High-Throughput Plasma Lipidomics: Detailed Mapping of the Associations with Cardiometabolic Risk Factors

Kevin Huynh,1,2 Christopher K. Barlow,1 Kaushala S. Jayawardana,1 Jacquelyn M. Weir,1 Natalie A. Mellett,1 Michelle Cinel,1 Dianna J. Magliano,1 Jonathan E. Shaw,1 Brian G. Drew,1 and Peter J. Meikle1,2,3,*

1Head Metabolomics Laboratory, Baker Heart and Diabetes Institute, 75 Commercial Road, Melbourne, VIC 3004, Australia
2Faculty of Medicine, Nursing and Health Sciences, Monash University, Melbourne, Australia
3Lead Contact
*Correspondence: peter.meikle@baker.edu.au
https://doi.org/10.1016/j.chembiol.2018.10.008

SUMMARY

High-throughput targeted lipid profiling with liquid chromatography-mass spectrometry (LC-MS) has been used extensively to identify associations between plasma lipid species and disease states. Such methods, used to characterize larger clinical cohorts, often suffer from an inability to differentiate isomeric forms of glycerophospholipids that are typically reported as the sum fatty acid carbons and double bonds. Here we report a chromatography gradient coupled with a detailed characterization of the human plasma lipidome to provide improved resolution and identification of 636 lipid species, including previously unreported species, in a 15-min analysis. We have utilized this method on a subset of the Australian Diabetes, Obesity, and Lifestyle Study and have detailed associations of plasma lipid species with anthropometric and blood glucose measures. These results highlight the importance and power of high-throughput lipidomics coupled with a detailed characterization of the lipidome to better understand lipid biology in a population setting.

INTRODUCTION

Lipidomics involves the global characterization of lipid species in biological systems and has advanced in recent decades due primarily to improving liquid chromatography-mass spectrometry (LC-MS) technologies. The two major lipidomic strategies are untargeted lipidomics, which aims to identify compounds after data analysis and targeted lipidomics, which focuses on identification prior to analysis, both strategies have their own advantages and limitations (Cajka and Fiehn, 2015).

Cardiometabolic disease presents with significantly altered lipid metabolism and is thus a prime candidate for lipidomic studies. Lipidomic characterization of relevant patient cohorts have the potential to identify new lipid biomarkers for risk assessment, for therapeutic monitoring or as therapeutic targets and limitations (Cajka and Fiehn, 2015). Cardiometabolic characterization of relevant patient cohorts have the potential to identify new lipid biomarkers for risk assessment, for therapeutic monitoring or as therapeutic targets and limitations (Cajka and Fiehn, 2015). Cardiometabolic characterization of relevant patient cohorts have the potential to identify new lipid biomarkers for risk assessment, for therapeutic monitoring or as therapeutic targets and limitations (Cajka and Fiehn, 2015).
RESULTS

Identification of Additional Lipid Species in Human Plasma

Starting with our existing method (Weir et al., 2013), our goal was to expand with additional lipid classes while maintaining a 15-min analysis time. We optimized and included into our method species of: G_{M2} ganglioside, sulfatide, lyssolecithin, phosphatidylcholine, lysoalkenylphosphatidylethanolamine, lysocephatidylcholine, alkyl-diacylglycerol, sterol ester derivatives, sphingolipids containing alternate bases (d16:1, d17:1, d18:2, d19:1, and d20:1, evident primarily in ceramide and sphingomyelin species), ubiquinone, and acylcarnitine (Table 1). A complete list of multiple reaction monitoring transitions and retention times are shown in Table S1.

Characterization of Glycerophospholipid Isomers

Through increased chromatographic separation of lipid species, we observed multiple sphingolipid and glycerophospholipid isomers (Figures 1A–1D). These isomers could not be fully characterized as their protonated or ammonium adducts and so we performed additional fragmentation analysis of the lithium adducts in positive ionization mode and of the deprotonated species in negative ionization mode using the plasma quality control (PQC) samples in independent runs. With these additional experiments we were able to assign the fatty acid composition to the majority of glycerophospholipids and sphingolipids (Figures 1A–1D).

We identified several lipid species with isomers that consisted of the same acyl combination which, in some instances, could be attributed to differing double bond positions such as the phosphatidylcholine species, PC(18:0_22:5) (Figure 1C). Such glycerophospholipid isomers esterified with polyunsaturated fatty acids with different bond positions were well separated and full characterization was achieved by the synthesis of these species. For example, the phosphatidylcholine species esterified with docosapentaenoic acid (DPA) (Figures S1A and S1B), where the earlier eluting peak was the all-cis-7,10,13,16,19-DPA isomer (omega-3) and the later eluting peak the all-cis-7,10,13,16-DPA (omega-6) (Figure S1A). Alkenylphosphatidylethanolamine (ethanolamine plasmalogen) species that contain an 18:1 fatty alcohol, such as the phosphatidylethanolamine plasmalogen, PE(P-18:1/22:6), exhibited chromatographic separation of two isomers presumably due to the n7 and n9 isofoms of 18:1. We have subsequently assigned the fatty acid isomers in other lipid classes including phosphatidylethanolamine, phosphatidylcholine, cholesteryl ester, and lysocephatidylcholine.

Identification and Characterization of Branched Chain Glycerophospholipids

We identified multiple isomers of odd-chain glycerophospholipid species that had the same acyl composition but different retention times, for example, PC(17:0_18:2) (Figure 1D). Positional isomers as in PC(17:0_18:2) and PC(18:2_17:0) were deemed unlikely as we did not observe such isomers for related species such as PC(16:0_18:2) and PC18:2/16:0). Possible isomers of odd acyl chains are monomethyl branched-chain fatty acids, which might be esterified into glycerophospholipid pools (Garcia Caraballo et al., 2014). We synthesized phosphatidylcholine species containing either a C17:0 straight acyl chain or the branched-chain species 15-methylhexadecanoic acid (15-MHDA) using thionyl chloride-treated fatty acids and soy lysocephatidylcholine. The straight-chain C17:0 containing PC(17:0_18:2) aligned with the peak with the later retention time (Figures S1C and S1D), while the earlier eluting peak showed alignment with the PC(15-MHDA_18:2) (Figures S1C and S1D). It should be noted that there is likely some 14-MHDA that does not completely resolve from the 15-MHDA peak under our chromatographic conditions.

We observed odd-carbon ether lipid species that exhibited double peaks of the same composition, in a similar fashion to odd-carbon diacyl species. These additional peaks were further confirmed as plasmalogen species by acid hydrolysis of the alkenyl bond (Figures S2A and S2B). These choline and ethanolamine plasmalogen species all exhibited doublet peaks consistent with the presence of both straight-chain and branched-chain isomers (Figures S2A, S3A and S3B). Product ion scanning of peaks corresponding to PE(P-17:0/20:4) at retention times of 7.77 and 7.96, respectively, yielded the same product ions (Figures S3C and S3D). We were unable to synthesize the corresponding branched plasmalogen species for validation, but propose that the earlier eluting peak represent the branched alkenyl chain species. Branched and odd-carbon aldehydes from plasmalogens have been identified previously in humans (O’Brien and Sampson, 1965).

Deconvolution of Lysoglycerophospholipid Species

As we observed separation of both branched-chain and double-bond isomers in the majority of our glycerophospholipid species, we hypothesized that we would observe the corresponding lysophosphatidylcholine species. Coupled with the separation of the sn1 and sn2 isomers, this would likely result in four isomers of each lysophosphatidylcholine species (e.g., LPC(17:0) and LPC(22:5)). Indeed, convoluted chromatographic peaks resulting from the four isomers were seen for these species (Figures 2A and 2B).

To deconvolute the signals for these species, we examined two product ions concurrently. The product ion at m/z 184.1 is characteristic of the phosphocholine head group and results in four peaks; however, the product ion at m/z 104.1 only occurs in lysophosphatidylcholine species esterified in the sn1 position (Figures 2B, 2D, and S1B). Examining these concurrently allowed the separate analysis of sn1 15-MHDA from the sn2 17:0 lysophosphatidylcholine species. We synthesized the corresponding lysospecies using 15-MHDA or heptadecanoic acid and glycerophosphorylcholine, and validated these assignments by analyzing them with our LC-MS/MS method (Figures 2C and 2D). Our method includes transitions utilizing both 104.1 and 184.1 for lysophosphatidylcholine species that show these convoluted chromatograms, namely LPC(17:0), LPC(19:0), LPC(17:1), LPC(18:3), and LPC(22:5) (Table S1).

