Running Title: Lipid signatures of diabetes risk

Lipidomic profiling reveals early-stage metabolic dysfunction in overweight or obese humans

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ABSTRACT:

Background: Advances in mass spectrometry and lipidomics techniques are providing new insights into the role of lipid metabolism in obesity-related diseases. However, human lipidomic studies have been inconsistent, owing to the use of indirect proxy measures of metabolic outcomes and relatively limited coverage of the lipidome. Here, we employed comprehensive lipid profiling and gold-standard metabolic measures to test the hypothesis that distinct lipid signatures in obesity may signify early stages of pathogenesis toward type 2 diabetes.

Methods: Using high-performance liquid chromatography-electrospray tandem mass spectrometry, we profiled >450 lipid species across 26 classes in 65 overweight or obese non-diabetic individuals. Intensive metabolic testing was conducted using direct gold-standard measures of adiposity (% body fat by dual X-ray absorptiometry), insulin sensitivity (hyperinsulinaemic-euglycaemic clamps), and insulin secretion (intravenous glucose tolerance tests), as well as measurement of serum inflammatory cytokines and adipokines (multiplex assays; flow cytometry). Univariable and multivariable linear regression models were computed using Matlab R2011a, and all analyses were corrected for multiple testing using the Benjamini-Hochberg method.

Results: We present new evidence showing a strong and independent positive correlation between the lysophosphatidylinositol (LPI) lipid class and insulin secretion *in vivo* in humans (β [95%CI]= 781.9 [353.3, 1210.4], p=0.01), supporting the insulinotropic effects of LPI demonstrated in mouse islets. Dihydroceramide, a sphingolipid precursor, was independently and negatively correlated with insulin sensitivity (β [95%CI]= -1.9 [-2.9, -0.9], p=0.01), indicating a possible upregulation in sphingolipid synthesis in obese individuals. These associations remained significant in multivariable models adjusted for age, sex, and % body fat. The dihexosylceramide class correlated positively with interleukin-10 before and after adjustment for age, sex, and % body fat (p=0.02), while the phosphatidylethanolamine class and its vinyl ether-linked (plasmalogen) derivatives correlated negatively with % body fat in both univariable and age- and sex- adjusted models (all p<0.04).

Conclusions: Our data suggest that these lipid classes may signify early pathogenesis toward type 2 diabetes and could serve as novel therapeutic targets or biomarkers for diabetes prevention.

Keywords: Lipid metabolism, lipidomics, diabetes, insulin secretion, metabolic dysfunction, obesity.

ABBREVIATIONS: AUC, area under the curve; **BH**, Benjamini-Hochberg; **DG**, diacylglycerol; **GPR-55**, G-protein coupled receptor-55; **HOMA- IR**, homeostatic model assessment of insulin resistance; **hsCRP**, high-sensitivity C-reactive protein, **IQR**, interquartile range; **LPI**, lysophosphatidylinositol; **MCP-1**, monocyte chemoattractant protein-1; **PE**, phosphatidylethanolamine; **RCT**, randomized controlled trial; **TG**, triacylglycerol.

1. INTRODUCTION

Lifestyle changes including the Westernization of diet, reduced physical activity, and sedentary living and work environments have contributed to the rapid propagation of metabolic disorders, namely obesity and type 2 diabetes [1]. Insulin resistance is a central feature of these conditions, yet the etiological mechanisms responsible for the development of insulin resistance in obesity are not fully understood. Compelling evidence suggests that defective lipid metabolism resulting from over-nutrition and/or impaired adipocyte lipolysis may contribute to muscle and liver insulin resistance, in part by promoting intracellular accumulation of lipids which impair insulin signal transduction [2]. However, conventional lipid profiling of triglycerides and total, high-, and low-density lipoprotein cholesterol, while useful, provides only a narrow snapshot of the dynamic processes of lipid metabolism, particularly in complex metabolic disorders.

In contrast, rapidly developing lipidomics mass spectrometry techniques are now able to sensitively, comprehensively, and simultaneously quantify hundreds of lipid species to provide a more global assessment of lipid metabolism [3]. With the advancement of these techniques, it has become apparent that dyslipidaemia in obesity extends beyond free fatty acids and, indeed, a number of plasma lipids have been identified as potential mediators of insulin resistance and inflammation. Human observational studies have revealed variations in ceramides, triacylglycerols, and ether phospholipids in individuals with general or abdominal obesity [4-6]. Lysophosphatidylinositol has been linked to impaired insulin secretion in animal studies [7], while sphingomyelin has been associated with insulin secretion in humans [8]. Sphingolipids, despite being a relatively minor component of the lipid milieu in most tissues, are proposed to be among the most pathogenic lipids in the sequelae associated with obesity. Ceramides in particular have been studied extensively and are implicated as major contributors to systemic and skeletal muscle insulin resistance [9-11], although the latter remains an area of

debate [12-14]. Circulating factors associated with obesity such as inflammatory cytokines have also been shown to selectively up-regulate sphingolipid biosynthesis, amplifying the effect of lipid oversupply [15], while adipokines such as adiponectin or leptin can prevent lipotoxicity in peripheral tissues [16, 17].

Despite an increasing number of human lipidomic studies, results remain inconsistent owing to differences in study populations, small sample sizes (n<30), varying degrees of methodological rigour, limited coverage of the lipidome (<200 species), and/or use of indirect proxy measures such as body mass index (BMI) and homeostatic model assessment of insulin resistance (HOMA-IR). Here, we aim to explore the relationship between the plasma lipidome and metabolic risk factors using direct gold-standard measures of adiposity, insulin sensitivity, and insulin secretion, in addition to comprehensive lipidomic profiling (>450 species) and measurement of inflammatory cytokines and adipokines. We test the hypothesis that distinct lipid metabolites will be associated with metabolic risk factors in overweight or obese individuals and may signify early stages of pathogenesis toward type 2 diabetes.

2. METHODS:

2.1. Study Population

This study is a cross-sectional analysis of baseline data from a previous randomized controlled trial (RCT) and a detailed protocol has been published [18]. Briefly, 65 participants were recruited from the local community in Melbourne, Australia, via print and online advertisements. Participants were aged 18-60 years, overweight or obese (BMI $\geq$ 25 kg/m^2), and otherwise generally healthy on medical screening. Exclusion criteria included use of medications, vitamins, or supplements; smoking or high alcohol use (>4 and >2 standard drinks/week for men and women, respectively); major diseases including any diabetes (type 1 or 2), known or newly diagnosed based on a 75g oral glucose tolerance test (OGTT) and World

Health Organization (WHO) criteria [19], or presence of acute inflammation. Pregnant, lactating, or peri- or post-menopausal women were excluded. The study was conducted according to the principles of the Declaration of Helsinki [20] and received ethical approval from the Monash University Human Research Ethics Committee (Protocol ID: CF13/3874-2013001988). All participants provided written informed consent prior to study entry.

2.2. Data Collection and Outcome Measures

Detailed descriptions of data collection and outcome measures are reported in our published protocol [18]. Briefly, participants who were eligible on phone screening attended a medical review which included a detailed physical examination and medical history, routine blood analyses, anthropometric assessments, and 75g OGTTs to exclude diabetes.

