# Pathobiochemical signatures of cholestatic liver disease in bile duct ligated mice

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**Running title:** Cellular and molecular signatures of cholestasis

# 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. 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. The count of CTGF-positive cells provided the most reliable overall measure for disease progression at the histological level, bilirubin at biochemical level, and metalloproteinase inhibitor 1 (Timp1) on transcript level. Prominent molecular events exhibited by strong transcript peaks are found for the transcriptional regulator Nr0b2 (Shp) and 1,25-dihydroxyvitamin D(3) 24-hydroxylase (Cyp24a1) at 6 h. Based on these clusters we constructed a decision tree to identify factor combinations that can be used as markers for specific time intervals of disease progression. Best prediction of onset of disease is achieved by fibronectin (Fn1), the initial phase by (Cyp1a2), transition to the perpetuation phase by collagen -1 I (Col1a1), and transition to the progression phase by interleukin 17-a (Il17a), with early and late progression separated by Col1a1. Notably, these predictions remained stable even for randomly chosen small sub-sets of factors from the clusters.

**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 additional studies are needed which have to include knowledge on transcription factor activation due to accumulation of bile salts and shared transcriptions factor binding motifs of genes belonging to the same transcript cluster.

**Keywords:** liver injury, mouse, systems biology, fibrosis, cell proliferation, bile duct ligation, cholestasis, morphological profiling, VirtualLiver Network

# 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}, which 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 observed at higher scales of tissue organization. Extracting from 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 sets itself apart from existing studies which either describe the time course of a limited number selected parameters after BDL {Georgiev2008, Huss2010} or provide gene expression profiling for a limited number of time points, thereby missing the acute damage after BDL in the first 24h and long-term effects after 7 days {Tanaka2006}. None of these preceding studies providing 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.

# Materials and Methods

**Ethic 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).

**Mice**

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 analyzed 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 dehydrogenase (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 α-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 correction was performed using the Holm procedure {Holm1979}. To specifically test for the initial changes a two-tailed unpaired t-test (Welch correction for nonhomogeneity of variance) was performed for all factors between the classes 0h and 6h.

*Correlation analysis*: Correlation between two factor 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 terms: 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 2 for definitions).

In the analysis the following weights were used: , , . All reported correlations are values in the interval [-1,1]:

Cluster analysis of the correlation matrix used complete-linkage hierarchical clustering with euclidian distance measurement. This combination of complete-linkage with provided the best enrichments on gene-expression time-series in a recent comparisons of methods {Jaskowiak2014, Jaskowiak2013}. The number of clusters , was selected as maximum number of clusters with all clusters containing more than one factor. Normalization of factors was performed separately for each factor for all time points and repeats with and via

*Decision Trees*: For the prediction of distinct time points of disease progression a regression tree with the mean normalized factor data of the 6 clusters as predictor variables and the log transformed time points as dependent variable was fitted based on recursive partitioning using *rpart* {Breiman1984}. Logarithmic transformation was performed to obtain approximately equal-distant time points.

The regression tree was fitted using the complete trainings set (), with the minimum number of observations in a node for which a split was computed being 6, the minimum number of observations in a terminal node as 2, and the complexity parameter . The splitting criterion used to decide which predictor variable gives the best split for nodes in the regression tree was , with the sum of squares for node T and and the sums of squares for the left and right child.

A leave-one-out approach was used to test the robustness of the predicted time classes and predictive performance: For each sample ( mice), the regression tree was generated under the exclusion of data from the sample, with subsequent prediction on the left out test data (see Supplement 2).

The predictive capacity of the regression tree was evaluated using all single combinations of individual factors from the clusters (88572), and a random subset of 10000 two factor combinations from each cluster. Predictions for a given combination of factors from the 6 clusters were scored using the root mean square distance on log scale *d*, with the best combination of factors minimizing d

All , and the full analysis available in S and from <https://github.com/matthiaskoenig/bdl-analysis>

# 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 is 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 sham operated mice. 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 constant over the time course of 14 d (Figure 1D). The systemic blood cell count showed 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). The typical appearance of liver tissue at representative time points after BDL using H&E staining is depicted in Figure 2B. 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, however, 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, among others, 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-β 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) fibrogenesis- (Figure 5B), and inflammation-related genes (Figure 5C) was made arbitrary based on state-of the art knowledge.