Summary of Lipidome Identification

The majority of sphingolipids, acylcarnitines, and sterol esters have specific product ions corresponding to their acyl components which were used to provide unique identification. Sterol esters measured using the m/z 367.3 product ion showed chromatographic alignment with the desmosterol ester standard.
Table 1. Conditions for Tandem Mass Spectrometry Analysis of Lipid Species

<table>
<thead>
<tr>
<th>Lipid Class/Subclass</th>
<th>Parent Ion</th>
<th>Fragmentation</th>
<th>Number of Features</th>
<th>Internal Standard</th>
<th>Internal Standard (pmol)</th>
<th>Collision Energy (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphingosine</td>
<td>[M + H]+[a]</td>
<td>NL, 18.0 Da</td>
<td>3</td>
<td>Sph(d17:1)</td>
<td>50</td>
<td>8</td>
</tr>
<tr>
<td>Sphingosine-1-phosphate</td>
<td>[M + H]+[a]</td>
<td>sphingoid specific[a]</td>
<td>5</td>
<td>Sph(d17:1)</td>
<td>50</td>
<td>11–16</td>
</tr>
<tr>
<td>Dihydroceramide</td>
<td>[M + H]+[a]</td>
<td>sphingoid specific[a]</td>
<td>11</td>
<td>dhCer(d18:0/8:0)</td>
<td>50</td>
<td>31</td>
</tr>
<tr>
<td>Ceramide</td>
<td>[M + H]+[a]</td>
<td>sphingoid specific[a]</td>
<td>49</td>
<td>Cer(d18:1/17:0)</td>
<td>100</td>
<td>23–29</td>
</tr>
<tr>
<td>Monohexosylceramide</td>
<td>[M + H]+[a]</td>
<td>sphingoid specific[a]</td>
<td>14</td>
<td>HexCer (d18:1/16:0) d3</td>
<td>50</td>
<td>33</td>
</tr>
<tr>
<td>Dihexosylceramide</td>
<td>[M + H]+[a]</td>
<td>sphingoid specific[a]</td>
<td>10</td>
<td>Hex2Cer(d18:1/16:0) d3</td>
<td>50</td>
<td>53</td>
</tr>
<tr>
<td>Trihexosylceramide</td>
<td>[M + H]+[a]</td>
<td>PI, m/z 264.3</td>
<td>6</td>
<td>Hex3Cer(d18:1/17:0)</td>
<td>50</td>
<td>57</td>
</tr>
<tr>
<td>GM3 ganglioside</td>
<td>[M + H]+[a]</td>
<td>sphingoid specific[a]</td>
<td>7</td>
<td>Hex3Cer(d18:1/17:0)</td>
<td>50</td>
<td>57</td>
</tr>
<tr>
<td>GM1 ganglioside</td>
<td>[M + 2H][a][a]</td>
<td>PI, m/z 366.2</td>
<td>1</td>
<td>Hex3Cer(d18:1/17:0)</td>
<td>50</td>
<td>9</td>
</tr>
<tr>
<td>Sulfatide</td>
<td>[M + H]+[a]</td>
<td>PI, m/z 264.3</td>
<td>6</td>
<td>sulfatide(d18:1/12:0)</td>
<td>50</td>
<td>56</td>
</tr>
<tr>
<td>Ceramide-1-phosphate</td>
<td>[M + H]+[a]</td>
<td>PI, m/z 264.3</td>
<td>1</td>
<td>Cer(d18:1/17:0)</td>
<td>50</td>
<td>29</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>[M + H]+[a]</td>
<td>PI, m/z 184.1</td>
<td>44</td>
<td>SM(d18:1/12:0)</td>
<td>200</td>
<td>25</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>[M + H]+[a]</td>
<td>PI, m/z 184.1</td>
<td>68</td>
<td>PC(13:0/13:0)</td>
<td>100</td>
<td>21</td>
</tr>
<tr>
<td>Alkylphosphatidylcholine</td>
<td>[M + H]+[a]</td>
<td>PI, m/z 184.1</td>
<td>22</td>
<td>PC(13:0/13:0)</td>
<td>100</td>
<td>21</td>
</tr>
<tr>
<td>Alkenylphosphatidylcholine</td>
<td>[M + H]+[a]</td>
<td>PI, m/z 184.1</td>
<td>26</td>
<td>PC(13:0/13:0)</td>
<td>100</td>
<td>21</td>
</tr>
<tr>
<td>Lyso phosphatidylcholine</td>
<td>[M + H]+[a]</td>
<td>PI, m/z 184.1 and m/z 104.1</td>
<td>61</td>
<td>LPC(13:0)</td>
<td>100</td>
<td>21</td>
</tr>
<tr>
<td>Lysoalkylphosphatidylcholine</td>
<td>[M + H]+[a]</td>
<td>PI, m/z 104.1</td>
<td>10</td>
<td>LPC(13:0)</td>
<td>100</td>
<td>21</td>
</tr>
<tr>
<td>Lysoalkenylphosphatidylcholine</td>
<td>[M + H]+[a]</td>
<td>PI, m/z 104.1</td>
<td>6</td>
<td>LPC(13:0)</td>
<td>100</td>
<td>21</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>[M + H]+[a]</td>
<td>NL, 141.0 Da</td>
<td>37</td>
<td>PE(17:0/17:0)</td>
<td>100</td>
<td>17</td>
</tr>
<tr>
<td>Alkylphosphatidylethanolamine</td>
<td>[M + H]+[a]</td>
<td>NL, 141.0 Da</td>
<td>14</td>
<td>PE(17:0/17:0)</td>
<td>100</td>
<td>17</td>
</tr>
<tr>
<td>Alkenylphosphatidylethanolamine</td>
<td>[M + H]+[a]</td>
<td>acyl specific</td>
<td>55</td>
<td>PE(17:0/17:0)</td>
<td>100</td>
<td>17</td>
</tr>
<tr>
<td>Lyso phosphatidylethanolamine</td>
<td>[M + H]+[a]</td>
<td>NL, 141.0 Da</td>
<td>14</td>
<td>PE(17:0/17:0)</td>
<td>100</td>
<td>17</td>
</tr>
<tr>
<td>Lysoalkenylphosphatidylethanolamine</td>
<td>[M + H]+[a]</td>
<td>NL, 171.9 Da</td>
<td>4</td>
<td>PE(17:0/17:0)</td>
<td>100</td>
<td>19</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>[M + NH4][a]</td>
<td>NL, 277.0 Da</td>
<td>27</td>
<td>PE(17:0/17:0)</td>
<td>100</td>
<td>17</td>
</tr>
<tr>
<td>Phosphatidylglycerol</td>
<td>[M + NH4][a]</td>
<td>NL, 277.0 Da</td>
<td>8</td>
<td>LPI(13:0)</td>
<td>100</td>
<td>17</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>[M + H]+[a]</td>
<td>NL, 185.0 Da</td>
<td>7</td>
<td>PS(17:0/17:0)</td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td>Free cholesterol</td>
<td>[M + NH4][a]</td>
<td>PL, m/z 369.3</td>
<td>1</td>
<td>COH d7</td>
<td>10,000</td>
<td>23</td>
</tr>
<tr>
<td>Cholesteryl ester</td>
<td>[M + NH4][a]</td>
<td>PL, m/z 369.3</td>
<td>27</td>
<td>CE(18:0) d6</td>
<td>1000</td>
<td>10</td>
</tr>
<tr>
<td>Other steroid ester derivative</td>
<td>[M + NH4][a]</td>
<td>PL, m/z 367.3</td>
<td>6</td>
<td>CE(18:0) d6</td>
<td>1000</td>
<td>12</td>
</tr>
<tr>
<td>Acylcarnitines</td>
<td>[M + H]+[a]</td>
<td>PL, m/z 85.1</td>
<td>14</td>
<td>acylcarnitine(16:0) d6</td>
<td>100</td>
<td>30</td>
</tr>
<tr>
<td>Diacylglycerol</td>
<td>[M + NH4][a]</td>
<td>NL, fatty acid</td>
<td>20</td>
<td>DG(15:0/15:0)</td>
<td>200</td>
<td>21</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>[M + NH4][a]</td>
<td>NL, fatty acid</td>
<td>44</td>
<td>TG(17:0/17:0/17:0)</td>
<td>100</td>
<td>21</td>
</tr>
<tr>
<td>Alkylacylglycerol</td>
<td>[M + NH4][a]</td>
<td>NL, fatty acid</td>
<td>3</td>
<td>TG(17:0/17:0/17:0)</td>
<td>100</td>
<td>21</td>
</tr>
<tr>
<td>Ubiquinone</td>
<td>[M + NH4][a]</td>
<td>PI, m/z 197.0</td>
<td>1</td>
<td>Hex3Cer(d18:1/17:0)</td>
<td>50</td>
<td>17</td>
</tr>
</tbody>
</table>

Pl, product ion; NL, neutral loss.
[a]Full product ion list in Table S1.
[b]External internal standard.
In summary we have identified 636 unique lipid measurements. Of these, 379 species were characterized at the level of acyl chain identity and position, 115 species at the level of acyl chain identity, and 126 species at the level of acyl chain sum composition. A further 16 measures represented mixtures of two species identified at the acyl chain identity level.

