

Molecular characterization of chronic liver disease dynamics: From liver fibrosis to acute-on-chronic liver failure

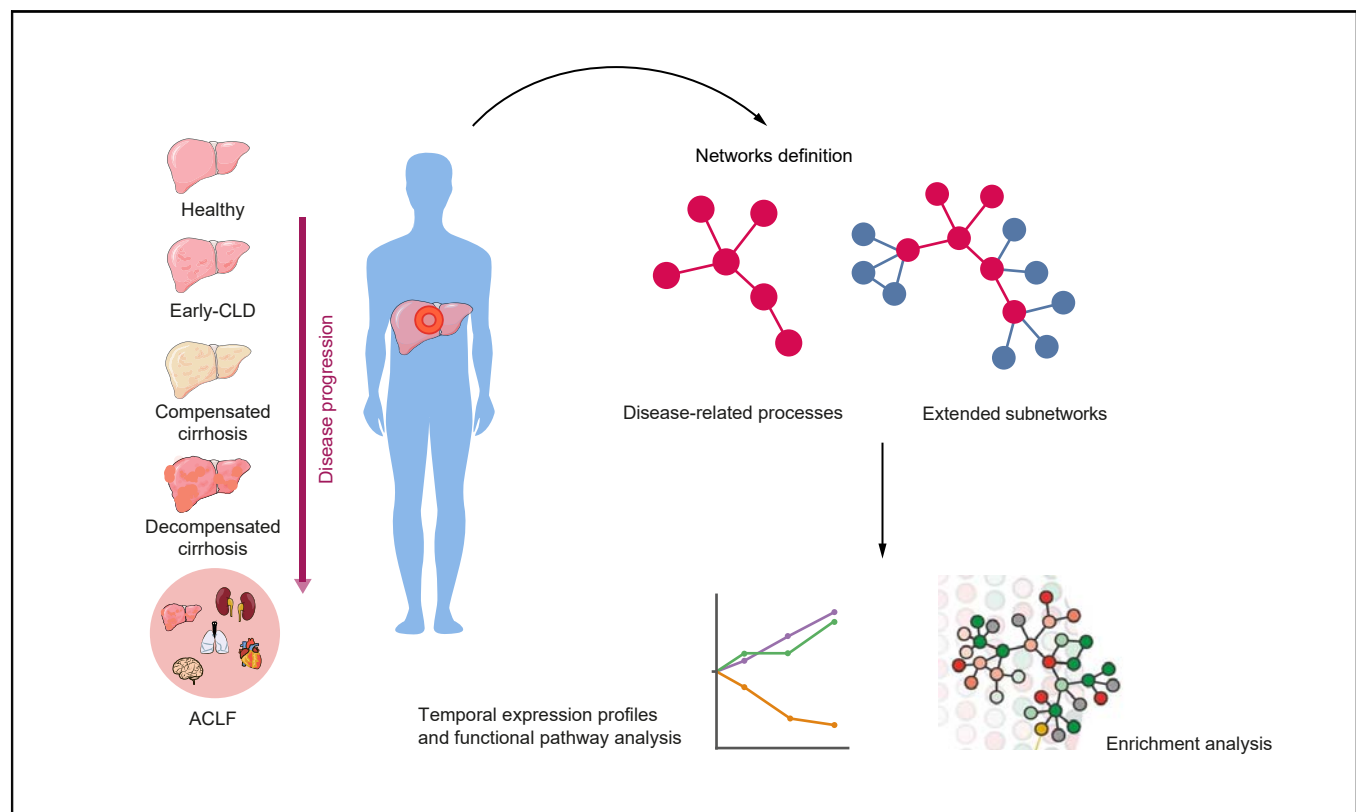
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Graphical abstract



Highlights

- We unveiled the molecular pathogenic mechanisms implicated in the progression of chronic liver disease to cirrhosis and ACLF.
- ACLF presents a specific hepatic gene expression pattern distinct from that of patients at earlier disease stages.
- Gene expression pattern of ACLF is mostly related to inflammation, fibrosis, angiogenesis, senescence and apoptosis pathways in the liver.

Lay summary

By using transjugular biopsies obtained from patients at different stages of chronic liver disease, we unveil the molecular pathogenic mechanisms implicated in the progression of chronic liver disease to cirrhosis and acute-on-chronic liver failure. The most relevant finding in this study is that patients with acute-on-chronic liver failure present a specific hepatic gene expression pattern distinct from that of patients at earlier disease stages. This gene expression pattern is mostly related to inflammation, fibrosis, angiogenesis, and senescence and apoptosis pathways in the liver.



Molecular characterization of chronic liver disease dynamics: From liver fibrosis to acute-on-chronic liver failure

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Background & Aims: The molecular mechanisms driving the progression from early-chronic liver disease (CLD) to cirrhosis and, finally, acute-on-chronic liver failure (ACLF) are largely unknown. Our aim was to develop a protein network-based approach to investigate molecular pathways driving progression from early-CLD to ACLF.

Methods: Transcriptome analysis was performed on liver biopsies from patients at different liver disease stages, including fibrosis, compensated cirrhosis, decompensated cirrhosis and ACLF, and control healthy livers. We created 9 liver-specific disease-related protein-protein interaction networks capturing key pathophysiological processes potentially related to CLD. We used these networks as a framework and performed gene set-enrichment analysis (GSEA) to identify dynamic gene profiles of disease progression.

Results: Principal component analyses revealed that samples clustered according to the disease stage. GSEA of the defined processes showed an upregulation of inflammation, fibrosis and apoptosis networks throughout disease progression. Interestingly, we did not find significant gene expression differences between compensated and decompensated cirrhosis, while ACLF showed acute expression changes in all the defined liver disease-related networks. The analyses of disease progression patterns identified ascending and descending expression profiles associated with ACLF onset. Functional analyses showed that ascending profiles were associated with inflammation, fibrosis, apoptosis, senescence and carcinogenesis networks, while descending profiles were mainly related to oxidative stress and genetic factors. We confirmed by qPCR the upregulation of genes of the ascending profile and validated our findings in an independent patient cohort.

Conclusion: ACLF is characterized by a specific hepatic gene expression pattern related to inflammation, fibrosis, apoptosis, senescence and carcinogenesis. Moreover, the observed profile is significantly different from that of compensated and decompensated cirrhosis, supporting the hypothesis that ACLF should be considered a distinct entity.

Lay summary: By using transjugular biopsies obtained from patients at different stages of chronic liver disease, we unveil the molecular pathogenic mechanisms implicated in the progression of chronic liver disease to cirrhosis and acute-on-chronic liver failure. The most relevant finding in this study is that patients with acute-on-chronic liver failure present a specific hepatic gene expression pattern distinct from that of patients at earlier disease stages. This gene expression pattern is mostly related to inflammation, fibrosis, angiogenesis, and senescence and apoptosis pathways in the liver.

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Introduction

Acute-on-chronic liver failure (ACLF) is a syndrome that occurs in patients with cirrhosis characterized by acute decompensation and development of organ failure(s) associated with high mortality.¹ To date, there is no effective approach to prevent progression to ACLF and its current management is based on organ support.² The absence of an effective treatment is mainly due to insufficient knowledge regarding the molecular pathogenesis of cirrhosis progression and ACLF development.

Patients with ACLF present marked increases in leukocyte count, C-reactive protein (CRP) and plasma cytokine levels.^{1,3,4}

More recently, the PREDICT study described the characteristics of patients with acute decompensated cirrhosis that develop ACLF and showed that patients with more severe systemic inflammation are those more prone to develop ACLF during follow-up.⁵ All these previous studies suggest that systemic inflammation plays a role in cirrhosis progression and ACLF development, but whether patients with ACLF have specific intrahepatic molecular alterations that may also play a role on ACLF development is not known.

