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Anti-miR-873-5p improves alcohol-related liver disease by enhancing hepatic deacetylation via SIRT1

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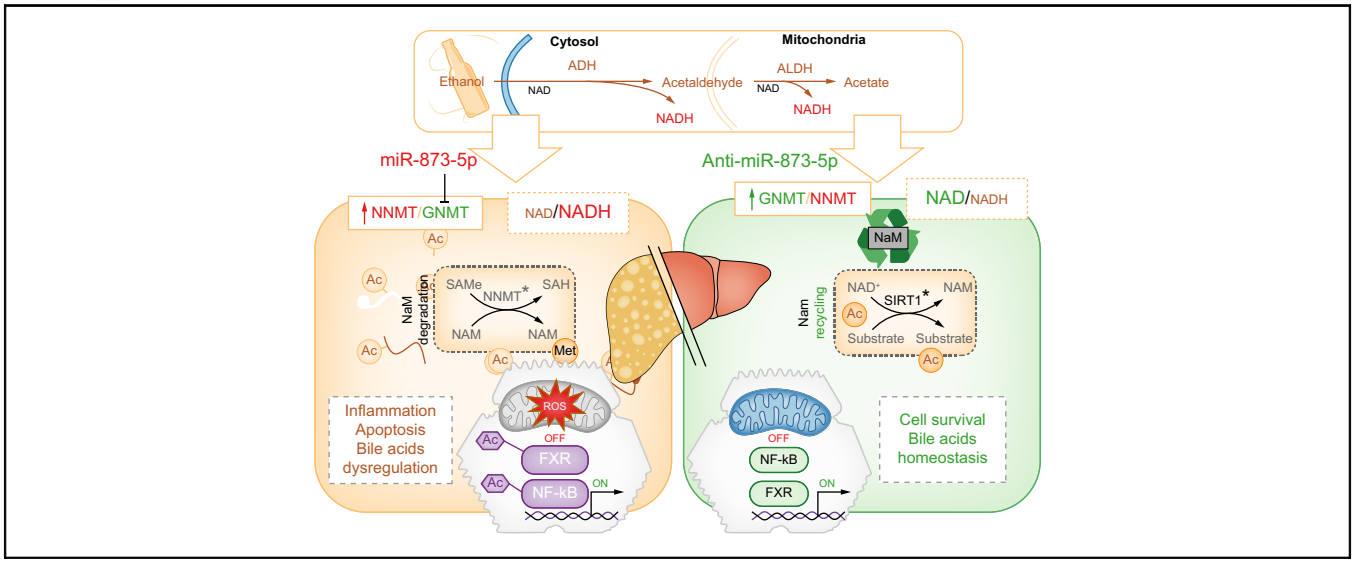
Graphical abstract



Highlights

- Patients with ALD exhibit upregulated hepatic miR-873-5p alongside a decrease in GNMT levels.
- Inhibition of miR-873-5p reduced hepatocyte death, ER stress, inflammation, and lipid accumulation.
- miR-873-5p promoted NNMT expression, altered NAD production, and affected SIRT1 activity.
- Elevated SIRT1 activity *in vivo* contributed to the restoration of bile acid homeostasis and reduction of inflammation.

Impact and implications

The role of miR-873-5p has not been explicitly examined in the progression of ALD, a pathology with no therapeutic options. In this study, inhibiting miR-873-5p exerted hepatoprotective effects against ALD through rescued SIRT1 activity and consequently restored bile acid homeostasis and attenuated the inflammatory response. Targeting hepatic miR-873-5p may represent a novel therapeutic approach for the treatment of ALD.

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Research article

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Anti-miR-873-5p improves alcohol-related liver disease by enhancing hepatic deacetylation via SIRT1

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Background & Aims: Current therapies for the treatment of alcohol-related liver disease (ALD) have proven largely ineffective. Patients relapse and the disease progresses even after liver transplantation. Altered epigenetic mechanisms are characteristic of alcohol metabolism given excessive acetate and NAD depletion and play an important role in liver injury. In this regard, novel therapeutic approaches based on epigenetic modulators are increasingly proposed. MicroRNAs, epigenetic modulators acting at the post-transcriptional level, appear to be promising new targets for the treatment of ALD.
Methods: MiR-873-5p levels were measured in 23 liver tissue from Patients with ALD, and GNMT levels during ALD were confirmed using expression databases (transcriptome n = 62, proteome n = 68). High-resolution proteomics and metabolomics in mice following the Gao-binge model were used to investigate miR-873-5p expression in ALD. Hepatocytes exposed to 50 mM alcohol for 12 h were used to study toxicity. The effect of anti-miR-873-5p in the treatment outcomes of ALD was investigated.
Results: The analysis of human and preclinical ALD samples revealed increased expression of miR-873-5p in the liver. Interestingly, there was an inverse correlation with NNMT, suggesting a novel mechanism for NAD depletion and aberrant acetylation during ALD progression. High-resolution proteomics and metabolomics identified miR-873-5p as a key regulator of NAD metabolism and SIRT1 deacetylase activity. Anti-miR-873-5p reduced NNMT activity, fuelled the NAD salvage pathway, restored the acetylome, and modulated the levels of NF-κB and FXR, two known SIRT1 substrates, thereby protecting the liver from apoptotic and inflammatory processes, and improving bile acid homeostasis.
Conclusions: These data indicate that targeting miR-873-5p, a repressor of GNMT previously associated with NAFLD and acetaminophen-induced liver failure. is a novel and attractive approach to treating alcohol-induced hepatoxicity.

Keywords: Alcohol-related liver disease; NIAAA model; microRNA; SIRT1; Nicotinamide adenine dinucleotide salvage pathway.
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Impact and implications: The role of miR-873-5p has not been explicitly examined in the progression of ALD, a pathology with no therapeutic options. In this study, inhibiting miR-873-5p exerted hepatoprotective effects against ALD through rescued SIRT1 activity and consequently restored bile acid homeostasis and attenuated the inflammatory response. Targeting hepatic miR-873-5p may represent a novel therapeutic approach for the treatment of ALD.

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Introduction

NAD is an important cofactor or substrate in a variety of redox and non-redox reactions.[1] Given the critical role of NAD in the biological functions of the cell, its life cycle is finely tuned. Although most NAD is recycled via the salvage pathway, its *de novo* synthesis occurs via the kynurenine pathway, starting from tryptophan (Trp) and the Preiss–Handler pathway. Interestingly, external metabolic stimuli can reprogramme NAD homeostasis, and its deficiency has been linked to ageing and diseases such as cancer.[2] Therefore, maintaining adequate redox homeostasis is critical for cell survival.

Alcohol-related liver disease (ALD) is a complex and multistage disorder that is considered one of the most common liver pathologies and the main cause of liver transplantation.[3] This disease is characterised by an altered redox state primarily caused by the hepatic oxidation of alcohol, resulting in increased NADH levels and the overproduction of toxic acetaldehyde and acetate.[4] The increased NADH/NAD^+ ratio leads to mitochondrial overactivation, in which respiration and oxygen consumption nearly double to restore NAD^+, whereas simultaneously attempting to metabolize toxic acetaldehyde. This is known as the Swift Increase in Alcohol Metabolism (SIAM) pathway.[5]

Chronic alcohol consumption modulates both metabolic and epigenomic homeostasis. Chronic alcohol consumption promotes the increase in acetyl-CoA as a degradation product of acetaldehyde, leading to abnormal acetylation and altered enzymatic activity. Furthermore, it overwhelms the oxidative capacity of mitochondria and impairs the oxidation of NADH and acetaldehyde, which accumulates and promotes the overproduction of reactive oxygen species (ROS).[6] The absence of NAD^+ disrupts the tricarboxylic acid cycle and fatty acid oxidation, promoting lipid synthesis and accumulation, which ultimately contributes to lipid peroxidation and additional ROS generation. [4]

Although alcohol abstinence is the cornerstone of early ALD care, most patients fail to comply. Few suboptimal therapeutic options for ALD are available, apart from liver transplantation[3,7], although there are few organ donors available. Thus, new therapeutic approaches are needed. In this direction, small non-coding microRNAs (miRNAs) are epigenetic modulators whose expression is altered in ALD[8], and whose regulation may open a new window for further exploration of their role in the treatment of ALD.

Previous work from our laboratory has shown that miR-873-5p post-transcriptionally modulates glycine N-methyltransferase (GNMT), the major hepatic methyltransferase for S-adenosylmethionine (SAMe). Its downregulation disrupts mitochondrial activity in non-alcoholic steatohepatitis (NASH).[9] Considering the common pathogenic features of non-alcoholic fatty liver disease (NAFLD) and ALD[10,11] and the involvement of miR-873-5p in several liver diseases[9,12] and hepatotoxicity[13], we explored the potential role of miR-873-5p in ALD.

