Linoleic Acid-Derived Oxylipins Differentiate Early Stage Alcoholic Hepatitis From Mild Alcohol-Associated Liver Injury

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Alcohol-associated liver disease (ALD) is a spectrum of liver disorders ranging from steatosis to steatohepatitis, fibrosis, and cirrhosis. Alcohol-associated hepatitis (AH) is an acute and often severe form of ALD with substantial morbidity and mortality. The mechanisms and mediators of ALD progression and severity are not well understood, and effective therapeutic options are limited. Various bioactive lipid mediators have recently emerged as important factors in ALD pathogenesis. The current study aimed to examine alterations in linoleic acid (LA)-derived lipid metabolites in the plasma of individuals who are heavy drinkers and to evaluate associations between these molecules and markers of liver injury and systemic inflammation. Analysis of plasma LA-derived metabolites was performed on 66 individuals who were heavy drinkers and 29 socially drinking but otherwise healthy volunteers. Based on plasma alanine aminotransferase (ALT) levels, 15 patients had no liver injury (ALT ≤ 40 U/L), 33 patients had mild liver injury (ALT > 40 U/L), and 18 were diagnosed with moderate AH (mAH) (Model for End-Stage Liver Disease score <20). Lipoxygenase-derived LA metabolites (13-hydroxy-octadecadienoic acid [13-HODE] and 13-oxo-octadecadienoic acid) were markedly elevated only in patients with mAH. The cytochrome P450-derived LA epoxides 9,10-epoxy-octadecenoic acid (9,10-EpOME) and 12,13-EpOME were decreased in all patients regardless of the presence or absence of liver injury. LA-derived diols 9,10-dihydroxy-octadecenoic acid (9,10-DiHOME) and 12,13-DiHOME as well as the corresponding diol/epoxide ratio were elevated in the mAH group, specifically compared to patients with mild liver injury. We found that 13-HODE and 12,13-EpOME (elevated and decreased, respectively) in combination with elevated interleukin-1β as independent predictors can effectively predict altered liver function as defined by elevated bilirubin levels. Conclusion: Specific changes in LA metabolites in individuals who are heavy drinkers can distinguish individuals with mAH from those with mild ALD. (Hepatology Communications 2021;5:947-960).

Long-term heavy alcohol consumption results in the development of a spectrum of liver disorders ranging from steatosis to steatohepatitis with varying degrees of fibrosis and cirrhosis, collectively known as alcohol-associated liver disease (ALD). ALD affects millions of patients worldwide...
each year,\(^1\) and up to 35% of heavy alcohol drinkers develop alcohol-associated hepatitis (AH).\(^2\) Acute AH is a distinct clinical subset of a severe form of ALD and is advanced and rapidly progressive liver injury accompanied by inflammation (often complicated by infection and multiorgan failure) resulting in substantial morbidity and mortality.\(^3\)-\(^5\) Moreover, there is no U.S. Food and Drug Administration-approved therapy for any stage of ALD, and effective therapeutic options for patients with AH are limited. The mechanisms of ALD development are not well understood, and identification of mediators and biomarkers of progression from mild ALD to early stage of AH and to more severe AH remains a clinical gap.

