Quantitative analysis of hepatic macro- and microvascular alterations during cirrhogenesis in the rat


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List of abbreviations:

- PHT = portal hypertension
- DALY = disability adjusted life year
- PV = portal vein
- IVC = inferior vena cava
- HV = hepatic vein
- IHVR = increased hepatic vascular resistance
- HA = hepatic artery
- TAA = thioacetamide
- VCC = vascular corrosion casting
- DTM = deep tissue microscopy
- µCT = micro-CT
- AA = abdominal aorta
- CVC = caudal vena cava
- RML = right medial lobe
- IHC = immunohistochemistry
- HVPG = hepatic venous pressure gradient
Abstract

Cirrhosis represents the end-stage of any persistent chronically active liver disease. It is characterized by the complete replacement of normal liver tissue by fibrosis, regenerative nodules, and complete fibrotic vascularized septa. The resulting angioarchitectural distortion contributes to an increasing intrahepatic vascular resistance, impeding liver perfusion and leading to portal hypertension.

To date, knowledge on the dynamically evolving pathological changes of the hepatic vasculature during cirrhogenesis remains limited. More specifically, detailed anatomical data of the vascular adaptations during disease development is lacking. To address this need, we studied the 3D architecture of the hepatic vasculature during induction of cirrhogenesis in a rat model. Cirrhosis was chemically induced with thioacetamide. At predefined time points, the hepatic vasculature was fixed and visualized using a combination of vascular corrosion casting and deep tissue microscopy. 3D reconstruction and data fitting enabled extracting cirrhogenic features at multiple scales, portraying the impact of cirrhosis on the hepatic vasculature. At the macrolevel, we noticed that regenerative nodules severely compressed pliant venous vessels from 12-week thioacetamide intoxication onwards. Especially hepatic veins were highly affected by this compression, with collapsed vessel segments severely reducing perfusion capabilities. At the microlevel, we discovered zone-specific sinusoidal degeneration with sinusoids located near the surface being more affected than those in the middle of a liver lobe. Our data sheds light on and quantifies the evolving angioarchitecture during cirrhogenesis. These findings may prove helpful for future targeted invasive interventions.

Keywords: cirrhosis, hepatic vasculature, 3D reconstruction, vascular corrosion casting, micro-CT-scanning, deep tissue microscopy, morphological analysis, rat liver
Introduction

Cirrhosis is the common end-point of any given progressive chronic active liver disease and can evolve to liver insufficiency and clinically significant portal hypertension (PHT) (Pinzani et al., 2011). PHT is responsible for the more severe and often lethal complications of cirrhosis such as bleeding oesophageal varices, ascites, renal dysfunction and hepatic encephalopathy. Because of the combined impact of these complications, PHT remains the most important cause of morbidity and mortality in patients with cirrhosis. Not surprisingly, cirrhosis therefore accounts for approximately 1.03 million deaths per year worldwide (Tsochatzis et al., 2014). Moreover, 31 million disability adjusted life years (DALYs), equivalent to 1.2% of the global DALY burden, are attributed to this chronic condition (Mokdad et al., 2014).

Since the common pathway to cirrhosis entails repetitive destruction and regeneration of liver tissue, morphological characteristics of cirrhosis comprise diffuse fibrogenesis and the conversion of the normal liver architecture into structural abnormal regenerative nodules (Anthony et al., 1978).

The morphological remodeling exerts a mechanical impact on each of the large hepatic venous vessels, as these pliant veins, i.e. the portal vein (PV), intrahepatic inferior vena cava (IVC) and hepatic veins (HVs) are highly amenable for mechanical compression (Levy AD, 2015, Ismail and Pinzani, 2009, Yamamoto et al., 1984). This architectural distortion contributes to an increased hepatic vascular resistance (IHVR) and is generally accountable for approximately 70% of the increase in portal pressure in liver cirrhosis (Hu et al., 2013, Laleman et al., 2005).