**Lipidomic Analysis of Human Plasma**

We applied our developed methodology to a subcohort (n = 640) from the AusDiab study, a prospective population study examining the prevalence and risk factors of type 2 diabetes (T2D) and cardiovascular disease in the Australian population (Dunstan et al., 2002). Details of the cohort are outlined in the STAR Methods and in Table S2.

We observed good reproducibility of quantification from repeated injections of our technical quality control (TQC) samples across the sample set (Table S3). In total there were 35 TQC injections spanning a period of 8 days. Median percentage coefficient of variation (%CV) for our technical replicates across the 636 analytes was 8.32%, with 541 of the analytes having a %CV of 15% or less (Table S3). All data for pooled PQCs, TQCs, blanks,
Associations between the Plasma Lipidome and Cardiometabolic Risk Factors

The majority of plasma lipid species are tightly coupled with circulating lipoprotein levels, which are themselves associated with cardiometabolic disease risk (DeFronzo and Ferrannini, 1991; Gordon et al., 1989), as well as the anthropometric measures under investigation. To identify lipid associations with cardiometabolic risk factors independent of lipoprotein levels we performed regression analyses with and without adjustment for clinical lipids (defined as the clinical measures of total cholesterol, high-density lipoprotein cholesterol [HDL-C], and triglyceride levels). Association studies for age, gender, and BMI were on the normoglycemic individuals only (n = 389), while association studies with fasting blood glucose (FBG) and 2-hr post load glucose (2h-PLG) were on the entire subcohort (n = 640).

Plasma Lipid Species Associate with Age

Aging is associated with a plethora of metabolic changes, so it is not surprisingly that many lipid species associate with age. Univariate regression analysis of age with plasma lipid species, adjusting for gender and BMI, identified 197 of the 636 measured lipid species as significantly associated after correction for multiple comparisons (Figure 3A; Table S4). After additional adjustment for the clinical lipid measures, 140 lipid species were associated with age (Figure 3B; Table S4). Concentrations of select individual species are presented in Figure 3D.

In general, sphingolipids were positively associated with age. The most significant associations typically involved species containing either a C16:0 or C24:1, and these were independent of the sphingoid base (Figures 3A and 3B; Table S4). The addition of the acyl chain to the sphingoid base in ceramide synthesis is controlled by a series of ceramide synthases, which show specificity for acyl chain length. Sphingolipids containing 24:1 are typically enriched in myelin, and knockout studies of ceramide synthase 2 have shown myelin defects and cerebellar degeneration (Imgrund et al., 2009), with circulatory 24:1 containing sphingolipid species possibly reflecting changes to myelin with age. Cer(d18:1/16:0) can be synthesized by ceramide synthases 5 and 6 (Mullen et al., 2012), and both Cer(d18:1/16:0) and Cer(d18:1/24:1) have recently been reported to be associated with increased risk of cardiovascular disease (Laaksonen et al., 2016).

Acylcarnitines were strongly associated with age, even after adjustment for clinical lipids. Acylcarnitine(14:2) had the

and the National Institute of Standards Technology (NIST) 1950 serum samples are presented in Table S3.

Figure 2. Characterization of Convoluted Lysophosphatidylcholine Species

(A) Chromatogram of lysophosphatidylcholine species showing the multiple reaction monitoring scans m/z 510.4/m/z 184.1 (black trace) from a human pooled plasma sample extract. The four observable peaks are LPC(15-MHDA) [sn2], LPC(17:0) [sn2], LPC(15-MHDA) [sn1], and LPC(17:0) [sn1].

(B) The same sample (black trace) examining the m/z 510.4/m/z 104.1 transition. Only LPC(15-MHDA) [sn1] and LPC(17:0) [sn1] are visible.

(C) Synthesized LPC(17:0) (green trace) and LPC(15-MHDA) (blue trace) examined using the m/z 510.4/m/z 184.1 transition.

(D) Synthesized LPC(17:0) (green trace) and LPC(15-MHDA) (blue trace) examined using the m/z 510.4/m/z 104.1 transition.
Figure 3. Associations between Age and Plasma Lipid Species

(A and B) Linear regression analyses between age and lipid species were performed on 389 normoglycemic individuals adjusting for BMI and gender (A) or BMI, gender, total cholesterol, HDL-C, and triglyceride levels (B). Gray open circles show non-significant species, dark gray circles show species with p < 0.05 after multiple comparisons correction. Red circles above graphs indicate that the change per year in age was statistically significant (p < 0.05). Significant changes are shown as blue broken lines in the network graph. Red circles below graphs indicate that the change per year in age was not statistically significant (p > 0.05). Significant changes are shown as green solid lines in the network graph.

Legend continued on next page.
strongest association (1.40% increase per year, corrected p value $1.81 \times 10^{-7}$, Table S4). A pathway highlighting acyl-carnitine metabolism is shown in Figure 3C. Dysregulation of this pathway with aging may lead to impairment of mitochondrial fatty acid oxidation, resulting in accumulation of intracellular acyl-carnitine (Schooneman et al., 2013), which is subsequently reflected in plasma (Nolands et al., 2009; Ramsay and Zammit, 2004). Acylcarnitine(14:2) is an atypical species (product ion spectra shown in Figure S4B) that has been reported in earlier studies (Costa et al., 1999), with its origin likely arising from partial beta oxidation of linoleic acid (18:2). Additional steps involving enoyl-coenzyme A (enoyl-CoA) isomerase are required to oxidize linoleic acid in the mitochondria as the double bonds prevent the normal oxidation process. We hypothesize that the isomerase is a limiting step leading to the build up of the 14:2 species. It may be possible that acylcarnitine(12:2) also exists, but this compound elutes with the solvent front under current chromatographic conditions.

Polyunsaturated fatty acids and their metabolites are a major area of research for their potential role in preventing many diseases, in particular neurological diseases of aging (Bazinet and Layé, 2014). As we were able to characterize acyl composition of the major glycerophospholipids, we observed a positive association of circulating omega-3 fatty acids and a negative association with omega-6 fatty acids in multiple glycerophospholipid classes with aging. It is unclear at this stage whether this reflects differences in dietary intake as we age, differences in fatty acid and/or glycerophospholipid biosynthetic pathways or differential metabolism omega-3 fatty acids to anti-inflammatory metabolites and omega-6 fatty acids into pro-inflammatory metabolites.

**Plasma Lipid Species Differ in Male and Female**

We identified 336 lipid species associated with gender after correction for multiple comparisons (Figure 4A; Table S5). After further adjustment for clinical lipids 297 lipid species were associated with gender (Figure 4B; Table S5). Unadjusted concentrations are presented in Figure 4C.

The most striking associations were observed between gender and sphingomyelin species containing a d18:2 sphingoid base, which showed a strong negative association with being male. The strongest individual association was with the sphingomyelin species, SM(d18:2/14:0), which was 28.9% lower in males even after adjustment for clinical lipids (corrected p value $1.81 \times 10^{-7}$, Figure 4B). A product ion spectra for SM(d18:2/14:0) is shown in Figure S4D. The precursor ceramide species, Cer(d18:2/14:0), was similarly associated (30.8% lower in males, corrected p value $2.59 \times 10^{-13}$, Figure 4B).

Early research has indicated the extra double bond in human plasma d18:2 sphingolipids is located on the cis-14 position (Renkonen and Hirvisalo, 1969) resulting in d18:2 (4E,14Z) sphingoid base, with recent research suggesting certain subclasses of sphingolipids only presenting a double bond in the 14Z position (Steiner et al., 2016). Our results suggest that the activity of this unknown desaturase is higher in women, resulting in elevated d18:2 sphingolipids. The exact mechanism for the formation of the double bond on the sphingolipid remains to be determined, but we observe the d18:2 base in all sphingolipid pools in human plasma (Table S3).

It has been suggested that the majority of palmitoleate is produced via de novo lipogenesis (DNL) (Hodson and Karpe, 2013) by the action of a steroyl-CoA desaturase and is typically a marker for adiposity. Nearly all species containing palmitoleate follow the same association as the d18:2 sphingomyelins and are higher in women than in men (Table S5). Similar to palmitoleic acid, myristic acid can also be synthesized through the DNL pathway (Wu et al., 2011). The incorporation of myristic acid and the d18:2 sphingoid base into the same pathway producing the ceramide and sphingomyelin species, Cer(d18:2/14:0) and SM(d18:2/14:0) may compound the difference of two different lipid pathways between males and females, leading to stronger associations of these species. Differences in DNL between male and female have been reported (Edens et al., 1993; Varlamov et al., 2014).

