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)[1]. 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[1,2]. 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[3]. The degree of NAFLD severity was determined by a hepatopathologist blinded to group identification, using validated

hepatic histopathological classification guidelines[1,2]. Patients were grouped based on the presence of NASH (presence of steatosis, inflammation, and ballooning) and fibrosis staging ≥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°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 4ºC. The upper-layer supernatant fraction was collected and centrifuged again at 2000 g for 15 min at 4ºC to pellet and remove any circulating cells or debris. Plasma aliquots were stored at -80ºC 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 ± SE. Differences were considered statistically significant at $P < 0.05$.

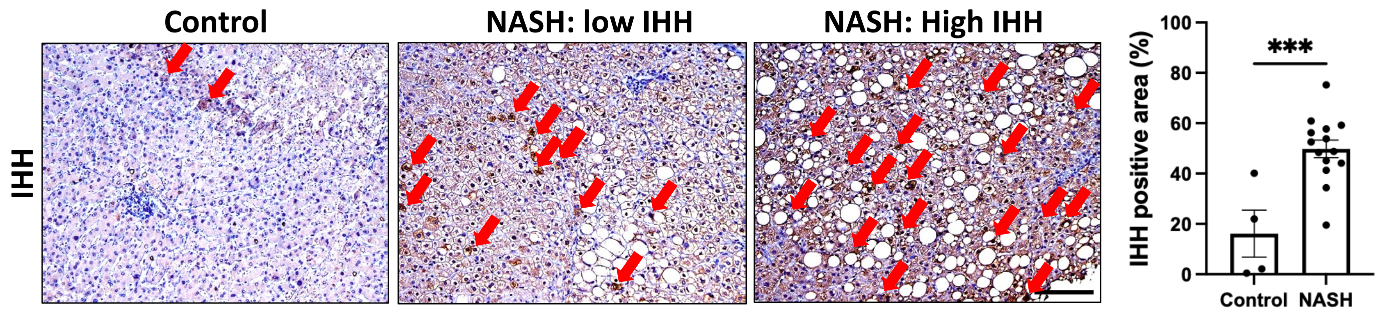

Fig. S1. Examples of liver IHH immunohistochemistry related to Figure 2G. Representative images using anti-IHH immunohistochemical staining of livers from Cohort 2, showing examples of control liver, NASH liver with low IHH, and NASH liver with high IHH (see Figure 2G) and accompanying quantitative data comparing control versus NASH subjects (n = 4-13) (Bar, 200 μm). 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)				
	Control	**Steatosis**	**NASH + Fibrosis**	**ANOVA (P value)**
	(n = 27)	(n = 15)	(n = 47)	
Age (years)	45±2	44±3	51±2	0.06
Sex (M/F)	(15/12)	(4/13)	(27/20)	-
BMI (kg/m2)	31.2±1.6	41.0±3.9*	35.5±1.2	**0.0149**
Smokers	n = 4	n = 5	n = 15	-
-	-	-	-	-
Glucose (mg/dL)	94±5	97±13	108±5	0.2949
Insulin	12±3	13±2	30±3	**0.0192**
HOMA-IR	2.8±1.0	2.9±0.5	8.0±0.9	**0.0226**
AST (U/L)	30±5	19±2	42±3&	**0.0015**
ALT (U/L)	33±5	26±5	63±5*,&	**<0.0001**
-	-	-	-	-
Cholesterol (mg/dL)	199±7	174±7	192±6	0.1760
Triglycerides (mg/dL)	89±7	100±12	179±23*	**0.0150**
HDLc (mg/dL)	63±6	52±4	46±2*	**0.006**
LDLc (md/dL)	125±6	104±6	122±7	0.2641
-	-	-	-	-
Steatosis	0.0±0.0	1.1±1.0*	2.4±0.1*&	**< 0.0001**
Lobular Inflammation	0.2±0.1	0.1±0.1	1.8±0.1*&	**< 0.0001**
Hepatocellular Ballooning	0.0±0.0	0.0±0.0	0.7±0.1*&	**< 0.0001**
NAFLD Activity Score	0.2±0.1	1.1±0.2*	4.9±0.1*&	**< 0.0001**
Fibrosis Score	0.1±0.1 F0 (n = 24) F1 (n = 3) F2 (n = 0) F3 (n = 0) F4 (n = 0)	0.0±0.0 F0 (n = 14) F1 (n = 1) F2 (n = 0) F3 (n = 0) F4 (n = 0)	1.8±0.1*& F0 (n = 0) F1 (n = 17) F2 (n = 23) F3 (n = 5) F4 (n = 2)	**< 0.0001**

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

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

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[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.