




Albumin Lipidomics Reveals Meaningful Compositional Changes in Advanced Cirrhosis and Its Potential to Promote Inflammation Resolution

Mireia Casulleras ^{1,2}, Roger Flores-Costa ^{1,2}, Marta Duran-Güell,^{1,2} Ingrid W. Zhang,^{1,2} Cristina López-Vicario,^{1,2} Anna Curto,¹ Javier Fernández,^{1,3} Vicente Arroyo,¹ and Joan Clària ^{1,2,4}

Albumin infusions are therapeutically used to revert hypoalbuminemia and to replace the extensively oxidized albumin molecule circulating in patients with acutely decompensated (AD) cirrhosis. Because albumin has high affinity for lipids, here we characterized the albumin lipidome in patients with AD and explored the albumin effects on the release of fatty acid (FA)-derived lipid mediators by peripheral leukocytes. Lipids and lipid mediators were measured by liquid chromatography–tandem mass spectrometry in albumin-enriched and albumin-depleted plasma fractions separated by affinity chromatography and in leukocyte incubations from 18 patients with AD and 10 healthy subjects (HS). Lipid mediators were also measured in 41 patients with AD included in an albumin therapy trial. The plasma lipidome associated with AD cirrhosis was characterized by generalized suppression of all lipid classes except FAs. In contrast to HS, albumin from patients with AD had lower content of polyunsaturated FAs (PUFAs), especially of the omega-3-PUFA docosahexaenoic acid. Consistent with this, the PUFA-derived lipid mediator landscape of albumin from patients with AD was dominated by lower content of monohydroxy FA precursors of anti-inflammatory/pro-resolving lipid mediators (i.e., 15-hydroxyeicosatetraenoic acid [15-HETE]). In addition, albumin from patients with AD was depleted in prostaglandin (PG) E₂, suggesting that this proinflammatory PG primarily travels disassociated to albumin in these patients. Incubation of leukocytes with exogenous albumin reduced PG production while inducing 15-lipoxygenase expression and 15-HETE release. Similar effects were seen under lipopolysaccharide plus *N*-formylmethionyl-leucyl-phenylalanine-stimulated conditions. Finally, PG levels were lower in patients with AD receiving albumin therapy, whereas 15-HETE was increased after albumin treatment compared with baseline. **Conclusion:** Our findings indicate that the albumin lipid composition is severely disorganized in AD cirrhosis and that administration of exogenous albumin has the potential to redirect leukocyte biosynthesis from pro-inflammatory to pro-resolving lipid mediators. (*Hepatology Communications* 2022;6:1443-1456).

Lipids not only represent an important energy source and are essential structural components of cell membranes, but they play critical roles in organelle homeostasis, cell metabolism, signaling and survival, and interorgan communication.⁽¹⁻⁴⁾ It is also recognized that lipids, especially fatty acids (FAs), govern immune responses.^(3,4) For example, polyunsaturated

FAs (PUFAs) serve as parent precursors for conversion through specific enzymatic pathways, predominantly expressed in immune cells, into a wide array of bioactive lipid mediators.⁽²⁾ The PUFAs consist of two distinct families: the omega-6 and the omega-3. The omega-6-PUFAs, of which arachidonic acid (AA) is the best representative, is the common substrate of the

Abbreviations: 14-HDHA, 14-hydroxydocosahexaenoic acid; 15-HETE, 15-hydroxyeicosatetraenoic acid; 17-HDHA, 17-hydroxydocosahexaenoic acid; 18-HEPE, 18-hydroxyeicosapentaenoic acid; AA, arachidonic acid; AD, acute decompensation; COX, cyclooxygenase; DHA, docosahexaenoic acid; DPBS, Dulbecco's phosphate-buffered saline; EPA, eicosapentaenoic acid; FA, fatty acid; fMLP, *N*-formylmethionyl-leucyl-phenylalanine; HS, healthy subjects; HSA, human serum albumin; LC/MS-MS, liquid chromatography coupled to tandem mass spectrometry; LOX, lipoxygenase; LPS, lipopolysaccharide; LT, leukotriene; LX, lipoxin; MUFA, monounsaturated FA; PBMC, peripheral blood mononuclear cell; PG, prostaglandin; PMN, polymorphonuclear neutrophil; PUFA, polyunsaturated FA; SMT, standard medical therapy; SPM, specialized pro-resolving mediator; TXB₂, thromboxane B₂.

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cyclooxygenase (COX) and lipoxygenase (LOX) pathways for the biosynthesis of potent pro-inflammatory and immunosuppressive lipid mediators such as prostaglandin (PG) and leukotrienes (LTs).^(2,5) On the other hand, the omega-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are converted by the same COX and LOX pathways into an array of anti-inflammatory lipid mediators involved in the resolution of inflammation, designated as specialized pro-resolving mediators (SPMs).⁽⁶⁾ AA can also be a precursor of SPMs, such as lipoxins (LXs).⁽⁶⁾ Fine equilibrium between omega-6-derived and omega-3-derived lipid mediators is critical for the regulation of immune and inflammatory processes and maintenance of tissue homeostasis.⁽⁷⁾ Consequently, an altered lipid composition can lead to immune-metabolic dysregulation and uncontrolled inflammation.

Albumin has high affinity for lipids, especially FAs, which are transported in the circulation attached to its molecule.⁽⁸⁾ Albumin, which is synthesized in the liver, is the most abundant protein in the bloodstream, is the main contributor to plasma colloid osmotic pressure, and is clinically used in conditions of hypovolemia and hypoalbuminemia.⁽⁹⁾ Indeed, infusion of exogenous albumin is an effective therapy in patients with acutely decompensated (AD) liver cirrhosis.^(9,10) Specifically, in these patients, short-term and long-term albumin administration prevents circulatory and renal dysfunction, the development of ascites,

and reduces the number of hospital readmissions and mortality.^(9,10) In addition to acting as a plasma volume expander, albumin is a potent immunomodulatory molecule, and its internalization by peripheral leukocytes from patients with AD cirrhosis has been shown to regulate endosomal toll-like receptor signaling and immune response to pathogens.⁽¹¹⁾ However, increased oxidative stress and high frequency of post-translational modifications of the albumin molecule are common findings in patients with AD cirrhosis.^(12,13) These features, on top of hypoalbuminemia, likely affect the lipid content of the albumin molecule and alter the circulatory lipid profile of patients with AD, which could contribute to the hyperinflammatory status and immune dysregulation characteristic of this condition. Taking all of these into account, in the current study we performed a lipidomics investigation of the albumin molecule by comparing the lipid composition of albumin-enriched and albumin-depleted plasma fractions from patients with AD cirrhosis with that of healthy subjects (HS). In addition, because albumin infusions are therapeutically used in patients with AD, we assessed the effects of exogenous albumin on the biosynthesis and release of FA-derived lipid mediators by peripheral blood mononuclear cells (PBMCs) and polymorphonuclear leukocytes (PMNs). Finally, we evaluated changes in the plasma levels of lipid mediators in patients with AD cirrhosis included in a therapeutic albumin trial.

