MONASH University

Plasma lipidomics to define the relationship of lipid metabolism with cardiometabolic risk factors and type 2 diabetes

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Abstract

Over the recent years, advances in liquid chromatography coupled with mass spectrometry have revolutionized the detailed mapping of all lipid species contained in a biological sample through lipidomics, including targeted and untargeted approaches. In particular, high-throughput targeted analysis of human plasma has become an increasingly popular approach to identify molecular lipid species associated with different diseases states. Lipidomics has a potential to predict disease risk, inform on the mechanism of disease pathogenesis, identify biomarkers and help monitor response to treatment of several disease conditions. While dyslipidaemia (high triglycerides or high total cholesterol and/or low high-density lipoprotein cholesterol) is a major risk factor for insulin resistance (IR) and type 2 diabetes (T2D), it does not represent the structurally and functionally diverse lipid classes/subclasses and species. Thus, detailed mapping of the plasma lipidome associated with these conditions and other cardiometabolic risk factors provides in-depth knowledge and better biological insight to help identify new biomarkers and/or therapeutic targets.

The overall objective of this thesis was to comprehensively examine the relationship of lipid metabolism with cardiometabolic risk factors and outcomes utilizing three independent human cohorts: 1) the Australian Diabetes, Obesity and Lifestyle Study (AusDiab, n=10, 339); 2) a cohort of young adults recruited in Melbourne (n=246) and 3) the Busselton Health Study (BHS) (n=4,207). Targeted lipidomics utilising liquid chromatography coupled with a tandem mass spectrometry (LC-MS/MS) was used to measure over 600 molecular lipid species across 36 classes/subclasses. We identified novel sex-specific lipidomic fingerprints of age and BMI in the AusDiab and validated these in a second population cohort, the BHS. Sex-specificity in the association of lipids with IR; suggesting the potential involvement of specific lipid species in the pathogenesis of IR and possible crosstalk between IR and sex-specific regulation of lipid metabolism was identified among young adults. Novel associations of alkyl-diacylglycerol species with change in WC (particularly in women) were identified. Using lipidomic data, we derived metabolic BMI scores (mBMI) and showed that metabolic discordant groups where those whose mBMI was greater than their real BMI displayed higher risk and unfavourable metabolic health profiles compared to those whose mBMI was less than their real BMI despite these

groups having a similar real BMI. Finally, a plethora of associations of lipid classes/subclasses and species with prevalent and incident T2D, including the negative association of lipid species containing monomethyl-branched chain fatty acids and linolenate (18:2) and positive associations with atypical sphingolipids such as the deoxyceramide species that have not been previously reported in this context. We also developed lipidomic risk models and showed improvement of these models upon traditional risk factors to predict the onset of T2D.

In summary, lipidomic analyses of large cohorts help not only identify biomarkers and but also asses the risk of cardiometabolic disorders. Indeed, the findings provide important insight into the role of lipids in health and disease.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Name: Habtamu B Beyene

Date: 27th April 2021

Publications during enrolment

1. **Habtamu B Beyene**, Steven Hamley, Corey Giles, Kevin Huynh, Alexander Smith, Michelle Cinel, Natalie A Mellet, Maria G Morales-Scholz, Danielle Kloosterman, Kirsten F Howlett, Greg M Kowalski, Christopher S Shaw, Dianna J Magliano, Clinton R Bruce, Peter J Meikle. Mapping the Associations of the Plasma Lipidome With Insulin Resistance and Response to an Oral Glucose Tolerance Test. **J Clin Endocrinol Metab 105**: 1–15, 2020

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Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 2 original articles published in peer reviewed journals and 3 manuscripts ready for submission. The core theme of the thesis is to elucidate the relationship between lipid metabolism and cardiometabolic risk and explore the role of age and sex in modifying these relationships. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Central Clinical School under the supervision of Prof. Peter Meikle and Prof. Dianna Magliano. The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of chapter 6, Corey Giles and Gavriel Olshansky helped me in developing modelling approaches for prediction of BMI. I performed lipidomic analysis and analysed the data. I characterised the relationship of metabolic BMI with cardiometabolic risk factors and outcomes. In the case of chapter 7 (diabetes prediction), Kevin Huynh worked with me on the R codes and the multivariate modelling (% contribution = 40%) and I performed lipidomic analysis on the samples and did the rest of the analysis. I performed all the write up described in chapters 1 and 2. In the case of chapter 3 and 4, my contribution to the work involved the following:

Thesis Chapter	**Publication Title**	**Status** *(published, in press, accepted or returned for revision, submitted)*	**Nature and % of student contribution**	**Co-author name(s) Nature and % of Co-author's contribution***	**Co-author(s), Monash student Y/N***
3	High-coverage	Published	Conducted the	**Gavriel Olshansky (1.5%)**	No to all

	plasma lipidomics reveals novel sex-specific lipidomic fingerprints of age and BMI: Evidence from two large population cohort studies			Lipid extraction, MS analysis, data processing and statistical analysis and, obtained all the data, interpretation of results, figure generation, manuscript writing **(65%)**	Intellectual input regarding statistical software, **Adam Alexander T. Smith (0.5%)** Statistical input and feedback of manuscript **Corey Giles (1.5%)** Intellectual input regarding statistical analysis **Kevin Huynh (1.5%)** Assistance in statistical analysis and lipid ontology **Cinel, Michelle (1.5%)** Assisting with sample preparation **Mellett, NA (1.5%)** Assisting with sample preparation and MS analysis **Gemma Cadby (0.5%)** Feedback regarding manuscript **Joseph Hung (0.5%)** Feedback regarding manuscript **Jennie Hui (0.5%)** Feedback regarding manuscript **John Beilby (0.5%)** Feedback regarding manuscript **Gerald F. Watts (0.5%)** Feedback regarding manuscript **Jonathan S. Shaw (0.5%)** Feedback regarding manuscript, coordinator of original clinical cohort study **Eric K. Moses (0.5%)** Feedback regarding manuscript **Dianna J. Magliano (5%)** Coordinator of original clinical cohort study, provided intellectual input into the manuscript, supervised student. **Meikle, PJ (18.5%)** Help develop the study, provided funding, provided feedback, intellectual input into all aspects of the analysis and finalised the manuscript	
4	Mapping the	Published	Conducted the	**Steven Hamley (0.5%)**	No to all

Associations of the Plasma Lipidome With Insulin Resistance and Response to an Oral Glucose Tolerance Test	Lipid extraction, MS analysis, data processing and statistical analysis and, obtained all the data, interpretation of results, figure generation, manuscript writing **(65%)**	Helped in the design, collection, data analysis **Corey Giles (1%)** Contributed in developing and improving MS methods and statistical analysis **Kevin Huynh (1%)** contributed in developing and improving MS methods and statistical analysis **Adam Alexander T. Smith (0.5%)** Contributed in developing scripts and statistical analyses **Michelle Cinel (1%)** Assisting with sample preparation **Natalie A. Mellet (1%)** Assisting with MS experiment and post-MS data pre-processing and analysis **Maria G. Morales-Scholz (0.5%)** Helped in data collection **Danielle Kloosterman (0.5%)** Data collection **Kirsten F. Howlett, (0.5%)** Data collection **Greg M. Kowalski (0.5%)** Helped in the design, collection, data analysis **Christopher S. Shaw (0.5%)** Helped in the design, collection, data analysis **Dianna J. Magliano (5%)** Contributed in interpretation of results and revision of the manuscript, supervised student. **Clinton R. Bruce (5%)** Designed the study, collected data, participated in data analysis, contributed plasma samples, and revised the manuscript. **Meikle, PJ (17.5%)**

	Help develop the study, provided funding, provided feedback, intellectual input into the project

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Student signature: Date: 27/04/2021

Main Supervisor signature: Date: 27/04/2021

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Abbreviations

Abbreviation	Name
AA	Arachidonic acid
ACAT2	Acetyl-CoA acetyltransferase
AIC	Akaike's information criterion
ApoB	Apolipoprotein B
AUC	Area under the curve
AusDaib	Australian Diabetes, Obesity and Lifestyle Study
BAT	Brown adipose tissue
BCAA	Branched-chain amino acids
BCAT	Branched-chain amino acid aminotransferase
BCKDK	Branched Chain Ketoacid Dehydrogenase Kinase
BH	Benjamini Hochberg
BHS	Busselton Health Study
BIC	Bayesian information criterion
BMI	Body mass index
BSA	Body surface area
BUME	Butanol-methanol
CABG	Coronary artery bypass grafting
CAD	Coronary artery disease
CDP	Cytidine diphosphate
CE	Cholestryl ester
Cer	Ceramide
Cer1P	Ceramide-1-Phosphate
CETP	Cholesteryl Ester Transfer Protein
CHD	Coronary heart disease
CID	Collision induced dissociation
COH	Cholesterol
CPT1	Carnitine palmitolyltransferase 1
CT	Computed tomography
CV	Coefficient of variation
CVA	Cerebrovascular accident
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DE	Dehydrocholesterol ester

Abbreviation	Name
deoxyCer	Deoxyceramide
DG	Diacylglycerol
DHA	Docosahexaenoic acid
dhCer	Dihydroceramide
DPA	Docosapentaenoic acid
DXA	Dual energy x-ray absorptiometry
ELOVL	Elongation of very long chain fatty acids
EPA	Eicosapentaenoic acid
ESI	Electrospray ionization
FADS3	Fatty acid desaturase 3
FAO	Fatty acid oxidation
FBG	Fasting blood glcuose
FDR	False discovery rate
FFA	Free fatty acid
GM1	GM1 ganglioside
GM3	GM3 ganglioside
HbA1c	Glycated Hemoglobin
HDL-C	High-density lipoprotein cholesterol
Hex2Cer	Dihexosylceramide
Hex3Cer	Trihexosylceramide
HexCer	Monohexosylceramide
HOMA-IR	Homeostatic model assessment of insulin resistance
HPLC	High performance liquid chromatography
HR	Heart rate
IDL-C	Intermediate-density lipoprotein cholesterol
IFN-γ	Interferon-gamma
IGT	Impaired glucose tolerant
IL-1β	Interleukin-1 beta
IL-6	Interleukin 6
IR	Insulin resistance
LASSO	Least absolute shrinkage and selection operator
LC	Liquid chromatography
LCAT	Lecithin cholesterol acyltransferase
LC-ESI-MS	LC electrospray ionisation mass spectrometry
LDL-C	Low-density lipoprotein cholesterol

Abbreviation	Name
LPC	Lysophosphatidylcholine
LPC(O)	Lysoalkylphosphatidylcholine
LPC(P)	Lysoalkenylphosphatidylcholine
LPE	Lysophosphatidylethanolamine
LPE(P)	Lysoalkenylphosphatidylethanolamine
LPI	Lysophosphatidylinositol
MAP	Mean arterial pressure
mBCAT	Branched-chain amino acid aminotransferase
mBMI	Metabolic BMI
MI	Myocardial infarction
mmBCFA	Monomethyl branched chain fatty acid
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MUFA	Monounsaturated fatty acid
NIST	National institute of standards technology
NL	Neutral loss
NMR	Nuclear magnetic resonance
NW-MH	Normal weight and metabolically healthy
OCFAs	Odd chain fatty acids
OGTT	Oral glucose tolerance test
O-MO	Obese and metabolically obese
OW-MOW	Overweight and metabolically overweight
PC	Phosphatidylcholine
PC(O)	Alkylphosphatidylcholine
PC(P)	Alkenylphosphatidylcholine (PC plasmalogen)
PE	Phosphatidylethanolamine
PE(O)	Alkylphosphatidylethanolamine
PE(P)	Alkenylphosphatidylethanolamine (PE plasmalogen)
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PLA2	Phospholipase A2
PLG / 2h-PLG	2 hour post load glucose
PQC	Plasma quality control sample
PS	Phosphatidylserine
PTCA	Percutaneous transluminal coronary angioplasty

Abbreviation	Name
PUFA	Polyunsaturated fatty acid
QQQ	Triple quadropole
ROS	Reactive oxygen species
S1P	Sphingosine-1-Phosphate
SBP	Systolic blood pressure
SCD-1	Stearoyl CoA desaturase 1
SFA	Saturated fatty acid
SM	Sphingomlyelin
sn	Stereospecifically numbered
Sph	Sphingosine
SPT	Serine palmitoyltransferase
T2D	Type 2 diabetes
TG	Triacylglycerol
TG(O)	Alkyldiacylglycerol
TNF-α	Tumor necrosis factor alpha
TQC	Technical quality control sample
UPLC	Ultra-performance liquid chromatography
UWA HREC	University of Western Australia Human Research Ethics Committee
VLDL-C	Very low density lipoprotein cholesterol
WAT	White adipose tissue
WC	Waist circumference
WHR	Waist/hip ratio

Chapter 1. Introduction

Preface

The section one of chapter 1 is a summary of the thesis followed by a literature review introducing the concept of lipidomics and its role in the characterisation of disease. It also reviews recent studies that have examined the dysregulation of lipid metabolism in insulin resistance, obesity and type 2 diabetes, followed by the hypothesis and aims of the project.

A specific introduction is also included in each chapter of the thesis.

1.1. Summary of thesis

Lipids are crucial components of biological membranes and lipoprotein particles. Lipidomics refers to a comprehensive mapping of molecular lipid species including metabolic pathways. Advances in liquid chromatography coupled with mass spectrometry over the recent years have revolutionized the detailed mapping of all lipid species contained in a biological sample through lipidomics, including targeted and untargeted approaches.

High-throughput targeted analysis of human plasma using liquid chromatography mass spectrometry has become an increasingly popular approach to identify molecular lipid species associated with different diseases states. While dyslipidaemia (high triglycerides or high total cholesterol and/or low high-density lipoprotein cholesterol) is a major risk factor for insulin resistance (IR) and type 2 diabetes (T2D), it does not represent the structurally and functionally diverse lipid classes/subclasses and species. Thus, detailed mapping of the plasma lipidome associated with these conditions and other cardiometabolic risk factors provides in-depth knowledge and better biological insight to help identify new biomarkers and/or therapeutic targets.

Large scale lipidomic analysis of population based cohorts provides statistical power for association studies and also offers an opportunity to determine the influence and interactions of age, sex, clinical and behavioural factors on lipid metabolism in health and disease. In chapter 3 of this thesis, we report the lipidomic profiling of a very large population cohort: The Australian Diabetes, Obesity and Lifestyle Study

(AusDiab, n = 10,339), measuring 706 distinct lipid species spanning 36 different classes/subclasses. We identified novel sex-specific lipidomic fingerprints of age and BMI and validated these in a second population cohort: The Busselton health study (BHS). In chapter 4, we identified, sex-specificity in the association of lipids with IR; suggesting the potential involvement of specific lipid species in the pathogenesis of IR and possible crosstalk between IR and sex-specific regulation of lipid metabolism.

In chapters 5, lipidomic profiles associated with the risk of increasing waist circumference (WC) or body mass index (BMI) over five years were investigated. Novel associations of alkyl-diacylglycerol species with change in WC (particularly in women) were identified. Branched and odd chain glycerophospholipids predicted change in WC and in BMI. In chapter 6, we derived a metabolic BMI score (mBMI) and examined its association with clinical risk factors and outcomes. The mBMI residuals predicted diabetes and cardiovascular disease (CVD). We identified metabolic discordant groups where those with mBMI was greater than their real BMI displayed higher risk and unfavourable metabolic health profiles compared to those whose mBMI was less than their real BMI despite these groups having a similar real BMI.

In chapter 7, we identified a plethora of associations of lipid classes/subclasses and species with prevalent and incident T2D, including the negative association of lipid species containing monomethyl-branched chain fatty acids and linolenate (18:2) and positive associations with atypical sphingolipids such as the deoxyceramide species which have not been previously reported. We were able to identify, differential associations of certain lipids with impaired fasting glucose (IFG) versus impaired glucose tolerant (IGT). Finally, we developed lipidomic risk models and showed improvement of these models upon traditional risk factors to predict the onset of T2D.

Chapter 8 then provides an overall discussion with future directions for the field

1.2. Obesity

Globally, the prevalence of obesity has increased dramatically over the last few decades (1, 2). Recent studies report that over 1.9 billion adults are overweight, of which over 650 million are obese. At least 2.8 million people die each year as a result of being overweight or obese (2). In Australia, two thirds (67%) of adults were overweight or obese in 2017/18 (3). The impact of obesity is immense; having adverse social, economic and health outcomes. There is a strong link between obesity and insulin resistance (IR), type 2 diabetes (T2D) (4, 5) and cardiovascular disease (CVD) (6).

Body mass index (BMI) is widely used as a measure for assessing adiposity and identifying patients at an increased risk of metabolic disease and related adverse health outcomes. However, BMI alone fails to account for body fat distribution across the viscera and the periphery. Body fat distribution (distinctly measured as total body fat, regional body fat or visceral fat) has been shown to explain cardiometabolic risk more effectively than BMI (7). One of the surrogate measures of central/abdominal fat mass is waist circumference (WC). Several studies have demonstrated that, central obesity (excess abdominal fat) associated with high WC is a stronger predictor of CVD than BMI (8-11). Interestingly, it has been shown that individuals having the same level of BMI and total body fat mass tend to have varying risk profiles of cardiometabolic outcomes. Of note, obese individuals with IR and atherogenic dyslipidaemia such as low levels of high density lipoprotein cholesterol (HDL-C), high apolipoprotein B, and high triglycerides and high cholesterol) were characterized by excess abdominal fat mass whereas the “metabolically healthy” counterparts showed low levels of abdominal adipose fat mass (12). It is likely that several other molecular lipid species will be associated with regional body fat distribution and thus aid in explaining the heterogeneity of adiposity phenotypes.

1.3. Insulin resistance

Insulin is a peptide hormone synthesized in pancreatic β cells in the islets of Langerhans within the pancreas. It plays a key role in regulating the blood glucose levels, lipolysis and fat mass (13). The key mechanisms of insulin action include 1) stimulating glucose uptake by skeletal and cardiac muscles, 2) inhibition of hepatic

glucose production and very low-density lipoproteins and 3) inhibition of free fatty acid release. These processes are required for proper cellular function and integrity. IR is a condition in which insulin is unable to trigger cellular glucose uptake leading to hyperglycaemia and abnormalities in glucose homeostasis; this is a functional defect of the cells. Increased circulating levels of insulin (a condition known as hyperinsulinemia) is a common phenomenon that occurs among young adults who are insulin resistant; and this has been shown to be due to increased insulin secretion and minimal clearance (14). The homeostasis model assessment of insulin resistance (HOMA-IR) is an index used to estimate insulin sensitivity and or IR (15).

The aetiology of hyperinsulinemia and IR is complex and largely unknown but is likely mediated by several factors. Dysregulation in lipid metabolism occurs in conjunction with IR and hyperinsulinemia. The key lipid mediators of IR include: free fatty acid, diacylglycerol, ceramide and phospholipids (16-19). Several other lipid classes including acylcarnitine, triacylglycerol and sphingomyelin have also been implicated (20-26). These will be discussed in the subsequent sections. Regardless of its aetiology and evolution, IR is often linked to several pathological conditions and metabolic sequelae. IR typically precedes the clinical onset of diabetes and is a key predictor of type 2 diabetes (4, 27, 28) and CVD (29).

1.4. Type 2 Diabetes

Diabetes is a complex metabolic disorder characterized by a persistently higher levels of blood glucose (hyperglycaemia). The criteria for the diagnosis of diabetes are as follows: fasting blood glucose (FBG) >7.0 mmol/L or two-hour post load glucose (2h-PLG) >=11.1 mmol/L during the oral glucose tolerant test (OGTT) (30). FBG between 5.6 mmol/L to 6.9 mmol/L indicates impaired fasting glucose (IFG), while 2h-PLG during an OGTT of 7.8 mmol/L to 11.0 mmol/L is indicative of impaired glucose tolerance (IGT). IFG and IGT are the “pre-diabetic” states where glucose level does not meet the criteria for diabetes but is too high to be considered normal (31). Hyperglycaemia usually results from defects in insulin secretion by pancreatic β-cells, defects in insulin action or both. Chronic hyperglycaemia in diabetes has been associated with a myriad of comorbidities and dysfunction of multiple organs;

the major adverse outcomes being microvascular and macrovascular complications (32).

T2D is the most prevalent type of diabetes accounting for 87-91% of all diabetes cases among adult population in the industrialized world. It is a significant public health challenge worldwide. Globally, an estimated 500 million prevalent cases of T2D were reported in 2018 (33). The Diabetes Atlas of the International Diabetes Federation reported that in 2017 nearly, 425 million adults aged between 20-79 years live with diabetes and 4 million died from diabetes. An estimated 212.4 million people (nearly 50% of all people) remained undiagnosed and some 352 million had impaired glucose tolerance (IGT). If the current trend continues, about 629 million people (aged 20-79 years), will have diabetes by 2045 (34). The rise in T2D is believed to be mostly due to increases in the prevalence of obesity, rates of urbanization and population ageing.

In Australia, the prevalence of T2D among adult Australians was estimated to be 5% in 2017-18, representing nearly 1.2 million Australians (35). Magliano *et al* conservatively estimated that there will be at least two million adults with T2D by 2025, (36, 37). This compounded with type 1 diabetes (which accounts for 12 cases per 100,000 population) (35) presents an escalating burden on health service resources in Australia.

1.5. Dyslipidaemia

Dyslipidaemia; a condition characterized by increased cholesterol, increased triglycerides, increased low density lipoprotein cholesterol (LDL-C) and decreased HDL-C, is strongly associated with metabolic diseases. It has been suggested that, adipose tissue lipolysis and a subsequent increase in FFA release to circulation ultimately leads to an increase in diacylglycerol, triacylglycerol and ceramide synthesis within the liver. The elevation in these metabolites in turn induces IR (25, 38). Several mechanisms through which lipids induce IR in liver and muscle has been documented (38, 39). In diabetes, dyslipidaemia is often characterized by elevated plasma triglyceride levels commonly referred as hypertriglyceridemia, or

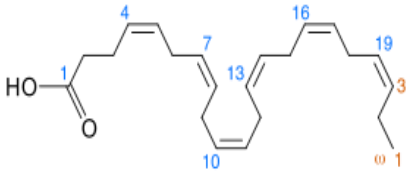
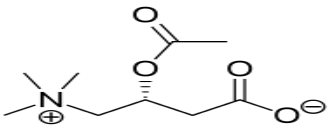
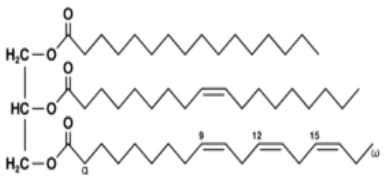
low levels of HDL-C and elevated small dense LDL-C (40). More, importantly dyslipidaemia associated with diabetes independently increases risk for CVD (41).

Peripheral IR associated with T2D is the major cause dyslipidaemia in diabetes. Dyslipidaemia, particularly hypertriglyceridemia in diabetes is mainly due to an increased flux of free fatty acid (FFA) arising primarily from insulin resistant adipocytes (42). Moreover, excessive intake of high calorie foods such as sugars and fats contribute to hypertriglyceridemia and elevated LDL-C levels (43). Excess FFAs that arise from either overconsumption of fats or via hepatic *de novo* lipogenesis (secondary to high carbohydrate intake or as a result of early onset of muscle IR) (44) lead to accumulation of fatty acids in the liver. In the liver, FFAs promote glycerolipid (diacyl- and triacylglycerol) synthesis with subsequent secretion of VLDL-C and apolipoprotein B (ApoB) into circulation. Alternatively, glycerolipids synthesized in the liver can be stored as lipid droplets in hepatocytes promoting hepatosteatosis; which in turn has been associated with T2D (45). Furthermore, sphingolipid and glycerophospholipd synthesis may take place depending on the nature of fats and the degree of excessive fat accumulation in the liver. The dysregulation in sphingolipid and glycerophospholipd pathways in addition to the traditional dyslipidaemia may better explain the pathophysiology of T2D.

1.6. Lipidomics

Lipids are defined as small hydrophobic or amphipathic molecules that arise entirely or partly by carbanion based condensations of thioesters as in fatty acyls, glycolipids, glycerophospholipids, sphingolipids, saccharolipids, and polyketides and/or by carbocation-based condensations of isoprene units such as phenol lipids and sterol lipids (46). Lipids play critical roles in several cellular processes including energy generation and storage, signal transduction, chemical communication and as structural components of cell membrane (47, 48). According to the LIPIDMAPS consortium, lipids fall into eight major categories (**Figure 1**). These include fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides (46). Each of these categories in turn consist of several classes/subclasses and many molecular species. Lipidomics refers to the “global” characterization of all lipid species contained in a cell or tissue (49). The

term “lipidome” signifies the complete set of lipids with in a cell, tissue, or organism. The lipidome is estimated to comprise tens of thousands to hundreds of thousands of lipid species at concentrations ranging from pM to mM in circulation (50). Recent advances and developments in analytical platforms such as mass spectrometry (MS) and high performance liquid chromatography have revolutionized the field of lipidomics (51, 52). These days, lipidomic approaches are gaining popularity for the comprehensive analysis of lipids in relation to metabolic disorders. There are two major strategies used in lipidomics; 1) shotgun lipidomics that involves direct infusion of samples to the mass spectrometer, i.e., without chromatographic separation and 2) chromatographic separation based approaches that employ either high performance liquid chromatography or gas chromatography to separate complex lipid mixture before the sample is subjected to mass analysis. Both approaches can be used for targeted and untargeted work. While shotgun lipidomics is often practiced in untargeted analyses in discovery studies (53, 54), the LC/GC based approaches are more applicable for targeted analyses of known lipids in validation studies.

Lipid category	Lipid class/subclass	Representative structure
Fatty acyls	Fatty acids and conjugates Octadecanoids Eicosanoids Docosanoids Fatty alcohols Fatty esters Fatty amides Fatty nitriles Acylcarnitines	 Docosahexaenoic acid  Acylcarnitine
Glycerolipids	Monoacylglycerols Diacylglycerols Triacylglycerols	 Triacylglycerol

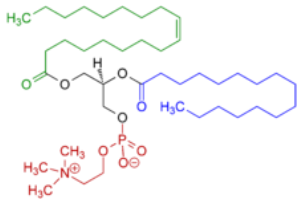
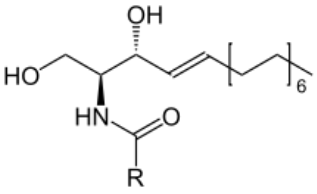
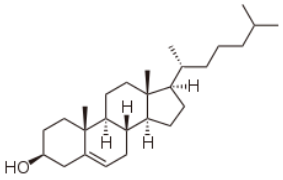
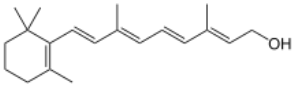
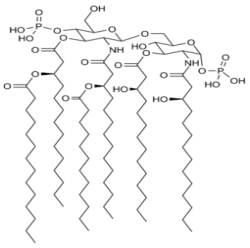
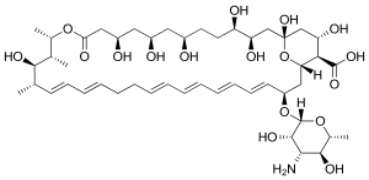
Lipid category	Lipid class/subclass	Representative structure
Glycerophospholipids	Phosphatidylcholines Phosphatidylethanolamindes Glycerophosphoserines Glycerophosphoglycerols Glycerophosphoinositols Glycerophosphates	 Phosphatidylcholine (PC)
Sphingolipids	Sphingoid bases Ceramides Phosphosphinolipids Neutralglycoshingolipids Acidicglycoshingolipids Basicglycoshphingolipids Amphoteric glycosphingolipids	 Ceramide; R – alkyl portion of fatty acid
Sterol lipids	Sterols Steroids Bile acids and its derivatives Secosteroids Steroid conjugates	 Cholesterol
Prenol lipids	Isoprenoids Quinones Polyprenols Hopanoids	 Vitamin A (retinol) – a prenol lipid
Saccharolipids	Acylaminosugars Lipid A Lipid X Lipopolysaccharides	 Lipid A
Polyketides	Linear polyketides Polyenes Polyether antibiotics Aflatoxins	 Nystatin – a polyene

Figure 1. Lipid category/classes and structures. Left column (major lipid categories), middle columns (lipid class/subclasses) and right column (a representative lipid structure within class/subclass).

Lipids are emerging as both early mediators of metabolic dysfunction leading to IR and T2D and as markers of the resulting inflammation and oxidative stress. Recently our understanding of the pathogenesis of IR, obesity and T2D has implicated a number of specific lipids that may prove to be useful biomarkers for the early detection disease onset and the monitoring of therapy (25, 26, 53-57). Understanding the diverse roles of lipids in health and diseases helps to elucidate the mechanisms of lipid related diseases, monitoring therapeutic response, and screening for potential biomarkers. As the most downstream end products of biological information flow, lipids represent the most amendable biological targets that have a potential to be used as predictive, diagnostic, and therapeutic biomarkers. Importantly, lipid metabolising enzymes may act as drug targets (58-60). As a result, modulation of lipid metabolism and or lipid metabolic pathways may confer a therapeutic benefit particularly for those conditions associated with dysregulation in lipid metabolism. For instance, lipid lowering drugs such as statins target cholesterol metabolism and alleviate CVD (61, 62). Now, it is becoming possible that lipid targets other than cholesterol can be identified as our understanding of the role of several molecular lipids in diseases is improving. The following section discusses the different classes/subclasses of lipids and their associations with cardiometabolic risk, IR, obesity and/or T2D.

1.5.1. Free fatty acids (FFAs)

Free fatty acids can be viewed as those that arise from 1) endogenous biosynthesis from acetyl-CoA as an initial substrate ultimately forming palmitoyl-CoA which can then undergo further modifications (63); including desaturation by Stearoyl-CoA desaturase-1 (SCD-1) to form palmitoleate (16:1 n7) or elongation to form stearate (18:0). Subsequent elongation and desaturation processes result in other forms of fatty acids. 2) Dietary polyunsaturated fatty acids (PUFAs) such as the omeg-3 and omega-6 are essential fatty acids required by the body. These class of fatty acids are completely derived from diet, and cannot be endogenously synthesised. Alpha linolenic acid (18:3 n3) and linoleic acid (18:2 n6) are the starting fatty acids that are further desaturated and elongated to form arachidonic acid (AA, 20:4 n6), docosapentaenoic acid (DPA, 22:5 n6), eicosapentaenoic acid (EPA, 20:5 n3) and

docosahexaenoic acid (DHA, 22:6 n3). Although these longer, more polyunsaturated forms are also taken up in the diet.

Higher levels of circulating FFAs have been associated with hypertension in obese and insulin resistant patients (64). For example, a study conducted on children and adults with normal glucose tolerance highlighted that there was an increase in plasma FFA levels associated with lower insulin secretion and higher incidence of IGT and T2D (65) suggesting that, FFAs might have a possible role in impaired pancreatic β-cell function. It is also postulated that flux of FFAs resulting from adipose tissue lipolysis aggravates IR in liver and muscle through altering insulin signalling pathways (66). Moreover, FFAs have been shown to modulate transcription by binding peroxisome proliferator-activated receptors leading to impaired glucose metabolism (24). In addition to their role in insulin signalling and glucose metabolism, FFAs have been also implicated in inflammatory pathways. Sears and Perry (67) investigated various inflammatory responses mediated by FFAs with resultant IR in different organs such as hypothalamus, liver, adipose tissue, skeletal muscle, pancreas and gastrointestinal environment. As a result of their diverse roles in pathophysiology, FFAs could serve as biomarkers of disease. A study that examined the relationship between serum FFAs, IR and cardiovascular risk factors in US, identified FFAs associated with IR starting early in young adulthood. However, the relationship between FFA and cardiovascular risk factors did not become significant until later adulthood (29). This study supports the role of ageing in contributing to metabolic changes including FFA metabolism. It has been also shown that, there is a linear correlation between FFAs and blood glucose and to hepatic glucose in diabetic patients. FFAs induce vascular markers of endothelial activation such as myeloperoxidase and PAI-1, vascular inflammation and thrombosis in obese and T2D patients (68).

1.5.2. Glycerolipids and cholesteryl esters

Glycerolipids of major biological importance include DG and TG. Cholesteryl esters (CEs) represent esterified cholesterol. Elevated levels of plasma TG, DG and CE have been implicated as markers of obesity (69-71), prediabetes and T2D (72-74). Using a high throughput plasma lipidomic technology, Meikle *et al* have reported that

elevated levels of DG and TG are associated with T2D (73, 75). Further, several longitudinal studies have demonstrated the ability of TG species to predict the risk of future T2D (72, 75-77). Interestingly, it has been demonstrated that the fatty acid composition of the triacylglycerol species determines the direction and strength of this association (78, 79). Specific species of plasma TG composed of fewer fatty acyl carbons (≤ 52) and fewer double bonds such as monounsaturated or saturated fatty acids are better markers of IR and increased risk of T2D, whereas TG species containing longer fatty acyl chain and polyunsaturated fatty acids are associated with decreased risk (79).

CE species have been implicated as markers of obesity and T2D; the direction of association depending on specific fatty acid composition. A positive association between serum levels of CE species with saturated or monounsaturated fatty acids such as (CE16:0), CE (16:1), CE (18:0) and obesity and T2D have been reported. Conversely, CE species composed of linoleic acid CE(18:2) have been found to be inversely associated with obesity (80) and with impaired glucose metabolism and T2D (81). This implies that the pathogenesis of obesity and T2D might be driven, to some extent, by altered cholesteryl ester metabolism driven by specific fatty acids.

In lipidomics research involving CVD outcomes, it has been well documented that dysregulation in metabolism of LDL, total cholesterol, TG and HDL underlies the pathogenesis of CVD. However, specific molecular lipid signatures associated with cardiometabolic risk factors and CVD remain largely unknown. A lipidome based approach to CVD risk prediction has begun to show great promise over recent years. Lipidomics enables the measurement of several hundreds to thousands of lipid species in a given sample and hence provides a detailed view of lipid classes, subclasses and individual molecular species. Consequently, advances in MS, provided a new platform for the identification of novel molecular lipid species associated with several disease conditions including CVD. Employing comparative lipidomics, Stegemann et al (82) have demonstrated heterogeneity in the plasma lipidome composition in patients with atherosclerotic lesions; accordingly they have reported combinations of lipid species that discriminate between patients with stable and unstable plaques. In a prospective population-based study, the levels of several

species of cholesteryl ester and triacylglycerol, particularly containing saturated or monounsaturated fatty acyl chains, were reported to be associated with incident CVD (83). Zshir *et al* examined the plasma lipidome using MS and identified several plasma lipid species associated with risk of future CVD and death; of these cholesteryl esters, triacylglycerol and diacylglycerol were the major constituents of the lipidomic profiles (84). For myocardial infarction (MI), multiple triacylglycerol species have been shown to discriminate patients with MI compared to controls (85).

Mechanisms linking dysregulation in lipid metabolism and atherosclerotic CVD have been proposed. As discussed above, CEs represent one of the major lipidomic biomarkers associated with the risk of CVD. CE carrying monounsaturated fatty acids (MUFA) are particularly associated positively with CVD events (83). In mammalian systems, enterocytes or hepatocytes expressing acyl-CoA: cholesterol acyltransferase 2 (ACAT2)/ Sterol O-Acyltransferase 2 (SOAT2) or SOAT1 (86) and plasma Cholesteryl Ester Transfer Protein (CETP) are the main sources of circulation CE. Lecithin–cholesterol acyltransferase (LCAT) is also involved in generating CE via HDL reverse cholesterol transport (87). ACAT 2 preferentially esterifies MUFA generating CEs such as (CE-16:1 & CE-18:1 carried in LDL particles), while CETP generates polyunsaturated CE species which are often carried in HDL particles. It has been suggested that LDL particles enriched with MUFA such as (CE-16:1) enhance atherosclerosis due to high affinity of such LDL variants to arterial proteoglycans (88). Further insight into the relationship between the perturbation of the lipidome and CVD risk in epidemiological studies is required.

1.5.3. Sphingolipids

1.5.3.1. Ceramides and dihydroceramides

A persistent FFA release into circulation induced by IR in obese and or insulin resistant subjects makes saturated FFAs available for the *de novo* synthesis of ceramides. Moreover, there is a strong proinflammatory immune response characterised by elevated IL-6 (89) and TNF-α (90) and decreased adiponectin (91) among other mediators associated with obesity. Inflammatory signals such as TNF-α activate sphingomyelinase and hence promote ceramide production by the turnover of sphingomyelin in metabolic conditions such as T2D (92). There are three potential

pathways that result in the generation of ceramides: the *de novo*, salvage and hydrolytic pathways; these are outlined in **Figure 2** below.

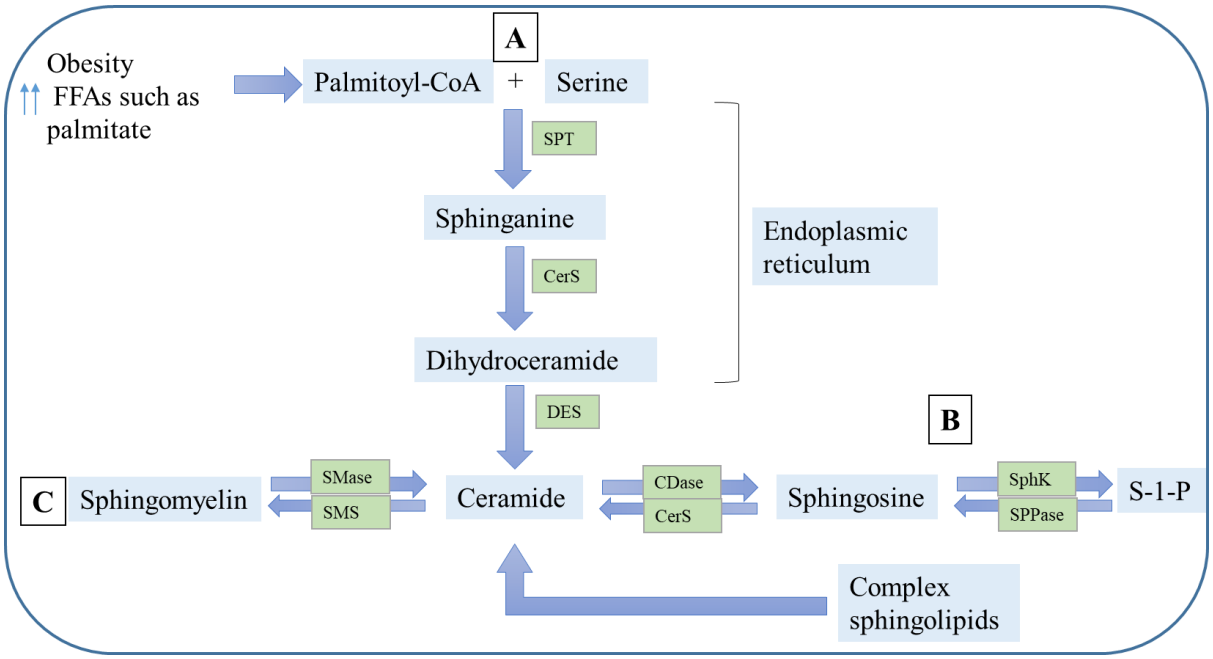


Figure 2. A simplified sphingolipid biosynthetic pathway. A) De novo pathway in which ceramide is synthesized from condensation of serine and palmitoyl-CoA in endoplasmic reticulum. **B)** Salvage pathway. Complex sphingolipids are degraded to ceramide which is then degraded to sphingosine. This can exit the lysosome and be phosphorylated to S-1-P or can be converted back into ceramide. **C)** Hydrolytic pathway takes place in membranes, Golgi, lysosomes and mitochondria. SMase (both acidic and neutral) convert sphingomyelin into ceramide. All substrates and enzymes are represented by light blue and green boxes respectively shown by light blue and enzymes. SMase, sphingomyelinase; SMS, sphingomyelin synthase; CDase, ceramidase; SPPase, sphingosine phosphate phosphatase; SphK, sphingosine kinase.

Ceramides are bioactive lipids that play a role in glucose metabolism, insulin signalling and diabetes. Plasma ceramides are often contained in circulating lipoproteins and are found to be elevated in obesity, IR and T2D (93, 94). Similarly, studies have shown higher levels of ceramide species such as Cer(18:1) or Cer (d18:1/18:1) in skeletal muscle to be associated with T2D (95). Ceramides have been shown to induce apoptosis of pancreatic beta-cells, mainly via inhibition of Akt

phosphorylation (22) (96). Apoptosis of beta cells in the islets is also induced by such cytokines as (TNF-α), interleukin-1 beta (IL-1β) and interferon-gamma (IFN-γ) secreted during inflammatory process (97). Moreover, ceramides impair insulin signalling and action, enhance inflammation (via activation of cytokines) resulting in IR and T2D. On the other hand, inhibition of ceramide synthesis improved insulin signalling and β-cell function (98, 99). The role of individual ceramide species in the pathogenesis of IR and T2D is yet to be fully defined.

Moreover, lipidomic studies targeting selected lipid classes have demonstrated strong associations with CVD risk. Of note, targeted lipidomic profiling has identified distinct serum ceramide species such as Cer(d18:1/16:0), Cer(d18:1/18:0), Cer(d18:1/24:0), and Cer(d18:1/24:1 that can predict major adverse cardiovascular events among apparently health people in a population based cohort in Finland (100). It has been suggested that ceramide molecular species with a specific acyl chain and double bond composition play a role as potential predictive biomarkers of CVD outcomes (101). In a plasma lipidomic profiling epidemiological study aimed at identifying abundant ceramide species, the ratio of Cer(C24:0)/(Cer C16:0) have been shown to be inversely associated with incident coronary heart disease and all-cause mortality (102).

Dihydroceramides are precursors of ceramides that play a major role in insulin signalling the pathogenesis of diabetes. Examining plasma lipids using shotgun lipidomics, researchers have reported dihydroceramide biomarkers that predicted progression to diabetes. A specific set of dihydroceramide species containing long chain fatty acids were significantly elevated in individuals who progressed to diabetes (103). Lipidomic profiling was performed on plasma of Mexican families in the US at high risk for obesity and T2D in the San Antonio Family Heart Study (104) and plasma dihydroceramide levels were shown to be strongly associated with WC independent of several cardiometabolic risk factors (105). Using targeted lipidomics we have also previously shown that plasma dihydroceramide levels predicted T2D (74).

1.5.3.2. Deoxysphingolipids

Deoxysphingolipids are a specific class of lipids that arise from a non-canonical sphingolipid synthetic pathway by the action of serine-palmitoyltransferase (SPT). They are formed from the condensation of palmitoyl-CoA with alanine instead of serine; hence in contrast to canonical sphingolipids, they lack a hydroxyl (OH) group at the C1 position, and so cannot be further modified by nor degraded via the classical sphingolipid pathway (106) (**Figure 3**). The 1-deoxysphingolipids have been shown to cause cell death of neurons and pancreatic β-cells (106). Indeed, the 1-deoxysphingolipids play a role as biomarkers in T2D (107-109). Othman *et al.* reported a significantly higher concentration of 1-deoxysphingolipids in patients with T2D comparted to normal individuals. The 1-deoxysphingolipids also independently predicted the risk of developing T2D over 8 years of follow up (109). During a five year follow up of non-obese, non-diabetic population in the CoLaus study, 1-deoxysphingolipids predicted incident T2D (108), signifying the relevance of these cytotoxic lipids as early biomarkers of T2D.

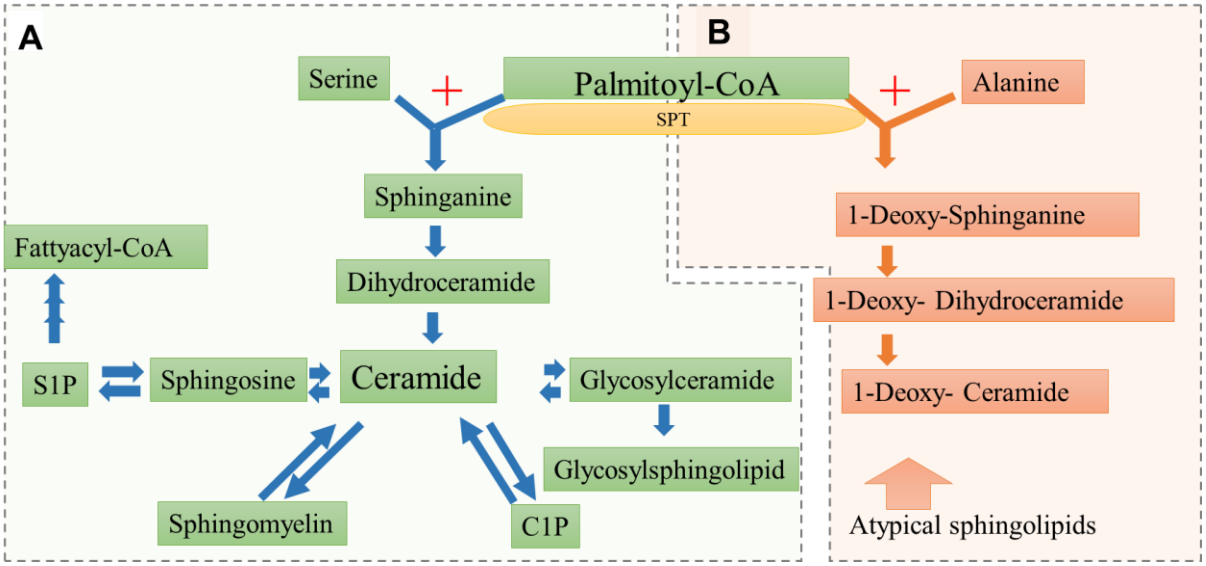


Figure 3. A simplified sphingolipid biosynthetic pathway. A) The usual (canonical pathway) where typical sphingolipids are synthesized. **B)**. Non-canonical pathway in which deoxyceramides are synthesized from condensation of palmitoyl-CoA with the amino acid alanine instead of serine.

1.5.3.3. Sphingomyelin

Sphingomyelin (SM) is the most abundant of all sphingolipids. SMs accounts for ~10% of all lipids in mammalian cells (110) and 18% of total plasma phospholipid

(111). They are important components of cell membrane and lipoproteins, and are enriched in myelin sheaths. The synthesis of SM is catalysed by sphingomyelin synthase; an enzyme that transfers phosphorylcholine from phosphatidylcholine to ceramide resulting in SM and diacylglycerol. Several studies indicate that SMs play a role as markers of cardiometabolic risk and associated outcomes, in particular, specific molecular species of SM, rather than the total SM pool have been shown to be associated with metabolic abnormalities. A study examining the relationship between individual SM species, with obesity IR and dyslipidaemia, has demonstrated that a distinct set of SM species containing saturated acyl chains such as C18:0, C20:0, C22:0 and C24:0) were positively associated with obesity and also strongly correlated with IR and atherogenic parameters (112). In line with this, it was reported in genetically obese mice that, metabolic enzymes promoting ceramide accumulation, such as SPT were significantly upregulated, resulting in elevated levels of SM, ceramides, sphingosine and sphingosine-1-phosphate in plasma (113). In a large population cohort, lipidomic profiling performed by Weir *et al.* identified a significant association of sphingomyelin and ceramide with age, gender and BMI (114). A study in rats has suggested that feeding conditions, gender and age also affect the plasma lipidomic profile (115). In an lipidomic study in healthy white adults which was stratified by age, investigators showed significantly higher levels of sphingomyelin species in females than in males, irrespective of age and matrix type (116). Dysregulation of the sphingomyelin metabolic pathway has been proposed to trigger hypertension in metabolic syndrome (117). SMs have been also implicated in identifying individuals with an increased risk for T2D. Using mass spectrometric analysis, Floegel *et al.* have found that, SM(d18:1/16:1) was associated with low risk of T2D in the European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam study with 800 incident cases of T2D and sub-cohort of 2,282 adults participants followed for 7 years (118). In a similar case-cohort study involving a population of high cardiovascular risk, species of sphingomyelin were found to be inversely associated with the risk of T2D. It is also likely that sphingomyelin metabolism is altered during atherosclerosis development. There is compelling evidence that shows altered SM levels associated with cardiovascular disease and related conditions. A recent human study has revealed higher plasma levels of SM as well as a higher SM to PC ratio among patients with coronary artery disease

(CAD) compared to the control group (119). These findings may suggest that sphingolipid metabolism particularly, the SM biosynthetic pathway would be a potential target for therapeutic intervention.

1.5.3.4. Hexosylceramides and G_{M3} gangliosides

Hexosylceramide (including mono-, di- and trihexosylceramide); precursors of broader glycosphingolipids and complex gangliosides are formed by the initial addition of glucose to ceramide (120). The G_{M3} ganglioside (monosialodi-hexosylganglioside) is a precursor for many complex ganglioside species and is the most studied in settings of metabolic diseases. Hyperinsulinemia, hyperlipidemia and hyperglycemia have been shown to be associated with elevated levels of specific serumm G_{M3} gangliosides; suggesting that G_{M3} ganglioside metabolism is affected by glucose homeostasis and lipid metabolism (121). Patients with T2D were also shown to have higher muscle G_{M3} ganglioside concentrations (122). Marked elevations of G_{M3} gangliosides in visceral tissue of obese women have been reported (123). This suggests that gangliosides may play a role in the pathogenesis of central obesity. Moreover, G_{M3} ganglioside and glucosylceramide impair insulin signaling (23, 124). Consequently, pharmacological interventions targeting G_{M3} gangliosides may pave the way for the treatment of IR and T2D. Of note, the absence of G_{M3} ganglioside synthesis in mutant mice lacking G_{M3} synthase, has resulted in animals with improved insulin sensitivity (125), decreased fasting blood glucose levels and improved glucose tolerance compared to wild-type mice (125). In addition, pharmacological inhibition of glucosylceramide synthase in mice has resulted in markedly reduced glucose levels, improved oral glucose tolerance, reduced glycated haemoglobin, improved insulin sensitivity (126). While there are accumulating data on the role of hexosylceramides and gangliosides in mediating or aggravating IR in animal models, epidemiological studies demonstrating the potential of these complex lipids as markers of cardiometabolic disease are scarce.

1.5.4. Glycerophospholipids

1.5.4.1. Phosphatidylcholines and Phosphatidylethanolamines

Glycerophospholipds are a diverse group of phospholipids. Glycerophospholipds typically contain a three carbon glycerol backbone with two long chain fatty acids and

a phosphatidic acid moiety attached. They make up the major components of biological membranes. Glycerophospholipids having choline and ethanolamine head groups bound to the phosphate group are termed phosphatidylcholine (PC) and phosphatidylethanolamine (PE) respectively. The fatty acid composition of glycerophospholipids varies in the carbon chain length and degree of unsaturation. In humans, the acyl chain lengths typically span between 12 to 26 carbons containing from none to one or more double bonds. *De novo* synthesised or dietary fatty acids provide the fatty acid content of these lipids. PC and PE are the most abundant phospholipids in mammalian membranes. The synthesis is mostly via the cytidine 5´-diphosphate (CDP)-choline pathway, and an analogous (CDP-ethanolamine) pathway. In addition, decarboxylation of phosphatidylserine by phosphatidylserine decarboxylase, a mitochondrial enzyme can result in the synthesis of PE (127, 128).

In several studies it has been documented that glycerophospholipids such as PC or PE are associated with IR and or T2D (129-131). As demonstrated with TGs, it is likely that the fatty acid carbon number and double bond content influence the relationship of PC with cardiometabolic diseases. Consequently, Zhao et al. have identified PC species contain docosahexaenoic acid (DHA) fatty acids such as PC(22:6_20:4) to be inversely associated with the risk of diabetes (130). Plasma lipidomic studies in human subjects have also demonstrated a strong association of PE with obesity (123), prediabetes and T2D (73). The relative abundance of PC and PE phospholipids may also play a role in glucose homeostasis and insulin signalling. One study has clearly demonstrated the distinct role of total PC and the PC to PE ratio (PC:PE); where the total muscle PC was positively associated with insulin sensitivity while the PC: PE ratio was inversely associated (132, 133).

Recently, LC-MS/MS based profiling of serum glycerophosphocholine metabolites has uncovered a novel association of glycerophosphocholine species with multiple CVD risk factors among 990 adolescents. In this population based cohort study, the phosphatidylcholine species PC(16:0/2:0) was shown to be negatively associated with visceral fat, blood pressure and fasting triglycerides. While, the lysophosphatidylcholine species PC(14:1/0:0) was positively associated with visceral

fat, fasting insulin levels, and triglycerides (134). A study conducted by Meikle *et al.* has reported a positive association of PE with T2D (73) and with CAD (135). In a population-based prospective epidemiological study, plasma lipidomic profiling revealed an association of baseline PE levels with increased risk of CVD (136). Dietary consumption of PC has been shown to be associated with increased risk of all cause and CVD mortality among US men and women (137). These studies suggest that the PC and PE phospholipids may either be positively or negatively associated with CVD risk depending on the specific acyl chain composition and double bond content. Moreover, the PC: PE ratio have been indicated as a key determinant in regulating cellular membrane integrity and playing a role in disease progression such as into steatohepatitis (138). The relative distribution of PC and PE across the membrane leaflet varies; outer leaflet having more PC compared to the inner leaflet. In contrast, inner leaflet has a higher PE level. Thus, a low PC: PE ratio presents a stress to membrane integrity by shifting more PE to outer leaflet. This phenomenon may alter membrane permeability to proteins leading to changes in the in downstream biological activities (26). For instance, improved insulin signalling associated with a lower PC:PE ratio has been demonstrated (139).

1.5.4.2. Lysoglycerophospholipids

Lysophosphatidylcholine (LPC) is the most common bioactive lipid belonging to lysoglycerophospholipids. There are several mechanisms in which LPC is generated in pathophysiological conditions; 1) hydrolysis of phosphatidylcholine (PC) by an enzyme called phospholipase A2 (PLA2) on LDL (140) , 2) cleavage of HDL-PC by endothelial lipases (EL) (141) and 3) by the action of lecithin cholesterol acyltransferase (LCAT) on to transfer of an acyl chain from phoshatidylcholine to cholesterol to produce cholesteryl esters in HDL particles (142). Circulating LPC levels has been implicated in the pathophysiology of several metabolic conditions including adiposity, T2D and CVD. An increase in LPC level in plasma associated with proinflammatory conditions and a decrease in ether phospholipids were reported in obese young adults (143). In another study, a reduction in plasma LPC levels were reported in high fat diet induced obesity in mice and humans with obesity and T2D (144). LPC also plays a role in inflammation (145), IR (146) and T2D (147-149). The relationship between LPC and IR and or diabetes remains unknown and

the results of previous studies from animal models and human cohorts are mixed. Of note, Sook Han *et al.* (146) reported LPC to induce FFA mediated IR. Others reported that LPC level is reduced in obesity and T2D (71, 150-152). The number and nature of LPC species measured vary greatly between studies; and diet and adiposity greatly affect LPC levels. This calls for diet-controlled studies in future to improve our understanding of the role of LPC in disease process. Indeed, the involvement of lysophospholipids in myriad pathological conditions as discussed above implies their potential role as biomarkers and therapeutic targets.