The current understanding of the molecular mechanisms underlying chronic liver diseases (CLDs) and ACLF development is limited to the analysis of circulating cytokines, blood metabolomics or studies performed in experimental animal models.^{6–10} Therefore, the characterization of gene expression profiles of cirrhosis progression to ACLF is a fundamental step towards better understanding of the complexity of this syndrome. However, although ‘omics’ techniques provide valuable and detailed information about the molecular mechanisms responsible of human diseases they are naïve to the underlying biological system since they lack the mechanistic description of how the individual components communicate to give rise to biological events.¹¹ Besides, complex diseases are rarely related to the malfunction of a single gene, but rather reflect the perturbation of several inter-related cellular components.¹² Biological networks represent a perfect scaffold where these data can be integrated providing more realistic mechanistic insights about how diseases perturb human physiology and may facilitate the prediction of potential targets. The value of network-based approaches to explore molecular mechanisms underlying complex diseases has been demonstrated in human breast cancer,¹³ neuronal damage,¹⁴ breast cancer metastasis prediction,¹⁵ and colorectal cancer.¹⁶

In the present study, we used gene expression arrays from patients with CLD at different disease stages to comprehensively interrogate the molecular changes occurring in the liver during progression of chronic liver diseases with special focus on ACLF. We used a systems biology approach and generated manually curated protein-protein interaction networks describing the main biological processes related to CLDs and used them as scaffolds to interrogate the changes observed in our transcriptome data. Then, applying a directed network-based approach coupled with enrichment statistical analyses, functional data and the information provided from the characteristic temporal expression profiles, we identified specific gene expression profiles describing the main molecular mechanisms related with ACLF.

Materials and methods

Study population

Cohort 1- Descriptive cohort: We included a group of 33 patients encompassing the whole spectrum of CLD stages followed at the Liver Unit of Hospital Clínic in Barcelona: 5 patients with early-CLD (with fibrosis but without cirrhosis, named early-CLD), 8 patients with compensated cirrhosis (CC), 12 patients with decompensated cirrhosis (DC) and 8 patients with ACLF (ACLF). Patients with early-CLD and CC were outpatients in whom liver biopsy was scheduled as part of work-up for diagnosis purposes. Patients with DC and ACLF were all hospitalized patients that underwent liver biopsy for diagnostic purposes to establish the etiology of the liver disease or in the case of ACLF to assess the extension of the liver injury and rule-out other causes of liver injury (toxic, infectious diseases, etc.). All patients included gave

written informed consent and 2 specimens of liver biopsy were collected, 1 for histopathological diagnosis and 1 for microarray analysis. Clinical, demographic and laboratory data were collected in all patients at the time of liver biopsy. Definition of disease stages was made according to histological, clinical and ultrasonography assessment. Metavir score¹⁷ was used to define fibrosis stage. Definition of acute clinical decompensation was made according to EASL clinical guidelines¹⁸ and ACLF diagnosis was made when patients met the criteria defined in the CANONIC study.¹⁹ A group of 6 healthy individuals was included as a control group. Healthy individuals were liver donors from our Liver Unit Transplant Program in whom a liver biopsy was obtained at the time of living-donor liver transplantation.

Cohort 2- Validation cohort: A second group of 8 patients with ACLF and liver biopsy was included as a validation cohort. The hepatic gene expression of this second cohort was performed on paraffin-embedded liver samples.

The protocol of the study was approved by the Institutional Review Board of the Hospital Clínic of Barcelona (code:2012/7977).

Liver disease-related protein-protein networks definition

The value of protein-protein network-based approaches to explore molecular mechanisms has been demonstrated in cancer and neuronal diseases.^{13–16} For our study, 9 different liver disease-related protein-protein interaction networks (LDRNs) containing the most relevant molecular events involved in liver disease initiation and progression were generated. By manual curation we selected from the literature proteins which are known to be involved in liver diseases. These proteins were named seeds ($n = 363$). We divided our seeds into 9 specific categories describing the main molecular mechanisms of liver disease: genetic factors ($n = 35$), oxidative stress ($n = 12$), fibrosis and resolution ($n = 122$), inflammatory response ($n = 44$), apoptosis of hepatocytes ($n = 23$), apoptosis of hepatic stellate cells (HSC) ($n = 32$), angiogenesis ($n = 30$), cellular senescence ($n = 26$) and carcinogenesis ($n = 39$). [Table S1](#) shows a complete list of genes (seeds) included in each category. We then used those seeds as a scaffold to build our 9 protein-protein interaction networks, which we named LDRNs. We extended each LDRN including all direct physical protein interactors of the previously defined seeds to generate a protein-protein interaction network for each category. Physical protein interactions with reported experimental evidence were retrieved from a curated database generated from merging the 6 major protein interaction public sources (see [supplementary section for extended details](#)). [Table S2](#) shows proteins included in each LDRN in the extended network.

Bioinformatics analysis: Enrichment analysis, temporal expression profile clustering and functional pathway analysis

Gene-set enrichment analysis (GSEA): GSEA was employed to identify if our LDRNs were significantly associated with each one of the disease stages under study.²⁰

Analyses of temporal expression profiles: STEM software²¹ was used to interrogate our transcriptome data and identify specific gene expression patterns describing the changes that appear through CLD progression.

Functional annotation and signaling pathway analysis: Gene ontology and pathway analyses were done to identify curated terms, signaling and metabolic pathways that were overrepresented in a set of identified genes of interest associated with CLD progression and ACLF.

Further details on bioinformatic and histological analysis are provided in the [supplementary information](#).

Results

Characteristics of the patient population

Table 1 shows the characteristics of patients included in cohort 1. The main etiology of liver disease was alcohol-related, followed by non-alcoholic fatty liver disease (NAFLD) and hepatitis C virus infection. As expected by group definition, patients with DC and ACLF had worse liver and renal function and significantly higher model for end-stage liver disease scores compared to the other groups (early-CLD and CC).

Patients with early-CLD and CC were all outpatients, while those with DC and ACLF were admitted due to acute decompensation of cirrhosis. Two-thirds of patients with DC had a previous diagnosis of cirrhosis and 58% had had a previous episode of acute decompensation (with ascites being the most frequent complication). Half of patients with ACLF had no previous diagnosis of liver disease and only 38% had had a previous episode of acute decompensation.

The main cause of hospital admission in the DC group was ascites (42%), followed by infection (17%), jaundice (17%), portal hypertensive gastrointestinal bleeding (17%) and acute kidney injury (8%). The majority of patients with ACLF were admitted due to jaundice (50%), followed by infection (25%) and alcoholic hepatitis (50%). Infection was present in 25% of patients with DC and 88% of patients with ACLF at the time of inclusion and, as expected, patients with ACLF had higher leukocyte count and higher CRP levels than the other groups. Infection was the most frequent precipitating factor of ACLF (5 out of 8), while alcoholic hepatitis was the precipitating factor in 1 patient, and infection on top of alcoholic hepatitis was in the remaining 2 patients. ACLF grade distribution in our cohort was: 3 patients with ACLF-1 (37%), 2 with ACLF-2 (25%) and 3 with ACLF-3 (37%).

Global transcriptome profile of CLDs and ACLF

Principal component analysis (PCA) and unsupervised hierarchical clustering performed with the transcriptional data (accessible on the public repository of NCBI Gene Expression Omnibus (GSE139602)) were used to explore and visualize strong global transcriptional patterns for liver disease stages and emphasize variation in the data (Fig. 1). In the PCA plot, after accounting for batch effects, the first principal component clearly separated healthy individuals from all patients with CLD (Fig. 1A). Moreover, all samples from the same disease stage clustered together with a minimum overlap between CC and DC. Likewise, the major branch of the clustering analysis also showed a clear separation between healthy individuals and disease stage groups (Fig. 1B). Interestingly, patients with ACLF clustered together and recognizably away from the other disease stages, suggesting that ACLF has a transcriptome profile that is markedly different from that of CC and DC. The rest of the branches clustered together, first CC and DC, and then early-CLD samples, thus confirming previous observations in the PCA.

We next analyzed the differentially expressed genes (DEGs) between liver disease stages and healthy individuals and found that DEGs increased in parallel with disease progression: 224 DEGs (69 up- and 155 downregulated) in early-CLD, 292 DEGs (112 up- and 180 downregulated) in CC, 517 DEGs (200 up- and 317 downregulated) in DC and 888 DEGs (336 up- and 552 downregulated) in ACLF when compared to healthy individuals (Fig. 2A). The overlap between the DEGs at each disease stage relative to each other was examined and visualized by Venn diagrams (Fig. 2B). DC and ACLF shared the largest number of common DEGs (83 genes), followed by 55 genes commonly upregulated between CC, DC and ACLF and 36 commonly upregulated between all disease conditions (early-CLD, CC, DC and ACLF). A similar pattern was observed for downregulated genes.