2.2.1. Anthropometric Measurements

Total % body fat was measured using the gold-standard dual-energy X-ray absorptiometry (DPX-L; Lunar Radiation, Madison, WI). BMI was calculated from weight (kg)/ height $(m)^2$, and waist and hip circumferences were used to establish the waist-to-hip ratio (WHR) as an additional index for body fat distribution (waist (cm)/ hip (cm)= WHR).

2.2.2. Glycaemic Measurements

Glucose tolerance was measured after a 12-hour overnight fast using 75g OGTTs and WHO criteria [19], and plasma glucose concentrations were determined at real-time using the glucose oxidase method (YSI 2300 STAT, YSI Inc., OH, USA). Insulin sensitivity was assessed using the gold-standard hyperinsulinaemic-euglycaemic clamp. This was initiated by intravenous bolus injection of insulin (9 mU/kg), after which insulin was constantly infused (40 mU.m-2.min) for at least 120 min, while glucose was variably infused, monitored every 5 minutes, and adjusted to maintain euglycaemia (constant 5 mmol/L value for the last 30 minutes). Insulin sensitivity (M-value) was defined as the weight-adjusted glucose infusion

rate during the last 30 minutes when a steady state was achieved. Acute insulin secretion was measured using intravenous glucose tolerance tests, where 50 ml of 50 % glucose was delivered intravenously over a 3 min period and insulin and glucose concentrations were measured at 3, 4, 5, 6, 8, 10, 15, 20, 25, and 30 min to determine insulin secretory response. Plasma insulin area under the concentration-time curve (AUC) was determined using the trapezoidal rule [21] and was calculated from 3-5, 10-30, and 0-30 minutes after the glucose bolus to determine first-phase, second-phase, and total insulin secretion, respectively.

2.2.3. Biochemical Analyses

Fasting venous blood samples were drawn using standard phlebotomy techniques, centrifuged immediately, and stored at -80°C until analysis. All blood samples were analyzed under blinded conditions using standard quality control systems (all results within ± 2 SD) by accredited laboratories. Insulin was measured by simultaneous immunoenzymatic sandwich assays (Access/DXI ultrasensitive insulin assay, Beckman Coulter [BC], Australia), with inter- and intra- assay CVs of <5% and <7%, respectively. Plasma high sensitivity C-reactive protein (hsCRP) was measured by highly sensitive near-infrared particle immunoassays on Synchron LX analyzers (BC, Australia) according to manufacturer's instructions, and inter- and intra-assay CVs were <3% and <5%, respectively.

Serum inflammatory markers and adipokines were measured using bead-based multi-analyte assays (LEGENDplex™, Biolegend, CA), and data acquisition was performed using LSRII flow cytometers and FACS DIVA software (Becton Dickinson™, San Diego, CA), as per manufacturer's instructions. These panels simultaneously quantified multiple cytokines/chemokines including tumor necrosis factor-*alpha* (TNF-α), monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-1β, IL-6, IL-8, and IL-10, as well as adipokines adiponectin, leptin, resistin, and adipsin. All analytes had inter- and intra-assay CVs <8% and <9%, respectively.

2.2.4. Lipidomics Analyses

Targeted lipidomics analysis was conducted as described elsewhere [22] and detailed development of this methodology with structural identification is published [23]. Briefly, plasma samples (10 µL) were extracted in a single-phase extraction with 20 volumes of CHCl3:MeOH (2:1) and 10 µL of an internal standard mix containing between 50 and 1000 pmol each of 23 non-physiologic or stable isotope-labelled lipid standards. Lipid analysis was performed using high-performance liquid chromatography electrospray ionization on an AB Sciex 4000 Q/TRAP mass spectrometer. Liquid chromatography was performed on a Zorbax Eclipse Plus C18 (Agilent Technologies, USA), 1.8 um, 100 x 2.1 mm column, using solvents A and B consisting of water:acetonitrile:isopropanol, 50:30:20 and 1:9:90 respectively, both containing 10mM ammonium formate. The column was heated to 60°C and the auto sampler regulated to 25°C. Lipid extracts (5 ul) were injected and separated under gradient conditions with a flow rate of 400 ul/min: 10% B to 55% B over 3 minutes; increased to 70% B over 8 minutes; increased to 87% B over 0.1 minutes; increased to 92% B over 5.2 minutes; increased to 100% B over 0.1 minutes; and held at 100% B for 0.3 minutes. The solvent was then decreased to 10% B over 0.1 and held at 10% B for 4.2 minutes until the next injection at 21 minutes. The first 1.5 minutes and final 0.9 minutes of each analytical run were diverted to waste.

A total of 489 lipid species across 26 classes were measured using scheduled multiple reaction monitoring. Results from chromatographic data were analysed using MultiQuant 2.1.1 (Sciex, Australia) where relative lipid abundances were calculated by relating the area under the chromatogram for each lipid species to the corresponding internal standard. Total lipid class concentrations were calculated using the sum of individual lipid species within each class. Assay performance was monitored by calculating CVs of quality control plasma samples across

the entire analytical run. Median CVs of the internal standard areas and plasma quality control samples were 6% and 8%, respectively, indicating high precision (< 15%).

2.2.5. Nomenclature

This study uses the lipid naming convention outlined by the Lipid Maps Consortium and by Liebisch and colleagues [24, 25]. Lipids containing two fatty acid chains without further characterisation are expressed as the sum composition of carbon atoms and double bonds (eg. PC (38:6)). Where acyl chains have been determined but the position is unknown, an underscore between the acyl chains is used (i.e. PC (38:6) is changed to PC (16:0_22:6)), otherwise a forward-slash between acyl-chains is used where their position is known (i.e. PC (16:0/22:6)). The same nomenclature is used for other lipid classes and subclasses. Species separated chromatographically but incompletely characterised are labelled with an (a) or (b), (eg. PC (P-17:0/20:4) (a) and (b)), where (a) and (b) represent the elution order. Some parent species which do not have specific sn-1 or sn-2 information are represented as the sum of the fatty acids (eg. PI (16:0_20:4) is the combination of PI (16:0/20:4) and PI (20:4/16:0)) [23].

2.3. Statistical Analyses

This is a cross-sectional analysis of baseline data from a previous RCT [18] and no formal sample size calculation was undertaken for this study. Participant characteristics are reported as mean ± standard deviation (SD) or median (interquartile range [IQR]) for non-normally distributed variables. Normality was assessed by visual inspection of histograms and using Shapiro-Wilk tests, and because lipids have a naturally skewed distribution, quantitative values for each lipid species were normalized to the base-10 or to the IQR prior to statistical analyses. Univariable and multivariable linear regression models were computed using Matlab R2011a (Mathworks, MA, USA) to determine associations between lipid species/classes and the outcomes of interest. Predetermined factors known to influence lipids including age, sex, and % body fat were included as covariates in the regression models. Relationships between lipid

species/classes and each variable are presented as β-coefficients with 95% confidence intervals (CIs). β-coefficients represent the change in outcome measure (eg: fasting glucose) associated with an IQR increase in the lipid measurement. Unless otherwise specified, all reported p-values are corrected for multiple comparisons using the Benjamini-Hochberg (BH) method [26]. A corrected two-tailed p-value <0.05 was considered statistically significant.

