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Research Article

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No competing interests reported.

Tables 1 and 2 are available in the supplementary files section

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Abbreviations

ACLF	Acute-on-Chronic Liver Failure
ALT	Alanine Aminotransferase
ANOVA	Analysis of variance

AST	Aspartate Aminotransferase
AUROC	Area under the Receiver Operating Characteristic Curve
CARS	Compensatory anti-inflammatory response syndrome
CE	Cholesteryl ester
CER	Ceramide
CHB	Chronic hepatitis B
CLD	Chronic Liver disease
CLIF-C	Chronic liver failure consortium organ failure
CTP	Child-Turcotte-Pugh
DAG	Diacylglycerides
DAMP	Damage-associated molecular patterns
EASL	European Association for the Study of the Liver
EASL-CLIF	European Association for the Study of the Liver-Chronic Liver Failure Consortium
EDTA	Ethylene diamine tetra acetic acid
GA	Garcinoic acid
HC	Healthy control
HE	Hepatic encephalopathy
INR	International Normalized Ratio
LPC	Lysophosphatidyl choline
LPE	Lysophosphatidyl ethanolamine
LPG	Lysophosphoglycerol
LPI	Lysophosphatidyl inositol
LPS	Lipopolysaccharide
LPS (lipid)	Lysophosphatidyl serine
MAG	Monoacylglycerides
MAPK	Mitogen-Activated Protein Kinase
MELD	Model for end-stage liver disease
MELD-NA	Model for end-stage liver disease-Sodium
MOF	Multiple organ failure
MRM	Multiple reaction monitoring
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NET	Neutrophil extracellular traps

NLR	Neutrophil to lymphocyte ratio
OPLS4	Optimized Potentials for Liquid Simulations, Version 4
PA	Phosphatidic acid
PAMP	Pathogen associated molecular patterns
PC	Phosphatidyl choline
PE	Phosphatidyl ethanolamine
PI	Phosphatidyl inositol
PLS-DA	Partial least squares-discriminant analysis
PMN	Polymorphonuclear
PS	Phosphatidyl serine
PUFA	Polyunsaturated fatty acid
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
QTRAP	Quadrupole Ion Trap
SAP	Serum Alkaline Phosphatase
SIRS	Systemic inflammatory response syndrome
SM	Sphingomyelin
SMILES	Simplified Molecular Input Line Entry System
SOFA	Sequential Organ Failure Assessment
TLC	Thin Layer Chromatography
TLR	Toll-like Receptor
VEGFA-	Vascular Endothelial Growth Factor A – Vascular Endothelial Growth
VEGFR	Factor Receptor
VIP	Variable importance projection

Declarations

Ethics approval and consent to participate

The study has been approved by the All India Institute of Medical Sciences, New Delhi ethics committee [Reference No. IEC/473/9/2016; IEC/369/7/2016; IEC-630/07.12.2018; RP-36/2018; IEC-145/11.04.2023]. All procedures are as per the declaration of Helsinki. All participants included in the study were > 18 years of age and were recruited in the study after informed consent.

Availability of data and material

This study generated datasets as part of its analyses. All data supporting the findings of this study are available within the paper and its supplementary files. Any additional information or clarifications regarding the datasets will be made available by the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests as defined by BMC, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

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Authors' contributions

R.S., S.M., N.G.: Methodology, Data curation, Investigations, Validation, Formal analysis, Writing- Original draft preparation, Writing- Review and Editing; **P.S., R.U., M.R.:** Methodology, Investigations, Validation, Formal analysis; **W.W., S.S.P., K.M., G.M., S.P.:** Formal analysis, Visualization, Writing-Original draft preparation; **Y.G.:** Data curation, Formal analysis; **A.N., V.S.:** Conceptualization, Formal Analysis, Visualization, Writing- Original Draft Preparation; **S.B., S.:** Investigation, Resources, Data Curation, Writing- Original draft

preparation; **P.A.:** Conceptualization, Methodology, Visualization, Data curation, Funding acquisition, Resources, Project administration, Supervision, Writing- Original draft preparation, Writing- Review and Editing.

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Abstract

Background

Acute-on-chronic liver failure (ACLF) is characterized by severe systemic inflammation, progressive organ failure, and high short-term mortality. Although numerous alterations in circulating lipids have been described in ACLF and other forms of critical illness, it remains unclear whether biologically meaningful information resides primarily in individual lipid species or emerges through coordinated molecular organization associated with disease progression. We hypothesized that reproducible disease-associated biology would become increasingly apparent at higher levels of lipid organization across independent patient cohorts.

Methods

We performed targeted LC-MS/MS lipidomic profiling of plasma from two temporally independent cohorts comprising 140 patients with ACLF and healthy controls. Cross-cohort reproducibility was evaluated at the levels of individual lipid species, lipid classes, and co-varying lipid modules. Associations with disease severity and organ failure were assessed using clinical correlation analyses and a composite Lipid Severity Score. Independent neutrophil proteomic datasets, published transcriptomic studies, and computational regulatory analyses were subsequently examined as complementary molecular observations to provide biological context for the identified lipid alterations.

Results

ACLF was characterized by reproducible coordinated alterations in the circulating lipidome across independent cohorts, including depletion of lysophospholipids and plasmalogens with enrichment of glycerophospholipids and triacylglycerols. Although overlap of individual lipid species between cohorts was modest, lipid class-level alterations demonstrated strong concordance, indicating that reproducible disease-associated information emerged more consistently at higher levels of molecular organization. Unsupervised network analysis identified a phospholipid- and plasmalogen-enriched lipid module whose progressive depletion correlated with MELD, MELD-Na, INR, serum creatinine, cumulative organ failure burden, and the Lipid Severity Score (LSS). Cross-disease lipidomic comparison identified a conserved

critical illness lipid signature, with three phosphatidylcholine/phosphatidylethanolamine species [PC(36:3), PC(38:4), and PE(36:4)] consistently shared across ACLF, trauma, and COVID-19 cohorts. Independent neutrophil proteomic and published transcriptomic datasets converged on biological processes related to neutrophil activation, degranulation, and innate immune responses.

Conclusions

ACLF is characterized by reproducible coordinated alterations in the circulating lipidome that are closely associated with progressive organ failure. Our findings suggest that reproducible disease-associated biology is more robustly represented at higher levels of molecular organization, including coordinated lipid classes and co-varying lipid modules, than by individual lipid species alone. This framework provides a biologically grounded approach for interpreting molecular heterogeneity in ACLF and may facilitate the identification of more reproducible molecular signatures of disease severity and progression.

Keywords: ACLF, lipidomics, proteomics, mortality, neutrophils, PPAR γ , organ failure, interorgan communication, metabolomics

INTRODUCTION

Acute-on-chronic liver failure (ACLF) is a rapidly progressing clinical syndrome characterized by rapid depletion of hepatic reserves and tissue necrosis, systemic inflammation, multiple organ failure, and high short-term mortality (1-4). Although substantial advances have been made in understanding the inflammatory and metabolic disturbances that accompany ACLF, the molecular basis underlying its remarkable biological complexity remains incompletely understood (5-7). Patients with comparable clinical severity frequently demonstrate striking differences in disease progression and outcomes, suggesting that ACLF reflects complex, coordinated biological processes rather than disruption of isolated molecular pathways (4, 8). Increasing evidence across complex human diseases indicates that biological information is often more robustly represented by coordinated molecular programs than by individual molecular alterations (9-12). Rather than acting independently, genes, proteins and metabolites function as interconnected biological networks whose coordinated behaviour reflects underlying physiological and pathological states. Consequently, systems-level approaches that identify reproducible molecular organization may provide more biologically meaningful insights into disease mechanisms than analyses focused exclusively on individual molecular features. Whether this principle extends to ACLF, however, remains largely unexplored.

Lipids occupy a central position at the interface between metabolism, inflammation, membrane biology and cellular signalling (13-14). Beyond their traditional role as structural components and energy stores, lipids regulate membrane organization, receptor function, oxidative stress responses, vesicular trafficking, and innate immune signalling (15-17). Plasma lipidomics therefore provides an opportunity to capture integrated alterations in systemic metabolism occurring during critical illness. Previous lipidomic studies in ACLF and related inflammatory conditions have identified numerous differentially abundant lipid species associated with disease severity and clinical outcomes (18). However, individual lipid species often demonstrate limited reproducibility across patient cohorts owing to biological variability, disease stage, analytical platform, and extensive lipid remodeling. It therefore remains unclear whether the molecular heterogeneity and variability in the circulating ACLF lipidome reflects stochastic perturbations of individual lipid species or reproducible patterns of coordinated lipid organization associated with disease progression.

To address this question, we performed targeted plasma lipidomic profiling in two independent cohorts of patients with ACLF and healthy controls. We evaluated reproducibility across progressively higher levels of biological organization, including lipid classes, co-regulated lipid modules, and their relationship with clinical severity. We further examined whether these

coordinated lipid programs converged with independent molecular observations derived from neutrophil proteomics, publicly available transcriptomic datasets, and computational regulatory analyses. We hypothesized that reproducible biological information in ACLF would emerge more clearly at the level of coordinated lipid programs than individual lipid species, providing a more robust framework for understanding molecular heterogeneity in this clinically complex syndrome.