In order to evaluate the extent of the cirrhosis effect, pairwise analyses were conducted between 3 disease stages: early-CLD,

Table 1. Baseline characteristics of patients categorized according to the stage of chronic liver disease.

	eCLD (n = 5)	CC (n = 8)	DC (n = 12)	ACLF (n = 8)	p value
Age, years	58±7	64±7	54±18	55±6	0.25
Male sex	1 (20)	6 (75)	5 (42)	6 (75)	0.1
Diabetes mellitus,	2 (40)	2 (25)	5 (42)	2 (25)	0.8
Arterial hypertension	2 (40)	6 (75)	1 (8)	2 (25)	0.007
Etiology of cirrhosis					
Alcohol	1 (20)	1 (12.5)	4 (33)	6 (75)	0.4
Hepatitis C	2 (40)	1 (12.5)	3 (25)	1 (12.5)	
NASH	2 (40)	4 (50)	1 (8)	1 (12.5)	
Mixed*	–	2 (25)	4 (33)	1 (12.5)	
Ascites	–	–	9 (75)	7 (87)	<0.001
HE	–	–	1 (8)	4 (50)	0.02
Infections	–	–	3 (25)	7 (87)	0.001
Serum bilirubin (mg/dl)	0.8 (0.6-1.3)	1.1 (0.6-2)	1.6 (0.9-3.3)	18 (14-29)	<0.001
Serum albumin (g/L)	41 (31-43)	43 (33-46)	32 (29-35)	28 (24-34)	0.01
INR	1 (0.9-1.04)	1.1 (1.0-1.2)	1.2 (1.2-1.37)	2 (1.7-2.5)	<0.001
Serum creatinine (mg/dl)	0.7 (0.6-1.2)	0.8 (0.6-1.7)	0.7 (0.6- 1.2)	1.8 (0.9-2.9)	0.26
Serum sodium (mEq/L)	141 (138-143)	140 (139-142)	137 (133-142)	135 (131-136)	<0.01
Leukocyte count (10 ¹² cells/L)	4.5 (3.1-8.6)	5.2 (2.4-6.4)	5.0 (3.3- 8.5)	12.4 (9.0-13.4)	<0.001
Platelet count (10 ⁹ cells/L)	215 (110-250)	151 (109-180)	157 (89- 253)	79 (57-189)	0.38
CRP (mg/dl)	0.02 (0.01-0.04)	0.2 (0.1-0.3)	1.09 (0.2-3.5)	5.9 (1.4-9.7)	<0.001
Child-Pugh score	5 (5-5.7)	5 (5-6)	7.5 (7-8.7)	11 (9.5-12)	<0.001
MELD score	6 (6-7)	8.6 (6-12)	9.2 (6-15)	32 (22-35)	<0.001

Values are numbers (percentages) or median (interquartile range).

* Combination of more than one etiology: for CC 2 patients with HCV + alcohol; for DC: 2 patients with HCV + alcohol and 2 cryptogenic; for ACLF 1 patient with NAFLD + alcohol. Healthy individuals: Mean age 35±12, Male 66%; ACLF, acute-on-chronic liver failure; CRP, C-reactive protein; eCLD, chronic liver disease with fibrosis without cirrhosis; HE, hepatic encephalopathy; INR, international normalization ratio; MELD, model for end-stage liver disease; NASH, non-alcoholic steatohepatitis.

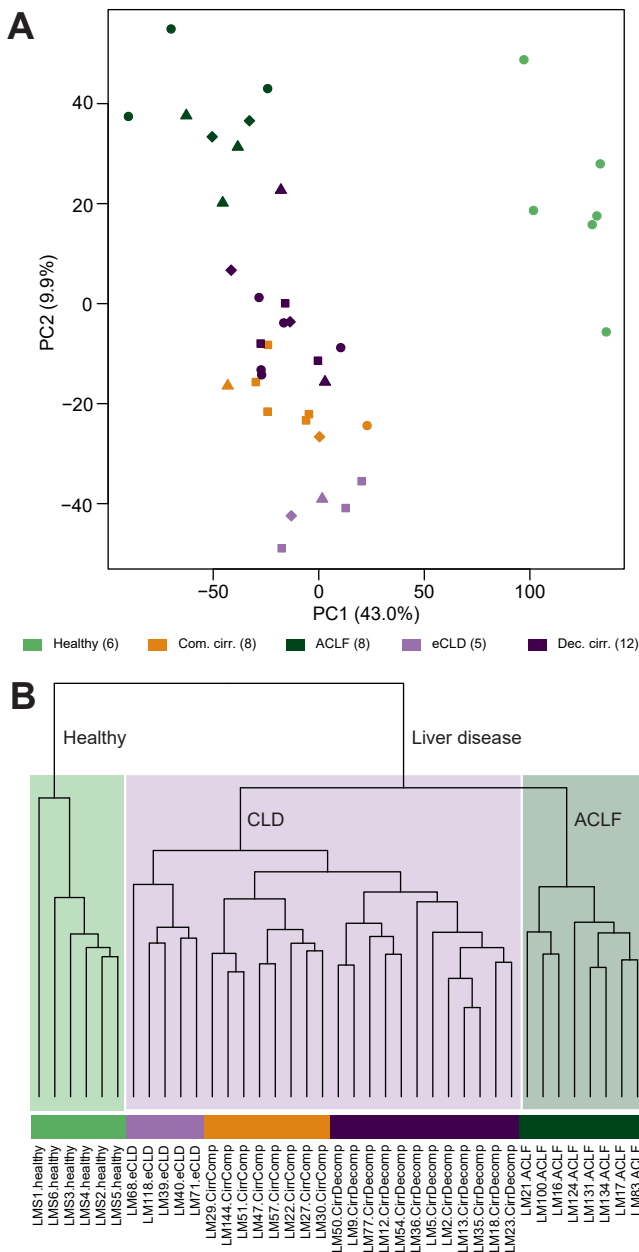


Fig. 1. Transcriptome analysis of patients with distinct liver chronic disease stages and ACLF. (A) PCA plots are presented for all 39 samples after including batch effects as covariates in the linear model. The first PC is displayed on the X axis and the second PC on the Y axis, with the corresponding percentage of total variance explained by each PC. Dots in the plot represent individual samples colored by disease condition: green for liver donors (healthy), pink early-CLD, orange compensated cirrhosis, blue decompensated cirrhosis and green ACLF, and shape indicating the sample processing date. (B) Unsupervised hierarchical clustering analysis of the 39 samples after normalization and batch adjustment. Each leave in the tree represents a liver sample from an individual and each color the disease condition. ACLF, acute-on-chronic liver failure; eCLD, early chronic liver disease; PC, principal component; PCA, principal component analysis.

CC and DC. These analyses revealed no significant DEGs after correcting p values for multiple hypothesis testing. This was consistent with the previously observed overlap between the 2 cirrhotic conditions in the PCA and clustering analyses. This

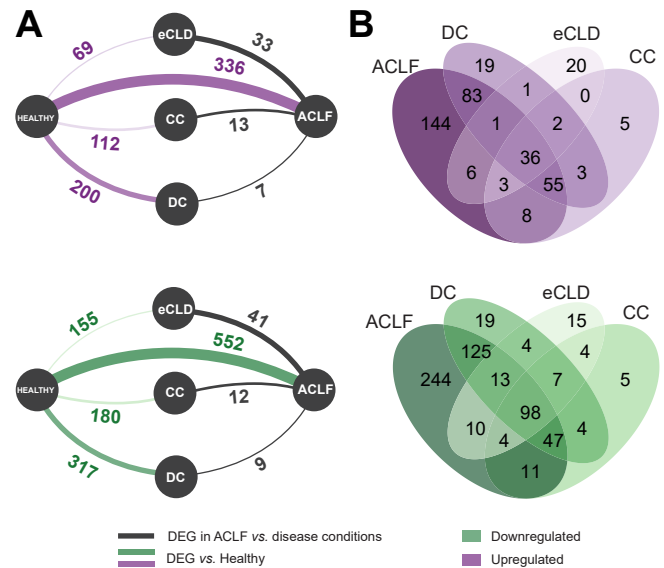


Fig. 2. Differentially expressed genes across liver disease stages and ACLF. DEGs in each disease condition at 1.5-fold change and adjusted p value <0.05 . (A) Total number of up- and downregulated genes between all pairs of comparisons are shown as a graph where nodes are sample groups and edges represent the number of DEGs between them. Disease conditions compared with healthy donors are highlighted in red and green for up and down-regulated genes respectively and the edge width corresponding to the change magnitude. ACLF compared to the other disease conditions is colored in black. (B) The Venn diagram represents the overlap between sets of DEGs in each disease condition compared with liver donors. ACLF, acute-on-chronic liver failure; CC, compensated cirrhosis; DC, decompensated cirrhosis; DEGs, differentially expressed genes; eCLD, early chronic liver disease.

observation suggests that the early-CLD stage as well as CC and DC are very similar to each other regarding their gene expression profiles and that the same processes might be altered in a similar way or remain constant throughout the natural history of the disease.

