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A liquid chromatography–mass spectrometry-based workflow for measurement of branched Fatty Acid esters of Hydroxy Fatty Acids (FAHFAs)

Tejia Zhang¹, Shili Chen¹, Ismail Syed², Marcus Ståhlman³, Matthew J. Kolar¹, Edwin A. Homan⁴, Qian Chu¹, Ulf Smith⁵, Jan Borén³, Barbara B. Kahn^{2,*}, and Alan Saghatelian^{1,*}

Tejia Zhang: tzhang@salk.edu; Shili Chen: shchen@salk.edu; Ismail Syed: isyed@bidmc.harvard.edu; Marcus Ståhlman: Marcus.Stahlman@wlab.gu.se; Matthew J. Kolar: mkolar@salk.edu; Edwin A. Homan: edwin.homan@gmail.com; Qian Chu: qchu@salk.edu; Ulf Smith: ulf.smith@medic.gu.se; Jan Borén: Jan.Boren@wlab.gu.se; Barbara B. Kahn: bkahn@bidmc.harvard.edu; Alan Saghatelian: asaghatelian@salk.edu

¹Salk Institute for Biological Studies, La Jolla, CA, USA

²Division of Endocrinology, Diabetes & Metabolism, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, USA

³Wallenberg Laboratory, Department of Molecular and Clinical Medicine, University of Gothenburg, Gothenburg, Sweden

⁴Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA, USA

⁵Lundberg Laboratory for Diabetes Research, Department of Molecular and Clinical Medicine, University of Gothenburg, Gothenburg, Sweden

Abstract

Branched Fatty Acid esters of Hydroxy Fatty Acids (FAHFAs) are a recently discovered class of endogenous mammalian lipids with anti-diabetic and anti-inflammatory effects. We identified 16 different FAHFA families, such as branched Palmitic Acid esters of Hydroxy Stearic Acids (PAHSA), and each family consists of multiple isomers where the branched ester is at different positions (e.g. 5- and 9-PAHSA). We anticipate increased need for PAHSA measurements as markers of metabolic and inflammatory diseases. In this protocol, we provide a detailed description of the extraction and subsequent liquid chromatography–mass spectrometry (LC–MS) of FAHFAs from human or mouse tissues. For a sample size of 6–12 the time frame is 2–3 days.

*Co-Corresponding authors: Barbara B. Kahn, tel.: (617) 735-3324, fax: (617) 735-3323, bkahn@bidmc.harvard.edu; or Alan Saghatelian, tel.: (858) 453-4100 × 1435, fax: (858) 552-1546, asaghatelian@salk.edu.

AUTHOR CONTRIBUTIONS: T.Z. developed the experimental design strategy (FAHFA extraction, SPE and LC–MS), the quality control strategy, performed sample measurements and wrote the manuscript. S.C. performed ongoing optimizations on the first-generation LC–MS method and sample measurements. I.S. performed sample measurements. M.S. developed additional offline HPLC methods for FAHFA enrichment. M.J.K. performed additional optimizations toward contaminant elimination and sample measurements. Q.C. performed *in vitro* activity assays to examine possible exogenous standard incorporation into FAHFAs during lipid extraction. E.A.H. performed initial global lipidomics and tandem MS. A.S., B.B.K., J.B. and U.S. conceived of, designed and supervised the experimental plan and interpreted experiments. A.S., B.B.K., T.Z., S.C., I.S. and M.S. edited the manuscript.

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INTRODUCTION

Type 2 diabetes mellitus (T2D) is a metabolic syndrome affecting more than 300 million people worldwide^{1–3}. Metabolic changes associated with insulin resistance and T2D are complex and involve interactions among many major branches of cellular metabolism^{4,5}. GLUT4 is the major insulin-sensitive glucose transporter and is expressed at highest levels in skeletal muscle, heart and adipocytes. GLUT4 mediates glucose uptake into tissues in response to a rise in circulating insulin levels following food ingestion^{6,7}. In humans with obesity, impaired glucose tolerance, or T2D, GLUT4 expression is lower in adipocytes but not in skeletal muscle^{8,9}. We generated mice with adipose-specific GLUT4 overexpression (AG4OX) to determine the impact of alterations in adipocyte GLUT4 expression on systemic insulin sensitivity. AG4OX mice display enhanced glucose tolerance in spite of obesity⁷. In these mice, expression of the glucose-regulated transcription factor, carbohydrate responsive element binding protein (ChREBP), is induced in adipose tissue and drives *de novo* lipogenesis, which plays a major role in the enhanced glucose tolerance in these mice^{7,8,10}.

Using liquid chromatography–mass spectrometry (LC–MS), we performed a comprehensive, unbiased comparison of lipid profiles from wildtype (WT) and AG4OX mice to determine the mechanisms by which GLUT4 upregulation affects lipid metabolism and how this promotes insulin sensitivity. Untargeted global lipidomics on WT and AG4OX adipose tissue led to the identification of a novel lipid class that is markedly upregulated in AG4OX adipose depots relative to WT⁶. Using a combination of tandem MS and chemical synthesis, the structure of these lipids was determined to be branched fatty acid esters of hydroxy fatty acids, which we refer to as Fatty Acid Hydroxy Fatty Acids (FAHFAs)⁶.

We also showed that FAHFAs have beneficial biologic effects including enhancing glucose tolerance, promoting secretion of glucagon-like peptide-1 and insulin, augmenting insulin-stimulated glucose transport, and reducing pro-inflammatory responses⁶. Using targeted lipidomics, we identified different FAHFA family members based on the structural variations of the component fatty acid and hydroxy fatty acid; the esterification of hydroxy stearic acid with palmitic acid, for example, yields the Palmitic Acid Hydroxy Stearic Acid (PAHSA) family of FAHFAs (Fig. 1a). Furthermore, for each FAHFA family, we identified multiple isomers that differ at the position of the ester group. The esterification of 5-hydroxy stearic acid and 9-hydroxy stearic acid with palmitic acid forms 5-PAHSA and 9-PAHSA, respectively (Fig. 1a)⁶. Due to identical chemical formulae for 5- and 9-PAHSAs, accurate quantification of different PAHSA isomers from sera and tissues by LC–MS requires significant optimization. We anticipate high interest in PAHSA quantification, because these are novel endogenous lipids with roles in a number of important physiological processes. This protocol describes the key steps for accurate and quantitative measurement of PAHSAs in sera and tissues.

Background

Metabolomics is a group of techniques that enables quantification of small-molecule metabolites and comparison of metabolic profiles across different biological samples¹¹. The two most commonly used detection methods in modern metabolomics are nuclear magnetic

resonance (NMR) and mass spectrometry (MS)¹¹. NMR has been employed in the analysis of a wide range of sample types including biofluids, tissues, plants and natural products¹². While the advantages of NMR are its ability to monitor different spin $\frac{1}{2}$ nuclei, detailed structural elucidation, and nondestructive data acquisition, the increased sensitivity associated with MS has resulted in wider metabolome coverage¹¹. Recent advancements in the sensitivity and resolution of mass analyzers such as time-of-flight, ion trap, Fourier transform and triple quadrupole systems have enabled both discovery of novel metabolites and pathways *via* untargeted metabolite profiling^{6,13}; and targeted, accurate metabolite quantification *via* Multiple Reaction Monitoring (MRM)^{14,15}.

In the case of MS, samples can be introduced either directly into the detector or chromatographically resolved prior to MS¹¹. The direct infusion method (also termed shotgun metabolomics) is highly amenable toward high-throughput applications¹⁶. Shotgun metabolomics performed on human plasma led to the detection of important metabolite classes such as amino acids, sugars, sphingolipids, and triglycerides¹⁷. While more time-consuming than the shotgun approach, coupling of liquid chromatography (LC), gas chromatography (GC) or capillary electrophoresis to MS allows separation and quantification of structurally similar metabolites that would not be resolved by direct infusion, and improves detection of low-abundance metabolites that could otherwise be obscured due to signal suppression¹⁸. GC–MS has been used in the measurement of a diverse range of molecules including amino acids, fatty acids, sugars, sugar alcohols and amines, although only volatile compounds are directly detectable and nonvolatile species require derivatization^{19,20}. By altering the column matrix and/or mobile phase, LC–MS could be tailored toward analysis of metabolites with a wide range of physical properties¹¹. Hydrophilic interaction chromatography–MS has been used to effectively resolve water-soluble components of central carbon, amino acid and nucleotide metabolism¹⁴, while reversed-phase chromatography is commonly employed in the analysis of lipids^{13,21–24}. Given the comprehensiveness and high sensitivity of targeted MRM-based LC–MS lipidomics, we have selected this technique for the detection and quantification of PAHSAs.

Experimental design

Optimization for PAHSA measurement is divided into two sections as follows: 1) optimization of PAHSA extraction from sera and tissues (general lipid extraction, solid phase extraction for PAHSA enrichment, and sample type-dependent preparations) and 2) optimization of PAHSA LC–MS settings (optimization of chromatography, optimization of MS detection, and LC–MS sample setup). These steps are addressed in detail in subsequent sections. We also present guidelines on identification of contaminant peaks and background subtraction.

