

Taking a transcriptomic perspective on cholestasis.

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Abstract

Background: Cholestasis is characterized by accumulation of toxic bile in the liver. However, it is unclear how the liver adapts to cholestatic conditions. This study aims to elucidate how hepatic tissue reacts to cholestatic conditions on a transcriptomic level.

Methods: Cholestatic, preoperatively drained and control tissues were analyzed to investigate transcriptomic differences. Both mRNA and miRNA data was processed and analyzed using a classical differential gene expression approach. Based on a FDR of 5% and absolute fold changes greater than 1.5, significantly different expressed genes were selected. Subsequently gene ontology enrichment and pathway enrichment analysis were combined with protein-protein interaction network construction to identify underlying biological mechanisms by which hepatic tissue adjusts to cholestatic conditions.

Results: It was found that drainage contributed largely to normalizing gene expression relatively to control subjects' gene expression profiles. Furthermore three main biological processes and pathways, namely bile secretion, extracellular matrix organization and inflammatory response, were found to have different gene expression levels in cholestatic subjects compared to control subjects. Accordingly, it was found that miRNA hsa-34a-5p which effects pro-apoptotic and pro-inflammatory genes was upregulated in cholestatic subjects. Interestingly it was found that OPN/SPP1 which is involved in ECM as well as neutrophil leukocyte attraction was highly upregulated.

Conclusion: Thus we hypothesize an underlying interplay between ECM re-organization, inflammatory response and hsa-34-5p upregulation in cholestasis. Research into this field will help elucidate the underlying mechanism of cholestasis and potentially lead to novel therapeutic strategies.

Introduction

Introduction

Cholestasis is characterized by impaired production and/or secretion of bile, resulting in accumulation of toxic bile substances in the liver.^{1,2} Prolonged cholestasis can cause severe liver injury due to chronic liver inflammation, ultimately leading to liver failure.¹ Gallstones or malignancies can physically obstruct bile, reducing bile flow and making secretion of bile difficult or impossible.³

Bile is an aqueous secretion that is uniquely formed in the liver.⁴ It mainly serves to secrete toxic compounds such as bile acids and bile salts, but also has several other functions, such as uptake of dietary fats and immune surveillance⁴. After formation by hepatocytes, the bile flows through bile ducts, where the composition is further modified by the bile duct epithelium.⁴ After leaving the liver, hepatic ducts transport bile either to the gallbladder, where it is concentrated, or directly into the duodenum.^{1,4}

During cholestasis, toxic bile compounds, such as bile acids are accumulated in the liver. A few previous studies have focused on how the liver adapts to these cholestatic conditions.^{5,6} Vendemiale *et al.* showed an increased production of toxic oxygen radicals, associated with impaired mitochondrial energy production.⁵ This further supported by the fact that elevated levels of oxidative stress markers are found in bile of drained cholestasis patients.⁶ The same study also demonstrated that the inflammatory markers tumor necrosis factor α (TNF- α) and interleukin 6 (IL-6) were

both present in higher concentrations in bile of drained patients, indicating an increased activity of the immune system.

However, both studies looked at compounds found in bile of drained patients.^{5,6} It is not yet clearly understood how the liver adjusts to these cholestatic conditions on a genetic level. In this study we took a state of the art data-driven approach to investigate the genetic profile of both drained and non-drained cholestatic patients.

Material and methods

Data collection

Normalized data was obtained from Maastricht University. In this study, 28 patients with pancreatic or liver cancer undergoing liver surgery at Maastricht University Hospital were included. Subjects can be divided into three groups; (1) cholestatic patients in which their bile ducts were obstructed due to their malignancy, (2) drained patients, which also had obstructed bile ducts due to malignancies, but underwent preoperative drainage several weeks prior to operation and (3) controls, who did not have obstructions in bile ducts. For an overview of included subjects see table 1. mRNA and miRNA was isolated from liver specimens from all subjects. Expression levels of both mRNA (Sureprint G3 Human GE 60K V2) and miRNA (Sureprint G2 Human miRNA V19) genes were determined using corresponding Agilent arrays. The study was approved by the local Medical Ethical Committee (Maastricht University Hospital).

mRNA statistical analysis

Statistical analysis was performed using the Linear Models for Microarray Data (LIMMA) package of R/Bioconductor⁷ in R. Prior to analysis, batch effects due to handling of samples were removed, as shown in figure 1. mRNA samples were measured on four different chips, which could account for a large proportion of variance in the dataset. Removing batch effects corrects for this variance.

According to the experimental design, a linear model was fitted. Fold changes between sample groups were computed using the linear model. The LIMMA package allows correction for multiple testing, using the “Benjamini and Hochberg” method. A threshold of a p-value smaller than 0.05 and an absolute value log2 fold change (log2 FC) greater than 1.5 was applied to select for differently expressed genes (DEGs).

Gene ontology enrichment analysis

The Database for Annotation, Visualization and Integrated Discovery (DAVID)^{8,9} was used to perform gene ontology (GO) enrichment analysis. This database allows for GO enrichment analysis adjusted for multiple testing. DAVID uses algorithms that assign gene names to pathways, gene ontologies and functional keywords. The results in DAVID were validated by performing GO enrichment analysis using the goana enrichment in the LIMMA package. Goana adjusts for gene length, but not for multiple testing.

