

RESEARCH ARTICLE



Comprehensive analysis of extracellular matrix remodelling via cyclophilin inhibition in human models of alcohol-related liver fibrosis

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Background and Purpose: Chronic liver disease and hepatic fibrosis constitute a threat to global health. Clinical translation of preclinical research has been limited, highlighting an urgent need for novel treatments. Cyclophilin inhibitors have shown beneficial effects in liver disease; however, the underlying mechanism of action and the effect across different aetiologies remain elusive. Here, we investigate the impact of a pan-cyclophilin inhibitor (rencofilstat, RCF) in human models of fibrosis and alcohol-related liver disease.

Experimental Approach: RCF was tested in human precision-cut liver slices (PCLS) and primary human hepatic stellate cells (HSCs). Fibrosis and cell activation were assessed using transcriptomic and protein analysis. A comprehensive characterisation of changes in extracellular matrix (ECM) biochemical and structural composition was performed in PCLS and HSC-derived matrix using proteomics, imaging and bioinformatic tools to study ECM alignment. PCLS stiffness upon treatment was assessed by atomic force microscopy.

Key Results: Transcriptomic and proteomic analyses of PCLS revealed a dramatic impact of RCF on ECM organisation and remodelling. Biochemical composition and fibre alignment analysis of the ECM obtained from HSCs showed a reduction in the amount of ECM core proteins and associated enzymes by RCF, reshaping the architecture of matrix fibres without affecting the HSC activation. The disordered matrix detected in RCF-treated HSC cultures reflected a less-stiff ECM, which was confirmed in the PCLS.

Abbreviations: ALD, alcohol-related liver disease; CDM, cell-derived matrix; CLD, chronic liver disease; DEGs, differentially expressed genes; ECM, extracellular matrix; HSCs, hepatic stellate cells; LC-MS, liquid chromatography-mass spectrometry; MASLD, metabolic-associated steatotic liver disease; PCLS, precision-cut liver slices; RCF, rencofilstat; SOX9, SRY-Box Transcription Factor 9.

Una Rastovic and Sara Campinoti contributed equally to this work.

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Funding information

Foundation for Liver Research; Hepion
Pharmaceuticals; Wellcome Trust,
Grant/Award Number: Early career award
(225125/Z/22/Z) : PhD studentship (218461/
Z/19/Z) : 107859/Z/15/Z; UK Engineering
and Physical Sciences Research Council,
Grant/Award Number: EP/V04723X/1

Conclusions and Implications: This work provides evidence for a novel mechanism linking cyclophilins and ECM remodelling in advanced 3D models of liver disease, with potential applications in therapeutic development.

KEYWORDS

cyclophilins, extracellular matrix, hepatic stellate cells, liver fibrosis, precision-cut liver slices

1 | INTRODUCTION

Chronic liver disease (CLD) is currently the 11th-leading cause of death worldwide, resulting in the loss of over two million lives each year (Devarbhavi et al., 2023). Among the main causes of CLD are alcohol misuse and metabolic dysfunction, which contribute to the high global incidence of steatotic liver disease (SLD), a group of CLD recently redefined by a Delphi-based consensus study (Rinella et al., 2023). SLDs encompass metabolic dysfunction-associated steatotic liver disease (MASLD), alcohol-related liver disease (ALD) and MetALD, a newly defined entity that accounts for the combined influence of alcohol and metabolic factors and their prevalence. Notably, this updated nomenclature recognises the frequent co-occurrence and synergistic effects of these contributors in driving liver disease, and it acknowledges the significant impact of alcohol in disease development, even at moderate levels (Arab et al., 2025).

The pathogenesis of ALD is complex and involves the activation of multiple disease processes, such as direct and indirect cellular alterations caused by **ethanol** metabolism, a systemic derangement of fatty acids, a general immune dysfunction, and a concomitant hyper-inflammatory state caused by an increased bacterial translocation from the gut (Yan et al., 2023). This highlights the need to incorporate these factors in experimental models to enhance translational value.

A key element in the clinical progression of ALD, which is shared among most CLD including MASLD, is the unbalanced activation of wound-healing processes, which drive liver fibrosis. Fibrosis is characterised by an excessive accumulation of extracellular matrix (ECM), primarily mediated by hepatic stellate cells (HSCs). Alongside aberrant ECM deposition by HSCs, dysregulated ECM enzymes and ECM-associated proteins exacerbate progressive scarring, leading to non-physiological changes in the ECM composition and its physical properties (Sørensen et al., 2022). These alterations contribute to liver stiffening and progressive loss of liver function. If left untreated, hepatic fibrosis ultimately leads to cirrhosis and is the most critical prognostic factor in CLD patients (Anstee et al., 2024; Degré et al., 2020).

What is already known?

- Hepatic fibrosis and alcohol-related liver disease constitute a global health challenge, but therapies are lacking.
- Cyclophilin inhibitors shown promising results in several diseases, but their mechanism of action remains elusive.

What does this study add?

- Pan-cyclophilin inhibitor rencofilstat exerts antifibrotic effects in human models of ALD/fibrosis remodelling extracellular matrix.
- Rencofilstat changed the orientation of extracellular-matrix fibres, leading to a reduction in liver tissue stiffness.

What is the clinical significance?

- Rencofilstat affects multiple pathological processes which are relevant in ALD patients.
- Multiparametric methodologies for the analysis of matrix organisation have been applied to 3D patient-derived models.

The current cornerstone of treatment for both MASLD and ALD is lifestyle intervention, but sustained improvements are achieved only by a minority of patients, indicating an urgent need for novel therapies. Despite the burden of alcohol consumption (Asrani et al., 2019; Huang et al., 2023), to date, pharmacological approaches targeting key mechanisms of disease progression in SLD, such as fibrosis, have

primarily focussed on MASLD. As mentioned, MASLD and ALD share several similarities in the pathogenesis, suggesting that therapies under investigation for MASLD may hold promise for ALD and MetALD as well. At the same time, important differences exist between these conditions that should be explored in preclinical models before moving into clinical studies.

Taken together, this underscores the need to investigate pathological mechanisms and test potential anti-fibrotic therapies in multi-factorial models that replicate aetiology-specific fibrosis and incorporate alcohol toxicity alongside synergistic factors to advance clinical development. These studies would inform not only about their potential in ALD but likely in metALD.

Despite advances in understanding fibrogenesis and several successful preclinical studies, the translation of anti-fibrotic into clinical practice has been disappointingly low (Friedman & Pinzani, 2022). Pharmacological approaches that have reached Phases 2 and 3 of clinical development for hepatic fibrosis primarily target growth factors and associated signalling pathways (i.e. TGF- β s, PDGFs and FGFs) (M. Zhao et al., 2022). Other strategies are aimed at modulating ECM cross-linking via the inhibition of enzymes such as lysyl oxidase-like proteins (LOXL2) (W. Zhao et al., 2018) and at regulating upstream disease processes such as metabolism and inflammation (i.e. **Toll-like receptors** and **glucagon-like peptide-1**) (M. Zhao et al., 2022).

Cyclophilins, a family of ubiquitous and evolutionary conserved proteins with **peptidyl-prolyl cis-trans isomerase** (PPIase) activity, have been implicated in key disease processes. They play a crucial role in protein folding and assembly of multidomain proteins (Göthel & Marahiel, 1999; Wang & Heitman, 2005). Inhibition of cyclophilins has shown therapeutic potential in different human pathologies, including liver disease in the context of viral hepatitis, MASLD, fibrosis and hepatocellular carcinoma (Naoumov, 2014; Stauffer, Goodman, & Gallay, 2024). **Rencofilstat** (RCF, previously known as CRV431 or CPI-431-32) is a pan-cyclophilin inhibitor that has shown a safe profile in Phases 1 and 2 clinical trials in healthy volunteers and subjects with fibrosis due to MASLD (Remenchik et al., 2023). However, the mechanism of action contributing to the therapeutic effect of cyclophilin inhibitors, including RCF, is unclear, and the role of cyclophilins in hepatic fibrogenesis and other components of CLD pathogenesis remains elusive.

In this study, we investigated the role of cyclophilins in liver disease by assessing the effects of cyclophilin inhibition via RCF in advanced 3D models of hepatic fibrosis and ALD, including primary human HSCs and organotypic cultures of precision-cut liver slices (PCLS). Previously, we demonstrated that PCLS effectively recapitulate clinically relevant features of ALD, underscoring their utility as a preclinical drug testing platform (Rastovic et al., 2023). Using multi-parametric methodologies, we elucidated that cyclophilin inhibition primarily mediates its anti-fibrotic effects through ECM remodelling rather than mere ECM deposition. Furthermore, our study reveals the pleiotropic therapeutic effects of RCF on multiple pathological processes in ALD patients. These findings elucidate the mechanisms of action of pan-cyclophilin inhibitors in hepatic fibrosis and ALD, highlighting the critical role of this isomerase family in ECM biology and associated disease processes.

2 | METHODS

The authors declare that all the relevant recommendations set out in the *British Journal of Pharmacology* (BJP) editorials to ensure transparency and scientific rigour in the statistical analysis were followed in this study.

2.1 | Patient recruitment and ethics

PCLS and HSCs were obtained from a portion of liver resected for the removal of colorectal cancer metastasis. Prior to use, the tissue was assessed as tumour free by a liver histopathologist. This study was conducted in accordance with the Declaration of Helsinki and approved by the local Research Ethics Committee established by the Health Research Authority (REC reference 17/NE/0340; IRAS project ID 222302). All the patients involved in the study provided written informed consent. Male and female patients were recruited in an unbiased manner. Surgeries spanned over a period from October 2018 to April 2023 and took place at King's College Hospital. The study participants did not have chronic liver disease; therefore, the specimen region far from the tumour was considered healthy. For the PCLS generation, specimens from seven patients were used whose main clinical characteristics are outlined in Table 1.

2.2 | Human PCLS preparation, culture and treatments

Liver specimens were collected immediately after resection, flushed with and kept in ice-cold sterile University of Wisconsin solution (ViaSpan; Bridge to Life Ltd, USA) until slicing. PCLS were obtained using a Krumdieck tissue slicer (Alabama R&D, USA), and their culture was set up exactly as previously described (Rastovic et al., 2023) in William's E Medium (Thermo Fisher Scientific, USA) supplemented with 5% human AB serum (Cambridge Bioscience, UK), 1% penicillin/streptomycin (Gibco, USA), 2 mM L-glutamine (Gibco, USA), ITS (10 mg l⁻¹ insulin + 5.5 mg l⁻¹ transferrin + 6.7 μ g l⁻¹ sodium selenite) (Gibco, USA), 1 nM **epidermal growth factor** (EGF; Gibco, USA), 100 nM **glucagon** (Sigma-Aldrich, USA) and 1 μ M **corticosterone** (Sigma-Aldrich, USA). The overall ex vivo culture length was 6 days in total. The day after slicing (day 0), PCLS were exposed to toxic insults: ethanol (EtOH, 250 mM, Sigma-Aldrich, USA), free fatty acids (FA, 0.1 mM mixture of **oleic acid** and **linoleic acid**, Merck, Germany) and **lipopolysaccharide** (LPS, 10 μ g ml⁻¹, Sigma-Aldrich, USA) in the following combinations: EtOH, or EtOH + FA, or EtOH + FA + **LPS**. Moreover, according to the experimental plan, slices were treated with RCF (Hepion Pharmaceuticals) either alone or simultaneously with the toxic insults. Two doses of RCF (1 and 5 μ M), previously screened for cytotoxicity in human cell cultures, were assessed in the PCLS model, and the highest dose (5 μ M) was selected for the study based on optimal response (Kuo et al., 2019). Slices and supernatants were collected after 1, 3 and 5 days from day 0 (start of the treatment). Media were changed daily, toxins and the drug were added freshly every day.

TABLE 1 Baseline characteristics of liver tissue donors for PCLS and HSCs preparation.

SUBJECT ID	Demographics				Background liver		Tumour Aetiology	Treatment (Y/N)	Alcohol	
	Gender	Age	Ethnicity	BMI	Fibrosis score	Other observations			Current/former	Units/week
PCLS-002-KCH	M	71	Caucasian	25.33	F2	No additional observation	CRLM	Y	Current	UA
PCLS-067-KCH	F	87	Caucasian	28.30	F0	50% steatosis	CRLM	Y	N	UA
PCLS-130-KCH	F	81	Caucasian	28.97	F0-F1*	Areas of parenchyma atrophy and F1 with mild CL PSF. 10% steatosis	CRLM	Y	N	UA
PCLS-132-KCH	M	39	Caucasian	UA	F0-F3*	Areas of F2–3, secondary to SOS with marked PSF and NRH 20% steatosis	CRLM	N	UA	UA
PCLS-149-KCH	F	37	Caucasian	19.36	F0	Rare pp delicate fibrous spurs	CRLM	Y	UA	UA
PCLS-156-KCH	F	69	Caucasian	17.3	F0*	Very focal pp delicate expansion and focal CL PSF	CRLM	Y	Current	<14
PCLS-159-KCH	M	40	Asian	24.8	F0	<5% steatosis	CRLM	N	N	UA
PCLS-190-KCH	M	60	Caucasian	26.7	F0	30% steatosis	CRLM	N	N	UA
PCLS-215-KCH	M	50	Caucasian	28.7	F0-F1	Variable pp delicate fibrosis and patchy CL PSF (mild SOS)	CRLM	N	UA	UA

Abbreviations: KCH, King's College Hospital; CRLM, colorectal liver metastasis; CL, centrilobular; PSF, perisinusoidal fibrosis; SOS, sinusoidal obstruction syndrome; NRH, nodular regenerative hyperplasia; UA, unavailable.

