**Materials** A pooled sample of human plasma compiled from hundreds of de-identified consented donors was obtained from the Red Cross of Michigan.

**Extraction/Reconstitution** Metabolites were extracted with four parts of 1:1:1 methanol:acetonitrile:acetone and one part human plasma in 15 mL polypropylene centrifuge tubes followed by vortexing for 20 seconds and allowing the suspension to rest on ice for an additional 10 minutes. The extract was centrifuged at 5000 x g for 10 minutes, and the supernatant was divided into 1.5 mL aliquots which were completely dried under a gentle stream of nitrogen gas. Dried samples were reconstituted in varied volumes of 90:10 water:methanol (for RPLC analyses) and 85:15 methanol:water (for HILIC).

**Separations** LC-MS separations were performed on an Agilent (Santa Clara, CA) 1290 Infinity II LC coupled to an Agilent 6545 quadrupole time of flight mass spectrometer. Instrument settings for positive ionization mode following electrospray ionization were as follows: Sheath gas flow rate, 11 L/min; Drying gas, 8 L/min; Drying gas temperature, 320 ºC; Nebulizer, 35 psi; Capillary voltage, 3500 V; Nozzle voltage, 1000 V; Fragmentor, 175 V; Skimmer, 65 V; Octupole 1 RF Vpp, 750 V; Collision energy, 20; Iterative MS/MS mass error tolerance, ± 20 ppm; Iterative MS/MS retention time exclusion tolerance, ± 0.5 min; Spectrum data type, centroid. Data dependent MS/MS parameters: Mass range, 25-1200 m/z; Rate, 2 spectra/s; Max precursor ions per cycle, 3; Absolute precursor threshold, 5000 counts; Relative precursor threshold, 0.001%; Active exclusion enabled after 2 spectra and released after 0.5 min; Isolation width, narrow (~1.3 m/z). Negative mode separations had the same mass spectrometer settings except the capillary voltage was set to -3500 V. For injection volumes exceeding 20 uL a multi-draw kit was installed.

RPLC separations were performed using a Waters (Milford, MA) Acquity UPLC HSS T3 column (2.1 x 100 mm; 1.8 um) with a matching Vanguard precolumn. The flow rate was set to 0.4 mL/min and mobile phases consisted of (A) water with 0.1% v/v formic acid and (B) methanol with 0.1% v/v formic acid.

HILIC separations were performed on a Waters Acquity UPLC BEH amide column (2.1 x 100 mm 1.7 um) with a matching Vanguard precolumn. The flow rate was set to 0.3 mL/min and mobile phases consisted of (A) 95:5 acetonitrile/water with 0.125% v/v formic acid and 10 mM ammonium formate and (B) 95:5 water/acetonitrile with 0.125% v/v formic acid and 10 mM ammonium formate. Mobile phase A requires sonication for 10 minutes or longer to ensure all ammonium formate is thoroughly dissolved.

The column compartment was maintained at 55 ºC for both separation types. Gradient parameters for RPLC and HILIC methods are described further in Tables 1 and 2 below.

**Table 1.** RP LC-MS methods on a Waters Acquity UPLC HSS T3 column (2.1 x 100 mm; 1.8 um). Methods are presented as a function of time (min) and percentage of mobile phase B. Mobile phase consisted of (A) water with 0.1% v/v formic acid and (B) methanol with 0.1% v/v formic acid. The flow rate was set to 0.4 mL/min for all methods.

| % B | 21-minute | 60-minute | 120minute | 150minute | 180minute | 240minute | 360minute |
|---|---|---|---|---|---|---|---|
| **0** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **99** | 10 | 30 | 60 | 75 | 90 | 120 | 180 |
| **99** | 17 | 51 | 102 | 127.5 | 153 | 204 | 306 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **0** | 17.1 | 51.1 | 102.1 | 127.6 | 153.1 | 204.1 | 306.1 |
| **0** | 21 | 60 | 120 | 150 | 180 | 240 | 360 |