Alterations in polyunsaturated fatty acid (PUFA)-derived bioactive lipid metabolites have been linked to the pathogenesis of various liver diseases, including ALD.\(^6\)-\(^12\) The majority of the PUFA-derived oxidized lipid products (also known as oxylipins) are generated through several metabolic pathways, e.g., by lipoxygenases (LOXs), cyclooxygenases (COXs), and cytochrome P450 (CYP450) epoxygenases or through nonenzymatic auto-oxidation mechanisms. Profound alterations in serum oxylipins derived predominantly from the n6 PUFA arachidonic acid (AA) and n3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were observed both in patients with alcohol use disorders and those with AH.\(^12\) In patients with AH, elevated levels of several oxylipins, e.g., 20-hydroxyeicosatetraenoic acid (20-HETE; a CYP450-derived product of AA), positively correlated with hepatic steatosis and polymorphonuclear leukocyte infiltration observed in liver biopsy and negatively correlated with 90-day survival.\(^12\) The importance of the PUFA-derived bioactive lipid metabolites generated through CYP450 has recently emerged in a variety of pathologies, including liver disease of different origins.\(^13\)-\(^17\) Multiple epoxides derived from AA, EPA, and DHA are also known for their potent cytoprotective, anti-oxidant, anti-inflammatory properties\(^18\)-\(^20\) and can promote resolution of inflammation.\(^21\) Epoxides can also be beneficial in preventing or ameliorating the severity of liver diseases, e.g., nonalcoholic fatty liver disease (NAFLD),\(^22\) and can promote tissue and organ regeneration, including the liver.\(^23\) In hepatocytes, primarily AA-derived epoxides improved autophagy and attenuated palmitate-induced accumulation of intracellular lipids and endoplasmic...
reticulum stress. However, less is known of the CYP450-derived metabolites of linoleic acid (LA), specifically how alcohol consumption affects their abundance and their potential role in ALD. Further, increased plasma levels of other AA-derived products, 5-, 12-, and 15-HETEs (LOX pathway) and 9- and 13-hydroxy-octadecadienoic acids (9- and 13-HODEs; LOX-derived oxidized metabolites of LA, [OXLAMs]), were found in the plasma of patients diagnosed with alcohol-associated cirrhosis. Of note, OXLAMs were proposed as a potential pathogenic link between fatty liver and type 2 diabetes and as biomarkers of liver injury in nonalcoholic steatohepatitis. The potential for OXLAMs as biomarkers of ALD severity has yet to be determined. Accumulating evidence from our laboratory and other groups demonstrated that dietary LA exacerbated liver injury in various animal models of ALD, which might partially be due to alterations in multiple LA-derived oxylipins. Importantly, LA is the most abundant n6-PUFA in the human diet, and its consumption has dramatically increased during the past several decades; however, studies examining alcohol-mediated alterations in LA metabolites and their role in human ALD are limited.

The aim of the current study was to examine the effects of alcohol consumption on circulating LA-derived bioactive metabolites in a cohort of individuals who were heavy drinkers with or without ALD. We tested the hypothesis that elevated plasma LA-derived lipoxins may facilitate progression from mild to more severe ALD. We postulated that the specific changes in LA-derived lipid mediators could effectively differentiate mild ALD from early stage AH. Our ultimate goal was to evaluate candidate lipid mediators for their potential to serve as biomarkers of clinical diagnosis/prognosis.

Patients and Methods

**HUMAN SUBJECTS AND CLINICAL CHARACTERIZATION**

The study included 66 patients who were heavy drinkers and alcohol dependent with or without ALD. Twenty-nine healthy individuals with no history or ongoing clinical diagnosis of ALD or alcohol abuse (both present or recent past) and without any inflammatory disease were enrolled as healthy controls (HCs). For the present study, de-identified plasma samples stored at −80°C were obtained from the University of Louisville biorepository. We performed analyses of variables of interest on samples obtained at baseline (day zero) for cohort 1 and cohort 2, as discussed below. This investigation was a single time point assessment. All data collected from the study participants were de-identified and coded.

Patient cohort 1 comprised 48 individuals who were heavy drinkers (34 men/14 women, aged 23-63 years) enrolled in a clinical investigation conducted at the National Institute of Alcohol Abuse and Alcoholism inpatient unit at the National Institute of Health Clinical Center (clinicaltrials.gov indexed protocol NCT#00106106). Patients were admitted to the alcohol withdrawal program, and all received standard inpatient medical management for alcohol detoxification. Written informed consent was obtained from all patients before collection of data and blood samples. This patient cohort also served as the study cohort in our previous studies. Detailed recruitment strategy, inclusion and exclusion criteria, alcohol consumption history, and clinical care have been reported in several publications from our group. Alcohol consumption was evaluated using Timeline Followback of the past 90 days. For the purpose of the current study, the subjects were stratified into two groups based on alanine aminotransferase (ALT) levels at admission. We set plasma ALT levels ≤ 40 U/L as within the normal reference range; plasma ALT levels > 40 U/L were considered clinically significant, indicating the presence of liver injury. Therefore, patients belonged to one of two groups: group 1 (Gr.1) comprised subjects who were alcohol dependent without liver injury and had normal ALT levels (ALT ≤40 U/L, n = 15); Gr.2 comprised subjects who were alcohol dependent with mild ALD (ALT > 40 U/L, n = 33). No patient had clinically evident AH in this cohort.

