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Journal

Journal of Lipid Research, 56(1)

ISSN

0022-2275

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Publication Date

2015

DOI

10.1194/jlr.p055640

Peer reviewed

Polyunsaturated fatty acid metabolites as novel lipidomic biomarkers for noninvasive diagnosis of nonalcoholic steatohepatitis[1]

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Abstract Lipotoxicity is a key mechanism thought to be responsible for the progression of nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH). Noninvasive diagnosis of NASH is a major unmet clinical need, and we hypothesized that PUFA metabolites, in particular arachidonic acid (AA)-derived eicosanoids, in plasma would differentiate patients with NAFL from those with NASH. Therefore, we aimed to assess the differences in the plasma eicosanoid lipidomic profile between patients with biopsy-proven NAFL versus NASH versus normal controls without nonalcoholic fatty liver disease (NAFLD; based on MRI fat fraction <5%). We carried out a cross-sectional analysis of a prospective nested case-control study including 10 patients with biopsy-proven NAFL, 9 patients with biopsy-proven NASH, and 10 non-NAFLD MRI-phenotyped normal controls. We quantitatively compared plasma eicosanoid and other PUFA metabolite levels between NAFL versus NASH versus normal controls. Utilizing a uniquely well-characterized cohort, we demonstrated that plasma eicosanoid and other PUFA metabolite profiling can differentiate between NAFL and NASH. The top candidate as a single biomarker for differentiating NAFL from NASH was 11,12-dihydroxy-eicosatrienoic acid (11,12-diHETrE) with an area under the receiver operating characteristic curve (AUROC) of 1. In addition, we also found a panel including 13,14-dihydro-15-keto prostaglandin D_2 (dhk PGD2) and 20-carboxy arachidonic acid (20-COOH AA) that demonstrated an AUROC of 1. This proof-of-concept study provides early evidence that 11,12-diHETrE, dhk PGD2, and 20-COOH AA are the leading eicosanoid candidate biomarkers for the noninvasive diagnosis of NASH.—Loomba, R., O. Quehenberger, A. Armando, and E. A. Dennis. **Polyunsaturated fatty acid metabolites as novel lipidomic biomarkers for noninvasive diagnosis of nonalcoholic steatohepatitis. *J. Lipid Res*. 2015.** 56: **185–192.**

Supplementary key words nonalcoholic fatty liver disease • hepatic steatosis • cirrhosis • liver disease • diagnostic test accuracy • eicosanoid profiling • mass spectrometry

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in the United States (1). It can be broadly subclassified into nonalcoholic fatty liver (NAFL), which is thought to have minimal risk of progression to cirrhosis, and nonalcoholic steatohepatitis (NASH), which is thought to have an increased risk of progression to cirrhosis (2) (3–5). The current diagnostic gold standard for differentiating whether a patient with NAFLD has NAFL versus NASH is liver biopsy (6, 7). However, liver biopsy is an invasive procedure, which is limited by sampling variability and cost and may be complicated by morbidity and rarely even death (7).

Accurate, noninvasive biomarkers for the detection of NASH are currently not available (1). Noninvasive diagnosis of NASH is a major unmet medical need (1). Previous studies have shown that lipotoxicity plays an important role in the pathogenesis of NASH (8–11). Recent data suggest that oxidized LDL as well as other lipid moieties have been implicated as increased in patients with NASH compared with those with NAFL (10, 12–14).

Oxidized PUFAs and their metabolites are implicated in a wide range of inflammatory diseases, and autooxidized linoleic and linolenic acids have been reported in NAFLD (11, 12). With the recent evolution of LC/MS-based

This work was supported in part by National Institutes of Health Grant K23 DK090303 (R.L.) and the LIPID MAPS "Glue" Grant GM U54 069338 (E.A.D.). Support was also provided by the American Gastroenterological Association (AGA) Foundation – Sucampo – ASP Designated Research Award in Geriatric Gastroenterology, a T. Franklin Williams Scholarship Award, Atlantic Philanthropies Inc., the John A. Hartford Foundation, the Association of Specialty Professors, and the AGA (R.L.).

Manuscript received 19 October 2014 and in revised form 14 November 2014.

Published, JLR Papers in Press, November 17, 2014
DOI 10.1194/jlr.P055640

Abbreviations: 11,12-diHETrE, 11,12-dihydroxy-eicosatrienoic acid; 14,15-diHETrE, 14,15-dihydroxy-eicosatrienoic acid; 20-COOH AA, 20-carboxy arachidonic acid; AA, arachidonic acid; COX, cyclooxygenase; CYP, cytochrome P450; dhk PGD2, 13,14-dihydro-15-keto prostaglandin D_2; LOX, lipoxygenase; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD activity score; NASH, nonalcoholic steatohepatitis; PDFF, proton-density-fat-fraction; PGD2, prostaglandin D_2; PGE2, prostaglandin E_2.

[1] Guest editor for this article was Arthur A. Spector, Carver School of Medicine, University of Iowa (Emeritus).