In a first step, a one-way analysis of variance (ANOVA) was applied to reduce the complete data set of biochemical, histochemical and transcript data consisting of 153 factors to a subset of those factors showing significant (pad j< 0.05) changes during the time course of disease progression (see section ANOVA in Supplement 2). This resulted in a subset of 90 factors comprising two biochemical markers (bilirubin, GLDH), five histochemical markers (BEC, NHC, Kupffer cells, Sirius red), three immunostainings by antibodies (CTGF, -SMA, S100a4) and 80 genes (14/47 ADME, 22/46 fibrosis, 44/47 inflammation panel). Many of the ADME genes and fibrosis genes were filtered out, whereas almost all of the inflammation panel was retained. The top significant factors were: Cyp1a2, serum bilirubin, Il10rb, Tgfb1, Ccl2, Cd86, Ccr2, and Mrc1. Within this subset, a bivariate time-dependent correlation analysis (see Methods and Supplement 2) was performed for all pairs of factors to identify those factors displaying similar temporal profiles (see Figure 7). The top correlations for the biochemical, histological and immunostainings ared depicted in Figure 8. Based on the obtained correlation matrix a hierarchical cluster analysis was applied resulting in 6 different clusters comprising between 2 - 61 factors with distinct time courses attaining their maximum at different time points (see Figure 9). Accordingly, the clusters can be assigned to the early, middle and late phase of disease progression. The found clusters comprise both ‘classical’ biochemical and histochemical factors and genes characteristic for a specific phase of disease progression.

## Correlations between transcripts and non-RNA factors

In what follows we discuss in more detail the biological significance of the correlations between different types of factors shown in Figure 8 thereby including already exiting knowledge.

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. In the following the top correlated factors with the biochemical, histological and immunostaining presented in Figure 8 are discussed. The main findings are summarized in Figure 11 bringing the different aspects, phases and markers together.

### Initial response

GLDH, ALT, Cluster 2 and 3

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. GLDH like ALT shows a strong initial increase, but in contrast to the latter increases further up to 18h, before it decreases gradually.

Among the up-regulated factors (see Supplement t-test for initial phase) are the members of cluster 3, i.e. GLDH (Figure 8B), Fn1 (Figure 1B) and Sult1a1 increase in the initial phase after BDL, and cluster 2, i.e. Cyp24a1 and Nr0b2.

GLDH shows no high correlation with any other parameter, while serum ALT clusters with Cyp7a1 by a negative correlation.

In addition to the cluster analysis, we tested specifically for changes in the initial time point (see Supplement). A marked down-regulation after 6h could be observed among others for Cdh2 and Pde4a and the main enzyme of bile acid synthesis Cyp7a1 (cholesterol-7-α-hydroxylase), which 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].

### 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 individual liver cell death and progressive development of confluent bile infarct areas. The total area of infarcts increases steadily with relatively high variance (Figure 2A).

The most correlated factor with bile infarcts is plasma bilirubin (Figure 8C).

### Loss of liver function

Albumin, bilirubin

The highest positive correlation with serum **bilirubin** levels can be observed for the transcipts Timp1, Cd14. Ccl2 and Ccl3. Ccl2 (chemokine C-C motif ligand 2) is a soluble biomarker for hepatic fibrosis in NAFLD [24].

A high negative correlation is found between bilirubin and **Slc10a** … discuss

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).

### Hepatic cell proliferative response

TODO: Discuss correlations of NHC, Kupffer, BEC cells.

BrdU-positive Kupffer cells, BECs and S100a4-positive cell numbers are clustered with the closely related RNAs of Gstm1 and Gsta2 (top left, purple, brown, light blue). BrdU-positive hepatocytes are clustered together with Notch1, Birc5, and Mki67 (bottom, cyan). CTGF and α-SMA positive cells are clustered with the RNA of Pparg and Gstp1 (top left, grey blue).

During disease progression, various hepatic cells start proliferating, 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, which is markedly triggered between the 30h and 2d time point (Figure 3D), with very similar time course to the increase in KC, BECs and NHCs. These are highly correlated with **Mki67**, encoding the antigen Ki-67, a known proliferation marker and **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 restructuring.

Liver macrophages (Kupffer cells) infiltrate the liver tissue, are activated, and proliferate starting at 30 h in our time course to reach a maximum at day 2 and to decrease again thereafter (Figure 3E).