Glycerophospholipids whose alkyl chain is linked to s1 position of glycerol via ether bond are termed alkylphospholipids aka ether lipids. A similar class of ether lipids called alkenylphospholipids (plasmalogens) containing a vinyl ether linkage at the sn1 position of glycerol, are of particular interest in settings of metabolic diseases. These lipid classes originate from peroxisomes and the endoplasmic reticulum via a complex synthetic pathway (55). Plasmalogens have been shown to play a protective role in inflammation (153). Using a high throughput plasma lipidomic profiling, Meikle *et al.* (73) have reported an inverse association of alkyl and alkenylphosphatidylcholine species in prediabetes and T2D. A case-cohort study examining 250 incident cases of T2D and 692 controls during 3.8 years of median follow-up period identified lysophospholipids and PC-plasmalogens to be associated with lower risk of T2D in the PREDIMED trial (154). Moreover, a decrease in PE plasmalogen and an increased in lipid peroxidation has been documented in the LDL of patients with T2D (155). Some studies have also indicated a negative association of alkylphosphatidylethanolamine (PE(O)), alkenylphosphatidylethanolamine (PE(P)), alkylphosphatidylcholine (PC(O)) and alkenylphosphatidylcholine (PC(P)) with stable CAD in clinical cohorts (135). Thus, modulation of plasmalogens is likely to confer a therapeutic benefit. In mice studies, plasmalogen supplementation protected from inflammation, oxidative stress and atherosclerosis (156, 157). Many of the unique roles of plasmalogens are related to their vinyl ether bonds that are susceptible to oxidation and so are thought to provide protection to other lipids and proteins against oxidative stress(158). It is also suggested that plasmalogens play a role in cholesterol homeostasis (159, 160) and cell survival (161).

1.5.5. Acylcarnitines

Acylcarnitines are esterified forms of L-carnitine with fatty acids (21). A mitochondrial enzyme; carnitine palmitoyltransferase 1 (CPT 1) catalyses the exchange of the acyl moiety of acyl-CoA with carnitine generating acylcarnitines followed by the translocation of acylcarnitine into the mitochondrial matrix by carnitine acylcarnitine translocase (CACT) (**Figure 4**) (21). Acylcarnitines are therefore, required to transport long chain fatty acyl-CoA across mitochondrial membrane for subsequent β-oxidation Dysregulation in acylcarnitine metabolism; leading to the accumulation of these lipids reflects a defect in mitochondrial function associated with disease conditions such as IR (21). Evidence from human studies have also shown a positive association of acylcarnitine species with prediabetes (162) and T2D (56, 163, 164). Acylcarnitines have emerged as important predictive biomarkers for T2D.It has been shown that acylcarnitines improved the early prediction of T2D based on baseline plasma levels in population-based studies (56, 165). In the diabetic state, mitochondrial function is impaired (166) and this may be linked to incomplete fatty acid oxidation (FAO) leading to the accumulation of the acylcarnitine. A study in mice fed with 4-thia fatty acid tetradecylthiopropionic acid have demonstrated a reduction in β-oxidation associated with hepatic mitochondrial function, and increased acylcarnitine levels (167). Moreover, acylcarnitine can be proinflammatory via the induction of cyclooxygenase-2 (20).

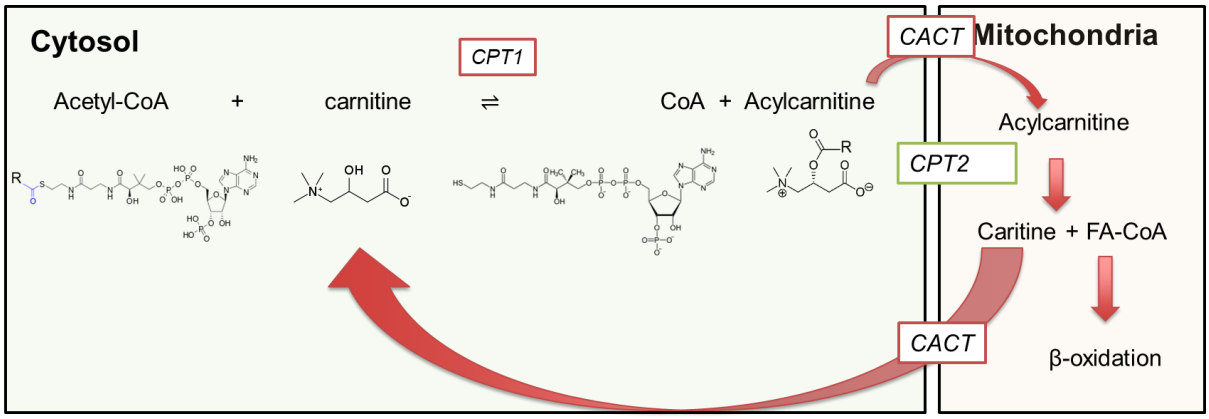


Figure 4. Acylcarnitine metabolism. CPT1 exchanges the CoA moiety for carnitine resulting in the formation of acylcarnitine (AC) in mammalian cells. Acylcarnitine is then transported into the mitochondrion by CACT. Inside, the mitochondrion, CPT2 breaks down acylcarnitine back to free carnitine and acyl-CoA which goes to β-

oxidation. The free carnitine can be transported back via mitochondrial membrane to cytosol to repeat the cycle.

1.7. Mass spectrometry and plasma lipidomics

1.7.1. The human plasma lipidome

Plasma is one of the most commonly evaluated biological materials in lipidomic studies. The plasma lipid species we measure using lipidomic approaches are primarily derived from circulating lipoprotein particles **(Figure 4).** The lipoprotein structure is a complex assembly with the most outer layer composed of apolipoproteins together with various phospholipids, sphingolipids and un-esterified cholesterol. The inner core is primarily composed of hydrophobic and neutral lipids such as triacylglycerol, diacylglycerol and cholesteryl esters **(Figure 5).** Conventionally, the lipoprotein particle as a whole has been viewed as either HDL-C "a good cholesterol" or LDL-a "bad cholesterol". However, detailed characterization based on size, density and lipid to protein ratio shows that there are several classes and subclasses of lipoproteins. These include but not limited to very low-density lipoprotein (VLDL) and intermediate-density lipoprotein (IDL). Each of these classes in turn encompass several subclasses based on size (e.g. LDL particle ranges from very large to small), and the VLDL ranges from very large to small VLDL particles (168, 169).

Conventional measures of HDL-C or triglycerides obtained as part of clinical lipid panel only reflect the sum of these lipids carried across lipoproteins. Further insight into the particle size and subclass concentration of lipoproteins is relevant in distinguishing various subtypes of cardiovascular disease (170). Yet, the lipoprotein class/subclass analysis, does not take into account the composition such as sphingolipids and phospholipids and hence fail to explain the complexity of the altered lipid metabolism associated cardiovascular risk. Several plasma lipidomic studies have shown association of molecular lipids with T2D and cardiovascular diseases independent of lipoprotein profile (83, 84, 171). The individual lipid profile of each lipoprotein particle is substantially different and therefore, a more detailed characterization of the association of lipid classes, subclasses and individual lipid species with cardiometabolic risk factors and outcomes could be more informative.

The introduction of analytical platforms such as the LC-MS/MS has made it possible to measure several hundreds to thousands of lipid species in clinical samples. A brief introduction on the LC-MS/MS methodology for lipidomic analysis is discussed in the subsequent section.

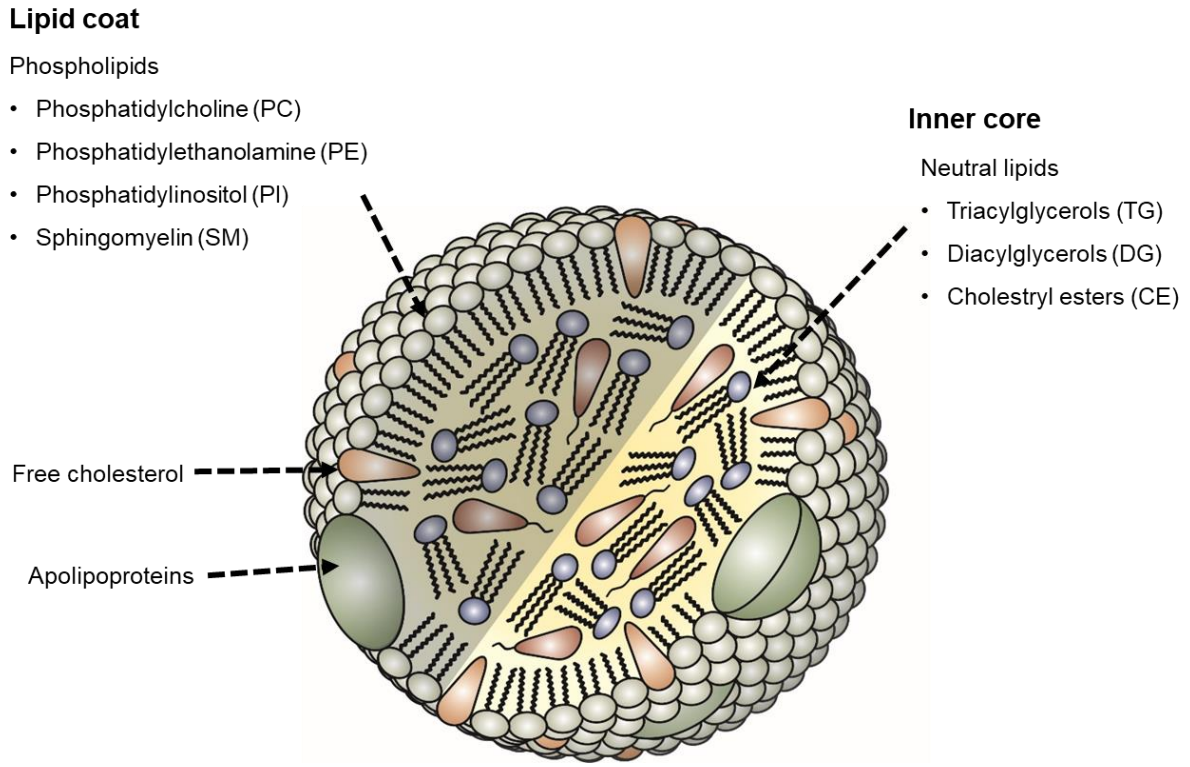


Figure 5. Lipoprotein structure. The surface coat of lipoprotein is composed of a mainly phospholipid monolayer. Phosphatidylcholine, phosphatidylethanolamine and sphingomyelin are the predominant phospholipids that make up the phospholipid monolayer of plasma lipoprotein particles while the inner core is essentially composed of neutral/non-polar lipids such as cholesteryl ester, triacylglycerol and diacylglycerol species.

1.7.2. LC-MS/MS based lipidomics

Analytical technologies such as MS coupled with complex chromatographic separation techniques (51, 172, 173), nuclear magnetic resonance (NMR) (174, 175), fluorescence spectroscopy (176) and microfluidic platforms (177) have been used in lipidomics in order to detect, identify and quantify lipids. Amongst, these analytical platforms, MS is widely used in lipidomic studies. Mass spectrometric based approaches primarily measure the mass of a given molecular lipid species

and produce information in the form of mass spectra; the intensity of which is directly related to the concentration of analyte present in a given sample.

The ultimate goal of lipidomics is to fully characterize, identify and quantify the molecular lipid species in a sample. Powerful analytical technologies are often required to characterize such diverse molecular lipid species. There are two main lipidomic strategies to effect this; “untargeted lipidomics” where the aim is to provide relative measurements of a wide range of lipids including unknown species for the purpose of identifying new biomarkers and “targeted lipidomics” in which a specific known set of lipids are quantified, typically using internal standards of some sort (54). Untargeted analyses involve the global examination of all detectable lipid species (both known and unknown) and are mainly meant for hypothesis generation and qualitative studies. In contrast, targeted lipidomics is aimed at identifying and quantifying known list of metabolites. Each of these approaches presents with its own advantages and disadvantages. Here, all steps involved in targeted lipidomic profiling are discussed in detail.

1.7.2.1. Lipid extraction and liquid chromatography

A typical LC-MS/MS analysis starts with lipid extraction from a given sample such as plasma (**Figure 6**). A single phase 1:1 (v/v) BUME extraction method is one of the popular techniques shown to be efficient to recover a wide range of polar and non-polar lipid species (178). This method is highly reproducible, less time consuming than its counterparts and thus is better suited for high throughput lipidomic analysis of large population cohorts. The singe phase BUME method is also suitable for automated lipid extraction (178). Several lipid extraction methods have been devised in the past; the ‘Folch’ method being the most popular classical approach (179). The ‘Folch’ method and its modified versions such as Bligh-Dyer (180) and acidic ‘Folch’ method (181) are based on bi-phasic liquid-liquid extraction using mixture of chloroform: methanol. These methods are less safe, time consuming and less reproducible compared to the butanol-methanol (BUME) method. Moreover, in the classical methods, lipid partition occurs between the phases or sit at the interface and therefore, this leads to poor and irreproducible recoveries.

Following extraction, the lipid extract is subjected to liquid chromatography (LC) system; such as ultra-performance liquid chromatography (UPLC). In HPLC reverse phase columns facilitate separation of individual lipids based on their hydrophobic interaction with a stationary and mobile phases. Recent developments in this area make use of column matrices of smaller particle size and higher operating pressure, and thereby provide superior resolving power and faster chromatography. The level of hydrophobicity of lipid species is influenced by the fatty acid chain length and degree of unsaturation; and hence enable us to separate molecules that vary in their acyl chain length and unsaturation (182).

Chromatographically separated metabolites need to be exposed to an ion source as MS essentially detects molecules once they are ionized. There are several techniques of ionization; a mechanism where gaseous phase ions/charged lipid molecules are generated (183). Electrospray ionization (ESI) techniques is the most commonly used interface to ionize molecules before they are subjected to MS (183). As the working condition of LC system (the liquid environment) and MS (the vacuum system) are incompatible, an interface is required to transfer the separated components from the LC column into the MS ion source. Moreover, the interface is required to remove a significant portion of the mobile phase used in LC while preserving the chemical identity of the chromatographic products. The advent of soft ionization techniques such as ESI and coupling this technique with tandem MS has revolutionized LC-MS based lipidomics.

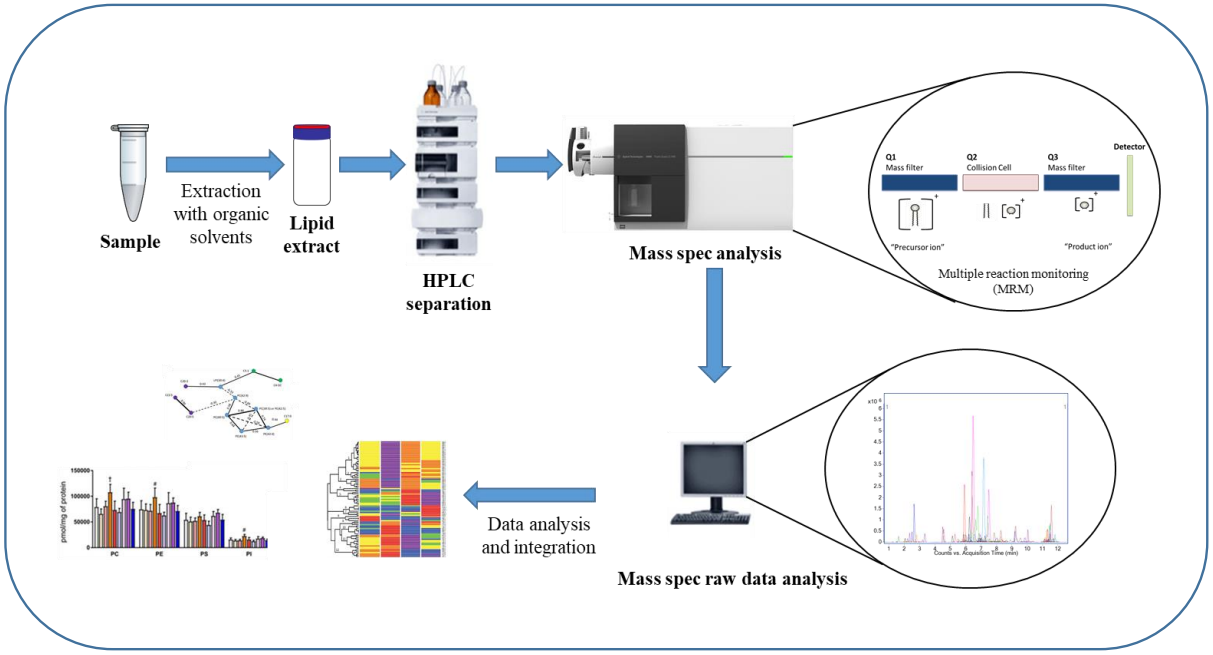


Figure 6. Overview of LC-MS based targeted lipidomics workflow. Lipid is extracted using organic solvents such as 1:1 (v/v) BUME from a complex biological matrix (e.g. human plasma). The crude lipid extract (a complex mixture of all lipids present in the sample) is separated using liquid chromatography (HPLC systems) and then subjected to MS for further analysis and detection. Identification and quantification of lipids is made using integration software, typically provided by the instrument vendor. Computational biology and statistical tools are applied to identify associations with disease outcomes, determine significant correlations, identify and validate potential biomarkers, and to integrate these with existing biological knowledge.

1.7.2.2. Targeted lipidomic analysis using LC-MS/MS

Targeted LC-MS/MS lipidomics combines liquid chromatographic separation with MRM experiments on a known set of lipids. It makes use of commercially available stable isotopes or non-physiological internal standards to quantify lipids with a known fragmentation pattern (184-186). In tandem MS like triple quadrupole (QQQ) instruments, scan modes such as the product ion scan, precursor ion scan and neutral ion loss are employed to identify lipids. Such MS instruments consist of two quadrupole mass analyzers (in Q1 and Q3) for sequential mass filtration and non-mass-resolving collision cell (Q2) between them. Product ion scan is used to analyse the masses of the fragments generated from a selected parent ion of interest from

collision-induced dissociation in quadrupole 2 (Q2). Whereas, precursor ion scanning is used to analyse the mass-to-charge ratio (*m/z*) of precursor ions that generate a selected daughter ion of interest. Similarly, neutral loss scan, is where the second mass analyser (Q3) detects daughter ions that are a specified mass smaller than the parent ion scanned in Q1 (**Figure 7**)

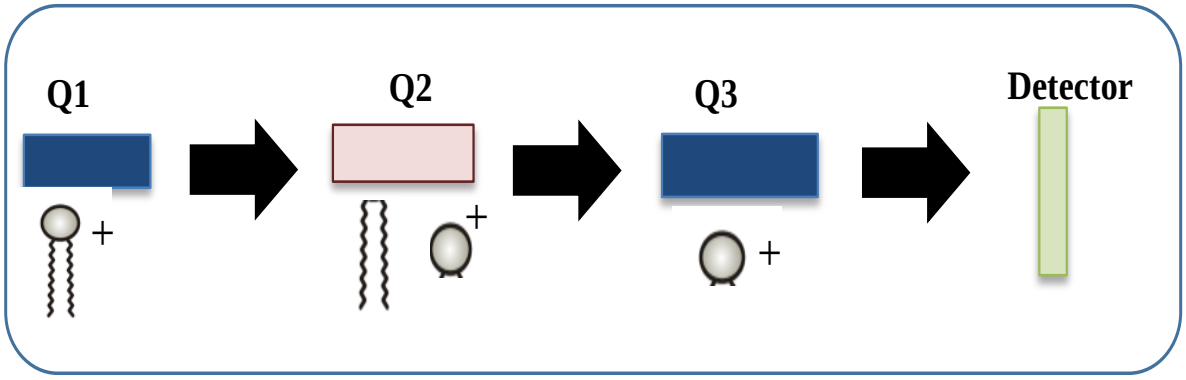


Figure 7. Analysis of lipids on QQQ MS; overview of MRM experiment. In the first mass analyser (Q1) a single *m/z* for the parent ion of interest is selected. The subsequent quadrupole (Q2) is essentially a collision cell where the parent ion of interest undergoes collision-induced dissociation (CID) resulting in fragmentation of the molecule. The 2nd mass filter/analyser (Q3) a single *m/z* of the selected daughter ion is monitored, and a signal is detected by the mass detector. In a typical QQQ mass spectrometer, several scan modes exist, e.g. a precursor ion scan involves scanning structurally similar ions (in Q1) with a common fragment ion selected in Q3. In a product ion scan mode, a selected m/z in Q1 is scanned for fragmentation patterns in Q3. In a MRM experiment, ion masses are fixed in the Q1 and Q3 for a sensitive detection of a targeted panel of analytes.

In the Metabolomics Laboratory at the Baker Institute, we employ LC-ESI-MS/MS with MRM experiments to identify and characterise over 700 individual lipid species belonging to 36 classes/subclasses in human plasma samples using a 15 minute analysis time (a single run in a positive ionization mode). This comprehensive targeted list of lipids was achieved iteratively over many years of development work in the Metabolomics Laboratory. We choose targeted lipidomics over untargeted lipidomics as our aim was to measure and quantify defined set of lipids and utilize these for assessment of cardiometabolic risk. We have performed lipid identification at least to the bond type level such as whether a given lipid species has an acyl,

alkyl or alkenyl linkage using different scan modes (84, 114) and subsequently rely on retention time coupled with MRM analysis to quantify these species. In the most recent methodology, chromatographic separation of the sn1 and sn2 isomers of glycerophospholipids as well as the n-3 and n-6 fatty acid containing lipids was demonstrated (187). In this methodology, information on fatty acid composition of each lipid species, i.e, the fatty acid chain length and or number of double bonds present in each fatty acyl chain can also be obtained, e.g. diacylglycerol (DG) 16:1/18:1, sphingomyelin (SM) SM(d18:2/14:0). Triacylglycerol (TG) species were measured as both the neutral loss (NL) of specific fatty acids and as the sum composition of all species of a specific *m/z* using single ion monitoring (SIM). Such NL measurements reveal the structural diversity in TG species and provide a comparative quantification of the different fatty acyl species. While the SIM measurements provide a more accurate absolute quantification of TG species.

Despite the level of characterisation of lipid species possible in current lipidomic research, there remain a number of analytical challenges (52). For instance, an in-depth structural characterization, such as to identify lipid species that differ, for e.g. in the double bond position of fatty acyl chain (e.g. 9Z or 11Z) is possible but not practical in a high throughput quantitative assay. Determining the precise structural composition all lipid species in a given cell, organ or bio-fluid remains a key limitation in lipidomic studies. This is because, the lipid extract by itself contains an extremely complex mixture of individual molecular lipid species with huge structural diversity. Moreover, accurate quantification of each lipid species in a given biological matrix presents a significant challenge, partly due to lack of stable isotope standards for each lipid species and that the concentrations of each lipid species ranges across multiple orders of magnitude. Furthermore, sample extraction conditions, ionization efficiencies, and the detector responses affect ion abundances, and hence the precise quantitative determination of individual lipid species.

Further to the analytical challenges, there are also issues related to the presentation and interpretation of lipidomic data. Lipid profile is affected not only by biological factors (such as age, gender, ethnicity, microbiome, diet and life style) (188-190) but also by other factors such as sample handling (sample collection techniques, freeze-

thaw cycle, storage time, temperature fluctuations etc.). Metabolomics data is also prone to unwanted variations due to batch effects and long run time with in each batch for large datasets. Thus, the interpretation of lipidomic data and its associations with outcomes needs to consider the aforementioned factors. To achieve biologically sound associations, some of the challenges such as unwanted variations in lipidomic data could be overcome by normalization approaches such as median centring using QC samples. In large population cohorts where the lipidomic analysis is run in many different batches, the batch effects can be corrected using a median centring approach utilising PQC (191). Many lipid species comprising the lipidome are correlated each other, hence the issue of collinearity presents a challenge in statistical modelling. To handle the collinearity problem, models such as the ridge regression could be utilized. With the use of linear models such as the ridge regression we have shown the potential of lipidomic data to generate metabolic risk scores that help assess cardiometabolic risk.

Despite the above limitations, quantification of lipids at class/subclass or species level as well as the possibility of identifying individual lipid species containing different fatty acids (that vary in acyl chain length and saturation level) by LC-ESI-MS/MS allows for a comprehensive understanding of lipid metabolism. Advances in analytical technologies over the past few decades have enabled detailed profiling of the individual lipid species present in the lipidome of a given biological system and also quantitative determination of known lipids (173, 187, 192). In addition, the possible elucidation of their biological roles and interactions with other metabolites is also explored with this technology (193, 194). Importantly, lipidomic profiling can facilitate our understanding of the comprehensive biochemical mechanisms underlying lipid metabolism and lipid associated diseases including obesity, T2D and CVD. Lipidomics plays an important role in discovery of lipid biomarkers for disease prediction, diagnosis, prognostic monitoring and new drug targets for therapeutic interventions (57). Furthermore, the lipidomic data has a potential to generate metabolic risk scores that help assess cardiometabolic risk.

1.8. Clinical significance of lipidomics

Dyslipidaemia (the raised total cholesterol, plasma triglycerides and LDL-cholesterol, and decreased HDL-cholesterol) is an independent risk factor for metabolic diseases. However, there is a compelling evidence that these 'classical' lipid measures alone do not adequately explain the complex dysregulation of lipid metabolism associated cardiometabolic risk factors, nor do they represent the structurally and functionally diverse lipid species associated with the pathogenesis of IR, obesity or T2D. As a consequence, these markers alone do not provide the full spectrum of metabolic effects on lipid metabolism and do not suffice as diagnostic, prognostic and predictive biomarkers.

Currently, comprehensive plasma lipidomic profiling on large population cohorts using robust analytical instrumentation is limited. There are relatively few studies that utilize high throughput lipidomic analysis but these studies highlighted the significance of such an approach for the identification of candidate lipidomic biomarkers in relation to cardiometabolic risk factors, IR and diabetes. Accumulating evidence also shows a strong relationship of plasma lipid species with prediabetes and T2D (73). The potential of a lipidomic risk score to predict T2D (74) and improve upon traditional risk factors to classify disease risk has also been documented (75, 195, 196). Most of the existing data is however, based on smaller sample sizes, models with a limited number of lipid species, and are derived from cross-sectional studies or studies with short follow up.

In this thesis, comprehensive plasma lipidomic analysis using the LC-MS/MS methodology was performed. Three independent human cohorts were utilised to define the relationship of lipid metabolism with cardiometabolic risk factors and outcomes; 1) a cohort of healthy young adults, 2) the Australian Diabetes, Obesity and Lifestyle Study (AusDiab) and 3) the Busselton Health Study cohort (BHS). These data were systematically analysed to define the relationship between plasma lipid classes/subclasses and species and different cardiometabolic traits and outcomes.

1.9. Hypothesis

The central hypotheses of this project is that the comprehensive examination of the human plasma lipidome is useful to identify new markers associated with cardiometabolic risk factors and outcomes.

The specific hypotheses include:-

- The human lipidomic profile is different between men and women and hence there is sex interaction in the association of lipidome with cardiometabolic risk factors.
- The lipidome is perturbed in hyperinsulinemia and insulin resistance among young adults with no known diabetes.
- Lipidomic profiling of incident obesity will provide insight into the metabolic drivers and consequences of obesity.
- Lipidomic data has the potential to provide metabolic measures of obesity that will be better a predictor of disease risk.
- Changes in the plasma lipid profile precede the clinical onset of T2D and will be useful biomarkers for risk assessment and early diagnosis of type 2 diabetes and prediabetes.

1.10. Aims

1. To perform a comprehensive plasma lipidomic analysis of a large population cohort to identify plasma lipid species associated with cardiometabolic risk factors including age, sex and BMI and health outcomes.
2. To examine the relationship between the plasma lipidome and IR in a cohort of healthy young individuals.
3. To identify baseline metabolic phenotype associated with future change in WC and BMI.
4. To construct a metabolic BMI score and examine its association with metabolic disease risk.
5. To examine the relationship of plasma lipidome with prediabetes and T2D. To determine whether lipids can improve upon existing markers to predict the risk of type 2 diabetes.

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Chapter 2. General Methods

The general methods used in this thesis are described below. The detailed description of methods relating to specific chapters and the statistical analysis can be found in each chapter.

Three independent human cohorts have been studied in this project: 1) the Australian Diabetes, Obesity and Lifestyle Study (AusDiab cohort) (chapters 3, 5, 6 and 7); 2) a cohort of young adults recruited in Melbourne (chapter 4) and 3) the Busselton Health Study cohort (BHS) (chapters 3 and 6). The general methodology for lipidomic profiling of each cohort remained the same although, the number of lipid species being measured varied in each cohort.

1.11. Lipid extraction

We extracted lipids in human plasma using a standard butanol-methanol method as described previously (1). The extraction process (including pipetting) was assisted by the liquid handling robot (MicroLAB STAR, Hamilton, Biosystems, Inc). Briefly, 10µL of plasma was collected by the machine and mixed with 100µL with 1-butanol and methanol (1:1 v/v) containing the relevant internal standard mix and 5mM ammonium formate (Chapter 3, Table 1). The resulting mixture was thoroughly vortexed for about 10 seconds before sonicated for 60 min at 25^{o}C in a sonic water bath and centrifuged at 16,000xg for 10 minutes maintain the temperature at 20^{o}C. The supernatant containing lipids was finally transferred to 0.2ml glass tubes with Teflon inserts. The extract was screw capped and stored at -80^{o}C.

Blanks (10 µl MilliQ water per each extraction with or without internal standards) and and two different types QCs (plasma quality control (PQC) and samples from the national institute of standards technology (NIST 1950 reference plasma samples), were included in the extraction process to monitor the quality of lipid extraction process. Technical quality control samples (TQCs) were included with the patient samples during the run in order to assess the performance of liquid chromatography-mass spectrometry analysis. Below is a simplified illustration of the robot assisted lipid extraction **(Figure 2.1).**

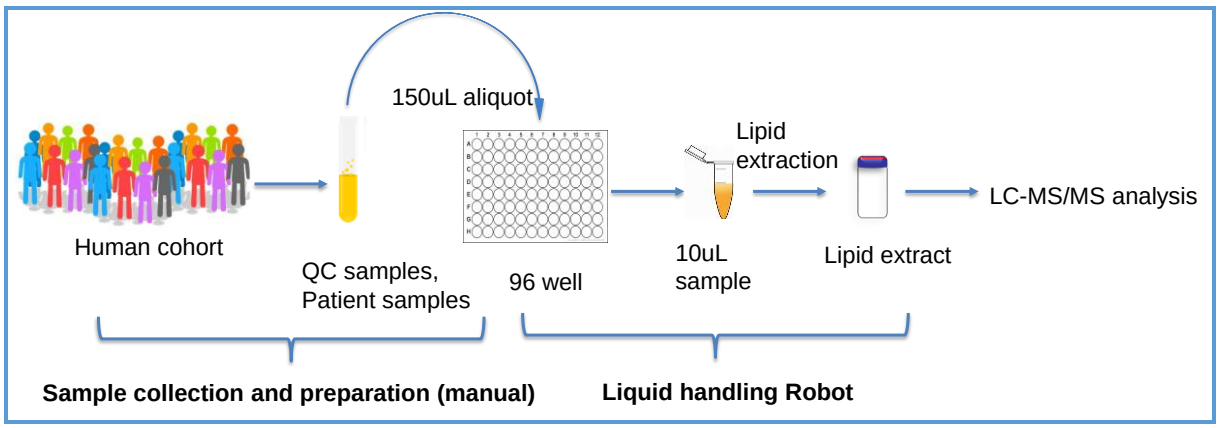

Figure 2.1. A simplified illustration of the robot assisted lipid extraction and mass analysis (applied for each cohort).

1.12. Liquid chromatography mass spectrometry (LC-MS/MS).

Prior to analysis, the complex mixture of lipids in the crude lipid extract was subjected to a high performance liquid chromatography (HPLC) (Agilent 1290 series HPLC system and a ZORBAX eclipse plus C18 column (2.1x100mm 1.8µm) to separate individual lipid species. Tandem mass spectrometry (MS/MS) (Agilent 6490 triple quadrupole (QQQ)) mass spectrometer with electrospray ionization interphase was utilized to analyse lipid species using positive ion mode. The mobile phase included solvent A and solvent B. Solvent A composed of 50% water, 30%, acetonitrile, 20% isopropanol (v/v/v) and solvent B with 1% water, 9% acetonitrile, 90% isopropanol (v/v/v) each containing 10mM ammonium formate were used applying a linear gradient over 14-minute run time per each sample **(Figure 2.2).** A 1µL of sample was injected to MS.

The chromatographic gradient starts with 10% B solvent at a flow rate of 0.4 mL/min linearly increasing to 45% over 2.7 min, then to 53% for 0.1 min, to 65% for 6.2 min, to 89% over 0.1 min, to 92% over 1.9 min and finally to 100% over 0.1 min before holding at 100% for 0.8 min. Equilibration was then achieved by decreasing 100% B to 10% B over 0.1 min and then held for an 0.9 min. Finally, the flow rate was switched to 0.6 ml/min for 1 min followed by holding to 0.4 mL/min over 0.1 min. The MS column temperature was set to a thermostat of 60°C. The following MS condition was used; gas temperature, 150oC, flow rate 17 L/min, nebulizer 20psi, sheath gas temperature 200oC and flow rate, 10L/min and capillary voltage of 3500V. The

detailed mass spectrometry settings and MRM transitions can be found elsewhere (2).

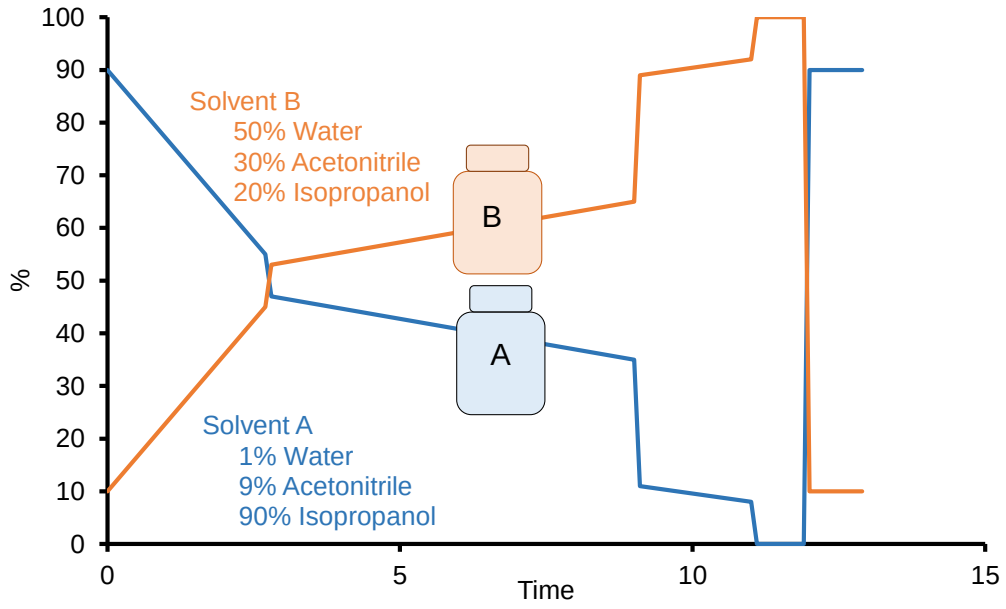

Figure 2.2. The solvent system and chromatography gradient profile for LC-MS/MS analysis.

1.13. Lipid quantification, batch correction and outlier detection

The chromatographic peaks were integrated using the Mass Hunter (B.07.00, Agilent Technologies) software. Each peak was assigned to the corresponding species it stands for based on MRM (precursor/product) ion pairs and retention time. The relative concentration of each lipid species was determined by comparing the peak areas to relevant internal standard (Chapter 3, Table 1). Response factors generated previously in same lab were applied for each of cholesteryl ester species to estimate their true concentrations (3). Batch correction was achieved using median centring for PQC samples, as explained in detail elsewhere (4).

Principal component analysis (PCA) and scatter plots of all samples were used to visualize the possible outliers. Each sample that falls outside the 99-percentile eclipse of the PCA plot were manually checked whether the peak peaking was correct, or whether the vials contain the right amount of extract and so on. For few samples where incorrect peak integration was detected, peak peaking was redone. Samples suspected of having technical problems including missed injection (associated with no peak or signal intensity as background), faulty sample volume,

empty vials were excluded. All other samples were real after checking and are included in the downstream analysis.

1.14. Statistical analysis

Statistical analyses were performed using R software (version 3.6.1), Excel, Graph Pad Prism 8 or STATA™ v14 (StataCorp LP, Inc., Texas, USA) as necessary. The lipidomic dataset was log10 transformed prior to statistical analysis and scaled to SD where necessary. The associations between lipids and cardiometabolic traits and outcomes were determined using linear regression, logistic regression, or cox regression adjusting for appropriate covariates. To identify associations independent of lipoprotein profile, models were adjusted for total cholesterol, HDL-C and triglycerides on top age, sex and BMI. The β-coefficients from linear regression analyses and 95% confidence intervals were converted to percentage differences (percentage difference = (10^β-coefficient – 1) x 100) where necessary. P-values were corrected for multiple comparisons using the Benjamini and Hochberg procedure (5). Linear models with regularization such as ridge, lasso and elastic net were employed to model BMI and the ridge regression to predict incident type 2 diabetes.

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Chapter 3. High-coverage plasma lipidomics reveals novel sex-specific lipidomic fingerprints of age and BMI: Evidence from two large population cohort studies

Preface

We sought to comprehensively analyse the human plasma lipidome (measuring over 700 lipid species) in large population cohorts to define the relationship of lipid metabolism with common anthropometric risk factors. This chapter investigated the complex effects of age and sex on lipid metabolism in a population setting. Utilizing two independent large population cohorts, we determined novel age and sex-specific lipidomic fingerprints of obesity. This chapter was also aimed at mapping enzyme pathways perturbed with disease risk by utilizing a specific lipid ratios as a proxy for enzyme activity.

Chapter 3 has been published in Plos Biology

To facilitate a better flow between chapters within this thesis, the Supplementary Tables for this chapter have been moved to the Appendix I.

PLOS BIOLOGY

RESEARCH ARTICLE

High-coverage plasma lipidomics reveals novel sex-specific lipidomic fingerprints of age and BMI: Evidence from two large population cohort studies

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Data Availability Statement: Because of the participant consent obtained as part of the recruitment process for the Australian Diabetes, Obesity and Lifestyle Study, it is not possible to make these data publicly available. Individual-level data will be made available upon reasonable written request to the study lead Professor Jonathan Shaw and the AusDiab Study Committee (Email: Jonathan.Shaw@baker.edu.au).

Abstract

Obesity and related metabolic diseases show clear sex-related differences. The growing burden of these diseases calls for better understanding of the age- and sex-related metabolic consequences. High-throughput lipidomic analyses of population-based cohorts offer an opportunity to identify disease-risk–associated biomarkers and to improve our understanding of lipid metabolism and biology at a population level. Here, we comprehensively examined the relationship between lipid classes/subclasses and molecular species with age, sex, and body mass index (BMI). Furthermore, we evaluated sex specificity in the association of the plasma lipidome with age and BMI. Some 747 targeted lipid measures, representing 706 molecular lipid species across 36 classes/subclasses, were measured using a high-performance liquid chromatography coupled mass spectrometer on a total of 10,339 participants from the Australian Diabetes, Obesity and Lifestyle Study (AusDiab), with 563 lipid species being validated externally on 4,207 participants of the Busselton Health Study (BHS). Heat maps were constructed to visualise the relative differences in lipidomic profile between men and women. Multivariable linear regression analyses, including sex-interaction terms, were performed to assess the associations of lipid species with cardiometabolic phenotypes. Associations with age and sex were found for 472 (66.9%) and 583 (82.6%) lipid species, respectively. We further demonstrated that age-associated lipidomic fingerprints differed by sex. Specific classes of ether-phospholipids and lysophospholipids (calculated as the sum composition of the species within the class) were inversely associated with age in men only. In analyses with women alone, higher triacylglycerol and lower lysoalkylphosphatidylcholine species were observed among postmenopausal women compared

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Competing interests: The authors have declared that no competing interests exist.

Abbreviations: ApoB, apolipoprotein B; AusDiab, Australian Diabetes, Obesity and Lifestyle Study; BHS, Busselton Health Study; BMI, body mass index; CE, cholesteryl ester; Cer, ceramide; Cer-1-P, ceramide-1-phosphate; CV, coefficient of variation; CVD, cardiovascular disease; DE, dehydrocholesterol; deoxyCer, deoxyceramide; DG, diacylglycerol; DHA, docosahexaenoic acid; dhCer, dihydroceramide; DPA, docosapentaenoic acid; ELOVL, elongation of very long chain fatty acids protein; EPA, eicosapentaenoic acid; FADS3, fatty acid desaturase 3; FDR, false discovery rate; GM1, G_{M1} ganglioside; GM3, G_{M3} ganglioside; HDL-C, high-density lipoprotein cholesterol; HexCer, monohexosylceramide; Hex2Cer, dihexosylceramide; Hex3Cer, trihexosylceramide; HRT, hormone replacement treatment; LCAT, lecithin cholesterol acyltransferase; LC-ESI-MS/MS, LC electrospray ionisation MS/MS; LC-MS/MS, liquid chromatography tandem mass-spectrometry; LDL-C, low-density lipoprotein cholesterol; LPC, lysophosphatidylcholine; LPC(O), lysoalkylphosphatidylcholine; LPC(P), lysoalkenylphosphatidylcholine; LPE, lysophosphatidylethanolamine; LPE(P), lysoalkenylphosphatidylethanolamine; LPI, lysophosphatidylinositol; NIST, National Institute of Standards and Technology; NL, neutral loss; PC, phosphatidylcholine; PC(O), alkylphosphatidylcholine; PC(P), alkenylphosphatidylcholine; PE, phosphatidylethanolamine; PE(O),

with premenopausal women. We also identified sex-specific associations of lipid species with obesity. Lysophospholipids were negatively associated with BMI in both sexes (with a larger effect size in men), whilst acylcarnitine species showed opposing associations based on sex (positive association in women and negative association in men). Finally, by utilising specific lipid ratios as a proxy for enzymatic activity, we identified stearoyl CoA desaturase (SCD-1), fatty acid desaturase 3 (FADS3), and plasmanylethanolamine Δ1-desaturase activities, as well as the sphingolipid metabolic pathway, as constituent perturbations of cardiometabolic phenotypes. Our analyses elucidate the effect of age and sex on lipid metabolism by offering a comprehensive view of the lipidomic profiles associated with common cardiometabolic risk factors. These findings have implications for age- and sex-dependent lipid metabolism in health and disease and suggest the need for sex stratification during lipid biomarker discovery, establishing biological reference intervals for assessment of disease risk.

Introduction

Cardiometabolic conditions including obesity, type 2 diabetes (T2D), and cardiovascular disease (CVD) are tightly associated with dysregulation of lipid metabolism [1–3], which contributes directly or indirectly to adverse metabolic outcomes. Elevated total cholesterol, triglycerides, and low-density lipoprotein cholesterol (LDL-C) and decreased high-density lipoprotein cholesterol (HDL-C) are used as measures of metabolic health status and disease risk [4–6]. However, such clinical lipid measures do not adequately explain the complex pathophysiology of metabolic disease, nor do they suffice as diagnostic, prognostic, or predictive biomarkers. Defining the relationship between cardiometabolic risk factors and individual lipid species helps to identify potential biomarkers associated with disease risk and to understand the metabolic basis and pathophysiology of cardiometabolic diseases.

The human plasma lipidome is composed of many hundreds to thousands of molecular lipid species, displaying an enormous structural and functional diversity [7–9]. Yet, there is limited understanding about the relationship of these molecular components with cardiometabolic risk. Many lipid species have been shown to be altered during the onset and progression of cardiometabolic diseases such as T2D and CVD [10–13]. However, whilst evidence of the association of lipid species with cardiometabolic risk factors or disease outcomes on small cohorts has been well documented [10, 13, 14], there have been relatively few studies involving large population-based cohorts. In a cohort of 1,000 participants, Weir and colleagues utilised liquid chromatography tandem mass-spectrometry (LC-MS/MS)–based targeted lipidomics and profiled major lipid classes (23) and species (312) in human plasma, identifying lipid classes and subclasses associated with cardiometabolic risk factors [15]. More recently, Huynh and colleagues reported detailed associations of lipid species with anthropometric and insulin resistance measures in a subcohort (n = 640) selected from the Australian Diabetes, Obesity and Lifestyle Study (AusDiab) [16]. In addition to the limitations of small sample numbers, most previous studies have used targeted mass-spectrometry–based lipidomics with a limited number of lipid species [14, 16–19]. Advances in high-throughput LC-MS/MS–based lipidomic profiling now allow the measurement of many hundreds of biologically relevant circulating molecular lipid species, providing a more complete picture of the lipidome [16, 20].

The interaction of age and sex with metabolism in disease settings is well recognised [21–23]. Of note, the risk at time of onset and pathology of CVD has been shown to vary depending

alkylphosphatidylethanolamine; PE(P), alkenylphosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PLA2, phospholipase A2; PQC, plasma quality control; PS, phosphatidylserine; SCD-1, stearoyl CoA desaturase 1; SM, sphingomyelin; sn, stereospecifically numbered; Sph, sphingosine; SPT, serine palmitoyltransferase; S-1-P, sphingosine-1-phosphate; TG, triacylglycerol; TG (O), alkyl-diacylglycerol; TQC, technical quality control; T2D, type 2 diabetes; UWA HREC, University of Western Australia Human Research Ethics Committee; VLDL-C, very low-density lipoprotein cholesterol; WC, waist circumference; WHR, waist/hip ratio.

on sex [24]. Cholesterol metabolism is regulated in a sex-specific manner and is associated with distinct risk profiles in men and women [25]. Several investigators have shown that metabolite fingerprints in men and women vary significantly in an age-specific manner [26, 27]. Understanding the age and sex interaction within lipid metabolism is important for biomarker identification and will be necessary for precision medicine. Whilst the levels of nonlipid metabolites and a few lipid or lipoprotein classes have been shown to differ in age- and sex-specific fashions, evidence on age- and sex-specific lipidomic fingerprints, as well as the effect of menopause, is lacking. The identification of sex-specific associations of lipid species with cardiometabolic risk factors is essential to understanding crosstalk between lipid metabolism and sex that may underlie differential risk, and to obtain a holistic view of lipid metabolism in these disease processes.

Using a targeted LC-MS/MS method, we measured 706 individual lipid species across 36 distinct lipid classes from the blood of 10,339 adult Australians, aged between 25 to 95 years, from the AusDiab study. We examined the associations between the plasma lipidome and common cardiometabolic risk factors including age, sex, and body mass index (BMI). We further tested whether the relationship between age or BMI and lipidomic profile differs by sex to gain biological insight into the potential mechanism of lipid dysregulation during obesity in men and women. We validated our results measuring 563 lipid species in 4,207 participants of the Busselton Health Study (BHS).

Results

The plasma lipidome is sex-specific

The variability in the levels of plasma lipid species between men and women was generally similar (% coefficient of variation [CV] = 14–170). However, the coefficient of variation for many diacylglycerol, triacylglycerol, and alkyl-diacylglycerol species tends to be higher in men (S1 Fig). The correlation structure of the lipidome showed strong correlations between species both within and between classes (S2 Fig, S1 Table). We could also observe a negative correlation structure, particularly for di- and triacylglycerol species against phospholipid and sphingolipid species. The correlation structure in men (S3 Fig, S2 Table) and women (S4 Fig, S3 Table) appeared similar. However, subtraction of one from the other identified many differences (S5 Fig, S4 Table).

At the lipid class level, 30 out of 36 classes/subclasses were significantly associated with sex after adjusting for age, BMI, total cholesterol, HDL-C, and triglycerides (corrected $p < 0.05$). "Corrected p" throughout this paper refers to false discovery rate (FDR) correction using the Benjamini–Hochberg procedure. Based on p-values, lysoalkenylphosphatidylethanolamine displayed the strongest difference between sexes (15.8% higher in men relative to women, corrected p-value = 1.25×10^{-15}) (Fig 1A).

Class-level analyses do not capture the detailed differences in the fatty-acyl chain length and double-bond content that characterise individual lipid species. Therefore, we extended our analysis in relation to molecular lipid species. A total of 583 (82.6%) lipid species were associated with sex ($p < 0.05$) after correction for multiple comparisons. Within the sphingolipid classes, being male was strongly associated with lower levels of sphingomyelin and higher deoxyceramide species (Fig 1B). Within the sphingomyelin class, SM(18:2/14:0) displayed the most significant difference between sexes (24.9% lower in men relative to women, $p = 1.79 \times 10^{-16}$). Deoxyceramide species were higher in men, with Cer(m18:1/24:0) showing the strongest association with sex (29.4% higher in men, $p = 9.87 \times 10^{-18}$). Ceramide species were generally lower in men except for those containing a 24:0 fatty-acyl chain, which showed a significant positive association with men (Fig 1B). From the glycerophospholipid category,

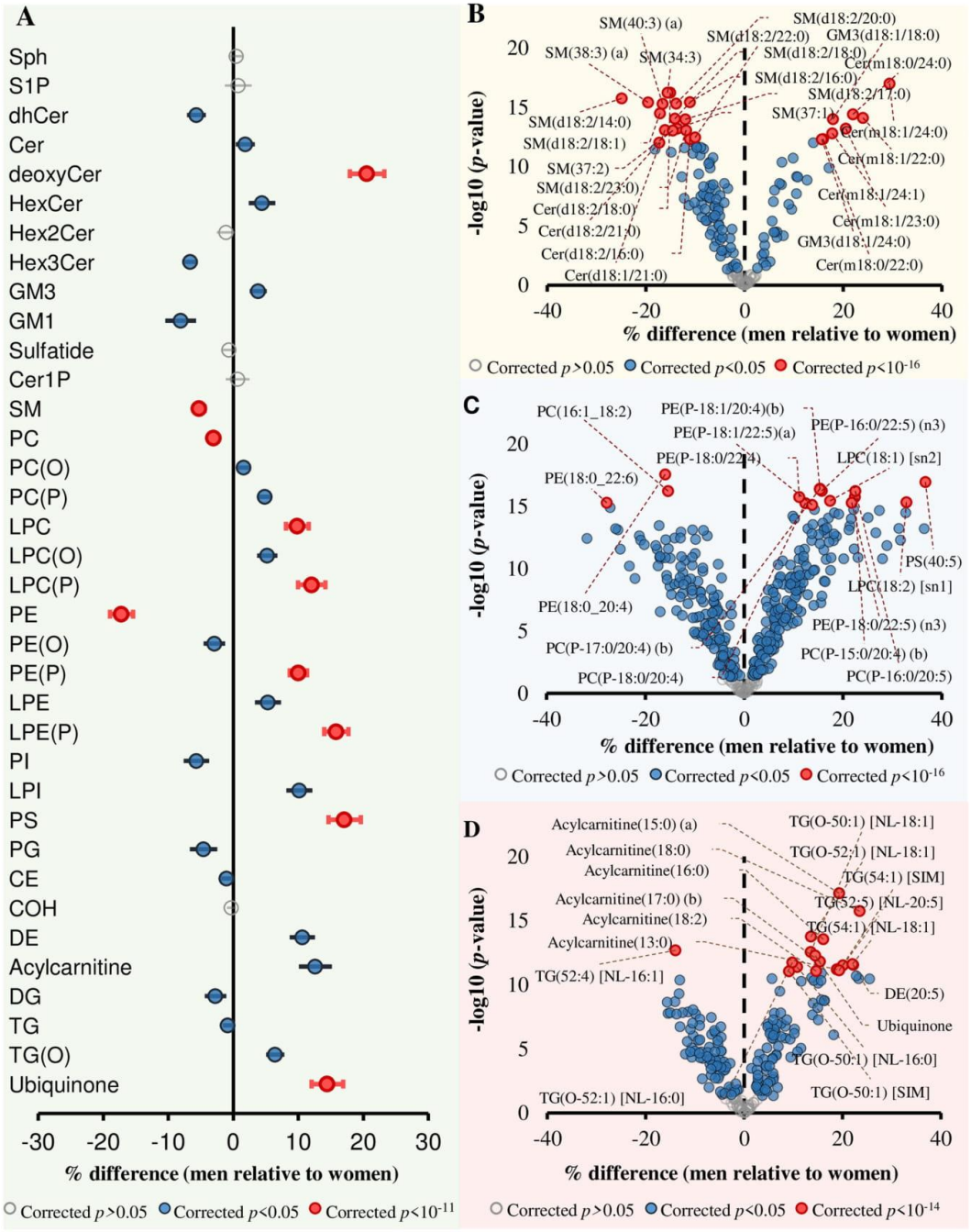
A
Sph
S1P
dhCer
Cer
deoxyCer
HexCer
Hex2Cer
Hex3Cer
GM3
GM1
Sulfatide
Cer1P
SM
PC
PC(O)
PC(P)
LPC
LPC(O)
LPC(P)
PE
PE(O)
PE(P)
LPE
LPE(P)
PI
LPI
PS
PG
CE
COH
DE
Acylcarnitine
DG
TG
TG(O)
Ubiquinone
-30 -20 -10 0 10 20 30
% difference (men relative to women)
Corrected p>0.05 Corrected p<0.05 Corrected p<10-11
B
-log10 (p-value)
SM(40:3) (a)
SM(38:3) (a)
SM(34:3)
SM(d18:2/22:0)
SM(d18:2/20:0)
GM3(d18:1/18:0)
SM(d18:2/18:0)
Cer(m18:0/24:0)
SM(d18:2/16:0)
SM(d18:2/17:0)
SM(37:1)
SM(d18:2/14:0)
Cer(m18:1/24:0)
SM(d18:2/18:1)
Cer(m18:1/22:0)
SM(37:2)
SM(d18:2/23:0)
Cer(m18:1/24:1)
Cer(d18:2/18:0)
Cer(m18:1/23:0)
Cer(d18:2/21:0)
GM3(d18:1/24:0)
Cer(d18:2/16:0)
Cer(m18:0/22:0)
Cer(d18:1/21:0)
-40 -20 0 20 40
% difference (men relative to women)
Corrected p>0.05 Corrected p<0.05 Corrected p<10-16
C
-log10 (p-value)
PC(16:1_18:2)
PE(P-18:1/20:4)(b)
PE(P-18:1/22:5)(a)
PE(P-16:0/22:5) (n3)
PE(P-18:0/22:4)
LPC(18:1) [sn2]
PE(18:0_22:6)
PS(40:5)
LPC(18:2) [sn1]
PE(18:0_20:4)
PE(P-18:0/22:5) (n3)
PC(P-17:0/20:4) (b)
PC(P-15:0/20:4) (b)
PC(P-18:0/20:4)
PC(P-16:0/20:5)
-40 -20 0 20 40
% difference (men relative to women)
Corrected p>0.05 Corrected p<0.05 Corrected p<10-16
D
-log10 (p-value)
Acylcarnitine(15:0) (a)
TG(O-50:1) [NL-18:1]
Acylcarnitine(18:0)
TG(O-52:1) [NL-18:1]
Acylcarnitine(16:0)
TG(54:1) [SIM]
Acylcarnitine(17:0) (b)
TG(52:5) [NL-20:5]
Acylcarnitine(18:2)
TG(54:1) [NL-18:1]
Acylcarnitine(13:0)
TG(52:4) [NL-16:1]
DE(20:5)
Ubiquinone
TG(O-50:1) [NL-16:0]
TG(O-52:1) [NL-16:0]
TG(O-50:1) [SIM]
-40 -20 0 20 40
% difference (men relative to women)
Corrected p>0.05 Corrected p<0.05 Corrected p<10-14

Fig 1. Associations between sex and lipid classes, subclasses, and species. Linear regression analysis between sex and log-transformed concentrations of each lipid species was performed adjusting for age, BMI, total cholesterol, HDL-C, and triglycerides (n = 10,339). The beta coefficients were converted to percentage difference for men relative to women. (A) Associations between sex and lipid classes/subclasses. (B) Associations between sex and sphingolipid species. (C) Associations between sex and phospholipid species. (D) Associations between sex and glycerolipid and fatty-acyl species. Grey circles show nonsignificant classes/subclasses and species, blue circles show classes/subclasses and species with corrected $p < 0.05$, and pink circles represent the most significantly associated classes/subclasses and species. Whiskers represent 95% confidence intervals. The underlying data can be found in S1 Data. BMI, body mass index; CE, cholesteryl ester; Cer, ceramide; Cer-1-P, ceramide-1-phosphate; COH, free cholesterol; DE, dehydrocholesterol; deoxyCer, deoxyceramide; DG, diacylglycerol; dhCer, dihydroceramide; GM1, G_{M1} ganglioside; GM3, G_{M3} ganglioside; HDL-C, high-density lipoprotein cholesterol; HexCer, monohexosylceramide; Hex2Cer, dihexosylceramide; Hex3Cer, trihexosylceramide; LPC, lysophosphatidylcholine; LPC(O), lysoalkylphosphatidylcholine; LPC(P), lysoalkenylphosphatidylcholine; LPE, lysophosphatidylethanolamine; LPE(P), lysoalkenylphosphatidylethanolamine; LPI, lysophosphatidylinositol; NL, neutral loss; PC, phosphatidylcholine; PC(O), alkylphosphatidylcholine; PC (P), alkenylphosphatidylcholine; PE, phosphatidylethanolamine; PE(O), alkylphosphatidylethanolamine; PE(P), alkenylphosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; SM, sphingomyelin; sn, stereospecifically numbered; Sph, sphingosine; S-1-P, sphingosine-1-phosphate; TG, triacylglycerol; TG(O), alkyl-diacylglycerol.

https://doi.org/10.1371/journal.pbio.3000870.g001

lower phosphatidylcholine and phosphatidylethanolamine and higher lyso- and ether-phospholipid levels were associated with being male (Fig 1C). The majority of acylcarnitine, triacylglycerol, and alkyl-diacylglycerol species were significantly higher in men compared with women (Fig 1D).

