Abstract

**Background:** Disrupted bile secretion leads to liver damage characterized by inflammation, fibrosis, eventually cirrhosis and hepatocellular cancer. As obstructive cholestasis often progresses insidiously, markers for the diagnosis and staging of the disease are urgently needed. To this end we compiled a comprehensive data set of serum markers, histological parameters and transcript profiles at 8 time points after bile duct ligation (BDL) in mice.

**Results:** Statistical analysis of the more than 6,000 data points revealed distinct temporal phases of disease. The count of CTGF-positive cells provided the most reliable overall measure for disease progression at the histological level. Putting time-courses of biochemical, histochemical and mRNA transcripts (=factors) together, time-series correlation analysis defined 6 distinct clusters related to different phases of disease progression. Onset of disease is marked best by fibronectin, transition to the perpetuation phase by interleukin 2, and the progression phase by interleukin 28-β. Prominent molecular events exhibited by strong transcript peaks are found for small heterodimer partner at 6 h and transin-2 at 18 h. Based on these clusters we constructed a decision tree to identify factor combinations that can be used as markers for a specific time interval of disease progression. Notably, these predictions remained stable even for randomly chosen small sub-sets of factors.

**Conclusion:** Our explorative study demonstrate the existence of a reproducible and well-coordinated temporal gene-expression program underlying disease progression in BLD cholestasis. To further elucidate the regulatory network behind this expression program further studies are needed wwhich have to include knowledge on transcription factor activation by the metabolic disorder caused by accumulation of bile salts and shared transcriptions factor binding motifs of genes belonging to the same transcript cluster.

Background

Cholestatic liver diseases are caused by an impaired flow of the bile from the liver to the duodenum. The main fraction of organic molecules in the bile are bile salts which are strong detergents required for the extraction of lipids from the apical membrane of hepatocytes into the bile fluid and for the emulgation of lipids in the gut. Moreover, the bile fluid, contains numerous endogenous end products (e.g. bilirubin) and potentially toxic compounds cleared by the liver. Hence, accumulation of bile compounds in the cholestatic liver causes an unspecific cell damage that initiates a cascade of inflammatory events: Necrosis of hepatocytes and cholangiocytes, activation of macrophages releasing of pro-inflammatory cytokines and chemokines, neutrophil infiltration, cholangiocyte and hepatocyte proliferation, stellate cell activation with progressive fibrosis causing secondary biliary cirrhosis, ultimately leading to liver failure {Wang2005}.

Multiple pathologies may represent the primary trigger of impaired bile flow as, for example, defects in the export of the bile from hepatocytes to the extracellular space (hepatocellular cholestasis) or obstruction of bile ducts by gall stones or local tumor impingement (extrahepatic cholestasis) {Woolbright2013}. Among the most common causes of cholestatic liver disease in the adult population are primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC), while biliary atresia and Alagille syndrome are commonly found in the pediatric population { Lindblad1977,Trautner1998, Trauner2004, Wang2013, Nguyen2014}.

One of the main causes of cholestasis is biliary tract obstruction, which is a common mechanism of hepatic injury in a variety of clinical settings, including obstructing neoplasms, post-operative bile duct injury, biliary atresia, sclerosing cholangitis, and primary biliary cirrhosis. The corresponding experimental model to induce obstructive cholestatic injury in mice and rats is surgical bile duct ligation (BDL) {Tag2015, Georgiev2008}. BDL results in stereotypical histopathological changes of cholestasis, The BDL experimental model has been well described and evaluated in rats and mice and is widely used to study cholestatic liver injury and fibrogenesis {Huss2010}.

Chronic liver diseases (CLD) like cholestasis represent with characteristic temporal morphologic, biochemical and molecular changes in liver and serum. For instance, in the BDL model of cholestasis an early phase of acute hepatocyte injury is followed by a proliferative response, up-regulation of pro-inflammatory cytokines and pro-fibrotic metabolic enzymes, and subsequent development of fibrosis after around 7 days {Georgiev2008, Huss2010}. Such alterations can be exploited to identify biomarkers reporting on specific stages of disease progression and severity of the disease. Hitherto semi-quantitative morphological scoring is the standard technique for grading in CLD. However, nowadays the availability of high-throughput technologies enables to flank the histological assessment of injured tissue with a comprehensive molecular profiling of gene transcripts, gene products (proteins) and metabolites. Such analyses not only provide a more detailed characterization and thus more refined staging of disease progression, they also lead to a deeper understanding of the molecular networks governing the histological and physical alterations overserved at higher scales of tissue organization. Extracting from large sets of high-throughput data relevant information for clinical translation is still in its beginnings. The identification of key processes triggering the transition between different phases of disease progression requires mathematical analyses which take into account multiple parallel processes, process dynamics, and experimental data from different levels of cellular organization [4].

In this study we explicitly wanted to systemize existing and newly acquired knowledge on the molecular biomarkers of cholestasis and to analyze the time-dependent disease progression following BDL in mice in a comprehensive manner. Our approach goes beyond previous studies considering the time course of only a limited number selected parameters after BDL {Georgiev2008, Huss2010} or performing gene expression profiling at a limited number of time points, thereby missing the acute damage phase after BDL in the first 24h and long-term effects after 7 days {Tanaka2006}. None of these preceding investigations provided predictive models based on the acquired time course data. Therefore, a central aim of our study was to identify molecular markers for the temporal progression of BDL cholestasis by correlating high-accuracy image data and transcriptional profiles of preselected targets with pathobiochemical markers, whereby all data were obtained at different time points after onset of obstructive cholestasis. We collected from 8 different time points after BDL more than 6,000 experimental data points, comprising immunohistochemistry, biochemistry and molecular profiling measures. Statistical methods were applied to unravel robust interrelations in this large-scale data set and find clusters of parameters corresponding to characteristic time profiles in cholestatic injury induced by BDL. We correlated level and timing of pathophysiological events with transcriptional changes in order to define molecular markers, and developed predictive decision trees that allow assessment of the different disease phases occurring during the development of cholestasis based on a subset of biomarkers.