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lipidomics techniques, our laboratory has developed a robust and comprehensive approach to the lipidomics analysis of hundreds of fatty acids, acylethanolamines, and inflammatory eicosanoids, including their numerous metabolites arising from an array of cyclooxygenases, lipoxygenases, cytochrome P450s, and nonenzymatic oxidation-producing isoprostanes, as well as combinations thereof (15). Particular attention has been focused on the eicosanoids derived from arachidonic acid (AA), and we can now routinely quantify >150 such metabolites and have used this approach to profile AA and other PUFAs as well as their metabolites in human plasma (15–17). We have now applied this approach to analyze the plasma of NAFLD patients.

The aim of this proof-of-concept study was to detect if plasma eicosanoid profiling can differentiate well-characterized patients with biopsy-proven NASH versus NAFL versus uniquely phenotyped normal controls by documenting liver fat content of <5% by proton-density-fat-fraction (MRI-PDFF), a novel MRI-based method.

EXPERIMENTAL PROCEDURES

Study design and participants

This study was a cross-sectional analysis derived from a prospective nested case-control study including three groups of uniquely phenotyped patients with biopsy-proven NAFLD (including NASH and NAFL) and normal non-NAFLD controls. All participants were derived from the University of California at San Diego (UCSD) NAFLD Research Clinic and were seen between January 2011 and November 2012 (18–20). All participants provided written informed consent and underwent a detailed standardized clinical research visit including medical history, alcohol use and quantification history (using Audit and Skinner questionnaire), physical examination, anthropometrics, fasting biochemical tests, and detailed exclusion of other causes of liver disease (see the inclusion and exclusion criteria described subsequently). A fasting plasma sample was collected in the morning of the clinical research visit and stored at −80°C freezer housed in the UCSD NAFLD Translational Research Unit. The study was approved by the UCSD Human Subjects Institutional Review Board.

Description of cohort

All cases of NAFLD included in this study had a liver biopsy-confirmed diagnosis of NAFLD.

Histologic description. Biopsy was scored by an experienced liver pathologist who was blinded to clinical data and lipidomic and imaging data. The NASH Clinical Research Network histologic scoring system was used to score biopsies (21). NAFLD activity score (NAS) and fibrosis score were recorded for all patients. NAS score ranges from 0 to 8 and is the summation of the degree of steatosis (0–3), lobular inflammation (0–3), and hepatocellular ballooning (0–2). Liver fibrosis ranges from 0 to 4 with 0 being no fibrosis and 4 indicating cirrhosis.

Definition of NAFL. Patients with biopsy-confirmed NAFLD who had predominantly zone-3 macrovesicular steatosis with or without minimal inflammation, absence of ballooning degeneration, and no fibrosis were classified as having NAFL.

Definition of NASH. Patients with biopsy-confirmed NAFLD who had predominantly zone-3 macrovesicular steatosis and lobular inflammation and the presence of classic ballooning degeneration were classified as having NASH.

Inclusion criteria for the NAFLD. Inclusion criteria included age at least 18 years during the consent process, ability and willingness to give written informed consent, minimal or no alcohol use history consistent with NAFLD (see Exclusion criteria), and collection of plasma within 90 days of the liver biopsy.

Exclusion criteria. Clinical or histologic evidence of alcoholic liver disease included the following: regular and excessive use of alcohol within the 2 years prior to interview defined as alcohol intake >14 drinks per week in a man or >7 drinks per week in a woman. Approximately 10 g of alcohol equals one "drink" unit. One unit equals 1 ounce of distilled spirits, one 12 ounce beer, or one 4 ounce glass of wine. Secondary causes of hepatic steatosis included previous surgeries, bariatric surgery, total parenteral nutrition, short bowel syndrome, steatogenic medications, evidence of chronic hepatitis B as marked by the presence of Hepatitis B surface antigen in serum, evidence of chronic hepatitis C as marked by the presence of anti-Hepatitis C virus antibody (HCV) or HCV RNA in serum, evidence of other causes of liver disease (such as α-1-antitrypsin deficiency, Wilson disease, glycogen storage disease, dysbetalipoproteinemia, known phenotypic hemochromatosis, autoimmune liver disease, or drug-induced liver injury), or concomitant severe underlying systemic illness that in the opinion of the investigator would interfere with the study.

Definition of normal controls. A novel aspect of this study was the inclusion of a uniquely well-characterized non-NAFLD normal control group. Participants were classified as normal non-NAFLD by accurate hepatic fat quantification by MRI-PDFF-derived fat fraction of <5% (18, 20). Liver biopsy is unethical in normal individuals. Other noninvasive measures such as ultrasound and computed tomography are inaccurate and lack sensitivity especially at liver fat fraction between 1% and 10%. Therefore, MRI-PDFF was utilized in this study for accurate diagnosis of absence of hepatic steatosis. MRI-PDFF is highly accurate, sensitive, reproducible, and precise. The detailed description of MRI-PDFF protocol has been published previously (18–20, 22–25).

Inclusion and exclusion criteria: normal (non-NAFLD) control cohort. Inclusion criteria in the healthy (non-NAFLD) control group included *1*) age greater than 18 years, *2*) liver MRI-PDFF <5%, and *3*) no history of known liver disease.

Exclusion criteria included *1*) age less than 18 years; *2*) significant systemic illness; *3*) inability to undergo MRI; and *4*) evidence of possible liver disease, including any previous liver biopsy, positive hepatitis B surface antigen, hepatitis C viral RNA, or autoimmune serologies, α-1 antitrypsin deficiency, hemochromatosis genetic testing, or low ceruloplasmin.

