Three-dimensional spatially resolved geometrical and functional models of human liver tissue reveal new aspects of NAFLD progression

Fabian Segovia-Miranda1, Hernán Morales-Navarrete1, Michael Kücken2, Vincent Moser3, Sarah Seifert1, Urska Repnik1, Fabian Rost2,4, Mario Brosch3,5, Alexander Hendricks6, Sebastian Hinz6, Christoph Röcken7, Dieter Lütjohann8, Yannis Kalaidzidis1,9, Clemens Schafmayer6, Lutz Brusch2, Jochen Hampe3,5* and Marino Zerial6,8,1*

Early disease diagnosis is key to the effective treatment of diseases. Histopathological analysis of human biopsies is the gold standard to diagnose tissue alterations. However, this approach has low resolution and overlooks 3D (three-dimensional) structural changes resulting from functional alterations. Here, we applied multiphoton imaging, 3D digital reconstructions and computational simulations to generate spatially resolved geometrical and functional models of human liver tissue at different stages of non-alcoholic fatty liver disease (NAFLD). We identified a set of morphometric cellular and tissue parameters correlated with disease progression, and discovered profound topological defects in the 3D bile canalicular (BC) network. Personalized biliary fluid dynamic simulations predicted an increased pericentral biliary pressure and micro-cholestasis, consistent with elevated cholestatic biomarkers in patients' sera. Our spatially resolved models of human liver tissue can contribute to high-definition medicine by identifying quantitative multiparametric cellular and tissue signatures to define disease progression and provide new insights into NAFLD pathophysiology.

High-definition medicine is emerging as an integrated approach to profile and restore an individual's health using a pipeline of multi-parametric analytical and therapeutic technologies. It relies on large datasets—for example, genomics and metabolomics—as well as imaging and computational modeling approaches to identify functional and structural abnormalities in organs and tissues associated with a disease state. Histology remains the method of choice to characterize pathological alterations of tissue structure. However, this technique has several disadvantages; for example, it is subjective (depends on the pathologist's skills), is often semi-quantitative and provides only two-dimensional (2D) information. In recent years, an increasing number of studies have highlighted the importance of considering 3D information for the histopathological examination of tissues. The liver is a pertinent example of complex 3D tissue organization. It consists of functional units, the liver lobuli, containing two intertwined networks, the sinusoids for blood flow and the bile canaliculi (BC) for bile secretion and flux. Sinusoids and BC run antiparallel along the central vein (CV)-portal vein (PV) axis. The hepatocytes are the major parenchymal cells and display a peculiar and unique type of cell polarity distinct from that of simple epithelia. Whereas in simple epithelia the apical surfaces of all cells face the lumen of the organ, hepatocytes are sandwiched between the sinusoidal endothelial cells and share the apical surface with multiple neighboring hepatocytes to form a 3D BC network. Such an architecture makes it difficult to grasp the 3D organization of cells, BC and sinusoidal networks, and overall tissue structure from 2D histological sections. Recent advances in optical clearing and multi-photon microscopy allow imaging thick sections of tissues such that 3D information can be captured. The image stacks can be processed to generate 3D digital reconstructions, that is, geometrical models, with subcellular resolution. The geometric models provide a detailed quantitative description of the different cells and microstructures forming the tissue and can be used to generate predictive models of tissue function, for example biliary fluid dynamics, thus gaining new insights into liver tissue organization and function. Thereby, geometrical models can be used to improve our understanding of liver (patho)biology.

NAFLD, defined as an accumulation of triglycerides and lipid droplets (LD) in the liver in the absence of alcohol intake, is rising to become the most common chronic liver disease worldwide. NAFLD includes a spectrum of liver diseases, ranging from simple steatosis to non-alcoholic steatohepatitis (NASH). Whereas steatosis is considered to be a 'non-progressive' status of the disease, NASH has the potential to progress to more severe stages, such as cirrhosis and hepatocellular carcinoma, leading eventually to liver failure and transplantation. Thus, the understanding of the transition from steatosis (STEA) to early NASH (eNASH) as a
To quantitively characterize the transition from simple STEA to eNASH, we stained, imaged and digitally reconstructed human liver tissue in 2D and 3D from biopsies of 25 people classified into four groups: normal control (NC, n = 6), healthy obese (HO, n = 4), steatosis (STEA, n = 8) and eNASH (n = 7). The demographic, clinical and histological details of the participants are summarized in Table 1 and Supplementary Table 1. We focused on lobule size, cell and nuclear morphology, LD and tissue features such as BC and sinusoids. For this, we tested 27 antibodies combining antigen retrieval that enabled the staining of sinusoids (fibronectin), nuclei (DAPI), LD (BODIPY) and cell borders (phalloidin). All images cover one complete CV–PV axis within a liver lobule. The axis was oriented according to the direction of bile flow\(^\text{12}\). Finally, we reconstructed the tissue using our open-source Motion Tracking software, as described previously\(^\text{7}\) (Fig. 1 and Supplementary Videos 1 and 2). The generation of geometrical models constituted the basis for the quantitative and structural characterization of the liver tissue in NAFLD biopsies.

### Nuclear-based analysis of NAFLD progression

We first quantified properties of hepatocytes nuclei, such as cell nuclearity and ploidy, since hepatocytes are heterogenous\(^\text{4,17}\). We quantified nuclear vacuolation given that it is a common histological characteristic in NAFLD\(^\text{18}\). Finally, we measured nuclear texture homogeneity, a feature associated with various pathological conditions\(^\text{19,20}\), methylation and acetylation status\(^\text{21}\) and, more recently, transcriptional activity\(^\text{22}\),. We observed differences in neither the proportion of mono/biniucleated cell nor in the ploidy between the groups (Extended Data Fig. 2b,c). The average values of several nuclear features showed only modest variations (Extended Data Fig. 2g–j). Therefore, our analysis reveals that the combined spatially distributed values of nuclear vacuolation and texture homogeneity could contribute to the differentiation of healthy patients from those with NAFLD.

### Morphometric parameters of LD correlate with disease progression

The finding that quantitative, spatially resolved analysis of nuclear parameters can reveal changes that are not evident in an average estimate prompted us to re-evaluate the morphometric characterization of LD. Even though LD are the hallmark of NAFLD, a detailed quantitative description of size and spatial localization within the liver lobule has not been achieved yet. Contrary to traditional histology\(^\text{1}\), immunostaining of thick tissue sections preserved most LD (Fig. 2a). In agreement with the histopathological description of NAFLD\(^\text{6,13}\), a major increase in LD between the second and fifth regions was observed in STEA and eNASH (Fig. 2b). However, the LD occupy a higher volume of the tissue in eNASH than STEA, consistent with ref.\(^\text{27}\). It is known that the LD can present massive differences in size\(^\text{28}\), and nuclear the LD occupy a higher volume of the tissue in eNASH than STEA, consistent with ref.\(^\text{27}\). It is known that the LD can present massive differences in size\(^\text{28}\), and nuclear vacuolation given that it is a common histological characteristic in NAFLD\(^\text{18}\). Finally, we measured nuclear texture homogeneity, a feature associated with various pathological conditions\(^\text{19,20}\), methylation and acetylation status\(^\text{21}\) and, more recently, transcriptional activity\(^\text{22}\),. We observed differences in neither the proportion of mono/biniucleated cell nor in the ploidy between the groups (Extended Data Fig. 2b,c). The average values of several nuclear features showed only modest variations (Extended Data Fig. 2g–j). Therefore, our analysis reveals that the combined spatially distributed values of nuclear vacuolation and texture homogeneity could contribute to the differentiation of healthy patients from those with NAFLD.