Patient cohort 2 comprised individuals who were heavy drinkers and alcohol dependent with a confirmed clinical diagnosis of the early stage of acute AH. Disease severity was determined using the Model for End-Stage Liver Disease (MELD) score < 20 (moderate AH, [mAH]). For the present study, 18 patients with mAH were defined as Gr.3 (12 men/6 women, aged 31-63 years). The patients
were enrolled in a clinical trial on ALD (clinicaltrials.gov indexed protocol NCT# 01809132) and approved by the University of Louisville Institutional Review Board (IRB #12.0427). Written informed consent was obtained from all patients before collection of data and blood samples. Inclusion criteria were age 21 years or older and reported heavy drinking for at least the past 6 months. Exclusion criteria were (a) unwilling or unable to provide informed consent; (b) significant comorbid conditions (heart, kidney, lung, neurologic or psychiatric illnesses, or sepsis) and active drug abuse; (c) women who were pregnant or lactating; (d) other known liver disease; (e) prisoners or other vulnerable subjects. At admission, a complete medical history was taken and a complete physical and routine laboratory examination was performed for all patients. Alcohol consumption history was evaluated using the Alcohol Use Disorders Identification Test (AUDIT) and the lifetime drinking history (LTDH) questionnaire.

BLOOD BIOCHEMICAL ASSESSMENT

The standard blood biochemical parameters, including ALT, aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatinine, total bilirubin (T-Bili), and albumin (Alb), were measured by the clinical laboratories at the respective clinical sites (cohort 1 at the National Institutes of Health and cohort 2 and the HC group at the University of Louisville, with the exception of ALT and AST for the HC group) and acquired from the clinical laboratory databases. ALT and AST levels for the HC group were measured by our research group using commercially available reagents from Thermo Fisher Scientific (Waltham, MA).

PLASMA LIPID METABOLITE MEASUREMENT

Targeted lipidomic analysis to measure plasma levels of bioactive oxidized metabolites of LA was performed by the Wayne State University Lipidomic Core Facility, as described. Briefly, samples in 150 µL methanol were spiked with 5 ng each of 15(S)-HETE-d8 and 14(15)-epoxyeicosatrienoic acid-d8 as internal standards for recovery and quantitation and mixed thoroughly. The samples were then extracted for PUFA metabolites using C18 extraction columns. The extracted samples were analyzed for metabolites of LA (hydroxy, epoxy, keto, and dihydroxy metabolites) by liquid chromatography–mass spectrometry using the QTRAP5500 mass analyzer (Sciex).

PLASMA CYTOKINE MEASUREMENT

Plasma levels of interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α) proinflammatory cytokines were analyzed using V-PLEX immunoassay proinflammatory panel 1 (catalog number K15049D), and data were collected on the Meso Sector S 600 instrument and analyzed with Discovery Workbench, version 4.0 (all from MesoScale Discovery, Rockville, MD).

STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism version 6.05 for Windows (GraphPad Software, Inc., La Jolla, CA). Data are expressed as mean ± SEM. Multiple group comparisons were performed using one-way analysis of variance followed by Tukey’s or Sidak’s multiple comparison post hoc tests. Associations between various subject characteristics were assessed using Spearman’s correlation analysis. The efficiency of LA metabolites to discriminate between individuals who were heavy drinkers with mild ALD and those with mAH was estimated for true positivity by receiver operating characteristic (ROC) curve analysis. Area under the ROC curve (AUROC) with corresponding P values were calculated. Multivariate analyses were performed with the stepwise elimination method and likelihood ratio tests to identify significant independent predictors. Associations are given as $R^2$ with the corresponding P values. $P < 0.05$ was considered statistically significant.

Results

CHARACTERISTICS OF STUDY PARTICIPANTS

The demographics and baseline study participant characteristics are summarized in Table 1. The study
investigated a cohort of individuals who were heavy alcohol drinkers and who were divided into three groups based on the presence or absence of alcohol-associated liver injury. Gr.1 included individuals without ALD (ALT < 40), Gr.2 included individuals with mild ALD (ALT > 40), and patients with early stage AH (mAH; based on MELD score < 20 [average MELD score, 17.0 ± 0.5]) comprised Gr.3. Twenty-nine healthy individuals comprised the HC group. There were more men in both the patient and HC groups, and there were no statistical differences in patient age between the study groups. As we reported, the duration of alcohol consumption was significantly higher in patients with mild ALD (Gr.2) compared to patients without ALD (Gr.1) (mean ± SEM, 17.6 ± 1.8 vs. 10.9 ± 1.6 years of alcohol drinking, respectively). The mean ± SEM length of alcohol consumption in mAH/Gr.3 was 18.3 ± 3.4 years (derived from the LTDH questionnaire), values very similar to those with mild ALD (Gr.2). Additionally, Gr.1 and Gr.2 patients consumed on average 15 drinks per day (according to NIH, 1 “standard drink” contains 14 grams of pure alcohol), as we reported. Similarly, mAH/Gr.3 also drank heavily, and their mean AUDIT score was 25.3 ± 1.9, indicating that all patients met the criteria of the dependence domain and heavy drinking (>20 drinks/week) during the assessment phase.

