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Original Article

Liver Characterization of a Cohort of Alpha-1 Antitrypsin Deficiency Patients with and without Lung Disease

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Abstract

Background and Aims: Alpha-1 antitrypsin deficiency (AATD) is a genetic disorder characterized by the misfolding and accumulation of the mutant variant of alpha-1 antitrypsin (AAT) within hepatocytes, which limits its access to the circulation and exposes the lungs to protease-mediated tissue damage. This results in progressive liver disease secondary to AAT polymerization and accumulation, and chronic obstructive pulmonary disease (COPD) due to deficient levels of AAT within the lungs. Our goal was to characterize the unique effects of COPD secondary to AATD on liver disease and gene expression. ***Methods:*** A subcohort of AATD individuals with COPD (n = 33) and AATD individuals without COPD (n = 14) were evaluated in this study from our previously reported cross-sectional cohort. We used immunohistochemistry to assess the AATD liver phenotype, and RNA sequencing to explore liver transcriptomics. We observed a distinct transcriptomic profile in liver tissues from AATD individuals with COPD compared to those without. ***Results:*** A total of 339 genes were differentially expressed. Canonical pathways related to fibrosis, extracellular matrix remodeling, collagen deposition, hepatocellular damage, and inflammation were significantly upregulated in the livers of AATD individuals with COPD. Histopathological analysis also revealed higher levels of fibrosis and hepatocellular damage in these individuals. ***Conclusions:*** Our data supports a relationship between the development of COPD and liver disease in AATD and introduces genes and pathways that may play a role in AATD liver disease when COPD is present. We believe addressing lung impairment and airway inflammation may be an approach to managing AATD-related liver disease.

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Introduction

Alpha-1 antitrypsin (AAT), an acute-phase protein, is the most abundant protease inhibitor in plasma. AAT plays an important role in limiting host tissue destruction by proteases at sites of inflammation.[1] Alpha-1 antitrypsin deficiency (AATD) is a genetic condition caused by pathogenic variants in the SERPINA1 gene. The most common disease-causing variant, PiZ, causes hepatic accumulation of the misfolded pathogenic variant of AAT, predisposing individuals with AATD to develop liver disease, ranging from liver fibrosis to cirrhosis and hepatocellular carcinoma.[2] AATD individuals are also susceptible to chronic obstructive pulmonary disease (COPD) due to low levels of AAT in the plasma.[1,3] In this context, increased pro-inflammatory mediators in the sputum and bronchoalveolar lavage fluid of AATD individuals indicate the presence of early airway inflammation.[4] Several studies have shown that COPD is a disease associated with systemic inflammation and higher levels of inflammatory markers in the blood, including C-reactive protein (CRP), and cytokines such as interleukin (IL)-6, TNF-alpha, and IL-8.[5]

The liver, the largest metabolic organ in the body, is recognized as a unique immune organ with distinctive immune characteristics. Due to its exposure to various foreign and inflammatory molecules carried by the portal vein and systemic blood, the liver's default immune status is anti-inflammatory or immune-tolerant.[6] However, a failure to mount a proper response to inflammatory stimuli or to properly regulate immune mechanisms can lead to liver disease.[7] Lung injury has been shown to impact hepatic function by aggravating liver diseases through systemic inflammatory responses.[8] This systemic inflammation is recently hypothesized to be one of the probable mechanisms involved in the progression of liver disease.[9]

The liver disease mediated by AATD is initiated by a toxic gain-of-function mechanism in which the misfolded mutant variant of AAT accumulates in hepatocytes, leading to liver disease.[2] However, the relationship between AATD-associat-

Keywords: Alpha-1 antitrypsin deficiency; Chronic obstructive pulmonary disease; Liver fibrosis; Liver biopsy; Liver histology; Transcriptomics.

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ed COPD and liver disease is not well understood, despite the high prevalence of COPD in AATD individuals.

In our study, we investigated the association between COPD and AATD-mediated liver disease in a prospectively recruited AATD cohort. We examined the liver phenotype using liver tissue histology and liver transcriptomic profiles from AATD individuals with and without COPD. Our histological evidence shows that AATD-associated liver inflammation and fibrosis are more prevalent in AATD individuals with COPD. The liver transcriptomic data also indicated different aspects of fibrogenesis, such as hepatic stellate cell activation and extracellular matrix deposition, in AATD individuals with COPD compared to those without COPD. These findings may pave the way for identifying new diagnostic markers of liver disease in AATD individuals with COPD and aid in developing novel preventative and therapeutic strategies for AATD-mediated liver disease.

Methods

Human subjects

The protocol for this study was approved by the Clinical Research Ethics Committee of the University of Florida (IRB202101148). Written informed consent was obtained from each subject in accordance with the Declaration of Helsinki Principles.[10] AATD individuals (n = 94) were recruited by pulmonologists specializing in AATD within the Division of Pulmonary, Critical Care and Sleep Medicine at the University of Florida, Shands Hospital. Subjects were recruited prospectively over three years. Recruitment methods included our own clinic as well as the AATD registry. Patients were followed for a year at our center, where all testing was performed. The diagnosis of AATD was established according to AAT genotyping, isoelectric focusing of serum proteins, and AAT serum level measurement using nephelometry (Behring Diagnostics, Marburg, Germany), with in-house standards and controls.[11] Pulmonary function tests were performed on AATD individuals, and a post-bronchodilator forced expiratory volume in 1 s/forced vital capacity ratio was calculated by a pulmonologist. A ratio of less than 70%, combined with evidence of emphysema and a history of smoking, was used to diagnose COPD.[12] Liver biopsy was performed as part of a separate clinical study (NCT01810458) on a well-characterized cross-section of adults with the AATD genotype (ZZ pathogenic variant), as we previously reported. Briefly, AATD individuals aged 21–71, from the US or Canada, who were willing to consent to a liver biopsy, were included.[13] Patients with decompensated liver disease and those who had received a liver or lung transplant were excluded from the study. We isolated whole RNA from all liver biopsy samples, and a subcohort of patients whose liver tissue yielded sufficient RNA was selected for liver transcriptomic analysis based on RNA availability (n = 47; age range 35–70 years; mean 57 years; male: female ratio 16:31). The STROBE study reporting guideline was followed for this manuscript. All authors had access to the study data and reviewed and approved the final manuscript.