Methods and Materials

*Ethical Statement*

All experiments were approved by the local government Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Voprommern (LALLF M-V/TSD/7221.3-1.2-049/09) and performed in accordance with the German legislation on protection of animals and the National Institutes of ‘‘Health Guide for the Care and Use of Laboratory Animals’’ (Institute of Laboratory Animal Resources, National Research Council; NIH publication 86-23 revised 1985).

Laboratory Animals

Male C57BL/6J (Charles River Laboratories, Sulzfeld, Germany) at 8–10 weeks of age with a body weight of 23–26 g were kept on water and standard laboratory chow ad libitum.

*Surgical procedure and experimental groups*

Mice were anesthetized by breathing isoflurane (1.5 vol%). BDL was performed after midline laparotomy. The common bile duct was ligated three times with 5-0 silk and transected between the two most distal ligations. Sham operation was performed similarly, except for ligation and transection of the bile duct (0 h, n=5). All surgical procedures were performed under aseptic conditions. Animals were allowed to recover from anesthesia and surgery under a red warming lamp and were held in single cages until subsequent experiments followed at postoperative hours 6, 12, 18 and 30 (n=5 animals per time point), and at 2, 5 and 14 days after BDL (n=5 animals per time point). Sham-operated animals without BDL served as controls (n=5). To analyze the regenerative response in regard to proliferation of different cell types, 5-bromo-2-deoxyuridine (BrdU; 50 mg/kg bw ip) was injected 1 h prior to harvest of liver tissue. BrdU incorporation into DNA was analysed by immunohistochemistry. To obtain blood and liver samples, mice were killed at the indicated time points. Liver tissue was partially embedded in paraffin for morphology analysis and snap frozen for molecular biology and biochemistry analyses. In addition, liver tissue served for the parallel Taqman qRT-PCR using microfluidic Fluidigm Biomark™ platform (Fluidigm, CA, USA).

*Hematological measurements and plasma enzyme levels*

Animals were anesthetized and exsanguinated by puncture of the vena cava inferior. Red blood cell and blood platelets count, hemoglobin, and hematocrit were assessed with an automated cell counter (Sysmex KX-21, Sysmex). Plasma activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and glutamate dehydro-genase (GLDH) were measured spectrophoto-metrically.

Assays

EDTA plasma served for the analysis of albumin as a parameter of liver function, which was determined with a commercially available enzyme-linked immunosorbent assay kit in accordance with the manufacturer’s instructions (Assaypro, MO, USA).

*Histopathology and image analysis*

Liver tissue samples were fixed in formalin for 2 to 3 days and embedded in paraffin. 5 μm sections were stained with hematoxylin and eosin (H&E) for routine examination and quantification of bile infarcts. Sirius red staining served for quantification of collagen deposition. All samples from a series of experiments were stained simultaneously and evaluated in a blinded manner. For histomorphometric analysis, images of 20 random low power fields (x10 magnification, Olympus BX 51, Hamburg, Germany) were acquired with a Color View II FW camera (Color View, Munich, Germany) and evaluated using an image analysis system (Adobe Photoshop, Adobe Systems, Uxbridge, UK). Fibrosis deposition was quantified as a percentage of Sirius red stained area compared with the total section area. The surfaces of large centrilobular veins and large portal tracts were excluded from this calculation. Bile infarcts were quantified in H&E-stained sections in a similar manner and the percentage of the focal necrosis surface to the whole liver section area was assessed.

*Immunohistochemistry and image analysis*

For analyzing DNA-incorporated BrdU in liver cells, 4 µm sections collected on poly-L-lysine-coated glass slides were incubated with monoclonal mouse anti-BrdU antibody (1:50; Dako Cytomation, Hamburg, Germany) overnight at 4°C, followed by horseradish-peroxidase (HRP)-conjugated goat anti-mouse immunoglobin (LSAB kit plus; Dako). Sites of peroxidase-binding were detected by 3,3`-diaminobenzidine (Dako). Sections were counterstained with hemalaun. BrdU-positive hepatocellular nuclei were counted in a blinded manner within 30 consecutive high power fields (HPF) (x40 objective, numerical aperture 0.65) and are given as cells/mm². In analogy, BrdU-expressing non-parenchymal cells were assessed and also given as cells/mm².

To specify the proliferative response of non-parenchymal cells upon BDL, we performed double immunohistochemistry of BrdU with specific markers for different liver cells: F4-80/BrdU for Kupffer cells and SM22α/BrdU for BEC. For each protocol, the immune-staining procedure for the specific marker was conducted after the BrdU staining protocol. Resident liver macrophages were analyzed using the F4-80 antigen (1:10; Serotec, Oxford, UK). Overnight incubation (4°C) with the first antibody (polyclonal rat anti-F4-80) was followed by alkaline-phosphatase (AP) conjugated mouse anti-rat immunoglobulin (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sites of AP-binding were detected using the chromogen fuchsin (Dako).