Network analysis of disease progression and ACLF

To provide biologically meaningful insights in our dataset, we used our 9 LRDNs as a scaffold and searched for significantly enriched gene sets at different disease stages. We used 2 different approaches. First, for each disease stage we performed an overrepresentation analysis with Fisher's exact test considering separately up- and downregulated genes in our LRDN (networks and extended networks). Second, we repeated the same analysis with GSEA.

Fig. 3 shows the overrepresentation analyses and GSEA for the 9 LRDNs along disease stages. Overall, there was no contradiction between both statistical tests. Overrepresented gene sets were found significantly enriched in the same direction in both statistical analyses as expected. Moreover, we also found consistency between gene sets containing only seeds and networks (containing seeds extended with their direct interactors). Patients with early-CLD presented a downregulation of oxidative stress and genetic factor networks and upregulation of inflammation, fibrosis and apoptosis of HSC networks compared to healthy individuals. These changes were also present when patients with CC and DC were compared to healthy individuals but, at these disease stages, apoptosis of HSC and angiogenesis

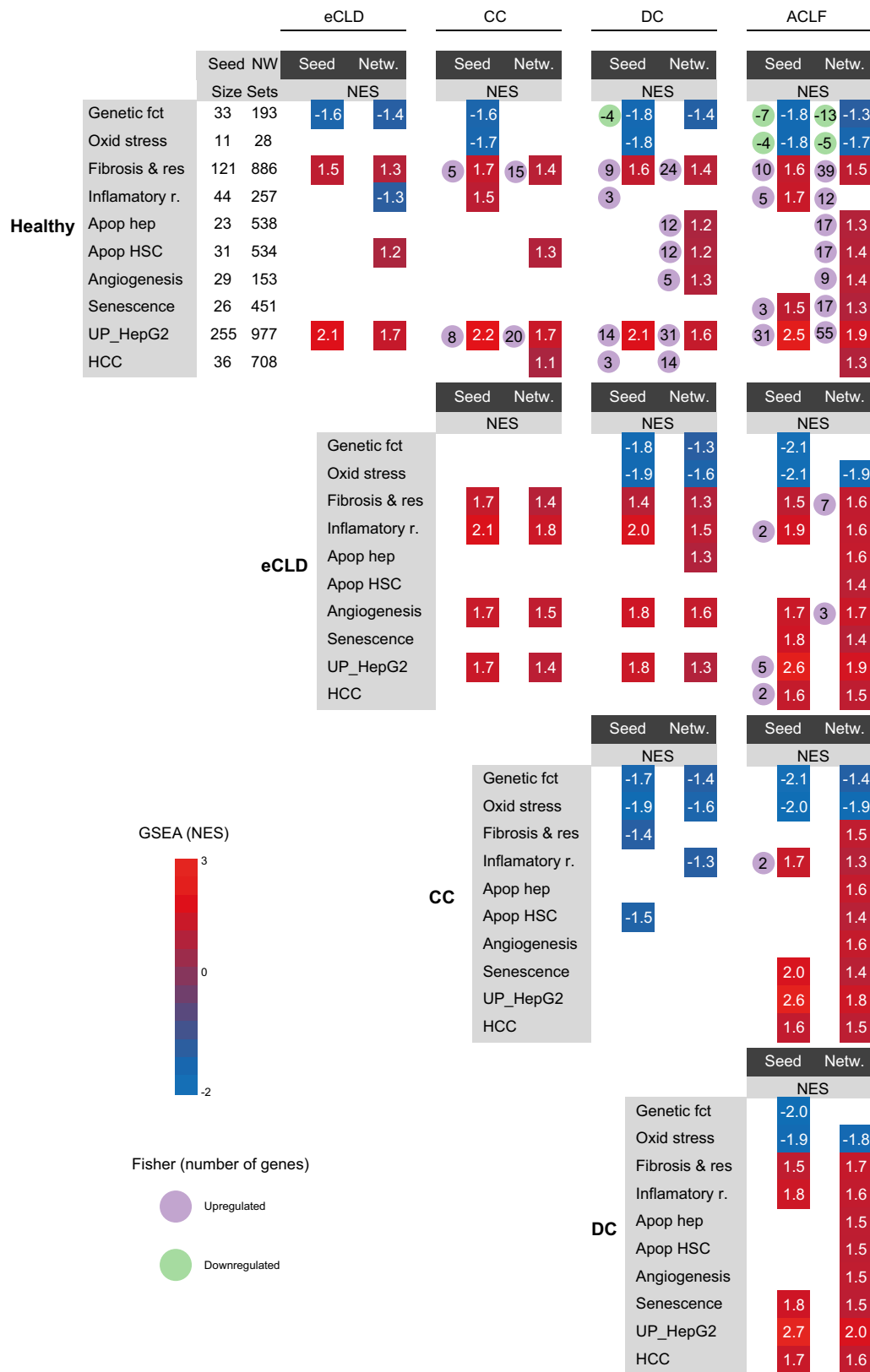


Fig. 3. Enrichment analyses of liver disease-related networks. Gene set enrichment analysis using as gene sets: i) the list of disease-related processes defined in the seeds or ii) the extended networks and as a dataset the pairwise comparisons between disease stages. Significantly enriched gene sets are colored ranked on NES showing only NES for tests with p value ≤ 0.05 and $FDR \leq 0.25$. Significantly up- and downregulated gene sets are highlighted in red and blue respectively. The number in the orange circles represent the number of genes included in the gene sets (seeds or Extended networks) significantly overrepresented (p value ≤ 0.05) while the number within the green circles indicate the number of genes found significantly under-represented. ACLF, acute-on-chronic liver failure; CC, compensated cirrhosis; DC, decompensated cirrhosis; eCLD, early chronic liver disease; FDR, false discovery rate; HCC, hepatocellular carcinoma; HSC, hepatic stellate cell; NES, normalized enrichment scores