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ARTICLE INFORMATION:

From the ¹European Foundation for the Study of Chronic Liver Failure, Barcelona, Spain; ²Biochemistry and Molecular Genetics Service, Hospital Clínic-IDIBAPS-CIBERhd, Barcelona, Spain; ³Liver Unit, Hospital Clínic, Barcelona, Spain; ⁴Department of Biomedical Sciences, University of Barcelona, Barcelona, Spain.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Joan Clària, PhD
Biochemistry and Molecular Genetics Service
Hospital Clínic/IDIBAPS/EF-Clif
Villarroel 170

08036 Barcelona, Spain
E-mail: jlclaria@clinic.cat
Tel.: +1-34-932-271-400

Experimental Procedures

PATIENTS WITH AD CIRRHOSIS AND HS

Eighteen patients with AD cirrhosis from the Liver Intensive Care Unit (ICU) of the Hospital Clinic (Barcelona, Spain) were enrolled in this study. Ten of these patients were included in plasma albumin fractionation experiments, and 8 were included in the experiments with isolated leukocytes. Samples and cells from HS ($n = 10$) aged 30-60 years were obtained from the Blood Bank of the Hospital Clinic of Barcelona integrated within the Blood and Tissue Bank of the Catalan Department of Health. In addition, biobanked plasma samples were obtained from patients with AD cirrhosis ($n = 41$) included in the INFECIR-2 study, a phase 4, randomized, open-label, parallel, multicenter therapeutic study aimed at comparing the effect of standard medical therapy with antibiotics (SMT) versus SMT plus human serum albumin (HSA) treatment on hospital mortality in patients with AD cirrhosis and infections other than spontaneous bacterial peritonitis (ClinicalTrials.gov: NCT02034279).⁽¹⁴⁾ Samples were collected before (basal) and after treatment (post) (SMT alone or SMT plus HSA). The selection of patients for these measurements among the whole cohort of the INFECIR-2 study was based on the availability of plasma samples at baseline and after treatment from the same patients. The INFECIR-2 study was approved by the corresponding Ethics Committee of each participating hospital, and informed consent included the potential use of biobanking material for plasma measurements. All participating patients provided signed informed consent. Approval for the use of biological samples from patients in the ICU was obtained from the Hospital Clinic internal review board (#HCB/2020/0138).

PREPARATION OF ALBUMIN-ENRICHED AND ALBUMIN-DEPLETED PLASMA FRACTIONS

Albumin depletion was performed with a Pierce Albumin depletion kit (Thermo Fisher Scientific, Waltham, MA) based on dye-ligand affinity chromatography. Briefly, a spin column was loaded with

200 μL of albumin depletion agarose resin and centrifuged for 1 minute at 12,000g. Then, 200 μL of binding buffer were added to the column, centrifuged, and the flow-through discarded before the addition of 50 μL of plasma from HS or patients with AD to the column. Samples were incubated for 2 minutes at room temperature and again centrifuged. The flowthrough was collected, and 50 μL of binding buffer was again added to the column before centrifugation; this process was repeated four times. After that, the first albumin-depleted fraction (albumin-depleted fraction 1) was obtained. The albumin-enriched fraction retained in the column was placed in a new collection tube and washed four times with 200 μL of elution buffer (20 mM Na_3HPO_4 and 250 mM NaSCN ; pH 7.2) by centrifugation, retaining the flowthrough in all cases. Because preliminary experiments showed that the albumin-depleted fraction 1 contained some albumin leftover; this fraction was added to a new column followed by the addition of binding buffer (50 μL) to the column and centrifugation. After repeating this process four times, the second albumin-depleted fraction (albumin-depleted fraction 2) was collected. The albumin-enriched fraction retained in this second column was also eluted as described previously. This methodological process is shown in Supporting Fig. S1.

AA MEASUREMENT BY COMPETITIVE ENZYME-LINKED IMMUNOSORBENT ASSAY

To test whether the preparation of albumin-enriched and albumin-depleted fractions by dye-ligand affinity chromatography affects the binding of lipids to this molecule, we monitored AA concentrations by enzyme-linked immunosorbent assay (see Supporting Materials).

ANALYSIS OF ALBUMIN LEVELS IN ALBUMIN-ENRICHED AND ALBUMIN-DEPLETED PLASMA FRACTIONS

The albumin levels in the different plasma fractions were assessed by western blot and polyethylene glycol-enhanced immunoturbidimetry (see Supporting Materials).

CELL ISOLATION AND INCUBATIONS

PBMCs and PMNs were isolated from 20 mL of peripheral blood collected in ethylene diamine tetraacetic acid tubes as described.⁽¹¹⁾ Blood samples were centrifuged at 200g for 10 minutes, plasma collected, and sedimented cells diluted with Dulbecco's phosphate-buffered saline (DPBS) without magnesium and calcium (DPBS^{-/-}) up to a volume of 20 mL. Diluted blood was coated over 13.3 mL of Ficoll-Hypaque PLUS (GE Healthcare Lifescience, Chicago, IL) and centrifuged at 500g for 25 minutes. The mononuclear cell layer was collected and washed with DPBS^{-/-} and centrifuged again at 400g for 5 minutes. The pellet containing the PMN was incubated with prewarmed ammonium-chloride-potassium lysis buffer for 10 minutes at room temperature to remove red blood cells and then centrifuged at 400g for 5 minutes. The red blood lysis procedure was repeated twice, and the resultant pellet was washed with DPBS^{-/-}. Isolated PBMCs and PMNs were enumerated and resuspended in Roswell Park Memorial Institute 1640 medium with penicillin (100 U/mL) and streptomycin (100 U/mL) and L-glutamine (4 mM) without fetal bovine serum. Cells were seeded at a density of 1.5×10^6 cells/mL and incubated with vehicle or human serum albumin (Albutein 20%; Grifols, Barcelona, Spain) at concentrations of 10 and 15 mg/mL for 30 minutes and then exposed to vehicle or lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 (1 μ g/mL; Merck, Darmstadt, Germany) for 30 minutes plus *N*-formyl-methionyl-leucyl-phenylalanine (fMLP, Merck, Darmstadt, Germany) (1 μ g/mL) for 15 minutes. At the end of the incubation period, samples were centrifuged at 1,500g for 5 minutes at 4°C, and supernatants and cell pellets were collected for further analysis.

LIPIDOMICS

Untargeted Lipidomics for the Analysis of Structural Lipids

Lipid extraction was accomplished by fractionating the plasma samples and fractions into pools of species with similar physicochemical properties, and analysis was performed using mass spectrometry coupled with ultrahigh performance liquid chromatography (see Supporting Materials).

Targeted Lipidomics for the Analysis of FA-Derived Lipid Mediators in Cell Supernatants

A total of 200 μ L of cell supernatants were spiked with an internal standard in acetonitrile, water, and 2,6-di-*tert*-butyl-4-methylphenol, and shaken vigorously. After centrifugation, supernatants were submitted to solid-phase extraction (SPE) in Bond Elute Certify II columns (Agilent Technologies, Santa Clara, CA). The eluate was evaporated and solid residues dissolved in 70 μ L acetonitrile/H₂O (50/50, vol/vol) and analyzed using an Agilent 1290 high-performance liquid chromatography (HPLC) system coupled with an Agilent 6495 Triplequad mass spectrometer with an electrospray ionization (ESI) source. The liquid chromatography coupled with tandem mass spectrometry (LC/MS-MS) conditions used to profile arachidonic acid-derived metabolites are described in Supporting Table S1. Quantification was performed using calibration curves with synthetic standards for each of the lipid mediators included in the analysis.

Targeted Lipidomics for the Analysis of FA-Derived Lipid Mediators and SPMs in Plasma

The extraction protocol and analysis of bioactive lipid mediators were performed as described by Le Faouder et al.,⁽¹⁵⁾ adapted by the Ambiotis SAS (Toulouse, France) standard operating procedure. Briefly, plasma (1 mL) was mixed with 0.4 mL of ice-cold methanol and held at -80°C for protein precipitation. Samples were then centrifuged and supernatants collected. After removal of the organic solvent under a stream of nitrogen, samples were suspended in methanol and rapidly acidified to pH 3.5 with HCl. Acidified samples were then loaded into C-18 SPE cartridges (Waters, Milford, MA), rapidly neutralized, and eluted with methyl formate. Eluate solvents were evaporated under a stream of nitrogen and residues suspended in mobile phase for LC/MS-MS analyses using an Exion LCAD U-HPLC system coupled with a Sciex QTRAP 6500+ MS-MS system (AB Sciex, Framingham, MA), equipped with an ESI source in negative ion mode. Quantification was performed using calibration curves with synthetic standards for each of the SPMs included in the analysis.

