



# Circulating indian hedgehog is a marker of the hepatocyte-TAZ pathway in experimental NASH and is elevated in humans with NASH

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**Background & Aims:** Non-alcoholic steatohepatitis (NASH)-induced liver fibrosis is emerging as the most common cause of liver disease. For evaluation of therapies, there is a pressing need to identify non-invasive, mechanism-based biomarkers. A pro-fibrotic process relevant to human NASH involves a pathway in which a transcriptional regulator called TAZ (WWTR1) in hepatocytes induces the secretion of pro-fibrotic Indian hedgehog (IHH). We therefore reasoned that circulating IHH may be a useful mechanism-based marker to assess changes in NASH fibrosis.

**Methods:** Circulating IHH was assessed in wild-type and hepatocyte-TAZ-silenced NASH mice and in three separate cohorts of patients with mild-moderate NASH.

**Results:** Circulating IHH was elevated in mice with diet-induced NASH compared with chow-fed mice or with NASH mice in which hepatocyte TAZ was silenced, which is an effective means to decrease NASH fibrosis. In patients with fatty liver disease with or without NASH, NASH fibrosis was associated with increased concentrations of circulating IHH.

**Conclusions:** The results of these analyses support further investigation to determine whether circulating IHH may be useful as a mechanism-based indicator of target engagement in anticipated future clinical trials testing NASH fibrosis therapies that block the IHH pathway.

**Impact and implications:** Non-alcoholic steatohepatitis (NASH)-induced liver fibrosis is a common cause of liver disease. Circulating biomarkers that reflect liver fibrosis in NASH would be very useful to evaluate therapies. One mechanism of NASH fibrosis with potential as a therapeutic target involves a liver-secreted protein called Indian hedgehog (IHH). We report that circulating levels of IHH in experimental and human NASH associates with NASH and NASH-associated liver fibrosis, providing the premise for further investigation into using circulating IHH to evaluate anticipated future NASH therapies that block the IHH pathway in liver.

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## Introduction

Non-alcoholic steatohepatitis (NASH) is emerging as the leading cause of cirrhosis worldwide.<sup>1-3</sup> Disease progression from simple

steatosis to NASH is caused by multiple insults that cause liver inflammation, hepatocellular death, and, most importantly, histological liver fibrosis, which correlates best with clinical outcome in NASH.<sup>4</sup> As there are no FDA-approved drugs to treat NASH, there is a critical need for novel therapies that can halt or reverse progression to liver fibrosis.<sup>5-8</sup> Accordingly, these efforts will require non-invasive approaches to identify patients at increased risk of NASH, assess disease progression, and monitor response to therapy.

A key process in NASH fibrosis is activation of collagen-producing hepatic stellate cells (HSCs).<sup>8-10</sup> One mechanism involves cholesterol-induced upregulation in hepatocytes of the transcriptional regulator TAZ (WWTR1), which induces the synthesis and secretion of the HSC activator, Indian hedgehog

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(IHH).<sup>11,12</sup> This pathway is supported by showing increased TAZ and IHH in human NASH vs. steatotic liver and by causation studies using primary human and mouse hepatocytes and mouse NASH models.<sup>11,12</sup> For example, silencing hepatocyte TAZ or IHH blocks NASH progression, and the improvement in NASH by silencing hepatocyte TAZ is abrogated by genetically restoring hepatocyte IHH.<sup>11</sup> From a therapeutic standpoint, silencing hepatocyte TAZ using GalNAc-siTaz, which is based on a platform currently in use in humans,<sup>13–18</sup> blocks the progression of liver fibrosis in experimental NASH.<sup>19</sup>

Given the importance of secretory IHH, we reasoned that circulating IHH may be a useful mechanism-based marker related to histological features of NASH and fibrosis. We show here that circulating IHH increases in NASH in mice in a hepatocyte-TAZ-dependent manner and associates with non-alcoholic fatty liver disease (NAFLD) activity score and liver fibrosis in humans with mild–moderate NASH. These findings support further investigation into whether plasma IHH may be useful as an indicator of target engagement in anticipated trials testing NASH fibrosis therapies that block the IHH pathway.