Kupffer cells show a high correlation to BrdU-positive BECs, with S100a4-positive cells, which itself has a higher correlation to BrdU-positive BECs and Mki67.

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).

Kupffer cells are also highly correlated with the number of S100a4-positive cells

**Slc10a1**, encoding the Na+-taurocholate co-transporting polypeptide, which transports bile acids through the basolateral membranes of hepatocytes is highly negatively correlated with BEC proliferation. A decrease of Slc10a1 has been shown to protect hepatocytes from cholestasis-induced injury [32].

### 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. Levels of have been reported significantly up-regulated in experimental liver fibrogenesis and human chronic liver disease patients of various etiologies [35; 36]. The number of CTGF-positive cells shows a steady increase with comparatively little variance (Figure 3F), as shown by the high significances in ANOVA analysis (see Supplement 2). The highest correlation with CTGF positive cell number shows **Tgfb2**, encoding the cytokine TGF-β2 (Figures 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].

The second 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.

Additionally, there are considerable correlations to several other genes such as **Tgfbr2**, encoding the transforming growth factor β receptor 2 [41], Cd14, Cxcl5, Timp1.

-SMA-positive cells, a marker of HSC activation, increase steadily during disease progression (Figure 3B), highly correlated to the number of CTGF-positive and S100a4-positive cells.

Notably, -SMA staining, but also CTGT and S100a, are highly negatively correlated to **Cyp1a2** (cytochrome P450 1A2, Figure 6A), known to be lowered in liver cirrhosis mediated by AhR [27], and to **Ppara** (Figure 8A)

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]. S100a4-positive cells show 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.

### Fibrosis

Sirius red-positive area (collagen) is quite isolated in the graph map, and most closely relates to the cytokines’ cluster (left, grey).

Both Col1a1 and Col3a1, fibrillar collagen1α1 and 3α1, which predominantly exist in fibrotic livers, showed up-regulation around 30h after BDL and a continuous increase with severity of liver fibrosis up to 14 days (Figures 6E, F). Among the peptide mediators, Tgfb1 and Tgfb2 (Tgf-β isoforms 1 and 2) expression was increasing after 2d, confirming its postulated role as fibrogenic master cytokine [14]. Associated with induction of fibrogenesis-related genes (Figure 5B), which particularly are representative for HSC activation, the dynamics of the inflammation gene signature (Figure 5C) nicely matches with the increase in the number of proliferating Kupffer cells (F4-80/BrdU staining values) observed from day 2 onwards (Figure 3E). Very low expression levels were present immediately after BDL, except for the chemokines Cxcl1 und 2. Starting at time points between 2 and 5 d after BDL, most cytokines and chemokines in the list were strongly upregulated for a longer period of time, until day 14.

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).

The progression of fibrogenesis is histomorphologically characterized by excessive deposition of extracellular matrix visible by Sirius red staining of liver slices (Figure 4BC). Surprisingly, Sirius red did not have high correlatonions to other factors, mainly due to the high variability in measurements from 0h to 2d and was in this study not a very reliable predictor for fibrogenesis.

## Markers of disease progression

The main focus of this study was to detect factors and factor combinations which characterize particular stages of the disease process best. In what follows we discuss in more detail the biological significance of the 6 clusters of timely correlated factors.

### Cluster 1

Cluster 1 (Figure 9A) decreases continuously over time.

Top correlations with the cluster mean time course are Cyp2c37

Top ANOVA results in the cluster are …

ADME decrease

**Cyp2c37**

**Cyp2e1**

**Cyp2c29**

**Ugt1a1**

**Cyp1a2**

### Cluster 2

**Nr0b2** (small heterodimer partner, SHP, Figure 6D) transcript abundance shows a strong peak at 6h. 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) also shows a strong peak the 6h time point. Cyp1a2 shows the highest significance in the ANOVA test (padj=2.9E-14). The gene product has already been observed to decrease upon bile obstruction [60].

### Cluster 3

The initial damage can be separated from the initial damage based on Fn1, GLDH, Sulf1a1.