Pathway enrichment analysis

Significantly changed pathways were identified using the clusterProfiler package in R.¹⁰ Adjustment for multiple testing was done using the “Benjamini and Hochberg” method. Pathways are considered significantly enriched at a p-value smaller than 0.05. Significant genes were mapped onto pathways employing the online pathway viewer tool from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.¹¹⁻¹³

Network analysis

String-DB, a database for known protein-protein interactions, was used to create protein interaction networks.¹⁴ Based on a list of genes discovered to be significantly changed, string-db computed a protein interaction network. The highest confidence parameter (0.9) and removal of unconnected nodes was selected. Only known interactions, i.e. based on experiments or curated databases as well as protein homologs were connected with an edge.

miRNA statistical analysis

The normalized miRNA data was statistically analyzed to identify DEGs, using the LIMMA package. The LIMMA package was used to produce log2 fold changes, p-value, and adjusted p-value for each miRNA gene. P-values were adjusted using the "Benjamini and Hochberg" method, which is already built-in in LIMMA. A miRNA gene is then defined as DEG if the adjusted p-value is smaller than 0.05 and absolute value of log2 FC is bigger than 1.5.

miRNA target prediction

miRNA predicted targets were obtained using the MultiMir package by Bioconductor.¹⁵ All MultiMir databases were searched with a prediction score cutoff of 10%. mRNA targets were limited to significantly changed mRNA in our dataset. Only validated and unique miRNA-mRNA pairs were selected for further investigation.

Results

mRNA statistical analysis

For each comparison, DEGS were selected based on a P.Value < 0.05 and absolute log2 FC > 1.5 (figure 2). The comparison cholestatic versus control yielded 249 DEG (supplementary table S1). The distribution of these DEGs has a volcano shaped distribution, shown in figure 3. The five most significantly changed genes are secreted phosphoprotein 1 (SSP1) (also called osteopontin (OPN)), matrix metalloproteinase-7 (MMP7), cystic fibrosis transmembrane conductance regulator (CFTR), cluster of differentiation 52 (CD52) and Ras homolog gene family, member C (RHOC). The comparison cholestatic versus drained yielded 43 DEG (figure 3 and supplementary table S2). The comparison drained versus control yielded only one DEG. Because drained and control conditions no substantial difference, the results for comparisons with the drained group are not shown.

GO enrichment analysis

The most significant terms in the DAVID GO enrichment analysis are involved inflammation processes, such as leukocyte migration and chemokine-mediated signaling (figure 4). These results are further supported by the goana results (supplementary table S3), which shows many inflammatory processes, such as leukocyte activation, leukocyte degranulation and granulocyte activation.

Furthermore, DAVID GO enrichment terms also indicate alterations in extracellular matrix (ECM) organization and cell adhesion processes.

Pathway enrichment analysis

Two pathways are significantly enriched, namely the bile secretion and ECM receptor interaction pathway, shown in figure 5 and 6 respectively. The ECM receptor interaction pathway contains six DEGs in our dataset and the bile secretion pathway contains seven DEGs, also shown in figure 5 and 6.

Protein-protein network analysis

The protein-protein network is shown in figure 7. Proteins in this network that are annotated to be involved in the inflammatory response are shown in red. The network presents a big cluster of genes involved in inflammation, among which our highly significant gene OPN/SPP1. Furthermore, we also find MMP7 connected to SPP1.

miRNA statistical analysis

The miRNA data was analyzed for significantly changed miRNA genes for each comparison separately (cholestatic-control, cholestatic-drained, drained-control). Only the cholestatic-control comparison revealed significantly changed miRNA genes, five in total. Of these five, three miRNA genes were downregulated; (1) miR-375, (2) miR-378a-5p and (3) miR-24-1-5p and two were upregulated (4) miR34a-5p and (5) miR-34b-5p. The miR-34b-5p has been wrongly annotated until recently, and is therefore not included in further analysis.¹⁶

miRNA target prediction

The Bioconductor MultiMir package was used to search databases for predicted targets of all significantly changed miRNA genes. Four miRNA genes could be matched to possible targets, with 21 validated pairs in total. For a list of all miRNA genes and their targets see supplementary table S4.

Discussion

Cholestasis is characterized by toxic bile accumulation in the liver. However, how the liver reacts to these cholestatic conditions is not yet clearly understood. This study aimed to generate hypothesis on how the liver adjust to cholestatic conditions, based on changes in mRNA and miRNA expression. We find three major processes changed in the expression levels of cholestatic patients, namely altered bile production and transportation, increased inflammation and changes in ECM organization.

Pathway enrichment analysis revealed seven genes that are differentially expressed in cholestatic patients compared to controls. Four of these genes are closely connected to bile acid metabolism in hepatocytes and will be discussed here in further detail.

The cytochrome P (CYP) 7A1 gene has previously been shown to be downregulated in cholestatic patients.¹⁷ CYP7A1 catalyzes the rate-limiting step in the synthesis of bile from cholesterol.¹⁸ We were able to reproduce the downregulation of CYP7A1 in cholestatic liver tissue (figure 5).

Organic solute transporter α and β (OST-α and -β) together form an ATP binding cassette (ABC) transporter, involved in transporting the major species of bile acids out of the hepatocyte into the systemic circulation.¹⁹ We found both of these subunits to be upregulated in cholestatic liver tissue (figure 5), suggesting a way of the hepatocytes to remove superfluous toxic bile acids from its cytosol.

Multidrug resistance protein 3 (MDR3), which is also known under the name phosphatidylcholine (PC) translocator ABCB4, actively transports PC from the hepatocyte cytosol into the bile canalculus.²⁰ In the bile canalculus PC forms micelles around bile acids and therefore protects canalculus epithelial cells from their toxic effects. We found MDR3 to be upregulated, indicating a mechanism to protect the canalculus epithelial cells from increased levels of toxic bile acids.

