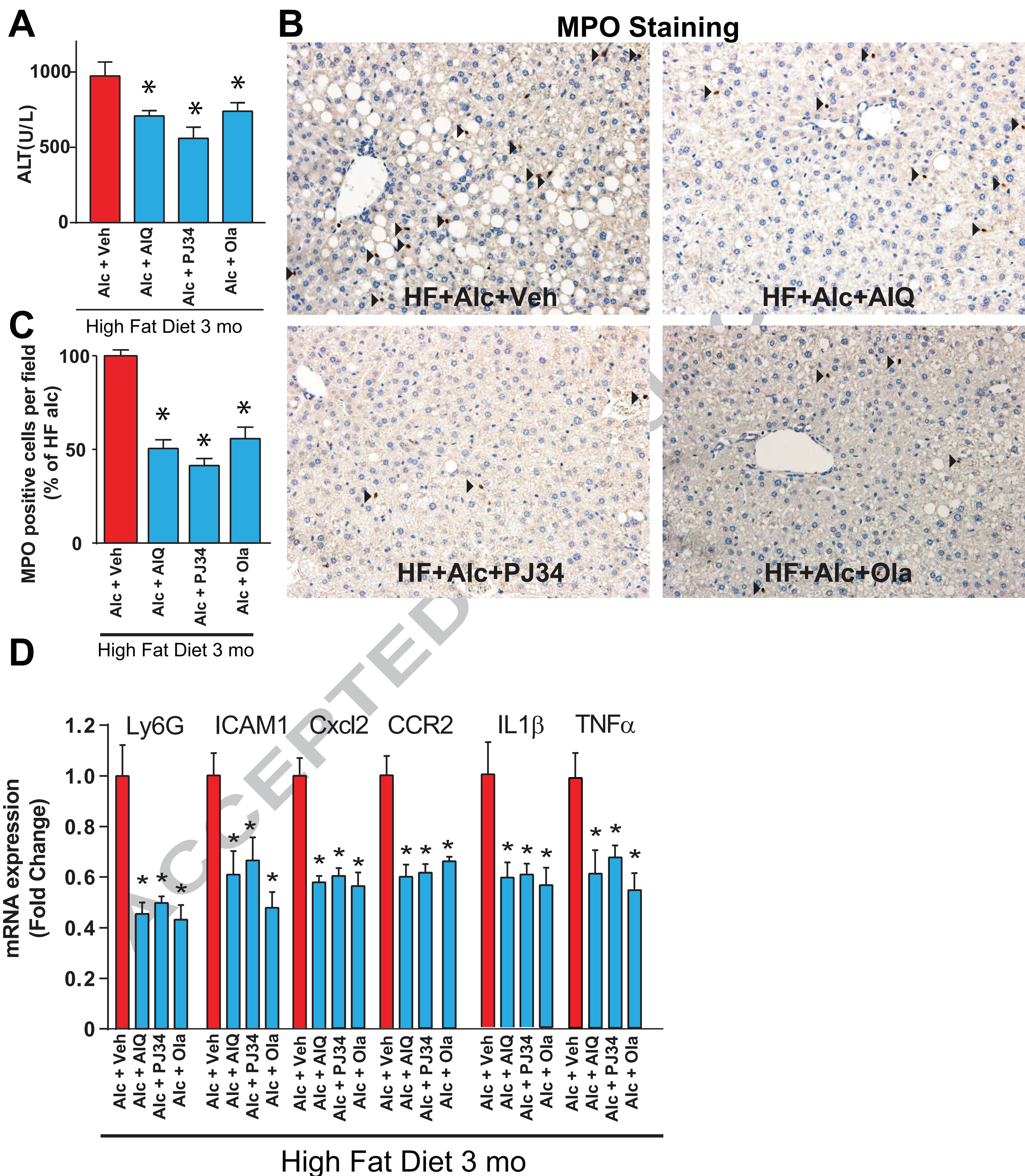

FIG 6