Markers of liver injury, including ALT and AST levels, in Gr.1 were similar to those of the HC group, while Gr.2 and mAH/Gr.3 had significantly elevated ALT and AST levels compared to HCs and to Gr.1 (Fig. 1A,B). Plasma T-Bili and Alb were comparable between Gr.1 and Gr.2 and were similar to the levels found in the HC group (Fig. 1C,D). Significantly elevated T-Bili and decreased Alb levels were observed in mAH/Gr.3 compared to all other groups (Fig. 1C,D). In addition, ALP levels were gradually elevated across ALD severity and were significantly higher in patients with mAH compared to all other groups (Fig. 1E). Creatinine levels were lower while international normalized ratio was significantly elevated in patients with mAH compared to Gr.1 and Gr.2 (Fig. 1F,G). The average ± SEM MELD score in mAH/Gr.3 was 17.0 ± 0.5. Because MELD score and clinical criteria (not liver biopsy) were used to diagnose mAH, we cannot exclude the possibility that some patients had underlying cirrhosis. However, in patients where follow-up laboratory data were available after reduction/cessation of drinking, tests, such as serum Alb and platelet count, were normalized.

We further evaluated several markers of systemic inflammation, including plasma IL-1β and TNF-α proinflammatory cytokines, commonly elevated in patients with ALD and AH. While IL-1β levels were similar in Gr.1 and Gr.2 and comparable to HCs, mAH/Gr.3 patients had significantly elevated IL-1β compared to HCs and to Gr.1 and Gr.2 (Fig. 1H). Plasma TNF-α was significantly elevated in all groups of individuals who were heavy drinkers compared to HCs and was similar in patients with mild liver injury (Gr.2) and mAH/Gr.3 (Fig. 1I). Both these groups also had markedly higher TNF-α levels compared to individuals who were heavy drinkers without ALD (Gr.1).

LOX PATHWAY METABOLITES
13-HODe ANd 13-OxO-OCTADECADIENOIC ACID WERE MARKEDLY ELEVATED ONLY IN mAH

We evaluated plasma levels of bioactive oxidized products of LA generated predominantly through LOX and the P450/soluble epoxide hydrolase (s-EH) enzymatic pathways (Fig. 2A). Among the

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**Table 1. Demographics and Clinical Characteristics of the Study Participants**

|                  | HCs | Group 1 ALT < 40 | Group 2 ALT > 40 | Group 3 mAH |
|------------------|-----|------------------|------------------|-------------|
| Number of subjects | 29  | 15               | 33               | 18          |
| Sex, men/women   | 20/9| 7/8              | 27/6             | 12/6        |
| Age, years       | 37.52 (2.50) | 40.44 (2.95)   | 44.45 (1.71)    | 48.17 (2.63)* |
| AUDIT            | na  | na               | na               | 25.3 (1.9)  |
| MELD             | na  | na               | na               | 17.0 (0.5)  |

Values are presented as mean (SEM).

*P < 0.05 versus healthy controls.

Abbreviation: na, not applicable.
**FIG. 1.** Liver injury markers and proinflammatory cytokine response in the study cohorts. (A) ALT, (B) AST, (C) total bilirubin, (D) albumin, (E) plasma ALP, (F) creatinine, (G) INR, (H) plasma IL-1β, and (I) plasma TNF-α. Data are presented as mean ± SEM; *P < 0.05. Abbreviation: INR, international normalized ratio.

**FIG. 2.** Plasma oxylipin levels of the LOX metabolic pathway across the spectrum of ALD. (A) Schematic presentation of LA metabolism showing the differences in (B) 9-HODE, (C) 9-OxoODE, (D) 13-HODE, and (E) 13-OxoODE in patients who were alcohol dependent with or without ALD. Data are presented as mean ± SEM; *P < 0.05.
metabolites of the LOX pathway, 9-HODE and 9-oxooctadecadienoic acid (9-OxoODE) were comparable among all groups, including HCs and individuals who were heavy drinkers with or without ALD/AH (Fig. 2B,C). Similarly, there were no significant differences in 13-HODE (the most abundant among the measured metabolites) or 13-OxoODE between HCs and Gr.1 and Gr.2. However, mAH/Gr.3 patients had significantly elevated levels of both 13-HODE and 13-OxoODE (~50% increase) compared to HCs, Gr.1, and Gr.2 (Fig. 2D,E), indicating clear associations between liver disease severity and levels of LOX-derived LA metabolites. There were no statistically significant differences between men and women for any of the LA metabolites (possibly due to a smaller number of women; Table 2); thus, we did not pursue in-depth sex-difference analysis.