Lipid extraction

Plasma samples for lipidomic profiling were obtained within 90 days of the liver biopsy and MRI-PDFF for cases and controls, respectively. All plasma samples were stored at −80°C, thawed once, and immediately used for free fatty acid and eicosanoid isolation as described previously (15, 17). Briefly, 50 µl plasma was spiked with a cocktail of 26 deuterated internal standards (individually purchased from Cayman Chemicals, Ann Arbor, MI) and brought to a volume of 1 ml with 10% methanol. The samples were then purified by solid phase extraction on Strata-X columns (Phenomenex, Torrance, CA), using an activation procedure consisting of consecutive washes

with 3 ml of 100% methanol followed by 3 ml of water. The eicosanoids were then eluted with 1 ml of 100% methanol, and the eluent was dried under vacuum, dissolved in 50 μl of buffer A [consisting of water-acetonitril-acetic acid, 60:40:0.02 (v/v/v)], and immediately used for analysis as follows: For free fatty acids analysis, 50 μl of plasma was spiked with deuterated fatty acid standards, and the free fatty acids were isolated by selective extraction with methanol and isooctane. The extracted fatty acids were derivatized and analyzed by gas chromatography and MS, as described (15).

Reverse-phase LC/MS

Eicosanoids in plasma were analyzed and quantified by LC/MS/MS as previously described (17, 26). Briefly, eicosanoids were separated by reverse-phase chromatography using a 1.7 μM 2.1 × 100 mm BEH Shield Column (Waters, Milford, MA) and an Acquity UPLC system (Waters). The column was equilibrated with buffer A, and 5 μl of sample was injected via the autosampler. Samples were eluted with a step gradient starting with 100% buffer A for 1 min, then to 50% buffer B (consisting of 50% acetonitril, 50% isopropanol, and 0.02% acetic acid) over a period of 3 min, and then to 100% buffer B over a period of 1 min. The LC was interfaced with an IonDrive Turbo V ion source, and mass spectral analysis was performed on a triple quadrupole AB SCIEX 6500 QTrap mass spectrometer (AB SCIEX, Framingham, MA). Eicosanoids were measured using electrospray ionization in negative ion mode and multiple reaction monitoring (MRM) using the most abundant and specific precursor ion/product ion transitions to build an acquisition method capable of detecting 158 analytes and 26 internal standards. The ionspray voltage was set at −4,500 V at a temperature of 550°C. Collisional activation of the eicosanoid precursor ions was achieved with nitrogen as the collision gas with the declustering potential, entrance potential, and collision energy optimized for each metabolite. Eicosanoids were identified by matching their MRM signal and chromatographic retention time with those of pure identical standards.

Quantitation of lipids

Eicosanoids and free fatty acids were quantitated by the stable isotope dilution method. Briefly, identical amounts of deuterated internal standards were added to each sample and to all the primary standards used to generate standard curves. To calculate the amount of eicosanoids and free fatty acids in a sample, ratios of peak areas between endogenous metabolite and matching deuterated internal standards were calculated. Ratios were converted to absolute amounts by linear regression analysis of standard curves generated under identical conditions.

Statistical analysis

The Chi-square (χ^2) test was used for comparisons between categorical variables, and the *t*-test was used for comparisons between continuous variables. We examined differences in the plasma eicosanoid profiles between normal controls, patients with biopsy-proven mild NAFL, and patients with biopsy-proven NASH. Finally, we examined the diagnostic accuracy of nine biomarkers that yielded significant differences as biomarkers to differentiate NAFL from NASH. A two-tailed *P* value ≤0.05 was considered statistically significant. Statistical analyses were performed using the SAS statistical software package version 9.4 (SAS Inc., Cary, NC).

TABLE 1. Baseline demographic, clinical, biochemical, and histologic characteristics of the patients in the study population

	Controls (n = 10)	NAFL (n = 10)	NASH (n = 9)	Control versus NAFL *P*	NAFL versus NASH *P*
Age	31.8 ± 15.66	48.90 ± 14.03	45.89 ± 12.94	**0.019**	0.633
Sex	40% male	44% male	40% male		
BMI	24.73 ± 4.17	29.49 ± 5.39	29.59 ± 5.01	**0.041**	0.966
Laboratory data					
Platelet	240,500 ± 48,808.58	264,900 ± 53,371.76	244,888.89 ± 52,312.63	0.300	0.400
WBC	7.05 ± 1.90	7.18 ± 2.28	5.96 ± 0.87	0.892	0.101
Alk P	71.9 ± 23.65	85.70 ± 40.42	78.78 ± 18.19	0.367	0.638
ALT	16.7 ± 8.51	61.10 ± 39.73	104.33 ± 61.79	**0.006**	0.053
AST	23.1 ± 8.71	35.00 ± 12.53	66.33 ± 32.69	**0.025**	**0.013**
D Bili	0.12 ± 0.04	0.12 ± 0.04	0.12 ± 0.04	1.000	0.628
T Bili	0.49 ± 0.30	0.53 ± 0.21	0.56 ± 0.25	0.732	0.763
GGT	18.8 ± 19.52	46.20 ± 24.03	72.89 ± 38.47	**0.012**	0.067
Glucose	88.7 ± 5.93	99.00 ± 13.41	97.11 ± 8.68	**0.046**	0.612
Hba1c	5.6 ± 0.30	5.79 ± 0.82	5.84 ± 0.45	0.505	0.947
Insulin	8.7 ± 4.35	13.64 ± 6.23	14.78 ± 10.31	**0.003**	0.608
PT	10.98 ± 0.58	10.59 ± 1.33	10.77 ± 0.80	0.411	0.657
Chol	172.9 ± 21.46	196.60 ± 33.20	229.67 ± 28.97	0.077	0.050
TG	87.5 ± 41.70	124.80 ± 52.37	221.22 ± 108.43	0.096	**0.034**
HDL	58.1 ± 12.38	55.00 ± 18.34	55.44 ± 24.78	0.664	0.847
LDL	97.6 ± 18.40	116.50 ± 129.00	129.00 ± 26.88	0.083	0.284
Liver histology					
Steatosis		0.75 ± 0.5	2.33 ± 0.82		**0.005**
Fibrosis		0 ± 0	1.60 ± 089		**0.016**
NAS		1.75 ± 0.5	6.33 ± 1.03		**0.0001**
Hepatocellular ballooning		0 ± 0	1.50 ± 0.84		**0.007**
Lobular inflammation		1 ± 0	2.17 ± 0.41		**0.001**
Portal inflammation		0.5 ± 0.55	0.17 ± 0.41		0.262