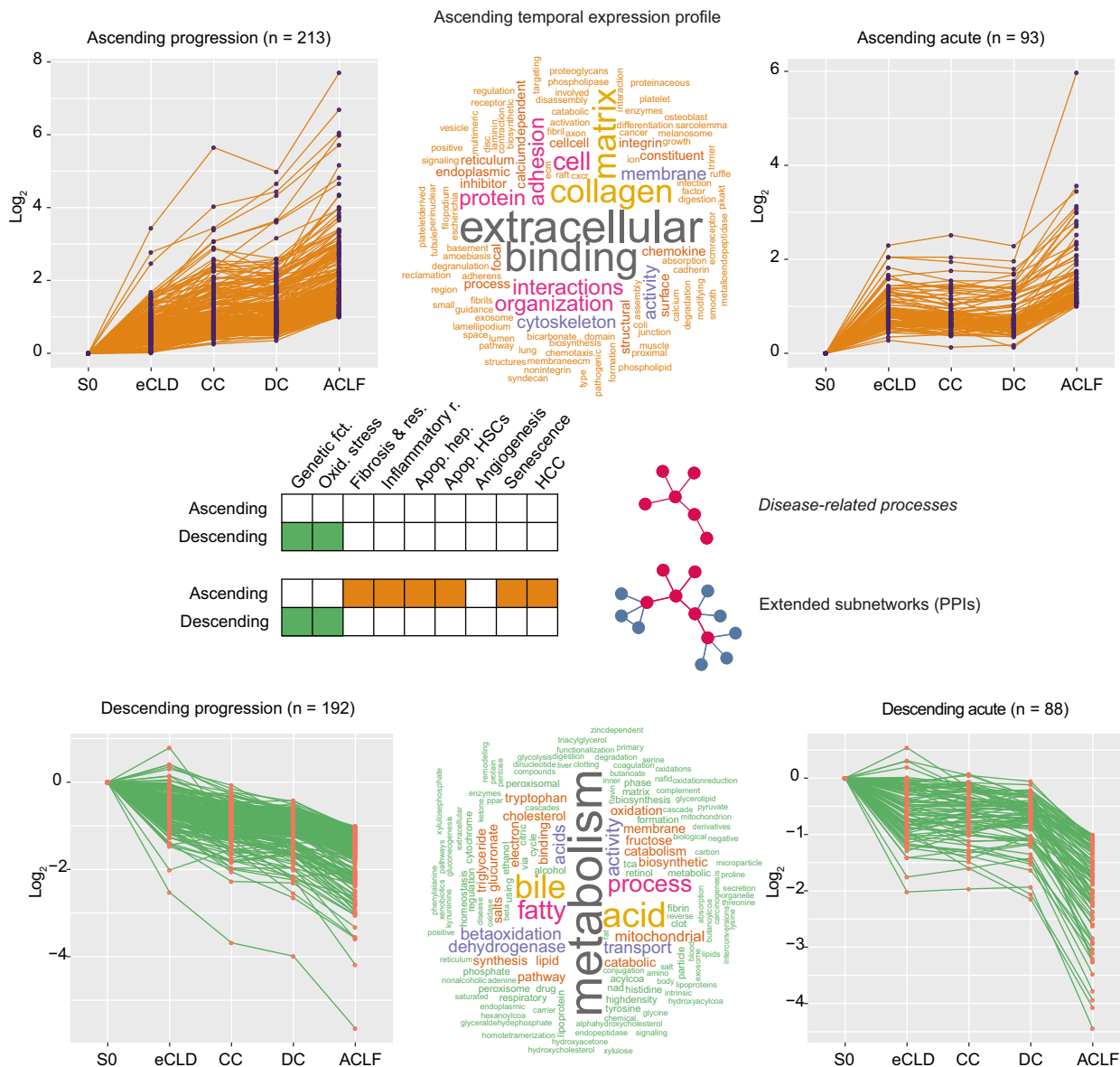


Fig. 4. Disease signatures. Temporal expression profiles were grouped into 4 signatures (top) ascending profiles, (bottom) descending profiles, (left) profiles associated with progressive disease progression and (right) profiles related to acute progression or related to ACLF development. Word Clouds between profiles visualize the most significantly enriched GO terms, KEGG and Reactome pathways using as input the ascending and descending signatures constructed. In the middle of the figure, we represent the curated disease-related processes significantly overrepresented by the ascending (red) and descending signatures (cyan), analyzing only the lists of disease-related genes (seeds) or the high-confidence binary subnetworks (wordcloud plot generated with `query.wordcloud` function from <http://www.sthda.com>). ACLF, acute-on-chronic liver failure; GO, gene ontology; HCC, hepatocellular carcinoma; HSC, hepatic stellate cell.

networks were also upregulated. Interestingly, as we observed in PCA and unsupervised clustering analysis, patients with CC and DC showed similarities and most LDRNs were not significantly differentially expressed between them. Finally, patients with ACLF had significantly different gene expression in all LDRNs: oxidative stress and genetic factors were downregulated compared to those of healthy individuals, whereas inflammation, apoptosis of hepatocytes and HSCs, angiogenesis, fibrosis and resolution, senescence, and carcinogenesis networks were

upregulated compared to patients with early-CLD, CC, and DC and to healthy individuals.

Our results suggest that our LRDNs capture most of the known changes occurring in CLDs. Although most changes were already detectable just by analyzing the list of seeds, by including the direct interactors of our predefined disease-related genes we increased the statistical power of our analysis and identified a larger number of significantly associated processes. Moreover, patients with ACLF exhibit specific hepatic gene expression that

is markedly different from that of healthy individuals and patients at other disease stages.

Temporal gene expression profiles involving disease progression to ACLF

The majority of patients that develop ACLF have previous decompensation and those with increased systemic inflammation are at greater risk of developing ACLF.⁵ However, the CANONIC study also showed that 23% of patients presented ACLF without previous decompensation. Due to the fact that ACLF may present in either patients with DC or CC, and because our interest was to understand the intrahepatic molecular events that drive patients to develop ACLF, we defined 2 different time courses for the transcriptomic data: i) short course: from early-CLD to CC and then ACLF, and ii) long course: from early-CLD to CC, DC and, finally, ACLF. We performed gene expression profile clustering with STEM software to identify common temporal expression patterns within and between both time courses.

Only genes with absolute log base 2-fold change greater than 1 in at least 1 stage in comparison to healthy livers were considered for clustering analysis. A total of 2,401 genes were selected for the short course and 2,464 for the long course. Using STEM software, 9 profiles involving 1,501 genes were identified as significant in the short course, while 6 significant profiles involving 1,197 genes were identified in the long course (Fig. S1). To assess profile similarity across both clinical courses we compared and visualized the gene assignment overlap with a chord diagram (Fig. S2). Profiles were classified as ascending or descending according to the direction of gene expression along disease progression and ACLF.

Because the main objective of our work was the characterization of the gene expression profile that drives disease progression to ACLF, we focused on gene profiles associated with higher gene expression changes relative to ACLF in both time

courses. Fig. 4 shows the representation of temporal expression profiles that were categorized into 4 different patterns defining 4 signatures: i) progressively ascending throughout the course of the disease up to ACLF development; ii) ascending but with sharp increase at the time of ACLF; iii) progressively descending throughout the course of the disease up to ACLF development; and iv) descending but with a sharp drop at the time of ACLF. Table S3 shows the complete list of genes associated with each signature. Functional analysis interrogating these 4 signatures on our manually curated LDRNs showed that genetic factors and oxidative stress-related networks were overrepresented in the descending profiles, while inflammatory response, fibrosis and resolution, apoptosis of hepatocytes and HSCs, senescence, and carcinogenesis networks were overrepresented in the ascending profiles (Fig. 4). We also performed functional pathway analysis using public databases and, as shown in Fig. 4, ascending profiles were associated with biological processes and pathways related to focal adhesion, extracellular matrix, collagen biosynthesis and fibril organization, cytoskeleton organization, and integrin binding, whereas descending profiles included genes involved in fatty acid degradation, metabolic pathways, cholesterol transport, oxidation-reduction process, lipid homeostasis, mitochondrion, fructose metabolism, and alcohol dehydrogenase activity.

Internal validation of transcriptome analysis

To confirm the results found in ACLF development profiles we chose 8 genes at the top of the list of the profiles, 4 from the ascending signature (*CXCL6*, *ITGA2*, *KRT18*, and *SPINK1*) and 4 from the descending signature (*AKR1D1*, *DGAT2*, *GSTA*, and *F13B*) and performed qPCR. Fig. 5 shows the hepatic expression of these 8 genes at different disease stages, from healthy individuals to patients with ACLF. Consistent with results found in gene profiles, there was a significantly increased gene expression in all 4 genes of the ascending signature; for the genes included