**Table 2.** HILIC LC-MS methods on a Waters Acquity UPLC BEH amide column (2.1 x 100 mm; 1.7 um). Methods are presented as a function of time (min) and percentage of mobile phase B. Mobile phases consisted of (A) 95:5 acetonitrile/water with 0.125% v/v formic acid and 10 mM ammonium formate and (B) 95:5 water/acetonitrile with 0.125% v/v formic acid and 10 mM ammonium formate.

| **% B** | **22-minute** | **60-minute** | **90-minute** | **120minute** | **150minute** | **180minute** | **240minute** |
|---|---|---|---|---|---|---|---|
| **0** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **0** | 0.7 | 2 | 3 | 4 | 5 | 6 | 8 |
| **15** | 6.7 | 20 | 30 | 40 | 50 | 60 | 80 |
| **15** | 8.7 | 26 | 39 | 52 | 65 | 78 | 104 |
| **72** | 16 | 48 | 72 | 96 | 120 | 144 | 192 |
| **72** | 16.7 | 50 | 75 | 100 | 125 | 150 | 200 |
| **0** | 16.8 | 50.1 | 75.1 | 100.1 | 125.1 | 150.1 | 200.1 |
| **0** | 22 | 60 | 90 | 120 | 150 | 180 | 240 |

**Data Processing and Compound Identification**

Iterative LC-MS/MS data acquired from the modified HILIC separation conditions were loaded into an in-house software tool, *MetIDTracker*. This software facilitates searching of experimental spectra against multiple libraries by providing a graphical front-end for both input and output from the NIST MSPepSearch tool (www.chemdata.nist.gov). It also enables results generated using "identity," "in-source," and "hybrid" strategies to be reviewed in a single interface. Hits from identity searches share the same precursor ion to the collected spectra, while in-source hits ignore the precursor ion and score alignment of the fragment ions only. Hybrid searches combine both direct peak matching and neutral-loss matching where the mass difference between collected precursor ions and database entries is calculated before fragment ions are conditionally shifted by that delta mass. Spectral search hits are ranked by score values and are visualized using head-to-tail plots. Spectral quality metrics including pattern recognition entropy (PRE) and total intensity are also calculated for all spectra. Comprehensive manual review of all spectra in a full data set was performed only for the modified HILIC method, as this remains a time-consuming effort, but individual RPLC spectra were reviewed as needed and match scores, PRE, and total intensity were examined for all datasets.

The impact of mass loading, gradient length, and iterative acquisition on compound identification performance was assessed by the number of unique compound identifications from LC-MS/MS features searched against NIST20 (www.nist.gov), LipidBlast,29 Metlin,30 and Massbank of North America (MONA, www.massbank.us) spectral libraries. MS/MS spectral search was performed using the following parameters: mass range, 25-1200 m/z; precursor ion tolerance, 0.05 m/z; fragment peak tolerance, 0.05 m/z; minimum match factor, 0; minimum peak intensity, 1. Thresholds utilized for automated compound ID comparison experiments were based on parameters determined from the manually reviewed dataset as defined in the SI Methods section. Up to 10 output hits for all search types were considered when manual review was performed, whereas only the top scoring hit was used when evaluating automated compound ID assignments. Manual review additionally involved assessing extracted ion chromatograms for acceptable peak shape before selecting the database match for a feature. LipidBlast in silico MS/MS library hits were additionally considered MSI level 2 identifications after validating compound class and accurate precursor m/z. Identifications made using the Metlin database were only counted when manual review was performed as the database could only be searched using proprietary Agilent software, which uses a different scoring system than our standard method. All lipid and acylcarnitine hits were consolidated to species level identifications through LipidLynxX. Degenerate features (e.g. multiple parent ions produced by in-source fragmentation or multiple adduct formation from a single compound) were "collapsed" into a single representative feature with the highest intensity and counted as one unique ID with the assistance of the data reduction tool Binner.