BECs and oval cells were detected by overnight incubation (4°C) with a polyclonal rabbit anti-SM22α antibody (1:100; Abcam, Cambridge, UK) followed by AP conjugated goat anti-rabbit immunoglobulin as secondary antibody (1:100; Dako). The sites of AP-binding were detected by Permanent Red (Dako).

Slides were viewed under a light microscope (Olympus BX 51) and the number of BrdU-positive cells co-expressing F4-80 or SM22α were counted in a blinded manner within 30 consecutive high power fields (HPF) (x40 objective, numerical aperture 0.65) and are given as cells/mm².

Antibodies for detection of -SMA in HSCs and of S100a4-positive cells were from DAKO (M0851 and A5114, 1:500 and 1:200 dilution, respectively). CTGF antibody was from Santa Cruz (sc-1439, 1:200 dilution). Sections were de-paraffinized in serial ethanol dilutions. After a PBS wash, sections were transferred into 10 mM sodium citrate buffer (pH 6.0) and antigen unmasking was performed in a microwave. After cooling down, sections were incubated in peroxidase blocking reagent (Dako) for 1 h and with primary antibodies overnight at 4oC. EnVision peroxidase (Dako) was applied for 1 h at room temperature after a PBS wash next day. Sections were developed with diaminobenzidine for 5 minutes. The number of α-SMA-, CTGF- and S100a4-positive cells was quantified under a Leica light microscope (x20) by counting three fields.

*High-throughput quantitative Taqman RT-PCR analysis*

Total RNA was isolated from the liver tissue samples using RNeasy Mini Kit including on column genomic DNA digestion with RNase free DNase Set (Qiagen, Hilden, Germany). RNA was reverse transcribed to cDNA with TaqMan Reverse Transcription Reagents (Applera GmbH, Darmstadt, Germany). For quantitative real-time PCR, we used the Fluidigm's Biomark high-throughput qPCR chip platform (Fluidigm Corporation, San Francisco, CA, USA) with pre-designed gene expression assays from Applied Biosystems, according to the manufacturer’s instructions [6]. Data were analyzed using the ddCt method and expression values were normalized to the expression levels of the Gapdh gene.

Statistical Data Analysis

Dimension reduction: A one-way analysis of variance (ANOVA) was applied to reduce the data set to the parameter subset showing significant (padj<0.05) up- or down-regulation during the time course. Multiple testing corrections were performed using the Holm procedure {Holm1979}.

Correlation analysis: Correlation between two parameter time courses was calculated via a modified correlation coefficient based similarity measure for clustering time-course gene expression data {Son2008}. The correlation between two factors *i* and j is the linear combination of three rterms: i) a classical correlation part based on Spearman correlation , ii) accounting for the similarity in changes between the two time courses, and iii) comparing the location of the minimum and maximum values of the time course (see {Son2008} and supplement for definitions).

is calculated on individual data points, and on the mean time courses averaged over the repeats per time point. In the analysis the following weights were used:

, ,

For the calculation of the similarity of changes within time courses, , we used Spearman (S) correlation which is more robust against outliers as Pearson (R) correlation:

Simple Pearson and Spearman correlation were calculated for comparison.

Cluster analysis of the correlation matrix used complete-linkage hierarchical clustering with euclidian distance measurement. This combination of methods provided the best enrichments on gene-expression time-series in a recent comparisons of methods {Jaskowiak2014, Jaskowiak2013}.

Normalization of factors was performed separately for each factor for all time points *i* and repeats *r* via

Decision trees: For the prediction of distinct time points of disease progression after BDL a regression tree model based on recursive partitioning {Breiman1984} was fitted using the mean normalized data of the 6 clusters as predictor variables and the log transformed time points as dependent variable

The logarithmic transformation was performed to obtain approximately equal intervals between time points. The resulting tree was fitted with the full trainings data set, i.e. all 5 repeats per time point. The predictive capacity of the model was evaluated using all single combinations of individual factors from the clusters, and a random subset of two factor combinations from each cluster.

All computations were performed in R with source code, data and the full analysis available in supplement S2.

Results and Discussion

***Temporal changes of biochemical, cellular and histochemical markers after BDL***

In mice, BDL over 14 days induces time dependently progressing stages of a secondary biliary CLD. The first week after BDL begins with an acute cholestatic injury associated with necroinflammation, followed by a chronic injury stage, comprising hepatitis and liver fibrosis. Macroscopically, marked dilation of the gallbladder and formation of bilioma are found, associated with weight loss and a mortality rate of 10% in the first week due to bile leakage and rupture of the gallbladder [7]. Pathophysiologically, BDL interferes with glandular liver function and hepatobiliary transport, which comprises its detoxification and secretion function including bile formation. Obstruction of the bile duct leads to afflux of newly generated bile fluid. The main components of the bile, bile acids and phospholipids, induce toxicity and damage towards hepatocytes and cholangiocytes, therewith initiating the disease process. Rapidly after BDL, mice develop obstructive jaundice and cholestasis, as displayed by markedly elevated serum transaminases and bilirubin levels (Figure 1), macroscopically evident from yellow ears and urine. Within the first 30 h, there was a massive release of liver enzymes, like ALT and GLDH, reflecting the initial hepatocyte damage as initial pathophysiological event in the process of BDL-induced liver fibrosis (Figures 1A, B). The plasma levels of diagnostic liver enzymes remained elevated over several days but then dropped to reach after 14 days values that were slightly above those of the sham operated liver. Concomitantly, liver detoxification capacity was deteriorated, as indicated by the rise of total bilirubin, a classical plasma marker of cholestasis (Figure 1C). Notably the plasma level of albumin, an important parameter for the evaluation of liver functionality, remained relatively almost constant over the time course of 14 days (Figure 1D). Analysis of the systemic blood cell count revealed constant levels of erythrocytes and platelets up to day 5 (Table 1). In contrast, leukocytes decreased by 50% during the first two days, reflecting intrahepatic cell entrapment, and recovered to values of sham operated animals within the subsequent observation period (Table 1). During progression of fibrosis, red blood cells, hemoglobin and hematocrit slightly decreased.