To validate our results, we utilised an independent cohort of 4,207 participants from the BHS, in which lipidomic data were available for 30 lipid classes/subclasses and 563 lipid species. For replication, we recalculated the lipid classes/subclasses using only the 563 common lipid species. Nineteen of the 27 classes/subclasses associated with sex in the AusDiab cohort were also significantly associated with sex, with similar effect sizes, in the Busselton cohort (Fig 2A). In addition, of the 563 lipid species common to both cohorts, 478 were associated with sex in the AusDiab cohort, and 408 of these were replicated in Busselton at a corrected p-value threshold of 0.05 (S5 Table). Only 8 lipid species showed opposing associations in the 2 cohorts. Of the 50 most significant species associated with sex in the AusDiab cohort (corrected $p < 7.76 \times 10^{-14}$), 49 were replicated in the Busselton cohort ($p < 6.60 \times 10^{-7}$). Only one species—PC(16:1_20:4)—was not significant in the Busselton cohort at a corrected p-value threshold of 0.05 (Fig 2B). Overall, there was a strong correlation between the regression coefficients for sex in the AusDiab and the Busselton cohorts ($r^2 = 0.840$, S6 Fig).

The association of age with the plasma lipidome

In the AusDiab cohort, linear regression of age against lipid species—adjusting for sex, BMI, total cholesterol, HDL-C, and triglycerides—identified a total of 472 plasma lipid species significantly associated with age after correction for multiple comparisons (Fig 3A). Age was strongly associated with ceramide species (Fig 3B) and deoxyceramide species, which arise from an atypical sphingolipid de novo synthesis pathway (Fig 3C). A specific set of triacylglycerol species, containing eicosapentaenoic acid (EPA) (20:5) fatty acids, were positively associated with age, even after adjusting for clinical measures of triglycerides (Fig 3A). Most of the associations seen in the AusDiab cohort were replicated in the Busselton cohort. Meta-analysis of the AusDiab and Busselton cohorts revealed remarkable positive associations of age with acylcarnitine and ceramide species; with acylcarnitine(14:2) displaying the strongest association (% difference per year = 1.368, corrected p-value = 1.024×10^{-240}). Ether-phospholipids—particularly alkylphosphatidylcholine, alkylphosphatidylethanolamine, and alkenylphosphatidylethanolamine species—were inversely associated with age (S7 Fig).

Age-related differences of circulating lipidomic profiles are sex-dependent

To explore age- and sex-related differences in lipidomic profile, we constructed heat maps of lipid classes/subclasses in the AusDiab cohort. The mean lipid level was computed for each

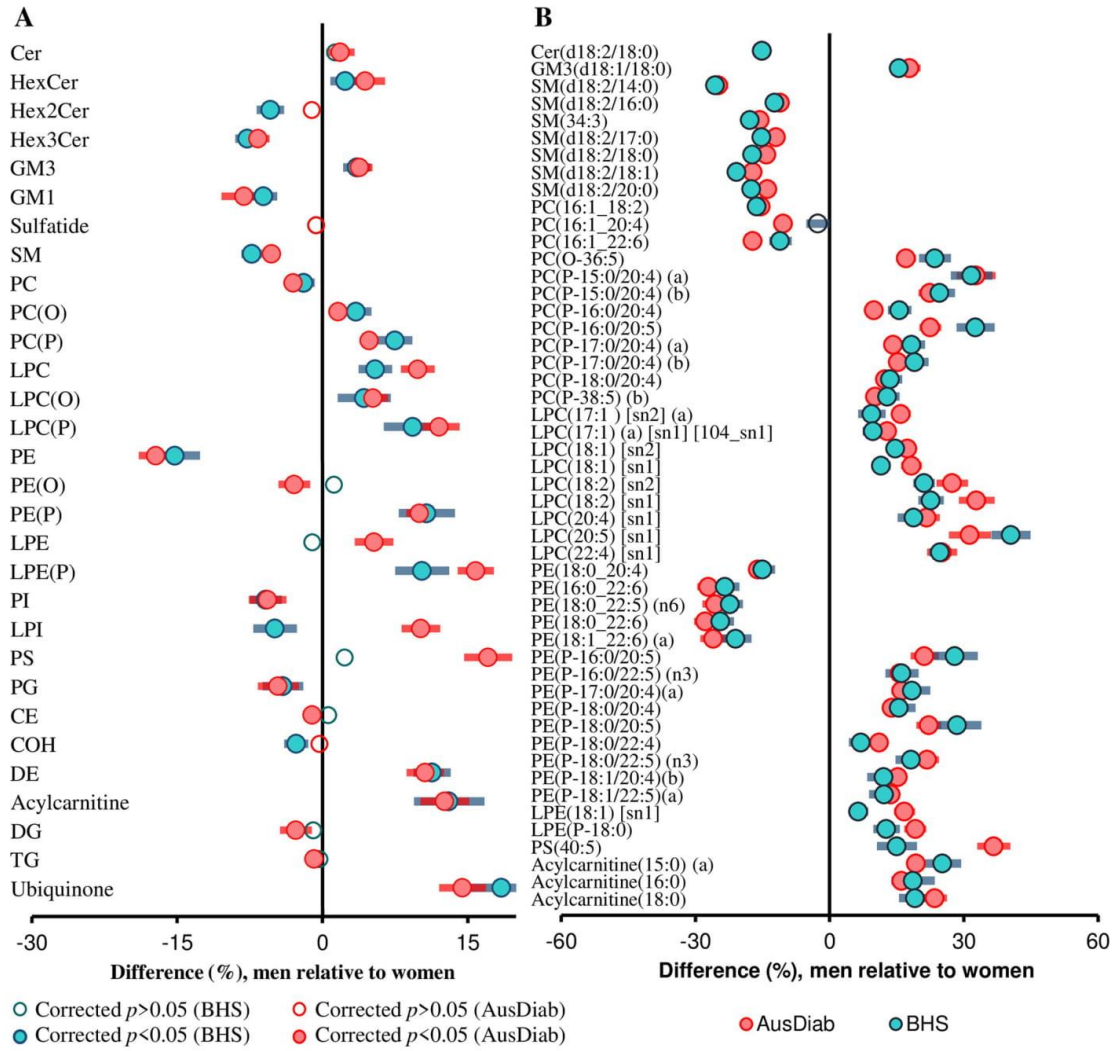

Fig 2. Validation of the associations between sex and plasma lipid classes and species. Linear regression analysis between sex and log-transformed lipid concentrations was performed adjusting for age, BMI, total cholesterol, HDL-C, and triglycerides on 10, 339 subjects in the AusDiab cohort and 4,207 in Busselton at a class level (A) and the 50 most significant lipid species (B). Blue (men) and pink (women) open circles show lipid classes/subclasses or species with corrected $p > 0.05$. Closed circles show classes/subclasses or species with corrected $p < 0.05$. Whiskers represent 95% confidence intervals. See S1 Data for the underlying data. AusDiab, Australian Diabetes, Obesity and Lifestyle Study; BMI, body mass index; CE, cholesteryl ester; Cer, ceramide; COH, free cholesterol; DE, dehydrocholesterol; DG, diacylglycerol; GM1, G_{M1} ganglioside; GM3, G_{M3} ganglioside; HDL-C, high-density lipoprotein cholesterol; HexCer, monohexosylceramide; Hex2Cer, dihexosylceramide; Hex3Cer, trihexosylceramide; LPC, lysophosphatidylcholine; LPC(O), lysoalkylphosphatidylcholine; LPC (P), lysoalkenylphosphatidylcholine; LPE, lysophosphatidylethanolamine; LPE(P), lysoalkenylphosphatidylethanolamine; LPI, lysophosphatidylinositol; PC, phosphatidylcholine; PC(O), alkylphosphatidylcholine; PC(P), alkenylphosphatidylcholine; PE, phosphatidylethanolamine; PE(O), alkylphosphatidylethanolamine; PE(P), alkenylphosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; TG, triacylglycerol.

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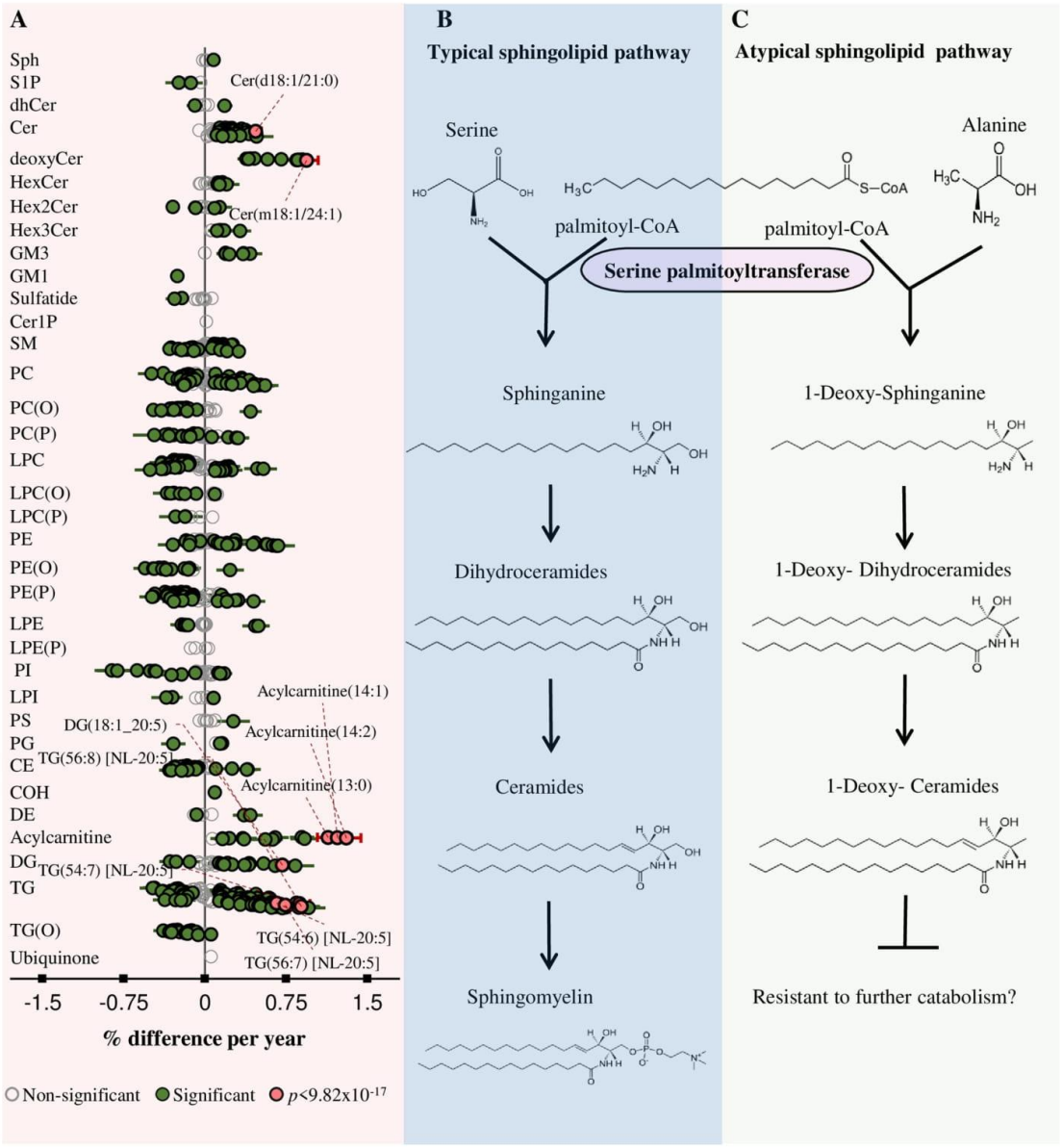

Fig 3. Associations between age and plasma lipid species. (A) Linear regression between age and log-transformed lipid species concentration on 10,339 individuals adjusting for sex, BMI, total cholesterol, HDL-C, and triglyceride levels. Grey circles show nonsignificant species, green show species with $p < 0.05$, and pink show the 10 most significant species after correction for multiple comparisons ($p < 9.82 \times 10^{-17}$). Whiskers represent 95% confidence intervals. (B) The typical sphingolipid pathway in which SPT joins canonical substrates serine and palmitoyl-CoA to produce the sphinganine base, a precursor for simple and complex sphingolipids. (C) SPT can also utilise alanine instead of serine to produce 1-deoxysphinganine and deoxyCers that lack an OH group at the C1-position. See S1 Data for the underlying data. BMI, body mass index; CE, cholesteryl ester; Cer, ceramide; Cer-1-P, ceramide-1-phosphate; COH, free cholesterol; DE, dehydrocholesterol; deoxyCer, deoxyceramide; DG, diacylglycerol; dhCer, dihydroceramide; GM1, G_{M1} ganglioside; GM3, G_{M3} ganglioside; HDL-C, high-density lipoprotein cholesterol; HexCer, monohexosylceramide; Hex2Cer, dihexosylceramide; Hex3Cer, trihexosylceramide; LPC, lysophosphatidylcholine; LPC(O), lysoalkylphosphatidylcholine; LPC(P), lysoalkenylphosphatidylcholine; LPE, lysophosphatidylethanolamine; LPE(P),

lysoalkenylphosphatidylethanolamine; LPI, lysophosphatidylinositol; NL, neutral loss; OH, hydroxyl; PC, phosphatidylcholine; PC(O), alkylphosphatidylcholine; PC(P), alkenylphosphatidylcholine; PE, phosphatidylethanolamine; PE(O), alkylphosphatidylethanolamine; PE(P), alkenylphosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; Sph, sphingosine; SPT, serine palmitoyltransferase; S-1-P, sphingosine-1-phosphate; TG, triacylglycerol; TG(O), alkyl-diacylglycerol.

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1-year age interval, then normalised to a 'metabolically healthy' reference group (25–34 years old) so that the number of standard deviations by which the mean level differed from the reference group was depicted for each lipid. Heat maps were adjusted for BMI by using the residuals of the linear regression of lipids against BMI in the model. The lipidomic profiles across age groups at the lipid class level in women (Fig 4, upper panel) was clearly distinct from men (Fig 4, lower panel), particularly for lyso- and ether-linked phospholipids and for the ether-linked glycerolipid alkyl-diacylglycerol, which tend to decrease with age in men but not in women.

Of those lipid classes that increased with age, the increases in deoxyceramide and G_{M3} ganglioside were stronger in women compared to men. Changes in the lipidomic profile of women appeared to occur at older ages than among men, with changes greater than 0.5 standard deviation units not occurring until 48 years of age in women but at about 38 years in men (Fig 4). Further adjustments for plasma total cholesterol, HDL-C, and triglycerides resulted in fewer lipid classes being different between men and women (S8 Fig). Heat maps for individual lipid species were also constructed, and these also showed differences between men and women (S9 Fig).

The heat maps presented both similarities and differences in the lipidomic profiles between men and women across age ranges. We therefore assessed the effect of sex on the association of age with individual lipid species by including an interaction term between sex and age for each lipid species and lipid classes/subclasses adjusting for sex, BMI, total cholesterol, HDL-C, and triglycerides. At the lipid class level, 26 out of the 36 lipid classes/subclasses displayed a significant interaction with sex (Fig 5A). Alkyl- and alkenylphosphatidylcholine and lysophosphatidylcholine tend to be negatively associated with age in both sexes, whilst alkenylphosphatidylethanolamine, lysoalkenylphosphatidylethanolamine, and lysophosphatidylinositol displayed opposing effects (positive in women and negative in men) (Fig 5A).

Several distinct lipid species belonging to the classes mentioned above were found to show opposing effects with age depending on sex. The interaction term was significant for 472 of the 706 lipid species measured (corrected p-value < 0.05) (Fig 5B). Species of lysophosphatidylcholine and ether-linked phospholipids showed strong differential association with age based on gender. In particular, omega-3 docosapentaenoic acid (DPA; 22:5) fatty-acid–containing species were highly dependent on age and sex; these species tend to be positively associated with age in women but were negatively associated in men (Fig 5B).

Association of lipid species with menopause

In a subgroup analysis of women, we assessed the association of menopause with the plasma lipidome. Menopausal status was assessed using an interview administered questionnaire. A total of 2,253 reported as postmenopausal and 2,383 as premenopausal; these were included in the analysis (S6 Table). Participants who were not sure about their menopausal status ($n = 192$) or were taking hormone replacement treatment (HRT), such as oestrogen ($n = 981$), were excluded. A multivariable linear regression adjusted for age, BMI, smoking status, and diabetes status was fitted against menopausal status. Lipid species across multiple lipid classes were associated with menopause (Fig 6). These associations were similar to those observed between age and lipid species, despite controlling for age in the analysis. We therefore performed a subanalysis of women in the menopausal transition window (40–60 years old, $n = 1,920$) to define the effect of menopause on the lipidome independent of age. We observed a significant association

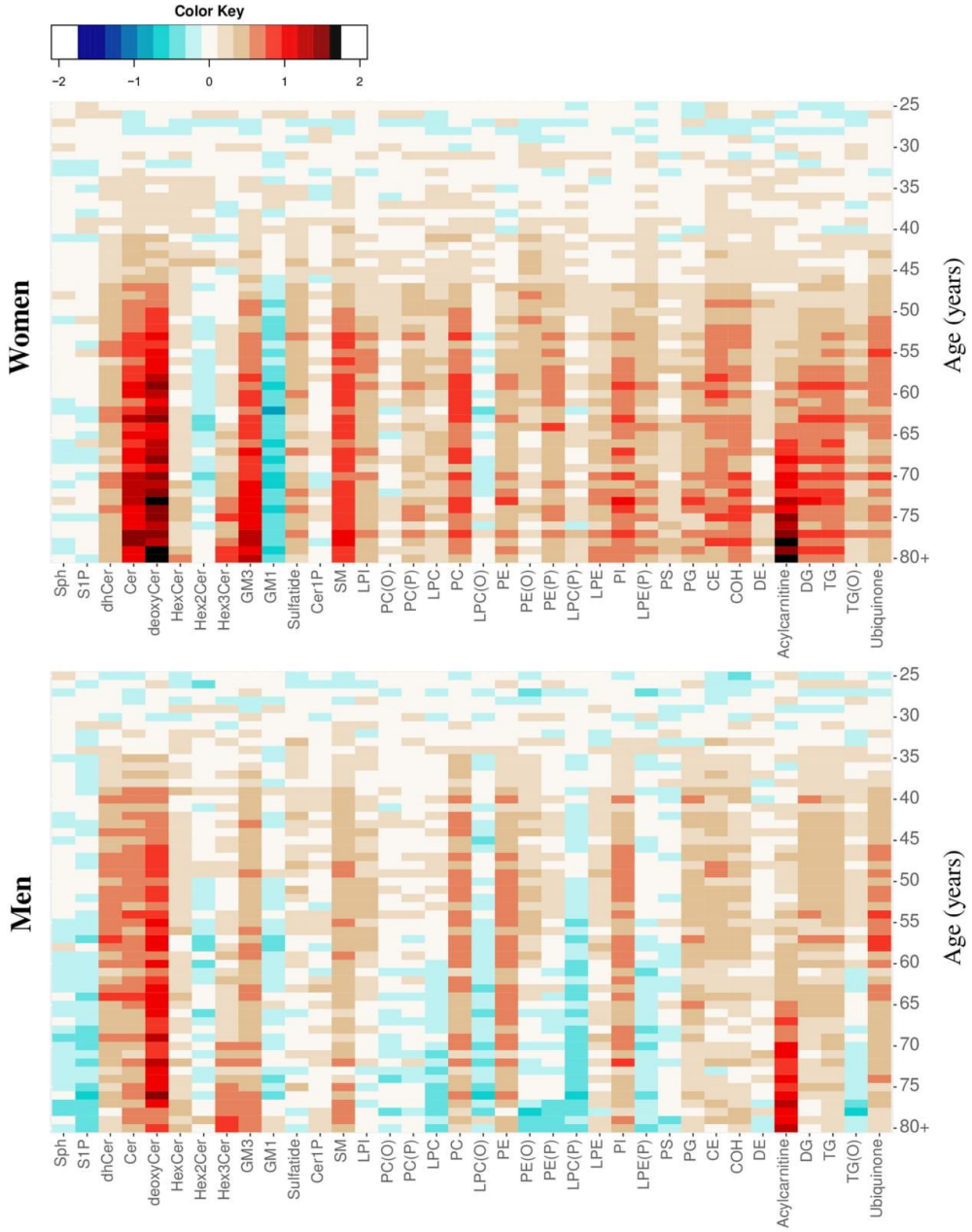

Fig 4. Age- and sex-related differences in plasma lipid classes in the AusDiab cohort. Heat maps showing differences in lipid class/subclass levels across a 1-year age interval constructed separately for men (lower panel) and women (upper panel) after adjusting for BMI. Average lipid class levels were calculated for each 1-year age interval and then centred and scaled to a 'reference' group corresponding to 25- to 34-year–old participants. Age groups (by 1-year intervals) are displayed on the *y* axis and the lipid classes on the *x* axis. Colour intensities represent the number of standard deviations away from the mean lipid class levels of the reference group. AusDiab, Australian Diabetes,

Obesity and Lifestyle Study; BMI, body mass index; CE, cholesteryl ester; Cer, ceramide; Cer-1-P, ceramide-1-phosphate; COH, free cholesterol; DE, dehydrocholesterol; deoxyCer, deoxyceramide; DG, diacylglycerol; dhCer, dihydroceramide; GM1, G_{M1} ganglioside; GM3, G_{M3} ganglioside; HexCer, monohexosylceramide; Hex2Cer, dihexosylceramide; Hex3Cer, trihexosylceramide; LPC, lysophosphatidylcholine; LPC(O), lysoalkylphosphatidylcholine; LPC(P), lysoalkenylphosphatidylcholine; LPE, lysophosphatidylethanolamine; LPE(P), lysoalkenylphosphatidylethanolamine; LPI, lysophosphatidylinositol; PC, phosphatidylcholine; PC(O), alkylphosphatidylcholine; PC(P), alkenylphosphatidylcholine; PE, phosphatidylethanolamine; PE(O), alkylphosphatidylethanolamine; PE(P), alkenylphosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; Sph, sphingosine; S-1-P, sphingosine-1-phosphate; TG, triacylglycerol; TG(O), alkyl-diacylglycerol.

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of menopause with 53 lipid species (Fig 6). Postmenopausal women showed significantly higher phosphatidylinositol, diacylglycerol, triacylglycerol, and alkyl-diacylglycerol levels compared with premenopausal women. Postmenopausal women had lower ether and lysophospholipids, particularly alkylphosphatidylcholine and lysoalkylphosphatidylcholine species (Fig 6).

The association between BMI and the plasma lipidome

Independent of age and sex, there were 577 lipid species associated with BMI (Fig 7A). In analyses adjusted for age, sex, total cholesterol, HDL-C, and triglycerides, 508 species were associated with BMI. Sphingolipids, including sphingosine, ceramide-1-phosphate, dihydroceramide, and deoxy-ceramide, were positively associated with BMI, whereas species of ganglioside, sulfatide, and mono-, di-, and trihexosylceramide were negatively associated (Fig 7A–7C). BMI was associated with most ceramide and sphingomyelin species, except those containing a relatively longer-chain fatty-acyl chain (Fig 7C). Most lysophospholipid species were negatively associated with BMI, as were ether-linked phospholipids (Fig 7A). Phosphatidylcholine species containing polyunsaturated omega-6 fatty acids such as 20:3, 20:4, or 22:4 were positively associated, whereas those containing the omega-6 fatty acid 18:2 and/or very long-chain omega-3 fatty acids were negatively associated (Fig 7B).

Many lipid species and lipid subclasses/classes associated with BMI in the AusDiab cohort were replicated in the Busselton cohort. At the class/subclass level, 16 out of 30 were significantly associated with BMI in the AusDiab cohort, and of these, 11 were significant in the same direction, 4 were not significant, and 1 subclass, lysoalkenylphosphatidylcholine, showed an opposite association in the Busselton cohort (Fig 8A). In addition, a total of 337 out of 563 common lipid species were replicated in Busselton with the effect sizes in the same direction as in the AusDiab discovery cohort (S7 Table). Among the 50 species with the strongest association in the AusDiab cohort ($p < 5.90 \times 10^{-11}$), 48 were replicated in the Busselton cohort (Fig 8B). The correlation between regression coefficients of each lipid in the AusDiab and the Busselton cohort was strong ($r^2 = 0.750$) (S10 Fig).

We further tested whether the association between BMI and circulating lipid classes/subclasses and species was sex dependent. We fitted a linear model including an interaction term for sex, adjusted for age, sex, total cholesterol, HDL-C, and triglycerides. At the class/subclass level, the associations of BMI with ceramide, dihydroceramide, G_{M3} ganglioside, and several phospholipid classes/subclasses were significantly different between men and women (interaction $p < 0.05$). Acylcarnitines were positively associated in women but negatively associated in men (Fig 9A). Analyses based on individual lipid species resulted in a total of 323 lipid species showing a significant sex interaction (corrected interaction p-value < 0.05) (Fig 9B). The association of phospholipids in general and lysophospholipids in particular with BMI was typically different between men and women.

Associations with waist circumference (WC) and waist/hip ratio (WHR)

BMI, WC, and WHR were correlated with each other (S11 Fig). We observed a similar association of lipids with BMI, WC, and WHR (S12 Fig). Of the 706 lipid species, some 357 lipid

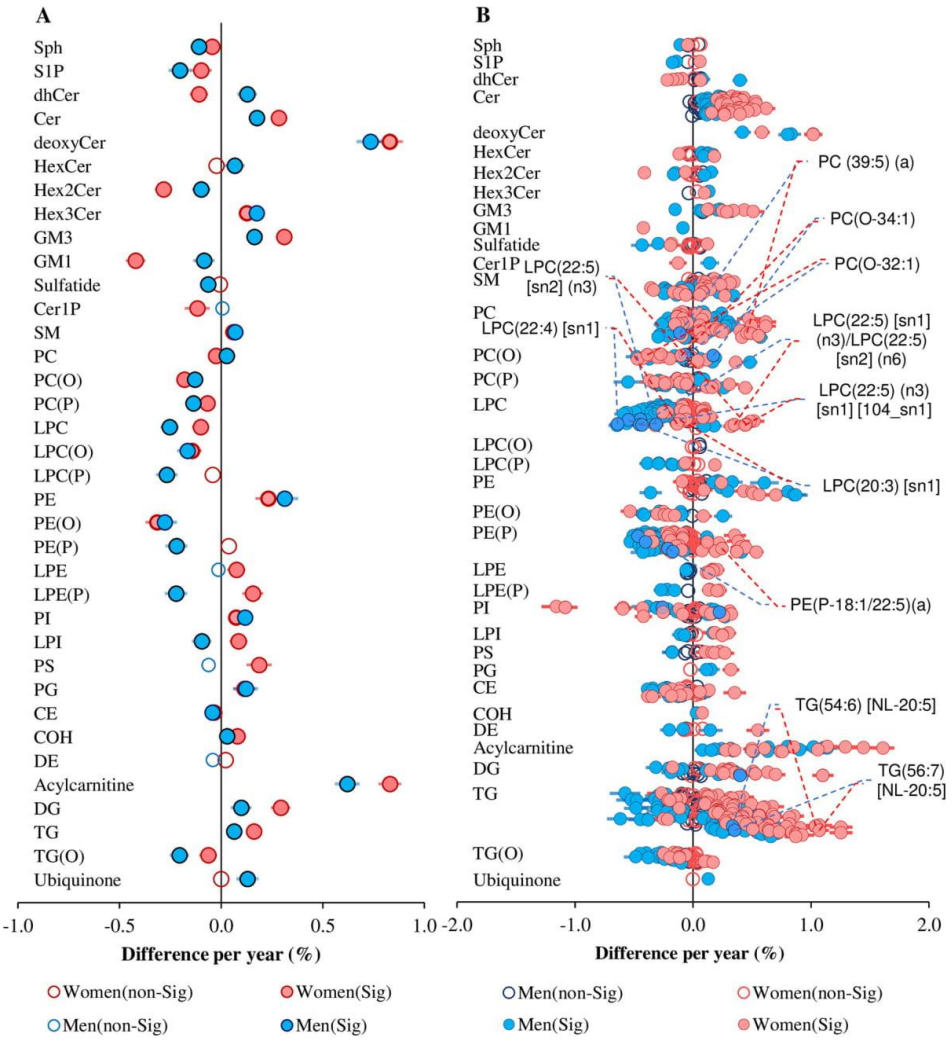

Fig 5. Sex-dependent associations of lipid classes/subclasses and species with age. Linear regression of age against lipid classes/subclasses (A) and lipid species (B) adjusting for sex, BMI, total cholesterol, HDL-C, and triglycerides and including an age × sex interaction term. Pink and blue solid circles show classes and species that are significantly associated with age in women and men respectively (corrected $p < 0.05$). Open circles represent nonsignificant classes/subclasses or species (corrected $p > 0.05$). All lipid classes/subclasses showing significant sex interaction (interaction $p < 0.05$) and the 10 most significantly different species between men and women (interaction p-value $< 1.0 \times 10^{-22}$) in the association with age are labelled. Whiskers represent 95% confidence intervals. See S1 Data for the underlying data. BMI, body mass index; CE, cholesteryl ester; Cer, ceramide; Cer-1-P, ceramide-1-phosphate; COH, free cholesterol; DE, dehydrocholesterol; deoxyCer, deoxyceramide; DG, diacylglycerol; dhCer, dihydroceramide; GM1, G_{M1} ganglioside; GM3, G_{M3} ganglioside; HDL-C, high-density lipoprotein cholesterol; HexCer, monohexosylceramide; Hex2Cer, dihexosylceramide; Hex3Cer, trihexosylceramide; LPC, lysophosphatidylcholine; LPC(O), lysoalkylphosphatidylcholine; LPC(P), lysoalkenylphosphatidylcholine; LPE, lysophosphatidylethanolamine; LPE(P), lysoalkenylphosphatidylethanolamine; LPI, lysophosphatidylinositol; NL, neutral loss; PC, phosphatidylcholine; PC(O), alkylphosphatidylcholine; PC(P), alkenylphosphatidylcholine; PE, phosphatidylethanolamine; PE(O), alkylphosphatidylethanolamine; PE(P), alkenylphosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; sn, stereospecifically numbered; Sph, sphingosine; S-1-P, sphingosine-1-phosphate; TG, triacylglycerol; TG(O), alkyl-diacylglycerol.

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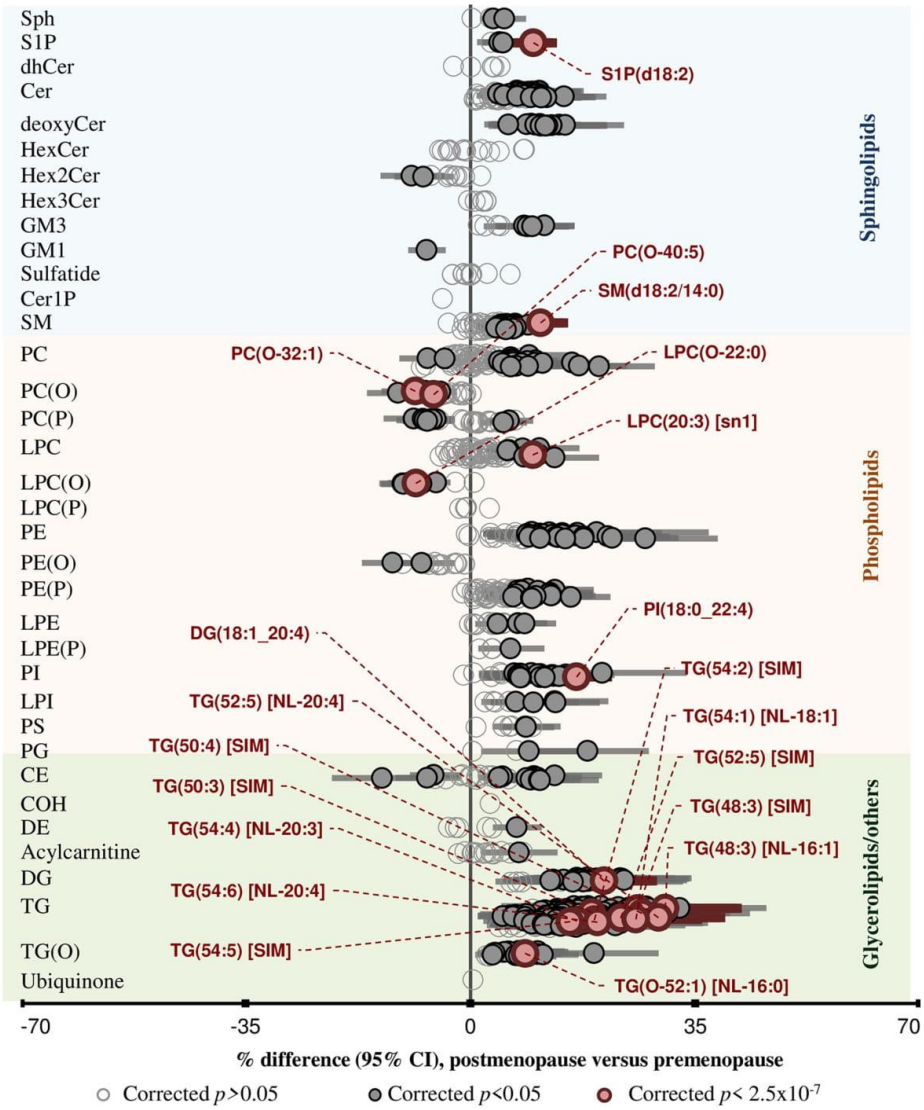

Fig 6. Association of lipid species with menopausal status. Multivariable linear regression analysis between menopausal status (post relative to pre) and $\log_{10}$-transformed lipid species concentrations was performed in women only, adjusting for age, BMI, smoking status, and diabetes status. Beta coefficients were converted to percentage difference of lipid level in postmenopausal relative to premenopausal women. Open circles show nonsignificant species ($p > 0.05$). Grey and pink closed circles show species with corrected $p < 0.05$ and corrected $p < 2.5 \times 10^{-7}$, respectively. See S1 Data for the underlying data. BMI, body mass index; CE, cholesteryl ester; Cer, ceramide; Cer-1-P, ceramide-1-phosphate; COH, free cholesterol; DE, dehydrocholesterol; deoxyCer, deoxyceramide; DG, diacylglycerol; dhCer, dihydroceramide; GM1, G_{M1} ganglioside; GM3, G_{M3} ganglioside; HexCer, monohexosylceramide; Hex2Cer, dihexosylceramide; Hex3Cer, trihexosylceramide; LPC, lysophosphatidylcholine; LPC(O), lysoalkylphosphatidylcholine; LPC(P), lysoalkenylphosphatidylcholine; LPE, lysophosphatidylethanolamine; LPE(P), lysoalkenylphosphatidylethanolamine; LPI, lysophosphatidylinositol; NL, neutral loss; PC, phosphatidylcholine; PC(O), alkylphosphatidycholine; PC(P), alkenylphosphatidylcholine; PE, phosphatidylethanolamine; PE(O), alkylphosphatidylethanolamine; PE(P), alkenylphosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SIM, single ion monitoring; SM, sphingomyelin; sn, stereospecifically numbered; Sph, sphingosine; S-1-P, sphingosine-1-phosphate; TG, triacylglycerol; TG(O), alkyl-diacylglycerol.

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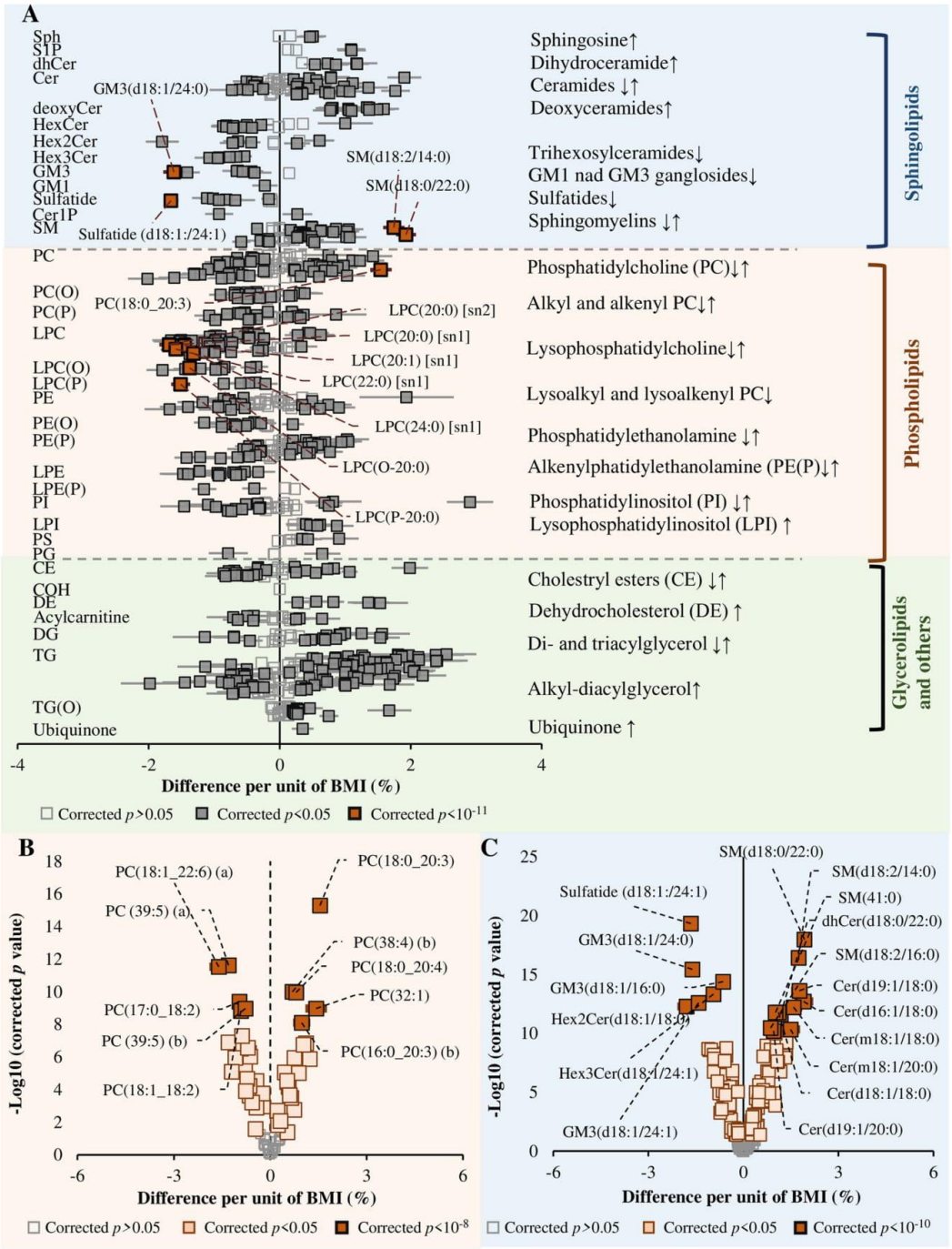
A
Sph
S1P
dhCer
Cer
deoxyCer
HexCer
Hex2Cer
Hex3Cer
GM3
GM1
Sulfatide
Cer1P
SM
PC
PC(O)
PC(P)
LPC
LPC(O)
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PE
PE(O)
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LPE
LPE(P)
PI
LPI
PS
PG
CE
COH
DE
Acylcarnitine
DG
TG
TG(O)
Ubiquinone
GM3(d18:1/24:0)
Sulfatide (d18:1:/24:1)
SM(d18:2/14:0)
SM(d18:0/22:0)
PC(18:0_20:3)
LPC(20:0) [sn2]
LPC(20:0) [sn1]
LPC(20:1) [sn1]
LPC(22:0) [sn1]
LPC(24:0) [sn1]
LPC(O-20:0)
LPC(P-20:0)
Sphingosine↑
Dihydroceramide↑
Ceramides ↓↑
Deoxyceramides↑
Trihexosylceramides↓
GM1 nad GM3 ganglosides↓
Sulfatides↓
Sphingomyelins ↓↑
Phosphatidylcholine (PC)↓↑
Alkyl and alkenyl PC↓↑
Lysophosphatidylcholine↓↑
Lysoalkyl and lysoalkenyl PC↓
Phosphatidylethanolamine ↓↑
Alkenylphatidylethanolamine (PE(P)↓↑
Phosphatidylinositol (PI) ↓↑
Lysophosphatidylinositol (LPI) ↑
Cholestryl esters (CE) ↓↑
Dehydrocholesterol (DE) ↑
Di- and triacylglycerol ↓↑
Alkyl-diacylglycerol↑
Ubiquinone ↑
Sphingolipids
Phospholipids
Glycerolipids and others
-4 -2 0 2 4
Difference per unit of BMI (%)
Corrected p>0.05
Corrected p<0.05
Corrected p<10-11
B
PC(18:1_22:6) (a)
PC (39:5) (a)
PC(17:0_18:2)
PC (39:5) (b)
PC(18:1_18:2)
PC(18:0_20:3)
PC(38:4) (b)
PC(18:0_20:4)
PC(32:1)
PC(16:0_20:3) (b)
-Log10 (corrected p value)
0 2 4 6 8 10 12 14 16 18
-6 -3 0 3 6
Difference per unit of BMI (%)
Corrected p>0.05
Corrected p<0.05
Corrected p<10-8
C
SM(d18:0/22:0)
Sulfatide (d18:1:/24:1)
GM3(d18:1/24:0)
GM3(d18:1/16:0)
Hex2Cer(d18:1/18:0)
Hex3Cer(d18:1/24:1)
GM3(d18:1/24:1)
SM(d18:2/14:0)
SM(41:0)
dhCer(d18:0/22:0)
SM(d18:2/16:0)
Cer(d19:1/18:0)
Cer(d16:1/18:0)
Cer(m18:1/18:0)
Cer(m18:1/20:0)
Cer(d18:1/18:0)
Cer(d19:1/20:0)
-Log10 (corrected p value)
0 5 10 15 20 25
-6 -3 0 3 6
Difference per unit of BMI (%)
Corrected p>0.05
Corrected p<0.05
Corrected p<10-10

Fig 7. The association between BMI and the plasma lipidome. (A) Linear regression analysis between BMI and log-transformed lipid concentration was performed on 10,339 subjects adjusting for age, sex, total cholesterol, HDL-C, and triglycerides. Open squares show nonsignificant species, and grey and yellow closed squares show species with corrected $p < 0.05$ and species showing most significant associations (corrected $p < 1 \times 10^{-11}$), respectively. Error bars represent the 95% confidence interval. (B) The association of BMI with PC species and (C) the association of BMI with sphingolipid species. In panels B and C, the grey, pale tan, and dark tan squares indicate lipid species with corrected *p*-value greater than 0.05 or less than 0.05 and most significant, respectively. See S1 Data for the underlying data. BMI, body mass index; CE, cholesteryl ester; Cer, ceramide; Cer-1-P, ceramide-1-phosphate; COH, free cholesterol; DE, dehydrocholesterol; deoxyCer, deoxyceramide; DG, diacylglycerol; dhCer, dihydroceramide; GM1, G_{M1} ganglioside; GM3, G_{M3} ganglioside; HDL-C, high-density lipoprotein cholesterol; HexCer, monohexosylceramide; Hex2Cer, dihexosylceramide; Hex3Cer, trihexosylceramide; LPC, lysophosphatidylcholine; LPC(O), lysoalkylphosphatidylcholine; LPC(P), lysoalkenylphosphatidycholine; LPE, lysophosphatidylethanolamine; LPE(P), lysoalkenylphosphatidylethanolamine; LPI, lysophosphatidylinositol; PC, phosphatidylcholine; PC(O), alkylphosphatidylcholine; PC(P), alkenylphosphatidylcholine; PE, phosphatidylethanolamine; PE(O), alkylphosphatidylethanolamine; PE(P), alkenylphosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; sn, stereospecifically numbered; Sph, sphingosine; S-1-P, sphingosine-1-phosphate; TG, triacylglycerol; TG(O), alkyl-diacylglycerol.

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species were associated with all 3 measures (BMI, WC, and WHR). In addition, there were many other species associated with BMI and WC or BMI and WHR or WC and WHR. A summary of this overlapped association is outlined in S13 Fig. There were differences in the strength of some associations—triacylglycerol species tended to be more strongly associated with WC or WHR compared with BMI. Deoxyceramide and lysoalkylphosphatidylcholine species showed a stronger association with WHR than with BMI. There were 40, 30, and 9 lipid species associated with only BMI, WC, or WHR, respectively. The list of these lipids can be found in S13 Fig.

Association of smoking with the plasma lipidome

In this study, smokers ($n = 1,623$) compared with nonsmokers ($n = 5,632$) had higher levels of saturated and monounsaturated fatty-acid–containing lipid species and lower levels of polyunsaturated fatty acid phospholipids and ether-linked lipid classes and subclasses, including alkylphosphatidylethanolamine and alkyl-diacylglycerol (S14 Fig).

Association of risk factors with the fatty acid composition of the lipidome

Our current method provides full characterisation of the individual fatty-acyl chain composition for the majority of lipid species. This can be informative, particularly for lipid species containing only a single fatty acid (e.g., lysophosphatidylcholine) where the association may be more readily interpreted. To investigate this, we summed the sn1 and sn2 isomers for each lysophosphatidylcholine species and performed a correlation analysis. Using compositional data (lipids expressed as the percentage of their class total), we performed regression analyses with age, sex, and BMI. The lysophospholipid species (representing fatty acids) showed a strong positive correlation between odd-chain fatty acids and, to a lesser extent, between even-chain fatty acids (S15 Fig). Linear regression analyses, adjusted for sex, BMI, total cholesterol, HDL-C, and triglycerides showed that age was positively associated with long-chain to very long-chain fatty acids and fatty acids in the omega-3 pathway. Medium-chain and odd-chain fatty acids, including 14:0 and 15:0 and the omega-6 pathway, showed strong inverse association with age (S16A Fig). Regression analyses with sex adjusted for age, BMI, total cholesterol, HDL-C, and triglycerides showed most polyunsaturated fatty acids to be higher in men, whereas saturated and some monounsaturated fatty acids, including the C16:1 species, were lower in men (S16B Fig). Regression analyses with BMI, adjusted for age, sex, total cholesterol, HDL-C, and triglycerides, showed negative association between most fatty acids and BMI. However, a few fatty acids such as myristic acid (14:0) and palmitoleic acid (16:1) were positively associated (S16C Fig).

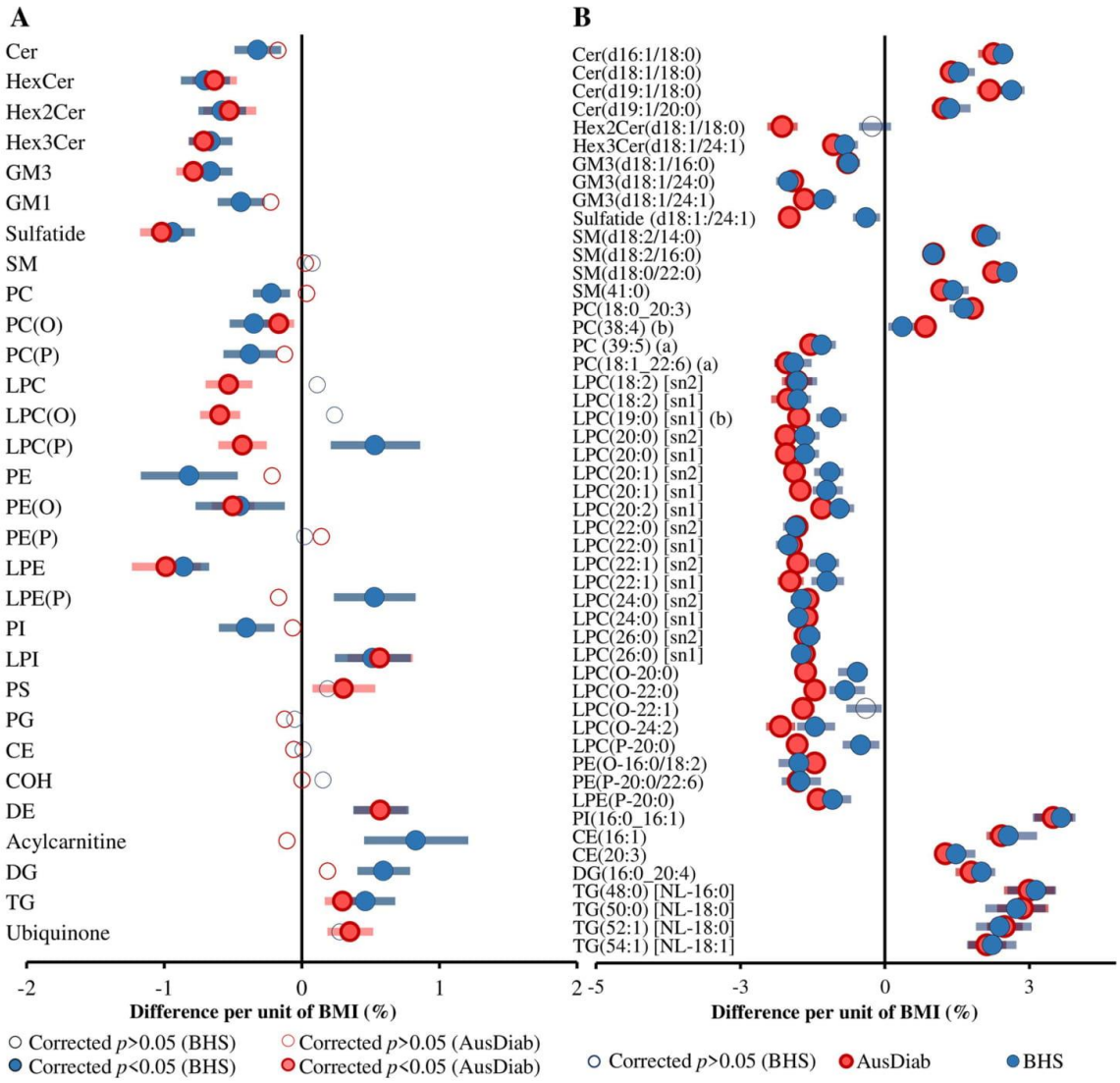

Fig 8. Validation of the association between BMI and plasma lipid classes/subclasses and species. (A) Linear regression analysis between BMI and log-transformed lipid class concentrations was performed on 10,339 AusDiab participants and 4,097 Busselton participants. (B) The same analyses were performed at the lipid species level, and the 50 most significant species in the AusDiab were selected based on lowest corrected *p*-values. The analyses were adjusted for age, sex, total cholesterol, HDL-C, and triglycerides. Pink and blue closed circles show lipid species/classes associated with BMI in AusDiab and Busselton cohort, respectively ($p < 0.05$). Open circles represent nonsignificant associations (corrected $p > 0.05$). Error bars represent the 95% confidence intervals. See S1 Data for the underlying data. AusDiab, Australian Diabetes, Obesity and Lifestyle Study; BMI, body mass index; CE, cholesteryl ester; Cer, ceramide; Cer-1-P, ceramide-1-phosphate; COH, free cholesterol; DE, dehydrocholesterol; deoxyCer, deoxyceramide; DG, diacylglycerol; dhCer, dihydroceramide; GM1, G_{M1} ganglioside; GM3, G_{M3} ganglioside; HDL-C, high-density lipoprotein cholesterol; HexCer, monohexosylceramide; Hex2Cer, dihexosylceramide; Hex3Cer, trihexosylceramide; LPC, lysophosphatidylcholine; LPC(O), lysoalkylphosphatidylcholine; LPC(P), lysoalkenylphosphatidycholine; LPE, lysophosphatidylethanolamine; LPE(P), lysoalkenylphosphatidylethanolamine; LPI, lysophosphatidylinositol; NL, neutral loss; PC, phosphatidylcholine; PC(O), alkylphosphatidylcholine; PC(P), alkenylphosphatidylcholine; PE, phosphatidylethanolamine; PE(O), alkylphosphatidylethanomine; PE(P), alkenylphosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; sn, stereospecifically numbered; TG, triacylglycerol.