## Materials and methods

### Animal studies

Male wild-type C57BL/6J mice (10 weeks old) were obtained from Jackson Laboratory (#000664; Bar Harbor, ME, USA) and assigned randomly to receive chow diet (PicoLab Rodent Diet 20, #5053; Lab Diet, St Louis, MO) or a NASH-inducing diet rich in fructose, palmitate, and 1.25% cholesterol (FPC; TD.160785, Envigo Teklad Diets; Madison, WI; with drinking water containing 23.1 g/L fructose and 18.9 g/L glucose).<sup>11</sup> In view of the poor absorption of dietary cholesterol in these mice,<sup>20</sup> 1.25% cholesterol is required to mimic the cholesterol content of human NASH liver.<sup>11</sup> This is important, as increased liver cholesterol contributes to NASH progression,<sup>21–23</sup> including promoting the increase in the pro-NASH TAZ-IHH pathway.<sup>12</sup> The feeding period was 16 or 28 weeks, with tail vein injection of AAV8-H1-shTaz virus or control AAV8-H1-shControl virus (Vector Biolabs, Malvern, PA;  $2 \times 10^{11}$  genome copies/mouse) at 8 weeks for the 16-week protocol. Plasma was collected and livers were harvested and snap-frozen or formalin fixed as previously described.<sup>11,12,24</sup> Experiments complied with guidelines of the Columbia Animal Care and Use Committee.

### Human samples

Three separate cohorts were included. Patients gave informed consent, and protocols were approved by the institutional review board of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico Milan (cohorts 1 and 2) or the University of Missouri (cohort 3) and conducted according to the World's Medical Association Declaration. Patient records were pseudo-anonymised and de-identified. See Supplementary information for description of tissue and blood collection and analyses.

## Results

### Plasma IHH is increased in experimental NASH and lowered by silencing hepatocyte TAZ

We assayed plasma IHH in mice fed the NASH-inducing FPC diet for 16 or 28 weeks and in mice in which hepatocyte TAZ was silenced between Weeks 8 and 16. Metabolic, biochemical, and histologic data for the mice have been previously published.<sup>11</sup> Plasma IHH

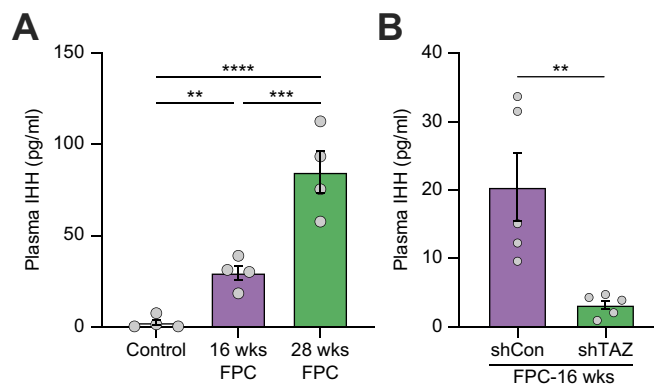
levels were very low in chow-fed mice but became markedly increased in mice fed the FPC diet for 16 and 28 weeks (Fig. 1A). Further, in 16-week-fed mice, silencing hepatocyte TAZ between Weeks 8 and 16, which lowers liver fibrosis and IHH,<sup>11</sup> markedly reduced plasma IHH (Fig. 1B). Thus, in experimental NASH, plasma IHH is elevated, and it is lowered by silencing hepatocyte TAZ.