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#### Table 1 | Summary of analyzed samples

|          | Control (NC) | Healthy obese (HO) | Steatosis (STEA) | Early NASH (eNASH) |
|----------|--------------|--------------------|-----------------|-------------------|
| n        | 6            | 4                  | 8               | 7                 |
| Male, %  | 33           | 25                 | 63              | 14                |
| Age, years | 69 (54–85) | 36.5 (29–68)       | 42 (34–51)      | 51 (39–58)        |
| BMI      | 25 (21–27)   | 40.5 (32–45)       | 45.5 (39–60)    | 51 (45–75)        |
| NAS score| 0 (0–1)      | 0.5 (0–1)          | 3 (1–3)         | 4 (3–5)           |
| NAS fat  | 0 (0–1)      | 0.5 (0–1)          | 2.5 (1–3)       | 2 (2–3)           |
| NAS ballooning | 0        | 0                  | 0 (0–1)         | 1 (0–1)           |
| NAS inflammation | 0  | 0                  | 0 (0–1)         | 1 (0–1)           |
| Fat content % | 0 (0–8) | 2.5 (0–5)         | 60 (25–80)     | 60 (40–80)        |
| Fibrosis | 0            | 0                  | 0               | 0 (0–1)           |
| GGT (U per L) | 27.5 (18–60) | 19 (17–20)       | 57 (21–112)    | 77 (28–139)       |
| AP (U per L) | 70 (63–118) | 55 (50–59)       | 76 (60–90)     | 86 (59–125)       |
| Bilirubin (µmol per L) | 7 (0–14) | 5 (3–6)           | 6.5 (5–8)      | 8 (6–18)          |
| ALT (U per L) | 25.5 (15–51) | 25 (15–27)     | 34 (23–55)     | 42 (18–71)        |
| AST (U per L) | 21 (11–44) | 15 (10–25)       | 61 (25–80)     | 57 (19–76)        |

The number of subjects in each phenotypic category is provided together with demographic and histologic characteristics and blood parameters. All numeric traits are shown as the median with the range provided in parenthesis. BMI, body mass index; NAS, NAFLD activity score.

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and percentage of cell volume occupied by LD. We found a ~50% reduction in the number of hepatocytes located between the CV and the middle zone of the CV–PV axis, in STEA and eNASH (Fig. 3a). Next, we estimated the number of hepatocytes per lobule section using the density of hepatocytes (Fig. 3a) and the radius of the liver lobule in 2D (Extended Data Fig. 1b). We observed a decrease in the number of hepatocytes in HO and STEA, which partially reverted to normal in eNASH (Fig. 3b). The reduction in the number of hepatocytes could be due to apoptosis, a phenomenon reported in NAFLD29. This reduction was compensated by a massive increase in cell volume (Fig. 3c). Hepatocytes were twice as large as the average size (Fig. 3c), reaching values up to ~100,000 µm³ for STEA and eNASH (twenty times larger than a small hepatocyte) (Fig. 3d). A population analysis of the hepatocytes based on volume revealed a characteristic distribution of different cell populations along the liver lobule (Fig. 3d–g). STEA and eNASH were characterized predominantly by small and large hepatocytes which are anti-correlated along CV–PV axis (Fig. 3e–g). Even though cell density and cell volume were practically indistinguishable between STEA and eNASH, we observed a remarkable phenotype regarding the fraction of cell volume occupied by LD (Fig. 3h–j). In eNASH, hepatocytes accumulated LD even in the perportal region (Fig. 3i,j and Supplementary Video 3), suggesting that LD accumulation progressively extends to the PV as the disease progresses.

Altogether, these data reveal profound quantitative morphological disparities in cell size and LD content along the CV–PV axis. Specifically, the percentage of cell volume occupied by LD in the PV and CV areas holds the potential to discriminate between STEA and eNASH.

Alterations in apical protein trafficking. The massive presence of LD that occupy a large portion of the cytoplasm raises the question of whether apical transport of proteins in hepatocytes is affected. We analyzed the localization of four apical proteins, aminopeptidase N (CD13), bile salt export pump (BSEP), multidrug resistant-associated protein-2 (MRP2) and dipeptidylpeptidase-4 (DPPIV), CD13, BSEP and MRP2 were correctly localized to the apical membrane in all conditions (Extended Data Figs. 1c–f and 3a,b). DPPIV was enriched on the apical membrane with a small fraction on the basal membrane in NC and HO (Extended Data Fig. 3c). Strikingly, DPPIV was redistributed to the lateral membrane in pericentral hepatocytes, showing already a clear trend in STEA that becomes statistically significant in eNASH, while retaining its normal localization in the peripoortal zone (Extended Data Fig. 3c–e). Considering that DPPIV follows the transcytotic route to the apical surface10, whereas BSEP and MRP2 do not26,27, the mislocalization of DPPIV suggests a possible disruption of this transport route in pericentral hepatocytes. This supports previous findings regarding the misregulation of membrane-protein trafficking in NAFLD29 and prompted us to evaluate whether the integrity and 3D organization of the BC could be affected during disease progression.

Bile canaliculi network shows geometrical and topological defects in NAFLD. We carried out a geometrical and topological characterization of BC and sinusoidal networks. Even though there is a tendency towards a reduction in volume fraction and total length of the sinusoidal network in STEA and eNASH (Extended Data Fig. 4b,e), no major defects in sinusoidal microanatomy were detected (Extended Data Fig. 4a,c,d,f–h). In contrast, we detected profound quantitative alterations in the architecture of the BC network between groups. Contrary to the packed and homogeneous appearance in NC and HO, the BC in STEA and eNASH displayed clear morphological defects, which were more pronounced in the pericentral region (Fig. 4a,b). A detailed analysis revealed a sustained increase in BC radius in STEA and eNASH that was especially...
pronounced toward the periporal region (Fig. 4d). In addition, in both STEA and eNASH, we observed a strong reduction in the total length and branches crossing regions of the BC towards the pericentral zone (Fig. 4c,l). Other geometrical properties, such as volume fraction and junction density, were unaffected (Fig. 4c,e). These changes are noteworthy because we found very little variability in the geometric and topological features of the BC network among liver lobules of the same participant and among lobules within the same group (Extended Data Fig. 5a–d).

To investigate the topological properties of the BC network, we performed an analysis of network connectivity. Surprisingly, we found a pronounced decay in the connectivity in STEA and eNASH towards the pericentral region (Fig. 4a,b,g,h and Supplementary Video 4). One possibility is that the alterations in BC connectivity are only apparent. As the volume of hepatocytes is increased (Fig. 3g), the sectioning may cut both the BC and sinusoidal networks and, therefore, give the impression of only an altered connectivity. However, this is not the case for the sinusoidal network, which indeed does not appear disrupted (Extended Data Fig. 4a,f,g), supporting the idea that the BC network is specifically affected. Thus, our data point at specific geometrical and topological alterations in the pericentral BC network in both STEA and eNASH.

These results suggest that an unbiased classification of people with NAFLD on the basis of quantitative morphometric and topological features of the BC network is feasible. To test for this, we analyzed the prognostic power of the BC network parameters to classify patients in different disease stages using a machine-learning framework. We used Support vector machine (SVM)\textsuperscript{34}, a supervised classifier that has been successfully applied to several classification problems of disease conditions\textsuperscript{35}. We analyzed 52 reconstructed BC networks from healthy tissue (NC and HO), STEA and eNASH. We used 5 features (volume fraction, radius, length per volume, fraction of connected network and branches crossing regions) at 10 regions along the CV–PV axis (Fig. 4c,d,f,g), resulting in a set of 49 parameters. Remarkably, our analysis showed that a subset of just 7 parameters, which are only measurable in 3D, gives the maximum predictive accuracy (86.96 ± 0.45%, mean ± s.e.m. ) (Fig. 4i). Almost no errors were found in the discrimination of healthy tissue, and few discrepancies were found between STEA and eNASH (Fig. 4k,l). These results suggest that a relatively small set of parameters describing the alterations of BC network may be sufficient to discriminate between disease stages.