This study shows that miR-873-5p is overexpressed in liver samples from patients with ALD and in several preclinical mouse models mimicking this pathology. By analysing unrelated human transcriptome and proteome databases, we found that GNMT levels were reduced during the progression of ALD, whereas nicotinamide N-methyltransferase (NNMT), another methyltransferase that consumes SAMe and the NAD precursor nicotinamide (NaM)[14], was increased, showing a key intersection between cellular metabolism and epigenetic regulation. The negative modulation of miR-873-5p is hepatoprotective in both *in vitro* and *in vivo* ALD models, fuelling NAD production and subsequently activating SIRT1 deacetylase activity. In fact, the recovery of the acetylome regulates key proteins such as NF-KB and Farnesoid X receptor (FXR), which exert antiapoptotic and anti-inflammatory effects. In ALD, the restoration of GNMT by the inhibition of miR-873-5p prevents NNMT activity and subsequent NaM oxidation, leading to NAD renewal via the salvage pathway.[14] Hepatoprotection by anti-miR-873-5p is counteracted when SIRT1 activity and the NAD salvage pathway are inhibited. Overall, our study showed that miR-873-5p is involved in epigenetic alterations and mediates the progression of ALD, thus supporting its application as a novel therapeutic target.

Materials and methods

Animal models and experimental protocol

CIC bioGUNE Animal Care and Use Committee and the local authority (Diputación de Bizkaia) in compliance with the European Union guidelines approved the animal procedures protocols. To apply the DUAL and ethanol-binge (NIAAA model) dietary mouse model of ALD described previously[15], male C57BL/6J mice (n = 12; 3 months old) were purchased from Charles River (St Germain sur l'Arbresle, France). Following a five-day acclimation period, the mice were divided into two groups. The first group (n = 8) received a 10-day *ad libitum* diet of 5% liquid ethanol, with subgroups receiving either anti-miR-873-5p or miR-control injections on days 4 and 8. The second control group (n = 4) received an isocaloric control diet. On day 11, mice were orally gavaged with ethanol or control solution containing FitC-dextran for permeability analysis. After 9 h, the mice were euthanized, and blood and liver specimens were collected for further analysis.

Human samples

In this study, human samples were obtained from various sources. For the analysis of has-miR-873-5p expression levels were determined as previously described;[16] human liver samples were obtained from the Hospital Clinic of Barcelona. The gene expression profile was obtained from a study conducted by Argemi *et al.*[17] Protein abundance data was sourced from an unrelated database provided by Niu *et al.*[18] All patients involved in the clinical study provided their consent in accordance with the principles of the Declarations of Helsinki and Istanbul.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad software, v.9.2.0, CA, USA), and values were

expressed as mean ± SD. Groups were compared by ANOVA, followed by *post-hoc* Bonferroni tests (for three or more groups) or Student's t tests (for two groups). A p value <0.05 was considered statistically significant. For further details regarding the materials and methods used, please refer to the CTAT Table and Supplementary Data.

Results

Targeting miR-873-5p in primary hepatocytes increased viability and exerted protective effects against alcohol-induced stress

Our group has previously shown that miR-873-5p is involved in mitochondrial dysfunction during the progression of NAFLD, and has also provided evidence supporting anti-miR-873-5p as a valuable therapeutic for many chronic liver diseases.[9] ALD, one of the most common liver diseases and a leading cause of liver transplantation, currently lacks effective treatment. Since mitochondrial dysfunction is one of the first signs of alcohol-induced liver injury[19], we explored the implication of miR-873-5p in ALD. Primary mouse hepatocytes incubated with 50 mM ethanol (EtOH) for 12 and 24 h showed upregulation of miR-873-5p expression (Fig. 1A) and downregulation of *Gnmt* mRNA (Fig. 1B) and protein (Fig. 1C). In line with previously reported findings[9,13], exposure to anti-miR-873-5p resulted in a partial reduction of miR-873-5p, leading to an increase in cellular *Gnmt* content (Fig. 1D and E).[9,13] Anti-miR-873-5p also reduced the extent of ethanol-induced cell death, a major feature of alcohol-induced liver injury[20], as determined by the TUNEL assay, Trypan blue staining (Fig. 1F and G), and expression of cell death markers (Fig. S1A).

Cell death is a highly coordinated phenomenon involving multiple signalling pathways, including oxidative stress, endoplasmic reticulum (ER) stress, and mitochondrial dysfunction.[21,22] As oxidative stress is a by-product of ethanol metabolism and a driver of cell injury, mitochondrial ROS and CYP2E1 activity were analyzed.[23,24] A decrease in mitochondrial ROS levels, determined by MitoSOX staining (Fig. 1H), and reduced CYP2E1 oxidative activity (Fig. 1I) were detected in the anti-miR-873-5p-treated hepatocytes exposed to ethanol, supporting its protective role.

Increased ROS production, including that induced by CYP2E1 activity, can directly affect the ER, promoting ER stress and even increasing K+ channels.[25,26] Therefore, we used a specific ER-tracker to stain K^+ channels and measured the ER response to alcohol exposure. The staining showed that the ethanol-induced ER perturbations were reversed when we negatively modulated miR-873-5p levels (Fig. 1J), which was also confirmed by changes in other ER stress signalling markers (Fig. S1B).[26] To study ER functionality, we examined Ca^{2+} release capacity using the method described by Grynkiewicz *et al.* in primary hepatocytes exposed to ethanol[27] with a specific Fura-2 acetoxymethyl (AM) ester label. Anti-miR-873-5p provided more ER protection than miR-Control in response to thapsigargin after an ethanol insult, as demonstrated by increased Ca2+ release[28] (Fig. 1K).

Interestingly, lipid droplet accumulation was partially reversed in anti-miR-873-5p-treated hepatocytes (Fig. S1C). Fructose was then added as another steatogenic source during EtOH exposure, as the combined effect poses a major challenge for the cells. Both steatosis and ER stress were ameliorated with anti-miR-873-5p (Fig. S1D and E).

Altogether, the inhibition of miR-873-5p reduced ethanol-induced cell death and attenuated ER stress and the progression of steatosis in primary hepatocytes.

Knockdown of miR-873-5p protected from chronic ethanol-induced liver injury *in vivo*

Regarding *in vivo* ALD models, the dysregulation of miR-873-5p was also confirmed in the DUAL and ethanol-binge (NIAAA model) dietary mice model. The DUAL model synergistically combined the effects of alcohol in the form of 10% vol/vol alcohol in sweetened drinking water and the Western diet for 10 and 23 weeks[1], whereas the ethanol-binge mice model described by Gao consists of 10 days of *ad libitum* oral feeding with a Lieber–DeCarli liquid ethanol diet (5% volume) and an ethanol-binge on day 11 (31.5 % volume).[29] As previously established, miR-873-5p specifically targets *Gnmt* expression in mouse hepatocytes.[12] Consistent with these findings, our current study shows a progressive increase in miR-873-5p expression in the liver of the different mouse models compared with the liquid diet control group, which was accompanied by a corresponding decrease in GNMT levels (Fig. 2A-C and Fig. S2A-C).

Given the promising results obtained in primary hepatocytes, the 10-day Gao-binge mouse model mimicking the early stage of ALD was used to investigate the potential benefits of anti-miR-873-5p administration *in vivo*. On days 4 and 8, mice were treated with either 60 μg anti-miR-873-5p, or an unrelated miR-control conjugated with *invivofectamine* via tail vein injection (Fig. S2D). The significantly reduced expression of miR-873-5p was confirmed in anti-miR-873-5p-treated mice, together with increased *Gnmt* mRNA and protein expression (Fig. 2D and E and Fig. S2E and F). No differences were observed in liver weight (Fig. S2G).

Moreover, ethanol-fed anti-miR-873-5p mice exhibited less liver damage, measured by TUNEL staining, haematoxylin and eosin, caspase-3 activity assays, Western blotting, and ApopTag staining (Fig. 2F–I and Fig. S2I), which was supported by lower BAX and higher BCL-2 levels (Fig. S2J and K). The analysis of serum transaminases showed that treatment with anti-miR-873-5p decreased the levels of alanine transaminase and aspartate transaminase, both known as serum markers of liver damage (Fig. 2J).

Regarding ethanol metabolism, anti-miR-873-5p-exposed mice showed a reduction in hepatic ethanol and a tendency towards reduced acetaldehyde levels (Fig. 2K and L). Together with alcohol dehydrogenase (ADH), CYP2E1 and catalase can also mediate ethanol oxidation.[24] As opposed to ROS-producing CYP2E1, catalase is an antioxidant enzyme that converts hydrogen peroxide into water and oxygen.[4] Interestingly, the activity of catalase was increased in mice with miR-873-5p silencing (Fig. 2M). In relation to the redox status, we found a decreased ratio of reduced glutathione (GSH)/oxidised glutathione disulfide (GSSG) in mice fed with ethanol, which showed a recovery trend in anti-miR-873-5p-treated mice (Fig. 2N). Furthermore, the relative mRNA expression levels of inflammatory markers (*Il6, Il1b, Cxcl1, Ccl2*) were decreased in the treatment group (Fig. 2O).