3. RESULTS:

Sixty-five participants (41 males/ 24 females) were included in the study. Demographic, anthropometric, clinical, and biochemical characteristics are presented in **Table 1**. The mean age of participants was 31.3 ± 8.5 years and approximately half (n= 35; 53%) were obese (BMI ≥30 kg/m^2). There were no associations between age and any lipid species or classes (all p≥0.05, data not shown). Of the 26 lipid classes measured, two sphingolipid classes were associated with sex (**Table 2**), with females having lower concentrations of ceramides and GM3 gangliosides compared with males (approximately -70% difference in each lipid).

3.1. Associations between lipids and anthropometric measures:

In univariable analyses, BMI and WHR were not associated with any lipid species or classes (all p≥0.1, Supplemental Material). However, total % body fat was inversely associated with ten lipid classes and subclasses including ceramides, monohexosylceramides, GM3 gangliosides, triacylglycerols (TGs), and lysophosphatidylcholines, as well as the phosphatidylethanolamine lipid class and its lyso- and ether-linked derivatives (lysophosphatidylethanolamine and alkylphosphatidylethanolamine) and vinyl ether-linked plasmalogens (lysoalkenyl- and alkenyl-phosphatidylethanolamine, Table 2).

In multivariable models adjusted for age and sex, associations between BMI or WHR and lipid species or classes remained non-significant (all p>0.05, Supplemental Material). Total % body fat remained inversely associated with the phosphatidylethanolamine lipid class in the

age- and sex-adjusted model (β [95%CI]= -0.2 [-0.4, -0.1], p=0.03, Table 2), as well as with the plasmalogen subclasses lysoalkenylphosphatidylethanolamine (β [95%CI]= -0.04 [-0.1, -0.02], p=0.04) and alkenylphosphatidylethanolamine (β [95%CI]= -0.5 [-0.7, -0.2], p=0.03, Table 2). However, associations between total % body fat and the remaining seven lipid classes were attenuated (all p>0.05, Table 2).

3.2. Associations between lipids and glycaemic measures:

In univariable analyses, total and second-phase insulin secretion were positively associated with the lysophosphatidylinositol (LPI) lipid class after BH correction (β [95%CI]= 781.9 [353.3, 1210.4], p=0.01 and β=521.3 [240.0, 802.5], p=0.01, respectively, **Figure 1, Table 3**), as well as with the individual species (LPI 18:0, 18:1, 18:2, and 20:4) at both sn-1 and sn-2 levels before BH correction. First-phase insulin secretion was only associated with the LPI lipid class before BH correction for multiple comparisons (β [95%CI]= 150.1 [51.6, 248.7], p=0.004), but was no longer significant after correction (p=0.1, Table 3). There were no associations between any lipid species or classes and fasting glucose, 2-hour post-OGTT glucose, fasting insulin (all p>0.1, Supplemental Material), or insulin sensitivity (Table 3).

After adjustment for age, sex, and body fat, total and second-phase insulin secretion remained positively associated with the LPI class (p=0.02 and p=0.01, respectively; Table 3) as well as with the individual LPI species (LPI 18:0, 18:1, 18:2, and 20:4) at both sn-1 and sn-2 levels, but the latter were only significant before BH correction. First-phase insulin secretion was only associated with LPI in the multivariable model before BH correction (β [95%CI]= 145.1 [45.2, 245.1], p=0.006), but not after correction (p=0.1; Table 3). Associations between lipid species or classes and fasting glucose, 2-hour post-OGTT glucose, or fasting insulin remained non-significant after adjusting for age, sex, and body fat (all p>0.1, Supplemental Material). However, insulin sensitivity became inversely associated with the dihydroceramide

lipid class (β [95%CI]= -1.9 [-2.9, -0.9], p=0.01, Table 3) as well as with several DG and TG lipid species (**Table 4** and Supplemental Material).

3.3. Associations between lipids and inflammatory markers and adipokines:

The dihexosylceramide lipid class was positively associated with IL-10 in univariable analysis (β [95%CI]= 4.5 [2.0, 6.9], p= 0.02), as well as after adjustment for age, sex, and body fat (β [95%CI]= 4.2 [1.8, 6.7], p=0.03, Supplemental Material). There was a positive association between the cholesterol ester lipid class and IL-1β (β [95%CI]= 11.9 [4.8, 19.0], p=0.046); however this was attenuated after adjustment for the above covariates (p=0.1, Supplemental Material). No associations were found between any lipid species or classes and other inflammatory markers measured including hsCRP, TNF-α, MCP-1, IL-6, or IL-8 (all p≥0.1 before and after adjustment for age, sex, and body fat, Supplemental Material). Similarly, leptin, resistin, and adipsin were not associated with any lipid species or classes in both univariable and multivariable analyses (all p>0.1, Supplemental Material). Adiponectin was inversely associated with four DG and four TG subspecies in univariable analyses; however these relationships were attenuated after adjustment for age, sex, and body fat (Table 4).

4. DISCUSSION

In a metabolically well-characterised cohort of overweight or obese and otherwise healthy individuals, we provide novel evidence that the LPI lipid class is associated with higher insulin secretion and may be a potential biomarker for early-stage β-cell dysfunction. We also confirm previous studies showing reduced ether phospholipids and plasmalogens in obesity and implicating sphingolipids and glycerolipids in the pathogenesis of insulin resistance and inflammation. Collectively, our findings suggest that distinct lipid classes present in obesity are associated with worse metabolic profiles and that targeting of such lipids could have substantial untapped therapeutic potential.

Total % body fat was inversely associated with the phosphatidylethanolamine (PE) lipid class and its vinyl ether-linked plasmalogen subclasses alkenyl-PE and lysoalkenyl-PE before and after adjusting for age and sex. PE is a multifunctional phospholipid and precursor for other lipids including phosphatidylcholine. Deficiency in PE impairs oxidative phosphorylation and alters mitochondrial function [27], whereas elevated hepatic PE is proposed to have beneficial glucose-lowering effects [28]; however, results are conflicting [29]. Similarly, plasmalogens exert antioxidant effects via preventing oxidation of cholesterol in phospholipid bilayers and regulating cholesterol esterification and efflux [30]. Lower plasmalogens have frequently been observed in conditions of elevated oxidative stress, including in obesity as demonstrated here and in previous studies [22], as well as in prediabetes and type 2 diabetes [29], cardiovascular diseases [31], and Alzheimer's disease [32]. Large population-based cohorts [22] and smaller studies in twins [5], non-diabetic adults [33] and obese women [34] have reported that PEs and plasmalogens correlate negatively with BMI [22], body fat by DEXA [5, 33], subcutaneous fat by MRI [5], and visceral fat measured by CT [34]. However, some studies did not adjust for age or sex, while others did not exclude smoking, comorbidities, or medication use, factors which can influence plasma lipidome composition [35, 36]. Indeed, associations between seven lipid classes and total % body fat in the present study were attenuated after adjustment for age and sex. Our findings strengthen current evidence that obesity, even in the absence of overt metabolic disease, is associated with deleterious alterations in lipid metabolism which may facilitate oxidative stress and development of type 2 diabetes.

