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

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## 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<sub>4</sub> 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<sub>4</sub> 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<sub>4</sub> together with LXA<sub>5</sub> (derived from eicosapentaenoic acid) and EKODE (derived from linoleic acid) associated with short-term mortality. Interestingly, LXA<sub>5</sub> 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<sub>2</sub> (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<sub>2</sub> (TXA<sub>2</sub>) and lipoxins (LXs) (5,6). With the exception of LXs, eicosanoids are considered to be proinflammatory, and some members such PGs and TXA<sub>2</sub> 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 F2 $\alpha$ -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-HOTrE $\gamma$ , LTE<sub>4</sub>, 20-HETE, 11-keto-TXB<sub>2</sub> and PGE<sub>1</sub> in the plasma of patients with AD. Among these, LTE<sub>4</sub> (a member of the slow-reacting substance of anaphylaxis and a pathway marker of pro-inflammatory cysteinyl-LT biosynthesis), 11-keto-TXB<sub>2</sub> (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<sub>2 $\alpha$</sub> , 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<sub>2</sub> from AA; 8-HETrE and PGE<sub>1</sub> from DGLA and 13-HOTrE $\gamma$  from  $\gamma$ -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<sub>4</sub> discriminate disease severity**

Among the 16 lipid mediators identified in the PCA analysis, two of them (PGF<sub>2 $\alpha$</sub>  derived from AA [**Figure 4B**] and 8-HDoHE derived from DHA) distinguished patients with ACLF from HS (**Table 1**). Importantly, LTE<sub>4</sub> 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<sub>4</sub> 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<sub>4</sub> 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<sub>4</sub> 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  $\text{LTE}_4$  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  $\text{LTE}_4$  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  $\text{LTE}_4$  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  $\text{LXA}_5$ , 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  $\text{LTE}_4$  and  $\text{LXA}_5$ .  $\text{LTE}_4$ , 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.  $\text{LTE}_4$  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  $\text{LTE}_4$  can be associated with the non-apoptotic form. On the other hand,  $\text{LXA}_5$  was inversely correlated to  $\text{LTE}_4$ , showed a negative correlation with IL-8 and did not associate with cK18 and K18. Interestingly,  $\text{PGE}_2$ , which has previously been associated with immunosuppression in cirrhosis (16), was positively correlated with IL-8 ( $p=0.526$ ,  $P<0.001$ ), 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  $\text{LXA}_5$  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,  $\text{LXA}_5$  together with EKODE constituted a minimal fingerprint of liver and coagulation failures, while  $\text{PGF}_{2\alpha}$  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  $\text{LXA}_5$ , which were the lipid mediators associated with at least two different organ failures. Moreover, increased EKODE and  $\text{LTE}_4$  together with reduced  $\text{LXA}_5$  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)  $\text{LTE}_4$  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,  $\text{LTE}_4$  levels followed the clinical course of the disease (levels increased when worsening and decreased when improving). Moreover,  $\text{LTE}_4$  positively correlated with markers of cell death (K18) and inflammatory cytokines (IL-8). 4)  $\text{LXA}_5$ , which was invariably reduced in patients, was the only lipid mediator that inversely correlated with IL-8. 5)  $\text{LTE}_4$  was part of a minimal plasma fingerprint for ACLF, whereas  $\text{LXA}_5$  and EKODE, discriminated organ failures. 6) Finally, increased  $\text{LTE}_4$  and EKODE together with decreased  $\text{LXA}_5$  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,  $\text{LTE}_4$  was the lipid mediator enzymatically generated from AA with the largest fold change in ACLF versus

1 HS.  $\text{LTE}_4$  is formed upon activation of 5-LOX, which converts AA into 5-HpHETE, an  
 2 intermediate in the generation of  $\text{LTA}_4$  (**Supplementary Figure 1A**).  $\text{LTA}_4$  is further  
 3 converted into  $\text{LTB}_4$  by  $\text{LTA}_4$  hydrolase or into  $\text{LTC}_4$  by  $\text{LTC}_4$  synthase.  $\text{LTC}_4$  is further  
 4 metabolized to  $\text{LTD}_4$  and  $\text{LTE}_4$  (generically termed as cysteinyl-LTs), which are potent  
 5 vasoconstrictors that were previously known as slow-reacting substances of anaphylaxis (17).

6  
 7 Cysteinyl-LTs are primarily generated by neutrophils, macrophages, eosinophils and mast  
 8 cells at sites of infection and/or inflammation and their release is enhanced by activation of  
 9 Toll-like receptors (18). Cysteinyl-LTs participate in a variety of diseases including arthritis,  
 10 inflammatory bowel disease, atherosclerosis and especially asthma and allergy, conditions in  
 11 which blockage of their receptors is used as therapy (19). In these conditions, cysteinyl-LTs  
 12 directly interact with cytokines (i.e. IL-6, IL-10 and  $\text{TNF}\alpha$ ) and chemokines (i.e. IL-8,  
 13 eotaxin and MIP-1 $\alpha$ ) (20). In addition to inflammation, cysteinyl-LTs might also be related to  
 14 cell death, since in our study  $\text{LTE}_4$  levels were strongly correlated with K18, which is a  
 15 marker of total cell death. Interestingly,  $\text{LTE}_4$  negatively correlated with the ratio between  
 16 cK18 (apoptosis) and K18 (apoptosis and necrosis), indicating that this lipid mediator is  
 17 related to non-apoptotic cell death, which potentially is more immunogenic (13). On the other  
 18 hand, cysteinyl-LTs induce hyperreactivity of the arterial vascular tissue to vasoactive  
 19 compounds (such as angiotensin II and norepinephrine), suggesting that these lipid mediators  
 20 may have pathological significance in the development of organ failures in ACLF (21).  
 21 Indeed, elevated urinary  $\text{LTE}_4$  levels were reported in patients with hepatorenal syndrome  
 22 and might contribute to the development of kidney dysfunction in patients with cirrhosis (22).