### Plasma IHH is increased in humans with NASH fibrosis

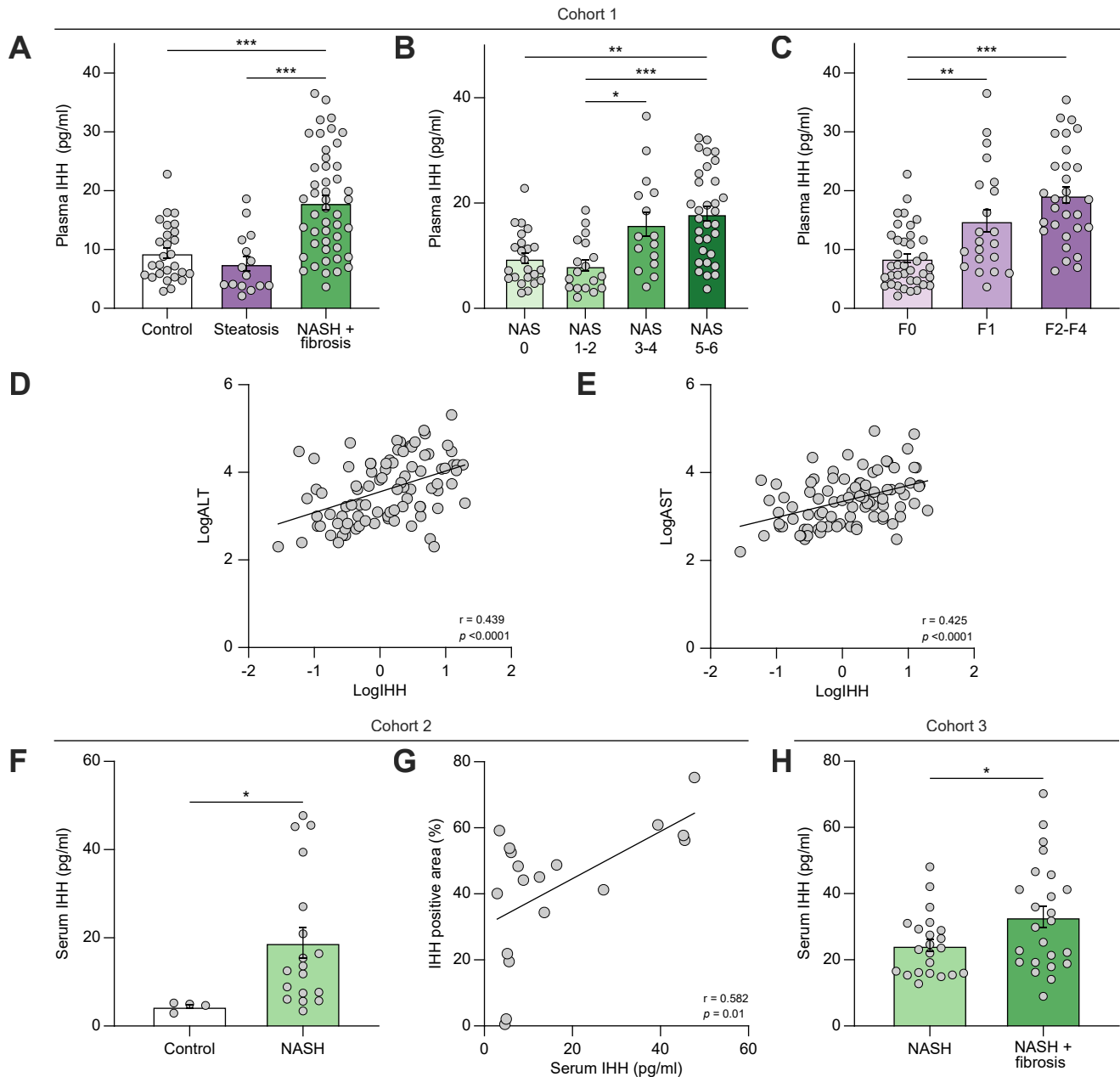
In cohort 1, which included 96 patients who underwent liver biopsy for suspected NASH, plasma IHH was approximately twofold higher in participants with histologically confirmed NASH and mild–moderate fibrosis vs. simple steatosis or without NAFLD (Fig. 2A and Table S1). Further, plasma IHH was higher in participants with higher NAFLD activity score (Fig. 2B) and in participants with NASH with liver fibrosis vs. participants with NASH without liver fibrosis (Fig. 2C). Plasma IHH also correlated positively with serum alanine aminotransferase and aspartate aminotransferase (Fig. 2D and E). The finding of increased circulating IHH in participants with NASH vs. control participants was reproduced in cohort 2, which included patients ( $n = 22$ ) who were overweight or obese and referred for liver biopsy to diagnosis NASH (Fig. 2F and Table S2). In addition, there was a positive correlation between IHH-positive area in immunostained liver sections and serum IHH (Fig. 2G). In cohort 3, which consisted of patients ( $n = 48$ ) with morbid obesity undergoing bariatric surgery as described,<sup>25</sup> serum IHH was higher in participants with NASH with histologically confirmed mild–moderate liver fibrosis vs. participants with NASH without fibrosis (Fig. 2H and Table S3). Although the wedge liver biopsies from these bariatric surgery patients may contain loci of capsular and subcapsular fibrosis, these findings support our findings in cohorts 1 and 2 that plasma and serum IHH is elevated in the setting of NASH fibrosis.

## Discussion

The premise for this study was based on recently published preclinical work, backed by studies of human NASH liver and human hepatocytes, suggesting that targeting the TAZ-IHH pathway in hepatocytes NASH may be a therapeutic option to



**Fig. 1. Plasma IHH is increased in experimental NASH and lowered by silencing hepatocyte TAZ.** (A) Plasma IHH of mice fed chow or FPC diet for 16 and 28 weeks ( $n = 4$  mice/group; \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ). Data were analysed using one-way ANOVA. (B) Plasma IHH of mice fed the FPC diet for 16 weeks, with AAV8-H1-shTaz or control (shCon) vector injected at Week 8 ( $n = 5$  mice/group, \*\* $p < 0.01$ ). Data were analysed using a two-tailed Student's  $t$  test. FPC, fructose, palmitate, and cholesterol; IHH, Indian hedgehog; NASH, non-alcoholic steatohepatitis.