**Fn1** (Fibronectin, see Figure 6G) a constituent of the ECM in liver fibrosis.

**Sulf1a1**

**GLDH**

### Cluster 4

continuous disease progression (fibrosis)

**CTGF**

**bilirubin**

**S100a4**

**Col1a1** (Collagen alpha-1(I) chain, cluster 4)

**Col3a1**

**Sparc**

**Tnfrsf1a**

**Gstm1**

### Cluster 5

late disease progression

The splitting by hierarchical clustering of cluster 4 and 5, with baseline values of Cluster 5 until 2 days indicates that large changes occur between day 2 and day 5 in the disease process.

**Gdf2**

**Il17a**

**Il28b**

### Cluster 6

**Cdh2** (cadherin 2)

**Bad1**

### Discussion

Most histologic parameters and cell count observations, as well as most genes related to inflammation and fibrogenesis increase with disease progression (about 2/3 of all factors studied) either in a continuous manner starting in the perpetuation phase after ~1 day or at the latest beginning at day 5 (cluster 4 and 5). Based on the strong increase between day 2 and 5 in cluster 5 (but also in cluster 4), we conclude that transition from day 2 to day 5 can be easily monitored, also in clinical practice, as serum bilirubin is among the top correlations of cluster 4, and the interleukins among cluster 5 (Il2, Il17a, Il28b, Figure 6H and K) as well as the growth factors in cluster 4 (Pdgfb, Tgfb1, Tgfb2, Figure 6I, Hgf) and contributors to the extracellular matrix among cluster 4 (Sparc, Col3a1, Col1a1, Figures 6F and E). All together encode excreted gene products, that should be detectable in blood samples.

Interestingly no histological, biochemical or immunostaining based factors are found in the clusters 1, 2 and 6. The transcripts in these clusters provide unique information which cannot be captured with the subet of analyzed histological markers.

## Decision trees for disease progression

We next asked the question, which of the analyzed factors are best suited as markers for particular stages of the disease process. To this end we constructed a decision tree based on the time courses of the normalized clusters (Figure 9). This regression tree allows the prediction of a specific time interval of the disease process based on the combination of time courses of factors from the clusters (Figure 10). The algorithm used for the construction of the decision tree aims at avoiding over-fitting of the data by balancing the number of knots against robustness of the classification tested by cross-validation. Consequently, the decision tree assigns a pattern of cluster to time intervals of disease progression rather than the discrete experimental time points, resulting in 6 time classes. Interestingly, mainly time points in the late initial phase and perpetuation phase (12h, 18h, 30h, 48h) were merged into time classes, whereas the control (0h), early initial phase (6h), and progression phase (5d, 14d) remained almost unchanged. The robustness of the predicted time classes and prediction performance were tested with a leave-one-out approach, resulting in reproducible time classes and good prediction performance on the left out test data (see Supplement 2).

The resulting regression tree exploits time course information from cluster c1, c3, c4, and c5, whereas clusters c2 and c6 are not used. Cluster 4 was found to possess the strongest discriminating power performing the important split between early phase after BLD (classes 0h, 6h and 14h with range 0h-21.8h) and the later perpetuation and progression phase (classes 34h, 6d,14d, with range 21.8h-14d): If the mean value of the factors within this cluster is smaller than -0.12 at a measured time point this time point of BLD induced disease is classified as being not larger than 21.8 hours. The more detailed assignment of the respective time interval requires to interrogate additional clusters: The value of c3 decides between control and initial phase with subsequent splitting based on c1 in early and late initial phase. Analogue, the value of c5 decides between perpetuation and progression phase with subsequent splitting in early and late progression phase based on c4. Note that the values of cluster 4 appear twice in the decision tree, owing to the monotonous increase after BDL.

The predictive performance of the regression tree on the mean cluster data is depicted in Figure 10B (blue), showing which experimental time points were classified in which classes. All samples of control, 6h, and 14d are assigned to their respective time classes 0h, 6h and 14d, whereas neighboring time points are combined in classes 14h, 34h and 6d. In addition, we evaluated the decision tree based on a subsets of factors from the time course clusters using either a single factor or two factors randomly chosen from each cluster and their values were used for prediction (Figure 10B, single and double factors). Even with only one factor selected from c1, c3, c4, and 5 surprisingly consistent classifications were achieved. As expected, the quality of predictions was improved by increasing the number of factors. The control, early initial phase and late progression phase can be predicted with high accuracy, whereas the intermediate phase shows more misclassifications in case of the single and double factors.