The *P* values in bold are statistically significant ($P \leq 0.05$). Differences between groups evaluated with *t*-test. Alk P, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Chol, cholesterol; D Bili, direct bilirubin; GGT, gammaglutamyl transferase; Hba1c, hemoglobin a1c; PT, protime; T Bili, total bilirubin; WBC, white blood count.

RESULTS

Cohort demographics

This study included 19 patients with NAFLD (10 NAFL cases and 9 cases of NASH) and 10 non-NAFLD normal controls. The detailed baseline characteristics including demographics, BMI, biochemical tests, lipid profile, MRI-PDFF for controls, and liver biopsy data on patients with NAFLD are described in **Table 1**. Non-NAFLD controls were younger, had lower BMI, and had lower serum ALT, AST, GGT, and glucose and insulin levels as expected. Routine liver-related and metabolic tests did not significantly differ between NAFL versus NASH (Table 1), except that plasma triglycerides were marginally higher in patients with NASH. Compared with patients with NAFL, patients with NASH had more severe liver histology with a higher degree of steatosis, ballooning degeneration, lobular inflammation, and fibrosis.

PUFA and metabolite lipidomics profiling

At present, there are no noninvasive biomarkers with sufficient specificity to distinguish NASH from other fatty liver states. Liver biopsy remains the benchmark to reliably identify NAFL and NASH, but the procedure is invasive and carries certain risks. Thus, there is great demand from the clinical community for the development of noninvasive procedures capable of accurately characterizing and staging NAFLD, as that furnishes valuable information on treatment options and prognosis. Inflammation and oxidative stress contribute to disease progression from steatosis with relatively benign outcome to NASH with risk of cirrhosis and hepatocellular carcinoma. Here we used LC/MS to profile and quantitate bioactive lipids and lipid peroxidation products in circulation that are characteristic of hepatic inflammation in NASH patients.

We established complete eicosanoid profiles and assessed the plasma levels of free eicosanoids derived from AA (20:4 n-6) and related PUFAs including linoleic acid (18:2 n-6), α-linolenic acid (18:3 n-3), dihomo-γ-linolenic acid (20:3 n-6), eicosapentaenoic acid (20:5 n-3), and docosahexaenoic acid (22:6 n-3) in well-characterized cohorts of patients with suspected NAFL or NASH, stratified according to their liver biopsy scores (Table 1). Our initial eicosanoid profile consisted of 158 individual metabolites that our analytical platform can reliably measure. Of these, we detected 26 eicosanoids that were present at measurable levels in the control, NAFL, or NASH plasma samples (**Table 2**). These mediators are generated through complex biosynthetic mechanisms and multiple routes for modification and degradation (27). As shown in **Figs. 1** and **2**, eicosanoids derived from all three major enzymatic pathways, the cyclooxygenase pathway (COX-1 and COX-2), the lipoxygenase pathway (5-LOX, 12-LOX, and 15-LOX), and the cytochrome P450 (CYP) pathway, were present at various amounts in the control, NAFL, and NASH samples. COX-derived thromboxane B_2 (TXB2) and 12-hydroxy-heptatrienoic acid (12-HHTrE), one of the primary AA metabolites produced by thromboxane synthase in