**Fig. 2. Plasma IHH is increased in patients with NASH and NASH-associated liver fibrosis.** (A) Plasma IHH concentrations in control participants (n = 29), participants with steatosis (n = 17), and participants with NASH + fibrosis (n = 50) from cohort 1. (B and C) Data from (A) were stratified based on NAS and fibrosis score. (D and E) Pearson R correlation of the relationship between plasma IHH (LogIHH) and serum ALT (LogALT) or AST (LogAST) in cohort 1. (F and G) Serum IHH in participants without NAFLD (control; n = 4) or with NASH (n = 18) and Pearson R correlation of the relationship between serum and liver IHH in participants from cohort 2. (H) Serum IHH in obese participants with NASH without or with fibrosis (n = 22 and 25, respectively) from cohort 3. Grouped data were analysed using one-way ANOVA. For (A)–(C), (F), and (H), the data are expressed as mean ± SE relative to the first group in each graph (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ). ALT, alanine aminotransferase; AST, aspartate aminotransferase; IHH, Indian hedgehog; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis.

block the progression of NASH fibrosis.<sup>11,12,19</sup> To summarise, TAZ and IHH are upregulated in human and experimental NASH; TAZ-induced secretory IHH is a direct activator of fibrosis-inducing HSCs; and silencing TAZ or IHH in hepatocytes blocks HSC activation and liver fibrosis in experimental NASH.<sup>11,12</sup> Moreover, we have shown that genetically targeting hepatocyte TAZ in NASH mice lowers liver fibrosis by blocking the IHH pathway,<sup>11</sup> and we obtained similar results by treating NASH

mice with GalNac-siTaz,<sup>19</sup> which uses a platform in use in humans for other diseases.<sup>13–18</sup> These preclinical studies and analyses of human NASH liver provide the rationale for future clinical trials to evaluate the effectiveness and safety<sup>26,27</sup> of this type of therapy in humans. Accordingly, we reasoned that a mechanism-based biomarker of target engagement involving the measurement of circulating IHH, that is, based on the fact that IHH is a secretory protein, might be useful as investigators begin

to consider such clinical trials. The combination of the preclinical and clinical data herein, namely, showing that plasma IHH is markedly decreased by silencing hepatocyte TAZ in NASH mice and that circulating IHH in humans associates with NAFLD activity score, mild–moderate fibrosis, and liver IHH in humans with mild–moderate NASH, provides preliminary support for our idea and provides the rationale for further work in this area. However, the ultimate value of plasma IHH as a marker of target engagement will not be known until such trials are initiated.

As to whether plasma IHH might be useful as a more general marker of liver fibrosis in NASH cannot be addressed by this study, as we do not have the data to compare plasma IHH with other plasma or imaging markers of NASH. Moreover, the cohorts were relatively small and did not include enough participants with advanced NASH to compare different fibrosis stages, and we did not have certain clinical data that would allow us to correct for all confounding factors, such as medications at the time of

biopsy. One interesting direction for a future prospective study would be to determine if plasma IHH, when added to another scoring scheme based on different mechanisms, may be able to improve the sensitivity and/or specificity for predicting the progression of early to advanced NASH.

### Conclusions

Circulating IHH is elevated in mice with diet-induced NASH and lowered by silencing hepatocyte TAZ, which blocks IHH-induced liver fibrosis in NASH. In humans, increased concentrations of circulating IHH associates with mild–moderate NASH fibrosis. Although these results should be confirmed in larger cohorts across the full spectrum of NASH and NASH-associated fibrosis, they provide the premise for further investigation into using circulating IHH as a mechanism-based indicator of target engagement in anticipated future trials testing NASH fibrosis-targeting therapies that lower liver IHH.

### Abbreviations

FPC, fructose, palmitate, and cholesterol; HSC, hepatic stellate cell; IHH, Indian hedgehog; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis.

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### Conflicts of interest

IT and XW have received research funding (unrestricted) from Takeda Pharmaceuticals. LV has been an invited speaker for MSD, Gilead, Alfa-Sigma, and AbbVie; consults for Gilead, Pfizer, Astra Zeneca, Novo Nordisk, Intercept pharmaceuticals, Diatech Pharmacogenetics, IONIS, Viatrix, and Boehringer Ingelheim; and has received research funding (unrestricted) from Gilead.