The morphological impact of cirrhosis is also conspicuous at the level of the hepatic microcirculation (Thabut and Shah, 2010). The microvascular phenotype is transformed from highly specialized porous sinusoids into continuous, more rigid capillaries, a process termed sinusoidal capillarization with a uniform defenestration of the endothelial cells and development of subendothelial basal membranes (Braet and Wisse, 2002, Huet et al., 1982).
addition, cirrhosis causes numerous microscopic vessel aberrations. Hepatic arterial (HA), PV and HV vessels may tangle with each other. Various distorted spatial arrangements have been reported in blood vessels of cirrhotic livers, such as sharp bends, anomalous branching patterns, abnormal branching angles and tortuosity. Severe damage (characterised by bridging fibrosis) results in stenosis and eventually loss of vessels. In contrast, new vessels may originate to support the blood supply and venous drainage of the regenerative nodules. The resulting neovasculature is primarily located in the fibrotic regions which bypasses functional liver tissue and further aggravates liver insufficiency (Debbaut, 2013, Kline et al., 2014, Hano and Takasaki, 2003, Van Steenkiste, 2010, Vanheule et al., 2008). Furthermore, cirrhosis is considered the principal cause of intrahepatic portosystemic shunts (Lutz et al., 2004). However, the majority of portosystemic shunts observed in portal hypertension are extrahepatic, attempting to alleviate the raised portal pressure (Bodner et al., 2002, Alexander et al., 2001, Mori et al., 1987). Non-tumorous arterioporal shunts have been described in cirrhosis, as are portal-to-portal venous shunts, although the latter are considered rare (Bodner et al., 2002, Kassissia et al., 1994, Bhargava et al., 2011).

Despite the irrefutable importance of the above described research, knowledge on the pathological alterations of the hepatic (micro)vasculature during the genesis of cirrhosis is scanty. In this context, animal models are valuable tools to analyze this disease process in the most appropriate way. The thioacetamide (TAA) model is a reproducible model of homogenous and macronodular cirrhosis, associated with all the typical features of cirrhosis, including PHT and a hyperdynamic circulatory state (Laleman et al., 2006).

We aimed at measuring the morphological changes of the hepatic vasculature at different time points during the TAA-induced cirrhogenesis. Detailed anatomical data of rat livers was obtained using two complementary, recently optimized techniques, namely vascular corrosion casting (VCC) and deep tissue microscopy (DTM) after immunofluorescence staining (Peeters...
et al., 2017). A quantitative description of the spatiotemporal impact of cirrhosis on the hepatic vasculature may facilitate a better understanding of the underlying mechanisms, contributing to the increasing IHVR which eventually leads to complications such as PHT (Laleman et al., 2006).
Materials and methods

A. Animals

Cirrhogenesis was induced by oral administration of TAA (Sigma-Aldrich, Bornem, Belgium). Prolonged TAA intoxication causes a stepwise process toward compensated cirrhosis (Laleman et al., 2006). At the start of the protocol, 0.03% TAA concentration was added to the drinking water. Thereafter, TAA concentrations were weekly adapted to keep individual body weights within the limits of 250 - 300g.

Male Wistar rats (n = 38) were randomly divided into 4 groups. Each group consisted of 9 animals, except for the fourth group where 2 extra animals were allocated to accommodate potential mortality. The animals were kept in cages at a constant temperature and humidity in a 12h controlled light/dark cycle, with food and water provided ad libitum. Group 1 served as control group, allowing the baseline description of normal hepatic characteristics. Groups 2 to 4 underwent TAA intoxication for 6, 12 and 18 weeks, respectively. After 6 weeks of administration, histopathological characteristics corresponded to steatohepatitis with significant fibrosis. At 12 weeks, the advanced fibrotic stage was attained and eventually, after 18 weeks of intoxication, animals showed homogeneous macronodular cirrhosis (Laleman et al., 2006).

At the different time points (0, 6, 12 and 18 weeks), the corresponding group (1-4, respectively) was sacrificed. Five animals of each group (6 in case of group 4) were assigned to the combination of VCC and micro-CT (µCT) imaging to study the macrocirculation. Four animals (5 in case of group 4) were allocated to DTM after immunofluorescence staining to capture the microcirculation.