In consequence of intrahepatic toxic bile accumulation, progressive development of confluent bile lakes is a hallmark of cholestasis. Histological quantification of bile infarcts, defined as clusters of injured hepatocytes, revealed a steady rise of infarct areas until day 14 after BDL (Figure 2A). Figure 2B shows the typical appearance of liver tissue at representative time points after BDL using H&E staining. Further histopathological changes of the livers after BDL included enlargement of portal tracts, accompanied by dilation of bile canaliculi and proliferation of BECs and oval cells (Figure 3A), resulting in formation of artificial bile ductules (Figure 2C), a cellular response termed ‘ductular reaction’ [5; 8]. However, recent data from lineage tracing experiments indicated that these cells do not contribute to the population of ECM producing/fibrogenic cells, which in the BDL model is largely consisting of HSCs [9].

The inflammatory response resulting from chronic hepatocyte injury resulted in the accumulation of immune cells in the liver, among others, T cells, macrophages and dendritic cells, which were mainly found within and around bile infarct areas (Figure 2C, asterisk) [10]. The inflammatory and fibrogenic response is initiated by resident liver cells, primarily liver macrophages (Kupffer cells) and activated hepatic stellate cells (HSCs) secreting a wide range of cytokines and chemokines which determine the quality and quantity of inflammatory and consequently fibrotic responses [11; 12]. Upon parenchymal damage, quiescent HSCs undergo phenotypical changes to myofibroblasts (MFBs). The most prominent role of MFBs is extracellular matrix (ECM) production and reorganization, as reflected by an increased synthesis of α-SMA, type I collagen and TIMPs. A marked increase of the number of α-SMA- and S100a4-positive cells, as measured by immunohistochemistry, was observed after BDL (Figures 3B, C). Migration of MFBs to the site of injury and their contractility contribute to liver scarring. This is accompanied by parenchymal cell proliferation, which began to rise at day 2 as regenerative response but decreased at day 14 (Figure 3D). With a slightly faster response as compared to HSCs, starting at 30 h upon BDL, Kupffer cells started to proliferate (Figure 3E).

The overall hepatic proliferative response as analyzed by immunohistochemistry was confirmed by elevated mRNA expression of Ki67 (Figure 4A).

Between days 5 and 14 after BDL, periportal alterations were associated with fibrotic changes. As demonstrated by Sirius red staining, extensive fibrosis, characterized by a several-fold increase of collagen deposition (Figure 4B), including bridging, was observed 5 days after BDL (Figure 4C). We further stained for connective tissue growth factor (CTGF), a prominent fibrogenic cytokine and enhancer of TGF- effects [13]. Appearance of CTGF-positive cells started as early as 12 h upon BDL and their count increased continuously (Figure 3F).

***Evidences for different time phases of diseases progression after BDL***

For the identification of distinct phases in disease progression induced by BDL we included besides biochemical and histochemical markers time-resolved transcriptomics profiles of three different gene panels related to hepatocyte metabolism fibrogenesis and inflammation using the Fluidigm platform (Figure 5). The selection of representative genes for ADME- (absorption, distribution, metabolism, and excretion) (Figure 5A) and fibrogenesis- (Figure 5B), and inflammation-related genes (Figure 5C) was based on state-of the art knowledge.

First, a one-way analysis of variance (ANOVA) was applied to reduce the complete data set of biochemical, histochemical and transcript data to a subset of those factors showing significant (pad j< 0.05) up- or down-regulation during the time course of disease progression (see Table X, supplement). This resulted in a subset of ? factors comprising ? biochemical markers (…), ? histochemical markers (…) and ? genes. Within this subset, a bivariate time-dependent correlation analysis (see Methods) was performed for all pairs of factors to identify those factors displaying similar temporal profiles (see Fig. 7). Based on the obtained correlation matrix a hierarchical cluster analysis was applied whereby the minimum cluster size was put to 2. This yielded 6 different clusters comprising between 2 – 61 factors with distinct time courses attaining their maximum at different time points (see Fig. X). Accordingly, the clusters can be assigned to the early, middle and late phase of disease progression. For example, mRNA levels of genes involved in metabolism (ADME, Cluster ?) such as members of the cytochrome P450 system are immediately induced during the first 6 h upon damage and then steadily decrease with time after BDL. This time course suggests that early after the insult the detoxification activity of hepatocytes is increased but than decreases owing to ongoing necrosis of hepatocytes and feedback inhibition of CYP 450 expression by accumulating bile salts.

The found clusters comprise both ‘classical’ biochemical and histochemical factors and genes characteristic for a specific phase of disease progression. The correlation between these two cardinally different types of factors is shown in Fig. 8. The time course of each of the ‘classical’ factors contained in the ANOVA-subset can be correlated with the expression time course of at least one gene. Only for GLDH and BrdU Sirius Red this correlation is weak (p=?).

In what follows we discuss in more detail the biological significance of the 6 clusters of timely correlated factors and the correlation between different types of factors shown in Fig. 8 thereby including already exiting knowledge.

*Initial response*

Immediately after BDL, there is a massive release of liver enzymes up to day 5, followed by a drop down to almost values of sham-operated livers (Figures 1A, B). It can be interpreted as the beginning breakdown of hepatocellular protein synthesis or the cease of cell necrosis.