RNA ISOLATION, REVERSE TRANSCRIPTION, AND REAL-TIME POLYMERASE CHAIN REACTION

Details for the isolation of total RNA from PBMCs and PMNs and analysis by real-time polymerase chain reaction are provided in the Supporting Materials.

STATISTICAL ANALYSIS

Continuous variables were analyzed using unpaired Student *t* tests with Welch's correction, if necessary, or one-way analysis of variance corrected with Tukey's correction if necessary, when comparing more than two groups. Data were expressed as mean \pm SEM. Data analyses were performed using GraphPad Prism software version 9 with α set at 0.05 and two-tailed test. Supervised heatmaps representing mean values for each group of the study were represented. Log₂ fold changes comparing groups were calculated and represented in Cleveland, and volcano plots together with $-\log_{10}$ transformed *P* values. An unpaired Student *t* test was used for single comparisons and Spearman correlation to assess the correlation of variables. Missing values of some lipid species were set to 0 to approximate no detection and added 0.01 before log₂ transform to assess normal distribution. Statistical analysis was performed using R version 4.0.2 (R-Foundation for statistical computing, www.Rproject.org).

Results

CHANGES IN PLASMA LIPID LEVELS IN PATIENTS WITH AD CIRRHOSIS

The clinical characteristics and standard laboratory data of the patients included in the plasma fractionation by affinity chromatography are given in Supporting Table S2. The circulating lipid profile of patients with AD cirrhosis was determined by LC/MS-MS and compared with that of HS. Consistent with a recent study,⁽⁴⁾ the variation in the plasma lipidome associated with AD cirrhosis was characterized by generalized lipid suppression,

especially in the glycerophospholipid family (Fig. 1A). FAs were the only lipid class that was not suppressed but rather significantly increased (Fig. 1A), probably reflecting the intense lipolysis present in these patients.⁽¹⁶⁾ The increased abundance of FAs in the circulation of patients with AD can also be appreciated by plotting the percentual contribution of each lipid class to the total plasma lipid pool (Fig. 1B). The analysis of the lipid abundance expressed as arbitrary units (corresponding to the chromatographic peak areas) revealed that, in patients with AD, FAs escalated from the last to the third position of the lipid class rank order (Supporting Fig. S2A). We next compared the plasma levels of each individual lipid species in a pairwise manner between patients with AD and HS and ranked their fold changes in a Cleveland plot. As shown in Fig. 1C, most lipids were reduced in patients with AD, being the 15 lipids with the highest fold-change reductions (phosphatidylethanolamines [PEs] and phosphatidylcholines containing the omega-6-PUFA AA (20:4) together with cholesteryl esters and PEs containing the omega-3 PUFAs EPA [20:5] and DHA [22:6]). Members of the sphingolipid family (i.e., sphingomyelin [d18:2/23:0] and ceramide [d18:1/23:0]) were also among the lipids more severely suppressed in plasma from patients with AD cirrhosis (Fig. 1C). In contrast, a small group of lipids were increased in patients with AD, among them FAs, which accounted for 8 of the 15 lipids with the highest fold-change increases (Fig. 1C). (See Supporting Table S3 for details of the FAs detected in our study.) This increase was primarily attributed to monounsaturated FAs (MUFAs), especially palmitoleic, oleic, and gadoleic acids together with the omega-9 PUFA mead acid (Fig. 1D and Supporting Fig. S2B). Of interest, the percent contribution of PUFAs to the plasma FA pool decreased in patients with AD (Supporting Fig. S2C).

SETUP OF A PLASMA FRACTIONATION METHOD TO IDENTIFY THE CONTENT OF LIPIDS IN THE ALBUMIN MOLECULE

Albumin has high avidity for lipids.⁽⁸⁾ Patients with AD cirrhosis present qualitative and quantitative

