Research Article
Cirrhosis and Liver Failure

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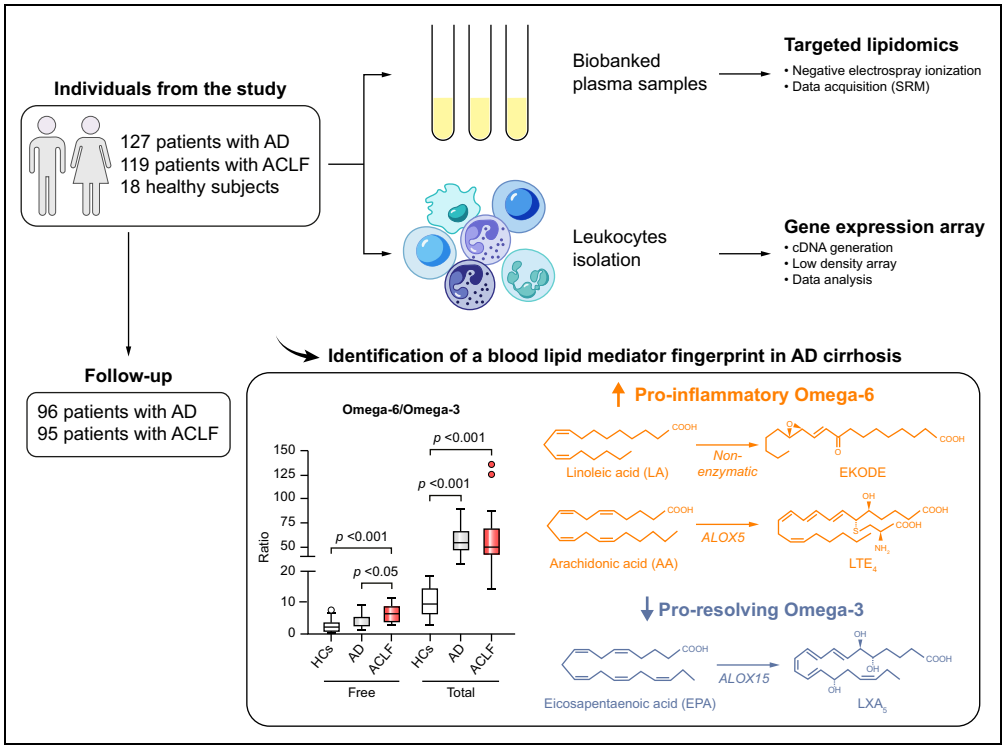
Targeted lipidomics reveals extensive changes in circulating lipid mediators in patients with acutely decompensated cirrhosis

Graphical abstract



Highlights

- Lipidomics was performed to assess the profile of lipid mediators in plasma from patients with and without ACLF.
- Measurements were prospectively repeated during a 28-day follow-up period.
- 59 lipid mediators were detected in plasma from cirrhotic patients, of which 16 were associated with disease status.
- Among these, leukotriene E_4 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.

Authors

Cristina López-Vicario, Antonio Checa, Arantxa Urdangarin, …, Vicente Arroyo, Craig E. Wheelock, Joan Clària

Correspondence

jclaria@clinic.cat
(J. Clària).

Lay summary

Acute-on-chronic liver failure (ACLF) is characterized by intense systemic inflammation, multiple organ failures and high short-term mortality. In the current study, we assessed the plasma lipid profile of 100 bioactive lipid mediators in healthy controls, patients with decompensated cirrhosis, and those who had developed ACLF. 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.

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

Cristina López-Vicario[1,2], Antonio Checa[3], Arantxa Urdangarin[4], Ferran Aguilar[1], José Alcaraz-Quiles[2], Paolo Caraceni[5], Alex Amorós[1], Marco Pavesi[1], David Gómez-Cabrero[4], Jonel Trebicka[1,6], Karl Oettl[7], Richard Moreau[1,8], Núria Planell[4], Vicente Arroyo[1], Craig E. Wheelock[3], Joan Clària[1,2,9,*]

[1]European Foundation for the Study of Chronic Liver Failure (EF-Clif) and Grifols Chair, Barcelona, Spain; [2]Biochemistry and Molecular Genetics Service, Hospital Clínic-IDIBAPS and CIBERehd, Barcelona, Spain; [3]Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden; [4]Translational Bioinformatics Unit, NavarraBiomed, Pamplona, Spain; [5]Department of Medical and Surgical Sciences, University of Bologna, Bologna, Italy; [6]J.W. Goethe University Hospital, Frankfurt, Germany; [7]Institute of Physiological Chemistry, Center of Physiological Medicine, Medical University of Graz, Graz, Austria; [8]Inserm, U1149, Centre de Recherche sur l'Inflammation (CRI), UMRS1149; Université Paris Diderot-Paris 7, Paris, France; [9]Department of Biomedical Sciences, University of Barcelona, Barcelona, Spain

Background & Aims: Acute-on-chronic liver failure (ACLF) is a newly described syndrome, which develops in patients with acute decompensation of cirrhosis, and is characterized by intense systemic inflammation, multiple organ failures and high short-term mortality. The profile of circulating lipid mediators, which are endogenous signaling molecules that play a major role in inflammation and immunity, is poorly characterized in ACLF.
Methods: In the current study, we assessed the profile of lipid mediators by liquid chromatography coupled to tandem mass spectrometry in plasma from patients with acute decompensation of cirrhosis, with (n = 119) and without (n = 127) ACLF, and from healthy controls (n = 18). Measurements were prospectively repeated in 191 patients with acute decompensation of cirrhosis during a 28-day follow-up period.
Results: Fifty-nine lipid mediators (out of 100) were detected in plasma from cirrhotic patients, of which 16 were significantly associated with disease status. Among these, 11 lipid mediators distinguished patients at any stage from healthy controls, whereas 2 lipid mediators (LTE_4 and 12-HHT, 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) were associated with short-term mortality. LXA_5 and EKODE formed a signature associated with coagulation and liver failures.
Conclusion: Taken together, these findings uncover specific lipid mediator profiles associated with disease severity and prognosis in patients with acute decompensation of cirrhosis.
Lay summary: Acute-on-chronic liver failure (ACLF) is characterized by intense systemic inflammation, multiple organ failures and high short-term mortality. In the current study, we assessed the plasma lipid profile of 100 bioactive lipid mediators in healthy controls, patients with decompensated cirrhosis, and those who had developed ACLF. 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 acute decompensation of cirrhosis (AD) frequently develop acute-on-chronic liver failure (ACLF).[1,2] ACLF is closely associated with multiple organ failures, recurrent infections and high short-term mortality and is mostly driven by innate immune system dysfunction, leading to exacerbated systemic inflammation, immune paralysis and tissue immunopathology.[3] Indeed, plasma cytokines are unusually elevated in patients with ACLF and their levels directly correlate with ACLF severity.[3] Moreover, a blood metabolite fingerprint specific for ACLF that is 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 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