B. Vascular corrosion casting

The procedure started with anaesthesia by intraperitoneal injection of 130µl/100g pentobarbital (Nembutal, Ceva Sante Animale, Brussels, Belgium) and careful exposure of the liver and
surrounding vessels. Anticoagulation was performed through intrasplenic administration of heparin (0.3 ml; 5000u/ml) (Heparine Leo, Leo Pharma, Lier, Belgium). The PV and abdominal aorta (AA) were injected sequentially and manually with 30 ml and 20 ml of a polyurethane-based casting resin, respectively. The resin mixture consisted of PU4ii (VasQtec, Zurich, Switzerland), hardener (VasQtec, Zurich, Switzerland), ethyl methyl ketone (EMK; Merckx, Darmstadt, Germany) and color dyes (yellow and blue for the HA and PV system, respectively). The radiocontrast agent Lipiodol (Guerbet, Roissy-CdG, France) was added to the AA resin to allow a clear distinction between the venous and arterial vascular trees on µCT images. The thoracic aorta and renal arteries were clamped prior to infusion to direct the resin flow. Immediately after injection, the thoracic caudal vena cava (CVC) and both inlet vessels were clamped to prevent resin leakage. The specimen was allowed to polymerize for 72 hours. Afterwards, the liver tissue was macerated in potassium hydroxide (25% KOH) for approximately 5 days. The resulting cast was then scanned with X-ray imaging at a resolution of 40 µm using an in-house developed high-resolution µCT scanner (HECTOR, Centre for X-ray Tomography (UGCT), Ghent University, Belgium). A more elaborate description of the VCC and µCT protocol was described earlier (Peeters et al., 2017).

C. Deep tissue microscopy

Animals were anaesthetized by intraperitoneal injection of 130µl/100g pentobarbital (Nembutal, Ceva Sante Animale, Brussels, Belgium) and subsequently underwent perfusion fixation with 4% phosphate-buffered paraformaldehyde. The liver was excised and cut into 350 µm thick slices by means of a vibratome (Microm HM650V; Thermo Scientific, Massachusetts, USA). Slices from the top (up to 2 mm from the surface) and mid (4 – 6 mm from the surface) region of the right medial lobe (RML) were selected for further processing. The obtained slices were permeabilized following a protocol adapted from Renier et al. (Renier et al., 2014). After permeabilization, the samples were immunostained using a generic endothelial marker antibody
(RECA-1; Serotec, Kidlington, UK). The limited antibody penetration and imaging depth inherent to traditional immunohistochemistry (IHC) was tackled by applying an adapted version of the CUBIC (clear, unobstructed brain imaging cocktails and computational analysis) clearing protocol after IHC (Susaki et al., 2014). Subsequent confocal laser scanning (Nikon A1R; Nikon, Tokyo, Japan) using a 40x Plan Fluor air lens with extra-long working distance (numerical aperture 0.6; working distance 3.6-2.8 mm; Nikon Instruments, Paris, France) provided detailed volumetric datasets of the microcirculation (voxel resolution of (0.63 x 0.63 x 1.4) µm³). The datasets were further processed and analyzed using in-house developed software. For more elaborate details on the DTM protocol, the reader is referred to (Peeters et al., 2017).

D. Data analysis of the macrocirculation

The µCT datasets were processed using the commercial software package Mimics (Materialise, Leuven, Belgium). Vascular trees (HA, PV and HV) were semi-automatically segmented as their grey value ranges differed in the µCT images. The arterially added contrast agent allowed distinguishing the PV from the HA system, and also assigned a different grey value range to the HV system due to mixing of the injected AA (with contrast agent) and PV resin. After segmentation, centerlines of the vascular trees were calculated and converted to graph structures using in-house developed software, which is based on TiQuant (Peeters et al., 2017, Friebel et al., 2015, Hammad et al., 2014). These graphs were used to quantify the branching topology and geometrical attributes (branch radius, length and number of vessels). A diameter-defined top-down ordering method was implemented, partially based on the method used by Jiang et al. (Jiang et al., 1994), to assign generation numbers to the different branches. As opposed to Jiang, inlets of each vascular tree (HA, PV and HV) were assigned generation “1” and daughter branches were allocated generation numbers higher than (or equal) to their parent vessel (Peeters et al., 2017). After data classification, exponential trend lines were fitted to the
morphological features (radius, length, number of vessels) as a function of their generation number. The fitting principle was similar to previous studies (Debbaut et al., 2012, Debbaut et al., 2014), and allowed quantifying the cirrhotogenic evolution of the parameters studied. Regarding the number of vessels for each generation, trend lines were fitted based on the first 4 generations of the PV and HV (and 3 in case of HA) and then further extrapolated. In this way, an inaccuracy of the number of vessels due to under-segmentation was limited.