Liver histology

A percutaneous liver biopsy was performed using a 16-gauge BioPince™ core biopsy needle. Liver tissues were fixed with 4% formaldehyde and embedded in paraffin blocks, and thin sections (4 μm) were prepared. The paraffin-embedded liver tissue sections were deparaffinized with xylene and a graded series of ethanol. Stains used included H&E,[14] Masson's Trichrome,[15] Periodic acid-Schiff with Diastase (PAS-D)[13] and Picrosirius Red. Histopathological analysis was performed by PAS-D globules present in a high-power field, which were scored from 0–3 as follows: 0 - None: no hepatocytes with globules, 1 - Rare: < 5 hepatocytes with globules, 2 - Few: 5–20 hepatocytes with globules, 3 - Numerous: ≥20 hepatocytes with globules.[13] Steatosis was graded using the non-alcoholic fatty liver disease activity score (NAS), which involves the evaluation of steatosis, lobular inflammation, and hepatocyte ballooning.[16,17] Steatosis was classified based on the percentage of hepatocytes involved, with S0 indicating no steatosis, S1 indicating < 5% steatosis, S2 indicating 5–33% steatosis, and S3 indicating >33% steatosis. In addition, the degree of inflammatory cell infiltration was assessed using the NAS system, which considers the presence and severity of lobular inflammation. The grading for inflammation ranged from 0–3, with 0 indicating no inflammation, 1 indicating mild inflammation (<2 inflammatory foci per 200x field), 2 indicating moderate inflammation (2–4 inflammatory foci per 200x field), and 3 indicating severe inflammation (>4 inflammatory foci per 200x field).[14] The assessment of hepatocyte ballooning, also using the NAS system, involved evaluating the presence of enlarged hepatocytes with cytoplasmic clearing. Ballooning was graded based on the severity and frequency of the observed ballooning cells within the liver parenchyma. The severity of fibrosis was evaluated using the METAVIR scoring system, which classifies fibrotic changes into five distinct stages, ranging from F0–F4 (indicative of cirrhosis). F0 denotes the absence of fibrosis; F1 represents minimal fibrosis restricted to the portal areas without septa formation; F2 indicates increased fibrosis extending beyond the portal areas with the presence of a few septa; F3 signifies significant fibrosis with numerous septa but without cirrhosis; and F4 represents advanced cirrhosis characterized by widespread fibrosis, nodular formation, and significant distortion of the liver architecture.[18] Each biopsy was scored by two pathologists, and images were acquired using a Keyence BZ-X710 microscope.[19]

To supplement the pathologists' scores, we used the BZ-X Analyzer (Version 1.3.1.1) image analysis software, which accompanies the Keyence BZ-X710 microscope. The Hybrid Cell Count module in this software uses image brightness and hue to separate positive staining from background tissue. Images of the stained liver biopsy tissue were captured at 4X or 10X magnification, in an X-Y stitching format, allowing for full capture of the entire tissue. Image tiles from this capture were then analyzed using a macroinstruction specific to the stain in question and applied across all samples. The output from this analysis included total stained tissue area and total tissue area, allowing for the final analysis measure, "total stained area", a ratio of the aforementioned measurements, given as a percentage.

RNA isolation and library preparation

Total RNA from AATD liver tissues was extracted using the RNeasy Plus Mini Kit (Qiagen, Germantown, Maryland).[20] The quantity and quality of the extracted RNA were determined using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). Total RNA was used for polyadenylation and synthesis of double-stranded cDNAs according to Illumina's TruSeq RNA Sample Prep guidelines (San Diego, CA). Liver tissue library preparation and RNA sequencing were performed at the University of Florida Interdisciplinary Center for Biotechnology Research.

RNA-sequencing and Bioinformatics

The quality of the RNA-Seq data was first evaluated using FastQC[21] prior to further downstream analysis. Low-quality

Table 1. Patient clinical and demographic characteristics collected from the 47 AATD (alpha-1 antitrypsin deficiency) individuals enrolled in this study

Patient characteristics	Lung disease (LD)	No lung disease (NLD)	*p*-value
Age (years, range)	59.3 (42–70) ± 8.0	51.1 (36–63) ± 8.0	0.006
Sex (% female)	57.6	85.7	N/A
BMI	26.1 ± 5.7	26.2 ± 4.8	0.834
FEV1/FVC (%)	46.0 ± 18.0	72.0 ± 7.5	<0.001
Smoking history (% smokers)	60.6	28.6	N/A
Genotype (SerpinA1)	ZZ	ZZ	N/A
Augmentation (%)	72.7	28.6	N/A
AST (Units/L)	26.0 ± 12.0	22.0 ± 8.5	0.073
ALT (Units/L)	24.0 ± 13.5	22.0 ± 13.0	0.407
GGT (Units/L)	24.0 ± 25.0	21.0 ± 17.0	0.324
ALP (Units/L)	72.3 ± 21.3	70.3 ± 18.0	0.938

BMI, body mass index; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase.

sequences were trimmed, and poor-quality reads were removed using Trimmomatic.[22] The Star Aligner[23] was used to map high-quality single-end reads to the GRCh38 genome.[24] Gene expression was obtained using RSEM.[25] The expected read counts and Fragments Per Kilobase of transcript per Million mapped reads were extracted for further analysis. The estimated read counts were used as input for edgeR[6] to perform differential expression (DE) analysis. Prior to the DE analysis, PCA was performed to identify outlier samples. No obvious outliers were found.

Functional pathway, upstream regulator, and network analyses

Pathway enrichment analysis was performed using ingenuity pathway analysis (IPA) (IPA System, Ingenuity Systems, Inc., Redwood, CA). IPA core analyses are based on previous knowledge of the associations of upstream regulators and their downstream target genes archived in the Ingenuity knowledge base.[19] An overlapping p-value is computed based on the significant overlap between the genes and their targets regulated by the transcriptional regulator, and an activation z-score is used to infer the probable activation or inhibition states of upstream transcriptional regulators. A z-score ≥2.0 (or ≤−2.0) and an overlap *p*-value ≤0.05 were considered indicative of significant activation or inhibition. Using mRNA sequencing data, enrichment analyses were also conducted to explore the functional pathways associated with various biological conditions. Specific genes of interest were identified based on statistical significance (adjusted *p*-value < 0.05) and substantial differential expression between groups.