We observe the same sphingomyelin species, SM(d18:2/14:0), associate with BMI even after adjusting for gender. These results together point to potential mechanisms whereby increased DNL in women, leading to higher subcutaneous adiposity, may contribute to reduced cardiometabolic risk, while increased DNL associated with BMI, leading to higher visceral adiposity, contributes instead to increased cardiometabolic risk (primarily in men).

**Plasma Lipid Species Are Associated with BMI**

Obesity is a major risk factor for several metabolic diseases, and lipid species associated with BMI might shed some light on the pathophysiology. We identified 338 plasma lipid species associated with BMI (Figure 5A; Table S6). Further adjustments for clinical lipids resulted in 147 lipid associations. BMI is typically associated with increased total cholesterol and triglycerides and decreased HDL-C (Brown et al., 2000), and so, as expected, adjustment for clinical lipids explained many lipid species associations. Unadjusted concentrations are presented in Figure 5D.

There were contrasting associations of simple sphingolipids (sphingosine, dihydroceramide, and ceramide) and complex sphingolipids such as mono, di-, and trihexosylceramides. Simple sphingolipids were positively associated with BMI, whereas complex sphingolipids displayed a negative association with BMI (Figure 5A; Table S6). Ceramide sits at the center of sphingolipid metabolism, and has been shown to be a contributing factor to cardiometabolic disease in both animal models and profiling studies (Meikle and Summers, 2017). As with our earlier profiling studies (Meikle et al., 2013; Weir et al., 2013), our current results suggest an upregulation of ceramide biosynthesis, which flows through to sphingomyelin synthesis but is blocked by a downregulation of the complex glycosphingolipid biosynthetic pathway. However, here we also observe that the ceramide profiles of simple sphingolipids are different between genders (Steiner et al., 2016). Our results suggest that the activity of this unknown desaturase is higher in women, resulting in elevated d18:2 sphingolipids. The exact mechanism for the formation of the double bond on the sphingolipid remains to be determined, but we observe the d18:2 base in all sphingolipid pools in human plasma (Table S3).

It has been suggested that the majority of palmitoleate is produced via de novo lipogenesis (DNL) (Hodson and Karpe, 2013) by the action of a steroyl-CoA desaturase and is typically a marker for adiposity. Nearly all species containing palmitoleate follow the same association as the d18:2 sphingomyelins and are higher in women than in men (Table S5). Similar to palmitoleic acid, myristic acid can also be synthesized through the DNL pathway (Wu et al., 2011). The incorporation of myristic acid and the d18:2 sphingoid base into the same pathway producing the ceramide and sphingomyelin species, Cer(d18:2/14:0) and SM(d18:2/14:0) may compound the difference of two different lipid pathways between males and females, leading to stronger associations of these species. Differences in DNL between male and female have been reported (Edens et al., 1993; Varlamov et al., 2014).

We observe the same sphingomyelin species, SM(d18:2/14:0), associate with BMI even after adjusting for gender. These results together point to potential mechanisms whereby increased DNL in women, leading to higher subcutaneous adiposity, may contribute to reduced cardiometabolic risk, while increased DNL associated with BMI, leading to higher visceral adiposity, contributes instead to increased cardiometabolic risk (primarily in men).

**Plasma Lipid Species Are Associated with BMI**

Obesity is a major risk factor for several metabolic diseases, and lipid species associated with BMI might shed some light on the pathophysiology. We identified 338 plasma lipid species associated with BMI (Figure 5A; Table S6). Further adjustments for clinical lipids resulted in 147 lipid associations. BMI is typically associated with increased total cholesterol and triglycerides and decreased HDL-C (Brown et al., 2000), and so, as expected, adjustment for clinical lipids explained many lipid species associations. Unadjusted concentrations are presented in Figure 5D.

There were contrasting associations of simple sphingolipids (sphingosine, dihydroceramide, and ceramide) and complex sphingolipids such as mono, di-, and trihexosylceramides. Simple sphingolipids were positively associated with BMI, whereas complex sphingolipids displayed a negative association with BMI (Figure 5A; Table S6). Ceramide sits at the center of sphingolipid metabolism, and has been shown to be a contributing factor to cardiometabolic disease in both animal models and profiling studies (Meikle and Summers, 2017). As with our earlier profiling studies (Meikle et al., 2013; Weir et al., 2013), our current results suggest an upregulation of ceramide biosynthesis, which flows through to sphingomyelin synthesis but is blocked by a downregulation of the complex glycosphingolipid biosynthetic pathway. However, here we also observe that the ceramide
protein phospholipase A2 activity, with the exception of species associated with BMI, presumably as a result of increased lipo-
dylcholine, and lysophosphatidylethanolamine) were negatively
dylcholine, lysoalkylphosphatidylcholine, lysoalkenyl(phosphati-
dylcholine, and lysophosphatidylethanolamine) were negatively
dyshed in Table S6).

Several classes of lysoglycerophospholipids (lysophosphatidylcholine, lysolaurylphosphatidylcholine, lysolenylphosphatidylcholine, and lysophosphatidylethanolamine) were negatively associated with BMI, presumably as a result of increased lipo-
protein phospholipase A2 activity, with the exception of species

appears to be linked to lipoprotein metabolism as the associa-
tion with ceramide species is largely lost when we adjust for clin-
ical lipids (Table S6).

Overlapped Associations between Plasma Lipids with Age, Gender,
and BMI

Out of the 636 lipid species measured, 58 lipid species were associated with age, gender, and BMI independent of each other (Table S7). Only 26 species remained

associated after further adjustments for clinical lipids. As lipids
arise from many interconnecting pathways it is not surprising
that some species have similar associations with various mea-
sures. The ceramide species, Cer(d18:1/21:0), for example, is
associated with age and BMI due to ceramide being increased with age and body mass as a class (Tables S4 and S6), whereas a strong negative association with being male was seen for cer-

Figure 4. Associations between Gender and Plasma Lipid Species
(A and B) Linear regression analysis between gender and lipid species was performed on 389 normogly-
cemic individuals adjusting for BMI and age (A) or BMI, age, total cholesterol, HDL-C, and triglyceride levels (B). Gray circles show non-significant species, orange and purple circles show species with p < 0.05 after correction for multiple comparisons (Benjamini-Hochberg). The top 15 species after correction for multiple comparisons are highlighted in red. (C) Boxplots visualizing the unadjusted concentra-
tion differences of specific lipid species between females (n = 197), males (n = 192), NIST sample (n = 10 replicates), and our pooled plasma quality control sample (n = 34 replicates). For full lipidomic associations, see Table S5. esterified with a 14:0 or 16:1 fatty acid (Figure 5A). In general 14:0 and 16:1 fatty acids are positively associated with BMI (likely relating to the increased lipogenesis), but lysoglycerophospholipids as a class are normally negatively associated with BMI. This results in a net zero association for these species. A similar effect is observed in the phosphatidylethanolamine species and is driven by clinical lipids (Figure 5B). Prior to adjustment for clinical lipids, many phosphatidylethanolamine species exhibit a positive association with BMI; however, these associations are not signif-
icant after adjustment. In contrast, lipid species containing branched, odd and 18:2 acyl chains are typically negatively associated with BMI, thus, before adjustment for clinical lipids, ethanolamine lipid species show a net zero association (a balance between the positive association of PE and the negative association of the fatty acids with BMI) and a significant negative association after adjustment for clinical lipids (Figure 5B, Table S6).
Figure 5. Associations between BMI and Plasma Lipid Species
(A) Linear regression analysis between BMI and lipid species was performed on 389 normoglycemic individuals adjusting for age and gender (light blue squares) or age, gender, total cholesterol, HDL-C, and triglyceride levels (red circles). Clusters corresponding to different classes are highlighted. Gray squares and circles show non-significant species, light blue squares and red circles show species with p < 0.05 after correction for multiple comparisons (Benjamini-Hochberg).
(B) The same analysis of the individual phosphatidylethanolamine species; error bars are 95% confidence intervals.
(C and D) Proposed pathway of branched-chain amino acid catabolism to produce branched-chain and odd fatty acids in humans (C). Boxplots visualizing the unadjusted concentration differences of specific lipid species between BMI of 19–25 (n = 156), BMI of 25–30 (n = 164), BMI of 30+ (n = 69), NIST sample (n = 10 replicates), and our pooled plasma quality control sample (n = 34 replicates) (D). For full lipidomic associations, see Table S6.
Associations between Plasma Lipids and Measures of Glucose Homeostasis and Tolerance

A total of 186 lipid species were associated with FBG. Strong negative associations were observed with lysophosphatidylcholine species esterified with branched- or odd-chain fatty acids. Lysoalkylphosphatidylcholine species were also negatively associated (Figure 6A; Table S8). Similar to associations with BMI, a strong positive association was observed for PE species particularly those esterified with polyunsaturated fatty acids (both omega-3 and omega-6), although these associations were independent of BMI.