Hepatocytes react to cholestatic conditions by downregulation synthesis of bile acids, increasing secretion of bile acids into the systemic circulation and protecting canalculus epithelial cells.

Another process we found enriched in ontologies and networks is inflammation. Activation of the immune system was enriched in both goana and DAVID GO enrichment analysis. In previous studies it has been shown that chronic inflammation plays an important role in clinical pathogenesis of cholestasis.²¹ Chronic inflammation can lead to fibrosis and ultimately to liver failure.

Previously, it has been shown that neutrophils are an important mediator of inflammation in cholestatic mice.²² Furthermore, OPN/ SPP1 is an important mediator of neutrophil influx in rat liver after induced inflammation.²³ Activity of SPP1 is more efficient in promoting cell migration after cleavage by MMP7.²⁴ We find both SPP1 and MMP7 to be greatly upregulated in cholestatic liver tissue (figure 3). We hypothesize that SPP1 and MMP7 are important mediators of inflammation in cholestasis, by promoting neutrophil influx in human liver tissue.

Matching the finding of increased immune response, pathway enrichment as well as GO analysis also showed that the ECM organization is changed (figures 4 and 6). Reduction of attachment to the ECM is a sign of structural weakening in hepatic tissue. We hypothesize this is an effect of the detergent effect of bile acids as well as inflammation. Further investigation in this topic is necessary to elucidate the underlying mechanism.

Weakening of hepatic tissue and cell death of hepatocytes is in agreement with our finding of upregulated miRNA hsa-34a-5p. MiRNA 34a-5p has been linked to cell survival and chemoresistance in several cancers.²⁵⁻²⁷ Furthermore, miRNA34a-5p has been shown to induce apoptosis and cell cycle arrest in an immortalized human hepatocyte cell line²⁸ as well as in rat liver.²⁹ Consequently, our miRNA target prediction (shown in supplementary table S4) reveals several targets involved in apoptosis and inflammation. However, determining whether the upregulation of this miRNA is a cause or a result of the undermined integrity of hepatic tissue needs additional research.

The presented results need to be viewed under the consideration that our study, like every study, has its advantages and disadvantages. Important to keep in mind is that all cases and controls have been diagnosed with pancreatic or liver cancer, this might affect our results. However, during removal, the tissues were carefully selected to be noncancer tissue.

Furthermore, databases have their limitations and are based on previous, perhaps biased, research and knowledge. If a disease is heavily researched, it will have a

higher default chance of showing up in database searches. By implementing a truly data-driven approach, we kept this bias to a minimum. Furthermore, we were able to reproduce our findings in multiple approaches, such as gene ontology and pathway enrichment analysis.

Lastly, it needs to be mentioned that a substantial amount of data points in our miRNA data were missing. This might be due to relatively low concentrations of miRNAs present in cells, making it more difficult to detect above background levels.

In conclusion, this study reveals three important mechanisms employed by the liver to adjust to cholestatic conditions. The liver actively alters its gene expression to protect itself from toxic cholestatic conditions. Important alterations can be found in bile acid production and transportation, induction of inflammation and ECM organization. Further research into these processes will bring us a step closer to targeted therapies for cholestasis.

References

1. Li, Y. *et al.* Bile acids and intestinal microbiota in autoimmune cholestatic liver diseases. *Autoimmun. Rev.* **16**, 885–896 (2017).
2. Gossard, A. A. Care of the Cholestatic Patient. *Clin. Liver Dis.* **17**, 331–344 (2019).
3. Xu, S. C. *et al.* Damage to mtDNA in liver injury of patients with extrahepatic cholestasis: The protective effects of mitochondrial transcription factor A. *Free Radic. Biol. Med.* **52**, 1543–1551 (2012).
4. Boyer, J. L. Bile Formation and Secretion. *Compr Physiol* **3**, 1035–78 (2013).
5. Vendemiale, G. *et al.* Hepatic oxidative alterations in patients with extra-hepatic cholestasis. Effect of surgical drainage. *J. Hepatol.* **37**, 601–605 (2002).
6. Shoda, J. *et al.* The expression levels of plasma membrane transporters in the cholestatic liver of patients undergoing biliary drainage and their association with the impairment of biliary secretory function. *Am. J. Gastroenterol.* **96**, 3368–3378 (2001).
7. Ritchie, M. E. *et al.* Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* **43**, e47 (2015).
8. Huang, DW. *et al.* Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* **37**, 1–13 (2009).
9. Huang, DW. *et al.* Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. *Nat. Protoc.* **4**, 44–57 (2009).
10. Yu, G. *et al.* clusterProfiler: an R Package for Comparing Biological Themes Among Gene Clusters. *Omi. A J. Integr. Biol.* **16**, 284–287 (2012).
11. Ogata, H. *et al.* KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* **27**, 29–34 (1999).
12. Kanehisa, M. *et al.* KEGG: New perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Res.* **45**, D353–D361 (2017).
13. Kanehisa, M. *et al.* New approach for understanding genome variations in KEGG. *Nucleic Acids Res.* **47**, 590–595 (2018).
14. Jensen, L. J. *et al.* STRING 8 - A global view on proteins and their functional interactions in 630 organisms. *Nucleic Acids Res.* **37**, 412–416 (2009).
15. Ru, Y. *et al.* The multiMiR R package and database: Integration of microRNA-target interactions along with their disease and drug associations. *Nucleic Acids Res.* **42**, (2014).
16. Engkvist, M. E. *et al.* Analysis of the miR-34 family functions in breast cancer reveals annotation error of miR-34b. *Sci. Rep.* **7**, 1–10 (2017).
17. Schaap, F. G. *et al.* High expression of the bile salt-homeostatic hormone fibroblast growth