Quantitative real-time PCR

Total RNA was extracted using a Qiagen RNeasy Plus Mini Kit and reverse transcribed into cDNA using SuperScript VILO Master Mix. Gene expression levels were analyzed by qPCR using an Applied Biosystems 7500 fast real-time PCR system and TaqMan Fast Advanced Master Mix. Relative expression levels of target mRNAs were determined using TaqMan probes normalized with 18S as endogenous control and analyzed by the $2^{-\Delta\Delta CT}$ method as previously described.[26]

Statistical analysis

Data are expressed as means ± SD when normally distributed, medians ± IQR when non-normally distributed, and percentages when reporting frequencies. Statistical analyses were performed using the Prism 9 software (GraphPad Software) using Student's t-test or Mann–Whitney U test. Values of $p < 0.05$ were considered statistically significant. For RNA-seq, false discovery rate (FDR)-adjusted *p*-values < 0.05 were considered significant. For some genes of interest, the non-adjusted *p*-value was also reported, even if the FDR-adjusted *p*-value was not significant to reduce the type II error rate (false negatives).[27]

Results

Clinical characteristics

We enrolled a total of 47 AATD individuals from across the U.S. and Canada in the current study. Liver tissues were collected from individuals with COPD (n = 33) and without COPD (n = 14) following a biopsy procedure. Demographic and clinical characteristics are shown in Table 1. A history of smoking was more prevalent among individuals with COPD compared to those without. No significant differences were observed in plasma levels of aspartate aminotransferase, alanine aminotransferase, or gamma-glutamyl transferase between the two groups. The mean values fell within normal limits. No concomitant viral hepatitis, autoimmune hepatitis, iron overload, or cholestatic liver diseases were identified (Table 1).

Liver histopathological characteristics

Accumulation of the pathogenic variant of AAT in hepatocytes is a hallmark of AATD-mediated liver disease, as measured by PAS-D staining of liver sections.[13] In our cohort, liver PAS-D scores were PAS-D0 = 10.6% (n = 5), PAS-D1 = 44.7% (n = 21), PAS-D2 = 21.3% (n = 10), and PAS-D3 = 23.4% (n = 11) (Fig. 1A). The prevalence of AATD-mediated liver fibrosis was 63.8% (n = 30). Individual fibrosis stages were F0 = 36.2% (n = 17), F1 = 40.4% (n = 19), F2 = 14.9% (n = 7), and F3 = 8.5% (n = 4) (Fig. 1B). The prevalence of hepatocyte ballooning was 42.6% (n = 20). Individual ballooning stages were stage 0 = 57.4% (n = 27), stage 1 = 38.3% (n = 18), and stage 2 = 4.3% (n = 2) (Fig. 1C). Portal inflammation, comprising immune cells, was observed in 31 cases of our study cohort

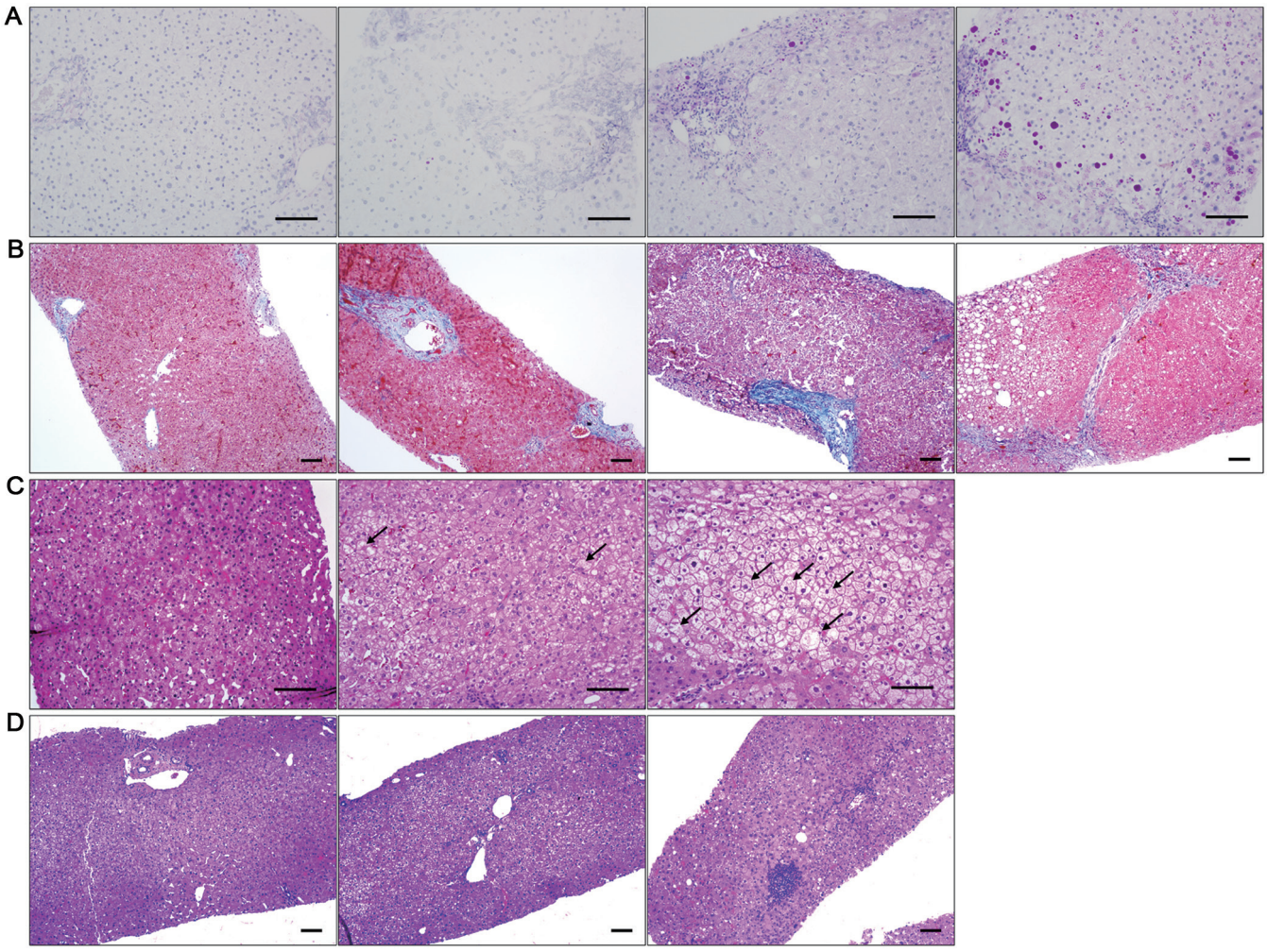

Fig. 1. Liver histopathology. Representative brightfield images of AATD (alpha-1 antitrypsin deficiency) liver tissue showing: A) PAS-D (periodic acid Schiff plus diastase) staining scored 0–3 from left to right, at 20X magnification; B) Masson's trichrome staining scored 0–3 from left to right, at 10X magnification; C) H&E (hematoxylin and eosin) staining scored 0–2 for hepatocyte ballooning from left to right, at 20X magnification (arrows indicate examples of ballooning hepatocytes); and D) H&E staining scored 0–2 for portal inflammation from left to right, at 10X magnification. All scale bars = 100 μm.