For 2h-PLG, a total of 223 lipids were associated after multiple comparison correction (Figure 6B; Table S8), with a common negative association observed with linoleic acid (18:2) esterified glycerophospholipids, which have been highlighted in multiple studies looking into insulin resistance and T2D (Hodge et al., 2007; Wu et al., 2017). These results also highlight the importance of separating and characterizing glycerophospholipid isomers, with species such as phosphatidylcholine, PC(18:2/18:2), showing strong negative associations, 4.6% change per mmol/L of 2h-PLG level, corrected p value 1.39 x 10^-6, in contrast to its isomer, PC(16:0_20:4), which had no association, 0.65%, corrected p value 2.49 x 10^-1. In general the downstream metabolite of linoleic acid, arachidonic acid (20:4), was not associated with 2h-PLG.
Interestingly, ceramides n-acylated with an odd fatty acid were negatively associated with 2h-PLG, in contrast to the many studies highlighting positive associations with ceramides and insulin resistance (Chaurasia and Summers, 2015). In addition, sphingolipid species with either a d17:1 or d18:2 sphingoid base were also negatively associated with 2h-PLG levels. Only 81 lipids were associated with both FBG and 2h-PLG, highlighting the major differences between these lipid profiles. Common negative associations include the lysophosphatidylcholine and lysoalkyl/phosphatidylcholine species, odd/ branched glycerophospholipid species, and complex sphingolipids, while common positive associations included phosphatidylethanolamine, diacylglycerol, and triacylglycerol species. Of particular interest were the differences in the lipid associations observed; while many alkyl- and alkenylphosphatidylethanolamine species showed a strong positive association with FBG, these were typically non-significant or negatively associated with 2h-PLG (Figures 6A and 6B). Phosphatidylinositol species also showed opposing associations with seven species negatively associated with FBG and five species positively associated with 2h-PLG, while acylcarnitine species were also negatively associated with FBG and non-significantly or positively associated with 2h-PLG (Figures 6A and 6B). Three species of alkylphosphatidylethanolamine showed significant opposite associations with FBG and 2h-PLG (PE(P-20:0/18:1), PE(P-20:0/ 20:4), and PE(P-20:0/22:6), Table S8).

Relationship of Our Quantification with Other Lipidomic Studies

A recent inter-laboratory lipidomics comparison project was conducted using the NIST 1950 human serum (Bowden et al., 2017). This massive study had 30 different laboratories examine the NIST 1950 human serum sample to report concentrations of individual lipids.

We have analyzed the same NIST 1950 sample on our platform (10 replicates, Table S3) and have performed a comparison of individual lipid species between our measures and the consensus measures from the published study (Table S8). We were able to align 200 lipid measures with single or pooled measures in our NIST profile. When we plotted the consensus concentration from the NIST study against our values we obtained a very strong correlation (y = 1.04x – 2.13, R² = 0.978). In general our results were close to the calculated consensus concentrations; for example, the lysophosphatidylcholine species, LPC(20:4), was reported to have a concentration of 6.0 µM (median of the laboratory means [MEDM]), n = 20 different laboratories with a standard uncertainty of 0.6, whereas our concentrations (sum of LPC(20:4) sn1 and sn2 isomers) of the same NIST sample was reported as 5.31 µM (Table S8). Species without class-specific internal standards such as phosphatidylinositol still performed within range, e.g., PI(18:0_22:6); Bowden et al. (2017) reported consensus concentration of 0.84 µM (MEDM, n = 12, uncertainty 0.16) versus our value of 0.67 µM. Species with calculated response factors and bordering linearity still performed well, for example, the most abundant cholesterol ester, CE(18:2), had a reported consensus concentration of 1,700 µM (MEDM, n = 26, uncertainty 430) versus our reported value of 1,870 µM (Table S8).

DISCUSSION

The associations with age, gender, BMI, FBG, and 2h-PLG identified in this study highlight the complex interactions between lipid metabolism and cardiometabolic risk. Importantly the level of detail provided with this lipidomic methodology facilitates our understanding of the lipid biology as well as potentially identifying useful clinical markers. Lipid species are complex molecules and it is evident that association strength with a given outcome is driven by multiple factors, including, but not limited to, the metabolic pathways producing the fatty acyl constituents, the lipid class and the pathways involved in lipid metabolism and turnover. By measuring not only lipid classes, but also isomeric and isobaric species within classes, our lipidomic approach provides a holistic view of the interconnecting pathways for a more detailed picture of their biological relevance.

One of the striking observations of this study was the variety of lipid isomers in human plasma. Not only do we see separation of acyl compositional isomers, we also observe separation of double-bond isomers and methyl branched species. The identification of branched-chain glycerophospholipid isomers raises the question on their route of synthesis. Branched- and odd-chain fatty acids have been described extensively in the literature, mainly pertaining to bacterial lips (Kaneda, 1991), ruminant fats (Månsén, 2008), and metabolites of gut flora (Ríos-Covián et al., 2016). It is likely that mammalian cells have similar capability to synthesize these species (Horning et al., 1961). A proposed pathway is shown in Figure 5C. Recently monomethyl branched-chain fatty acid have been identified in human adipose tissue and shown to correlate with muscle insulin sensitivity (Su et al., 2015). Importantly, branched-chain amino acid catabolism has also been demonstrated to fuel adipocyte lipogenesis of odd- and branched-chain fatty acids and account for up to 30% of the lipogenic acyl-CoA pool, thereby marking this pathway as a major contributor to lipogenesis in mammalian systems (Green et al., 2016). The biological relevance of negative associations between branched-chain fatty acids with BMI, FBG, and 2h-PLG remain to be elucidated, but impairments to BCAA metabolism has been linked to obesity-related disease such as T2D (Newgard et al., 2009).

With the large cohort analyzed in this study we were able to characterize, in detail, the associations between plasma lipid species and prediabetes including many atypical lipid species and several uncommon pathways. In total, 328 lipid species were significantly associated with either FBG, 2h-PLG, or both, highlighting the comprehensive dysregulation of lipid homeostasis that precedes the onset of T2D. Importantly, we observed a marked difference in associations between the lipidome and measures of glucose homeostasis and insulin resistance. The prediabetic state is clinically defined as either having FBG of 6.1–6.9 (resulting in impaired fasting glucose [IFG]) or 2h-PLG of 7.8–11.1 (resulting in impaired glucose tolerance [IGT]) (Unwin et al., 2002). Existing literature suggests that individuals with IFG and IGT have differing pathophysiology reflecting central and peripheral insulin resistance (Meyer et al., 2006). Our findings here highlight major differences in the dysregulation of lipid metabolism between these two states that also reflect the differences between central (hepatic) from peripheral (skeletal muscle) insulin resistance. In particular, dysregulation of glycerophospholipid...
linoleic acid levels appears to be isolated to IGT, while dysregulation of ether phospholipids, particularly those containing polyunsaturated fatty acids, is more tightly associated with IFG. Since both IFG and IGT are precursors to the development of T2D, these findings have important implications as to how we might use lipid species as biomarkers for prediction of T2D and how we might seek to modulate lipid metabolism to reduce the risk of progression to T2D. These findings argue for a precision medicine approach where IFG and IGT are recognized early and treated accordingly. Further studies will be required to assess whether these lipid metabolic pathways may represent therapeutic targets to prevent progression to T2D.

**Quantification and Limitations of the Method**

Our strategy focuses on the development of high-throughput lipidomics for association analyses of large population and clinical cohorts. The internal standards available for our analysis were limited. While we incorporated 18 internal standards, representing most lipid classes and subclasses, there were some classes for which suitable standards were unavailable at the time. While our quantitation appears to align well with the NIST consensus plasma results, absolute quantification will require consideration of other factors including isotopic distribution, changes in matrix effects over time, and other differential response factors between analytes and the respective standards.

Limitations in the methodology to identify the acyl composition of glycerophospholipid species must also be noted. Our direct measurements of individual plasma samples utilize common non-acyl identifying fragments for most compounds (i.e., m/z184.1) and rely on chromatographic separation inferring results from our pooled plasma samples. Minor amounts of other coeluting fatty acyl chains may be present, as our assignments are limited to what we can detect. In-depth characterization using complementary techniques such as ozonolysis (Poad et al., 2017) in conjunction with high-resolution MS, which has already shown promising results with phosphatidylcholine species, could provide specific identification and assignment of these isomers. However, quantification of these species using in-depth characterization on a high-throughput comprehensive platform has yet to be reported.

**Considerations When Comparing Lipidomic Studies**

Comparisons between lipidomic studies is becoming much more difficult with identification of additional isomeric/isobaric species and as knowledge about the human lipidome grows. The abundance of odd-carbon glycerophospholipids and sphingolipids in the mammalian lipid pools has only recently become apparent. Depending on the techniques used, reported species may be convoluted with isotopic, isomeric, or isobaric signals leading to substantial differences in reported concentrations as seen in the NIST study (Bowden et al., 2017). We would stress that careful consideration of techniques, instrument limitations, and the level of structural detail inferred, be made when comparing lipidomic studies.