Optimization of PAHSA extraction from sera and tissues

General lipid extraction—Extraction solvents determine the type of metabolites entering the analysis sample and require careful consideration. A widely used protocol for extraction of common lipid classes (e.g. fatty acids, phospholipids, sphingolipids, acylglycerols, cholesterol and cholesterol esters) is the Bligh-Dyer method, which relies on a 1:1:2 mixture of aqueous buffer:methanol:chloroform²⁵. We applied the Bligh-Dyer method with slight

modifications toward extraction of lipids from murine sera and tissues, using citric acid buffer (100 mM sodium citrate tribasic dihydrate, 1 M sodium chloride, pH 3.6) as the aqueous phase; the increased salt content of this buffer enhances separation between organic and aqueous phases while the reduced pH allows protonation of acidic lipids, facilitating their movement into the organic phase. We have successfully applied this method toward lipid isolation for comparative lipidomics in several biological settings^{21,26,27}.

When we used this method to extract lipids from 130 mg WT murine perigonadal white adipose tissue (PG WAT) as preliminary test, we were able to detect 13/12, 11, 10 and 9-PAHSAs by LC–MS, but the ion counts were low for all species (Supplementary Fig. 1). This was due to high concentrations of neutral lipids such as triglycerides (TGs) in WAT²⁸. TGs are present as an oil at room temperature, and make up a large proportion of the volume of the reconstituted sample. Their high concentration limits the amount of sample that can be injected while increasing ion suppression. TGs have poor solubility in water or methanol, which restricted the carrier solvent to chloroform and prevented injection of large sample volumes because chloroform has unfavorable effects on the chromatographic separation on C18 stationary phase (see *Optimization of chromatography* section). To address this problem, we chose to fractionate the lipid extract prior to LC–MS to enrich the PAHSAs.

Solid phase extraction for PAHSA enrichment—Solid phase extraction (SPE) is a widely used technique that separates analytes in solution based on their interactions with a solid support²⁹. In the context of metabolomics, SPE enables enrichment of specific metabolite pools while removing other metabolites and contaminants that could cause signal suppression and/or impair chromatographic resolution^{30,31}. Careful choice of solid support and elution solvent allows selective enrichment of metabolites with a wide range of structures and polarities. We developed an analytical-scale SPE protocol based on our hexane:ethyl acetate flash chromatography method employed during synthesis of PAHSA standards^{5,6}. In this step, a silica extraction cartridge is used. The cartridge is first washed with hexane, neutral lipids such as TGs and cholesterol esters are removed with 95:5 hexane:ethyl acetate, and then FAHFAs are eluted with ethyl acetate, collected in 1 ml fractions (**PROCEDURE**, steps 6–16). The resulting samples can be reconstituted in methanol, which was previously unfeasible due to the poor solubility of TGs, enabling analysis of larger sample quantities through injection of higher sample volumes.

We tested this method on a solution of synthetic 12, 11, 10, 9, ¹³C-9, ¹³C-8, 6, 5 and 4-PAHSA standards, and have included details on the optimization process and results in Supplementary Note 1.

Sample type-dependent preparations—In our initial experiments, we analysed murine serum, subcutaneous (SQ) and perigonadal (PG) WATs, brown adipose tissue (BAT) and liver (chromatograms are in **ANTICIPATED RESULTS**). In principle any liquid or homogenizable semi-solid or solid material could be used. The main points to consider are sample stability and quantity for extraction. Biological materials such as sera and tissues should be flash frozen immediately in liquid nitrogen upon collection, and stored at –80 °C. To minimize sample degradation, we recommend thawing biofluids on ice. Frozen tissue aliquots should be transferred into a precooled tissue grinder on ice and homogenized

immediately in the Bligh-Dyer solvent to minimize thawing prior to lipid isolation. It is also important to optimize the quantities of sample and internal standard for each new sample type to ensure adequate LC–MS signal for quantification without saturating the SPE column during extraction.

Our procedure specifies the use of $^{13}\text{C}_{16}$ -9-PAHSA as internal standard for quantification (**PROCEDURE**, step 2). To ensure consistency in the amount of standard added to each sample, the total amount of internal standard calculated for all samples to be extracted should be dissolved in chloroform first, and the chloroform distributed equally among the samples. A list of serum volume, tissue weights and standard quantities is provided in Table 1 as guide; the exact quantity of standard to be added depends on the biological sample and could vary based on human subject or mouse strain, and should therefore be empirically determined by the user. Typically, ion intensity of the added standard should fall within one order of magnitude of the intensity of the corresponding endogenous lipid to ensure accurate quantification. In cases where this is not possible, ^{13}C -9-PAHSA standard should be adjusted such that its ion intensity falls in the middle of the highest and lowest PAHSA isomer intensities. While only the ^{13}C -9-PAHSA internal standard was used within the scope of our current protocol, incorporation of additional ^{13}C -PAHSA standards to accommodate the entire range of PAHSA isomer concentrations could improve the accuracy of quantification.

To ensure efficient lipid extraction, the aqueous buffer:methanol:chloroform ratio should be maintained at 1:1:2 (v:v:v) or as close to this ratio as possible; when extracting lipids from serum, the volume of the serum should be taken into account as a portion of the aqueous volume. SPE was performed for all sera and tissue lipids prior to LC–MS. The use of more than 150 mg adipose tissue or 80 mg liver is not recommended, as this could cause column overloading and carryover of neutral lipids (visible as a clear to yellow oil) into the ethyl acetate fraction during elution. Typical serum volume for extraction is 150–300 μL . A larger serum volume can be used if not limited by sample availability; however, care should be taken to ensure no overloading of SPE column (we have previously extracted up to 900 μL human serum successfully).

Optimization of PAHSA LC–MS settings

Optimization of chromatography—PAHSAs were initially identified by global lipidomics of WT and AG4OX mice in negative ionization mode, using a reversed-phase water-isopropanol LC gradient and C4 or C18 columns^{6,27}. This chromatographic method is optimized for lipidomics of a wide range of lipid families and does not separate different PAHSA isomers. We therefore needed to develop new liquid chromatography conditions for the more challenging separation of PAHSA isomers.

The conditions described in the **PROCEDURE** (step 27) were optimized using a solution containing 12, 11, 10, 9, ^{13}C -9, ^{13}C -8, 6, 5 and 4-PAHSA standards on a C18 column (3 μm , 100 $\text{\AA}$, 250 $\times$ 2.0 mm)⁶. This column is longer and the particle sizes is smaller than those in the columns we use for global lipidomics. The increased column length allows more time for the peaks to separate, and the smaller particle size usually results in sharper peaks. More details on the development of this LC method can be found in Supplementary Note 2.

Optimization of MS detection—The low biological abundance of PAHSAs relative to major lipid families such as fatty acids and acylglycerols presented a challenge for measurement of these lipids in sera and across multiple tissue types. To reduce quantity of sample necessary for analysis and improve quantification, we developed a targeted approach using a triple quadrupole mass spectrometer operated in Multiple Reaction Monitoring (MRM) mode.

MRM has emerged as a powerful analytical technique that enables targeted, simultaneous quantification of multiple metabolites with high sensitivity^{14,15,27,32,33}. By subjecting the metabolite of interest (precursor ion) to an optimized set of fragmentation conditions and monitoring a specific fragment (product ion) that consistently originates from the precursor, MRM takes advantage of the characteristic precursor-to-product ion transition specific to each metabolite, allowing unambiguous identification and detection of known metabolites with high signal-to-noise ratio (Fig. 1b)^{6,15,32}. As most metabolites with distinct structures will generate unique transitions, it is possible for many species to be monitored at once. Given many metabolites fragment to yield multiple product ions at consistent ratios, multiple transitions could be optimized and monitored for each metabolite to increase the confidence of identification.

Tandem MS of 9-PAHSA (m/z 537) in negative ionization mode yielded three characteristic product ions at m/z 255, 281 and 299 (Fig. 1c), corresponding to precursor-to-product ion transitions m/z 537 $\rightarrow$ 255, m/z 537 $\rightarrow$ 281 and m/z 537 $\rightarrow$ 299 (Fig. 1d). Fragmentor voltage for the precursor ion and collision energies for all fragments were empirically optimized to maximize signal-to-noise ratio for each transition.

Of the fragments observed, m/z 255 corresponds to palmitic acid, m/z 299 to hydroxystearic acid, and m/z 281 to C18:1 fatty acid (Fig. 1c)⁶. 9-PAHSA consistently fragments with the relative fragment abundances m/z 255 > 281 > 299, which is reflected in the relative abundances of the corresponding precursor-to-product ion transitions (m/z 537 $\rightarrow$ 255 > m/z 537 $\rightarrow$ 281 > m/z 537 $\rightarrow$ 299) (Fig. 1d). The m/z 537 $\rightarrow$ 255 transition yielded the highest ion count in all PAHSA (12, 11, 10, 9, 6, 5 and 4) standards tested (Fig. 1e, f), a likely consequence of stability of the palmitic (m/z 255) ion relative to the more chemically labile hydroxystearic (m/z 299) ion, which undergoes further fragmentation to yield C18:1 fatty acid (m/z 281). It is worth noting that while m/z 537 $\rightarrow$ 255 is the major transition for all PAHSA standards, relative ratios of detected transitions depend on isomer position (Fig. 1e, f). A lower 255/281 ratio was found for 6-PAHSA relative to 9-12-PAHSAs while m/z 537 $\rightarrow$ 255 was the only significantly detectable transition for 4-PAHSA (Fig. 1e, f). The m/z 537 $\rightarrow$ 255 transition is therefore the most reliable choice for use in quantification; however, we included all three transitions in our measurements given the ratios among these transitions are FAHFA-dependent and provide a simple method to exclude non-PAHSA contaminants.