**DISTINCT ALTERATIONS OF LA METABOLITES DERIVED THROUGH CYP450 AND s-EH PATHWAYS**

The levels of LA metabolites generated through the CYP450 pathway 9,10- and 12,13-epoxy-octadecenoic acid (9,10-EpOME and 12,13-EpOME) were significantly lower in all individuals who were heavy drinkers compared to the HC group (Fig. 3A,B). Further, the levels of 9,10-dihydroxy-octadecenoic acid (9,10-DiHOME), the diol formed through the action of s-EH on the corresponding epoxide 9,10-EpOME, were similar in HCs, Gr.1, and Gr.2. However, 9,10-DiHOME was significantly elevated in mAH/Gr.3 patients compared to all other groups (Fig. 3C). Interestingly, another diol, 12,13-DiHOME, formed through s-EH from 12,13-EpOME, had the same levels in HCs as in individuals who were heavy drinkers without liver injury (Gr.1) but was significantly reduced in patients with mild liver injury (Gr.2) compared to HCs (Fig. 3D). Notably, 12,13-DiHOME was significantly elevated in mAH/Gr.3 patients compared to patients with mild liver injury (Gr.2). The ratio between corresponding diol:epoxide pairs is an indirect measure of s-EH activity, and an increase in this ratio has been associated with various pathologies, including liver diseases and conditions associated with inflammation.\(^{14,19,37}\) Compared to HCs, there were no differences in the 9,10-DiHOME/9,10-EpOME and 12,13-DiHOME/12,13-EpOME ratios either in Gr.1 or Gr.2 (Fig. 3E,F). However, mAH/Gr.3 patients had a significantly elevated diol:epoxide ratio for both pairs compared to the HC group. In addition, the 9,10-DiHOME/EpOME ratio was higher in mAH/Gr.3 compared to Gr.1, and the 12,13-DiHOME/EpOME ratio was higher in mAH/Gr.3 compared to both Gr.1 and Gr.2. Collectively, our data demonstrated that 1) alcohol consumption resulted in a reduction of LA-CYP450-derived epoxides regardless of the presence/absence of liver injury and 2) the corresponding diols were significantly elevated in patients with more severe liver damage (in patients with mAH).

**TABLE 2. LIPID METABOLITES IN THE STUDY GROUPS**

| Metabolites      | HCs | Group 1 (ALT < 40) | Group 2 (ALT > 40) | Group 3 (mAH) |
|------------------|-----|--------------------|--------------------|---------------|
|                   | M   | F                  | M                  | F             | M             | F               |
|                   | n = 20 | n = 9              | n = 7              | n = 8         | n = 27        | n = 6           |
| 9-HODE           | 1.8 ± 0.24 | 2.07 ± 0.34        | 1.47 ± 0.17        | 1.20 ± 0.30   | 1.23 ± 0.18   | 2.06 ± 0.57     |
| 13-HODE          | 7.7 ± 0.95 | 8.42 ± 1.59        | 8.44 ± 0.63        | 5.98 ± 1.51   | 7.54 ± 1.22   | 9.90 ± 2.60     |
| 9-OxoODE         | 0.38 ± 0.05 | 0.55 ± 0.18        | 0.53 ± 0.11        | 0.34 ± 0.11   | 0.47 ± 0.07   | 0.83 ± 0.20     |
| 13-OxoODE        | 0.80 ± 0.11 | 1.16 ± 0.38        | 1.06 ± 0.19        | 0.62 ± 0.14   | 0.77 ± 0.08   | 1.11 ± 0.14     |
| 9,10-EpOME       | 2.88 ± 0.43 | 2.95 ± 0.47        | 1.62 ± 0.24        | 1.22 ± 0.22   | 1.50 ± 0.17   | 1.70 ± 0.08     |
| 12,13-EpOME      | 2.06 ± 0.26 | 2.54 ± 0.46        | 1.88 ± 0.45        | 0.92 ± 0.15   | 1.14 ± 0.09   | 1.37 ± 0.09     |
| 9,10-DiHOME      | 1.55 ± 0.25 | 1.81 ± 0.37        | 2.28 ± 0.65        | 0.57 ± 0.09   | 1.17 ± 0.34   | 0.97 ± 0.35     |
| 12,13-DiHOME     | 2.06 ± 0.23 | 2.64 ± 0.63        | 3.00 ± 0.65        | 1.04 ± 0.21   | 1.35 ± 0.27   | 1.51 ± 0.55     |

All metabolites are expressed as mean ± SEM (n/mL plasma).