Keywords: Lipidomics; Decompensated cirrhosis; Systemic inflammation; Lipid mediators.
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* Corresponding author. Address: Hospital Clínic, EF-Clif, Travessera de Gràcia 11, 08021 Barcelona, Spain. Tel.: +34 932 271 400.
E-mail address: jclaria@clinic.cat (J. Clària).
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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 produced intracellularly 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 3 enzymatic families: the cyclooxygenases (COXs), the lipoxygenases (LOXs) and the cytochrome P450 epoxygenases (CYP450), to produce a large array of lipid mediators (Fig. S1A).[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 (TX)A_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 non-steroidal anti-inflammatory drugs.[7] In addition to AA, the same enzymes can effectively metabolize its parent precursor, linoleic acid (LA) (Fig. S1A), 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-hydroxyeicosapentaenoic acid, 17-hydroxydocosahexaenoic acid (HDoHE) and 14-HDoHE that are involved in the resolution of inflammation (Fig. S1B).[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 liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) in 246 patients with AD 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 AD 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, plasma samples were available 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 Fig. S2. The investigation also included 18 healthy controls (HCs, 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 Table S1.

Further details regarding the 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 are provided in the supplementary information and references.[3,12,13]

Statistical analysis

Among the 100 lipid mediators screened (Table S1), 39 were below the detection limits of the method in the 3 study groups. Among the 61 lipid mediators detected, 2 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 information.

Results

Baseline clinical and standard laboratory data are given in Table S2. C-reactive protein levels and white blood cell count were significantly increased in patients with ACLF compared to those with AD. Platelet count was significantly reduced in patients with 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 (1 organ failure), 44 (36.9%) had ACLF grade 2 (2 organ failures), and 18 (15.1%) had ACLF grade 3 (≥3 organ failures). The frequency of failing organs in these patients is also shown in Table S2. 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 transition and comparison of retention time to that of authentic standards (Tables S3 and S4). Annotated lipid mediators were mostly derived from PUFAs of the omega-6 (AA and its precursor LA) and omega-3 (DHA and EPA) families (Fig. 1A). As shown in Fig. 1B, plasma levels of free PUFAs were similar across the 3 study groups, except for the AA, whose levels were slightly increased in patients with AD and in those with ACLF. However, the total content of PUFAs (all PUFAs 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 (Fig. 1B). In both cases (free and total PUFAs), the AA/EPA ratio, which is a surrogate marker of systemic inflammation,[14] was significantly increased in both groups of patients with cirrhosis (Fig. 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 (Table S5). 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 downregulated, respectively (Fig. 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 Fig. S1 for an overview of the biosynthetic pathways and Tables S6-8 for a comprehensive classification of each lipid mediator according to its biosynthetic precursor and

