Fecal Lipidomics Profiling and Structural Identification using High Resolution LC-MS and HCD Fragmentation

Susan S. Bird[1, 2]; Katherine E. Gregory[1, 3]; Vera S. Gross[4]; Vasant R. Marur[1]; Alexander V. Lazarev[4]; W. Allen Walker[2, 5]; Bruce S. Kristal[1, 2]

[1]Brigham and Women's Hospital, Boston, MA; [2]Harvard Medical School, Boston, MA; [3]Boston College, Boston, MA; [4]Pressure BioSciences, South Easton, MA; [5]Massachusetts General Hospital, Boston, MA

Overview

Purpose: HRAM LC-MS with HCD fragmentation allows for the structural characterization of intact, unique, fecal lipid species not currently found in standard lipid databases. The techniques developed herein open the door to the use of fecal lipid profiling for both scientific and clinical applications.

Methods: Lipids from premature infant fecal samples were analyzed by high resolution LC-MS for profiling and a pooled sample was run with LC-MS with all ion HCD fragmentation for characterization. Lipid identifications for novel fecal species are highlighted.

Results: We have identified 304 endogenous lipid species in fecal material, covering 6 of the LIPID MAPS[1] categories and their related classes and subclasses (**Table 1.**). The fecal lipidome had increased incorporation of odd chained FAs, such as FA 17:0, 17:1, 15:0, and 19:1, into intact lipid side chains as well as FA dimers - likely resulting from the dairy-based premature infant diet.

Introduction

Methodologies for the analysis of intact lipids from fecal matter will facilitate the understanding of gut (patho)physiology and its role(s) in health and disease while directly impacting patient care. Because lipids are central to intestinal biology, more stable than many metabolites, and more conserved across microbiota than proteins, they will provide information relevant to the microbiome, host, and environment.

We developed a fecal extraction method that utilizes two separate, complementary extraction chemistries, dichloromethane (DCM) and a methyl tert-butyl ether/hexafluoroisopropanol mixture (MTBE), alone or with high-pressure cycling (PCT), to provide robust and comprehensive lipidome coverage[2].

Extracts were assessed by LC-MS profiling in both positive and negative ionization modes with unknown lipid characterization performed using all ion HCD fragmentation and chromatographic alignment. Using two complementary extraction chemistries alone or with PCT, prior to LC-MS analysis yielded more robust and broad lipidome coverage. MTBE preferentially extracted the more polar lipid species, while the more hydrophobic compounds preferred DCM.

Methods

Premature Infant Sample Collection

Approximately 1 g of stool sample was collected from the diapers of premature infants who were born prior to 32 weeks gestation and cared for in the Neonatal Intensive Care Unit at Brigham and Women's Hospital. (**Figure 1.)**

Fecal Lipid Extraction

Lipids were extracted from 0.67 mg of fecal material from premature infants using two extraction approaches: (i) dichloromethane/methanol and water liquid-liquid extraction following Bligh and Dyer volumes (DCM) and (ii) a methyl tert-butyl ether-hexafluoroisopropanol mixture (MTBE). Samples from both methods were processed with and without PCT. The PCT samples were placed in a Pressure BioSciences Barocycler at 35,000 psi for 30 cycles (each cycle consisting of 20 seconds at high pressure and 10 seconds at ambient pressure for a total run time of ~15 mins). The lipid rich organic phase was then collected and dried under vacuum. A pool sample was created from a small aliquot of all 4 extraction methods.

Liquid Chromatography

Samples were dissolved in 300 µL of 65:35:5 ACN:IPA:H20 5 µg/mL PG(17:0/17:0). Accela 1250 chromatograph aInd Open Accela auto sampler, 10µL Injection. Column: 2.1 x 100mm C18, 2.7µm was operated at 260 µL/min and 55 °C. The RP HPLC method is described in detail in S. Bird, et al., Anal. Chem. **2011**, *83*, 940–949, 6648–6657[3].

Mass Spectrometry

Thermo Scientific Exactive MS operated in high resolution mode (60 K, 2 Hz scan speed), scanning between *m/z* 120-2000 in both positive and negative ionization mode (separate scans). For qualitative/structural ID, HCD fragmentation was done by alternating each full scan with either 30 eV, 60 eV or 100 eV all ion fragmentation scans;

Data Analysis Software

SIEVE v 1.3 (Thermo Scientific)

Results

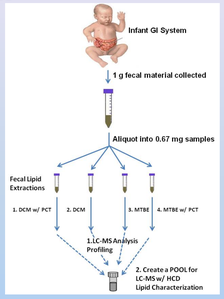


FIGURE 1. Premature infant fecal lipid extraction methodology. A 1 g sample was split into 4 equal aliquots which were then extracted separately using 1. DCM, 2. DCM along with Pressure Cycling Technology (PCT) (see Methods), 3. MTBE and 4. MTBE along with PCT. These samples are then either analyzed by non-targeted LC-MS profiling or pooled together to create a sample used for unknown characterization by LC-MS and HCD fragmentation.