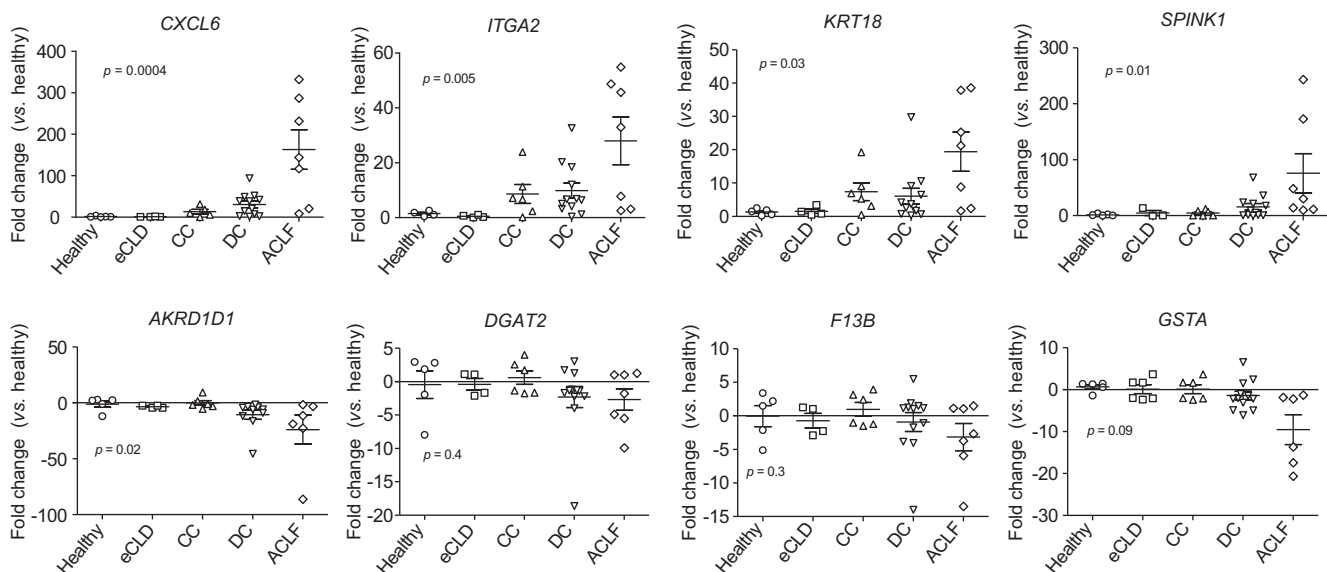


Fig. 5. Hepatic gene expression of selected genes from the ascending and descending ACLF profiles along disease progression. Hepatic gene expression levels of genes included in the ascending signature: *CXCL6*, *ITGA2*, *KRT 18*, *SPINK-1* and the descending signature: *AKR1D1*, *DGAT2*, *F3B*, *GSTA* were assessed by qPCR. The analysis includes patients with early-CLD (n = 5), CC (n = 8), DC (n = 12), ACLF (n = 8) and healthy controls (n = 6). Data are represented as as mean ± SEM. Levels of significance: *p <0.05; (Mann-Whitney U test). ACLF, acute-on-chronic liver failure; CC, compensated cirrhosis; DC, decompensated cirrhosis; eCLD, early chronic liver disease.

in the descending signature we observed a trend to a decreased expression in all evaluated genes but only *AKR1D1* was found significantly reduced in ACLF.

External validation of ACLF profiles

To confirm our results and to assess whether the identified profiles in our cohort could be safely generalized, we repeated the analyses on an independent cohort of patients with ACLF (n = 8). We performed GSEA using our 4 profiles in this new cohort of patients with ACLF and found that genes of the ascending and

descending profiles were significantly up- and downregulated in the new ACLF cohort. Reassuringly, the set of genes contributing the most to the enrichment signal (i.e. at the extremes of the distribution) show a sharp deregulated expression in ACLF (up and down), suggesting a high correlation with the pathological condition (Fig. S3).

Histological analyses of patients with ACLF

Next, we evaluated, by immunohistochemistry, if there was an increase in inflammation, angiogenesis and fibrosis at the

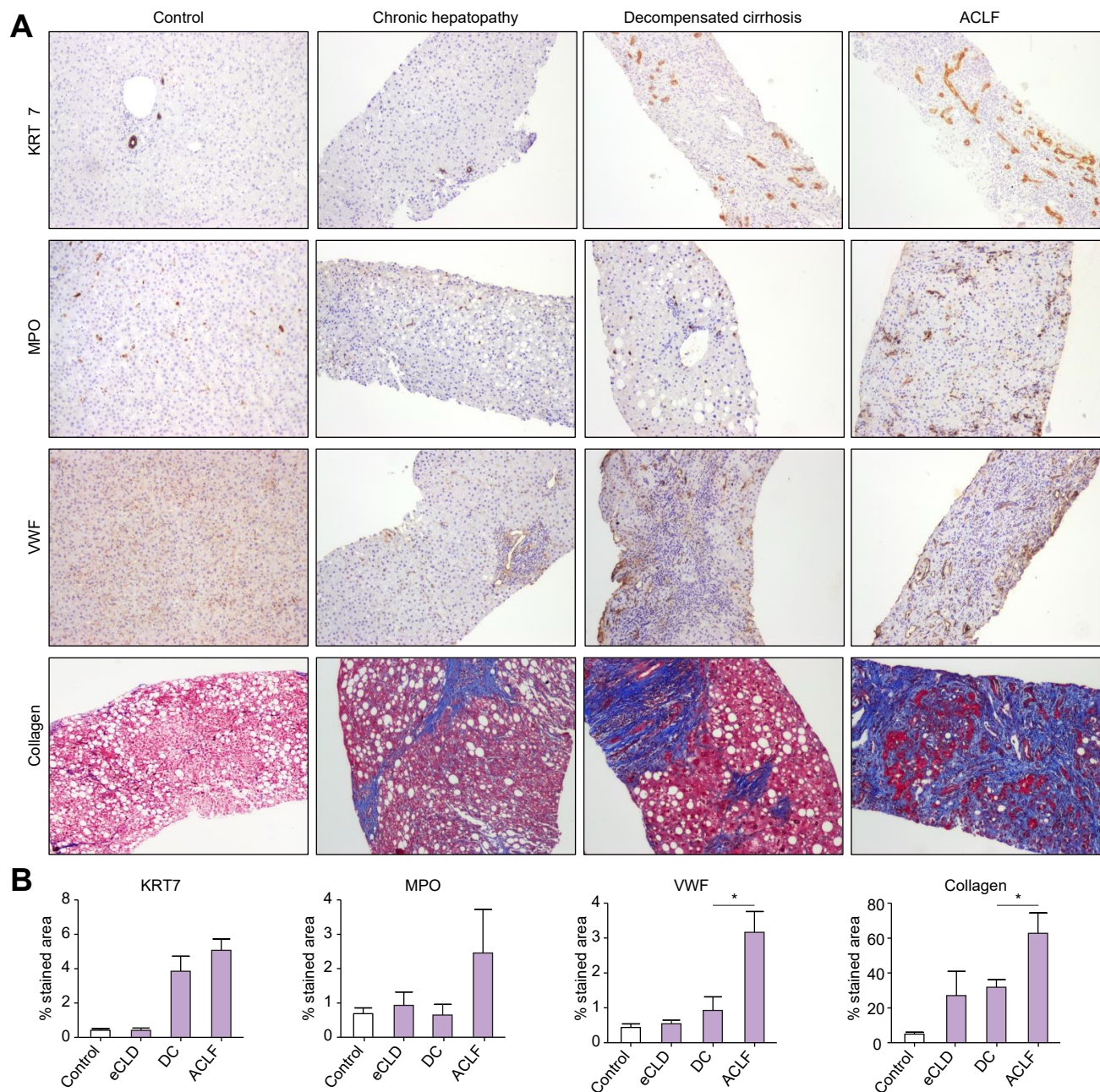


Fig. 6. Histological analysis of the presence of ductular reaction, inflammation, angiogenesis and fibrosis along disease progression. (A) KRT7, MPO, VWF and collagen staining from a representative liver section obtained from patients with eCLD, DC and ACLF and control. (B) Quantification of stained area per field of view. Patients included in the analysis presented early-CLD (n = 4), CC (n = 4), ACLF (n = 4) and healthy controls (n = 4). The bars of all graphs represent the mean ± SEM. Levels of significance: *p < 0.05; (Mann-Whitney U test). ACLF, acute-on-chronic liver failure; CLD, chronic liver disease; DC, decompensated cirrhosis; eCLD, early chronic liver disease; KRT7, keratin-7; MPO, myeloperoxidase; VWF, von Willebrand factor.

histological level to confirm the results found in the transcriptomic analysis. We used trichromic staining to evaluate fibrosis, myeloperoxidase as a marker of neutrophils, keratin-7 as a well-known marker of ductular reaction and von Willebrand factor as an angiogenesis marker. As shown in Fig. 6, patients with ACLF presented a significantly higher expression of all these markers compared to patients in the other liver disease stages. It is important to note that patients with ACLF presented a higher degree of lobular inflammation, with neutrophils being the predominant inflammatory cell in the liver compared to in other disease stages.

Discussion

The results of the current study show that patients with ACLF have a characteristic hepatic transcriptomic profile different from that of patients with cirrhosis, either compensated or decompensated. The nature of our disease data set, encompassing the whole spectrum of CLDs, allowed us to identify specific gene profiles for ACLF development that come from 2 distinct clinical courses, either the compensated or decompensated stage. Furthermore, with a network-based approach we found that pathways related to inflammation, fibrosis, angiogenesis, senescence, apoptosis of hepatocytes and HSCs, and liver carcinogenesis are key events in ACLF development.

Omics technologies have provided detailed information about potential molecular mechanisms underlying human diseases and have contributed to a better understanding of human pathophysiology²² and CLDs.²³ However, although informative, these data are not representative of the underlying complexity of the cellular networks governing biological systems. Systems biology has emerged as an integrative and holistic approach to deciphering the complexity of biological systems and their perturbations.¹² In the current work, the use of an integrated systemic biology approach allowed us to visualize in a network context the gene perturbations found in ACLF and build an intrahepatic molecular hypothesis of ACLF development. In our study, out of the 9 LRDNs, the most highly overexpressed in ACLF were inflammation, senescence, fibrosis, angiogenesis, apoptosis, and carcinogenesis networks, thus suggesting that these are the main pathogenic events in ACLF development.