To investigate whether the hepatoprotective mechanism of miR-873-5p alleviates ER stress, we examined different markers involved in this signalling pathway. Anti-miR-873-5p inhibited the activation of IRE1a (Fig. 2P and Fig. S2O), affecting Xbp1

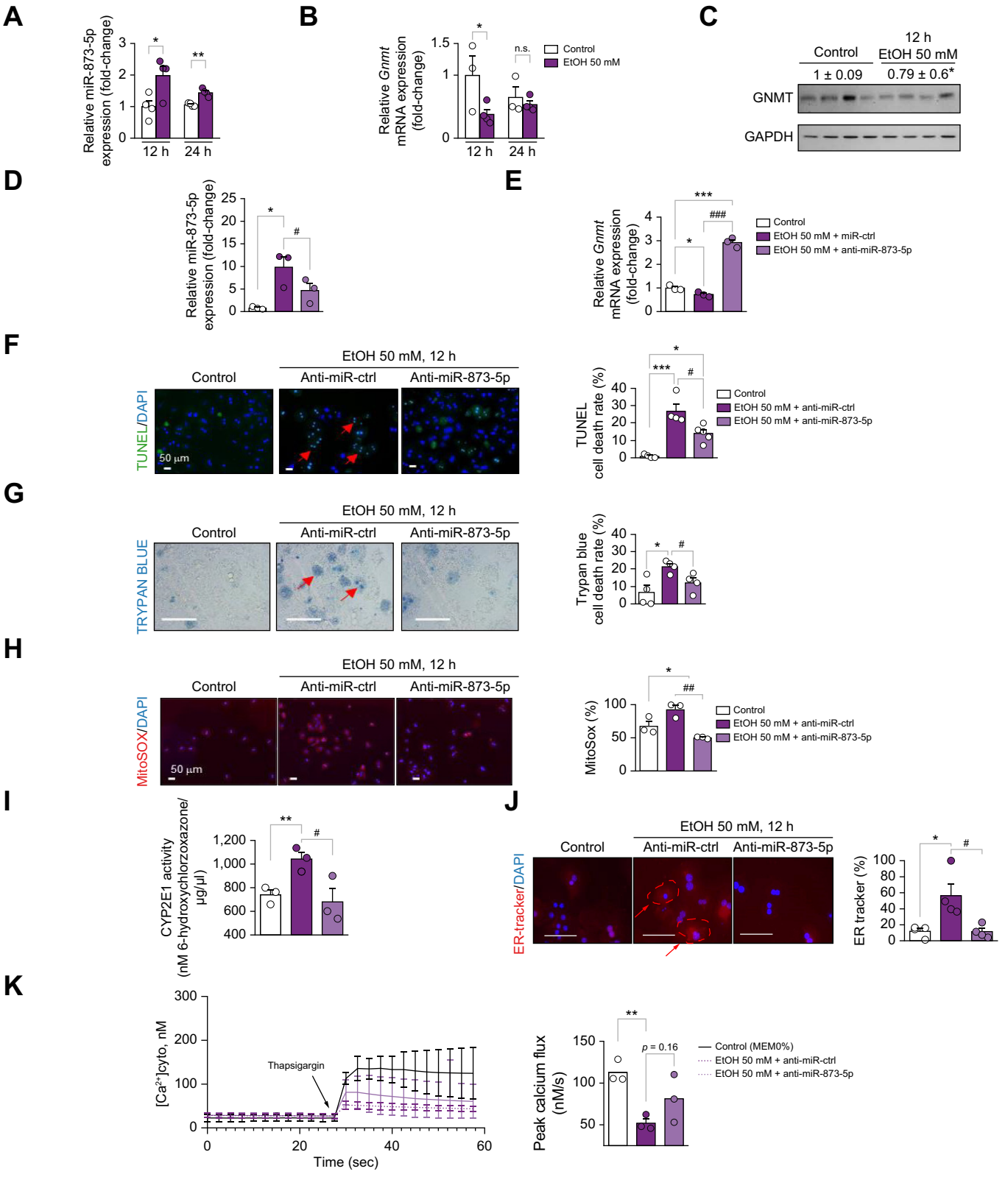

Fig. 1. Anti-miR-873-5p protects against alcohol toxicity in primary hepatocytes exposed to 50 mM EtOH for 12 h. (A) Relative mRNA expression of miR-873-5p in primary hepatocytes after 12 and 24 h with 50 mM ethanol. GNMT downregulation was also confirmed by (B) mRNA expression and (C) western blotting. (D) Cell death rates determined by TUNEL staining (blue colour for nuclear saining DAPI/green colour for TUNEL positive nuclei. Red arrows indicate positive apoptotic nuclei) and (E) Trypan blue (the blue staining passes through porous membranes of dead cells). (F) Mitochondrial ROS production measured by MitoSox (blue colour for nuclear staining DAPI, red colour for MitoSOX staining). (G) CYP2E1 activity in primary hepatocytes measured by 6-hydroxychlorzoxazone production. (H) ER stress was measured with ER-tracker dye (blue indicating nuclear staining with DAPI and red indicating ER -tracker staining). Two

expression (Fig. 2Q). In addition, other ER stress markers such as *Atf4, Atf6,* and *Xbp1s* were decreased in ethanol-fed mice treated with anti-miR-873-5p (Fig. 2Q and Fig. S2O).

Since alcohol disrupts lipid metabolism and leads to lipid accumulation[30,31], we studied the effects of anti-miR-873-5p on lipid metabolism. Silencing of miR-873-5p reduced liver steatosis, as measured by Sudan red staining, hepatic triglycerides, and cholesterol levels (Fig. 2R-T and Fig. S2P).

Consequently, the inhibition of miR-873-5p facilitates ethanol clearance, promotes cell survival, and shows anti-oxidant and anti-inflammatory effects, and ultimately prevents steatosis.

Proteomic insights into the Gao-binge mouse model treated with an miR-873-5p antagonist

To further characterise the mechanism by which anti-miR-873-5p therapy improves ALD, we mapped the proteome of ethanol-binge mice model liver tissues using Liquid chromatography–mass spectrometry (LC-MS) technology. The overall differences in the proteome between groups are represented as a volcano plot in Fig. 3A and Fig. S3A. In total, 959 proteins were detected in the liver as being differentially expressed between the non-treated and anti-miR-control ethanol-fed group. Specifically, 223 proteins differed in expression between the anti-miR-873-5p and anti-miR-control groups; a heatmap of the 25 top upregulated and downregulated proteins resulting from miR-873-5p silencing is shown in Fig. 3B. An in-depth study using Ingenuity Pathway Analysis (IPA) revealed the canonical pathways affected in mice receiving an alcohol-fed diet compared with control mice. Processes affected included the sirtuin signalling pathway, NAD, glutathione metabolism, and fatty acid metabolism (Fig. S3B). Considering the predicted activation, chronic ethanol consumption activates signalling pathways such as EIF2, nicotine degradation, or mTOR and decreases activity in insulin secretion/receptor signalling or cholesterol biosynthesis (Fig. S3C). In addition, ethanol degradation has some impact in this model, as it is predicted to increase acetate, NADH, and acetyl-CoA levels (Fig. S3D) as described previously.[4]

IPA analysis revealed the canonical pathways with proteins differentially expressed in the miR-Ctrl and anti-miR-873-5p groups. Among them, NAD synthesis and sirtuin signalling pathways were highlighted (Fig. 3C). Furthermore, considering their activation z-score, ethanol degradation II, xenobiotic metabolism by PXR and AHR, and the sirtuin signalling pathway were the canonical processes activated by anti-miR-873-5p therapy in the ethanol-binge mice model (Fig. 3D and Fig. S3E). In contrast, necroptosis, liver fibrosis, ferroptosis, IL-8, and EIF2 signalling pathways appeared to be diminished in the anti-miR-873-5p group of the ethanol-binge mice model compared with the anti-miR-control group (Fig. 3D). Interestingly, the IPA results suggest SIRT1 as one of the top predicted upstream regulators in the anti-miR-873-5p group (z-score 2.385) (Fig. 3E and Fig. S3F).

These findings suggest that alcohol degradation and sirtuin signalling pathways are important mechanisms affecting anti-miR-873-5p-mediated protection.

Anti-miR-873-5p modulated SIRT1 activity and the acetylome in ALD

It is known that the activity of SIRT1 is impaired with the progression of ALD.[32] Considering that SIRT1 may be the upstream regulator of the hepatoprotective effects observed in the anti-miR-873-5p treatment group and given the importance of SIRT1 during ALD progression[33], we examined the role of SIRT1 in anti-miR-873-5p-treated and anti-miR-control ethanol-fed mice. The treated group exhibited higher hepatic SIRT1 activity compared with miR-control mice, as shown in Fig. 4A, with no changes in *Sirt1* mRNA expression levels (Fig. 4B), suggesting that anti-miR-873-5p has a transcriptional-independent effect. Given that SIRT1 is an important deacetylase enzyme, we performed a comprehensive acetylome analysis. As depicted in Fig. 4C, treatment with anti-miR-873-5p led to a significant reduction in the acetylated proteome in the livers of the ethanol-binge mouse model. Furthermore, mass spectrometry analysis revealed 10 acetylated proteins with a mass addition of +42.01 that displayed significant differences between the control and the anti-miR-control groups, and five proteins between the ethanol groups treated with or without anti-miR-873-5p (Fig. 4D and E and Table S6).[34,35]

Enhanced SIRT1 activity restored bile acid and inflammation homeostasis through its direct targets

The maintenance of adequate bile acid (BA) homeostasis is essential. Patients with advanced ALD show a shift in their BAs pool towards more toxic species, together with cholestasis and increased plasma levels of BAs.[35–37] Farnesoid X receptor (FXR) plays a key role in the aforementioned phenotype[35], as chronic alcohol consumption causes the hyperacetylation of FXR, altering its activity.[35,38] The silencing of miR-873-5p in ethanol-fed mice resulted in decreased FXR acetylation levels (Fig. 5A), which is consistent with the restored expression pattern of BA synthesis genes (Fig. 5B). The liver BA composition in ethanol-fed mice treated with anti-miR-873-5p showed increased levels of hydrophilic species, such as alpha-murocholate; beta-MCA; and omega-MCA (non-conjugated), and tauro-alpha-MCA (conjugated) (Fig. 5C and Fig. S5A), potentially attenuating the toxicity of BAs. On the other hand, anti-miR-873-5p restored primary conjugated glycocholic acid and taurocholic acid, together with secondary conjugated BAs (taurodeoxycholic acid, tauroursodeoxycholic acid and taurolitholic acid), to their control levels (Fig. 5D and E, Fig. S5A).