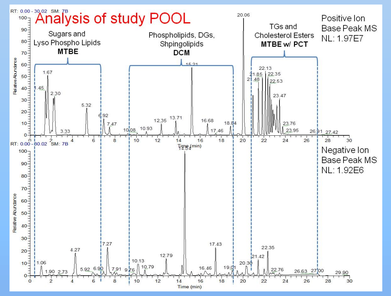


FIGURE 2. LC-MS Chromatograms of Fecal Lipid POOL in both Positive and Negative Ion mode. Representing lipids extracted from all 4 methods and the extraction preferences of regions of the chromatogram.

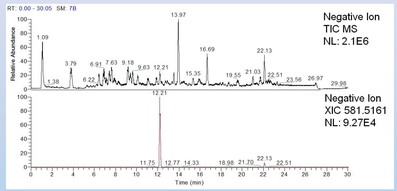
Lipid Class	Unique Lipids**	- Ionization	+ Ionization
Glycerophospholipids (GP)			
Phosphocholine (PC)	22	[M+FormAcid]-	[M+H]+
plasmalogenphosphocholine (plsPC)	4	[M+FormAcid]-	[M+H]+
lysoPhosphocholine (LysoPC)	6	[M+FormAcid]-	
Phosphoethanolamine (PE)	29	[M+H]-	[M+H]+
plasmalogenphosphoethanolamine (plsPE)	7	[M+H]-	[M+H]+
lysoPhosphoethanolamine (LysoPE)	3	[M+H]-	[M+H]+ / [M+NH4]+
Phosphoserine (PS)	6	[M+H]-	
Phosphoinositol (PI)	9	[M+H]-	
Phosphoglycerol (PGs)	14	[M+H]-	
lysoPhosphoglycerol (LysoPG)	2	[M+H]-	
Phosphatidic Acid (PA)			
lysoPhosphatidic Acid (LysoPA)	2	[M+H]-	
Fatty Acyls (FA)			
Free Fatty Acids (FFA)	19	[M+H]-	[M+NH4]+
Sphingolipids (SL)			
Sphingomyelins (SM)	13	[M+FormAcid]-	[M+H]+
Ceramides (Cer)	50	[M+H]-	[M+H]+
Gangliosides (Gan)	5	[M+H]-[M+H2O+H]-	
Glycerolipids (GL)			
Diacylglycerol (DG)	19		[M+NH4]+
Triacylglycerol (TG)	88		[M+NH4]+
Sterol Lipids (ST)			
Bile acids and derivatives	2		[M+H]+ / [M+NH4]+
Cholesterol Esters (CE)	2		[M+H]+ / [M+NH4]+
Prenol Lipids (PL)			
coenzyme-Q (CoQ)	2		[M+H]+ / [M+NH4]+

Table 1. LIPIDMAPS Categories, Classes, Subclasses of the 304 Unique Fecal Lipids Identified[2]. IDs, including fatty acid moieties, were identified by monoisotopic mass and fragmentation; IDs, not including fatty acid moieties, were identified by monoisotopic mass, chromatographic retention time and quantitation pattern across the different extraction methods and dilutions.

****defined as having a unique mass and retention time pair that represents a distinct species. Those species that are observed in both ionization modes are only counted once.**

IDENTIFICATION OF UNKNOWN FECAL LIPIDS

3A.



3B.

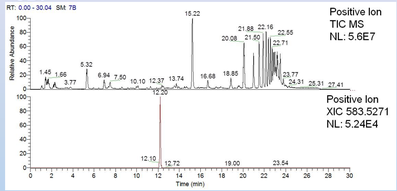
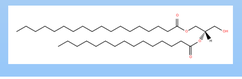


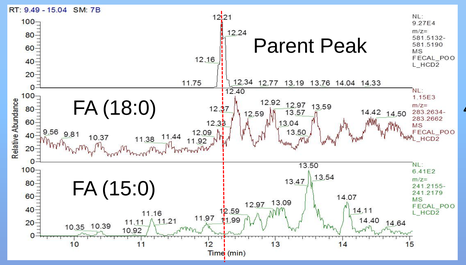
FIGURE 3. Total Ion Chromatogram (TIC) of the POOL lipid sample in both Negative and Positive Ion analysis are shown on the top panel of both A. and B. The Extracted Ion Chromatogram (XIC) of an unknown lipid is shown in the bottom panel. The unknown has an exact mass of 582.5223 daltons.

METLIN DATABASE SEARCH OF EXACT MASS SUGGESTS:

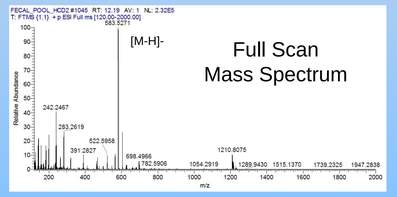
DG (18:0/15:0)



4A.



4B.



4C.