23  
 24 Another LOX-derived lipid mediator that could be relevant for the understanding of the  
 25 pathophysiology of the ACLF syndrome is  $\text{LXA}_5$ , for which plasma levels were significantly  
 26 reduced in patients with ACLF but not in those with AD.  $\text{LXA}_5$  is an EPA (omega-3-PUFA)-  
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derived lipid mediator that belongs to the family of specialized pro-resolving mediators promoting the timely resolution of inflammation (10). Biosynthesis of LXA<sub>5</sub> 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<sub>5</sub> 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<sub>4</sub>) and anti-inflammatory omega-3-derived (i.e. LXA<sub>5</sub>) lipid mediators in patients with AD and ACLF. These two lipid mediators showed opposite relationships (positive for LTE<sub>4</sub> and negative for LXA<sub>5</sub>) 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<sub>2</sub>, which was related to immunosuppression 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<sub>2α</sub>, 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<sub>2</sub> synthase in an equimolar ratio to TXA<sub>2</sub>, 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<sub>2</sub>, although recent studies indicate that this lipid mediator induces chemotaxis of immune cells by binding to LTB<sub>4</sub> 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<sub>4</sub> 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<sub>5</sub> and EKODE). Moreover, LTE<sub>4</sub> 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<sub>5</sub> 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.

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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,  $\gamma$ -linolenic acid (GLA), dihomo- $\gamma$ -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<sub>4</sub> 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<sub>2</sub> $\alpha$  and LTE<sub>4</sub> levels in the study groups. (D) LTE<sub>4</sub> in ACLF patients according to severity (grade-1, n=57; grade-2, n=44 and grade-3, n=18). (E) LTE<sub>4</sub> according to the absence (n=219) or presence (n=22) of bacterial infection. (F) LTE<sub>4</sub> according to the absence (n=68) or presence (n=177) of ascites. (G) LTE<sub>4</sub> according to the absence (n=133) or presence (n=133) of esophageal varices. (H) Differences in LTE<sub>4</sub> 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<sub>4</sub> with cK18 and K18, LTE<sub>4</sub> and LXA<sub>5</sub> with IL-8 and LTE<sub>4</sub> with LXA<sub>5</sub>.

**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<sub>5</sub> levels in patients with (n=55) and without (n=191) liver failure. (C) EKODE and LXA<sub>5</sub> in patients with (n=44) and without (n=202) coagulation failure. (D) Association analysis of EKODE, LXA<sub>5</sub> and LTE<sub>4</sub> 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≤0.05 was considered statistically significant.

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

Lipid mediator	Kruskal-Wallis		HS - AD		HS - ACLF		AD - ACLF	
	<i>p</i> -val	Adj <i>p</i> -val	<i>p</i> -val	Adj <i>p</i> -val	<i>p</i> -val	Adj <i>p</i> -val	<i>p</i> -val	Adj <i>p</i> -val
Significant changes between HS vs. AD								
4-HDoHE	<0.01	0.02	<b>&lt;0.01</b>	<b>0.04</b>	0.03	1.00	<0.01	0.18
Significant changes between HS vs. AD and ACLF								
12(13)-EpOME	<0.01	<0.01	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>	0.26	1
9(10)-EpOME	<0.01	<0.01	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>	0.01	0.55
EKODE	<0.01	<0.01	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>	0.03	1.00
8-HETE	<0.01	<0.01	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>	0.05	1.00
20-HETE	<0.01	<0.01	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>	0.15	1.00
8-HETrE	<0.01	<0.01	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>	0.01	0.54
11,12-DiHETrE	<0.01	<0.01	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>	0.44	1.00
14,15-DiHETrE	<0.01	<0.01	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>	0.16	1.00
13-HOTrE $\gamma$	<0.01	<0.01	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>	0.76	1.00
11-keto-TXB <sub>2</sub>	<0.01	<0.01	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<b>0.03</b>	0.10	1
PGE <sub>1</sub>	<0.01	0.03	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<b>0.01</b>	0.61	1
Significant changes between HS vs. ACLF								
PGF <sub>2<math>\alpha</math></sub>	<0.01	<0.01	<0.01	0.11	<b>&lt;0.01</b>	<b>0.01</b>	<0.01	0.24
8-HDoHE	<0.01	<0.01	<0.01	0.09	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<0.01	0.09
Significant changes between AD vs. ACLF								
12-HHT	<0.01	<0.01	0.09	1.00	0.65	1.00	<b>&lt;0.01</b>	<b>&lt;0.01</b>
LTE <sub>4</sub>	<0.01	<0.01	<b>&lt;0.01</b>	<b>0.01</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>

*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 ( $p < 0.05$ ).