- factor 19 in the liver of patients with extrahepatic cholestasis. *Hepatology* **49**, 1228–1235 (2009).
18. Chiang, J. Y. L. Bile acids: regulation of synthesis. *J. Lipid Res.* **50**, 1955–1966 (2009).
 19. Xia, X. *et al.* Bile acid interactions with cholangiocytes TOPIC HIGHLIGHT. *World J Gastroenterol World J. Gastroenterol. ISSN* **12**, 3553–3563 (2006).
 20. Dröge, C. *et al.* Sequencing of FIC1, BSEP and MDR3 in a large cohort of patients with cholestasis revealed a high number of different genetic variants. *J. Hepatol.* **67**, 1253–1264 (2017).
 21. Yadav, K. *et al.* Mitochondrial dysfunction in cholestatic liver diseases. *Fish Physiol Biochem.* **38**, 1035–45. doi: 10.1007/s10695-011-9588-7. Epub 2011 (2012).
 22. Gujral, J. S. *et al.* Neutrophils aggravate acute liver injury during obstructive cholestasis in bile duct-ligated mice. *Hepatology* **38**, 355–363 (2003).
 23. Apte, U. M. *et al.* Role of osteopontin in hepatic neutrophil infiltration during alcoholic steatohepatitis. *Toxicol. Appl. Pharmacol.* **207**, 25–38 (2005).
 24. Agnihotri, R. *et al.* Osteopontin, a Novel Substrate for Matrix Metalloproteinase-3 (Stromelysin-1) and Matrix Metalloproteinase-7 (Matrilysin). *J. Biol. Chem.* **276**, 28261–28267 (2001).
 25. Bianchi, E. *et al.* Role of miR-34a-5p in hematopoietic progenitor cells proliferation and fate decision: Novel insights into the pathogenesis of primary myelofibrosis. *Int. J. Mol. Sci.* **18**, 1–22 (2017).
 26. Pu, Y. *et al.* The miR-34a-5p promotes the multi-chemoresistance of osteosarcoma via repression of the AGTR1 gene. *BMC Cancer* **17**, 1–9 (2017).
 27. Alvarez-Garcia, I. *et al.* A uniform system for microRNA annotation. *Nucleic Acids Res.* **17**, 1–10 (2014).
 28. Li, C. *et al.* Inhibition of miR-34a-5p alleviates hypoxia-reoxygenation injury by enhancing autophagy in steatotic hepatocytes. (2018).
 29. Liu, C. *et al.* Upregulation of miR-34a-5p antagonizes AFB1-induced genotoxicity in F344 rat liver. *Toxicon* **106**, 46–56 (2015).

Tables and figures

	Cholestatic	Drained	Control
n	9	10	9
Age (SD)	63,6 (11,9)	68,7 (10,4)	50,6 (16,3)
Male (n, %)	5 (55,6%)	7 (70,0%)	4 (44,4%)
Drainage time (weeks)	-	12,5	-

Table 1. Overview of included subjects.

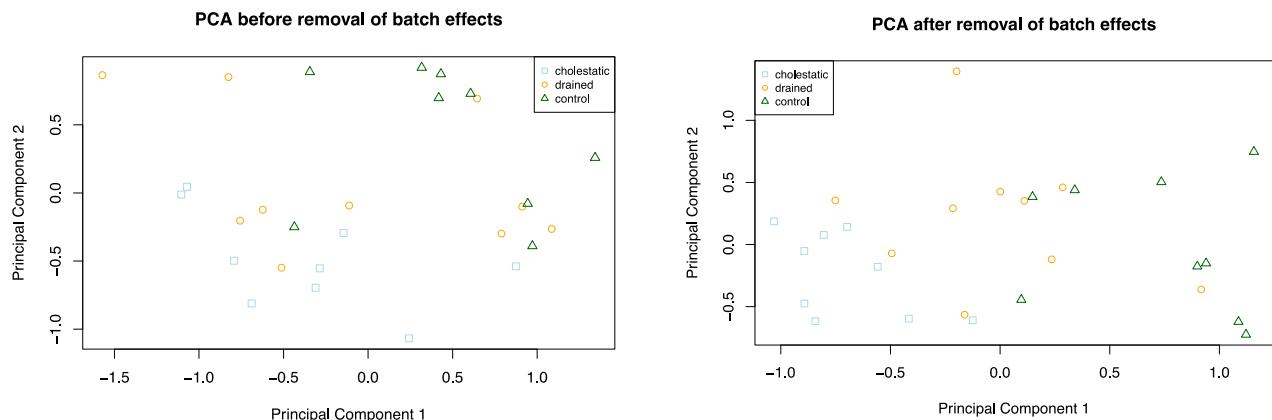


Figure 1. Removal of batch effects. mRNA samples were measured on four different chips. The graph on the left shows PCA before removal of batch effect and on the right PCA after removal of batch effects. After correction for these batches, cholestatic subjects cluster together. Drained and control overlap more.