2.3 | Histological staining of PCLS

PCLS were fixed in 10% neutral buffered formalin (NBF, CellPath) overnight at 4°C, dehydrated in ascending grades of ethanol (70%, 95%, 100%, Thermo Fisher Scientific, USA), cleared in xylene (Fisher Chemical, USA) and embedded in paraffin (VWR, USA). The paraffin blocks were cut at a thickness of 4 µm using a microtome (pfm medical, Germany). Prior to staining, tissue sections were deparaffinised in xylene (Fisher Chemical, USA), rehydrated in descending grades of ethanol (100%, 96%, 70%, Thermo Fisher Scientific, USA) and rinsed in distilled H₂O. For haematoxylin and eosin (H&E) staining, the tissue sections were incubated in Modified Harris Haematoxylin (Epredia, USA), differentiated in acid alcohol (1% HCl in 70% ethanol) and incubated in eosin (Sigma-Aldrich, USA). Stained sections were dehydrated in ascending grades of ethanol solutions (70%, 96%, 100%, Thermo Fisher Scientific, USA), cleared in xylene (Fisher Chemical, USA) and mounted with DPX mounting medium (Sigma-Aldrich, USA). Picrosirius Red staining (Polysciences, USA) was performed according to the manufacturer's protocol.

2.4 | TWOMBLI analysis on Picrosirius red stained PCLS

Picro Sirius Red staining on PCLS was performed as described earlier, and collagen ECM patterns were analysed using The Workflow Of

Matrix BioLogY Informatics (TWOMBLI) as described before (Wershof et al., 2021). The analysis was conducted on two PCLS samples/conditions produced from one donor. For the analysis, the entire PCLS were imaged as a single frame-image (MIA) or as single snaps at 40X magnification, using an Olympus BX43 fluorescence microscope (Olympus, Japan). Parameters used for Sirius Red TWOMBLI analysis are displayed in Figure S1.

2.5 | Bulk RNA sequencing of PCLS

For RNA extraction, PCLS were processed with Allprotect Tissue Reagent (Qiagen, Germany) at the time of sample collection and following the manufacturer's recommendations. Total RNA was extracted using Qiagen RNeasy Mini kit following the manufacturer's instructions (Qiagen, Germany) by automated extraction. RNA samples were quantified using a Qubit 4.0 Fluorometer (Life Technologies Invitrogen, USA), and RNA integrity was checked with the RNA Kit on an Agilent 5300 Fragment Analyser (Agilent Technologies, USA). RNA sequencing libraries were prepared using the NEBNext Ultra RNA Library Prep Kit for Illumina, following manufacturer's instructions (New England Biolabs, USA). Briefly, mRNAs were first enriched with Oligo (dT) beads. Enriched mRNAs were fragmented for 15 min at 94°C. First-strand and second-strand cDNAs were subsequently synthesised. cDNA fragments were end-repaired and adenylated at 3'ends, and universal adapters were ligated to cDNA fragments,

followed by index addition and library enrichment by limited-cycle PCR. Sequencing libraries were validated using the NGS kit on the Agilent 5300 Fragment Analyser (Agilent Technologies, USA) and quantified by using a Qubit 4.0 Fluorometer (Invitrogen, USA). The sequencing libraries were multiplexed and loaded on the flow cell on the Illumina NovaSeq 6000 instrument according to manufacturer's instructions. The samples were sequenced using a 2×150 Pair-End (PE) configuration v1.5. Image analysis and base calling were conducted by the NovaSeq Control Software v1.7 on the NovaSeq instrument (Illumina, USA). Raw sequence data (.bcl files) generated from Illumina NovaSeq (Illumina, USA) was converted into fastq files and de-multiplexed using Illumina bcl2fastq programme version 2.20. One mismatch was allowed for index sequence identification. After investigating the quality of the raw data, sequence reads were trimmed to remove possible adapter sequences and nucleotides with poor quality using Trimmomatic v.0.36. The trimmed reads were mapped to the *Homo sapiens* reference genome available on ENSEMBL using the STAR aligner v.2.5.2b, generating BAM files. Unique gene hit counts were calculated by using feature counts from the Subread package v.1.5.2. Only unique reads that fell within exon regions were counted. After the extraction of gene hit counts, the gene hit counts table was used for downstream differential expression analysis. Using DESeq2, a comparison of gene expression between the groups of samples was performed. The Wald test was used to generate Log2 fold changes and *P*-values, also adjusted for multiple comparisons. Genes with adjusted *P*-values < 0.05 and absolute Log2 fold changes > 0.5 were regarded as differentially expressed genes (DEGs) in the untargeted transcriptomic analysis, whereas genes with *P*-values < 0.05 and absolute Log2 fold changes > 0.5 were considered as DEGs in the additional differential analysis performed within samples coming from the same donor. A principal component analysis (PCA) was performed using the 'plotPCA' function within the DESeq2 R package. The top 500 genes, selected by highest row variance, were used to generate the plot. An overrepresentation analysis was conducted to identify enriched biological pathways associated with the DEGs using the 'enrichPathway' function in R (v4.3.2) within Reactome database (Yu & He, 2016) (<https://reactome.org/> v1.46.0), with the significance threshold for gene enrichment set at 0.05 and a false discovery rate (FDR) threshold set at 0.10. Additionally, the Ingenuity Pathway Analysis (IPA software, Qiagen, Germany) was performed to assess the directionality of the enriched pathways, including prediction of upstream regulators and integration of omic datasets using comparison analysis.

2.6 | HSC isolation

HSCs were derived from 4 to 9 g of liver tissue. Upon collection, tissue was minced in HBSS (Hanks' balanced salt solution, Sigma-Aldrich, USA) containing 1% penicillin/ptreptomycin (P/S, Sigma-Aldrich, USA). After mechanical disruption, the tissue was then digested using 30–50 ml of Collagenase-P (0.05 M, Sigma-Aldrich, USA) prepared in DMEM (Dulbecco's Modified Eagle's Medium, Sigma-Aldrich, USA),

supplemented with 1% P/S, for 1 h at 37°C using a magnetic stirrer set at 100 rpm. After dissociation, the digested tissue was first cleaned removing leftover pieces using a metal sieve and then filtered with 100-mm cell strainers. After filtration, the tissue was centrifuged twice at $50 \times g$ for 10 min and at each time, the pellet of hepatocytes was discarded. Then, the cell suspension was centrifuged three times at $250 \times g$ for 10 min. After each spin, the pellet was collected and pooled together as 'HSC pellet'. The cells that did not pellet after the three $250 \times g$ spins were discarded. The pooled HSC pellet was washed once in 50 ml of DMEM + 1% P/S and 10% foetal bovine serum (FBS, Gibco), centrifuged at $500 \times g$ for 10 min and plated for HSC culture.

2.7 | HSC culture

HSCs were cultured in HSC Medium, composed of DMEM, 10% FBS, 1% P/S, 1% Glutamine (Gibco, USA) and 10 ng ml⁻¹ of EGF (Qkine, UK) in 5% CO₂ incubator at 37°C. HSCs were seeded at a density of 4000 cells cm⁻² in tissue culture flasks coated with rat tail Collagen-I solution (62.5 µg ml⁻¹, Sigma-Aldrich, USA) diluted 1:40 in phosphate buffer saline (PBS, Gibco, USA). HSCs were fed every other day and passaged when they reached 70–80% of confluence (approximately every 4–5 days). To subculture HSCs, media was removed, and the flask was washed twice with PBS. 1X Trypsin–EDTA (Gibco, USA) was added and incubated for 5 min at 37°C. Cell suspension was then collected, and fresh medium was added to neutralise the trypsin. Cell suspension was spun down at $250 \times g$ for 5 min. The supernatant was discarded, and the pellet was resuspended for counting. HSCs were cryopreserved in 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) in FBS and stored long term in a liquid nitrogen tank.

2.8 | HSC activation and RCF treatment

HSCs between passages 6 and 10 of culture were seeded in 24-well plates (8,000 cells per well for immunofluorescence and RNA and 20,000 cells per well for cell-derived matrix [CDM] analysis) on circular coverslips (VWR, USA, cover glasses, 13 mm diameter, 1.5 thickness). Prior to seeding with cells, the coverslips were first sterilised in 70% ethanol for 20 min and then rinsed twice with PBS. For immunofluorescence and gene expression analysis, the coverslips were coated with rat tail Collagen-I solution (62.5 µg ml⁻¹, Sigma-Aldrich, USA). For the experiments on cell-derived matrix, the coverslips were prepared following established protocols (Kaukonen et al., 2017; Kenny et al., 2023; Marcotti et al., 2021). Briefly, the ethanol-sterilised coverslips were covered with 1 ml of 0.2% sterile gelatine type B (Merck, Germany) and incubated at 37°C for 1 h. Then, the gelatine solution was gently aspirated and replaced with 500 µl of 1% glutaraldehyde (Sigma-Aldrich, USA) solution for 30 min at room temperature to allow cross-linking of the gelatine. After, the coverslips/wells were rinsed twice with PBS and then incubated with 1 ml of 1 M glycine (Sigma-Aldrich, USA) solution for 20 min at room temperature to

quench the gelatine cross-linking. Once incubated for 20 min, they were rinsed twice with PBS and immersed in 1 ml of HSC base medium for 30 min at 37°C. Finally, the coverslips/wells were rinsed once in PBS and either immediately used or stored at 4°C for up to 2 weeks. Twenty-four hours after plating, HSC medium was changed to HSC starvation medium (containing 0.5% FBS instead of 10%) for 24 h. Then, the media were replaced with HSC medium (with 10% FBS) with 2.5 ng/ml of **transforming growth factor-β1** (TGF-β1, QKine, UK), 5 μM RCF or both. Treatments were conducted for 5 days, with feeding of the cells at day 2. A graphical schematic of the 2D in vitro experiments can be found in Figure 4b.

2.9 | QuantiGene gene expression analysis of PCLS

The PCLS used for gene expression analysis were collected and processed with Allprotect tissue reagent (Qiagen, Germany) following the manufacturer's instructions. The tissue samples were processed with the QuantiGene Sample Processing Kit (Thermo Fisher Scientific, USA), and individual slices or baseline tissue were homogenised with Precellys homogeniser (Bertin Instruments, France) at 4°C in 600 μl of homogenising solution with 6 μl Proteinase K using CK14 ceramic beads (Bertin Instruments, France); programme: 2 cycles × 15 s at 5000 rpm with 1 min break on ice. The gene expression analysis was performed using the QuantiGene Plex Assay (Thermo Fisher Scientific, USA) for the following genes: *SOX9*, *ACTA2*, *TIMP1*, *COL1A1*, *PDGFRB*, and *RG55*. The relative gene expression was calculated as the ratio of the median fluorescence intensity (MFI) of each target gene versus the geometric mean of the MFI of four housekeeping genes (*RPS29*, *HPRT1*, *RPLP0* and *TBP*). Then, the fold-change for each target gene was calculated by dividing the relative expression value of each sample by the geometric mean relative expression value from the control samples.

2.10 | Analysis of soluble markers in culture supernatants

Slice culture supernatants were collected and centrifuged at $21,300 \times g$ for 10 min at 4°C to remove cell debris and were stored at -80°C until analysis. Both M65 epitope (total cell death) and caspase-cleaved M30 epitope (apoptotic death) of cytokeratin-18 were measured in PCLS supernatants by enzyme-linked immunosorbent assay (ELISA; Peviva, Sweden). The procedure was performed as per manufacturer's instructions, and the total PCLS lysate was measured as maximal cytokeratin-18 release. TIMP-1 and TGF-β1 were measured by ELISA (DTM100, DB100C, Biotechne, USA). In order to quantify the release of active form of TGF-β1, no acid activation was performed on culture supernatants. Release of N-terminal propeptide of type III collagen was measured in PCLS culture supernatants by NordicPRO-C3™ assays (Nordic Bioscience, Denmark) and by a pro-collagen type III N-terminal Propeptide ELISA (PIIINP, NBP2-76434, Biotechne, USA).

2.11 | RNA extraction from cultured HSCs, reverse transcription and quantitative polymerase chain reaction (qPCR)

RNA from HSCs was extracted using ReliaPrep™ RNA Cell Miniprep protocol (Promega, USA). Purified RNA was washed and diluted with nuclease-free water (NFW) following the manufacturer's instructions. Yield and purity of the samples were measured spectrophotometrically using a Nanodrop (Thermo Fisher Scientific, USA) at 260, 280 and 230 nm. Isolated RNA from the samples were converted to cDNA using Promega GoScript Reverse transcription kit (Promega, USA) and stored at -20°C. Real-time polymerase chain reaction (RT-PCR) was carried out to check the gene expression profile of different samples. A 20 ng of cDNA was added to 5 μl of Precision Plus Master Mix (PrecisionPLUS-R-Primerdesign Ltd., UK) for each qPCR reaction with 1X Taqman qPCR probes (Integrated DNA Technology, list of probes used in Table 2) and NFW to reach a final volume of 10 μl to be added in each well of a MicroAmp Fast Optical 96-well Reaction Plates (Starlab, Belgium). The samples were centrifuged in a plate centrifuge for 1 min to ensure the reaction mixture was fully mixed. PCR reaction cycle was then performed with 3 min of denaturation at 95°C, followed by 10 s of annealing at 95°C and 1 min extension at 60°C, which was repeated for 40 cycles using the AriaMx Real-time PCR System (Agilent Technologies, USA).