**Personalized model of bile flow predicts increase in bile pressure in the pericentral region.** The observed alterations of BC-network architecture are likely to have consequences for liver tissue function, particularly for bile flow. However, it is not yet possible to measure bile flow in the BC of human liver. We recently developed a computational model of bile fluid dynamics, validated its quantitative predictions in mouse models and demonstrated that bile velocity and bile pressure distributions along the liver lobule strongly depend on BC geometry\textsuperscript{32}. Here, we extended this model\textsuperscript{32} in a spatially heterogenous fashion (Fig. 5a) to handle the extreme inhomogeneity of BC density in STEA...
and eNASH. Briefly, the refined model is based on conservation of mass for water and osmolytes and Darcy’s Law for laminar flow. Since we obtained morphometric data from individual subjects, we can now aim at developing personalized models—that is, those parameterized by integrating previously reported values (viscosity\(^3\), permeability\(^3\)\(^7\)\(^8\)\(^9\)\(^1\) and osmolyte secretion rate\(^3\(^9\)\(^1\)\) with individual geometrical and topological BC measurements (Fig. 4c, d, g, and Extended Data Fig. 6). No free parameters remained and, hence, no parameter fitting was needed. Next, we applied this model to predict bile velocity, flux and pressure across the liver lobule for individuals from all four groups.

The model predicts similar bile velocities (5–10 \(\mu\)m sec\(^{-1}\)) and flux (10–35 \(\mu\)m\(^2\) sec\(^{-1}\)) for the different groups in the periportal area (Fig. 5b–e). Comparable velocities have been reported in mice\(^1\(^2\)\(^3\). The percentage of cellular volume occupied by the different populations is shown in (small (a), medium (b) and large (c)). Percentage of the cell volume occupied by LD: distribution (a) and statistics along the CV–PV axis and overall (b). Hepatocytes with percentage of LD volume lower than 0.001% are not presented in the distributions, which were normalized such that their integrals are equal to 1,000. We analyzed 11,278 cells from 16 reconstructions (NC = 5 samples, HO = 3 samples, STEA = 4 samples, eNASH = 4 samples). Spatially resolved quantification represented by median + MAD per region and overall quantifications by box-plots (median values as red lines, 25th and 75th percentiles as blue bottom and top edges of the boxes, extreme data points by whiskers). One-tailed Wilcoxon rank-sum test. * \(P < 0.05\), ** \(P < 0.01\), *** \(P < 0.001\) j. Representative cells reconstructed in 3D and selected from region 3 and 8. Apical, basal and lateral surface are shown in green, magenta and grey, respectively. LD are shown in red. Scale bar, 10 \(\mu\)m. We repeated 16 reconstructions (NC = 5 samples, HO = 3 samples, STEA = 4 samples, eNASH = 4 samples) independently with similar results.

None of the pericentral pressures in the NC and HO subjects exceeded 2,000 Pa, but this was the case for 8 patients (53%) in the STEA and eNASH groups. In 4 of the STEA and eNASH subjects (27%), the pericentral pressure exceeded 3,000 Pa and in 2 patients (13%) even 4,000 Pa (Fig. 5d,e). Therefore, our model predicts an increase in pericentral bile pressure in STEA and eNASH conditions, spanning different levels of severity, depending on the BC geometry and topology of individuals.

We next set out to validate the model predictions. As it is impossible to measure bile flow and pressure at this resolution in the human liver, we considered possible consequences of changes in bile pressure. Increased bile pressure is a hallmark of cholestasis\(^6\)\(^5\)\(^6\)\(^1\). Therefore, as readout of increased bile pressure, we analyzed the most commonly used cholestatic and liver damage biomarkers in serum, including bilirubin, gamma glutamyl transpeptidase (GGT), alkaline phosphatase (AP), total and primary bile acids (BAs), aspartate aminotransferase (AST) and alanine aminotransferase (ALT). To increase the statistical power, we analysed additional sera samples for the different groups (NC, n = 22; HO n = 27; eNASH n = 4).
STEA, n = 31; and eNASH, n = 24 samples) (Supplementary Table 1). Whereas GGT was elevated in STEA and eNASH, and primary BAs in only eNASH, we did not detect significant changes in the levels of bilirubin, AP and total BAs between the groups (Extended Fig. Data 7).

Finally, we analyzed the correlation between the predicted pericentral bile pressure and the biomarkers for individual participants (Extended Data Fig. 8a–f). Strikingly, we found a strong correlation for GGT and a significant correlation for AP (Fig. 5f and Extended Data Fig. 8b). There was a significant correlation for primary BAs...
One of its pillars is the combination of image analysis and computational modeling to uncover tissue alterations at different structural and functional levels. During the past few years, there has been an urge to gain a better understanding of NAFLD establishment and progression because of its growing impact on public health. A lot of attention has been drawn to signaling pathways, the microbiome, metabolism, genetic risk factors, BAs and so on. However, a major challenge is to understand how the molecular alterations detected are an expression of the organ dysfunction, manifested as morphological and functional alterations of cells and tissue architecture. The classical histological analysis has provided insights into fundamental aspects of NAFLD. However, a quantitative description of the 3D tissue morphology is indispensable, particularly for the liver. Here, we used high-resolution multiphoton microscopy and 3D digital reconstructions to generate a comparative dataset of structural changes of human liver tissue from participants sorted into NC, HO, STEA and eNASH groups. We identified a set of spatially distributed morphological alterations, such as a characteristic size distribution of LD, nuclear texture homogeneity and BC morphology, that could be used as tissue biomarkers to resolve different stages of NAFLD progression. Although several morphological defects could be inferred from 2D images, topological characterization of the BC and sinusoidal networks can only be extracted from a 3D reconstruction. Indeed, our 3D digital reconstruction of human liver tissue provided the first evidence that BC integrity is disrupted in NAFLD, bringing BC integrity and the mechanisms involved in its maintenance and homeostasis (polarized transport, bile flow, BAs turnover and so on) into focus for future studies. Quantitative 3D features of BC architecture are therefore candidate parameters for unbiased classification of tissue samples.

**Discussion**

High-definition medicine provides a new approach to understanding human health of individuals with unprecedented precision. One of its pillars is the combination of image analysis and computational modeling to uncover tissue alterations at different structural and functional levels. During the past few years, there has been an urge to gain a better understanding of NAFLD establishment and progression because of its growing impact on public health. A lot of attention has been drawn to signaling pathways, the microbiome, metabolism, genetic risk factors, BAs and so on. However, a major challenge is to understand how the molecular alterations detected are an expression of the organ dysfunction, manifested as morphological and functional alterations of cells and tissue architecture. The classical histological analysis has provided insights into fundamental aspects of NAFLD. However, a quantitative description of the 3D tissue morphology is indispensable, particularly for the liver. Here, we used high-resolution multiphoton microscopy and 3D digital reconstructions to generate a comparative dataset of structural changes of human liver tissue from participants sorted into NC, HO, STEA and eNASH groups. We identified a set of spatially distributed morphological alterations, such as a characteristic size distribution of LD, nuclear texture homogeneity and BC morphology, that could be used as tissue biomarkers to resolve different stages of NAFLD progression. Although several morphological defects could be inferred from 2D images, topological characterization of the BC and sinusoidal networks can only be extracted from a 3D reconstruction. Indeed, our 3D digital reconstruction of human liver tissue provided the first evidence that BC integrity is disrupted in NAFLD, bringing BC integrity and the mechanisms involved in its maintenance and homeostasis (polarized transport, bile flow, BAs turnover and so on) into focus for future studies. Quantitative 3D features of BC architecture are therefore candidate parameters for unbiased classification of tissue samples.