METHODS

To determine whether reproducible disease-associated biology in ACLF emerges through coordinated molecular organization rather than individual lipid alterations, we performed targeted plasma lipidomics in two independent cohorts and integrated these observations with clinical phenotyping, network analysis, neutrophil proteomics, published transcriptomic datasets, and computational analyses.

An overall scheme of the study design and work plan are provided in Figure 1. The study strictly adhered to the tenets of the Declaration of Helsinki and was approved by the institutional ethics committee (Ethics Committee Ref. No. IEC/473/9/2016; IEC/369/7/2016; IEC-630/07.12.2018; RP-36/2018; IEC-145/11.04.2023). The European Association for the Study of the Liver-Chronic Liver Failure (EASL-CLIF) definition was used for the diagnosis of ACLF (19). The EASL definition includes patients with acute decompensation of cirrhosis (CLD-AD), and classifies them as ACLF based on the CLIF-Organ Failure (OF) scoring assigned for 6 organ systems (liver, kidney, brain, coagulation, circulation, and respiration). This definition takes into account non-hepatic organ failure, such as kidney and brain for ACLF classification and importance is given to severity (ACLF grades) and association with short-term mortality (28-day). The following parameters are used for classifying each organ failure, (i) Liver- bilirubin ≥ 12 mg/dL; (ii) Kidney-creatinine ≥ 2 mg/dL or requiring renal replacement therapy; (iii) Brain- West Haven grade III/IV for hepatic encephalopathy (HE) (20); (iv) Coagulation-INR ≥ 2.5 ; (v)Circulatory- requiring vasopressors; and Respiratory-PaO₂/FiO₂ ≤ 200 or SpO₂/FiO₂ ≤ 214 .

Sample Size for Lipidomics Experiment

An expected 28-day mortality rate of ~50% in ACLF guided the inclusion of 100 patients recruited across two independent time periods (60 in Cohort 1 and 40 patients in Cohort 2, respectively), together with 40 healthy controls in the plasma lipidomics analysis. This sample

size provides ~80% power to detect moderate-to-large differences between survivors and non-survivors (Cohen's $d \geq 0.6$) at $\alpha = 0.05$, consistent with the effect sizes commonly observed in lipidomic biomarker studies. Sample size considerations were guided by standard two-sample effect size calculations.

$$n_{per\ group} \approx \frac{2(Z_{1-\alpha/2} + Z_{1-\beta})^2}{d^2},$$

For $\alpha = 0.05$ and 80% power, approximately 44 subjects per group are required to detect a moderate effect size (Cohen's $d = 0.6$), which is consistent with commonly observed effect sizes in lipidomic biomarker studies. To further assess the adequacy of precision, Hanley and McNeil's AUC variance approximation indicates that clinically meaningful prognostic performance ($AUC \approx 0.80$) can be estimated with acceptable 95% confidence intervals in a cohort of this size. The use of two temporally independent cohorts, combined with false-discovery rate control and penalized regression, enhances reproducibility and supports the robustness of the identified lipid biomarkers.

Patient Recruitment and Sample Collection

ACLF patients were recruited on the day of admission (Day 0) or the next day (Day 1), from the Department of Gastroenterology at AIIMS, New Delhi (Nov 2022-Nov 2024) based on EASL criteria (19). Patients were recruited immediately after diagnosis. Blood collection was done on day 0 or 1, in the morning for all patients, to minimize diurnal variation in plasma lipid levels. Peripheral blood (~6 mL, EDTA) was processed for plasma and other analyses in the Department of Biochemistry. Patients were followed up for 28-day outcomes and classified as survivors (S) or non-survivors (NS). Healthy age- and sex-matched controls were recruited from the Blood Bank. In plasma lipidomics analysis the Cohort 1 included 60 ACLF patients (30 S, 30 NS) and 30 healthy controls; the Cohort 2 included 40 ACLF patients (20 S, 20 NS) and 10 controls (Figure 1). Plasma lipidomics, and PMN proteomics were performed with independent patient cohorts and the baseline clinical parameters of each cohort have been provided in Supplementary Table_1.

Plasma lipidomics analysis

A total of 100 ACLF plasma samples were analyzed for comparative lipidomic profiling across two temporally independent cohorts (Cohort 1 and Cohort 2; Figure 1). Blood samples were processed within 2 hours, plasma was separated using standard protocols, and stored at $-80\ ^\circ\text{C}$

until analysis. Total lipid extraction was performed using a modified Bligh and Dyer method with a triphasic dichloromethane/methanol/water system (2:2:1, v/v) (Supplementary Methods M1) (21-22).

Targeted LC-MS/MS was conducted using a Waters ACQUITY™ UPLC BEH HILIC XBridge Amide column coupled to a Sciex QTRAP 6500+ system (23) (Supplementary Methods M2). Peak mapping and integration were performed using MultiQuant 3.0.3. A total of 1218 lipid species across 17 classes were identified, including (+ve MS/MS) SM, Cer, CE, MAG, DAG, TAG, and (−ve MS/MS) PA, LPC, PC, LPE, PE, LPI, PI, LPG, PG, LPS, and PS. Within PE species, ‘O-’ denotes alkyl ether and ‘P-’ denotes plasmalogen substituents.

Cohort 1 included ACLF patients (n=58; 30 S, 28 NS) and HC (n=29) passing QC. Findings were validated in an independent Cohort 2 (ACLF n=40; HC n=10), recruited at different time points under identical criteria at AIIMS New Delhi. Both cohorts were processed using identical LC-MS platforms, sample preparation, and data pipelines to ensure analytical consistency.

Statistical analyses were performed using R(v4.3.1) and MetaboAnalyst6.0 (Supplementary Methods M3-M4). Data preprocessing included 70% detection filtering, MissForest imputation, $\log_2$ transformation, and ComBat batch correction. Feature selection was performed using the Boruta algorithm, followed by supervised analyses for ACLF vs HC, ACLF-NS vs ACLF-S, and sex-stratified comparisons.

PLS-DA was used for exploratory multivariate analysis, with model robustness assessed by 7-fold cross-validation and permutation testing (n=1000). Differential lipid abundance was evaluated using unpaired t-tests (ACLF vs HC) with Benjamini–Hochberg FDR correction (significance threshold FDR <0.05), and Wilcoxon rank-sum tests for ACLF-NS vs ACLF-S. As no lipids remained significant after FDR correction in survival analysis, these comparisons were treated as exploratory (p<0.05), with emphasis on class-level trends.

The same analytical workflow was applied to Cohort 2 to identify reproducible lipid species and concordant class-level changes. Data visualization, including t-statistic distributions, cross-cohort concordance plots, and lipid class summaries, was performed in R using ggplot2.

Unsupervised Lipid Co-regulation Module Analysis

To investigate higher-order lipid organization and identify biologically coordinated lipid programs, unsupervised hierarchical clustering was performed on all 137 lipid species.

Pairwise lipid correlations were calculated across all samples and converted into a distance matrix: “Distance=1-Correlation”

Agglomerative hierarchical clustering was performed using average linkage, and the dendrogram was partitioned into four major modules (k = 4). Each lipid module was summarized using a module eigenscore, defined as the first principal component (PC1) of all lipids belonging to that module. Module eigenscores represent the dominant abundance pattern shared by all lipids within a module and provide a single quantitative metric describing coordinated lipid behavior.

Module eigenscores were subsequently correlated with clinical traits, including MELD, MELD-Na, INR, creatinine, bilirubin, and albumin using Spearman rank correlation analysis. Differences between survivors and non-survivors were evaluated using Wilcoxon rank-sum testing.

Construction of a Lipid Severity Score (LSS)

To derive a clinically interpretable lipid signature associated with disease severity, reproducible lipids from Cohort 1 and Cohort 2, were selected to construct a composite Lipid Severity Score (LSS). For each lipid, abundance values were standardized across subjects using z-score transformation:

$$Z_i = \frac{x_i - \mu_i}{\sigma_i}$$

where:

- x_i = lipid abundance in an individual sample
- μ_i = cohort mean abundance
- σ_i = cohort standard deviation.

For downregulated lipids, which decreased with increasing disease severity, z-scores were multiplied by -1 so that higher values represented a more severe lipid phenotype. Associations between the Lipid Severity Score (LSS) and clinical variables were evaluated using Spearman correlation, while differences between survivors and non-survivors were assessed using Wilcoxon rank-sum testing.

Associations between module eigenscores, LSS, and clinical parameters were assessed using Spearman's rank correlation coefficients. Differences between categorical variables were evaluated using the Wilcoxon rank-sum test or Kruskal-Wallis test, as appropriate.

Composite organ failure burden was calculated as:

$$OF_{Burden} = OF_{Liver} + OF_{Kidney} + OF_{Brain} + OF_{Coagulation} + OF_{Circulation} + OF_{Respiration}$$

yielding a score ranging from 0 to 6.