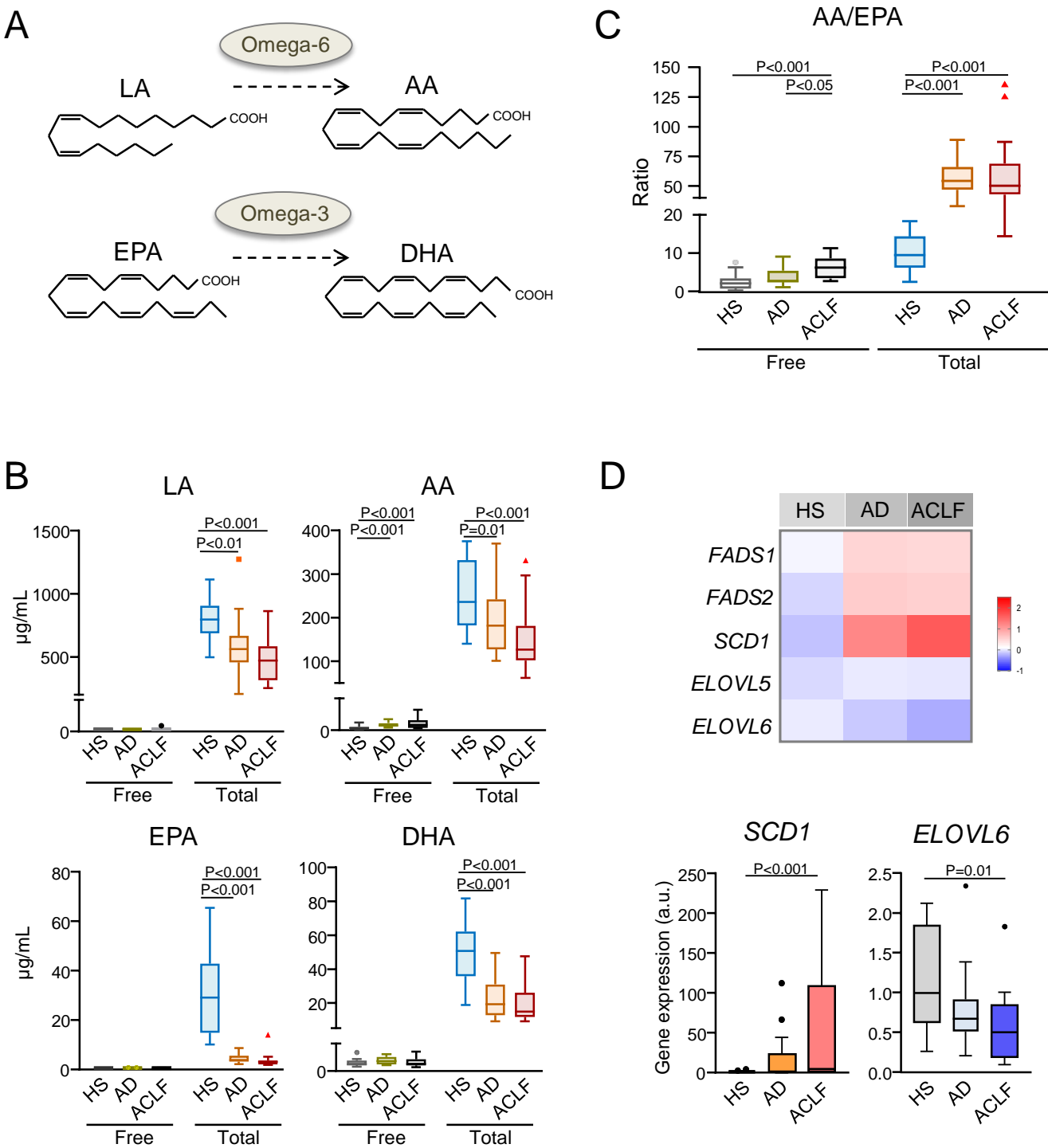
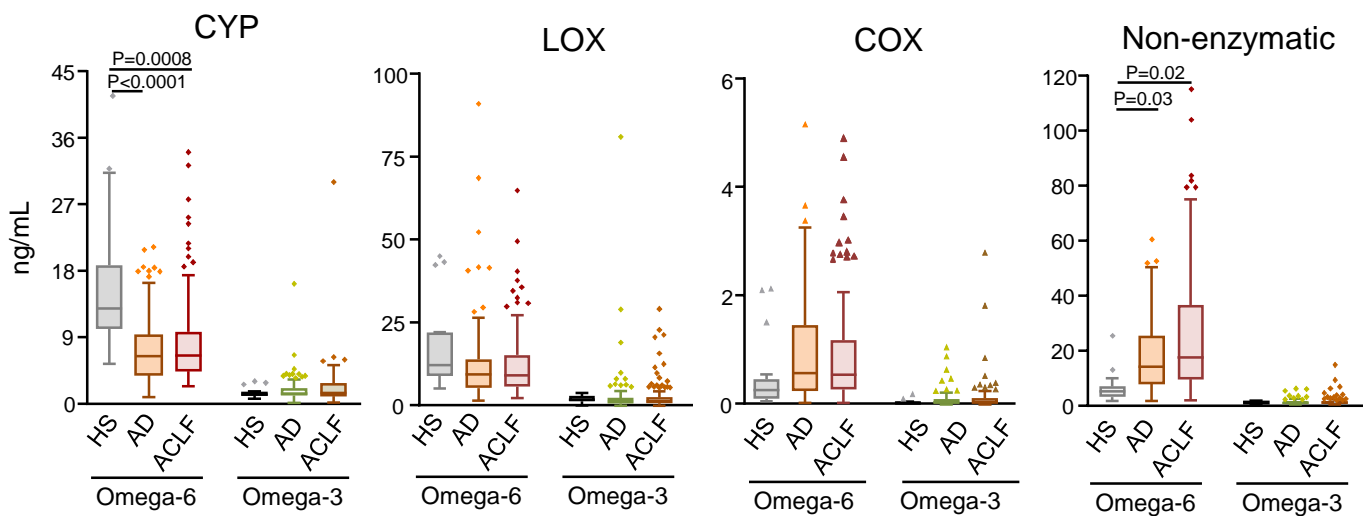
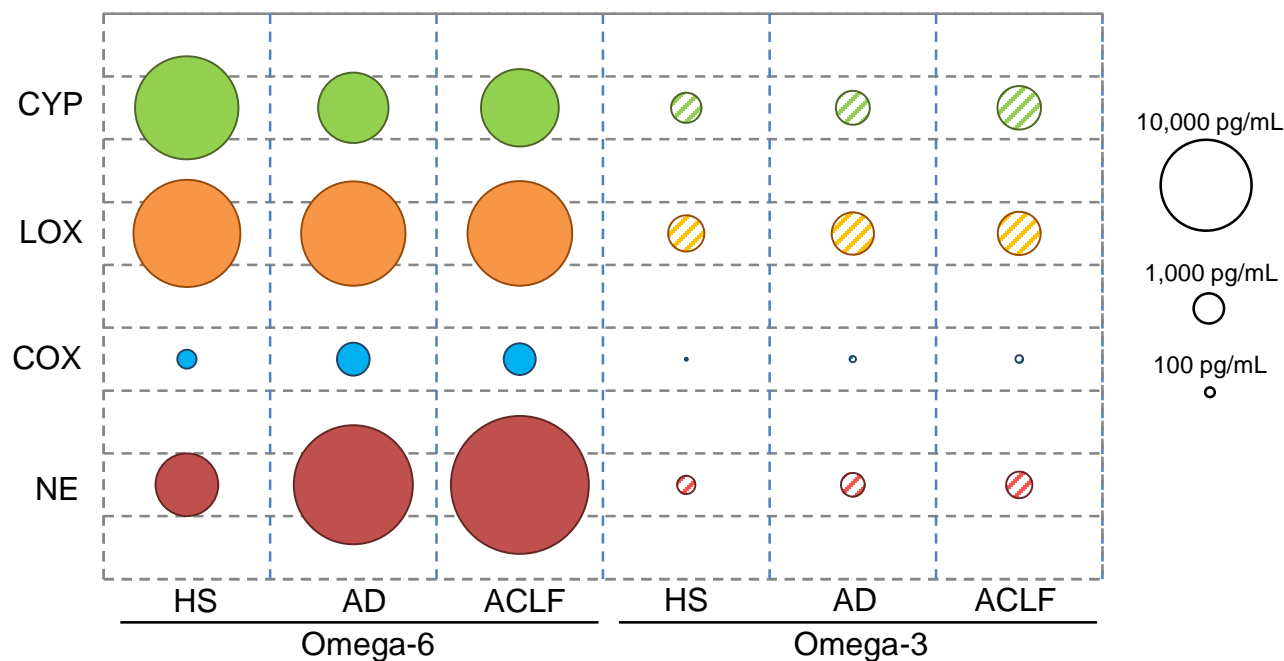