The best performing decision tree based on a single transcript from each cluster (Figure 10A) splits on Col1a1(Collagen alpha-1(I) chain, c4), Fn1(Fibronectin, c3), Cyp1a2(Cytochrome P450 1A2, c1), and Il17a(Interleukin-17A, c5), all important factors involved in disease progression (discussed above). The best performing decision tree based on all factors, i.e. histological, biochemical, immunostaining factors and transcripts, resulted in a highly similar tree, with the single change of using S100a4 instead of Col1a1 for c4 splitting, and providing GLDH as alternative factor to Fn1 to perform the c3 splitting. By selecting a subset of good performing transcripts very good prediction performance already on a small subset of factors can be achieved (Figure10B red). Our approach allows the prediction of progression after BDL from an arbitrary subset of measured parameters.

## Individual variability

We observed a variability of many .example 5, between and(Figure 2A) measured by Sirius red between (Figure 4B) Similar high variability can be observed in the proliferative response (BEC Figure 3A, Kupffer Figure 3E) or expression of collagen (Col1a1 Figure 6E, Col3a1 Figure 6F), to name a few. These large variation in parameters during the perpetuation and especially progression phase are an intriguing finding, considering the homogeneity of the experimental system (see also heatmap of time courses in Supplement 2). A possible explanation could be a different individual pace of disease progression due to variations in susceptibility to the damage caused by BDL, for instance due to different genetic background. As a consequence heterogenous time courses develop, with highly affected mice showing strong signs of fibrosis earlier.

Another hypothesis are variable routes of disease progression: One route is characterized by a strong increase in necrotic tissue and a weaker activation of HSC and lower expression change of inflammation factors. The other route is characterized by strong activation of fibrogenesis factors, and finally macroscopic collagen tissue. Both routes are similarly connected to loss of liver function, however, the histopathological display is quite different: the former contains a large amount of necrotic tissue while the latter contains large fibrotic tissue. Such alternate disease routes can be of far-reaching importance for an individualized therapy, as obviously the medical interventions avoiding necrosis differ from interventions reducing overshooting fibrosis. The design of the study, which included the sacrifice of mice after a specific time, did not allow to answer whether the alternate developments, shown macroscopically in later time points, can also be observed in earlier time points.

# Conclusion

The time-resolved analysis of a wide range of parameters in bile duct ligated mice has shown that many factors share the pattern of an increase throughout disease progression (Figure 9). Particularly, pronounced changes were observed during the transition from perpetuation to progression phase 2 to 5 days after BDL, characterized by strong increase of parameters, such as Il17A, Il2, Il28b or Il13. This information has strong clinical relevance, as it indicates a switching point, and human homologs of the respective interleukins are top candidates to be used as clinical markers.

Our experiments also found previously unknown molecular events which are probably elements of a transcriptional program. For instance, there are strong transcript peaks for SHP (Nr0b2) at 6h, which now need a focused analysis to delineate the chain of molecular interactions causing it and the functional consequences for disease onset and progression.

Based on time course correlation analysis we found a distinct number of time course patterns following BDL. Based on these results, we propose a decision tree from a subset of parameters, such as in Figure 10, as a promising tool for assessment of disease progression. Therefore, as next step, suitability of the parameters selected from the mouse model need to be confirmed for human patients as in [69].

Many of the measured parameters The here suggested approach of pooling information from factors falling in the same time course classes could be a possible solution for more robust predictors.

response ,resulting in an

# Availability of supporting data

The data sets supporting the results of this article are included within the article and its additional files.

Dataset S1. Measurements in bile duct ligated mice.

Dataset S2. Statistical analysis.

# List of Abbreviations

CTGF: connective tissue growth factor; BDL: bile duct ligation; SHP: small heterodimer partner; CLD: chronic liver disease; HSC: hepatic stellate cells; KC: Kupffer cells; ADME: absorption, distribution, metabolism, and excretion; ALT: alanine aminotransferase; GLDH: glutamate dehydrogenase; EDTA: ethylenediaminetetraacetic acid; H&E: hematoxylin and eosin;

**Competing Interests**

The authors declare that they have no conflicts of interest.

**Author Contributions**

Conceived and designed the experiments: KA AH MT IM BV SD. Performed the experiments: KA MT IM HW. Analyzed the data: KA MK AH MT IM ME HW HH UMZ BV SD. Wrote the paper: KA MK AH MT ME HW HH UMZ BV SD. All authors read and approved the final manuscript.

