Supplementary data

Glisson's capsule matrix structure and function is altered in patients with cirrhosis irrespective of etiology

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Supplementary Methods

Histology & Staining:

For diaminobenzidine (DAB) labelling, sections were incubated in 3% H_2O_2 to quench endogenous peroxidases (Sigma Aldrich, Rockville, MA), and then blocked for avidin/biotin (Vector Laboratories, Newark, CA) followed by StartingBlock™ T20/phosphate buffered saline (PBS) Blocking Buffer (Thermo Fisher Scientific, Waltham, MA). Samples were incubated in primary antibodies (staining buffer-0.2% Triton X-100, 0.1% bovine serum albumin, in PBS) overnight at 4°C. After washing in PBS, samples were washed and incubated with appropriate biotinylated secondary antibodies (Vector Laboratories) for 1 h at room temperature. Hyaluronic acid/hyaluronan (HA) was stained overnight using biotinylated HA binding protein (Millipore, Burlington, MA; 385911). All sections were washed and incubated with VECTASTAIN® ABC-HRP kit (Vector Laboratories). Staining was developed with a DAB substrate kit (Vector Laboratories). Elastic fibers were stained using an elastic stain kit (Sigma-Aldrich, HT25A-1KT) per the manufacturer's instructions. Sections were imaged by brightfield microscopy using a Nikon E600 and NIS-elements software (Nikon, Melville, NY).

For fluorescence staining, sections were blocked with StartingBlock™ T20/phosphate buffered saline (PBS) Blocking Buffer before incubation in primary antibodies (staining buffer) overnight at 4°C, followed by washing and incubation with fluorescent secondary antibody for 1 h at RT in the dark. Staining was imaged on a Zeiss Axio Observer 7 inverted microscope (Zeiss Axiocam 701 monochrome CMOS camera) and Zen blue software (Zeiss, White Plains, NY). All antibodies used are listed in Supplementary Table 1.

For picrosirius red staining, paraffin-embedded sections were dewaxed and rehydrated as above. Sections were then incubated in PSR stain (Poly Scientific R&D, Bay Shore, NY) for 1 h at RT. Sections were then quickly washed in acidified water (0.5% acetic acid in H_2O), and dehydrated in 3 washes of 100% ethanol before clearing in Xylene and mounting.