(66.0%). Individual portal inflammation stages were 0 = 34.0% (n = 16), stage 1 = 57.4% (n = 27), and stage 2 = 8.5% (n = 4) (Fig. 1D, Table 2).

Liver disease stages

Masson's Trichrome staining of liver biopsy samples revealed significantly higher fibrosis scores (p = 0.0201) in AATD individuals with COPD compared to those without COPD (Fig. 2A). This finding was also confirmed by collagen-specific stains (Picrosirius Red), which showed significantly higher amounts of collagen deposition in the livers of AATD individuals with COPD compared to those without COPD (1.8 ± 1.4% vs 4.0 ± 4.3%) (Supplementary Fig. 1A and B). Liver damage, as defined by hepatocyte ballooning using H&E stain, was also significantly higher (p = 0.0118) among AATD individuals with COPD compared to those without COPD (Fig. 2B). Furthermore, liver portal inflammation, defined by infiltration of immune cells using H&E stain, was found to be significantly higher (p = 0.0240) among AATD individuals with COPD compared to those without COPD (Fig. 2C). In-

Table 2. Liver histopathology characteristics. Distribution of scores for the major liver histopathology characteristics assessed from the 47 AATD (alpha-1 antitrypsin deficiency) individuals enrolled in this study, reported as frequencies

Liver histopathology characteristic	Score 0	Score 1	Score 2	Score 3	Overall distribution
PAS-D	10.6% (n = 5)	44.7% (n = 21)	21.3% (n = 10)	23.4% (n = 11)	89.4% (n = 42)
Fibrosis	36.2% (n = 17)	40.4% (n = 19)	14.9% (n = 7)	8.5% (n = 4)	63.8% (n = 30)
Hepatocyte ballooning	57.4% (n = 27)	38.3% (n = 18)	4.3% (n = 2)	N/A	42.6% (n = 20)
Portal inflammation	34.0% (n = 16)	57.4% (n = 27)	8.5% (n = 4)	N/A	66.0% (n = 31)

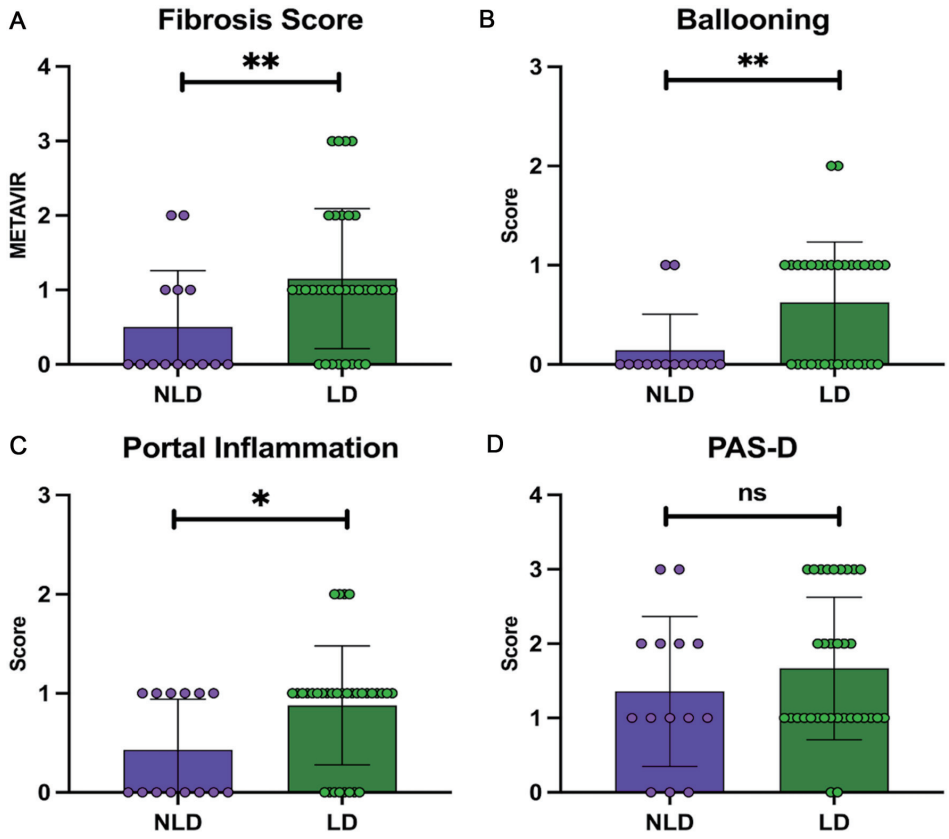

Fig. 2. Quantitative assessment of histopathology. Quantitative assessment of liver histopathology using pathological scoring. Comparison of cohorts of AATD (alpha-1 antitrypsin deficiency) individuals with lung disease (LD) to those without (NLD) revealed: A) significantly higher fibrosis scores; B) significantly higher hepatocyte ballooning phenotype; and C) portal inflammation in LD, although D) PAS-D (periodic acid Schiff plus diastase) was not significant. *$p < 0.05$ and **$p < 0.01$.

terestingly, our data showed no significant difference in PAS-D scores between liver tissues from AATD individuals with and without COPD. This finding suggests that the differences in liver damage, fibrosis, and collagen deposition between AATD individuals with and without COPD are independent of hepatic accumulation of AAT, as indicated by PAS-D staining (Fig. 2D).

Liver transcriptome changes in AATD individuals with COPD vs. individuals without COPD

To understand the potential effect of COPD on the liver transcriptomics of AATD individuals, bulk RNA-seq was performed on liver tissues obtained from AATD individuals (n = 47). Statistical analysis revealed 339 genes with significantly different expression (with thresholds of p-value < 0.05 and fold change >2), the majority of which were protein-coding genes (Supplementary Table 1). Of the 339 differentially expressed genes (DEGs), 198 were upregulated in the liver tissues of AATD individuals with COPD, while 141 were downregulated (Fig. 3A, B). The most significant expression changes were observed in *TFF3, DES, CDK, MYL2, ACTA1, TPM1, TPM2*, and *ASCL1* (Fig. 3C). Next, we performed RT-qPCR to validate the expression of selected genes from our RNA-seq dataset. Our results showed that collagen (*COL1A1*) and smooth muscle actin (*SMA*) levels were elevated in the group with lung disease, with *COL1A1* showing a statistically significant increase. Additionally, *TFF3* was found to be significantly decreased in AATD individuals with lung disease, while *DES* expression did not show a significant difference (Fig. 3D). It is important to note that only samples with sufficient remaining RNA were analyzed, which may affect the qPCR data. A larger and more representative sample size from this cohort could provide a more robust validation of the RNA-seq results.