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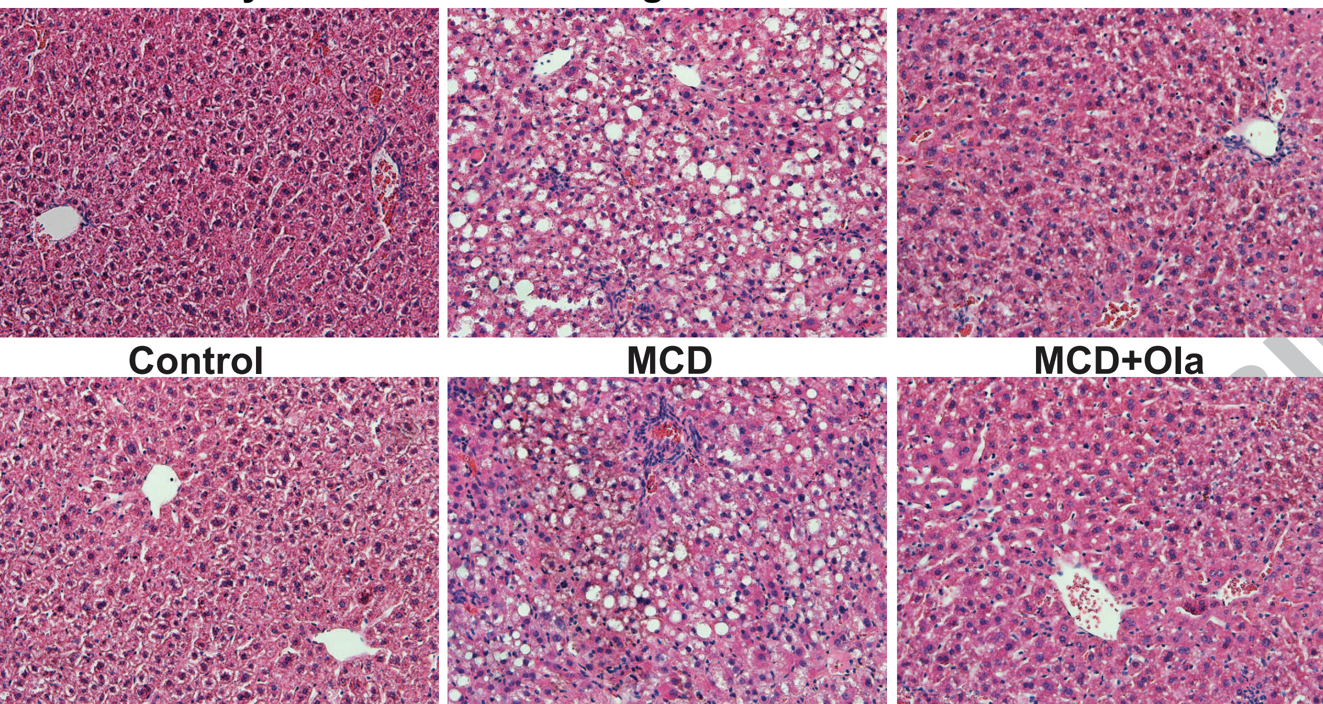