The novel finding of the present study is the strong independent positive association between total and second phase insulin secretion and the LPI lipid class. LPI is a bioactive lipid generated by the phospholipase-A (PLA) family of lipases, primarily by PLA_2 enzymes. Group IV calcium-dependent PLA_2 ($cPLA_2$) are the main enzymes responsible for LPI synthesis, although the Group VI cytosolic calcium-independent PLA_2 ($iPLA_2$) has also been shown to

produce LPI [37]. LPI has been implicated in several biological processes including cell growth, differentiation, and motility in various cell-types [38]. Two early studies [7, 39] showed that LPI stimulated the release of insulin by rat pancreatic islets, suggesting that LPI may have potent insulinotropic effects. In 2007, Oka et al. [40] uncovered the first receptor target for LPI, the G protein-coupled receptor-55 (GPR-55), which is present in pancreatic β-cells [38]. Several animal models and human cell-culture studies have since reported the potential role of LPI in metabolism and glucose homeostasis [41, 42], suggesting that it may influence insulin secretion indirectly via GPR-55 activation or directly via inducing intracellular calcium release and potentiating ion channels [38]. To our knowledge, no previous studies have demonstrated a relationship between LPI and insulin secretory responses *in vivo* in humans. Existing cross-sectional studies have only examined insulin secretion in relation to sphingolipids [8, 43], namely ceramides, while intervention studies examined insulin secretory responses following lipid infusion [44], pharmacotherapy [43], or dietary free fatty acids such as high palmitic or oleic acid diets [45]. Here, we present new evidence for an association between LPI and insulin secretion *in vivo* in humans, supporting the possible insulinotropic effects of LPI demonstrated in earlier animal models. Elevated LPI may be a novel biomarker for impaired insulin secretion and early-stage β-cell dysfunction in obese individuals, and further investigation is warranted.

Negative associations were observed between insulin sensitivity and the dihydroceramide class and several DG and TG glycerolipid subspecies after adjustment for age, sex, and body fat. Glycerolipids are proposed to blunt insulin signalling pathways and reduce insulin sensitivity by phosphorylating insulin receptor substrate-1 (IRS-1), either directly or via activation of the inhibitor of kappa-B kinase (IKKβ) and Jun N-terminal kinase (JNK) [46]. Dihydroceramides, the precursors for complex sphingolipids, have been implicated in multiple biological processes associated with insulin action including autophagy, apoptosis, mitochondrial function, and oxidative stress [47]. Cross-sectional studies using HOMA-IR [4,

43, 48-50], Matsuda [4], or insulin sensitivity index [8, 43] have reported contradictory results for dihydroceramides [8, 43, 49], muscle DGs and TGs [4, 48], and mono vs linoleic acid TGs in serum, respectively [50]. It is posited that specific TG and DG moieties are metabolically active or that their actions on muscle insulin resistance are fibre type-specific [9, 50]. In studies using hyperinsulinaemic clamps, some report negative associations with dihydroceramides [9] or glycerolipids [5], consistent with our study, while others report no association [10, 51], the latter likely due to small sample sizes (n<22). We previously reported that insulin sensitivity by clamp was negatively associated with TG and DG subspecies, and with ceramides, but not dihydroceramides, in obese non-diabetic adults [33]. However, participants were older than in the present study (mean age >55 versus 31 years) with potentially greater metabolic abnormalities. We speculate that elevated dihydroceramides may indicate an initial upregulation of sphingolipid synthesis, after which a shift in sphingolipid metabolism occurs leading to greater ceramide accumulation in more insulin resistant states. Indeed, ceramides are nearly two-fold higher in patients with type 2 diabetes compared with lean [11, 52] or obese controls [8, 10], and higher dihydroceramides in healthy adults predicted a two-fold increased risk of diabetes after 3 years [53]. While this is an intriguing hypothesis, there were no associations with other sphingolipids in this study (likely due to the small sample size), thus further corroborating evidence is needed. Dihydroceramides may represent a marker of early-stage metabolic dysfunction, yet, whether they can be combined with other biochemical or genetic biomarkers to improve risk prediction and prevention of diabetes remains to be evaluated.

We found that dihexosylceramide, a complex glycosphingolipid class, was positively associated with the anti-inflammatory cytokine IL-10, independent of age, sex, and body fat. Dihexosylceramide mediates cholesterol biosynthesis and is reported to be lower in conditions associated with chronic low-grade inflammation including obesity [22], prediabetes, and type

2 diabetes [29]. We postulate that dihexosylceramide may function synergistically with IL-10 to regulate anti-inflammatory responses; however, further investigation is needed to corroborate this hypothesis. A positive association between IL-1β and the cholesterol ester class was also identified, but was attenuated after adjustment for covariates. Nevertheless, animal studies report that cholesterol accumulation in islets leads to macrophage recruitment and increased IL-1β expression, as well as defective glucose stimulated insulin secretion [54]. Whether dysregulated cholesterol drives 1L-1β secretion and metabolic inflammation awaits further study. No associations were found for the other inflammatory markers measured, consistent with previous studies reporting no correlations with TNF-α and hsCRP in plasma [49] or TNF-α in muscle [13]. However, others report that muscle and plasma ceramides and DGs correlate positively with TNF-α [11, 55] and IL-6 [49]. The latter studies were in patients with existing diseases including type 2 diabetes [11], sepsis [55], and coronary heart disease [49], in whom inflammation is exacerbated. Participants in the present study had relatively normal ranges of inflammatory markers which may explain the lack of associations observed.

Finally, we report inverse associations between adiponectin and several DG and TG subspecies, which were attenuated after adjustment for covariates. In experimental and animal models, overexpression of adiponectin and its receptors (AdipoR1/R2) enhances lipid clearance in liver and adipose tissue, promotes ceramidase activity, and prevents accumulation of deleterious lipid metabolites including ceramides and DGs [17]. We previously reported inverse associations between adiponectin and similar DG and TG subspecies in non-diabetic adults [33], and studies in both adults and children also support the antilipotoxic properties of adiponectin [43, 52]. Conversely, no associations were found between leptin, adipsin, or resistin and any lipids. Few human lipidomic studies have examined leptin and resistin and, to our knowledge, no studies have examined adipsin in relation to the plasma lipidome. In older men, leptin was inversely associated with lysophospholipids [56], and both leptin and resistin

were inversely associated with plasmalogens in lean non-diabetic adults [57]. Leptin has also been shown to stimulate fatty acid oxidation in muscle from lean, but not obese, humans [16], suggesting that obesity induces peripheral leptin resistance leading to the accumulation of intramuscular lipids and development of insulin resistance [16]. Although we found no associations between these adipokines and any lipids, this may be due to the small sample size. Further investigation is needed to clarify whether adipokines influence lipidomic pathways and their relative contributions to the overall functioning of the lipidome.