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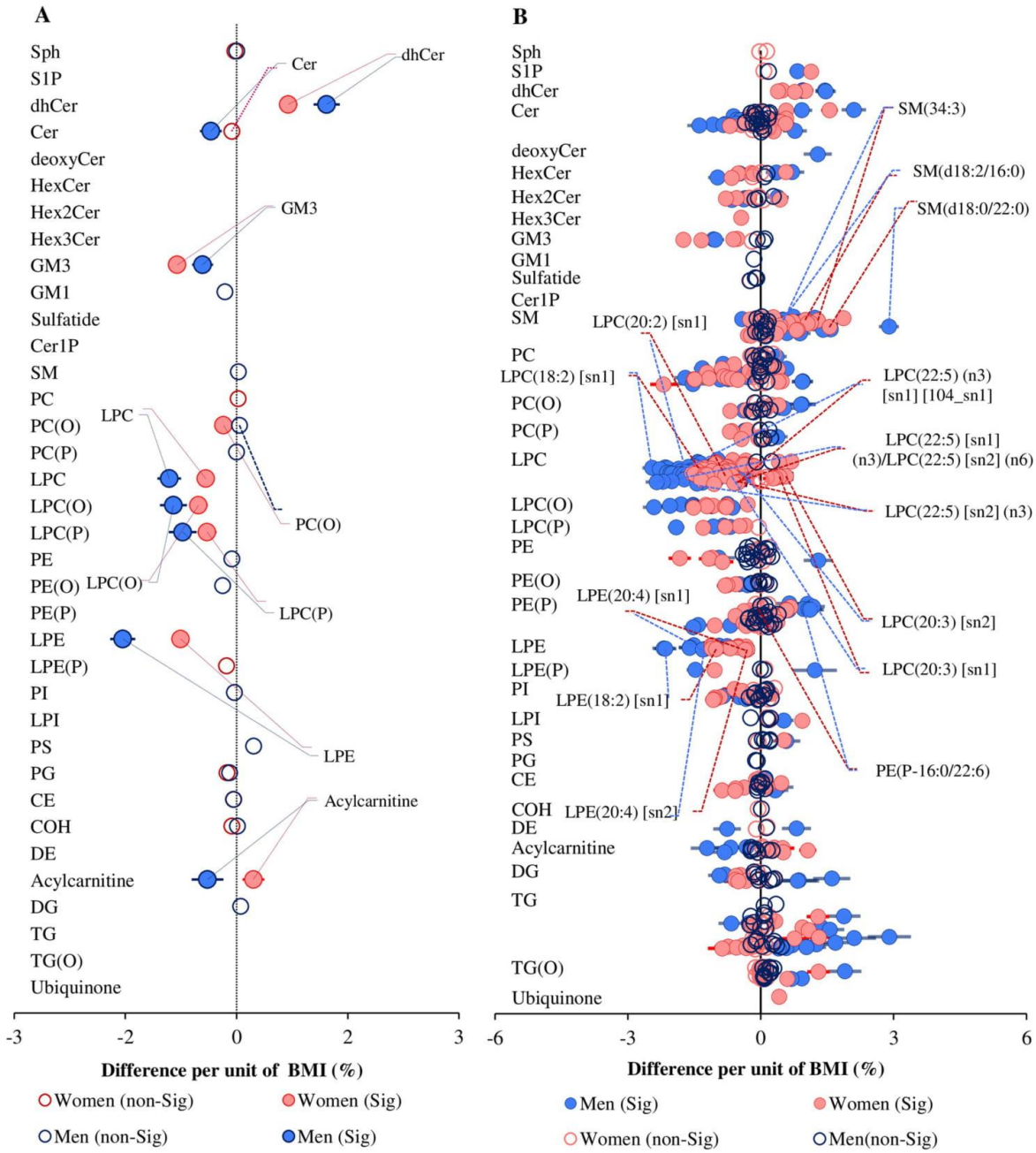

Fig 9. Sex-specific association between BMI and plasma lipid classes and species. (A) Linear regression analysis between BMI and log-transformed lipid class concentrations on 10,339 participants (men = 4,654 and women = 5,685) adjusting for age, sex, total cholesterol, HDL-C, and triglycerides and including a sex × BMI interaction term. (B) Linear regression analyses between BMI and log-transformed lipid species concentrations including sex interaction, adjusting for age, sex, total

cholesterol, HDL-C, and triglycerides. Associations with lipid species that showed a significant sex interaction (corrected $p < 0.05$) were plotted separately for women (pink) and men (blue). Closed circles show significant associations (corrected $p < 0.05$). Open circles show corrected $p > 0.05$. Error bars represent 95% confidence intervals. See S1 Data for the underlying data. BMI, body mass index; CE, cholesteryl ester; Cer, ceramide; Cer-1-P, ceramide-1-phosphate; COH, free cholesterol; DE, dehydrocholesterol; deoxyCer, deoxyceramide; DG, diacylglycerol; dhCer, dihydroceramide; GM1, G_{M1} ganglioside; GM3, G_{M3} ganglioside; HDL-C, high-density lipoprotein cholesterol; HexCer, monohexosylceramide; Hex2Cer, dihexosylceramide; Hex3Cer, trihexosylceramide; LPC, lysophosphatidylcholine; LPC(O), lysoalkylphosphatidylcholine; LPC(P), lysoalkenylphosphatidylcholine; LPE, lysophosphatidylethanolamine; LPE(P), lysoalkenylphosphatidylethanolamine; LPI, lysophosphatidylinositol; PC, phosphatidylcholine; PC(O), alkylphosphatidylcholine; PC(P), alkenylphosphatidylcholine; PE, phosphatidylethanolamine; PE(O), alkylphosphatidylethanolamine; PE(P), alkenylphosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; sn, stereospecifically numbered; Sph, sphingosine; S-1-P, sphingosine-1-phosphate; TG, triacylglycerol; TG(O), alkyl-diacylglycerol.

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The use of lipid ratios to define metabolic pathways

To gain further insight into the relationship of lipid metabolic pathways with age, sex, and BMI, we performed an unbiased association analyses of with all lipid ratios adjusted for covariates (278,631 lipid ratios). We utilised the *p*-gain value (lowest *p*-value of the lipid species used in the ratio divided by the *p*-value of the lipid ratio) to identify lipid ratios that were providing new information and considered the *p*-gain values significant if they exceeded 10 × the number of ratios tested (2.79×10^6) as described by Petersen and colleagues [28]. There were 38,519 lipid ratios with a significant *p*-gain value for BMI (S8 Table). When these were sorted (based on *p*-gain values), we observed the top-ranked lipid ratios contained lipid species that were not clearly related in metabolic pathways [e.g., SM(34:3)/PC(17:0_18:2), *p*-gain 1.50×10^{258}; SM(d18:2/14:0)/LPC(26:0)[sn1], *p*-gain 1.69×10^{248}] and were likely the result of internal normalisation of technical variance in the data set. However, some lipid ratios did appear to relate through metabolic pathways [e.g., SM(d18:2/16:0)/SM(d18:1/16:0), *p*-gain 2.85×10^{224}; PE(18:0_22:6)/PE(16:0_22:6), *p*-gain 6.82×10^{213}] (S8 Table). In each case, we noted a high correlation between the lipid species within the ratios. We subsequently filtered the lipid ratios to those with a correlation coefficient >0.7 between the lipid species within the ratio and then again ranked by *p*-gain. This provided a shorter list of 560 lipid ratios in which the lipid species within each ratio were more closely related.

Among the top-ranked ratios, we observed lipid pairs differing by specific fatty acids (e.g., 16:0/18:0; 16:0/16:1; 16:1/18:1) showing strong *p*-gain values in their association with BMI. We mapped some of these ratios to known metabolic pathways. We searched the data set for additional ratios in which the lipid species differed in a single feature (e.g., 16:0 to 18:0 or d18:1 to d18:2) and used this to identify supporting ratios for each of our biologically relevant top-ranked lipid ratios. The ratio of PE(18:0_22:6)/PE(16:0_22:6) (Fig 10A) was positively associated with BMI, as were 4 additional lipid ratios differing only in the 16:0 to 18:0 fatty acid (S9 Table). However, one ratio PI(18:0_20:4)/PI(16:0_20:4) was negatively associated with BMI, with a *p*-gain value of 5.87×10^{11} (S9 Table). Lipid ratios defined by a 16:1/16:0 fatty acid difference were also positively associated with BMI (Fig 10B), whereas the ratios defined by 18:1/16:1 (Fig 10C), 18:1/18:0 (Fig 10D), 20:0/18:0 (Fig 10E), and 22:0/18:0 (Fig 10F) were negatively associated with BMI. The mapping of the monounsaturated fatty acid pathway is shown in Fig 10G. Some of the same fatty acid pathways were also associated with age (S9 Table, S10 Table) and sex (S9 Table, S11 Table).

We also observed ratios of dihydroceramide/ceramide species (S17A Fig and S17B Fig), hexosylceramide/ceramide species (S17C Fig), and ceramide/sphingomyelin species (S17D Fig) associated with BMI that could be mapped to the sphingolipid biosynthetic pathway (S17 Fig, S12 Table). Ratios of sphingolipid species differing in their sphingoid base; e.g., d18:2 and d18:1-containing species, Hex2Cer(d18:2/16:0)/Hex2Cer(d18:1/16:0) (S17E Fig) and SM (d18:2/16:0)/SM(d18:1/16:0) (S17F Fig), were strongly associated with BMI and map to the fatty acid desaturase 3 (FADS3) (S17H Fig). The ratios of d18:2/d18:1 were also strongly

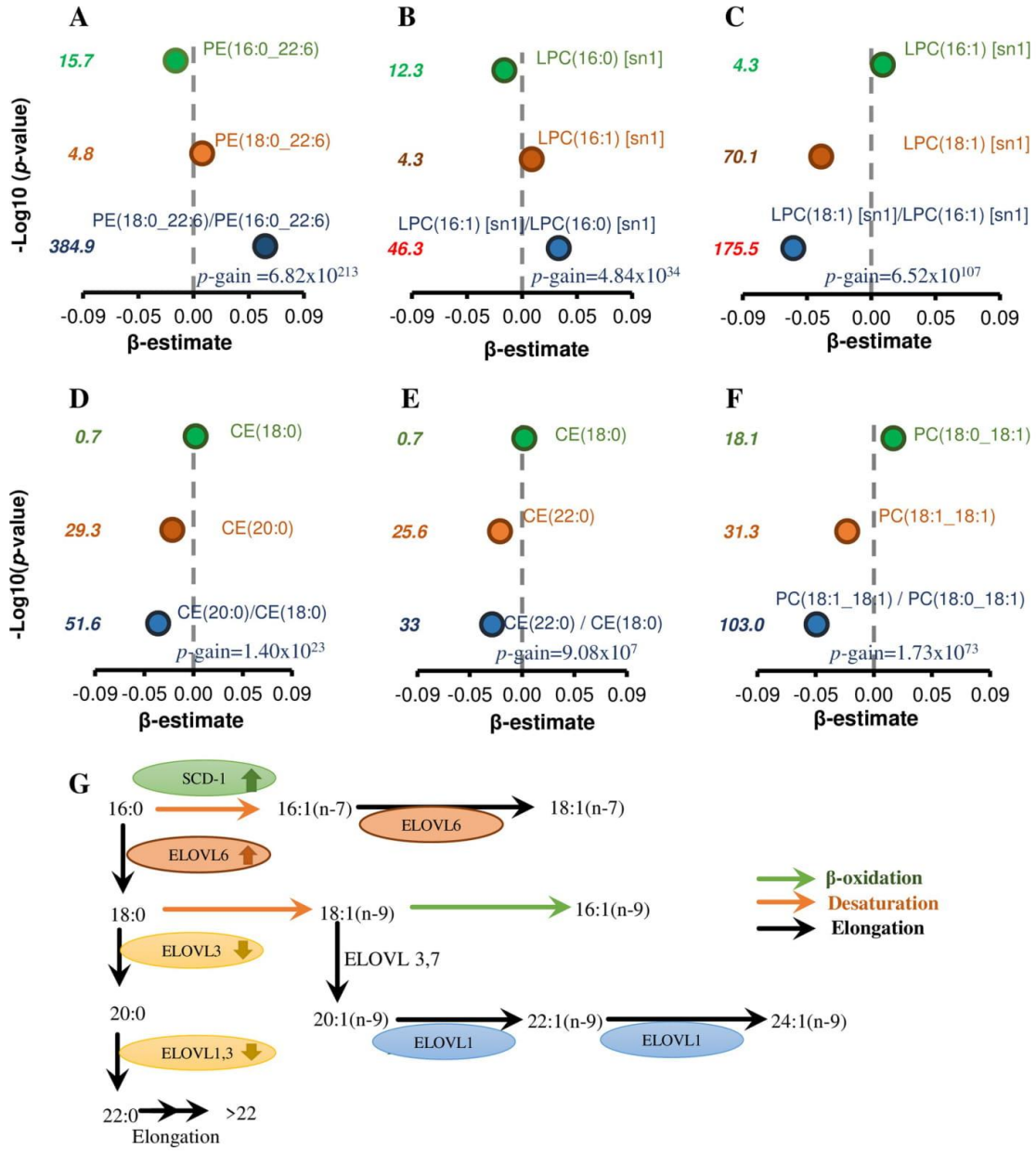

Fig 10. Plasma fatty acid ratios and enzyme pathways associated with BMI. A linear regression adjusted for age, sex, total cholesterol, HDL-C, and triglycerides was performed between individual lipid species or lipid ratios and BMI (panels A–F). The strength of associations with ratios relative to individual lipid species is indicated by *p*-gain values. (G) The de novo monounsaturated fatty acid pathway in mammals. The proposed increase or decrease in enzyme activities with increasing BMI is shown by arrows. BMI, body mass index; CE, cholesteryl ester; ELOVL, elongation of very long chain fatty acids protein; HDL-C, high-density lipoprotein cholesterol; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SCD-1, stearoyl CoA desaturase 1; sn, stereospecifically numbered

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associated with age (S13 Table) and women (S18A Fig). Similarly, ratios of alkenylphosphatidylethanolamine/alkylphosphatidylethanolamine species (e.g., PE(P-16:0/22:6)/PE(O-16:0/22:6)) were also strongly associated with BMI (S19 Fig, S14 Table), age, and sex (S14 Table) and signify the specific activity of plasmanylethanolamine Δ1-desaturase enzyme.

We have provided rationale and examples for our approach to mining the lipidomics data and provide full summary results of all lipid ratios with significant *p*-gain values for age, sex, and BMI as a reference tool and resource. We have also provided the correlation structures between lipid species in the entire cohort (S2 Fig, S1 Table), for men (S3 Fig, S2 Table), and for women (S4 Fig, S3 Table), as well as the differences in correlation coefficients (men relative to women) (S5 Fig, S4 Table).

Discussion

This study is the first of its kind, to our knowledge, to examine over 700 circulating molecular lipid species in a large population-based cohort using a targeted lipidomics approach [16]. We demonstrated complex associations of the plasma lipidome with age, sex, and BMI, as well as with WC and WHR and smoking, and have mined these data to identify lipid metabolic pathways. We have further shown that the association between the plasma lipidome and age or BMI were sex- dependent. These findings provide the basis for understanding the dysregulation of lipid metabolism in aging and obesity and how this is influenced by sex. Many of the results were replicated in the BHS cohort, an independent cohort composed of participants with a comparable age range and ethnicity to the discovery cohort.

The plasma lipidome differs between men and women

Sphingolipids are structurally diverse lipids characterised by a sphingoid backbone, the most abundant base in humans being the 18-carbon sphingosine (d18:1) accounting for 57%, followed by sphingadienine (d18:2, 21%) [29]. Sphingolipids containing the d18:2 sphingoid base showed strong associations with sex (higher levels in women). The dienic base contains an extra double bond at *cis*-14 [30] in addition to the 4E double bond, giving rise to d18:2 (4E,14Z). The enzyme responsible for the incorporation of this extra double bond was recently reported as FADS3 [29]. They reported a higher level of d18:2 sphingomyelin species (30%) in women relative to men and a corresponding higher level of FADS3 activity [29]. We performed regression analysis of lipid ratios with sex. By filtering the ratios based on the correlation between the lipid species and sorting based on the *p*-gain, we identified biologically relevant lipid ratios that captured the same FADS3 signal (S18B Fig). The association of sex with SM(d18:2/24:0) and SM(d18:1/24:0) had *p*-values of 4.26×10^{-7} and 2.28×10^{-49}, respectively, whereas the ratio of SM(d18:2/24:0)/SM(d18:1/24:0) had a *p*-value of 1.26×10^{-139}; *p*-gain = 2.80×10^{91}, clearly reflecting the differential activity of the FADS3 enzyme in men and women.

Atypical sphingolipids that arise from noncanonical sphingolipid pathways, such as the 1-deoxyceramide species, were higher in men than in women. Deoxyceramide is synthesised from the condensation of palmitoyl-CoA with alanine by the action of serine palmitoyltransferase (SPT), leading to the formation of atypical ceramides lacking a hydroxyl group at the C1 position (53). Phosphorylation of the C1 hydroxyl group is requisite for catabolism of sphingolipids. Thus, it is not clear how such metabolites are catabolised, leading to the speculation they may accumulate throughout life. It is, unclear at this point whether difference in substrates (alanine/serine) levels as previously reported [31] or a difference in rate of synthesis and/or turnover is responsible for the observed variation in the levels of deoxyceramide species between men and women. In disease conditions, deoxyceramide species have emerged as

biomarkers of metabolic syndrome and T2D [32, 33]. Gender- and age-related differences (also observed in this study) have been reported in the risk, pathophysiology, and complications of T2D [22, 34]. Our findings highlight the possibility that dysregulation in atypical sphingolipid metabolism with age and sex may contribute to cardiometabolic risk.

Lysophosphatidylcholine and ether-linked phospholipids were significantly elevated in the plasma of men compared with women, suggesting there is a sexual dimorphism in phospholipid metabolism, which could be partly due to differences in the activity of phospholipases and/or complex hormonal, dietary, and lifestyle factors. Two phospholipid-metabolising enzymes, lecithin cholesterol acyltransferase (LCAT) [35] and lipoprotein-associated phospholipase A2 (PLA2) [36], have been reported to differ between men and women and likely contribute to these lipidomic associations. Indeed, increased activity and/or mass of lipoprotein-associated PLA2 is an independent risk factor for stroke and cardiovascular disease [37, 38].

The plasma lipidome is associated with age

The positive association of acylcarnitine species with age, independent of sex, BMI, and clinical lipids is a key finding of our study. Huynh and colleagues have reported a similar finding [16]. The elevated circulating acylcarnitine species may reflect impaired mitochondrial β-oxidation [39], which has previously been associated with age [40, 41]. In a mice model, Bodil and colleagues have reported an increase in plasma acylcarnitine levels caused by inhibition of mitochondrial fatty acid oxidation [42]. In clinical studies, elevated plasma acylcarnitine levels have been shown to be associated with age-related cardiometabolic conditions such as the risk of T2D and CVD, independently of age [43–46]. Notwithstanding these observations, other explanations are possible, and further studies are required to validate our findings.

A striking finding was the negative association between age and most ether lipids, including alkylphosphatidylcholine, alkenylphosphatidylcholine, alkylphosphatidylethanolamine, and ether-linked triacylglycerol species. Reduced levels of ether lipids, particularly plasmalogens, have been associated with oxidative stress and age-related metabolic disorders [47, 48]. Lipidomic studies have reported that plasmalogens are inversely associated with obesity, T2D, CVD, and Alzheimer's disease [14, 49, 50]. Chaleckis and colleagues demonstrated that the levels of antioxidant compounds were reduced in elderly compared with young adults [51], and so higher levels of oxidative stress may contribute to oxidation and turnover of plasmalogens or species containing polyunsaturated fatty-acyl groups, which are both susceptible to oxidation. We also observed multiple species of ether-phospholipids (alkylphosphatidylcholine and alkylphosphatidylethanolamine) and ether glycerolipids (alkyl-diacylglycerol) that did not contain polyunsaturated acyl chains negatively associated with age. This suggests an alternative hypothesis: down-regulation of the de novo synthesis resulting from peroxisomal dysfunction with aging. This concept has been proposed previously [52, 53] and is based primarily on the associations between peroxisomal dysfunction as measured by decreased plasmalogens and other peroxisomal-derived lipids and age-related diseases such as diabetes, CVD, and Alzheimer's disease [14, 54, 55].

Interestingly, we observed that some ether-phospholipids containing n3 fatty acyls, including docosahexaenoic acid (DHA) and EPA, were positively associated with age, possibly as a result of higher dietary intake in the older population as has been reported [56]. However, whilst omega-3 supplementation can increase these species of ether lipids, it does not increase the total ether lipid content of plasma and so is unlikely to attenuate the peroxisomal driven defects associated with aging [57]. Such peroxisomal defects are also supported by the strong positive association of age with saturated and monounsaturated very long-chain fatty acids, including 22:1, 24:0, and 26:0, which are metabolised via β-oxidation exclusively in

peroxisomes. In line with our findings, elevated 24:0 fatty acid levels with age have also been documented [58]. The effect of diet on omega-3 fatty acid levels was further supported by the fatty-acyl side-chain–specific analyses, in which fatty acids of the n3 pathway, including 20:5 and 22:6 (DHA) as well as the 22:5(n3), were positively associated with age, whereas the n-6 fatty acid species 20:3, 22:4, and 22:5(n6) showed a negative association with age. Elongation of very long chain fatty acids protein (*ELOVL2*) and *ELOVL5* genes play a key role in the biosynthesis of very long-chain polyunsaturated fatty acids. However, the recent report of a methylation-induced decrease in the expression of *ELOVL2* with age [59], combined with the opposing associations of the n3 and n6 fatty acids, would suggest the n3 increases observed here were not due to the up-regulation of the biosynthetic pathway, but rather a dietary effect.

The association between age and the plasma lipidome is sex-dependent

In sex-stratified analysis, we observed a clear difference in lipidomic profiles across age groups in men and women. These results suggest that there could be a differential impact of aging on lipid metabolism in men compared to women; understanding these complex interactions will help identify sex-specific lipidomic biomarkers for more personalised interventions. In women, the age fingerprint was marked by a shift starting at about 48 years of age (coinciding with menopause) as opposed to an earlier shift (at about 38 years) in men.

Profiles of lysophosphatidylcholine, lysoalkylphosphatidylcholine, lysoalkenylphosphatidylcholine, alkylphosphatidylcholine, alkylphosphatidylcholine, alkylphosphatidylethanolamine, and alkenylphosphatidylethanolamine with age were particularly different between men and women. These lipids showed a stronger negative association with age in men relative to women, suggesting that age has a differential effect on lyso- and ether-phospholipid metabolism based on sex. As discussed earlier, hydrolysis of phosphatidylcholine by the enzymatic action of PLA2 and LCAT results in higher levels of lysophosphatidylcholine in men. However, it is not clear whether there is a greater decrease in PLA2 and LCAT activity or increased clearance of lysophosphatidylcholine with age in men compared to women.

Lysophospholipids containing omega-3 fatty acids such as DPA (22:5n-3) showed the strongest age–sex interaction (being negatively associated with age in men and positively associated in women). This may reflect differences in the omega-3 synthetic pathway to produce 22:5n-3 [60], in diet, or in both.

Our findings also suggest that aging has a differential effect on peroxisomal ether-phospholipid biosynthesis in men and women. Ether lipids including the alkyl-ether and plasmalogens (alkenyl-ethers) are subclasses of glycerophospholipids that have the same initial step in their biosynthesis [47]. Here, we demonstrated age to be associated with ether-phospholipids, particularly plasmalogens such as alkenylphosphatidylethanolamine and alkenylphosphatidylcholine, differently in men and women. This strong age and sex effect on the circulating plasmalogens suggests that specific mechanisms exist that regulate ether-phospholipid biosynthetic pathway differently between men and women based on their age. Whilst aging has been shown to be associated with a substantial reduction in plasmalogens [61], this has not been shown to be sex-specific. Whether age- and sex-related differences in the action and/or expression of enzymes involved in ether-phospholipid biosynthesis exist or whether sex hormones play a role in driving these differences needs to be further investigated.

Menopausal status is associated with the lipidomic profile

We observed a significant difference in the lipidomic profile between post- and premenopausal women. Menopause is accompanied by hormonal changes, which in turn are associated with increased abdominal fat and risk of cardiometabolic diseases [62, 63]. Menopause status has

been reported to contribute to the rise in risk of CVD, mainly due to changes in atherogenic lipids [64], oxidative stress [65], or endothelial dysfunction in women [66]. Elevated levels of traditional plasma lipid measures including total cholesterol, LDL-C, and triglycerides have been reported in association with menopause [67, 68]. The higher levels of phosphatidylcholine, phosphatidylinositol, sphingomyelin, phosphatidylethanolamine, ceramide, and di- and triacylglycerol species observed in postmenopausal women in the present study may reflect the effect of hormonal changes during menopause on liver lipoprotein metabolism [69]. Previous studies have shown that menopause and abdominal obesity are major determinants of hepatic lipid metabolism [70], and thus, it is not surprising to observe plasma lipidomic changes in relation to menopause. In agreement with our findings, a menopause-associated increase in atherogenic lipoprotein profile, including plasma total cholesterol, LDL-C, triglycerides, very low-density lipoprotein cholesterol (VLDL-C), and apolipoprotein B (ApoB) [27, 67, 68] as well as phosphatidylcholine [27], has been reported. We have also observed an inverse association of alkyl chain containing ether-phospholipids, particularly alkylysophosphatidylcholine, alkylphosphatidylcholine, and alkylphosphatidylethanolamine species. Decreased levels of ether-phospholipids in conditions of oxidative stress, including Alzheimer's disease [49], T2D [14], and CVD [71] have been documented. Menopause may contribute to neurodegenerative and cardiometabolic risk by modulating the metabolism of ether-phospholipids via either impaired liver lipid metabolism or peroxisomal dysfunction.

The association of BMI and smoking with the plasma lipidome

BMI is the most widely used measure to define obesity and is a risk factor for multiple cardiometabolic diseases, including fatty liver disease and T2D. Here, we report BMI associated with 577 lipid species in age- and sex-adjusted models and 508 lipid species in age-, sex-, and clinical lipids (total cholesterol, HDL-C, and triglyceride)-adjusted models. Adjustment for clinical lipids serves to remove the associations driven by the changes in circulating lipoprotein levels (increased VLDL and LDL and decreased HDL) and so reveals the underlying associations with lipid metabolism. Smoking is a potential confounder of the association of BMI with lipid species [72]. However, adjustment for smoking had little effect on these associations.

In the current study, complex glycosphingolipids, including mono-, di-, and trihexosylceramide as well as G_{M3} ganglioside and sulfatide species, showed negative associations, whereas sphingomyelin, dihydroceramide, and deoxyceramide showed positive associations with BMI. These associations confirm and expand on our and others earlier reports of lipid species associated with BMI [16, 19, 73]. Examination of the associations of BMI with lipid ratios revealed highly significant *p*-gain values for many lipid ratios. Of note, the *p*-gain value for the lipid ratio SM(d18:2/16:0)/SM(d18:1/16:0) was 2.85×10^{224}, suggesting an up-regulation of FADS3 activity with increasing BMI. This highlights the interaction of sex with lipid metabolism because FADS3, which converts d18:1 sphingolipids to d18:2, is expressed in higher levels in women (defined by the lipid ratio SM(d18:2/24:0)/SM(d18:1/24:0)) and as reported previously [29].

LPC species were negatively associated with BMI, with the exception of 14:0 or 16:1 fatty-acid–containing species, which showed positive associations. The negative association of LPC with BMI could be due to increased PLA2 activity. A study examining over 1,000 metabolites by Cirulli and colleagues showed a strong metabolome perturbation was associated with BMI and metabolic risk and identified several lysophosphatidylcholine species showing the same direction of association as observed in this study [74]. The positive associations observed for LPC(14:0) and LPC(16:1) likely reflect the increased de novo fatty acid synthesis associated with BMI that produces 14:0 and 16:1 fatty acids. This was highlighted in analyses with the fatty acid composition of the lysophosphatidylcholine species, in which these species showed

the strongest positive association with BMI. Lipogenesis and the subsequent metabolism of fatty acids was also captured in the lipid ratio analyses. The strength of the association of BMI with (16:1/16:0) compared with either of the 2 fatty acids, e.g., signifies a biological role of the stearoyl CoA desaturase 1 (*SCD-1*) enzyme that converts 16:0 fatty acid to 16:1 fatty acid [75]. Increased activity of *SCD-1* has been associated with obesity and insulin resistance [76]. The ratio of 16:1/16:0 has also been associated with waist gain and long-term risk of metabolic syndrome [77]. We also observed certain saturated fatty acid ratios strongly associated with BMI. The conversion of the fatty acid 16:0 to 18:0, captured in the lipid ratio PE(18:0_22:6)/PE(16:0_22:6) (p-gain 6.82×10^{213}) was strongly associated with BMI and likely represents an up-regulation of *ELOVL6* activity. Analyses of related lipid ratios further suggests a down-regulation of *ELOVL1* and/or *ELOVL3* as depicted in Fig 10. However, not all lipid ratios provided clear signals of enzyme activities; the ratio PC(18:0_18:1)/PC(18:1_18:1), which might also be thought to capture the SCD-1 activity, indicated a down-regulation of the conversion of 18:0 to 18:1, in contradiction to the 16:0 to 16:1 conversion. As indicated in Fig 10, other sources of 18:1, including diet, and turnover of 18:1 may also impact these ratios and confound biological interpretation. Thus, caution must be exercised in such interpretations.

One clear lipid ratio signal associated with BMI was the ratio between alkenylphosphatidylethanolamine/alkylphosphatidylethanolamine species; e.g., the PE(P-16:0/22:6)/PE(O-16:0/22:6) ratio displayed a p-gain of 2.57×10^{33}, which suggests the up-regulation of the plasmanylethanolamine delta 1-desaturase enzyme, responsible for the introduction of a characteristic vinyl ether double bond into plasmalogens, with increasing BMI [78]. Notwithstanding the limitations described above, our findings highlight the potential of lipid ratios to identify enzymatic pathways being altered in response to changes in adiposity and other metabolic traits. Our results agree with the findings of previous studies reporting that the strength of associations with metabolic phenotypes increases when using metabolite ratios rather than individual metabolites [79].

In the present study, we found a negative association between BMI and lysophosphatidylcholine, alkylphosphatidylcholine, alkenylphosphatidylcholine, and lysophosphatidylethanolamine; these associations were stronger in men than in women. A study by Gerl and colleagues revealed similar findings [80]; in particular, the stronger negative association of lysophospholipids with BMI in men compared with women was consistent with our findings. Acylcarnitine species showed contrasting associations with BMI based on sex (i.e., negative association in men and positive association in women), suggesting a sex-specific regulation of acylcarnitine. Men and women have been shown to differ in many metabolic aspects; body fat distribution and muscle mass represent well-known contributors to sex differences [81, 82], and these may drive the differential regulation of acylcarnitine and energy metabolism.

Finally, we show that smoking had a strong effect on the plasma lipidome. Our observations of negative associations with lipids containing polyunsaturated fatty acids, particularly omega-3 species such as DHA (C22:6), and positive association with saturated or monounsaturated species agree with previous reports [83, 84]. Smoking modulates essential fatty acid metabolism and results in a reduction of polyunsaturated fatty acid levels through depletion of antioxidants and subsequent increase in lipid peroxidation [85]. These changes in fatty acid metabolism may in turn relate to the significant reduction in HDL-C [86] and the shift towards an atherogenic lipid profile [83, 87].

Comparison with commercial lipidomics solutions

The targeted lipid panel reported in this study constitutes 706 distinct lipid species that span 36 lipid classes/subclasses. For 506 of these, we provide alkyl, alkenyl, and acyl-chain resolution

(e.g., PE(P-18:0/20:4)), whereas only 83 species are reported as the sum totals (e.g., PC(38:2)), with the remainder showing some level of fatty acid definition (e.g., TG(48:2) [neutral loss (NL)-14:0]). Comparable platforms that are able to perform larger cohort studies include the Biocrates AbsoluteIDQ p180 kit, which has been used extensively to provide data on multiple large cohort studies [88, 89] but covers a smaller number of lipid classes, with limited structural resolution owing to technical limitations [90, 91]. However, we do observe general alignment with our findings; Trabado and colleagues measured 185 plasma metabolites in 924 healthy individuals using the AbsoluteIDQ p180 kit and showed higher lysophosphatidylcholine and lower sphingomyelin and phosphatidylcholine species in men relative to women [91]. More recent expanded platforms such as the Biocrates MxP Quant 500 platform and the SCIEX Lipidyzer platform have provided expanded coverage of the lipidome. Although structural resolution is still limited on the MxP Quant 500 platform, greater structural resolution of lipid species is available from the SCIEX Lipidyzer platform. Larger cohorts using these more recent platforms have not yet been reported.

Here, we provide machine-readable lipid identifiers for 91.3% of the lipid species from the SwissLipids database to facilitate comparison of our results with other studies (S15 Table).

Limitations and strengths of the study

The major strengths of this study are 1) the large population-based sample size of the cohort, comprising over 10,000 participants in the discovery cohort and over 4,000 in the replication set and 2) improved lipidome coverage (over 700 species across 36 classes/subclasses). This study now provides a powerful resource for further lipidomic studies. The major limitation is, however, the cross-sectional nature of the present study, which did not allow us to determine causality of the relationships. In addition, the response rate of the AusDiab cohort was relatively low; hence further studies are needed to prove the generalisability our findings to other population groups and ethnicities. Despite the limitations, our study has uncovered novel, to our knowledge, associations of lipidomic biomarkers with common cardiometabolic risk factors, thus improving our current understanding of lipid biology. The sex-specific nature of the association of the lipidome with age and BMI may underpin the sex differences in the pathogenesis of age-related cardiometabolic diseases.

Our findings pave the way for further evaluation of the associations of plasma lipidomic profiles with disease risk factors in a sex-specific manner. We suggest that sex plays an important role in lipid metabolism associated with cardiometabolic risk, and understanding this will be essential for sex-specific biomarker discovery and precision medicine. Indeed, it is important to consider age- and sex-specific stratification during the design and analysis of cohort studies involving lipidomics.

Materials and methods

Study cohorts

AusDiab. We utilised all baseline fasting plasma samples from the AusDiab cohort (n = 10,339). The AusDiab cohort is the largest population-based prospective population study that was established to study the prevalence and risk factors of diabetes and CVD in the Australian adult population. The baseline survey was conducted in 1999–2000, with 11,247 participants aged ≥25 years from randomly selected areas from the 6 states and the Northern Territory, comprising 42 urban and rural areas of Australia, using a stratified cluster sampling method. The detailed description of study population, methods, and response rates of the AusDiab cohort is found elsewhere [92]. Measurement techniques for clinical lipids, including fasting serum total cholesterol, HDL-C, and triglycerides, as well as for height, weight, BMI,

and other behavioural risk factors, have been described previously [93]. Here, we performed a comprehensive plasma lipidomic analysis on a total of 10,358 baseline fasting plasma samples after excluding samples from pregnant women ($n = 19$), those with missing data ($n = 279$), or those whose fasting plasma samples were unavailable or of inadequate amount for lipid extraction ($n = 591$). Following lipidomic analysis, additional samples ($n = 19$) were excluded from the final analysis because of technical issues. Thus, 10,339 participants (5,229 [51%] women) were included in the present analysis. The mean (range) age was 49 (25–91) and 47 (25–95) for women and men, respectively. The baseline characteristics of participants can be found in supplementary file (S16 Table).

Busselton study cohort. A total of 4,492 participants in the 1994/95 survey of the ongoing epidemiological study (the BHS) were included. The BHS is a population-based study in the town of Busselton, Western Australia; the participants are predominantly of European origin. The brief description of study subjects is found elsewhere (S16 Table). The details of the study characteristics and measurements for HDL-C, LDL-C, triglycerides, total cholesterol, and BMI are described elsewhere [94].

Ethics statement. This study used data sets from the AusDiab biobank (project grant APP1101320) approved by the Alfred Human Research Ethics Committee, Melbourne, Australia (project approval number, 41/18) and the BHS cohort (informed consent obtained from all participants, and the study was approved by the University of Western Australia Human Research Ethics Committee [UWA HREC; approval number, 608/15]). Both studies were conducted in accordance with the ethical principles of the Declaration of Helsinki.

Lipid extraction. Lipid extraction and analysis was carried out in a total of 28 batches; each run batch comprised 384 patient samples, 26 technical quality control (TQC) samples, 21 plasma quality control (PQC) samples, and 8 National Institute of Standards and Technology (NIST) samples. For each batch, a robot-assisted lipid extraction was carried out using the butanol/methanol method; the details are as described previously [95]. Briefly, 10 μL of plasma was mixed with 100 μL of butanol/methanol (1:1) with 10 mM ammonium formate. A standard mix containing some 22 internal standards (Table 1) representing 36 lipid classes were also included in the extraction solvent. The list of all internal standards is found in supplementary file (S17 Table). Samples were vortexed thoroughly, followed by sonication for 60 min at room temperature. Each sample was then centrifuged (14,000 × *g*, 10 min, 20˚C). After centrifugation is complete, the supernatant containing the crude lipid extract was collected and transferred into Teflon glass vials with glass inserts.

LC-MS analysis

Lipidomic analysis was performed using LC electrospray ionisation MS/MS (LC-ESI-MS/MS). An Agilent 6490 triple quadrupole (QQQ) mass spectrometer (Agilent 1290 series HPLC system and a ZORBAX eclipse plus C18 column [2.1 × 100 mm × 1.8 μm; Agilent, Santa Clara, CA, USA]) in positive ion mode was used (details of the method and chromatography gradient have been described previously [16]). The solvent system consisted of solvent A, 50% H_2O/30% acetonitrile/20% isopropanol (v/v/v) containing 10 mM ammonium formate, and solvent B, 1% H_2O/9% acetonitrile/90% isopropanol (v/v/v) containing 10 mM ammonium formate. We used a linear gradient with a 14-minute cycle time and a 1-μL sample injection per sample. The following mass spectrometer conditions were used: gas temperature, 150˚C; gas flow rate, 17 L/min; nebuliser, 20 psi; sheath gas temperature, 200˚C; capillary voltage, 3,500 V; and sheath gas flow, 10 L/min. Given the large sample size, samples were run across several batches, as described above.

Table 1. LC-MS/MS instrument conditions for targeted lipidomic analysis.

Lipid Class/Subclass	Parent Ion	Fragmentation	No. of Lipid Species	ISTD	ISTD (pmol)/ Sample	Collision Energy (V)
Sphingosine	[M + H]+	NL, 18.0 Da	4	Sph(d17:1)	20	8
Sphingosine-1-phosphate	[M + H]+	sphingoid-base specific	4	Sph(d17:1)	25	10
Dihydroceramide	[M + H]+	sphingoid-base specific	6	dhCer(d18:0/8:0)	50	31
Ceramide	[M + H]+	sphingoid-base specific	50	Cer(d18:1-d7/17:0)	50	25
Deoxyceramide	[M + H]+	sphingoid-base specific	11	Cer(d18:1/17:0)	50	25
Monohexosylceramide	[M + H]+	sphingoid-base specific	14	HexCer (d18:1/16:0) d3	50	33
Dihexosylceramide	[M + H]+	sphingoid-base specific	10	Hex2Cer(d18:1/16:0) d3	50	53
Trihexosylcermide	[M + H]+	PI, m/z 264.3	6	Hex3Cer(d18:1/17:0)	25	57
G_{M3} ganglioside	[M + H]+	sphingoid base	8	Hex3Cer(d18:1/17:0)	25	57
G_{M1} ganglioside	[M + 2H]2 +	PI, m/z 366.2	1	Hex3Cer(d18:1/17:0)	25	9
Sulfatide	[M + H]+	PI, m/z 264.3	10	sulfatide(d18:1/12:0)	10	56
Ceramide-1-phosphate	[M + H]+	PI, m/z 264.3	1	Cer(d18:1/17:0)	50	29
Sphingomyelin	[M + H]+	PI, m/z 184.1	44	SM(d18:1/12:0)	100	25
Phosphatidylcholine	[M + H]+	PI, m/z 184.1	70	PC(13:0/13:0)	100	21
Alkylphosphatidylcholine	[M + H]+	PI, m/z 184.1	22	PC(13:0/13:0)	100	21
Alkenylphosphatidylcholine	[M + H]+	PI, m/z 184.1	26	PC(13:0/13:0)	100	21
Lysophosphatidylcholine	[M + H]+	PI, m/z 184.1 and m/z 104.1	61	LPC(13:0)	100	21
Lysoalkylphosphatidylcholine	[M + H]+	PI, m/z 104.1	10	LPC(13:0)	100	21
Lysoalkenylphosphatidylcholine	[M + H]+	PI, m/z 104.1	6	LPC(13:0)	100	21
Phosphatidylethanolamine	[M + H]+	NL, 141.0 Da	37	PE(17:0/17:0)	100	17
Alkylphosphatidylethanolamine	[M + H]+	NL, 141.0 Da	14	PE(17:0/17:0)	100	17
Alkenylphosphatidylethanolamine	[M + H]+	acyl-chain specific	54	PE(17:0/17:0)	100	17
Lysophosphatidylethanolamine	[M + H]+	NL, 141.0 Da	14	PE(17:0/17:0)	100	17
Lysoalkenylphosphatidylethanolamine	[M + H]+	NL, 171.9 Da	4	PE(17:0/17:0)	100	19
Phosphatidylinositol	[M + NH4] +	NL, 277.0 Da	27	PE(17:0/17:0)	100	17
Lysophosphatidylinositol	[M + NH4] +	NL, 277.0 Da	8	LPI(13:0)	20	17
Phosphatidylserine	[M + H]+	NL, 185.0 Da	7	PS(17:0/17:0)	50	25
Phosphatidylglycerol	[M + NH4] +	NL, 189.0 Da	4	PG(17:0/17:0)	50	21
Free cholesterol	[M + NH4] +	PI, m/z 369.3	1	COH d7	10,000	23
Dehydrocholesterol	[M + NH4] +	PI, m/z 369.4	4	CE(18:0) d6	1,000	23
Cholesteryl ester	[M + NH4] +	PI, m/z 369.3	28	CE(18:0) d6	1,000	10
Acylcarnitines	[M + H]+	PI, m/z 85.1	14	Acylcarnitine(16:0) d3	10	30
Diacylglycerol	[M + NH4] +	NL, fatty acid	25	DG(15:0/15:0)	200	21
Triacylglycerol	[M + NH4] +	NL, fatty acid	112	TG(17:0/17:0/17:0)	100	21
Alkyl-diacylglycerol	[M + NH4] +	NL, fatty acid	28	TG(17:0/17:0/17:0)	100	21

(*Continued*)

Table 1. (Continued)

Lipid Class/Subclass	Parent Ion	Fragmentation	No. of Lipid Species	ISTD	ISTD (pmol)/ Sample	Collision Energy (V)
Ubiquinone	[M + NH4] +	PI, m/z 197.0	1	Hex3Cer(d18:1/17:0)	50	21

Abbreviations: CE, cholesteryl ester; Cer, ceramide; Cer-1-P, ceramide-1-phosphate; COH, free cholesterol; DE, dehydrocholesterol; deoxyCer, deoxyceramide; DG, diacylglycerol; dhCer, dihydroceramide; GM3/1, G_{M3}/G_{M1} ganglioside; HexCer, monohexosylceramide; Hex2Cer, dihexosylceramide; Hex3Cer, trihexosylceramide; ISTD, internal standard; LC-M/MS, liquid chromatography tandem mass-spectrometry; LPC, lysophosphatidylcholine; LPC(O), lysoalkylphosphatidylcholine; LPC(P), lysoalkenylphosphatidylcholine; LPE, lysophosphatidylethanolamine; LPE(P), lysoalkenylphosphatidylethanolamine; LPI, lysophosphatidylinositol; NL, neutral loss; PC, phosphatidylcholine; PC(O), alkylphosphatidylcholine; PC(P), alkenylphosphatidylcholine; PE, phosphatidylethanolamine; PE(O), alkylphosphatidylethanolamine; PE(P), alkenylphosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; Sph, sphingosine; S-1-P, sphingosine-1-phosphate; TG, triacylglycerol; TG(O), alkyl-diacylglycerol.

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Quality control

As part of monitoring the quality of sample extraction and LC-MS/MS analysis, PQC samples consisting of a pooled set of 10 healthy individuals were incorporated into the analysis at a rate of 1 PQC per 20 plasma samples. Additional, quality control samples utilising the NIST 1950 reference plasma sample (obtained from NIST, Gaithersburg, MD, USA) were inserted every 40 patient samples to facilitate future alignment with other studies. TQC samples consisted of PQC sample extracts that 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/ 20 plasma samples. TQCs were used to monitor for changes in peak area, width, and retention time to determine the performance of the LC-MS/MS analysis.

Lipid quantitation and statistical analysis

Chromatographic peaks for each lipid were integrated using the Mass Hunter (B.07.00, Agilent Technologies) software. Relative quantification of lipid species was determined by comparing the peak areas of each lipid in each patient sample with the relevant internal standard (Table 1). Whilst most lipid isotopes were resolved chromatographically, we note that CE (22:5) (n3) was not resolved from CE(22:6), and so the signal for this species also contained the M + 2 isomer for CE(22:6). Quantification of lipid classes was determined as the sum composition of the lipid species within each class. For validation of lipid classes, only the 563 lipid species common to both data sets were used for the calculation of lipid classes. Over 90% of the lipid species were measured with a coefficient of variation <20% (based on PQC samples). Batch effects were corrected using a median centring approach utilising PQC samples [96]. Outliers deemed to be of technical origin, such as missed injections (n = 19), were excluded from the downstream analysis. Prior to statistical analysis, lipid data were $\log_{10}$ transformed. A multivariable linear regression adjusted for sex and BMI was performed to evaluate the associations with age. Similarly, sex-related differences in lipid species were assessed by linear regression adjusting for age and BMI or age, BMI, total cholesterol, HDL-C, and triglycerides. We included clinical lipid measures in the model to be able to identify individual lipid species associated with phenotypes independently of lipoprotein metabolism. Furthermore, we performed age–sex interaction analyses (including an age- and sex-interaction term in the model). For interpretation of results, β-coefficients from linear regression analysis and 95% confidence intervals associated with these were converted to percentage differences (% difference = $(10^{\beta\text{-coefficient}} - 1) \times 100$). To account for false discovery rate, p-values were corrected for multiple comparisons using the Benjamini and Hochberg procedure [97]. Sex-stratified heat maps were

constructed to visualise lipid classes/species levels across age groups, averaging the lipid levels of participants into 1-year intervals. The averaged lipid class/species levels were normalised to a reference group consisting of the 25- to 34-year–olds. The mean value of each lipid in the reference group was subtracted from all 1-year interval means for that lipid, then the resulting values were scaled by the standard deviation for both sexes in the reference group ([lipid mean in a given age group − reference mean]/reference standard deviation). Lipid ratios (747 × [747 − 1]/2 = 278,631) were generated on the log scale (ratio of lipid a/lipid b is log[a/b] = −log[b/a], so we can refer to ratios as lipid pairs). The association of lipid pairs with phenotypes was assessed using linear models, with the lipid–lipid pair used as the outcome. To evaluate the difference in using lipid ratios compared to individual lipids, the *p*-gain statistic was calculated, defined as the lower phenotype *p*-value from the 2 lipids in the ratio divided by the phenotype *p*-value when using the lipid ratio as the outcome. Here, we applied a conservative cutoff point for the *p*-gain as described previously [28]. All statistical analyses were performed in R (3.5.2).

Nomenclature of lipids

The naming system for all lipid species reported in this paper follows guidelines developed by LIPID MAPS and others [8, 9]. Some species are reported with the (a), (b), or (c) notations because it is not possible to provide full structural detail for such species. The (a), (b), and (c) denote the elution order from chromatographic column. PE(P-17:0/22:6)(a), e.g., refers to the species that elutes prior to PE(P-17:0/22:6)(b). For most glycerophospholipids that have 2 FA chains, the naming may be based on total FA carbon and double-bond content, e.g., PC(38:6); when the FA chain composition is known whilst the sn1 and sn2 positions are undetermined, PC(38:6) can be expressed as PC(16:0_22:6), or when sn1 and sn2 positions are known, as PC (16:0/22:6). For TG species, we used NL MS scans, and therefore, these species are expressed as total FA composition and the specific NL associated with it; e.g., a TG species composed of 56 FA carbons and 2 double bonds in which the NL corresponds to an 18:2 FA is expressed as TG(56:2) [NL-18:2].