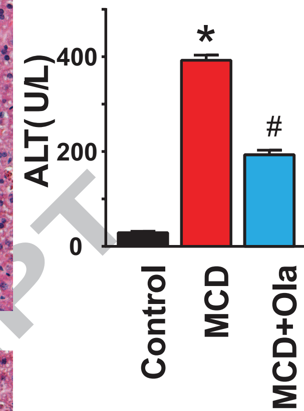

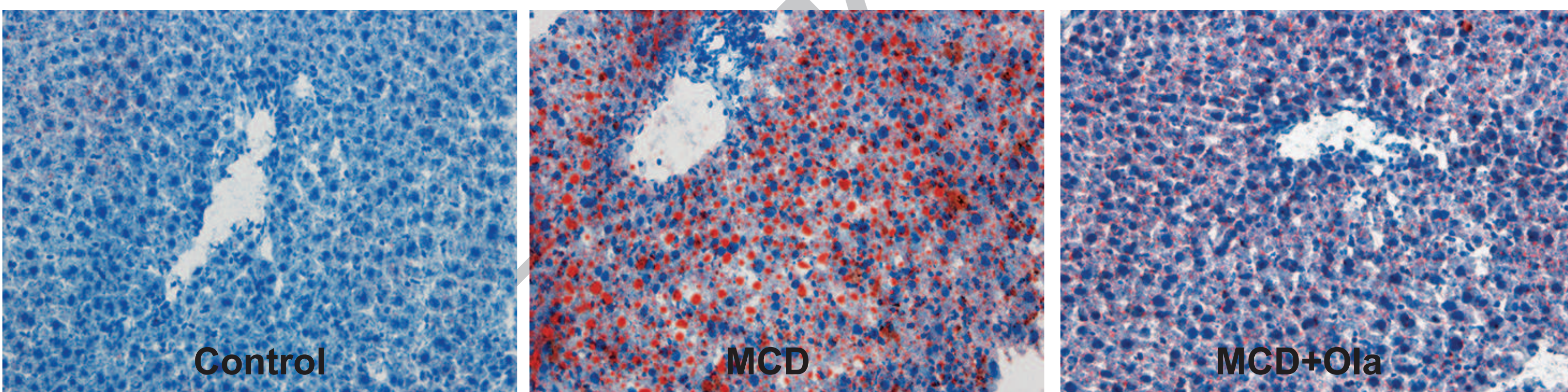

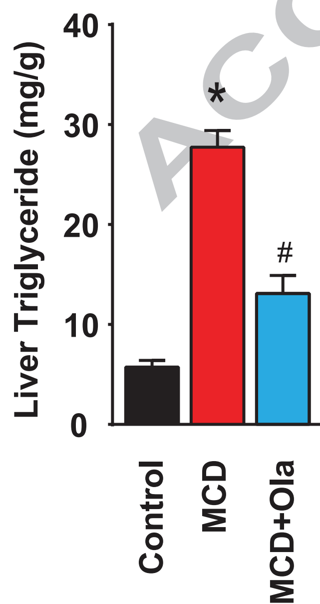

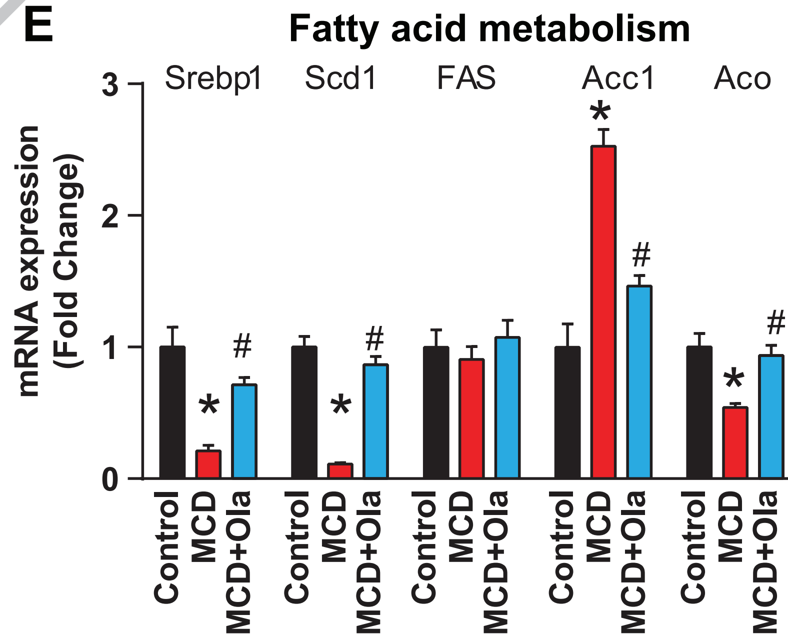

FIG 7