Abbreviations: F, female; M, male.
In order to determine the true positivity of our findings, we performed analysis of the ROC curves obtained for comparison between mAH/Gr.3 and the HC group, Gr.1 patients, and Gr.2 patients. This analysis suggested that the candidate LA-derived metabolites, namely 13-HODE, 13-OxoODE, 9,10-EpOME, 12,13-EpOME, and 9,10-DiHOME, could efficiently distinguish HCs from mAH/Gr.3 (Fig. 4). Further, these same LA-derived metabolites, namely 13-HODE, 13-OxoODE, and 9,10-DiHOME, could also effectively distinguish the individuals who were heavy drinkers who had yet to develop liver injury (Gr.1) from those with mAH/Gr.3 (Fig. 5). Importantly, analyses of the ROC curves obtained for the comparison of patients with mild liver injury (Gr.2) and mAH/Gr.3 indicated that they were also significantly different based on several LA-derived products (Fig. 6). Specifically, the metabolites of the LOX pathway, 13-HODE (difference of 0.7579; \( P = 0.0034 \)) and 13-OxoODE (difference of 0.7817; \( P = 0.0014 \)), and metabolites of the CYP450/s-EH pathway, 9,10-DiHOME (difference of 0.8452; \( P = 0.0001 \)) and 12,13-DiHOME (difference of 0.8373; \( P = 0.0001 \)), were significantly different. Thus, our data indicate that specific changes in LA-derived metabolites can distinguish individuals who are heavy drinkers with mAH from those without or with mild ALD.

**MODELING OF POTENTIAL LINKS AMONG ALCOHOL-INDUCED ALTERATIONS IN PLASMA OXLAMs, SYSTEMIC INFLAMMATION, AND LIVER FUNCTION**

Lastly, we examined the potential links among LA metabolites, systemic inflammation, and liver injury...
Fig. 4. AUROC curve analyses to identify the degree of true positivity of the difference in candidate oxylipins between healthy controls and patients with mAH. (A) Comparison of 9-HODE, 9-OxoODE, 13-HODE, and 13-OxoODE (LOX pathway metabolites) between the two groups. (B) Comparison of 9,10-EpOME, 12,13-EpOME, 9,10-DiHOME, and 12,13-DiHOME (CYP450/s-EH pathway metabolites) between the two groups. Significant true positivity was set at $P < 0.05$.

Fig. 5. AUROC curve analyses to identify the degree of true positivity of the difference in candidate OXLAMs between individuals who were heavy drinkers without liver injury (Group 1) and patients with mAH (Group 3). (A) Comparison of 9-HODE, 9-OxoODE, 13-HODE, and 13-OxoODE (LOX pathway metabolites) between the two groups. (B) Comparison of 9,10-EpOME, 12,13-EpOME, 9,10-DiHOME, and 12,13-DiHOME (CYP450/s-EH pathway metabolites) between the two groups. Significant true positivity was set at $P < 0.05$. 
in the study cohorts. First, we performed univariate regression analysis and found no direct significant association between any LA metabolites, individual proinflammatory cytokines as markers of immune activation/inflammation, or markers of liver injury and function (data not shown). This could be due to the possibility that LA-derived metabolites did not directly or as individual molecules activate the immune system or cause liver injury in our patients. In addition, it is well known that ALD pathogenesis is multifactorial and multiple molecules and mechanisms can simultaneously contribute to liver injury. Therefore, bioactive lipid molecules most likely act as part of a complex mechanism underlying the inflammatory responses and ethanol-associated liver injury and altered function. Next, we used multiple regression models to tease out the potential links among alcohol-induced alterations in plasma oxylipins, systemic inflammation, and liver injury/dysfunction. Because ALT and AST frequently do not correlate well with disease severity, we focused on elevated T-Bili levels as a critical feature of altered liver function in patients with AH. Importantly, LA metabolites as well as any of the cytokines alone as independent variables did not show any statistically significant associations with T-Bili. For example, decreased 12,13-EpOME in Gr.1 (Fig. 7A) or a combination of decreased 12,13-EpOME and elevated IL-1β levels in Gr.2 (Fig. 7B) were not correlated with T-Bili. However, a cluster of decreased 12,13 -EpOME and elevated IL-1β levels in combination with elevated 13-HODE were cumulatively associated with elevated T-Bili levels in patients with mAH ($R^2 = 0.5085; P = 0.0164$) (Fig. 7C). These data suggest that an indicator defined by 12,13-EpOME/13-HODE/IL-1β may serve as independent predictor of liver function noted by T-Bili levels. We found that 13-HODE was elevated only in patients with mAH and appeared to be a key factor in the IL-1β/12,13-EpOME/13-HODE cluster. Thus, 13-HODE may play a key role within this group of factors.