Supporting information

S1 Fig. CV (%). The CVs for each lipid species were computed separately for men (blue circles) and women (pink circles) as follows: (SD/mean concentration) × 100. Each circle represents individual lipid species. See S1 Data for underlying data. CV, coefficient of variation
(TIF)

S2 Fig. Pearson's correlation coefficients between all lipid species in the whole cohort (men and women combined). Pearson's correlation coefficients were calculated between all pairs of lipid species. The correlation coefficients were plotted as a heat map. The colour scale illustrates the magnitude and direction of correlation between lipid species: red, positive correlations and blue, negative correlations.
(ZIP)

S3 Fig. Pearson's correlation coefficients between all lipid species in men. Pearson's correlation coefficients were calculated between all pairs of lipid species. The correlation coefficients were plotted as a heat map. The colour scale illustrates the magnitude and direction of correlation between lipid species: red, positive correlations and blue, negative correlations.
(ZIP)

S4 Fig. Pearson's correlation coefficients between all lipid species in women. Pearson's correlation coefficients were calculated between all pairs of lipid species. The correlation coefficients were plotted as a heat map. The colour scale illustrates the magnitude and direction of

correlation between lipid species: red, positive correlations and blue, negative correlations.
(ZIP)

S5 Fig. Differences (men relative to women) in the Pearson's correlation coefficients between all lipid species. The differences in the Pearson's correlation coefficients were calculated for all pairs (men correlation coefficients subtracted from women's correlation coefficients). The differences in the correlation coefficients were plotted as a heat map. The colour scale illustrates the magnitude and direction of difference in the correlation of lipid species in men and women.
(ZIP)

S6 Fig. Correlation between regression coefficients of lipids associated with sex. The regression coefficients on *x* axis (AusDiab) and *y* axis (Busselton) cohorts have an $R^2 = 0.8398$. See S1 Data for underlying data. AusDiab, Australian Diabetes, Obesity and Lifestyle Study.
(TIF)

S7 Fig. Associations between age and plasma lipid species. A random-effect meta-analysis between age and log-transformed lipid species concentration was performed on 10,339 individuals (from the AusDiab cohort) and 4,207 participants (from the BHS cohort) adjusting for sex, BMI, and cholesterol, HDL-C, and triglyceride levels. The pooled effect size as percentage difference per year for each lipid species is displayed on the *x* axis. Open circles show nonsignificant species, grey circles show species with $p < 0.05$, and brown circles show the 30 most significant species after correction for multiple comparisons (6.29×10^{-60}). Whiskers represent 95% confidence intervals. See S1 Data for the underlying data. AusDiab, Australian Diabetes, Obesity and Lifestyle Study; BHS, Busselton Health Study; BMI, body mass index; HDL-C, high-density lipoprotein cholesterol.
(TIF)

S8 Fig. Age-related sex differences in plasma lipid class levels in the AusDiab cohort. The cohort was stratified into women ($n = 5,685$, top panel) and men ($n = 4,654$, bottom panel). Average lipid class levels were calculated for each 1-year age interval group and then centred and scaled to a 'reference' group (25- to 34-year–old participants). Age groups (by 1-year intervals) are displayed on the *y* axis and the lipid class on the *x* axis. The analysis was adjusted for BMI and clinical lipids. Colour intensities represent the number of standard deviations away from the mean lipid levels of the reference group (25–34 years old). AusDiab, Australian Diabetes, Obesity and Lifestyle Study; BMI, body mass index.
(TIF)

S9 Fig. Age-related, sex differences in plasma lipid species levels in the AusDiab cohort. The cohort was stratified into men ($n = 4,654$, left panel) and women ($n = 5,685$, right panel). Average lipid species levels were calculated for each 1-year age interval and then centred and scaled to a 'reference' group corresponding to the 25- to 34-year–old participants. Age groups (by 1-year intervals) are displayed on the *x* axis and the lipid species on the *y* axis. Colour intensities represent the number of standard deviations away from the mean lipid levels of the reference group (25–34 years old). AusDiab, Australian Diabetes, Obesity and Lifestyle Study.
(EPS)

S10 Fig. Regression coefficients between lipid species and BMI in the AusDiab and Busselton cohorts. The correlation between regression coefficients of each lipid species associated with BMI in the AusDiab (*x* axis) and in the Busselton cohort (*y* axis) was examined. See S1 Data for underlying data. AusDiab, Australian Diabetes, Obesity and Lifestyle Study; BMI,

body mass index.
(TIF)

S11 Fig. Correlation between metabolic risk factors. Pearson's correlation coefficients were calculated for each pair of risk factors. Colour intensities show the strength of correlation. Significant correlations at p-value $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, respectively.
(TIF)

S12 Fig. Association of BMI, WC, and WHR with lipid species. Linear regression analyses of BMI (A), WC (B), and WHR (C) with lipid species were performed adjusting for age, sex, cholesterol, HDL-C, and triglycerides. Open grey symbols, closed grey symbols, and closed orange symbols show lipid species with corrected p-values >0.05, <0.05, and $<1 \times 10^{-11}$, respectively. Whiskers represent 95% confidence intervals. See S1 Data for the underlying data. BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; WC, waist circumference; WHR, waist/hip ratio.
(TIF)

S13 Fig. Association between BMI, WC, or WHR and the plasma lipidome. A linear regression between log-transformed lipid concentration and BMI or WC or WHR was performed on 10,339 subjects adjusting for age, sex, total cholesterol, HDL-C, and triglycerides. (A) Venn diagram showing overlaps and unique associations of lipid species with BMI, WC, and WHR. (B), (C), and (D) show lipid species significantly associated with BMI only, WHR, only, and WC only, respectively. See S1 Data for the underlying data. BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; WC, waist circumference; WHR, waist/hip ratio.
(TIF)

S14 Fig. Association of smoking with plasma lipidomic profile. A logistic regression analysis between smoking status and $\log_{10}$-transformed lipid species concentrations was performed adjusting for age, sex, BMI, total cholesterol, HDL-C, and triglycerides. Grey circles show nonsignificant species ($p > 0.05$), and grey and pink circles show species with $p < 0.05$ and $p < 2.95 \times 10^{-9}$, respectively, after correction for multiple comparisons. The whiskers represent 95% confidence intervals. See S1 Data for underlying data. BMI, body mass index; HDL-C, high-density lipoprotein cholesterol.
(TIF)

S15 Fig. Correlation matrix between fatty acid composition of lysophospholipids. Pearson's correlation analysis was performed between 27 fatty acids. Blue coloured eclipses in each square represent positive correlations, and orange show negative correlations. See S1 Data for the underlying data.
(TIF)

S16 Fig. Association between age, sex, and BMI with plasma lysophosphatidylcholine fatty acids. Multivariable linear regression analysis of age (A), sex (B), and BMI (C) with log-transformed lipid composition data was performed on 10,339 AusDiab participants adjusting for BMI, age, and sex (as appropriate) together with total cholesterol, HDL-C, and triglycerides. Open grey circles represent nonsignificant fatty acids (corrected $p > 0.05$). Orange circles show fatty acids associated with BMI, age, or sex (corrected $p < 0.05$). Bars represent the 95% confidence intervals. See S1 Data for the underlying data. AusDiab, Australian Diabetes, Obesity and Lifestyle Study; BMI, body mass index; HDL-C, high-density lipoprotein cholesterol.
(TIF)

S17 Fig. Selected lipid ratios and sphingolipid metabolic pathway associated with BMI. A linear regression adjusted for age, sex, total cholesterol, HDL-C, and triglycerides was

performed between individual lipids or lipid concentration ratios and BMI. Each of the panels from (A)–(F) represent association of BMI with a given lipid ratio and individual lipids species that make up the ratio. (G) An overview of the sphingolipid biosynthetic pathway. (H) FADS3 as a sphingoid base desaturase responsible for increased d18:2/d18:1 sphingolipid ratio. BMI, body mass index; DEGS, dihydroceramide desaturase; FADS3, fatty acid desaturase 3; GCS, glucosylceramide synthase; HDL-C, high-density lipoprotein cholesterol; SMase, sphingomyelinase; SMS, sphingomyelin synthase.
(TIF)

S18 Fig. The d18:2 to d18:1 ratios of sphingolipid species associated with sex. A linear regression adjusted for age, BMI, total cholesterol, HDL-C, and triglycerides was performed between individual lipids or lipid concentration ratios and sex. (A) represents association of sex with the ratio between d18:2/d18:1 sphingolipid. (B) FADS3 as a sphingoid base desaturase responsible for the conversion of d18:2/d18:1 sphingolipid. BMI, body mass index; FADS3, fatty acid desaturase 3; HDL-C, high-density lipoprotein cholesterol.
(TIF)

S19 Fig. Association of BMI with the ratio between PE(P)/PE(O). A linear regression adjusted for age, sex, total cholesterol, HDL-C, and triglycerides was performed between BMI and individual lipid or lipid concentration ratio. BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; PE(O), alkylphosphatidylethanolamine; PE(P), alkenylphosphatidylethanolamine.
(TIF)

S1 Table. The correlation structure between all lipid species in the whole cohort (men and women combined).
(XLSX)

S2 Table. The correlation structure between all lipid species in men.
(XLSX)

S3 Table. The correlation structure between all lipid species in women.
(XLSX)

S4 Table. Differences in the Pearson's correlations (men relative to women) for all lipid species.
(XLSX)

S5 Table. Validation of the association between sex and lipid species.
(XLSX)

S6 Table. Characteristics of women participants.
(XLSX)

S7 Table. Validation of the association of BMI with lipid species. BMI, body mass index.
(XLSX)

S8 Table. Association of BMI with lipid concentration ratios. BMI, body mass index.
(XLSX)

S9 Table. Association of plasma phospholipid fatty acid composition with BMI, age, or sex. BMI, body mass index.
(XLSX)

S10 Table. Association of age with lipid concentration ratios.
(XLSX)

S11 Table. Association of sex with lipid concentration ratios.
(CSV)

S12 Table. Association of sphingolipid ratios with BMI, age, or sex. BMI, body mass index.
(XLSX)

S13 Table. Association of the d182:1/d181 sphingoid base ratios with BMI, age, or sex. BMI, body mass index.
(XLSX)

S14 Table. Association of the PE(P)/PE(O) ratios with BMI, age, or sex. BMI, body mass index; PE(O), alkylphosphatidylethanolamine; PE(P), alkenylphosphatidylethanolamine.
(XLSX)

S15 Table. SwissLipids identifiers for lipid species.
(XLSX)

S16 Table. Characteristics of study participants.
(XLSX)

S17 Table. MRM transitions and conditions for examined lipid species. MRM, multiple reaction monitoring.
(XLSX)

S1 Data. Numerical data underlying figures and supplemental figures.
(XLSX)

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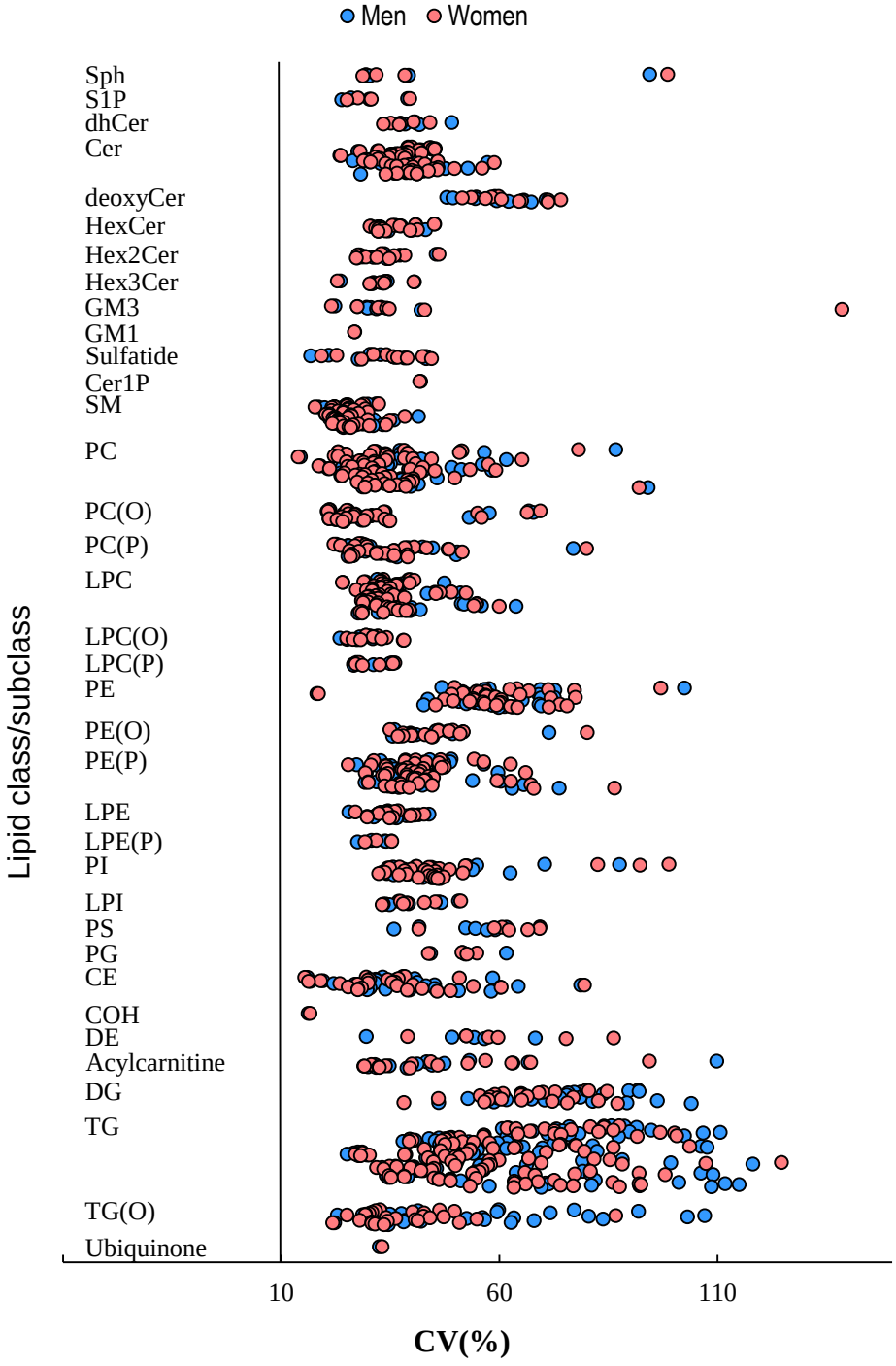


S1 Fig. CV (%). The CVs for each lipid species were computed separately for men (blue circles) and women (pink circles) as follows: (SD/mean concentration) × 100. Each circle represents individual lipid species. See S1 Data for underlying data. CV, coefficient of variation.

Chapter 3. S2 Fig. Pearson's correlation coefficients between all lipid species in the whole cohort (men and women combined).

Pearson's correlation coefficients were calculated between all pairs of lipid species. The correlation coefficients were plotted as a heat map. The colour scale illustrates the magnitude and direction of correlation between lipid species: red, positive correlations and blue, negative correlations. https://doi.org/10.1371/journal.pbio.3000870.s002

Chapter 3. S3 Fig. Pearson's correlation coefficients between all lipid species in men.

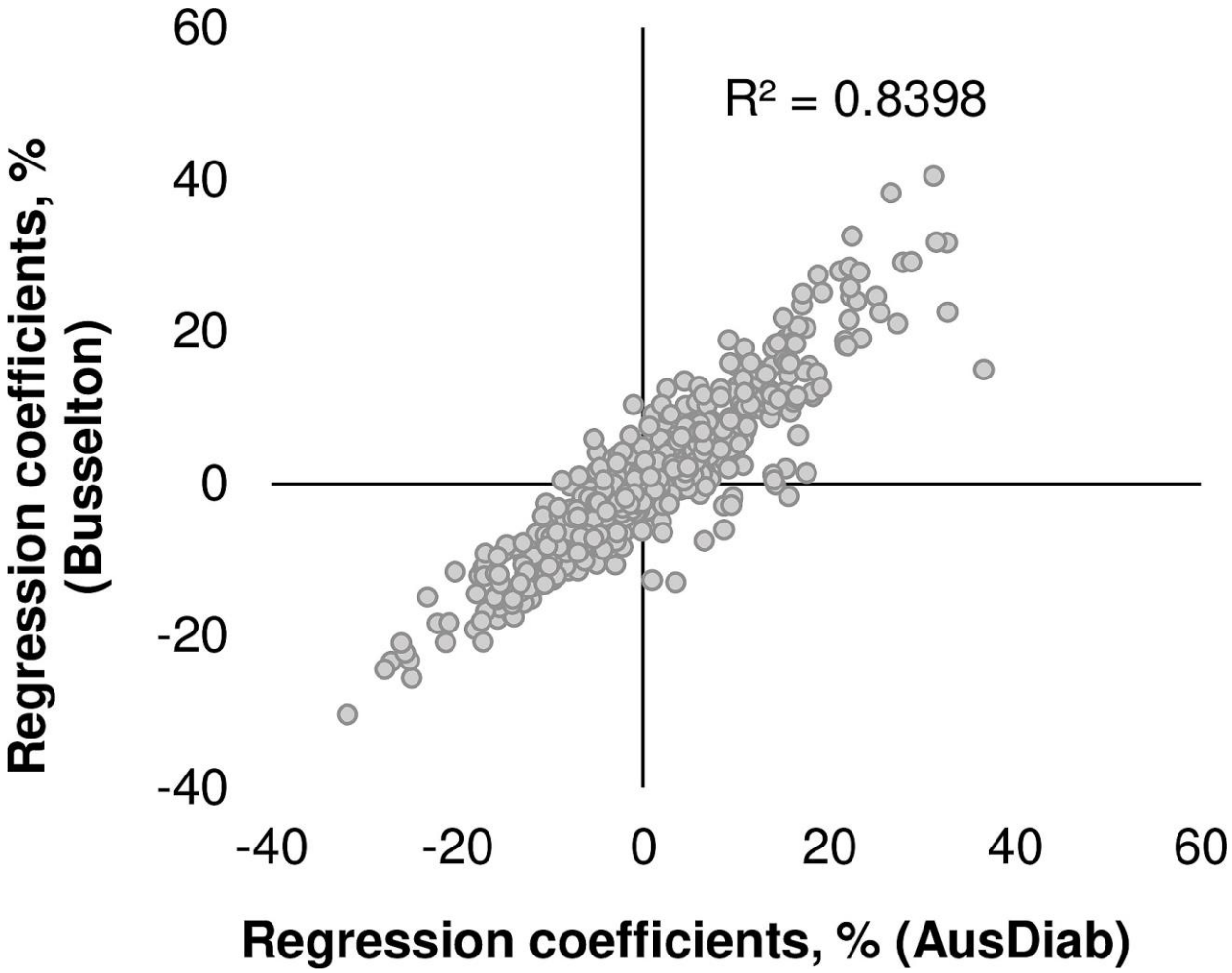
Pearson's correlation coefficients were calculated between all pairs of lipid species. The correlation coefficients were plotted as a heat map. The colour scale illustrates the magnitude and direction of correlation between lipid species: red, positive correlations and blue, negative correlations. https://doi.org/10.1371/journal.pbio.3000870.s003

Chapter 3. S4 Fig. Pearson's correlation coefficients between all lipid species in women.

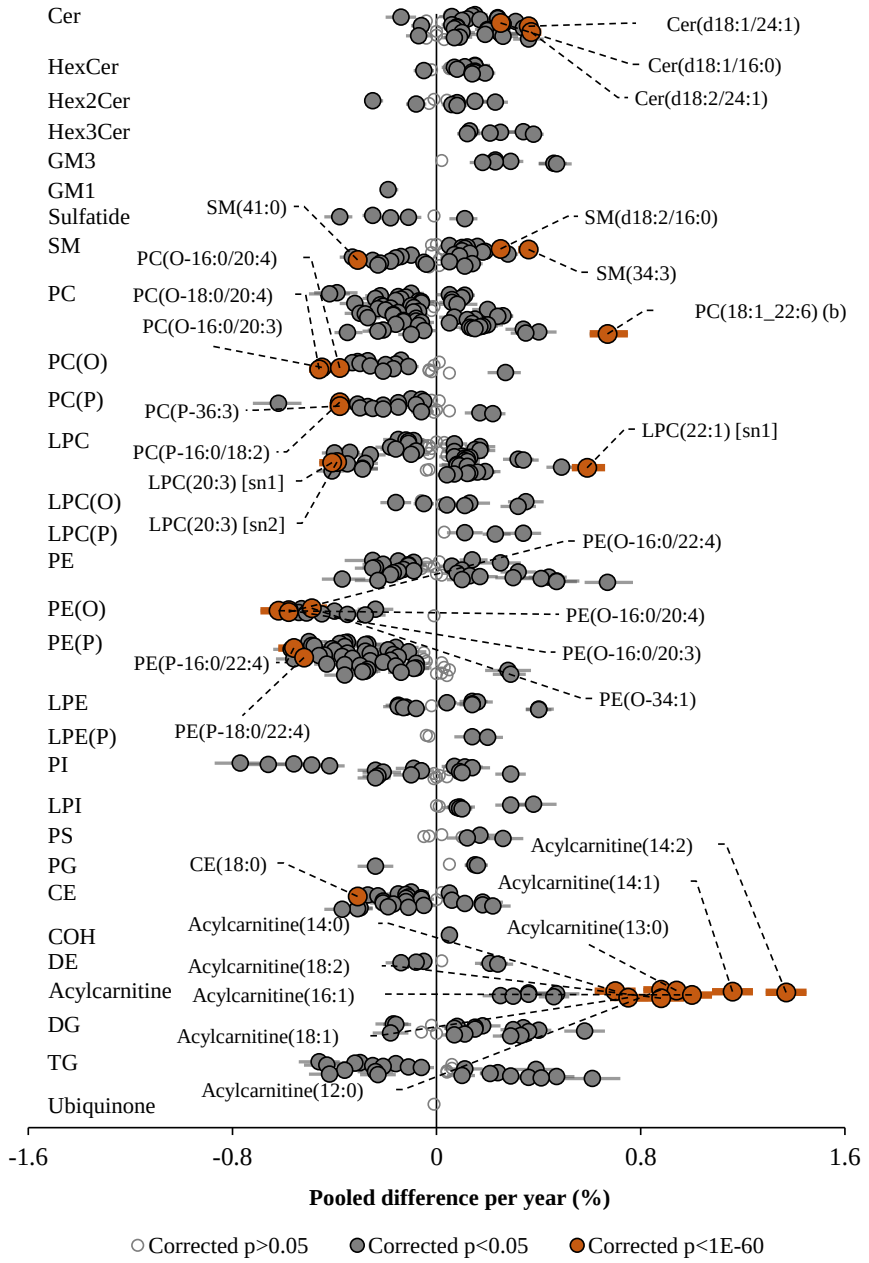
Pearson's correlation coefficients were calculated between all pairs of lipid species. The correlation coefficients were plotted as a heat map. The colour scale illustrates the magnitude and direction of correlation between lipid species: red, positive correlations and blue, negative correlations. https://doi.org/10.1371/journal.pbio.3000870.s004

Chapter 3. S5 Fig. Differences (men relative to women) in the Pearson's correlation coefficients between all lipid species.

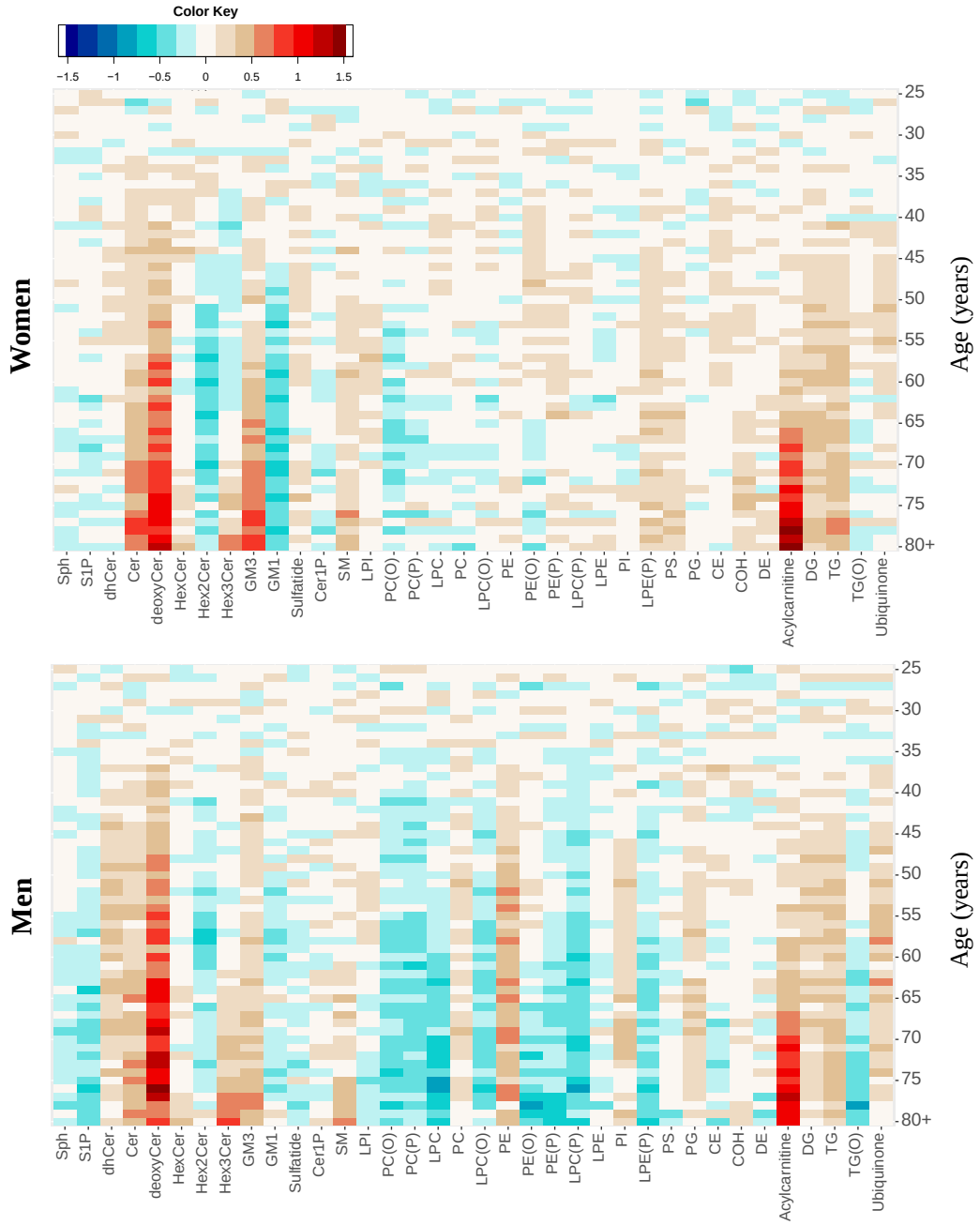
The differences in the Pearson's correlation coefficients were calculated for all pairs (men correlation coefficients subtracted from women's correlation coefficients). The differences in the correlation coefficients were plotted as a heat map. The colour scale illustrates the magnitude and direction of difference in the correlation of lipid species in men and women. https://doi.org/10.1371/journal.pbio.3000870.s005



S6 Fig. Correlation between regression coefficients of lipids associated with sex. The regression coefficients on x axis (AusDiab) and y axis (Busselton) cohorts have an R2 = 0.8398. See S1 Data for underlying data. AusDiab, Australian Diabetes, Obesity and Lifestyle Study.



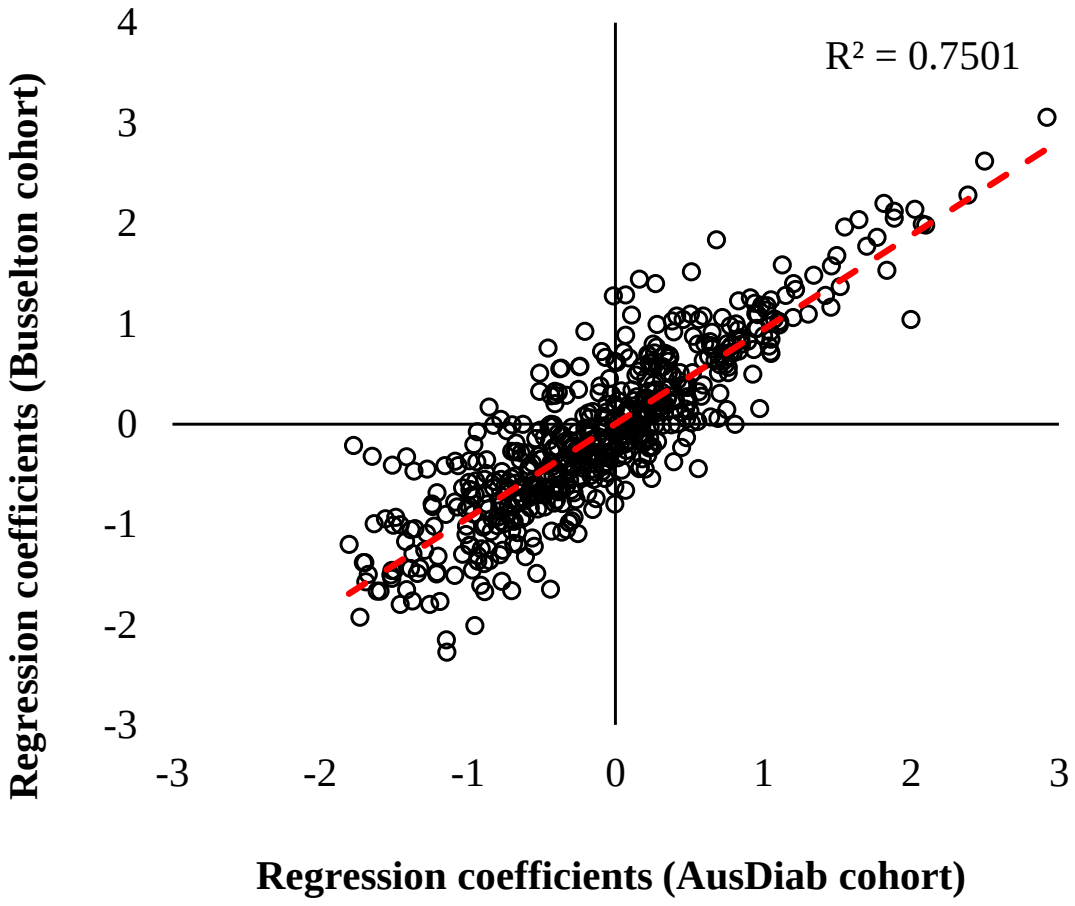
S7 Fig. Associations between age and plasma lipid species. A random-effect meta-analysis between age and log-transformed lipid species concentration was performed on 10,339 individuals (from the AusDiab cohort) and 4,207 participants (from the BHS cohort) adjusting for sex, BMI, and cholesterol, HDL-C, and triglyceride levels. The pooled effect size as percentage difference per year for each lipid species is displayed on the *x* axis. Open circles show nonsignificant species, grey circles show species with $p < 0.05$, and brown circles show the 30 most significant species after correction for multiple comparisons (6.29×10^{-60}). Whiskers represent 95% confidence intervals. See S1 Data for the underlying data. AusDiab, Australian Diabetes, Obesity and Lifestyle Study; BHS, Busselton Health Study; BMI, body mass index; HDL-C, high-density lipoprotein cholesterol.



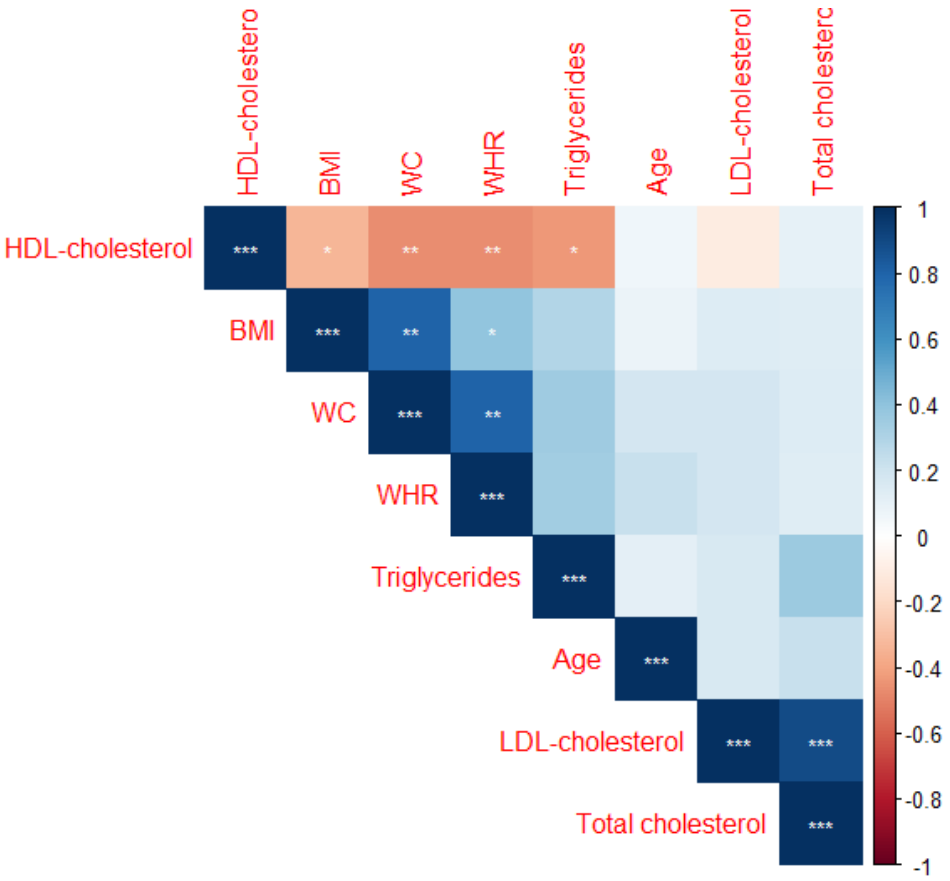
S8 Fig. The cohort was stratified into women (*n* = 5,685, top panel) and men (*n* = 4,654, bottom panel). Average lipid class levels were calculated for each 1-year age interval group and then centred and scaled to a 'reference' group (25- to 34-year–old participants). Age groups (by 1-year intervals) are displayed on the *y* axis and the lipid class on the *x* axis. The analysis was adjusted for BMI and clinical lipids. Colour intensities represent the number of standard deviations away from the mean lipid levels of the reference group (25–34 years old). AusDiab, Australian Diabetes, Obesity and Lifestyle Study; BMI, body mass index.

Chapter 3. S9 Fig. Age-related, sex differences in plasma lipid species levels in the AusDiab cohort.

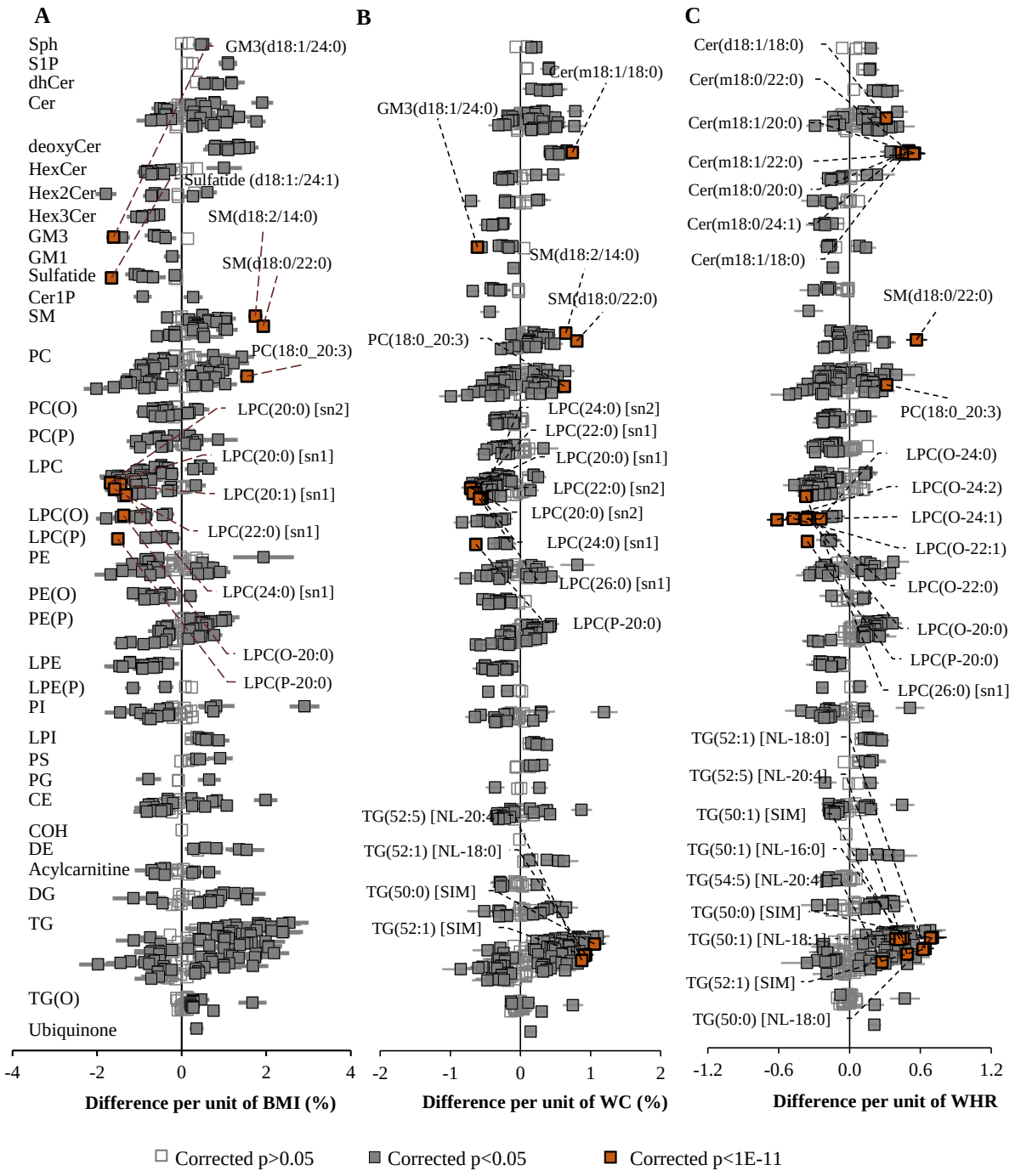
The cohort was stratified into men (*n* = 4,654, left panel) and women (*n* = 5,685, right panel). Average lipid species levels were calculated for each 1-year age interval and then centred and scaled to a 'reference' group corresponding to the 25- to 34-year–old participants. Age groups (by 1-year intervals) are displayed on the *x* axis and the lipid species on the *y* axis. Colour intensities represent the number of standard deviations away from the mean lipid levels of the reference group (25–34 years old). AusDiab, Australian Diabetes, Obesity and Lifestyle Study. https://doi.org/10.1371/journal.pbio.3000870.s009



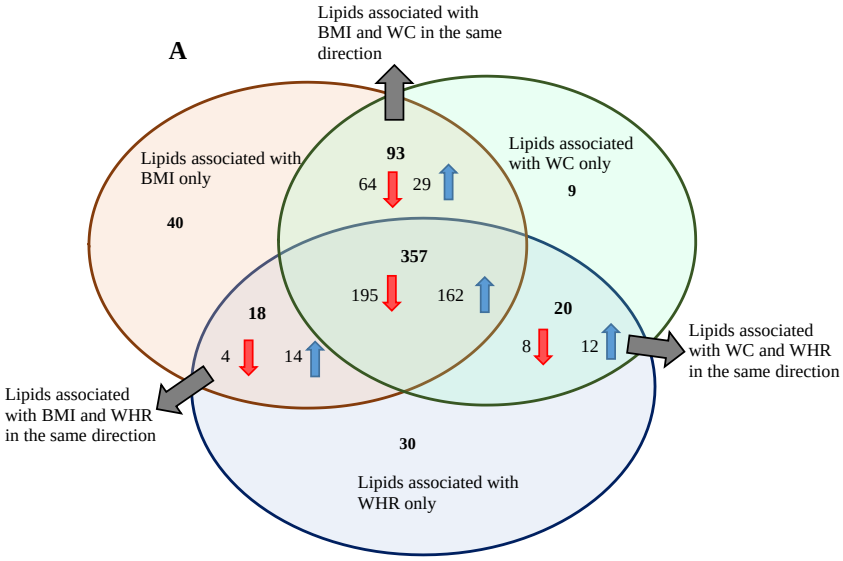
S10 Fig. Regression coefficients between lipid species and BMI in the AusDiab and Busselton cohorts. The correlation between regression coefficients of each lipid species associated with BMI in the AusDiab (x axis) and in the Busselton cohort (y axis) was examined. See S1 Data for underlying data. AusDiab, Australian Diabetes, Obesity and Lifestyle Study; BMI, body mass index. https://doi.org/10.1371/journal.pbio.3000870.s010



S11 Fig. Correlation between metabolic risk factors. Pearson's correlation coefficients were calculated for each pair of risk factors. Colour intensities show the strength of correlation. Significant correlations at p-value *p < 0.05, **p < 0.01, and ***p < 0.001, respectively



S12 Fig. Association of BMI, WC, and WHR with lipid species. Linear regression analyses of BMI (A), WC (B), and WHR (C) with lipid species were performed adjusting for age, sex, cholesterol, HDL-C, and triglycerides. Open grey symbols, closed grey symbols, and closed orange symbols show lipid species with corrected p-values >0.05, <0.05, and <1 × 10−11, respectively. Whiskers represent 95% confidence intervals. See S1 Data for the underlying data. BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; WC, waist circumference; WHR, waist/hip ratio.



B
Lipids associated with BMI only

AcylCarnitine(12:0)
AcylCarnitine(16:0)
AcylCarnitine(17:0) (a)
CE(20:4)
CE(20:5)
Cer(d18:2/16:0)
Cer(d18:2/21:0)
DE(16:0)
DG(18:2_20:4)
Hex2Cer(d18:1/22:0)
LPC(16:1) [sn1]
LPC(18:0) [sn2]
LPC(20:4) [sn1]
LPC(22:4) [sn1]
LPC(22:4) [sn2]
PC(38:2)
PC(38:5) (a)
PC(O-16:0/20:3)
PC(O-16:0/20:4)
PC(O-32:2)
PC(P-15:0/20:4) (b)
PC(P-16:0/20:4)
PC(P-17:0/20:4) (a)
PC(P-38:5) (a)
PE(P-18:1/18:3)
PI(18:0_20:3) (b)
SM(37:1)
SM(38:3) (a)
SM(44:1)
SM(44:2)
SM(d18:1/17:0)/SM(d17:1/18:0)
Sulfatide (d18:1:/18:0(OH))
TG(54:7) [NL-20:5]
TG(56:7) [NL-20:5]
TG(56:8) [NL-20:4]
TG(O-50:2) [NL-18:1]
TG(O-52:1) [NL-16:0]
TG(O-52:2) [NL-16:0]
TG(O-52:2) [NL-18:1]
TG(O-54:2) [NL-18:1]

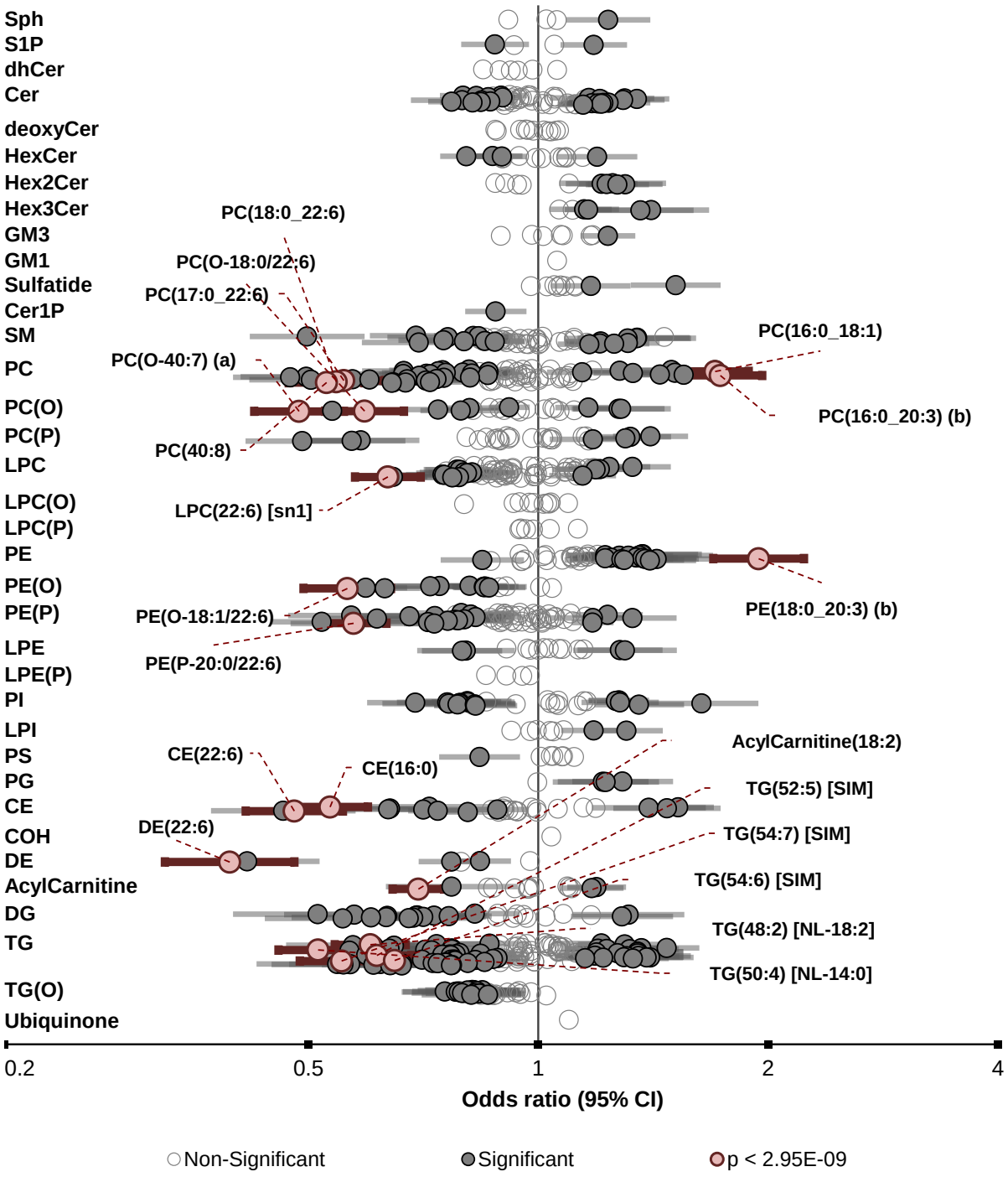
C
Lipids associated with WHR only

CE(20:2)
Cer(d18:1/14:0)
DG(18:0_18:2)
GM3(d20:1/18:0)
LPE(P-16:0)
LPI(18:0) [sn1]
PC(15:0_20:3)
PC(16:0_18:1)
PC(16:1_18:2)
PC(31:0) (b)
PC(O-32:1)
PC(P-16:0/14:0)
PE(O-38:5) (a)
PE(P-16:0/18:3)
PE(P-18:0/18:1)
PE(P-18:1/18:1)
PE(P-18:1/18:1)(a)
PE(P-18:1/18:1)(b)
PE(P-18:1/20:5)(a)
PE(P-18:1/20:5)(b)
PE(P-18:1/22:4)
PI(16:0_20:3) (a)
PI(34:0)
PS(40:5)
SM(35:2) (b)
SM(37:2)
SM(d17:1/16:0)
SM(d17:1/24:1)
SM(d18:2/23:0)
TG(54:2) [NL-20:1]

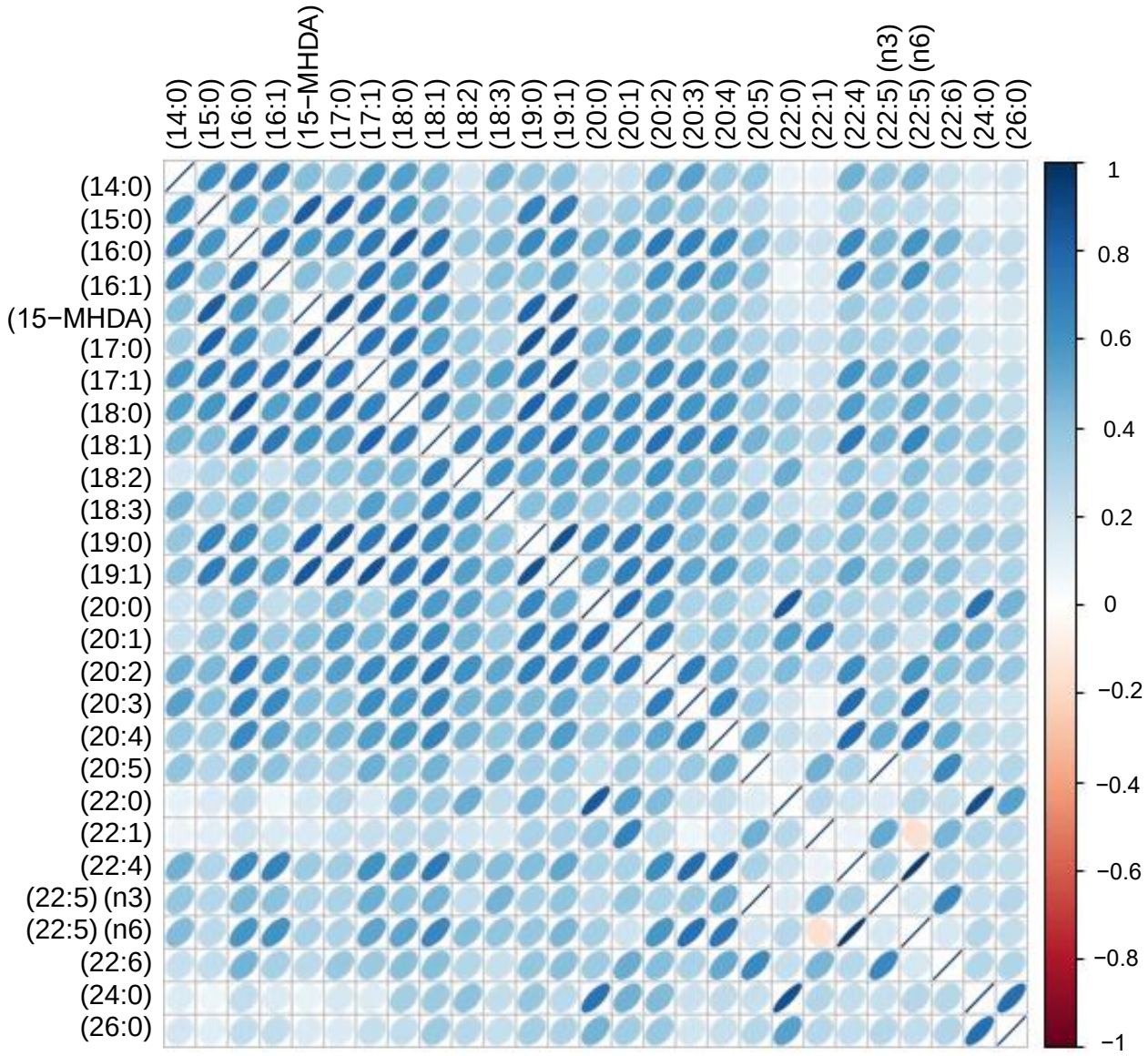
D
Lipids associated with WC only

DG(18:1_22:6)
DG(18:2_22:6)
dhCer(d18:0/16:0)
Hex2Cer(d16:1/24:1)
PE(O-38:5) (b)
PE(P-20:1/20:4) TG(56:9) [NL-22:6] TG(58:8) [NL-22:6] TG(O-50:2) [NL-18:2]

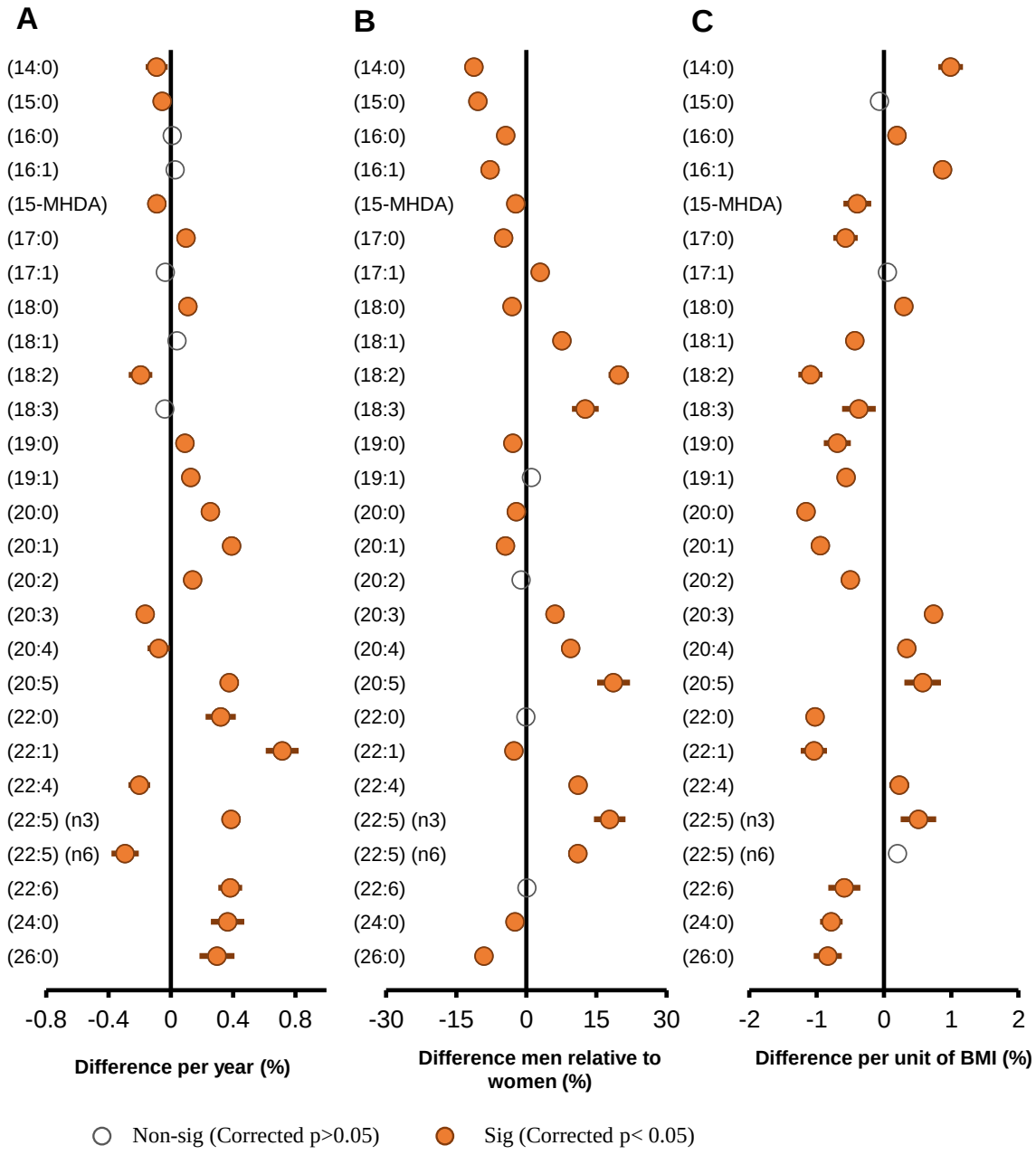
S13 Fig. Association between BMI, WC, or WHR and the plasma lipidome. A linear regression between log-transformed lipid concentration and BMI or WC or WHR was performed on 10,339 subjects adjusting for age, sex, total cholesterol, HDL-C, and triglycerides. (A) Venn diagram showing overlaps and unique associations of lipid species with BMI, WC, and WHR. (B), (C), and (D) show lipid species significantly associated with BMI only, WHR, only, and WC only, respectively. See S1 Data for the underlying data. BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; WC, waist circumference; WHR, waist/hip ratio.



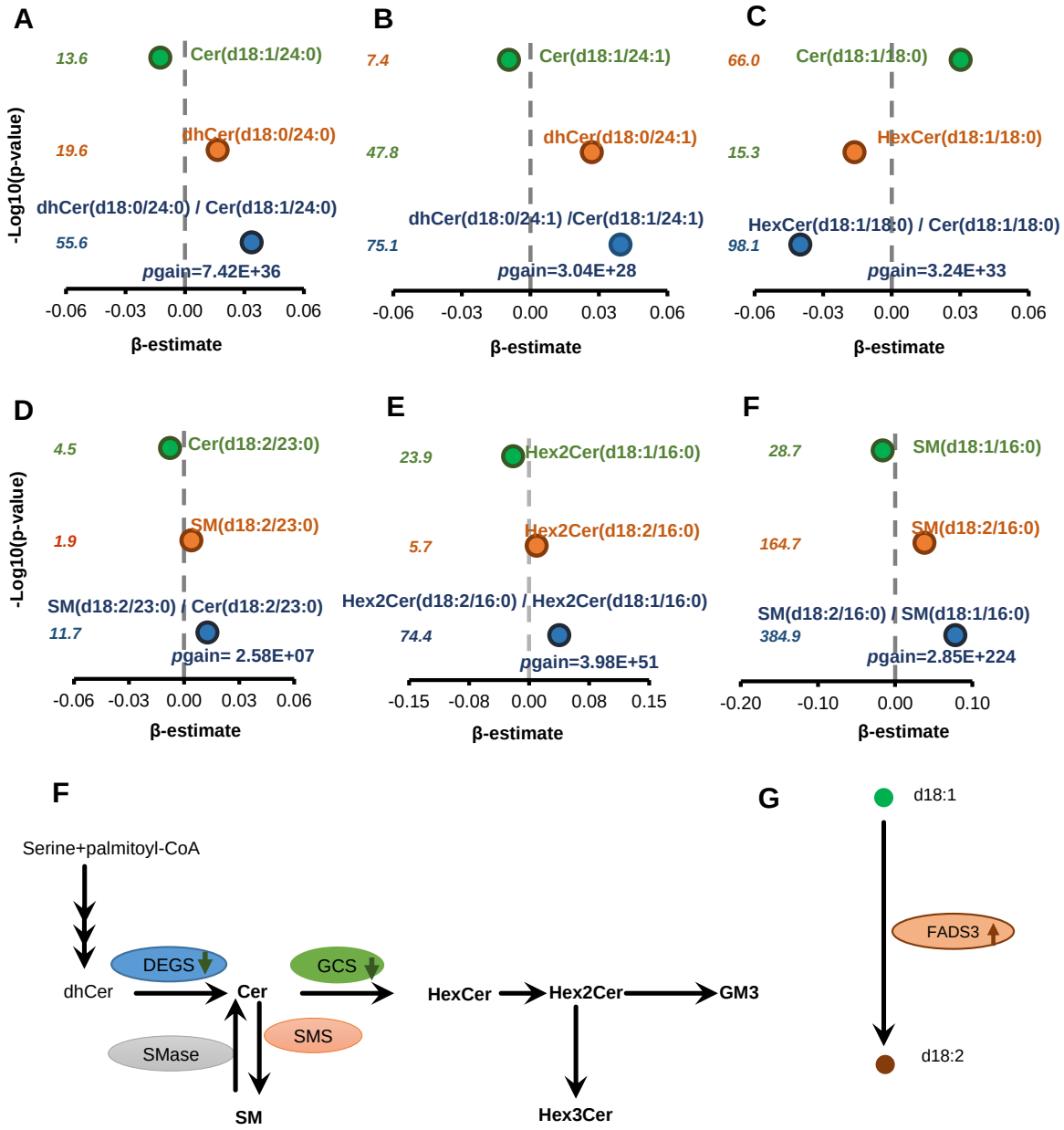
S14 Fig. Association of smoking with plasma lipidomic profile. A logistic regression analysis between smoking status and $\log_{10}$-transformed lipid species concentrations was performed adjusting for age, sex, BMI, total cholesterol, HDL-C, and triglycerides. Grey circles show nonsignificant species ($p > 0.05$), and grey and pink circles show species with $p < 0.05$ and $p < 2.95 \times 10^{-9}$, respectively, after correction for multiple comparisons. The whiskers represent 95% confidence intervals. See S1 Data for underlying data. BMI, body mass index; HDL-C, high-density lipoprotein cholesterol.



