Targeted lipidomics reveals extensive changes in circulating lipid mediators in patients with acutely decompensated cirrhosis

Targeted Lipidomics Reveals Extensive Changes in the Blood Lipid Mediator Profile in Acutely Decompensated Cirrhosis

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Keywords: Lipidomics; decompensated cirrhosis; systemic inflammation; lipid mediators

Electronic word count: 5975

Abstract word count: 273

Number of figures: 6

Number of tables: 1

Conflict of interest statement: None

Financial support: Supported by MEC (SAF15-63674-R) and EF-Clif, a non-profit private organization that receives unrestricted donations from Cellex Foundation, Grifols and the European Union's Horizon 2020 research and innovation programme. Our laboratory is a

Consolidated Research Group recognized by the Generalitat de Catalunya (2017SGR1449). CIBERehd is funded by the Instituto de Salud Carlos III. C.L-V received funding from the Horizon 2020 grant agreement Nº 825694.

Author Contributions: Study concept and design (CLV, JC, RM, VA); acquisition of lipidomics data (CLV, AC, CEW); bioinformatics and statistical analysis (AU, FA, AA, MP, DGC, NP); integration of clinical and biological results and interpretation of data (CLV, JC, AC); drafting of the manuscript (CLV, JC); writing of the final manuscript (CLV, JC, RM); critical revision of the manuscript for important intellectual content (JAQ, PC, JT, KO, VA); study supervision (CLV, JC).

Abstract

Acute-on-chronic liver failure (ACLF) is a newly described syndrome, which develops in patients with acutely decompensated cirrhosis, and is characterized by intense systemic inflammation, multi-organ failure and high short-term mortality. The profile of circulating lipid mediators, which are endogenous signaling molecules generated from polyunsaturated fatty acids released from membrane phospholipids that play a major role in inflammation and immunity, is poorly characterized in ACLF. In the current study, we assessed the profile of lipid mediators by liquid chromatography coupled to tandem mass spectrometry in plasma from patients with acutely decompensated cirrhosis, with (n=119) and without (n=127) ACLF, and from healthy subjects (HS, n=18). Measurements were prospectively repeated in 191 patients with acutely decompensated cirrhosis during a 28-day follow-up period. Fifty-nine lipid mediators (out of 100) were detected in plasma from cirrhotic patients, of which, 16 were significantly associated with the disease status. Among these, 11 lipid mediators distinguished patients at any stage from HS, whereas two lipid mediators (leukotriene [LT] E_4 and 12-hydroxyheptadecatrienoic acid, both derived from arachidonic acid) shaped a minimal plasma fingerprint that discriminated patients with ACLF from those without. Levels of LTE_4 distinguished ACLF grade 3 from ACLF grades 1 and 2, followed the clinical course of the disease (increased with worsening and decreased with improvement) and positively correlated with markers of inflammation and non-apoptotic cell death. Moreover, LTE_4 together with LXA_5 (derived from eicosapentaenoic acid) and EKODE (derived from linoleic acid) associated with short-term mortality. Interestingly, LXA_5 and EKODE formed a signature profile associated with coagulation and liver failures. Taken together, these findings uncover specific lipid mediator profiles associated with severity and prognosis of patients with acutely decompensated cirrhosis.

Lay summary

Acute-on-chronic liver failure (ACLF) is characterized by intense systemic inflammation, multi-organ failure and high short-term mortality. In the current study, we assessed by targeted lipidomics using a LC-MS/MS-based platform the plasma profile of 100 bioactive lipid mediators, which are endogenous signaling molecules generated from polyunsaturated fatty acids that play a major role in inflammation and immunity. We identified lipid mediator signatures associated with inflammation and non-apoptotic cell death that discriminate disease severity and evolution, short-term mortality and organ failures.

Introduction

Patients with acutely decompensated (AD) cirrhosis frequently succumb to the onset of multi-organ failure, a syndrome known as acute-on-chronic liver failure (ACLF) (1,2). ACLF is closely associated with recurrent infections and high short-term mortality and is mostly driven by dysfunctional innate immune system leading to exacerbated systemic inflammation, immune paralysis and tissue immunopathology (3). Indeed, plasma cytokines are unusually elevated in ACLF patients and their levels directly correlate with ACLF severity (3). Moreover, a blood metabolite fingerprint specific for ACLF closely associated with the levels of inflammatory markers and the presence of organ failures has recently been identified (4). Overall, these studies reinforce the concept that systemic inflammation and tissue/organ injury in ACLF is triggered by the concerted actions of cytokines/chemokines and amino acid-derived factors that act as metabotoxins. However, the contribution of lipid mediator species in the pathogenesis of systemic inflammation and development of organ failures in ACLF remains at present unexplored.

Lipid mediators are signaling molecules with potent and diverse actions on blood and tissue homeostasis and responses to stress and injury. These compounds comprise a vast number of species, whose biosynthetic pathways form a complex network of multiple substrates transformed via multiple enzymes (5,6). In general, several enzymes can act on a single substrate, and conversely, multiple substrates can be metabolized by the same enzyme. The majority of the bioactive lipid mediators are intracellularly produced from polyunsaturated fatty acids (PUFAs) released from membrane phospholipids by the action of phospholipase A_2 (5). After the release of free PUFAs to the cytosol, they are rapidly metabolized by three enzymatic families: the cyclooxygenases (COXs), the lipoxygenases (LOXs) and the cytochrome P450 epoxygenases (CYP450), to produce a large array of lipid mediators

(**Supplementary Figure 1A**) (5,6). The most common substrate for these enzymes is arachidonic acid (AA), an omega-6-PUFA that is the precursor of eicosanoids such as prostaglandins (PGs), leukotrienes (LTs), thromboxane A_2 (TXA_2) and lipoxins (LXs) (5,6). With the exception of LXs, eicosanoids are considered to be proinflammatory, and some members such PGs and TXA_2 are targeted by NSAIDs (7). In addition to AA, the same enzymes can effectively metabolize its parent precursor, linoleic acid (LA) (**Supplementary Figure 1A**), which is abundant in low-density lipoproteins and inner mitochondrial membrane phospholipids (8). LA derivatives are traditionally considered to exert detrimental actions on renal, respiratory and cardiovascular systems (9). In contrast, the omega-3-PUFAs eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids give rise to an array of lipid mediators such as 18-HEPE, 17-HDoHE and 14-HDoHE that are involved in the resolution of inflammation (**Supplementary Figure 1B)** (10). Finally, all PUFAs are susceptible to non-enzymatic oxidation, yielding epoxides, ketones and hydroxylated derivatives, which in general are considered oxidative stress markers (11).

In view of the magnitude and diversity of the lipid mediator network, the analysis and identification of these molecules in complex diseases requires an "omics" approach. This study reports the profiling of 100 lipid mediators using LC-MS/MS in 246 patients with AD cirrhosis of whom 127 did not have ACLF and 119 had ACLF (hereafter called patients with ACLF). Measurements were prospectively repeated in 191 patients during a 28-day follow-up period.

Patients and methods

The investigation was performed in plasma samples from 246 patients with acutely decompensated cirrhosis of whom 119 had ACLF (57 with ACLF-1, 44 with ACLF-2 and 18 with ACLF-3) from the CANONIC cohort (1). In 191 out of the 246 patients with AD cirrhosis, there was availability of plasma samples with enough volume to perform the measurements during the 28-day follow-up. All these individuals or their legal representatives and the ethics committee of each hospital involved in the study gave informed consent for omics investigations in the biobanked material. A flow chart of the patients from the CANONIC study included in the targeted lipidomics is shown in **Supplementary Figure 2**. The investigation also included 18 healthy subjects (HS, age: 45-65 years).

Analysis of lipid mediators by targeted LC-MS/MS

Plasma levels of 100 lipid mediators were determined by LC-MS/MS. Common and systematic nomenclature of these lipid mediators are detailed in **Supplementary Table 1**.

Analysis of PUFA, isolation of leukocytes, analysis of gene expression by TaqMan low-density arrays and measurement of cytokines, chemokines and oxidative stress and cell death (keratin 18 [K18] and caspase-cleaved K18 [cK18]) markers. See **Supplementary Material** and references 3, 12 and 13.

Statistical analysis

Among the 100 lipid mediators screened (**Supplementary Table 1**), 39 were below the detection limits of the method in the three study groups. Among the 61 lipid mediators detected, two were excluded (5-iso-prostaglandin F2α-VI and 12-KETE) because they did not

meet the quality control criteria. Therefore, the final analysis included a total of 59 lipid mediators. See **Supplementary Material**.

Results

Baseline clinical and standard laboratory data are given in **Supplementary Table 2**. C-reactive protein levels were higher in patients with AD relative to HS and much higher in those with ACLF. White blood cell count was significantly increased in patients with ACLF as compared to those with AD. Platelet count and serum albumin were significantly reduced in patients with AD and ACLF. There were significant differences between patients with AD and ACLF in serum bilirubin and creatinine levels. Among patients with ACLF, 57 (47.9%) had ACLF grade 1 (one organ failure), 44 (36.9%) had ACLF grade 2 (two organ failures), and 18 (15.1%) had ACLF grade 3 (three organ failures or more). The frequency of failing organs in these patients is also shown in **Supplementary Table 2**. Patients with ACLF had higher MELD and CLIF organ failure and Child-Pugh scores and greater 28-day mortality than patients with AD.