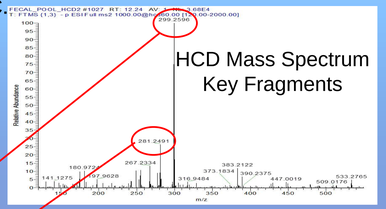
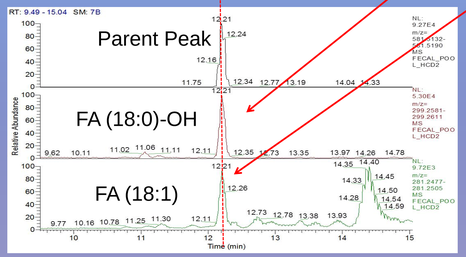


FIGURE 4. The top panel is the XIC of the parent [M-H]- ion, shown in 4B. The next two panels represent the XIC of FA(18:0) and FA(15:0) ions which are NOT found in the HCD spectra in 4C.

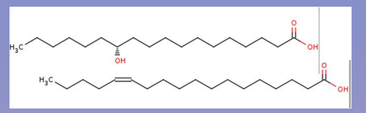
NOT DG(18:0/15:0)!!!



What is it?

FIGURE 5. The top panel is the XIC of the parent [M-H]- ion, shown in 4B. The next two panels represent the XIC of the two circled ions in figure 4C and which chromatographically align with the parent and suggest the unknown species to be a fatty acid dimer.

The structure below is not necessarily the 3 dimensional orientation, however, these two fatty acids comprise the species eluting at RT 12.21 minutes, with an exact mass of 582.5223 daltons and this option of FA dimer was no found in any database.



Conclusions

Non-targeted profiling of premature infant fecal samples using HRAM LC-MS on a Thermo Exactive benchtop Orbitrap MS combined with HCD all ion fragmentation allowed for the identification of intact lipid species from a biological matrix previously not studied.

Analytical Impact

- First report of intact lipids from fecal material, previous reports focused on GC-MS analysis of FAs where much of the lipidome diversity is lost.
- LC-MS profiling on the Thermo Exactive benchtop orbitrap MS found over 500 lipophillic species
- LC-MS w/ HCD AIF identified 304 unique species from 6 LIPID MAPS Categories **(Table 1.)**.
- Two extraction methodologies provide enhanced lipidome coverage **(Figure 2.)**:
  - More polar lipids preferentially extract using MTBE
  - Less polar lipids preferentially extract using DCM.
- Lipid species not readily found in standard databases were characterized by HCD AIF including:
  - Fatty acid dimers **(Figures 3-5.)**
  - PLs and TGs with odd-chained FA side-chains

Clinical Importance

- The method provides a reproducible, generally-applicable, broad based, quantitatively driven, and non-invasive technique for establishing gut function in patients regardless of age or disease.
- Using fecal lipidomic profiling to define gut function in health and disease will help physicians assess intestinal function, determine gut response to nutrition, and recognize existing inflammation/pathology.
- Specific to premature infants, this study represents a new paradigm for determining the impact of initial intestinal colonization and assessing nutritional, infectious, and inflammatory status in this vulnerable patient population.

Future Directions

- Examine the origin of the identified intact fecal lipids as either microbial or human.
- Annotate all FA dimers found in these samples, there are definitely more.
- Pursue the characterization of other still unknown biological lipids found in fecal matter
- Investigate patient populations by assessing the differences in their fecal lipidome and how these differences may relate to overall gut function and health..

References

1. LIPID MAPS comprehensive classification system for lipids. Fahy E., Subramaniam S., Murphy R., Nishijima M., Raetz C., Shimizu T., Spener F., van Meer G., Wakelam M., and Dennis E., J. Lipid Res. **2009**, *50*, S9-S14. doi: 10.1194/jlr.R800095-JLR200.
2. Method Development for Fecal Lipidomics Profiling Gregory KE, Bird SS, Gross VS, Marur VR, Lazarev AV, Walker WA, Kristal BS. Anal Chem. **2013** Jan 15;85(2):1114-23. doi: 10.1021/ac303011k.
3. Lipidomics profiling by high-resolution LC-MS and high-energy collisional dissociation fragmentation: focus on characterization of mitochondrial cardiolipins and monolysocardiolipins. Bird SS, Marur VR, Sniatynski MJ, Greenberg HK, Kristal BS. Anal Chem. 2011 Feb 1;83(3):940-9. doi: 10.1021/ac102598u.

Acknowledgements

The research reported was supported by K23 NR011320 (PI-KEG), U01-ES16048 (PI-BSK), the Harvard Clinical Nutrition Research Center, Pilot/Feasibility Funding (P30-DK040561 PI-Walker), Brigham and Women's Hospital Department of Neurosurgery Lamas Hydrocephalus Research Initiative (BSK) and Intramural Funding from Boston College (KEG). The authors (SSB, BSK), thank ThermoFisher for the loan of an Exactive Benchtop orbitrap for demonstration testing and financial support for scientific meeting attendance.