LC–MS sample setup—We recommend performing LC–MS on five biological replicates for each sample type to check that there is no drastic shift in the retention times of the peaks. There should be no significant carryover in a blank sample analysed after the fifth biological sample. Recommended injection volumes and an example injection order (worklist) are

detailed in **PROCEDURE** (step 27). Blank samples should be run at the beginning and end of each worklist to completely remove carryover from previous and for subsequent datasets. A solution of 12, 11, 10, 9, ^{13}C -9, ^{13}C -8, 6, 5 and 4-PAHSA standards (IS_L) should also be run before and after the biological samples to confirm no loss of column resolution.

Assuming that you are confident that there is no significant carryover following five sample runs (~10 h), we recommend running a blank sample at the end of every five to six biological samples (rather than after each sample). The column can also be washed with isopropanol at the end of the experiment to thoroughly dissociate any remaining bound lipids before storage in solvents recommended by the manufacturer. If comparison between two groups of samples (e.g. wildtype vs. knockout) is necessary, samples from different groups should be alternated in the worklist to minimize any time-dependent fluctuations in acquisition conditions.

While retention time should not change drastically within each dataset, we find that there is some variability in retention time across datasets run on separate days. This effect can be reduced by preparing separate bottles of A (100 % water) and B (100 % methanol) mobile phase solutions and using the LC solvent mixer to achieve the final elution percentage. It is also acceptable to prepare one bottle of 93 % methanol in water; however, this could lead to more retention time drift across datasets due to measuring inaccuracies. Samples from different datasets can be aligned based on retention time of the ^{13}C -9-PAHSA internal standard in each sample. Given solvent evaporation over time, we recommend reconstitution of no more than two samples in chloroform and reconstitution of no more than four samples in methanol at once for LC-MS, assuming 10 μL volume per sample; solvent addition should be staggered corresponding to the order of the worklist and all prepared samples should be kept at 10 $^\circ\text{C}$ to minimize solvent evaporation.

Contaminants and background subtraction

While MRM targets the precursor-to-product ion transition unique to each metabolite, false positives are still possible in cases where two different metabolites share similar precursor and product ion masses, because the mass spectrometer is not set up to obtain the exact masses of the ions (in MRM). Therefore, monitoring of multiple transitions per metabolite increases the confidence of identification. For the majority of PAHSA isomers, appearances of peaks for all three transitions at identical retention times and with the major peak corresponding to m/z 537 $\rightarrow$ 255 are important criteria for the PAHSA structure assignment, and simultaneous monitoring of all three transitions enables identification of contaminant peaks. We provide an example of the identification of false positives under **ANTICIPATED RESULTS**, where the sphingolipid ceramide shares all three transitions with PAHSAs but can be distinguished from the latter based on differences in the ratio of the three transitions and different retention times.

In addition to non-FAHFA contamination that could be introduced during the FAHFA isolation process, it is also important to note that use of SPE columns and aqueous buffers during the FAHFA extraction process yields some background signal in the FAHFA MRM channel. We have observed signals that correspond to ~15 % of the total serum signal as background (Fig. 6). This background signal is negligible for liver and adipose tissues,

which have much higher FAHFA levels (**ANTICIPATED RESULTS**). Consequently, for serum analysis we recommend carrying out a blank (water instead of serum) extraction, followed by SPE and LC–MS. To ensure accurate quantification the background signal from this blank can then be subtracted from the sample signal. We have also found that the instrument, especially the injector, needs to be rigorously cleaned or replaced to ensure that there is no residual background due to injecting large amounts of standards. The procedure provides instructions for lipid extraction, fractionation and LC–MS of 6–12 samples.

MATERIALS

! CAUTION Handle all toxic and flammable chemicals in fume hood. Wear appropriate chemical-resistant protective equipment

▲ CRITICAL Use same grade of solvent as LC mobile phase for cleaning of spatulas and glassware related to LC–MS. Avoid all detergents for cleaning, as this will cause significant instrument contamination. It is highly recommended that a set of weighing equipment and glassware be set aside specifically for LC–MS use. Use glassware instead of plastics whenever possible. Transfer solvents using either Pasteur pipettes or glass serological pipettes.

! CAUTION For all work involving laboratory animals, adhere to all guidelines and regulations under protocols approved by the investigator's institutional animal research review committee. All personnel working with laboratory animals should be trained in animal handling and use appropriate protective equipment.

REAGENTS

- Human serum **! CAUTION** Adhere to all guidelines and regulations associated with collection and use of human serum. All personnel working with human serum samples should be informed of risks associated with potential blood-borne pathogens and be trained in correct sample handling techniques. Use appropriate personal protective equipment
- WT and AG4OX mice **! CAUTION** Adhere to all guidelines and regulations under protocols approved by the investigator's institutional animal research review committee, all personnel working with laboratory animals should be trained in animal handling and use appropriate protective equipment (i.e. gloves, face mask and protective clothing) as set by the institution's animal facility
- Water for citric acid buffer (molecular biology grade, Fisher Scientific, cat. no. MT-46-000-CM)
- Water for LC–MS (B&J® Brand Multipurpose ACS, HPLC, VWR, cat. no. BJ365)
- Methanol (B&J® Brand Multipurpose ACS, HPLC, VWR, cat. no. BJ230) **! CAUTION** Methanol is toxic and highly flammable and should be handled in the fume hood

- Methanol for offline HPLC, step 6(B) (hypergrade for LC–MS LiChrosolv®, Merck Millipore, cat. no. 1060351000)
- Isopropanol (B&J® Brand Multipurpose ACS, HPLC, VWR, cat. no. BJ323) ! **CAUTION** Isopropanol is flammable and should be handled in the fume hood
- Isopropanol for offline HPLC, step 6(B) (LC-MS CHROMASOLV®, Fluka, cat. no. 34965)
- Hexane (B&J® Brand Multipurpose, VWR, cat. no. BJ212) ! **CAUTION** Hexane is harmful, dangerous for the environment and highly flammable and should be handled in the fume hood
- Ethyl acetate (B&J® Brand Multipurpose ACS, HPLC, VWR, cat. no. BJ100) ! **CAUTION** Ethyl acetate is highly flammable and an irritant and should be handled in the fume hood
- Chloroform (contains amylene preservative, VWR, cat. no. BJ049) ! **CAUTION** Chloroform is harmful and should be handled in the fume hood
- Chloroform for offline HPLC, step 6(B) (HPLC grade, Rathburn Chemicals, cat. no. RH1009)
- n-Heptane (HPLC Grade, 99+%, Rathburn Chemicals, cat. no. RH1004) ! **CAUTION** Hexane is harmful, dangerous for the environment and highly flammable and should be handled in the fume hood
- Tetrahydrofuran (UV, HPLC Grade, 99.7+% min, unstab., Alfa Aesar, cat. no. 22904) ! **CAUTION** Tetrahydrofuran is harmful and flammable and should be handled in the fume hood; this product is inhibitor-free and may form explosive peroxides overtime, discard or test for peroxide formation after one year
- Acetone (HPLC Grade, Rathburn Chemicals, cat. no. RH1021) ! **CAUTION** Acetone is highly flammable and should be handled in the fume hood
- Dichloromethane (HPLC Grade, Rathburn Chemicals, cat. no. RH1010) ! **CAUTION** Dichloromethane is harmful and should be handled in the fume hood
- Dimethyl sulfoxide (DMSO) (B&J® Brand Multipurpose ACS, HPLC, VWR, cat. no. BJ081) ! **CAUTION** DMSO can permeabilize skin to additional chemicals, wear DMSO-resistant protective equipment when handling
- Diethyl ether anhydrous (ACS reagent, 99.0%, contains BHT as inhibitor, Sigma-Aldrich, cat. no. 673811) ! **CAUTION** Diethyl ether is harmful and extremely flammable and should be handled in the fume hood
- Ammonium hydroxide (28 % ammonia in water, 99.99 % trace metals basis, Sigma-Aldrich, cat. no. 338818) ! **CAUTION** Ammonium hydroxide is corrosive and dangerous for the environment and should be handled in the fume hood ▲ **CRITICAL** Ammonia solution is volatile and should be stored tightly capped at 4 °C, when stored at this temperature no change in data quality was observed for at least 6 months