Patients with AD and those with ACLF have increased plasma levels of AA and higher AA/EPA ratio

The final database obtained from the targeted LC-MS/MS analysis included a total of 59 lipid mediators (see Patients and methods for the criteria used in the selection of compounds). The identity of each lipid mediator was assessed by both the selected reaction monitoring (SRM) transition and comparison of retention time to that of authentic standards (**Supplementary Tables 3 and 4**). Annotated lipid mediators were mostly derived from PUFAs of the omega-6 (AA and its precursor LA) and omega-3 (DHA and EPA) families (**Figure 1A**). As shown in **Figure 1B**, plasma levels of free PUFA were similar across the three study groups, except for the AA, which levels were slightly increased in patients with AD and in those with ACLF. However, the total content of PUFA (all PUFA from triglycerides, phospholipids and cholesterol esters after saponification), which represents the actual PUFA pool in plasma,

were considerably reduced in patients with AD and also in patients with ACLF (**Figure 1B**). In both cases (free and total PUFA), the AA/EPA ratio, which is a surrogate marker of systemic inflammation (14), was significantly increased in both groups of patients with cirrhosis (**Figure 1C**). This finding was consistent with the fact that patients with ACLF exhibited an increased systemic inflammatory burden, as reflected by the presence of augmented plasma levels of cytokines/chemokines (**Supplementary Table 5**). Genes related to the desaturation and elongation of fatty acids were not dramatically altered in leukocytes from patients with cirrhosis, except for *SCD1* and *ELOVL6*, which were up- and down-regulated, respectively (**Figure 1D**).

Distinct plasma distribution of lipid mediators in patients with AD and in those with ACLF

The biosynthesis of lipid mediators from PUFA involves a complex network of LOX, COX and CYP450 enzymes (see **Supplementary Figure 1** for an overview of the biosynthetic pathways and **Supplementary Tables 6-8** for a comprehensive classification of each lipid mediator according to its biosynthetic precursor and enzymatic route). **Figure 2A** shows a graphical representation of the plasma abundance of lipid mediators categorized into two families (omega-6 and omega-3) and classified by each enzymatic pathway. Circles represent the absolute amount of lipid mediators within the pathway (upper panel), and the box plots represent individual values for each subject included in the analysis (lower panel). The most abundant lipid mediators in HS were derived from CYP450 and LOX pathways followed by non-enzymatic metabolites and minor quantities of COX derivatives. The abundance of CYP450-derived lipid mediators of the omega-6 family significantly decreased in patients with AD and ACLF whereas those derived from LOX remained steady. The amount of COX-derived lipid mediators slightly increased in patients with cirrhosis, but changes did not reach

statistical significance. Notably, the levels of lipid mediators produced from non-enzymatic routes (i.e. free radical lipid oxidation) abruptly increased in patients with AD and culminated in those with ACLF. This increase was predominantly in the omega-6 family and in particular in the LA-derived lipid mediator EKODE (**Supplementary Table 7**). The increased levels of these non-enzymatic products were consistent with the presence of an intense degree of systemic oxidative stress, as reflected by the plasma levels of HNA1 and HNA2 (**Supplementary Table 5**), which are established markers of systemic oxidative stress in patients with cirrhosis (15).

Leukocyte gene expression of lipid mediator-generating enzymes differs between patients and HS

We next investigated the expression of genes coding for enzymes responsible for the conversion of PUFA precursors to the individual lipid mediators in leukocytes from patients and from HS. **Figure 2B** shows the relative distribution of each of the three enzymatic pathways (i.e. CYP450, LOX and COX) and **Figure 2C** shows the expression of individual representative genes among these pathways in leukocytes from HS, patients with AD and patients with ACLF. In agreement with results described earlier, the expression of the main CYP450 enzyme involved in PUFA metabolism, *CYP2C8*, was markedly down-regulated in patients with AD and in those with ACLF, relative to HS. In contrast, the expression of LOXs, specifically *ALOX5*, which codes for the 5-LOX enzyme involved in the production of inflammatory LTs, was remarkably up-regulated in patients with AD and in those with ACLF. Expression of enzymes of the COX pathway, especially COX-2 (*PTGS2*) and mPGES-1 (*PTGES1*), was also up-regulated in patients relative to HS.

Distinct profile of lipid mediators in patients with AD and in those with ACLF relative to HS

Next, we grouped the 59 lipid mediators detected in the plasma of patients according to their cognate chemical families and calculated for each family the fold changes in AD vs HS and in ACLF vs HS. Finally, we ranked fold changes from the highest to the lowest values and the results were visualized in a Cleveland plot (**Figure 3A)**. This analysis revealed that LTs, epoxy-keto fatty acids, AA/DHA epoxides, PGs and TX were increased in patients (either AD or ACLF) as compared to HS. In contrast, LXs, which are anti-inflammatory and pro-resolving lipid mediators, LA diols and LA epoxides were remarkably reduced in cirrhosis.

We then calculated fold changes for each individual lipid mediator in AD vs HS and in ACLF vs HS and ranked and plotted the results in a Cleveland plot (**Figure 3B**). This analysis uncovered that 24 out of a total of 59 lipid mediators were significantly increased (fold change >1.5) in AD as compared to HS, of which 4 were further increased in ACLF as compared to AD (**Figure 3B** and **Supplementary Table 9**). On the other hand, the plasma levels of 9 lipid mediators were significantly decreased (fold change <0.5) in AD as compared to HS and none of them was further reduced in ACLF as compared to AD. A complete list of fold-changes between ACLF and AD for all the 59 lipid mediators included in the analysis is provided in **Supplementary Table 9**. Changes in circulating levels of lipid mediators in patients affected indistinctly all PUFA families as shown on the left side of **Figure 3B**, where each lipid mediator is color coded by its biosynthetic precursor.

We next plotted in volcano plots the fold changes in the levels of lipid mediators in patients with AD and in those with ACLF relative to HS, taking into account the statistical significant differences (P values). As shown in **Figure 3C**, this analysis identified increased levels of 8-

HETE, 14,15-DiHETrE, 12,13-epoxy-9-keto-10(trans)octadecenoic acid (EKODE), 11,12-DiHETrE, 8-HETrE, 13-HOTrEγ, LTE_4, 20-HETE, 11-keto-TXB_2 and PGE_1 in the plasma of patients with AD. Among these, LTE_4 (a member of the slow-reacting substance of anaphylaxis and a pathway marker of pro-inflammatory cysteinyl-LT biosynthesis), 11-keto-TXB_2 (a prothrombotic marker) and 20-HETE (a potent renal vasoconstrictor) have pathophysiological significance in these patients. On the other hand, 9(10)-epoxyoctadecenoic (9(10)-EpOME) and 12(13)-epoxyoctadecenoic (12(13)-EpOME) acids, which are generated by neutrophils during oxidative burst and are markers of bactericidal activity, were remarkably suppressed in patients with AD (**Figure 3C**). In patients with ACLF, the lipid mediators whose levels were significantly increased were the same as in patients with AD, but the profile was enriched in two additional lipid mediators ($PGF_{2\alpha}$, a COX-derived vasoconstrictor, and 8-HDoHE, a product of DHA autoxidation) (**Figure 3D**). Of note, the fold changes achieved by some lipid mediators such as 8-HETE, which was increased more than 16-fold, or 9(10)-EpOME and 12(13)-EpOME, which were reduced by 8-fold, indicate that PUFA metabolism is severely disrupted in patients with cirrhosis.

Unbiased identification of a lipid mediator signature specific of AD cirrhosis

To reduce the dimension of our dataset, we next explored whether any combination or combinations of lipid mediators could serve as a fingerprint of patients with AD cirrhosis. To address this question, we performed an unbiased PCA analysis on the 59 lipid mediators collected at baseline in the entire study cohort. As shown in **Figure 3E**, the PCA analysis yielded a clear distinction between patients and HS. After adjusting for gender and age, we refined from 59 to 16 the number of lipid mediators separating the different stages of the disease. The plasma levels of these 16 lipid mediators can be visualized in a heatmap, which reveals that, with the exception of 9(10)-EpOME and 12(13)-EpOME, changes in lipid

mediators were highly heterogeneous in our cohort of patients (**Figure 3F)**. Of interest, one out of these 16 lipid mediators, the DHA product 4-HDoHE, distinguished patients with AD from HS, whereas 11 lipid mediators discriminated HS from patients at any stage (either AD or ACLF) (**Table 1**). These 11 lipid mediators were 9(10)-EpOME, 12(13)-EpOME and EKODE from LA; 8-HETE, 20-HETE, 11,12-DiHETrE, 14,15-DiHETrE and 11-keto-TXB_2 from AA; 8-HETrE and PGE_1 from DGLA and 13-HOTrEγ from γ-LA. Box plots for 9(10)-EpOME and 12(13)-EpOME are shown in **Figure 4A**. Although 9(10)-EpOME, 12(13)-EpOME are biologically relevant and their levels presented the largest reductions in the volcano plots, these two lipid mediators were not associated with any clinical outcome (**Supplementary Figure 3**). Lipid mediators that were not significantly associated and did not discriminate the different stages of the disease are listed in **Supplementary Table 10**.