**SIGNIFICANCE**

Traditional lipidomics typically report sum compositions of many lipid species, which limits the ability to visualize metabolic pathways. By improving our separation of isomeric glycerophospholipids and sphingolipids, and further characterizing isomers using a combination of several existing techniques, our lipidomic method allows us to assign additional detail to our measured species for human studies. In addition, we have characterized many atypical lipid species that may provide insight into different pathways of human lipid metabolism. This method was used to examine a subset of an Australian population cohort to emphasize the significance of improved structural characterization. We report associations between the plasma lipidome and basic anthropometric measures that have not been described thus far. This LC-MS/MS method, in conjunction with an efficient and fast lipid extraction procedure, will allow for lipidomic analysis of large clinical cohorts that will be essential in defining relationships and understanding biology between the lipidome, lipid metabolism, and cardiometabolic disease.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **CONTACT FOR REAGENT AND RESOURCE SHARING**
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
- **METHOD DETAILS**
  - Lipid Extraction
  - External Reference Standard
  - Liquid Chromatography Mass Spectrometry
  - Nomenclature
  - Expanding the Analysis of Lipid Species in Human Plasma
  - Characterisation of Fatty Acid Species in Glycerophospholipids and Sphingolipids
  - Acid Hydrolysis for Verification of Plasmalagens Species
  - Synthesis of Glycerophospholipids
  - Linearity Experiment
- **QUANTIFICATION AND STATISTICAL ANALYSIS**
- **DATA AND SOFTWARE AVAILABILITY**

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes five figures and nine tables and can be found with this article online at https://doi.org/10.1016/j.chembiol.2018.10.008.

**ACKNOWLEDGMENTS**

The AusDiab study co-coordinated by the Baker Heart and Diabetes Institute, gratefully acknowledges the study participants. J.E.S., D.J.M., and P.J.M. are supported by Senior Research Fellowships from the National Health and Medical Research Council of Australia. K.H. was supported by a Dementia Australia Research Foundation Scholarship.

**AUTHOR CONTRIBUTIONS**

P.J.M., C.K.B., and J.M.W. developed the previous lipidomics method. K.H., B.G.D., P.J.M., D.J.M., and J.E.S. designed the updated methodology and the study. K.H. and C.K.B. performed structural characterization and identified
atypical species. The AusDiab cohort was coordinated by D.J.M. and J.E.S., who also provided clinical characteristics and samples. K.H., N.A.M., and M.C. performed the lipidomic analysis of the AusDiab samples. Statistical analysis was performed by K.H., K.S.J., and J.M.W. K.H. and P.J.M. prepared the figures and wrote the manuscript. All authors have provided feedback on the manuscript and have approved the final version.

DECLARATION OF INTERESTS

K.H., D.J.M., J.E.S., and P.J.M. are named inventors on a provisional patent application on lipidomic markers for type 2 diabetes.

Received: October 4, 2017
Revised: April 6, 2018
Accepted: October 5, 2018
Published: November 8, 2018

REFERENCES


### KEY RESOURCES TABLE

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biological Samples</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIST SRM 1950 Human Plasma</td>
<td>National institute of Standards and Technology</td>
<td>NIST1950</td>
</tr>
<tr>
<td><strong>Chemicals, Peptides, and Recombinant Proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thionyl chloride</td>
<td>Sigma-Aldrich</td>
<td>Cat#230464</td>
</tr>
<tr>
<td>14-Methylhexadecanoic acid</td>
<td>Sigma-Aldrich</td>
<td>Cat#M3164</td>
</tr>
<tr>
<td>15-Methylhexadecanoic acid</td>
<td>Sigma-Aldrich</td>
<td>Cat#M6531</td>
</tr>
<tr>
<td>Lyso PC L-a-lyosphatidylcholine (Soy)</td>
<td>Avanti Polar Lipids</td>
<td>Cat#840072P</td>
</tr>
<tr>
<td>Heptadecanoic acid</td>
<td>Sigma-Aldrich</td>
<td>Cat#H3500-1G</td>
</tr>
<tr>
<td>Docosapentaenoic acid (all-cis-7,10,13,16,19)</td>
<td>Larodan</td>
<td>Cat#10-2205</td>
</tr>
<tr>
<td>Docosapentaenoic acid (all-cis-4,7,10,13,16)</td>
<td>Larodan</td>
<td>Cat#10-2265</td>
</tr>
<tr>
<td>L-a-glycerophosphorylcholine</td>
<td>Sigma-Aldrich</td>
<td>Cat#G5291</td>
</tr>
<tr>
<td>Dihydroceramide (dhCer) 8:0</td>
<td>Avanti Polar lipids</td>
<td>Cat#860626P</td>
</tr>
<tr>
<td>Sphingomyelin (SM) 12:0</td>
<td>Avanti Polar lipids</td>
<td>Cat#860583P</td>
</tr>
<tr>
<td>Sphingosine (Sph) 17:1 base</td>
<td>Avanti Polar lipids</td>
<td>Cat#860640P</td>
</tr>
<tr>
<td>Phosphatidylcholine (PC) 13:0 13:0</td>
<td>Avanti Polar lipids</td>
<td>Cat#850340P</td>
</tr>
<tr>
<td>Phosphatidylethanolamine (PE) 17:0 17:0</td>
<td>Avanti Polar lipids</td>
<td>Cat#830756P</td>
</tr>
<tr>
<td>Phosphatidylglycerol (PG) 17:0 17:0</td>
<td>Avanti Polar lipids</td>
<td>Cat#830456P</td>
</tr>
<tr>
<td>Phosphatidylserine (PS) 17:0 17:0</td>
<td>Avanti Polar lipids</td>
<td>Cat#840028P</td>
</tr>
<tr>
<td>Lysophosphatidylcholine (LPC) 13:0</td>
<td>Avanti Polar lipids</td>
<td>Cat#855476P</td>
</tr>
<tr>
<td>Diacylglycerol (DAG) 15:0 15:0</td>
<td>Santa Cruz Biotech</td>
<td>Cat#sc-213512</td>
</tr>
<tr>
<td>Triacylglyceride 17:0 17:0</td>
<td>Sigma Aldrich</td>
<td>Cat#T2151</td>
</tr>
<tr>
<td>Cholesterol (D7)</td>
<td>Avanti Polar lipids</td>
<td>Cat#700041P</td>
</tr>
<tr>
<td>CE 18:0 (d6)</td>
<td>CDN isotopes</td>
<td>Cat#D-5823</td>
</tr>
<tr>
<td>Lysophosphatidylethanolamine 14:0</td>
<td>Avanti Polar lipids</td>
<td>Cat#856735P</td>
</tr>
<tr>
<td>sulphatide d18:1/12:0</td>
<td>Avanti Polar lipids</td>
<td>Cat#860573P</td>
</tr>
<tr>
<td>Trihexosylceramide 17:0</td>
<td>Matreya LLC</td>
<td>Cat#1523</td>
</tr>
<tr>
<td>Lysophosphatidylinositol 13:0</td>
<td>Avanti Polar lipids</td>
<td>Cat#850101</td>
</tr>
<tr>
<td>Lactosylceramide 16:0 d3</td>
<td>Matreya LLC</td>
<td>Cat#1534</td>
</tr>
<tr>
<td>Cholesterol ester (17:0)</td>
<td>Mp Biomedicals Australasia</td>
<td>Cat#219033125</td>
</tr>
<tr>
<td>Triacylglyceride 12:0_12:0_14:0</td>
<td>Sigma-Aldrich</td>
<td>Cat#D2907</td>
</tr>
<tr>
<td>Ceramide d18:1/20:0</td>
<td>Avanti Polar Lipids</td>
<td>Cat#860520P</td>
</tr>
<tr>
<td>Dihydroceramide (d18:1/16:0)</td>
<td>Avanti Polar Lipids</td>
<td>Cat#860634P</td>
</tr>
<tr>
<td>Lysophosphatidylcholine 17:0</td>
<td>Avanti Polar Lipids</td>
<td>Cat#855676P</td>
</tr>
<tr>
<td>Phosphatidylcholine 20:0/20:0</td>
<td>Avanti Polar Lipids</td>
<td>85036BP</td>
</tr>
<tr>
<td>Phosphatidylcholine 21:0/21:0</td>
<td>Avanti Polar Lipids</td>
<td>850370P</td>
</tr>
<tr>
<td>Phosphatidylcholine 17:0/17:0</td>
<td>Avanti Polar Lipids</td>
<td>850360P</td>
</tr>
<tr>
<td>Phosphatidylethanolamine 16:1/16:1</td>
<td>Avanti Polar Lipids</td>
<td>850706P</td>
</tr>
<tr>
<td><strong>Deposited Data</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quality Control and National Institute of Standards Technology 1950 Plasma Lipidomics Data</td>
<td>Table S3</td>
<td>#N/A</td>
</tr>
</tbody>
</table>

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to the Lead contact, Peter Meikle (Peter.Meikle@baker.edu.au).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

We utilized plasma samples from the Australian Diabetes, Obesity and Lifestyle (AusDiab) study, a prospective population study examining the prevalence and risk factors of T2D and CVD in the Australian population (Dunstan et al., 2002). We performed lipidomic analysis on 640 individuals which represented 389 normoglycemic individuals (fasting plasma glucose <6.1 mM, 2-h post load glucose <7.8 mM) and 251 individuals with prediabetes, made up of 147 individuals with impaired fasting glucose (FBG 6.1–6.9 mM) and 183 individuals with impaired glucose tolerance (2h-PLG 7.8–11.0 mM). There were 79 individuals who had both IFG and IGT (Table S2). This study was approved by the Alfred Hospital, Ethics Committee.