- Ammonium acetate (99.999 % trace metals basis, Sigma-Aldrich, cat. no. 372331) **▲ CRITICAL** Ammonium acetate is hygroscopic and should be stored in a drybox (see “Dry-Keeper™ desiccator cabinet” under EQUIPMENTS section) with desiccant (see “DRIERITE®” under REAGENTS section), water absorption will occur over time and the bottle should be replaced when significant stickiness and clumping are observed
- Sodium citrate tribasic dihydrate (for molecular biology, 99.0 %, Sigma-Aldrich, cat. no. C8532)
- Sodium chloride (Fisher BioReagents, 99.5 %, Fisher Scientific, cat. no. BP358-212)
- Ethanolamine (CTL Scientific Supply Corp., cat. no. 103254P)
- Hydrochloric acid (GR ACS, 36.5–38.0 %, EMD Millipore, cat. no. HX0603) ! **CAUTION** Hydrochloric acid is corrosive and should be handled in the fume hood
- Acetic acid (anhydrous, Merck Millipore, cat. no. 1000631000) ! **CAUTION** Acetic acid is corrosive and should be handled in the fume hood
- DRIERITE®, regular (8 mesh), non-indicating (calcium sulphate, VWR, cat. no. JTL056)
- DRIERITE®, indicating (8 mesh) (calcium sulphate with indicator (cobalt (II) chloride), VWR, cat. no. JTL058) ! **CAUTION** Indicating DRIERITE is toxic and dangerous for the environment and should be handled in the fume hood
- Sodium sulfate, ACS reagent, 99.0 %, anhydrous, granular (Sigma-Aldrich, cat. no. 239313)
- ESI-L low concentration tuning mix (Agilent Technologies, cat. no. G1969-85000) ! **CAUTION** Tuning mix contains mass standards in 95: 5 acetonitrile: water, acetonitrile is harmful and highly flammable and should be handled in the fume hood **▲ CRITICAL** Tuning mix should be stored at 4 °C and small portions of the tuning mix removed as needed for MS calibration
- $^{13}\text{C}_{16}$ -5-PAHSA, $^{13}\text{C}_{16}$ -9-PAHSA (Fig. 2) and non-labeled 12- through 4-PAHSA standards were chemically synthesized following standard synthetic techniques.¹ $^{13}\text{C}_4$ -9-PAHSA standard is commercially available (Cayman Chemical, cat. no. 17725) **▲ CRITICAL** heavily deuterated PAHSA standards display a significant forward retention time shift relative to endogenous lipids when analyzed using our LC method (Fig. 2), consequently we strongly recommend the use of ^{13}C standards when retention time alignment is necessary for isomer identification

EQUIPMENT

- Agilent 6410 Triple Quadrupole mass spectrometer (Agilent Technologies)

- Agilent 1200 Series LC (Agilent Technologies) ▲ **CRITICAL** We recommend operation of the indicated LC–MS model as the minimal spec required for PAHSA quantification
- Agilent MassHunter software package for acquisition and analysis, version B.03.01 (Agilent Technologies)
- Agilent Quaternary 1200 pump for offline HPLC, controlled by Chromeleon 6.80
- Upchurch Scientific® PEEK™ tubing, IDEX Health & Science (red, 0.005 in × 1/16 in, 7000 psi, VWR, cat. no. 21511-392) ▲ **CRITICAL** Due to sensitivity of the chromatographic separation the internal diameter of the tubing should be kept consistent between the LC and column, and between column and MS
- PK one-piece fitting for 1/16 in OD tubing (IDEX Health & Science, cat. no. PK-120BLCx)
- Sure-Lok fitting tightening tool (aluminum, Phenomenex, cat. no. AQ0-8530)
- Union (webbed, 1/16 in, 0.020 in thru hole, PEEK™, IDEX Health & Science, cat. no. P-704)
- KrudKatcher ULTRA HPLC in-line filter, 0.5 µm depth filter × 0.004 in ID (Phenomenex, cat. no. AF0-8497)
- Luna® 3 µm C18(2) 100 Å LC column, 250 × 2.0 mm (Phenomenex, cat. no. 00G-4251-B0)
- Spherisorb silica column, 80 Å, 5 µm, 100 × 4.6 mm (Waters, cat. no. PSS830112)
- Abrasive mess 4000 grit (Agilent Technologies, cat. no. 8660-0827)
- Capillary cleaning wire (500 ft spool, Agilent Technologies, cat. no. G1946-80054)
- Cotton-tipped applicator (VWR, cat. no. 89133-814)
- KIMAX* graduated cylinder, 10 mL (Kimble Chase, class A, serialized and certified, VWR, cat. no. 89001-666)
- KIMAX* graduated cylinder, 50 mL (Kimble Chase, class A, serialized and certified, VWR, cat. no. 89001-676)
- KIMAX* graduated cylinder, 250 mL (Kimble Chase, class A, serialized and certified, VWR, cat. no. 89001-674)
- KIMAX* graduated cylinder, 500 mL (Kimble Chase, class A, serialized and certified, VWR, cat. no. 89001-678)
- KIMAX* graduated cylinder, 1000 mL (Kimble Chase, class A, serialized and certified, VWR, cat. no. 89001-670)
- Storage bottle for LC mobile phase, 500 mL (VWR, cat. no. 89000-238)
- Storage bottle for LC mobile phase, 1 L (VWR, cat. no. 89000-240)

- Nalgene™ Teflon™ FEP wash bottle, 125 mL (Thermo Scientific, cat. no. 2403-0125) ▲ **CRITICAL** LC–MS solvents should be stored in phthalate-free Teflon wash bottles; plastic wash bottles should be avoided to prevent leaching of impurities into solvents
- Solvent filtration unit (100 mesh stainless steel support, VWR, cat. no. 26316-728)
- Nylon membrane filter (hydrophilic, 0.20 µm, 47 mm, white, plain, EMD Millipore, cat. no. GNWP04700)
- MS sample insert (250 µL polyspring, glass conical, Thermo Scientific, cat. no. C4010630)
- Blue screw cap (Agilent Technologies, cat. no. 5182-0717)
- Autosampler vial (amber glass with ID patch, 1.8 mL, VWR, cat. no. 46610-726)
- KONTES® Dounce tissue grinders (Kimble Chase, 7 mL, VWR, cat. no. KT885300-0007)
- KONTES® Dounce tissue grinders (Kimble Chase, 15 mL, VWR, cat. no. KT885300-0015)
- KIMAX® sample vial (with PTFE-lined screw cap, Kimble Chase, 4 mL, VWR, cat. no. 66009-981)
- KIMAX® sample vial (with PTFE-lined screw cap, Kimble Chase, 8 mL, VWR, cat. no. 66009-984) ▲ **CRITICAL** It is highly recommended that samples are extracted and stored in vials with PTFE-lined caps instead of caps with paper-based liners to prevent sample contamination from solvent erosion of cap liners
- 20 mL disposable glass vials (VWR, cat. no. 66022-106)
- Glass tubes (12 mL, 16 × 110 mm, BergmanLabora, cat. no. 005-1430)
- 1.2 mL glass vials (Fisher Scientific, cat. no. 212-0206)
- 0.2 mL crimp top tapered vial (Chromacol, cat. no. 02-CTVG)
- Crimp cap (Chromacol, cat. no. 8-AC-ST15)
- 700 Series MICROLITER® Syringes, Hamilton, 25 µL (Model 702 N, 22s gauge, point style 3, VWR, cat. no. 60373-957)
- 700 Series MICROLITER® Syringes, Hamilton, 50 µL (Model 705 N, 22s gauge, point style 3, VWR, cat. no. 60373-968)
- 700 Series MICROLITER® Syringes, Hamilton, 100 µL (Model 710 N, 22s gauge, point style 3, VWR, cat. no. 60373-979)
- Disposal serological pipets (glass, 2 mL, VWR, cat. no. 93000-742)
- Fisherbrand™ microhematocrit capillary tube (heparinized, Fisher Scientific, cat. no. 22-362-566)
- Centrifuge tube, 50 mL (polypropylene, sterile, VWR, cat. no. 93000-032)

- Disposable Pasteur pipette (flint glass, 9 in, VWR, cat. no. 14672-380)
- Small latex bulbs (2 mL, VWR, cat. no. 82024-554)
- General use syringe, 1 mL (BD, cat. no. 309659)
- PVC tubing (1/4 in (ID) × 3/8 in (OD), 1/16 wall thickness, VWR, cat. no. 60985-528)
- HyperSep™ silica SPE column (500 mg bed weight, 6 mL column volume, Thermo Scientific, cat. no. 60108-411)
- Extraction manifold (20 position, 16 × 75 mm tubes, Waters, cat. no. WAT200608)
- Magnetic stir bar (VWR, cat. no. 58948-171)
- Nalgene™ Rapid-Flow™ sterile disposable filter unit (0.2 µm SFCA membrane, Thermo Scientific, cat. no. 161-0020)
- Razor blades (0.009 RD, Fisher Scientific, cat. no. S17302)
- Aluminum foil roll (Fisher Scientific, cat. no. 01-213-104)
- Six port mini-vap evaporator/concentrator (7.5 in (L) × 1.2 in (W), for use with 1–250 mL containers, Sigma-Aldrich, cat. no. 22971)
- Dry-Keeper™ desiccator cabinet (vertical, non-electric, Cole-Parmer, cat. no. EW-08909-52)
- Bath sonicator (Branson Branson™ CPX series ultrasonic cleaner, 2.8 L capacity, Fisher Scientific, cat. no. 15-336-131)
- Analog vortex mixer (VWR, cat. no. 58816-121)
- Cimarec™ digital stirring hotplate (Thermo Scientific, cat. no. SP131325Q)
- B10P benchtop pH meter (VWR, cat. no. 89231-662)
- pH electrode (VWR, cat. no. 89231-580)
- Eppendorf 5810R centrifuge (with adaptors for 15 mL and 50 mL conical tubes, Fisher Scientific, cat. no. 05-413-402)

REAGENT SETUP

Murine serum and tissues

All AG4OX and WT mice (C57BL/6J strain) were age- and sex-matched. Animals were kept on a 12-hour light, 12-hour dark schedule and fed *ad libitum*. For tissue collection, euthanize animals with carbon dioxide and quickly dissect tissues, flash freeze immediately with liquid nitrogen and store at –80 °C. Collect blood in heparinized tubes and centrifuge at 16168 g for 10 min at 4 °C. Flash freeze supernatant immediately with liquid nitrogen and store at –80 °C. Sera and tissues are stable for at least one year when maintained at –80 °C. ! **CAUTION** Adhere to all guidelines and regulations under protocols approved by the investigator's institutional animal research review committee.