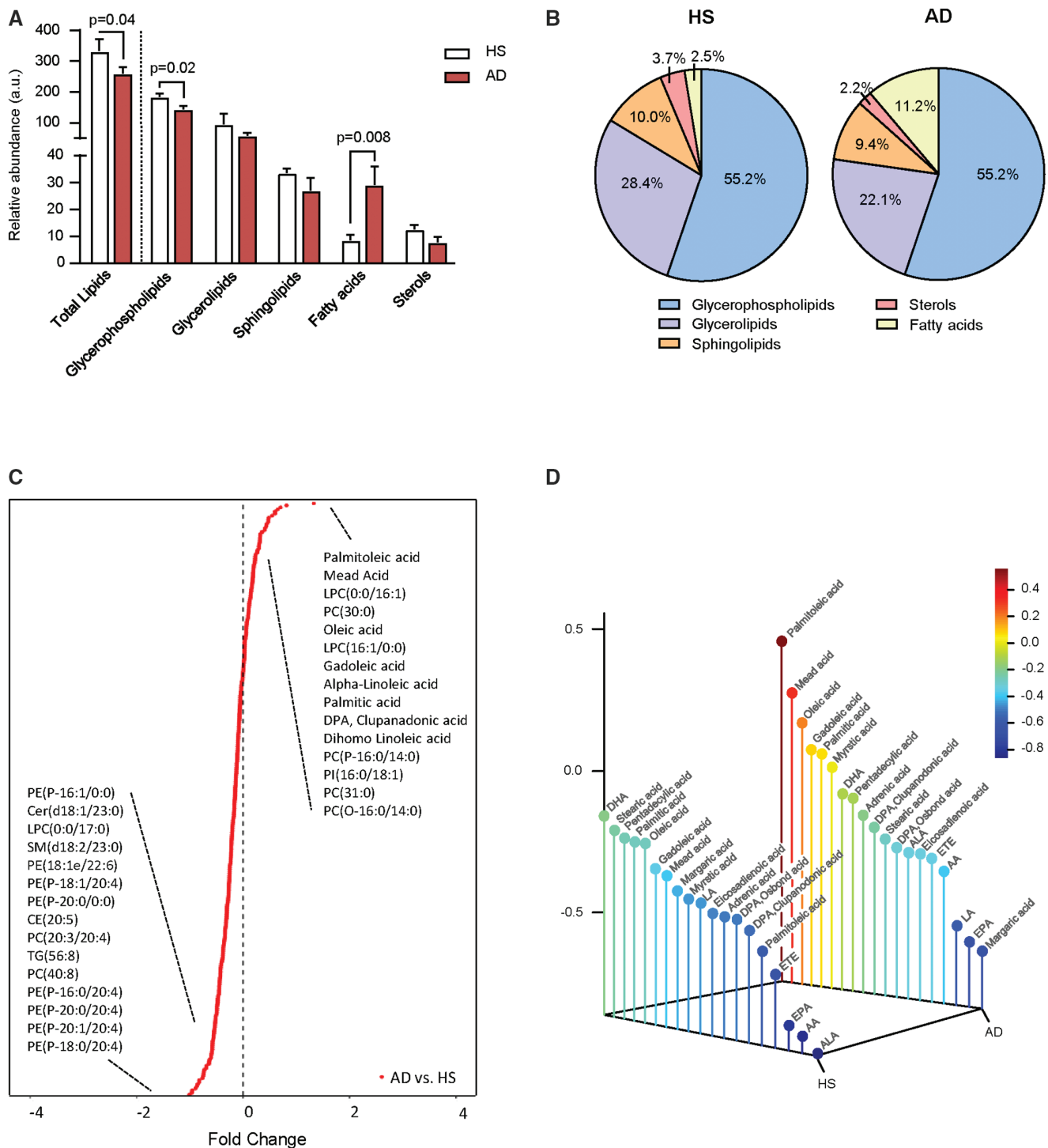


FIG. 1. Lipidomic profile in patients with AD cirrhosis compared to HS. (A) Levels of total lipids and the five lipid classes measured by LC/MS-MS in total plasma from patients with AD cirrhosis ($n = 10$) and HS ($n = 5$). Results are expressed as mean \pm SEM. (B) Pie charts representing the percentage of each lipid class with respect to total plasma lipid content in patients with AD cirrhosis and HS. (C) Cleveland plot of the whole set of 342 lipids measured in total plasma ranked according to the fold changes between patients with AD versus HS. Upper-right inset: zooming on the 15 lipids more abundant in AD compared to HS. Lower left inset: zooming on the 15 lipids with the highest fold-change reductions in AD compared to HS. (D) Three-dimensional plot of \log_{10} -converted values of fatty acids measured by LC/MS-MS in total plasma from HS and patients with AD ranked by their abundance. Abbreviations: ALA, Alpha-Linolenic acid; CE, Cholesteryl Ester; Cer, Ceramide; DPA, Docosapentaenoic acid; ETE, Eicosatrienoic acid; LA, Linoleic acid; LPC, Lysophosphatidylcholine; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PI, Phosphatidylinositol; SM, Sphingomyelin; TG, Triacylglycerol.

changes in the albumin molecule. For example, these patients present extensive posttranslational albumin modifications^(12,13) together with hypoalbuminemia (Supporting Fig. S3A), features that likely contribute to altering their plasma lipidome. To investigate this, we fractionated the patient's plasma by affinity chromatography into albumin-enriched and albumin-depleted fractions and assessed their lipid composition. As described previously, the first albumin-depleted fraction contained some albumin leftover; therefore, the depletion process was repeated (see Materials and Methods and Supporting Fig. S1 for details). Following this procedure, proper separation of the two fractions was achieved as confirmed by western blot analysis (Supporting Fig. S3B). This fractionation process was specific for albumin, as other proteins abundant in serum such as immunoglobulin Gs were detected in the albumin-depleted fraction but not in the enriched one (Supporting Fig. S3C). The quantification of the albumin concentration by polyethylene glycol-enhanced immunoturbidimetry also confirmed proper separation in both HS and patients with AD (Supporting Fig. S3D). Indeed, albumin enrichment was 99.96% and 98.27% in HS and patients with AD, respectively (Supporting Fig. S3E). Furthermore, we selected AA as a representative lipid species to prove the ability of our system to detect lipids in the two albumin fractions (Supporting Fig. S3F).

LOWER ABUNDANCE OF FAS IN THE ALBUMIN-ENRICHED FRACTION FROM PATIENTS WITH AD CIRRHOSIS

We next performed a supervised cluster analysis of the lipids detected in the albumin-enriched and albumin-depleted fractions. As shown in Fig. 2A,B and Supporting Fig. S4, lipids were primarily found in the albumin-enriched fraction in both HS and patients with AD. The only lipid that was not primarily found in this fraction was the steroid hormone cortisol (Supporting Fig. S4). Mirroring that described in total plasma (Fig. 1A), the lipid profile of the albumin-enriched and albumin-depleted fractions from patients with AD was characterized by a generalized suppression (Fig. 2A,B). The distribution of FAs between albumin-enriched and albumin-depleted fractions varied between HS and patients with AD. In

HS, saturated FAs were similarly distributed between the two fractions, whereas MUFAs and PUFAs were found primarily in the albumin-enriched fraction (Fig. 2C). In patients with AD, higher content of MUFAs and PUFAs was detected in the albumin-depleted fraction, although these FAs appeared to travel mostly associated with albumin in the bloodstream of these patients (Fig. 2D). Moreover, a shift in the distribution of omega-6 and omega-3 PUFAs between the two fractions was observed in patients with AD. Specifically, whereas in HS the omega-3 DHA was the most significant PUFA in the albumin-enriched fraction, this leading position was taken by the omega-6-PUFAs adrenic acid and AA in patients with AD (Fig. 2E,F).

ALTERED PLASMA PROFILE OF PUFA-DERIVED LIPID MEDIATORS IN AD CIRRHOSIS

Bioactive lipid mediators are recognized as important endogenous cell signaling molecules. These lipid mediators originate primarily from PUFAs released from plasma membrane phospholipids into the cytoplasm, where they are converted by COX and LOX pathways into PGs, LTs and LXs in the case of AA, and into resolvins, protectins, and maresins in the case of DHA and EPA (Fig. 3A). PGs and LTs carry potent inflammatory properties, whereas LXs, resolvins, protectins, and maresins have anti-inflammatory and pro-resolution activities and are collectively known as SPMs.^(5,6) In our study, the overall plasma concentrations of lipid mediators were significantly reduced in patients with AD cirrhosis—a reduction mostly attributed to the suppression of the monohydroxy FAs 12-hydroxyicosatetraenoic acid (12-HETE) and 14-hydroxydocosahexaenoic acid (14-HDHA) as well as thromboxane B₂ (TXB₂) (Fig. 3B,C). This observation could be related to the characteristic thrombocytopenia prevalent in cirrhosis, as all of these lipid mediators are derived from platelets.⁽¹⁷⁾ PGs showed a decrease trend in leukocytes from patients with AD, which was not associated with changes in PG-generating enzymes such as *PTGS1* but rather with increased expression of the PG-degrading enzyme 15-hydroxyPG dehydrogenase (Fig. 3B,D). In contrast, plasma levels of pro-inflammatory LTs showed an increase trend in patients with AD, which was not associated with changes in *ALOX5* expression