E. Data analysis of the microcirculation

Prior to microvascular segmentation, DTM datasets were post-processed to reduce lipofuscin-like autofluorescence, which was abundantly present from 12 weeks of TAA administration onwards. Practically, autofluorescence was separately recorded for every sample. The resulting autofluorescence signal was subsequently subtracted from the signal of the vasculature. In addition, we applied contrast enhancement, denoising and deconvolution to eliminate imaging noise and reattribute out-of-focus components (Schindelin et al., 2012, Luisier et al., 2011). Segmentation of the microcirculation was executed automatically using in-house developed software. The segmented datasets allowed for accurate 3D reconstructions of the intertwined and interconnected blood vessels. Centerlines were calculated for each vessel to extract and quantify various morphological parameters. The radius was measured using a best-fit diameter approach, which was achieved by measuring the radius in 8 radially evenly distributed directions. By averaging over the 8 radii, the best-fit radius was able to account for the ellipsoidal character of blood vessels. Branch lengths were calculated as the cumulative distance between vessel intersections. The tortuosity of a branch was defined as the ratio of the total branch length to the distance between the start and end point of the branch. The 3D porosity of the vascular network was calculated as the total vascular volume divided by the volume of its envelope. More information on the segmentation and analysis pipeline can be found in (Peeters et al., 2017).
Statistical analyses were performed in R (open source language). Non-parametric Kruskal-Wallis tests were executed with Holm-Bonferroni adjustment to assess the sinusoidal remodeling during cirrhogenesis. Differences with a p-value below 0.05 were considered statistically significant. Post hoc pairwise multiple comparison used the Conover-Iman test, which is robust for small sample sizes.
Results

As illustrated by Fig. 1A-B, rat livers were excised and casted at different time points during cirrhogenesis. The macroscopic expression of the liver clearly evolved from normal over an irregular ‘salt & pepper’-like appearance (6 weeks) to an emerging nodular (12 weeks) and eventually macronodular liver at 18 weeks. These changing appearances were accurately captured by the casting procedure, as nodules appeared at the liver’s surface from 12 weeks onwards.

A. Macrocirculation

Cirrhosis affects mainly the hepatic venous vessels

For each time point, two liver casts were processed down to a 3D reconstruction of all vascular trees (HA, PV, and HV) (Fig. 1C-E). From 12-week intoxication onwards, regenerative nodules started to mechanically compress their surroundings, which aggravated due to their continuously growing dimensions. The pliant HV branches were largely affected by this mechanical compression, and even appeared to collapse as evidenced from the scanned casts (Fig. 2A). In the cirrhotic stage, the PV system was also affected by the nodular compression, albeit to a lesser extent, and several portosystemic shunt vessels were detected, connecting the trunk of PV with the CVC (Fig. 2B, color-coded in magenta). Furthermore, we observed that HA branches became more tortuous due to cirrhosis as sudden sharp bends appeared which were not observed in the control group. However, the HA cross-sections remained unaffected by the nodular compression, most likely because arterial vessel walls include a thick muscle layer (Fig. 2C).

Regenerative nodules mechanically compress the hepatic venous trees
The vascular trees were classified according to their diameter-defined branching topology. For each generation, the mean radius, length and number of vessels were measured (Fig. 3). Due to the restricted µCT resolution, fewer blood vessel generations were measured for the HA systems, as HA branches normally have smaller diameters than venous branches.

At the 6-week time point, all vascular trees (HA, PV, HV) appeared unaffected as their morphological parameters (radii, length, and number vessels) were comparable to control values. From the 12-week time point, when regenerative nodules began to grow in expansive manner, radii of the HV gradually decreased. Illustrative is the significant decline of the CVC radius, dropping from 3.01 mm (healthy) to 1.39 mm (most severe case of cirrhosis) (see supplementary material Table 1).