The pathophysiology of ACLF is not known but increasing evidence suggests that systemic inflammation participates in ACLF development. Patients with ACLF have marked alterations in their systemic inflammatory response^{3,4} and more recently, the PREDICT study has shown that patients with acute decompensation and a higher degree of systemic inflammation are more prone to develop ACLF and that the increase in the levels of inflammatory markers accompanies the transition from DC to ACLF.⁵ Our data provides evidence that intrahepatic inflammation may also play a significant role, with the inflammatory network being one of the most upregulated LRDNs in ACLF. A closer look at the genes of the ascending ACLF profiles found in the temporal expression analysis showed that at least 5 genes (LCN2, CCL20, CCL2, CXCL5, and SPP) have previously been reported to play a role in intrahepatic inflammatory processes²⁴⁻²⁶ and one of them, LCN2, has been shown to be a good biomarker of ACLF.²⁴ These findings support a role for intrahepatic inflammation being a driver event in ACLF development.

Besides inflammation, senescence and carcinogenesis networks were specifically upregulated in ACLF. The role of senescence in CLDs is poorly understood. In fact, in NAFLD it has

been shown that senescent HSCs produce less extracellular matrix components.²⁷ The role of senescence in ACLF development would require further investigation but we hypothesize that hepatic senescence could impair liver regeneration promoting hepatocyte cell arrest, as has been shown in alcoholic hepatitis.²⁸ Regarding the liver carcinogenesis network, we confirmed the upregulation of CXCL6, SPINK-1, and ITGA2 in ACLF by qPCR. CXCL6 is a chemokine with neutrophil chemoattractant and angiogenic properties that attracts granulocytes and stimulates the secretion of matrix metalloproteinase-9.²⁹ It has also been shown that CXCL6 has a role in promoting HCC invasion and metastasis through its pro-angiogenic effect.³⁰ SPINK-1 is a serine peptidase inhibitor involved in tumorigenesis that has been found upregulated in a number of tumors, including HCC, where it may promote epithelial to mesenchymal transition phenomena.³¹ Finally, ITGA2 is a transmembrane receptor for collagens and related proteins that mediates the adhesion of platelets and other cell types to the extracellular matrix and has been shown to be one of the key genes involved in HCC development.³² In fact, patients with NAFLD and NAFLD-related HCC have increased platelet activation and adhesion within the liver and blockade of platelet activation prevents HCC development in a murine model of NAFLD.³³ The specific roles of CXCL6, SPINK-1 and ITGA2 in ACLF are not known but our results suggest that upregulation of these pathways may indicate the activation of pro-angiogenic events, cycle deregulation and metaplasia present in the context of ACLF and may represent potential therapeutic targets.

With respect to fibrosis and apoptosis of hepatocytes or HSCs, we found that all networks were increasingly upregulated along disease stages, suggesting that these processes are linked to disease progression but also present in ACLF development. Specifically, we found that keratin-18 (KRT-18) was significantly upregulated in the liver of patients with ACLF. KRT-18 is one of the cytoplasmic intermediate filament proteins present in the liver.³⁴ KRT-18 can be considered as a hepatocyte stress protein due to its induction upon liver injury and has cytoprotective effects in preventing hepatocyte apoptosis and other forms of injury. KRT-18 is increased in CLDs³⁵ and a recent study demonstrated increased levels of KRT-18 in patients with ACLF compared to those with DC without ACLF.³⁶ On the other hand, downregulation of the oxidative stress and genetic factor disease networks found in our study points toward an impairment of metabolic functions of the liver as the disease progresses. In this regard, we found that AKR1D1 – an enzyme encoding Δ 4-3-oxosteroid-5 β -reductase that participates in the synthesis of bile acids³⁷ – was significantly downregulated in patients with ACLF. Mutations in this enzyme have been described as inborn errors and associated with liver failure in the presence of hepatocellular and canalicular cholestasis.³⁷ An intriguing finding of the present study is that while ACLF had a specific hepatic gene expression pattern, compensated and decompensated cirrhosis stages show similar differential expression profiles compared to healthy individuals. This suggests that progression from compensated to decompensated cirrhosis may not be driven by activation of specific hepatic genes.

The current study has some limitations that should be mentioned. First, the sample size is relatively small. Nevertheless, both gene set profiles, ascending and descending, were validated in an independent cohort of patients with ACLF by GSEA. Second, the study was cross-sectional; therefore, different

stages of CLD analyzed corresponded to different patients. Although the ideal design would have consisted of a longitudinal study assessing the liver biopsies of the same patients through different stages of the disease, such a design is unrealistic because it would require liver biopsies from the same patient at different time periods. Finally, alcohol consumption was the most common etiological factor in patients with ACLF in this study, reflecting the most common etiology of ACLF in many areas of the world.³⁸ Whether the findings of the study apply to other etiologies of ACLF, such as hepatitis B infection, remains to be determined.

Abbreviations

ACLF, acute-on-chronic liver failure; CC, compensated cirrhosis; CLD, chronic liver disease; CRP, C-reactive protein; DC, decompensated cirrhosis; DEGs, differentially expressed genes; GSEA, gene set-enrichment analyses; HCC, hepatocellular carcinoma; HSC, hepatic stellate cells; KRT-18, keratin-18; LDRN, liver disease-related protein-protein interaction network; NAFLD, non-alcoholic fatty liver disease; PCA, principal component analysis.

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Conflict of interest

PG reports Investigator Research grant and Advisory Board work from Grifols, Investigator Research grant and Advisory Board from Gilead, Investigator Research grant from Mallinckrodt, Advisory Board for Promethera, Advisory Board for Martin-Pharmaceuticals, grants from Ferring-Pharmaceuticals, grants and Advisory Board Work from Sequana, outside the submitted work. IG has received lecture-fees from Gilead and Novartis. No other authors have any declared interests.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

IG and LI equally participated in the design of this study, performed experiments, the analysis and drafted the manuscript; M.C. supervised the experiments and critically reviewed the manuscript; A.D. performed the histological analysis of all liver biopsies and contributed to the critical review of the manuscript; E.P., J.V., T. R-T, C.M-S. M.L.L., TR participated in the gene expression and immunohistochemistry experiments, analysis and critically reviewed the manuscript, P.H., R.M., C.S., E.S., C.F. recruited patients for the study and contributed to the critical review of the manuscript. J.J.L. participated in the bioinformatic analysis and interpretation of the data and contributed to the critical review of the manuscript. P.S.B. participated in the conceptual design of the study, interpreted data and contributed to the critical reviewed of the manuscript; P.G. and P.A. conceived and designed the study, critically reviewed the manuscript and supervised the study.

In conclusion, the results of the current study indicate that ACLF is characterized by a specific hepatic gene expression pattern different from that of compensated and decompensated cirrhosis. This supports the concept that ACLF should be considered a distinct entity in the clinical course of cirrhosis and that intrahepatic molecular mechanisms are involved in the pathogenesis of ACLF. The network biology approach is a powerful method to gain insights into the mechanistic details of ACLF and provides a perfect scaffold to construct new hypotheses regarding ACLF; in the future, it may assist in finding new biomarkers and therapeutic targets.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author.