Some limitations of our study should be noted. This was a secondary analysis of baseline data from a previous RCT, hence, the study was not powered for lipidomics analyses and our sample size may have been too small to detect significant correlations for some variables. The false discovery rate of 0.05 may have been too conservative and a more liberal rate may have identified additional correlations. As per protocol [18], we studied overweight or obese non-diabetic adults and our results may not be generalizable to other populations. Future studies are needed to validate our findings against other cohorts, including lean individuals. The cross-sectional design means we cannot establish causality or directions of the relationships observed. We also cannot determine temporal changes in lipids which occur as a result of altered diet or circadian rhythms. However, our standardized collection of plasma samples in the morning after a 12-hr overnight fast from all participants minimizes variation caused by these factors. Moreover, lipids were measured in plasma rather than in target tissues and while this provides insight into lipid metabolism (predominantly within the liver), it also reflects lipoprotein production, circulatory metabolism, and turnover, and results should be interpreted in light of this. We addressed this issue to some extent by adjusting for demographic covariates known to influence lipoprotein levels, including age, sex, and body fat. TG concentrations were measured based on the neutral loss of multiple fatty acids. Hence, some isomeric TG species would have been excluded, although we suspect this to be minimal and limited to low abundant

species. Finally, grouping the results into lipid classes may lead to over-interpretation of our findings since lipid classes consist of several chemical entities with varied and often counteracting biological functions, thus results should be interpreted with caution.

Notwithstanding these limitations, we report the novel finding that LPI was associated with insulin secretion *in vivo* in humans, which has not previously been reported. Our targeted lipidomics platform covered >450 species and 26 classes with known identity and quantitative measurements, allowing for a comprehensive assessment of the plasma lipidome. We employed direct gold-standard measures of obesity, insulin sensitivity, and insulin secretion, and rigorous statistical analyses including adjustment for important covariates and standardization of each lipid measurement to the interquartile range prior to analysis. Finally, our sample comprised a metabolically well-characterized cohort of overweight or obese, otherwise healthy adults, where there was no confounding by disease status or use of medications, supplements, or substances.

5. CONCLUSIONS

Taken together, our data suggest that distinct lipid classes are associated with poorer metabolic profiles in overweight or obese individuals. We hypothesize that these lipids may alter signalling or differentiation pathways and ultimately result in type 2 diabetes, a hypothesis which needs to be confirmed by large-scale prospective studies. Our novel findings substantiate previous animal data and further implicate LPI as a potential culprit in early-stage β-cell dysfunction. Similarly, dihydroceramides may indicate an upregulation of sphingolipid synthesis at the early stages of metabolic dysfunction prior to disease onset. The newly recognised roles of these lipids, if validated in larger studies, could see their introduction as biomarkers for identifying high-risk individuals and for developing preventive and therapeutic strategies in future.

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6.1. Author Contributions Statement

AM conducted the research, performed data analysis and interpretation and wrote the first draft of the manuscript. NN conducted the research and contributed to data interpretation and writing and editing the manuscript. JJ, NM, and PJM performed the laboratory analyses and contributed to data interpretation and writing and editing the manuscript. BdC is the chief investigator of the trial and planned and designed the study, obtained funding, oversaw data collection and analysis, and contributed to data interpretation and writing and editing the manuscript. BdC is the guarantor of this work and takes responsibility for data integrity and accuracy. All authors meet the ICMJE criteria for authorship, and have approved the final version of the manuscript.

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6.3. Conflicts of Interest: none declared.

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Table 1. Participant Characteristics:

Characteristics	Mean ± SD or median (IQR)
Male, n (%)	41 (63%)
Age (years)	31.3 ± 8.5
Body mass index (kg/m^2)	30.1 (27.8, 33.3)
Waist circumference (cm)	101 (96, 108)
Waist-to-hip-ratio	0.93 ± 0.06
Total body fat (%)	40.1 ± 8.7
Fat mass (kg)	36.0 ± 11.0
Fat-free mass (kg)	53.4 ± 12.4
Fasting glucose (mmol/L)	4.6 ± 0.5
2-hour post OGTT glucose (mmol/L)	5.6 ± 1.6
Fasting insulin (mU/L)	8.9 (6.7, 12.8)
Insulin sensitivity (M-value; mg/kg/min)	6.6 ± 2.8
Total insulin AUC (mU/L)	1972.2 ± 1302.1
First-phase insulin AUC (mU/L)	385.0 ± 291.7
Second-phase insulin AUC (mU/L)	1303.6 ± 856.8
High-sensitivity C-reactive protein (mg/L)	1.7 (0.9, 4.4)
Tumor necrosis factor-α (pg/ml)	32.4 (16.9, 63.5)
Monocyte chemoattractant protein-1 (pg/ml)	631.6 (425.4, 982.8)
Interleukin-1β (pg/ml)	21.2 (10.7, 35.9)
Interleukin-6 (pg/ml)	22.1 (13.1, 44.4)
Interleukin-8 (pg/ml)	15.7 (9.8, 22.7)
Interleukin-10 (pg/ml)	8.7 (6.5, 16.3)
Adiponectin (ng/ml)	4749.4 (2348.1, 11243.4)
Leptin (ng/ml)	2.8 (1.5, 15.8)
Resistin (ng/ml)	1058.2 ± 914.7
Adipsin (ng/ml)	0.4 (0.3, 1.2)

Data are expressed as mean ± standard deviation, or median (interquartile range) for non-normally distributed variables, unless otherwise specified. **OGTT,** oral glucose tolerance test; **AUC**, area under the curve.

Table 2. Associations between lipid classes and sex and total % body fat:

Lipid Class	Sex*		Total % body fat			
	Unadjusted β (95% CI)	*P*	Unadjusted β (95% CI)	*P*	Adjusted† β (95% CI)	*P*
Trihexosylceramide	0.2 (-0.1, 0.5)	0.4	-0.0002 (-0.02, 0.02)	0.9	-0.02 (-0.04, 0.01)	0.4
Triacylglycerol	-4.9 (-8.3, -1.5)	0.06	**-0.3 (-0.5, -0.1)**	**0.02**	-0.2 (-0.5, 0.1)	0.3
Sphingomyelin	0.4 (-0.1, 1.4)	0.7	0.01 (-0.04, 0.01)	0.7	-0.004 (-0. 1, 0.1)	0.9
Phosphatidylserine	0.1 (-0.7, 0.8)	0.9	-0.01 (-0.1, 0.03)	0.6	-0.05 (-0.1, 0.01)	0.3
Phosphatidylinositol	-0.2 (-1.4, 1.0)	0.8	-0.03 (-0.1, 0.04)	0.4	-0.04 (-0.1, 0.1)	0.6
Phosphatidylglycerol	-0.1 (-0.5, 0.3)	0.7	-0.02 (-0.04, 0.01)	0.3	-0.03 (-0.1, 0.01)	0.2
Alkenylphosphatidylethanolamine	-1.9 (-4.9, 1.1)	0.4	**-0.3 (-0.4, -0.1)**	**0.01**	**-0.5 (-0.7, -0.2)**	**0.03**
Alkylphosphatidylethanolamine	-0.8 (-2.1, 0.5)	0.4	**-0.1 (-0.2, -0.02)**	**0.04**	-0.1 (-0.2, -0.03)	0.08
Phosphatidylethanolamine	-0.5 (-2.3, 1.3)	0.7	**-0.1 (-0.2, -0.03)**	**0.045**	**-0.2 (-0.4, -0.1)**	**0.03**
Alkenylphosphatidylcholine	-0.5 (-1.8, 0.7)	0.6	-0.01 (-0.2, -0.02)	0.05	-0.1 (-0.2, -0.04)	0.07
Alkylphosphatidylcholine	-0.8 (-1.9, 0.4)	0.4	-0.1 (-0.1, -0.004)	0.1	-0.1 (-0.2, -0.02)	0.2
Phosphatidylcholine	-1.8 (-5.2, 1.5)	0.5	-0.2 (-0.4, -0.03)	0.07	-0.3 (-0.1, 0.01)	0.2
Monohexosylceramide	-0.5 (-0.8, -0.1)	0.1	**-0.03 (-0.05, -0.01)**	**0.02**	-0.03 (-0.1, -0.004)	0.1
Lysophosphatidylinositol	-0.4 (-0.9, 0.1)	0.3	-0.02 (-0.05, 0.005)	0.2	-0.004 (-0.05, 0.04)	0.9
Lysoalkenylphosphatidylethanolamine	-0.2 (-0.5, 0.1)	0.4	**-0.02 (-0.04, -0.01)**	**0.02**	**-0.04 (-0.1, -0.02)**	**0.04**
Lysophosphatidylethanolamine	-0.4 (-1.0, 0.2)	0.4	**-0.04 (-0.08, -0.01)**	**0.04**	-0.1 (-0.1, -0.01)	0.09
Lysoalkenylphosphatidylcholine	-0.2 (-0.4, 0.1)	0.4	-0.01 (-0.03, 0.00003)	0.1	-0.02 (-0.04, 0.002)	0.2
Lysoalkylphosphatidylcholine	-0.5 (-0.9, -0.02)	0.2	-0.03 (-0.1, -0.01)	0.05	-0.03 (-0.1, 0.004)	0.3
Lysophosphatidylcholine	-3.1 (-5.4, -0.8)	0.08	**-0.2 (-0.3, -0.1)**	**0.01**	-0.2 (-0.4, 0.004)	0.2
G_{M3} ganglioside	**-0.5 (-0.8, -0.3)**	**0.01**	**-0.03 (-0.04, -0.02)**	**0.004**	-0.02 (-0.04, 0.01)	0.3
Dihydroceramide	0.1 (-0.4, 0.6)	0.8	-0.01 (-0.04, 0.02)	0.5	-0.03 (-0.1, 0.01)	0.3
Dihexosylceramide	-0.3 (-0.6, 0.1)	0.3	-0.02 (-0.04, -0.003)	0.07	-0.03 (-0.1, 0.004)	0.3
Diacylglycerol	-5.7 (-9.9, -1.5)	0.08	-0.3 (-0.5, -0.01)	0.06	-0.2 (-0.5, 0.2)	0.2
Free Cholesterol	-0.03 (-0.1, 0.02)	0.4	-0.003 (-0.01, -0.0001)	0.1	-0.003 (0.01, 0.001)	0.6
Ceramide	**-0.5 (-0.8, -0.2)**	**0.04**	**-0.03 (-0.05, -0.02)**	**0.006**	-0.03 (-0.1, -0.002)	0.2
Cholesterol ester	0.6 (-0.4, 1.5)	0.4	0.02 (-0.03, 0.1)	0.5	0.01 (-0.1, 0.1)	0.9

Data presented as β-coefficients and corresponding 95% confidence intervals (CI), with β-coefficients representing change in outcome variable associated with an interquartile range increase in lipid class. All lipids were log-transformed to the base 10 prior to analyses, and all p-values represent significance of associations after correcting for multiple comparisons using the Benjamini-Hochberg method.

* Males used as the reference group in examining associations between lipids and sex.

† Multivariable model adjusted for age and sex.

Table 3. Univariable and multivariable associations between lipid classes and insulin sensitivity and secretion:

Lipid Class	Insulin Sensitivity		Total Insulin Secretion		First Phase Insulin Secretion		Second Phase Insulin Secretion	
	Unadjusted β (95% CI)	Adjusted* β (95% CI)	Unadjusted β (95% CI)	Adjusted* β (95% CI)	Unadjusted β (95% CI)	Adjusted* β (95% CI)	Unadjusted β (95% CI)	Adjusted* β (95% CI)
Trihexosylceramide	0.8 (-0.1, 1.7)	0.8 (-0.03, 1.7)	-430.5 (-848.6, -12.4)	-472.0 (-900.2, -43.7)	-78.6 (-173.3, 16.1)	-97.6 (-191.3, -4.0)	-279.7 (-555.1, -4.4)	-472.0 (-900.2,-43.7)
Triacylglycerol	-0.4 (-1.4, 0.6)	-1.1 (-2.0, -0.1)	459.5 (6.0, 913.0)	529.2 (51.8, 1006.5)	82.8 (-20.0, 185.5)	94.0 (-11.3, 199.4)	327.7 (30.9, 624.4)	529.2 (51.8, 1006.5)
Sphingomyelin	-0.2 (-0.9, 0.6)	-0.2 (-0.9, 0.4)	-183.1 (-512.8, 146.7)	-166.7 (-494.5, 161.2)	-45.4 (-119.2, 28.3)	-42.8 (-114.0, 28.4)	-84.2 (-302.3, 133.8)	-166.7 (-494.5, 161.2)
Phosphatidylserine	-0.3 (-1.2, 0.7)	-0.6 (-1.5, 0.3)	-116.6 (-562.7, 329.6)	-17.9 (-481.1, 445.3)	-40.7 (-140.3, 59.0)	-19.1 (-120.0, 81.7)	-19.6 (-313.8, 274.5)	-17.9 (-481.1, 445.3)
Phosphatidylinositol	-0.2 (-1.2, 0.9)	-0.6 (-1.5, 0.4)	275.8 (-184.0, 735.6)	305.2 (-163.2, 773.7)	46.1 (-57.5, 149.6)	45.5 (-57.3, 148.4)	217.0 (-84.2, 518.1)	305.2 (-163.2, 773.7)
Phosphatidylglycerol	-0.3 (-1.3, 0.7)	-0.9 (-1.8, 0.01)	249.9 (-195.6, 695.5)	323.5 (-131.7, 778.8)	73.0 (-26.2, 172.2)	88.3 (-10.0, 186.5)	162.8 (-130.5, 456.0)	323.5 (-131.7, 778.8)
Alkenylphosphatidylethanolamine	0.1 (-1.0, 1.1)	-1.1 (-2.1, -0.2)	-125.8 (-585.8, 334.1)	85.3 (-436.9, 607.5)	-26.8 (-129.9, 76.3)	17.1 (-96.7, 130.9)	-99.2 (-401.6, 203.2)	85.3 (-436.9, 607.5)
Alkylphosphatidylethanolamine	-0.02 (-1.0, 0.9)	-0.9 (-1.8, 0.02)	-177.9 (-608.7, 252.8)	-10.2 (-483.0, 462.6)	-38.5 (-135.1, 58.0)	0.7 (-102.3, 103.7)	-128.0 (-411.2, 155.1)	-10.2 (-483.0, 462.6)
Phosphatidylethanolamine	-0.2 (-1.1, 0.7)	-1.0 (-1.8, -0.1)	241.4 (-162.4, 645.1)	392.3 (-32.6, 817.3)	65.4 (-24.6, 155.4)	91.2 (-1.1, 183.4)	154.1 (-111.7, 420.0)	392.3 (-32.6, 817.3)
Alkenylphosphatidylcholine	0.6 (-0.3, 1.5)	-0.1 (-1.0, 0.8)	-136.3 (-571.6, 299.0)	-80.0 (-540.8, 380.8)	-23.1 (-120.8, 74.5)	-17.9 (-118.3, 82.5)	-121.9 (-407.6, 163.8)	-80.0 (-540.8, 380.8)
Alkylphosphatidylcholine	0.3 (-0.5, 1.2)	-0.3 (-1.1, 0.5)	-165.3 (-554.3, 223.7)	-84.4 (-495.9, 327.1)	-36.0 (-123.2, 51.1)	-18.8 (-108.4, 70.9)	-116.8 (-372.6, 138.9)	-84.4 (-495.9, 327.1)
Phosphatidylcholine	-0.1 (-1.2, 0.9)	-0.8 (-1.8, 0.2)	274.8 (-206.4, 756.0)	285.4 (-221.9, 792.8)	53.7 (-54.4, 161.8)	44.7 (-66.4, 155.8)	192.3 (-124.0, 508.5)	285.4 (-221.9, 792.8)
Monohexosylceramide	0.8 (-0.2, 1.8)	-0.1 (-1.1, 0.9)	-176.1 (-634.3, 282.2)	-144.6 (-664.3, 375.2)	-33.5 (-136.3, 69.3)	-25.4 (-138.8, 87.9)	-132.8 (433.9, 168.3)	-144.6 (-664.3, 375.2)
Lysophosphatidylinositol	-0.9 (-1.9, 0.1)	-1.2 (-2.2, -0.3)	**781.9 (353.3, 1210.4)**	**798.3 (353.7, 1242.9)**	150.1 (51.6, 248.7)	145.1 (45.2, 245.1)	**521.3 (240.0, 802.5)**	**798.3 (353.7, 1242.9)**
Lysoalkenylphosphatidylethanolamine	0.1 (-0.9, 1.1)	-1.0 (-1.9, 0.02)	-79.6 (-541.5, 382.4)	97.5 (-415.1, 610.2)	-22.1 (-125.5, 81.5)	15.6 (-96.2, 127.3)	-43.8 (-347.9, 260.3)	97.5 (-415.1, 610.2)
Lysophosphatidylethanolamine	0.5 (-0.4, 1.4)	-0.03 (-0.9, 0.8)	15.0 (-403.8, 433.7)	83.8 (-350.6, 518.3)	20.9 (-72.7, 114.6)	33.3 (-61.1, 127.7)	-13.3 (-288.8, 262.2)	83.8 (-350.6, 518.3)
Lysoalkenylphosphatidylcholine	0.7 (-0.2, 1.6)	0.3 (-0.5, 1.2)	-178.4 (-593.0, 236.3)	-189.3 (-618.2, 239.6)	-30.5 (-123.6, 62.6)	-35.2 (-128.8, 58.4)	-141.1 (-413.3, 131.1)	-189.3 (-618.2, 239.6)
Lysoalkylphosphatidylcholine	0.4 (-0.6, 1.4)	-0.2 (-1.2, 0.7)	-205.5 (-650.0, 239.0)	-141.9 (-620.1, 336.4)	-38.3 (-138.1, 61.5)	-19.5 (-123.9, 84.9)	-139.3 (-431.7, 153.0)	-141.9 (-620.1, 336.4)
Lysophosphatidylcholine	0.4 (-0.4, 1.2)	-0.2 (-1.0, 0.6)	56.6 (-304.7, 417.8)	85.5 (-316.6, 487.5)	15.9 (-65.0, 96.8)	19.3 (-68.3, 106.9)	25.2 (-212.6, 263.0)	85.5 (-316.6, 487.5)
G_{M3} ganglioside	0.9 (0.04, 1.8)	0.3 (-0.6, 1.3)	-173.3 (-598.0, 251.4)	-258.4 (-751.6, 234.9)	-35.5 (-130.7, 59.7)	-56.3 (-163.8, 51.2)	-111.9 (-391.4, 167.6)	-258.4 (-751.6, 234.9)
Dihydroceramide	-1.3 (-2.5, -0.1)	**-1.9 (-2.9, -0.9)**	314.4 (-247.9, 876.7)	471.0 (-95.5, 1037.5)	34.3 (-92.6, 161.2)	64.0 (-61.1, 189.1)	264.8 (-103.0, 632.5)	471.0 (-95.5, 1037.5)
Dihexosylceramide	0.2 (-0.6, 0.9)	0.01 (-0.6, 0.7)	-315.1 (-637.8, 7.6)	-331.6 (-652.6, -10.6)	-68.4 (-140.8, 4.1)	-74.8 (-144.5, -5.0)	-184.6 (-398.2, 29.0)	-331.6 (-652.6, -10.6)
Diacylglycerol	-0.4 (-1.6, 0.7)	-1.2 (-2.3, -0.2)	432.3 (-76.4, 940.9)	562.6 (26.6, 1098.5)	70.1 (-45.0, 185.3)	99.0 (-19.2, 217.3)	316.0 (-17.0, 649.0)	562.6 (26.6, 1098.5)
Free Cholesterol	-0.2 (-1.04, 0.6)	-0.6 (-1.4, 0.1)	136.7 (-243.0, 516.5)	136.4 (-252.8, 525.6)	15.1 (-70.2, 100.5)	7.3 (-77.8, 92.5)	115.1 (-134.1, 364.4)	136.4 (-252.8, 525.6)
Ceramide	0.3 (-0.6, 1.2)	-0.6 (-1.5, 0.4)	28.4 (-385.3, 442.1)	134.6 (-342.5, 611.7)	7.4 (-85.2, 100.1)	32.9 (-71.0, 136.8)	18.8 (-253.4, 291.0)	134.6 (-342.5, 611.7)
Cholesterol ester	0.02 (-0.9, 1.0)	0.2 (-0.7, 1.0)	-491.6 (-907.0, -76.1)	-516.6 (-934.8, -98.3)	-110.0 (-203.1, -16.9)	-120.4 (-210.8, -29.9)	-295.1 (-570.4, -19.8)	-516.6 (-934.8, -98.3)

Data presented as β-coefficients and corresponding 95% confidence intervals (CI), with β-coefficients representing change in outcome variable associated with an interquartile range (IQR) increase in lipids. All analyses were conducted with log-IQR transformation to control for skewness of lipids, and all analyses were corrected for multiple comparisons using the Benjamini-Hochberg (BH) method. Bold values indicate significance at two-tailed $p<0.05$ after BH correction.

*Multivariable models adjusted for age, sex, and total % body fat.