The 3D spatially resolved quantitative analysis of human liver samples revealed a set of unknown morphological features, ranging from the subcellular to tissue level, that seem to be perturbed during NAFLD progression. First, we detected changes in nuclear texture in pericentral hepatocytes, which have been reported in several diseases, and may reflect changes in transcriptional activity. Second, although LD accumulation is a characteristic feature of NAFLD, our analysis revealed quantitative changes in their size distribution, with the medium-sized LD enriched in the periporal region in eNASH. In healthy conditions, the LD number and size are accurately regulated, and changes in their distribution point to cell-specific alterations in the mechanisms regulating LD biogenesis and catabolism. Third, and most striking, we observed alterations of the apical plasma membrane of hepatocytes and of the
BC network. The large pericentral hepatocytes showed mislocalization of DPPIV, pointing towards a dysregulation in apical protein trafficking[2]. Interestingly, not all apical proteins were missorted, suggesting that trafficking defects could be pathway (transcytosis) and/or cargo specific. Transcytosis defects may be due to physical constraints caused by the large LD in the swollen hepatocytes. In addition, the uneven size of hepatocytes in the pericentral zone may introduce perturbations in cell packing, leading to disruption of BC connectivity. The unaltered architecture of the sinusoidal network makes it unlikely that the reduction in BC connectivity is due to the sectioning of the enlarged hepatocytes within the thickness of the tissue slice, since this should have then affected both BC and sinusoidal networks in a similar fashion.

The weaker BC network connectivity and dilated BC radius are partly compensatory and counteracting effects. Whereas the first hinders bile flow, the latter eases it. Therefore, without a mathematical model, it would have been impossible to predict the combined effect. On the basis of the geometrical and topological information extracted from the BC, our computational personalized model predicted high bile pressure in the pericentral area in people with STEA or eNASH. This suggests that STEA and eNASH livers are affected by a pericentral microcholestasis, a prediction that was supported by the detection of cholestatic biomarkers in serum. Cholestasis is reflected by a range of biomarkers. The strong correlation of systemic GGT levels to pericentral biliary pressure may reflect localized apical membrane stress that does not lead to a bilirubin excretion problem at the organ level. Therefore, GGT may be an indicator of more advanced tissue microalterations that lead to bile pressure increase.

In recent years, a lot of research has been devoted to the role of BAs and BA receptor FXR in NALFD[26–27]. However, there is currently no explanation for the alterations in BA composition in blood, the decreased ratio of secondary/primary BAs observed by us (Extended Data Fig. 7f–j) and others[16,29,30], and whether it correlates with changes in tissue morphology[31]. Our data shed new light on this problem. The altered BC microanatomy and consequent pericentral microcholestasis may hamper BA secretion into BC, as apical pumps (BSEP, MRP2) have to operate against elevated luminal BA concentrations. This would lead to back-flux of primary BAs into the blood, reducing their availability for conversion into secondary BAs by the intestinal microbiota (Extended Data Figs. 7) and 8g), thus contributing to the changes in BAs composition observed in NALFD[26,42,43]. Bile flow is essential for normal liver function. Bile accumulation, due to its detergent-like properties, can cause liver damage[20,31] and bile pressure can affect metabolism[32]. Indeed, the accumulation of LD[33,34] and BAs[35] could induce oxidative stress and trigger apoptosis[36], which is consistent with the reduction in pericentral hepatocytes observed in STEA and eNASH. The occurrence of pericentral microcholestasis is a new piece in the NALFD pathophysiology puzzle that contributes to clarify some aspects of the disease so far without explanation, for example increase of GGT levels[37], BAs in serum[38], upregulation of MRP3 in NASH[39] and the beneficial effect of UDCA treatment in NALFD[40], all signs of ongoing cholestasis[31,37].

The combination of experimental data with computational models of tissues has proven successful in elucidating pathogenic mechanisms using animal models[2,29]. However, animal models very often fail to mimic human diseases, including NALFD[41]. In this study, the geometrical models of liver tissue from human biopsies combined with spatially heterogeneous computational simulations revealed new aspects of NALFD pathology. A firm distinction between STEA and SASHI will require further integration of molecular analysis and 3D morphological tissue parameters. This approach may help to identify new biomarkers for early disease diagnosis and predict the functional status of the tissue with potential applications in high-definition medicine[42,43].

Online content
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Methods

Human liver samples. Liver samples were obtained intraoperatively from people in whom an intraoperative liver biopsy was indicated on clinical grounds, such as exclusion of liver malignancy during major oncologic surgery or assessment of liver histology during bariatric surgery. Standardized histopathologic assessment was performed by a single pathologist (C. R.) based on the NAFLD activity score, the NASH-CRN. The samples were divided into 4 groups: NC, HO, STEA and eNASH based on the following criteria. NC samples showed steatosis ≤5%, no inflammation, no ballooning and no fibrosis and were obtained during liver resections or biopsy in non-hepatobiliary malignancy in people with a body mass index (BMI) <30 kg/m². Livers were either free of metastases or were at a distance of at least 2 cm from the next metastasis observed. Samples for the HO, STEA and eNASH categories were obtained in people undergoing bariatric surgery with a BMI >30, and subgroups were defined by liver histology as follows: HO samples showed a normal liver histology, such as that of the NC samples; STEA samples had >5% fat, no inflammation, fibrosis ≤1 and ballooning ≤1; eNASH samples were characterized by >5% fat, an inflammation grade of at least 1, fibrosis ≤1 and ballooning ≤1. All participants were of European descent. None of the individuals underwent preoperative chemotherapy, and liver histology demonstrated absence of both cirrhosis and malignancy. Biopsy specimens were fixed immediately in 4% paraformaldehyde 0.1% Tween-20/PBS for 2–3 days. People with evidence of viral hepatitis, hemochromatosis or alcohol consumption greater than 20 g day⁻¹ for women and 30 g day⁻¹ for men were excluded. All participants provided written informed consent. The study protocol was approved by the institutional review board (Ethikkommission der Medizinischen Fakultät der Universität Kiel, D425/07, A111/99) before study commencement.

Immunolabeling, optical clearing and imaging. For the immunostainings where heat-induced epitope retrieval was required, liver slices were transferred to an Eppendorf tube with citrate buffer pH 6.0 (Sigma-Aldrich cat. no. C9999) and heated for 30 min at 80°C. Next, immunolabeling (Supplementary Table 2) and optical clearing were performed as described previously.

Liver samples were imaged (0.3-µm voxel size) in an inverted multiphoton laser-scanning microscope (Zeiss LSM 780 NLO) using a 63x/1.3 numerical aperture glycerol immersion objective (Zeiss). DAPI and phalloidin A647 were excited at 790 nm using a Chameleon Ti-Sapphire 2-photon laser and detected with non-descanned detectors (NDD). Alexa Fluor 488, 555 and 594 were excited with 488, 561 and 594 laser lines and detected with Gallium arsenide phosphide (GaAsP) detectors.