Features that had high-quality spectral matches to one or more curated libraries, had a precursor ion match (± 20 ppm), and included at least two fragment ion matches (± 0.01 Da) were counted as Metabolomics Standards Initiative (MSI) level 2 identifications. If two or more features satisfying these criteria yielded the same compound ID, only the feature with the highest NIST score was counted as a unique identification. This approach causes chromatographically resolved isomers that could not be definitively distinguished by MS/MS to be counted only once. Although many of our MSI2 identifications technically qualify as MSI level 1 because the parent ion mass and the obtained MS/MS spectrum can be considered "orthogonal criteria," we typically reserve MSI1 to describe identified features with retention time confirmed using authentic standards. "In-source" (in-source fragment of precursor ion) and "hybrid" (neutral loss compatible) spectral matches were only accepted as a MSI2 compound ID after manual review and only if we confirmed coelution with the anticipated parent ion mass for the unfragmented molecule. Precursor ions observed to be present at levels less than twice their abundance in the solvent blank were removed from the dataset without further analysis, as were spectra that yielded only one or no fragment ions. Additionally, compound class annotations (MSI level 3) were assigned to features with identity, in-source, and hybrid search results that demonstrated good fragmentation alignment without acceptable precursor ion agreement by similar quantile thresholds determined from manual review. ClassyFire ontology was included for all MSI2 and MSI level 3 annotations when InChIKeys were available. Remaining features were considered MSI level 4 ("unknowns").

To validate as many of our identifications as possible we analyzed 95 authentic standards in our in-house library that matched compounds identified by MS/MS in our human plasma sample. We also ran plasma samples spiked with the same standards. After processing and performing database searching, we used retention time alignment and MS/MS spectra to confirm or refute compound identifications.

**Determination of High-Priority Unknowns**

After review and classification of features with *MetIDTracker*, linear discriminant analysis (LDA) was performed on confirmed identified metabolites (MSI2) and annotated or unknown compounds (MSI3

& MSI4) with retention time, precursor m/z, PRE, and total intensity standardized by the Z-score method to attempt to describe global trends between the two groups. The LDA model was supervised with an 80:20 training to testing split utilizing R packages MASS, car, and caret, while accuracy was calculated according to original feature classification and the model prediction. After ten trials to assess overall accuracy and standard deviation, the eleventh trial's model coefficients were multiplied by the four standardized variables to create the 4d space for K-nearest neighbor analysis (kNN). kNN was then performed with R package class 50 times by randomly generating a training:testing data split with a 2:1 likelihood respectively each trial, ensuring that all features were tested at least 5 times. The seven nearest neighbors were used to classify a given feature. Unknown spectral features surrounded by neighbors consisting of more confirmed identifications than unidentified features for a majority of the times tested were considered to be high-priority unknowns. Resulting high-priority unknown features from the model were consolidated by a +/- 20 ppm m/z and +/- 0.05 min retention window before loading into SIRIUS where plausible formulas and structures were assigned utilizing built-in CSI:FingerID.

**Alignment of Identified Features with Higher-Throughput LC-MS Data**

Metabolite identifications from the modified RPLC and HILIC conditions were mapped to features (both identified and unidentified) detected in the high-throughput (i.e. conventional) methods. Feature detection for both conditions was performed with MZmine2 through a combination of targeted (modified) and untargeted (high-throughput) analysis modules. The resulting feature lists were then aligned using *metabCombiner*, an R based package. Alignment of all feature pairs was scored from 0 to 1 (excellent alignment) based on differences in m/z, retention time fitting error, and relative abundance, with specific weights 60, 10, and 0.1 respectively. *metabCombiner* scores above 0.75 were classified as high-confidence alignments, while scores below 0.5 were rejected. Alignment scores between 0.5 and 0.75 were classified as possible matches for which manual validation is recommended.