Functional and pathway enrichment analysis

To identify the functional pathways enriched among DEGs in both groups, we performed pathway enrichment analysis using IPA. We found that DEGs were mostly enriched in pathways related to cellular stress and tissue injury (Fig. 4A). All 339 DEGs were enriched in cellular responses to stress and inflammatory signaling pathways, including acute phase response, autophagy, ERK/MAPK, p38/MAPK, hepatic fibrosis, extracellular matrix remodeling, actin cytoskeleton organization, and activation of hepatic stellate cells (Fig. 4B). Additionally, pathway enrichment analysis revealed the top 10 liver disease-associated categories that demonstrated statistical significance. These categories were visualized using Cytoscape software and included liver disease-associated conditions such as liver failure, hyperplasia/hyperproliferation of the liver, and hepatocellular carcinoma (Fig. 5).

Upstream regulator and network analyses

Upstream regulator analysis was performed using enriched pathways and FDR values computed by the IPA literature review algorithm. The IPA upstream regulator analysis identified upstream transcriptional regulators and mechanistic

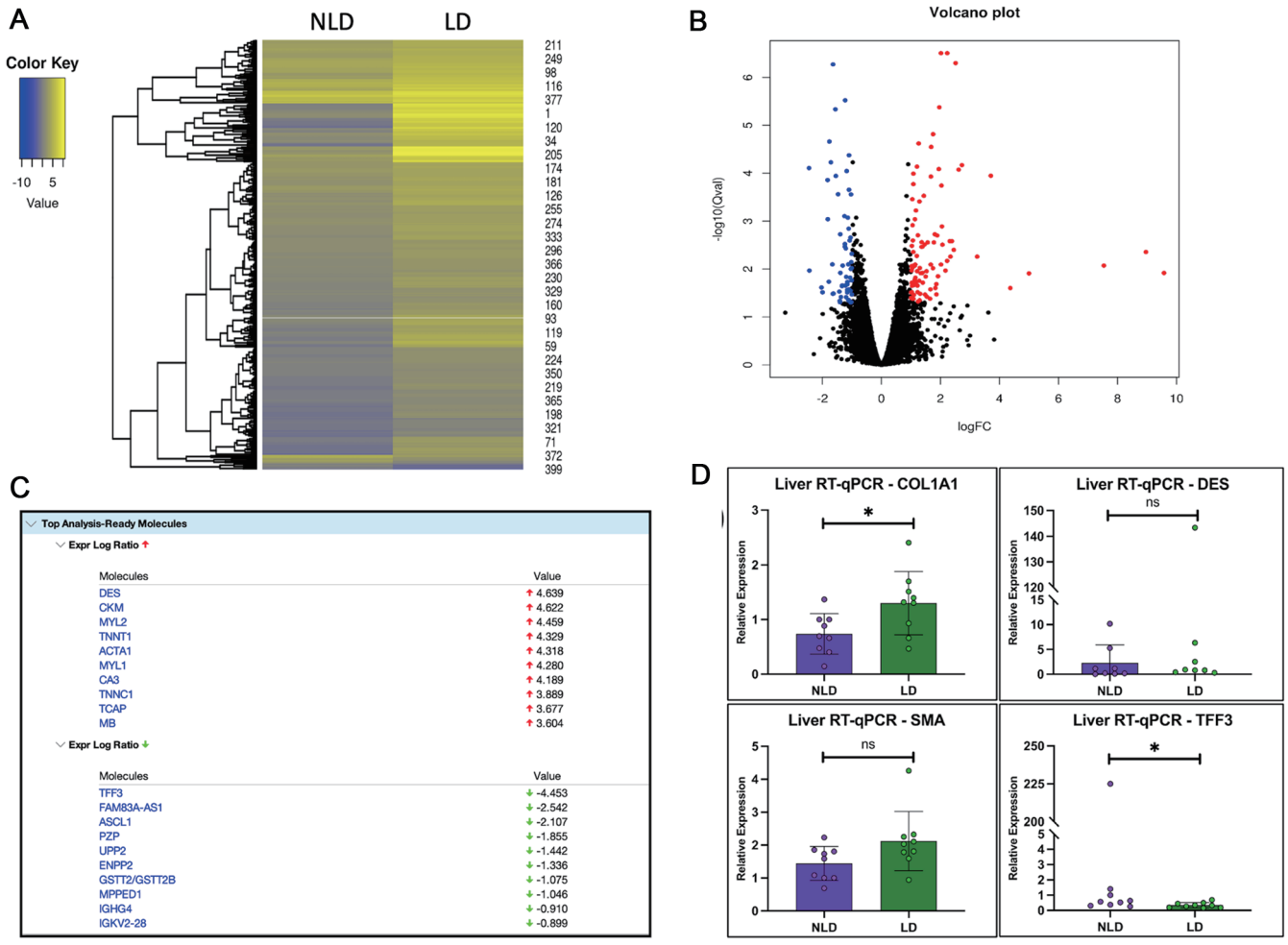

Fig. 3. Differential gene expression. Bulk RNA-seq was performed on all AATD (alpha-1 antitrypsin deficiency) liver tissues (n = 47). Statistical analysis revealed 339 genes with significantly different expressions between NLD (no lung disease) and LD (lung disease) groups. Of these, 198 genes were upregulated in LD and 141 were downregulated, as shown by: A) heatmap and B) volcano plot. C) This table shows genes with the highest observed expression changes: *TFF3, DES, CDK, MYL2, ACTA1, TPM1, TPM2*, and *ASCL1*. ↑ indicates upregulation and ↓ indicates downregulation. D) RT-qPCR validated RNA-seq results by showing an increase in fibrotic markers for collagen (*COL1A1*) and smooth muscle actin (*SMA*), although only *COL1A1* was significantly different. Genes with the highest observed expression changes were also measured in *TFF3* and *DES*, with only *TFF3* showing a significant difference. *$p < 0.05$.

networks that could explain the DEGs observed in the livers of AATD individuals with COPD compared to those with normal lung function. A total of 142 upstream regulators were predicted to be activated, and 146 upstream regulators were predicted to be inhibited (Supplementary Table 1) in the livers of AATD individuals with COPD. Several dysregulated genes and their relationships related to liver fibrogenesis were identified in our dataset, including genes whose changes are predicted to increase and upregulate migration (Fig. 6A) and activation of hepatic stellate cells (Fig. 6B). Additionally, our results showed that the dysregulation of genes related to cell death is predicted to inhibit the apoptosis of hepatic stellate cells (Fig. 6C). We also found that the MAPK signaling pathway was one of the top upstream regulators in our dataset, regulating many genes in the liver, including those related to liver inflammation and fibrosis (Fig. 6D). IPA also predicted the liver steatosis pathway to be upregulated in AATD individuals with COPD, based on DEGs from our dataset (Supplementary Fig. 2A). However, we did not observe significant differences in the steatosis scores between the study groups (Supplementary Fig. 2B).