Proteomics Analysis of ACLF-derived Neutrophils

Peripheral blood polymorphonuclear cells (PMNs) (ACLF n=9; HC n=9) were isolated using a double-density gradient centrifugation protocol (Supplementary Methods File_M6) (24). Proteomic profiling of neutrophil samples was performed at vProteomics (New Delhi, India). PMN samples were pooled (3 samples per pool; total 3 pools) to increase protein input. Samples were analyzed using an Easy-nLC 1000 system coupled to an Orbitrap Exploris 240 mass spectrometer (Thermo Fisher Scientific).

Raw data were processed using Proteome Discoverer v2.5 and searched against the UniProt human reference database using Sequest and Amanda search engines (Supplementary Methods File_M7). Functional enrichment analysis was performed using Metascape.

Kinase and transcription factor enrichment analyses

Differentially expressed PMN genes and proteins from previously published (24) and the present study, were subjected to kinase and transcription factor enrichment analysis using the KEA3 (version 3) and X2Kweb tool (ChEA and ENCODE databases) (<https://maayanlab.cloud/X2K/>) respectively. Enriched kinases and TFs were cross-referenced against our original lists of differentially expressed genes (DEGs) and proteins (DEPs) (Saha et. al., 2021 and present study respectively).

To evaluate potential binding sites on key genes by the TF PPAR γ , transcription factor binding motif analysis was performed using the Search Motif Tool available through the Eukaryotic Promoter Database (EPD; <https://epd.epfl.ch/>). The genomic regions from –1000 base pairs (bp) upstream to +100 bp downstream relative to the transcription start site (TSS) were selected for each gene. PPAR γ motifs were searched using the JASPAR CORE vertebrates transcription factor motif library, applying a p-value cut-off of 0.001. The location of each predicted PPAR γ

binding site was recorded relative to the TSS and visualized using the graphical output provided by the EPD motif tool. Genes without any predicted binding sites within the specified region were also noted. Genes assessed included-*TLR1*, *LR10*, *SLC25A4*, *SLC2A1*, *CXCL1*, *CD177*, *FFAR3*, *MAPK1*, *MAPK14*, and *MT3*, along with neutrophil degranulation protein list.

***In Silico* Studies for investigation of ACLF lipid-PPAR γ interaction**

Molecular docking was performed using Maestro Version 13.7 (Schrödinger Suite, Release 2023-3). The crystal structure of PPAR γ in complex with garcinoic acid (GA) (PDB ID: 7AWD) was prepared using the Protein Preparation Wizard with default parameters. ACLF-associated lipid molecules were prepared using LigPrep with the OPLS4 force field. Protonation states were assigned using Epik at pH 7.0, and relevant tautomeric forms were generated.

The receptor grid was defined at the ligand-binding site after removal of one GA molecule, and docking was performed using Glide (Release 2023-3) with the OPLS4 force field. Prepared lipid ligands were docked into the PPAR γ binding pocket, and docking poses were evaluated based on standard Glide scoring functions.

A subset of cohort-reproducible lipid species were used for docking analysis. Structural similarity among lipid species was assessed using RDKit (v2022.9.5) and mapchiral (v0.0.7) in Python. Molecules were parsed from SMILES strings and converted to RDKit Mol objects. Molecular fingerprints were generated using Morgan fingerprints (radius = 2, nBits = 2048) and MAP4Chiral fingerprints²⁶ (maxradius = 2, npermutations = 2048). Pairwise similarity was quantified using Tanimoto coefficients (DataStructs.TanimotoSimilarity) and Jaccard similarity (mapchiral.jaccard_similarity).

Similarity matrices were exported to R (v4.4.1) for downstream analysis. Tanimoto similarity values were converted to distance matrices ($1 - \text{similarity}$), and hierarchical clustering was performed using the hclust function with complete linkage.

Statistical Analysis

All statistical analyses were performed in GraphPad Prism 10, Jamovi (version 2.7.6), Python, and R (version 4.3.1). Data distribution was assessed using normality tests. Continuous variables were summarized as mean $\pm$ SD or median (IQR) and compared using t-test or Mann–Whitney U test, while categorical variables were compared using the χ^2 test or Fisher's exact

test. For comparisons involving more than two groups, one-way ANOVA or the Kruskal-Wallis test was used.

All statistical tests were two-sided, with $p < 0.05$ considered statistically significant.

RESULTS

Baseline characteristics of lipidomic cohorts

ACLF patients ($n=100$) were enrolled for targeted plasma lipidomics, in the initial phase as Cohort 1 ($n=60$) and in the next phase as a distinct temporally independent Cohort 2 ($n=40$) (Figure 1, Figure 2A, Supplementary Table_1). In the Cohort 1, 58 samples [30 survivors (S), 28 non-survivors(NS)] passed batch correction and quality control, whereas in Cohort 2 all the samples passed batch correction (Supplementary Methods File_M3). Baseline demographic, clinical, organ failure, and outcome variables were comparable and well-matched between ACLF cohort 1 and cohort 2, with no statistically significant differences observed across parameters ($p>0.05$) (Table 1, Supplementary Table 1, Supplementary Table S1). Both cohorts had higher proportion of male patients 47 (81.0%) in Cohort 1 and 31 (77.5%) in Cohort 2 (Table 1). A separate survivor vs non-survivor analysis restricted to male patients was performed to account for potential confounding effects of estrogen on lipid distribution (Supplementary Figures S2 and S3).

In cohort 1, ACLF non-survivors had significantly higher ($p\leq 0.05$) total leukocyte counts (TLC mm^3) (8220 vs 13295), INR (1.90 vs 2.83), urea levels mg/mL (51.0 vs 71.5), MELD (31 vs 35), and MELD-Na (31 vs 35) scores at admission compared with survivors (Table 2). In this cohort, ACLF non-survivors showed a significant higher presence of active infection at admission compared with survivors (67.9% vs 25.9%, $p = 0.002$), along with higher brain organ failure (64.3% vs 36.0%, $p = 0.040$). Other organ failures, including liver, kidney, coagulopathy, circulation, and respiratory failure, as well as gender distribution and ascites grade, were comparable between survivors and non-survivors (Supplementary Table S2). In contrast, baseline parameters in Cohort 2 did not differ significantly between survivors and non-survivors; however, non-survivors showed a consistent trend toward greater disease severity, including higher INR, urea, creatinine, bilirubin, and MELD scores ($p > 0.05$) (Table 2).

Hepatic encephalopathy severity differed significantly, with non-survivors having higher HE grades at admission compared with survivors ($p = 0.005$) (Supplementary Table S2).

ACLF is characterized by reproducible coordinated alterations in the circulating lipidome

Two temporally independent ACLF cohorts were studied to evaluate the reproducibility of circulating lipid alterations (Figure 2A). Targeted LC-MS/MS profiling identified 1,218 lipid species spanning 17 lipid classes in both cohorts. Multivariate analysis demonstrated clear separation between ACLF patients and healthy controls in each cohort (Figure 2A), indicating extensive remodeling of the circulating lipidome.

Inspection of the variable importance in projection (VIP) scores and corresponding heatmaps identified consistent patterns across both cohorts (Figure 2B-C). ACLF was characterized by coordinated depletion of multiple lysophospholipid species, particularly polyunsaturated lysophosphatidylcholines and lysophosphatidylethanolamines, together with relative enrichment of several glycerophospholipids and triacylglycerols. These findings suggest that lipid alterations in ACLF occur as coordinated changes across lipid classes rather than isolated perturbations of individual lipid species.

Differential abundance analysis identified 339 and 356 significantly altered lipid species ($FDR < 0.05$) in Cohorts 1 and 2, respectively (Supplementary Table 2). Despite the large number of altered lipid species, class-wise distributions of t-statistics demonstrated striking concordance between the two independent cohorts (Figure 2D). Lysophosphatidylcholines (LPC), lysophosphatidylethanolamines (LPE), and plasmalogen phosphatidylethanolamines (PE-P) consistently showed negative shifts in ACLF, whereas lysophosphatidylinositols (LPI) and triacylglycerols (TAG) were relatively enriched, indicating reproducible alterations at the lipid-class level.

Cross-cohort comparison identified 137 lipid species that were significantly altered following FDR correction in both cohorts and changed in the same direction (Supplementary Table S3). Although this represented only a subset of all differentially abundant lipids, these shared species demonstrated strong concordance in directionality between cohorts (Figure 2D), supporting the reproducibility of the core circulating lipid alterations associated with ACLF.

Outcome-associated lipidome alterations demonstrate greater reproducibility at the lipid-class level

To determine whether lipid alterations associated with 28-day mortality were reproducible across independent ACLF cohorts, survivor (S) and non-survivor (NS) comparisons were

performed separately in Cohorts 1 and 2. No individual lipid species remained significant following false discovery rate correction, indicating that outcome-associated differences at the level of individual molecular species were modest and should be interpreted as exploratory.