The PV system was less affected by cirrhosis. The PV inlet radius marginally dilated from 1.28 mm (rat 1 and 2) to 1.71 mm (rat 7) with increasing intoxication time. However, in rat 8 (presumably a more advanced cirrhotic stage), the PV inlet radius narrowed to 1.01 mm (shown in supplementary material Table 2). Moreover, a clear widening of the arterial tree was measured during cirrhogenesis, as the HA inlet radius dilated from 1.71 x 10^{-1} mm (rat 2) to 3.01 x 10^{-1} mm (rat 7) (depicted in supplementary material Table 3).

We did not observe any trends in the progression of cirrhogenesis for the number of vessels and length in function of the generation number. The mean lengths did not even show clear-cut declining trends in the first generation(s), but started to decrease in higher generations. In addition, the length of the first generation was underestimated as it was partially cut during resection of the liver. Therefore, the length of the first generation was not considered when fitting the trend lines in Fig. 3B.
B. Microcirculation

Cirrhogenesis instigates remodeling of the microcirculation

For each time point, ten to maximally thirteen DTM samples (randomly selected from four livers with a minimum of two samples/liver) were post-processed, 3D reconstructed and subjected to pairwise comparison (Fig. 4A-B). For the 12-week and 18-week samples, we differentiated between sinusoids in regenerative nodules and shunt vessels in the fibrotic septa, both constituting the microcirculation. This was achieved by pre-imaging the slices at a lower resolution (2.48 µm; x10 magnification), allowing visual recognition of nodules and vascularized septa. Samples of their respective microcirculation were subsequently gathered by scanning both structures individually at a higher resolution (0.63 µm; x40 magnification).

Histograms of the sinusoidal radii during disease progression are displayed in Fig. 4C. The mean radius decreased from 4.45 ± 0.23 µm in the control animals to 3.87 ± 0.47 µm at week 18 (p = 0.0047). In addition, the porosity (i.e. the sinusoidal volume per unit of volume) steadily declined from 20.43 ± 1.92% (control) to 11.12 ± 3.06% (week 18) (p < 10^-8) (see Fig. 4D). The sinusoidal tortuosity and length increased slightly but significantly (p < 10^-5) during cirrhogenesis going from 1.12 ± 0.01 to 1.19 ± 0.05 and 18.67 ± 0.83 µm to 23.95 ± 4.39 µm, respectively from week 0 to week 18 (see Fig. 5).

Cirrhosis affects the sinusoidal network zone-specifically

Samples sectioned near the top (n >= 5) were compared to samples located in the middle of the RML (n >= 5). We found that sinusoids situated in the core of the lobe typically had larger radii and appeared less affected by the cirrhogenic process compared to those near the surface (Fig. 6). Pairwise comparison of the 18-week samples demonstrated significantly different radii in these 2 zones (p = 0.048). Variations between the porosity of mid- and top-located samples
existed, but did not vary significantly. Similar observations were made for the branch length and tortuosity.

Cirrhosis is characterized by the formation of highly vascularized fibrous septa

We analyzed several samples consisting primarily of vascular septa in case of cirrhosis (n=3) and compared their radial histogram with those of cirrhotic sinusoids (Fig. 4D). The skewed distribution of the vascular septa indicated the presence of very large blood vessels (radius > 10 µm) acting as intrahepatic shunts. The septa also comprised a substantial amount of smaller intertwined blood vessels, which probably originated to support blood supply. Both vessel types are embedded in fibrotic tissue and thus separated from the hepatocytes. They act as bypasses guiding the blood flow directly from the portal tract into the central vein.
Discussion

The present study is, to the best of our knowledge, the first to analyze the main remodeling events of the hepatic vascular architecture during TAA cirrhogenesis in rat. Solely using static techniques (VCC and DTM), we were able to study and quantify the dynamic transition of this pathological process. At 4 discrete time points during the progression toward cirrhosis, VCC and DTM were used to capture, 3D reconstruct and morphologically analyze the intricate hepatic vasculature across multiple length scales. Our data demonstrated various anatomical abnormalities attributable to cirrhosis, which are likely to underlie the increase of total IHVR as previously characterized hemodynamically in this model and at the same given time points (Laleman et al., 2006).