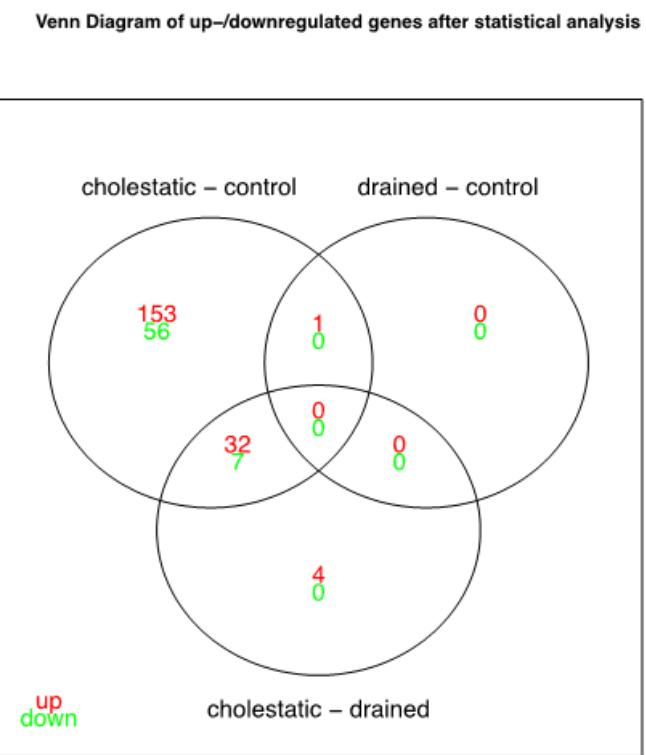


Figure 2. Venn diagram after differential gene expression analysis. Red indicates upregulated genes per comparison. Green indicates downregulated genes per comparison.

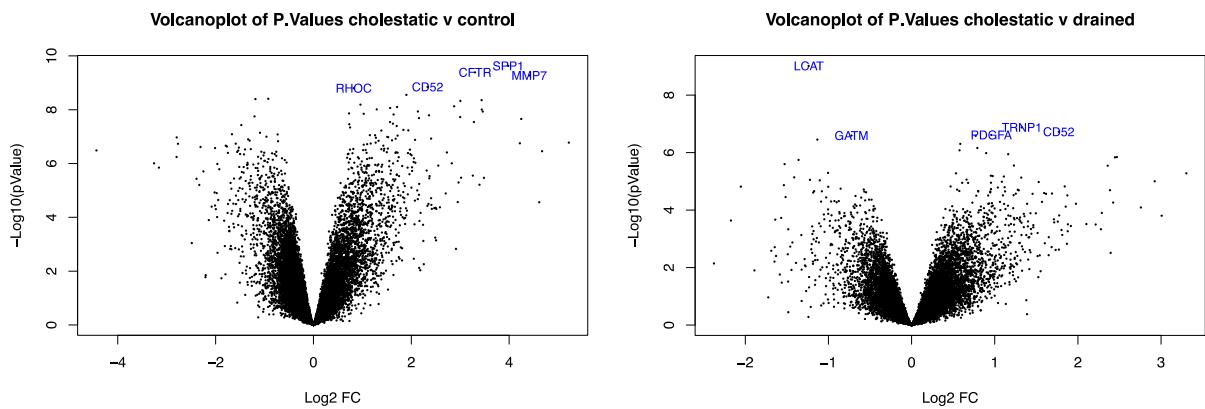


Figure 3: -log10 p-values plotted against log2 fold changes per gene. *P*-values are not adjusted for multiple testing. 5 genes with the lowest *p*-value are highlighted in each plot. Comparisons cholestatic versus control (left) and cholestatic versus drained (right) are shown.