Exploratory PLS-DA demonstrated partial separation between survivors and non-survivors in both cohorts (Figure 3A-B; Supplementary Figure S3), consistent with the expectation that both groups represented patients with advanced ACLF (grades 2-3). Similarly, Wilcoxon rank-sum analysis identified 50 nominally significant lipid species in Cohort 1 and 39 in Cohort 2 ($p < 0.05$; Supplementary Table 4). However, unsupervised clustering of these lipids did not clearly distinguish survivors from non-survivors within either cohort (Figure 3A-B), highlighting the substantial heterogeneity of individual lipid species associated with clinical outcome.

Despite this limited overlap at the level of individual lipid species, outcome-associated alterations demonstrated markedly greater concordance at the lipid-class level. Stacked bar plots summarizing the distribution of nominally differential lipids across classes revealed highly comparable patterns between cohorts, with coordinated alterations involving dihydroceramides (DCER), diacylglycerols (DAG), lysophosphatidylcholines (LPC), lysophosphatidylethanolamines (LPE), phosphatidic acids (PA), phosphatidylcholines (PC), phosphatidylethanolamines (PE and PE.O), phosphatidylglycerols (PG), phosphatidylinositols (PI), phosphatidylserines (PS), and triacylglycerols (TAG) (Figure 3C). These findings suggest that reproducible biological information associated with outcome is more consistently preserved at the level of coordinated lipid classes than individual molecular species.

Unsupervised lipid co-expression analysis identifies coordinated lipid modules associated with ACLF severity

Unsupervised hierarchical co-expression analysis identified four coordinated lipid modules comprising 18, 24, 74, and 21 lipid species, respectively (Figure 3D; Supplementary Table 5). The modules displayed distinct lipid compositions, with Module 1 enriched in neutral and signalling lipids, Module 2 comprising a heterogeneous mixture of diacylglycerols and ether phospholipids, Module 3 representing the largest network enriched in phosphatidylplasmalogens, phosphatidic acids, lysophosphatidylethanolamines, and phosphatidylcholines, and Module 4 consisting predominantly of membrane-associated phospholipids, including lysophospholipids, phosphatidylcholines, phosphatidylethanolamines, phosphatidic acids, ether phospholipids, and plasmalogens.

Among these, Module 4 demonstrated the strongest clinical associations. Module eigenscores correlated inversely with MELD ($\rho = -0.330$, $p = 0.0013$), MELD-Na ($\rho = -0.346$, $p = 0.00068$), INR ($\rho = -0.267$, $p = 0.012$), and serum creatinine ($\rho = -0.245$, $p = 0.016$), while no significant association was observed with serum albumin ($\rho = 0.198$, $p = 0.054$) (Figure 3E). Although Module 4 abundance showed only a trend towards lower levels in non-survivors ($p = 0.0705$) (Figure 3F), its consistent inverse relationship with established markers of hepatic dysfunction, coagulopathy, and renal impairment suggests that depletion of coordinated membrane-associated lipid networks accompanies progressive organ failure in ACLF. Notably, the relationship between Module 4 eigenscores and MELD-Na was preserved in both survivors and non-survivors (Figure 3G), supporting the robustness of this association across clinical outcome groups.

Composite Lipid Severity Score (LSS) correlates with ACLF severity and outcome

To determine whether coordinated lipid alterations were associated with disease severity, we generated an exploratory composite Lipid Severity Score (LSS) from six lipid species that remained significantly associated with clinical parameters after FDR correction (Figure 4A). The score, derived from z-score normalized lipid abundances, was oriented such that higher values reflected greater disease severity. The LSS demonstrated significant positive correlations with established markers of ACLF severity, including INR ($\rho = 0.481$, $p = 2.45 \times 10^{-6}$), MELD ($\rho = 0.441$, $p = 9.57 \times 10^{-6}$), MELD-Na ($\rho = 0.456$, $p = 4.32 \times 10^{-6}$), and serum creatinine ($\rho = 0.228$, $p = 0.025$) (Figure 4B-C). Consistent with progressive clinical deterioration, the score was significantly higher in non-survivors than survivors ($p = 0.017$) and increased with cumulative organ failure burden (Figure 4D-E).

Although not developed as a predictive model, the LSS provides a simple summary measure of coordinated lipid alterations associated with progressive systemic dysfunction in ACLF, supporting the relationship between coordinated changes in the circulating lipidome and clinical disease severity.

Comparative analysis of ACLF lipid signatures with published critical illness lipidomes

To determine whether these coordinated lipid alterations extended beyond ACLF, we compared our findings with published lipidomic datasets from ACLF (18) (Figure 5A), trauma (25) (Figure 5B), and COVID-19(26) (Figure 5C) using a harmonized lipid nomenclature. Although overlap of individual lipid species varied considerably across studies, several

membrane-associated lipid classes, including lysophosphatidylcholines, phosphatidylcholines, phosphatidylinositols, and phosphatidylethanolamines, displayed consistent patterns across independent cohorts and disease states. ACLF1 shared 22 lipid species with the independent ACLF2 cohort, 26 lipid species with the COVID-19 dataset, and 6 lipid species with the trauma dataset. Three lipid species PC(36:3), PC(38:4), and PE(36:4) were common across all four datasets, suggesting a conserved lipid signature associated with critical illness (Figure 5D). Together, these observations suggest that while individual lipid species remain disease- and cohort-dependent, coordinated alterations in membrane-associated lipid classes represent a more reproducible feature of severe systemic illness.

Independent molecular analyses provide biological context for coordinated lipid alterations

To provide a molecular context for the coordinated lipid alterations identified in ACLF, we profiled circulating polymorphonuclear neutrophils (PMNs) from patients with ACLF and healthy controls (Figure 6A). Differential proteomic analysis identified widespread alterations in the ACLF PMN proteome, with neutrophil degranulation emerging as the dominant enriched biological process (Figure 6B–C). This pathway was consistently represented across the PMN proteome and independent transcriptomic datasets, suggesting convergence on innate immune activation as a characteristic feature of ACLF.

To explore candidate regulatory mechanisms underlying these convergent molecular observations, we integrated the PMN proteomic dataset with a published ACLF PMN transcriptomic dataset (Saha *et al.*, 2021). Integrated pathway analysis identified MAPK signalling as a prominent regulatory axis, with enrichment of MAPK1-, MAPK14-, and MAP2K-regulated networks (Supplementary Table S4). Among transcription factors, PPAR γ , together with CEBPB and CEBPD, was consistently upregulated, whereas GATA1 and GATA2 were downregulated (Supplementary Table S4). Network analysis further identified MAPK1- and MAPK14-centred regulatory modules involving inflammatory and transcriptional regulators (Supplementary Figure S4).

Given the established role of PPAR γ in lipid sensing and inflammatory regulation, we next examined whether genes implicated in lipid signalling and ACLF pathogenesis contained putative PPAR γ binding motifs. Promoter analysis identified predicted PPAR γ binding sites in genes encoding MAPK1, MAPK14, FFAR3, CD177, Toll-like receptors, and metallothioneins, as well as in 96 of 129 genes comprising the neutrophil degranulation pathway (Supplementary

Tables S3-S4), suggesting that PPAR γ may represent one potential regulatory node linking coordinated lipid alterations with inflammatory gene networks.

Finally, to examine whether ACLF-associated lipids could plausibly engage this regulatory pathway, we performed molecular docking using lipid species that were reproducibly altered across both patient cohorts. Several phospholipids demonstrated favourable predicted binding to the orthosteric pocket of PPAR γ , with docking scores comparable to the endogenous ligand garcinoic acid (Figure 7A–C; Supplementary Figure S5, Supplementary Table S5). Structural clustering further demonstrated that these candidate ligands belonged to three distinct lipid families (Figure 7D), indicating that multiple structurally diverse phospholipids possess the potential to interact with PPAR γ . Collectively, these computational analyses nominate PPAR γ as a biologically plausible candidate regulator linking coordinated lipid alterations with convergent neutrophil inflammatory pathways in ACLF.

DISCUSSION

Acute-on-chronic liver failure (ACLF) is a clinically heterogeneous syndrome characterized by profound systemic inflammation, progressive organ failure, and high short-term mortality (1-3, 27, 28). Although numerous molecular abnormalities have been described in ACLF, it remains unclear whether these represent independent pathological perturbations or coordinated biological processes that reproducibly accompany disease progression. By integrating plasma lipidomics from two independent patient cohorts with clinical phenotyping, network analysis, neutrophil proteomics, published transcriptomic datasets, and regulatory pathway analyses, we demonstrate that ACLF is characterized by highly reproducible remodeling of the circulating lipidome that is most evident at the level of coordinated lipid classes and network modules rather than individual molecular species. These findings suggest that complex biological organization, rather than isolated lipid alterations, provides the most robust representation of the molecular architecture of ACLF.