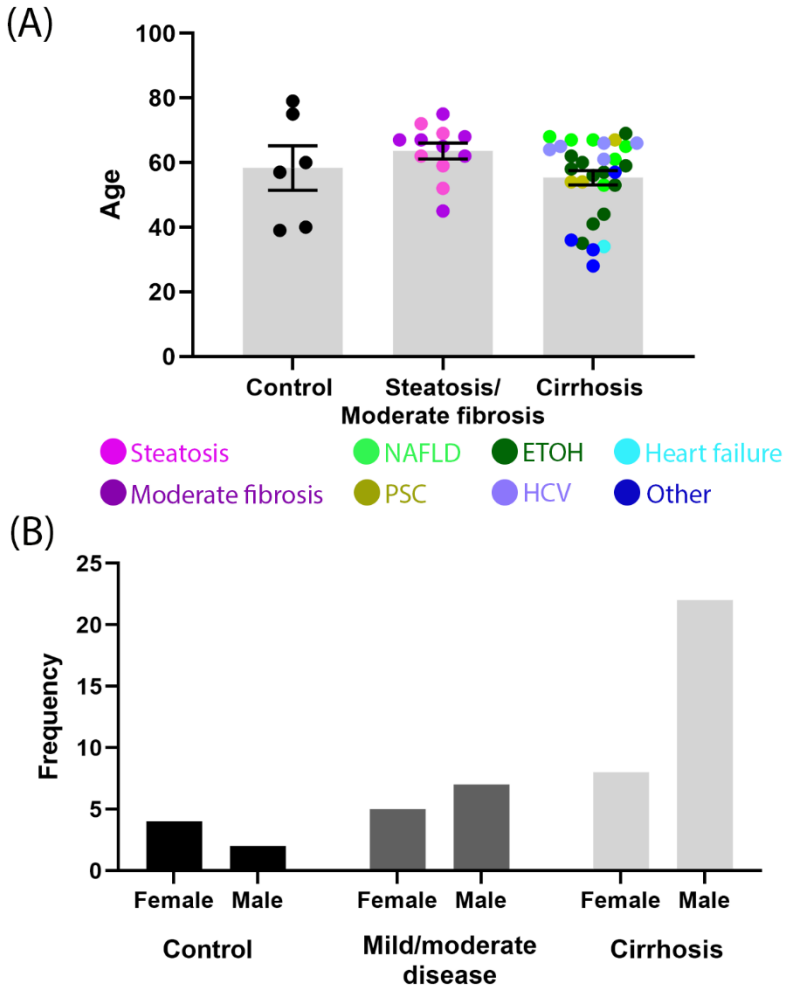
Image analysis

Thickness, percentage area and cell counting were analyzed using ImageJ (1). 3-5 images per liver were taken using the same parameters and analyzed using manually-determined regions of interest. Thickness was measured manually, with 5 measurements per image. Quantification of percentage area for DAB-stained slides was carried out using the deconvolution tool H DAB followed by thresholding. Percentage area of collagen in SHG cross-sections was also done using thresholding. Capsule areas were divided into two areas, the upper 70 μm and a lower layer (anything further than 70 μm from the

surface), using separate regions of interest. Cells were counted using the particle analysis tool. CD31 structures were counted manually using the count tool. Structures were defined as 2 or more clustered cells; some of these formed vessel-like structures.

Picro Sirius red staining was analyzed under polarized light with 6-10 images analyzed per sample. The PSR dye enhances the natural birefringence of the collagen when exposed to polarized light (2). PSR images were used to estimate a uniformity index of collagen fiber distribution, as previously described (3). This index calculates values between 1 (when the objects are distributed in a regular array) and 0 (when maximal clustering occurs). In order to apply this method, a subsampled binary image of the collagen pattern was obtained by extracting pixels corresponding to the points of a superimposed regular/systematic grid.

Crimping of collagen fibers was assessed on z stacks of SHG images. Depth and length were manually measured using the line tool in ImageJ. Approximately 20 fibers were measured in 3-8 images per sample. Orientation and alignment of collagen fibers were also assessed on SHG Z-stacks using a previously described method (4).



1. Sex and age of patient samples. (A) Patient ages for the control, steatosis/moderate fibrosis and cirrhotic samples used. (B) Sex distribution of patients for the control, steatosis/moderate fibrosis and cirrhotic samples used.