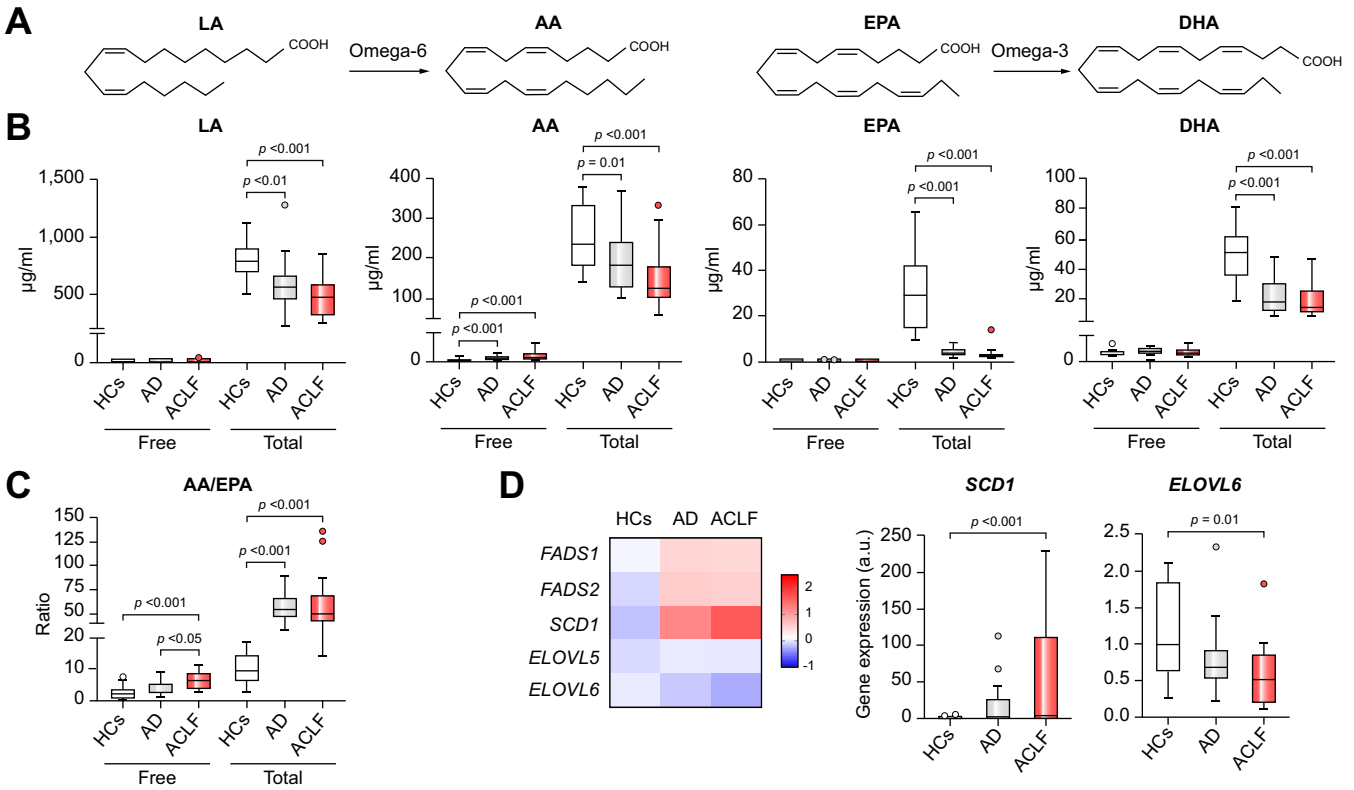

Fig. 1. Unbalanced omega-6/omega-3 ratio in patients with acute decompensation of cirrhosis. (A) Schematic diagram and chemical structures of omega-6 (LA and AA) and omega-3 (EPA and DHA) PUFA families. (B) Plasma levels of free and total PUFAs in HCs (n = 38) and AD (n = 34) and ACLF (n = 25) patients. (C) AA/EPA ratio of free and total PUFA content. (D) Changes in gene expression of desaturases (*FADS1*, *FADS2* and *SCD1*) elongases (*ELOVL5* and *ELOVL6*) in leukocytes from HCs (n = 14) and patients with AD (n = 14) and ACLF (n = 14). Changes in *SCD1* and *ELOVL6* expression are shown at the bottom. Statistical analyses were performed with two-tailed Mann-Whitney test or one-way ANOVA for multiple comparisons test. An adjusted *p* value ≤0.05 was considered statistically significant. Results are expressed as median and IQR. AA, arachidonic acid; ACLF, acute-on-chronic liver failure; AD, acute decompensation; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HCs, healthy controls; LA, linoleic acid; PUFAs, polyunsaturated fatty acids.

enzymatic route). Fig. 2A shows a graphical representation of the plasma abundance of lipid mediators categorized into 2 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 patient included in the analysis (lower panel). The most abundant lipid mediators in HCs 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 12,13-epoxy-9-keto-10(trans)octadecenoic acid (EKODE) (Table S7). 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 (Table S5), 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 HCs

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 HCs. Fig. 2B shows the relative distribution of each of the 3 enzymatic pathways (*i.e.* CYP450, LOX and COX) and Fig. 2C shows the expression of individual representative genes among these pathways in leukocytes from HCs, 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 downregulated in patients with AD and in those with ACLF, relative to HCs. In contrast, the expression of LOXs, specifically *ALOX5*, which codes for the 5-LOX enzyme involved in the production of inflammatory LTs, was remarkably upregulated 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 upregulated in patients relative to HCs.

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

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.* HCs and in ACLF *vs.* HCs. Finally, we ranked fold changes from the highest to