The dominant feature of the circulating lipidome in ACLF was coordinated depletion of membrane-associated phospholipids, lysophospholipids, and plasmalogens, accompanied by relative enrichment of selected glycerophospholipids and triacylglycerols (Figure 2, Figure 3). These alterations remained highly consistent across two temporally independent patient cohorts despite the inherent biological heterogeneity of ACLF (Figure 2D). Rather than representing isolated metabolic abnormalities, these coordinated alterations are consistent with systematic changes in circulating membrane-associated lipids during severe systemic illness, as reflected by the reproducible depletion of lysophospholipids, phospholipids, and plasmalogens across

independent patient cohorts, their organization into coordinated lipid modules, and their association with organ failure severity (Figures 3 C-G, Figure 4). Membrane phospholipids regulate membrane architecture, intracellular signalling, lipid trafficking, and receptor organization, while plasmalogens contribute to membrane dynamics and represent important endogenous antioxidants because of their highly reactive vinyl ether bond (29-30). Experimental studies have further demonstrated that plasmalogens are abundant in innate immune cells, influence membrane organization and oxidative stress responses, and participate in inflammatory signalling (31-32). An important observation emerging from our study was that reproducibility increased with increasing levels of biological organization, i.e., although overlap of individual lipid species between independent cohorts and previously published lipidomic studies was modest, concordance at the level of lipid classes was substantially greater (Figure 3, Figure 4). This distinction can be biologically explained. Individual lipid species are influenced by multiple variables including dietary intake, acyl-chain remodeling, enzymatic activity, disease stage, and analytical platform, whereas coordinated changes in lipid classes reflect broader alterations in membrane composition and lipid metabolism (33-34). Similar principles have emerged across other systems biology disciplines, where reproducibility is often greatest at the level of pathways, modules, and biological processes rather than individual molecular features (35-37). Our findings therefore suggest that coordinated lipid programs provide a more stable representation of disease biology than isolated lipid species, particularly in clinically heterogeneous syndromes such as ACLF.

This concept was further reinforced by unsupervised network analysis (Figure 3 D-G). Rather than behaving as independent molecules, reproducible lipids self-organized into distinct co-regulated modules, among which the phospholipid- and plasmalogen-enriched Module 4 demonstrated the strongest relationship with disease severity (Figure 3E). Progressive depletion of this module closely paralleled increasing MELD, MELD-Na, INR, serum creatinine, and cumulative organ failure burden, indicating that coordinated membrane remodeling accompanies progressive clinical deterioration (Figure 3, Figure 4). Plasmalogens and ether phospholipids are increasingly recognized as regulators of membrane integrity, redox homeostasis, and inflammatory responses, and reduced plasmalogen abundance has been described across several inflammatory and critical illness states (38-40). Rather than identifying individual prognostic lipids, our findings suggest that collapse of coordinated membrane-associated lipid networks may represent a more biologically meaningful indicator of disease progression.

To summarize these coordinated alterations in a clinically interpretable manner, we developed a composite Lipid Severity Score (LSS) from reproducible lipid species associated with disease severity (Figure 4). It is important to note that this score was designed as an integrated biological summary rather than as a prognostic model. Nevertheless, its close association with MELD, MELD-Na, INR, serum creatinine, and cumulative organ failure burden suggests that coordinated lipid remodelling captures clinically relevant aspects of systemic dysfunction in ACLF (Figure 3 D-G, Figure 4). Whether such network-derived lipid signatures improve patient stratification beyond established clinical scores will require evaluation in larger prospective multicentre cohorts.

To contextualize these findings, we next examined whether lipid remodelling observed in ACLF reflects disease-specific alterations or broader responses to systemic inflammation. Comparative analyses with an independent ACLF lipidomics dataset and other inflammatory conditions, including trauma, and COVID-19 (Figure 5), revealed limited overlapping lipid species. The conserved phosphatidylcholine and phosphatidylethanolamine species, particularly PC(36:3), PC(38:4), and PE(36:4), may represent a core inflammatory lipid response associated with immune activation, membrane remodelling, and altered phospholipid metabolism during severe systemic injury (Figure 5D). At the same time, the substantially larger number of unique lipid alterations observed in ACLF indicates disease-specific metabolic reprogramming beyond the shared inflammatory response. These findings support the concept that while ACLF exhibits a conserved lipidomic framework characteristic of critical illness, its distinct lipid perturbations may reflect the unique pathophysiological consequences of acute hepatic decompensation and multi-organ dysfunction. From a clinical perspective, these class-level lipid signatures represent stable, quantifiable biomarkers, as lipids are chemically stable in biofluids and can be measured with high analytical precision using targeted LC-MS workflows (41-42).

Integration of plasma lipidomics with neutrophil proteomics and previously published transcriptomic datasets consistently identified biological processes related to innate immune activation, neutrophil degranulation, inflammatory signalling, and cellular stress responses (Figure 6). These datasets were generated using different patient cohorts, different molecular platforms, and different analytical approaches, yet converged on related biological processes. Although these observations do not yet establish causal relationships, such orthogonal convergence substantially increases confidence that the observed molecular programs reflect reproducible features of ACLF biology rather than cohort-specific findings.

The coordinated nature of the observed lipid alterations suggests the presence of upstream regulatory mechanisms rather than independent perturbations of individual lipid species. Integrative regulatory analyses nominated several lipid-responsive pathways, among which PPAR γ emerged as one plausible candidate (Figure 7). Given its established role in lipid sensing and inflammatory regulation, PPAR γ represents a biologically credible hypothesis that warrants experimental evaluation (43, 44).

Several limitations should be acknowledged. First, this study was observational and therefore cannot establish causal relationships between lipid remodeling and immune dysfunction. Second, the regulatory analyses identify candidate mechanisms but require experimental validation. Finally, although reproducibility was demonstrated across two independent cohorts, additional multicentre studies incorporating longitudinal sampling will be necessary to determine the temporal evolution and generalizability of these coordinated lipid programs.

Overall, our findings suggest that the circulating lipidome in ACLF may reflect a coordinated molecular state rather than independent perturbations of individual lipid species. By demonstrating that reproducibility increased from individual lipid species to lipid classes, co-regulated lipid modules, and convergent molecular pathways, our study highlights the importance of considering higher-order biological organization when interpreting complex human omics datasets. Defining disease through reproducible molecular programs rather than isolated biomarkers may provide a more robust framework for understanding biological heterogeneity in ACLF, improving molecular disease classification, and guiding the future development of clinically relevant biomarker panels and mechanistic studies.

In a broader context, our observations have implications beyond ACLF. In biologically heterogeneous diseases, molecular reproducibility may be more effectively captured through coordinated networks than through individual molecular features, providing a conceptual framework for interpreting human omics datasets.

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Figures and Figure Legends:

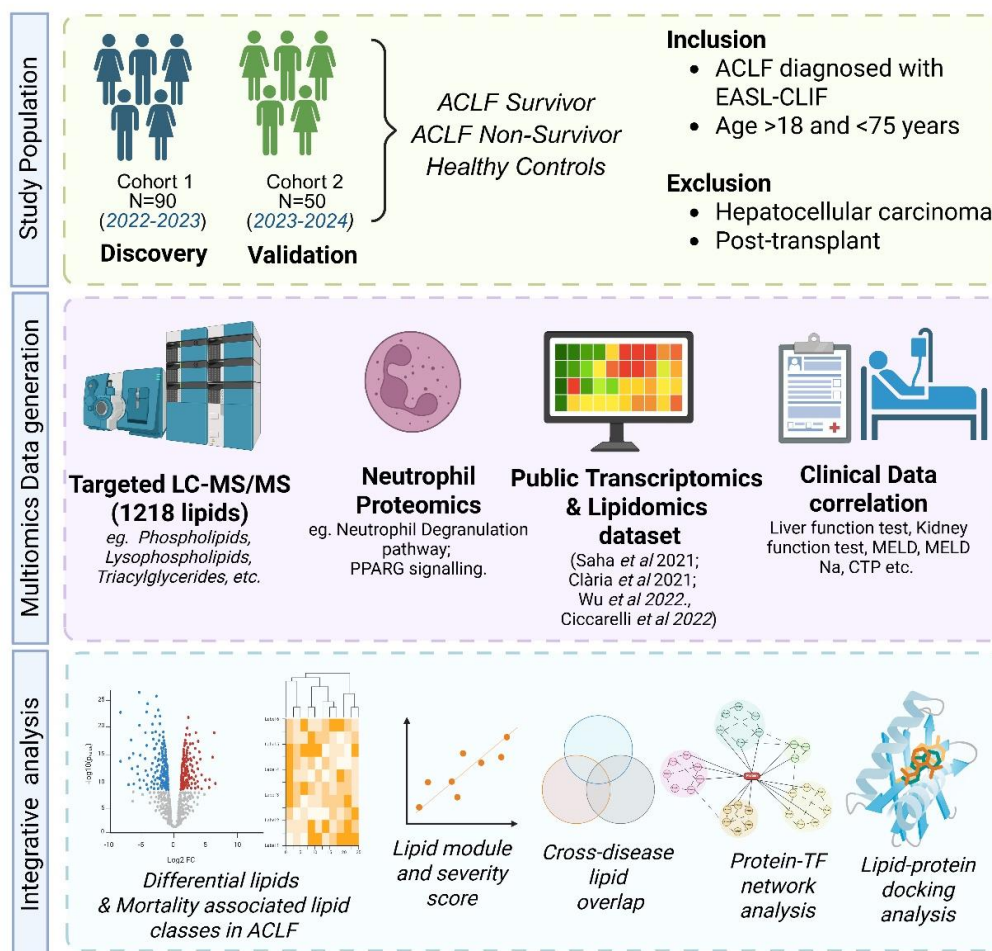


Figure 1. Study design and analytical work flow. The study design combined multi-omics, *ex vivo*, and *in silico* approaches. Plasma samples, and polymorphonuclear cells (PMNs), were obtained from ACLF patients and healthy controls. Plasma lipid profiling was conducted in Cohort 1 (ACLF $n = 60$; Healthy $n = 30$) and a temporally distinct Cohort 2 (ACLF $n = 40$; Healthy $n = 10$), with subgroup analyses of survivors and non-survivors. Untargeted proteomics was performed on PMNs, with pathway enrichment using Metascape. Analytical workflows included peak mapping, batch correction, and multivariate analyses (PLS-DA, VIP scoring, correlation heatmaps). *In silico* analyses combined transcription factor and kinase enrichment to identify key upstream regulators, highlighting PPARG as a central transcription factor linked to lipid-mediated immune dysfunction. Lipid docking (Tanimoto analysis) was then used to evaluate binding interactions of validated differential lipids with PPAR γ , integrating lipidomics with regulatory pathway discovery.

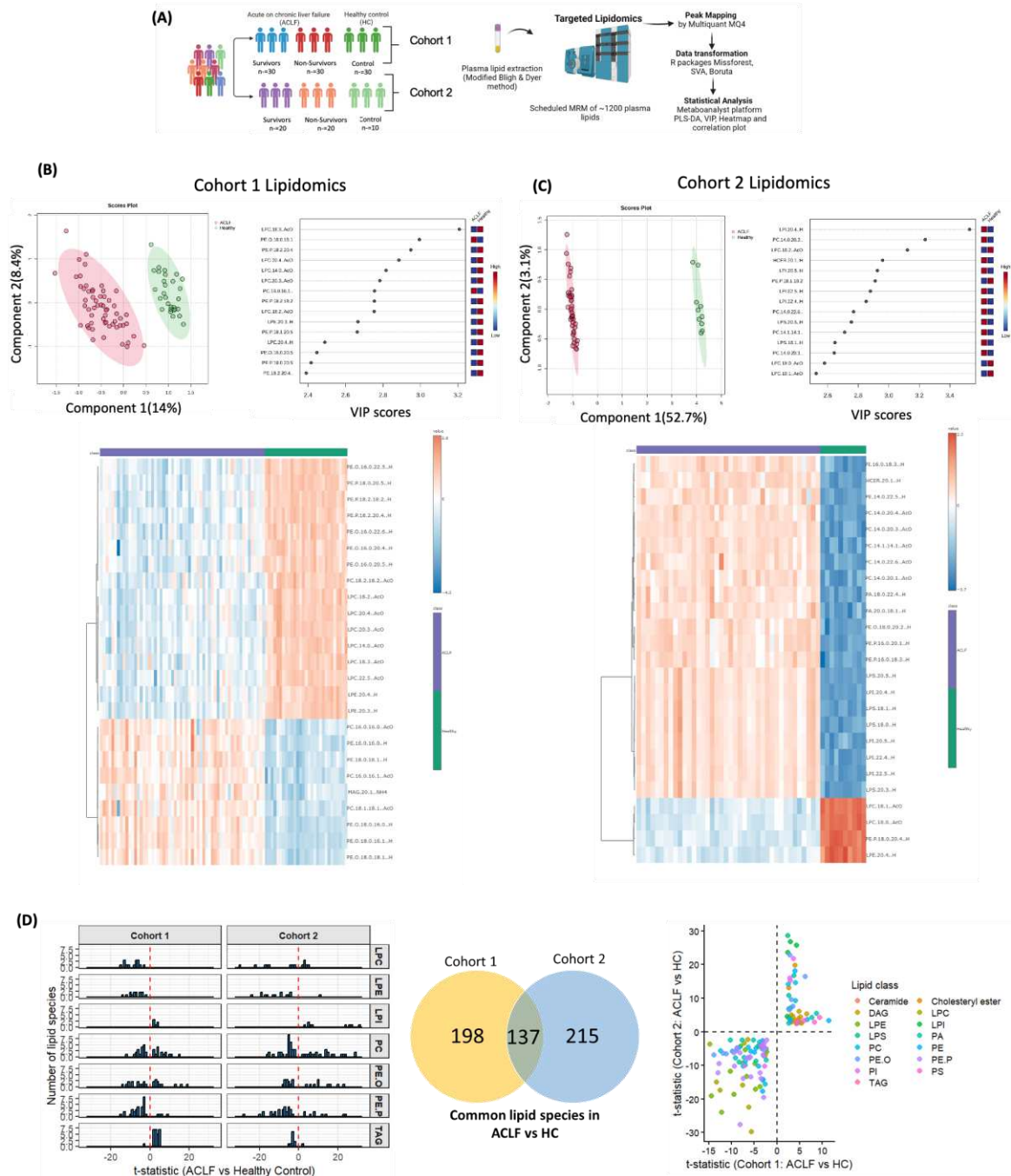


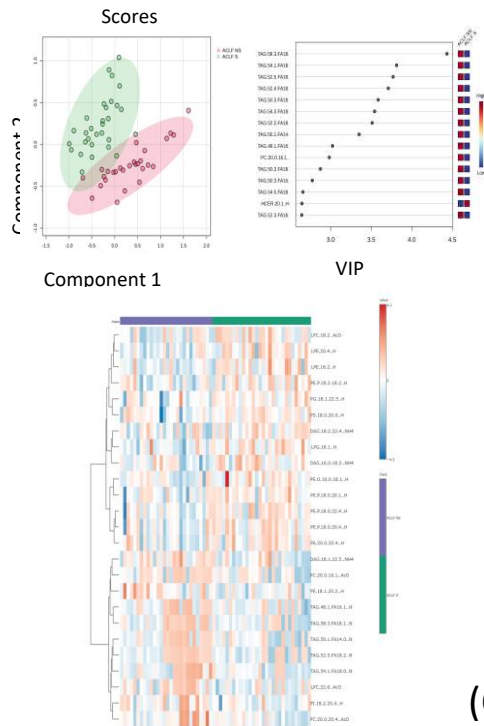
Figure 2. Targeted plasma lipidomic profiling identifies lipid classes associated with ACLF.

(A) Overall study design of the lipidomics analysis using two temporally independent cohorts (Cohort 1 and Cohort 2). Plasma lipid extraction was followed by a standardized targeted LC-MS/MS lipidomic workflow. Data were analyzed using R (v4.3.1) and MetaboAnalyst 6.0.

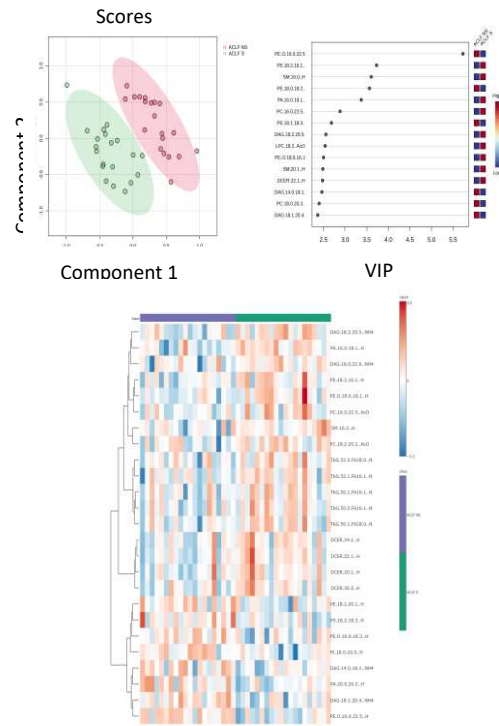
(B-C) Partial least squares-discriminant analysis (PLS-DA) plots showing separation between ACLF and healthy controls (HC), corresponding variable importance in projection (VIP) plots highlighting the top discriminatory lipid species, and heatmaps of the top 25

differentially abundant lipid species in Cohort 1 **(B)** and Cohort 2 **(C)**. **(D)** Left, face histograms showing the distribution of t -statistics across lipid classes for FDR-significant lipid species (ACLF vs healthy controls) in both cohorts, with the red dashed line indicating no change. Middle, Venn diagram showing the overlap of significantly altered lipid species between cohorts, identifying 137 shared lipid species. Right, scatter plot of t -statistics for the 137 shared lipid species in the two cohorts, with points colored by lipid class.