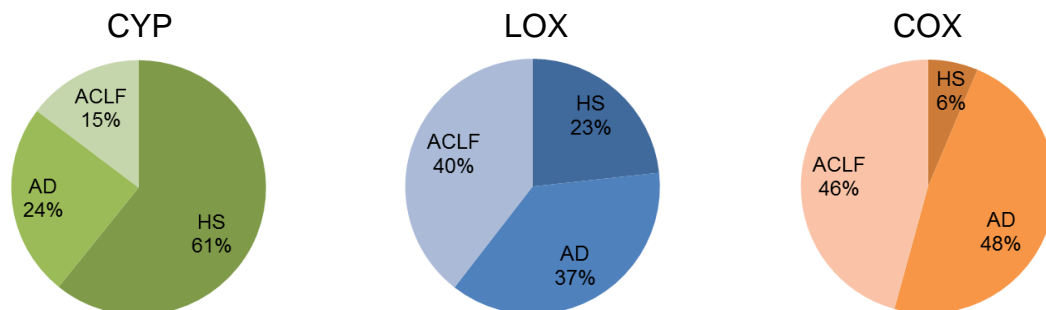
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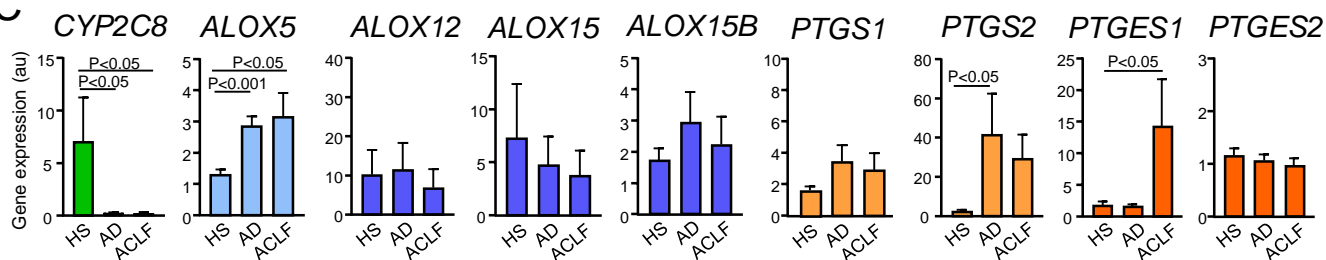