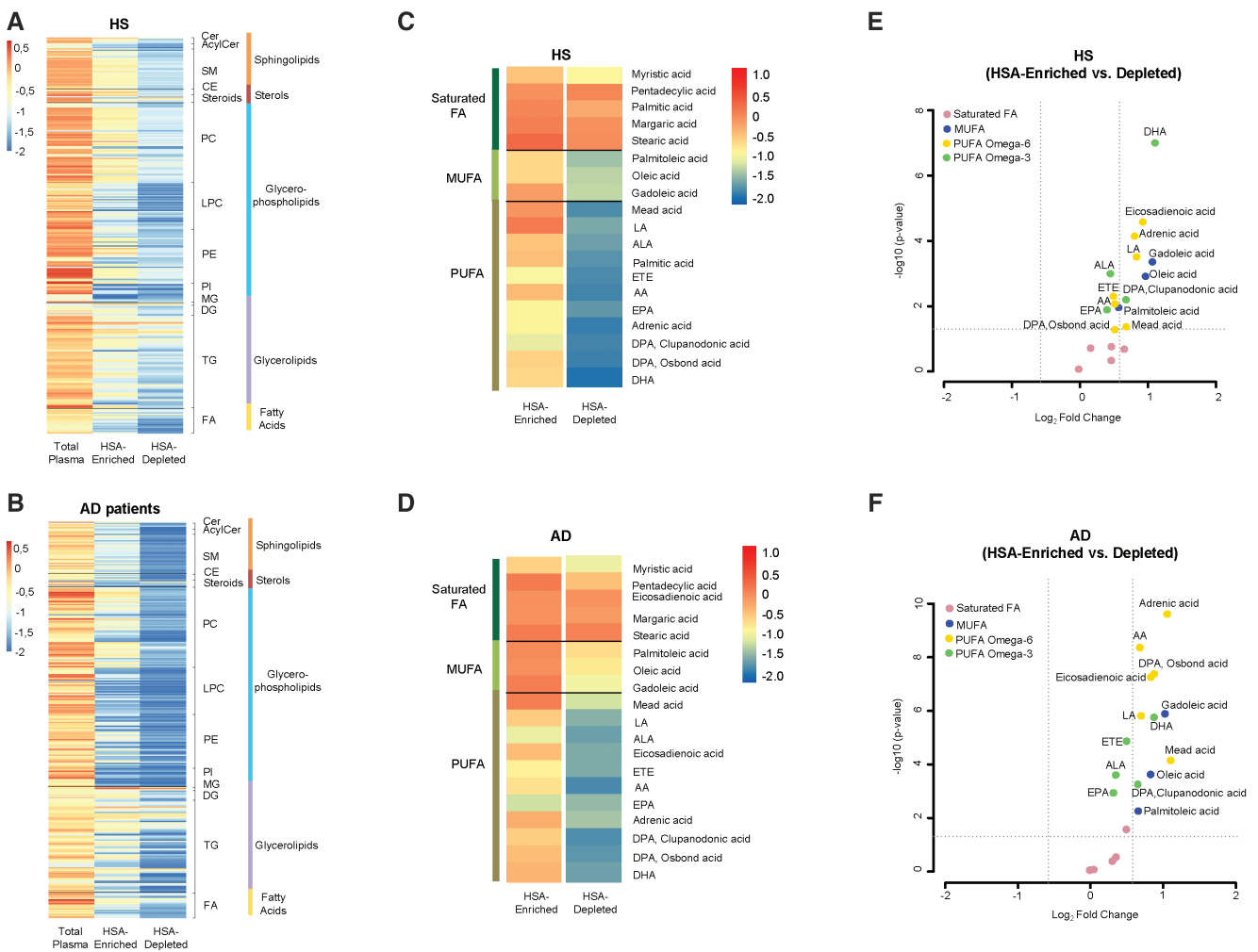


FIG. 2. Lipidomic profile after plasma albumin fractionation in patients with AD and HS. (A) Supervised heatmap of lipids in total plasma and in albumin-enriched and albumin-depleted fractions in HS ($n = 5$). Mean values of \log_{10} -converted amount of lipids are represented. (B) Same as (A) but for lipids in patients with AD ($n = 10$). (C) Zooming in the supervised heatmap of FAs in plasma albumin fractions represented by \log_{10} -transformed mean values for HS. (D) Same as (C) but for FAs in patients with AD. (E) Volcano plot of FAs in albumin-enriched versus albumin-depleted fractions from HS. (F) Same as (E) but for FAs in patients with AD. \log_2 -fold changes comparing groups were calculated together with $-\log_{10}$ -transformed P values, considered as significant when a \log_2 -fold change was >0.5 and a $-\log_{10} P$ value was > 1.5 . Abbreviations: Acylcer, Monohexosylceramide; ALA, Alpha-Linolenic acid; CE, Cholesteryl Ester; Cer, Ceramide; DG, Diacylglycerol; ETE, Eicosatrienoic acid; DPA, Docosapentaenoic acid; DPA, Docosapentaenoic acid; LA, Linoleic acid; LPC, Lysophosphatidylcholine; MG, Monoacylglycerol; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PI, Phosphatidylinositol; SM, Sphingomyelin; TG, Triacylglycerol.

(Fig. 3B,E). Finally, SPM levels were similar in HS and patients with AD (Fig. 3B), but the ratio between LTs and SPMs was higher in patients with AD cirrhosis (Supporting Fig. S5A), likely reflecting a preponderance of an inflammatory environment in this condition. To understand to what extent the circulating lipid mediator network was altered in AD cirrhosis, we created a supervised clustered correlation matrix of lipid mediators. As shown in Supporting

Fig. S5B, two different large clusters, including SPMs, were identified in HS. One cluster was associated with PGs and the other with LTs, reinforcing the view that lipid mediators that promote the onset of inflammation (i.e., PGs and LTs) also dictate its resolution (i.e., SPMs) as previously described by Serhan and Savill.⁽¹⁸⁾ Of note, these SPM clusters were not clearly seen in patients with AD, suggesting a profound alteration of the lipid mediator networks in these patients.