2.12 | Cell-derived matrix preparation

At time points of treatment of HSCs, the coverslips were decellularised, leaving only the ECM deposited, following established protocols (Kaukonen et al., 2017; Kenny et al., 2023). Firstly, the media was removed from the cells and replaced with 1 ml of prewarmed decellularisation buffer composed of 10 mM ammonium hydroxide (Sigma-Aldrich, USA) and 0.25% Triton X100 in PBS and incubated at 37°C for 4 min. Next, the decellularisation buffer was aspirated and replaced with PBS. The PBS was removed and replaced with decellularisation buffer for 4 min at 37°C once more before being placed in PBS again. The coverslips/wells were then covered in 1 ml of DNase I (Roche, Switzerland; 10 μg ml⁻¹ in saline containing Ca²⁺) and incubated for 30 min at 37°C to remove the residual cellular DNA. Once all the residual DNA fragments were removed, the DNase I solution was removed, and the coverslips/wells were rinsed with 1 ml of PBS.

TABLE 2 Taqman qPCR probes (from integrated DNA technology [IDT]).

Gene name	Ref Seq number	IDT assay name
ACTA2	NM_001613	Hs.PT.56a.2542642
COL1A1	NM_000088	Hs.PT.58.15517795
HPRT1	NM_000194	Hs.PT.58v.45621572
SOX9	NM_000346	Hs.PT.58.38984663
TIMP1	NM_003254	Hs.PT.58.27632928

The coverslips were stored at 4°C for up to 2 weeks to run immunofluorescence (IF) staining. The wells were then ready to extract ECM protein for proteomics.

2.13 | Immunofluorescence on HSC and CDM

The immunofluorescence analysis conducted and experimental details provided conform with BJP guidelines (Alexander et al., 2018). The coverslips containing HSCs were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich, USA) for 30 min at room temperature (RT), followed by three PBS washes for 5 min each. The coverslips containing fixed HSCs or CDM were incubated with Blocking Buffer for 1 h at RT (Blocking Buffer composed of PBS, 0.1% Triton-X 100 (Sigma-Aldrich, USA), 1% bovine serum albumin [BSA, Sigma-Aldrich, USA]). Coverslips were then incubated overnight at 4°C with primary antibodies (Table 3) diluted in Antibody Dilution Buffer, which was composed of PBS, 0.01% Triton-X 100 and 1% BSA. The next day, coverslips were washed three times with PBS for 5 min each wash. Subsequently, secondary antibodies (Table 4) were prepared in Antibody Dilution Buffer and added for 1 h at RT. Both blocking and antibody dilution buffers were prepared freshly and only used once. They were then washed with PBS three times for 5 min each wash. A few drops of mounting media containing 4',6-diamidino-

2-phenylindole (DAPI; (Abcam, UK) were added to each coverslip, and then rectangular slides (VWR, USA) were placed on coverslips. Slides were left to dry overnight and stored at 4°C prior to imaging.

2.14 | HSC imaging and analysis

For HSCs imaging, five different areas of interest were chosen from each coverslip with the same microscope setting, including laser brightness, exposure time and camera gain. All IF images were taken with an Olympus BX43 fluorescence microscope (Olympus, Japan). Representative images were taken for each channel at 40X magnification and analysed by Image J (<http://imagej.nih.gov/ij.1.53e>).

Images were analysed based on the marker used. Expression of vimentin and collagens was quantified by measuring the intensity of fluorescence in ImageJ using the measurement tool. The intensity values were then normalised to the average intensity and compared to the control group (-TGF- β , -RCF). α SMA and SOX9 were quantified discretely as there are distinct regions and patterns of fluorescence. α SMA expression was quantified by counting cells that were positive for α SMA stress fibre organisation, as opposed to diffused α SMA expression, which is not a hallmark of the myofibroblast phenotype (Hillsley et al., 2021).

TABLE 3 Primary antibodies used for immunofluorescent staining.

	Primary antibodies						
	Epitope	Company	Host/isotype and clone	Cat number	Lot number	Dilution	RRID
Antibodies for HSC staining	α -SMA	Invitrogen	Mouse/IgG2a, clone 1A4	14-9760-82	2798495	1:500	AB_2,572,996
	VIM	R&D	Rat/IgG2A clone 280,618	MAB2105	UUQO223061	1:500	AB_2241653
	SOX9	Millipore	Rabbit/IgG polyclonal	AB5535	3450032	1:500	AB_2239761
	Collagen I/II/III/IV/V	Novus	Rabbit/IgG polyclonal	PA1-85324	H00218	1:200	AB_930067
Antibodies for CDM staining	Collagen I/II/III/IV/V	Novus	Rabbit/IgG polyclonal	PA1-85324	H00218	1:200	AB_930067
	Collagen I	Proteintech	Mouse/IgG1 clone 1E9A7	67288-1-Ig	10020286	1:200	AB_2882554
	Collagen III	Proteintech	Rabbit/IgG polyclonal	22734-1-AP	00086215	1:500	AB_2879158
	Collagen IV	Novus	Goat/IgG polyclonal	NBP1-26549	H2915-RM83	1:75	AB_1853202
	FN	Abcam	Rabbit/IgG polyclonal	AB23750	GR3386400-1	1:500	AB_447655

TABLE 4 Secondary antibodies used for immunofluorescent staining.

Secondary antibodies							
Epitope, isotype, fluorochrome conjugated	Company	Host/isotype and clone	Cat number	Lot number	Dilution	RRID	
Anti-mouse IgG (H + L), Alexa Fluor 555	Thermo Fisher	Donkey/IgG polyclonal	A32773	WG322206	1:500	AB_2762848	
Anti-rat IgG (H + L), Alexa Fluor 555	Thermo Fisher	Donkey/IgG polyclonal	A48270	GR3257148	1:500	AB_2896336	
Anti-rabbit IgG (H + L), Alexa Fluor 488	Thermo Fisher	Donkey/IgG polyclonal	A32790	XG350189	1:500	AB_2762833	
Anti-goat IgG (H + L), Alexa Fluor 488	Thermo Fisher	Donkey/IgG polyclonal	A32814	WF319539	1:500	AB_2762838	
Anti-rabbit IgG (H + L), Alexa Fluor 555	Thermo Fisher	Donkey/IgG polyclonal	A32794	XH355383	1:500	AB_2762834	

2.15 | CDM imaging and analysis

To quantify the amount of CDM-proteins deposited, two coverslips were stained and imaged in six different ROIs at 20X using an Olympus BX43 fluorescence microscope. The average intensities of stained ECM protein were normalised against a negative control (secondary antibody only) stained CDM from the same HSCs line.

For ECM Fibrillar Feature Alignment using AFT (Alignment by Fourier Transform) workflow, two coverslips were stained per condition and 12 Z-stack images using a 60X objective lens were taken/coverslip using a Nikon A1R Confocal Microscope (Nikon, Japan). The 24 images were then analysed using the MATLAB workflow AFT developed by Marcotti and colleagues (Marcotti et al., 2021), following their guidelines. This workflow allows the quantification of the alignment of fibrillar features of 2D images using AFT-Alignment by Fourier Transform algorithm to generate an order parameter value, representing the overall fibre alignment of the image (Kenny et al., 2023; Marcotti et al., 2021).

Finally, for a deeper analysis of more fibrillar features of CDM proteins, the FIJI macro TWOMBLI was run on the same CDM images used for AFT analysis. TWOMBLI parameters of immunofluorescent stained CDM proteins are summarised in the table in Figure S1.

2.16 | Extraction of ECM proteins from CDM for proteomics

To recover ECM proteins from CDM, whilst preventing their degradation, RIPA buffer was used by combining 150 mM sodium chloride (Sigma-Aldrich, USA), 50 mM TRIS-HCL (pH 8) (Thermo Fisher Scientific, USA), 1% Triton-X 100 (Sigma-Aldrich, USA), 0.5% sodium deoxycholate (Sigma-Aldrich, USA), 0.1% sodium dodecyl sulphate (Sigma-Aldrich, USA) and 5 mM ethylenediaminetetraacetic acid (EDTA, Thermo Fisher Scientific, USA) in distilled water. After the decellularisation of cell cultures, as described earlier, 100 µl RIPA buffer was used to collect CDM proteins from two wells, using a rubber scraper (Starlab, Belgium). The RIPA buffer containing ECM proteins was collected into a microcentrifuge tube, 0.1X cOmplete™ Protease Inhibitor Cocktail (Merck, Germany) was added and the samples were stored at −80°C until proteomic analysis.

2.17 | Proteomic analysis on PCLS and CDM

2.17.1 | Sample preparation for proteomic analysis

Precision-cut liver slices processed with Allprotect Tissue Reagent (Qiagen, USA) and protease inhibitor cocktail (cOmplete protease inhibitor Cat# CO-RO, Merck, Germany) were treated with 500 µl of 8 M urea containing phosphatase (PhosSTOP, Merck, Cat# 4906845001) and protease inhibitors (cOmplete protease inhibitor Cat# CO-RO, Merck, Germany), following the manufacturer's instructions. Samples

were lysed using a sonic probe in 3 × 3-s bursts and immediately placed on ice to cool. All samples were diluted 1:50 in 8 M urea buffer, and protein concentration was measured by Pierce BCA Protein assay. CDM was processed with 600 µl RIPA buffer, and the protein concentration was measured by Nanodrop (Thermo Fisher Scientific, USA) at 280 nm with baseline correction at 340 nm. PCLS samples were normalised to 50 mg total protein, while CDM lysates were normalised to 100 mg total protein. All samples were balanced to 100 µl with 0.2 M EPPS buffer solution at pH 8.0 (Alfa Aesar J61476.AE).

2.17.2 | Reduction, alkylation and enzymatic digestion

Samples were reduced using tris(2-carboxyethyl)phosphine (TCEP) hydrochloride (Sigma-Aldrich, USA; C4706, MW 286.65) at a concentration of 200 mM in 0.2 M EPPS. Following reduction, samples were alkylated with iodoacetamide (IAM; Sigma-Aldrich, USA; A3221, MW 184.96) at a concentration of 20 mM in 0.2 M EPPS. To prevent unwanted alkylation reactions with amino acids other than cysteine, the IAM was quenched briefly (10-min incubation at RT) with 6 µl 200 mM TCEP to give a final concentration of 20 mM. Prior to enzymatic digestion, the protein content was precipitated with methanol-chloroform (in 4:1 ratio). Finally, 300-µl ultrapure water was added and briefly vortexed. The mixture was centrifuged at 14,000 rpm for 1 min. The top layer of liquid was removed, and the protein layer was stuck to the side of the tube for easier removal of the liquid layer below the protein disk. Samples were washed with 400 µl methanol and centrifuged at 14,000 rpm for 5 min. The supernatant was discarded, and the samples were dried briefly in a SpeedVac. The protein pellets were resuspended in 100 µl 0.2 M EPPS by vortexing and sonication in an ultrasonic water bath for 10 min. Enzymatic digestion was performed using a trypsin/LysC mix (Promega, USA; V5073) to cleave at arginine and lysine residues in a 1:100 enzyme:substrate ratio. Samples were incubated at 37°C overnight. Digested peptides were cleaned with Pierce peptide desalting columns CD18 spin columns (5 µg to 5 mg Cat. #89852, Thermo Fisher Scientific, USA) following the manufacturer's instructions. Samples were acidified to a final concentration of 0.1% trifluoroacetic acid (TFA) and checked with pH strips (Dosatest pH 0–14, VWR #85410.101) to determine pH less than 2. Samples were completely dry-eluted in a SpeedVac. The eluted peptide mixture was resuspended in 100 µl 0.2 M EPPS, with 25 µl (25 mg) of sample taken for TMT labelling.

2.17.3 | Peptide labelling with TMTpro

The TMT16pro isobaric label reagent (5 mg), (Thermo Fisher Scientific, USA; A44521) (Lot# XA339839) was used to tag the samples chemically. The reference sample was produced by combining 2 µl of each liver tissue sample in the experiment prior to labelling to reduce the technical error between the two TMT16 pro kits used in the analysis.

2.17.4 | Liquid chromatography with tandem mass spectrometry (LC-MS/MS) TMT ratio check

The TMT labelled peptide mix was resuspended in 50 ml of resuspension buffer (2% ACN in 0.05% TFA) with 6 ml sample (2.9 mg on column) for analysis by Orbitrap Fusion Lumos. Chromatographic separation was performed using a U3000 UHPLC NanoLC system (Thermo Fisher Scientific, USA). Peptides were resolved by reversed phase chromatography on a 75 mm C18 Pepmap column (50 cm length) using a linear gradient of 80% acetonitrile in 0.1% formic acid. The gradient was delivered to elute the peptides at a flow rate of 250 nl min⁻¹ over 60 min and ionised by electrospray ionisation using an Orbitrap Fusion Lumos (Thermo Fisher Scientific, USA) operating under Xcalibur v4.4.16.14.

2.17.5 | Determination of TMT labelling efficiency

MS/MS raw data were processed and searched against the current Human Uniprot database (51,079 entries) using the Sequest (Eng et al., 1994) searching algorithm within Proteome Discoverer version 2.5. Labelling efficiency has been determined at 1% FDR with variable modifications including oxidation, (M), TMTpro (K, N-term) and carbamidomethylation (C) as a static modification. Once labelling efficiency had been determined, the ratio of TMT reporter ion S/N intensity values for each channel was summed and plotted prior to mixing all channels in a 1:1 ratio.

2.17.6 | High pH reversed-phase peptide fractionation of TMT peptides and enriched phosphopeptides

The Pierce High pH Reversed-Phase Fractionation Kit #84868 was used following the manufacturer's instructions, with the modification of replacing the supplied columns with the C18 peptide desalting columns from Thermo Fisher Scientific, USA; #89852 (5–5 mg). Samples were solubilised in 300 ml 2% TFA, vortexed and sonicated in an ultrasonic bath for 10 min. Around 300 µl of sample were added to a pre-conditioned C18 column in a 2.0 ml Lo-bind tube and centrifuged at 3000 × g for 2 min, retaining the flow through. The sample was reloaded to the column, following which the column was washed with 300 ml of ultrapure water and eluted with the ascending concentration of acetonitrile and decreasing concentrations of triethylamine for a total of eight washes. All eluted fractions were dried to completion in a SpeedVac.