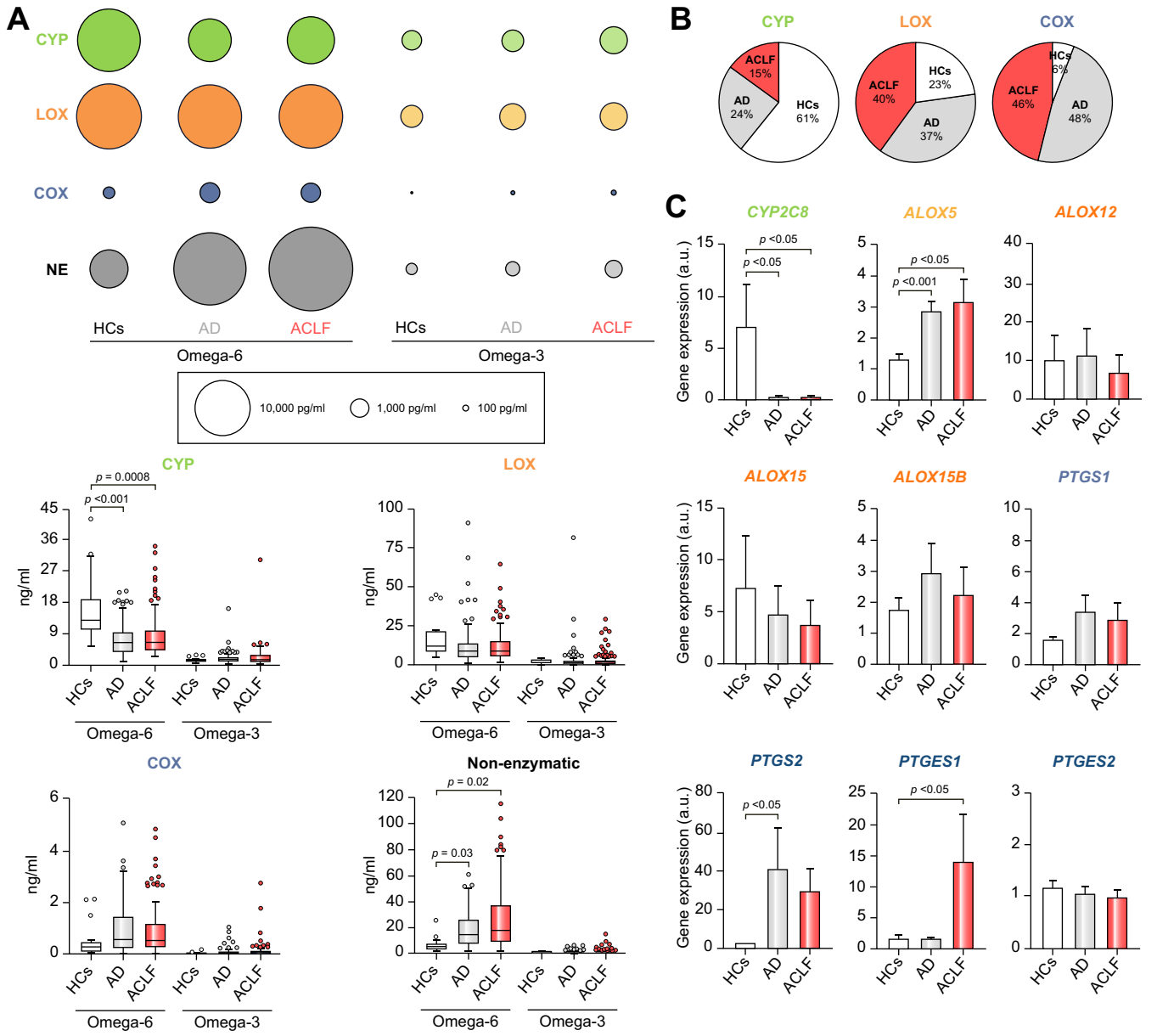

Fig. 2. Altered biosynthesis of lipid mediators in leukocytes of patients with acute decompensation of cirrhosis. (A) Total amount (upper panel) and box plots of individual values (lower panel) of lipid mediators from omega-6 and omega-3 PUFA families generated by CYP, LOX, COX and NE pathways in plasma from HCs (n = 18) and patients with AD (n = 127) and ACLF (n = 119). (B) Percent expression of genes of the CYP, LOX and COX pathways in leukocytes from HCs (n = 14) and patients with AD (n = 14) and ACLF (n = 14). (C) Gene expression of individual CYP, LOX and COX enzymes in leukocytes from HCs and patients with AD and ACLF. Statistical analyses were performed with two-tailed Mann-Whitney test or one-way ANOVA for multiple comparisons test. An adjusted *p* value ≤0.05 was considered statistically significant. Results are expressed as median and IQR. ACLF, acute-on-chronic liver failure; AD, acute decompensation; COX, cyclo-oxygenase; CYP, cytochrome; HCs, healthy control; LOX, lipoxygenase; NE, non-enzymatic; PUFA, polyunsaturated fatty acid.

the lowest values and the results were visualized in a Cleveland plot (Fig. 3A). This analysis revealed that LTs, epoxy-keto fatty acids, AA/DHA epoxides, PGs and TX were increased in patients (either AD or ACLF) compared to HCs. 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.* HCs and in ACLF *vs.* HCs and ranked and plotted the results in a Cleveland plot (Fig. 3B). This analysis revealed that 24 out of a total of 59 lipid mediators were significantly increased (fold change >1.5) in patients with AD compared to HCs, of which 4 were further increased in patients with ACLF compared to AD (Fig. 3B and Table S9). On the other hand, the plasma levels of 9 lipid mediators were significantly decreased (fold change <0.5) in patients with AD compared to HCs and none of them was further reduced in patients with ACLF compared to AD. A complete list of fold changes between ACLF and AD for all 59 lipid mediators included in the analysis is provided in Table S9. Changes in circulating levels of lipid mediators indistinctly affected all PUFA families as shown on the left side of Fig. 3B, where each lipid mediator is color coded by its biosynthetic precursor.