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Supplementary data

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References

- [1] Hernaez R, Solà E, Moreau R, Ginès P. Acute-on-chronic liver failure: an update. *Gut* 2017 Mar;66(3):541–553.
- [2] Zaccherini G, Weiss E, Moreau R. Acute-on-chronic liver failure: definitions, pathophysiology and principles of treatment. *JHEP Reports Innov Hepatol* 2021 Feb;3(1):100176.
- [3] Solé C, Solà E, Morales-Ruiz M, Fernández G, Huelin P, Graupera I, et al. Characterization of inflammatory response in acute-on-chronic liver failure and relationship with prognosis. *Sci Rep* 2016 Aug;6:32341.
- [4] Clària J, Stauber RE, Coenraad MJ, Moreau R, Jalan R, Pavesi M, et al. Systemic inflammation in decompensated cirrhosis: characterization and role in acute-on-chronic liver failure. *Hepatology* 2016 Oct;64(4):1249–1264.
- [5] Trebicka J, Fernandez J, Papp M, Caraceni P, Laleman W, Gambino C, et al. The PREDICT study uncovers three clinical courses of acutely decompensated cirrhosis that have distinct pathophysiology. *J Hepatol* 2020 Oct;73(4):842–854.
- [6] Fischer J, Silva TE, Soares E Silva PE, Colombo BS, Silva MC, Wildner LM, et al. From stable disease to acute-on-chronic liver failure: circulating cytokines are related to prognosis in different stages of cirrhosis. *Cytokine* 2017 Mar;91:162–169.
- [7] López-Vicario C, Checa A, Urdangarin A, Aguilar F, Alcaraz-Quiles J, Caraceni P, et al. Targeted lipidomics reveals extensive changes in circulating lipid mediators in patients with acutely decompensated cirrhosis. *J Hepatol* 2020 Oct;73(4):817–828.
- [8] Moreau R, Clària J, Aguilar F, Fenaille F, Lozano JJ, Junot C, et al. Blood metabolomics uncovers inflammation-associated mitochondrial dysfunction as a potential mechanism underlying ACLF. *J Hepatol* 2020 Apr;72(4):688–701.
- [9] Tripathi DM, Vilaseca M, Lafoz E, Garcia-Calderó H, Viegas Haute G, Fernández-Iglesias A, et al. Simvastatin prevents progression of acute on chronic liver failure in rats with cirrhosis and portal hypertension. *Gastroenterology* 2018 Nov;155(5):1564–1577.
- [10] Pose E, Solà E, Lozano JJ, Juanola A, Sidorova J, Zaccherini G, et al. Treatment with simvastatin and rifaximin restores the plasma metabolomic profile in patients with decompensated cirrhosis. *Hepatol Commun* 2021 Dec.
- [11] Sun YV, Hu Y-J. Integrative analysis of multi-omics data for discovery and functional studies of complex human diseases. In: *Advances in genetics*; 2016. p. 147–190.

- [12] Barabási A-L, Gulbahce N, Loscalzo J. Network medicine: a network-based approach to human disease. *Nat Rev Genet* 2011 Jan;12(1):56–68.
- [13] Pujana MA, Han J-DJ, Starita LM, Stevens KN, Tewari M, Ahn JS, et al. Network modeling links breast cancer susceptibility and centrosome dysfunction. *Nat Genet* 2007 Nov;39(11):1338–1349.
- [14] Casas C, Isus L, Herrando-Grabulosa M, Mancuso FM, Borrás E, Sabidó E, et al. Network-based proteomic approaches reveal the neurodegenerative, neuroprotective and pain-related mechanisms involved after retrograde axonal damage. *Sci Rep* 2015 Mar;5(1):9185.
- [15] Chuang H-Y, Lee E, Liu Y-T, Lee D, Ideker T. Network-based classification of breast cancer metastasis. *Mol Syst Biol* 2007 Oct;3:140.
- [16] Nibbe RK, Koyutürk M, Chance MR. An integrative -omics approach to identify functional sub-networks in human colorectal cancer. Price ND, editor *PLoS Comput Biol* 2010 Jan;6(1):e1000639.
- [17] Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. *Hepatology* 1996 Aug;24(2):289–293.
- [18] Angeli P, Bernardi M, Villanueva C, Francoz C, Mookerjee RP, Trebicka J, et al. EASL Clinical Practice Guidelines for the management of patients with decompensated cirrhosis. *J Hepatol* 2018;69(2):406–460.
- [19] Moreau R, Jalan R, Gines P, Pavesi M, Angeli P, Cordoba J, et al. Acute-on-chronic liver failure is a distinct syndrome that develops in patients with acute decompensation of cirrhosis. *Gastroenterology* [Internet] 2013 Jun;144(7):1426–1437. , 1437.e1-9 [cited 2015 Jun 12]. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/23474284>.
- [20] Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* 2005 Oct;102(43):15545–15550.
- [21] Ernst J, Bar-Joseph Z. STEM: a tool for the analysis of short time series gene expression data. *BMC Bioinformatics* 2006 Apr;7(1):191.
- [22] Halu A, De Domenico M, Arenas A, Sharma A. The multiplex network of human diseases. *NPJ Syst Biol Appl* 2019;5:15.
- [23] Govaere O, Cockell S, Tiniakos D, Queen R, Younes R, Vacca M, et al. Transcriptomic profiling across the nonalcoholic fatty liver disease spectrum reveals gene signatures for steatohepatitis and fibrosis. *Sci Transl Med* 2020 Dec;12(572).
- [24] Ariza X, Graupera I, Coll M, Solà E, Barreto R, García E, et al. Neutrophil gelatinase-associated lipocalin is a biomarker of acute-on-chronic liver failure and prognosis in cirrhosis. *J Hepatol* 2016 Jul;65(1):57–65.
- [25] Affò S, Morales-Ibanez O, Rodrigo-Torres D, Altamirano J, Blaya D, Dapito DH, et al. CCL20 mediates lipopolysaccharide induced liver injury and is a potential driver of inflammation and fibrosis in alcoholic hepatitis. *Gut* [Internet] 2014 Nov;63(11):1782–1792 [cited 2014 Oct 30]. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/24415562>.
- [26] Morales-Ibanez O, Domínguez M, Ki SH, Marcos M, Chaves JF, Nguyen-Khac E, et al. Human and experimental evidence supporting a role for osteopontin in alcoholic hepatitis. *Hepatology* [Internet] 2013 Nov;58(5):1742–1756 [cited 2014 Sep 26]. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/23729174>.
- [27] Papatheodoridi AM, Chrysavgis L, Koutsilieris M, Chatzigeorgiou A. The role of senescence in the development of nonalcoholic fatty liver disease and progression to nonalcoholic steatohepatitis. *Hepatology* 2020 Jun;71(1):363–374.
- [28] Sharma S, Maras JS, Das S, Hussain S, Mishra AK, Shasthry SM, et al. Pre-therapy liver transcriptome landscape in Indian and French patients with severe alcoholic hepatitis and steroid responsiveness. *Sci Rep* 2017 Jul;7(1):6816.
- [29] Mehrad B, Keane MP, Strieter RM. Chemokines as mediators of angiogenesis. *Thromb Haemost* 2007 May;97(5):755–762.
- [30] Tian H, Huang P, Zhao Z, Tang W, Xia J. HIF-1 α plays a role in the chemotactic migration of hepatocarcinoma cells through the modulation of CXCL6 expression. *Cell Physiol Biochem* 2014;34(5):1536–1546.
- [31] Shek FH, Luo R, Lam BYH, Sung WK, Lam T-W, Luk JM, et al. Serine peptidase inhibitor Kazal type 1 (SPINK1) as novel downstream effector of the cadherin-17/ β -catenin axis in hepatocellular carcinoma. *Cell Oncol (Dordr)* 2017 Oct;40(5):443–456.
- [32] Wang J, Tian Y, Chen H, Li H, Zheng S. Key signaling pathways, genes and transcription factors associated with hepatocellular carcinoma. *Mol Med Rep* 2018 Jun;17(6):8153–8160.
- [33] Malehmir M, Pfister D, Gallage S, Szydłowska M, Inverso D, Kotsiliti E, et al. Platelet GPIb α is a mediator and potential interventional target for NASH and subsequent liver cancer. *Nat Med* 2019 Apr;25(4):641–655.
- [34] Ku N-O, Strnad P, Zhong B-H, Tao G-Z, Omary MB. Keratins let liver live: mutations predispose to liver disease and crosslinking generates Mallory-Denk bodies. *Hepatology* 2007 Nov;46(5):1639–1649.
- [35] Guldiken N, Usachov V, Levada K, Trautwein C, Ziolk M, Nahon P, et al. Keratins 8 and 18 are type II acute-phase responsive genes overexpressed in human liver disease. *Liver Int* 2015 Apr;35(4):1203–1212.
- [36] Macdonald S, Andreola F, Bachtiger P, Amoros A, Pavesi M, Mookerjee R, et al. Cell death markers in patients with cirrhosis and acute decompensation. *Hepatology* 2018 Mar;67(3):989–1002.
- [37] Sundaram SS, Bove KE, Lovell MA, Sokol RJ. Mechanisms of disease: inborn errors of bile acid synthesis. *Nat Clin Pract Gastroenterol Hepatol* 2008 Aug;5(8):456–468.
- [38] Mezzano G, Juanola A, Cardenas A, Mezey E, Hamilton JP, Pose E, et al. Global burden of disease: acute-on-chronic liver failure, a systematic review and meta-analysis. *Gut* 2022 Jan;71(1):148–155.