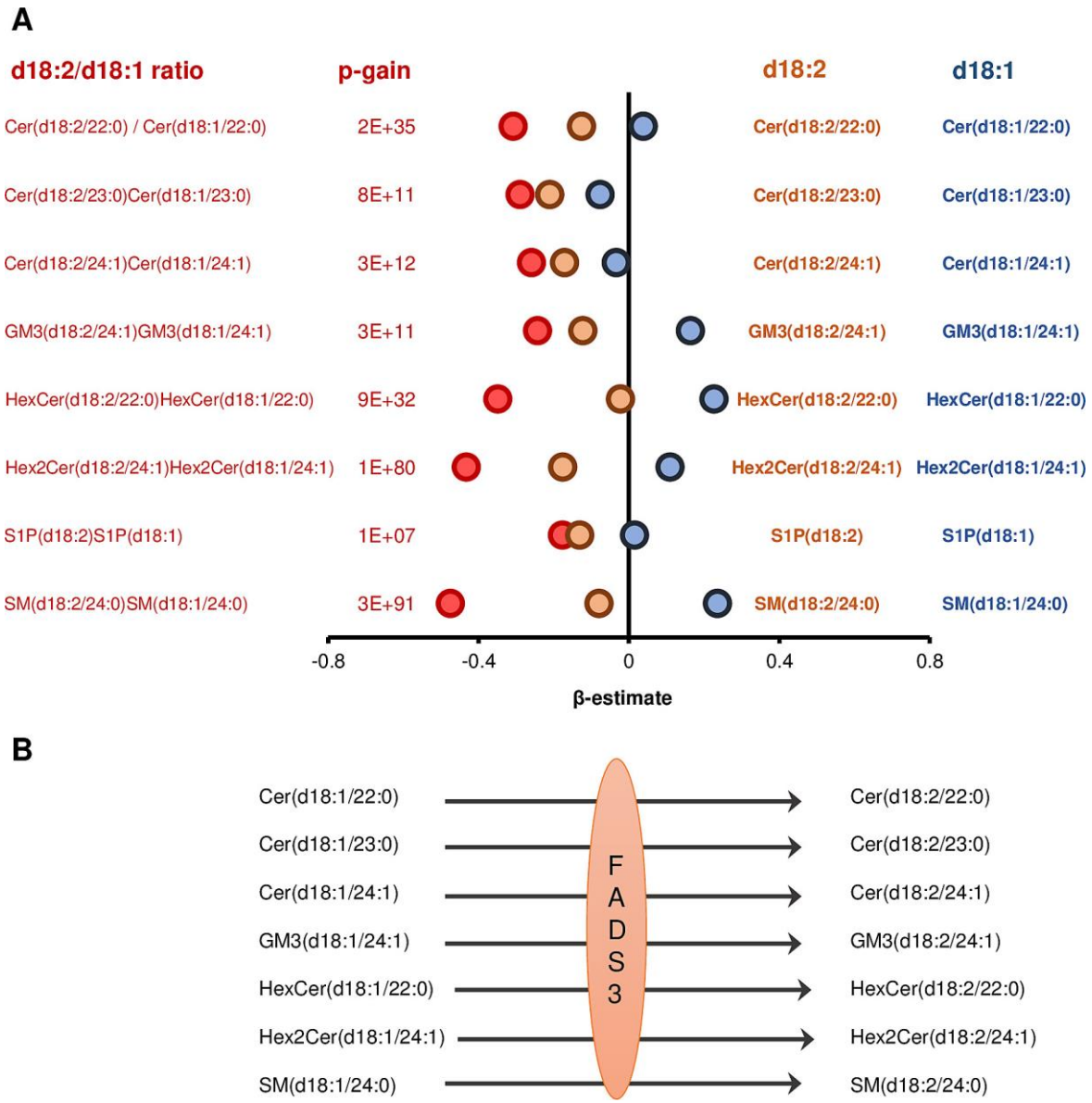
S15 Fig. Correlation matrix between fatty acid composition of lysophospholipids. Pearson's correlation analysis was performed between 27 fatty acids. Blue coloured eclipses in each square represent positive correlations, and orange show negative correlations. See S1 Data for the underlying data.



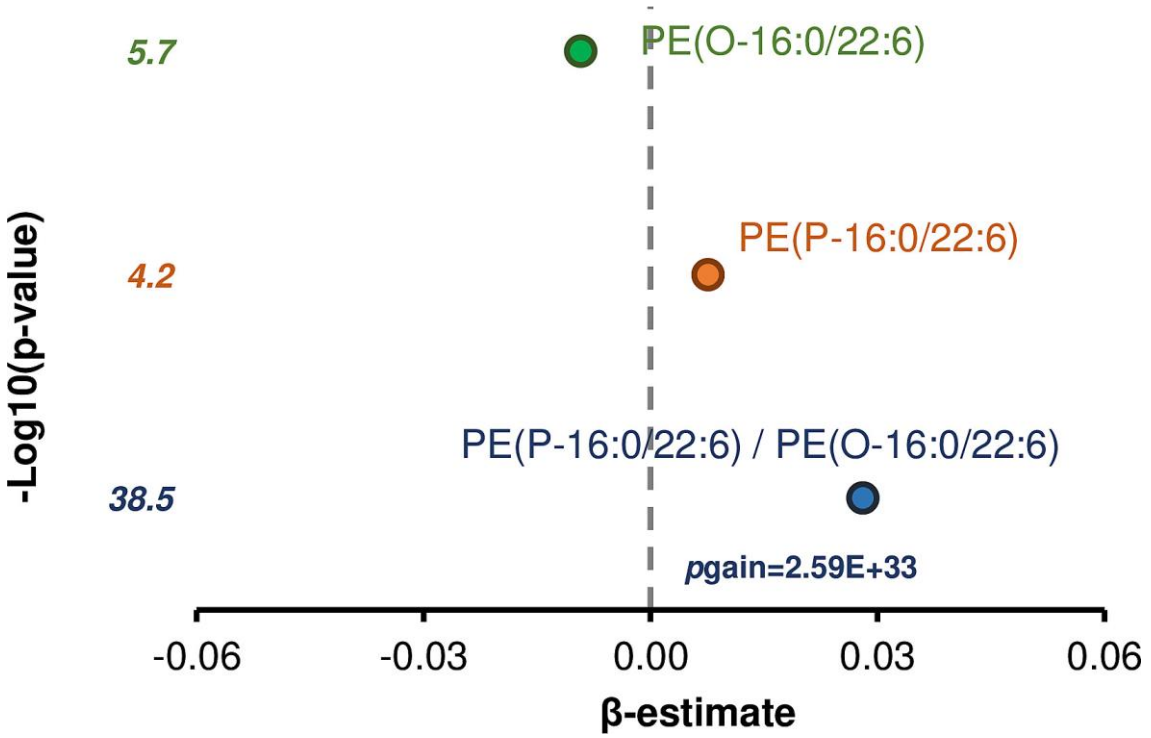
S16 Fig. Association between age, sex, and BMI with plasma lysophosphatidylcholine fatty acids. Multivariable linear regression analysis of age (A), sex (B), and BMI (C) with log-transformed lipid composition data was performed on 10,339 AusDiab participants adjusting for BMI, age, and sex (as appropriate) together with total cholesterol, HDL-C, and triglycerides. Open grey circles represent nonsignificant fatty acids (corrected $p > 0.05$). Orange circles show fatty acids associated with BMI, age, or sex (corrected $p < 0.05$). Bars represent the 95% confidence intervals. See S1 Data for the underlying data. AusDiab, Australian Diabetes, Obesity and Lifestyle Study; BMI, body mass index; HDL-C, high-density lipoprotein cholesterol.



S17 Fig. Selected lipid ratios and sphingolipid metabolic pathway associated with BMI. A linear regression adjusted for age, sex, total cholesterol, HDL-C, and triglycerides was performed between individual lipids or lipid concentration ratios and BMI. Each of the panels from (A)–(F) represent association of BMI with a given lipid ratio and individual lipids species that make up the ratio. (G) An overview of the sphingolipid biosynthetic pathway. (H) FADS3 as a sphingoid base desaturase responsible for increased d18:2/d18:1 sphingolipid ratio. BMI, body mass index; DEGS, dihydroceramide desaturase; FADS3, fatty acid desaturase 3; GCS, glucosylceramide synthase; HDL-C, high-density lipoprotein cholesterol; SMase, sphingomyelinase; SMS, sphingomyelin synthase.



S18 Fig. The d18:2 to d18:1 ratios of sphingolipid species associated with sex.A linear regression adjusted for age, BMI, total cholesterol, HDL-C, and triglycerides was performed between individual lipids or lipid concentration ratios and sex. (A) Represents association of sex with the ratio between d18:2/d18:1 sphingolipid. (B) FADS3 as a sphingoid base desaturase responsible for the conversion of d18:2/d18:1 sphingolipid. BMI, body mass index; FADS3, fatty acid desaturase 3; HDL-C, high-density lipoprotein cholesterol.



S19 Fig. Association of BMI with the ratio between PE(P)/PE(O). A linear regression adjusted for age, sex, total cholesterol, HDL-C, and triglycerides was performed between BMI and individual lipid or lipid concentration ratio. BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; PE(O), alkylphosphatidylethanolamine; PE(P), alkenylphosphatidylethanolamine.

Chapter 4. Mapping the Associations of the Plasma Lipidome with Insulin Resistance and Response to an Oral Glucose Tolerance Test

Preface

A perturbation in lipid metabolism associated with insulin resistance in adults with established disease such as type 2 diabetes is well documented. However, it is not well known whether molecular lipid metabolism is dysregulated in young healthy adults with hyperinsulinemia during the oral glucose tolerant test (OGTT). In Chapter 4 of this thesis, we explored the link between lipidomic signatures and insulin resistance and the oral glucose challenge during the OGTT. Several molecular lipid species perturbed in response to OGTT and species associated in a sex-specific manner with insulin resistance were identified. In addition, the changes in lipid levels during the OGTT were also studied.

Chapter 4 has been published in the Journal of Clinical Endocrinology and Metabolism (JCEM).

To improve the flow between chapters within this thesis, the Supplementary Tables for this chapter has been moved to the Appendix II.

Mapping the Associations of the Plasma Lipidome With Insulin Resistance and Response to an Oral Glucose Tolerance Test

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Context: Insulin resistance (IR) remains a global health challenge. Lipidomics offers an opportunity to identify biomarkers and better understand mechanisms of IR associated with abnormal lipid metabolism.

Objective: The objective of this article is to determine plasma lipid species associated with indices of IR and evaluate the lipidome response to an oral glucose tolerance test (OGTT).

Design and setting: This study was community based and cross-sectional.

Participants and sample: Plasma samples (collected at 0 and 120 min during an OGTT) from nonobese, young adults age 18 to 34 years (n = 246) were analyzed using liquid chromatography–tandem mass spectrometry.

Main outcome measures: The associations between indices of IR and lipid classes and species (with a sex interaction term), or changes in lipid levels during an OGTT, were tested using linear models (adjusted for age, sex, body mass index, total cholesterol, high-density lipoprotein cholesterol, and triglycerides).

Results: Some (213) and (199) lipid species were associated with the homeostatic model assessment of insulin resistance and insulin area under curve (AUC), respectively. Alkylphosphatidylcholine (10), alkenylphosphatidylcholine (23), and alkylphosphatidylethanolamine (6) species were associated with insulin AUC in men only. Species of phosphatidylcholine (7) and sphingomyelin (5) were associated in women only. In response to an OGTT, a perturbation in the plasma lipidome, particularly in acylcarnitine species, was observed; and the changes in many lipid species were associated with insulin AUC.

Conclusions: The plasma lipidome and changes in lipid levels during an OGTT were associated with indices of IR. These findings underlie the involvement of molecular lipid species in the pathogenesis of IR and possibly crosstalk between IR and sex-specific regulation of lipid metabolism. **(*J Clin Endocrinol Metab* 105: e1041–e1055, 2020)**

Key Words: high-throughput lipidomics, insulin resistance, OGTT, young adults, lipid metabolism

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Abbreviations: AUC, area under the curve; BCAA, branched-chain amino acids; BMI, body mass index; Cer, ceramide; deoxyCer, deoxyceramide; DG, diacylglycerol; dhCer, dihydroceramide; HOMA-IR, homeostatic model assessment of insulin resistance; LPC, lysophosphatidylcholine; MRM, multiple reaction monitoring; MS, mass spectrometer; OGTT, oral glucose tolerance test; PC, phosphatidylcholine; PC(O), alkylphosphatidylcholine; PC(P), alkenylphosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; S1P, sphingosine-1-phosphate; SM, sphingomyelin; TG, triacylglycerol.

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The antecedents of type 2 diabetes and cardiovascular disease such as insulin resistance (IR) may occur early in young adults. The common measures of clinical lipids such as total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides have been used as markers of metabolic risk and disease (1, 2). But these measures alone do not reflect the complex dysregulation of lipid metabolism involving many molecular lipid species. Compelling evidence shows that lipid species other than the clinical lipid measures, including acylcarnitine, glycerophospholipid, sphingolipid, and glycerolipid species are associated with metabolic risk factors and predicting disease outcomes (3-7).

The early detection of metabolite signatures associated with metabolic risk in young adults is of paramount importance for prompt intervention because the progression to full-blown disease outcomes may become inevitable after a certain stage. IR and subsequent type 2 diabetes are accompanied by aberration in circulating lipid and lipoprotein profiles in young adults (2, 8). We hypothesized that the dysregulation of lipid metabolism associated with IR (9, 10) is reflected in the plasma lipidome, and that lipidomic profiling might provide insight into the relationship with cardiometabolic risk and disease. We also hypothesized that the plasma lipidome is perturbed during an oral glucose tolerance test (OGTT), and such a perturbation is associated with indices of IR.

Lipidomic profiling using high-throughput analytical technologies such as liquid chromatography–mass spectrometry is a promising approach to measure several hundred to thousands of lipid species in biological samples. Application of such a technology on relevant population cohorts has the potential to identify novel lipid biomarkers that may serve as tools for risk assessment, disease monitoring or as therapeutic targets. Although the role of plasma lipid species has been demonstrated in disease conditions including type 2 diabetes (3, 11, 12) and cardiovascular disease (4, 13), studies assessing lipidomic signatures associated with metabolic health of nonobese and diabetes-free young adults are lacking. Furthermore, the response of the plasma lipidome to an OGTT, and whether such a response is associated with indices of IR, has not been clearly defined. Moreover, whether sex differences mediate the association between IR and plasma lipid species has not been thoroughly investigated.

The main aim of this study was therefore to determine whether plasma lipid species are associated with 2 indices of insulin action: the homeostatic model assessment of insulin resistance (HOMA-IR) and the integrated plasma insulin response (insulin AUC) during an OGTT in nonobese young adults. We further investigated the role of sex on these associations to assess whether the dysregulation of lipid metabolism associated with IR is different in men and women. A secondary aim was to evaluate the effect of an OGTT on the plasma lipidome and how that related to IR.

Materials and Methods

Participants and setting

The detailed description of the study cohort is available from a previously published study (14). Briefly, a total of 246 nonobese (body mass index [BMI] < 30 kg/m^2) young adults age 18 to 35 years without diabetes were recruited from Deakin University and surrounding areas to participate in this study (Table 1). The Deakin University Human Research Ethics Committee approved all studies, which were conducted in accordance with the Declaration of Helsinki. The purpose, nature, and potential risks were explained in a plain-language statement, and participants gave written consent and completed a medical questionnaire to determine eligibility. Participants arrived at the clinical research laboratory at approximately 9 AM after an overnight fast (no food after 9:30 PM) and were asked to avoid vigorous physical activity for 48 hours before the study. A trained phlebotomist then inserted a 22-gauge cannula into a forearm vein for blood sampling and a fasting blood sample was taken, followed by a second fasting blood sample taken 10 minutes later. Following the fasting blood sample, participants were given a glucose drink containing 75 g of glucose in 225 mL of water (75 gm Glucose Tolerance Test, CLEAR, Daniels Health). Participants were given 5 minutes to consume the glucose drink. Additional blood samples were drawn at 10, 20, 30, 60, 90, 120, 150, and 180 minutes after completion of the drink. Blood was placed on ice, centrifuged (4°C; 4400 revolutions per minute; 10 min), and plasma was stored at –80°C.

Plasma Hormones and Metabolite Analysis

Plasma glucose was determined using the glucose oxidase method. Plasma insulin was measured by enzyme-linked immunosorbent assay according to the manufacturer's instructions (ALPCO). HOMA-IR was computed from fasting insulin and glucose measures as follows: HOMA-IR = (glucose in mmol/L × insulin in μU/ml/22.5 mmol/L). Insulin AUC was calculated from the –10- to 180 (–10, 0, 10, 20, 30, 60, 90, 120, 150, and 180)-minute time points using the trapezoidal rule with the mean of the 2 basal samples (–10 and 0 min) used for baseline. The Matsuda insulin sensitivity index was calculated from fasting samples (obtained at 0 min) using the following formula [10 000/square root of (fasting glucose × fasting insulin) × (mean glucose × mean insulin during OGTT)] (15). Plasma triglycerides were measured by a plate modified microassay from a commercially available triglycerides kit (Roche Diagnostics). Total plasma cholesterol was measured spectrophotometrically by an enzymatic colorimetric

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Table 1. Characteristics of study participants

Characteristics	Women n = 141 (57.3%) Mean (SD)/Median (IQR)	Men n = 105 (42.7%) Mean (SD)/Median (IQR)
Age, y	23 (21-26)	23.5 (21.5-28)
BMI, kg/m^2	22.8 (3.1)	25.3 (3.4)
BSA, m^2	1.7 (0.2)	2.0 (0.2)
SBP, mm Hg	115.8 (12.3)	125.4 (12)
DBP, mm Hg	74.7 (9.3)	76.5 (9.0)
MAP, mm Hg	88.4 (9.6)	92.8 (9.0)
HR, beats/min	67.1 (13.7)	60.3 (11.2)
Glucose, mmol/L	5.3 (0.5)	5.5 (0.6)
Total C, mmol/L	4.3 (0.8)	4.0 (0.8)
HDL-C, mmol/L	1.5 (0.3)	1.3 (0.3)
Non–HDL-C, mmol/L	2.8 (0.8)	2.6 (0.8)
Total C:HDL-C, mmol/L[a]	2.8 (2.4-3.3)	2.7 (2.3-3.4)
HDL-C:TAG, mmol/L	2.2 (0.9)	2.1 (1.0)
TG, mmol/L[a]	0.8 (0.6-0.9)	0.7 (0.6-0.9)
Insulin, uU/mL	3.5 (2.9)	4.3 (7.5)
Log, insulin AUC	3.6 (0.2)	3.5 (0.3)
HOMA-IR	0.8 (0.7)	0.95 (0.7)
Matsuda index value	15.2 (0.5)	13.4 (0.4)

Abbreviations: AUC, area under the curve; BMI, body mass index; BSA, body surface area; C, cholesterol; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance; HR, heart rate; IQR, interquartile range; MAP, mean arterial pressure; SBP, systolic blood pressure; TAG, triacylglycerides; TG, triacylglycerol.
[a]Data in median (IQR) for variables not normally distributed in men and women.

assay according to the manufacturer's instructions (Cholesterol E Kit: Wako Chemicals), except that 10 µL of standards and samples were added to each well instead of 3 µL to improve accuracy. Plasma HDL-C was measured spectrophotometrically by an enzymatic colorimetric assay according to the manufacturer's instructions (HDL-Cholesterol Kit: Crystal Chem), except that 10 µL of standards and samples were added to each well instead of 3 µL to improve accuracy.

Plasma lipidomics

Lipid extraction and lipidomic analysis.

Plasma samples for lipidomic analyses were collected before and 120 minute after oral glucose ingestion during an OGTT. Plasma lipids were extracted as described previously (16). However, the method employed in this study was assisted by an automated liquid handling robot (MicroLAB STAR, Hamilton, Biosystems, Inc). 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. Samples were vortexed thoroughly and immediately sonicated in a sonicator bath for 1 hour maintained at (< 20°C) and then centrifuged (at 14 000 *g*, 10 min, 20°C). Finally, 100 µL of lipid extract was transferred to sample vials with glass inserts. A targeted lipidomic approach using liquid chromatography–mass spectrometry was employed to measure individual plasma lipid species on an Agilent 6490 triple-quadrapole mass spectrometer (MS) with an Agilent 1290 series high-performance liquid chromatography system and a ZORBAX eclipse plus C18 column (2.1 × 100 mm 1.8 µm, 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 are shown in the supplementary document. All supplementary tables and figures are located in digital research materials repositories (17, 19). The solvent system consisted of i) 50% H_2O/30% acetonitrile/20% isopropanol (v/v/v) containing 10 mM ammonium formate and solvent, and ii) 1% H_2O/9% acetonitrile/90% isopropanol (v/v/v) containing 10 mM ammonium formate. A linear gradient with a 15-minute cycle time as described elsewhere (5) was used. The MS was set to gas temperature, 150°C, gas flow rate 17 L/min, nebulizer 20 psi, sheath gas temperature 200°C, capillary voltage 3500 V, and sheath gas flow 10 L/min. Plasma quality control samples were incorporated into the analysis at 1 plasma quality control sample per 18 plasma samples. Technical quality control samples were included in the run to monitor technical variation attributable to the MS instrument.

Data processing and statistical analysis

The chromatographic peaks of lipid species acquired from the MS were integrated using the Mass Hunter (B.07.00, Agilent Technologies) software and assigned to

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a specific lipid species based on MRM (precursor/product) ion pairs and retention time. The relative concentration of each lipid species was determined by comparing their peak areas to the relevant internal standard (17).

Lipid data were $\log_{10}$-transformed (when necessary) before statistical analysis. The association between indices of IR and plasma lipid species or changes in lipid species was determined by using multivariable linear regression, adjusting for either 1) age, sex, and BMI, or 2) age, sex, BMI, total cholesterol, HDL-C, and triglycerides. Interactions between lipids and sex were investigated using interaction terms between each lipid and sex. A P value of .05 was considered as suggestive of an interaction. These analyses allowed examination of sex-dependent associations between IR indices and lipid species. β coefficients and 95% CI were converted to percentage difference (percentage difference = $(10^{\wedge\beta\ \text{coefficient}} - 1) \times 100$). A paired student t test was performed to determine significance of the change in lipid concentrations 2 hours after the glucose load relative to fasting levels; and the results expressed as mean percentage changes. All P values were corrected for multiple comparisons using the Benjamini-Hochberg procedure (18). Statistical analyses were performed using R version 3.4.4.

Results

Association of homeostatic model assessment of insulin resistance with lipid species

A total of 662 plasma lipid species across 4 major lipid categories (sphingolipids, phospholipids, sterol lipids, and glycerolipids) and 36 lipid classes/subclasses were measured (17) among young adults without diabetes. The characteristics of the study participants are shown in Table 1. Analyses were performed before and after excluding individuals with prediabetes (n = 13), both cases for which the results were similar. HOMA-IR was associated with 213 lipid species (corrected $P < .05$); of which, 12 ceramide (Cer), 10 deoxyceramide (deoxyCer), 22 phosphatidylethanolamine (PE), 6 phosphatidylserine (PS), 19 diacylglycerol (DG), and 42 triacylglycerol (TG) species showed strong positive associations independent of age, sex, and BMI. Of the positively associated lipid species, DG(18:0/18:1) displayed the strongest association (34.1% increase per HOMA-IR unit, P = 1.90E-12) followed by DG(16:0/16:0) (19.5% increase per HOMA-IR unit, P = 6.08E-12) (Fig. 1A) (17).

HOMA-IR was inversely associated with 3 alkylphosphatidylcholine (PC[O]), 16 alkenylphosphatidylcholine (PC[P]), 6 medium- to long-chain acylcarnitine, 9 cholesteryl ester containing omega-3 and omega-6 polyunsaturated fatty acids, and 9 hexosylceramide (monohexosylceramide, 2; dihexosylceramide, 2; trihexosylceramide, 5) species, where the cholesteryl ester CE(20:4) displayed the strongest association (17.8% decrease per unit HOMA-IR, P = 7.93E-08) (Fig. 1A) (17). Further adjustments for cholesterol, HDL-C, and TGs resulted in fewer associations (Fig. 1B) (17). The direct correlation between selected lipid species based on the lowest corrected P values and HOMA-IR is also shown (Fig. 1C).

Relationship between Matsuda index of insulin sensitivity and plasma lipidome

The Matsuda index showed similar but opposite associations to the plasma lipid species as HOMA-IR, indicating the same associations to insulin sensitivity. There were 120 lipid species associated with the Matsuda index (17). As expected, the TG species (37), DG (16) were among those showing negative association with the Matsuda index. The ether-linked phospholipids and plasmalogens such as species of PC(O) (8), and PC(P) (17) were positively associated (Fig. 2A) (17). Similar patterns of associations were observed even after controlling for clinical lipids in addition to age, sex, and BMI (Fig. 2B) (17).

Association of integrated insulin response (insulin area under the curve) with lipid species

Insulin AUC was significantly associated with a total of 199 lipid species (corrected $P < .05$) independent of age, sex, and BMI. The associations with species of dihydroceramide (dhCer) (4), deoxyCer (7), TG (49), DG (20), and lysophosphatidylinositol (5) were strongly positive (Fig. 3A) (17). The association with DG(16:0/18:2) was the strongest: 24.3% increase per SD of insulin AUC, P = 1.00E-10) (Fig. 3A) (17). Lysophosphatidylcholine (LPC) (15), lysoalkylphosphatidylcholine (5), lysoalkenylphosphatidylcholine (4), PC(O) (8), and PC(P) (24) species displayed negative associations. The associations between insulin AUC and most lysophospholipids were also significant after adjustment for clinical lipids (Fig. 3B) (17). Fig. 3C depicts the unadjusted lipid concentrations across quartiles of HOMA-IR for selected species based on the lowest corrected P values.

Overlap in associations of lipid species with homeostatic model assessment of insulin resistance and insulin area under the curve

Independently of age, sex, and BMI, 138 lipids showed an overlap in their association with HOMA-IR and insulin AUC. Some 29 species were negatively associated both

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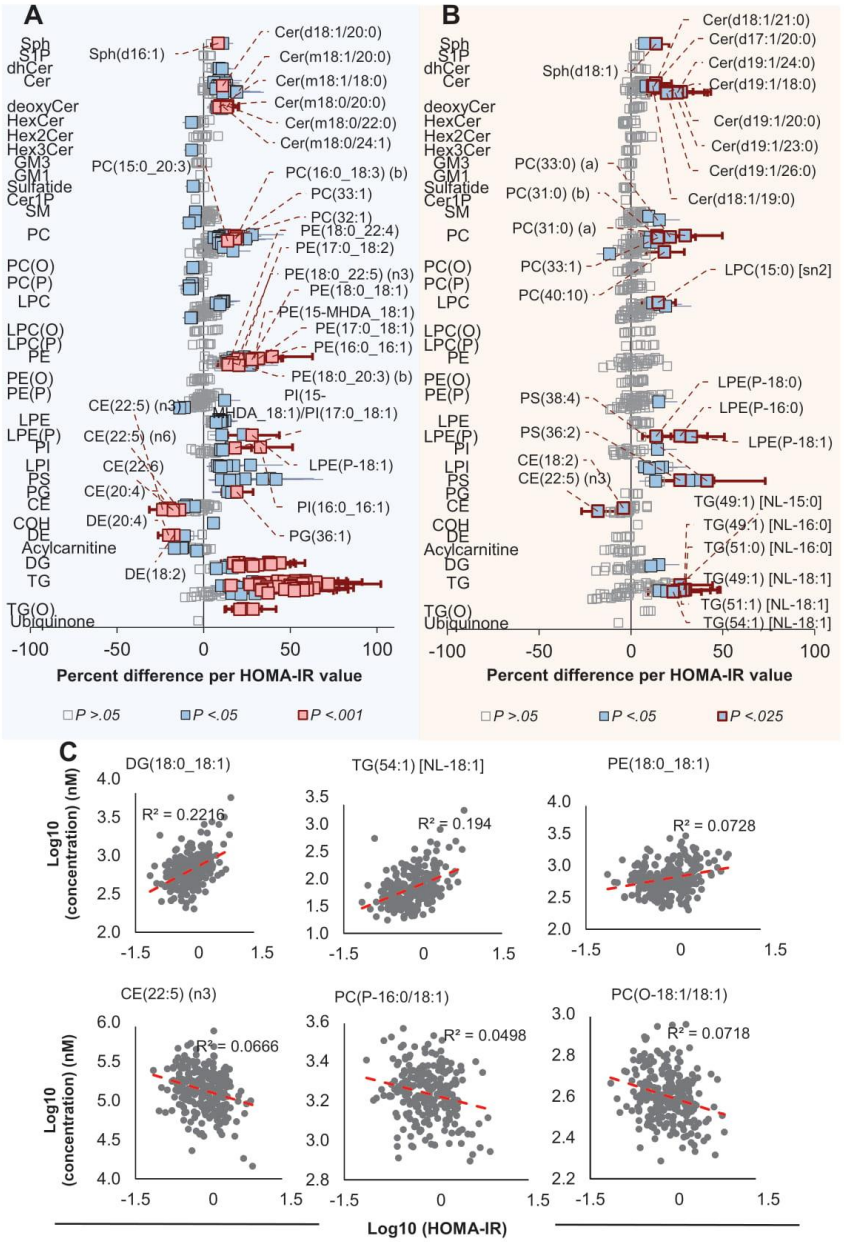

Figure 1. Association of the homeostatic model assessment of insulin resistance (HOMA-IR) with plasma lipid species. Linear regression of HOMA-IR against each lipid species was performed and the β coefficients were converted to percentage difference per HOMA-IR unit. A, Analyses adjusted for age, sex, and body mass index (BMI). B, Analyses adjusted for age, sex, BMI, total cholesterol, high-density lipoprotein cholesterol, and triglycerides. Gray squares show lipid species with *P* less than .05 after correction for multiple comparison. Blue squares show lipid species with *P* less than .05; A, pink squares represent lipid species with *P* less than .001, and B, blue with pink borders show *P* less than .025. Whiskers represent 95% CI. C, Scatterplots of the unadjusted linear associations between selected lipid species and HOMA-IR. Of the positively and negatively associated lipid species, we selected the 3 species with the lowest corrected *P* values.

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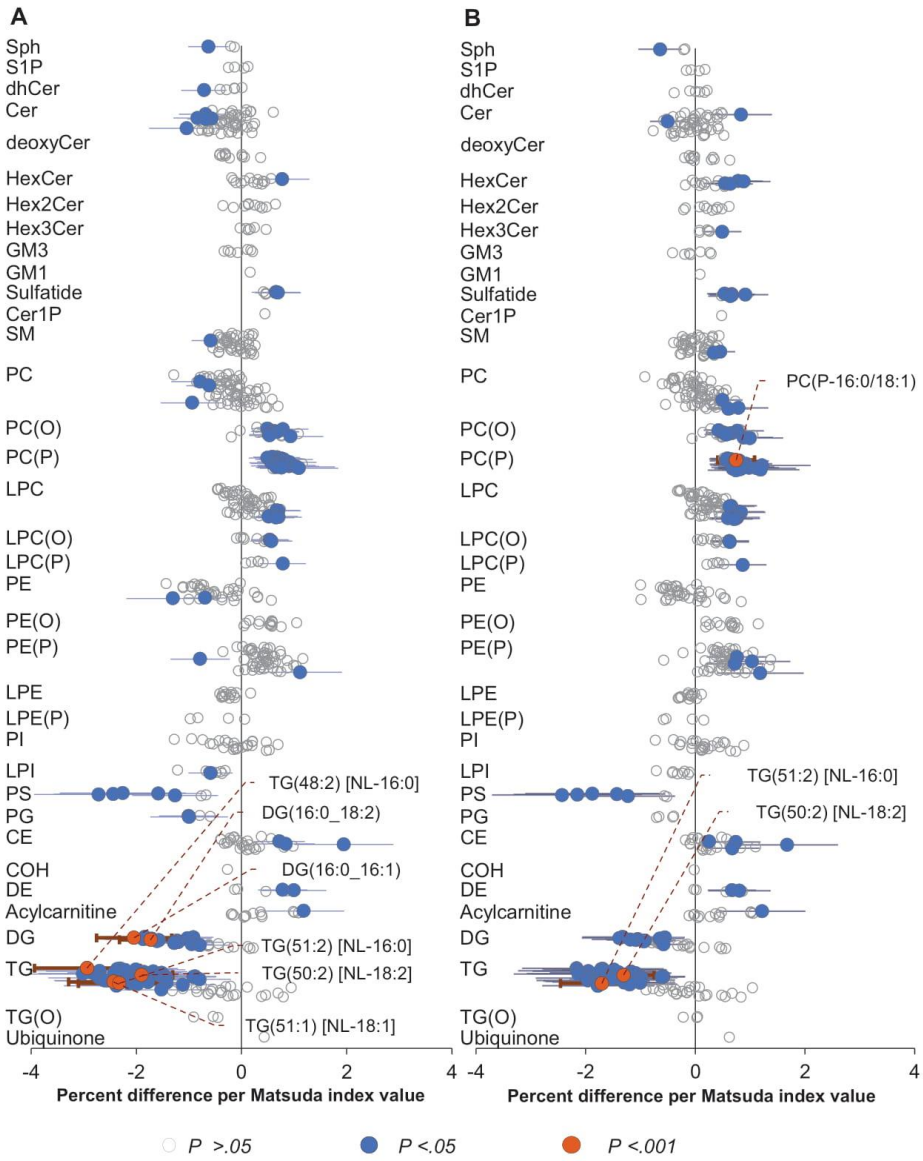

Figure 2. Relationship between Matsuda index of insulin sensitivity and plasma lipid species. Linear regression between Matsuda index and each lipid species was performed, A, adjusting for age, sex, and body mass index (BMI) and B, adjusting for age, sex, BMI, total cholesterol, high-density lipoprotein cholesterol, and triglycerides. Percentage differences of lipids per Matsuda index value are indicated on the x-axis. Gray open circles represent *P* greater than .05; blue and brown represent *P* less than .05 and *P* less than 9.00E-06 after correction for multiple comparisons. Whiskers show 95% CI. Top significant species were selected based on lowest corrected *P* values (brown circles).

with HOMA-IR and insulin AUC (corrected $P < .05$). The remaining 109 out of 138 were positively associated both with HOMA-IR and insulin AUC) (Fig. 4A), most of which were DG and TG species (17). In general, the effect sizes for HOMA-IR were strongly correlated with the effect sizes for insulin AUC ($r^2 = 0.7608$) (Fig. 4B).

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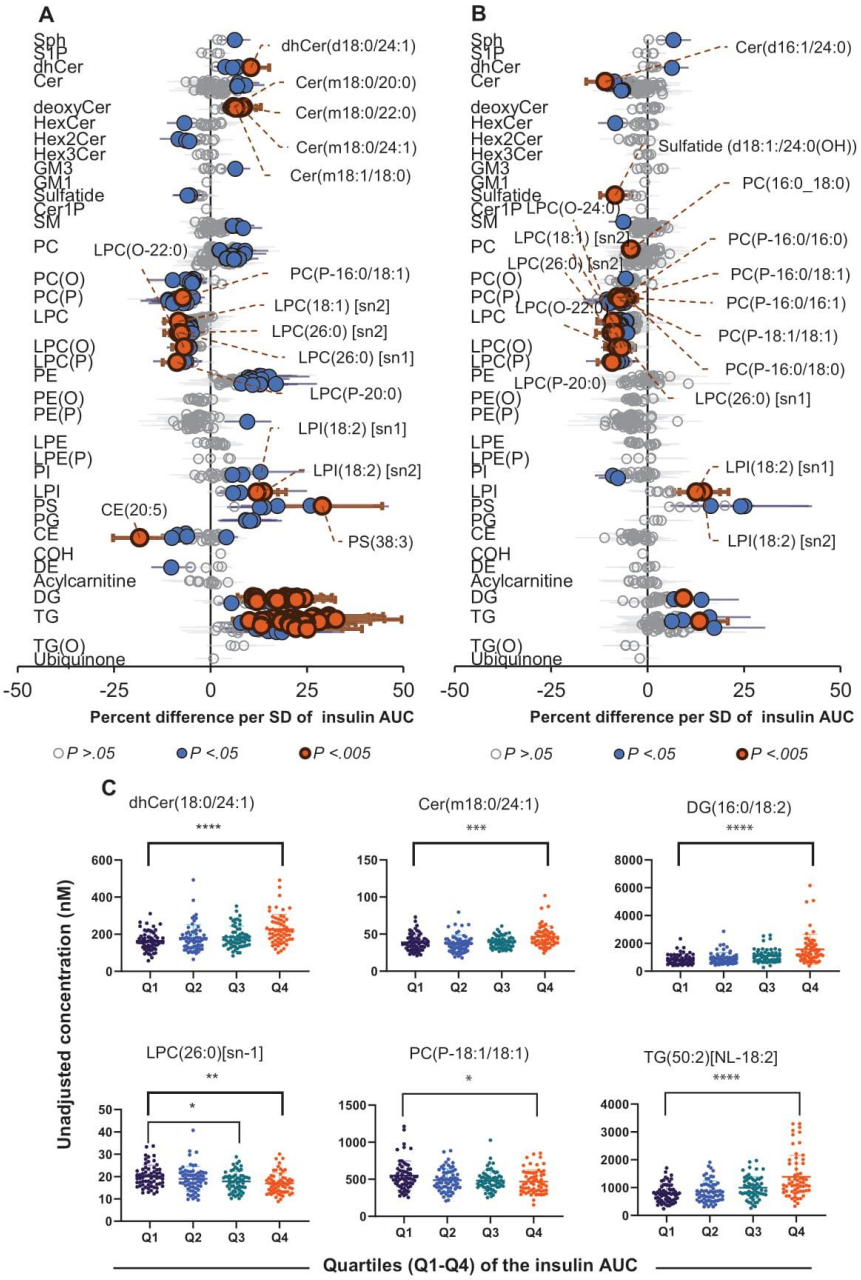

Figure 3. Association of insulin resistance with plasma lipid species. A, Linear regression between insulin area under the curve (AUC) and each lipid species was performed controlling for age, sex, and body mass index (BMI) and B, controlling for age, sex, BMI, total cholesterol, high-density lipoprotein cholesterol, and triglycerides. Gray circles represent *P* greater than .05, blue represents *P* less than .05, and brown represents top ranked species significantly associated with insulin AUC (*P* < .005) during oral glucose tolerance test. Whiskers show 95% CI. All *P* values are corrected for multiple comparisons using the Benjamin Hochberg procedure. C, Sina plots showing the unadjusted concentration of selected lipid species (based on lowest *P* values) across quartiles of the insulin AUC; the lowest quartile (Q1, n = 60), lower middle quartile (Q2, n = 63), upper middle quartile (Q3, n = 63), and the highest quartile (Q4, n = 60). Significances at *P* values of .05, .01, .001, and .0001 are shown by *, **, *** and ****, respectively.

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Sex-specific associations of insulin area under the curve with lipid classes/subclasses and species

We evaluated whether the association of IR indices with lipid classes/subclasses and species is dependent on sex. At a class level analysis adjusted for age and BMI, 5 out of the 36 lipid classes showed a nominally significant difference in the association with insulin AUC based on sex. Phosphatidylcholine (PC) was associated positively in women only, whereas the ether lipids alkyl- and PC(P) and alkenylphosphatidylethanolamine showed no effect in women, but were associated in men (17, 19).

At a species level, independently of age and BMI, species of sphingomyelin (SM), PC, PE, and phosphatidylinositol species were positively associated in women but not in men. Of note, the SM species SM(d18:2/14:0) was positively associated in women (9.4% increase per SD of insulin AUC, corrected P = 2.4E-02), whereas in men it did not show a statistically significant association. PC species such as PC(17:1/18:2) show a positive association in women (9.7% increase per SD of insulin AUC, corrected P = 4.2E-02) but no significant association in men.

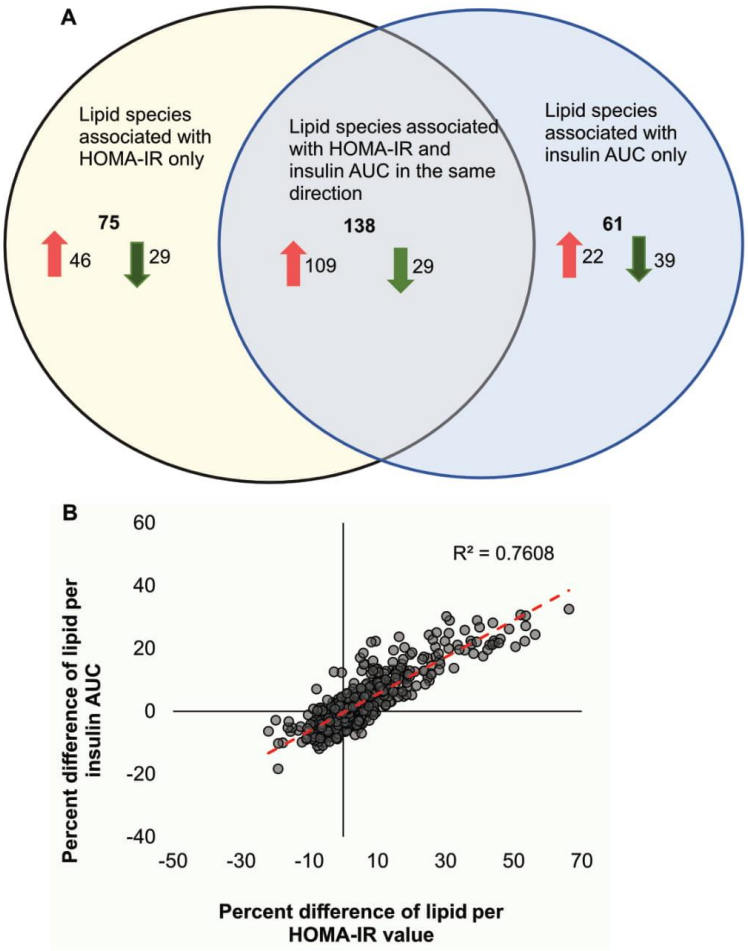

Figure 4. Comparison of the associations of homeostatic model assessment of insulin resistance (HOMA-IR) and insulin area under the curve (AUC) with lipid species. A, Summary of overlapped and distinct associations of HOMA-IR and insulin AUC with lipid species adjusted for age, sex, and body mass index (BMI). Red arrows indicate positive associations and green show negative associations after correction for multiple comparisons using the Benjamin Hochberg procedure. B, The relationship between effect sizes in percentage difference (HOMA-IR) and effect sizes in percentage difference (insulin AUC) associated with each lipid. Linear regression of HOMA-IR and insulin AUC on lipid species adjusted for age, sex, and BMI were performed. Each data point represents a pair of the effect sizes (HOMA-IR and insulin AUC) for a given lipid species.

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Among men, species of ether phospholipids, particularly PC(O), PC(P), alkylphosphatidylethanolamine, and alkenylphosphatidylethanolamine, were negatively associated in men but not in women (Fig. 5) (17).

The effect of an oral glucose tolerance test on circulating lipid species

Of the 662 lipid species measured, 405 were significantly perturbed following a standard 2-hour glucose tolerance test. All sphingolipids with the exception of sphingosine-1-phosphate (S1P) and SM species were decreased after an oral glucose load. Acylcarnitine species showed a sharp decline in response to OGTT, with acylcarnitine (14:2) displaying the strongest change (a decrease of 72.9%, corrected *P* value = 6.89E-111). Most lysophospholipid species, including LPC, lysophosphatidylethanolamine, and lysophosphatidylinositol, showed a strong decrease, although the lysoetherphospholipid species increased in response to an oral glucose load. Several DG and TG species containing monounsaturated or saturated fatty acids were also decreased (Fig. 6A) (17).

Changes in lipid levels associated with insulin area under the curve

The change in the level for each lipid species following the oral glucose load was computed as: change (Δ) = log (concentration at 120 min) – log (concentration at 0 min). A linear regression analysis after adjustment for age, sex, and BMI showed changes in the levels of 99 plasma lipid species being significantly associated with insulin AUC. The changes in the species of medium-chain acylcarnitine (5), TG (25), DG (10), LPC (15), PE (10), and PS (4) were significantly associated with insulin AUC. The change in the TG species TG(52:3) [NL-16:1] in response to OGTT was the strongest predictor of insulin AUC (β = 1.8, corrected *P* = 1.25E-07) (Fig. 6B) (17). Further adjustments for total cholesterol, HDL-C, and triglyceride levels resulted in fewer lipid species, particularly DG and medium-chain acylcarnitine species containing 12:0 and 13:0 fatty acids, being associated with insulin AUC (17). Fold-changes in the levels of selected lipid species based on lowest *P* values in relation to different quartiles of the insulin AUC are shown as sina plots (Fig. 6C). With regard to HOMA-IR, only 7 lipid species—acylcarnitine (3), PE (2), PC (1), and lysophosphatidylethanolamine (2)—were associated (data not shown).

Discussion

The novel aspect of the present study is that we observed striking associations between 3 different indices of IR (HOMA-IR, the Matsuda index, and insulin AUC) and individual circulating lipid species in apparently healthy young adults. The sex-specific nature of many of these associations is a further novel finding. Furthermore, we observed that oral glucose ingestion had a profound effect on the plasma lipidome: Multiple lipid pathways appear to be significantly altered following an OGTT and many of the changes over the 2 hours of the OGTT were associated with the indices of IR.

Although the HOMA-IR of our study participants was within a relatively narrow range, HOMA-IR was associated with multiple molecular lipid species across several lipid classes, including sphingolipids, phospholipids, and glycerolipids. In age-, sex-, and BMI-adjusted linear models, HOMA-IR was positively associated with species of Cer and deoxyCer composed of saturated or monounsaturated fatty acyl chains. On adjustment for clinical lipids, associations with deoxyCer species were lost, suggesting that this might be driven by plasma lipoprotein levels. However, the association with Cer species, particularly those containing 19:0, 21:0, and 26:0 fatty acids, were independent of lipoprotein levels. Cers in general are implicated in visceral adiposity, IR, and development of type 2 diabetes (20). Although higher levels of Cer containing 16:0, the most abundant fatty acid in plasma reported to be associated with obesity and the risk of incident diabetes (21-23) as well as HOMA-IR in a relatively older cohort (an average age of 38 years) (24), we did not observe such an association in young adults. A C16:0 Cer could be a consequence of more severe IR, which may explain why we did not find a significant association in younger cohort. These results suggest selective or differential levels of upregulation of Cer synthase 2 (CerS2) in young adults with lowered insulin sensitivity. CerS2 plays a catalytic role in synthesizing longer acyl-chain (C20-C26) fatty acyl Cer species but not C16 (25). In line with our current finding, a strong positive correlation of plasma fasting insulin and HOMA-IR with plasma Cers containing saturated fatty acids such as 20:0 and 24:0 has been demonstrated (24).

Consistent with our findings, Luukkonen et al have shown elevated DG, Cer, and dhCer species with saturated and monounsaturated fatty acyl chains in the liver of IR individuals (26), suggesting that liver is the likely source of these Cers released as very low-density lipoprotein (VLDL), which also contributes to the elevated plasma TG species in the IR state. These VLDL particles will subsequently be converted to LDL particles and so the elevated Cer species are likely to present primarily in VLDL and LDL. We have previously reported elevated LDL Cer in individuals with type 2 diabetes (27). We further observed that LDL Cer secretion from hepatocytes is elevated in obese mice compared to lean mice, indicating that the liver is the primary source of these

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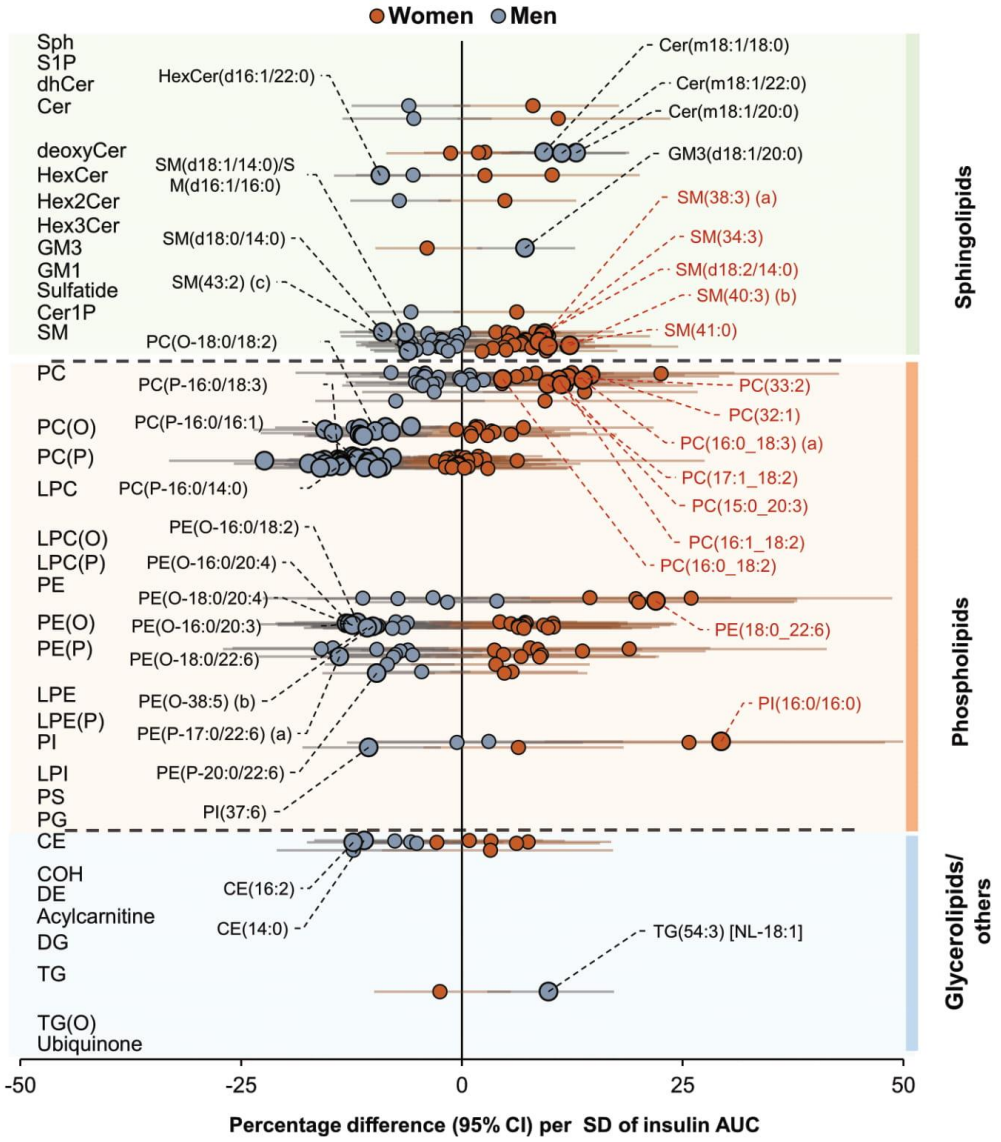

Figure 5. Effect of sex on the association of insulin area under the curve (AUC) with lipid species. Linear regression including a sex*insulin AUC interaction term further adjusted for age and body mass index was performed. Percentage difference per SD of insulin AUC is displayed on the x-axis. Blue circles (in men) and brown (in women) show lipid species presenting an interaction *P* value of less than .05. Whiskers show 95% CI. Large circles show the most significant species that differed between men and women selected based on lowest interaction *P* values.

circulating Cer (27). The Matsuda index showed similar but opposite associations to the plasma lipid species as HOMA-IR, indicating the same associations to insulin sensitivity. Overall, the number of significantly associated lipids was lower for the Matsuda index (120 compared to 213); however, further adjustment for clinical lipid measures had little effect on the Matsuda index associations (115 compared to 120) but a larger effect on the HOMA-IR associations (36 compared to 213). This would suggest that the HOMA-IR measure is more tightly related to the clinical lipid measures than the Matsuda index.

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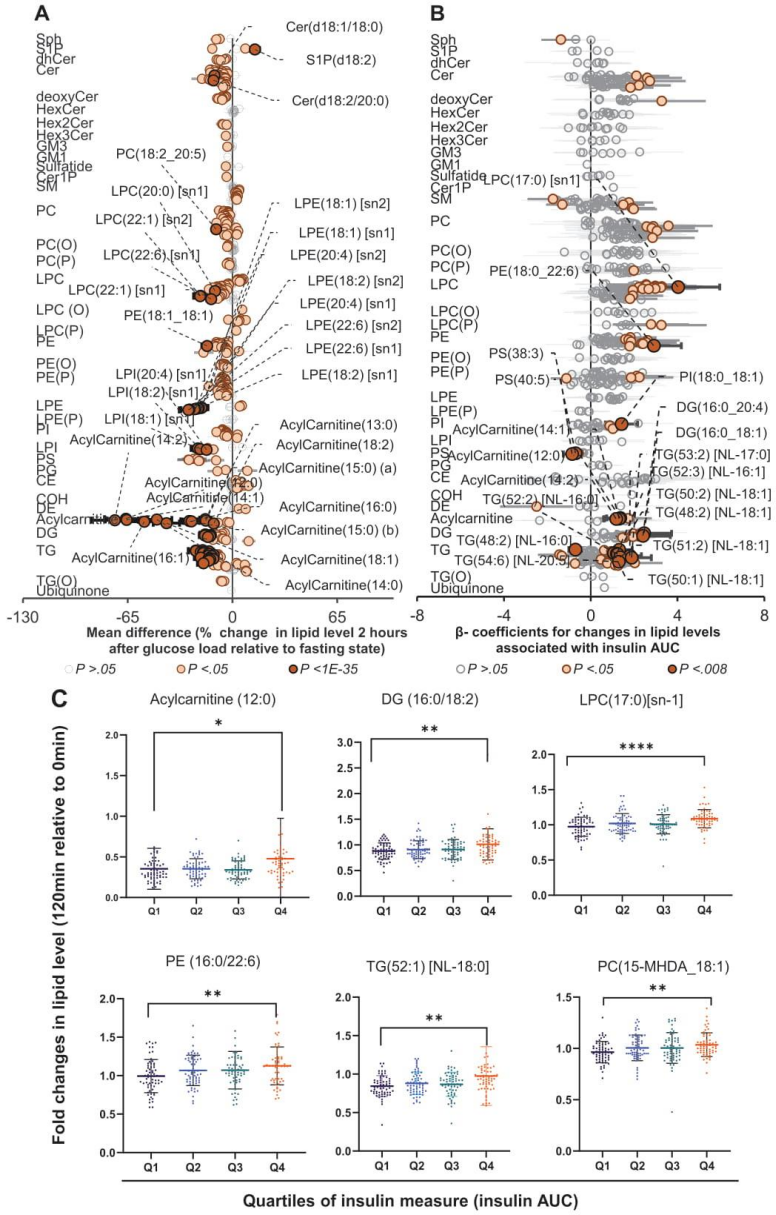

Figure 6. Plasma lipidome response to oral glucose tolerance test and changes in lipid levels associated with insulin AUC. A, Effect of oral glucose intake on plasma lipid species is expressed as the mean percentage change in lipid levels 2 hours after glucose load relative to the fasting state. B, β Coefficients from linear regression of insulin AUC with changes in lipid species adjusted for age, sex, and body mass index. Gray circles show lipid species with *P* greater than .05 after correction for multiple comparison. Yellow circles show lipid species with *P* less than .05. Dark yellow circles represent lipid species with A, *P* less than 1E-35 and B, *P* less than .008. Whiskers show 95% CI. C, Sina plots showing changes in the selected lipid levels across quartiles of insulin AUC. Species were selected based on lowest *P* values among lipids representing major lipid classes. Significances at *P* values of .05, .01, and .0001 are shown by *, **, and ****, respectively.