Discussion

The present study demonstrated differential alterations in LA-derived metabolites in individuals

FIG. 6. AUROC curve analyses to identify the degree of true positivity of the difference in candidate OXLAMs between individuals who were heavy drinkers with mild liver injury (Group 2) and patients with mAH (Group 3). (A) Comparison of 9-HODE, 9-OxoODE, 13-HODE, and 13-OxoODE (LOX pathway metabolites) between the two groups. (B) Comparison of 9,10-EpOME, 12,13-EpOME, 9,10-DiHOME, and 12,13-DiHOME (CYP450/s-EH pathway metabolites) between the two groups. Significant true positivity was set at $P < 0.05$. 
who were heavy drinkers without liver injury, with mild ALD, and with mAH. One of our major findings was that the LOX-derived LA metabolites 13-HODE and 13-OxoODE but not 9-HODE and 9-OxoODE were only markedly elevated in patients with mAH. This suggests that 13-HODE but not 9-HODE might be a critical LA-derived metabolite contributing to the development of a more severe form of ALD. The mechanisms linking 13-HODE to alcohol-associated liver damage may be related to its ability to induce proinflammatory responses in macrophages\(^2\) and to induce production of reactive oxygen species, endoplasmic reticulum stress, and apoptosis in hepatocytes.\(^2\) Our observation that 13-HODE was elevated in patients with a more severe form of ALD is in line with a previous report demonstrating elevated levels of 9-HODE and 13-HODE in plasma of patients with alcohol-associated cirrhosis.\(^7\) The authors of that study did not differentiate 9-HODE from 13-HODE, and therefore it is possible that the elevated OXLAM levels in those patients with cirrhosis were driven by 13-HODE. It is important to mention that LA oxidation and metabolism is a sequential process, and 13-HODE can be further converted to 13-OxoODE by the action of nicotinamide adenine dinucleotide phosphate (reduced form)-dependent fatty acid dehydrogenases.\(^4\) Interestingly, 13-OxoODE is an endogenous ligand for peroxisome proliferator-activated receptor gamma and can exert anti-inflammatory effects, e.g., in colonic epithelial cells.\(^4\) Whether this effect occurs in monocytes/macrophages and/or in hepatocytes remains to be determined.

Another important finding of our study was that heavy chronic alcohol drinking resulted in a significant decrease in the LA-CYP450-derived epoxides 9,10-EpOME and 12,13-EpOME, regardless of presence or absence of liver injury. Interestingly, the LA-derived diols 9,10-DiHOME, 12,13-DiHOME, and the corresponding diol/epoxide ratios were elevated only in patients with mAH when compared to HCs and patients who were heavy drinkers with or without mild liver injury. This could potentially result from either significantly decreased expression of CYP450 enzymes involved in epoxide production or a markedly elevated activity of s-EH, the enzyme converting epoxides into diols,\(^1\) or a combination of both. Of note, in our pilot study, we observed significantly reduced messenger RNA (mRNA) levels of several CYP450 enzymes, such as CYP2J2, CYP2C19, and CYP4A1, and elevated s-EH mRNA expression in explanted liver tissue samples obtained from patients with AH (unpublished data). This observation may partially explain the low epoxide/high diol levels observed in our study. However, the role of specific LA-derived epoxides and diols in ALD has yet to be examined. A recent comprehensive review\(^4\) summarized the role of EpOMEs and DiHOMEs in a variety of biological functions, including immune responses, pain perception, and cytotoxic processes. For example, 9,10-DiHOME can induce mitochondria toxicity\(^4\), whether this