2.17.7 | LC-MS/MS analysis of prepared fractions

All fractionated samples were resuspended in 30 ml of 2% ACN/0.05% TFA, vortexed well and centrifuged at 14,000 rpm for 1 min. Samples were injected at a volume of 6 ml using an HCD SPS MS3 method over a 2-h LC gradient run.

2.17.8 | Database searching

Raw mass spectrometry data were processed and searched as previously described with the following parameters:

Spectrum Selector

Min. Precursor mass:350 Da
Max. Precursor mass:10,000 Da
S/N Threshold (FT-only):1.5

Sequest

Database:Uniprot Human
Enzyme:Trypsin
Missed cleavage:2
Precursor mass tol:20 ppm
Fragment mass tol:0.6 Da
Static modifications:TMTpro (K)
TMTpro (Peptide N-Term)
Carbamidomethyl (C)
Variable modifications:Oxidation (M)

Peptide validator

Target FDR (strict):0.01
Target FDR (relaxed):0.05

Reporter ion quantifier

Integration window tol.:20 ppm
Integration method:Most confident centroid

Within the consensus processing module, the reporter ion intensity values (signal-to-noise) for each peptide spectral match were grouped with other identified peptides and calculated at the protein level identification as a grouped abundance. All grouped abundances at the protein level were normalised using the total peptide amount calculated as the total sum of the abundance values for each channel over all peptides identified within a file corrected by the channel with the highest total abundance as a reference.

2.17.9 | Differential abundance analysis

Processed data were analysed using MATLAB 2023a (Mathworks). The two TMT samples referring to TG samples were corrected for batch effect by ComBat algorithm (Bindea et al., 2009; Johnson et al., 2007; Shannon et al., 2003). PCA was performed by alternating least squares (ALS) algorithm to handle proteins with missing values. Paired two-sided t-test was performed to detect variations between relevant conditions, according to an uncorrected *P*-value less than 0.05 and an absolute fold change larger than 1.5. Differentially expressed proteins are reported in the Supporting Information data files and visualised in volcano plots, Venn diagrams, and heat maps after hierarchical clustering with Euclidean distance and complete linkage. Enrichment analysis of differentially expressed proteins in pair comparisons was performed in ClueGO v. 2.5 (Bindea et al., 2009) within Cytoscape v. 3.9 (Shannon et al., 2003) environment for category clustering (according to κ -score) and network visualisation. The analysis was performed within Gene Ontology - Biological Processes and Cellular Components (GO-BP and GO-CC, <https://www.ebi.ac.uk/GOA>) and Reactome (<https://www.ebi.ac.uk/Reactome>)

[reactome.org](https://www.reactome.org)) databases, selecting categories with a *P*-value, corrected by Benjamini–Hochberg method, less than 0.05. Gene Ontology category number was reduced by GO Term Fusion option of ClueGO.

2.17.10 | Atomic force microscopy (AFM) force spectroscopy measurements and data analysis

200- to 250- μm thick tissue sections were thawed in PBS before being placed in 60 mm dishes using a diagonally cut Pasteur pipette. Excess PBS was removed by carefully blotting with tissue paper. The 150- μm sections of 1.5% (w/v) agarose were cut using a vibratome and kept hydrated in PBS prior to use. To hold tissue samples in place for AFM measurements, agarose sections were blotted with tissue paper to remove excess PBS, and two small windows were created using a 2-mm biopsy punch. Gorilla glue was then added to the edges of the agarose section using a pipette tip before it was carefully placed over the tissue sample in the 60-mm dish and submerged in PBS.

A JPK CellHesion 300 was used to perform AFM force spectroscopy measurements. Tipless triangular silicon nitride cantilevers from Bruker (MLCT-O10) with a spring constant (K) $\approx 0.03 \text{ N m}^{-1}$ were modified with 37 μm polystyrene spherical beads (Microparticles GmbH, cat. no. PS-R-37.0), which were attached using UV curable Loc-tite superglue (Norman et al., 2021). The cantilever was allowed to equilibrate for 20–30 min in PBS, before calibration was performed using the thermal noise method. A DinoLite camera was mounted on top of the AFM head for sample visualisation, which allowed for measurements to be collected on the tissue through the agarose sampling window. Four force maps (100 $\mu\text{m} \times 100 \mu\text{m}$, 64 force curves per map) were generated for each sample at a setpoint of 2.6 nN and a loading rate of 5 $\mu\text{m s}^{-1}$. Force curves were evaluated for quality (Figure S2), and the Hertz model was used to calculate Young's modulus of accepted curves using proprietary JPK software (DP version 6.4.12).

2.18 | Materials

Rencofilstat (RCF) was obtained from Hepion Pharmaceuticals (Edmonton, Canada). Details of other materials and suppliers are provided in specific subsections in Methods.

2.19 | Data and analysis

Statistical tests were performed in accordance with the BJP's recommendations and requirements on experimental design and analysis (Curtis et al., 2022). The studies were designed to generate groups of equal size, with samples being randomly allocated to each treatment group and analysis carried out in blind whenever possible. The experimental design of this study included patient-matched PCLS and primary HSC cell lines derived from the same donor. Multiple slices from each donor were exposed to all tested conditions, enabling within-

donor comparisons and enhancing the rigour and robustness of the findings. Because of inherent heterogeneity of the liver tissue, which is preserved in PCLS, individual PCLS samples were considered independent. Intra-individual donor specificity implicit in this design was accounted for in the statistical models. If necessary, raw data were log-transformed to reduce skew, as indicated in the figures. No outliers were excluded in this study. For relative gene expression in PCLS experiments, data were normalised to the mean of the untreated controls. For HSC experiments, data were normalised by pairwise matching each treated sample to its corresponding untreated control. In the manuscript, *N* (slices) indicates the number of independent samples per condition and *n* (donors) indicates the number of individual liver tissue donors from whom PCLS and HSCs were obtained. Group sizes were determined based on feasibility and consistency with established practices in the literature, using human-derived experimental models. Each group included multiple biological replicates obtained from a minimum of three and up to seven tissue donors. This approach aligns with standard practices reported in comparable studies in the field. Statistical analysis was performed only when the group size was $n \geq 5$ independent samples (slices or corresponding supernatants) originated from multiple tissue donors. When these criteria were not met because of limited tissue availability, no statistical test was applied and these results were referred to as preliminary (N [slices] < 5 Figure 1d and for HSC cultures, Figures 4 and 5). All the continuous variables measured on PCLS were compared by two-way analysis of variance (ANOVA) model, for data normally distributed per Shapiro–Wilk test. All the tests were performed at two-tailed 5% significance level ($\alpha = 0.05$). Post-hoc tests were run only if *F* achieved $P < 0.05$ and there was no significant variance inhomogeneity. For significant models, individual multiple comparisons between treatments and conditions were then carried out with a Šidák's post hoc multiple comparisons adjustment. All the analyses were conducted using GraphPad Prism (10.2.3).

2.20 | Nomenclature of targets and ligands statement

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <https://www.guidetopharmacology.org>, and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/2024 (Alexander, Christopoulos, et al., 2023; Alexander, Fabbro, et al., 2023; Alexander, Kelly, et al., 2023).

3 | RESULTS

3.1 | Cyclophilin inhibition via RCF affects pathways associated with hepatic fibrosis

To evaluate the impact of pan-cyclophilin inhibition by RCF in ALD, organotypic cultures of human PCLS were exposed to ethanol alone (EtOH) or in combination with fatty acids (EtOH + FA) and

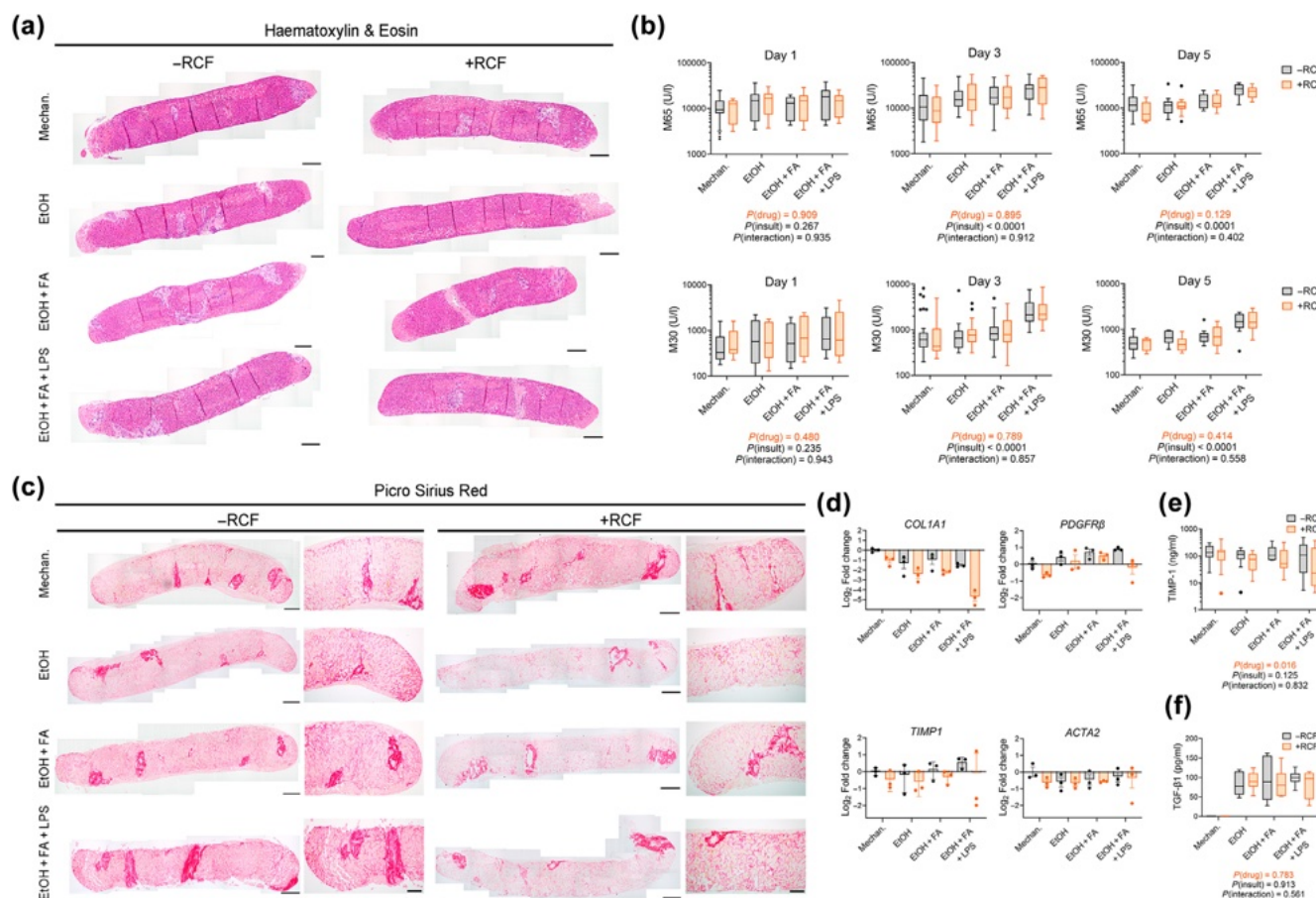


FIGURE 1 Treatment with RCF impacts fibrosis in PCLS. (a) Haematoxylin and eosin (H&E) staining of PCLS at day 3 of the culture. Manually stitched image acquisition of the whole slice using multiple image alignment (MIA) option in Olympus cellSens Standard 2.3 software (scale bar = 200 μ m). (b) Longitudinal cytokeratin-18 release in PCLS supernatants indicative of total epithelial cell death (M65 epitope) and apoptosis (M30 epitope) measured by ELISA. For day 1: $N(\text{slices}) = 9$ per condition and $n(\text{donors}) = 3$. For day 3: $N(\text{slices}) > 18$ per condition and $n(\text{donors}) = 7$. For day 5: $N(\text{slices}) = 12$ per condition and $n(\text{donors}) = 4$. Statistical analysis: two-way ANOVA model. Graph type: Tukey boxplot. (c) Picrosirius red staining of PCLS at day 5 of the culture. Manually stitched image acquisition of the whole slice using multiple image alignment (MIA) option in Olympus cellSens Standard 2.3 software, and a selected area (scale bar = 100 μ m). (d) Preliminary QuantiGene analysis of gene expression of selected pro-fibrogenic markers in PCLS at day 5. $N(\text{slices}) = 3$ per condition and $n(\text{donors}) = 3$. Data normalised to the mean gene expression of the control untreated slices (Mechan., -RCF). Mean $\pm$ SEM. (e) TIMP-1 concentration measured by ELISA in PCLS supernatants at day 5 $N(\text{slices}) > 12$ per condition and $n(\text{donors}) = 4$. Statistical analysis: two-way ANOVA model. Graph type: Tukey boxplot. (f) Active TGF- β 1 concentration in PCLS supernatants at day 3. $N(\text{slices}) = 6$ per condition and $n(\text{donors}) = 3$. Statistical analysis: two-way ANOVA model. There was no TGF- β 1 detected in mechanical only condition so it was not considered in statistical analysis. Graph type: Tukey boxplot.

lipopolysaccharide (EtOH + FA + LPS) to mimic the lipid derangement and inflammatory components of advanced disease and clinically relevant features observed in patients (Rastovic et al., 2023). Control slices not exposed to any toxic insults were referred to as “mechan.”, which is an intrinsic response activated by the mechanical injury, and observed in all PCLS. Firstly, to investigate the impact of RCF on liver histology, PCLS exposed to the drug with/without ethanol insults and stained by H&E were assessed blindly by an expert liver histopathologist. The histological examination focussed on parenchyma organisation, tissue appearance and pathological features such as steatosis, fibrosis and cell death. Previously shown features associated with the model (Rastovic et al., 2023) were observed in both untreated and RCF-treated slices. These included minimal coagulative necrosis located parallel with the cut surface of the PCLS, which did not follow

the lobular pattern (Figure 1a). In both groups, the portal tracts were maintained with the occasionally attenuated portal vein and the presence of ductular reaction irrespective of drug treatment. Lastly, signs of hepatocyte changes appearing as granular pale cytoplasm were observed in sporadic areas in PCLS treated with RCF, but this was not associated with increased apoptosis or necrosis. Indeed, in accordance with the histological analysis, the levels of cytokeratin-18 measured after 1, 3 and 5 days of treatment in culture supernatants as an indication of total epithelial cell death (M65 epitope) or apoptosis (M30 epitope) were not significantly different between the slices exposed, or not, to the drug (Figure 1b).