Discussion

Liver fibrosis, caused by excessive deposition of extracellular matrix proteins in the liver, occurs in most types of chronic liver diseases. The prevalence of advanced liver fibrosis in AATD patients is more than 35%.[13] Many antifibrotic therapies have been developed to inhibit and control liver fibrogenesis. Although many of these therapeutic interventions are effective in experimental models of liver fibrosis, their clinical deployment has achieved limited success.[28] Therefore, it is crucial to improve our knowledge about liver fibrosis mediated by AATD to develop better therapeutic strategies. Our data demonstrated that the liver transcriptome and associated functional annotations are largely overlapping in AATD individuals with COPD and those without, indicating that COPD did not act as a confounding factor in the evaluation of disease-associated transcriptome signatures in AATD liver disease. However, we observed that AATD individuals with COPD have distinct liver transcriptome signatures compared to AATD individuals with normal lung function. The liver transcriptomics of AATD individuals with COPD showed dysregulated expression of genes associated with aggravated liver

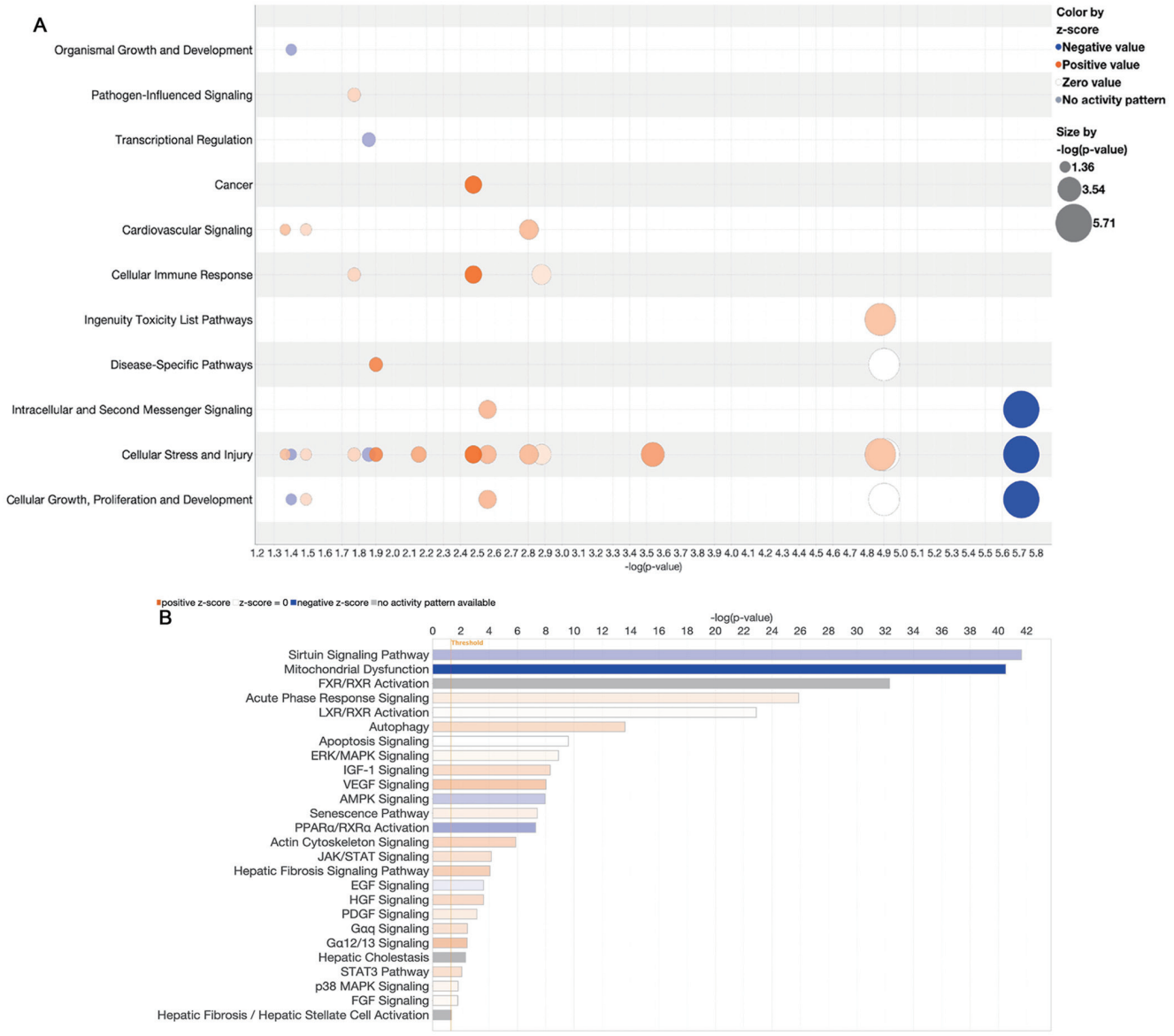

Fig. 4. Functional pathway enrichment analysis. Functional pathway enrichment analysis revealed that DEGs (differentially expressed genes) in the LD (lung disease) cohort were mostly enriched in pathways related to cellular stress and injury, as shown in A) the bubble chart. All 339 genes were enriched in various cellular responses and signaling pathways involved in hepatic injury, as shown in B) the bar chart.

fibrogenesis and damage compared to AATD individuals with normal lung function. We also observed that liver transcriptome signatures in AATD individuals with and without COPD are independent of hepatic accumulation of AAT and PAS-D scores. Consistent with liver transcriptomic data, quantitative histopathology analysis of liver tissues also revealed that AATD individuals with COPD have significantly higher liver fibrosis scores compared to those without. Hepatocellular damage, as indicated by the presence of hepatocyte ballooning, also appeared to be significantly more severe in AATD individuals with COPD compared to those without. These findings suggest that the active pathogenic process occurring in the lungs of AATD-deficient individuals may be associated with the development of liver disease.