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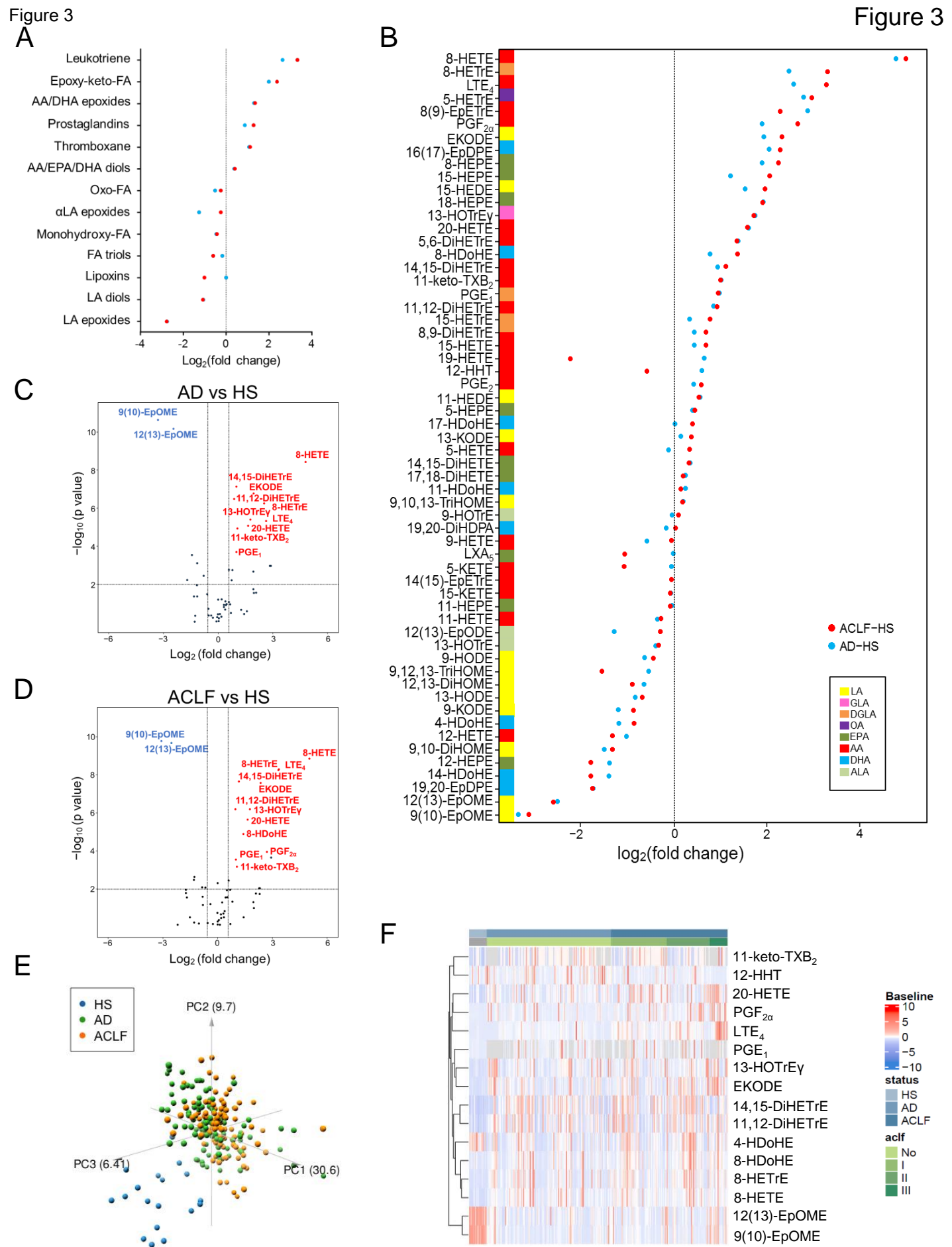