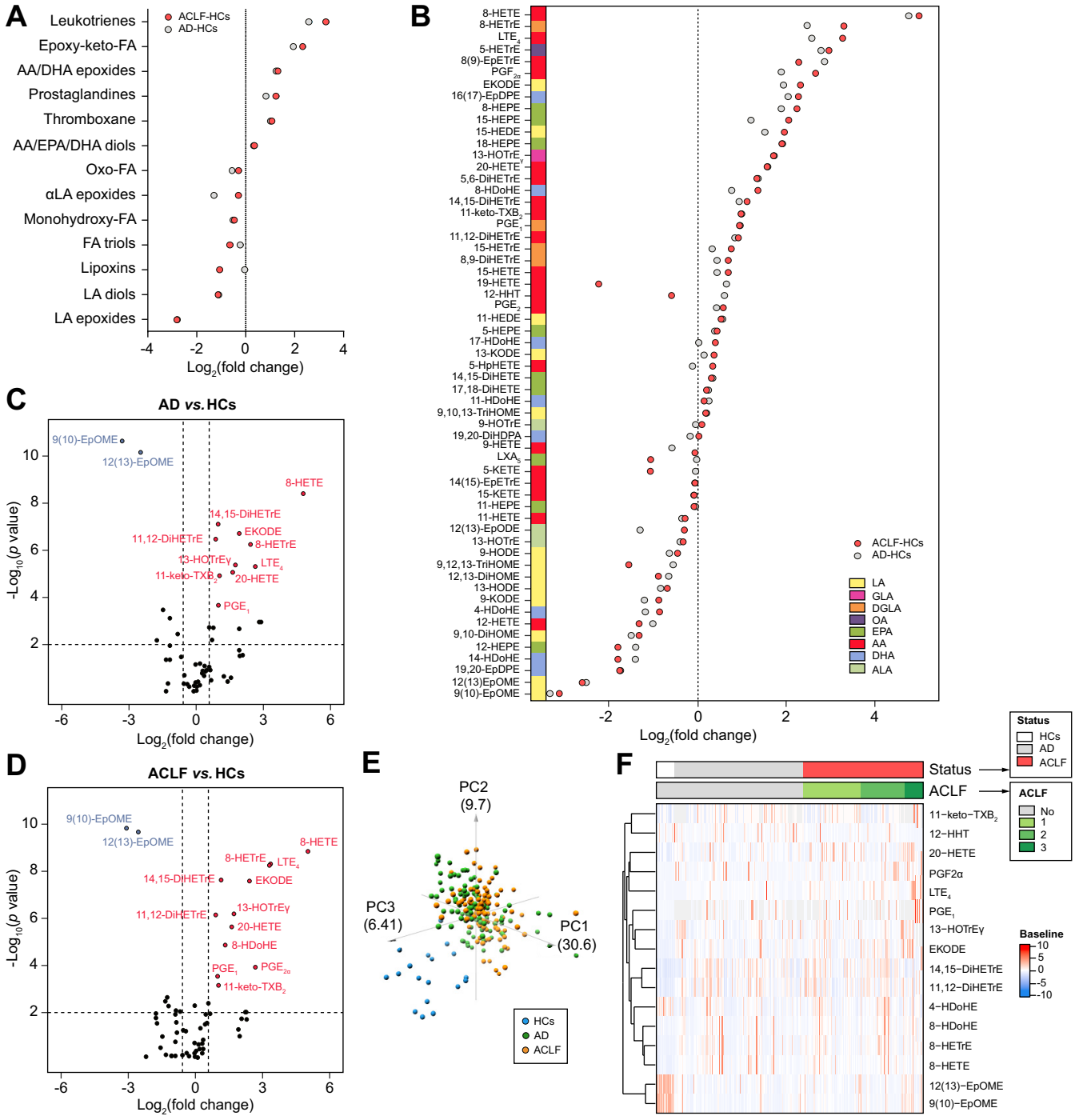

Fig. 3. Identification of a lipid mediator signature of acute decompensation. (A) Cleveland dot plot showing a ranked log2 transformation of fold changes of plasma levels of lipid mediators categorized by chemical families (LTs, epoxy-keto-FAs, AA and DHA epoxides, PGs, TX, AA/EPA/DHA diols, oxo-FA, ALA epoxides, monohydroxy-FA, FA triols, LXs, LA diols and LA epoxides). Blue and red dots represent the fold changes between patients with AD (n = 127) and ACLF (n = 119), with respect to HCs (n = 18), respectively. (B) Cleveland dot plot of the analyzed 59 lipid mediators ranked by fold change between patients with AD and ACLF *vs.* HCs. Color coding on the left represents each biosynthetic precursor ((LA, GLA, DGLA, OA, EPA, AA, DHA and ALA)). (C) Volcano plot representing the levels of lipid mediators up- or downregulated in patients with AD with respect to HCs. Lipid mediators with significant changes are presented in red (significant increase) or in blue (significant decrease). (D) Volcano plot representing changes in patients with ACLF with respect to HCs. (E) 3D-PCA of lipid mediators in plasma from HCs and patients with AD and ACLF. (F) Heatmap of the 16 lipids associated with patient status at enrollment (unsupervised analysis). Rows represent individual lipid mediators and columns represent individual HCs, and patients with AD and ACLF. AA, arachidonic acid; ACLF, acute-on-chronic liver failure; AD, acute decompensation; ALA, αLA; dgla, dihomo-γ-linolenic acid; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DiHETrE, dihydroxyeicosatrienoic acid; DiHOME, dihydroxyoctadecenoic acid; EKODE, 12,13-epoxy-9-keto-10(trans)octadecenoic acid; EPA, eicosapentaenoic acid; EpOME, epoxyoctadecenoic acid; FA, fatty acid; GLA, γLA; HCs, healthy controls; HDoHE, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; HETrE, hydroxyeicosatrienoic acid; HHT, hydroxyheptadecatrienoic acid; HOTrE, hydroxyoctadecatrienoic acid; KETE, oxo-eicosatetraenoic acid; KODE, oxo-octadecadienoic acid; LA, linoleic acid; LT, leukotriene; LX, lipoxin; OA, oleic acid; PCA, principal component analysis; PG, prostaglandin; PUFA, poly-unsaturated fatty acid; TX, thromboxane.

We next plotted fold changes (using volcano plots) in the levels of lipid mediators in patients with AD and in those with ACLF relative to HCs, considering the statistically significant differences (*p* values). As shown in Fig. 3C, this analysis identified increased levels of 8-hydroxyeicosatetraenoic acid (HETE), 14,15-dihydroxyeicosatrienoic acid (DiHETrE), EKODE, 11,12-DiHETrE, 8-hydroxyeicosatrienoic acid (HETrE), 13-hydroxyoctadecatrienoic acid (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 proinflammatory 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 (EpOME) and 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 (Fig. 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 2 additional lipid mediators ($PGF_{2\alpha}$, a COX-derived vasoconstrictor, and 8-HDoHE, a product of DHA autoxidation) (Fig. 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 for AD

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. 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 Fig. 3E, the PCA analysis yielded a clear distinction between patients and HCs. After adjusting for gender and age, we identified 16 lipid mediators that distinguished between 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 (Fig. 3F). Of interest, one out of these 16 lipid mediators, the DHA product 4-HDoHE, distinguished patients with AD from HCs, whereas 11 lipid mediators discriminated HCs 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 dihomo-γ-linolenic acid and 13-HOTrEγ from γ-LA. Box plots for 9(10)-EpOME and 12(13)-EpOME are shown in Fig. 4A. Although 9(10)-EpOME, 12(13)-EpOME are biologically relevant and their levels presented the largest reductions in the volcano plots, these 2 lipid mediators were not associated with any clinical outcome (Fig. S3). Lipid mediators that were not significantly associated and did not discriminate the different stages of the disease are listed in Table S10.

Plasma levels of LTE_4 discriminate disease severity

Among the 16 lipid mediators identified in the PCA analysis, 2 of them ($PGF_{2\alpha}$ derived from AA (Fig. 4B) and 8-HDoHE derived from DHA) distinguished patients with ACLF from HCs (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 2 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 patients with AD compared to HCs and in patients with ACLF compared to those with AD (Fig. 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 (Fig. 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 (Fig. 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 Tables S11 and S12). The dynamics of LTE_4 were 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 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 had ACLF (Table S13A-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 from ACLF to AD and significant increase in those who worsened from AD to ACLF) (Fig. 4H). However, sensitivity analysis in AD patients developing ACLF revealed that LTE_4 has low predictive value (area under the receiver-operating characteristic 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 Fig. 5, lipid mediators generally had a positive correlation among themselves, except LXA_5, which is an anti-inflammatory and pro-resolution lipid mediator that was inversely correlated with other lipid mediators. In general, lipid mediators had a weak correlation with cytokines/chemokines, except LTE_4 and LXA_5. LTE_4, part of the minimal ACLF fingerprint, was the only lipid mediator with a distinct positive correlation with inflammatory cytokines/chemokines, in particular with interleukin (IL)-1RA and IL-6, and specially with IL-8. LTE_4 also positively correlated with markers of cell death in patients with AD and ACLF (Fig. 5 and Fig. S4). This correlation was stronger with K18 than with cK18, suggesting that LTE_4 could be associated with the non-apoptotic form. On the other hand, LXA_5 was inversely correlated with 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 (Fig. S5 and Tables S11 and S12).