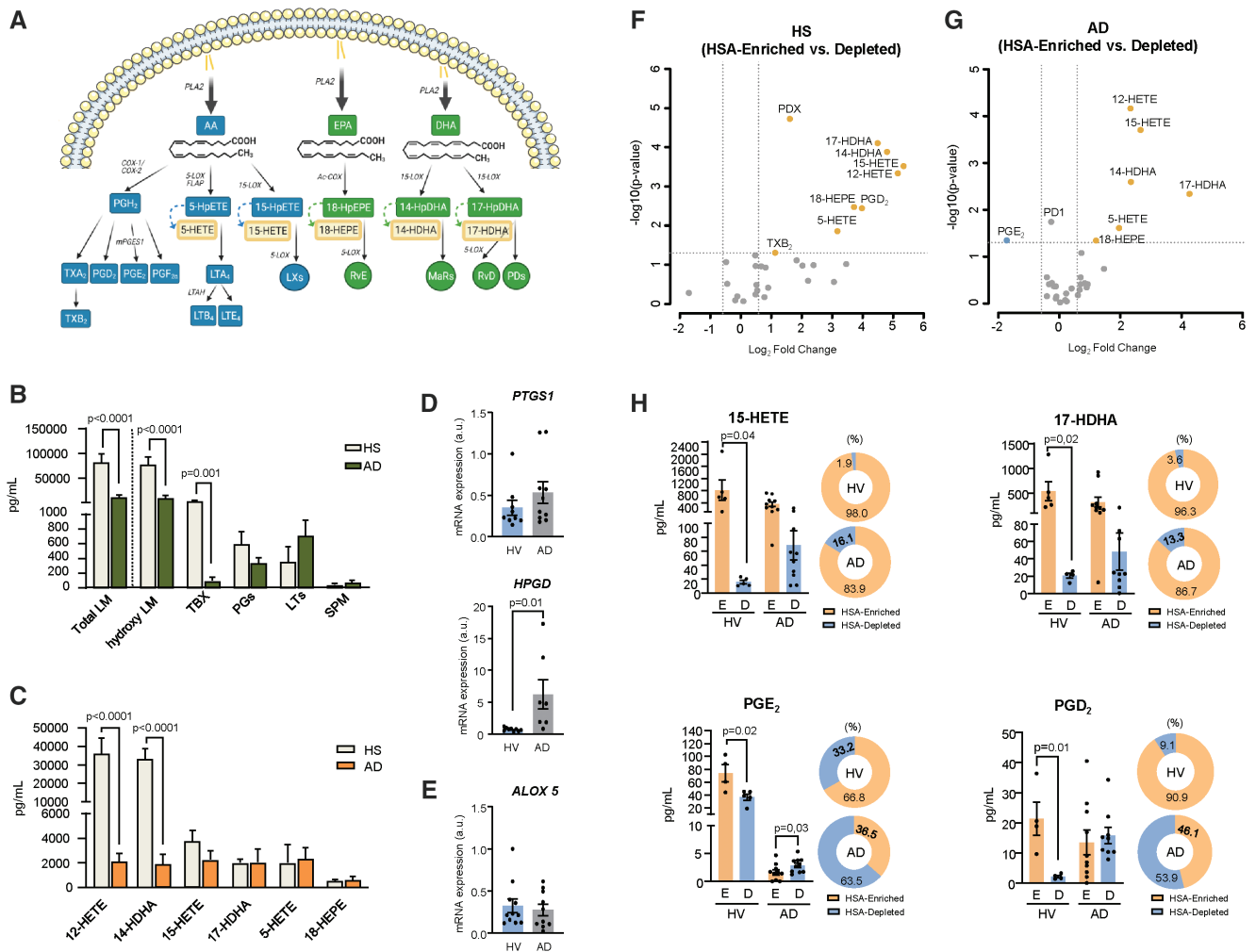


FIG. 3. Distribution of bioactive lipid mediators between albumin-enriched and albumin-depleted fractions. (A) Schematic diagram summarizing the lipid mediator biosynthetic pathways from AA (omega-6-PUFA) (blue) and EPA and DHA (omega-3-PUFA) (green). (B) Concentrations of total lipid mediators and lipid mediator families were obtained by LC/MS-MS in total plasma from HS ($n = 5$) and patients with AD cirrhosis ($n = 10$). (C) Detail of the monohydroxy FA family. (D) Messenger RNA (mRNA) expression of *PTGS1* (COX-1) and *15-hydroxyPG dehydrogenase* in PBMCs from HS ($n = 9$) and patients with AD ($n = 9$). (E) Same as in (D) but for *ALOX5* (5-LOX) mRNA expression. (F) Volcano plot of lipid mediators in albumin-enriched versus albumin-depleted fraction from HS ($n = 5$). Log_2 fold changes comparing groups were calculated together with $-\log_{10}$ -transformed P values, considered as significant with a log_2 fold change >0.5 and a $-\log_{10} P$ value > 1.5 . (G) The same as in (F) but for patients with AD ($n = 10$). (H) Absolute concentrations and percent distribution of 15-HETE, 17-HDHA, PGE_2 , and PGD_2 in albumin-enriched (denoted as “E”) and albumin-depleted (denoted as “D”) fractions from HS ($n = 5$) and patients with AD ($n = 10$). Results are expressed as mean \pm SEM. Abbreviation: *HPGD*, 15-hydroxyPG dehydrogenase. Abbreviations: *ALOX5*, arachidonate 5-lipoxygenase (5-LOX); *PTGS1*, prostaglandin-endoperoxide synthase 1 (COX-1); LM, Lipid mediators; Mar, Maresins; PD, Protectins; RvD, D-series Resolvins; RvE, E-series Resolvins; 5-HETE, 5-Hydroxyicosatetraenoic acid; 12-HETE, 12-Hydroxyicosatetraenoic acid.

DIFFERENTIAL PARTITIONING OF PUFA-DERIVED LIPID MEDIATORS IN AD CIRRHOSIS

We next compared the distribution of lipid mediators into the albumin-enriched and albumin-depleted

plasma fractions, and the results were plotted in volcano plots comparing fold changes between the two fractions in HS on one side and in patients with AD on the other. In HS, the albumin fraction was enriched with monohydroxy FAs, which are intermediate precursors and/or pathway markers of the biosynthesis of SPMs such

as 17-hydroxydocosahexaenoic acid (17-HDHA), 14-HDHA, 15-hydroxyeicosatetraenoic acid (15-HETE), and 18-hydroxyeicosapentaenoic acid (18-HEPE) (Fig. 3F). The lipid mediator that reached the highest statistical significance was PDX, a 17-HDHA-derived SPM (Fig. 3F). Other AA-derived eicosanoids were present at a higher extent in the albumin-enriched fraction, including 12-HETE, PGD₂, 5-HETE, and TXB₂ (Fig. 3F). On the other hand, albumin from patients with AD had a lower content of precursors/markers of SPMs (Fig. 3G). Notably, in these patients, a significantly higher content of PGE₂ was found in the albumin-depleted fraction, suggesting that this proinflammatory and immunosuppressive PG primarily travels disassociated to albumin in the circulation of patients with AD (Fig. 3G). Figure 3H shows the absolute values and the percent distribution between the two plasma fractions of two SPM precursors (15-HETE and 17-HDHA) and two PGs (PGE₂ and PGD₂), reinforcing the view of profound changes in the composition of lipid mediators in the albumin molecule from patients with AD cirrhosis. In fact, the clustering of PGs with SPMs was severely disturbed in the albumin-enriched fraction from patients with AD cirrhosis (Supporting Fig. S6). The distribution of other monohydroxy FAs, prostanoids, and SPMs between the albumin-enriched and albumin-depleted fractions was also altered in patients with AD cirrhosis (Supporting Fig. S7).

ALBUMIN MODULATES THE BIOSYNTHESIS OF PUFA-DERIVED LIPID MEDIATORS BY HUMAN LEUKOCYTES

A recent study demonstrated that albumin inhibits the release of cytokines by peripheral leukocytes from patients with AD cirrhosis.⁽¹¹⁾ Therefore, we wondered whether albumin could also modulate the release of lipid mediators by peripheral leukocytes. To investigate this, we incubated PBMCs and PMNs from HS with exogenous albumin. The lipid composition and the content of lipid mediators of the commercial albumin used in our experiments are described in Supporting Table S4 (see separate Excel file) and Supporting Tables S5 and S6. The lipid mediator 17-HDHA, which levels in the commercial albumin, exceeded those present in the leukocyte culture media and was excluded from the analysis. As shown in Supporting Fig. S8A, incubation of resting PBMCs with albumin translated into reduced

PGE₂, PGD₂, PGF_{2 α} , and TXB₂ levels in the supernatant. Unexpectedly, albumin triggered the release of 15-HETE and, to a lower extent, 18-HEPE, which are precursors of anti-inflammatory SPMs (Supporting Fig. S8A). Consistent with this, albumin predominantly up-regulated 15-LOX-2 expression in PBMCs (Supporting Fig. S8B). The effects of albumin on the release of lipid mediators were confirmed in LPS/fMLP-primed PBMCs—a condition that mimics the inflammatory conditions encountered by peripheral leukocytes in patients with AD cirrhosis. In comparison to HS, stimulation of PBMCs from patients with AD cirrhosis with LPS/fMLP induced a higher prostanoid production with no significant changes in the levels of SPM-forming monohydroxy-FAs (Supporting Fig. S8C). When albumin was added to these incubations, a prominent inhibition of the LPS/fMLP-induced release of PGE₂, PGD₂, PGF_{2 α} , and TXB₂ was observed in PBMCs from both HS and patients with AD (Fig. 4A). Furthermore, albumin triggered the formation of 15-HETE and 18-HEPE in a concentration-dependent manner in LPS/fMLP-elicited PBMCs, independently of whether these cells were isolated from either HS or patients with AD (Fig. 4B). The effects of albumin on the release of lipid mediators by PMN followed a similar trend to that observed in PBMCs from HS, but the magnitude of changes was lower (Supporting Fig. S9).