Plasma levels of LTE_4 discriminate disease severity

Among the 16 lipid mediators identified in the PCA analysis, two of them ($PGF_{2\alpha}$ derived from AA [**Figure 4B**] and 8-HDoHE derived from DHA) distinguished patients with ACLF from HS (**Table 1**). Importantly, LTE_4 and 12-hydroxyheptadecatrienoic (12-HHT) derived from AA shaped a minimal plasma fingerprint that discriminated patients with ACLF from patients with AD **(Table 1)**. Between these two lipid mediators, LTE_4 appeared to have a robust discriminative power and its levels gradually increased in parallel with the severity of the disease, being significantly higher in AD cirrhosis compared to HS and in ACLF compared to AD **(Figure 4C**). In addition, LTE_4 levels were higher in patients with ACLF grade 3 than in those with ACLF grade 1 and ACLF grade 2 (**Figure 4D**), suggesting that in terms of this lipid mediator, ACLF-1 and ACLF-2 are indistinguishable. Similar LTE_4 levels in plasma were observed when patients were categorized according to the presence or absence of bacterial infections, portal hypertension, ascites and esophageal varices (**Figure**

4E-G). The association of lipid mediators with bacterial and fungal infections, development of bacterial infection during hospitalization, portal hypertension, ascites and esophageal varices is detailed in **Supplementary Tables 11** and **12**). The dynamics of LTE_4 was also investigated in a subset of 191 patients (96 AD and 95 ACLF at enrollment) who underwent follow-up for a maximum of 28 days. Twenty-one percent of patients improved the status from ACLF to no ACLF, 10.5% became worse (from AD to ACLF), 19 patients presenting ACLF at inclusion increased the degree of ACLF and 7 reduced the ACLF degree but still having ACLF (**Supplementary Table 13A-B**). Paired sample tests between baseline and follow-up measurements showed that plasma levels of LTE_4 paralleled the course of the disease (significant reduction in patients who improved the clinical status from ACLF to AD and significant increase in those who worsened the condition from AD to ACLF) (**Figure 4H**). However, sensitivity analysis in AD patients secondary developing ACLF revealed that LTE_4 has low predictive value (area under the ROC curve=0.304).

A specific lipid mediator profile associates with markers of inflammation and cell death in patients with AD and in those with ACLF

To investigate the association of lipid mediators collected at baseline with markers of inflammation (cytokines and chemokines) and cell death (cK18 and K18), we constructed a correlation matrix plot including the whole group of patients with cirrhosis. As shown in **Figure 5**, lipid mediators had in general a positive correlation among them, except LXA_5, which is an anti-inflammatory and pro-resolution lipid mediator that was inversely correlated. In general, lipid mediators had a weak correlation with cytokines/chemokines, except LTE_4 and LXA_5. LTE_4, which composes the ACLF fingerprint, was the only lipid mediator with distinct positive correlation with inflammatory cytokines/chemokines, in particular with IL-1RA and IL-6, and specially with IL-8. LTE_4 also positively correlated with markers of cell

death in AD and ACLF patients (**Figure 5** and **Supplementary Figure 4**). This correlation was stronger with K18 than with cK18, suggesting that LTE_4 can be associated with the non-apoptotic form. On the other hand, LXA_5 was inversely correlated to LTE_4, showed a negative correlation with IL-8 and did not associate with cK18 and K18. Interestingly, PGE_2, which has previously been associated with immunosuppresion in cirrhosis (16), was positively correlated with IL-8 (ρ=0.526, P<0.001), although this lipid mediator was not associated with infection or any other clinical variable (**Supplementary Figure 5** and **Supplementary Tables 11 and 12**).

A specific lipid mediator signature associates with organ failures and short-term mortality in patients with ACLF

We finally investigated the association of the 59 lipid mediators collected at baseline with the most frequent organ failures (i.e. circulatory, brain, coagulation, liver, kidney and respiratory) in patients with AD and ACLF. The heatmap in **Figure 6A** shows that LXA_5 was the lipid mediator with the strongest association, in particular with liver failure. Other lipid mediators associated with liver failure were autooxidation products such as 9-KODE, 8-HETrE, 8-HDoHE, 4-HDoHE, 11,12-DiHETrE and EKODE. Of interest, LXA_5 together with EKODE constituted a minimal fingerprint of liver and coagulation failures, while $PGF_{2\alpha}$ was significantly associated with circulatory failure. None of the lipid mediators associated with brain, kidney or respiratory failures. **Figures 6B and C** show the presence of increased EKODE in the context of reduced LXA_5, which were the lipid mediators associated with at least two different organ failures. Moreover, increased EKODE and LTE_4 together with reduced LXA_5 significantly associated with 28-day mortality **(Figure 6D** and **Supplementary Table 12**).

Discussion

The current study investigated the profile of lipid mediators in plasma from patients of the CANONIC study (1). By LC-MS/MS we screened 100 lipid mediators derived from PUFAs in plasma from 246 patients with AD cirrhosis of whom 119 had ACLF. Our major findings were the following: 1) Patients with AD, and to a greater extent patients with ACLF, showed increased ratio between AA (omega-6-PUFA that serves as substrate precursor for inflammatory and vasoconstrictor lipid mediators) and EPA (omega-3-PUFA that serves as substrate precursor for anti-inflammatory and pro-resolving lipid mediators), which is a surrogate marker of systemic inflammation (14). 2) ACLF was associated with higher circulating levels of LTs, PGs, epoxy-keto fatty acids and TX families, in parallel with reductions in LXs and epoxy fatty acids. 3) LTE_4 was one of the top differentially regulated lipid mediators and gradually increased from HS to AD and ACLF, as well as in ACLF-3 as compared with ACLF-1 and -2. In addition, LTE_4 levels followed the clinical course of the disease (levels increased when worsening and decreased when improving). Moreover, LTE_4 positively correlated with markers of cell death (K18) and inflammatory cytokines (IL-8). 4) LXA_5, which was invariably reduced in patients, was the only lipid mediator that inversely correlated with IL-8. 5) LTE_4 was part of a minimal plasma fingerprint for ACLF, whereas LXA_5, and EKODE, discriminated organ failures. 6) Finally, increased LTE_4 and EKODE together with decreased LXA_5 was associated with higher 28-day mortality. Collectively, these findings capture a specific lipid mediator profile in patients with AD cirrhosis, adding value to recent studies within the frame of CANONIC, describing a characteristic metabolomic fingerprint in these patients (4).

There are findings in our study that deserve particular attention. For example, LTE_4 was the lipid mediator enzymatically generated from AA with the largest fold change in ACLF versus

HS. LTE_4 is formed upon activation of 5-LOX, which converts AA into 5-HpHETE, an intermediate in the generation of LTA_4 (**Supplementary Figure 1A**). LTA_4 is further converted into LTB_4 by LTA_4 hydrolase or into LTC_4 by LTC_4 synthase. LTC_4 is further metabolized to LTD_4 and LTE_4 (generically termed as cysteinyl-LTs), which are potent vasoconstrictors that were previously known as slow-reacting substances of anaphylaxis (17). Cysteinyl-LTs are primarily generated by neutrophils, macrophages, eosinophils and mast cells at sites of infection and/or inflammation and their release is enhanced by activation of Toll-like receptors (18). Cysteinyl-LTs participate in a variety of diseases including arthritis, inflammatory bowel disease, atherosclerosis and especially asthma and allergy, conditions in which blockage of their receptors is used as therapy (19). In these conditions, cysteinyl-LTs directly interact with cytokines (i.e. IL-6, IL-10 and TNFα) and chemokines (i.e. IL-8, eotaxin and MIP-1α) (20). In addition to inflammation, cysteinyl-LTs might also be related to cell death, since in our study LTE_4 levels were strongly correlated with K18, which is a marker of total cell death. Interestingly, LTE_4 negatively correlated with the ratio between cK18 (apoptosis) and K18 (apoptosis and necrosis), indicating that this lipid mediator is related to non-apoptotic cell death, which potentially is more immunogenic (13). On the other hand, cysteinyl-LTs induce hyperreactivity of the arterial vascular tissue to vasoactive compounds (such as angiotensin II and norepinephrine), suggesting that these lipid mediators may have pathological significance in the development of organ failures in ACLF (21). Indeed, elevated urinary LTE_4 levels were reported in patients with hepatorenal syndrome and might contribute to the development of kidney dysfunction in patients with cirrhosis (22).

Another LOX-derived lipid mediator that could be relevant for the understanding of the pathophysiology of the ACLF syndrome is LXA_5, for which plasma levels were significantly reduced in patients with ACLF but not in those with AD. LXA_5 is an EPA (omega-3-PUFA)-

derived lipid mediator that belongs to the family of specialized pro-resolving mediators promoting the timely resolution of inflammation (10). Biosynthesis of LXA_5 from endogenous sources of EPA is initiated by 15-LOX and mainly occurs in cells bearing 15-LOX activity, such as those of the immune system. Since circulating LXA_5 levels were suppressed in patients with ACLF without changes in 15-LOX, this impairment was likely related to limited access to EPA. Indeed, EPA abundance was significantly reduced in plasma from patients with AD cirrhosis, who also presented an unbalanced AA/EPA ratio. In agreement with this, we identified unbalanced formation between pro-inflammatory omega-6-derived (i.e. LTE_4) and anti-inflammatory omega-3-derived (i.e. LXA_5) lipid mediators in patients with AD and ACLF. These two lipid mediators showed opposite relationships (positive for LTE_4 and negative for LXA_5) with IL-8, suggesting that this imbalance might be a contributory factor for unresolved systemic inflammation in these patients. It is worth mentioning that Schwarzkopf et al. (23) reported no changes in the plasma levels of omega-6 and -3 PUFA in patients with cirrhosis with and without ACLF. However, these authors did not include a group of healthy subjects and the comparisons were made between patients with AD and ACLF with respect to a group of patients with compensated cirrhosis. Moreover, these authors determined the levels of free PUFA whereas in our study we determined not only free PUFA but also the total PUFA content (all PUFA from triglycerides, phospholipids and cholesterol esters after saponification), which more accurately represents the actual PUFA pool in plasma.