PCLS samples were then stained with Picrosirius Red (PS) to evaluate the impact of the cyclophilin inhibitor on fibrosis. RCF appeared to decrease PS staining (Figure 1c), but the quantification was

inconclusive because of heterogeneity between tissue areas and the risk of sampling bias. However, a preliminary screening on selected key proteins involved in liver fibrosis highlighted a consistent impact of RCF on fibrogenesis supported by a trend of decrease in the gene expression of *COL1A1* and *PDGFRB* (Figure 1d) and significantly reduced secretion of the pro-fibrogenic marker tissue metalloprotease inhibitor TIMP-1 (Figure 1e) in PCLS treated with RCF (with/without alcohol insults). Similarly, preliminary investigation of secreted N-terminal propeptide of type III collagen (measured as PIINP and PRO-C3) showed a reduction in PCLS exposed to EtOH + FA + LPS induced by RCF, in three out of four patients (Figure S3). Treatment with the cyclophilin inhibitor did not affect the levels of the active form of TGF- β 1, a major driver of fibroblast activation and ECM production, in the supernatants from PCLS exposed to RCF when compared to untreated slices, suggesting an effect downstream of TGF- β 1 (Figure 1f).

3.2 | RCF affects collagen deposition and ECM remodelling pathways at a transcriptional level

To achieve a more comprehensive analysis of the impact of the cyclophilin inhibitor RCF on liver fibrosis, bulk RNA sequencing was performed in PCLS cultured for 3 days and grouped in three conditions: (1) mechanical only, (2) EtOH alone and (3) EtOH + FA + LPS.

In slices not exposed to toxic insults (mechanical only), PCA showed separation between the untreated and RCF-treated samples. The exploratory untargeted analysis of the RNAseq dataset identified 773 differentially expressed genes (DEGs) (Figure 2a), while the over-representation analysis of DEGs with ClusterProfiler highlighted an over-representation of genes involved in several pathways within the Reactome dataset associated with liver fibrosis (Figures S4 and S5).

In accordance, the top 15 pathways in the ingenuity pathway analysis (IPA, Qiagen) showed down-regulation of 'extracellular matrix organisation', 'collagen biosynthesis and modifying enzymes', 'pulmonary fibrosis idiopathic signalling pathway', 'integrin cell surface interactions', 'assembly of collagen fibrils and other multimeric structures', 'collagen chain trimerisation' and 'collagen degradation' in PCLS (mechan.) treated with RCF (Figure 2b). Additionally, the analysis of the upstream regulators predicted the inhibition of the main transcription factors and activators of fibrogenesis and inflammation, such as *TGFB1*, *TNF*, *ERBB2*, lipopolysaccharide and *SMAD3*. At the same time, the analysis predicted the activation of some regulators involved in reducing inflammation (e.g. *dexamethasone*) or promoting liver function (e.g. *HNF4A*) (Figure 2c).

In PCLS exposed to RCF in the presence of insults (EtOH or EtOH + FA + LPS), the primary determinant shaping the grouping of samples in the PCA appeared to be the patient. Specifically, donor origin exerted a stronger influence on the pathway analysis outcomes than the drug treatment (Figure S6). However, when the impact of RCF was analysed in each patient separately, approximately 100 DEGs were identified in each comparison. Few DEGs were shared among patients (Figure S7), but over-representation analysis within the

Reactome database highlighted that most pathways affected by RCF were common to all three patients across the conditions. Among these, 'extracellular matrix organisation', 'ECM proteoglycans', 'collagen formation', 'collagen degradation' and 'integrin cell interactions' were commonly identified in all patients and conditions (Figure 2d). This mirrored the effect of RCF on mechanically induced fibrosis in the PCLS.

Given the strong impact of RCF on collagen-related pathways revealed by the over-representation analysis, we assessed the effect of RCF on the gene expression of different collagens and **fibronectin** (from the Matrisome database (Hynes & Naba, 2012) in our dataset. RCF treatment caused the down-regulation in the expression of many collagen genes, including *COL1A1*, *COL1A2*, ***COL3A1***, ***COL4A1***, *COL4A2*, *COL4A3*, *COL4A4*, *COL4A5* and fibronectin (*FN1*) in all three conditions and despite inter-patient variability (Figure 2e).

Similarly, given the prevalence of pathways affected by RCF such as 'ECM organisation' and 'collagen degradation', we explored the expression of selected matrisome proteins and enzymes linked to ECM remodelling (Figure 2e). A trend of down-regulation induced by RCF confirmed the impact of the drug on ECM reorganisation.

Taken together, these findings showed the impact of pan-cyclophilin inhibition on genes involved in collagen turnover and ECM remodelling.

3.3 | Down-regulation of proteins associated with ECM organisation and remodelling is induced by pan-cyclophilin inhibition

To further dissect the mechanism(s) by which RCF is exhibiting an anti-fibrotic effect, we performed an exploratory LC-MS proteomic analysis in PCLS exposed to the drug. RCF had a distinct impact on the protein signatures in the three PCLS cultures (mechanical only, EtOH and EtOH + FA + LPS), with PCA plots showing a clear separation of samples in all conditions (Figure 3a). Proteomics analysis identified differentially expressed proteins following treatment with RCF across the three experimental conditions with varying degrees of overlap (Figure 3b). Interestingly, the pattern of down-regulated/up-regulated proteins was dependent on the type of insult (Figure 3c), suggesting that RCF affects different proteins in the presence of different cellular stressors associated with CLD. Proteins involved in ECM organisation and remodelling were consistently found down-regulated by RCF, including key protagonists of hepatic fibrosis such as **collagen type I, III, IV** and VI, **fibronectin (FN1)**, *EMILIN1*, *decorin (DCN)*, *tenascin-C (TNC)*, *tenascin-X (TNXB)*, *biglycan (BGN)* and *lumican (LUM)* in the core matrisome, and **galectins (LGALS1, LGALS3, LGALS4)**, *syndecan 4 (SDC4)*, *tissue inhibitor of metalloproteinases 1 (TIMP1)* and **cathepsin D (CTSD)** in the ECM associated proteins (Figure 3d,e).

Enrichment analysis within the Reactome and Biological Processes databases confirmed the down-regulation of pathways involved in ECM organisation, collagen formation and organisation, and ECM proteoglycans, among other metabolic pathways in all three

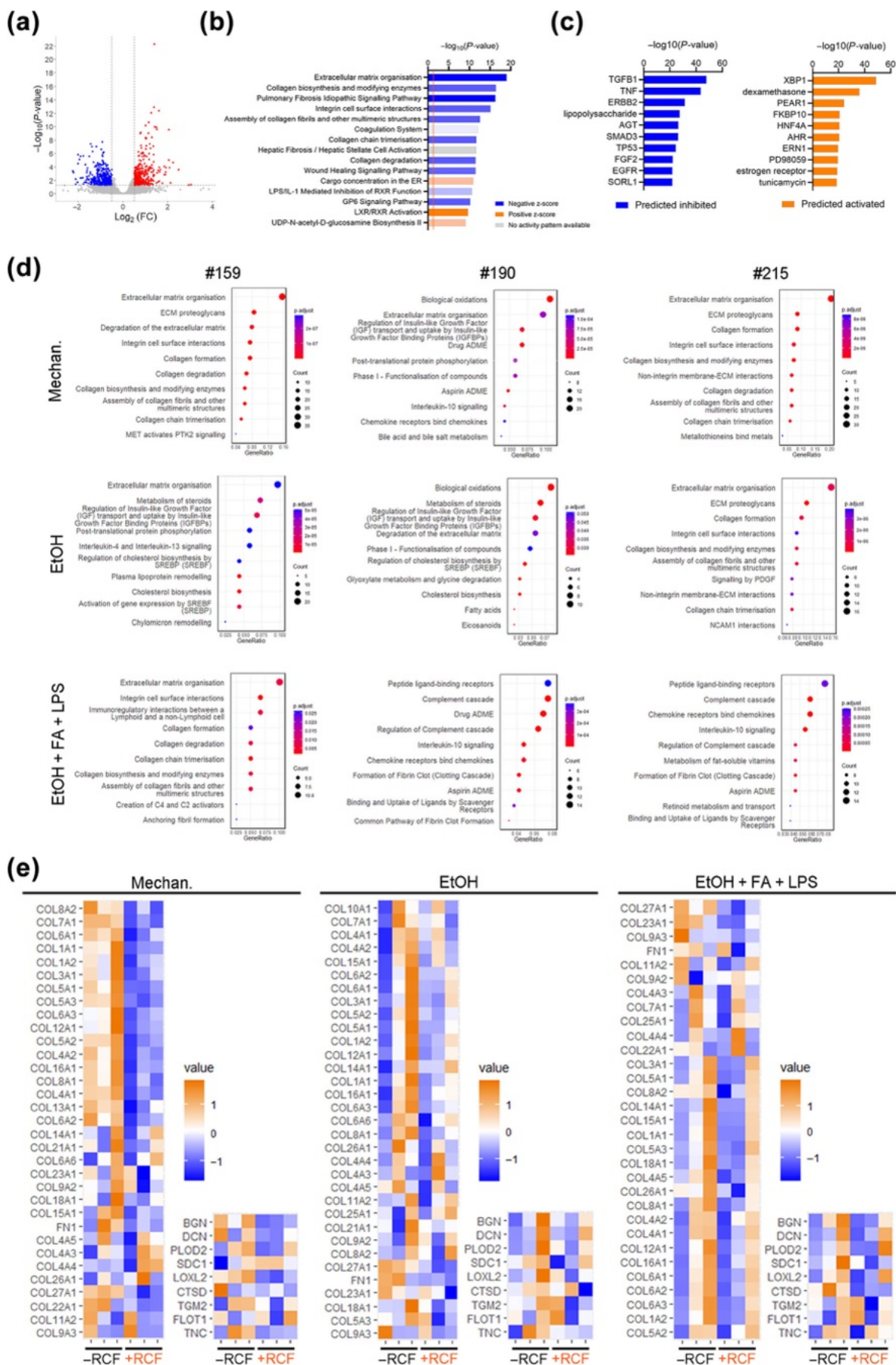


FIGURE 2 Legend on next page.

conditions (Figures S8–S10), consistent with our observations during the transcriptomic analyses. Interestingly, RCF had an additional effect in the presence of ethanol, leading to down-regulation of the pathways involved in apoptotic execution phase and plasma lipoprotein remodelling (Figure S9), suggesting beneficial effects of the drug in reducing cell toxicity and regulating lipoprotein metabolism in ALD. Furthermore, in the EtOH + FA + LPS condition, in addition to down-regulating ECM proteoglycan pathways, RCF also up-regulated the antioxidant responses mediated by NRF2 (*NFE2L2* gene) (Figure S10) (Fuentes-Agudo et al., 2023), which is known to play an important protective role against acute alcohol-induced hepatic damage (Sun et al., 2018).

Additionally, in all three conditions, IPA comparison analysis identified that the pathways and the upstream regulators affected the most by RCF treatment based on activation score (Figure 3f,g). Once again, we detected a down-regulation of pathways involved in ECM organisation; integrin cell surface interactions; collagen biosynthesis; and modifying enzymes, as well as predicted inhibition of SMAD3, TGF β 1, *TEAD1*, *CCR2* and MRTFB, critical regulators involved in liver fibrosis, inflammation, HSC activation and integration of mechanical cues in response to tissue stiffness. The down-regulation of pathways involved in ECM organisation, fibrosis and collagen synthesis by RCF was further corroborated by integrative analysis of the transcriptomic and proteomic data (Figure S11). Analysis of the Extracellular Matrix Organisation pathway using IPA revealed that RCF influenced multiple proteins across all three conditions (Figures S12–S14). Specific affected proteins varied among conditions, showing consistent down-regulation. This outcome suggests that although RCF targets distinct proteins under different cellular stressors and stimuli, its overall effect converges on shared biological processes, primarily ECM remodelling. Overall, these results indicate that RCF exhibited anti-fibrotic effects in patient-derived models of fibrosis induced by ethanol and other insults, by decreasing the deposition of collagen and affecting the expression of hepatic ECM proteins involved in matrix remodelling.

Next, we explored the effect of RCF on ECM fibre alignment in $N = 12$ liver slices derived from one patient using the Workflow Of Matrix BioLogY Informatics—TWOMBLI pipeline (Wershof et al., 2021) (Figure S1). Preliminary analysis showed that pancyclophilin inhibition by RCF affected the collagen fibre alignment and showed a trend of reduction of alignment in EtOH and EtOH + FA + LPS conditions (Figure S15), suggesting substantial ECM remodelling by the drug in the context of liver fibrosis.