In addition, the mRNA levels of *Ntcp*, a BA uptake transporter[35], were restored in the anti-miR-873-5p group (Fig. 5F), which could facilitate the uptake of BAs from serum, suggesting that anti-miR-873-5p can prevent the EtOH-induced serum overaccumulation of BAs (Fig. 5G and Fig. S5A). This idea is also sustained by the trend seen in major secondary BAs species in the livers of anti-miR-873-5p mice (Fig. 5H). Similarly, anti-miR-873-5p restored the expression of the transporters *Mdr1* and *Mrp2,* whereas the expression of *Mrp3* was not affected (Fig. 5F). These data suggest that anti-miR-873-5p promotes proper homeostasis in the enterohepatic circulation.

positive cells have been highlighted by red arrows and the intense area is demarcated by a dotted line. (I) ER calcium release after addition of thapsigargin followed by maximal release. For all graphical bar charts, upper bars represent SD. Student's t tests were used for statistical analysis between two groups and one-way ANOVA, followed by *post hoc* Bonferroni tests for three or more groups. Statistical significance is represented as $*p$ <0.05; $**p$ <0.01 and $***p$ <0.001 *vs.* Ctrl are shown; #*p* <0.05; ##*p* <0.01 and ###*p* <0.001 *vs.* miR-Ctrl are shown.ER, endoplasmic reticulum; GNMT, glycine N-methyltransferase; ROS, reactive oxygen species.

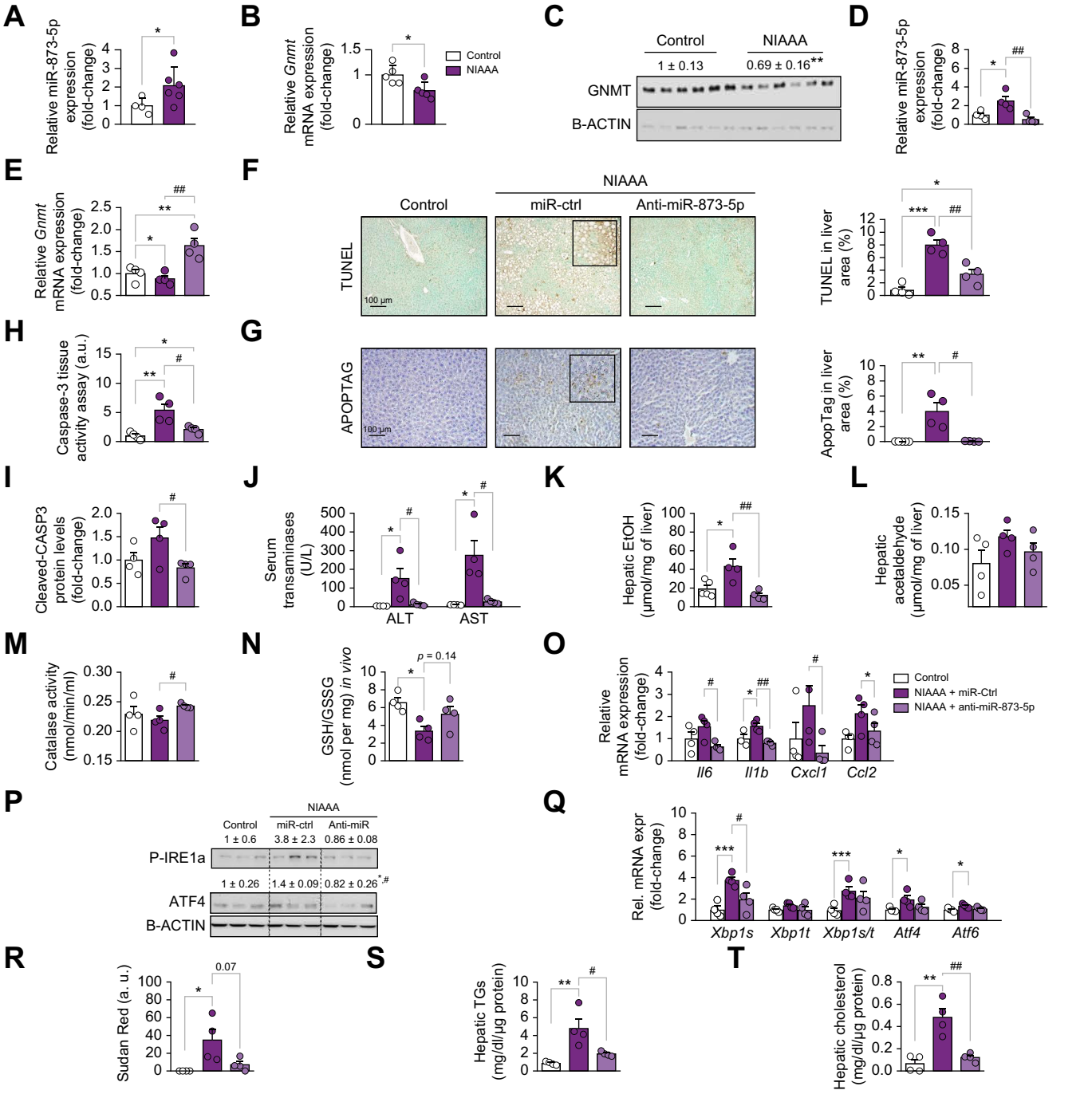

Fig. 2. Anti-miR-873-5p protects against alcohol toxicity in a mouse model of chronic and binge ethanol consumption. (A) The relative miRNA expression of miR-873-5p in mice with chronic ethanol consumption. Anti-miR-873-5p effect on *Gnmt* was confirmed by (B) mRNA expression and (C) protein levels. Transfection with anti-miR-873-5p effect is shown by (D) miRNA levels analysis and its effect on *Gnmt* was also confirmed by (E) mRNA expression. (F and G) Liver damage resulting from cell death was demonstrated by TUNEL staining and ApopTag staining of the liver parenchyma. Magnification of the image in the anti-miR control condition allows differentiation of staining between nuclear/cytoplasmic regions. (H) Caspase 3 activity as a marker of cell death was used (I) Western blotting analysis of cleaved Caspase 3. (J) Activity of serum transaminases: alanine aminotransferase and aspartate aminotransferase. Analysis of alcohol metabolism by measuring levels of (K) ethanol and (L) acetaldehyde. (M) Catalase activity assay and (N) GSH/GSSG ratio as markers of inflammatory response. (O) relative mRNA expression of the proinflammatory markers IL6 and IL1b. ER stress markers were measured by (P) western blotting and (Q) mRNA expression. Quantification of lipid metabolism was performed by (R) Sudan red staining quantified from liver parenchyma staining, furthermore, quantification of (S) liver triglycerides and (T) cholesterol. For all graphical bar charts, upper bars represent SD. Student's t tests were used for statistical analysis between two groups and one-way ANOVA, followed by *post hoc* Bonferroni tests for three or more groups. Statistical significance is represented as $^{*}p$ <0.05; $^{**}p$ <0.01 and $^{***}p$ <0.001 *vs.* Ctrl are shown; #*p* <0.05; ##*p* <0.01 and ###*p* <0.001 *vs.* miR-Ctrl are shown. GSH/GSSG ratio, reduced glutathione /oxidised glutathione disulphide ratio.