TABLE 2. Eicosanoid metabolites in normal controls versus NAFL versus NASH

	Controls (n = 10)	NAFL (n = 10)	NASH (n = 9)	Control versus NAFL P^a	Control versus NASH P^a	NAFL versus NASH P^a	Compare All Groups P^b
AA-derived metabolites (units = pmol/ml)							
TXB2	0.23 ± 0.22	4.53 ± 8.52	5.47 ± 9.86	0.1452	0.1498	0.8261	0.2704
12-HHTrE	1.06 ± 0.78	9.06 ± 16.00	23.07 ± 37.70	0.1485	0.1181	0.3242	0.1291
PGE2	0.05 ± 0.06	0.05 ± 0.07	14.63 ± 0.12	0.9899	**0.0437**	**0.0485**	**0.0408**
dhk PGD2	0.25 ± 0.16	0.29 ± 0.11	0.72 ± 0.27	0.4895	**0.0002**	**0.0011**	**<.0001**
11-HETE	0.22 ± 0.15	0.63 ± 0.62	0.75 ± 0.80	0.0681	0.0830	0.7134	0.1270
5-HETE	1.04 ± 0.47	3.06 ± 3.65	1.85 ± 0.87	0.1156	**0.0211**	0.3300	0.1427
12-HETE	5.47 ± 4.04	14.15 ± 14.04	13.42 ± 14.77	0.0883	0.1521	0.9141	0.2167
Tetranor 12-HETE	0.24 ± 0.11	0.25 ± 0.23	0.42 ± 0.20	0.8992	**0.0196**	0.0956	0.0714
15-HETE	1.32 ± 0.35	2.08 ± 0.63	1.03 ± 0.53	**0.0036**	0.1684	**0.0011**	**0.0004**
5,6-diHETrE	0.33 ± 0.09	0.53 ± 0.19	1.25 ± 1.20	**0.0109**	0.0521	0.1126	**0.0180**
11,12-diHETrE	0.34 ± 0.18	0.41 ± 0.13	1.11 ± 0.31	0.2949	**<.0001**	**<.0001**	**<.0001**
14,15-diHETrE	0.95 ± 0.31	1.14 ± 0.30	1.64 ± 0.45	0.1847	**0.0011**	**0.0101**	**0.0009**
20-COOH AA	7.41 ± 2.57	10.13 ± 3.45	38.94 ± 42.14	0.0607	0.0552	0.0747	**0.0131**
Alternative substrate-derived metabolites (units = pmol/ml)							
13-HODE	13.27 ± 12.07	10.18 ± 5.24	12.41 ± 8.80	0.4721	0.8629	0.5059	0.7419
9-HODE	7.03 ± 6.14	6.25 ± 3.80	8.82 ± 6.51	0.7384	0.5444	0.3018	0.5984
9-oxoODE	1.59 ± 1.19	1.40 ± 2.00	4.60 ± 4.60	0.7955	0.0887	0.0801	**0.0420**
9,10-EpOME	4.08 ± 2.06	3.72 ± 2.78	3.73 ± 2.57	0.7446	0.7458	0.9928	0.9349
9,10-diHOME	6.67 ± 6.00	3.14 ± 1.27	4.70 ± 2.57	0.0991	0.3611	0.1268	0.1450
12,13-EpOME	1.89 ± 1.53	1.39 ± 1.63	5.64 ± 5.72	0.4936	0.0882	0.0592	0.2048
12,13-diHOME	5.56 ± 2.55	3.59 ± 1.36	4.65 ± 2.07	**0.0446**	0.4103	0.1976	0.1187
9-HOTrE	0.35 ± 0.47	0.25 ± 0.13	0.37 ± 0.22	0.5436	0.8789	0.1520	0.6628
15-HETrE	—	—	0.04 ± 0.06	—	—	—	—
12-HEPE	0.40 ± 0.37	1.40 ± 2.61	1.16 ± 0.90	0.2618	**0.0391**	0.7909	0.3771
14-HDoHE	2.81 ± 2.21	5.71 ± 8.35	3.14 ± 2.70	0.3125	0.7725	0.3765	0.4233
16-HDoHE	0.01 ± 0.02	0.11 ± 0.14	0.18 ± 0.16	0.0542	**0.0139**	0.3312	**0.0201**
19,20-DiHDPA	1.71 ± 0.52	2.39 ± 1.72	3.14 ± 1.28	0.2558	**0.0101**	0.2996	0.0671

Data are expressed as mean ± standard deviation. DiHDPA, dihydroxydocosapentaenoic acid; diHOME, dihydroxyoctadecenoic acid; EpOME, epoxyoctadecenoic acid; HDoHE, hydroxydocosahexaenoic acid; HOTrE, hydroxyoctadecatrienoic acid; oxoODE, oxooctadecadienoic acid.

[a] Differences between groups evaluated with *t*-test.

[b] Comparison between all groups assessed using ANOVA. The *P* values in bold are statistically significant ($P \leq 0.05$).