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Tables

**Table 1.** Analysis of systemic blood cell count of sham-operated mice (S) and mice underwent BDL. Values are given as means ± SEM.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | erythrocytes | | platelets | leukocytes | hemoglobin | | hematocrit |
| S | 8.4±0.1 | 1177±60 | | 7.5±0.3 | 7.9±0.1 | 44.8±0.7 | |
| 6h | 8.1±0.1 | 1061±39 | | 4.2±0.5 | 7.7±0.1 | 42.8±0.7 | |
| 12h | 8.2±0.1 | 1036±47 | | 4.5±0.4 | 7.7±0.1 | 43.0±0.6 | |
| 18h | 8.7±0.3 | 856±110 | | 4.1±0.2 | 8.1±0.3 | 45.6±1.9 | |
| 30h | 8.5±0.5 | 1071±100 | | 5.8±0.9 | 7.9±0.5 | 44.9±2.8 | |
| 2d | 8.7±0.2 | 1117±65 | | 4.7±1.3 | 6.5±1.7 | 45.9±0.9 | |
| 5d | 8.7±0.3 | 1295±107 | | 7.6±1.2 | 7.8±0.3 | 46.5±1.6 | |
| 14d | 7.6±1.4 | 1362±58 | | 7.4±1.1 | 6.6±0.2 | 38.4±1.3 | |

# Figure legends

**Figure 1:** **Analysis of liver injury and-function.** Plasma activities of alanine aminotransferase (ALT) (**A**) and glutamate dehydrogenase (GLDH) (**B**) and concentrations of plasma bilirubin (**C**) and albumin (**D**) at multiple time points after BDL. Values are given in means ± SEM of five independent experiments per time point.

**Figure 2:** **Quantification of bile infarcts in H&E-stained liver sections at multiple time points after BDL (A).** Values are given in means ± SEM of five independent experiments per time point. Representative H&E stainings of paraffin-embedded liver sections for each time point after BDL (**B**; arrows indicate bile lakes; magnification x10) with higher magnifications (x40) in (**C**), displaying cellular infiltrates (asterisk) and formation of artificial bile ductules (arrowhead).

**Figure 3: Analysis of the proliferative and cellular response at multiple time points after BDL.** Quantitative immunohistochemical analysis of BrdU-positive biliary epithelial cells (**A**), liver cells positive for α-SMA (**B**) and S100a4 (**C**), BrdU-positive hepatocytes (**D**) and Kupffer cells (**E**) and CTGF-positive cells (**F**). Values are given in means ± SEM of five independent experiments per time point. Corresponding representative immunohistochemical stainings are shown in the right panel (magnifications x40).

**Figure 4:** **Analysis of proliferation and extracellular matrix accumulation.** mRNA quantification of the proliferation marker Ki67 (**A**) by Fluidigm real-time PCR. Values are given in means ± SEM of five independent experiments per time point. Quantitative analysis of extracellular matrix deposition (**B**) and representative histological images (**C**; magnification x10) of Sirius red-positive areas at multiple time points after BDL. Values are given in means ± SEM of five independent experiments per time point.

**Figure 5:** **Heat maps displaying gene expression pattern at multiple time points after BDL.** Gene expression relative to Gapdh gene, obtained from Fluidigm qPCR, are shown as fold changes to sham mice (0 h) and are displayed in log2 scale. Red colour indicates up-regulation (2), blue down-regulation (-2) and white transcription levels about 1. (**A)** selected ADME genes, (**B**) selected fibrogenesis genes, and (**C)** selected cytokine genes.

**Figure 6:** **mRNA quantification of different selected genes by Fluidigm real-time PCR displayed in log2 scale.** (**A**) Cyp1a2, (**B**) Cyp24a1, (**C**) Gstm1, (**D**) Nr0b2, (**E**) Col1α1, (**F**) Col3α1, (**G**), Fn1, (**H**) Mmp10, (**I**) Tgfb2, (**J**) Il2, (**K**) Il28b, (**L**) Tnfrsf1a. Values are given in means ± SEM of five independent experiments per time point.