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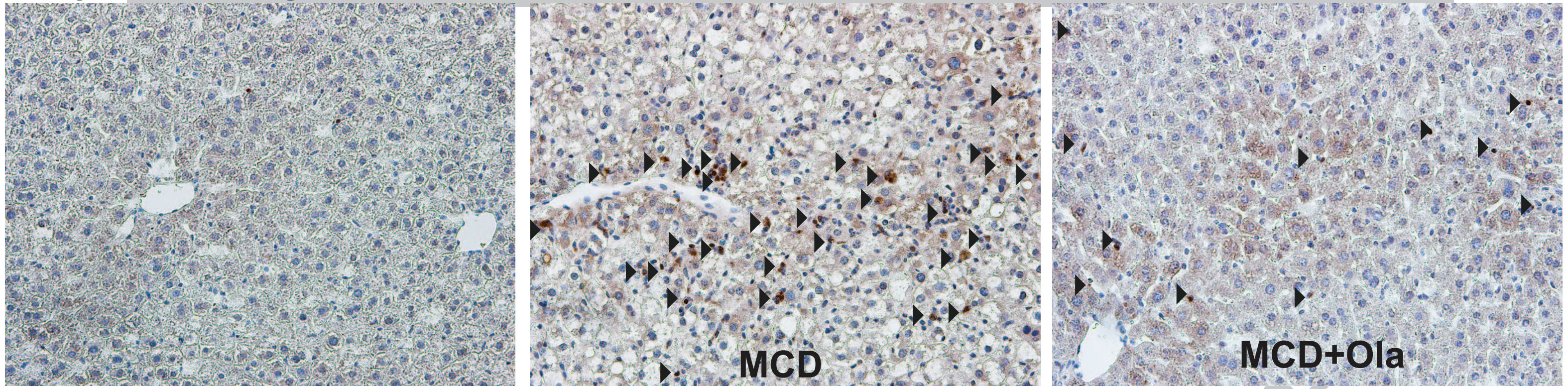
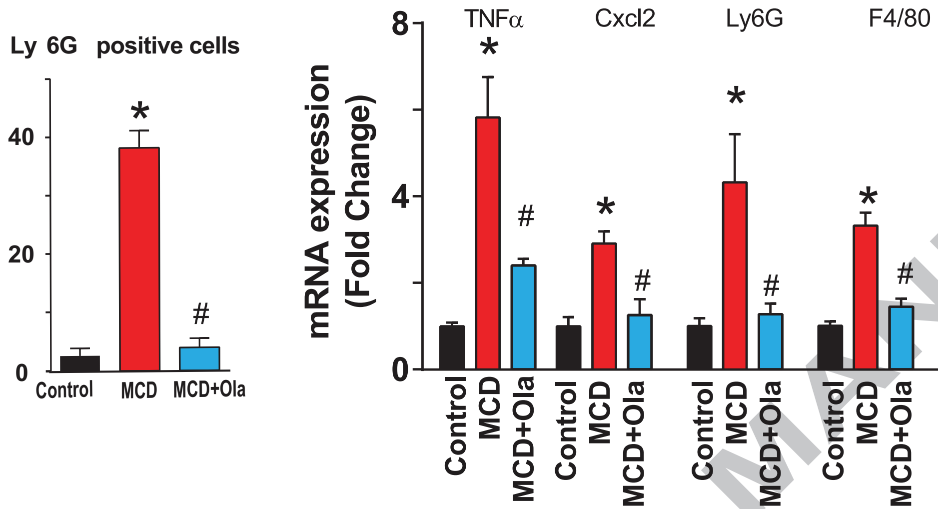
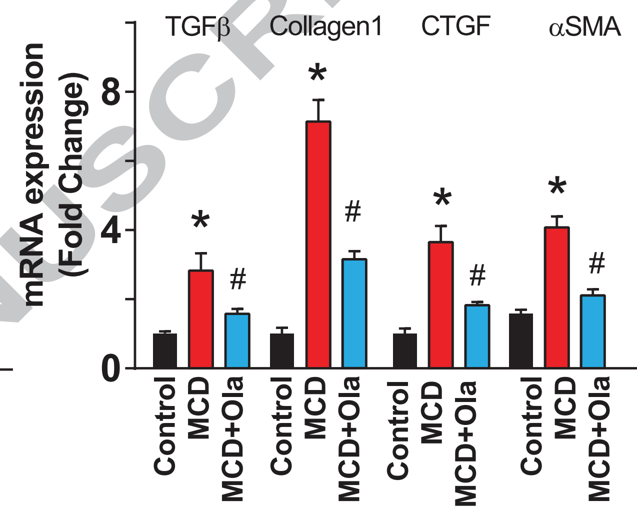