Citric acid buffer

This buffer contains 100 mM sodium citrate tribasic dihydrate, 1 M sodium chloride and is adjusted to pH 3.6. Carefully weigh 23.53 ± 0.03 g sodium citrate tribasic dihydrate and 46.75 ± 0.03 g sodium chloride into a clean, dry 1 L glass storage bottle with stir bar, add 800 mL water and stir resulting mixture until fully dissolved. Adjust pH to 3.6 with hydrochloric acid (36.5–38 %). **! CAUTION** Hydrochloric acid is corrosive and should be handled in the fume hood. Wear appropriate personal protective equipment. **▲ CRITICAL** This buffer is stable at room temperature for at least one year. Store buffer at a cool, dry place away from sunlight to minimize bacterial and/or mold growth.

PAHSA internal standard (IS) solutions

! CAUTION Handle all harmful and flammable chemicals in fume hood. Wear appropriate personal protective equipment.

IS_I (individual PAHSA standard solution)—▲ CRITICAL To ensure accuracy of SI solutions, all synthesized PAHSAs should be dried under high vacuum overnight and quality of standard verified by ¹H NMR and MS.

Carefully weigh out between 5 to 10 mg PAHSA standard, transfer into 4 mL sample vial and record exact weight registered on scale. Add chloroform based on recorded standard weight to final PAHSA concentration of 10 mM (5.38 mg/mL non-heavy-labeled standard). Store SI solutions tightly capped at –80 °C. When stored at this temperature no change in data quality was observed for at least six months. Some precipitation may occur at –80 °C, which should re-dissolve upon equilibration to room temperature and vortexing. To prevent solvent evaporation over time, small aliquots of the 10 mM chloroform solution could be further diluted to 100 μM with DMSO, and the resulting solution stored at –80 °C.

IS_L (PAHSA ladder solution)—The PAHSA ladder solution contains a mixture of non-labeled 12, 11, 10, 9, 6, 5 and 4-PAHSAs and ¹³C₁₆-labeled 9 and 8-PAHSAs. Dilute IS_I solutions (in chloroform or methanol) in 1: 1 methanol: chloroform to create the working ladder solution containing 200 nM of each PAHSA standard in a final volume of 0.5–1.0 mL. Store IS_L at –80 °C and use 4 μL for each LC–MS run. When stored at this temperature no change in data quality was observed for at least six months. **▲ CRITICAL** 4 μL IS_L standard yields on average an area count of 10⁵ (m/z 537 → 255 transition) per PAHSA species on the Agilent 6410 LC/MS system; amount of IS_L for injection should be determined empirically by the user for different instrument models.

▲ CRITICAL In cases where IS_I solutions are in DMSO, the DMSO needs to be removed to minimize interference with chromatographic resolution. To remove DMSO, mix all standards and dry under a gentle stream of nitrogen until all volatile solvents have evaporated and only the standards in DMSO remain. Add equal volumes of water and diethyl ether to vial (1.5 mL of each solvent for lipids from IS_L method above), shake vigorously for at least 30 s to enable extraction of PAHSAs into the ether layer. Centrifuge resulting emulsion (2200 g, 6 min, 4 °C) to separate ether (top) and water (bottom) layers and carefully remove ether layer with glass Pasteur pipette. Filter ether layer through sodium

sulfate to remove residual water and dry under a gentle stream of nitrogen. Reconstitute remaining lipids in 0.5 mL 1:1 methanol: chloroform. Use 4 μ L IS_L for each LC–MS run.

Mobile phase solutions

! CAUTION Handle all toxic and flammable chemicals in fume hood. Wear appropriate chemical-resistant protective equipment **▲ CRITICAL** 1.7 mL mobile phase A and 22.3 mL mobile phase B are required per sample for the Luna column separation. The total volume of mobile phases should be calculated based on the number of samples and the amount of additional solvent (100–200 mL) necessary for blanks and purging LC lines. An example sample worklist is provided under **PROCEDURE** (step 27). All solutions should be prepared fresh. Fresh solutions can be used for up to 48 h with no change in data quality.

10 % (v) ammonia hydroxide solution—Carefully transfer 0.1 mL ammonia hydroxide (28 % ammonia in water) to 0.9 mL water in sample vial with PTFE-lined cap using the syringe-Pasteur pipette setup in Fig. 3a. Mix by vortexing and use immediately **▲ CRITICAL** To minimize contamination, vortex carefully to maximize mixing while not allowing any solvent to reach the vial cap.

Mobile phase A: Carefully weigh 192.7 ± 0.5 mg ammonium acetate into a clean, dry 1 L glass bottle. Filter 500 mL water through solvent filtration unit with 0.2 μ m nylon membrane and transfer water into glass bottle containing ammonium acetate, sonicate for 20 min in room temperature water bath to further degas and ensure complete solvation of ammonium acetate. Using the syringe-Pasteur pipette setup in Fig. 3a, add to resulting solution 0.5 mL 10 % (v) ammonia hydroxide solution to achieve a final ammonium hydroxide concentration of 0.01 % (v). Mix thoroughly by gently swirling bottle without allowing any solvent to reach the bottle cap. Use immediately.

Mobile phase B: Carefully weigh 385.4 ± 0.5 mg ammonium acetate into a clean, dry 1 L glass bottle. Filter 1 L methanol through solvent filtration unit with 0.2 μ m nylon membrane and transfer methanol into glass bottle containing ammonium acetate, sonicate for 20 min in room temperature water bath to further degas and ensure complete solvation of ammonium acetate. Using the syringe-Pasteur pipette setup in Fig. 3a, add to resulting solution 1 mL 10 % (v) ammonia hydroxide solution to achieve a final ammonium hydroxide concentration of 0.01 % (v). Mix thoroughly by gently swirling bottle allowing any solvent to reach the bottle cap. Use immediately **▲ CRITICAL** Solubility of ammonium acetate is higher in water than methanol, examine mobile phase B to confirm complete solvation of ammonium acetate before use.

Column wash buffer—Filter 1 L isopropanol through solvent filtration unit with 0.2 μ m nylon membrane. Refill as needed.

Column storage buffer—Prepare 1 L solution of 65:35 acetonitrile:water, filter through solvent filtration unit with 0.2 μ m nylon membrane. Refill as needed.

Needle wash buffer—Filter 1 L isopropanol through solvent filtration unit with 0.2 μm nylon membrane. Refill as needed.

EQUIPMENT SETUP

Syringe-Pasteur Pipette system

Setup as shown in Fig. 3a.

LC-MS

Equipment setup is instrument-dependent and should be performed by the user based on manufacturer's instructions. Detailed initial setup and maintenance procedures are provided for the Agilent 6410 LC/MS system as example in Supplementary Note 3.

PROCEDURE

! CAUTION Handle all toxic, harmful and flammable chemicals in fume hood. Wear appropriate chemical-resistant protective equipment. Follow all relevant ethical guidelines and regulations for the collection and use of human sera, and handling of laboratory animals

▲ CRITICAL Use glassware instead of plastics whenever possible. Transfer solvents using either Pasteur pipettes (use the syringe-pipette setup in Fig. 3a for improved control) or glass serological pipettes

Prepare reagents and glassware for extraction ● TIMING 30 min

- 1 | Remove glass vial of 10 mM or 100 μM IS_I $^{13}\text{C}_{16}$ -9-PAHSA solution from $-80\text{ }^{\circ}\text{C}$ storage and equilibrate to room temperature for 20 min
- 2 | Prepare a working solution of $^{13}\text{C}_{16}$ -9-PAHSA (0.5–5 pmol per sample) in chloroform (2–3 mL per sample) and store at $4\text{ }^{\circ}\text{C}$ prior to extraction **▲ CRITICAL STEP** Calculate the total chloroform volume for number of samples + 1, using 2–3 mL chloroform per sample. Amount of $^{13}\text{C}_{16}$ -9-PAHSA needed is sample-dependent and should be determined empirically by the user, recommended values for selected WT murine tissues and human or WT murine serum are provided in Table 1.
- 3 | Cool sample vials and tissue grinders on ice for at least 5 min prior to performing the extraction **▲ CRITICAL STEP** Cooling glassware for too long could lead to water condensation inside tissue grinders

Prepare sera and tissue samples ● TIMING 30 min

- 4 | Follow either option (A) or option (B) for preparation of murine tissues or sera, respectively
 - A. Murine tissue
 - i. Keep tissues on dry ice as much as possible. Cut quantity of tissue (50–150 mg) needed with razor blade, quickly weigh on scale at room temperature before transferring sample back onto dry ice.