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In addition to using HOMA-IR and the Matsuda index as measures of IR, the insulin response as measured by insulin AUC during the OGTT was also used because it has been reported to be the best surrogate index of IR (28). The systemic insulin response (ie, AUC) represents the balance between insulin secretion and clearance, and hence elevated AUC could be due to either increased insulin secretion, reduced clearance, or both. In this study, OGTT insulin responses vary widely within otherwise healthy young adults with normal glucose tolerance. Hyperinsulinemia (indicative of a reduction in insulin action) in normoglycemic individuals was manifested because of the combined effects of increased insulin secretion and reduced insulin clearance (14). It therefore appears that there is a reciprocal mechanism that integrates insulin secretion and clearance to produce the plasma insulin profile required to maintain normal glucose tolerance. This highlights that the insulin AUC does not necessarily define glucose tolerance; that is, differences in plasma insulin responses can occur independent of any change in the plasma glucose response during an OGTT (29). Moreover, because there are no definitive criteria to classify IR, we chose to use the insulin AUC because it has been shown that this provides the best surrogate index of insulin action (28).

As with HOMA-IR, the insulin AUC shows a similar pattern of associations with lipid species independent of age, sex, and BMI. However, although most associations between HOMA-IR and lipid species were lost after additional adjustments for total cholesterol, HDL-C, and triglycerides, the association of insulin AUC with lipid species, particularly PS, ether phospholipids, and lysophospholipids, remained unchanged, suggesting that the insulin AUC would better capture metabolic perturbations associated with IR. An increase in PS with HOMA-IR and insulin AUC could be explained by platelet activation accompanied by an increase in plasma insulin (30). Platelet activation in turn has been shown to induce externalization of PS from the inner mammalian plasma membrane to the cell surface (31), potentially leading to elevated levels in plasma as well.

Insulin AUC and HOMA-IR were inversely associated with lysophospholipids, including LPC species. In support of this, other groups have found LPC species to be inversely associated with IR (10). An inverse association of the lyso and ether phospholipids with prediabetes (11) and type 2 diabetes (3) has been also demonstrated. Circulating LPC species are generated from lipoprotein/membrane PCs by the action phospholipase A2 (PLA_2). Endothelial lipases also generate LPCs from PCs and HDL or oxidized LDL by lecithin-cholesterol acyltransferase (32). It is not clear whether these enzymes are downregulated with an increase in IR, but it appears from this study and previous data (10, 12, 33) that LPCs could be good markers of subclinical disease such as prediabetes. However, further investigation is required to establish a causal relationship as to whether insulin sensitivity influences phospholipid metabolism or phospholipid metabolism plays a role in regulating insulin sensitivity.

In contrast to LPC, lysophosphatidylinositol species, particularly LPI (18:2) and LPI (20:4), were positively associated with insulin AUC. LPI plays an important role in insulin release and signaling, glucose homeostasis, and regulation of adipogenesis (34). LPI is a bioactive signaling molecule and is produced by PLA_2 as LPC. Moreover, a fatty acyl chain–specific phospholipase DDHD domain-containing protein 1 (DDHD1) has been implicated in LPI homeostasis. DDHD1 is a variant of phospholipase A_1 involved in the biosynthesis of arachidonic acid (AA) containing LPI species such as LPI (20:4) (35). Thus, the positive association of insulin with LPI (18:2), a precursor of AA and LPI (20:4) itself, complements the above report. Furthermore, plasma PLA_2 concentrations have been positively correlated with IR (36). Further studies are needed to fully explain the contrasting associations of LPC and LPI species with IR.

Next we sought to examine whether there were sex-specific associations of indices of IR with lipid classes, subclasses, and species. Sex-specific associations between plasma free fatty acids and markers of IR (37), branched-chain amino acids (BCAA), uric acid, acylcarnitine species, and IR (38) as well as phospholipids with metabolic syndrome (39) have been previously reported. Here we observed a striking sex-specific association of insulin AUC with lipid classes and species. At the species level analyses adjusted for age and BMI, ether phospholipids, particularly alkyl and PC(P), PC, deoxyCer, and SM species, showed nominal sex-dependent associations. Although these were not significant after correction for multiple comparisons, the grouping of these species into specific classes supports the biological relevance of these observations. Multiple species of ether phospholipids displayed a negative association with insulin AUC in men, but no association in women. This was also evident at the class level for alkyl-, PC(P), and alkylphosphatidylethanolamine. Because ether lipids are synthesized in peroxisomes (40), the negative association of ether lipids with IR may indicate peroxisomal dysfunction. Our findings suggest that peroxisomal function is decreased in men but not in women in the early stages of IR.

In women, species of PC and SM were positively associated while showing no association in men. At the class level SM did not show a sex-dependent association

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and the effect size for PC was small; when this is taken in context with the majority of species showing a sex-specific association containing odd or branched chain fatty acids, this suggests that the effect is a reflection of differential fatty acid metabolism between men and women with IR. Branched and odd chain fatty acids have recently been identified to result from the catabolism of BCAA within the mitochondria, particularly in adipose tissue, and so reflect mitochondrial function (41, 42). Our observations suggest that women have enhanced mitochondrial function in the early stages of IR, whereas men do not. This is further supported by the earlier report of sex-dependent associations with BCAA (8, 37, 43). This may be an important differentiating factor of how men and women adapt to changing metabolic status. Indeed, the lipidomic profiles forming sex-specific imprints of IR call for sex-stratified analyses in future physiological or biomarker discovery studies.

Phospholipid and SM species have previously been associated with IR (10); however, these associations have not been shown to be sex dependent. This is the first time that a sex-specific association of the lipidomic profile with indices of IR at a class and species level in young adults has been reported. The reason for sex-specific differences in the metabolic response to the early stages of IR remain unclear, but reflect possible differences in hormonal, adiposity, genetic, and lifestyle factors (44). Further studies are required to define the exact mechanism driving sex-specific regulation of lipid metabolism in IR.

For the first time, using a targeted lipidomics approach, we have demonstrated profound perturbations in several lipid species in response to an OGTT. An increase in the concentration of S1P following glucose ingestion was expected. Studies in cell and mouse models have shown that glucose stimulates the rise in S1P by activation of sphingosine kinase 2 in pancreatic β cells (33). A correlation between glucose-induced insulin secretion and elevated plasma S1P level have also been demonstrated (45). We observed a sharp decrease in response to glucose ingestion, which is likely a reflection of altered mitochondrial β oxidation. A similar decline in plasma acylcarnitine during OGGT has been reported (46). During fasting, there is a greater reliance on adipose tissue lipolysis resulting in mobilization of free fatty acids for subsequent β oxidation in other tissues (47). During the OGTT, however, this phenomenon may not take place because glucose is a preferred substrate for oxidation and adenosine triphosphate production. Thus, the insulin-mediated suppression of lipolysis and a subsequent drop in free fatty acids or the glucose oxidation in preference to fatty acids resulting in a decrease in acylcarnitine for mitochondrial fatty acid transport may explain the observed decline in circulating acylcarnitine species following OGTT.

Another novel aspect of our study is the association between changes in plasma lipid levels with insulin response during OGTT. The changes in DG, TG, and medium-chain acylcarnitine and PE showed significant associations with insulin AUC. Nowak and colleagues have reported a blunted decline of medium-chain acylcarnitine species in participants with worse IR during an OGTT (48). In people with and without IR, changes in nonlipid metabolites such as amino acids showed an association with the insulin excursion during an OGTT (49) have also been documented. However, no previous studies had explored the association of changes in lipidomic profiles during an OGTT with indices of IR. Differential metabolic perturbations depending on the IR status during OGTT inform future studies to investigate whether changes in lipid species during an OGTT predict conditions such as prediabetes and improve our understanding of the underlying pathophysiology of IR. Further mechanistic studies are required to better define the effect of oral glucose administration in the association of lipid species with IR.

The present study had several strengths. First, we applied our state-of-the-art high-throughput lipidomic methodology. This approach enabled us to measure a large number of lipid species (more than 600) and tested their associations with IR; this improves our understanding of the relationship between individual molecular lipid species and IR. Second, we tested the associations of plasma lipid species using 2 different indices of IR—HOMA-IR and the insulin AUC—which enabled a more holistic view of lipid metabolism in relation to early changes in insulin sensitivity among young adults. The insulin AUC, a non–steady-state index of IR, strongly correlates with clamp-derived measures of glucose utilization and is a superior index for the assessment of IR compared to fasting indices (50). Limitations of our study include the cross-sectional design and convenient sampling that did not allow us to establish a causal relationship between lipid metabolism and IR. As such, the focus of the study is mainly hypothesis generation. A second limitation was the lack of a standardized meal before sample collection. It has been documented that the intake of saturated fat in large amounts affects the liver and potentially the plasma lipid profile (51, 52), although all participants in this study fasted, which would have minimized the effect of their previous meal on the plasma lipidome. In summary, our findings have important implications in understanding the possible role of molecular lipid species in the pathogenesis of IR in young adults. We have also

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demonstrated sex-specific lipidomic fingerprints associated with indices of IR, suggesting that abnormal lipid metabolism associated with IR could be driven by sex-related differences. Finally, glucose ingestion had a profound effect on the plasma lipidome, the extent of which appears to be dependent on IR status.

Acknowledgments

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Author Contributions: G.M. collected data. S.H. designed the study, collected data, and participated in data analysis. D.K. collected data. K.H. designed the study and collected data. G.K. designed the study, collected data, and participated in data analysis. C.S. designed the study, collected data, and participated in data analysis. H.B.B. extracted plasma samples, performed MS analysis, analyzed the data, and wrote the manuscript. A.S. contributed in developing scripts and statistical analyses. C.G. and K.H. contributed in developing and improving MS methods and statistical analysis. M.C. contributed in developing extraction protocols and extracted plasma samples. N.M. contributed in MS experiment and post-MS data preprocessing and analysis. D.J.M. contributed in interpretation of results and revision of the manuscript. C.B. designed the study, collected data, participated in data analysis, contributed plasma samples, and revised the manuscript. P.J.M. oversaw this work and revised the manuscript. P.J.M. and C.B. are the guarantors of this work and take responsibility for full access to and integrity of the data.

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Disclosure Summary: The authors have nothing to disclose.

Data Availability: All data generated or analyzed during this study are included in this published article or in the data repositories listed in References.

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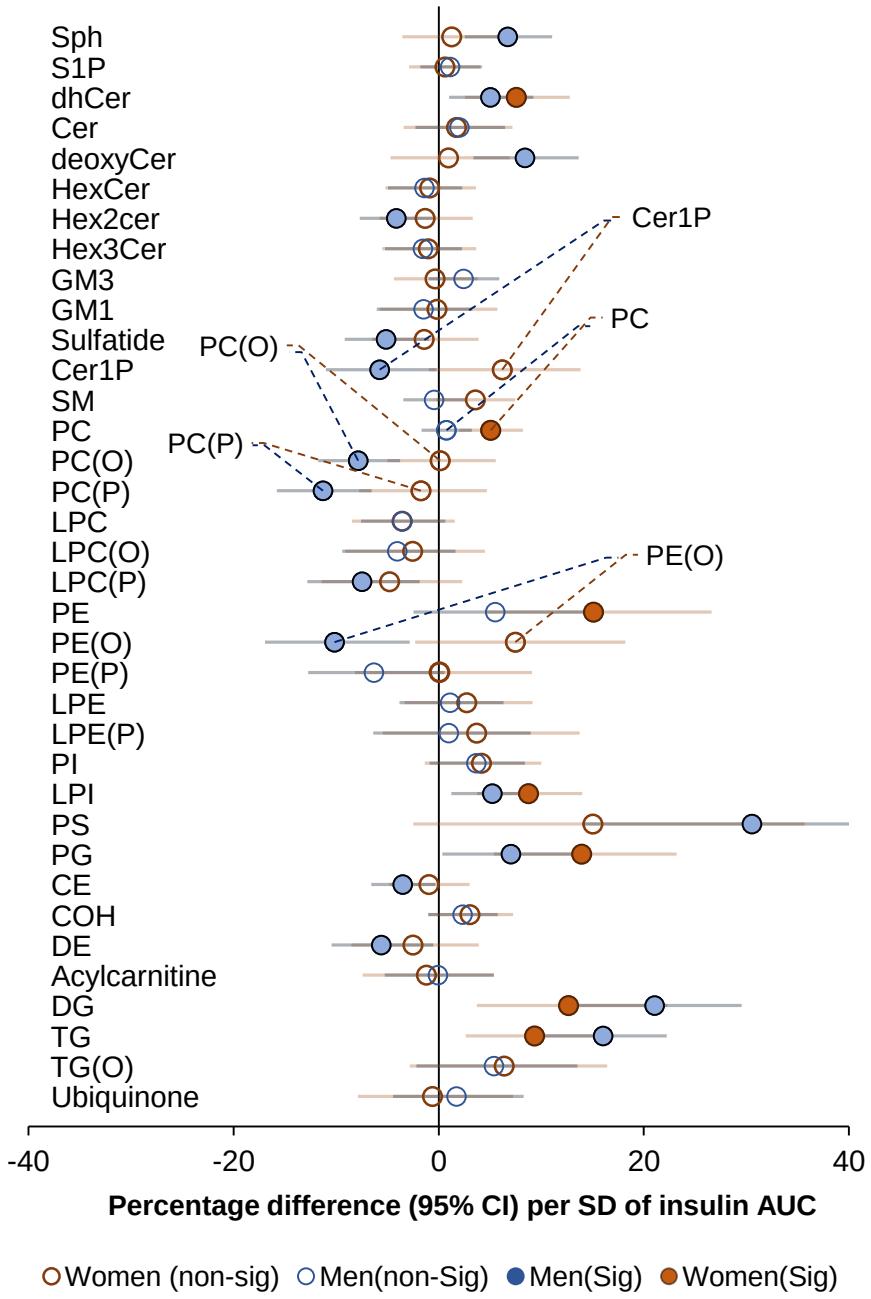
Chapter 4. Supplementary Figure



Chapter 4. Figure S1. Sex-specific association of the insulin AUC with lipid classes. Linear regression between insulin AUC including a sex*insulin AUC interaction term further adjusted for age and BMI was performed. Percentage difference per SD of insulin AUC is displayed on x-axis. Blue and brown circles represent associations in men and women respectively. Non-significant classes are denoted by open circles. Lipid classes with interaction p-value <0.05 are labeled. Whiskers show 95% confidence intervals.

Chapter 5. Circulating lipidomic signatures of changes in adiposity: A large prospective study of 5,849 adults from the AusDiab cohort

Preface

Chapter 5 explores the relationship between the plasma lipidomic profile and change in WC and in BMI over a 5-year follow-up in the AusDiab longitudinal study. The chapter focuses on understanding the baseline metabolic phenotype associated with waist or BMI gain. We also examined the interaction of sex in these associations. Finally, we attempted to build multivariate models including lipid species and asked whether these models improve upon the traditional risk factors to predict the risk of gaining waist. The outcomes from this chapter provide an overview of the plasma lipid associations and the potential of metabolic phenotype to predict obesity.

Chapter 5 has been prepared for submission to the International Journal of Obesity (IJO)

The Supplementary Tables for this chapter are presented separately (Appendix III).

Circulating lipidomic signatures of changes in adiposity: a large prospective study of 5,849 adults from the AusDiab cohort

Running title: Lipidomic profiles of changes in obesity measures

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Abstract

Background

Lipid metabolism is tightly linked to adiposity and weight gain. Previously, we have reported a profound perturbation in lipid metabolism associated with measures of obesity. Here, we investigated the association between the human plasma lipidome and changes in waist circumference (WC) and body mass index (BMI) over time to understand how baseline lipidomic signatures associate with risk of gaining weight or increasing WC. We also evaluated whether the baseline metabolic profile predicts the risk of increase in WC. The identification individuals at a greater risk of gaining weight based on their basal metabolic state could benefit targeted prevention.

Methods and findings

Adults (2,653 men and 3,196 women), 25-95 years old who attended the baseline survey of the Australian Diabetes, Obesity and Lifestyle Study (AusD iab) and the 5-year follow-up were included. A targeted lipidomic approach was used to quantify 706 distinct molecular lipid species in the plasma samples. The relationship between the baseline lipidomic profile and changes in two different measures of obesity (WC and BMI) over a 5-year follow-up were estimated using multiple linear regression models adjusted for baseline age, sex, WC or BMI, clinical lipid profiles and other covariates. P-values were corrected for multiple comparisons using the Benjamini-Hochberg procedure. Metabolic scores for change in WC were generated using a ridge regression model. We identified lipid species associated with change in WC (134 species) and BMI (156 species). Alkyl-diacylglycerol levels at baseline were associated with WC gain, with TG(O-50:2) [NL-18:1] displaying the strongest association (β- coefficient=0.125 SD increment per 1 cm increase in WC, p = 2.78E-11). Many lipid species containing linoleate (18:2) fatty acids were negatively associated with both WC and BMI gain. While associations with changes in BMI also showed strong associations with lipid species containing odd carbon number or branched chain fatty acids. The association of alkyl-diacylglycerols with WC gain showed a strong sex interaction (associated in women only). Finally, we report that multivariate models containing lipid species compared to traditional models identify individuals at a greater risk of gaining WC. Models including clinical variables and

the lipid species predicted the risk of gaining WC; top quintile relative to bottom quintile (odds ratio, 95% CI = 5.5, 4.2 – 7.2 for women and 2.4, 1.6 – 2.8 for men).

Conclusions

Our findings define metabolic profiles that characterise individuals at risk of weight gain or WC increase and represent potential biomarkers for these outcomes. Detailed mechanistic studies are now needed to better understand the interplay between lipid metabolism (as defined by the lipidomic profiles) and changes in weight and WC. Further investigations are also need to clarify the potential utility of the baseline metabolic profile for guiding weight gain management programs.

Introduction

The prevalence of obesity has increased dramatically over the past few decades and now represents up to 25% of the population in developed countries (1). Obesity and weight gain significantly increase the risk of diabetes and cardiovascular disease (CVD). Obesity can be defined using different approaches; the simplest measures include but are not limited to body mass index (BMI) (2), waist circumference (WC) and waist to hip ratio (WHR). Measures such as WC and WHR are better able to inform on body fat distribution such as abdominal fat; WC in particular correlates well with computed tomography (CT) or dual energy x-ray absorptiometry (DXA) that reflect intraperitoneal adiposity. WC and WHR show stronger associations with risk of type 2 diabetes compared to BMI (3, 4).

Lipidomics enable the measurement of several hundreds to thousands of molecular lipid species and can facilitate the identification of biomarkers for the assessment of disease risk, including weight gain (5). High throughput lipidomic analysis in large population cohorts can be useful not only to identify biomarkers but also to better understand the underlying lipid metabolism in obesity and associated co-morbidities. Recently, Lamichhane and colleagues have demonstrated the relationship of plasma lipid species with weight gain in patients with psychosis (6). In addition, a study on the metabolic signature of obesity has highlighted that the human metabolome is a stronger predictor of metabolic health compared to genetic estimates of obesity and BMI (7).

Traditional clinical chemistry approaches or nuclear magnetic resonance (NMR) technologies have been used in a number of studies to define the associations between metabolite levels and prospective changes in measures of adiposity (8-10). However, few studies have reported the association of molecular lipid species with changes in BMI (10, 11). These reports were based on small sample sizes and limited coverage of the lipidome and have not adequately defined how baseline lipidomic measures relate to changes in adiposity over time.

The aim of this study was therefore to determine the relationship between baseline lipidomic profiles and the 5-year change in WC and BMI utilizing a large population cohort; the Australian Diabetes, Obesity and Lifestyle Study (AusDiab).

Methods

Study design and participants

The AusDiab is a population-based cohort study of diabetes and associated risk factors on Australian adults. During the baseline survey conducted in 1999/2000, 11 247 subjects who were >= 25 years were recruited (23). In the subsequent follow-up during 2004/2005, 6 400 participants were re-examined (24). In this analysis, of the 10 358 subjects who had a baseline fasting plasma sample, 4 509 were excluded due to (1) being loss to follow-up (n=4 458), (2) having insufficient amount of plasma samples for analysis (n=13), (3) technical issues during MS analysis (n=19). Thus, 5 849 subjects who had a complete data at baseline and at the 5-year follow-up were included. Annual change in each metric (BMI or WC) was calculated as the difference in the metric between the baseline and 5-year follow-up divided by the follow-up duration in years.

Data collection and laboratory measurements

The collection of demographic and behavioural characteristics of the participants has been described in detail elsewhere (11, 12). Fasting blood samples were taken and BMI, weight and WC were measured at baseline and follow-up (12). Collection of physical activity levels (13) and TV viewing time (14) have been described previously. Smoking status as smokers (current) and non-smokers (never smoked)

and (ex-smokers) and daily energy intake (kJ/day) have also been described (15). Fasting plasma cholesterol and lipoprotein concentration including total cholesterol, high density cholesterol, (HDL-C), low density lipoprotein (LCL-C) and triglycerides, fasting plasma glucose (FPG) and 2 hour post load glucose (2h-PLG) were measured using standard protocols (16). Dietary information was collected using a validated food frequency questionnaire (FFQ) (11). The study was approved by the Human Research Ethics Committee at the Alfred Hospital, Melbourne, Australia.

Plasma lipidomic profiling

Lipid extraction

Plasma lipids were extracted as described previously (17) but was assisted by an automated liquid handling robot (MicroLAB STAR, Hamilton, Biosystems, Inc.). In brief, 10µL of plasma was mixed with 100µL of butanol:methanol (1:1) containing 10mM ammonium formate in Eppendorf tubes. Internal standards (22) representing major lipid classes (Table S1) were included in the extraction mix. Samples were vortexed thoroughly and sonicated in a sonicator bath for 1 hour maintained at (< 20^{o}C) and then centrifuged (14,000g, 10 min, 20^{o}C). A 100µL aliquot of the lipid extract was then transferred into 2mL vials with glass inserts.

Liquid chromatography tandem mass spectrometry (LC-MS/MS)

Lipid analysis was performed by LC ESI-MS/MS using a triple quadruple mass spectrometer (Agilent 6490 QQQ mass spectrometer with an Agilent 1290 series HPLC system and a ZORBAX eclipse plus C18 column (2.1x100mm x 1.8mm). Mass spectrometry analysis was performed in a positive ion mode with dynamic scheduled multiple reaction monitoring (MRM) as described previously (18). TGs were monitored as SIMs associated with a specific fatty acid neutral loss (NL). There were however, some modification to our previous method; we utilized a dual column setup (in which one of the column is set to equilibrate while the other is running a sample). The temperature was reduced to 45^{o}c from 60^{o}c with modifications to the chromatography to enable similar level of separation as in previous method. The mobile phase included solvent A and solvent B. Solvent A composed of 50% water, 30%, acetonitrile, 20% isopropanol (v/v/v) and solvent B with 1% water, 9% acetonitrile, 90% isopropanol (v/v/v) each containing 10mM

ammonium formate were used. Starting at 15% solvent B and increasing to 50% B over 2.5 minutes, then quickly ramping to 57% B for 0.1 minutes. For 6.4 minutes, %B was increased to 70%, then increased to 93% over 0.1 minutes and increased to 96% over 1.9 minutes. The gradient was quickly ramped up to 100% B for 0.1 minutes and held at 100% B for a further 0.9 minutes. This is a total run time of 12 minutes. The column is then brought back down to 15% B for 0.2 minutes and held for another 0.7 minutes prior to switching to the alternate column for running the next sample. The column that is being equilibrated is run as follows: 0.9 minutes of 15% B, 0.1 minutes increase to 100% B and held for 5 minutes, decreasing back to 15% B over 0.1 minutes and held until it is switched for the next sample. We used a 1-µL injection per sample with the following mass spectrometer conditions were used: gas temperature, 150˚C; gas flow rate, 17 L/min; nebuliser, 20 psi; sheath gas temperature, 200˚C; capillary voltage, 3,500 V; and sheath gas flow, 10 L/min. The detailed mass spectrometry conditions are presented in the supplementary file (Table S1). Given the large sample size, samples were run across several batches, as described above. Quality control samples; Plasma Quality Control samples (PQC) and Technical Quality Control samples (TQC), were included in the run to assess the assay performance.

Lipid classes/subclasses and species

A total of 706 distinct molecular lipid species excluding SIMs across 36 classes/subclasses, representing sphingolipids, glycerophospholipids, glycerolipids and sterols were measured (Table S2). The number of lipid species in each class/subclass ranged from 1 free cholesterol (COH) to 77 triacylglycerol (TG) species.

Data processing and statistical analysis

The 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. The relative concentration of each lipid species was determined by comparing their peak areas to the relevant internal standard (Table S1). Previously generated response factors for each species were used (19). Lipid data were $\log_{10}$ transformed and standardised prior to analysis. Each

species was examined for the association with annualized changes in WC or BMI using linear models adjusted for baseline age, sex and baseline WC/BMI and additionally for the same covariates plus total cholesterol, HDL-C, triglycerides, exercise time, smoking status and TV viewing time. Additional models were adjusted for daily total energy, dairy, saturated fat, fibre and protein intake to observe whether the associations were independent of dietary intake. We also examined if the associations between changes in WC or BMI were sex-specific by testing for sex interactions (i.e. by including sex as an interaction term in the models). P-values were corrected for multiple comparisons using the Benjamini-Hochberg procedure (20). A nominal p-value of 0.05 was considered as suggestive of an interaction.

Multivariate model development

Lipids were scaled to the standard deviation after mean centring prior to modelling. We used ridge regression model to generate metabolic scores. A 10-fold cross validation was employed for the generation metabolic scores (i.e. models trained on the 9/10th and used to predict the metabolic score in holdout 1/10th of the cohort). This was iterated so that each sample obtained a metabolic score. Metabolic scores for change in WC were derived from either traditional clinical risk factors (age, sex, WC, total cholesterol, HDL-C, triglycerides, education, smoking, exercise time, TV viewing time and energy intake) (Model 1) or these factors plus lipid species (Model 2) for men and women separately and for men and women combined. Following the generation of the scores, participants were stratified into quintiles. The relative risk (i.e. the risk of gaining >5% WC from baseline) adjusted for age, sex and baseline WC, was then computed for each quintile. All statistical analyses were performed in R version 3.6.1.

Results

Characteristics of subjects

The participant's characteristics at baseline (1999-2000) and changes in obesity metrics are shown in Table 1. The overall mean annual BMI change was 0.16 kg/m^2/year (95% CI = 0.15 – 0.17) and that of WC was 0.43 cm/year (95% CI = 0.40 – 0.46). The annual change in BMI for women was 0.18 kg/m^2/year and 0.14 kg/m^2/year for men, p= 3.00E-04. On average, WC increased by 0.51 cm/year and

0.33 cm/year, p= 3.54E-08 in men and women, respectively. The mean annual increase in BMI and WC differed by age group (Fig. S1, Table 1).

Table 1. Baseline characteristics of participants associated with changes in WC or BMI

Characteristics	n	Annualized BMI change (Kg/m^2) (SD)	^P value	Annualized WC change (cm) (SD)	^*P* value
Overall	5,849	0.16 (0.41)		0.43 (1.30)	
Sex					
Men	2,653	0.14 (0.38)	3.00E-04	0.33 (1.51)	3.54E-08
Women	3,196	0.18 (0.40)		0.51 (1.40)	
Age group					
>=55	2,241	0.09 (0.34)	2.20E-16	0.29 (1.32)	1.03E-10
<55	3,608	0.20 (0.41)		0.51 (1.26)	
Education					
High-school and below	2,178	0.17 (0.37)	7.53E-01	0.46 (1.31)	3.88E-01
Certificate and diploma	2,544	0.16 (0.39)		0.41 (1.30)	
Bachelor degree and above	1,129	0.16 (0.37)		0.42 (1.27)	
BMI category*					
Normal	2,207	0.18 (0.30)	8.20E-03	0.49 (1.19)	5.18E-03
Overweight	2,397	0.15 (0.38)		0.39 (1.29)	
Obese	1,245	0.16 (0.52)		0.37 (1.47)	
WC category#					
Low risk	2,336	0.18 (0.30)	5.50E-03	0.67 (1.12)	6.47E-34
Moderate risk	1,529	0.15 (0.36)		0.36 (1.23)	
High risk	2,015	0.15 (0.49)		0.19 (1.47)	
Smoking					
Current smoker	664	0.22 (0.55)	2.90E-05	0.54 (1.39)	8.23E-04
Ex-smoker	1,694	0.13 (0.39)		0.33 (1.29)	
Non-smoker	3,401	0.17 (0.37)		0.45 (1.28)	
TV viewing time (minutes per week)					
Tertile 1 (less than 420)	1,960	0.20 (0.36)	8.36E-07	0.51 (1.28)	3.01E-04
Tertile 2 (420-900)	1,938	0.15 (0.37)		0.41 (1.26)	
Tertile 3 (> 900)	1,929	0.14 (0.42)		0.36 (1.34)	
Diabetes					
Yes	327	0.06 (0.41)	4.67E-06	0.33 (1.24)	8.03E-02
No	5,522	0.17 (0.38)		0.44 (1.30)	
Exercise status based on exercise time (min/week)					
Sedentary (zero min)	909	0.17 (0.41)	5.32E-01	0.39 (1.28)	5.45E-01
Insufficient (0-150)	1,793	0.17 (0.39)		0.45 (1.29)	
Sufficient (over 150min)	3,127	0.16 (0.38)		0.43 (1.27)	
Total energy intake (KJ/day)					
Tertile 1 (<6430.5)	1,836	0.17 (0.40)	8.00E-02	0.51 (1.35)	4.15E-04
Tertile 2 (6430.5- 8671)	1,901	0.17 (0.39)		0.42 (1.27)	
Tertile 3 (> 8671)	1,900	0.15 (0.37)		0.36 (1.27)	

^P values are derived from Student's t-test for dichotomous characteristics, or one way ANOVA as necessary
** BMI category: Normal weight, 18.5–24.9 kg/m2; overweight, 25–29.9 kg/m^2; obese: ≥30 kg/m^2.*
 # WC categories: low risk, <94 cm for men, <80 for women; moderate risk: 94–101.9 for men, 80–87.9 for women; high risk: ≥102 for men, ≥88 for women.

Association of lipid species with change in WC

There were 134 lipid species (excluding TG measures using single ion monitoring) associated with WC change after adjusting for baseline age, sex and WC in the whole cohort. These include species of acylcarnitine (7), diacylglycerol (8), lysophosphatidylcholine (12), phosphatidylcholine (13), triacylglycerol (29 including SIMs) and alkyl-diacylglycerol (14 including SIMs) (Fig. 1A, Table S3). Glycerophospholipid species containing 18:2 or 18:3 fatty acids typically showed a strong negative association with increasing WC (i.e. lower baseline levels predicted an increase in WC) (Fig. 1A and 1B). The species positively associated with WC change were dominantly acylcarnitine and glycerolipids (diacylglycerol, triacylglycerol and alkyl-diacylglycerol species) with TG(O-50:2) [NL-18:1] representing the most significant species (0.125 SD increment per 1 cm increase in WC change per year, p= 2.78E-11). Additional adjustment for total cholesterol, HDL-C, triglycerides, exercise time, educational attainment, smoking and television viewing time resulted in fewer significant lipid species and lowering of effect sizes (Fig. 1B, Table S3). While some phospholipid and most TG and DG signatures were lost upon adjustment for clinical lipids, the TG(O) and the 18:2 fatty acid profile were not substantially modified (Fig. 1B, Table S3).

The baseline metabolic phenotype associated with increasing WC is independent of dietary intake (Fig. S2). The correlation of beta-coefficients of lipids before and after accounting for diet is shown in Fig. S2. In secondary analyses performed after excluding individuals who lost more than 5% of their WC from baseline (n=748), we found many significant associations (Table S4, Fig S3); the pattern of which is similar to what was observed in the whole cohort except for few species. The lipidomic signatures associated with change in WC and the signature associated with the baseline WC were not highly correlated (r^2 = 0.163) (Fig. S4). Generally, the lipidomic associations with baseline WC were stronger and span across all lipid classes/subclasses with 561 species significantly associated with baseline WC (Table S5) compared to associations with change in WC.

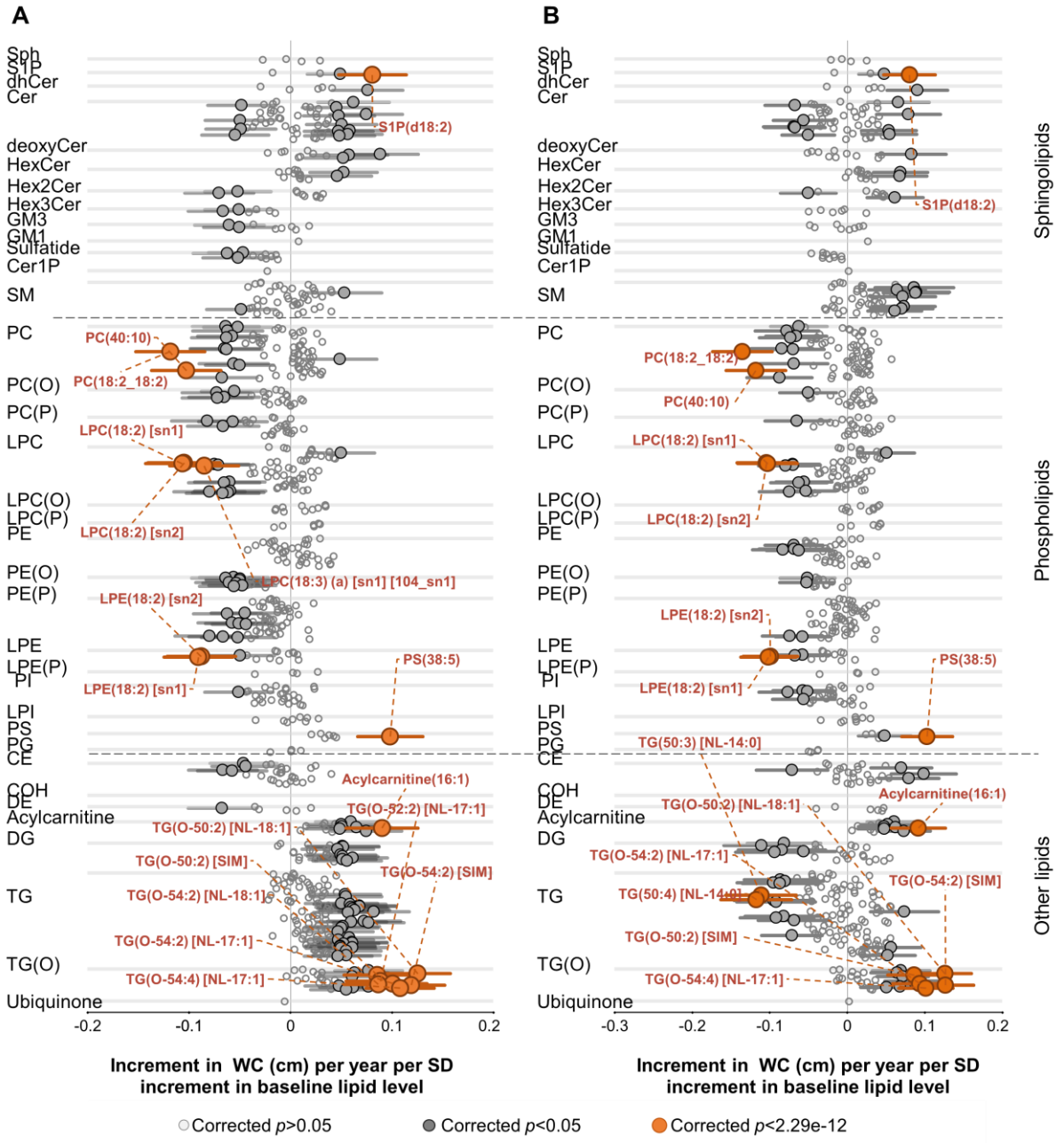

Fig. 1 Association of lipid species with change in waist circumference. Linear regression analyses of SD normalised lipid species against annualized change in waist circumference were performed, adjusting for baseline age, sex and baseline WC (panel A) or for baseline age, sex, baseline WC, total cholesterol, HDL-C, triglycerides, smoking status, education, exercise time and television viewing time (panel B). The β-coefficients (95% CIs) represent the change in WC per year associated with a SD difference of the lipid species at baseline. Open circles show lipid species with corrected p>0.05, closed circles show corrected p<0.05 and

orange circles show lipid species with the lowest corrected p-values. Whiskers represent the 95% confidence intervals.

Association of lipid species with change in BMI

After adjusting for baseline age, sex and BMI, there were 63 lipid species positively associated with change in BMI and 93 lipid species showing a negative association. (Fig. 2A, Table S6). LPC(O-22:1) displayed the strongest association (annual change in BMI = 0.027 kg/m^2 per SD increase, corrected p-value = 7.70E-05) (Fig. 2A, Table S6). Strong associations were observed with species of triacylglycerol, deoxyceramide, and several phospholipid classes. A model adjusted for age, sex, BMI, total cholesterol, HDL-C, triglycerides, smoking status, education, physical activity time and TV viewing time resulted in relatively weaker associations (Fig 2B). A further adjustment for dietary intake (including daily total energy, dairy, saturated fat, fiber and protein intake) did not materially change the pattern. The cross-sectional associations of the lipidome with baseline BMI were different from associations with changes in BMI over time (Fig. S5, Table S7).

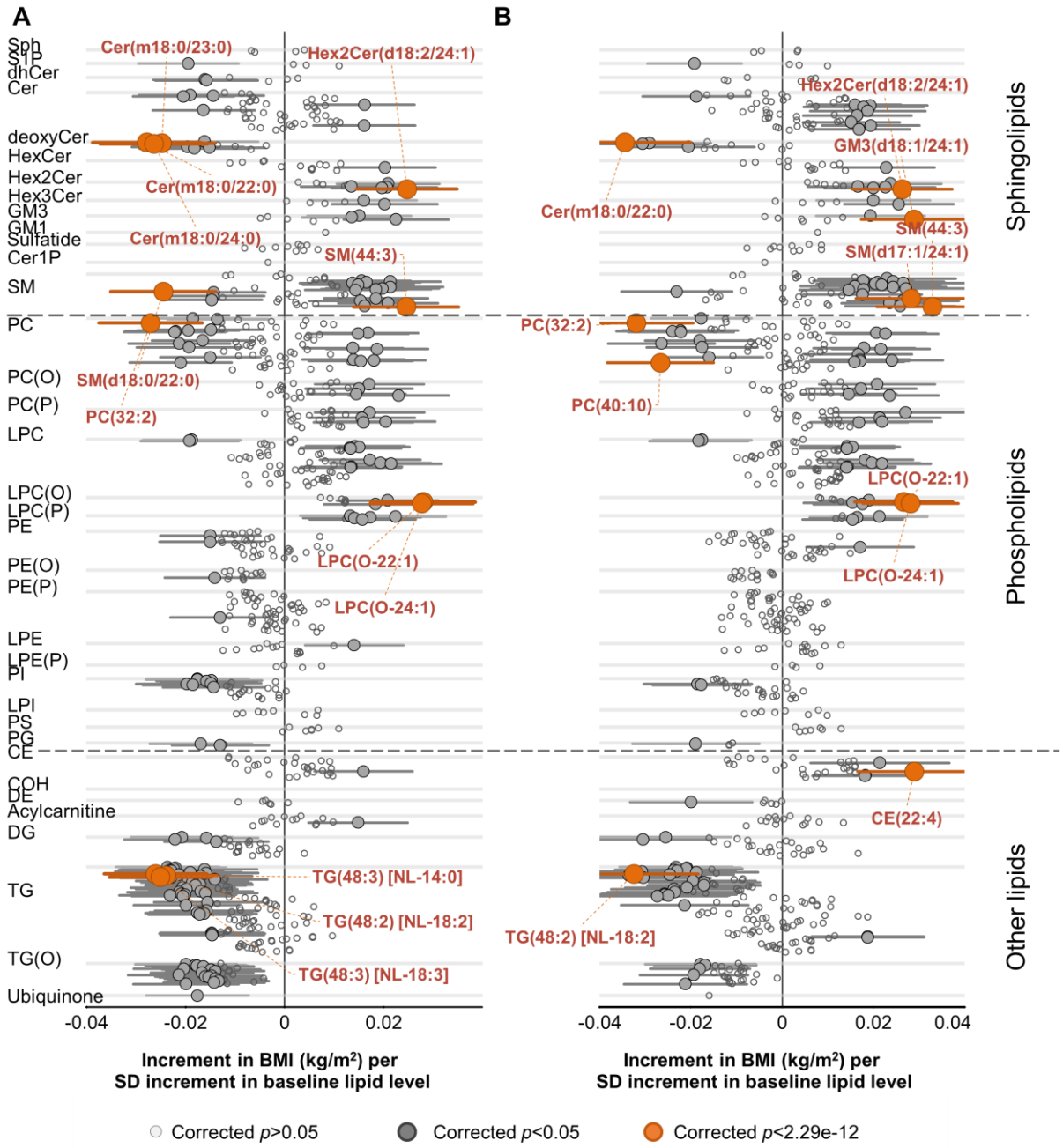

Fig. 2 Association of lipid species with change in BMI. Linear regression analyses of SD normalised lipid species against annualized change in BMI were performed adjusting for baseline age, sex and baseline BMI (panel A) or for baseline age, sex, baseline BMI ,total cholesterol, HDL-C, triglycerides, education, smoking status, exercise time and television viewing time (panel B). The β-coefficients (95% CIs) represent the change in BMI per year associated with a SD difference of the lipid species level at baseline. Open circles show lipid species with corrected p>0.05, closed circles show corrected p<0.05 and orange circles show lipid species with the lowest corrected p-values. Whiskers represent the 95% confidence intervals.

Overlapping associations of lipid species with change in WC and change in BMI over time

Change in BMI and change in WC over follow up were significantly correlated (R^2 = 0.48) (Fig. S6). We assessed whether the pattern of association between lipid species and change in WC was different from that with change in BMI. In age, sex and baseline WC or BMI adjusted models, 22 species were associated with both change in BMI and WC; 8 TG(O) species (enriched for O-18:1 alkyl chains, identified as [NL-17:1] species in Tables S3) in opposite direction and the rest in same direction (Fig S7). While 30 species were associated with both change in WC and BMI in age, sex, baseline BMI or WC plus other risk factors adjusted model (Fig 3) excluding lipid measurements based on SIMs which represent composite measures. While lipid species, particularly those containing the 18:2 fatty acid, were more strongly associated with changes in WC than with changes in BMI (Table S3, Table S6, Fig. 3), the odd and branched chain fatty acid containing lipid species were associated mainly with change in BMI. The associations of lipid species with changes in WC were correlated with the associations with changes in BMI as shown in Fig 3 (R^2 = 0.3).

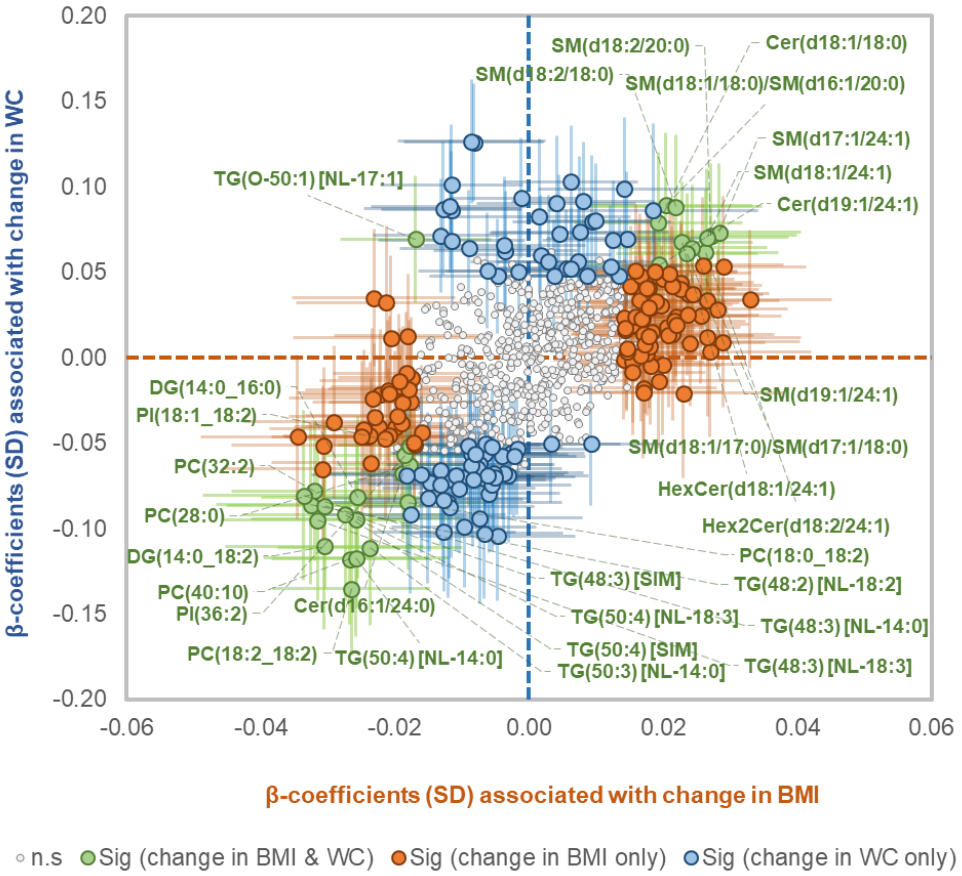

Fig. 3 Association of lipid species with change in waist circumference and BMI. Linear regression analyses of SD normalised lipid species concentrations against annualized change in WC or BMI were performed, adjusting for baseline age, sex, baseline WC/BMI, total cholesterol, HDL-C, triglycerides, education, smoking status, exercise time and television viewing time. The β-coefficients of the associations with change in BMI were plotted against the β-coefficients of the associations with change in WC. β-coefficients for lipid species that were associated with both a change in WC and change in BMI are shown in green. β-coefficients for lipid species that were associated only with a change in BMI are orange and only with a change in WC are blue. The whiskers represent 95% confidence intervals.

The associations between baseline lipidomic profiles and change in WC were sex-specific. A total of 54 lipid species (excluding TG and TG(O) species monitored by SIMs) showed a nominally significant interaction with sex in predicting change in WC (Table S8). All TG(O) species showed a positive association with change in WC in women, while no species showed a significant association in men. Certain phospholipids and ether-linked phospholipid species, enriched in the 20:4 fatty acid

tend to be negatively associated with change in WC in men but positively associated in women (Fig 4, Table S8). In contrast to WC, only 25 lipid species showed a nominal sex interaction in the association with change in BMI after controlling for age, sex, baseline BMI, total cholesterol, HDL-C, triglycerides, education, smoking status, exercise time and television viewing time (Table S9).

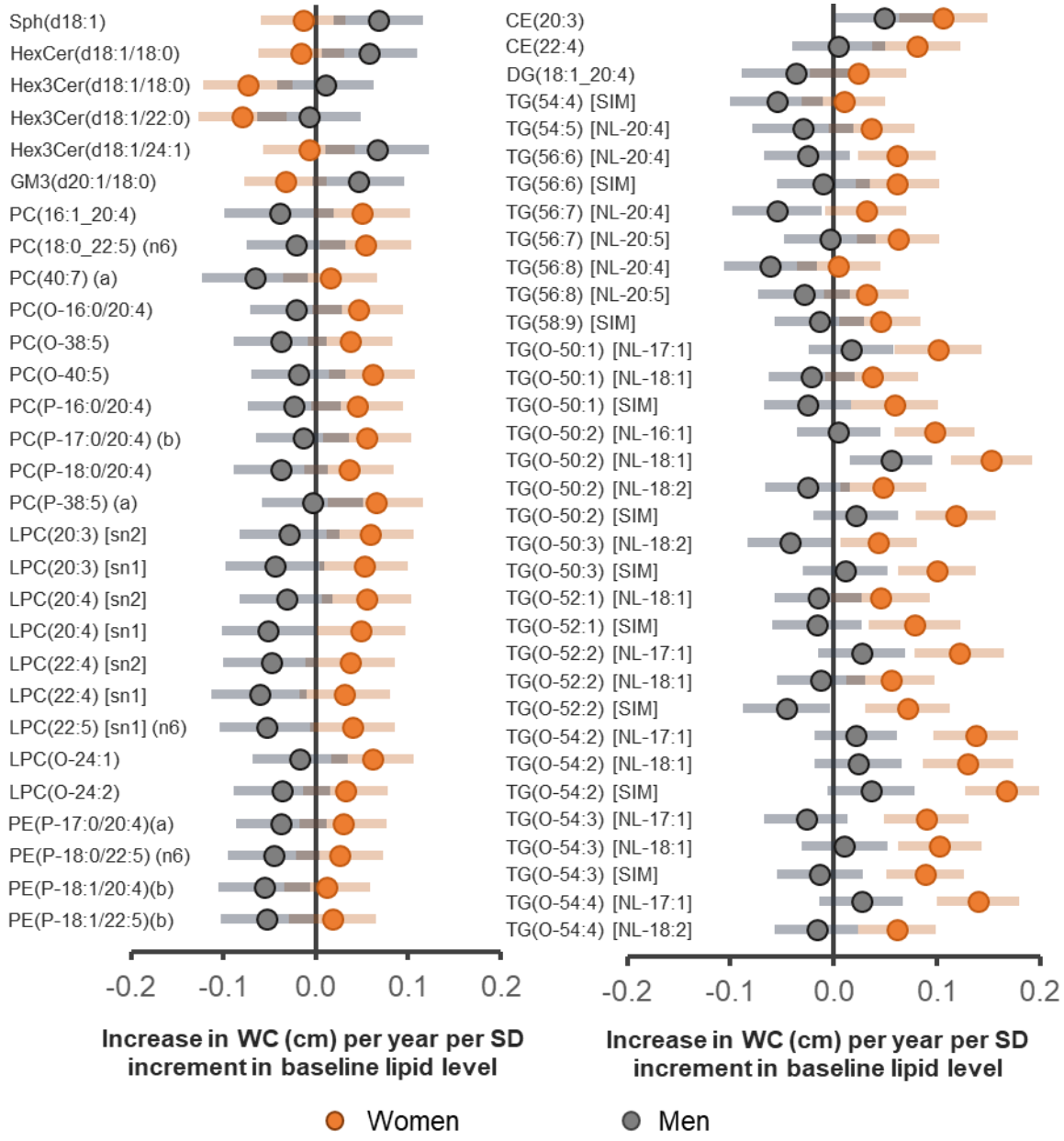

Fig. 4 The interaction of sex in the associations of lipid species with change in WC. Linear regression analyses of lipid species with change in WC were performed in models adjusting for age, sex, baseline WC, cholesterol, HDL-C, triglycerides, smoking, education, physical exercise time and TV viewing time including an interaction term for sex. The change in WC (an outcome) was annualized and each

lipid (a predictor) was scaled to SD prior analysis. The β- coefficients for each sex where the associations with change in WC showed a significant interaction (interaction $p<0.05$) are plotted. Grey circles (for men) and orange (for women) represent 1cm increase in WC per year per SD increase in the lipid predictor. Error bars show 95% confidence intervals.

Multivariate modelling to predict change in waist circumference

We developed multivariate models to generate scores for change in WC and subsequently predicted the risk of gaining waist circumference (>5% increase from baseline) during the 5-year follow up time. The scores for change in WC were derived either from: (1) the base model (Model 1) including baseline age, sex, WC, total cholesterol, HDL-C, triglycerides, education, smoking, exercise time, TV viewing time and energy intake or (2) Model 2 (Model 1 plus lipid species). The risk of gaining WC by more than 5% was 2.1 times higher among men in the top quintile relative to those in the bottom quintile (odds ratio, 95% CI = 2.1, 1.6 – 2.8). Base on the Model 2, the risk of >5% WC gain in the Q5 relative to Q1 was 2.4 times higher (Odds ratio, 95% CI = 2.4, 1.6 – 2.8) which is comparable to the base model (Table 2).

Table 2. Multivariate models predicting gain in WC in men

Quintiles of WC change score	Age years (SD)	Baseline WC cm mean (SD)	Follow up WC cm mean (SD)	Change in WC % (SD)	Risk >5% WC change relative to Q1 (Odds ratio, 95% CI)
Model 1 (Base model)					
Q1 (N=531)	59.5 (12.3)	109.5 (10.0)	110.3 (11.9)	0.7 (5.9)	1.0 (reference)
Q2 (N=531)	55.3 (12.0)	100.8 (7.0)	101.9 (8.7)	1.1 (5.6)	1.1 (0.9 – 1.5)
Q3 (N=531)	52.3 (11.3)	97.1 (6.6)	98.4 (8.3)	1.4 (5.6)	1.1 (0.8– 1.5)
Q4 (N=530)	49.5 (10.6)	92.2 (6.8)	94.2 (8.6)	2.3 (6.2)	1.4 (1.1– 1.8)*
Q5 (N=530)	42.4 (10.9)	86.5 (7.4)	89.5 (8.7)	3.3 (6.2)	2.1 (1.6 – 2.8)*
Model 2 (Model 1+lipids#)					
Q1 (N=531)	55.2 (12.2)	102.6 (10.9)	102.9 (12.5)	0.32 (5.7)	1.0 (reference)
Q2 (N=531)	53.4 (12.3)	100.0 (10.0)	101.0 (11.3)	0.98 (5.6)	1.2 (0.9– 1.6)
Q3 (N=531)	52.6 (12.6)	97.4 (10.3)	98.7 (11.0)	1.4 (5.8)	1.4 (1.1– 1.9)*
Q4 (N=530)	50.4 (12.5)	94.9 (9.6)	97.3 (10.7)	2.7 (5.8)	1.8 (1.4 –2.4)*
Q5 (N=530)	47.4 (12.9)	91.1 (10.0)	94.2 (11.0)	3.5 (6.4)	2.4 (1.6– 2.8)*

Base model: age, sex, WC, total cholesterol, HDL-C, triglycerides, smoking, exercise time, TV viewing time, energy intake.
#lipids - all the lipid species associated with change in WC in the univariate analyses
**Significant at p<0.05*

The WC trajectory across the quintiles derived from Model 1 (Fig. 5A) and Model 2 (Fig 5B) are only slightly different for men. The risk of gaining >5% of WC was greater among the four quintiles (Q2 to Q5) relative to Q1 in Model 2 compared to the Model 1 (Fig 5C).