Our study also identified profound alterations in the circulating levels of lipid mediators derived from LA. Among these, 9(10)-EpOME and 12(13)-EpOME, which are produced by the activity of CYP450 in leukocytes during oxidative burst (24), were invariably reduced in patients with AD and in those with ACLF. Since a defect in leukocyte oxidative burst is a

hallmark of ACLF (25), these LA-derived lipid mediators could serve as circulating biomarkers of decreased bactericidal activity in these patients. However, no significant differences in the levels of these lipid mediators were seen between patients with infections and those without. In contrast, nonenzymatic autooxidation products of LA were found invariably increased in patients with AD and in those with ACLF. One of these products was EKODE, which modulates aldosterone, corticosterone and dehydroepiandrosterone secretion by human adrenal cells (26), suggesting that it might drive adrenal dysfunction in patients with cirrhosis.

Finally, COX-derived PGs, which are widely distributed and formed at sites of inflammation, deserve some comments. For example, PGE_2, which was related to immunosuppresion in cirrhosis (16), was not significantly associated with the presence of infections at the time of inclusion or with the risk of developing infections during hospitalization. In contrast, $PGF_{2\alpha}$, which is involved in contraction of bronchial, vascular smooth muscle, renin secretion and blood pressure regulation (27), was associated with circulatory failure in patients with ACLF. On the other hand, 12-HHT, which is biosynthesized by TXA_2 synthase in an equimolar ratio to TXA_2, was part of the minimal plasma fingerprint discriminating patients with ACLF from those with AD. In the past, 12-HHT was considered a mere byproduct of the biosynthesis of the potent vasoconstrictor TXA_2, although recent studies indicate that this lipid mediator induces chemotaxis of immune cells by binding to LTB_4 receptor 2 (28).

In summary, the current study provides a comprehensive analysis of the plasma levels of 100 PUFA-derived lipid mediators in a well-clinically defined cohort (i.e. CANONIC) of patients with AD cirrhosis with and without ACLF. By using an agnostic approach to data analysis, we identified 11 lipid mediators that distinguished healthy from cirrhotic patients at any stage

(either AD or ACLF), 2 lipid mediators (LTE_4 and 12-HHT) that discriminated patients with ACLF from those with AD and 2 other lipid mediators that shaped a minimal plasma fingerprint of liver and coagulation failures (LXA_5 and EKODE). Moreover, LTE_4 distinguished ACLF grade 3 from ACLF grades 1 and 2 and its plasma levels followed the clinical course of the disease and together with LXA_5 and EKODE associated with short-term mortality. Overall, our study provides useful insights on the role of bioactive lipid mediators in the initiation and progression of systemic inflammation and organ failures in patients with AD cirrhosis.

Acknowledgements

We are indebted to the IDIBAPS Biobank, integrated in the Spanish National Biobank Network, for samples and data procurement.

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Figure legends

Figure 1. Unbalanced omega-6/omega-3 ratio in AD cirrhosis. (A) Schematic diagram and chemical structures of omega-6 (LA and AA) and omega-3 (EPA and DHA) PUFA families. (**B**) Plasma levels of free and total PUFA in HS (n=38) and AD (n=34) and ACLF (n=25) patients. **(C)** AA/EPA ratio of free and total PUFA content. **(D)** Changes in gene expression for delta-5 (*FADS1*), delta-6 (*FADS2*) and delta-9 (*SCD1*) desaturases and ELOVL fatty acid elongase 5 (*ELOVL5*) and 6 (*ELOVL6*) in leukocytes from HS (n=14) and AD (n=14) and ACLF (n=14) patients. Changes in *SCD1* and *ELOVL6* expression are shown at the bottom. Results are expressed as median and IQR.

Figure 2. Altered biosynthesis of lipid mediators in leukocytes of patients with AD cirrhosis. (A) Total amount (upper panel) and box plots of individual values (lower panel) of lipid mediators from omega-6 and omega-3 PUFA families generated by CYP, LOX, COX and non-enzymatic (NE) pathways in plasma from HS (n=18) and AD (n=127) and ACLF (n=119) patients. **(B)** Percent expression of genes of the CYP, LOX and COX pathways in leukocytes from HS (n=14) and AD (n=14) and ACLF (n=14) patients. (**C**) Gene expression of individual CYP, LOX and COX enzymes in leukocytes from HS and AD and ACLF patients. Results are expressed as median and IQR.

Figure 3. Identification of an AD cirrhosis lipid mediator signature. (A) Cleveland dot plot showing a ranked log2 transformation of fold changes of plasma levels of lipid mediators categorized by chemical families (LTs, epoxy-keto-fatty acids (FA), AA and DHA epoxides, PGs, TX, AA/EPA/DHA diols, oxo-FA, ALA epoxides, monohydroxy-FA, FA triols, LXs, LA diols and LA epoxides). Blue and red dots represent the fold changes between AD (n=127) and ACLF (n=119) patients with respect to HS (n=18), respectively. **(B)** Cleveland

dot plot of the analyzed 59 lipid mediators ranked by fold change of AD and ACLF patients versus HS. Color coding on the left represents each biosynthetic precursor ((LA, γ-linolenic acid (GLA), dihomo-γ-linolenic acid (DGLA), oleic acid (OA), EPA, AA, DHA and ALA)). **(C)** Volcano plot representing the levels of lipid mediators up- or down-regulated in AD patients with respect to HS. Lipid mediators with significant changes are presented in red (significant increase) or in blue (significant decrease). **(D)** Volcano plot representing changes in ACLF patients with respect to HS. **(E)** 3D-PCA of lipid mediators in plasma from HS and AD and ACLF patients. **(F)** Heatmap of the 16 lipids associated with patient status at enrolment (unsupervised analysis). Rows represent individual lipid mediators and columns represent HS, AD and ACLF individuals.

Figure 4. LTE_4 discriminates disease severity. **(A)** Plasma levels of 9(10)-EpOME, 12(13)-EpOME in the HS (n=18), AD (n=127) and ACLF (n=119) groups at enrollment. **(B, C)** $PGF_2\alpha$ and LTE_4 levels in the study groups. **(D)** LTE_4 in ACLF patients according to severity (grade-1, n=57; grade-2, n=44 and grade-3, n=18). (**E)** LTE_4 according to the absence (n=219) or presence (n=22) of bacterial infection. (**F**) LTE_4 according to the absence (n=68) or presence (n=177) of ascites. (**G)** LTE_4 according to the absence (n=133) or presence (n=133) of esophageal varices. (**H**) Differences in LTE_4 levels between baseline and follow-up measurements in plasma samples from patients with AD and ACLF according to the course of the disease: steady (n=105), improvement (n=47) or worsening (n=39). Mann-Whitney test was used. Bonferroni correction was applied to correct for multiple-testing. An adjusted P-value$\leq$ 0.05 was considered statistically significant.

Figure 5. Association of lipid mediators with markers of inflammation and cell death. Correlation matrix plot between lipid mediators and cytokines/chemokines/cell death markers

in patients with AD (n=127) and ACLF (n=119). Shades of blue indicate increasing positive correlation coefficient; shades of red indicate increasing negative correlation coefficient. Correlations of LTE_4 with cK18 and K18, LTE_4 and LXA_5 with IL-8 and LTE_4 with LXA_5.

Figure 6. Association of lipid mediators with organ failures and short-term mortality. **(A)** Heatmap representation of the 59 lipid mediators analyzed in the study and their association with organ failures in patients with AD (n=127) and ACLF (n=119). Grey color corresponds to low association (high p-values) and red color to high association (low p-values). **(B)** EKODE and LXA_5 levels in patients with (n=55) and without (n=191) liver failure. **(C)** EKODE and LXA_5 in patients with (n=44) and without (n=202) coagulation failure. (**D**) Association analysis of EKODE, LXA_5 and LTE_4 levels with 28-day mortality (survivors [n=206], non-survivors [n=40]). Mann-Whitney test was used. Bonferroni correction was applied to correct for multiple-testing. An adjusted p-value$\leq$0.05 was considered statistically significant.

Table 1. Association analysis of lipid mediators with the status of the patients at enrollment.