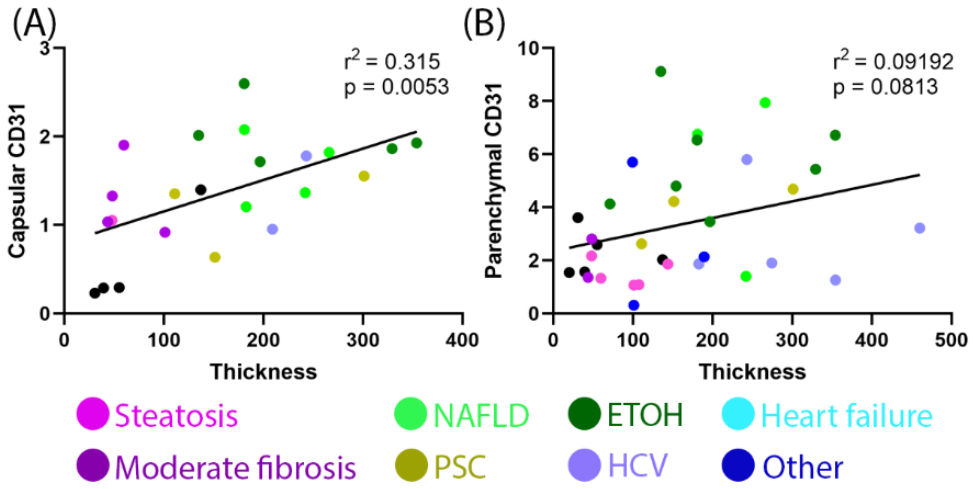


Fig. S2. Capsule thickness correlates with capsular CD31 but not parenchymal CD31. (A) Correlation between capsular thickness and capsular CD31 structures. (B) Correlation between capsular thickness and parenchymal CD31 percentage staining. Correlation analyzed by Pearson's correlation.

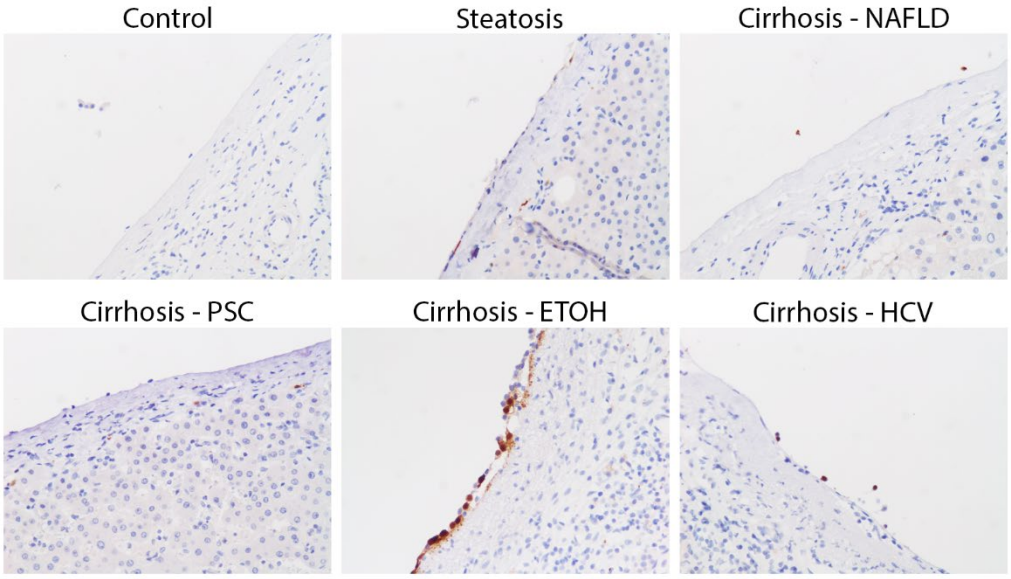


Fig. S3. Mesothelial cells are found sporadically on the capsule surface. Representative staining for calretinin in control and steatotic samples as well as cirrhotic samples of different etiologies.

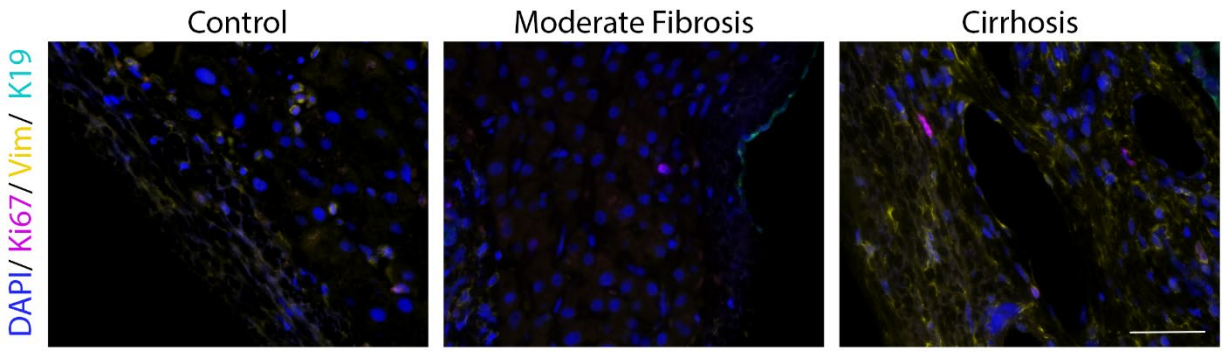


Fig. S4. Proliferation is unchanged in disease. Representative images of control, moderately fibrotic and cirrhotic samples (NAFLD in this example) stained for DAPI (nucleus; blue), Ki67 (proliferation, magenta), vimentin (fibroblasts; yellow), and K19 (BECs; cyan). Scale bars 50 μm.