MODULATION OF THE PLASMA LEVELS OF PUFA-DERIVED LIPID MEDIATORS IN PATIENTS WITH AD CIRRHOSIS RECEIVING ALBUMIN THERAPY

We finally determined the levels of lipid mediators in plasma samples from patients with AD cirrhosis receiving albumin therapy included in the INFECIR-2 study. The baseline clinical characteristics and standard laboratory data from these patients are described in Fernández et al.⁽¹⁴⁾ The results of these measurements are shown in Fig. 5. Under baseline conditions, plasma levels of PGE₂, PGD₂, and PGF_{2 α} were not statistically different between patients of the SMT group and patients who received SMT plus albumin (Fig. 5A). However, patients who received albumin showed lower levels of PGE₂ in the circulation as compared with those receiving SMT alone (Fig. 5A). If expressed as percent change versus basal, PGE₂ levels increased after SMT, something that was not observed in patients receiving SMT

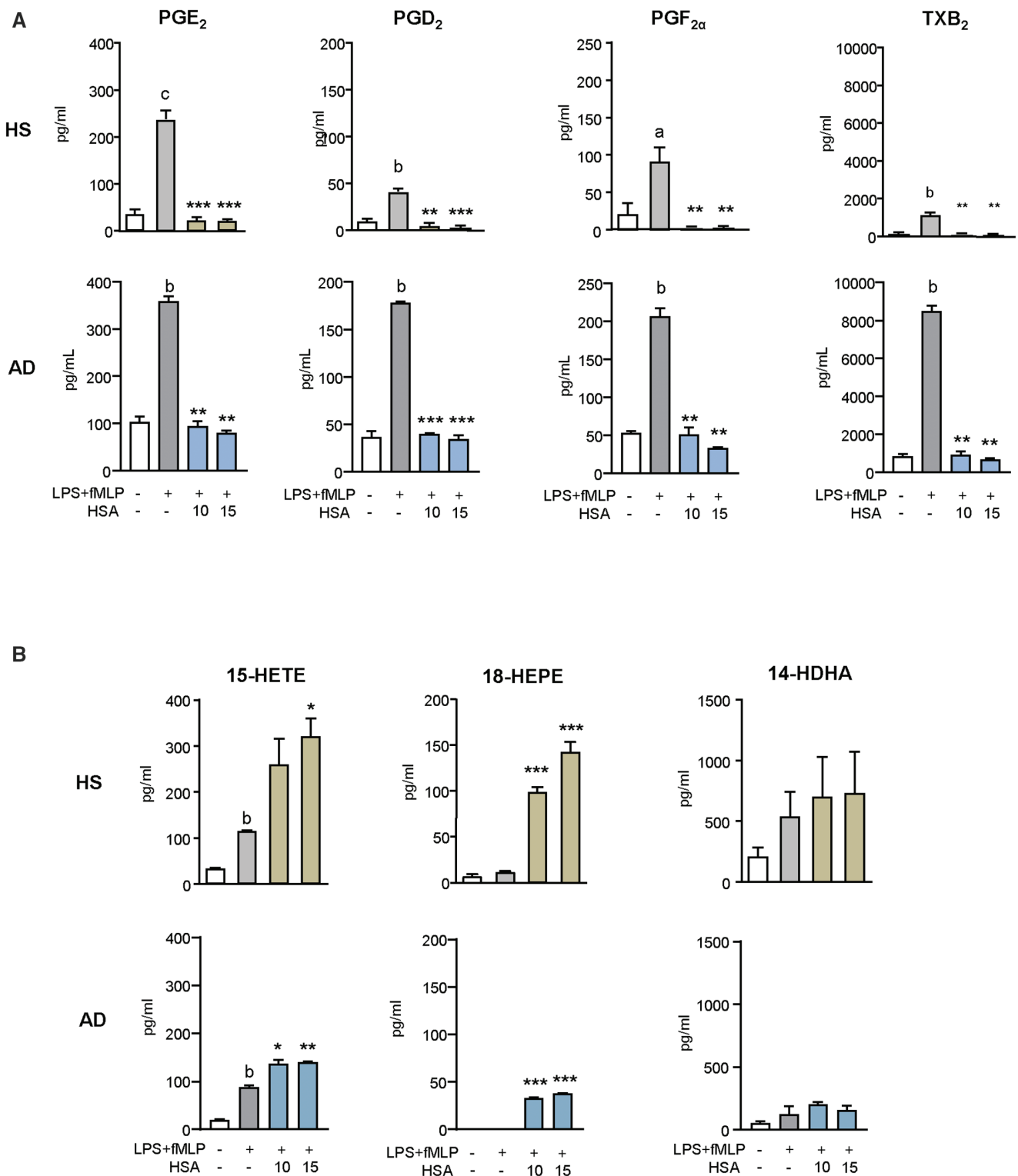


FIG. 4. Effect of exogenous albumin on the release of lipid mediators by PBMCs. (A) PGE₂, PGD₂, PGF_{2α}, and thromboxane TXB₂ levels measured by LC/MS-MS in supernatants of PBMCs from HS (upper panel) and patients with AD (lower panel) incubated with albumin (HSA, 10 and 15 mg/mL) for 30 minutes and then stimulated with LPS (1 μg/mL for 30 minutes) plus fMLP (1 μg/mL for 15 minutes) (n = 3 in duplicate). (B) 15-HETE, 18-HEPE, and 14-HDHA levels measured by LC/MS-MS in the same experimental conditions as in (A). Results are expressed as mean ± SEM. ^aP < 0.05, ^bP < 0.005, ^cP < 0.001 as LPS + fMLP versus vehicle, and *P < 0.05, **P < 0.005, and ***P < 0.001 as LPS + fMLP plus HSA versus LPS + fMLP alone.

plus albumin (Supporting Fig. S10A). Patients who received SMT plus albumin also had significantly lower levels of PGD_2 (Fig. 5A). In addition, patients receiving SMT plus albumin presented a significant increase in the plasma concentration of the pro-resolving monohydroxy FA 15-HETE (Fig. 5B). The percent change data versus basal is also shown in Supporting Fig. S10B. Nevertheless, the response of patients with AD to albumin administration was very heterogeneous, indicating that these findings need to be confirmed in other studies, including a larger number of patients with AD cirrhosis as well as with other commercial albumins that might have different lipid compositions.

Discussion

The results of the current study support the concept that albumin has the ability to modulate the

lipid composition of human plasma—an effect that is relevant to patients with AD cirrhosis in whom albumin infusions are used as therapy. The most important findings of our study are the following: (1) Circulating lipid levels in patients with AD cirrhosis are remarkably suppressed in comparison to HS. This suppression affects all major lipid families except FA, which are increased. (2) In patients with AD, albumin shows a characteristic lipid composition, especially of PUFAs and PUFA-derived lipid mediators. In particular, in comparison to HS, the albumin-enriched fraction of patients with AD has a lower content of pro-resolving monohydroxy FAs (i.e., 15-HETE). (3) In contrast, the albumin-depleted fraction of patients with AD has an increased content of PGE_2 , suggesting that this proinflammatory and immunosuppressive PG primarily travels disassociated to albumin in the circulation of patients with AD. (4) Albumin modulates the biosynthesis of lipid mediators by