	Kruskal-Wallis		**HS - AD**		**HS - ACLF**		**AD - ACLF**	
Lipid mediator	*p-val*	*Adj p-val*	*p-val*	*Adj p-val*	*p-val*	*Adj p-val*	*p-val*	*Adj p-val*
Significant changes between HS vs. AD								
4-HDoHE	<0.01	0.02	**<0.01**	**0.04**	0.03	1.00	<0.01	0.18
Significant changes between HS vs. AD and ACLF								
12(13)-EpOME	<0.01	<0.01	**<0.01**	**<0.01**	**<0.01**	**<0.01**	0.26	1
9(10)-EpOME	<0.01	<0.01	**<0.01**	**<0.01**	**<0.01**	**<0.01**	0.01	0.55
EKODE	<0.01	<0.01	**<0.01**	**<0.01**	**<0.01**	**<0.01**	0.03	1.00
8-HETE	<0.01	<0.01	**<0.01**	**<0.01**	**<0.01**	**<0.01**	0.05	1.00
20-HETE	<0.01	<0.01	**<0.01**	**<0.01**	**<0.01**	**<0.01**	0.15	1.00
8-HETrE	<0.01	<0.01	**<0.01**	**<0.01**	**<0.01**	**<0.01**	0.01	0.54
11,12-DiHETrE	<0.01	<0.01	**<0.01**	**<0.01**	**<0.01**	**<0.01**	0.44	1.00
14,15-DiHETrE	<0.01	<0.01	**<0.01**	**<0.01**	**<0.01**	**<0.01**	0.16	1.00
13-HOTrEγ	<0.01	<0.01	**<0.01**	**<0.01**	**<0.01**	**<0.01**	0.76	1.00
11-keto-TXB_2	<0.01	<0.01	**<0.01**	**<0.01**	**<0.01**	**0.03**	0.10	1
PGE_1	<0.01	0.03	**<0.01**	**<0.01**	**<0.01**	**0.01**	0.61	1
Significant changes between HS vs. ACLF								
$PGF_{2\alpha}$	<0.01	<0.01	<0.01	0.11	**<0.01**	**0.01**	<0.01	0.24
8-HDoHE	<0.01	<0.01	<0.01	0.09	**<0.01**	**<0.01**	<0.01	0.09
Significant changes between AD vs. ACLF								
12-HHT	<0.01	<0.01	0.09	1.00	0.65	1.00	**<0.01**	**<0.01**
LTE_4	<0.01	<0.01	**<0.01**	**0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**

p-value and adjusted p-value after Bonferroni correction for multiple testing are shown for each test (Kruskal-Wallis or pairwise Wilcoxon test). All lipids are statistically significant according to Kruskal-Wallis test ($p<0.05$).

Figure 1

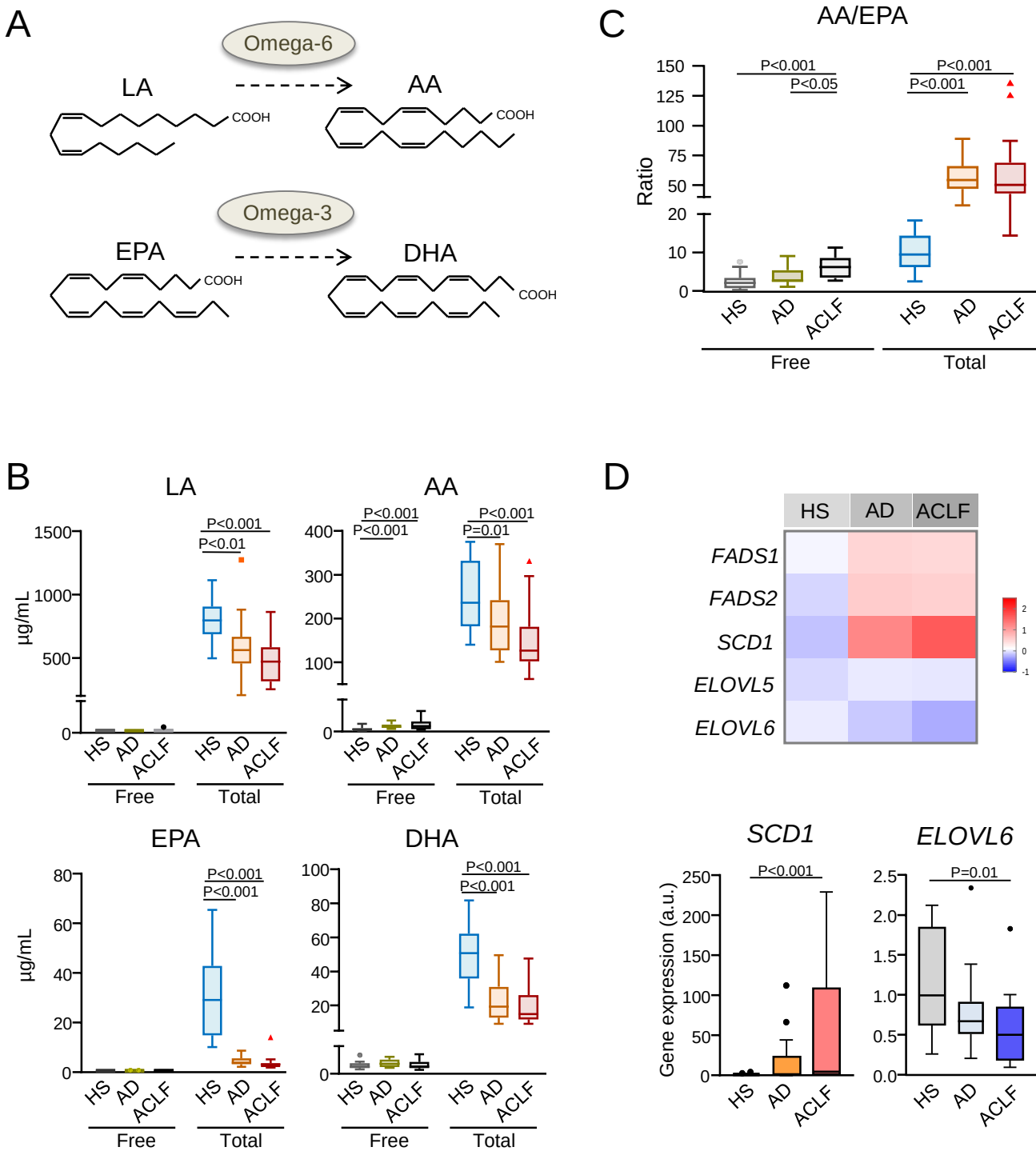

A
Omega-6
LA
AA
COOH
COOH
Omega-3
EPA
DHA
COOH
COOH
C
AA/EPA
Ratio
150
125
100
75
50
20
10
0
P<0.001
P<0.05
P<0.001
P<0.001
HS
AD
ACLF
HS
AD
ACLF
Free
Total
B
LA
AA
µg/mL
1500
1000
500
0
P<0.001
P<0.01
400
300
200
100
0
P<0.001
P<0.001
P<0.001
P=0.01
HS
AD
ACLF
HS
AD
ACLF
Free
Total
EPA
DHA
µg/mL
80
60
40
20
0
P<0.001
P<0.001
100
80
60
40
20
0
P<0.001
P<0.001
Free
Total
D
HS
AD
ACLF
FADS1
FADS2
SCD1
ELOVL5
ELOVL6
2
1
0
-1
SCD1
ELOVL6
Gene expression (a.u.)
250
200
150
100
50
0
P<0.001
2.5
2.0
1.5
1.0
0.5
0.0
P=0.01

Figure 2

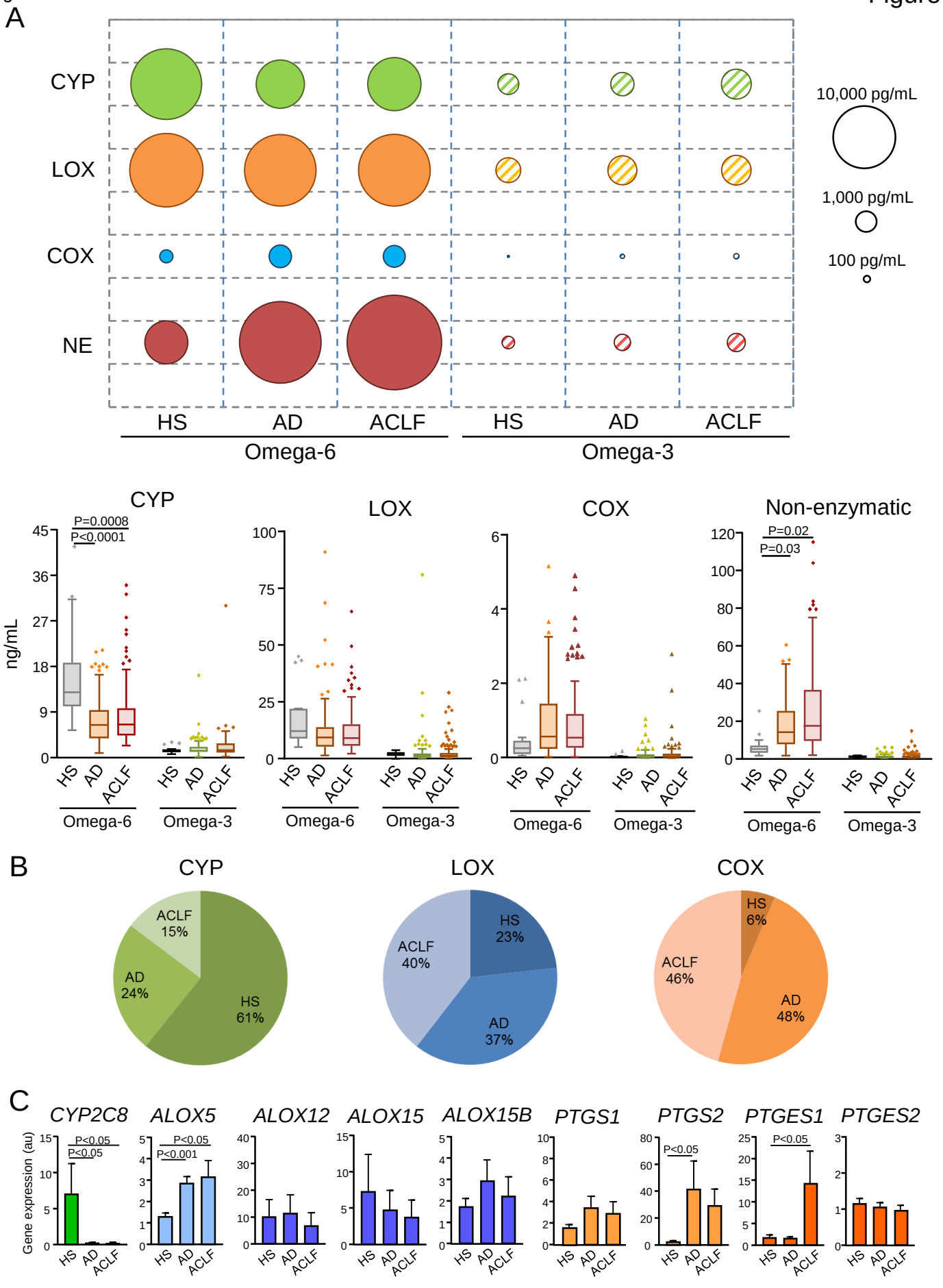

A
CYP
LOX
COX
NE
HS
AD
ACLF
HS
AD
ACLF
Omega-6
Omega-3
10,000 pg/mL
1,000 pg/mL
100 pg/mL
CYP
LOX
COX
Non-enzymatic
ng/mL
P=0.0008
P<0.0001
P=0.02
P=0.03
Omega-6
Omega-3
B
CYP
ACLF 15%
AD 24%
HS 61%
LOX
HS 23%
ACLF 40%
AD 37%
COX
HS 6%
ACLF 46%
AD 48%
C
CYP2C8
ALOX5
ALOX12
ALOX15
ALOX15B
PTGS1
PTGS2
PTGES1
PTGES2
Gene expression (au)
P<0.05
P<0.05
P<0.05
P<0.001
P<0.05
P<0.05
HS
AD
ACLF