METHOD DETAILS

Lipid Extraction

Lipid extraction was performed as described previously (Alshehry et al., 2015). In brief, 10 µL of plasma was mixed with 100 µL of butanol:methanol (1:1) with 10 mM ammonium formate which contained a mixture of internal standards (Table 1). Samples were vortexed thoroughly and set in a sonicator bath for 1 hour maintained at room temperature. Samples were then centrifuged (14,000xg, 10 min, 20°C) before transferring the into sample vials with glass inserts for analysis.

External Reference Standard

For alignment of our results with existing literature, the NIST (National Institute of Standards and Technology) human plasma standard reference material 1950 were extracted and run together with our plasma quality control samples in a secondary run.

Liquid Chromatography Mass Spectrometry

Analysis of plasma extracts was performed on an Agilent 6490 QQQ mass spectrometer with an Agilent 1290 series HPLC system and a ZORBAX eclipse plus C18 column (2.1x100mm 1.8µm, Agilent) with the thermostat set at 60°C. Mass spectrometry analysis was performed in positive ion mode with dynamic scheduled multiple reaction monitoring (MRM). Mass spectrometry settings and MRM transitions for each lipid class, subclass and individual species are shown in Tables 1 and S1.

The solvent system consisted of solvent A) 50% H2O / 30% acetonitrile / 20% isopropanol (v/v/v) containing 10mM ammonium formate and solvent B) 1% H2O / 9% acetonitrile / 90% isopropanol (v/v/v) containing 10mM ammonium formate. We utilized a stepped linear gradient with a 15-minute cycle time per sample and a 1µL sample injection.

The gradient was as follows; starting with a flow rate of 0.4ml/minute at 10% B and increasing to 45% B over 2.7 minutes, then to 53% over 0.1 minutes, to 65% over 6.2 minutes, to 89% over 0.1 minute, to 92% over 1.9 minutes and finally to 100% over 0.1 minute. The solvent was then held at 100% B for 0.8 minutes (total 11.9 minutes). Equilibration was as follows, solvent was decreased from 100% B to 10% B over 0.1 minute and held for an additional 0.9 minutes. Flow rate was then switched to 0.6 ml/minute for 1 minute before returning to 0.4 ml/minute over 0.1 minutes. Solvent B was held at 10% B for a further 0.9 minutes at 0.4ml/minute for a total cycle time of 15 minutes.

The following mass spectrometer conditions were used; gas temperature, 150°C, gas flow rate 17L/min, nebulizer 20psi. Sheath gas temperature 200°C, capillary voltage 3500V and sheath gas flow 10L/min. Isolation widths for Q1 and Q3 were set to “unit” resolution (0.7 amu).

PQC samples consisting of a pooled set of 6 healthy individuals were incorporated into the analysis at 1 PQC per 18 plasma samples. TQC consisted of PQC extracts which were pooled and split into individual vials to provide a measure of technical variation from the mass spectrometer only. These were included at a ratio of 1 TQC per 18 plasma samples. TQCs were monitored for changes in
peak area, width and retention time to determine the performance of the LC-MS/MS analysis and were subsequently used to align for differential responses across the analytical batches. Quantification of lipid species was determined by comparison to the relevant internal standard (Table 1). As previous described (Weir et al., 2013) response factors were generated for each cholesteryl ester species to better approximate their true concentrations. Response factors generate is provided in Table S1. Similarly, species with non-class specific internal standards had response factors generated as previously described (Weir et al., 2013).

Nomenclature
The lipid naming convention used here follows the guidelines established by the Lipid Maps Consortium and added to by Liebisch et al. (Fahy et al., 2005; Fahy et al., 2009; Liebisch et al., 2013). Glycerophospholipids typically contain two fatty acid chains and in the absence of detailed characterisation are expressed as the sum composition of carbon atoms and double bonds (i.e. PC(38:6)). However, where the acyl chains have been determined but the position is unknown, this is reflected by an underscore between the acyl chains (i.e. PC(38:6) is changed to PC(16:0_22:6)). Where the position of the acyl chains is known the acyl chains are separated by a / with the sn1 and sn2 acyl chains in order (i.e. PC(16:0_22:6) is changed to PC(16:0/22:6)). This is also extended into other lipid classes and subclasses. Species separated chromatographically but incompletely characterised were labelled with an (a) or (b), for example PC(17:0/20:4) (a) and (b) where (a) and (b) represent the elution order.

Expanding the Analysis of Lipid Species in Human Plasma
Starting with the list of 312 lipid species across 23 lipid classes and subclasses including sphingolipids, glycerophospholipids, glycerolipids and cholesterol esters, as reported previously (Weir et al., 2013), we sought to improve the coverage of this lipidomic profiling methodology in positive ion mode such that it would retain suitability for high-throughput analyses. We utilized a longer column, higher running temperatures and a different solvent system (as described above).

We selected additional lipid classes, based on those likely to be relevant to metabolic disease characterisation, which were missing from our original methodology. Plasma quality control samples were screened for the major species within each new class using a series of targeted MRM transitions derived from existing standards, literature and known fragmentation patterns.

The following standards were characterised and used to optimise the mass spectrometry parameters and identify suitable molecular transitions: Gm1 ganglioside species were obtained in a Gm1 mix (Avanti Polar Lipids) and plasma species were identified as [M+2H]^2+ molecular ions with a common product ion of m/z 366.2. Sulfatide species were obtained as a sulfatide mix (Avanti Polar Lipids) and plasma species identified by the common product ion of m/z 264.3, with additional qualifier transitions corresponding to a neutral loss of 98 Da and 260 Da. A single lysoalkenylphosphatidylcholine species LPC(P-18:0) (Avanti Polar Lipids) and plasma species identified by the common product ion m/z 104.1. Lysoalkenylphosphatidylethanolamine LPE(P-18:0) (Avanti Polar Lipids) was characterised to undergo a neutral loss of 171.9 Da and lysophosphatidylinositol LPI(13:0) (Avanti Polar Lipids) was characterised to undergo a neutral loss of 277.0 Da and these transitions were subsequently used to identify plasma species of these subclasses. Acylcarnitine species were identified with reference standards (Cambridge Isotope Laboratories) and characterised by the product ion m/z 85.1.

Cholesteryl ester derivatives where the cholesterol has an additional double bond were characterised with the product ion m/z 367.4 with precursors monitored as ammonium adducts similar to unmodified cholesteryl esters. Desmosterol 18:1 d6 (Avanti) was used to optimise collision energies and characterise elution time for dehydrocholesterol species.

We previously used a neutral loss of 141.0 Da to measure alkenylphosphatidylethanolamine (plasmalogen) species. Zemski Berry et al. identified additional product ions in positive ion mode which provide better sensitivity and acyl specific identification (Zemski Berry and Murphy, 2004). We extrapolated these transitions to identify alkenylphosphatidylethanolamine species with alkyl chains and acyl chains of 16 to 22 carbons with 0 to 6 double bonds.

It has been demonstrated previously that lysoglycerophospholipids with a single esterified fatty acid in either the sn1 or sn2 position elute at different retention times under reverse phase conditions (Creer and Gross, 1985). We re-examined our lysophosphatidylcholine, lysophosphatidylethanolamine and lysophosphatidylinositol for separation of these positional isomers.

Sphingolipids (with the exception of sphingomyelin) with different sphingoid bases were identified using product ions corresponding to the different sphingoid bases: d16:1, m/z 236.3, d17:1, m/z 250.3, d18:1, m/z 264.3, d18:2, m/z 262.3, d19:1, m/z 278.3, d20:1, m/z 292.3.