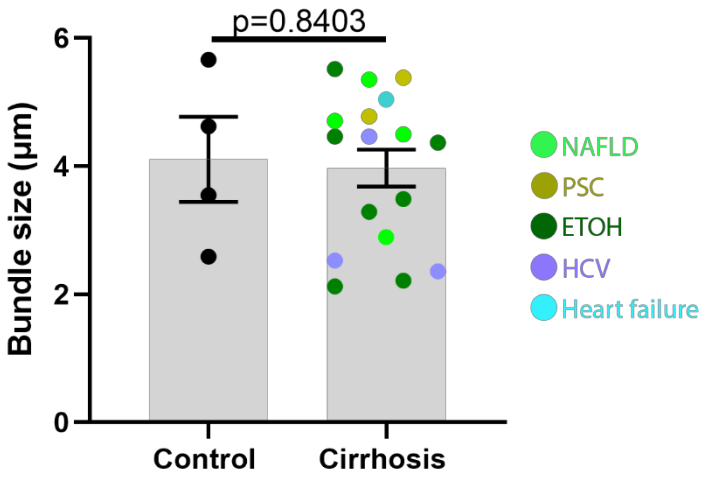


Fig. S5. Collagen bundling is unaffected by disease. Quantification of collagen bundle size. Data were analyzed with an unpaired two-tailed student t test and are shown as mean ± SEM. N=4-17