We then evaluated the impact of RCF in clinically relevant disease processes identified in patients with ALD, using publicly available datasets. We compared the differentially expressed proteins following the treatment with RCF in the PCLS model to the liver proteomic dataset from patients with ALD with increasing severity of fibrosis (Niu et al., 2022). Preliminary analysis showed that 25 proteins dysregulated in ALD patients were identified as differentially expressed in slices treated with RCF and overlapping among any combination of insults (Figure 3h). When the insults were analysed separately against the ALD patient cohort, 74 proteins were found altered by RCF in PCLS exposed to mechanical injury, 104 in EtOH condition, and 79 in slices exposed to EtOH + FA + LPS (Figure S16). This analysis showed that RCF in the liver slices reversed the pathological signatures observed in ALD patients, by down-regulating the expression of ECM proteins and regulators involved in ALD-specific fibrosis, such as biglycan (BNG), collagen type IV, EMILIN1, fibrillin (FBN1), TGF β -induced protein (TGFB1) and tenascin-C (TNC), and by up-regulating expression of different enzymes involved in metabolic processes, including aldehyde dehydrogenase ALDH8A1.

Overall, these results demonstrate that RCF drives changes in the expression of proteins that are critical in the pathogenesis and progression of ALD.

3.4 | Cyclophilin inhibition affects ECM deposition by transdifferentiated HSCs

To further elucidate the mechanism of action of RCF in reducing fibrosis through modulation of ECM remodelling, we studied the effect of cyclophilin inhibition on the phenotype and matrix deposition patterns in activated primary HSCs obtained from the same patient samples used for PCLS cultures. HSCs are the main cell population involved in ECM deposition and reorganisation during wound healing and fibrogenesis. Based on the results from PCLS that predicted inhibition of TGF β signalling with RCF (Figure 4a) and given the known role of TGF β in driving fibrosis by HSCs, we investigated the effect of RCF on HSCs exposed to TGF β for up to 4 days of culture. Primary HSCs isolated from human liver tissue were cultured on collagen-coated plastic with TGF β and RCF; changes to cell phenotype and ECM remodelling patterns were analysed (Figure 4b).

Preliminary results indicated that primary HSCs exposed to TGF β transdifferentiated into a myofibroblast phenotype as determined by an increased proliferation rate, protein expression of α -SMA and

FIGURE 2 Exploratory transcriptomic analysis reveals that RCF treatment downregulates pathways related to ECM organisation and impacts the gene expression of ECM components and various ECM regulators. (a) Volcano plot showing differentially expressed genes ($\text{Log}_2(\text{FC}) > |0.5|$ and adjusted P -value < 0.05) following the treatment with RCF in PCLS at day 3 – Mechan. only condition. The red dots represent the up-regulated genes, and the blue dots represent the down-regulated genes. $N(\text{slices}) = 3$ per condition and $n(\text{donors}) = 3$. (b) Top 15 pathways affected by RCF identified by IPA analysis in PCLS at day 3 – Mechan. only condition. P -value threshold 0.05 (red line). (c) Top 10 predicted upstream regulators by IPA analysis (Qiagen) in PCLS at day 3 – Mechan. only condition. Blue – predicted inhibited, Orange – predicted activated. (d) Dot plots showing the top 10 over-represented pathways per patient ($n = 3$; patient ID: #159, #190, #215) and type of insult following treatment with RCF at day 3 ordered per gene ratio. (e) Heatmaps showing the effect of RCF on collagens, fibronectin and ECM regulators gene expression in PCLS per type of insult at day 3.

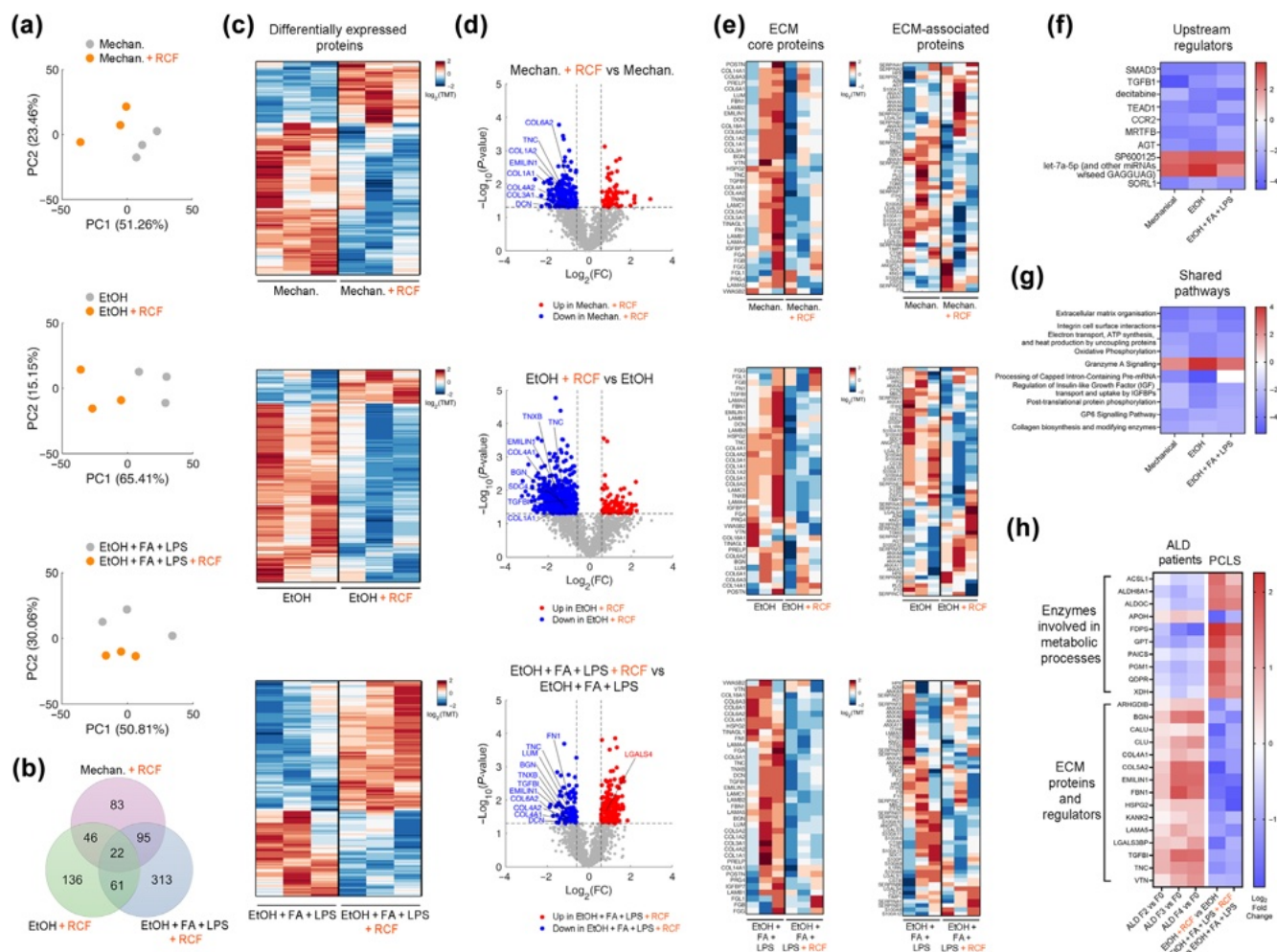


FIGURE 3 Exploratory proteomic analysis suggests that the anti-fibrotic effects of RCF are exerted through ECM remodelling. (a) Principal component analysis (PCA) of protein abundance in PCLS treated with RCF for 5 days in three conditions: mechanical, EtOH, EtOH + FA + LPS. *N* (slices) = 3 per condition and *n*(donors) = 3. (b) Venn diagram of significantly differentially expressed proteins after treatment with RCF. (c) Heatmaps of differentially expressed proteins in all three conditions (TMT: tandem mass tag). (d) Volcano plots of differentially expressed proteins in PCLS with ECM core and associated proteins indicated on the graphs. The red dots represent the statistically significant up-regulated proteins, and the blue dots represent the statistically significant down-regulated proteins. FC: fold change (e) Heatmaps showing the relative abundance of different ECM core and ECM-associated proteins following the treatment with RCF (TMT). (f) Top 10 upstream regulators identified by IPA comparison analysis shared in all three conditions ordered per z-score. (g) Top 10 shared enriched pathways identified by IPA comparison analysis in all three conditions ordered per z-score. (h) Heatmap of differentially expressed proteins in ALD patients with different stages of fibrosis significantly affected by RCF treatment in the PCLS model.

collagens (type I, II, III, IV and V), up-regulation of fibrogenic genes *ACTA2*, *COL1A1* and *SOX9* and change in morphology (Figure S17a–e). Primary HSC-derived myofibroblasts showed strong ECM deposition (cell derived matrix; CDM) in vitro as a result of transdifferentiation. ECM proteins, known to be accumulated in fibrotic livers (Arteel & Naba, 2020), were produced in large amounts by cultured HSCs only in the presence of TGF- β , showing that without this fibrogenic factor, their culture on collagen-coated plastic maintained low transdifferentiation levels. This CDM included core matrisome proteins such as FN1 and collagens, which were absent in cells cultured without TGF- β (Figure S17f). For this reason, only HSCs cultured in the presence of TGF- β were exposed to RCF for further

ECM deposition and remodelling analysis. Treatment of primary HSCs with RCF but without TGF- β had minimal effects on cell activation, cell morphology and expression of activation markers (i.e. α -SMA, SOX9, TIMP1 and vimentin) (Figure S18a–c). In the exploratory analysis of HSCs derived from four donors, only collagen abundance indicated by immunostaining was strongly reduced by RCF, although no changes in *COL1A2* gene expression were detected (Figure S18b,c). Primary HSC activation was moderately reduced when cells were treated with RCF together with TGF- β . While no change in cell proliferation was detected, both α -SMA gene and protein expression showed a trend of down-regulation induced by RCF (Figure 4c,d). Expression of other activation markers TIMP1, SOX9, vimentin and

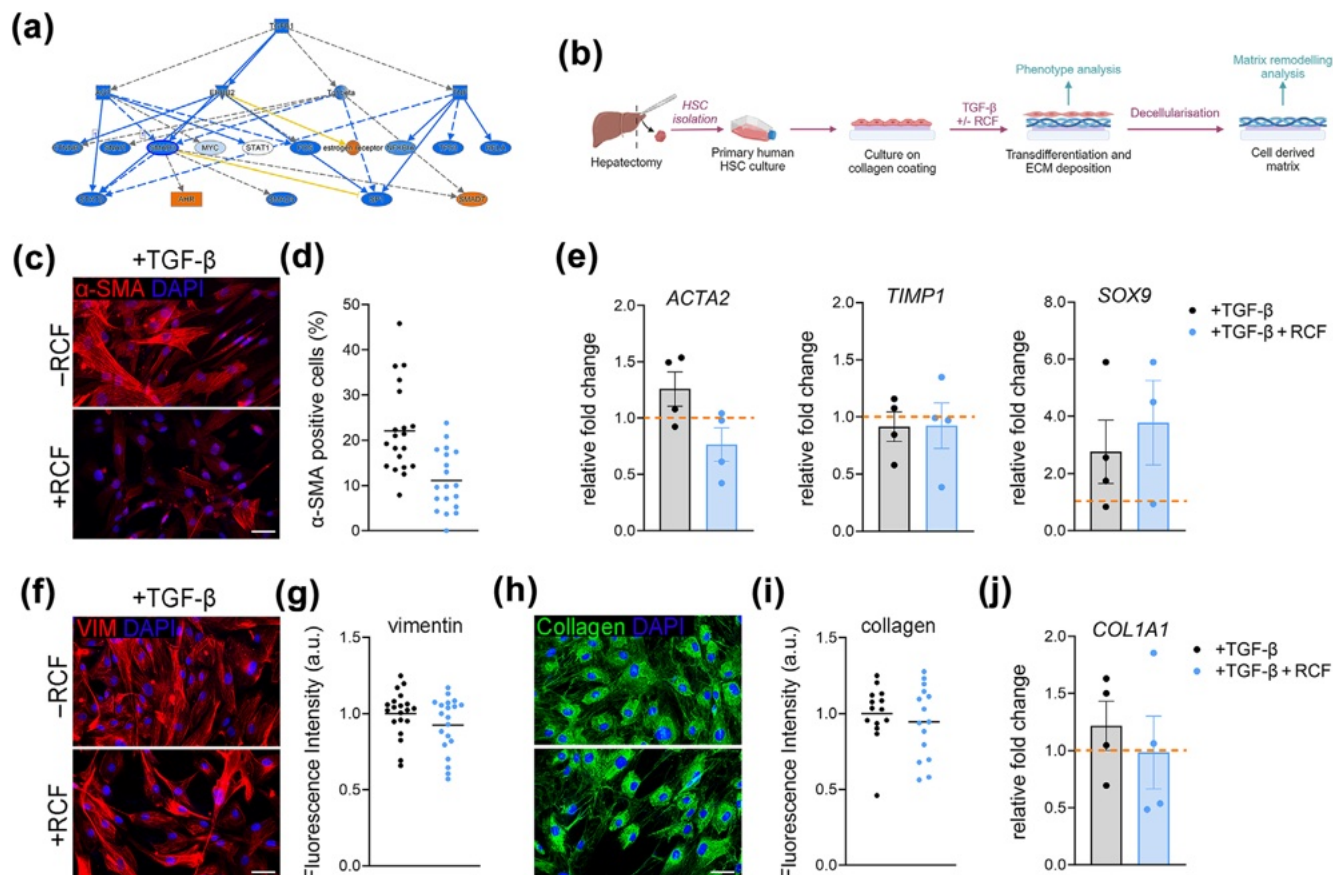


FIGURE 4 Hepatic stellate cells activation in response to co-treatment with TGF- β and RCF. (a) Predicted inhibition of TGF- β signalling pathway via RCF in the PCLS model. Blue: down-regulation by RCF. Orange: up-regulation by RCF. Image produced with IPA software (Qiagen). (b) Schematic of HSC isolation and culture for phenotypic and matrix remodelling analysis (made with Biorender). (c and d) Representative immunofluorescence images of activated HSCs after treatment with RCF stained for myofibroblast marker α -SMA and quantification over DAPI + total cell count (scale bar = 50 μ m, N = 4 cell lines, 15–20 ROI/cell line). (e) Gene expression analysis of activation genes ACTA2, TIMP1 and SOX9 (N = 4 cell lines, data normalised against housekeeping gene HPRT1 and untreated HSCs as control). (f and g) Representative immunofluorescence images of HSCs stained for fibroblast marker vimentin (VIM) and quantification measured with intensity of fluorescence (scale bar = 50 μ m, N = 4 cell lines, 15–20 ROI/cell line). (h and i) Representative immunofluorescence images of HSCs stained with an antibody targeting multiple collagen isoforms (I/II/III/IV/V) and quantification measured with intensity of fluorescence (scale bar = 50 μ m, N = 4 cell lines, 15–20 ROI/cell line). (j) Gene expression analysis of COL1A1 gene in activated HSCs after treatment with RCF (N = 4 cell lines, data normalised against housekeeping gene HPRT1 and untreated HSCs per respective cell line as control).

collagen seemed not to be affected at transcriptional or protein level by RCF treatment (Figure 4e–j).