With VCC, we examined the circulatory changes at the macrolevel. From 12-week TAA intoxication onwards, regenerative nodules started to grow in an expansive manner. It is commonly assumed that this tissue growth exerts a compressive force on the surrounding blood vessels (Fig. 2). This compression narrowed the pliant venous systems (PV and HV), but we found that especially HVs were highly impacted by this compression, with collapsed vessel segments severely impeding hepatic outflow (Fig. 3). The mean diameters of the HV across the first generations nearly halved. As resistance of a vessel scales inversely with its radius to the fourth power following Poiseuille’s law, it is not surprising that an increase of the total IHVR was documented in TAA-induced cirrhotic livers (Laleman et al., 2006).

Furthermore, the HA dilated with increasing intoxication time. The diameter of the PV most likely increased up until the moment the mechanical impact of the regenerative nodules outweighed the internal portal pressure and forced the PV to narrow, as observed in rat 8 (more advanced stage of cirrhosis). Progressive narrowing of the HV and PV vasculature contributed to the ever-increasing IHVR and hepatic venous pressure gradient (HVPG) over the liver in cirrhosis (Laleman et al., 2006).
At the microscopic level, sinusoidal remodeling was abundantly present. Capillarization and the impaired intrahepatic balance between vasodilators and -constrictor presumably caused the diameters and number of sinusoids to decrease (Thabut and Shah, 2010). We observed that, from 12 weeks onwards, the microvascular porosity and sinusoidal radii differed significantly from control data at week 0 (Fig. 4C and Fig. 5A-B). Even though macrocirculatory changes were still limited, PHT was already measured at 12 weeks as document earlier in the animal model (Laleman et al., 2006). This suggests that the increase of the IHVR is initiated at the microlevel and is further aggravated by alterations at the macroscale later on. At 18 weeks, PHT became associated with a hyperdynamic circulatory state (another pathophysiological hallmark of cirrhosis), which may have further contributed to the increasing portal pressure (Laleman et al., 2006).

At 18 weeks of TAA intoxication, the presence of complete fibrous vascularized septa was previously reported (Laleman et al., 2006). In the present work, we observed that these portal-portal and portal-central septa, encapsulating regenerative nodules, comprised a considerable amount of small vessels as well as larger intrahepatic shunt vessels (Fig. 4C). Additionally, intrahepatic portosystemic shunts were detected between the trunk of the PV and CVC (Fig. 2C). These shunt vessels most likely developed in an attempt to alleviate the elevated portal pressures, but at the cost of shunting large amounts of blood directly into the systemic circulation without contact with the hepatocytes, incapacitating as such synthetic and detoxification liver functions.

Although the workflow of this study is straightforward, some aspects are very labor intensive and time consuming. This is particularly the case for the segmentation of the µCT datasets of the vascular corrosion casts, which is why only two liver casts were fully segmented for each cirrhogenic stage. Therefore, it is necessary to consider the reported numerical data as indicative. Moreover, liver casts should ideally be µCT-scanned at a sufficiently high resolution.
to allow reconstructing the HA accurately up to the same generation as the PV. Since diameters of HA branches are typically smaller than PV branches, this was technically impossible with the current computational capabilities. As a consequence, the number of HA vessels for each generation was underestimated as smaller ramifying branches remained undetected. Since the HA runs in parallel with the PV, we can only assume that the number of vessels should be at least equal to or even higher (due to PV being flanked by more than one parallel HA vessels) than the PV (Debbaut et al., 2014).

The hypothesis that the casting resin caused significant tissue shrinkage was discredited. We presumed that the known shrinkage of the cast resin was compensated by the pressure exerted during the injection of the polymer (Debbaut et al., 2014, Krucker et al., 2006, Meyer et al., 2007).