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

### Authors’ contributions

Involved in the study conception and experimental design: MPM, XW, IT. Conducted mouse experiments, liver analyses, and plasma and serum assays: XW, MPM, HS. Were involved in conducting and analysing the human studies and providing important intellectual contributions: MM, AC, LR, EJP, JAI, RSR, LV, PD. Drafted the manuscript: MM, XW, IT. Revised the manuscript and approved the final version: all authors.

### Data availability statement

Further information and requests for resources, reagents, and data should be directed to, and will be fulfilled by, the lead contact, IT ([iat1@cumc.columbia.edu](mailto:iat1@cumc.columbia.edu)).

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### Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhepr.2023.100716>.

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**Supplemental information**

**Circulating indian hedgehog is a marker of the hepatocyte-TAZ pathway in experimental NASH and is elevated in humans with NASH**

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## Supplementary Description of Human Cohorts

**Cohort 1:** The first cohort included 96 adult individuals with NAFLD who were consecutively enrolled at the Metabolic Liver Disease Laboratory outpatient service at Fondazione IRCCS Ca' Granda Ospedale Policlinico Milan, Italy. Patients underwent liver biopsy for suspected NASH. Individuals with excessive alcohol intake (men, >30 g/day; women, >20 g/day), viral and autoimmune hepatitis, hereditary hemochromatosis, and alpha-1 antitrypsin deficiency or other causes of liver disease were excluded. For all patients, liver biopsy was performed by needle gauge and plasma samples were collected at the time of histological assessment of liver damage. Informed written consent was obtained from each patient, and the study protocol was approved by the Ethics Committee of the Fondazione IRCCS Ca' Granda, Milan (protocol code CE 401; 28/02/2019). Body mass index (BMI) was measured using standard procedures. T2D was diagnosed when impaired fasting glucose (IFG) tolerance was present and fasting glucose was >110 mg/dL. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides, total cholesterol, HDL, and LDL were measured by an automated analyzer (CobasC 702, Roche, Switzerland) at Fondazione IRCCS Cà Granda. Steatosis was graded according to the percentage of affected hepatocytes as 0 (0%- 4%), 1 (5%- 32%), 2 (33%- 65%), and 3 (66%- 100%). Disease activity was assessed according to the NAFLD activity score with systematic evaluation of steatosis (0 = steatosis <5%, 1 = steatosis 5-33%, 2 = >33% to 66%, 3 = >66%), hepatocellular ballooning (0 = none, 1 = few balloon cells, 2 = many cell), and necroinflammation (0 = no foci, 1 = <2 foci/x200 field, 2 = 2-4 foci/x200 field, 3 = >4 foci/x200 field); fibrosis was also staged according to recommendations of the NAFLD Clinical Research Network (1 = mild to moderate portal/periportal or pericellular fibrosis, 2 = mild to moderate pericellular and portal/periportal fibrosis, 3 = bridging fibrosis, 4 = cirrhosis)<sup>1</sup>. Scoring of liver biopsies was performed by independent pathologists unaware of patient status. NASH was diagnosed when steatosis, lobular inflammation, and/or ballooning were concomitantly present.



**Cohort 2:** In the second cohort, individuals were consecutively enrolled at the Precision Medicine Lab, Biological Resource Center outpatient service at Fondazione IRCCS Ca' Granda Ospedale Policlinico Milan, Italy. They were n = 18 individuals with obesity who underwent liver biopsy for suspected NASH and n = 4 individuals with other causes of liver disease (viral hepatitis, drug induced liver disease, liver metastasis of lung cancer). Liver biopsy was performed by needle gauge, and serum samples were collected at the time of histological assessment of liver damage. Liver samples were fixed in 1% formaldehyde for histological examination. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose, triglycerides, total cholesterol, HDL, and LDL were measured by an automated analyzer (CobasC 702, Roche, Switzerland) at Fondazione IRCCS Cà Granda. Histological scoring was performed by an expert pathologist unaware of patients' status. Steatosis was graded based on the percentage of affected hepatocytes as 0: 0%–5%, 1: 6%–33%, 2: 34%–66% and 3: 67%–100%. Disease activity was assessed according to the NAFLD Activity Score (NAS) with systematic evaluation of hepatocellular ballooning and necroinflammation; fibrosis was staged according to clinical guidelines<sup>1,2</sup>. NASH was defined as the concomitant presence of steatosis, lobular inflammation, and ballooning. Informed written consent was obtained from each patient, and the study protocol was approved by the Ethics Committee of the Fondazione IRCCS Ca' Granda, Milan (protocol code CE 692-2021; 18/06/2021).