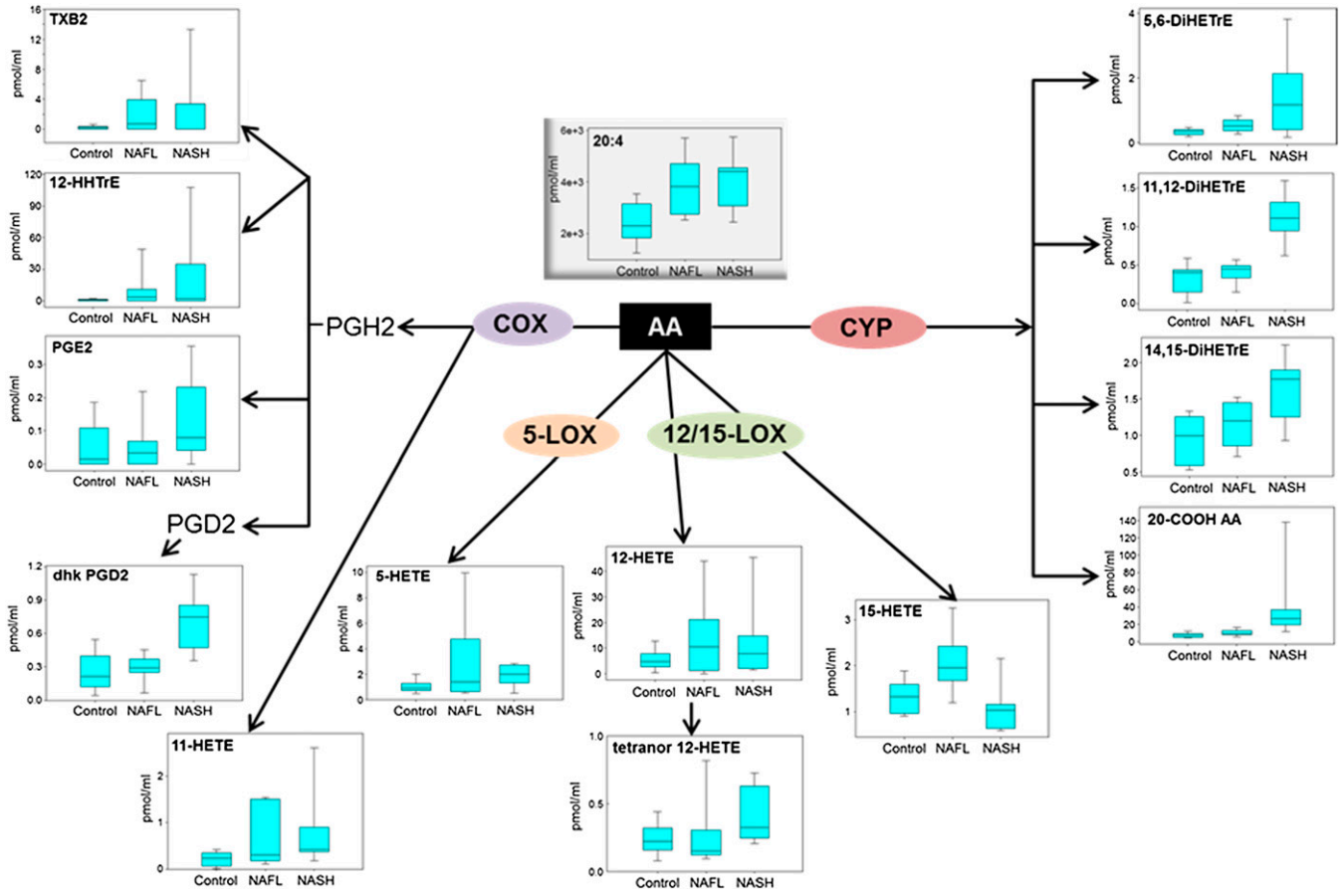

Fig. 1. AA-derived metabolites. The quantitative amounts of plasma free AA and its metabolites derived from COX, 5-LOX, 12/15-LOX, and CYP activities are shown in the three clinical arms for each metabolite. The results are displayed as box-whisker plots with the lower boundary of the box indicating the 25th percentile, the line within the box indicating the median, and the upper boundary of the box indicating the 75th percentile. The whiskers indicate the lower and upper extremes of the data range. The significant differences in amount of the metabolite for intergroup comparison are shown in Table 2.

human platelets, were detected at low levels in the control samples but were significantly higher in both the NAFL and NASH samples; however, no significant difference was found between NAFL and NASH. In contrast, prostaglandin E_2 (PGE2) was elevated only in the NASH samples, and no differences were observed between the controls and NAFL (Fig. 1). Prostaglandin D_2 (PGD2) was not detected in any of the samples, but the degradation product 13,14-dihydro-15-keto PGD2 (dhk PGD2) was significantly higher in NASH compared with NAFL (P value <0.0011) or control (P value <0.0002) (see Fig. 1).

LOX-derived metabolites were also increased in NAFLD. Of note, while the AA-derived products of 5-LOX and 12/15-LOX pathways appear to be highest in NAFL (Fig. 1), the metabolites from related PUFAs including linoleic acid, α-linolenic acid, dihomo-γ-linolenic acid, EPA, and DHA were generally higher in NASH (Fig. 2). Similarly, the AA-derived metabolites of the CYP pathway were predominantly elevated in NASH but unchanged in NAFL compared with healthy controls (Figs. 1, 2). In particular, 11,12-dihydroxy-eicosatrienoic acid (11,12-diHETrE) and 14,15-dihydroxy-eicosatrienoic acid (14,15-diHETrE) were significantly elevated in NASH compared with NAFL or controls. These metabolites are produced by the action of soluble epoxide hydrolase (sEH) on epoxyeicosatrienoic acids, which are the primary products of the epoxygenase pathway of CYP on the initial substrate AA. A number of biological effects have been ascribed to epoxyeicosatrienoic acids including cardioprotective vasodilation and leukocyte antimigratory and anti-inflammatory actions (28). Conversion of the epoxides to their corresponding diols by the sEH decreases their functional levels and thereby diminishes the associated health benefits. Similarly, 20-HETE, an AA metabolite synthesized by the CYP hydroxylase pathway, is reported to have important vasoactive properties (29). We did not detect 20-HETE in plasma, but we found a consistent increase of 20-carboxy arachidonic acid (20-COOH AA) in NASH samples. However, it did not reach statistical significance when comparing NAFL versus NASH (Table 2). The conversion of 20-HETE to 20-COOH AA is catalyzed by CYP enzymes and is responsible for reduced bioactivity.