The factor with the strongest negative correlation to ALT in serum is Cyp7a1 (Figure 8A). As Cyp7a1 mRNA encodes cholesterol-7-α-hydroxylase, down-regulation of bile acid synthesis can be seen as a fast and straightforward response to cholestasis. As underlying mechanism, activation of the JNK/c-Jun pathway has been reported [15]. In later time phases Cyp7a1 increases again, most likely SREBP-mediated, since plasma cholesterol concentration increases simultaneously [16]. The second highest correlated factor to ALT is Hmox1, encoding heme oxygenase, which was reported as increased upon BDL [17]. It therefore also can be defined as “early response” parameter, which subsequently remains at increased levels as compared to healthy liver. GLDH is another serum marker for hepatocellular injury that positively correlates to ALT, but less significant than Cyp7a1 and Hmox1, indicating that ALT and GLDH are suitable as independent measures. Additionally, a high correlation is found for ALT with Cebpb, which encodes CCAAT/enhancer-binding protein β, a regulator of the inflammatory response, e.g. via up-regulating Il6 [18]. The two chemokines Cxcl1, encoding neutrophil-activating protein 3, and Cxcl2, encoding macrophage inflammatory protein 2-α, are positively correlated to ALT. As both proteins are excreted, it is likely that they can be detected in the plasma and thus may be further investigated as potential diagnostic marker.

GLDH like ALT shows a strong initial response but in contrast to the latter as increases further up to 18h, before it decreases gradually (Figure 8B). It is highly correlated to ALT, but shows a low correlation to all other factors. In particular, the anti-correlation of GLDH to Cyp7a1, the 2nd highest correlation, is considerably lower than the ALT-Cyp7a1 correlation (see Supporting Information Dataset S3, section 4.2). Further, it is correlated to RNA levels of Fibronectin (Fn1, see Figure 6G), but only in time frames including the 18h time point (T18h, 6-18h, Perp).

Macroscopic organ damage

As documented with the H&E staining in Figure 2B, necroinflammation is caused by the BDL-induced intrahepatic toxic bile accumulation with death of liver cells and progressive development of confluent bile infarct areas. The total area of infarcts increases steadily with relatively high variance (Figure 2A). The most consistently positively correlated factor with bile infarcts is plasma bilirubin, which occurs in most time frames and even at single time points. The correlation is not very strong and the significance level is <5%, valid only for 3/11 time frames and 2/7 time points (see Supporting Information Dataset S3, section 4.10). The two parameters are biologically related as the infarct area inversely accounts for the number of functional hepatocytes.

Next most correlated is the RNA level of Il17a encoding interleukin-17A (Figure 8C), which plays a pivotal role in cholestatic liver fibrosis in mice by activation of both the KCs and HSCs [19]. The correlation is high only for later time points. Il17a is switched on between 2d and 5d to very high RNA levels, and as it is a secreted protein, it is likely to be detectable in the blood, thus representing a candidate diagnostic marker. Expression of Notch3 is initially negatively and later positively correlated with bile infarcts. This corresponds to the pattern of Notch3 expression. It drops below the level of untreated mice and is increased between days 2 and 5. Notch3 is reported to be significantly up-regulated in fibrotic liver tissues, most supposedly by regulating the activation of HSCs [20]. Next, Prom1, encoding prominin 1 (CD133), is reported to be increased in alcoholic hepatitis [21] and chronic liver injury [22], and was dedicated to be regulated by the DNA methylation in HSCs [23]. Many of the selected genes were positively correlated to a similar extent, with the highest values occurring for the later time frames. Among those, Col3a1 (see Figure 6F), Sparc, Col8a1, Wisp1, and Edn1 show a similar pattern as Notch3 for the initial anti-correlation. Remarkably, all genes whose expression values have a high consensus correlation to the infarct area show a high correlation for the 5d time point and the time frames covering day 5, while there is low correlation for the earlier time points and frames (Figure 8C). In addition, 5 days is the time point with the highest variance of the infarct area (Figure 2A). This indicates that different routes of the disease process exist, defined by the infarct area at day 5 and marked by expression of genes such as Il17a, Notch3, Prom1, Col3a1 (see Figure 6F), Actb, Sparc, Tnc, and Tgfbr2.

Loss of liver function

There is a large variability of bilirubin levels among different mice from the same time point. As bile cannot leave the liver via the biliary system due to cholestasis, differences in the measured serum bilirubin show the capacity of the hepatocytes and the bile lakes to store conjugated bilirubin. The highest correlation with serum bilirubin levels can be observed for Tnfrsf1b, encoding tumor necrosis factor receptor 2 (Figure 8D). Next, a high correlation is found for Ccl2, encoding chemokine (C-C motif) ligand 2, a soluble biomarker for hepatic fibrosis in NAFLD [24]. The 3rd highest correlation is found for Il10rb, encoding the interleukin 10 receptor β subunit, which was found up-regulated in NASH with fibrosis [25]. Intriguingly, the 12 factors most correlated to bilirubin levels show a negative correlation for the final time frame 14d (see Supporting Information Dataset S3, section 4.4). For instance, the mouse with the lowest bilirubin levels at day 14 (IM2, 9.12, see Supporting Information Dataset S1) has the highest Tnfrsf1b expression, leading to the hypothesis of alternate progression courses in the final disease stage.