**Cohort 3:** The third cohort consisted of a single liver wedge sample collected from morbidly obese patients at the time of their bariatric surgery at the University of Missouri Hospital. Before inclusion, all participants (n = 48) gave written informed consent to the protocol, which was approved by the Institutional Review Board of the University of Missouri (protocol #2008258). Dietary history before surgery is not known, but the patients were instructed to follow their physician's standard of care. These samples are also part of a previous study registered at ClinicalTrials.gov (Identifier: NCT03151798) and previously published<sup>3</sup>. The degree of NAFLD severity was determined by a hepatopathologist blinded to group identification, using validated

hepatic histopathological classification guidelines<sup>1,2</sup>. Patients were grouped based on the presence of NASH (presence of steatosis, inflammation, and ballooning) and fibrosis staging  $\geq 1$ . Blood was drawn before administration of anesthesia and immediately processed for biochemical measurements according to standard procedures by a Clinical Laboratory Improvement Amendments–standardized laboratory. Lipid measurements (total cholesterol [TC], triglycerides [TGs], low-density lipoprotein cholesterol [LDLc], and high-density lipoprotein cholesterol [HDLc]) were performed by an autoanalyzer (Roche Cobas 8000 System; coefficient of variation [CV], 0.6%–0.9%; Roche Diagnostics, Indianapolis, IN), using an electrochemiluminescent immunoassay. Liver enzymes (aspartate transaminase [AST], alanine aminotransferase [ALT], and alkaline phosphatase [ALP]) were measured using UV Absorbance (Roche Cobas 8000 System; CV, 0.5%–3.2% for AST and 0.5%–3.1% for ALT; Roche Diagnostics). Samples were collected as follows: After the initiation of anesthesia, a liver biopsy was obtained, according to standardized protocols. Liver samples were fixed in 1% formaldehyde for histological examination. Blood samples were drawn ahead of anesthesia. Serum and plasma were also collected and stored at  $-80^{\circ}\text{C}$  for future analysis of IHH. Human liver histological analyses were conducted by an experienced pathologist using hematoxylin-eosin (H&E) and Masson’s trichrome staining.

## **Supplemental Materials and Methods**

### **Histopathological Analysis**

For immunohistochemistry, paraffin sections were rehydrated and subjected to antigen retrieval by placing in a pressure cooker for 10 mins in Antigen Unmasking Solution (Vector Laboratories, H-3300). The slides were then treated with 3% hydrogen peroxide for 10 min and then blocked with Serum-Free Protein Block (Dako, X0909) for 30 min. Sections were incubated overnight with IHH primary antibodies (Proteintech, #13388) and then developed with DAB substrate kit (Cell Signaling, #8059). Images were captured randomly, and quantification was conducted without knowledge of cohort assignment.

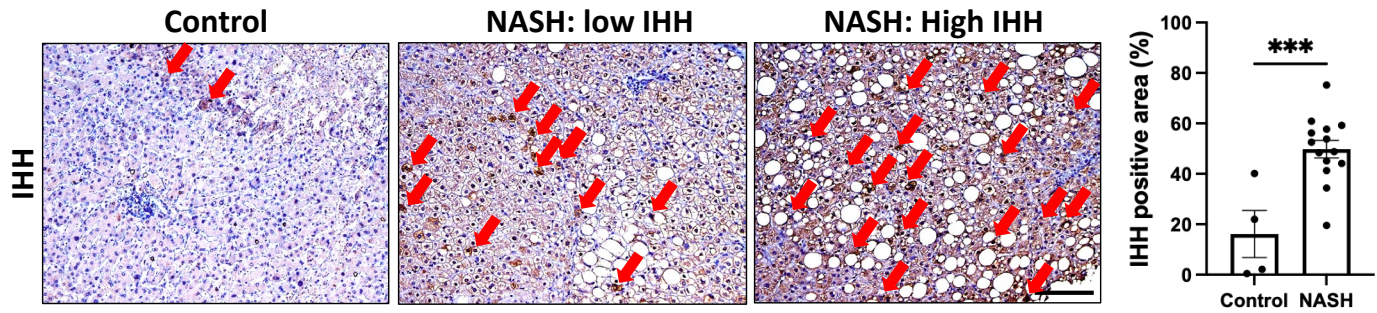
### **Plasma and Serum Analysis**

Blood samples were collected in separating gel vacuum collection tubes and centrifuged at 2000 g for 15 min at 4°C. The upper-layer supernatant fraction was collected and centrifuged again at 2000 g for 15 min at 4°C to pellet and remove any circulating cells or debris. Plasma aliquots were stored at -80°C until analysis, and only samples with less than 3 freeze-thaw cycles were used for IHH measurement. Plasma or serum IHH was measured by ELISA in mice (Cat#: EKU37089; Biomatik, Wilmington, Delaware, USA) and in humans (Cat#: EKU34122; Biomatik, Wilmington, Delaware, USA) according to manufacturer instructions. We obtained comparable numbers from plasma and serum.