3D tissue reconstruction. The different components of live tissue (that is, BC and sinusoidal networks, nuclei, LDIs and hepatocytes) were reconstructed from high-resolution (voxel size, 0.3×0.3×0.3 µm) fluorescent image stacks (100-µm depth) of fixed liver tissue stained by specific antibodies and/or small fluorescent molecules (CD13, fibronectin, DAPI, BODIPY and LDLR). To cover the entire CV–PV axes, tiles of 2 × 2 or 3 × 3 image stacks were stitched using the image stitching plug-in of Fiji62. In general, one CV–PV axis was imaged per subject. Variability among lobules and within subjects was tested by comparing the geometric and topological BC parameters used for the bile-flow model, from three distant CV–PV axes from different parts of the biopsies for 13 subjects, covering the four groups. Then, all images were reconstructed using the software MotionTracking (http://motiontracking.mpib-cbg.de) as described in ref. 62. Briefly, all stitched images were denoised using the Bayesian foreground/background discrimination (BFBD) de-noising algorithm proposed in ref. 62. Next, the tubular structures (BC and sinusoidal networks) as well as nuclei were segmented using a maximum entropy local thresholding algorithm. Artifacts generated by the segmentation (holes and tiny isolated objects) were removed by standard morphological operations (opening/closing). The triangulation mesh of the segmented structures was generated by the cube marching algorithm and tuned using an active mesh approach. In the case of tubular structures, representations of the “medial axis” or “skeleton”, also called central lines, of the networks were generated. Central lines are represented as 3D graphs. Finally, the shape of the cell surface (hepatocytes) was found using an active mesh expansion from the reconstructed nuclei. For details, refer to ref. 62.

Morphological spatial analysis. To account for the variability of different morphological parameters along the liver lobule, the CV–PV axis was computationally divided in ten equidistant zones. The zones were defined in terms of the distances to the closest CV and PV, as follows:

\[ z_{\text{zones}} = \left\lfloor \frac{d_v}{d_{\text{CV}} + d_{\text{PV}}} + 1 \right\rfloor \]

Where \( i = 1, 2, \ldots, n \), \( n \) is the number of zones, \( \lfloor \cdot \rfloor \) is the floor function, \( d_v \) and \( d_{\text{CV}} \) are the distances to the closest CV and PV, respectively. The average value of the different morphological parameters was calculated in every region.

Nuclear vacuolation. To determine whether nuclei are vacuolated, we calculated the DAPI mean intensity in the middle of the nucleus (inside a sphere of 1.2 µm located at the center of the nucleus) as well as in the inner surface of it (over a layer of thickness 0.6 µm). If the inner DAPI intensity was three times lower than the DAPI intensity in the surface, the nucleus was defined as vacuolated.

Nuclear texture. We analyzed the nuclear texture based on the Haralick texture features measured over images of DAPI. We examined the four features related to the texture homogeneity or heterogeneity of the image: homogeneity (angular second moment), local homogeneity (inverse difference moment), contrast and entropy. To avoid boundary artifacts, we analyzed only the intensity of DAPI inside a cube located in the center of the nucleus and of a length equal to half of the nucleus radius. All features were extracted from the normalized gray-level co-occurrence matrix (256 gray levels) averaged over the 13 directional co-occurrence matrices (in 3D) at distances from 1 to 3 pixels. Vacuolated nuclei were excluded from the analysis.

Network connectivity. Two approaches were used to quantify the connectivity of the tubular networks (BC and sinusoidal networks). The first one, also referred as ‘fraction of connected network’, is based on the central lines of the network and defined as the ratio between the length of the largest connected graph of the network and the total length of the network. The second one is based on the segmented 3D image of network and calculates the connectivity density using the Euler characteristic of the network (maximum number of branches that can be removed before the network is separated in two parts). The former calculation was performed in Fiji using the plugin BoneJ64.

Lobule radius. Liver slices were stained with DAPI and GS antibody to facilitate the visual discrimination between CV and PV. Then, the sections were imaged with a Plan-Apochromat 10x/0.45 M27 objective at 2-µm pixel size. The distance between the CV to the closest portal tract were measured in each 2D image. The lobule radius is reported as the median of all the measurements for each 2D image.

Hepatocytes per lobule. In order to estimate the number of hepatocytes per lobule section, we used the 3D information of the hepatocyes's density \( \delta \) and an approximation of the lobule section \( V_\text{lob} \), assuming the lobule to be hexagonal prism, as follows:

\[ n_s = N_s V_\text{lob} = \delta \times \frac{3\sqrt{3}}{2} \times R_i^2 \times H \]

where \( n_s \) is the number of hepatocytes per lobule section, \( \delta \) is the numerical density of hepatocytes (number of hepatocytes per tissue volume), \( R_i \) is the radius of the lobule and \( H \) is the height of the lobule section. We used the estimated radius on the lobule (in 2D) for \( R_i \), and \( H = 100 \mu m \).

Optimal set of parameters for cluster analysis. In order to find the most relevant parameters for the classification, we used a greedy algorithm to obtain an optimal subset (OS) of parameters, as described in ref. 66. Briefly, the values of individual parameters were systematically added to OS based on the classifier predictive accuracy. The multi-class classification was performed using an error-correcting output codes (ECOC) model for SVM, and the predictive accuracy was measured using ten-fold cross-validation.

Quantification of DPPIV localization. We stained 100-µm liver slices with DAPI, Phalloidin, BSEP and DPPIV antibodies. At 20 µm under the tissue surface, single-plane images were acquired with an LCI Plan-Neofluar 63x/1.3 objective and 0.3-µm pixel size, and using the same microscope settings for all images. The cell borders and apical domains were segmented using a threshold-based segmentation algorithm. Since no specific staining for the basolateral membrane was present, the basolatal domains were segmented manually on the basis of phalloidin staining and morphology. To analyze changes in the localization of DPPIV, the mean intensity of DPPIV was measured at each domain (apical, basal and lateral) and in an area covering a radius of covering 125 µm of tissue around each vein.

Electron microscopy. For the ultrastructural analysis of bile canaliculi, we used 100-µm-thick vibratome sections of human liver biopsy tissue originally fixed with 4% PFA for several days and then stored in PBS. Before embedding, vibratome sections were fixed with 1% glutaraldehyde in 200 mM HEPES at least overnight and then were cut into small pieces. Next, tissue was postfixed with 1% osmium tetroxide prepared in 1.5% potassium ferricyanide, for 1 h at room temperature. After washing with water, tissue was incubated with 1% tannic acid dissolved in 100 mM HEPES, pH 7.0 for 20 min, followed by incubation with 1% disodium sulfate for 5 min, and then by several washes with water. After that, tissue was incubated with 2% aqueous uranyl acetate for 2 h at room temperature and protected from light. A graded ethanol series—70%, 80%, 90%, 96%, each for 10 min—was used for dehydration, which was followed by absolute ethanol, 4 × 15 min. Infiltration was progressively infiltrated with embedding epon resin over 24 h. Finally, tissue pieces were flat embedded between two teflon-coated glass slides and heat polymerized overnight. Embedded tissue pieces were remounted.
for longitudinal sectioning. Periportal or pericentral regions were selected on 1-μm sections, stained with methylene blue-azur II and examined in a light microscope. Then 70- to 80-nm-thick sections were cut. These were stained with 0.4% lead citrate for 1 min and imaged in the Tecnaï T12 transmission electron microscope (ThermoFisher), equipped with an axial Tietz CCD camera (TVIPS). Images of bile canaliculi were taken at 6,800x magnification by systematic and random screening. Bile canaliculi with poorly preserved microvilli were not included in the analysis.

To estimate a fraction of free lumen (that is, lumen not occupied by microvilli) in bile canaliculi, we applied stereological point counting (the Cavalieri estimator) using Fiji software. A test grid of vertical and horizontal lines (area per point, 5,000 nm²) was laid over images. Cross points over total lumen profile area and over free lumen profile area were separately counted. For each set of samples and each region (central or portal vein) a minimum of five EM images was used, and counts obtained from individual images were summed up to obtain total counts. The analysis was performed in a blinded way.