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**Fig. 7.** Representation of pathway response of the arrangement of candidate OXLAMs. Shown are 13-HODE and 12,13 EpOME (involved with LOX pathway and CYP450, respectively) and proinflammatory immune response as characterized by IL-1β on the liver injury (function) marker (total bilirubin) in (A) Group 1, (B) Group 2, and (C) Group 3 patients with mAH. Schema presented as the output of univariate and multivariable regression analyses model. Effect sizes for direct (univariate) and cumulative (multivariable) response have been noted.
mechanism is involved in hepatocyte cell death is under active investigation in our laboratory. Further, it has been shown that DiHOMEs suppress the neutrophil respiratory burst, a critical process in immune-mediated elimination of microorganisms. Given that neutrophils play important roles in ALD, specifically in AH, the significance of elevated DiHOME levels in patients with mAH needs to be further investigated.

Lastly, a key observation of our study is that the clinical markers ALT and AST were not effective in distinguishing mild liver injury from mAH. Interestingly, our results demonstrated that alterations in specific LA-derived lipid metabolites could effectively distinguish mild ALD from the more advanced mAH. Specifically, 13-HODE and 13-OxoODE from the LOX pathway and 9,10-DiHOME and 12,13-DiHOME from the CYP450/s-EH pathway showed a high and significant true positivity of differences between the mAH group and the group of heavy drinkers with mild liver injury. Further, our study revealed that the association of a specific group of LA-derived oxylipins and cytokines and liver function as defined by elevated T-Bili levels could serve as a predictor of liver injury in patients with ALD/AH and could distinguish mAH from the ALD groups. It is well documented that ongoing inflammation exacerbates ALD, with IL-1β playing a critical role in inflammasome-mediated proinflammatory responses. IL-1β stood out as the most potent cytokine in our study that, in conjunction with the elevated 13-HODE and low levels of 12,13 EpOME, could effectively predict clinically high levels of T-Bili separating mAH from other groups of heavy drinkers who do not have such a clinical liver status. The fact that 13-HODE was elevated only in patients with mAH and appeared to be a key player in the IL-1β/12,13 EpOME/13-HODE cluster positions 13-HODE as an important LA-derived lipid mediator contributing to the progression of ALD to a more severe form. The detailed molecular mechanisms mediated by 13-HODE in ALD progression and liver dysfunction warrant further investigation.

One of the limitations of this study was our inability to correlate blood and hepatic LA-derived metabolite levels. Because a liver biopsy is not routine for patients with ALD and it is not standard-of-care in the United States, matching plasma and liver samples were not available for this study. It is possible that alterations in some plasma and liver lipid metabolites have a similar trend in patients with ALD, e.g., plasma and liver 9-HODE and 13-HODE. Elevated plasma 9- and 13-HODEs were accompanied by an increase in hepatic 5-LOX, 15-LOX-1, and 15-LOX-2 mRNA in patients with alcohol-associated cirrhosis, suggesting the activated enzymatic machinery producing OXLAMs in the liver of these patients. In addition, a strong positive correlation between serum and liver tissue 9-HODE and 13-HODE levels has been shown in a mouse model of NAFLD. However, it also has to be acknowledged that nonhepatic tissues may also contribute to circulating LA-derived metabolites. Another limitation was the smaller number of women compared to men in all but Gr.1/ALT < 40, and thus sex differences could not be accurately determined. We also did not have a disease control group, for example, nonalcoholic steatohepatitis, that could have provided information on the usefulness of LA-derived oxylipins in differential diagnosis from other disease conditions with similar pathways and inflammatory responses involved.

In summary, the results obtained in the current study advance our knowledge regarding alcohol-mediated alterations of bioactive lipid metabolites, specifically those derived from LA, in individuals who were heavy drinkers with or without ALD. We demonstrated that specific changes in LA metabolites can distinguish individuals with mild ALD from those with more advanced forms, specifically mAH. We identified a cluster of 13-HODE and 12,13 EpOME (elevated and decreased, respectively) linked with the immune response characterized by elevated IL-1β as an independent group predictor of altered liver function as defined by elevated T-Bili levels in patients with mAH. Lastly, the current study also provides novel data for the generation of innovative hypotheses for future mechanistic studies and for identification of new pharmacologic approaches to modulate LA-derived oxylipin production or action that may lessen the burden of liver disease in patients who are heavy alcohol drinkers. Our study also raised several important questions that need to be further investigated, such as whether there are any sex differences in ethanol-mediated alterations of lipid metabolites and whether our results could be extrapolated to another subgroup of patients with ALD or those with NAFLD.

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