### **Statistical Analysis**

Data were tested for normality using the Kolmogorov-Smirnov test, and statistical significance was determined using GraphPad Prism software and IBM SPSS Statistics software (version 28.0.1.0; IBM Corp., Armonk, NY, USA). Normally distributed data were analyzed using Student's *t* test for two groups with one variable tested and equal variances. Non-parametric data were analyzed using a Mann-Whitney *t*-test. For all human cohorts (1, 2, and 3), patient

characteristic data were analyzed using one-way analysis of variance, and, if significant, followed with a Tukey's post-hoc analysis. For the analysis of IHH comparisons, IHH data was log-transformed, and a one-way analysis of covariance (ANCOVA) was performed, with sex, age, diabetes status (or HbA1c), and body mass index as covariates. For groups of three or more, statistically significant differences were followed up with Sidak's post-hoc analysis. Differences were considered statistically significant at  $P \leq 0.05$ . Correlation analysis data were log-transformed and analyzed using Pearson's correlation. Data were graphed using GraphPad Prism software (version 9.0; GraphPad Software Inc., Cary, NC, USA), and analyses were performed using IBM SPSS Statistics software (version 28.0.1.0; IBM Corp., Armonk, NY, USA). All data are presented as means  $\pm$  SE. Differences were considered statistically significant at  $P < 0.05$ .



**Fig. S1.** Examples of liver IHH immunohistochemistry related to Figure 2G. Representative images using anti-IHH immunohistochemical staining of livers from Cohort 2, showing examples of control liver, NASH liver with low IHH, and NASH liver with high IHH (see Figure 2G) and accompanying quantitative data comparing control versus NASH subjects (n = 4-13) (Bar, 200  $\mu$ m). Data were analyzed with a Student's *t* test. Red arrows indicate examples of positive IHH staining.

**Table S1. Subject characteristics of Cohort 1 (Mean ± SE)**

	<b>Control</b>	<b>Steatosis</b>	<b>NASH + Fibrosis</b>	<b>ANOVA (P value)</b>
	(n = 27)	(n = 15)	(n = 47)	
<b>Age (years)</b>	45±2	44±3	51±2	0.06
<b>Sex (M/F)</b>	(15/12)	(4/13)	(27/20)	-
<b>BMI (kg/m<sup>2</sup>)</b>	31.2±1.6	41.0±3.9*	35.5±1.2	<b>0.0149</b>
<b>Smokers</b>	n = 4	n = 5	n = 15	-
-	-	-	-	-
<b>Glucose (mg/dL)</b>	94±5	97±13	108±5	0.2949
<b>Insulin</b>	12±3	13±2	30±3	<b>0.0192</b>
<b>HOMA-IR</b>	2.8±1.0	2.9±0.5	8.0±0.9	<b>0.0226</b>
<b>AST (U/L)</b>	30±5	19±2	42±3 <sup>&amp;</sup>	<b>0.0015</b>
<b>ALT (U/L)</b>	33±5	26±5	63±5 <sup>*,&amp;</sup>	<b>&lt;0.0001</b>
-	-	-	-	-
<b>Cholesterol (mg/dL)</b>	199±7	174±7	192±6	0.1760
<b>Triglycerides (mg/dL)</b>	89±7	100±12	179±23*	<b>0.0150</b>
<b>HDLc (mg/dL)</b>	63±6	52±4	46±2*	<b>0.006</b>
<b>LDLc (md/dL)</b>	125±6	104±6	122±7	0.2641
-	-	-	-	-
<b>Steatosis</b>	0.0±0.0	1.1±1.0*	2.4±0.1* <sup>&amp;</sup>	<b>&lt; 0.0001</b>
<b>Lobular Inflammation</b>	0.2±0.1	0.1±0.1	1.8±0.1* <sup>&amp;</sup>	<b>&lt; 0.0001</b>
<b>Hepatocellular Ballooning</b>	0.0±0.0	0.0±0.0	0.7±0.1* <sup>&amp;</sup>	<b>&lt; 0.0001</b>
<b>NAFLD Activity Score</b>	0.2±0.1	1.1±0.2*	4.9±0.1* <sup>&amp;</sup>	<b>&lt; 0.0001</b>
<b>Fibrosis Score</b>	0.1±0.1	0.0±0.0	1.8±0.1* <sup>&amp;</sup>	<b>&lt; 0.0001</b>
	F0 (n = 24)	F0 (n = 14)	F0 (n = 0)	
	F1 (n = 3)	F1 (n = 1)	F1 (n = 17)	
	F2 (n = 0)	F2 (n = 0)	F2 (n = 23)	
	F3 (n = 0)	F3 (n = 0)	F3 (n = 5)	
	F4 (n = 0)	F4 (n = 0)	F4 (n = 2)	

Data were analyzed using a one-way analysis of variance. \*P < 0.05 vs Control, &P < 0.05 vs. Steatosis.