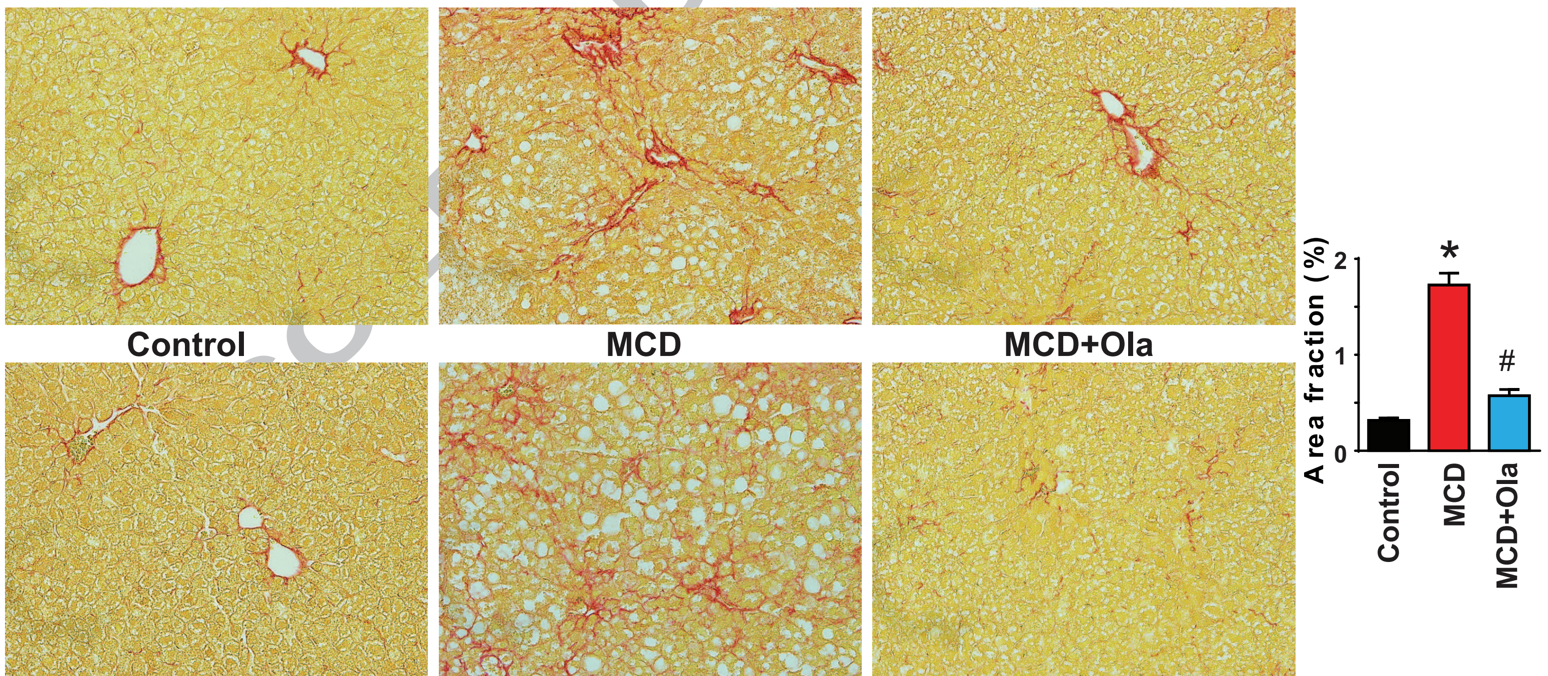
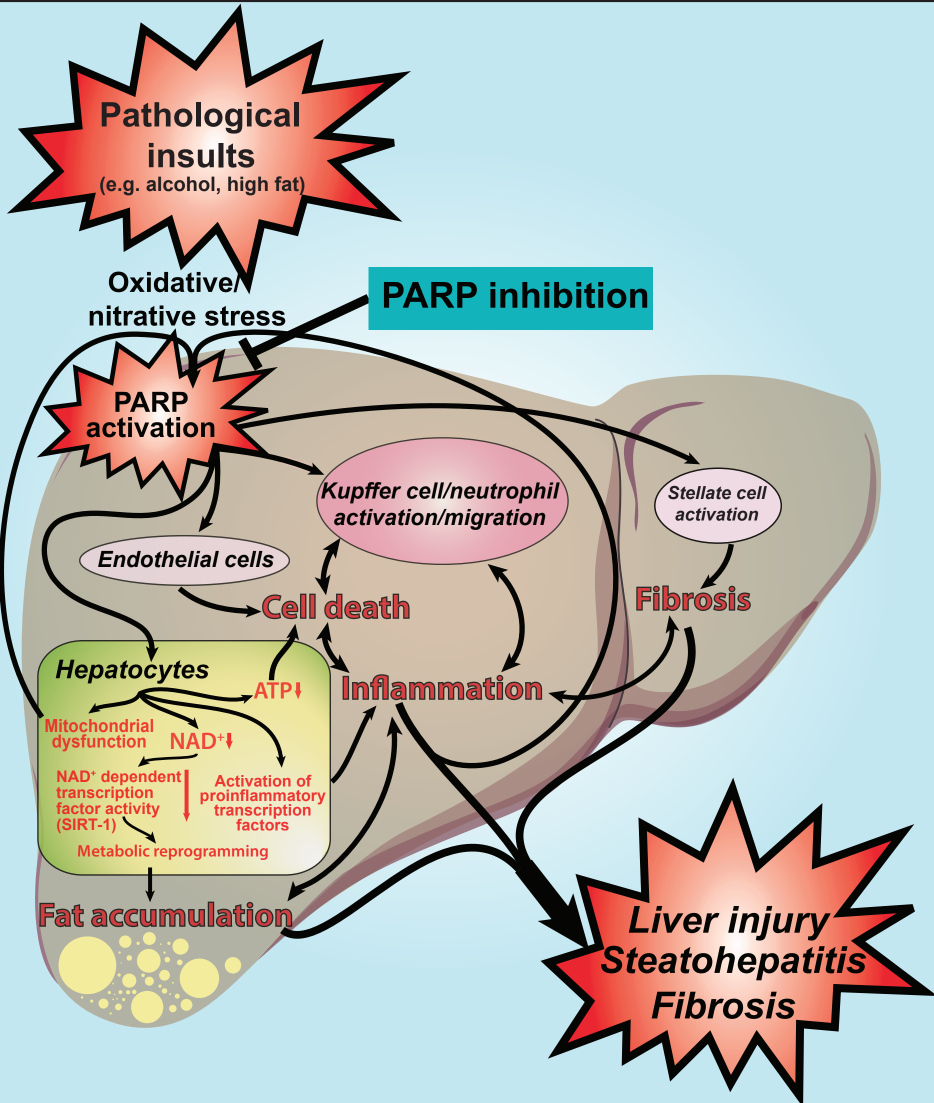


FIG 8



Pathological insults
(e.g. alcohol, high fat)
Oxidative/ nitrative stress
PARP inhibition
PARP activation
Kupffer cell/neutrophil activation/migration
Stellate cell activation
Endothelial cells
Cell death
Fibrosis
Hepatocytes
ATP↓
Inflammation
Mitochondrial dysfunction
NAD+↓
NAD+ dependent transcription factor activity (SIRT-1)
Activation of proinflammatory transcription factors
Metabolic reprogramming
Fat accumulation
Liver injury
Steatohepatitis
Fibrosis