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

	Kruskal-Wallis		HCs – AD		HCs – ACLF		AD – ACLF	
Lipid mediator	***p* value**	**Adjusted *p* value**	***p* value**	**Adjusted *p* value**	***p* value**	**Adjusted *p* value**	***p* value**	**Adjusted *p* value**
Significant changes between HCs *vs.* AD								
4-HDoHE	<0.01	0.02	<0.01	0.04	0.03	1.00	<0.01	0.18
Significant changes between HCs *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 HCs *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 ($p < 0.05$).
ACLF, acute-on-chronic liver failure; AD, acute decompensation; DGLA, dihomo-γ-linolenic acid; DiHETrE, dihydroxyeicosatrienoic acid; DiHOME, dihydroxyoctadecenoic acid; EKODE, 12,13-epoxy-9-keto-10(trans)octadecenoic acid; EpOME, epoxyoctadecenoic acid; HCs, healthy controls; HDoHE, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; HETrE, hydroxyeicosatrienoic acid; HHT, hydroxyheptadecatrienoic acid; HOTrE, hydroxyoctadecatrienoic acid; KETE, oxo-eicosatetraenoic acid; KODE, oxo-octadecadienoic acid; LT, leukotriene; LX, lipoxin; PG, prostaglandin; TX, thromboxane.

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 Fig. 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. Fig. 6B,C show the presence of increased EKODE in the context of reduced LXA_5, which were the lipid mediators associated with at least 2 different organ failures. Moreover, increased EKODE and LTE_4 together with reduced LXA_5 significantly associated with 28-day mortality (Fig. 6D and Table S12).

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 of whom 119 had ACLF. Our major findings were the following: i) Patients with AD, and to a greater extent patients with ACLF, had an 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] ii) 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. iii) LTE_4 was one of the top differentially regulated lipid mediators and gradually increased from HCs to AD and ACLF, as well as in ACLF-3 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). iv) LXA_5, which was invariably reduced in patients, was the only lipid mediator that inversely correlated with IL-8. v) LTE_4 was part of a minimal plasma fingerprint for ACLF, whereas LXA_5, and EKODE, discriminated organ failures. vi) Finally, increased LTE_4 and EKODE together with decreased LXA_5 were associated with higher 28-day mortality. Collectively, these findings capture a specific lipid mediator profile in patients with AD, adding value to recent studies within the framework 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 *vs.* HCs. LTE_4 is formed upon activation of 5-LOX, which converts AA into 5-HpHETE, an intermediate in the generation of LTA_4 (Fig. S1A). 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

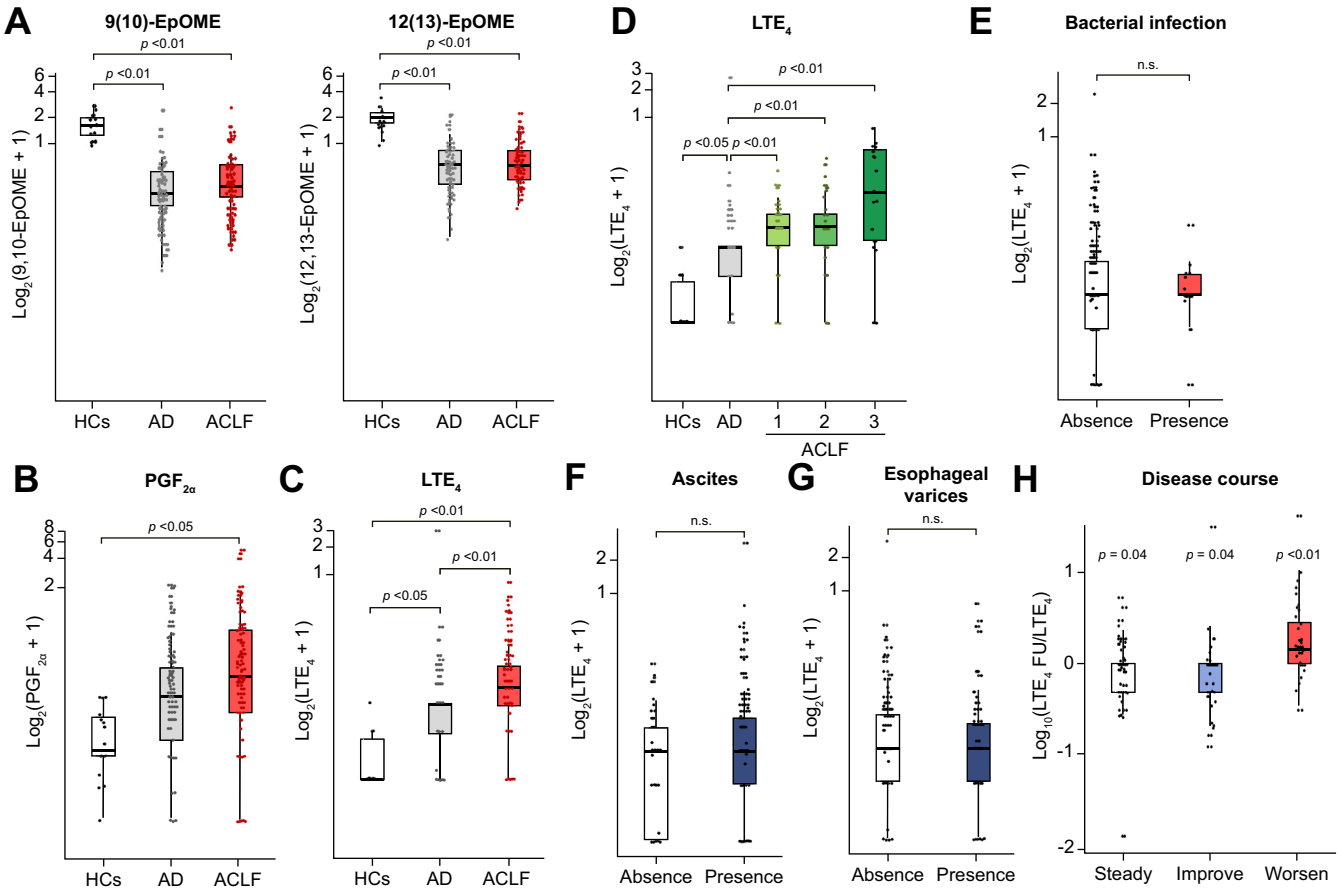

Fig. 4. LTE_4 discriminates disease severity. (A) Plasma levels of 9(10)-EpOME, 12(13)-EpOME in the HCs (n = 18), AD (n = 127) and ACLF (n = 119) groups at enrollment. (B, C) $PGF_2\alpha$ and LTE_4 levels in the study groups. (D) LTE_4 in ACLF patients according to severity (grade-1, n = 57; grade-2, 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 *U* test was used. Bonferroni correction was applied to correct for multiple testing. An adjusted *p* value ≤0.05 was considered statistically significant. ACLF, acute-on-chronic liver failure; AD, acute decompensation; EpOME, epoxyoctadecenoic acid; HCs, healthy controls; LT, leukotriene; PG, prostaglandin.