Surprisingly, albumin synthesis is maintained relatively constant over the observation period of 14 days (Figure 1D). Apparently, hepatic degradation of albumin is affected at similar levels as its synthesis. Due to this finding, it is not surprising that albumin is poorly correlated to the dynamics of other factors investigated (Figure 8E). Cyp2b10 shows high correlations to Albumin only for separate time frames and time points.

Hepatic cell proliferative response

In later phase of the disease process, different hepatic cells proliferate, underlined by co-immunostaining with BrdU and specific markers for hepatocytes, KC, and BECs (Figure 3), and indirectly reflected by the marked up-regulation of Ki67 mRNA (Figure 4A).

As compensatory activity of the liver to restore the damaged parenchyma, hepatocytes proliferate, monitored by the parameter BrdU-positive hepatocytes. It is markedly triggered between the 30h and 2d time point (Figure 3D). It is highly correlated with Mki67 expression (Figure 9B), encoding the antigen Ki-67, a known proliferation marker (Figure 9B). The 2nd highest correlated gene is Birc5, encoding survivin. Yes-associated protein has been reported to regulate the hepatic response after bile duct ligation via modulation of survivin [28], underlining the role of survivin in hepatic tissue restructure. The 3rd highest correlation is found for Notch1, a transmembrane receptor involved in developmental processes, and its increase can as well be seen as a sign of cell plasticity and tissue restructuring. Next in line is Cdh2, encoding cadherin-2, which is normally associated to cancerous cells. The correlation is high at the time points 30h to 5d. Then Lama1, encoding laminin subunit α-1, the next most correlated factor, follows an opposite pattern, as the correlation is high at early (6h) and late (2d-14d) time points. Lama1 was found to be increased in nonalcoholic fatty liver disease [29]. Further, Timp2, encoding tissue inhibitor of metalloproteinases 2, an antagonist for degradation of extracellular matrix (ECM), also correlates with the hepatocyte proliferative response and reflects increased ECM deposition and buildup of fibrotic tissue.

Liver macrophages infiltrate the liver tissue, are activated, and proliferate, monitored by the parameter “BrdU-positive Kupffer cells”. This starts at 30 h in our time course to reach a maximum at day 2 and to decrease again thereafter (Figure 3E). There is no strong correlation with other parameters, yet the highest with Gsta2, encoding glutathione S-transferase A2 (Figure 9A), which facilitates bilirubin import [26]. The correlation is confined to the middle time points (30h-2d), the time frame of the strongest increase of KC proliferation, while in the beginning (6h-12h) and the end (5d-14d) a slight anti-correlation is found. The next highest correlation is to BrdU-positive BECs, again in the middle time frames (30h-2d), and with S100a4-positive cells, which itself has a higher correlation to BrdU-positive BECs. The same is true for the RNA level of the cell cycle gene Mki67. The factor Por, encoding cytochrome P450 reductase, is negatively correlated in the time frames and time point from 30h on, thus, resembles the up-and-down pattern inversely. Por is reported to be down-regulated in liver cirrhosis via the aryl hydrocarbon receptor AhR [27].

Also biliary epithelial cells (BEC) proliferate, monitored by the parameter “BrdU-positive BECs”. The main increase occurs between days 2 and 5, where a minor increase can also be observed after 30 h (Figure 3A). Further, a remarkable variability of BEC proliferation in the different mice can be seen at day 14. The highest correlation of this event is observed for the number of S100a4-positive cells (Figure 9C), which is representative for the number of KC and is in line with the recent finding about feed-forward and feedback regulation of the two cell types BEC and KC in liver fibrogenesis. Only the time frames near the perpetuation phase show a high correlation. Not surprisingly, the gene expression most correlated to BrdU-positive BECs was found for Col1a1 (Figures 9C and 6E), encoding the collagen deposited in ECM in large quantities. The 3rd highest correlation is found for Tgfb, encoding the cytokine TGFβ, which is well known to correspond with the fibrotic process in a positive feedback loop [30]. Interestingly, the negative correlation at the 6h time point shows high significance suggesting a negative feedback regulation. The 4th highest correlation is found for Il10rb, encoding the β-subunit for the Il10 receptor, which was described to be increased in rat liver fibrosis [31]. Slc10a1, encoding the Na+-taurocholate co-transporting polypeptide, which transports bile acids through the basolateral membranes of hepatocytes is negatively correlated with BEC proliferation, mainly in the later time frames (30h-14d). A decrease of Slc10a1 has been shown to protect hepatocytes from cholestasis-induced injury [32]. Another negatively correlated factor is Ugt1a1, encoding UDP-glucuronosyl-transferase 1A, the main enzyme for conjugation of bilirubin, whose down-regulation is considered protective against the increased concentration of conjugated bilirubin in hepatocytes.

Increase in fibrogenic cells

In accordance to the proliferative activity of HSCs, immunohistochemical analyses demonstrate the gradual rise in CTGF-, α-SMA- and S100a4-expressing cells (Figure 3), reflecting activated HSC, activated KC and trans-differentiated hepatocytes (such that they are producing hepatokines). CTGF is a highly pro-fibrogenic protein expressed by HSCs, BECs and hepatocytes [33; 34] and mediates extracellular matrix inducing properties. It is reported that levels of this protein are significantly up-regulated in experimental liver fibrogenesis and human chronic liver disease patients of various etiologies [35; 36]. The number of CTGF-positive show a steady increase with comparatively little variance (Figure 3F), as shown by the high significances in ANOVA analysis (see Supplementary File 4). The highest correlation with CTGF positive cell number shows Tgfb2, encoding the cytokine TGF-β2 (Figures 9D and 6I). TGF-β is the major stimulus for CTGF expression in hepatocytes [37] and elevated levels of Tgfb2 were reported for bile duct ligated rats [38]. This is the result of a direct interaction [39], and the correlation is close to 1 for several time frames (30h-14d) and all time points, except for 30h. The next highest correlation is found for Pdgfb, encoding platelet-derived growth factor subunit B, which is reported to be up-regulated in liver inflammation and fibrosis [40]. The number of cells containing CTGF and -SMA is also highly correlated. There is also a strong correlation to Tgfbr2, encoding the transforming growth factor β receptor 2 [41]. Additionally, there are considerable correlations to several other genes such as Timp1, Cxcl5, Sparc, and Col8a1.