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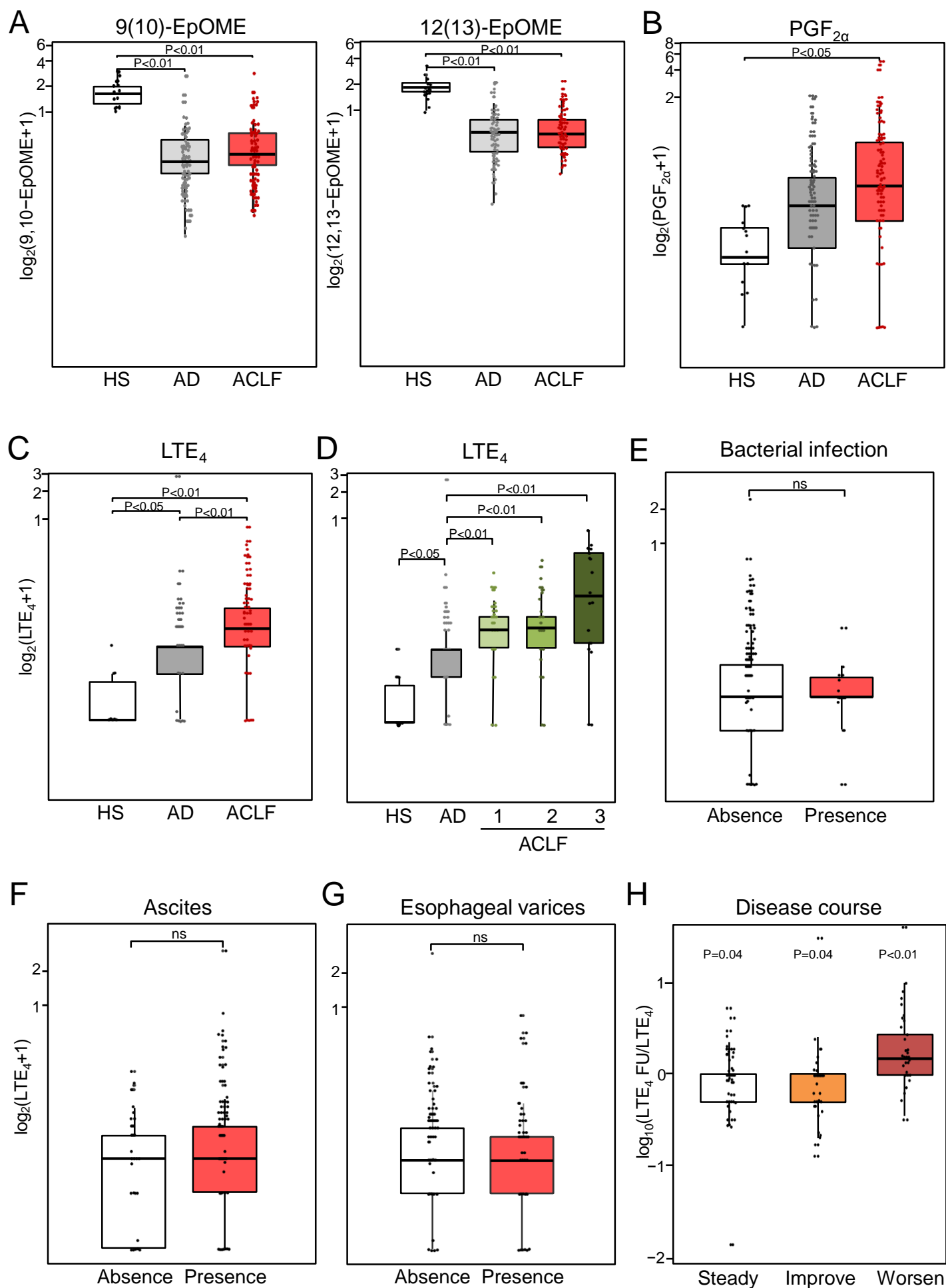