Characterisation of Fatty Acid Species in Glycerophospholipids and Sphingolipids
The fatty acid composition of glycerophospholipids was characterised by either collision induced dissociation (CID) in negative ionization mode, for neutral or negatively charged species, or by CID of lithium adducts in positive ionization mode for positively charged species. Fragmentation in negative mode enabled the identification of fragment ions corresponding to the fatty acyl chains. Fragmentation of the lithium adducts in positive ion mode enabled identification of fragment ions corresponding to the loss of the fatty acyl constituents (Hsu and Turk, 2003). Characterisation of the glycerophospholipid fatty acids was performed on whole lipid extracts of POC samples. For the analysis of lithium adducts of phosphatidylcholine, alkylphosphatidylcholine, alkylphosphatidylcholine and sphingomyelin the 10mM ammonium formate in the extraction buffer and running solvents were substituted with 200μM lithium acetate, which provided the ability to monitor for lithium adducts for additional structural information (Hsu et al., 2003).
Acid Hydrolysis for Verification of Plasmalogens Species

To confirm several atypical plasmalogen species, two identical samples were analysed following acid hydrolysis of one sample. Plasmalogens are susceptible to acid hydrolysis which completely removes the alkenyl chain resulting in their corresponding lyso species. Lipid extracts of pooled plasma samples were dried down in glass vials and exposed to concentrated HCl vapour for 5 minutes, before reconstitution in butanol:methanol (1:1) and subsequent lipidomic analysis by LC-MS/MS.

Synthesis of Glycerophospholipids

Acyl chlorides of selected fatty acid species were prepared by mixing 25 to 250 nmol of either 15-methylhexadecanoic acid (Sigma-Aldrich), 14-methylhexadecanoic acid (Sigma-Aldrich), heptadecanoic acid (Sigma-Aldrich), docosapentaenoic acid (all-cis-7,10,13,16,19, Larodan) or docosapentaenoic acid (all-cis-4,7,10,13,16, Larodan) with 20 μL of 0.2M thionyl chloride diluted in dichloromethane for 10 minutes at room temperature. Solvent was then removed with N2 gas at 60 °C before the addition of a 10% molar ratio of soy lysophosphatidylcholine (Avanti Polar Lipids) or L-α-glycerophosphorylcholine (Sigma-Aldrich) in 100μL dichloromethane and incubating for 10 minutes at room temperature. The solvent was then removed under N2 gas at 60 °C and the residue containing lipid species was reconstituted in butanol:methanol (1:1 v/v) with 10mM ammonium formate. Samples were then analysed by LC-MS/MS as described above.

Linearity Experiment

To assess linearity of response of the most abundant lipid classes, CE(17:0) was acquired from MP biomedicals, CE(24:1), DG(16:0_16:0) and TG(12:0_12:0_14:0) were acquired from Sigma-Aldrich. Cer(d18:1/20:0), dhCer(d18:1/16:0), LPC(17:0), PC(20:0/20:0), PC(21:0/21:0), PC(17:0/17:0), PE(16:1/16:1) were acquired from Avanti Polar Lipids. Lipid standards were dissolved together to their desired upper concentrations in chloroform:methanol (1:1 v/v), serially diluted (1:1) 11 times before a 10 μL aliquot was spiked into an extracted TQC with internal standards. A TQC with internal standards spiked with solvent was used as the baseline. Injection volume and running conditions are as previously described. Response was determined from the areas of the tested lipid species, normalized to their internal standards. Linearity was assessed by calculating an R² value from the linear plot of concentration against normalized area. The linear range of each lipid was determined as the range in which the R² was 0.99 or greater (Figure S5).

QUANTIFICATION AND STATISTICAL ANALYSIS

Following characterisation of lipid species as described above, chromatographic peaks were integrated using the Mass Hunter (B.07.00, Agilent Technologies) software and assigned to a specific lipid species based on MRM (precursor/product) ion pairs and retention time. Quantification was achieved by using the ratio of each analyte peak with the corresponding internal standard outlined in Table S1. Additional response factors were calculated as described previously (Weir et al., 2013).

Statistical analysis was carried out on Matlab 2013a or R (3.4.0). Plasma lipidomic data was log10 transformed prior to statistical analysis. Associations between participant characteristics (age, gender and BMI) and lipid species were determined using linear regression, adjusting for the other covariates in each analysis. β-coefficients and 95% confidence intervals were then converted to percentage change (percentage change = (10^β-coefficient – 1) x 100) for interpretation of results. These associations were also re-examined with further adjustments for clinical lipids (total cholesterol, HDL-C and triglycerides). All p-values were corrected for multiple comparisons using the method of Benjamini and Hochberg (Benjamini and Hochberg, 1995). Simple correlations (Pearson) and box plots comparing lipid concentrations were prepared using GraphPad Prism 7.

DATA AND SOFTWARE AVAILABILITY

Individual and summarised lipidomic results of quality control samples and the National Institute of Standards Technology 1950 plasma are presented in Table S3. Additional data can be requested for the Lead contact. Software used is outlines in the Key Resource Table.
Supplemental Information

High-Throughput Plasma Lipidomics: Detailed
Mapping of the Associations
with Cardiometabolic Risk Factors

Kevin Huynh, Christopher K. Barlow, Kaushala S. Jayawardana, Jacquelyn M. Weir, Natalie A. Mellett, Michelle Cinel, Dianna J. Magliano, Jonathan E. Shaw, Brian G. Drew, and Peter J. Meikle
Figure S1 – Additional characterisation of glycerophospholipid isomers. **A** – Chromatograms of PC(18:0_22:5) from: human pooled plasma sample (black trace); synthesized PC(18:0_22:5) (n3) (green trace); synthesized PC(18:0_22:5) (n6) (red trace); the peak at 7.09 is an isotopic peak of PC(40:6). **B** – Chromatogram of LPC(22:5) from a human pooled plasma sample showing PI m/z 184.1, specific for any lysophosphatidylcholine species (black trace); PI m/z 104.1, specific for lysophosphatidylcholine species esterified in the sn1 position only (blue trace). Two peaks with 22:5 esterified in the sn1 position at retention times 2.35 and 2.53 are shown, likely to be n3 and n6 isomers respectively. Peaks at 2.04 and 2.15 minutes are M+2 isotopic peaks of LPC(22:6). **C** – Chromatogram of PC(35:2) showing the MRM scan m/z 772.6 / m/z 184.1 [M+H] from a human pooled plasma sample extract (black trace); synthesized PC(15-MHDA_18:2) spiked into the same extract (green trace) synthesized PC(15-MHDA_18:2) spiked into the same extract (orange trace). **D** – Chromatogram of synthesized PC(17:0_18:2) (green trace) and PC(15-MHDA_18:2) (orange trace) run under the same chromatography.
Figure S2 – Characterisation of plasmalogens by acid treatment. A – Chromatogram of PC(P-17:0/20:4) showing the MRM scan m/z 780.6 / m/z 184.1 [M+H] from a human pooled plasma sample extract (black trace) and the same extract following acid treatment (red trace). Two peaks corresponding to plasmalogens can be seen at retention times 7.35 and 7.53. B – Chromatogram of LPC(O-18:1) and LPC(P-18:0), showing the MRM scan m/z 508.6 / m/z 104.1 [M+H] from a human pooled plasma sample extract (black trace) and the same extract following acid treatment (red trace). The signal from the LPC(P-18:0) at RT 3.57 is lost upon acid treatment.
Figure S3 – Characterisation of odd chain alkenylphosphatidylethanolamine species.
Alkenylphosphatidylethanolamine (ethanolamine plasmalogens) exhibit fatty acyl specific product ions which were used to identify odd chain alkenyl species. A – Extracted chromatogram of 17-carbon alcohol chain species of plasmalogens. B – Extracted chromatogram of 15-carbon alcohol chain species of plasmalogens. C – Product ion spectra of PE(P-17:0/20:4) (a) (black, left) and PE(P-17:0/20:4) (b) (blue, right). Product ion spectra were obtained from product ion scans using the same chromatography and collision energy as outlined in Table S1.

**Figure S3 – Relating to the results section**
Figure S4 – Product ion spectra of two atypical lipid species associated with anthropometric measures. A – Product ion spectra of Acylcarnitine (14:2) at 1.22 minutes. The m/z 85.2 product ion is characteristic of an acylcarnitine species. B – Product ion spectra of SM(d18:2/14:0) at retention time 4.69 minutes. Product ion scanning was performed on the lithiated adduct. Product ions 262.4 and 252.1 are characteristic of the C14:0 and d18:2 sphingoid base respectively, indicating SM(d18:2/14:0).
**Figure S5 – Linearity plot of selected lipid species.** Standards were spiked into an extracted plasma QC at their respective concentrations to generate response curves. Red solid lines show concentration at which there was a linear response. $R^2$ values were calculated with this line. Blue line, concentration at which the response becomes non-linear.

**Figure S5 – Relating to STAR methods**