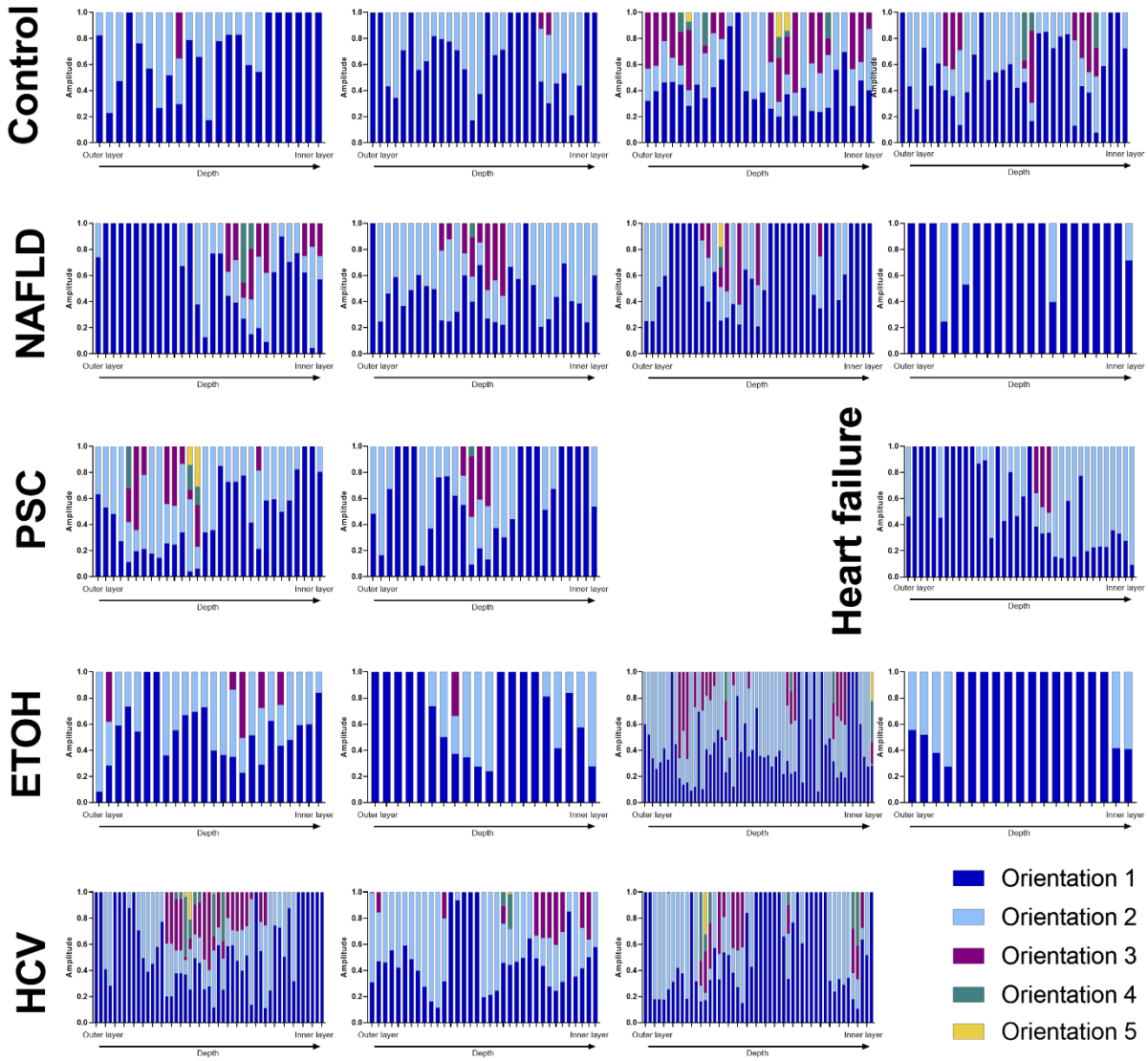


Fig. S6. Amplitude of collagen orientation families. Fibers from SHG z stacks placed into families of similar orientation for control and cirrhotic liver samples of different etiologies.

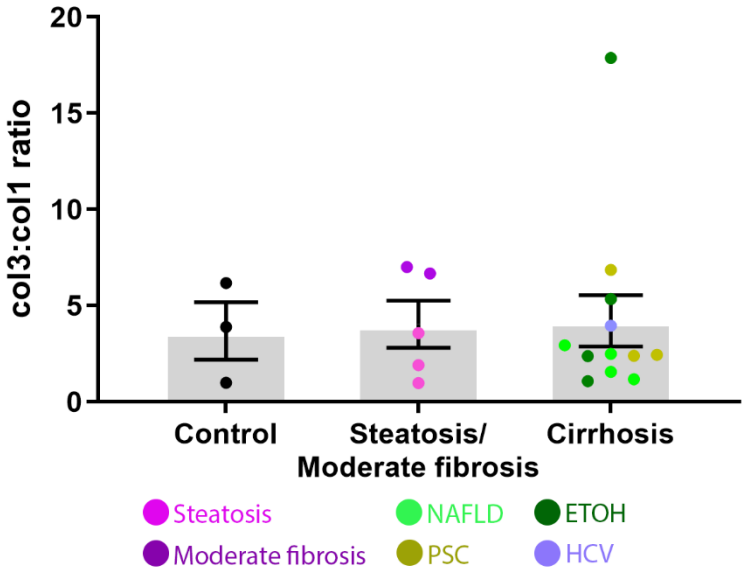


Fig. S7. Collagen 3 to collagen 1 ratio is unaltered in capsules in diseased livers. Quantification of collagen 3 to 1 ratio. Data were analyzed by one-way ANOVA and are shown as mean ± SEM. N=5-14.