3.5 | RCF treatment induces ECM remodelling in HSC cultures by altering the ECM fibre protein composition and organisation

Using the CDM model from primary human HSCs stimulated with TGF- β , we explored the effects of cyclophilin inhibition. Preliminary data showed that RCF treatment induced a decrease in the deposited amount of FN1 and collagen type I and III. Conversely, RCF had no effect on collagen type IV in the CDM, which represented the least produced core ECM protein among the ones quantified by immunofluorescence (Figure 5a). In accordance with these data, an explorative analysis by LC-MS of the biochemical composition of CDMs

suggested that several proteins were up-regulated and down-regulated (minimum 1.5-fold change) by RCF treatment (Figure 5b). We detected an overall down-regulation of processes involved in collagen formation, collagen assembly and ECM organisation (enrichment analysis) (Figure 5c). This was accompanied by changes in relative amounts of ECM core proteins and proteins involved in matrix remodelling in the CDM obtained from HSCs treated with both TGF- β and RCF: down-regulation of ECM proteins and regulators LOXL2, FLOT1 and collagen type VI, and up-regulation of ECM-degrading enzymes such as PIP, DCD and CTSD (Figure 5b).

Exploratory hierarchical clustering of selected proteins belonging to the ECM core and ECM-associated protein categories (Hynes & Naba, 2012) revealed that the RCF effect on the presence of these proteins was patient dependent. Overall, a down-regulation of ECM core proteins was induced by RCF, confirming our previous ECM protein quantifications via immunofluorescence analysis, as evidenced by

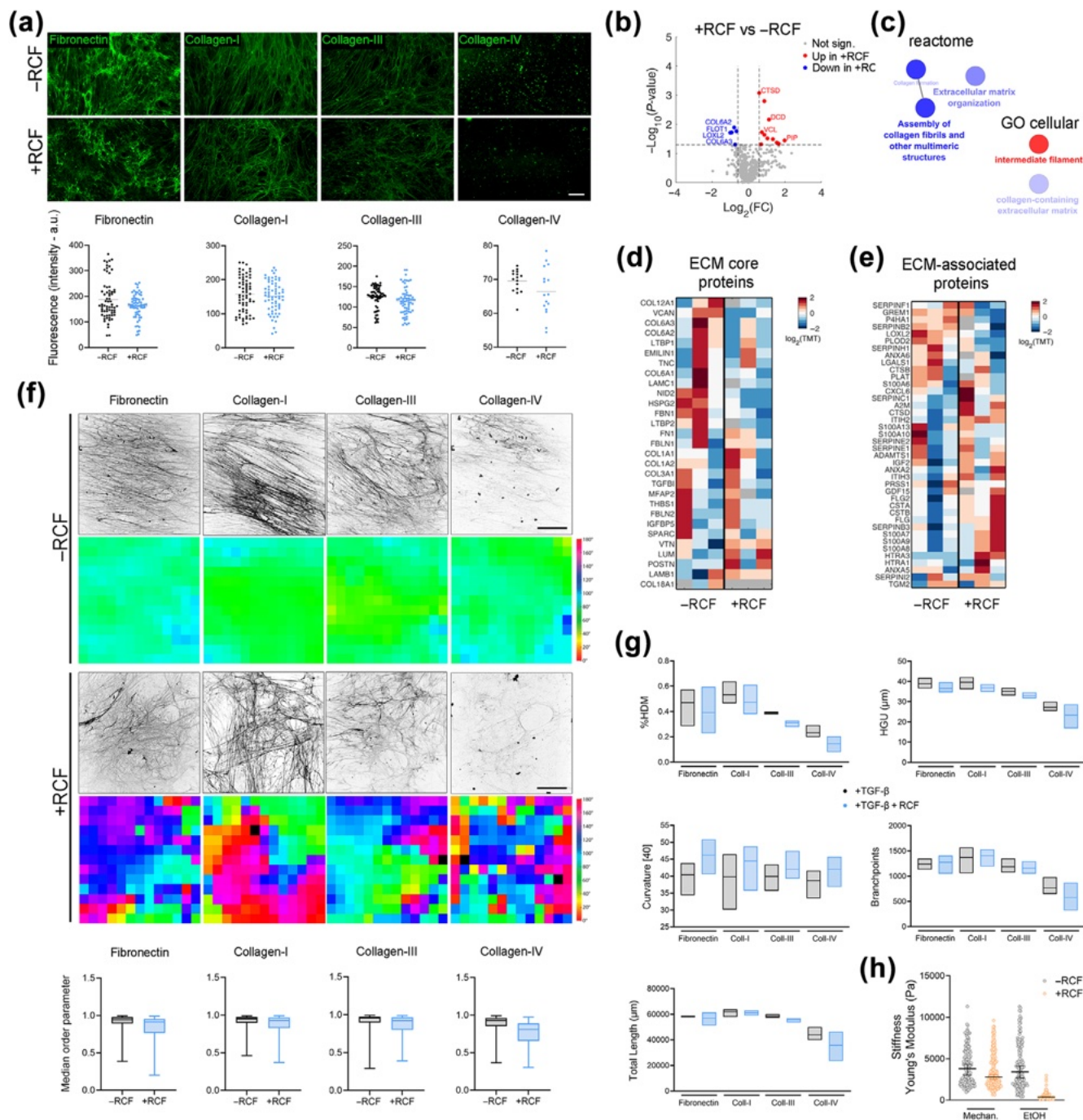


FIGURE 5 Characterisation of CDM from activated HSCs indicates a significant impact of RCF on ECM deposition and organisation.

(a) Representative immunofluorescence images of cell-derived matrix (CDM), stained for fibronectin, collagen-I, collagen-III and collagen-IV from activated HSCs in the absence or presence of RCF (scale bar = 50 μ m). Quantification of deposited CDM measured as intensity of fluorescence of stained ECM proteins ($N = 4$ cell lines, 6–12 ROI/cell line). (b) Volcano plot showing up-regulated and down-regulated ECM proteins in the CDM of HSCs treated with RCF. (c) Reactome and GO cellular analysis of CDM proteome. (d) Hierarchical clustering with heatmap visualisation of core ECM proteins in CDM of activated HSCs in the absence or presence of RCF. (e) Hierarchical clustering with heatmap visualisation ECM-associated proteins in CDM of activated HSCs in the absence or presence of RCF. (f) Quantification of CDM fibrillar alignment using alignment by Fourier transform (AFT) workflow. The panel shows stained CDM and the corresponding heatmap of the order parameter of each individual window size used for analysis (250 px). Subsequently, the mean order parameter is calculated and plotted to assess the overall fibrillar alignment ($N = 4$ cell lines, 24–37 ROI/cell line). (g) TWOMBLI workflow analysis for the assessment of different fibrillar parameters of CDM generated by activated HSCs treated or not with RCF, showing percentage of high-density matrix (%HDM), HGU and branchpoints and curvature (lines in the graphs indicate the mean value). $N = 3$ cell lines, 23–24 ROI/cell line). (h) Stiffness (measured as Young's modulus) of PCLS treated with RCF exposed to mechanical injury only or EtOH for 5 days (N [measurements] > 300, N [slices] = 3 per condition and n [donor] = 1).

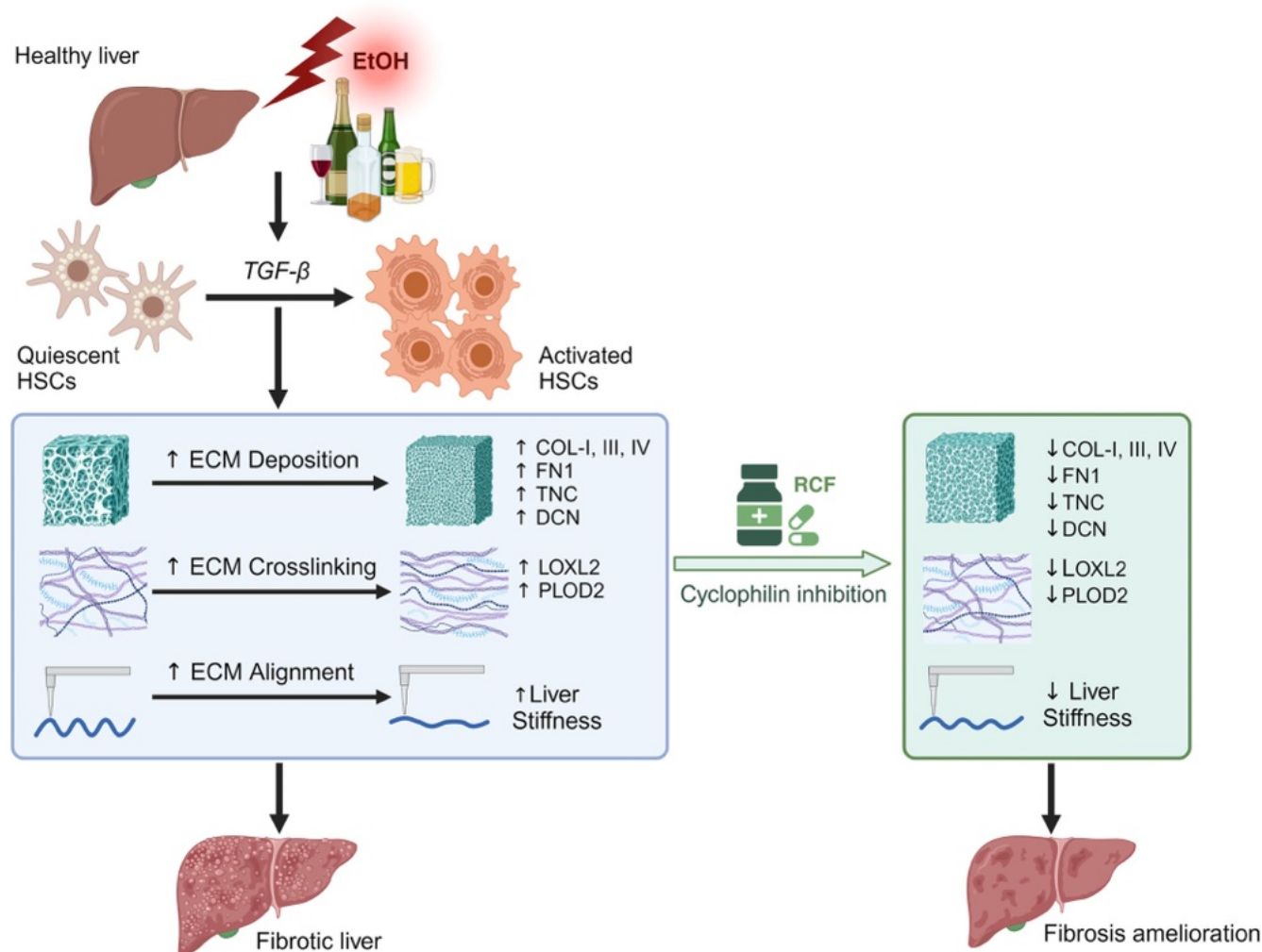


FIGURE 6 Mechanism of action of RCF in ameliorating liver fibrosis. Made with Biorender.

the quantification of collagens (type III, VI and XII), VCAN, FN1, LTBP1 and LTBP2, EMILIN1, TNC, and FBN1. The effect on other proteins was less consistent, with some cell lines showing a decrease and others an increase in protein amounts (e.g. collagen type I, SPARC, LUM and POSTN) (Figure 5d). This reflects the variability in patient-specific response highlighted in PCLS experiments and previous HSC analyses (Figures 1–4).

Similarly, several ECM-associated proteins in the CDM were reduced by RCF, for example, proteins involved in ECM deposition and cross-linking (LOXL2, PLOD2 and P4HA1) and proteins involved in cell-ECM communication (SERPINs and LSGAL1) (Figure 5e). Overall, these data show a blanket down-regulation of key ECM core and associated proteins, consistent with our findings in the PCLS models, that go beyond collagen deposition, depicting a broad reorganisation of the ECM network.