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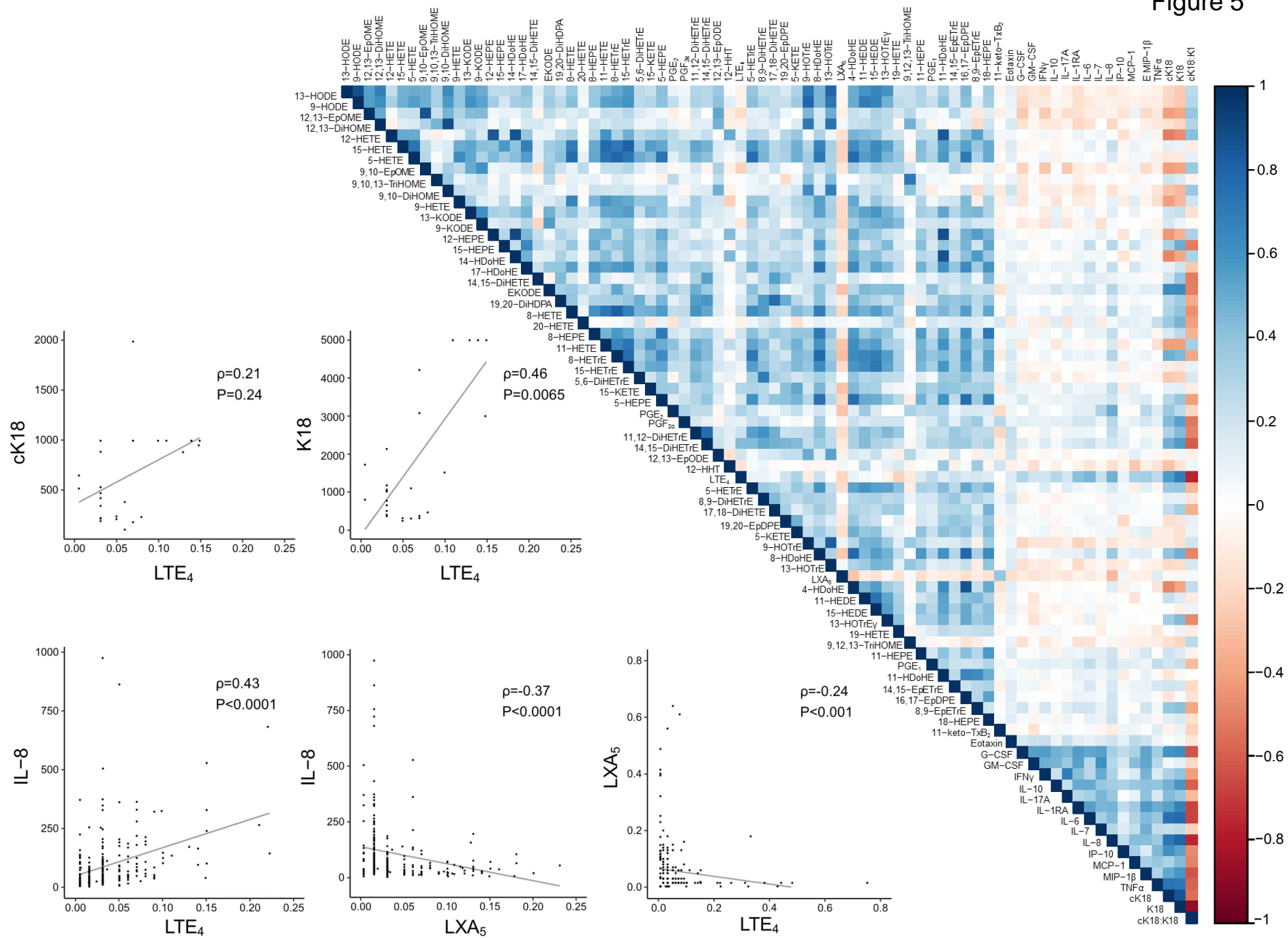
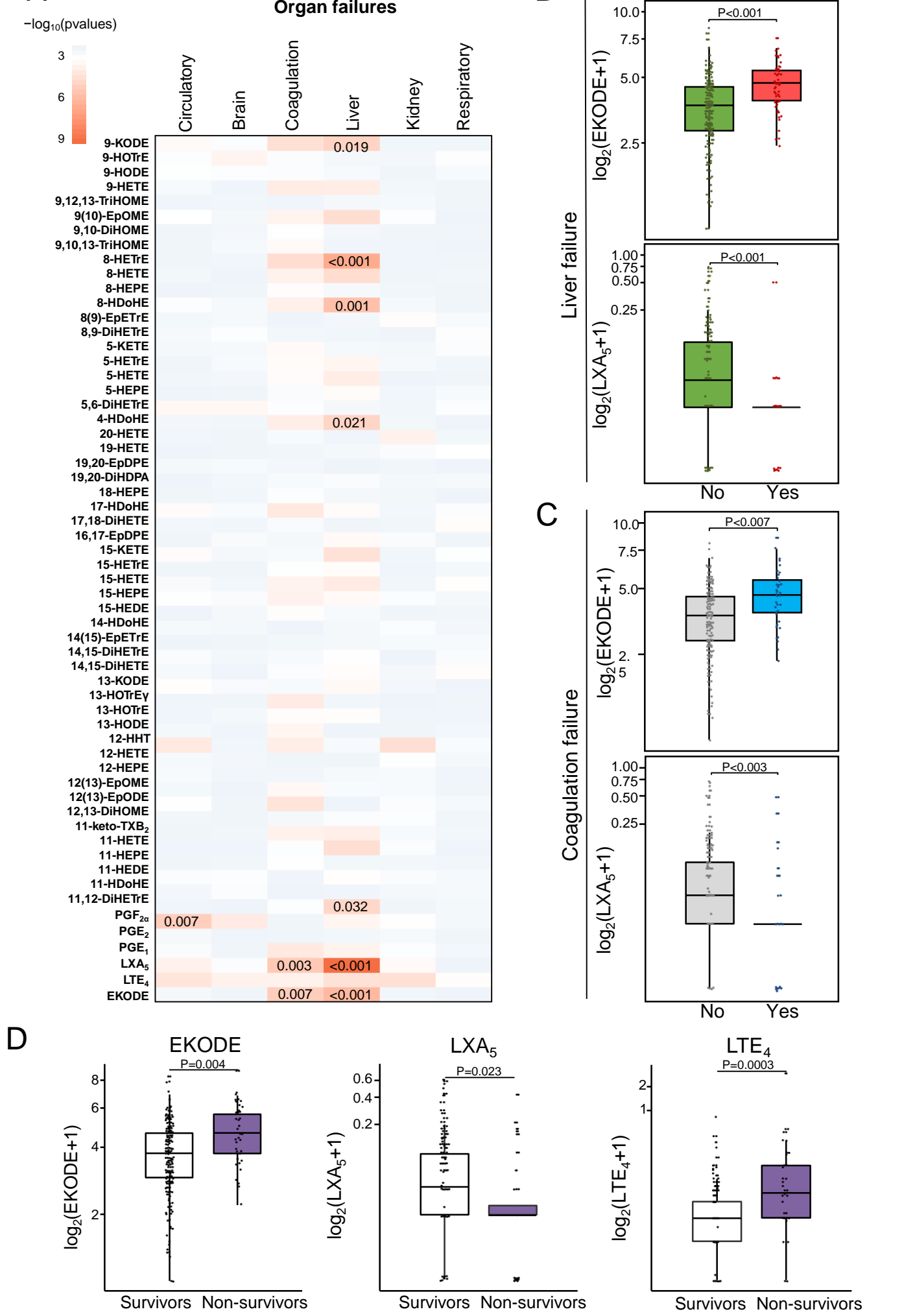
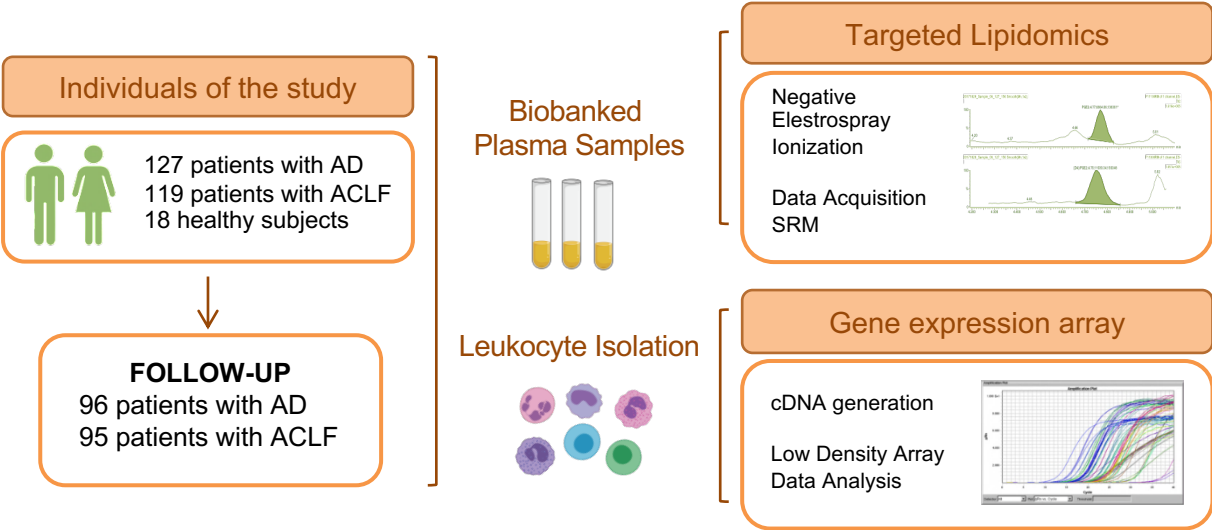