- ii. Record weight and keep all weighed tissues on dry ice prior to extraction **▲ CRITICAL STEP** Cover or wrap weighed tissues in aluminum foil to prevent frost formation while on dry ice

B. Serum

- i. Remove sera samples (typical volume for extraction is 150–300 μ L) from -80°C storage and leave on ice (~ 30 min) until thawed

Lipid extraction

- 5 | Follow either option (A) or option (B) for lipid extraction from tissues or sera, respectively. An image of the required glassware and summary of workflows are in Fig. 3a–c **▲ CRITICAL** the inclusion of a solvent-only negative control from this step onward is strongly recommended, as this enables background subtraction and more accurate quantification if any background signal is present **! CAUTION** To minimize risk of injury by broken glass when using the syringe-Pasteur pipette setup in Fig. 3a, take caution when connecting or removing pipette to and from the Teflon tubing connected to the syringe

(A) Extract lipids from murine tissues (Fig. 3b) ● TIMING 2 h

- i. Transfer weighed murine tissues into precooled tissue grinders on ice
- ii. Using the syringe-Pasteur pipette setup in Fig. 3a or glass serological pipet, immediately add 1.5 mL citric acid buffer, followed by 1.5 mL methanol and 3.0 mL chloroform containing $^{13}\text{C}_{16}$ -9-PAHSA standard (see step 2 and Table 1 for preparation of standard in chloroform) **▲ CRITICAL STEP** Tissue samples should be extracted immediately without thawing. Prolonged keeping of samples in aqueous buffer could lead to potential additional FAHFA metabolism. Given rapid extraction of lipids upon solvent addition, buffer and solvents can be used at room temperature; however, the chloroform containing standard should be kept at 4°C prior to extraction to minimize change in standard concentration due to evaporation
- iii. Homogenize by performing 40 strokes on ice using pestle A (we have previously observed no difference in extraction efficiency using pestle A or B; however, pestle A is less tight-fitting than pestle B and tends to be easier to use in cases of tougher tissues such as liver) **! CAUTION** Splashing may occur, some initial resistance may be felt especially in the case of liver, homogenize slowly and wear appropriate personal protective equipment
- iv. Carefully pour homogenized mixtures into precooled 8 mL sample vials with PTFE-lined caps on ice
- v. Using the syringe-Pasteur pipette setup in Fig. 3a or glass serological pipette, add 0.5 mL chloroform without standard to each tissue grinder, homogenize 3–5 times with pestle A to wash pestle and grinder
- vi. Combine 0.5 mL chloroform washes with previously homogenized lipid mixtures (~ 6 mL) in 8 mL vials on ice

- vii. Vortex each vial for 15 s at room temperature ! **CAUTION** Cap vials tightly to prevent spilling
- viii. Transfer sample vials into clean 50 mL centrifuge tubes, centrifuge at 2200 g, 6 min, 4 °C ! **CAUTION** Glass vials will tolerate this spinning speed without shattering. In the rare event of a vial breaking, dispose of appropriately while wearing personal protective equipment. Recover sample only if this does not present a risk to the user
- ix. Place centrifuge tubes containing vials back on ice. One sample at a time, gently remove chloroform (bottom) layer using the syringe-Pasteur pipette setup in Fig. 3a, transfer chloroform into a clean 4 mL sample vial at room temperature ?

TROUBLESHOOTING

- x. Transfer chloroform containing lipids (step 5ix) again into a second 4 mL sample vial at room temperature. Residual water will stick to the sides of the first glass vial during transfer, thus allowing further removal of aqueous contaminants ▲ **CRITICAL STEP** The second transfer can be performed with Pasteur pipette attached to a bulb to enhance transfer speed and minimize solvent evaporation
- xi. Dry samples for 30–45 min at room temperature under a gentle stream of nitrogen, proceed directly to fractionation (step 6) or briefly flush each sample with nitrogen and store tightly capped at –80 °C ? **TROUBLESHOOTING**

■ **PAUSE POINT** Dried lipids are stable at –80 °C for at least six months

(B) Extract lipids from human or murine sera (Fig. 3c) ● TIMING 1.5 h

- i. Transfer thawed sera (typical volume for extraction is 150–300 µL) to 8 mL sample vial on ice, add citric acid buffer to a final volume of 1 mL ▲ **CRITICAL STEP** Extraction solvent volume should be increased if using higher sera volumes (i.e. 1.5–2.0 mL aqueous:1.5–2.0 mL methanol:3.0–4.0 mL chloroform for 300–900 µL sera), maintain aqueous fraction:methanol:chloroform ratio at 1:1:2 (v:v:v)
- ii. Using the syringe-Pasteur pipette setup in Fig. 3a or glass serological pipette, immediately add 1 mL methanol followed by 2 mL chloroform containing ¹³C₁₆-9-PAHSA standard (see step 2 and Table 1 for preparation of standard in chloroform) ▲ **CRITICAL STEP** Solvents should be added quickly. Prolonged keeping of samples in aqueous buffer could lead to potential additional FAHFA metabolism
- iii. Shake each vial vigorously for 30 s by hand at room temperature ! **CAUTION** Cap vials tightly to prevent spilling
- iv. Vortex each vial for 15 s at room temperature ! **CAUTION** Cap vials tightly to prevent spilling
- v. Transfer sample vials into clean 50 mL centrifuges, centrifuge at 2200 g, 6 min, 4 °C ! **CAUTION** Glass vials will tolerate this spinning speed without shattering. In the rare event of a vial breaking, dispose of appropriately while wearing personal protective equipment. Recover sample only if this does not present a risk to the user

- vi. Replace samples back on ice, one sample at a time, gently remove chloroform (bottom) layer using the syringe-Pasteur pipette setup in Fig. 3a, transfer chloroform into clean 4 mL sample vial at room temperature ?

TROUBLESHOOTING

- vii. Transfer chloroform containing lipids again into a second 4 mL sample vial at room temperature to remove residual aqueous contaminants **▲ CRITICAL STEP** The second transfer can be performed with Pasteur pipette attached to a bulb to enhance transfer speed and minimize solvent evaporation
- viii. Dry samples for 30–45 min at room temperature under a gentle stream of nitrogen, proceed directly to fractionation (step 6) or briefly flush each sample with nitrogen and store tightly capped at -80°C ? **TROUBLESHOOTING**

■ PAUSE POINT Dried lipids are stable at -80°C for at least six months

FAHFA enrichment *via* fractionation (Fig. 4a–b) ● TIMING 4 h

- 6 | Remove tissue or serum lipids (see step 5(A) or 5(B) for lipid extraction from tissue or serum, respectively) from -80°C storage and equilibrate to room temperature for 20 min **▲ CRITICAL STEP** All steps are performed at room temperature. Image of required glassware and summary of workflow are in Fig. 4a–b
- 7 | While equilibrating samples, connect SPE columns to extraction manifold (Fig. 4a)
- 8 | Condition SPE column with 15 mL hexane, allow solvent to flow through column by gravity and collect flow through in 20 mL glass vials **▲ CRITICAL STEP** Do not disturb column bed with pipette. Gently pipet toward column wall and allow solvent to flow onto column bed for all steps ?
TROUBLESHOOTING
- 9 | While conditioning column, reconstitute each lipid sample in 200 μL chloroform, vortex 20 s at room temperature
- 10 | Check that hexane from step 9 has completely flown through column before loading sample. Load sample onto column, allow solvent to flow through column by gravity and collect flow through in 20 mL glass vials, vials from step 9 can be kept in place and reused for this step ? **TROUBLESHOOTING**
- 11 | Wash each sample vial with 100 μL chloroform, vortex 10 s, load wash onto column, allow solvent to flow through column by gravity and collect flow through in 20 mL glass vials, vials from step 10 can be kept in place and reused for this step, discard 20 mL vials containing hexane and chloroform and replace with new 20 mL vials for step 12 ? **TROUBLESHOOTING**
- 12 | Check that chloroform from step 11 has completely flown through column before elution. Elute neutral lipids with 16 mL 95:5 (v:v) hexane:ethyl acetate, allow solvent to flow through column by gravity and collect elution in 20 mL glass vials. Replace vials with new 20 mL vials for step 13. This fraction

contains neutral lipids (e.g. triglycerides and cholesterol esters) and can be dried and reconstituted for LC–MS if these lipids are of interest ?

TROUBLESHOOTING

- 13 |** Check that solvents from step 12 have completely flown through column before elution. Elute FAHFAs with 16 mL ethyl acetate, allow solvent to flow through column by gravity and collect in new 20 mL glass vials ?