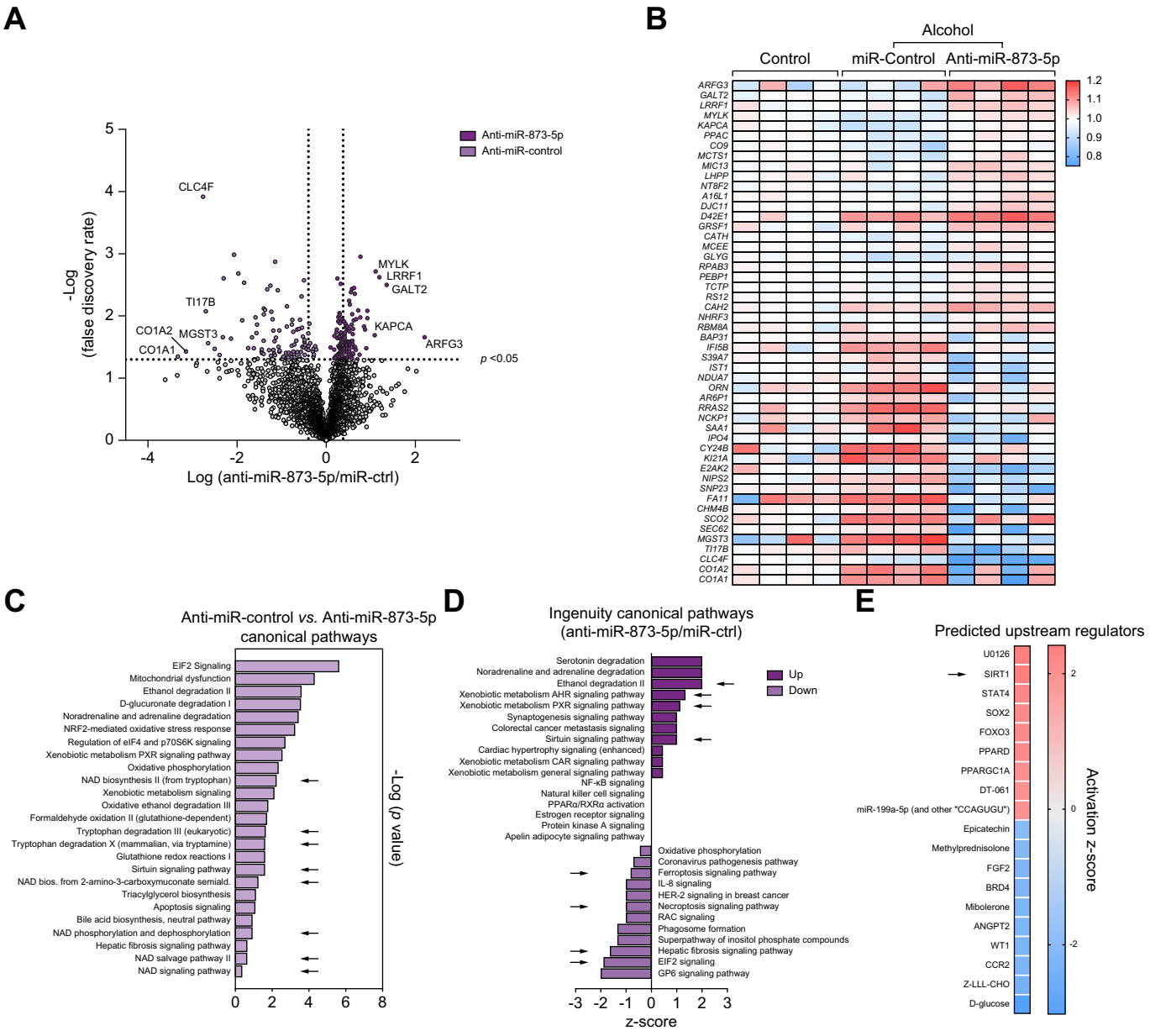

Fig. 3. Proteomic profile in ethanol-binge mice model, anti-miR-873-5p *vs.* miR-control effects. (A) Volcano plot of total proteins detected with fold change over ±0.8 (red: upregulated, for anti-miR-873-5p group and blue: downregulated proteins for miR-control group) between miR control and anti-miR-873-5p. (B) Heatmap showing the top-50 up- and downregulated proteins between miR control and anti-miR-873-5p, including proteins from control group (C) Canonical pathways of proteins selected based on fold change and statistically significant between groups (p <0.05). (D) The 29 most enriched canonical pathways analysed by IPA determined by z-score (anti-miR-873-5p *vs.* anti-miR-control), red indicates pathways predicted to be activated under anti-miR-873-5p administration. (E) Top upstream regulators, arrow highlights SIRT1 whose activation is predicted. IPA, Ingenuity Pathway Analysis.

Regarding inflammation, alterations in the function of the NF-κB regulatory complex are observed in alcoholic liver disease.[39] Naturally, acetylation affects NF-κB activity.[20] In this study, anti-miR-873-5p restored acetylated NF-κB back to control levels (Fig. S5B), possibly via SIRT1 activity[40], as is consistent with an improvement in the inflammatory response observed in the ethanol-binge mice model.

These results suggest that treatment with anti-miR-873-5p activates SIRT1 deacetylase activity, restores the acetylome, and modulates targets such as FXR and NF-κB involved in the physiopathology of ALD.

NAD synthesis and recycling increased SIRT1 activity in ethanol-fed mice

As mentioned above, SIRT1 is a NAD-dependent deacetylase, and so it relies on NAD to perform its function. NAD, which can either be oxidised to NADH or recycled to NaM via the salvage pathway, is depleted by alcohol metabolism, decreasing the overall NAD/NADH ratio and subsequently several metabolic pathways. Consistent with the literature[2,41,42] and in line with the results obtained in the IPA analysis, treatment with anti-miR-873-5p increased NAD+ (Fig. 6A) and decreased NADH levels (Fig. 6B) in ethanol-fed mice, leading to a restored

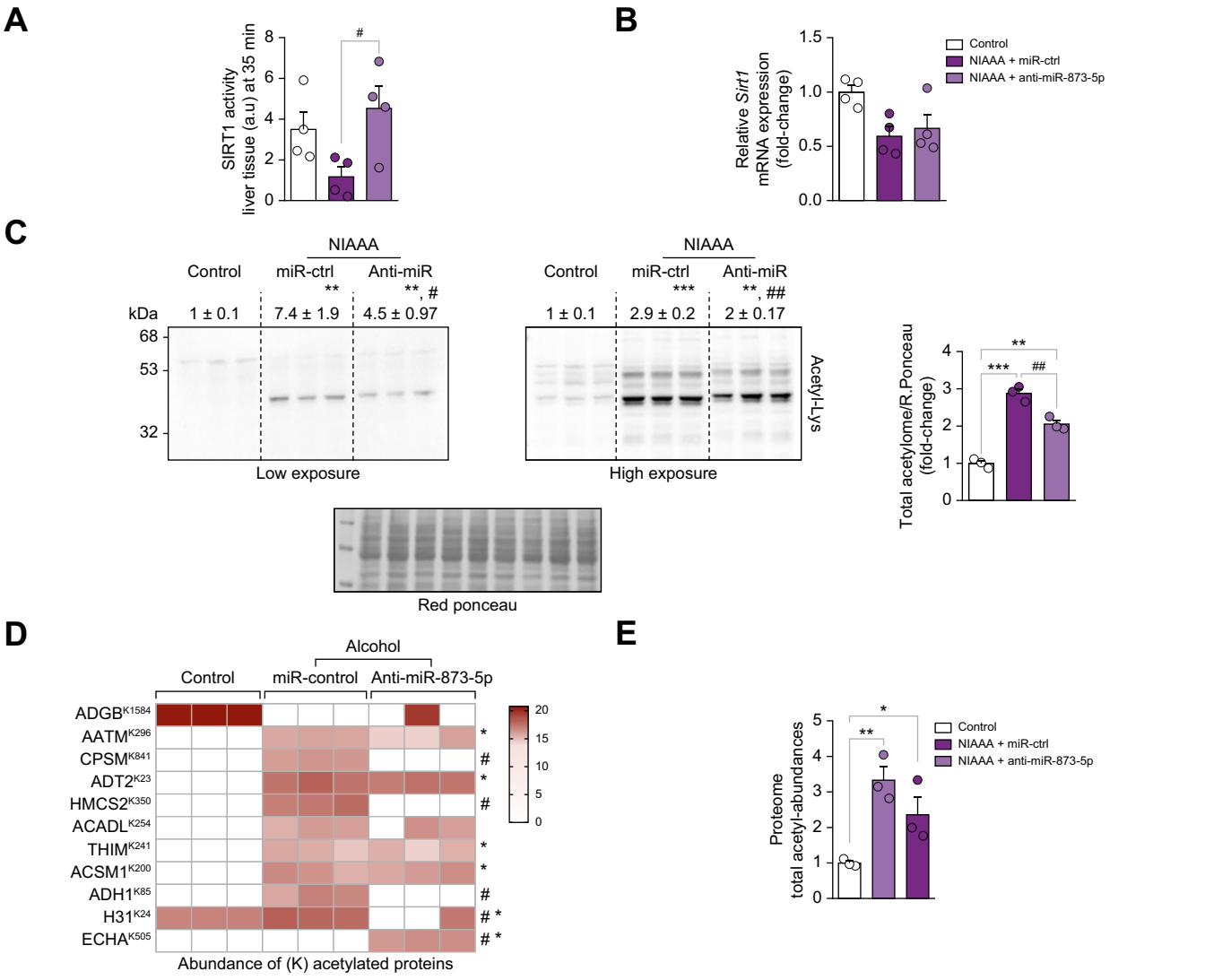

Fig. 4. SIRT1 activity is enhanced by anti-miR-873-5p, resulting in changes throughout the acetylome. (A) Nuclear SIRT1 activity from liver tissue and its (B) corresponding relative mRNA levels between mice groups. (C) Acetyl-lysine western blotting and quantification by densitometry of whole liver tissue lysate. Red Ponceau was used as a loading control. (D) Heatmap analysis of acetylation-related proteins (+42 Da shift) in proteomics in the ethanol-binge mice model detected by LC-MS and (E) Total sum of all abundances corresponding to the acetylated proteins. For all graphical bar charts, upper bars represent SD. The statistical analysis between groups for all graphical charts represented is one-way ANOVA, followed by *post-hoc* Bonferroni tests for three or more groups. Statistical significance is represented as $^{*}p$ <0.05; $^{**}p$ <0.01 and $^{***}p$ <0.001 *vs.* Ctrl are shown; #*p* <0.05; ##*p* <0.01 and ###*p* <0.001 *vs.* miR-Ctrl are shown.LC-MS, liquid chromatography–mass spectrometry.