Bile-flow model in the hepatic lobule. The disease phenotype of zonal cell swelling entails weaker BC network connectivity but also dilated BC radii, potentially indicative of increased BC fluid pressure. These are partly compensatory and counteracting effects as the first hinders flow but the latter eases it. Therefore, without the help of a mathematical model, it would be difficult to predict the combined effect on liver function (here bile flow) by all the observed alterations of the BC micro-anatomy (which are only attainable through a 3D imaging approach). Our mechanistic model integrates 6 quantitative data sets (both structural and functional). We considered the altered BC micro-anatomy (which are only attainable through a 3D imaging approach) in a compartmental model. Our mechanistic model integrates 6 quantitative data sets (Extended Data Fig. 6b), (4) fraction of connected BC (excluding dead-end branches that do not carry flow) (Fig. 4g), (5) canaliculi tortuosity \( t \) and (6) apical surface density \( \rho_{\text{As}} \). These are partly higher canaliculi bile-acid concentrations for people with fatty-liver disease. When multiple samples were available for a patient, the median of the simulated values was determined (Extended Data Fig. 5).

We consider a porous medium in a 3D domain \( \Omega \) with an osmolyte-influx density \( g(x) \) and a fluid-influx density \( \bar{g}(x) \), leading to a lumped concentration profile \( c(x,t) \) for osmolytes throughout the bulk of the domain. The bulk velocity field of the fluid is determined by Darcy’s law, according to which velocity \( \bar{v} \) is proportional, with a resistance coefficient \( -K(x) \), to the pressure gradient \( \rho(t) \). This approach is similar to previous models of blood circulation through the sinusoidal network\(^{107} \). At steady state, this system is described by conservation of mass for the osmolytes as well as the fluid and the connection between pressure gradient and velocity as follows:

\[
\nabla \rho(x) = f(x)
\]

\[
\nabla (c(x)\bar{v}(x)) - DNc(x) = g(x)
\]

\[
\rho(x) = K(x)\bar{v}(x)
\]

As an approximation, the liver lobule can be assumed to have cylindrical symmetry. Therefore, we take \( \Omega \) as a cylindrical domain with radial symmetry and radius \( L \), approximating a single prismatic liver lobule. Hence, we need to consider only the radial variable which will be denoted by \( \rho \). Following ref. \(^{107} \), we consider the fluid influx \( j \) as proportional to the difference in osmotic pressure \( (RTc - RT\bar{c}) \) and hydrostatic pressure \( p \). Here, \( c \) parametrizes the osmotic background pressure in the cytosol of surrounding hepatocytes. Osmolyte diffusion with diffusion constant \( D \) along BC can be neglected (by setting \( D = 0 \) for uniform osmolyte influx density \( g(x) \) and the parameters chosen here. Below, we will quantify the concentration term in the equation for \( w \) as follows:

\[
(w')/p = \kappa A(RT/(G/\rho w) - c) - p' = -K(p)w
\]

\[
(w')/p = \kappa A(RT/(G/\rho w) - c) - p - w/\rho
\]

or written

\[
(w')/p = \kappa A(RT/(G/\rho w) - c) - p = w/\rho
\]

\[
\rho_0(G/\rho w)/w
\]

\[
\lim_{\rho \to \rho_0} G = \rho_0 G/\rho \]

which modifies the equations as follows (introducing \( G = G/L^2 \) and immediately dropping the bars):

\[
w' = \kappa A(LG/\rho w - c) - p - w/\rho
\]

\[
p' = -Lkw
\]

Note that there is no singularity at \( p = \rho_0 \), even though \( w \) approaches zero and appears in the denominator because we have, after applying L'Hospital's rule:

\[
\lim_{\rho \to \rho_0} G = \rho_0 G/\rho
\]

and hence a finite limit for \( p = \rho_0 \).

The equations are solved numerically using the shooting method. For this method, we set the initial values to \( w(p) = 0 \) (the proper condition) and \( p(p) = p^* \) with some arbitrarily chosen value \( p^* \). We solve the ODEs for \( w \) and \( p \) with an implicit integration scheme till \( p = 1 \). Next, we iteratively updated \( p^* \) until \( p(1) = p_0 \). To avoid any division by \( w = 0 \) at \( p = p_0 \) in the first integration step, we make use of the above equation for the limit \( p = p_0 \). This results in an algebraic equation for \( w(p_0) \):

\[
(w(p_0)) = -s/2 + \sqrt{s^2/4 + 1}
\]

with

\[
t = \kappa A(LT(p_0)/\rho w)/w)
\]

\[
\kappa = \kappa A(T(p_0) + p(p_0))
\]

The equations were integrated using an implicit Euler method (for the freely available source code, see https://github.com/MichaelKuecken/bileflow).

For the determination of \( K \), we consider a network of tubes with radii \( r(p) \) (Fig. 4d) and tortuosity \( t(p) \) that cause a bulk porosity of \( \epsilon(p) \). The porosity \( \epsilon(p) \) is determined from subject data as the product of the BC volume fraction (Fig. 4c) and the BC connectivity profile (Fig. 4g), amounting to pruning of branches that do not relay the flow. Where BC connectivity was quantified as 0, we set it to 0.01 assuming that at least some remote connection may exist and thereby potentially underestimate the pressure profile. We take into account that a sizable portion of the BC lumen is taken up by microvilli. From our EM measurements, we know the fraction of free to total lumen \( A(p) \), therefore the effective porosity and radius are given by \( \epsilon_m(p) = A(p) t(p) \). To determine the effective BC radius, we consider two extreme cases. In the first scenario, all microvilli are considered to be pushed to
the canaliculi walls by the flow and we have \( r_{BC}(p) = r_{BC}(0) \), where \( r_{BC} = r_{BC}(0) \) is approximately 0.4 to 0.6 here. In the other scenario all microvilli behave like a porous medium inside each BC and there is no free lumen in the middle of the canaliculi. In this case porous media theory \(^\text{12}\) predicts a free radius of \( a(p) r_{BC} / (1 - a(p)) \) where \( r_{BC} = 6 \times 10^{-4} \text{m} \) is the radius of the microvilli \(^\text{12}\). In this case we obtain an effective radius of \( r_{BC}(p) = r_{BC}(0) / (1 - a(p)) \) which is close to the viscosity of water. The osmolyte loading of hepatocytes is chosen similar to that of blood and bile, \( \rho = 0.4 \text{L} / \text{s} \). We consider an arrangement. Therefore, there is a convergent flow of bile towards the bile ducts which originates from the flow around the microvilli in the canaliculi. This contribution is comparable to that of the osmotic pressure \( \rho \cdot \Delta p \) which is close to the viscosity of water. The data supporting the findings of this study are available within the paper and its Supplementary Tables 1 and 3.

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Bile acids and serum markers. Individual serum bile acids and their precursors were analyzed either by gas-liquid chromatography mass spectrometry (GLC–MS) or liquid chromatography (HPLC) as described \(^\text{11}\). Statistical analysis. For the spatially resolved quantifications (that is, statistics of data from along the CV–PV axis), the median ± median absolute deviation (MAD) values per region are shown. MAD was calculated as follows: MAD = median (\( |X - \text{median}(X)| \)), where \( X \) is any variable. Global quantifications (between enzymes, bile acids, and tissue/cell parameters) are shown using box plots. Median values are shown as red lines, and the 25th and 75th percentiles are represented by the blue bottom and top edges of the boxes, respectively. The whiskers extended up to the most extreme data points that are not considered outliers. The statistical significance analysis was performed using: (1) Kruskal–Wallis (non-parametric method for testing whether samples originate from the same distribution), one second comparison of variance (ANOVA) on ranks, to compare the data within conditions; (2) Wilcoxon rank-sum test to compare data between conditions (test all conditions against the normal control). The results of the correlation analysis are now reported as Spearman correlation coefficients with Wilcoxon rank-sum test values. One-sided hypothesis testing was used if a priori knowledge suggested a one-sided hypothesis, otherwise a two-sided test was applied. In both cases, unequal variances were assumed. Median, MAD, number of samples and \( P \) values for all figures are provided in Supplementary Table 3. All statistical analysis was performed using MATLAB 2018b.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data Availability
The data supporting the findings of this study are available within the paper and its Supplementary Tables 1 and 3.