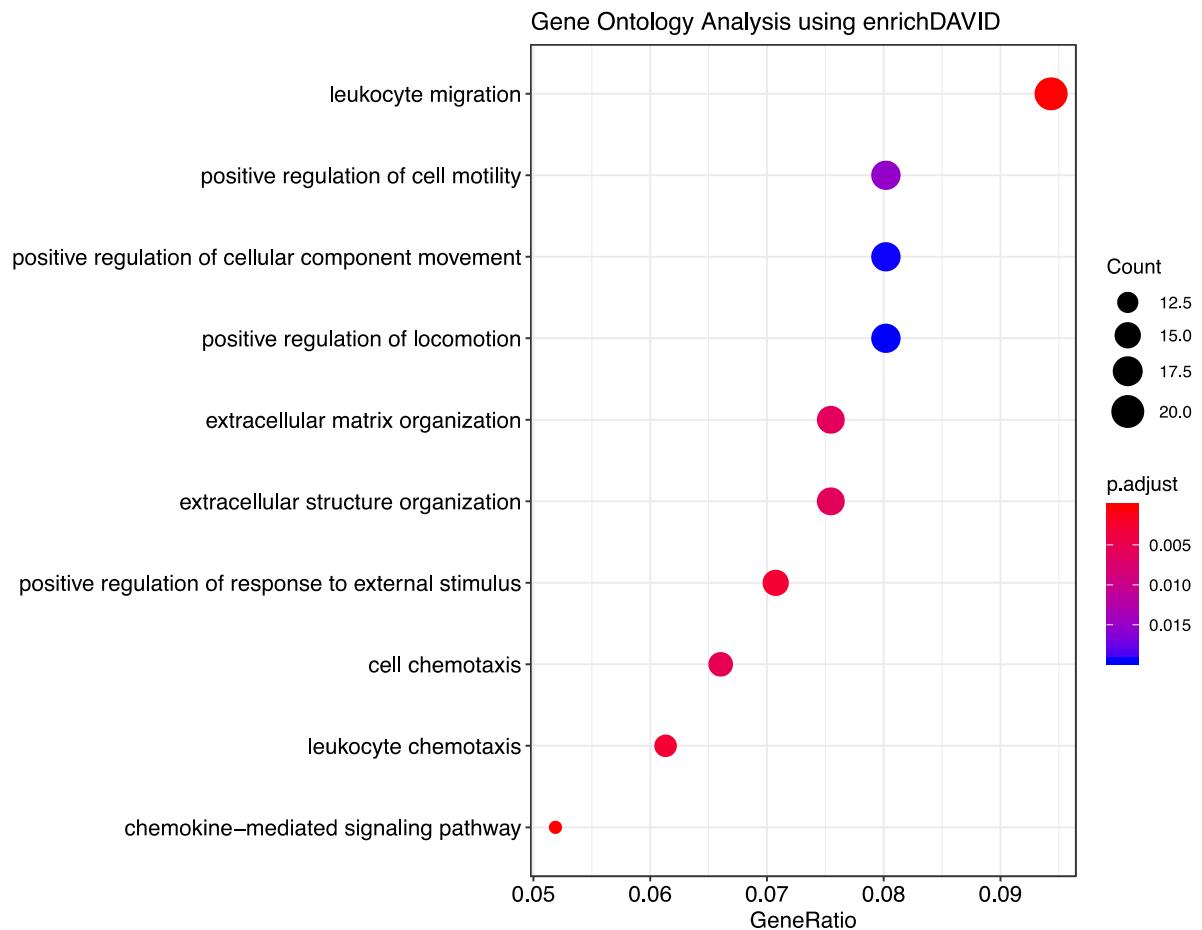


Figure 4. DAVID gene ontology results. There are two main biological processes in the ontologies enriched in our cholestatic versus control DEGs. These are inflammation and extracellular matrix organization

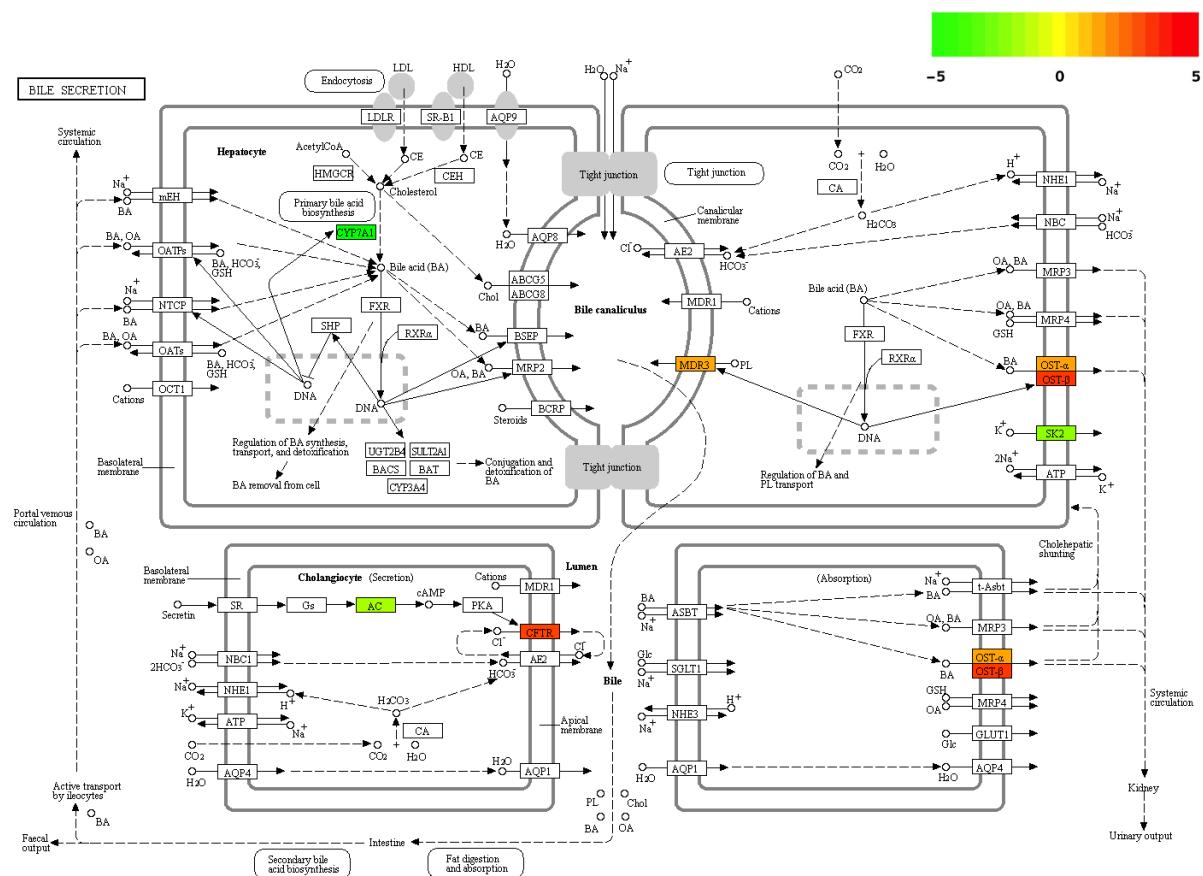


Figure 5. Bile secretion pathway. This pathway is significantly enriched in cholestatic conditions compared to controls. Genes depicted in green are downregulated, genes in red are upregulated. The liver reacts to cholestatic conditions by altering bile production and transportation.