Figure 3

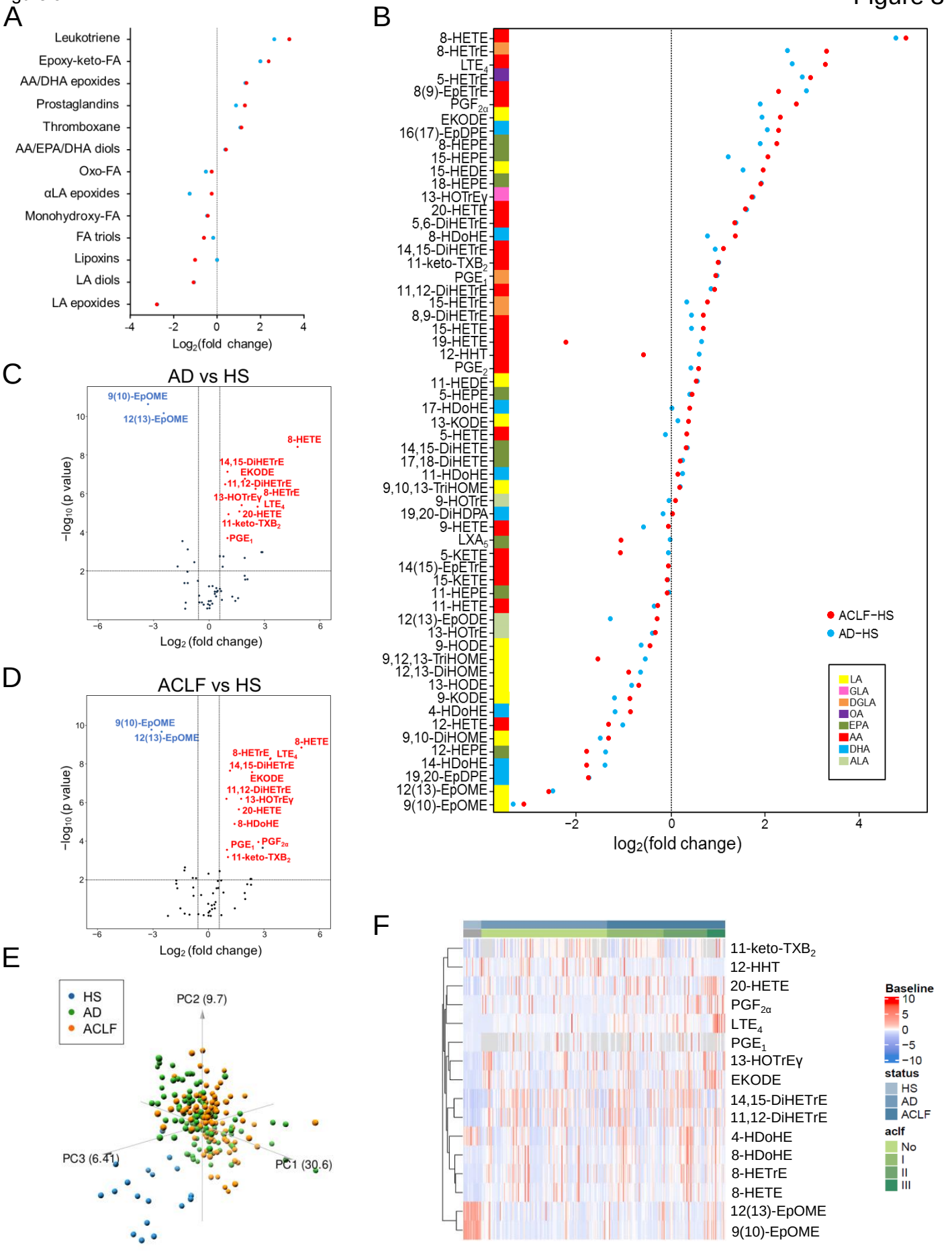

A
Leukotriene
Epoxy-keto-FA
AA/DHA epoxides
Prostaglandins
Thromboxane
AA/EPA/DHA diols
Oxo-FA
αLA epoxides
Monohydroxy-FA
FA triols
Lipoxins
LA diols
LA epoxides
Log2(fold change)
B
8-HETE
8-HETrE
LTE4
5-HETrE
8(9)-EpETrE
PGF2α
EKODE
16(17)-EpDPE
8-HEPE
15-HEPE
15-HEDE
18-HEPE
13-HOTrEγ
20-HETE
5,6-DiHETrE
8-HDoHE
14,15-DiHETrE
11-keto-TXB2
PGE1
11,12-DiHETrE
15-HETrE
8,9-DiHETrE
15-HETE
19-HETE
12-HHT
PGE2
11-HEDE
5-HEPE
17-HDoHE
13-KODE
5-HETE
14,15-DiHETE
17,18-DiHETE
11-HDoHE
9,10,13-TriHOME
9-HOTrE
19,20-DiHDPA
9-HETE
LXA5
5-KETE
14(15)-EpETrE
15-KETE
11-HEPE
11-HETE
12(13)-EpODE
13-HOTrE
9-HODE
9,12,13-TriHOME
12,13-DiHOME
13-HODE
9-KODE
4-HDoHE
12-HETE
9,10-DiHOME
12-HEPE
14-HDoHE
19,20-EpDPE
12(13)-EpOME
9(10)-EpOME
log2(fold change)
ACLF−HS
AD−HS
LA
GLA
DGLA
OA
EPA
AA
DHA
ALA
C
AD vs HS
9(10)-EpOME
12(13)-EpOME
8-HETE
14,15-DiHETrE
EKODE
11,12-DiHETrE
13-HOTrEγ
8-HETrE
LTE4
20-HETE
11-keto-TXB2
PGE1
−log10 (p value)
Log2 (fold change)
D
ACLF vs HS
9(10)-EpOME
12(13)-EpOME
8-HETE
8-HETrE
LTE4
14,15-DiHETrE
EKODE
11,12-DiHETrE
13-HOTrEγ
20-HETE
8-HDoHE
PGE1
PGF2α
11-keto-TXB2
−log10 (p value)
Log2 (fold change)
E
HS
AD
ACLF
PC2 (9.7)
PC3 (6.41)
PC1 (30.6)
F
11-keto-TXB2
12-HHT
20-HETE
PGF2α
LTE4
PGE1
13-HOTrEγ
EKODE
14,15-DiHETrE
11,12-DiHETrE
4-HDoHE
8-HDoHE
8-HETrE
8-HETE
12(13)-EpOME
9(10)-EpOME
Baseline
10
5
0
−5
−10
status
HS
AD
ACLF
aclf
No
I
II
III