therapy.[19] In these conditions, cysteinyl-LTs directly interact with cytokines (*i.e.* IL-6, IL-10 and tumor necrosis factor-α) and chemokines (*i.e.* IL-8, eotaxin and macrophage inflammatory protein-1α).[20] In addition to inflammation, cysteinyl-LTs might also be related to cell death, since 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 is potentially 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, who also presented an unbalanced AA/EPA ratio. In agreement with this, we identified unbalanced formation between proinflammatory 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 2 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 controls and the comparisons

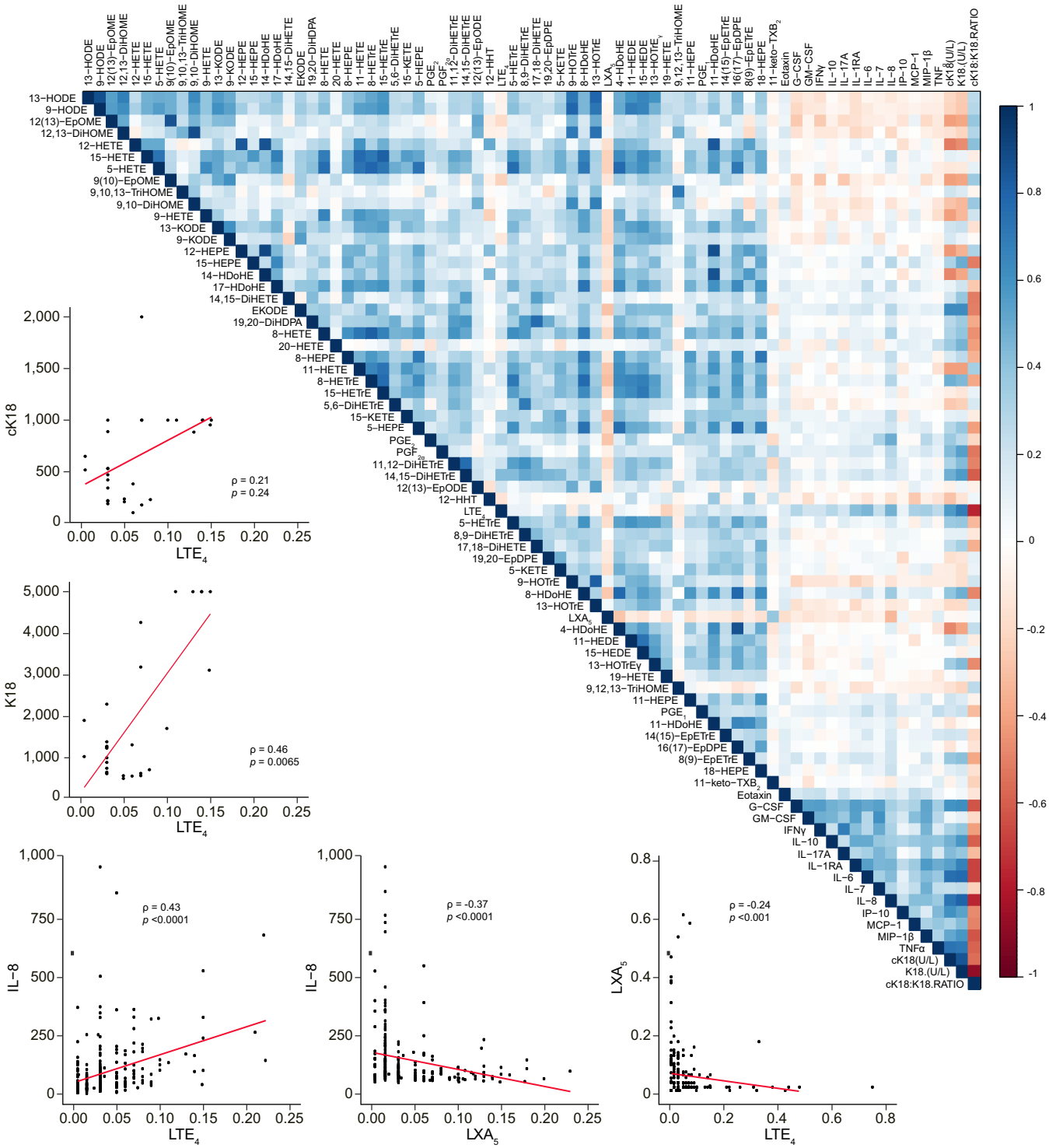

Fig. 5. Association of lipid mediators with markers of inflammation and cell death. Correlation matrix plot between lipid mediators and cytokines/chemokines/cell death markers in patients with AD (n = 127) and ACLF (n = 119). Shades of blue indicate increasing positive correlation coefficient; shades 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. Spearman correlation was performed and an adjusted *p* value ≤0.05 was considered statistically significant. ACLF, acute-on-chronic liver failure; AD, acute decompensation; DGLA, dihomo-γ-linolenic acid; DiHETrE, dihydroxyeicosatrienoic acid; DiHOME, dihydroxyoctadecenoic acid; EKODE, 12,13-epoxy-9-keto-10(trans)octadecenoic acid; EpOME, epoxyoctadecenoic acid; HCs, healthy controls; HDoHE, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; HETrE, hydroxyeicosatrienoic acid; HHT, hydroxyheptadecatrienoic acid; HOTrE, hydroxyoctadecatrienoic acid; IL-, interleukin; KETE, oxo-eicosatetraenoic acid; KODE, oxo-octadecadienoic acid; LT, leukotriene; LX, lipoxin; PG, prostaglandin; TX, thromboxane.