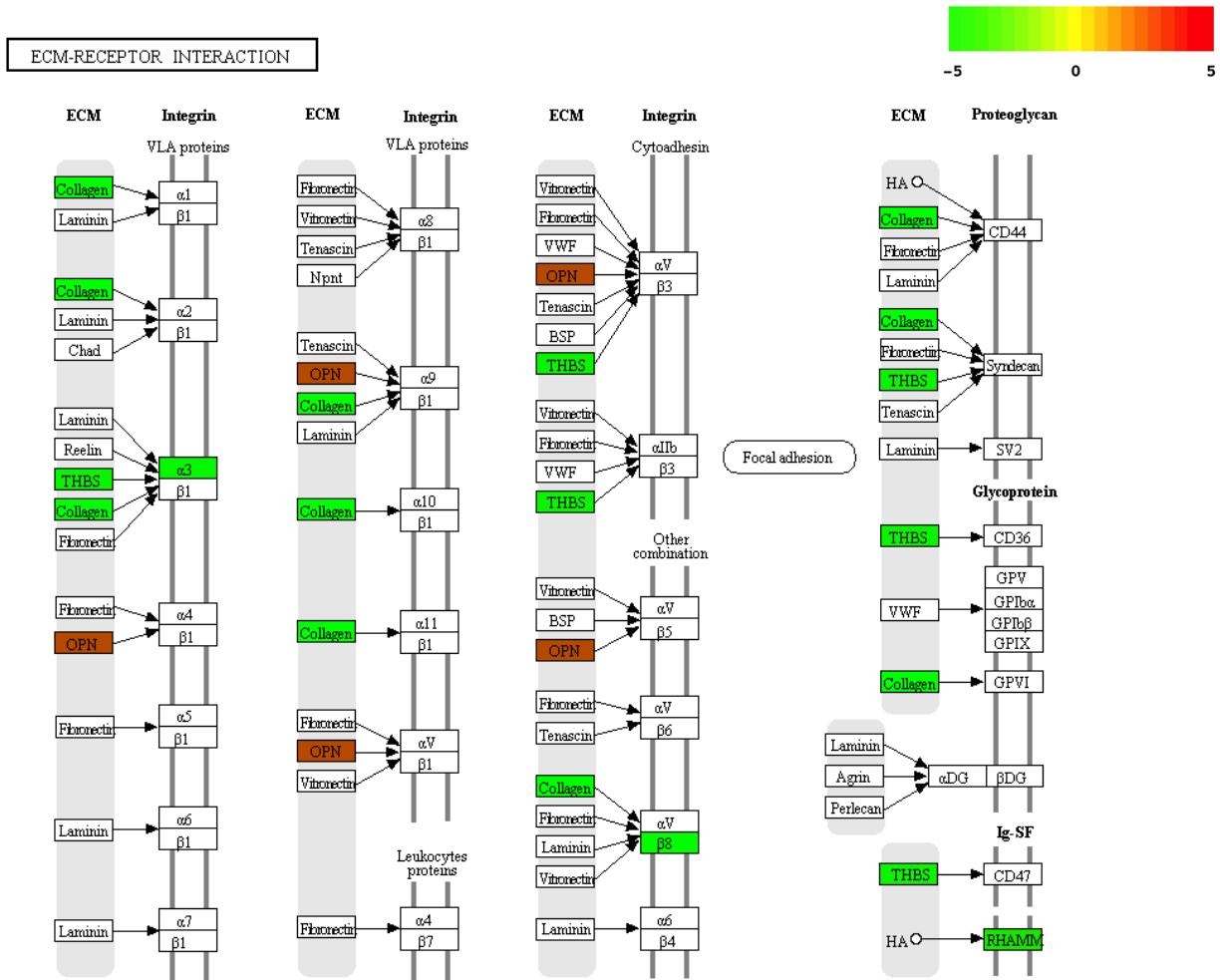
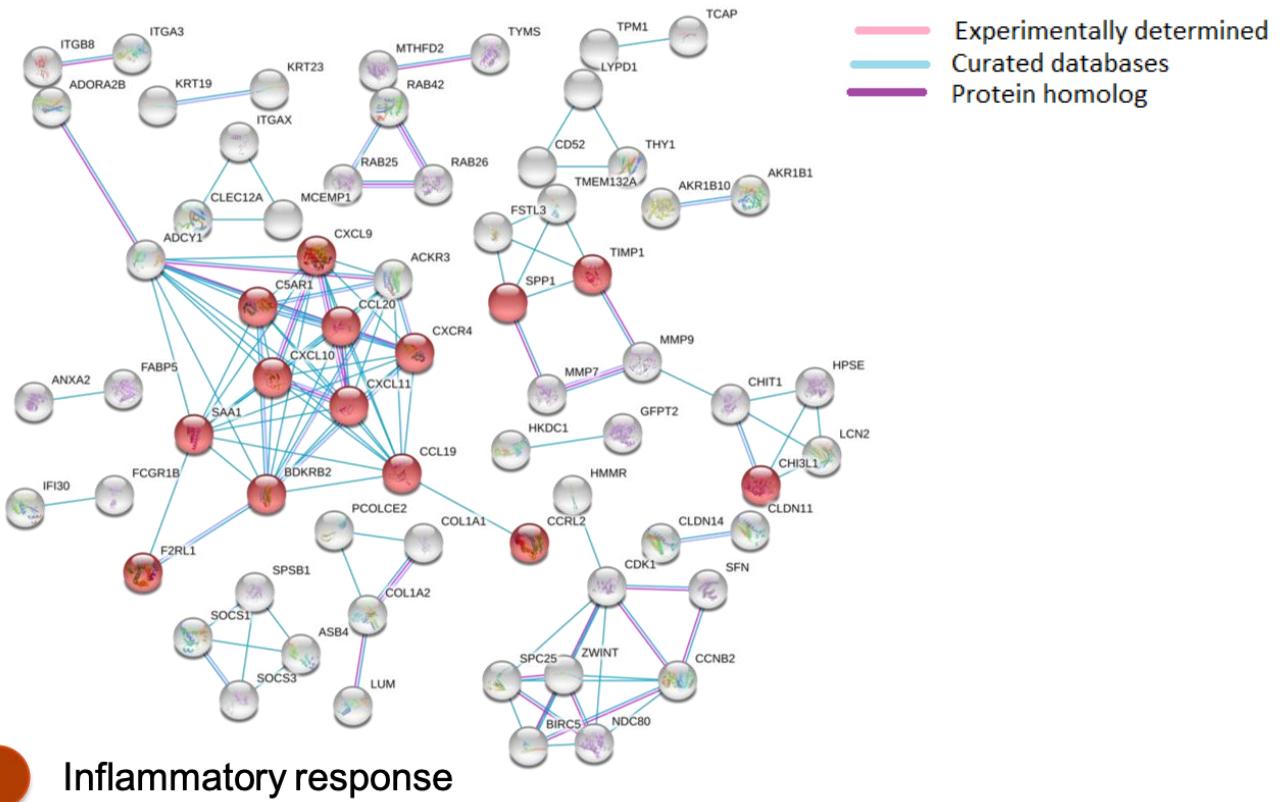