Figure 4

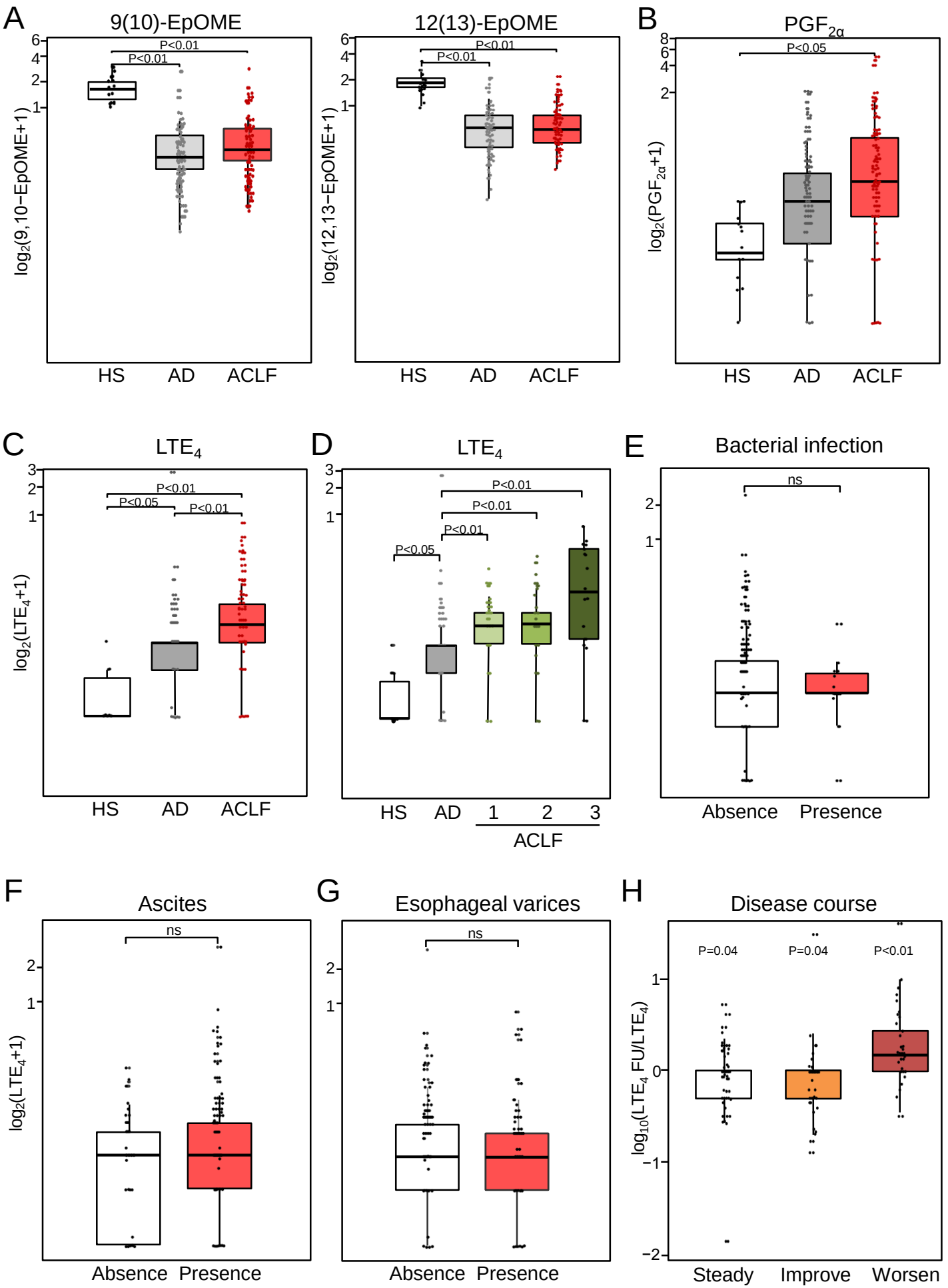

A
9(10)-EpOME
log2(9,10−EpOME+1)
P<0.01
P<0.01
HS
AD
ACLF
12(13)-EpOME
log2(12,13−EpOME+1)
P<0.01
P<0.01
HS
AD
ACLF
B
PGF2α
log2(PGF2α+1)
P<0.05
HS
AD
ACLF
C
LTE4
log2(LTE4+1)
P<0.01
P<0.05
P<0.01
HS
AD
ACLF
D
LTE4
P<0.01
P<0.01
P<0.01
P<0.05
HS
AD
1
2
3
ACLF
E
Bacterial infection
ns
Absence
Presence
F
Ascites
log2(LTE4+1)
ns
Absence
Presence
G
Esophageal varices
ns
Absence
Presence
H
Disease course
log10(LTE4 FU/LTE4)
P=0.04
P=0.04
P<0.01
Steady
Improve
Worsen

Figure 5

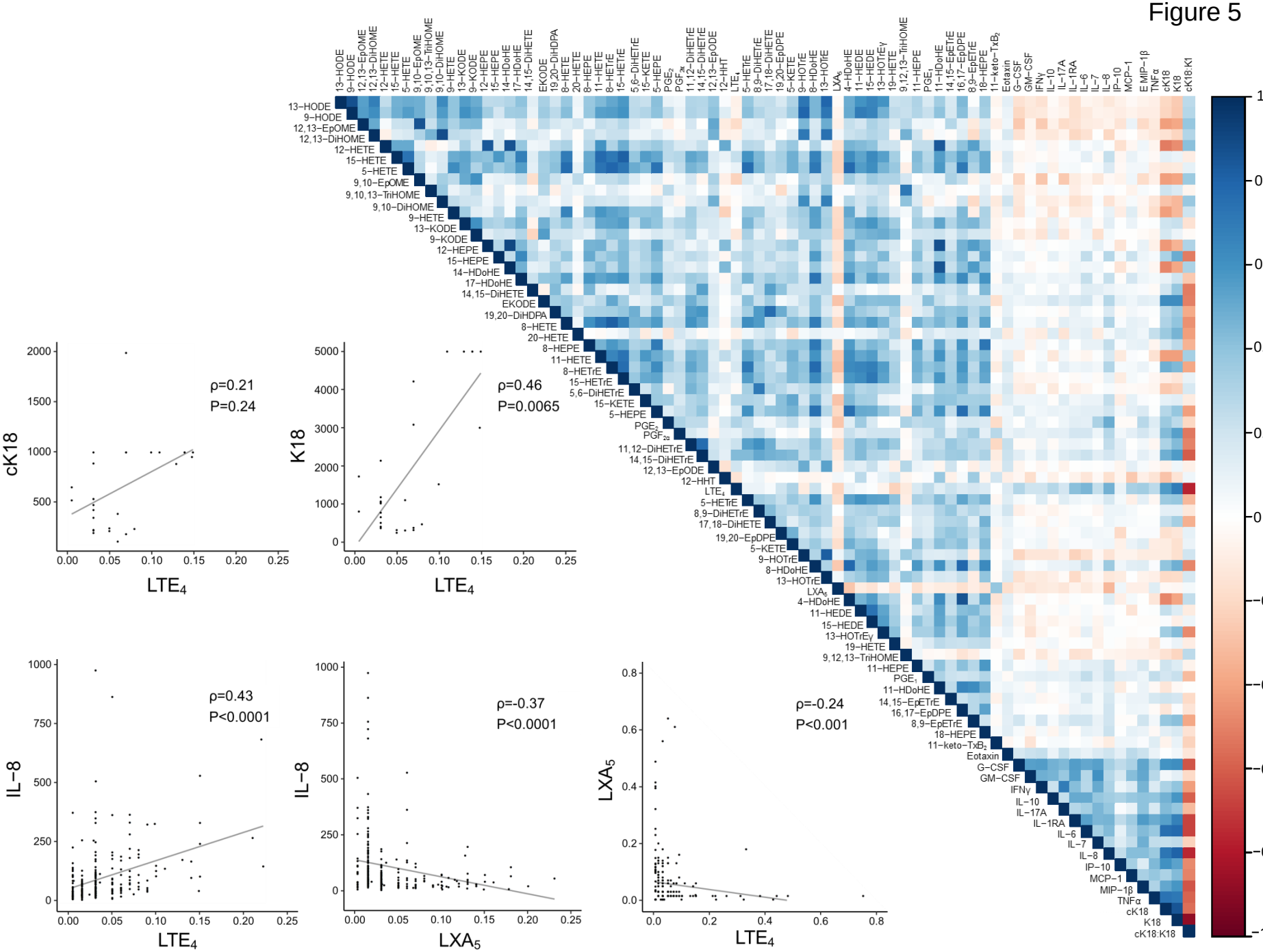
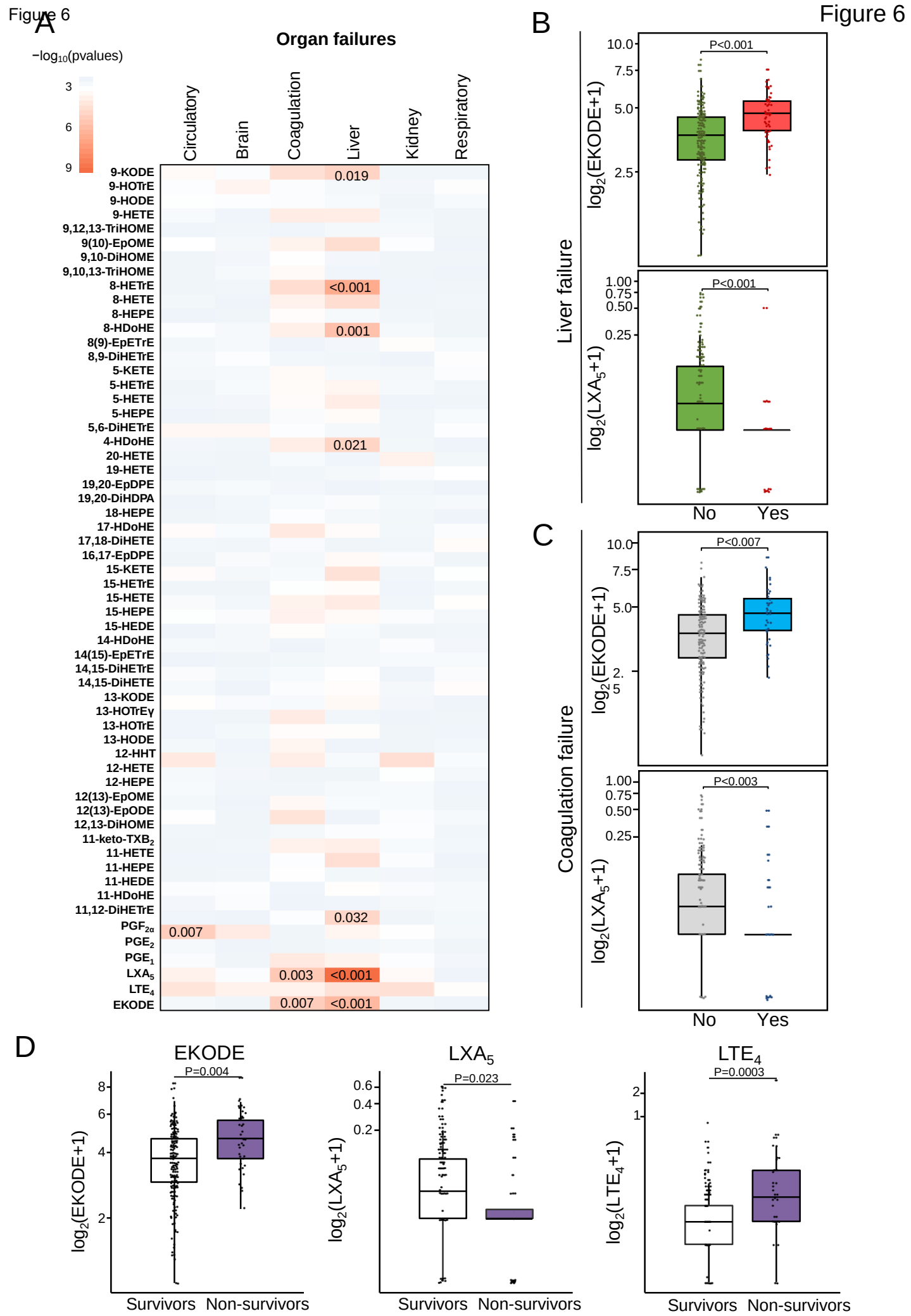
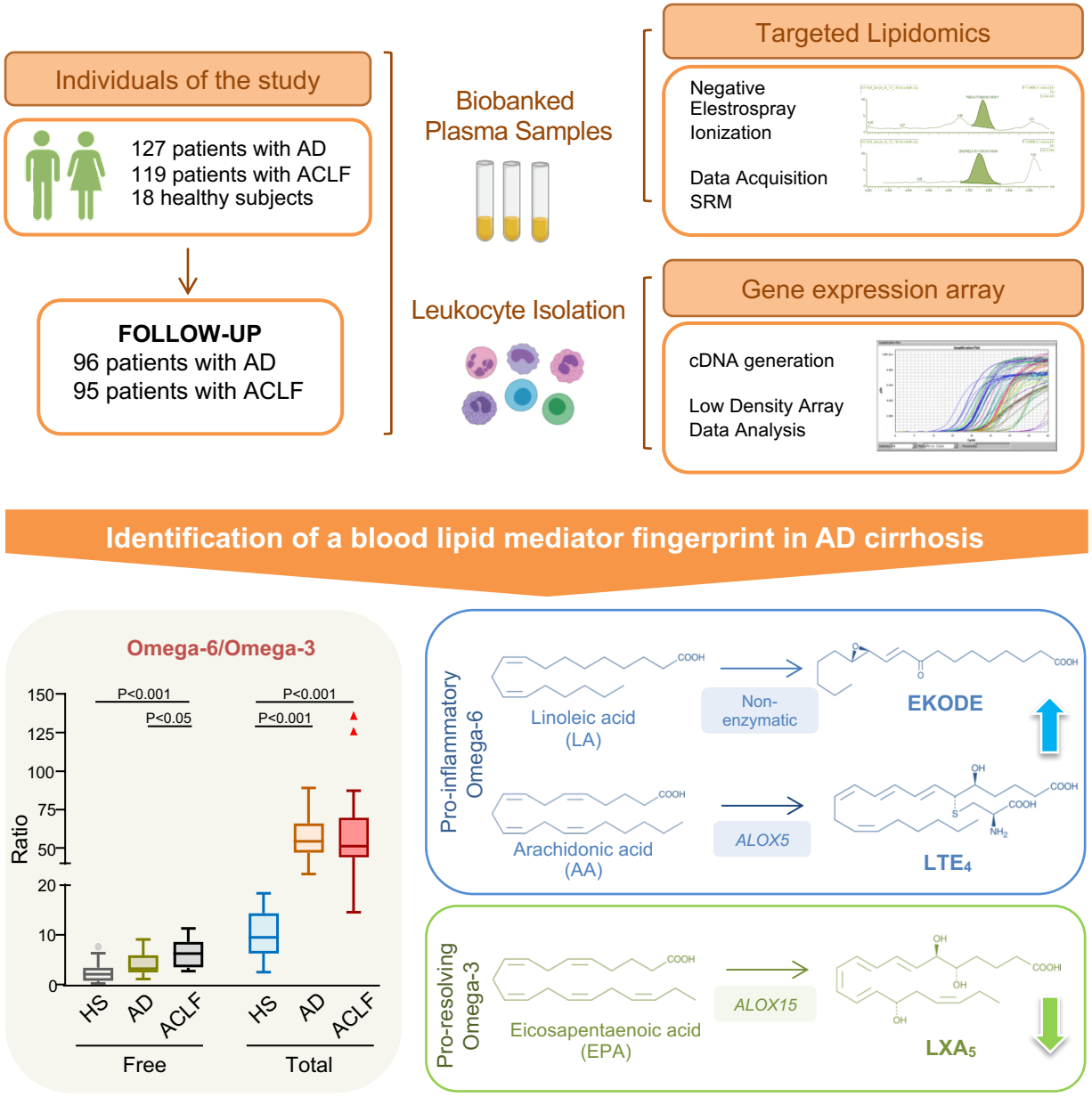