Table 4. Univariable and multivariable associations between selected lipid species and insulin sensitivity and adiponectin:

Insulin sensitivity				
Selected Lipid Species	**Unadjusted β (95% CI)**	***P***	**Adjusted* β (95% CI)**	***P***
Cer (d18:0/22:0)	-1.3 (-2.4, -0.2)	0.5	**-1.9 (-2.9, -1.0)**	**0.04**
Cer (d18:0/24:0)	-1.1 (-2.1, -0.1)	0.5	**-1.6 (-2.5, -0.7)**	**0.04**
DG (16:0_16:0)	-1.7 (-2.8, -0.7)	0.5	**-2.0 (-2.9, -1.1)**	**0.03**
DG (16:0_18:1)	-1.0 (-2.1, -0.1)	0.6	**-1.7 (-2.6, -0.7)**	**0.04**
PC (36:0)	-1.3 (-2.2, -0.4)	0.5	**-1.5 (-2.3, -0.7)**	**0.04**
TG (48:0) [NL-16:0]	-1.4 (-2.4, -0.4)	0.5	**-1.6 (-2.5,-0.7)**	**0.04**
TG (50:0) [NL-18:0]	-1.2 (-2.2,-0.1)	0.5	**-1.8 (-2.8, -0.8)**	**0.04**
TG (50:1) [NL-18:1]	-1.2 (-2.2, -0.2)	0.5	**-1.6 (-2.5, -0.7)**	**0.04**
TG (52:1) [NL-18:0]	-0.9 (-2.0, 0.3)	0.8	**-1.9 (-2.9, -0.8)**	**0.04**
Adiponectin				
Selected Lipid Species	**Unadjusted β (95% CI)**	***P***	**Adjusted* β (95% CI)**	***P***
DG (14:0_18:2)	**-7407.6 (-11350.3, -3464.9)**	**0.04**	-6951.4 (-11109.6, -2793.1)	0.1
DG (16:0_16:0)	**-9002.6 (-13382.2, -4623.1)**	**0.03**	-8835.2 (-13382.5, -4288.0)	0.1
DG (18:0_18:1)	**-7406.2 (-11571.7, -3240.8)**	**0.04**	-7334.2 (-11956.6, -2711.7)	0.1
DG (18:0_18:2)	**-8083.8 (-12524.8, -3642.8)**	**0.04**	-7950.1 (-12894.9, -3005.4)	0.1
PC (18:0_20:3)	**-7640.6 (-11768.6, -3512.5)**	**0.04**	-7203.0 (-11521.4, -2884.7)	0.1
TG (50:0) [NL-18:0]	**-8321.3 (-12704.6, -3938.1)**	**0.04**	-8321.3 (-13081.1, -3561.6)	0.1
TG (50:1) [NL-14:0]	**-7889.8 (-12315.7, -3463.9)**	**0.04**	-7752.4 (-12567.7, 2937.1)	0.1
TG (50:1) [NL-18:1]	**-7455.2 (-11472.1, -3438.3)**	**0.04**	-7169.4 (-11436.5, -2902.2)	0.1
TG (50:2) [NL-18:2]	**-8085.5 (-12012.8, -4158.2)**	**0.03**	-7748.0 (-11856.7, -3639.2)	0.1

Data presented as β-coefficients and corresponding 95% confidence intervals (CI), with β-coefficients representing change in outcome variable associated with an interquartile range (IQR) increase in lipids. All analyses were conducted with log-IQR transformation to control for skewness of lipids, and all p-values represent significance of associations after correcting for multiple comparisons using the Benjamini-Hochberg method.
*Multivariable models adjusted for age, sex, and total % body fat.
Cer, ceramides; **DG**, diacylglycerol; **PC**, phosphatidylcholine; **TG**, triacylglycerol.

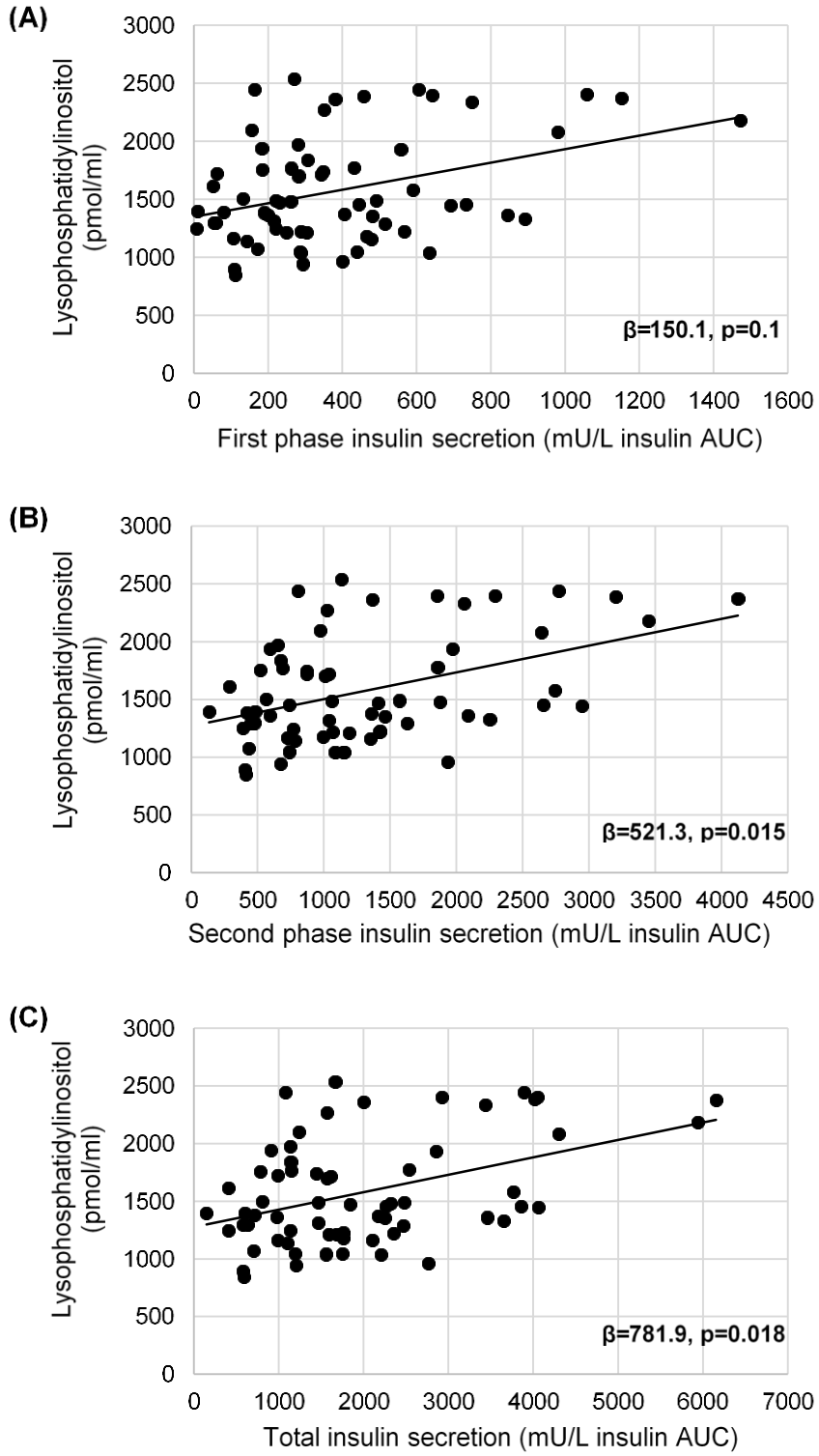

Figure 1. Associations between the lysophosphatidylinositol (LPI) lipid class and first-phase (A), second-phase (B), and total (C) insulin secretion. Scatter plots represent raw data (uncorrected), where **β**=change in insulin secretion associated with an interquartile range (IQR) increase in LPI. **P**-values represent significance of associations after correction for multiple comparisons using the Benjamini-Hochberg method. **AUC**, area under the curve.