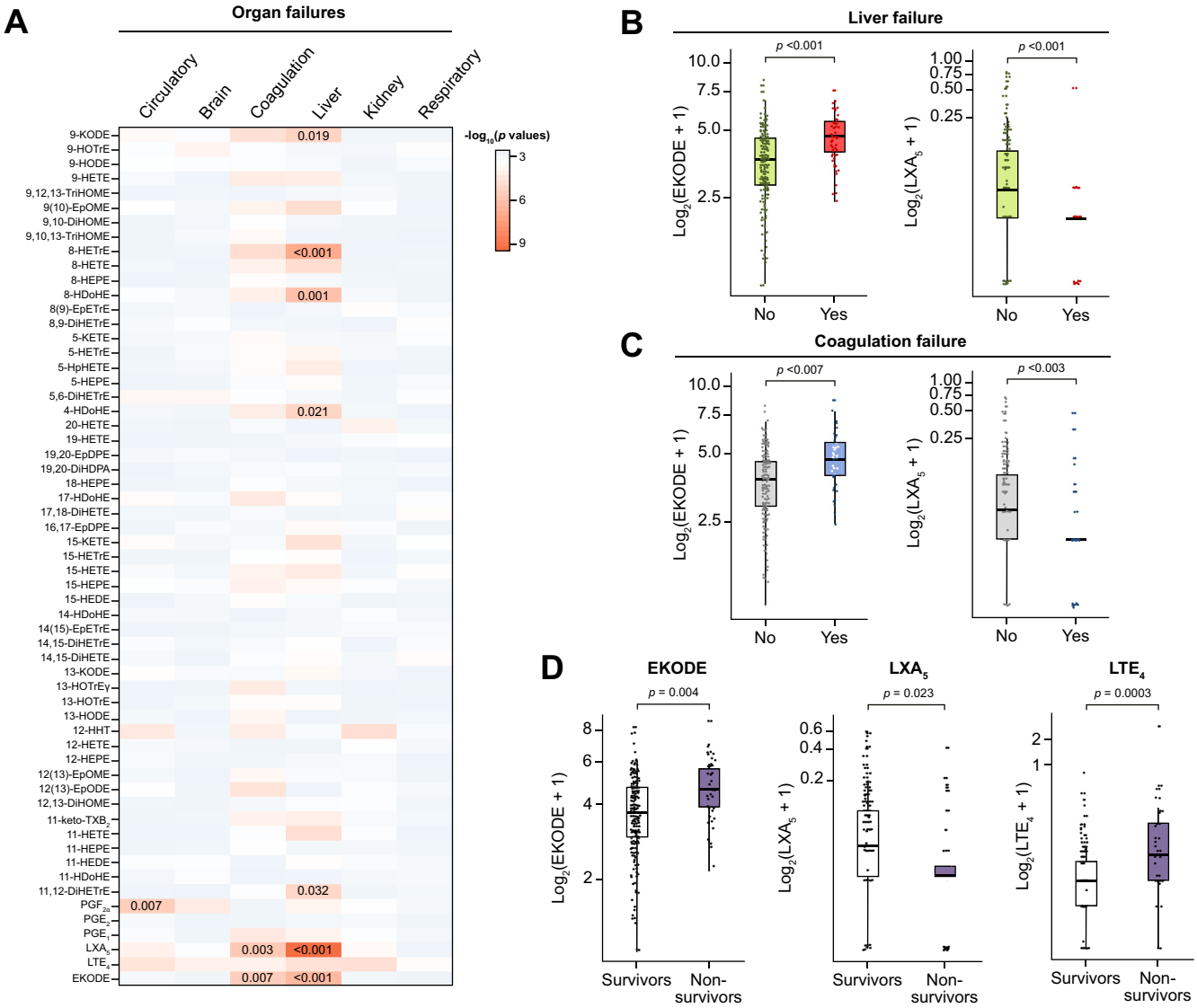

Fig. 6. Association of lipid mediators with organ failures and short-term mortality. (A) Heatmap representation of the 59 lipid mediators analyzed in the study and their association with organ failures in patients with AD (n = 127) and ACLF (n = 119). Grey color corresponds to low association (high *p* 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 *U* test was used. Bonferroni correction was applied to correct for multiple testing. An adjusted *p* value ≤0.05 was considered statistically significant. ACLF, acute-on-chronic liver failure; AD, acute decompensation; DGLA, dihomo-γ-linolenic acid; DiHETrE, dihydroxyeicosatrienoic acid; DiHOME, dihydroxyoctadecenoic acid; EKODE, 12,13-epoxy-9-keto-10(trans)octadecenoic acid; EpOME, epoxyoctadecenoic acid; HCs, healthy controls; HDoHE, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; HETrE, hydroxyeicosatrienoic acid; HHT, hydroxyheptadecatrienoic acid; HOTrE, hydroxyoctadecatrienoic acid; KETE, oxo-eicosatetraenoic acid; KODE, oxo-octadecadienoic acid; LT, leukotriene; LX, lipoxin; PG, prostaglandin; TX, thromboxane.

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.

Herein, we 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, non-enzymatic 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 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 were associated with short-term mortality. Overall, our study provides useful insights into the role of bioactive lipid mediators in the initiation and progression of systemic inflammation and organ failures in patients with AD.

Abbreviations

AA, arachidonic acid; ACLF, acute-on-chronic liver failure; AD, acute decompensation of cirrhosis; ALA, α-linoleic acid; cK18, caspase-cleaved keratin 18; COX, cyclooxygenase; CYP450, cytochrome P450; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DiHETrE, dihydroxyeicosatrienoic acid; DiHOME, dihydroxyoctadecenoic acid; EKODE, 12,13-epoxy-9-keto-10(trans)octadecenoic acid; ELOVL, elongation of very long chain fatty acids; EPA, eicosapentaenoic acid; EpOME, epoxyoctadecenoic acid; FA, fatty acid; FADS, fatty acid desaturase; GLA, γ-linoleic acid; HCs, healthy controls; HDoHE, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; HETrE, hydroxyeicosatrienoic acid; HHT, hydroxyheptadecatrienoic acid; HOTrE, hydroxyoctadecatrienoic acid; IL-, interleukin; K18, keratin 18; KETE, oxo-eicosatetraenoic acid; KODE, oxo-octadecadienoic acid; LA, linoleic acid; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; LOX, lipoxygenase; LT, leukotriene; LX, lipoxin; MELD, model for end-stage liver disease; mPGES, microsomal prostaglandin E synthase; NE, non-enzymatic; OA, oleic acid; PCA, principal component analysis; PG, prostaglandin; PUFA, polyunsaturated fatty acid; SCD, stearoyl-CoA desaturase; TX, thromboxane.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' 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).

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2020.03.046.

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