**Table S2. Subject characteristics of Cohort 2 (Mean ± SE)**

	<b>Control</b>	<b>NASH</b>
	(n = 4)	(n = 18)
<b>Age (years)</b>	59±11	51±2
<b>Sex (M/F)</b>	(4/0)	(15/3)
<b>Weight (kg)</b>	92.5±17.5	101.0±4.4
-	-	-
<b>Glucose (mg/dL)</b>	93±4	103±3
<b>AST (U/L)</b>	127±60	38±4*
<b>ALT (U/L)</b>	130±43	63±9*
-	-	-
-	-	-
<b>Steatosis</b>	0.2±0.2	1.9±0.2*
<b>Lobular Inflammation</b>	0.2±0.1	1.17±0.1
<b>Hepatocellular Ballooning</b>	0.0±0.0	1.0±0.0
<b>NAFLD Activity Score</b>	0.5±0.5	4.0±0.2*
<b>Fibrosis Score</b>	1.0±0.7	0.9±0.2
	F0 (n = 2)	F0 (n = 6)
	F1 (n = 1)	F1 (n = 10)
	F2 (n = 0)	F2 (n = 1)
	F3 (n = 1)	F3 (n = 0)
	F4 (n = 0)	F4 (n = 1)

Normally distributed data were analyzed using a Student's *t* test. Non-parametric data were analyzed using a Mann-Whitney *t* test. \*P<0.05 NASH vs. control.

**Table S3. Subject characteristics of Cohort 3 (Mean ± SE)**

	<b>NASH</b> (n = 23)	<b>NASH + Fibrosis</b> (n = 25)
<b>Age (years)</b>	46±2	44±2
<b>Sex (M/F)</b>	(6/17)	(2/23)
<b>Weight (kg)</b>	135.2±6.0	141.7±5.5
<b>BMI (kg/m2)</b>	47.6±1.4	50.0±1.7
-	-	-
<b>Glucose (mg/dL)</b>	103±7	112±7
<b>HbA1C (%)</b>	6±0.3	7±0.3
<b>AST (U/L)</b>	33±2.9	55±11.9
<b>ALT (U/L)</b>	40.0±4.5	63.0±12.1
-	-	-
<b>Cholesterol (mg/dL)</b>	162±8	184±8
<b>Triglycerides (mg/dL)</b>	128±11	144±10
<b>HDLc (mg/dL)</b>	41±2	42±2
<b>LDLc (md/dL)</b>	96±8	113±8
-	-	-
<b>Steatosis</b>	1.9±0.2	2.4±0.2*
<b>Lobular Inflammation</b>	1.2±0.1	1.3±0.1
<b>Hepatocellular Ballooning</b>	1.1±0.1	1.4±0.1*
<b>NAFLD Activity Score</b>	4.2±0.2	5.1±0.2*
<b>Fibrosis</b>	0.0±0.0	1.2±0.2
	F0 (n = 23)	F0 (n = 0)
	F1 (n = 0)	F1 (n = 16)
	F2 (n = 0)	F2 (n = 3)
	F3 (n = 3)	F3 (n = 4)
	F4 (n = 0)	F4 (n = 2)

Normally distributed data were analyzed using a Student's *t* test. Non-parametric data were analyzed using a Mann-Whitney *t* test. \*P < 0.05 NASH vs. NASH + Fibrosis.



## **Supplementary references**

[1] Brunt EM, Kleiner DE, Wilson LA, Belt P, Neuschwander-Tetri BA. Nonalcoholic fatty liver disease (NAFLD) activity score and the histopathologic diagnosis in NAFLD: distinct clinicopathologic meanings. *Hepatology* 2011;53:810-820.

[2] Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005;41:1313-1321.

[3] Moore MP, Cunningham RP, Meers GM, Johnson SA, Wheeler AA, Ganga RR, et al. Compromised hepatic mitochondrial fatty acid oxidation and reduced markers of mitochondrial turnover in human NAFLD. *Hepatology* 2022;76:1452-1465.