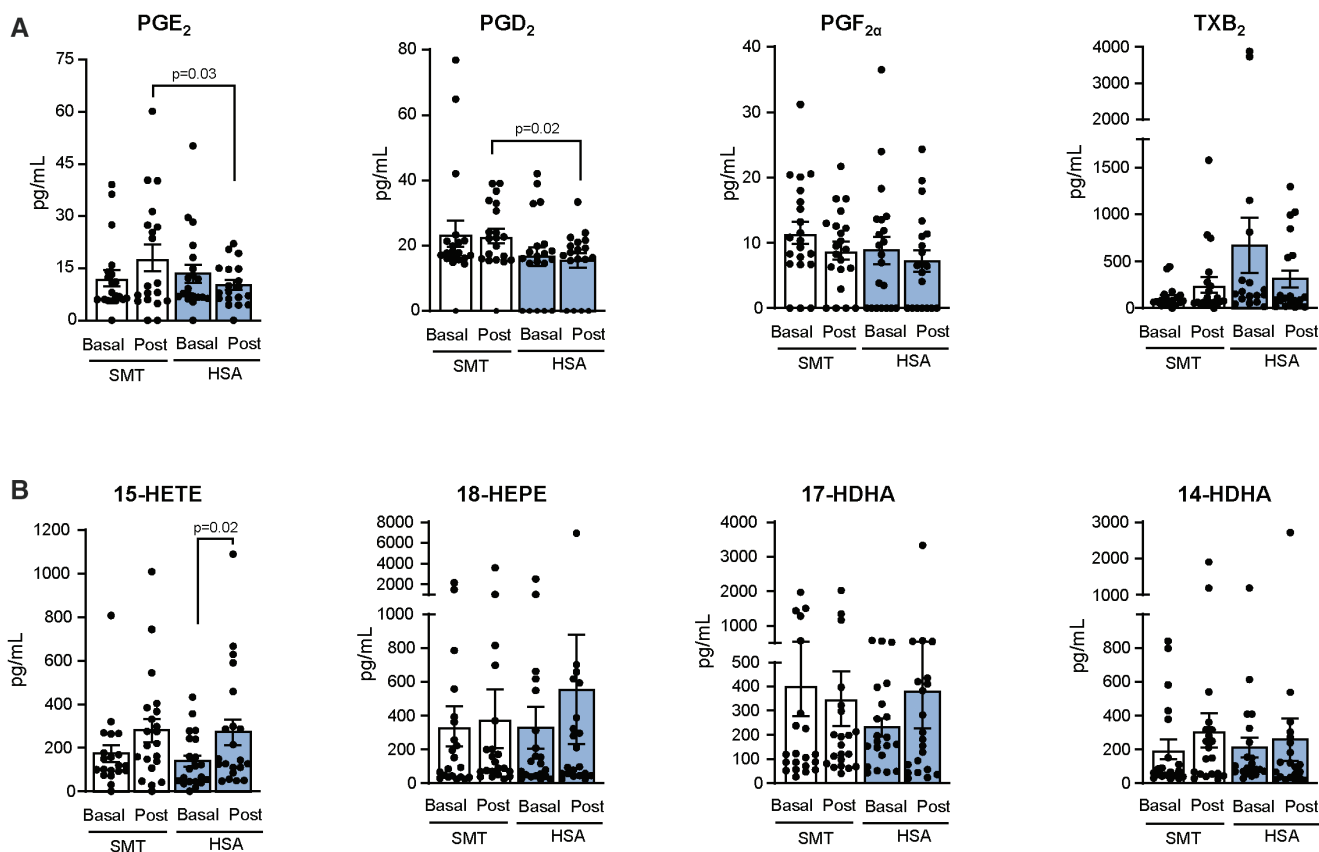


FIG. 5. Plasma levels of lipid mediators in patients with AD cirrhosis receiving albumin therapy. (A) PGE_2 , PGD_2 , and $\text{PGF}_{\alpha\alpha}$ concentrations were measured by LC/MS-MS in plasma from patients with AD included in the INFECIR-2 study under basal conditions and after 7 days of receiving SMT ($n = 21$) alone or in combination with albumin infusions (HSA, $n = 21$). (B) 15-HETE, 18-HEPE, and 17-HDHA concentrations in the same conditions as in (A). Results are expressed as mean \pm SEM.

circulating leukocytes by reducing pro-inflammatory PG production, while inducing the release of pro-resolving monohydroxy FAs, such as 15-HETE. (5) Lower plasma PG levels and higher concentrations of 15-HETE were observed in patients with AD cirrhosis receiving albumin therapy.

Many of the physiological functions of albumin are based on its ability to reversibly bind soluble molecules, contributing to their transportation in the plasma to different tissues and organs.^(9,10) In particular, the albumin molecule contains up to seven binding sites for long chain FAs, some of which are located in the critical Sudlow's sites I and II.⁽¹⁹⁾ Albumin can also bind esterified FAs in phospholipids, especially lysoPC.⁽²⁰⁾ This is important because the major part of the FA pool in the human plasma is esterified with a minor proportion actually free in solution.⁽²¹⁾ Albumin can also bind the sphingolipid sphingosine-1-phosphate⁽²²⁾ (Supporting Table S4). Although the binding of acylglycerols by albumin has been previously reported,⁽²⁰⁾ in our analysis of commercial human serum albumin we did not detect any member of this lipid family (Supporting Table S4). Any alteration of the albumin chemical structure has a direct impact on its ligand-binding function.⁽²³⁾ Although there is no direct evidence that links the structural alterations of albumin with its ability to bind FAs, oxidation of the Cys-34 residue in the albumin molecule has been shown to reduce the binding of molecules in the Sudlow's sites, where some of the FA binding sites are located.⁽²³⁾ This fact, together with the presence of lower concentrations of albumin, which in addition is highly oxidized in patients with AD cirrhosis,^(10,13) might explain the existence of higher levels of free FAs in the bloodstream of these patients. This is particularly relevant because albumin binds omega-6 and omega-3 PUFAs, especially AA and DHA.⁽²⁴⁾ Of note, commercial albumin is more enriched in DHA than in AA (Supporting Table S5). Moreover, albumin binds biologically active lipid mediators derived from PUFAs, including PGs,⁽²⁵⁾ LTs,^(26,27) and monohydroxy FAs.^(28,29) All of these data can help to understand the observed changes in the lipid composition of the albumin molecule in patients with AD cirrhosis.

Albumin has been reported to sequester PGs and accelerate the decomposition kinetics of these lipid mediators—an effect that is dose-dependent.⁽²⁸⁾ Our finding that albumin inhibits PGE₂ production by leukocytes from both HS and patients with AD *in vitro* is consistent with previous studies demonstrating that

albumin reduces PGE₂ levels in patients with AD cirrhosis.^(30,31) PGE₂ is a lipid mediator that drives immunosuppression, and therefore is relevant to AD cirrhosis because it can potentially increase the risk of infection in these patients. In our study, we could not confirm that PGE₂ levels were increased in patients with AD cirrhosis, but we observed that PGE₂ levels were reduced in the albumin-enriched fraction of patients with AD. On the other hand, albumin has been reported to favor lipoxygenation of PUFAs and to increase the formation of LOX-derived products.^(29,32) Consistent with this, we detected a significant increase in the 15-LOX-derived product 15-HETE in leukocytes from HS and patients with AD incubated with albumin. The enhanced formation of 15-HETE cannot be attributed to the presence of this monohydroxy FA in the commercial albumin used in our experiments because its levels in cell supernatants further exceeded those attached to the commercial albumin preparation (Supporting Table S6). Importantly, increased 15-HETE levels were observed in plasma from patients with AD cirrhosis after receiving albumin therapy. Together, our findings provide evidence that albumin is not merely transporting lipid mediators in the bloodstream but rather a molecule that modulates their biosynthesis and release by circulating immune cells.

Our study has some limitations. For instance, the results of our study do not allow us to bring any conclusion on the source of the circulating FAs in patients with AD cirrhosis. However, similar to other liver disease conditions, such as metabolic-associated fatty liver disease, circulating FAs in patients with AD patients are likely to be derived from white adipose tissue lipolysis. In addition, at present, there is scarce information about whether altered lipid levels in the systemic circulation of patients with AD is related to impaired lipoprotein production by the diseased liver of these patients. Finally, we recognize the preliminary nature of our results in patients with AD cirrhosis receiving albumin therapy. Because the response of patients with AD to albumin administration was very heterogeneous in terms of changes in lipid mediators, it is necessary to confirm these findings in other studies, including a larger number of patients and with proper stratification. Furthermore, these findings need to be confirmed with other commercial albumins that may have a different lipid composition.

In summary, this investigation provides a comprehensive analysis of the composition of lipids and lipid mediators in the albumin molecule from patients with AD cirrhosis, identifying a characteristic pattern of

PUFA and PUFA-derived lipid mediators in these patients. Furthermore, we provide evidence that exogenous albumin modulates the biosynthesis of lipid mediators in circulating leukocytes by redirecting the production of pro-inflammatory and immunosuppressive PGs to the activation of endogenous pro-resolving lipid mediator pathways.

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Author names in bold designate shared co-first authorship.

Supporting Information

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