NADH/NAD ratio (Fig. 6C). In addition, genetic analysis showed that the *de novo* and recycling (or salvage) pathways were upregulated in the anti-miR-873-5p treatment group compared with the miR-control group (Fig. 6D). The same phenotype was observed in ethanol-treated primary hepatocytes when miR-873-5p expression was silenced (Fig. 6E), suggesting that the increased SIRT1 activity may be driven by increased NAD synthesis.

Nnmt, a methyltransferase that degrades NaM using SAMe in response to ER stress[43], appears to be increased during ALD progression. Treatment with anti-miR-873-5p prevented this upregulation (Fig. 6F) and decreased its activity (Fig. 6G) in the ethanol-binge mice model. This methyltransferase determines the link between NAD metabolism and the methionine cycle (Fig. S6A), and its elevation is associated with worse effects.[44]

In line with this evidence, the quantitative analysis of liver metabolites from NAD synthesis and recycling revealed increased Trp, decreased nicotinamide mononucleotide (NaMN), and increased nicotinamide (NaM) levels, two precursors of NAD via the salvage pathway, upon treatment with anti-miR-873-5p (Fig. 6H). These effects are expected when NNMT is downregulated.

To further confirm that both sirtuin (SIRT1) activity and the nicotinamide adenine dinucleotide (NAD) salvage pathway are essential to the hepatoprotective effect of anti-miR-873-5p, we modulated SIRT1 and nicotinamide phosphoribosyltransferase (NAMPT) activities with EX527 and FK866 inhibitors, respectively. The ability of anti-miR-873-5p to exert protection in primary hepatocytes was largely abrogated when SIRT1 and NAMPT activities were blocked (Fig. 6I).

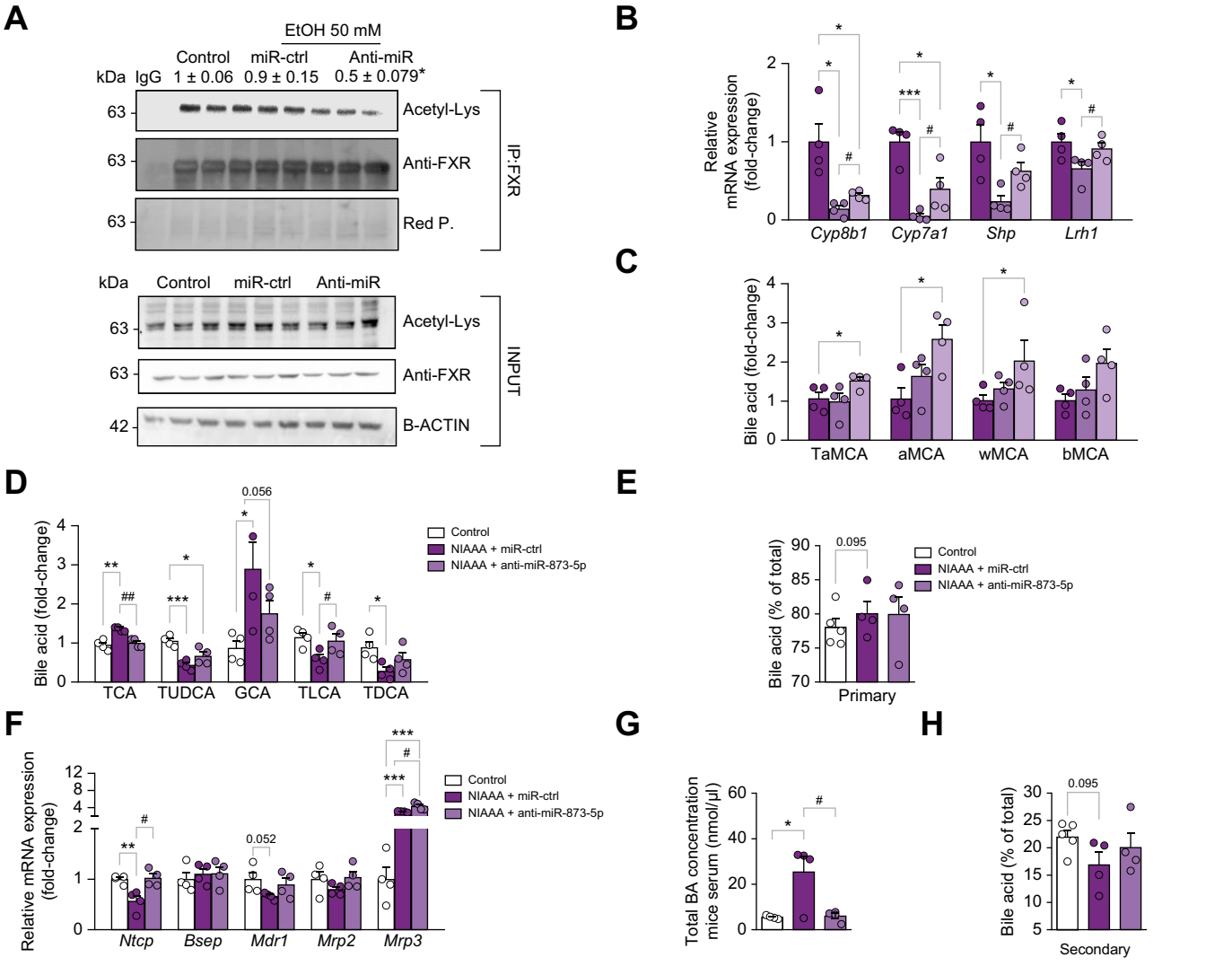

Fig. 5. SIRT1 activity deacetylates FXR and restores BA homeostasis. (A) Immunoprecipitation analysis of primary hepatocytes using beads conjugated with anti-FXR antibody. IgG was used as a negative control. Western blotting to evaluate acetyl-lysine levels was then performed. (B) The effect of SIRT1 and FXR was analysed by mRNA expression of markers involved in liver BA homeostasis. (C) BA species potentiated and (D) restored by anti-miR-873-5p (E) Total primary BA species. (F) Relative mRNA expression of some of the major BA transporters and (G) Total amount of circulating BAs. (H) Total amount of secondary BAs. For all graphical bar charts, upper bars represent SD. The statistical analysis between groups for all graphical charts represented is one-way ANOVA, followed by *post-hoc* Bonferroni tests for three or more groups. Statistical significance is represented as $^{*}p$ <0.05; $^{**}p$ <0.01 and $^{***}p$ <0.001 *vs.* Ctrl are shown; #*p* <0.05; ##*p* <0.01 and ###*p* <0.001 *vs.* miR-Ctrl are shown. BA, bile acid; FXR, farnesoid X receptor.

Overall, anti-miR-873-5p treatment rescued the NAD salvage pathway, increased both NAD synthesis and SIRT1 deacetylase activity, and restored the acetylome, delaying ALD progression. Therefore, our study introduces anti-miR-873-5p as a novel therapeutic approach to ALD.

Dysregulation of miR-873-5p and its targets was associated with impaired NAD metabolism and upregulation of NNMT in patients with ALD

To investigate whether miR-873-5p could also be associated with ALD progression in humans, we measured the expression of miR-873-5p in liver biopsies from control subjects (healthy tissue from resected patients) (n = 8) and patients with ALD (n = 15). A marked elevation in miR-873-5p levels was observed in the livers of patients with ALD when compared with healthy controls. Simultaneously, there was a notable decrease in *GNMT*, affirming a distinct inverse relationship in human subjects (Fig. 7A). Furthermore, the analysis of another cohort from an independent data repository showed significantly reduced levels of *GNMT* during the progression of ALD (Fig. 7B). These results are consistent with previous reports on the modulation of one-carbon metabolism in this chronic liver disease.[9,12]

Decreased *SIRT1* mRNA expression was also found in livers from patients with ALD relative to healthy controls (Fig. 7C). The progression of ALD is known to impair SIRT1 activity[32], which could be related to defects in NAD metabolism. In this sense, we observed that most of genes related to the synthesis or recycling of NAD were affected during the progression of ALD in patients (Fig. 7D), and the proteome database analysis showed that some enzymes were also affected at advanced stages of ALD (Kleiner score)[45] (Fig. 7E).