Figure 5
13−HODE
9−HODE
12,13−EpOME
12,13−DiHOME
12−HETE
15−HETE
5−HETE
9,10−EpOME
9,10,13−TriHOME
9,10−DiHOME
9−HETE
13−KODE
9−KODE
12−HEPE
15−HEPE
14−HDoHE
17−HDoHE
14,15−DiHETE
EKODE
19,20−DiHDPA
8−HETE
20−HETE
8−HEPE
11−HETE
8−HETrE
15−HETrE
5,6−DiHETrE
15−KETE
5−HEPE
PGE2
PGF2α
11,12−DiHETrE
14,15−DiHETrE
12,13−EpODE
12−HHT
LTE4
5−HETrE
8,9−DiHETrE
17,18−DiHETE
19,20−EpDPE
5−KETE
9−HOTrE
8−HDoHE
13−HOTrE
LXA5
4−HDoHE
11−HEDE
15−HEDE
13−HOTrEγ
19−HETE
9,12,13−TriHOME
11−HEPE
PGE1
11−HDoHE
14,15−EpETrE
16,17−EpDPE
8,9−EpETrE
18−HEPE
11−keto−TxB2
Eotaxin
G−CSF
GM−CSF
IFNγ
IL−10
IL−17A
IL−1RA
IL−6
IL−7
IL−8
IP−10
MCP−1
MIP−1β
TNFα
cK18
K18
cK18:K18
1
0.8
0.6
0.4
0.2
0
−0.2
−0.4
−0.6
−0.8
−1
cK18
LTE4
ρ=0.21
P=0.24
K18
ρ=0.46
P=0.0065
IL−8
ρ=0.43
P<0.0001
ρ=−0.37
P<0.0001
LXA5
ρ=−0.24
P<0.001


A
Organ failures
−log10(pvalues)
3
6
9
Circulatory
Brain
Coagulation
Liver
Kidney
Respiratory
9-KODE
9-HOTrE
9-HODE
9-HETE
9,12,13-TriHOME
9(10)-EpOME
9,10-DiHOME
9,10,13-TriHOME
8-HETrE
8-HETE
8-HEPE
8-HDoHE
8(9)-EpETrE
8,9-DiHETrE
5-KETE
5-HETrE
5-HETE
5-HEPE
5,6-DiHETrE
4-HDoHE
20-HETE
19-HETE
19,20-EpDPE
19,20-DiHDPA
18-HEPE
17-HDoHE
17,18-DiHETE
16,17-EpDPE
15-KETE
15-HETrE
15-HETE
15-HEPE
15-HEDE
14-HDoHE
14(15)-EpETrE
14,15-DiHETrE
14,15-DiHETE
13-KODE
13-HOTrEγ
13-HOTrE
13-HODE
12-HHT
12-HETE
12-HEPE
12(13)-EpOME
12(13)-EpODE
12,13-DiHOME
11-keto-TXB2
11-HETE
11-HEPE
11-HEDE
11-HDoHE
11,12-DiHETrE
PGF2α
PGE2
PGE1
LXA5
LTE4
EKODE
0.019
<0.001
0.001
0.021
0.032
0.007
0.003
<0.001
0.007
<0.001
B
Liver failure
log2(EKODE+1)
P<0.001
log2(LXA5+1)
P<0.001
No
Yes
C
Coagulation failure
log2(EKODE+1)
P<0.007
log2(LXA5+1)
P<0.003
No
Yes
D
EKODE
P=0.004
log2(EKODE+1)
Survivors
Non-survivors
LXA5
P=0.023
log2(LXA5+1)
Survivors
Non-survivors
LTE4
P=0.0003
log2(LTE4+1)
Survivors
Non-survivors

Graphical Abstract



Highlights

- Lipidomics was performed to assess the profile of lipid mediators in plasma from patients with acutely decompensated cirrhosis with and without ACLF.
- Measurements were prospectively repeated during a 28-day follow-up period.
- Fifty-nine lipid mediators were detected in plasma from cirrhotic patients, of which, 16 were significantly associated with the disease status.
- Among these, leukotriene E_4 derived from arachidonic acid was part of a minimal plasma fingerprint that discriminated disease severity and evolution.
- This lipid mediator positively correlated with markers of inflammation and non-apoptotic cell death.