Identification of a panel of eicosanoids as a diagnostic tool for detecting NASH

Based on Table 2, we found nine biomarkers to be significant in the assessment of NAFLD. We assessed their individual diagnostic test performances using AUROC

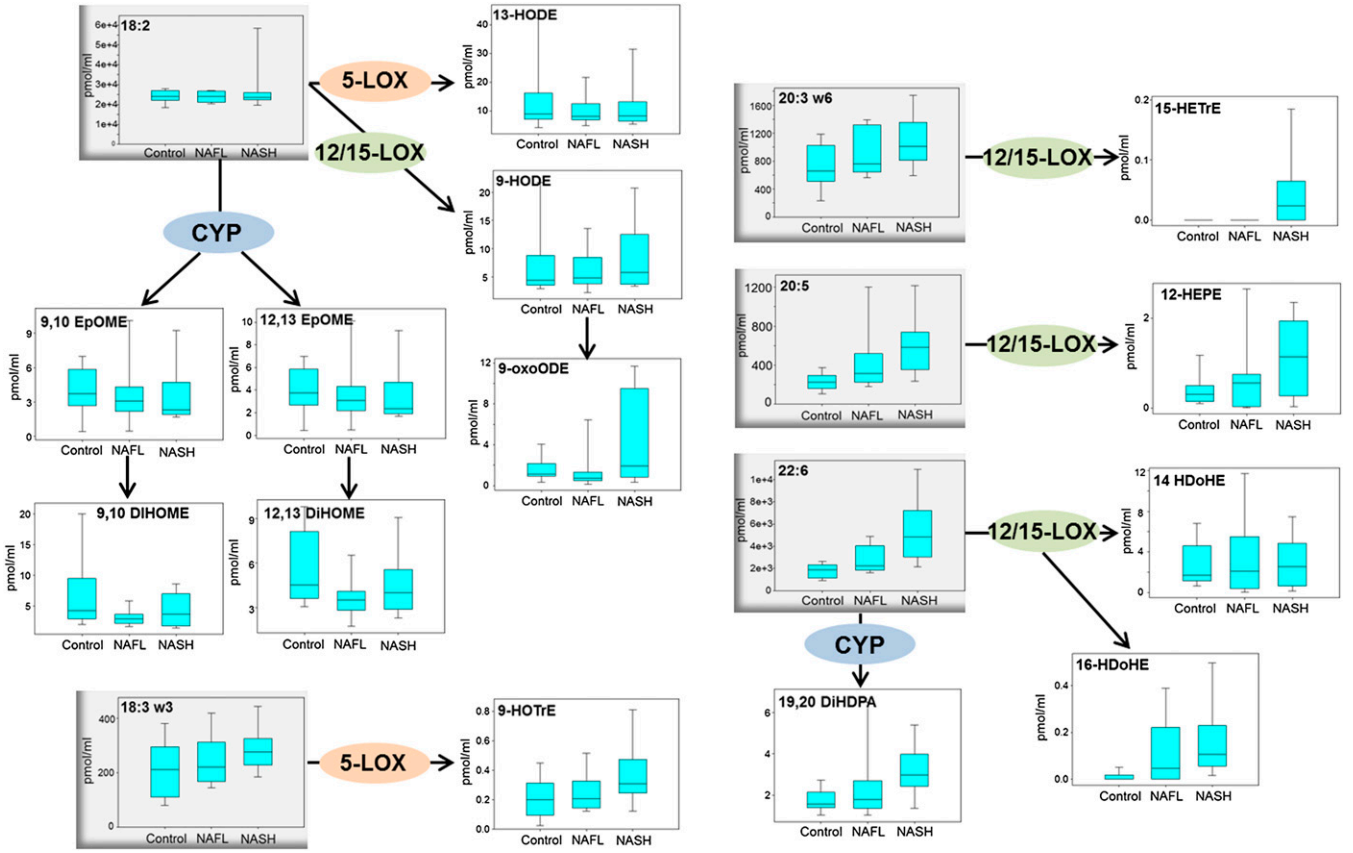

Fig. 2. Alternative substrate-derived metabolites. The quantitative amounts of plasma free linoleic acid, α-linolenic acid, eicosatrienoic acid, EPA, and DHA and their metabolites derived from 5-LOX, 12/15-LOX, and CYP activities are shown in the three clinical arms for each metabolite. The results are displayed as box-whisker plots with the lower boundary of the box indicating the 25th percentile, the line within the box indicating the median, and the upper boundary of the box indicating the 75th percentile. The whiskers indicate the lower and upper extremes of the data range. The significant differences in amount of the metabolite for intergroup comparison are shown in Table 2.

and reported them in **Table 3**. The top candidate as a single biomarker for differentiating NAFL from NASH was 11,12- diHETrE with an AUROC of 1. In addition, we also found a panel including dhk PGD2 and 20-COOH AA that demonstrated an AUROC of 1. These novel, hitherto unrecognized biomarkers need to be confirmed in larger studies and need to be validated.