TROUBLESHOOTING

- 14 |** Dry FAHFA fractions at room temperature under a gentle stream of nitrogen (~2 h) ? **TROUBLESHOOTING**

- 15 |** Reconstitute dried lipids in 1 mL chloroform, vortex 15–20 s, transfer chloroform with lipids into 4 mL vial and repeat this step two more times to ensure complete transfer of all lipids into the 4 mL vial, dry vial at room temperature (~30 min) under a gentle stream of nitrogen

- 16 |** Reconstitute dried lipids from step 15 in a small volume of chloroform (100–200 µL), vortex for 20 s to rinse lipids from sides of the vial (this will enable thorough sample reconstitution in small solvent volumes for LC–MS), dry at room temperature (~15 min) under a gentle stream of nitrogen, briefly flush each sample with nitrogen and store tightly capped at –80 °C

■ **PAUSE POINT** Dried lipids are stable at –80 °C for at least six months

Optimization of FAHFA detection by MRM ● TIMING 1.5 h

- 17 |** Optimize MRM parameters for PAHSA detection ▲ **CRITICAL** MRM parameters are instrument-dependent and should be optimized by the user if using a different instrument model. Optimization of instrument parameters (e.g. fragmentor voltage and collision energy) and precursor-to-product ion transitions can be performed manually or using an optimization software such as Agilent MassHunter Optimizer. A manual method with the Agilent 6410 LC/MS system is provided in Supplementary Note 4 as guide, and optimized MS parameters for the Agilent 6410 LC/MS system are in Table 4. Refer to EQUIPMENT SETUP and Supplementary Note 3 for initial instrument setup procedure

■ **PAUSE POINT** Save optimized method in acquisition software for future use

Sample preparation ● TIMING 30 min—▲ CRITICAL To minimize plastic contamination we recommend the use of low-volume glass syringes for sample reconstitution and transfer

- 18 |** Remove lipid samples from –80 °C storage, equilibrate to room temperature for 20 min ▲ **CRITICAL STEP** Samples can be thawed and prepared simultaneously as LC–MS setup.
- 19 |** Add to each sample 25 µL methanol, vortex 20 s, transfer 10 µL into insert with vial and immediately transfer onto autosampler maintained at 10 °C. Inject 10

μL for analysis **▲ CRITICAL STEP** Do not reuse sample vial caps. When stored tightly capped at 10 °C no visible evaporation was observed for at least 6 h for methanol samples and at least 2 h for chloroform samples. Given each sample run is 2 h, it is recommended that no more than two samples in chloroform and no more than four samples in methanol be reconstituted in solvent at once for LC–MS ? **TROUBLESHOOTING**

- 20 | Dry remaining samples under a gentle stream of nitrogen and restore at –80 °C

LC–MS setup and data acquisition ● TIMING 2 d—▲ CRITICAL An example setup is provided for the Agilent MassHunter acquisition software. Acquisition software is instrument-dependent and should be operated by the user following manufacturer's instructions. Refer to EQUIPMENT SETUP and Supplementary Note 3 for initial instrument setup procedure

- 21 | Load optimized method and tune files
- 22 | Connect mobile phases to LC lines and purge all lines
- 23 | Connect in-line filter to column **▲ CRITICAL STEP** Follow manufacturer's instructions and do not over-tighten. For convenience the in-line filter can remain attached to the column when not in use
- 24 | While purging LC lines, thoroughly clean the spray shield, areas around the spray shield and the interior of the spray chamber (see INSTRUMENT SETUP and Supplementary Note 3)
- 25 | Set flow rate to 0 mL/min and connect column with attached in-line filter to LC **▲ CRITICAL STEP** Prior to connecting column, confirm that all incompatible solvents from previous sample runs have been fully displaced
- 26 | Set solvent to 7 % A, 93 % B, start flow rate at 0.1–0.15 mL/min and gradually increase to 0.2 mL/min. Equilibrate column at 0.2 mL/min for at least 30 min and confirm that pressure has stabilized prior to sample run. Depending on the LC setup the pressure should stabilize between 210 and 240 bars
- 27 | Set up LC method and worklist while equilibrating column. LC method, recommended injection volumes and an example worklist are provided in Table 3 and Table 4 **▲ CRITICAL STEP** We recommend injection of same solvent type and volume as sample for Blanks at beginning and middle of worklist, and injection of 12–15 μL chloroform for Blanks at the end of worklist to allow more efficient washing of column and injection assembly
- 28 | Run sample

■ PAUSE POINT Back up acquisition files

Data analysis ● TIMING 30 min—▲ CRITICAL Data analysis is software-dependent and should be performed by the user following manufacturer's instructions. A manual integration method using the Agilent MassHunter Qualitative Data Analysis software (version B.03.01) is provided as example

- 29 | Open files (.d format) in MassHunter
- 30 | Go to Extract Chromatographs, set Type to MRM, MS level to MS/MS, Scans to Multiple reaction monitor and Transition to All to view peaks for all precursor-to-product ion transitions included in method, set Single m/z expansion for this chromatogram to Symmetric (m/z) $\pm$ 0.2
- 31 | Integrate peaks with the manual integration tool and record peak areas

? TROUBLESHOOTING

● TIMING

- Steps 1–3, 30 min
- Step 4, 30 min
- Step 5(A), 2 h
- Step 5(B), 1.5 h
- Steps 6–16, 4 h
- Step 17, 1.5 h
- Steps 18–20, 30 min
- Steps 21–28, 2 d
- Steps 29–31, 30 min

? TROUBLESHOOTING

Troubleshooting guidelines can be found in Table 5.

ANTICIPATED RESULTS

PAHSA extracted ion chromatograms are shown for murine serum, WATs, BAT and liver in Fig. 5. Individual data sets are aligned based on the retention time of the $^{13}\text{C}_{16}$ -9-PAHSA internal standard. Endogenous 13, 12, 11, 10, 9, 8, 7 and 5-PAHSAs were observable *via* our method, with 9-PAHSA being the major isomer in majority of samples (Fig. 5a, b). Highest total PAHSA level is found in BAT, with adipose tissues displaying widest isomeric range; 13/12, 9 and 8-PAHSAs were the only detectable isomers in liver (Fig. 5b). PAHSA isomeric distribution is tissue-dependent and could be affected by additional factors such as animal strain and metabolic state. Details on the expansion of PAHSA MS parameters toward additional FAHFA families are provided in Supplemental Note 3.

As was previously mentioned (“Contaminants and background subtraction” section), we have detected a small amount of background signal as a result of the FAHFA extraction process, which occupies ~15 % of the serum signal (Fig. 6), but is negligible in the case of liver and adipose tissues given the high PAHSA signals in those tissues (Fig. 5b). Consequently, the inclusion of a solvent-only negative control, especially for sera, will allow background subtraction and improve the accuracy of PAHSA quantification.

While MRM enables detection of specific metabolites based on their unique MS/MS fragmentations, it is important to note that false positives are still possible due to the lack of exact mass. We have found during our analysis that the sphingolipid ceramide can be detected with PAHSA transitions. As Fig. 7a illustrates, LC-MS of murine serum and tissues revealed a highly abundant peak at ~95 min with higher m/z 537 $\rightarrow$ 281 ion count than m/z 537 $\rightarrow$ 255. Tandem MS of the 95 min peak yielded fragments closely matching those from d18:1/16:0 ceramide (Fig. 7b). Given the loss-of-proton precursor ion for d18:1/16:0 ceramide is m/z 536, the 95 min peak (detected under PAHSA MRM parameters with m/z 537 as precursor) represents naturally occurring isotopes of C16 ceramide (i.e. ^{13}C -d18:1/16:0 ceramide) (Fig. 7b). Co-injection with a d18:1/16:0 ceramide standard and WT murine gastrocnemius muscle (GNM) validated this ion as a d18:1/16:0 ceramide (Fig. 7c). Presence of ceramide in fractionated murine lipids was not surprising, given ethyl acetate elution during SPE was not FAHFA-specific, and could lead to co-elution of additional lipids with similar lipid-matrix interactions. A more thorough survey of fractionation conditions could improve the specificity of FAHFA isolation and minimize co-elution of additional lipids.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

PAHSA isomers detection by Multiple Reaction Monitoring. **(a)** Structure of 9-Palmitic Acid Hydroxy Stearic Acid (9-PAHSA); all possible locations of hydroxylation are numbered. **(b)** Schematic of Multiple Reaction Monitoring. A product ion of interest (a_1) from a designated precursor ion (a) is monitored to yield quantification for compound a via the compound-specific precursor-to-product ion transition $a \rightarrow a_1$. **(c)** Tandem MS of 9-PAHSA yields three major fragments at m/z 255, m/z 281 and m/z 299. **(d)** MRM of 9-PAHSA; relative ratios of the three PAHSA-specific transitions are a direct reflection of the relative ratios of the corresponding product ions during tandem MS. **(e)** Ratios among

PAHSA-specific transitions for 12, 11, 10, 9, 6, 5 and 4-PAHSAs; transition ratios are isomer-dependent. (f) Finalized LC method enabling resolution of 12, 11, 10, 9, ^{13}C -9, ^{13}C -8, 6, 5 and 4-PAHSA standards.

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Figure 3.

Lipid extraction from serum and tissues. **(a)** Glassware for lipid extraction. **(b)** Workflow for lipid extraction from murine tissues, corresponding to **PROCEDURE** step 5(A). **(c)** Workflow for lipid extraction from human or murine serum, corresponding to **PROCEDURE** step 5(B) ! **CAUTION** Follow all relevant ethical guidelines and regulations for the collection and use of human serum, and handling of laboratory animals.

Figure 4. Solid phase extraction. **(a)** SPE setup. **(b)** Workflow for solid phase extraction of serum or tissue lipids using silica SPE column, corresponding to **PROCEDURE** steps 6–16.

Figure 5. PAHSA isomer distribution in WT murine sera and tissues. **(a)** Detected PAHSAs in murine WT PG WAT; insets show 7 and 5-PAHSAs. **(b)** PAHSA isomer distribution in WT murine sera and tissues. Extracted ion chromatograms are shown for the m/z 537 $\rightarrow$ 255 transition; all chromatograms are aligned based on retention time of the ^{13}C -9-PAHSA internal standard.

Figure 6. FAHFA background. (a) ~15 % of the total serum signal has been detected as background in the lipid extraction/SPE procedure. Inclusion of an extraction negative control enables background subtraction and more accurate quantification. All chromatograms are extracted MRMs.