The parameter “-SMA-positive cells”, a marker of HSC activation, increases steadily with the course of disease progression (Figure 3B). Not surprisingly, it is positively correlated to the number of CTGF-positive and S100a4-positive cells (Figure 9E). Interestingly, CTGF staining is highly anti-correlated to Cyp1a2 (Figure 6A), encoding cytochrome P450 1A2, known to be lowered in liver cirrhosis mediated by AhR [27]. The next most correlated gene is Pparg, encoding peroxisome proliferator-activated receptor gamma, but the most relevant correlation occurs in the early time frames, that is 18h-30h. PPAR- inhibits HSC activation [42]. As Pparg is increasing only in the early stages, we conclude that TGF-β and Pparg form a threshold system, where HSC activation is controlled in the first stage and overshooting in later time frames. Cyp2c37 shows a negative correlation in the early time frames (6h-18h), but the correlation of -SMA-positive cells to S100a4 is higher, as outlined below.

The S100a4 protein has been dedicated to myofibroblasts upon epithelial mesenchymal transition of hepatocytes in liver [43; 44]. This was however disproved in a careful analysis, indicating that the protein marks Kupffer cells [45]. The parameter “S100a4-positive cells” shows a steady rise until day 2, after which it stays constant (Figure 3C). Not surprisingly, it is positively correlated to the BrdU-positive BECs and -SMA-positive cells, but significant correlations do not occur after day 2 (Figure 9F). Expression of the gene Gstm1 (Figure 6C), encoding glutathione S-transferase Mu 1 is highly correlated. Genetic variation increases the susceptibility to alcoholic liver disease [46], but no connection between its regulation and other types of liver diseases has been reported. Cyp2c37 is negatively correlated, especially in the early time frames. The time course of Cyp2c37 expression shows a strong initial decline until 12 h, a plateau until day 2, and a final decline. Egfr, encoding epidermal growth factor receptor is also negatively correlated. Interestingly, both, but especially Egfr, show a higher anti-correlation for single time points than for time frames. Thus, although Egfr does not change much in the time course, it is a marker for increase of S100a4 positive cell number.

Fibrosis

During the perpetuation phase (18h-2d), paracrine and autocrine cytokines amplify hepatic inflammation and HSC activation resulting in continued ECM remodeling, being characterized by the enhanced mRNA expression of both fibrillar collagen1α1 and 3α1 (Figures 6E and F). Additionally, the progression stage of fibrogenesis is histomorphologically characterized by excessive deposition of extracellular matrix (Figure 4B), as analyzed by Sirius red staining of liver slices (Figure 4C). Significant correlations of “Sirius red positive area” are found after day 2 (Figure 8F). The most correlated parameter is Osmr, encoding the oncostatin M receptor, which transduces, among others, Il31 and oncostatin signaling events. Interestingly, it has been reported that in cirrhotic liver, Osmr is not expressed [47]. Thus, expression of this gene may be a candidate distinguishing factor for liver cirrhosis and cholestasis-induced liver injury, which however has to be confirmed with further investigations. Also Osm, encoding oncostatin M itself, is correlated, showing a combined induction of the ligand and its receptor [48]. Next most correlated with Sirius red staining is Ccr5, encoding C-C chemokine receptor type 5, which is a regulator of inflammation as well as macrophage recruitment and trafficking [49], thus representing a general promoter of hepatic fibrosis [50]. Additionally, blood bilirubin levels are correlated, as described above. Further correlated factors are Tnfrsf1b, encoding tumor necrosis factor receptor 2, and Tnfrsf1a, encoding isoform 1 of the receptor (see Figure 6L). The latter was previously reported as necessary for liver fibrosis in mice [51]. Then Cxcr1, encoding interleukin 8 receptor α, is reported to be highly up-regulated in chronic liver disease [52] and is found correlated with Sirius red (Figure 8F).

***Markers of disease progression***

We next asked the question, which of the analyzed factors are best suited as markers for particular stages of the disease process. One-way ANOVA identifies the parameters whose values at different time points are different in a general perspective. The calculated p-values allow a ranking how good the parameters deviate in the time course, the top significant are: Cyp1a2, serum bilirubin, Il10rb, Tgfb1, Ccl2, Cd86, Ccr2, and Mrc1.

In a more specific analysis, parameters were identified which have the largest difference of values in a particular time frame, a consecutive series of time points or a single time point. A separator for a particular time frame is a parameter, whose value range for mice belonging to the respective time frame is disjoint from the value range of mice outside this time frame. The quality of a separator is measured by the distance between the ranges in relation to the full range, the separation gap (see Supporting Information Dataset S3 for details).