DISCUSSION

Main findings

Utilizing a uniquely well-characterized cohort of patients with biopsy-confirmed NAFLD including NAFL and NASH, as well as non-NAFLD normal controls, we showed herein that plasma eicosanoid profiling can differentiate between NAFL and NASH. The only previous PUFA metabolites suggested to be elevated in NAFLD over controls were observed for the total plasma content of 9-HODE and 13-HODE, derived by nonenzymatic autooxidation of linoleic acid and linolenic acid, respectively (13). Although in the present study, we discovered several eicosanoid moieties that were significantly different between normal versus NAFL versus NASH, 11,12-diHETrE, dhk PGD2, and 20-COOH AA were greatly elevated in NASH patients versus NAFL versus normal controls in a dose-dependent manner. This proof-of-concept study provides early evidence that 11,12-diHETrE, dhk PGD2, and 20-COOH AA are the leading eicosanoid candidate biomarkers for the noninvasive diagnosis of NASH. Because these findings are derived from a pilot, single-center study, further validation studies should include testing of all three potential biomarkers. Thus, these novel findings, although preliminary in nature, provide the justification to carry out a large, well-designed, multicenter biomarker validation study in noninvasive detection of NASH.

In the context of the published literature

Our results were different than those that were observed by Feldstein and colleagues due to differences in the assessment of the metabolites. Feldstein and colleagues (13) measured total metabolites, that is, both free and those esterified to phospholipids, triglycerides, and so forth. For this they treated plasma with concentrated KOH at high pH to hydrolyze the complex lipids and to liberate the oxidized fatty acids that were then measured. Our approach was completely different; we measured the metabolites that were present in their free form and without KOH treatment. Even though the levels of the free

TABLE 3. Diagnostic accuracy of serum biomarkers in differentiating NAFL from NASH

Biomarker	AUROC	95% CI	P^a
PGE2	0.81	0.60-1.00	**0.0043**
dhk PGD2	0.93	0.82-1.00	**<0.0001**
Tetranor 12-HETE	0.81	0.59-1.00	**0.0077**
15-HETE	0.91	0.76-1.00	**<0.0001**
11,12-diHETrE	1.00	—	—
14,15-diHETrE	0.82	0.62-1.00	**0.0022**
20-COOH AA	0.96	0.86-1.00	**<0.0001**
9-oxoODE	0.73	0.48-0.99	0.0732
12,13-EpOME	0.87	0.68-1.00	**0.0001**
Panel			
dhk PGD2 + 20-COOH AA	1.00	—	—

[a]The *P* values in bold are statistically significant ($P < 0.05$).

eicosanoids are much lower than the eicosanoids esterified to lipids, our approach captures many more metabolites and allows for a much broader profiling strategy. We know from recent studies that KOH destroys all prostaglandins as well as a number of LOX-derived metabolites, thus, it is plausible that Feldstein et al. missed these metabolites in their experiments.

In NAFLD, normal lipid metabolism is disrupted leading to increased levels of free fatty acids and triglyceride synthesis (30–32). Free fatty acids have been shown to elicit hepatotoxicity and may stimulate the progression from NAFL to NASH via several mechanisms (12). They can be directly cytotoxic and stimulate the production of inflammatory pathways in hepatocytes (11, 12). These fatty acids also serve as precursors for inflammatory eicosanoids. Consistent with this, the plasma levels of several free PUFAs were consistently higher in NAFL and NASH compared with healthy controls (Figs. 1, 2). However, there were no significant differences between NAFL and NASH suggesting that plasma free fatty acids are poor markers for differentiating between the various stages of NAFLD. By contrast, their conversion to eicosanoids may constitute a critical mechanism in disease progression, and the analysis of eicosanoid levels, rather than fatty acid levels, may be a useful clinical tool to discriminate between NAFL and NASH.

The strengths of the study include utilization of well-characterized patients with biopsy-confirmed NAFLD, including patients with NAFL and NASH, as well as MRI-PDFF-confirmed non-NAFLD controls; plasma having been stored at −80°C under identical conditions for all the participants included in the study; and utilization of uniform criteria for the diagnosis of NAFLD. Limitations include the cross-sectional nature of the study, the small sample size of this proof-of-concept study, and the lack of a large validation cohort. We plan to undertake a large, longitudinal validation cohort study in the future to confirm these findings. Further studies are needed to assess the association between the gene expression of enzymes and their plasma metabolite concentration differentiating patients with NAFL from NASH. Finally, additional studies are needed to assess the association of these biomarkers in differentiating primary NAFLD from secondary causes of NAFLD such as viral hepatitis and alcohol use.

Plasma eicosanoid profiling may provide a novel biomarker candidate for noninvasive detection of NASH. Further research is needed to assess the accuracy and reliability of these candidate biomarkers obtained by a relatively noninvasive blood lipidomic profiling for the diagnosis of NASH. Specifically, it will be important to establish whether a single eicosanoid biomarker will have sufficient diagnostic capacity or whether it should be used along with other eicosanoids as a panel of biomarkers. Large, multicenter, longitudinal clinical trials will be needed to assess the utility of these lipidomic biomarkers for diagnosis as well as clinical follow- up, and eventually to document whether they predict long-term clinically meaningful outcomes such as development of cirrhosis, hepatocellular carcinoma, or liver-related death.

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