Figure 6. ECM-receptor interaction pathway. This pathway is significantly enriched in cholestatic conditions compared to controls. Genes depicted in green are downregulated, genes in red are upregulated. Genes related to extracellular matrix organization, such as THBS and collagen are downregulated. OPN (also called SPP1) is known as an activator of inflammation and upregulated in cholestatic conditions.



Inflammatory response

Figure 7. Protein-protein interaction network. The network was created using String DB database at its highest confidence threshold. Proteins annotated as involved in the inflammatory response are depicted in red. According with GO results from DAVID, we find many inflammatory proteins in this network, among which SPP1.

Author contributions

Both authors had equal contributions to the presented study. PRS conducted mRNA analysis and network analysis. SEH conducted miRNA analysis and target prediction and literature research. Both authors were involved in gene ontology and pathway enrichment analysis and manuscript preparation. Furthermore, discussion and interpretation of all results was a combined effort of both authors.

Supplementary

See excel sheet for supplementary table 1 and 2. The tables were too big to show in word. Titles and descriptions for both tables are shown below.

Supplementary table S1. DEGs in cholestatic subjects compared to control. Statistical analysis was performed using the LIMMA package in R. P-values were adjusted for multiple testing using the “Benjamini and Hockberg” method.

Supplementary table S2. DEGs in cholestatic subjects compared to drained. Statistical analysis was performed using the LIMMA package in R. P-values were adjusted for multiple testing using the “Benjamini and Hockberg” method.

Term	n	n up	n down	P-value Up	P-value Down	GO ID
cell activation	1145	296	75	3,6E-47	1,0	GO:0001775
immune response	1650	378	108	1,5E-46	1,0	GO:0006955
immune system process	2406	487	162	2,4E-46	1,0	GO:0002376
immune effector process	997	265	54	1,7E-41	1,0	GO:0002252
response to stimulus	6941	1010	700	2,2E-41	1,0	GO:0050896
leukocyte activation	1021	263	62	2,8E-41	1,0	GO:0045321
leukocyte activation involved in immune response	605	186	31	1,1E-37	1,0	GO:0002366
cell activation involved in immune response	608	186	31	2,5E-37	1,0	GO:0002263
myeloid leukocyte activation	567	177	28	1,3E-36	1,0	GO:0002274
leukocyte mediated immunity	669	195	34	7,7E-36	1,0	GO:0002443
myeloid cell activation involved in immune response	486	158	25	2,4E-34	1,0	GO:0002275
leukocyte degranulation	476	156	23	3,2E-34	1,0	GO:0043299
myeloid leukocyte mediated immunity	492	158	27	1,7E-33	1,0	GO:0002444
cell communication	4947	743	477	6,8E-33	1,0	GO:0007154
regulated exocytosis	690	192	43	1,1E-32	1,0	GO:0045055
signaling	4915	737	475	2,1E-32	1,0	GO:0023052
signal transduction	4562	697	433	3,6E-32	1,0	GO:0007165
granulocyte activation	456	146	25	1,4E-30	1,0	GO:0036230
regulation of response to stimulus	3334	551	289	7,5E-30	1,0	GO:0048583
neutrophil activation	450	143	23	1,3E-29	1,0	GO:0042119

Supplementary table S3. GO enrichment analysis results by goana. The main upregulated ontologies are involved in inflammation, which corresponds with the ontologies found by DAVID. The total number genes in each ontology is represented by n. N up and n down represent how many of the total number of genes in each ontology are up- or downregulated, respectively.

MiRNA	Target	Validation experiment
miR-34a-5p	TPM1	Proteomics
miR-34a-5p	CD24	Immunoblot
miR-34a-5p	TYMS	Proteomics
miR-34a-5p	AKR1B1	Proteomics
miR-34a-5p	BIRC5	qRT-PCR/Western blot
miR-34a-5p	AREG	Microarray/qRT-PCR
miR-34a-5p	CAPG	Proteomics
miR-34a-5p	CRYAA	Proteomics
miR-34a-5p	SFN	Proteomics
miR-34a-5p	CXCL9	Proteomics
miR-34a-5p	MMP7	ELISA/qRT-PCR
miR-34a-5p	SAA1	Proteomics
miR-24-1-5p	ZWINT	PAR-CLIP
miR-378a-5p	MELK	HITS-CLIP
miR-378a-5p	CMBL	HITS-CLIP
miR-375	LHFPL2	Microarray
miR-375	FSTL3	Microarray
miR-375	SEMA3C	Microarray
miR-375	WWC1	Microarray
miR-375	RASD1	Luciferase transporter assay
miR-375	NCOA7	Microarray

Supplementary table S4. MiRNA target predictions. MiRNA targets were predicted using the MultiMir package in R. All targets shown are experimentally validated.