Code Availability
Code of the shooting solver is available from https://github.com/MichaelKuecken/bileflow.
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Author contributions

F.S.-M., J.H. and M.Z. conceived the project. F.S.-M., V.M. and S.S. performed the immunofluorescence experiments and imaging. H.M.-N. and Y.K. developed the image analysis algorithms. F.S.-M., V.M. and H.M.-N. performed the 3D tissue reconstructions. H.M.-N. and F.S.-M. performed the data analysis and interpretation of the results. U.R. performed the electron microscopy. A.H., S.H., C.R., C.S and M.B. obtained the samples and characterized the patients. D.L. measured bile acids. M.K., F.R., Y.K. and L.B. conceived and developed the mathematical model. M.K and F.R. programmed and simulated the mathematical model and performed statistical analysis. M.K. and L.B. interpreted results and wrote the model description. F.S.-M., H.M.-N., M.K., Y.K., L.B., J.H. and M.Z. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Extended data is available for this paper at https://doi.org/10.1038/s41591-019-0660-7. Supplementary information is available for this paper at https://doi.org/10.1038/s41591-019-0660-7.

Correspondence and requests for materials should be addressed to J.H. or M.Z.

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Extended Data Fig. 1 | Immunofluorescence of human liver tissue. a, Human liver sections were stained for glutathione synthetase (GS) to visualize CV and DAPI. Scale bar, 1,000 µm. Representative images from NC = 4 samples and eNASH = 5 samples. b, 2D analysis of liver lobule radius represented by box-plots (median values as red lines, 25th and 75th percentiles as blue bottom and top edges of the boxes, extreme data points by whiskers). NC = 4 samples, HO = 4 samples, STEA = 7 samples, eNASH = 5 samples. One-sided Wilcoxon rank-sum test. *P < 0.05, **P < 0.01, ***P < 0.001. c-f, Liver sections (~100 µm thick) were stained for bile canaliculi (CD13), sinusoids (fibronectin), nucleus (DAPI), LDs (BODIPY) and cell border (LDLR), optically cleared with SeeDB and imaged at high resolution using multiphoton microscopy (0.3 µm x 0.3 µm x 0.3 µm per voxel). Orthogonal view of NC (c), HO (d), STEA (e) and eNASH (f). Scale bar, 50 µm. Representative images from NC = 5 samples, HO = 3 samples, STEA = 4 samples, eNASH = 4 samples.
Extended Data Fig. 2 | Morphometric features of the nuclei. a, Representative IF images of fixed human liver tissue sections stained with DAPI. Shown is a single-plane covering an entire CV–PV axis. Arrowhead indicates some examples of vacuolated nuclei. Representative images from NC = 5 samples, HO = 3 samples, STEA = 4 samples, eNASH = 4 samples. b,c, Quantitative characterization of hepatocytes nuclei with respect to the proportion of mono/binuclear cells (b) and ploidy (c). Only the four major populations (1 × 2n, 1 × 4n, 2 × 2n and 2 × 4n), which account for >90% of the hepatocytes, are shown. d, Definition of the regions within the liver lobule. The CV–PV axis was divided in 10 equidistant regions. Regions 1 and 10 are adjacent to the CV and PV, respectively. Quantitative characterization of hepatocytes nuclear elongation (e) and texture based on their DAPI intensity (see Methods for details): nuclear vacuolation (f), homogeneity (Angular Second Moment) (g), local homogeneity (inverse difference moment) (h), contrast (i) and entropy (j). NC = 5 samples, HO = 3 samples, STEA = 4 samples, eNASH = 4 samples. Spatially resolved quantification represented by median ± MAD per region and overall quantifications by box plots (median values as red lines, 25th and 75th percentiles as blue bottom and top edges of the boxes, extreme data points by whiskers). One-tailed Wilcoxon rank-sum test. *P < 0.05, **P < 0.01, ***P < 0.001.
Extended Data Fig. 3 | Mislocalization of DPPIV in pericentral hepatocytes in STEA and eNASH. a–c, Representative confocal microscopy images of human liver sections stained for the apical markers BSEP (a), MRP2 (b) and DPPIV (c). Merged images of the apical markers, phalloidin and DAPI are shown in the right panels. Arrowhead indicates the lateral membrane. Scale bar, 10 µm. NC = 3 samples, STEA = 4 samples, eNASH = 4 samples were repeated independently with similar results. d,e, Large field images of a single-plane of liver tissue stained with DPPIV (d). Scale bar, 50 µm. Apical, basal and lateral membrane of the hepatocytes were segmented based on BSEP (not shown), DPPIV and phalloidin (not shown) in an area covering a radius of 125 µm around the CV and PV. DPPIV intensity was quantified and normalized to the area covered by the different sub-domains (e). NC = 3 samples, STEA = 4 samples, eNASH = 4 samples. Quantifications by box plots (median values as red lines, 25th and 75th percentiles as blue bottom and top edges of the boxes, extreme data points by whiskers). One-tailed Wilcoxon rank-sum test. *P < 0.05, **P < 0.01, ***P < 0.001.
Extended Data Fig. 4 | Structural and topological characterization of the sinusoidal network. a. Representative IF images of fixed human liver tissue sections stained with fibronectin after CAAR. Shown is a maximum projection of a 30-µm z-stack covering an entire CV–PV axis. Representative images from NC = 5 samples, HO = 3 samples, STEA = 5 samples, eNASH = 3 samples. b–h. Quantification of the tissue volume fraction occupied by the sinusoids (b), radius (c), number of junctions (d), total length per unit tissue volume (e), fraction of connected network (f) connectivity density (g) and branches crossing regions (h) for the sinusoidal network along the CV–PV axis and overall. NC = 5 samples, HO = 3 samples, STEA = 5 samples, eNASH = 3 samples. Spatially resolved quantification represented by median ± MAD per region and overall quantifications by box plots (median values as red lines, 25th and 75th percentiles as blue bottom and top edges of the boxes, extreme data points by whiskers). Two-tailed Wilcoxon rank-sum test.

\*P < 0.05, \**P < 0.01, \***P < 0.001.
Extended Data Fig. 5 | Geometric and topological variability of the BC network among liver lobules. BC network was reconstructed from three CV-PV axes from different lobules for each patient. NC = 4 patients, HO = 3 patients STEA = 3 patients, eNASH = 3 patients. Quantification of the tissue volume fraction occupied by the BC, radius, total length per unit tissue volume and fraction of connected network (a–d) along the CV–PV axis and overall. Spatially resolved quantification represented by median ± MAD per region and overall quantifications by box plots (median values as red lines, 25th and 75th percentiles as blue bottom and top edges of the boxes, extreme data points by whiskers). Two-tailed Kruskal–Wallis test. *P < 0.05, **P < 0.01, ***P < 0.001.
Extended Data Fig. 6 | Estimates for a fraction of free lumen in total volume of a bile canaliculus. **a**, Representative images of bile canaliculi for NC and eNASH liver tissue samples, used for making the estimates. Microvilli are well preserved. A red dashed line indicates lumen of a bile canaliculus. TJ, tight junction. NC = 3 samples, HO = 3 samples, STEA = 3 samples, eNASH = 3 samples. Scalebar, 500 nm. **b**, Estimation of fraction of free lumen by stereological point counting (the Cavalieri estimator). For each set of samples and each region (central / portal vein) a minimum of five EM images was used. NC = 3 samples, HO = 3 samples, STEA = 3 samples, eNASH = 3 samples, median ± MAD.