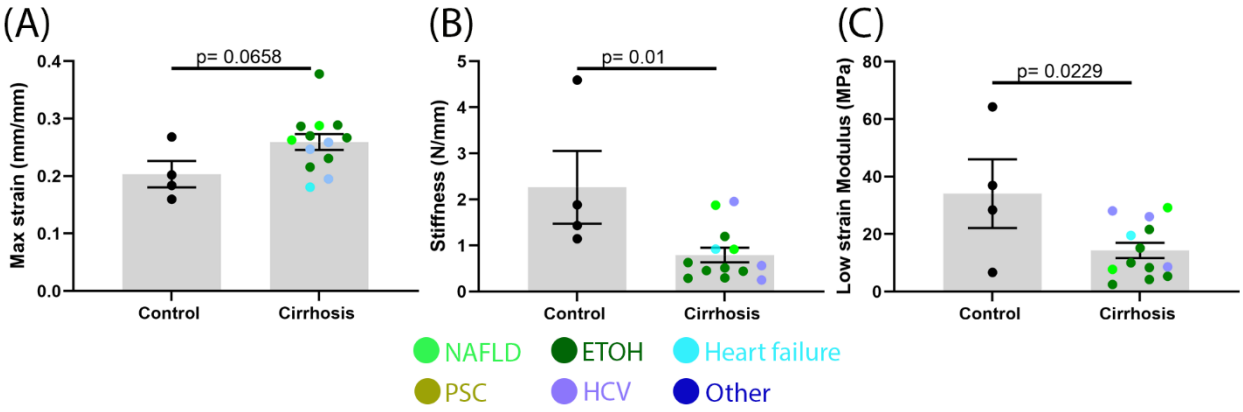


Fig. S8. Liver capsule mechanics are altered in cirrhotic patients. (A) Maximum strain at which tissue starts to fail. (B) Calculated stiffness. (C) Calculated moduli at low strain (approx. 4-6%). Data were analyzed with an unpaired two-way student t test and are shown as mean ± SEM. N=4-14.

Primary antibodies			
Antigen	**Species**	**Dilution**	**Source**
αSMA	Mouse	1:100	Sigma, A2547
Collagen 3	Goat	1:100	Southern Biotech ,1330-01
Cytokeratin 7 (K7)	Rabbit	1:200	Abcam, ab68459
Cytokeratin 19 (K19)	Mouse	1:200	Abcam, ab7754
Ki67	Rabbit	1:100	Abcam, ab16667
CD45	Rat	1:50	Novus, NB100-77417SS
Vimentin	Chicken	1:500	Novus, NB300 223
CD68	Mouse	1:100	Abcam, ab955
CD31	Rabbit	1:100	Novus, NB100-2284
Calretinin	Rabbit	1:100	Thermofisher, PA5-32287
Versican	Rabbit	1:200	Novus, NBP1-85432
Biotinylated anti-Rabbit	Donkey	1:200	Vector Laboratories, BA-1000
Biotinylated anti-Goat	Donkey	1:200	Vector Laboratories, BA-9500
Cy 2 anti-mouse	Donkey	1:500	Vector Laboratories ,715-225-150
Cy 3 anti-rabbit	Donkey	1:500	Vector Laboratories, 711-165-152
Cy3 anti-rat	Donkey	1:500	Vector Laboratories, 712-165-153
Cy5 anti-chicken	Donkey	1:500	Vector Laboratories, 703-605-155

Supplementary Table 1: Antibodies used

Supplementary References:

1. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods. 2012;9(7):676-82.
2. Montes GS, Junqueira LC. The use of the Picrosirius-polarization method for the study of the biopathology of collagen. Mem Inst Oswaldo Cruz. 1991;86 Suppl 3:1-11.
3. Guidolin D, Nico B, Crivellato E, Marzullo A, Vacca A, Ribatti D. Tumoral mast cells exhibit a common spatial distribution. Cancer Lett. 2009;273(1):80-5.
4. Witte M, Jaspers S, Wenck H, Rübhausen M, Fischer F. General method for classification of fiber families in fiber-reinforced materials: application to in-vivo human skin images. Sci Rep. 2020;10(1):10888.