Our results demonstrated several transcriptomic changes that are implicated in the altered deposition of extracellular matrix and cytoskeletal rearrangement in the livers of AATD individuals with COPD. Upregulation of genes related to extracellular matrix remodeling has been previously described in the progression of liver fibrosis.[29] For example, *de novo* expression of smooth muscle *ACTA2*, *DES*, and collagen are common features of hepatic stellate cell activation and initiation of liver fibrogenesis.[30] Upregulation of *TIMP-1* is also considered to promote fibrosis by inhibiting matrix metalloproteases.[20] Here, we noted upregulation of *DES, COL3A1, TIMP1, and ACTA2* in the livers of AATD individuals with COPD. Furthermore, *actin, profilin,* and myosin light chain phosphatase were also upregulated in the livers of AATD individuals with COPD. F-actin and profilin cytoskeleton reorganization are associated with hepatic stellate cell activation.[31] In addition, high activity of myosin light chain phosphatase has been implicated in liver cirrhosis.[32] Thus, our results con-

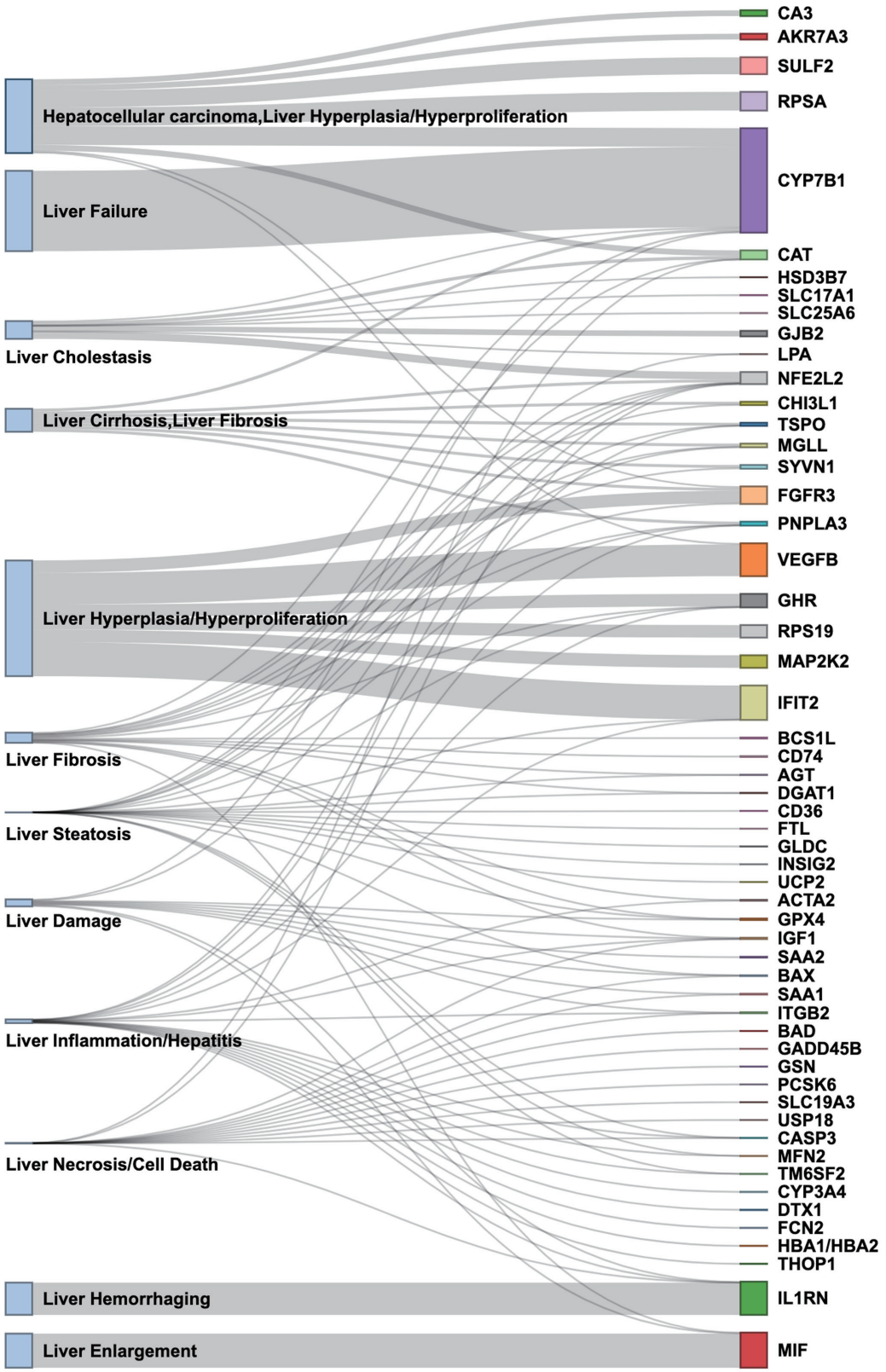

Fig. 5. Sankey diagram of functional pathway analysis. Sankey diagram illustrating the specific DEGs (differentially expressed genes) involved in liver disease-associated pathways identified during functional pathway enrichment analysis. The significance of the enrichment for each category is represented by -log10(*p*-value), which determines the size of the chords forming the link to each DEG as well as the size of the rectangle at each node.

Fig. 6. Upstream regulator analysis. IPA upstream regulator analysis identified transcriptional regulators and mechanistic networks related to liver fibrogenesis that were upregulated in AATD (alpha-1 antitrypsin deficiency) individuals in the LD (lung disease) cohort, such as: A) migration and B) activation of hepatic stellate cells. C) Apoptosis was inhibited. D) *MAPK* was identified as one of the top upstream regulators.

firm that dysregulation of the extracellular matrix and cytoskeletal rearrangement genes are particularly important in AATD-mediated liver fibrosis, specifically in those subjects who have developed impaired lung function secondary to AATD. These results are consistent with our immunohistopathology observation of the greater METAVIR fibrosis score in the livers of AATD individuals with COPD compared to those with normal lung function.

Hepatic transcriptomes of AATD individuals also revealed that pathways related to cellular stress and injury are activated in the livers of individuals with COPD compared to those with normal lung function. Pathway enrichment analysis showed that the DEGs in our dataset correlate with the upregulation of ERK/MAPK, p38/MAPK, VEGF, and PDGF pathways in the livers of AATD individuals with COPD. ERK signaling pathways, along with PDGF, are critical pathways in modulating the phenotypic responses of liver myofibroblasts. These pro-fibrogenic cells have been shown to significantly contribute to the advancement of fibrosis.[33] According to recent studies, ERK signaling pathways play major roles in mediating liver fibrotic responses, as elicited by a number of extracellular signals,[34] thus potentially revealing them as regulators of liver transcriptional changes in response to fibrotic stimuli. Our lab and others have also shown how ERK dysregulation promotes inflammatory signaling in airway epithelial cells and macrophages expressing Z-AAT,[35,36] highlighting a clear connection between a mechanism that perpetuates airway inflammation and modulates the progression of liver fibrosis.