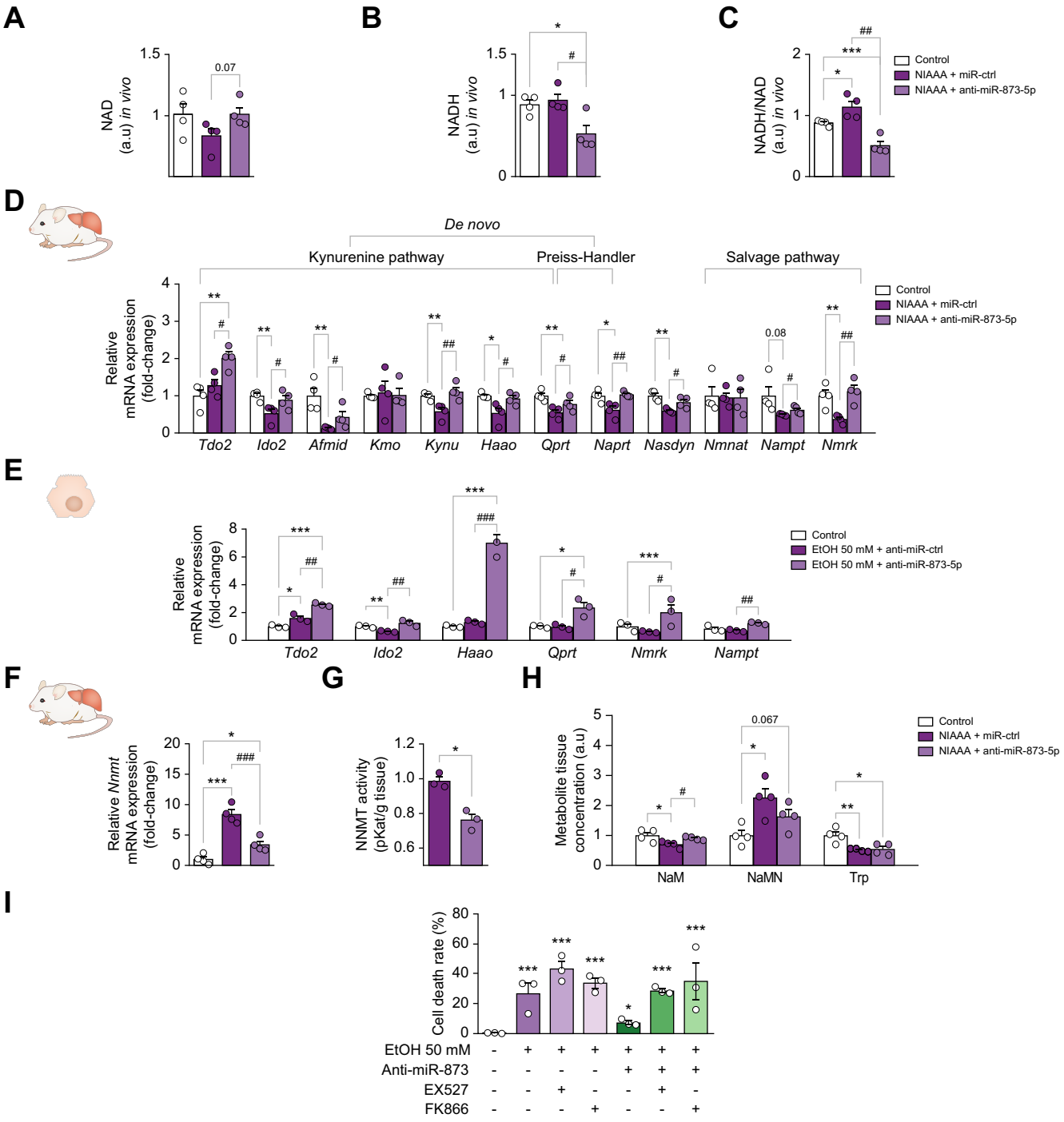

Fig. 6. The supply of NAD is increased in the liver of ethanol fed mice with anti-miR-873-5p. (A) Measured NAD levels and its reduced form in the liver of mice with ethanol consumption (B) NADH, then its (C) NADH/NAD ratio. (D) mRNA expression of enzymes from Kynurenine, Preiss-Handler, and NAD Salvage metabolism in samples from the ethanol-binge mice model. (E) mRNA expression of some major enzymes of NAD metabolism in primary hepatocytes. Furthermore, in livers from the ethanol fed mice, we measured the (F) *Nnmt* Relative mRNA levels and (G) activity (Student's t tests). (H) Means LC / MS analysis of metabolites involved in the synthesis of NAD from the liver of ethanol-binge mice model and (I) TUNEL in primary hepatocytes from a loss-of-function experiment with the chemical inhibitors FK866 as NAMPT inhibitor and EX527 as SIRT1 inhibitor; blue colour for nuclear saining DAPI/ green colour for TUNEL positive nuclei. Upper bars represent SD. For all graphical bar charts, except for Fig. 6G (Student's t tests, statistic comparison for two groups), the statistical analysis between groups for all graphical charts represented is one-way ANOVA, followed by *post-hoc* Bonferroni tests for three or more groups. Statistical significance is represented as $^{*}p$ <0.05; $^{**}p$ <0.01 and $^{***}p$ <0.001 *vs.* Ctrl are shown; #*p* <0.05; ##*p* <0.01 and ###*p* <0.001 *vs.* miR-Ctrl are shown.

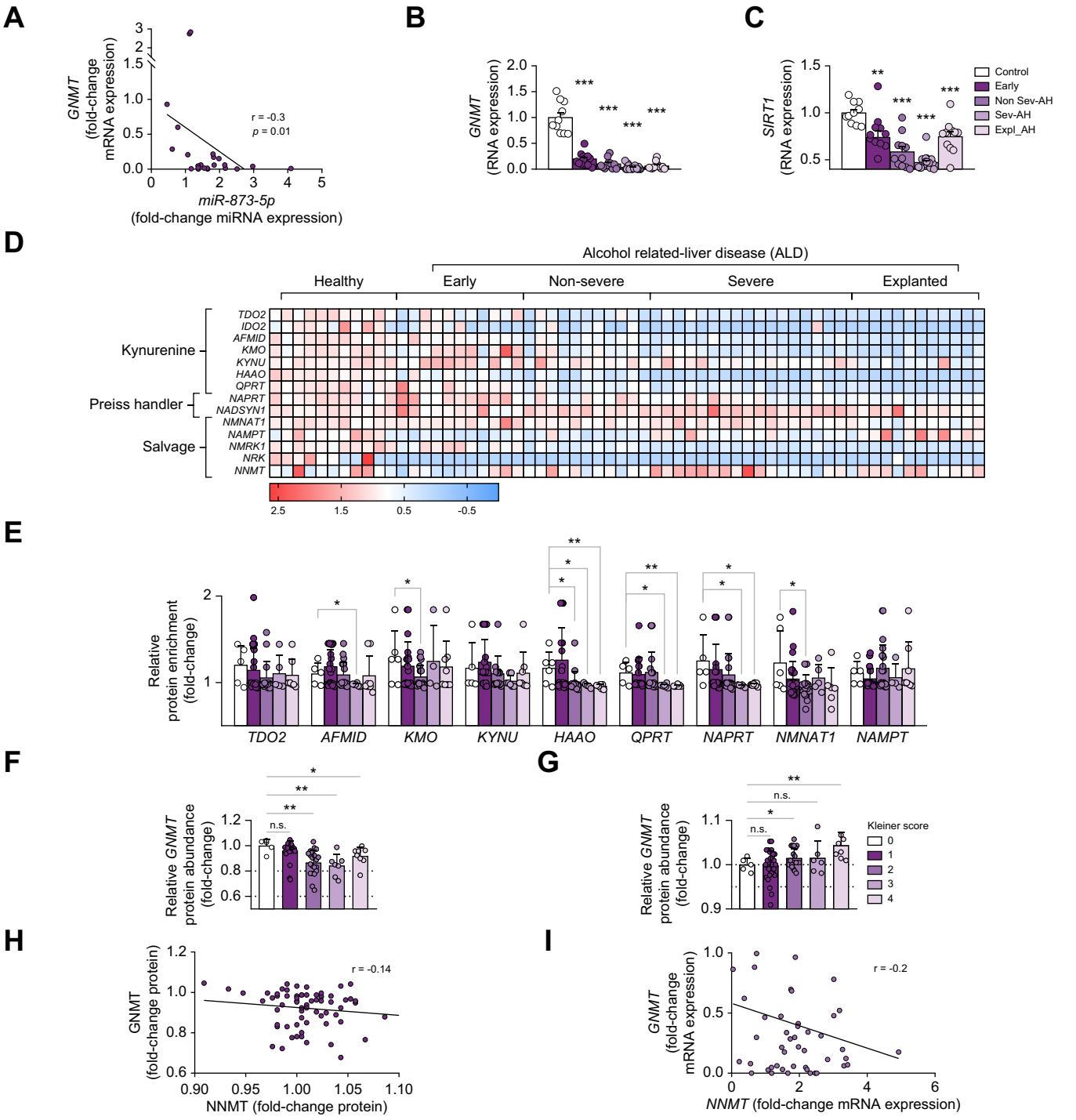

Fig. 7. The Impact of miR-873-5p on GNMT, NAD Pathways, and SIRT1 in patients with ALD. (A) Correlation between relative miR-873-5p expression and GNMT levels across diverse individuals. (B) Extending findings in an independent cohort of patients showing the effect of different degrees of ALD on *GNMT* expression. (C) The effect of ALD on *SIRT1* expression was confirmed by measuring expression in liver tissue along the progression of ALD. (D) Relative mRNA expression of genes associated with NAD synthesis pathways from human liver tissue during ALD progression. (E) Protein abundance of enzymes of NAD synthesis from human liver tissue (ALD stage defined as Kleiner score). (F) Relative protein abundance of GNMT and (G) NNMT at different stages of ALD according to Kleiner score. (H) Correlations between GNMT and NNMT at protein and (I) mRNA levels in the different patient cohorts of ALD during disease progression. For all graphical bar charts, upper bars represent SD. The statistical analysis between groups provided is Student's t tests (for two groups) and one-way ANOVA, followed by *post-hoc* Bonferroni tests for three or more groups. Statistical significance is represented as $^{*}p$ <0.05; $^{**}p$ <0.01 and $^{***}p$ <0.001 *vs.* Ctrl are shown; #*p* <0.05; ##*p* <0.01 and ###*p* <0.001 *vs.* miR-Ctrl are shown. ALD, alcohol-related liver disease.