**Conclusion**

Cirrhotic changes of the hepatic vasculature were analyzed and quantified in the TAA cirrhotic rat model. At predefined time points, two techniques (VCC and DTM) were used to accurately capture and 3D reconstruct the hepatic vasculature across different scales, ranging from the largest blood vessels down to the sinusoids. Their complementarity allowed to provide a comprehensive overview of the impact of cirrhosis on the vasculature, revealing static mechanisms behind the increased IHVR which were hemodynamically characterized earlier at similar time points and in the same model (Laleman et al., 2006). The combined impact of this work, both hemodynamically as angioarchitecturally, might be of interest for targeted liver interventions both pharmaceutically, surgically and angiographically.
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Figures

Fig. 1. A. Rat livers were excised at different time points during cirrhogenesis. The right medial lobe (RML) was sectioned prior to immunostaining. The macroscopic expression of the liver transformed from normal over an irregular ‘salt & pepper’-like appearance at 6 weeks to an emerging nodular liver at 12 weeks and ultimately macronodular liver at 18 weeks. B. Vascular replicas obtained using vascular corrosion casting (VCC). Blue pigmented resin was injected via the PV and yellow dye was added to the arterial resin. C-E. 3D reconstructions of the
mascroscopic hepatic veins (HV), portal veins (PV) and hepatic arteries (HA), respectively, obtained using VCC.

Fig. 2 Accurate 3D reconstruction of the macrocirculation of a cirrhotic liver (18 weeks). A. The amendable hepatic veins (HV) in the medial lobe are significantly compressed by regenerative nodules and some branches even appeared to collapse. B. Porto-systemic shunts (arrows) were detected, shunting directly from the root of the PV into the HV (CVC). Branching trees from the PV and HV were cut and the view was rotated to visualize the shunts. C. Due to cirrhosis, arterial branches became more tortuous, resulting in sudden sharp bends (arrows).
Fig. 3 The macrovascular trees – hepatic vein (HV), portal vein (PV), and hepatic artery (HA) – were classified according to their diameter-defined branching topology. For each liver intoxicated with TAA during different weeks (0w, 6w, 12w, 18w), the mean radius (A), length (B), and number of vessels (C) were measured as function of the generation number and exponential trend lines were fitted. Due to mechanical compression of the nodules, cirrhosis appeared to have a high impact on the radius of the HV.
Fig. 4. A. Example of a stack of 2D images acquired through deep tissue microscopy (DTM). B. The dataset was automatically processed to segment the sinusoidal network and convert it to a graph. Here, the network graph is colored according to the mean radius of the branches. The graph allowed extracting other morphological parameters, including the length, tortuosity and porosity. C. Histograms of the sinusoidal radii during the different cirrhogenic stages. The values visibly shift to the left, when progressing from a normal to a cirrhotic liver. At 12 weeks and 18 weeks, we differentiated between the sinusoids in regenerative nodules and the microvascular vessels in the vascular septa. These vascular septa consisted of a substantial number of smaller vessels, but also a considerable number of large shunt vessels (diameter > 10 µm). D. 3D reconstructions of the intricate sinusoidal network obtained with DTM (140 µm thick samples). The volume of blood vessels per volume unit (=porosity) decreased with increasing intoxication time from 19% (normal) over 16% (significant fibrosis) and 9% (advanced fibrosis) to 7% (cirrhosis).
Fig. 5 Boxplots for the radius (A), porosity (B), branch length (C), and tortuosity (D) of the microcirculation in function of TAA intoxication time. The radius and porosity differed significantly between normal and cirrhotic livers (p<0.05). Both parameters decreased gradually during cirrhotic progression, contributing to the increased intrahepatic vascular resistance.
**Fig. 6** Boxplots for the radius (A), porosity (B), branch length (C), and tortuosity (D) of the microcirculation as a function of TAA intoxication time and location within the lobe. Slices (350 µm) were taken near the top (up to 2 mm from the surface) and mid (4 – 6 mm from the surface) region of the right medial lobe (RML). Sinusoids situated in the core of the lobe appeared to be less affected by the cirrhogenic process, as their mean radii and porosity were typically larger than those near the surface. When comparing the 18-week intoxicated samples pairwise, the radii even differed significantly between the top and mid region (p = 0.048).