**Figure 7: Correlation matrix of factors.** Correlation matrix (YS3) of the subset of significantly changed factors determined by ANOVA. Positive correlation depicted in blue, negative correlation in red according to color key. Side dendrogram depicts the hierarchical clustering results with the 6 time course clusters marked in the color sidebar (see Figure 9 for the time courses corresponding to clusters c1-c6). Histological factors marked with H, immunostainings with A, and biochemical factors with B.

**Figure 8: Histological (H), biochemical (B), and immunostaining (A) correlations.** All correlations are YS3 correlation between the respective factors with numerical values provided in Supplement 2. (**A**) Correlation between histological, biochemical and antibody factors and gene transcripts. Only genes with at least one YS3 correlation of abs(YS3)>=0.6 are shown. Positive correlation in blue, negative correlation in red analog to Figure 7, with the area of circles corresponding to the correlation coefficients. (**B**) Correlation between histological, biochemical and antibody factors. (**C**) Highest absolute correlations between histological, biochemical and antibody factors and all other ANOVA filtered factors. Data sorted from left to right by absolute value of correlation. Color and size of the filled pie corresponds to the respective correlation value.

**Figure 9: Time course clusters in BDL.** Time course clusters based on YS3 correlation with hierarchical clustering based on complete linkage corresponding to clusters in Figure 8. Mean cluster time course (averaged over all factors and repeats) depicted in blue, normalized factor representatives of cluster in grey. Shaded grey area corresponding to SD of all mean factor time courses. Top correlations based on YS3 between the factors in the cluster and the mean cluster time course are shown above the time course. Histological factors marked with H, immunostainings with A, and biochemical factors with B. Cluster members are fully enumerated in the figure for all classes with exception of cluster 4 with the members and respective correlation to mean cluster time course being: Timp1 (0.94), bilirubin (B 0.92), Ccr2 (0.92), CTGF (A 0.91), Tgfbr2 (0.89), α-SMA (A 0.89), Ccl5 (0.88), Tgfb1 (0.88), Ccl3 (0.87), Tnc (0.87), Cd14 (0.87), Ccl2 (0.86), Cd86 (0.86), Pdgfb (0.86), Col1a1 (0.86), Cxcl3 (0.86), Ccl4 (0.85), Cxcl5 (0.85), Il10ra (0.85), Col3a1 (0.85), Il10rb (0.84), Ccl7 (0.82), Cd69 (0.82), Ifnar1 (0.82), Tnf (0.82), Osm (0.81), Sparc (0.8), Il6 (0.8), Tnfrsf1b (0.8), Cxcr2 (0.78), Il1b (0.78), Timp2 (0.77), Ifnar2 (0.77), Ccr5 (0.77), Il10 (0.76), Osmr (0.75), Gsta2 (0.74), Il4 (0.71), Ifng (0.71), Ccl8 (0.71), Hgf (0.7), Bak1 (0.7), Mrc1 (0.69), Tgfb2 (0.69), Ccr3 (0.68), Actb (0.68), S100a4 (A 0.66), Il13 (0.66), Met (0.66), bile infarcts (H 0.65), Il6st (0.63), Tnfrsf1a (0.63), Mki67 (0.62), Birc5 (0.6), Ctgf (0.58), BEC (H 0.56), Bax (0.56), Notch1 (0.54), Cxcr1 (0.51), Gstm1 (0.45), Cdh1 (0.42)

**Figure 10: Decision tree for disease progression.** (**A**) Regression tree for the prediction of time phases after BDL based on mean cluster time courses. The best gene representatives from the clusters are depicted above the decision rules. The regression tree classifies the data in 6 time classes 0h, 6h, 14h, 24h, 6d, 14d with information on mean time value, range, and number and percentage of samples falling given in the respective class. The best tree based on genes, histological, biochemical and antibody factors is highly similar to the best gene tree, with the single change of using S100a4 instead of Col1a1 for the decision on cluster c4 and allowing GLDH as equally good alternative to Fn1 in c3. (**B**) Predictive performance of decision tree. The predictive performance of the regression tree was evaluated using all single factor combinations from the individual clusters (white), a random sample (N=10000) of two factors from each cluster (gray), the best gene representative tree (red), and the mean cluster data (blue, trainings data).

**Figure 11: Outline of the disease process.** Each box is dedicated to a specific disease aspect (first line) which is represented by a commonly known marker (second line) or several markers. Below (in small font) the genes are shown whose expression is correlated to the factor above.