Figure 7. Contaminant identification. **(a)** Extracted ion chromatograms of WT murine serum and PG WAT; a highly abundant contaminant peak elutes at ~95 min. **(b)** Tandem MS of d18:1/16:0 ceramide standard and the contaminant peak at ~95 min, identifying the contaminant as naturally-occurring heavy isotopes of d18:1/16:0 ceramide. **(c)** Co-injection of WT GNM FAHFA fraction with d18:1/16:0 ceramide standard monitored by ceramide-characteristic and PAHSA-characteristic transitions identifies contaminant as ceramide.

Table 1

Recommended sample and internal standard quantities for human or WT murine serum, and WT murine tissues.

Sample type	Sample [mg or μ L]	Internal standard [pmol]	Solvent [mL aqueous: mL methanol: mL chloroform]
Serum	175	1	0.825: 1: 2
Perigonadal white adipose tissue	150	5	1.5: 1.5: 3
Subcutaneous white adipose tissue	100	2.5	1.5: 1.5: 3
Brown adipose tissue	75	2.5	1.5: 1.5: 3
Liver	75	1.5	1.5: 1.5: 3

Table 2

Optimized MS parameters for PAHSA detection

MS parameters				
Instrument	Agilent 6410 triple quadrupole mass spectrometer			
Ionization	Negative electrospray ionization			
Mode	Multiple Reaction Monitoring			
Capillary voltage	4000 V			
Drying gas temperature	350 °C			
Drying gas flow rate	8 L/min			
Nebulizer pressure	35 psi			
Skimmer voltage	15 V			
Compound-specific parameters				
Compound name	Precursor ion	Product ion	Fragmentor voltage	Collision energy
PAHSA	537.5	255.2	205 V	30 V
PAHSA	537.5	281.2	205 V	25 V
PAHSA	537.5	299.3	205 V	23 V
¹³ C ₁₆ -PAHSA	553.5	271.3	205 V	30 V
Dwell time/transition	100–300 ms			
MS1 resolution	Wide			
MS2 resolution	Unit			
EMV	400 V			

Table 3

Optimized LC parameters for PAHSA isomer resolution.

LC parameters	
HPLC	Agilent 1200 Series
Column	Luna C18(2), 3 μm , 100 $\text{\AA}$, 250 $\times$ 2.0 mm
Running buffer	93:7 methanol:water, 0.01 % ammonium hydroxide, 5 mM ammonium acetate, 0.2 mL/min for 120 min, 25 $^{\circ}\text{C}$
Column wash	100 % isopropanol, 0.1 mL/min for 210 min, 25 $^{\circ}\text{C}$
Column storage	65:35 acetonitrile:water, 0.1 mL/min for 60 min, 25 $^{\circ}\text{C}$

Table 4

Recommended injection volumes and example worklist for PAHSA measurement.

Recommended injection volumes	
100 % methanol (preferred)	10 µL
50:50 (v:v) methanol:chloroform	4 µL
100 % chloroform	2 µL
Example worklist	
Sample number	Sample description
1	Blank
2	Extraction/fractionation negative control
3	PAHSA ladder solution (IS _L)
4	Blank
5	Wildtype replicate 1
6	Knockout replicate 1
7	Wildtype replicate 2
8	Knockout replicate 2
9	Wildtype replicate 3
10	Knockout replicate 3
11	Blank
12	Wildtype replicate 4
13	Knockout replicate 4
14	Wildtype replicate 5
15	Knockout replicate 5
16	Wildtype replicate 6
17	Knockout replicate 6
18	PAHSA ladder solution (IS _L)
19	Blank
20	Column wash
21	Column storage

Table 5

Troubleshooting table.

Step	Problem	Possible causes	Solution
5Aix, 5Bvi	No phase separation	Insufficient centrifugation	Centrifuge samples at 2000–2500 g for 6–10 min at 4 °C to improve phase separation; in a few instances thick precipitates and lack of phase separation have been observed for human serum samples, vortex these samples thoroughly and re-spin for clear organic phase
		Incorrect aqueous: organic ratio	Check buffer-to-solvent ratios. Maintain aqueous:methanol:chloroform ratio at 1:1:2 to ensure efficient phase separation and lipid extraction
		Insufficient solvent volume for quantity of tissue or serum used	Follow Table 1 for recommended sample quantities and solvent volumes. Excessive amounts of sample could negatively impact phase separation and extraction efficiency
5Axi, 5Bviii	Sample not drying completely	Insufficient nitrogen	Increase nitrogen gas pressure to facilitate drying, volume of chloroform (2–3 mL) used in extraction should dry fully within 30–45 min
		Fatty tissues	Adipose tissues contain high concentrations of fatty acids and triglycerides, many of which are clear to semi-opaque oils at room temperature. Following evaporation of chloroform (30–45 min), store oils at –80 °C. Oils from fatty tissues will solidify at –80 °C and should be fully equilibrated to room temperature prior to reconstitution in chloroform for SPE
		Aqueous contamination	Small quantity of aqueous carryover can be dried using nitrogen with increased drying time and gas pressure; drying of aqueous carryover typically leaves behind white solids (salts from citric acid buffer) that will have low solubility in chloroform or 9:1 heptane:isopropanol and therefore only minimally enter the SPE or HPLC column; trace amounts of salts that do enter the columns will be separated from the FAHFA fraction and typically have little effect on FAHFA enrichment. In case of significant aqueous carryover, it is advisable that the extraction be repeated with new sample. Take care when isolating chloroform layer from extraction mixture and use the syringe-Pasteur pipette setup in Fig. 3a for improved control
5Axi, 5Bviii	Dried lipids appear pink to dark red in color	Blood pigments	The relatively low pH of citric acid buffer will lead to extraction of blood pigments from tissues, consequently lipids from highly vascularized tissues and sera containing residual red blood cells will appear pink to dark red upon drying. This can be minimized by quickly washing freshly excised tissues with PBS prior to freezing; blood pigments will also be removed during SPE or offline HPLC
8, 10–13	No solvent flow	Insufficient solvent volume	Load column to full capacity (6 mL), wait briefly for solvent to travel through column by gravity
		Clogged or defective column	Confirm that sample has been completely solubilized in chloroform with no significant amount of precipitates. Pull vacuum very gently to induce solvent flow, restart with new column if solvent still not flowing
14, 19	Sample is an oil and/or oil droplets are observed during reconstitution in methanol for LC–MS	Carryover of neutral lipid fraction into FAHFA fraction	Reduce sample quantity for lipid extraction. Do not use more than 80 mg liver or more than 150 mg adipose tissue. Follow Table 1 for recommended tissue weights
		Improper elution	Ensure that all solvents from the prior fraction have traveled through column before loading new solvents
31	Sub-optimal peak resolution	Insufficient column equilibration	Equilibrate column for at least 30 min with mobile phase prior to running sample and confirm that column pressure (210–240 bars for Agilent 1200 Series LC) has completely stabilized. To ensure complete

Step	Problem	Possible causes	Solution
			equilibration we suggest running Blank as the first sample in the worklist. See Table 4 for example worklist
		Dirty column/column overloading	Wash column with 100 % isopropanol overnight prior to starting worklist. To ensure no contamination of subsequent runs, wash column for 3–4 h in 100 % isopropanol at the end of each worklist. See Table 4 for example worklist For fatty tissues such as WATs and BAT, do not inject unfractionated samples; high abundance of neutral lipids in these samples will lead to column overloading and significant contamination of both column and LC lines, all of which adversely affect chromatography
		Old column	Replace with new column. To prolong column lifetime, do not store column in mobile phase containing acid or base; equilibrate column with storage buffer recommended by manufacturer at the end of each worklist. While degradation in column resolution could occur suddenly, we have observed gradual increase in column backpressure with use, consequently column backpressure could be an indication of column condition
		Incorrect mobile phase percentage	Verify that mobile phases have been mixed correctly. We recommend preparing A (100 % water) and B (100 % methanol) mobile phases in separate bottles and using the LC mixer to achieve the desired solvent ratio (93 % methanol)
		Inaccurate pH	pH of mobile phase affects peak resolution, verify that correct volume of ammonium hydroxide has been added to mobile phase. Ammonium hydroxide is volatile and solvents containing this additive should always be prepared fresh before use
		Injection volume too high/ incompatible carrier solvent	To ensure optimal peak separation, do not inject higher than 10 µL methanol, higher than 2 µL chloroform or higher than 4 µL 50:50 methanol:chloroform. Whenever possible, inject samples in methanol instead of chloroform given chloroform is significantly less well tolerated by the current mobile phase system
31	Ceramide contaminant peak overlaps with FAHFAs	Inaccurate pH	pH of mobile phase significantly affects retention time of ceramide contaminant; insufficient ammonium hydroxide typically causes the ceramide peak to move forward. Prepare mobile phases containing fresh ammonium hydroxide immediately before use, replace with new bottle of ammonium hydroxide for best results
31	Uncertainty about peak or extremely large peak	Contaminant	Check all three transitions to ensure that the ratio is consistent with FAHFAs, exclude all peaks with non-FAHFA-characteristic ratios
31	Low ion count	LC–MS dirty	Clean nebulizer needle and sample capillary. Tune instrument and ensure all instrument parameters are within accepted range prior to starting experiment