Notably, our findings reveal a significant increase in NNMT levels during the progression of ALD in patients, as shown in Fig. 7G. Additionally, we observed an inverse correlation between NNMT and GNMT expression at the protein (Fig. 7H) and mRNA levels (Fig. 7I). These results suggest that the dysregulation of the methionine cycle is associated with elevated NNMT expression, which may be involved in reducing SAMe excess and restoring the SAMe/S-adenosylhomocysteine (SAH) methyl donor ratio.[46] However, this mechanism comes at the cost of decreased NAM levels and subsequently diminished overall NAD production.

Overall, the findings from this study in patients with ALD present compelling evidence that the dysregulation of miR-873-5p and its target genes, particularly GNMT, significantly contribute to the pathogenesis of ALD. These dysregulations have a direct impact on NAD metabolism and the activity of SIRT1, further emphasising their importance in the disease process.

Discussion

Despite the increased public awareness of alcohol-related hepatotoxicity, alcohol consumption is increasing at an alarming rate. For decades, treatment options for ALD were limited to symptom elimination or alcohol abstinence, with dietary and lifestyle modifications represent cornerstones.[47,48] ALD accounts for approximately 3.8% of deaths worldwide, making it the leading cause of death from liver disease[49,50] and is the main indication for liver transplantation in the USA.[47] As a result, pharmacotherapies for ALD remain an unmet need that must be addressed.

Altered epigenetic mechanisms are a remarkable side effect of alcohol abuse caused by excessive acetate and NAD depletion, and play an important role in the progression of ALD. Among epigenetic modifiers, miRNA expression is known to be altered in this pathology.[8] Here, we show that hepatic levels of miR-873-5p are increased during the progression of ALD in both patients and mouse models, highlighting it as a novel therapeutic target. Together with elevated miR-873-5p levels, the expression of *Gnmt*, its direct target, was markedly downregulated with disease progression in mice.

The presence of GNMT and its regulation by anti-miR-873-5p in diverse cell types, such as cholangiocytes, suggests that the modulation of miR-873-5p could have implications beyond its impact on specific cell populations, indicating its potential significance in diverse biological processes.[12] Notably, the treatment of hepatocytes with anti-miR-873-5p does not affect cell survival[13], but protects the liver against chronic and acute alcohol consumption *in vivo*. Importantly, the presence of *Gnmt* is crucial for the effect of anti-miR-873-5p in hepatocytes, as previous results have shown that the silencing of miR-873-5p in *Gnmt*$^{-/-}$ mice has no protective effects.[9,12] In mice treated with anti-miR-873-5p, ER stress markers, inflammation, cell death, and overall liver injury were downregulated, whereas alcohol clearance and antioxidant activity improved.

In this study, we used a proteomics approach to identify SIRT1, a NAD-dependent deacetylase, as an upstream regulator of the signalling pathways modulated in ethanol-fed, anti-miR-873-5p-treated mice. The liver metabolises 80% of ingested alcohol, producing harmful metabolites such as acetaldehyde via ADH and acetate/acetyl-CoA via mitochondrial aldehyde dehydrogenase (ALDH). Both reactions require NAD^+ as the major cofactor.[4] As ethanol metabolism depletes the NAD supplies, NAD^+-dependent enzymes, such as sirtuin deacetylases, lose their functionality in ALD[33], triggering aberrant acetylation that leads to pathophysiological consequences. Interestingly, anti-miR-873-5p therapy restored not only SIRT1 deacetylase activity, but also hepatic NAD levels and the acetylome.

Regarding the hepatic NAD pool, although most of NAD is recycled via the salvage pathway, its *de novo* synthesis occurs via the kynurenine pathway, starting from Trp and the Preiss–Handler pathway.[2] Consistent with our proteomic analysis, metabolomic studies using LC-MS showed higher NaM levels, a precursor for the NAD salvage pathway, together with lower NaMN and higher Trp contents in anti-miR-873-5p-treated mice, suggesting that compared with the anti-miR-control group, the recycling metabolism is fuelled to restore NAD levels without depleting Trp stores.

Although the role of NaM as a potential SIRT1 inhibitor is controversial, this molecule can be used to rapidly synthesise NAD and induce the deacetylase activity of SIRT1 via the salvage pathway.[51] Our data confirm that the inhibition of miR-873-5p restores the NAD/NADH ratio and enhances SIRT1 deacetylase activity via the salvage pathway. However, the treatment loses its protective effect in the presence of FK866, an inhibitor of NAMPT, the rate-limiting enzyme from the NAD recycling pathway.

To understand how anti-miR-873-5p boosts the salvage pathway, it is worth noting that GNMT is a major hepatic methyltransferases and a direct target of miR-873-5p.[9,12,13] NNMT is another methyltransferase that not only inhibits NAD synthesis by degrading NaM in response to ER stress, but also appears elevated in patients with ALD during disease progression.[2,43,44] Herein, when GNMT decreases, NNMT is augmented. This was confirmed following the analysis of the human proteome and genome databases. In ethanol-fed mice treated with anti-miR-873-5p, the expression of *Nnmt* was decreased, corresponding to higher levels of *Gnmt* together with reduced NNMT activity. It is plausible that the anti-miR-873-5p-dependent expression of *Gnmt* competes with *Nnmt* for available SAMe, thereby preventing the consumption of NaM and, thus, promoting the salvage pathway.

Consistently, the rescuing of hepatic NAD levels and NAD-dependent SIRT1 deacetylase activity restores the liver acetylome in anti-miR-873-5p-treated mice, modulating the levels and activity of those proteins regulated through acetylation. We analysed two major hallmarks of the progression of ALD: cholestasis and inflammation. FXR is a nuclear receptor that controls the synthesis, secretion, and reabsorption of BAs, and its deficiency is associated with cholestasis in ALD.[35] SIRT1 acts as a positive regulator of FXR and prevents its hyperacetylation.[35,52] The study of BA species in the liver of Gao-binge model mice by LC-MS revealed that anti-miR-873-5p modulates the pool of BA to a less hydrophobic, less toxic level, possibly through FXR activation. The increase in secondary BAs (taurodeoxycholic acid, tauroursodeoxycholic acid and taurolitholic acid) also results from the improved enterohepatic circulation of BAs in the anti-miR-873-5p group. The overall reduction in serum total BAs in the anti-miR-873-5p treated group supports this hypothesis.

SIRT1 has also been identified as a master regulator of inflammatory and oxidative stress processes by inhibiting the NF-

κB P65 subunit through the modulation of Lys-310 acetylation.[53,54] Consistently, the silencing of miR-873-5p reduced acetyl–NF–κB levels in ethanol-fed mice, together with the expression of inflammatory markers *Il1b, Il6*, *Cxcl1*, and *Ccl2*. Since knocking down miR-873-5p also has a hepatoprotective effect by maintaining mitochondrial activity[9], this may additionally explain how the GSSG/GSH ratio was restored and mitochondrial ROS levels were reduced in the anti-miR-873-5p mice group.

Therefore, the use of anti-miR-873-5p provides new solutions to the unmet pharmacological need in ALD, by boosting the NAD salvage pathway and restoring the hepatic acetylome during ALD progression.

Abbreviations

ALD, alcohol-related liver disease; BA, bile acid; ER, endoplasmic reticulum; FXR, farnesoid X receptor; GNMT, glycine N-methyltransferase; GSH/GSSG ratio, reduced glutathione/oxidised glutathione disulfide ratio; IPA, Ingenuity Pathway Analysis; LC-MS, liquid chromatography–mass spectrometry; miRNAs, microRNAs; NaM, NAD precursor nicotinamide; NNMT, nicotinamide N-methyltransferase; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; ROS, reactive oxygen species; SAMe, S-adenosylmethionine; SAH, S-adenosylhomocysteine; SIAM, Swift Increase in Alcohol Metabolism; Trp, tryptophan.

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Conflicts of interest

MLMC advises for Mitotherapeutix LLC. All other authors have nothing to disclose.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Conception or design of the work: RRA, NGU, MLMC. Acquisition, analysis, or interpretation of data: RRA, IGR, MSM, MB, PP, DB, JMH, MMG, CMRG, SLO, CGP, MA, AM, ALS, FE, CM, YAN, FJC, TCD, JA, RB, REC, JMB, PSB, MAA, JJ, RJ, JM, JS, NGU, MLMC. Drafted the work: RRA, JS, NGU, MLMC. Substantively revised the work: JMB, REC, KS, RRA, JS, NGU, MLMC.

Data availability statement

This study did not generate any unique datasets or code.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2023.100918.

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Author names in bold designate shared co-first authorship

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