Two bioinformatic pipelines were used to analyse donor-specific fibrillar organisation and alignment of ECM proteins deposited by the cultured HSC cells, because these morphologic characteristics are known to have a direct impact on tissue stiffness in vivo (Taufalele

et al., 2019). The AFT pipeline analysed the alignment of ECM proteins by splitting confocal images into neighbourhoods and calculating the median order parameter generated by the average angle of the fibres in that neighbourhood. A median order close to 1 occurs when fibres are more organised (anisotropic) and close to 0 when less organised (isotropic) (Marcotti et al., 2021). We selected the same four core ECM proteins analysed previously (FN, collagen type I, III and IV) based on the knowledge that their amount and alignment can strongly impact organ stiffness (G. Chen et al., 2019). High-definition immunofluorescence z-stack images were processed to identify ECM protein patterns, showing a clear change in the organisation of fibres when CDMs were collected from HSCs treated with RCF together with TGF- β . In response to RCF, ECM fibres were less organised in parallel aligned bundles, which was confirmed by quantification via median order parameter analysis (Figure 5f). These preliminary data indicate that when HSCs are exposed to RCF during the fibrogenic process, they produce an isotropic and less dense ECM compared to untreated myofibroblasts. To further confirm this proposal, we used a second bioinformatic pipeline to measure other ECM organisation

parameters, similar to what was performed for the PCLS model. Confocal images of FN and collagen type I, III and IV in CDMs were analysed with TWOMBLI (Figure S1), which confirmed a clear fibre remodelling induced by RCF. FN and collagen type I fibres were less abundant in RCF treated samples (as indicated by percentage of high-density matrix [%HDM]) and presented higher curvature and hyphal growth unit (HGU, a measure of the number of endpoints per unit length), describing a more isotropic ECM network with less aligned fibres. Collagens type III and IV were also lower in relative amount in RCF samples (%HDM), but only collagen type IV showed an overall trend in changing patterns, with decreased branchpoints, HGU and fibre total length induced by RCF (Figure 5g). The suitability of TWOMBLI for the analysis of fibrillar CDMs was validated using a known inhibitor (ZDON) of an ECM remodelling enzyme (transglutaminase 2; TG2) in HSC cultures with TGF- β (Figure S19).

These preliminary results elucidate that the changes in fibre alignment, matrix density and organisation were mechanisms of ECM remodelling induced by RCF in the PCLS model. Because ECM remodelling towards a less organised network of fibres is generally thought to be associated with more pliant 3D matrix structures, we ultimately measured the stiffness of PCLS treated with RCF using atomic force microscopy-based force spectroscopy. There was minimal reduction of liver tissue stiffness in PCLS upon RCF exposure in the absence of alcohol; however, a much more pronounced reduction in liver tissue stiffness with RCF was seen in EtOH-treated PCLS (Figure 5h). Taken together, these findings demonstrate an anti-fibrotic effect induced by RCF in the PCLS models and confirm the mechanism of ECM reorganisation, shedding light on the involvement of cyclophilins in ECM remodelling.

4 | DISCUSSION AND CONCLUSIONS

This study uncovers a novel role for cyclophilins in liver disease, revealing their involvement in ECM organisation and remodelling. It provides clinically relevant insights into how pan-cyclophilin inhibition ameliorates hepatic fibrosis by impacting ECM remodelling, including core matrisome and associated proteins crucial for ECM turnover. Cyclophilins have been previously studied for their involvement in many cellular processes associated with the pathogenesis of several disorders, including liver diseases. Whereas cyclophilin inhibitors show therapeutic potential in fibrosis (Alqudah et al., 2023; Naoumov, 2014; Stauffer, Goodman, & Gallay, 2024), their mode of action remains unclear. Here, we reveal that cyclophilin inhibition consistently down-regulates ECM core proteins (collagen isoforms I, III, IV, V, VI, XII, FN1, DCN, TNC, TNXB, BGN, LUM, TGFBI and FBN1), which are prominent players in modulating ECM remodelling, organ stiffness, loss of hepatocyte function and hepatocarcinogenesis observed in liver fibrosis (Altrock et al., 2015; Bhattacharyya et al., 2016; W. Chen et al., 2014; Lorena et al., 2004; Luangmonkong et al., 2023; McQuitty et al., 2020; Sørensen et al., 2022; Williams et al., 2022). Additionally, cyclophilin inhibition by RCF affects ECM-associated regulators (e.g. cathepsins, galectins, LOXL2, DCD, PIP and PLOD2), modulating ECM deposition,

crosslinking and overall 3D organisation. These proteins contribute to liver initiation, development, and resolution via collagenolytic and crosslinking activities (Ikenaga et al., 2017; Radić et al., 2023; Ruiz-Blázquez et al., 2021; Yue et al., 2024). Whilst most studies to date primarily report reduced collagen burden following cyclophilin inhibition (Stauffer, Bobardt, Ure, et al., 2024) without a comprehensive analysis of the pathways involved, our findings elucidate broader fibrogenic pathways (transcription, ECM production, deposition and alignment), supporting RCF as a potential treatment to tackle CLD progression. One of the mechanisms underpinning the anti-fibrotic effect of RCF is via the blockade of *cis-trans* isomerisation of collagen proline residues, a process catalysed by cyclophilin B and that constitutes the rate-limiting step in the triple helix formation of collagens (Onursal et al., 2021; Steinmann et al., 1991). Our RNAseq analysis in PCLS corroborated this finding, showing ‘collagen helix trimerisation’ among the most down-regulated pathways. This effect may lead to increased ER retention of unfolded collagens, as suggested by the intracellular collagen staining in HSCs and supported by the up-regulation of genes involved in ‘cargo concentration in the ER’ in the PCLS transcriptomic datasets. Targeting collagen trafficking and the unfolded protein response has previously been proposed as a viable anti-fibrotic strategy (Mayers et al., 2017). We can speculate that this effect on collagen biosynthesis is associated with the downstream regulation of a wide range of enzymes associated with ECM turnover and remodelling.

This study provides preclinical evidence of the efficacy of cyclophilin inhibition and specifically the inhibitor RCF as a valid therapeutic approach for ALD, prompting clinical development. Although cyclophilin inhibition has shown safety and benefits in viral hepatitis and MASLD (Naoumov, 2014), preclinical and clinical studies are lacking in the context of ALD. Interestingly, studies on cyclophilins in the context of alcohol toxicity are very limited. To our knowledge, only one prior study had reported increased **cyclophilin D** hepatic expression and associated mitochondrial dysfunction in ethanol-fed rats, suggesting that lowering cyclophilin D levels may be beneficial in preventing liver injury (King et al., 2010). In our study, TWOMBLI and AFT in PCLS and CDM showed a trend of reduced ECM alignment upon RCF treatment. CDM analysis of activated HSCs confirmed ECM rearrangement involving collagen I, III and FN1, correlating with reduced PCLS stiffness and validating the anti-fibrotic potential of RCF. Finally, the translational value of these results was enhanced by the comparison between dysregulated protein datasets from ALD patients at different fibrosis stages (Niu et al., 2022) to PCLS treated with RCF. Our results also revealed that besides decreasing fibrosis, RCF modulated multiple pathways including apoptosis, plasma lipoprotein remodelling and antioxidant responses, which previous studies have shown to be beneficial targets in ALD (X. Chen, 2010; J. S. Lee et al., 2019; Ma et al., 2021).

Additional novelty of this study includes the use of organotypic 3D patient-derived cultures, gold standards for studying liver fibrosis dynamics (Y. S. Lee & Seki, 2023), combined with multi-omics and multiparametric imaging analysis. Fibrosis is associated with altered ECM architecture, more aligned and bundled matrix, modifying biomechanical properties. Multi-omics and quantitative imaging algorithms

revealed ECM changes impacting matrix deposition (quantitative/qualitative), remodelling (alignment/network organisation) and biomechanical properties (stiffness) (Kenny et al., 2023; Verhaegen et al., 2009; Wershof et al., 2021) (Figure 6). Crosslinking enzymes (e.g. PLODs, LOXs, TG2) mediate ECM stiffening, driving hepatic cell behavioural and phenotypic shifts. Here, we show that TWOMBLI and AFT techniques can be used to dissect ECM architecture in both PCLS and CDM, describing reduced order of alignment of ECM fibres upon RCF treatment. The characterisation of CDM from activated HSCs confirmed the dramatic re-arrangement of the ECM architecture involving collagen I, III and FN1. These events led to a resultant softer ECM, which was confirmed by the reduced stiffness of PCLS exposed to RCF, further validating the promising use of this drug to reduce fibrosis.

A limitation of our study is the inability to determine which cyclophilin isoforms drive ECM and fibrosis effects by RCF. Previous studies suggest cyclophilin B, but not **cyclophilin A**, mediates fibrosis amelioration in animal models (Stauffer, Goodman, Bobardt, et al., 2024). We speculate that the multi-isoform inhibition of RCF enhances therapeutic efficacy by targeting multiple pathways, making it a promising CLD treatment. Omics analyses highlighted patient-specific variability, a drawback of patient-derived models. However, despite patient heterogeneity, DEG-associated pathways were conserved, strengthening result robustness and real-world relevance.

In conclusion, our study demonstrates RCF pleiotropic therapeutic effects in patient-derived 3D ALD models and patient-matched in vitro fibrotic models, elucidating the anti-fibrotic mechanism of cyclophilin inhibition. We also validate multiparametric ECM analyses of matrix patterns and remodelling in models of liver disease, showing that RCF down-regulates ECM core protein deposition, reverses dysregulated ECM remodelling enzymes and restores physiological ECM organisation. This work bridges preclinical research and medical applications, igniting the path for the translation of our findings into new ways of treating ALD and fibrosis.

AUTHOR CONTRIBUTIONS

U. Rastovic: Formal analysis (lead); investigation (equal); methodology (equal); visualisation (lead); writing—original draft (Equal), and Writing—review and editing (equal). **S. Campinoti:** Formal analysis (lead); investigation (equal); methodology (equal); visualisation (lead); writing—original draft (equal); writing—review and editing (equal). **L. Wei:** Investigation (supporting); writing—original draft (supporting). **B. Almeida:** Investigation (supporting). **S. F. Bozzano:** Investigation (supporting); writing—original draft (supporting). **R. Amiri:** Formal analysis (supporting); writing—original draft (supporting). **N. Harris:** Investigation (supporting); writing—review and editing (supporting). **O. Ajayi:** Investigation (supporting). **T. S. Koay:** Investigation (supporting). **C. Kerins:** Investigation (supporting). **F. N. Kenny:** Investigation (supporting). **A. Zamalloa:** Resources (supporting). **L. Adofina:** Resources (supporting). **R. Miquel:** Formal analysis (supporting); investigation (supporting); resources (supporting). **Y. Zen:** Formal analysis

(supporting); resources (supporting). **P. Srinivasan:** Resources (supporting). **K. Menon:** Resources (supporting). **N. Heaton:** Resources (supporting). **C. Luni:** Formal analysis (equal); writing—review and editing (supporting). **E. Gentleman:** Methodology (equal); writing—review and editing (supporting). **T. Shaw:** Methodology (equal); writing—review and editing (supporting). **D. Ure:** Resources (equal); writing—review and editing (supporting). **S. Chokshi:** Conceptualisation (lead); funding acquisition (lead); methodology (equal); project administration (supporting); supervision (lead); writing—original draft (supporting); writing—review and editing (equal). **L. Urbani:** Conceptualisation (lead); funding acquisition (lead); methodology (lead); project administration (lead); supervision (lead); validation (equal); visualisation (equal); writing—original draft (lead); writing—review and editing (lead). **E. Palma:** Conceptualisation (lead); funding acquisition (lead); methodology (lead); project administration (lead); supervision (lead); validation (equal); visualisation (equal); writing—original draft (lead); writing—review and editing (lead).

ACKNOWLEDGEMENTS

The authors would like to thank all the staff at King's College Hospital Liver Histopathology Department and King's College Hospital Liver Surgical theatres for their help. We thank George Chennell and Chen Liang, from the Wohl Cellular Imaging Centre, and Steven Lynham from the Proteomics facility at King's College London. Finally, we are extremely grateful to the patients who consented and donated samples to this study.

The authors acknowledge that the work described in this manuscript was funded by the Foundation for Liver Research and Hepion Pharmaceuticals. S.Ca. was supported by a Wellcome Trust 'Early career award' (225125/Z/22/Z). C.K. was supported by a Wellcome Trust 'Advanced Therapies for Regenerative Medicine' PhD studentship (218461/Z/19/Z). E.G. acknowledges funding from the UK Engineering and Physical Sciences Research Council (EP/V04723X/1). F.N.K. was supported by the Wellcome Trust (grant no. 107859/Z/15/Z).

CONFLICT OF INTEREST STATEMENT

Dr Daren Ure is the former Chief Scientific Officer at Hepion Pharmaceuticals. Other authors declare no potential competing interests.

DATA AVAILABILITY STATEMENT

All raw data used in the study are available from the corresponding authors upon reasonable request.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for [Design and Analysis](#) and [Immunoblotting and Immunochemistry](#) and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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How to cite this article: Rastovic, U., Campinoti, S., Wei, L., Almeida, B., Bozzano, S. F., Amiri, R., Harris, N., Ajayi, O., Koay, T. S., Kerins, C., Kenny, F. N., Zamalloa, A., Adofina, L., Miquel, R., Zen, Y., Srinivasan, P., Menon, K., Heaton, N., Luni, C., ... Palma, E. (2025). Comprehensive analysis of extracellular matrix remodelling via cyclophilin inhibition in human models of alcohol-related liver fibrosis. *British Journal of Pharmacology*, 1–23. <https://doi.org/10.1111/bph.70139>