We identified 41 separators for specific time frames. For 35 of these separators, the values of the factor show a single transition, while for 6 separators, an internal time range is marked. 12 separators select a single time point, 9 of which separate the 0h time point (the control) from ligated mice. The transcript abundance for Nr0b2 (small heterodimer partner, SHP, see Figure 6D) is strongly decreased only for mice at the 6h time point. SHP is a repressor of retinoid and thyroid hormone, as well as estrogen receptors and by being diminished in human cirrhotic livers, it is suggested to be associated to cirrhosis and hepatic tumors [53]. This is also supported by the fact that cholestatic liver fibrosis induced by BDL was increased in SHP-/- mice [54]. Cyp24a1 (mitochondrial 1,25-dihydroxyvitamin D3 24-hydroxylase, see Figure 6B) is also a separator for the 6h time point, with a lower separation strength. It had been found increased in hepatoma cells [55]. Mmp10 (Stromelysin-2/transin-2, see Figure 6H) is a separator for the 18h time point, the only other internal time point with such a separator in the investigated parameter set. By degrading proteoglycans [56] and fibronectin [57], the metalloproteinase contributes to ECM break down [58] and is found increased after liver injury [57] and at intoxication [59]. The transcript of Cdh2 (cadherin 2) has increased levels only for 6h and 12h. Il28b and Il2 (Figures 6J and K) display a similar pattern, but the separation gap is small.

Among the 9 separators for untreated mice (0h), serum ALT is the strongest separator. Serum bilirubin, Hmox1 (decycling heme oxygenase 1), and serum GLDH have a separation >1%, while -SMA-positive cells, Cxcl2 (chemokine (C-X-C motif) ligand 2), Cd14 (CD14 antigen), Timp1 (tissue inhibitor of metalloproteinase 1), and Mmp10 (Figure 6H) have smaller separation values.

CTGF-positive cell number is the separator with a large separation gaps for several time frames of transition (6h/12h, 12h/18h, 30h/2d, 5d/14d). Thus, it is clearly the best candidate from those selected to monitor the disease progress. It is also among the parameters with the highest ANOVA significance (which is particularly remarkable as only 3 could be analyzed compared to 5 for other parameters, See Supplementary File 4, section 1). S100a4-positive cells is a similarly good separator for two transitions (12h/18h, 30h/2d). The parameter -SMA-positive cells is a good separator for the transitions 0h/6h, 6h/12h, 12h/18h, thus it can be considered as the best marker for the initial disease process.

Cyp1a2 (cytochrome P450 1A2, Figure 6A) was identified as best separator for the 6h/12h transition, whereby transcript levels are initially high and steeply decrease between 6h and 12h. Cyp1a2 shows the highest significance in the ANOVA test, and parameters pairs with Cyp1a2 as one partner yield the highest significances in MANOVA tests which show its robustness as a marker, see Supplementary File 4. The gene product has already been observed to decrease upon bile obstruction [60]. The two other RNA separators for this transition (Cd14, Ccl2) are poor.

There are no suitable RNA markers for 12h/18h, 18h/30h and 30h/2d transitions. For the 18h/30h transition, there are no separators at all, which supports the definition of a perpetuation phase represented by the 18h and 30h time points. Strong separators are abundantly available for the 2d/5d transition, which indicates that large qualitative changes occur between day 2 and day 5 of the experimental disease process. The best separator is Il28b (interleukin 28B; Figures 11 and 6K). Based on this investigation, we conclude that transition from day 2 to day 5 can be easily monitored, also in clinical practice, as serum bilirubin is among the best separators, and the interleukins (Il28b, see Figure 6K, Il13, Il17a) as well as the growth factors (Pdgfb, Tgfb2, see Figure 6I, Hgf) and contributors to the extracellular matrix (Sparc, Col3a1, Col1a1, Figures 6F and E), all together encode excreted gene products, that should be detectable in blood samples. Sparc (secreted acidic cysteine rich glycoprotein), a known indicator of chronic liver disease [53] and a mediator of fibrosis [61], also has a large separation gap.

For the 5d/14d transition, CTGF-positive cell numbers and mRNA expression of Ctgf are the only separators, which is an argument in favor of the combination of the 5 and 14 day time point in the progression phase.

To this end we constructed a decision tree that assigns specific patterns of factors to a specific time interval of the disease process (Fig. 11). The range of values covered by the factors in a single clusters during the observed time of 14 days was normalized to the interval [x,y] ? and the values within interval were used to formulate decision rules at each k not of the tree. Cluster 4 was found to possess the strongest discriminating power: If the mean value of the factors within this cluster is smaller than - 0.12 at any time point this time point of BLD induced disease is classified as being not larger than 14 hours. The more detailed assignment of the respective time interval requires to interrogate the values of other clusters. Note that the values of cluster 4 appear twice in the decision tree whereas the values of clusters 2 and 6 are not exploited at all. Note that the algorithm used for the construction of the decision tree aims at avoiding overfitting of the data in balancing the number of knots against robustness of the classification tested by cross-validation. As the number of decisions remains smaller than the number of discrete time points for which observations of the factors are available the decision tree assigns a pattern of cluster to time intervals of disease progression rather than discrete time points.

The blue bars in Fig. 12 and the 2 x 2 contingency table in the legend depict the performance of the decision tree. As the tree was constructed on the basis of cluster means it is obvious that single factors showing the best concordance with the cluster mean (indicated on top of the panels in Fig. X) represent the best markers for different disease phases (green bars).

We tested the robustness of the decision tree against a random choice of a smaller number of factors. Instead of using the cluster mean, either a single factor or two factors were randomly chosen from each cluster and their values used in the classification tree. The quality of predictions is shown in Fig. 12. Even with 4 factors randomly selected from clusters 1.3.4 and 5 are surprisingly consistent classifications was achived. As expected, the quality of predictions was improved by increasing the number of factors.