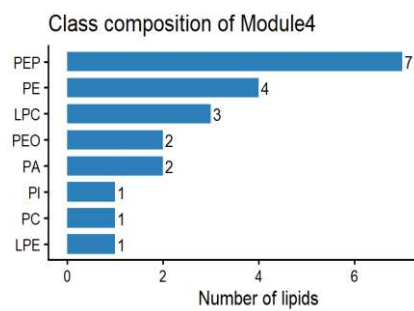
(A) COHORT



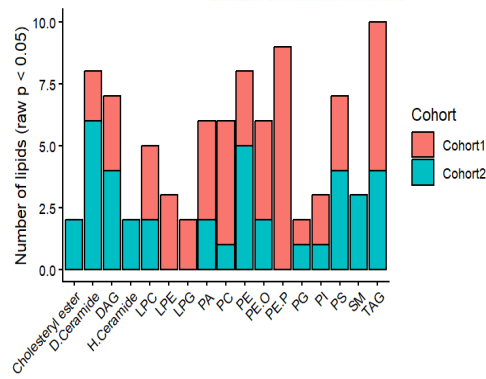
(B) COHORT



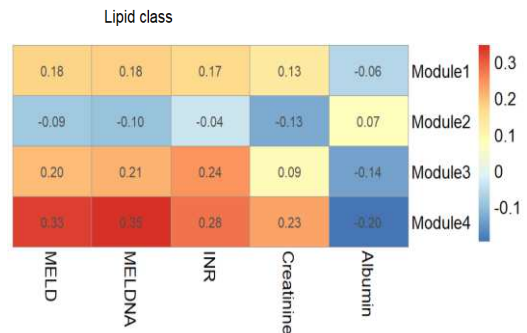
(D)



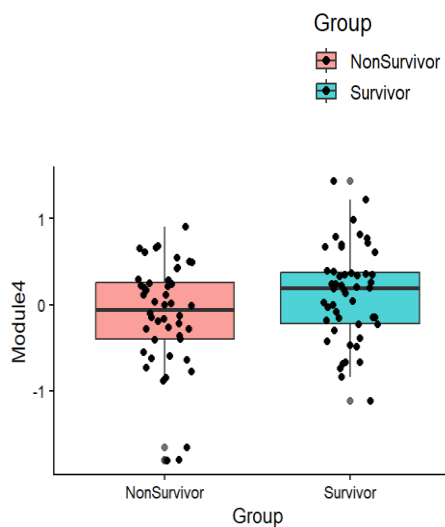
(C)



(E)



(F)



(G)

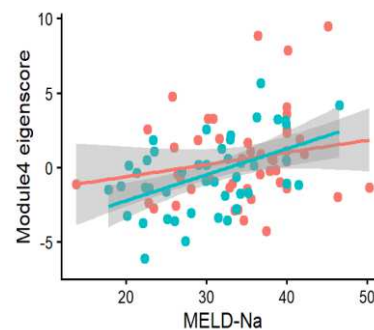


Figure 3. Lipidomic differences between survivors and non-survivors in ACLF across two independent cohorts

(A) Partial least squares-discriminant analysis (PLS-DA) score plot illustrating separation between survivors (S) and non-survivors (NS) in Cohort 1, along with the corresponding variable importance in projection (VIP) scores and heatmap of differential lipid species contributing to group discrimination. (B) PLS-DA score plot, VIP ranking, and heatmap showing survivor–non-survivor lipidomic differences in Cohort 2. (C) Stacked bar plot summarizing the number of differentially abundant lipid species (raw $p < 0.05$) across lipid classes in both cohorts, with Cohort 1 shown in orange and Cohort 2 shown in teal blue. Lipid classes are indicated on the x-axis, and bar height represents the number of significant lipid species per class. (D) Lipid class composition of Module 4, generated by Euclidean distance and complete linkage clustering, resulting into four lipid modules ($k=4$). Lipids belonging to each module were annotated according to their lipid class and module eigenscores were calculated as the mean standardized abundance of all lipids within a given module. (E) Module 4-trait correlation with MELD Na, MELD, INR, Creatinine, Albumin. (F) Module 4 eigenscore distribution in non-survivor vs survivor. (G) Module 4 eigenscore in ACLF non-survivor (orange) and ACLF survivor (green) versus MELD-Na.

(A)

$$\text{Lipid Severity Score} = -\frac{Z_{\text{PS}(16:0/18:1)} + Z_{\text{LPC}(16:1)} + Z_{\text{LPE}(16:1)} + Z_{\text{PE}(P-18:0/20:1)} + Z_{\text{PC}(16:0/20:3)} + Z_{\text{PC}(18:2/16:1)}}{6}$$

where

$$Z_i = \frac{x_i - \mu_i}{\sigma_i}$$

is the z-score normalized abundance of each lipid species.

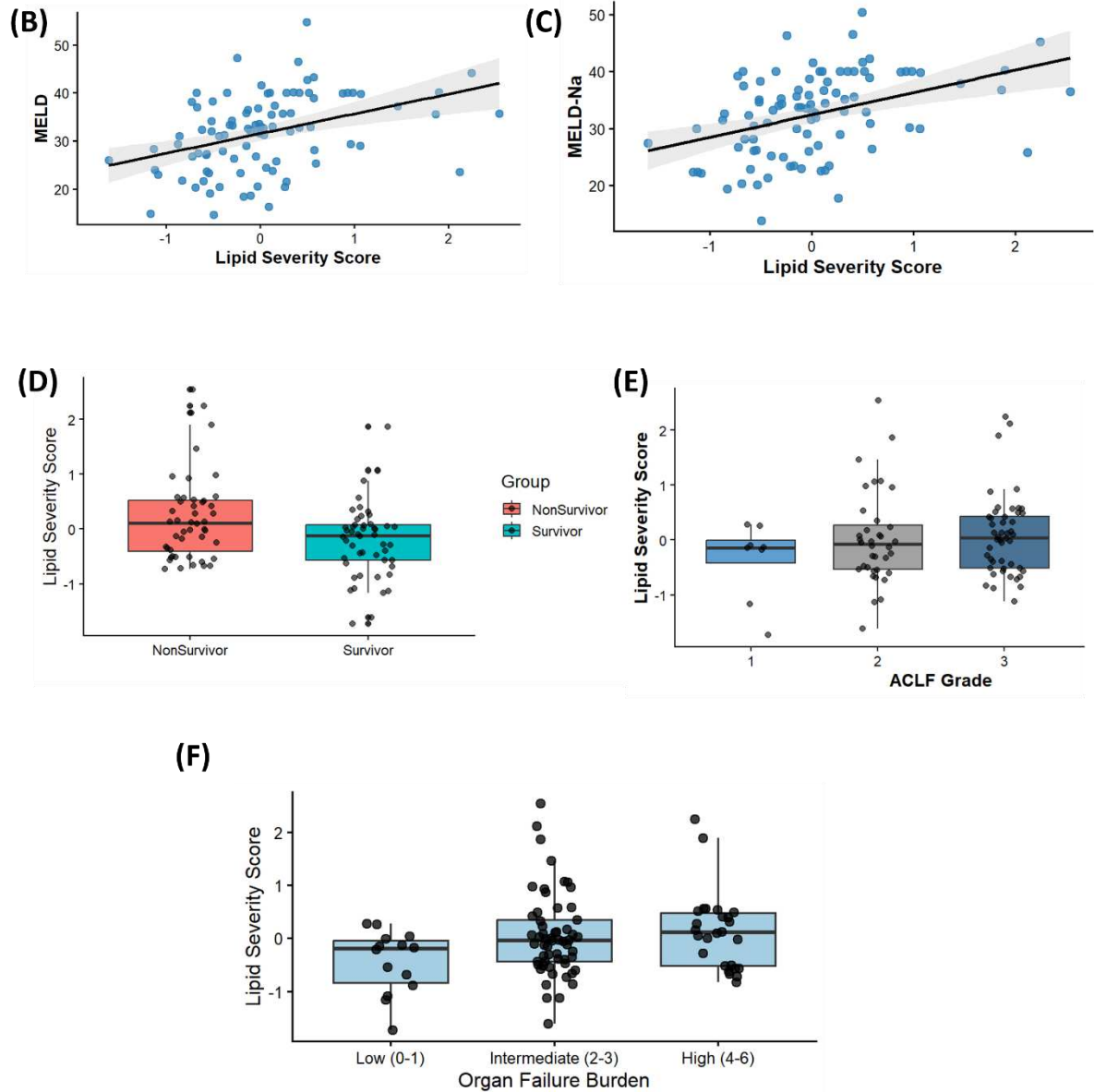


Figure 4. Composite Lipid Severity Score is associated with clinical severity in ACLF.

(A) Distribution of the Lipid Severity Score (LSS) across the study cohort. (B) Correlation between the Model for End-Stage Liver Disease (MELD) score and LSS. (C) Correlation between the Model for End-Stage Liver Disease-Sodium (MELD-Na) score and LSS. (D) Comparison of LSS between survivors and non-survivors. (E) Distribution of LSS across ACLF grades. (F) Association between LSS and cumulative organ failure burden.