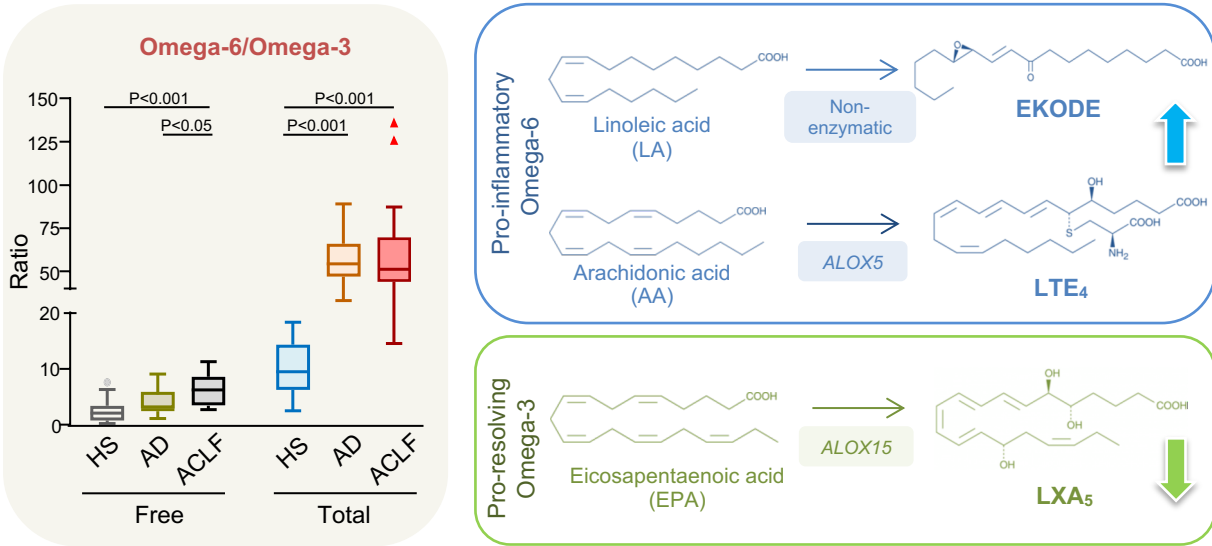
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Figure 6





Identification of a blood lipid mediator fingerprint in AD cirrhosis



## 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<sub>4</sub> 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.