Biological pathways, including mitochondrial dysfunction and sirtuin signaling, were uncovered by IPA in the livers of AATD individuals with COPD, highlighting disturbances in key processes linked to liver fibrogenesis.[37] Dysregulated mitochondrial biogenesis, along with increased production of reactive oxygen species, may cause irreversible cell growth arrest or senescence.[38] Senescent hepatic stellate cells have previously been identified in liver fibrosis.[39] The predictions from the liver transcriptomes of AATD individuals with COPD revealed signaling pathways similar to those previously reported in liver fibrosis. In addition to genes related to mitochondrial dysfunction, we also found downregulation of *IGF-1*, which has anti-fibrotic effects through attenuation of hepatic stellate cell activation, and upregulation of *MIF* and *AGT*, consistent with published evidence of their role in promoting the activation of hepatic stellate cells.[40,41] However, there were differences between our dataset and clinical cases of liver fibrosis regarding TGFβ, Hedgehog, and Wnt/β-catenin signaling pathways,[42] suggesting that the fibrotic insult in AATD individuals with COPD has a distinct pathogenesis compared to other fibrotic liver diseases.

Hepatocyte ballooning indicates hepatocellular damage, partly mediated by ER stress, and is associated with the enlargement and swelling of injured hepatocytes.[43,44] Our results revealed higher levels of hepatocyte ballooning in the livers of AATD individuals with COPD compared to those without. We also identified liver transcriptome changes in AATD individuals with COPD who presented with higher levels of hepatocyte ballooning, including pathways associated with impaired cell death and increased cellular viability.[45] Consistent with previous reports demonstrating that hepatocyte ballooning is associated with greater fibrosis,[15] our study also supports an association between hepatocyte ballooning, liver fibrosis stage, and liver-related outcomes.

It has been shown that the prevalence of hepatic steatosis in AATD is about 40%, which is higher than in the general population.[13] Here, we identified transcriptome changes in the livers of AATD individuals with COPD, indicating changes in lipid metabolism, including downregulation of *TFF3*. *TFF3* has been shown to upregulate peroxisome proliferator-activated receptor alpha and subsequently reduce hepatic steatosis by increasing fatty acid oxidation.[46] While we found no significant differences in hepatic steatosis scores between AATD individuals with and without COPD, the downregulation of *TFF3* suggests a compensatory enhancement of fatty acid oxidation to limit lipotoxicity in the livers of AATD individuals with COPD. This observation also underscores the multifaceted nature of hepatic steatosis, prompting consideration of the diverse array of variables, including alcohol consumption and obesity, that may contribute to its pathogenesis and progression.[47]

COPD, defined by airflow limitation caused by chronic bronchitis or emphysema,[48] is a systemic inflammatory disease. Subjects with COPD exhibit evidence of systemic inflammation and high levels of CRP concentration that correlate with the level of airway obstruction.[49,50] In this context, we have previously shown evidence of systemic inflammation[26] in addition to lung neutrophilic inflammation in AATD individuals.[4] Systemic inflammation has also been shown to play a causal role in the development of liver injury.[49] Here, we found upregulation of the CRP gene in the livers of AATD individuals with COPD (Supplementary Fig. 3A). Increased expression of CRP in the livers of AATD individuals with COPD raises the possibility that inflammatory processes contribute to the association between COPD and increased liver fibrogenesis in AATD individuals. However, the difference in plasma levels of CRP was not significant between our study groups (Supplementary Fig. 3B). This observation may be explained by the fact that CRP continues to be a nonspecific marker of systemic inflammation, and we acknowledge the active variability of CRP as an inflammatory marker. Our AATD liver transcriptome and functional annotations also revealed DEGs related to inflammation (*IL1RN, IL6R, TNFR, IGF2,* and *EGR1*) in the livers of AATD individuals with COPD. In agreement with the liver transcriptomic profile, portal inflammation was observed in the livers of these individuals. The presence of portal inflammation in the liver has been shown to be associated with advanced fibrosis.[14] This suggests that portal inflammation in AATD may be associated with fibrosis progression in AATD individuals with COPD. This knowledge is crucial for developing comprehensive, targeted therapies that improve patient outcomes and advance scientific progress in managing AATD-related liver disease. Moreover, broader implications could emerge. Unraveling lung-liver interactions could shed light on important mechanisms of inflammation-related liver conditions with multi-organ effects.

The strengths of our study include the recruitment of a phenotypically characterized patient cohort and clinical samples, including liver biopsy and plasma. However, this study has several limitations that need to be addressed. First, the small cohort size of AATD individuals without COPD could have influenced the significance of our results. Second, liver gene expression profiles are largely driven by the effects of hepatocytes, which represents a limitation when analyzing bulk liver transcriptomics. Therefore, single-cell RNA sequencing may be helpful in identifying the key regulators of liver fibrogenesis. Third, due to the cross-sectional nature of the AATD disease, it is not possible to make any causal inferences, and prospective research is necessary to elucidate their exact connection. Fourth, since this study examines a subcohort, it may not be fully representative of the characteristics of the entire cross-sectional cohort. Fifth, the mechanisms behind these findings have not been explored experimentally in this study, which is why our results are mostly descriptive. This will be investigated in future studies.

Finally, the presence of AAT augmentation, as well as differences in sex and age, could potentially confound the analysis of liver transcription.

Conclusions

Liver transcriptome signatures from AATD individuals with COPD identified several DEGs and further validated enhanced liver fibrogenesis, along with differences in liver fibrogenesis mechanisms compared to AATD individuals without COPD. We also demonstrated quantitative changes in histopathological fibrosis markers in AATD liver tissues from those with COPD compared to those without. Our results are consistent with previous studies indicating that impaired pulmonary function is associated with the development of liver fibrosis. Understanding the communication between the lungs and liver in AATD can provide potential targets for effective treatment strategies and reduce healthcare burdens. This also suggests that future treatment strategies for AATD individuals with liver disease may need to consider the lung function of these individuals.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (NM, RO, VC, JL, MB, NK), performance of experiments (NM, RO, TG, VC), analysis and interpretation of data (RO, TG, VC, JL, NA, GM, NK), manuscript writing (NM, RO, JL, GM, NK), critical revision (NM, RO, TG, VC, JL, NA, MB, NK), and statistical analysis (NM, RO, TG, GM, NK). All authors have made significant contributions to this study and have approved the final manuscript.

Ethical statement

The protocol for this study was approved by the Clinical Research Ethics Committee of the University of Florida (IRB202101148). Written informed consent was obtained from each subject in accordance with the Declaration of Helsinki Principles.

Data sharing statement

All data discussed in this publication are available from the corresponding author upon request. The sequencing data can be found at: https://doi.org/10.6084/m9.figshare.24763950.v1.

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