|                | Pericentral region | Periporal region |
|----------------|--------------------|------------------|
|                | median  | MAD   | median  | MAD   |
| NC             | 29.9    | 0.15  | 28.4    | 2.10  |
| HO             | 28.3    | 0.27  | 28.2    | 0.25  |
| STEA           | 25.0    | 2.47  | 26.7    | 0.39  |
| eNASH          | 26.0    | 0.02  | 22.3    | 0.53  |
Extended Data Fig. 7 | Profile of serum cholestatic and liver injury biomarkers as well as bile acids during disease progression. a–j, The levels of bilirubin (a), GGT (b), AP (c), AST (d), ALT (e), BA precursors (cholesterol, 7α-hydroxycholesterol and 27-hydroxycholesterol) (f), individual (CA, CDCA) and total primary BAs (g), individual (DCA, LCA, UDCA) and total secondary BAs (h), total BAs (i) and ratio secondary to primary BAs (j) were measured in the serum of the subjects and represented in box plots (median values as red lines, 25th and 75th percentiles as blue bottom and top edges of the boxes, extreme data points by whiskers). NC = 22 samples, HO = 27 samples, STEA = 31 samples, eNASH = 24 samples. One-tailed Wilcoxon rank-sum test. *P < 0.05, **P < 0.01, ***P < 0.001.
Extended Data Fig. 8 | Scatter plots and regression analysis of measured liver biomarkers and bile acids. a–g. Bilirubin (a), AP (b), total BAs (c), primary BAs (d), AST (e), ALT (f) and ratio secondary to primary BAs (g) measured in the serum versus the model-derived pericentral pressure in individual patients from all groups. Arrow indicates an outlier for primary BAs (h72S2). NC = 6 samples, HO = 4 samples, STEA = 8 samples, eNASH = 7 samples. P values and Spearman correlation coefficient are indicated in the plot.
Reporting Summary

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☐ ☑ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

1. MotionTracking V8.92 (http://motiontracking.mpi-cbg.de/get/).
2. Fiji with ImageJ 1.52p, Java 1.80_172.
3. Morpheus, v2.0.1, RRID:SCR_014975 (Starruss et al. 2014), open source code at https://gitlab.com/morpheus.lab/morpheus.
4. Code of the shooting solver is available from https://github.com/MichaelKuecken/bileflow.

Data analysis

MATLAB2018b, Excel 2016.

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The data supporting the findings of this study are available within the paper and its Supplementary Tables 1 and 3.
Field-specific reporting

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Because of the exploratory study design, the sample size was determined on the basis of the number of samples that could be processed in a time frame available. The results show, that indeed profound new insights into the biology of NAFLD are possible with this approach. |
| Data exclusions | Some antibodies did not work for some samples. Therefore, we had to remove those samples from the corresponding 3D analysis, e.g. in samples where CD13 antibody did not work, the bile canaliculi analysis was not performed. |
| Replication | We used only biological replications (different human liver tissue biopsies) but not technical ones. All samples and stainings meeting the technical quality criteria were included in the analysis. As this was an exploratory analysis, no formal replication step was included. The primary data analysis and all data points are represented in the Figures. |
| Randomization | Standardized histopathologic assessment was performed by a single pathologist based on the NAFLD activity score (NAS). The samples were divided into 4 groups: normal control (NC), healthy obese (HO), steatosis (STEA) and early NASH (eNASH). Samples were processed sequentially according to their clinical availability. This procedure is naturally random with respect to studied disease and histological parameters. |
| Blinding | The major histopathological markers used for standardized scoring (namely steatosis, fibrosis, inflammation) are of course also visible in 3D histology. Thus, analysis staff could not be blinded to these parameters. |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
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| n/a | Involved in the study | n/a | Involved in the study |
| ☒ | Antibodies | ☒ | ChIP-seq |
| ☒ | Eukaryotic cell lines | ☒ | Flow cytometry |
| ☒ | Palaeontology | ☒ | MRI-based neuroimaging |
| ☒ | Animals and other organisms | ☒ | Human research participants |
| ☒ | Clinical data | ☒ | Clinical data |

Antibodies

| Antibodies used | Validation |
|-----------------|------------|
| 1) Mouse monoclonal antibody against CD13, 1/50, Santa Cruz sc-136484. 2) Goat polyclonal antibody against DPPIV, 1/100, R&D systems AF1180. 3) Rabbit polyclonal antibody against BSEP, 1/2000, Sigma-Adrich HPA019035. 4) Mouse monoclonal antibody against MRP2, 1/100, Abcam ab3373. 4) Rabbit polyclonal antibody against fibronectin, 1/1000, LifeSpan BioScience LS-B2318. 5) Chicken polyclonal antibody against LDLR, 1/150, Sigma-Adrich SAB3500286. 6) Rabbit polyclonal antibody against Glutamine Synthetase, 1/1000, Sigma-Adrich G2781. 7) Donkey anti mouse Alexa Fluor 555, 1/1000, Invitrogen A31570. 8) Donkey anti rabbit Alexa Fluor 555, 1/1000, Invitrogen A31572. 9) Donkey anti goat Alexa Fluor 568, 1/1000, Invitrogen A11057. 10) Donkey anti goat Alexa Fluor 594, 1/1000, Invitrogen A11058. 11) Donkey anti rabbit Alexa Fluor 594, 1/1000, Invitrogen A21207. 12) Donkey anti mouse Alexa Fluor 647, 1/1000, Invitrogen A31571. 13) Donkey anti rabbit Alexa Fluor 647, 1/1000, Invitrogen A31573. 14) Donkey anti chicken Alexa Fluor 647, 1/25, Millipore AP1945A6. |
| CD13 was validated by Western blot (Jurkat and RAW 264.7 whole cell lysates) and immunoperoxidase staining (kidney tissue). DPPIV was validated by Western blot (lysate of PBMC), ELISA (recombinant human DPPIV) and immunoperoxidase (Human Psoriatic Skin). BSEP was validated by immunoperoxidase (human liver tissue) and the Human Protein Atlas (HPA) project. MRP2 was validated by immunoperoxidase (human liver tissue). Fibronectin was validated by immunoperoxidase (human breast). LDLR was validated by Western blot and immunoperoxidase of human liver tissue. GS was validated by Western blot (rat brain cytosolic fraction extract) and immunohistochemistry (formalin-fixed, paraffin-embedded sections of rat brain). |
Human research participants

| Policy information about studies involving human research participants |
| Population characteristics | Patients were aged ≥18 years, male and female. Liver samples were obtained intraoperatively in patients in whom an intraoperative liver biopsy was indicated on clinical grounds such as exclusion of liver malignancy during major oncologic surgery or assessment of liver histology during bariatric surgery. None of the individuals underwent preoperative chemotherapy and liver histology demonstrated absence of both cirrhosis and malignancy. Patients with evidence of viral hepatitis, hemochromatosis, or alcohol consumption greater than 20 g/day for women and 30 g/day for men were excluded. All patients provided written informed consent. |
| Recruitment                  | Consecutive participants were recruited by site-specific co-investigators at their respective institutions based on protocol eligibility criteria and patient consent and verified by the study coordinator. No other criteria (that potentially could introduce bias) were employed. |
| Ethics oversight             | The study protocol was approved by the institutional review board (Ethikkommission der Medizinischen Fakultät der Universität Kiel, D425/07, A111/99) before study commencement. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.