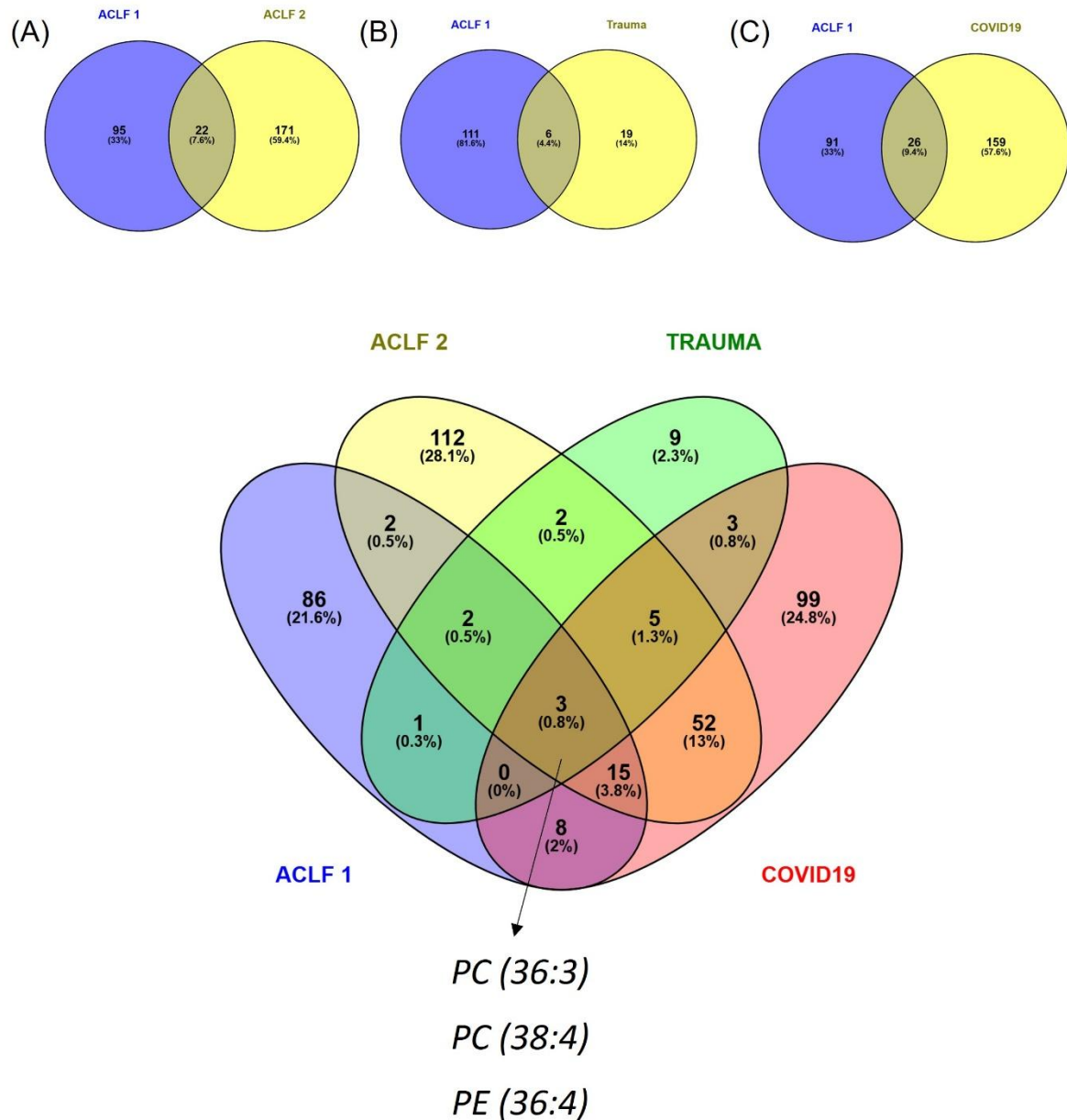


Figure 5. Cross-disease comparison identifies conserved membrane-associated lipid alterations.

Pairwise Venn diagrams showing the overlap of significantly altered lipid species between the ACLF discovery cohort (ACLF1) and an independent (A) ACLF cohort (ACLF2), (B) Trauma, and (C) COVID-19 datasets. (D) Three lipid species, PC(36:3), PC(38:4), and PE(36:4), were common across all four datasets, suggesting a conserved lipid signature associated with critical illness.

Figure 7

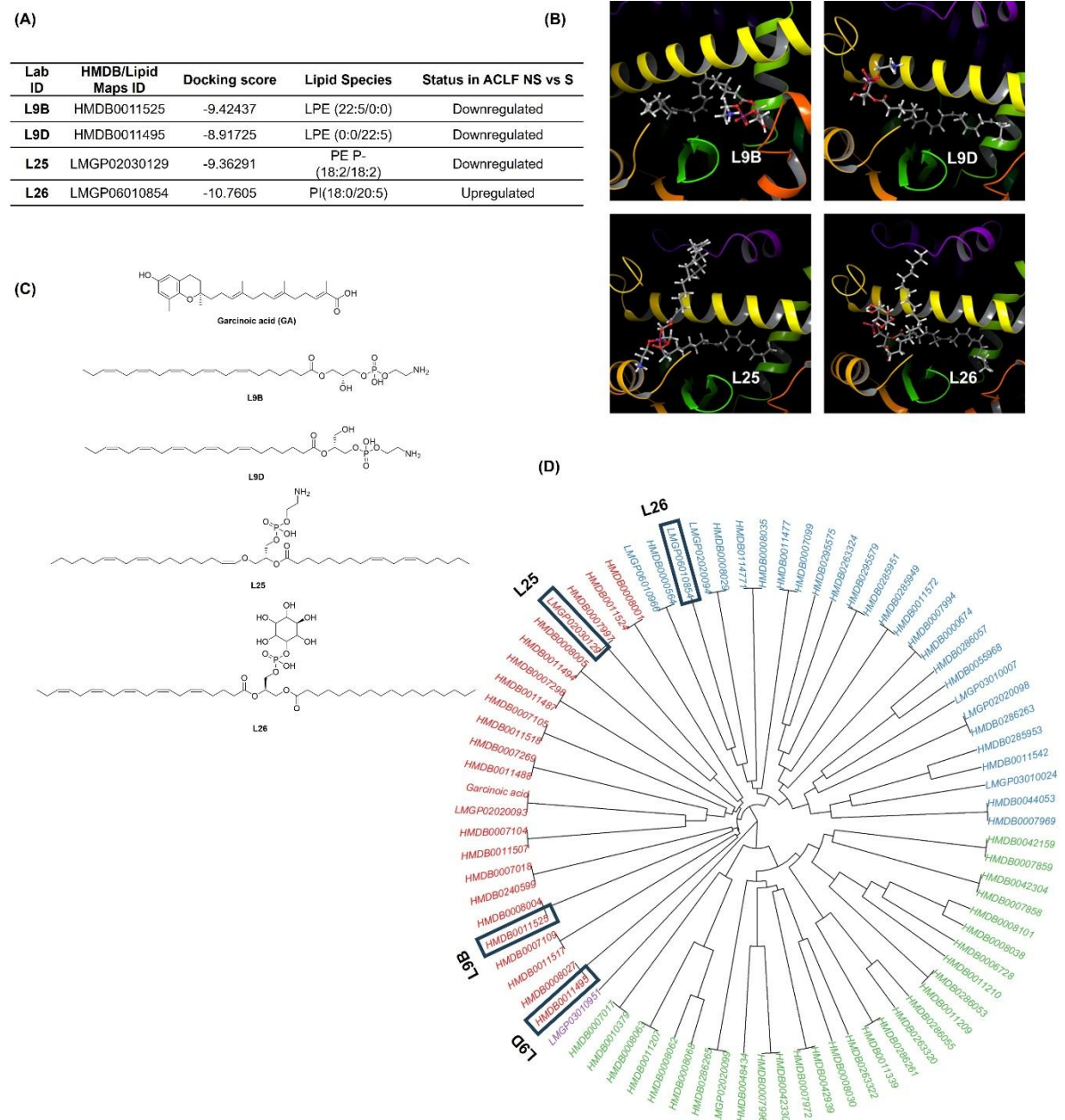


Figure 7. Computational docking of ACLF-associated lipids to PPAR γ and structural clustering of candidate lipid ligands.

(A) The four ACLF-associated lipid species with the highest predicted binding affinity (lowest docking scores) for PPAR γ . (B) Predicted binding poses of the four top-ranked lipid species within the ligand-binding pocket of PPAR γ . The receptor is shown as a ribbon representation and the docked lipid ligands are shown in gray. (C) Chemical structures of the four top-ranked lipid species used for docking analysis. (D) Circular dendrogram illustrating structural relationships among 80 ACLF-associated lipid species based on Jaccard similarity of molecular fingerprints. The four highest-ranking docked lipid species are highlighted.

Supplementary Files

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- [SupplementaryFigureS1.docx](#)
- [SupplementaryFigureS2.docx](#)
- [SupplementaryFigureS3.docx](#)
- [SupplementaryFigureS4.docx](#)
- [SupplementaryFigureS5.docx](#)
- [SupplementaryMethodsFile.docx](#)