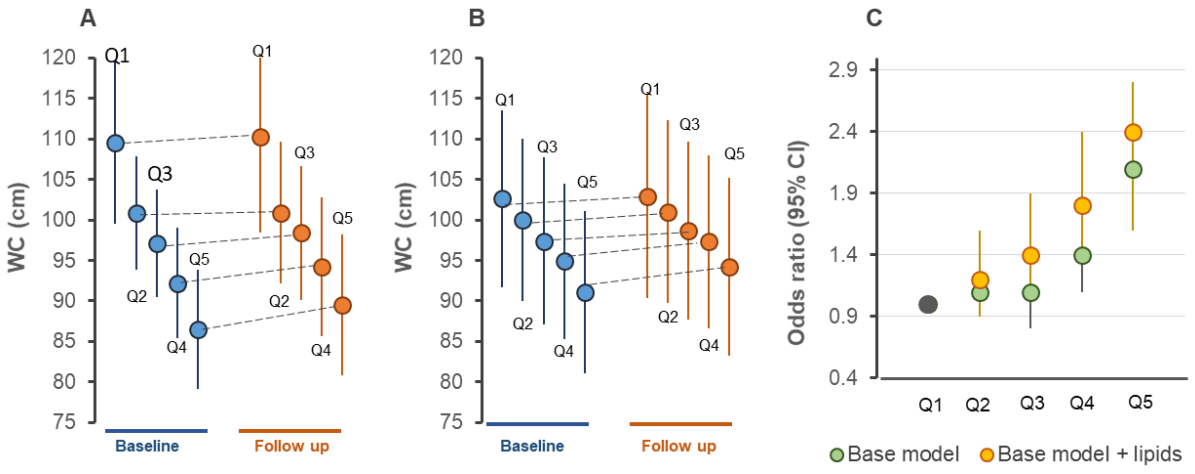

Fig 5. Risk of gaining WC across quintiles of metabolic scores in men. (A) Baseline and follow up WC measures (mean ± SD) across quintiles of the metabolic score

derived from the base model, Model 1; and (B) from the base model plus lipids, Model 2. (C) The relative risk of a >5% WC change across quintiles of the metabolic score. Base model, age, sex, WC, total cholesterol, HDL-C, triglycerides, smoking, exercise time, TV viewing time, energy intake; lipids, all the lipid species associated with change in WC.

Based on Model 1, the risk of >5% WC change in Q2-Q5 relative to Q1 was consistently higher among women relative to men. The addition of lipids to the base model particularly improved the risk of gaining WC among Q5 to Q1 (odds ratio, 95% CI = 5.5, 4.2 – 7.2) (Table 3).

Table 3. Multivariate models predicting gain in WC in Women

Quintiles of WC change score	Age years (SD)	Baseline WC cm mean (SD)	Follow up WC cm mean (SD)	Change in WC % (SD)	Risk >5% WC change relative to Q1 (Odds ratio, 95% CI)
Model 1 (Base model)					
Q1 (N=640)	58.6 (12.1)	103.0 (9.9)	103.3 (12.2)	0.3 (7.7)	1.0 (reference)
Q2 (N=639)	55.5 (11.8)	90.2 (6.3)	91.8 (9.1)	1.8 (7.8)	1.6 (1.2 – 2.1)*
Q3 (N=639)	52.9 (11.2)	83.1 (6.1)	86.2 (9.2)	3.7 (8.2)	2.3 (1.8 – 3.0)*
Q4 (N=639)	47.7 (10.3)	77.5 (5.5)	80.7 (7.8)	4.3 (8.1)	2.7 (2.1– 3.4)*
Q5 (N=639)	41.2 (9.0)	70.9 (5.0)	75.4 (7.3)	6.5 (8.3)	3.7 (2.9 – 4.8)*
Model 2 (Model 1+lipids#)					
Q1 (N=640)	55.8 (12.4)	95.7 (13.8)	94.9 (15.0)	-0.7 (8.1)	1.0 (reference)
Q2 (N=639)	53.8 (12.6)	87.1 (11.5)	88.9 (13.2)	2.1 (7.5)	1.9 (1.5 – 2.5) *
Q3 (N=639)	51.3 (12.4)	84.0 (11.9)	86.6 (12.4)	3.3 (7.7)	2.5 (1.9 – 3.3) *
Q4 (N=639)	49.1 (11.3)	81.6 (10.5)	85.4 (11.4)	4.8 (7.4)	3.8 (2.9 –4.9)*
Q5 (N=639)	46.0 (11.3)	76.3 (9.0)	81.6 (10.5)	7.2 (8.5)	5.5 (4.2 – 7.2) *

Base model: age, sex, WC, total cholesterol, HDL-C, triglycerides, smoking, exercise time, TV viewing time, energy intake.
#lipids - all the lipid species associated with change in WC in the univariate analyses
**Significant at p<0.05*

The WC trajectories across the quintiles derived from Model 1 (Fig. 6A) and Model 2 (Fig 6B) for women show similar pattern as for men but magnitudes were different. Model 2 compared to Model 1 identified higher proportion of individuals with >5% WC change across quintiles of the metabolic score (Fig 6C).

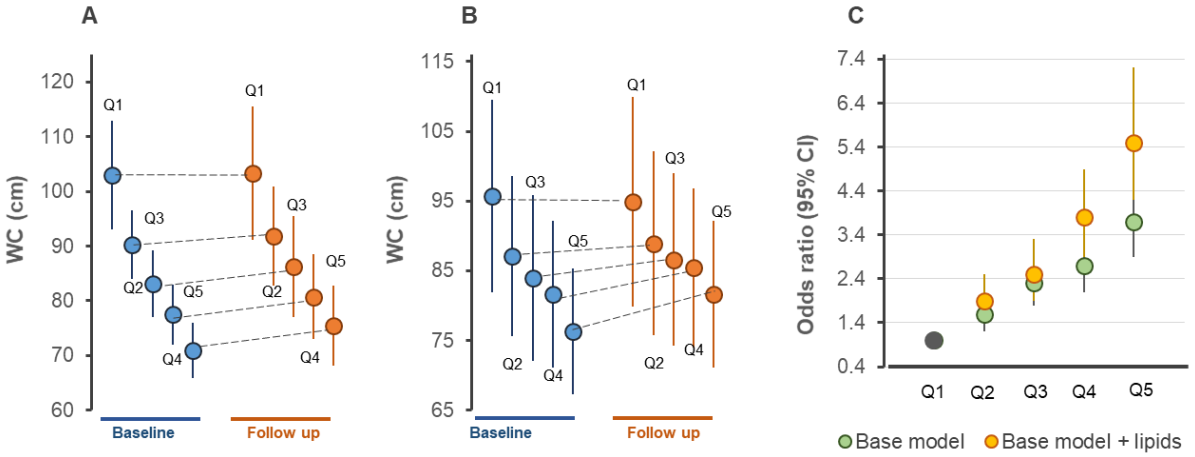

Fig 6. Risk of gaining WC across quintiles of metabolic scores in women. (A) Baseline and follow up WC measures (mean ± SD) across quintiles of the metabolic score derived from the base model, Model 1; and (B) from the base model plus lipids, Model 2. (C) The relative risk of a >5% WC change across quintiles of the metabolic score. Base model (age, sex, WC, total cholesterol, HDL-C, triglycerides, smoking, exercise time, TV viewing time, energy intake);
lipids, all the lipid species associated with change in WC.

In the men and women combined analysis, the addition of lipid species to the base model captured a larger proportion of individuals with the risk of gaining >%5 WC particularly in the top quintile. We observed a 4.5 fold higher risk (Q5 vs Q1) using Model 2 compared to only a 3.2 fold increased risk using the Model 1 (Tables S10).

Examining the baseline and follow up WC by quintiles, we observed a wide spread WC between quintiles in Model 1 (Fig. S8A). In contrast there is a narrower range in the WC measure between quintiles when lipid species were included in the model (Model 2, Fig. S8B). The odds of gaining >5% of WC was greater in Q2 to Q5 relative to Q1 in Model 2 (containing lipid species) compared to the base model with no lipid species (Model 1, Table S10 and Fig S8C).

Discussion

Perturbations across-multiple lipid classes were associated with change in WC and change in BMI over five years. Many of these lipid classes and subclasses have been associated with cardiometabolic disorders such as obesity (7, 21-24) and diabetes (23, 25-27). However, the profiles associated with change in WC and BMI

did not resemble the profiles associated with baseline WC and BMI. We also found that the baseline lipidomic profile associated with change in WC or BMI appears to be sex-dependent. Some of the lipid species (particularly in the alkyl-diacylglycerol class) showed contrasting associations with WC change and change in BMI, although the direction of association of most lipid species were common between the two measures of obesity. Importantly, we show that the lipidomic profile associated with change in BMI or WC was independent of dietary intake. Overall, our findings suggest that the risk of increasing WC or BMI is associated with a specific metabolic phenotype in the general population and this might be predicted using baseline lipidomic profiles.

Several phosphatidylcholine species, particularly those containing linoleic acid (18:2) such as PC(18:0_18:2), PC(18:2_18:2) and PC(18:2_20:5) were negatively associated with both change in BMI and WC suggesting a common mechanism. Indeed circulating linoleic acid (LA) levels have been shown to be inversely associated with type 2 diabetes (25, 28). LA is an essential fatty acid completely derived from diet (such as vegetable oils). Upon dietary intake LA can be esterified to form lipids such as triacylglycerols, cholesteryl esters and phospholipids. Although, we have no dietary information for LA in our cohort, adjusting out for common dietary variables such as energy, fat and protein did not affect the observed association of LA with change in obesity. While the negative association of LA with change in obesity suggests a protective role against waist or BMI gain, this will require testing in clinical trials in order to make dietary recommendations.

Phosphatidylcholine and other phospholipid species such as lysophosphatidylcholine containing odd and monomethyl branched chain fatty acids (mmBCFAs) like PC(15-MHDA_18:1) and LPC(15-MHDA) were positively associated, mainly with changes in BMI) but also with change in WC. This suggests not only the potential role of these fatty acids as biomarkers for predicting future waist and BMI gain but also as drivers of changes in obesity. The altered odd and mmBCFAs with changes in WC and BMI could be related to mitochondrial function. Although previously thought to be entirely derived from ruminant diary fat (29), odd chain fatty acids can be endogenously synthesized from the products of branched chain amino acid (BCAA) catabolism

which occurs in the mitochondria mainly in adipose tissue. Using differentiating 3T3-L1 adipocytes, Green *et al* have demonstrated the biosynthesis of C15:0 and C17:0 fatty acids from propionyl CoA produced from mitochondrial BCAA catabolism (30). Other groups have reported that the adipose tissue mitochondrial BCAA metabolism modulates circulating levels of mmBCFAs (31). Thus, the imbalance in mitochondrial BCAA catabolism in adipose tissue associated with altered levels of odd and branched chain fatty acids appear to precede changes in adiposity. Indeed, altered BCAA catabolism (i.e increased levels) have been implicated in insulin resistance and type 2 diabetes (32, 33) while we observed negative associations with WC change. Serum levels of odd and branched chain fatty acids have been also associated with excessive weight (34). Whether the altered mitochondrial function or the mmBCFA themselves might be causal in the subsequent weight gain remains to be determined.

A further novel observation in this study was that the baseline plasma alkyl-diacylglycerol levels and some ether lipids strongly predicted future waist gain, especially in women. Alkyl-diacylglycerols contain an alkyl group and two acyl chains attached to a glycerol backbone. Alkylglycerols are ether lipids mostly found in high abundance in shark liver oil (35); but in humans, they have not been well characterized. Studies show that alkylglycerols exhibit anti-diabetic and anti-inflammatory properties (36, 37). However, data on the role of alky-diacylglycerols in weight gain and obesity is lacking. Ether lipids in general are derived from peroxisomes that are implicated in the regulation of adipocyte thermogenesis and dynamics (38). Peroxisomes are particularly abundant in brown adipose tissue (BAT) relative to white adipose tissue (WAT) and alkylglycerols have been shown to maintain beige adipose tissue in early life in a mouse model (39). It has been shown that there is a sex-difference in the abundance of BAT (higher in women compared to men) (40) and women had a higher WC increase compared to men. Thus, it could be the interplay between peroxisomal dynamics associated with altered ether lipid metabolism (presumably in abdominal fat) that is driving changes in WC in women. The strong positive association of alkyl-diacylglycerol (mainly the O-18:1 species) with change in WC in women may indicate a differential metabolic regulation of these lipids (O-18:1 alkyl-diacylglycerols) with WC change according to sex.

We observed sex-specificity of the association of many lipid species with change in WC and BMI. Age and sex-specificity in the association of lipidome with changes in obesity had not been previously documented. However, sex-specificity in the association of free fatty acids with insulin resistance (41), and phospholipids with metabolic syndrome have been reported (42, 43). Further to these reports, a recent study identified sex-differences of metabolic profiles in BAT and WAT (44). There is a growing interest on sex- dimorphism particularly for those disorders associated with metabolic perturbations. There is a sex-specificity in the risk of CVD (45, 46) and lipid metabolism in obesity (43, 47). Such differences in disease risk are potentially reflected in metabolic profiles. The underlying mechanism for sex-specificity in the association of lipids with WC or BMI change observed here is not clear but it is possible that the endocrine system and genetics underpin these differences. Generally, younger participants, particularly women tend to gain WC and BMI over time (48) thus, it is likely that the underlying metabolic state associated with changes in obesity in men and women or in younger compared to older people are different. These sex-specific associations have important implications for sex-stratification during biomarker discovery and personalized interventions in weight loss management.

Multivariate models developed to generate risk scores for change in WC over a 5-year follow-up showed that the addition of lipid species (comprising of a mixture of sphingolipid, phospholipid, glycerolipid and other lipids) to the base model improved the stratification of individuals at high and low risk of gaining >5% WC. Our modelling suggests that the base model primarily captures the effect of age, sex and baseline WC on the risk of WC change, while the model containing lipid species captures the metabolic disturbance associated with weight gain. A logistic regression analyses between quintiles of risk revealed that the addition of lipid species to the traditional risk factors identified individuals in the top quintile with a higher odds of gaining WC. In an earlier study in the European Prospective Investigation into Cancer and Nutrition (EPIC) study a C-statistic of only 0.57 was reported using risk models with common cardiometabolic risk factors to predict a substantial weight gain (as defined by more than 10% increase from baseline during 5 year follow up) (49). However,

the EPIC study lacked the metabolic profile component that would have improved the predictive power of the model. Weight or waist gain are highly dynamic and complex processes driven by genetics, environment, dietary and life style factors. The human lipidome captures aspects of all these factors and therefore may be useful to identify those individuals at greater risk of waist gain to aid clinicians to make informed early decisions on appropriate interventions. Indeed, WC has become a vital marker in clinical practice and is an important target for reducing adverse health outcomes (50, 51).

The strengths of this study include the large national population-based sample which facilitates the generalizability of our findings. In addition to this, the plasma lipidome coverage was broad (measuring over 700 lipid species across 36 lipid classes/subclass) using the state-of-the-art LC-MS/MS technology. The potential limitations include, lack of a similar population-based cohort for replication of our findings and the fact that lipidomic profiles were measured only at baseline as opposed to the measures of obesity which were obtained at baseline and 5-year follow-up. Moreover, low initial response rate and modest loss to follow up in the AusDiab study may have led to a selection bias. Nevertheless, this study is the first of its kind to explore the potential of baseline lipidomic profiles to define and predict future gain in measures of obesity (WC and BMI) and to further explore whether these are sex-specific.

In conclusion, our study identified plasma lipidomic signatures that were potential predictors of future WC or BMI gain in a large population cohort. The observed associations between the baseline lipid levels and longitudinal changes in weight and WC may have implications not only for identification of individuals who are at risk of gaining weight or WC but also to shed light on the metabolic basis of obesity progression over time. The distinct nature of the lipidomic profiles that predict change in BMI compared to those that predict change in WC in the present study suggest the metabolic dysregulation preceding each is quite distinct and further studies may provide insight into effective interventions.

Acknowledgments

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Author Contributions

HBB extracted plasma samples, performed LC-MS/MS analysis, analysed the data and wrote the manuscript. GO & AATS developed R scripts and provided statistical support. CG and KH developed LC-MS/MS methods and provided support for the LC-MS/MS analysis and statistical analysis. MC developed extraction protocols and extracted plasma samples. NM supported the LC-MS/MS experiment and data pre-processing and analysis. JES and DJM, coordinated the AusDiab data, interpreted results and revised the manuscript. PJM oversaw this work and revised the manuscript. PJM and DJM are the guarantors of this work and shall take the responsibility for the full access and integrity of the data. All authors have approved the final version of the manuscript.

Competing interests

Authors have no competing interests associated with this submission

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Chapter 5. Supplementary Figures

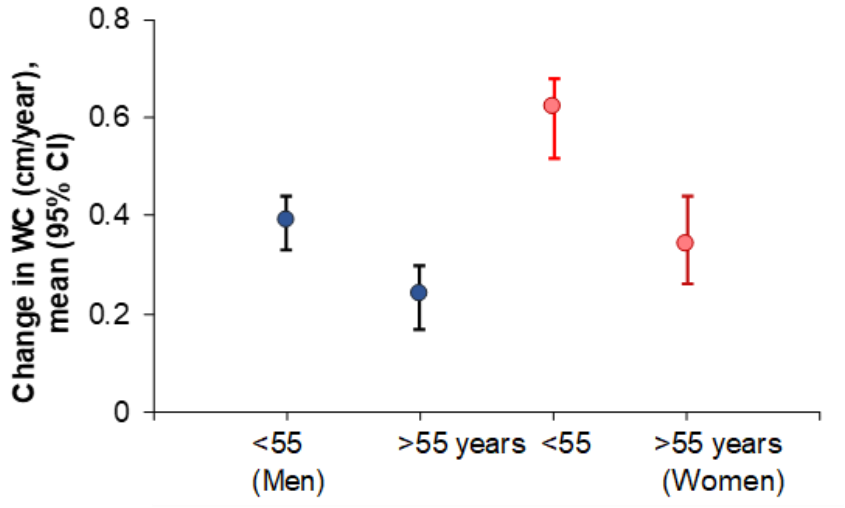

Fig. S1. Annual waist circumference (WC) change by age and sex. Mean annualized WC change (cm/year) is shown on y-axis for men and women further stratified by age (< 55 years and >55 years)

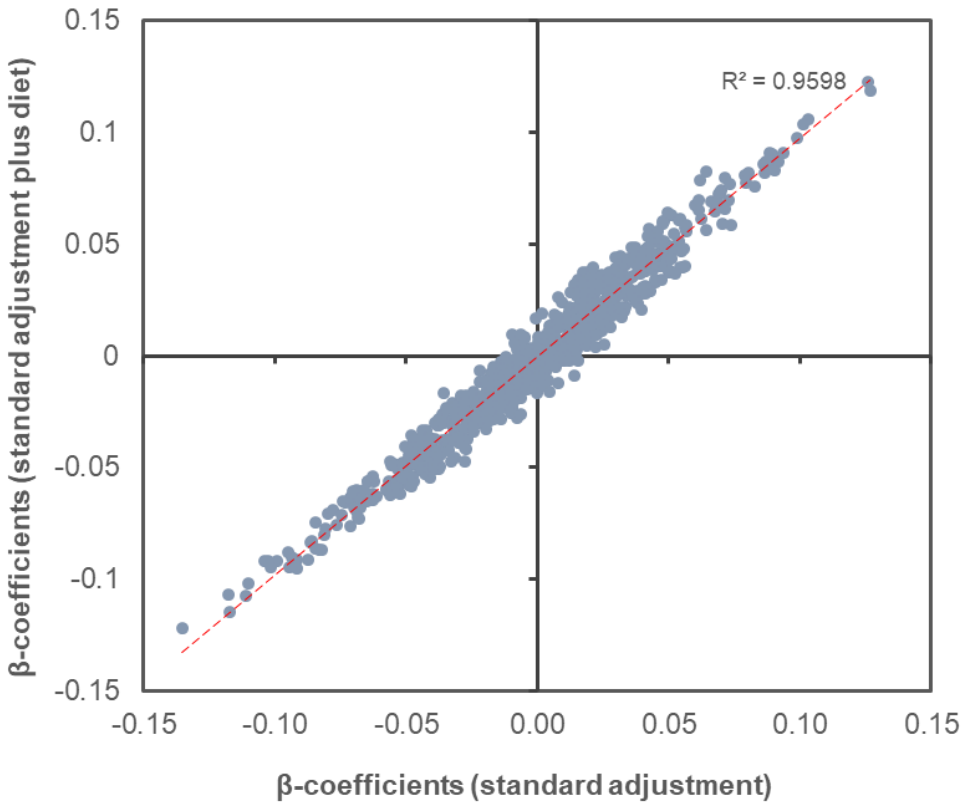

Fig. S2 the correlation between beta-coefficients of lipids associated with change in WC before and after accounting for diet. Linear regression analyses of SD normalised lipid species against annualized change in waist circumference were performed, adjusting for age, sex, baseline WC, total cholesterol, HDL-C, triglycerides, smoking status, education, exercise time and television viewing time, energy intake, total fat, saturated fat, protein, dairy and fiber (y-axis). Linear regression analyses of SD normalised lipid species against annualized change in waist circumference were performed, adjusting for age, sex, baseline WC, total cholesterol, HDL-C, triglycerides, smoking status, education, exercise time and television viewing time (y-axis). The β-coefficients represent the change in WC per year associated with a SD difference of the lipid species at baseline.

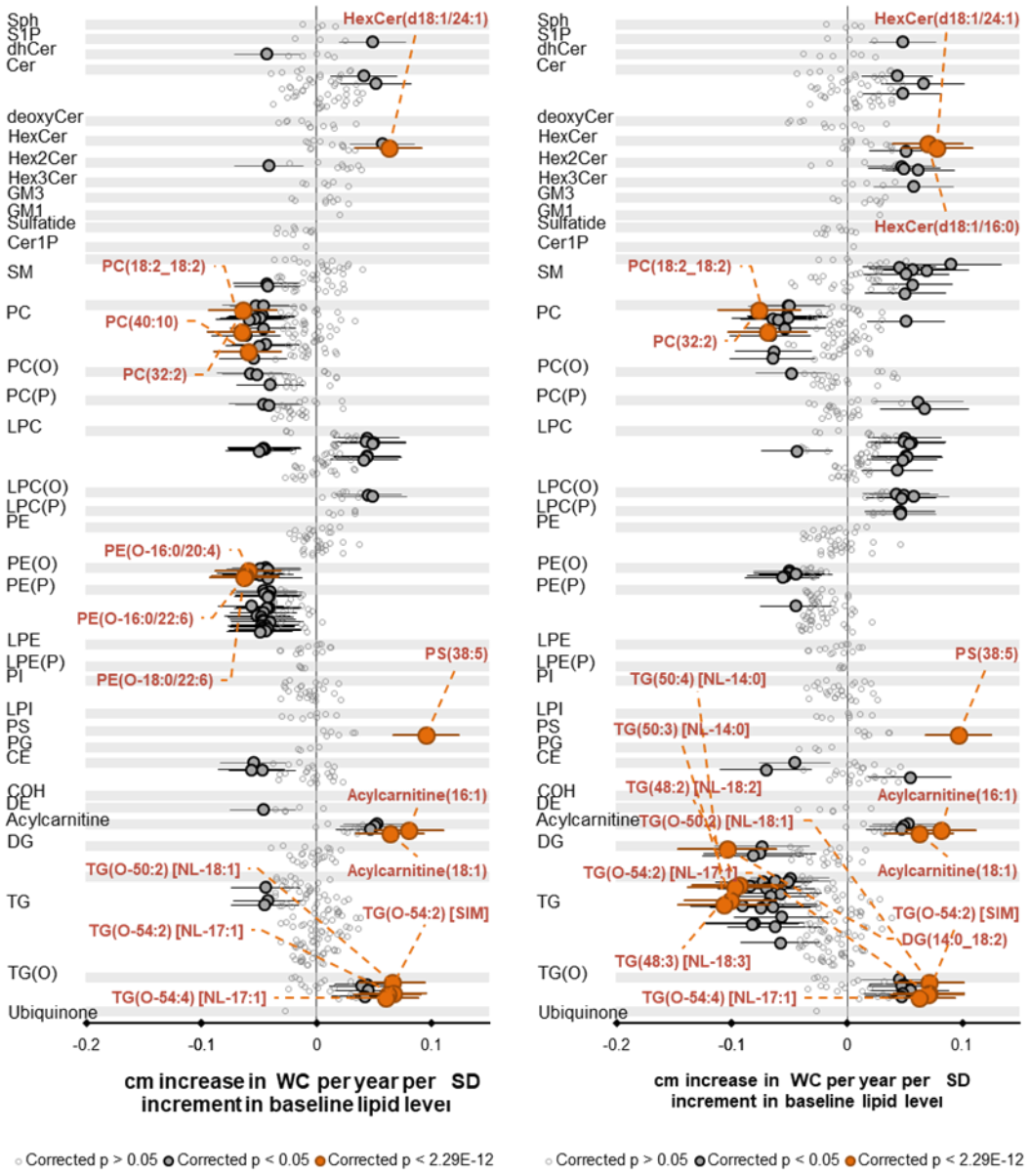

Fig. S3 Association of lipid species with change in WC. Linear regression analyses of SD normalised lipid species against annualized change in waist circumference were performed, adjusting for age, sex and baseline WC (left panel) and for age, sex, baseline WC , total cholesterol, HDL-C, triglycerides, smoking status, education, exercise time and television viewing time (excluding those subjects who lost more than 5% of their WC from the baseline) (right panel). The β-coefficients (95% CIs) represent the change in WC per year associated with a SD difference of the lipid species at baseline. Open circles show lipid species with corrected p>0.05, closed circles show corrected p<0.05 and orange circles show lipid species with the lowest corrected p-values. Whiskers represent the 95% confidence intervals.

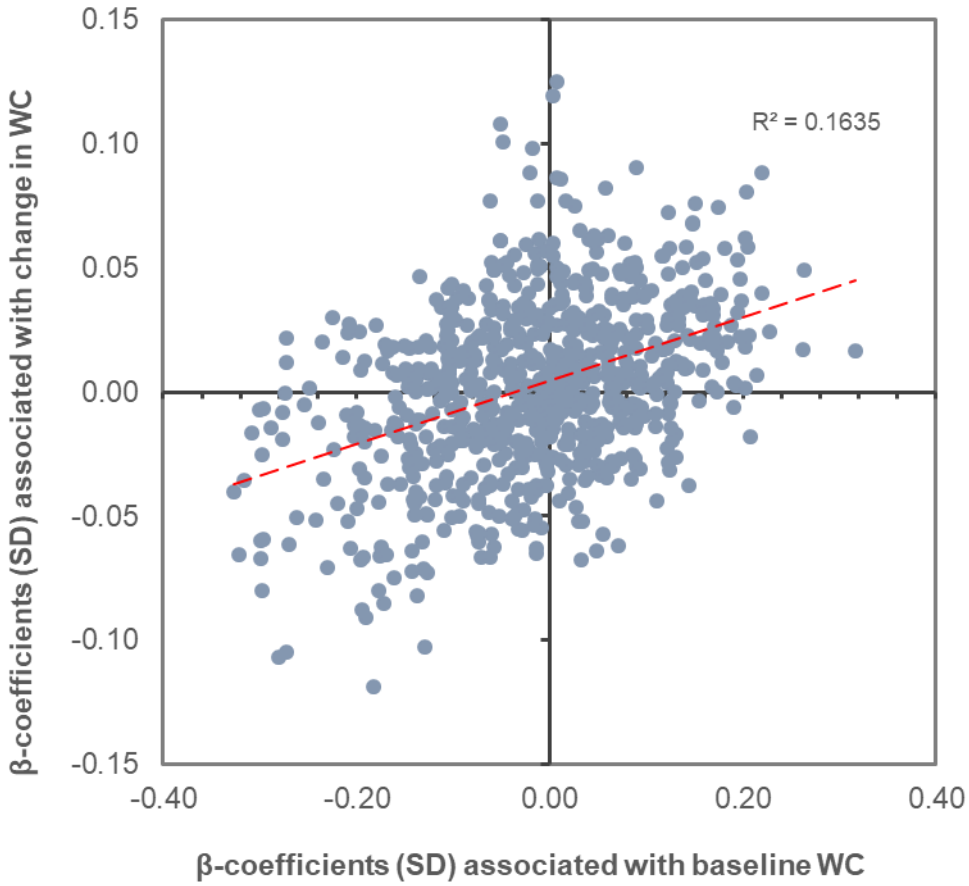

Fig. S4 the correlation between beta-coefficients for WC and change in WC. Linear regression analyses of SD normalised lipid species against baseline WC were performed, adjusting for age, sex, total cholesterol, HDL-C, triglycerides, smoking status, education, exercise time and television viewing time. Linear regression analyses of SD normalised lipid species against annualized change in WC were performed, adjusting for age, sex, baseline WC, total cholesterol, HDL-C, triglycerides, smoking status, education, exercise time and television viewing time (y-axis).

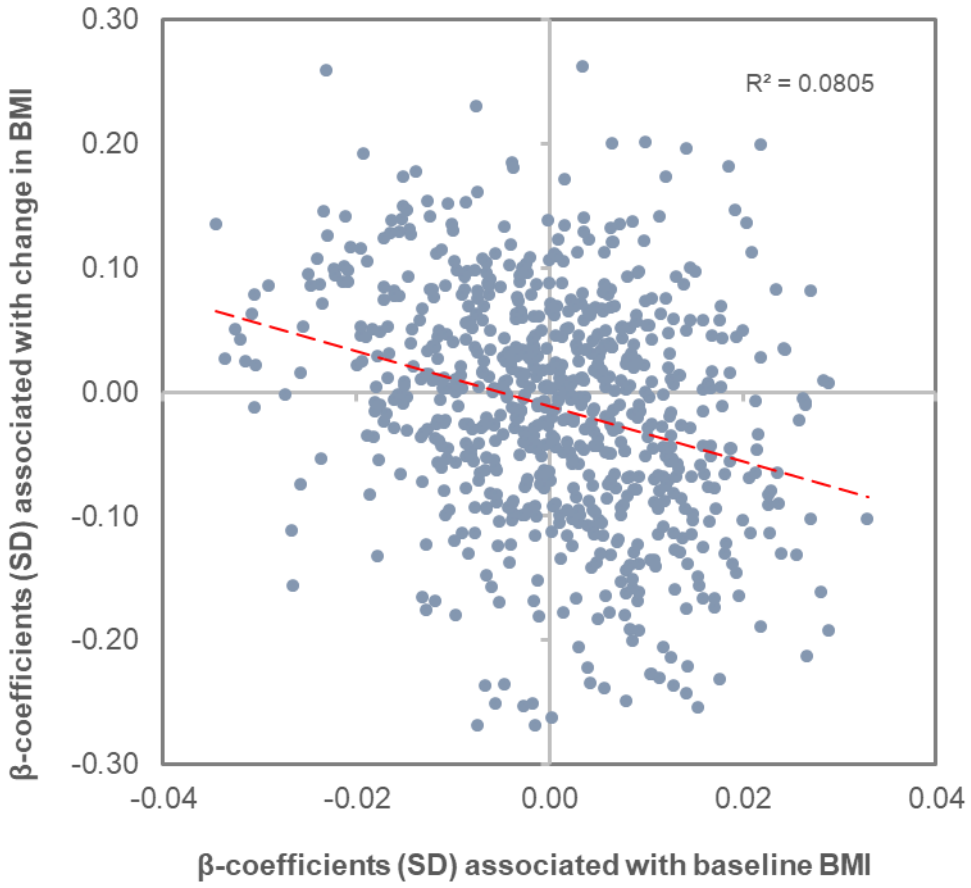

Fig. S5 the correlation between beta-coefficients for baseline BMI and change in BMI. Linear regression analyses of SD normalised lipid species against change in BMI were performed, adjusting for age, sex, baseline BMI, total cholesterol, HDL-C, triglycerides, smoking status, education, exercise time and television viewing time (y-axis). Linear regression analyses of SD normalised lipid species against baseline BMI were performed, adjusting for age, sex, total cholesterol, HDL-C, triglycerides, smoking status, education, exercise time and television viewing time (x-axis).

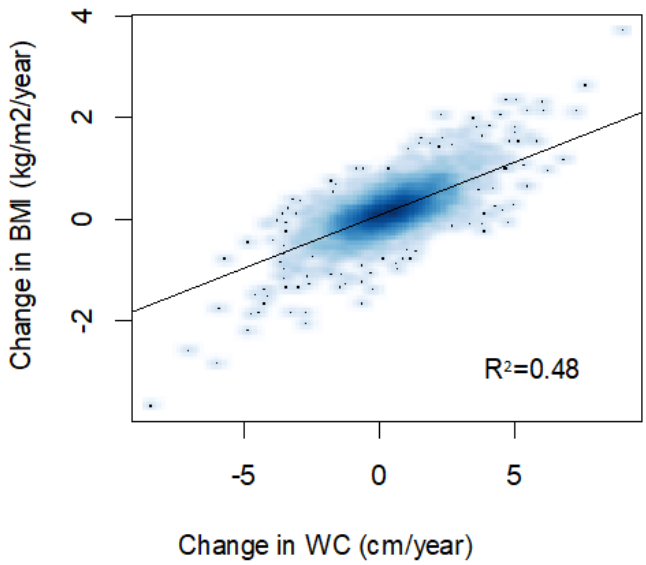

Fig S6. The correlation between change in BMI and change in WC. A linear relationship between change in BMI (kg/m2/year on y-axis) and the change in WC (cm/year on x-axis) was assessed by smooth scatter plot

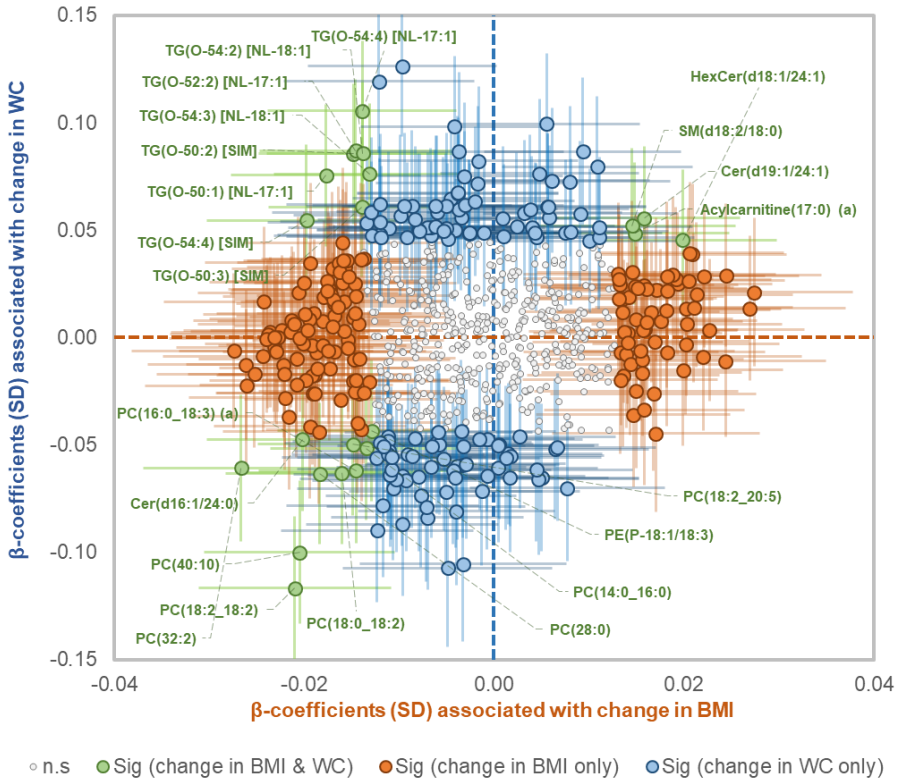

Fig. S7 Association of lipid species with change in WC and BMI. Linear regression analyses of SD normalised lipid species concentrations against annualized change in WC (adjusting for baseline age, sex and WC) or BMI (adjusting for baseline age, sex and BMI) were performed. The β-coefficients of the associations with change in BMI were plotted against the β-coefficients of the associations with change in WC. β-coefficients for lipid species that were associated with both a change in WC and change in BMI are shown in green. β-coefficients for lipid species that were associated only with a change in BMI are orange and only with a change in WC are blue

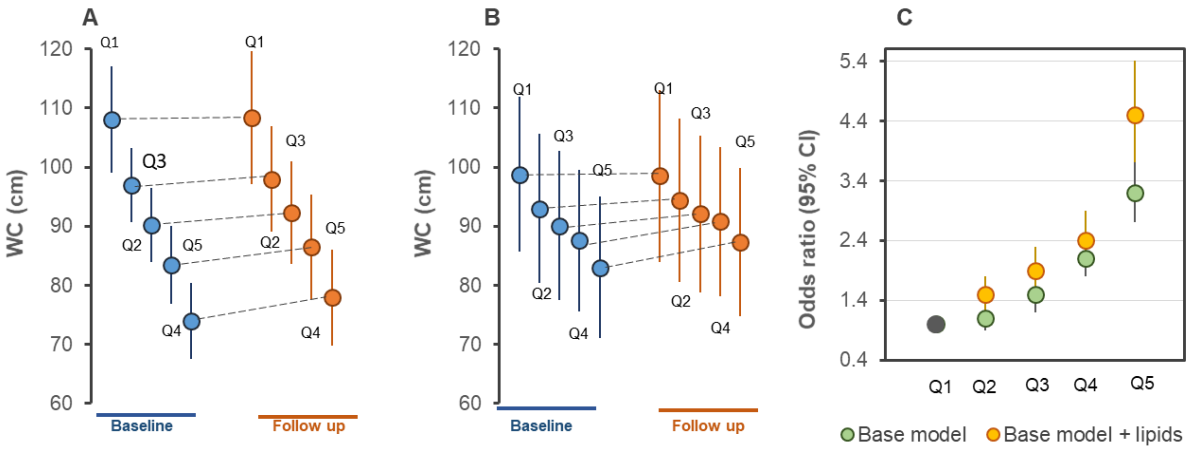

Fig. S8 Risk of gaining WC across quintiles of metabolic scores. (A) Baseline and follow up WC measures (mean ± SD) across quintiles of the metabolic score derived from the base model, Model 1; and (B) from the base model plus lipids, Model 2. (C) The relative risk of a >5% WC change across quintiles of the metabolic score.

Chapter 6. Metabolic Phenotyping of BMI to Assess Cardiometabolic Risk

Preface

BMI is strongly associated with cardiometabolic risk. Yet, two individuals with the same BMI can display different health risk profiles. This variance can be captured in part by the individual's metabolic profile. In Chapter 6 we derived a metabolic BMI score (mBMI) using lipidomic dataset in the AusDaib and validated the model on the BHS cohort. We, then examined at the associations of mBMI residuals with disease outcomes independent of the real/measured BMI. Findings from this study signify the role of mBMI to improve up on BMI in assessing health risk and in identifying a specific group of people at higher risk for cardiometabolic diseases.

Chapter 6 is being prepared for submission to the journal of clinical investigation (JCI).

To facilitate a better flow between chapters within this thesis, the Supplementary Tables for this chapter has been presented separately (Appendix IV).

Metabolic Phenotyping of BMI to Characterize Cardiometabolic Risk

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Abstract

Body mass index (BMI) is one of the key determinants of cardiometabolic disorders. Yet, two individuals with similar BMI can display varying risk profiles. This makes the use of BMI alone an imperfect measure of obesity and the associated metabolic disturbance. Here, we derived a metabolic BMI score, mBMI using lipidomic data and assessed it's utility to phenotype obesity and characterize metabolic health risk. The mBMI modelling improves our understanding of the metabolic basis of obesity and this in turn facilitates the identification of a specific group of individuals who at high risk for personalized interventions

Introduction

Obesity is strongly associated with an increased risk of cardiometabolic disorders including type 2 diabetes (T2D) (1, 2) and cardiovascular disease (CVD) (3, 4). The increasing burden of excess body weight is partly explained by high calorie intake coupled with insufficient physical exercise (5, 6). Several studies have identified metabolic signatures including lipids to be associated with obesity (7-9). In addition, we have previously shown that the plasma lipidome is strongly associated with BMI (where several hundreds of plasma lipid species in large population cohorts were identified) (10, 11).

Body mass index (BMI) is an accessible surrogate measure of obesity. Compared to "gold standard" measures of obesity, BMI is a simple to measure and interpret, using WHO classifications. Despite not directly measuring body composition, BMI strongly associates with cardiometabolic outcomes (12). Yet, it has been recognized that not all individuals who are obese/overweight just based on measured BMI present with an increased risk of metabolic complications (13). The fact that there is a substantial variability among individuals with the same BMI to develop different disease complications (14, 15) calls for new insight towards obesity estimation to improve disease phenotyping and characterization of cardiometabolic risk in large population cohorts. Such an approach is of paramount importance to facilitate identification of obesity markers for personalized management (15-17).

Genetics so far explains <3% of phenotypic variation of BMI (18). Metabolic profiling, on the other hand, explain up to 47% of BMI variability (8, 9). To precisely estimate the risk of cardiometabolic diseases and improve the precision of diagnosing obesity and quantifying associated risk, better surrogate markers such as the metabolome based risk scores are needed. The human metabolome contains several hundreds to thousands of molecular species that can be utilized to estimate metabolome based BMI score; the "metabolic BMI". Metabolic BMI (mBMI), as opposed to the real BMI can reflect metabolic health status better and be a more precise target for intervention of obesity as it helps select a specific group of people who are at higher risk of disease complications regardless of what their real BMI is (8).

Using the human metabolome, Cirulli *et al* showed that metabolomics approaches could develop models that effectively predict BMI in a way that better captures the metabolic disturbance associated with body fat (8). Cirulli *et al* devised mBMI score and demonstrated that the metabolome profile was a stronger indicator of metabolic health compared to the genetic risk score and BMI. The human lipidome also captures obesity-related metabolic alterations more accurately compared to classical clinical risk factors (9). Existing studies on this approach are very few and are limited by small number of participants or lack of validation on external independent cohorts. Thus, there is a need to build up on the previous studies and further information on the ability of the metabolome to predict BMI and whether this BMI better defines cardiometabolic health compared to the measured BMI

Here we extend upon the existing evidence to demonstrate in two large population studies that a metabolic BMI not only captures metabolic disturbance associated with obesity but that this disturbance is associated with poor cardiometabolic outcomes. We hypothesize that the plasma lipidome could provide a novel measure of obesity related metabolism - the mBMI score - which captures the metabolic health risk of individuals that BMI fails to do. The mBMI will also improve characterization of cardiometabolic risk, i.e., better discrimination of groups that differ in their health outcomes. Based on these hypotheses, we aimed 1) to construct mBMI using the human plasma lipidomic data in the AusDiab study cohort and validate this on an independent cohort; the Busselton Health Study cohort (BHS); 2) assess whether

mBMI and or mBMI residuals better correlate with metabolic traits (WC, FBG, 2h-PLG, HbA1C, HOMA-IR, cholesterol, LDL-C, HDL-C, triglycerides compared to true BMI; 3) to examine the relationship of mBMI or mBMI residuals and cardiometabolic outcome (prevalent diabetes, incident diabetes and CVD events).

Methods

Participants

The AusDiab study

The AusDiab cohort is a prospective population study that was established to study the prevalence and risk factors of diabetes and CVD in an Australian adult population. The baseline survey was conducted in 1999/2000 with 11,247 participants aged ≥ 25 years randomly selected from the six states and the Northern Territory comprising 42 urban and rural areas of Australia using a stratified cluster sampling method. The detailed description of study population, methods, and response rates of the AusDiab study is found elsewhere (19). Measurement techniques for clinical lipids including fasting serum total cholesterol, HDL-C and triglycerides as well as for height, weight, BMI and other behavioural risk factors have been described previously (20). We utilized all baseline fasting plasma samples from the AusDiab cohort (n = 10,339) after excluding samples from pregnant women (n =19), those with missing data (n= 279), or whose fasting plasma samples were unavailable (n=591). The mean (SD) age was 51.3 (14.3) years with women constituting 55% of the cohort. In the AusDiab Study cohort, 590 incident cardiovascular events (including fatal and non-fatal events) were recorded over 10 years of follow-up (Table S1). Additionally there were 577 prevalent CVD (history of heart attack and stroke combined) at baseline (Table S2). All the CVD events were ascertained through linkage to the National Death Index and medical records. The specific end points included fatal events, myocardial infarction (MI), cerebrovascular accident (CVA), coronary artery bypass grafting (CABG) and percutaneous transluminal coronary angioplasty (PTCA). The detailed baseline characteristics of the participants is presented in Table 1. The present study was approved by the Alfred Human Research Ethics Committee, Melbourne, Australia (project approval number, 41/18).

The Busselton health study (BHS)

The Busselton Health Study is a community-based study in the town of Busselton, Western Australia; the participants are predominantly of European origin. A total of 4,492 subjects in the 1994/95 survey of the ongoing epidemiological study were included. The mean (SD) age was 50.8 (17.4) years with women constituting 56% of the cohort. The details of the study and measurements for HDL-C, LDL-C, triglycerides, total cholesterol and BMI are described elsewhere (21, 22). The characteristics of study participants is shown in Table 1. We utilized the Busselton Health Study (BHS) cohort as a validation cohort. The BHS cohort consisted of 575 CVD events. The baseline characteristics of events and controls are summarized in Table S1. The BHS study was approved by the University of Western Australia Human Research Ethics Committee (UWA HREC).

Lipidomic data

Lipid extraction

A butanol/methanol extraction method described previously (23) was used to extract lipids from human plasma. Briefly, 10µL of plasma was mixed with 100µL of a 1-butanol and methanol (1:1 v/v) solution containing 5mM ammonium formate and the relevant internal standards (Table S3). The resulting mix was vortexed (10 seconds) and sonicated (60 min, 25°C) in a sonic water bath. Immediately after sonication, the mix was centrifuged (16,000xg, 10 mins, 20°C). The supernatant was transferred into samples tubes containing 0.2ml glass inserts and Teflon seals. The extracts were stored at -80ºC until analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS).

Liquid chromatography mass spectrometry

Targeted lipidomic analysis was performed using liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). An Agilent 6490 triple quadrupole (QQQ) mass spectrometer [(Agilent 1290 series HPLC system and a ZORBAX eclipse plus C18 column (2.1x100mm 1.8µm, Agilent)] in positive ion mode was used [details of the method and chromatography gradient have been described previously (10)]. Compared to our earlier study, we modified the methodology to enable a dual column setup (while one column runs a sample, the other is

equilibrated). In brief, the temperature was reduced to 45^{o}c from 60^{o}c with modifications to the chromatography to enable similar level of separation. Starting at 15% solvent B and increasing to 50% B over 2.5 minutes, then quickly ramping to 57% B for 0.1 minutes. For 6.4 minutes, %B was increased to 70%, then increased to 93% over 0.1 minutes and increased to 96% over 1.9 minutes. The gradient was quickly ramped up to 100% B for 0.1 minutes and held at 100% B for a further 0.9 minutes. This is a total run time of 12 minutes. The column is then brought back down to 15% B for 0.2 minutes and held for another 0.7 minutes prior to switching to the alternate column for running the next sample. The column that is being equilibrated is run as follows: 0.9 minutes of 15% B, 0.1 minutes increase to 100% B and held for 5 minutes, decreasing back to 15% B over 0.1 minutes and held until it is switched for the next sample. We used a 1-µL injection per sample with the following mass spectrometer conditions were used: gas temperature, 150˚C; gas flow rate, 17 L/min; nebuliser, 20 psi; sheath gas temperature, 200˚C; capillary voltage, 3,500 V; and sheath gas flow, 10 L/min. Given the large sample size, samples were run across several batches, as described above. The LC-MS/MS conditions and settings with the respective MRM transitions for each lipid can be found in Table S3.

Data pre-processing

Integration of the chromatograms for the corresponding lipid species was performed using Agilent Mass Hunter version 8.0. Relative quantification of lipid species was determined by comparing the peak areas of each lipid in each patient sample with the relevant internal standard (Table S3). A median centring approach was carried out to correct for batch effect using PQC samples (24). More than 90% of the lipid species were measured with a coefficient of variation <20% (based on PQC, samples). Only technical outliers (n = 19) were excluded from the downstream analysis.

Statistical analysis

Lipid data was scaled to unit variance prior to statistical analysis. A ridge regression model including age, sex and the lipidome (comprising 569 lipid species common to the AusDiab and the BHS cohorts) was employed to predict BMI. In addition, elastic

net and least absolute shrinkage and selection operator (LASSO) models were developed to predict BMI. A 10-fold cross validation was employed for the generation metabolic scores (i.e. models trained on the 9/10th and used to predict BMI in holdout 1/10th of the cohort). This was iterated so that each sample obtained a predicted BMI score using the *glmnet* R package. Models were developed using the AusDiab cohort and validated in the BHS cohort. A metabolic BMI (mBMI) was derived from the predicted BMI as follows: mBMI = BMI + (predicted BMI – line of best fit). The difference between the mBMI and the BMI, termed the 'mBMI residual', was then used to stratify individuals into five groups (obese-metabolically obese, overweight-metabolically overweight and normal weight-metabolically healthy in these cases the BMI was within the standard BMI ranges (18.5-25.0; 25.0-30.0; >30.0), while the mBMI fell between -2 and 2 Kg/m^2 of the BMI. The two remaining groups were those with mBMI>BMI (mBMI > BMI + $2Kg/m^2$) and mBMI<BMI (mBMI < BMI - $2Kg/m^2$). A logistic regression model was used to assess the relationship between BMI or the mBMI residual and T2D (prevalent and incident) and prevalent CVD adjusted for age and sex or age and sex plus clinical lipids. Cox regression models were fitted to compute hazard ratios (HRs) associated with future CVD events, using age as the time scale and adjusting for sex, smoking status and T2D with and without clinical lipids. Analyses were performed in STATATM v14 (StataCorp LP, Inc., Texas, USA) or R (version 3.6.1).

Results

Participants' characteristics

The baseline characteristics for the study subjects in the AusDiab and the Busselton cohorts are presented in Table 1.

Table 1. Characteristics of the study populations

	AusDiab	**BHS**
Characteristics	**(N=10,339)**	**(N=4,492)**
Age (years)	51.3 (14.3)	50.8 (17.4)
Women, n (%)	5685 (55.0)	2516 (56.0)
BMI (kg/m^2)	26.9 (4.9)	26.0 (4.1)
WC (cm)	90.8 (13.8)	86.1 (12.8)
Cholesterol (mmol/L)	5.7 (1.1)	5.6 (1.1)
LDL-C (mmol/L)	3.5 (0.9)	3.6 (1.0
HDL-C (mmol/L)	1.4 (0.4)	1.4 (0.4)
Triglycerides (mmol/L)*	1.3 (1.0)	1.1 (0.8)
SBP (mmHg)	129.2 (18.6)	123.5 (18.0)
DBP (mmHg)	70.1 (11.7)	74.5 (10.4)
Diabetes (%)	6.6	5.7
Smoking (%)	15.9	14.4

* data in median (IQR)

Modelling metabolic BMI

We (10, 11) and other groups have previously reported the associations of BMI with the plasma lipidome (25, 26). Here, we constructed linear models predicting BMI from the lipidomic data and used these to derive a mBMI. Models using ridge regression, elastic net and LASSO were performed and these resulted in comparable performances in terms of R^2 values for prediction of BMI (Table S4). The ridge model was chosen for further analyses as it provides beta coefficients for all lipid species and hence captures more biology at the same time it is highly suitable to handle multicollinearity in lipidomic data . The mBMI was predicted from the whole plasma lipidome (lipid species, n=569 common to the AusDiab and BHS cohorts) using ridge regression (lambda = 0.017), including age and sex. The correlation analysis between mBMI and BMI in the test population (AusDiab) yielded a R^2 = 81% (Fig 1A) and in the validation cohort (BHS) the same model produced a R^2 = 65% (Fig 1B). Modelling of BMI using the common risk factors (age, sex, HDL-C, total cholesterol and triglycerides) explained only 15.6 and 10.2% variability of the variation of BMI in the AusDiab and in the BHS cohort respectively (Table S4).

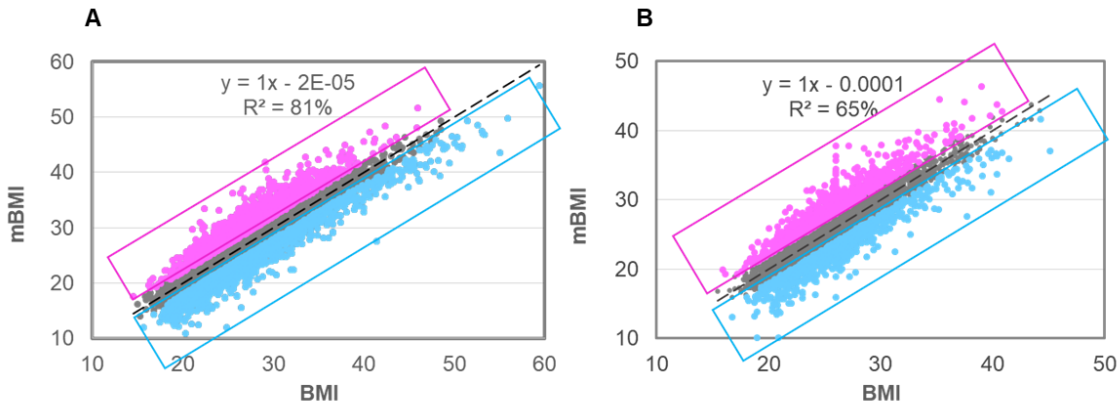

Fig 1. The correlation between mBMI and BMI. A ridge regression model was created using the whole lipidome (569 lipids species common to the AusDiab and BHS cohorts) to predict BMI, adjusting for age, sex, total cholesterol, HDL-C and triglycerides. A) The mBMI was plotted against the BMI for all individuals in the AusDiab cohort (n=10,339, and B) the same model was used to determine mBMI for individuals in the Busselton cohort (n = 4,492). The metabolic discordant groups are highlighted: pink (mBMI>BMI) and blue (mBMI<BMI). Blue and pink represent discordant groups: mBMI<BMI and mBMI>BMI respectively.

Characterizing obesity

In order to assess the relationship between mBMI and related risk factors, we classified the participants in to 5 groups based on the mBMI residual cut of +/-2 BMI units (Fig 2). The first three groups constitute subjects whose lipidome accurately predicted their BMI (i.e., mBMI residuals between -2 and 2 BMI units of their measured BMI). These include, normal weight-metabolically healthy (NW-MH, 18.5< BMI <25.0), overweight-metabolically overweight (OW-MOW, 25 ≤ BMI < -30.0 and obese-metabolically obese (O-MO, BMI ≥30) individuals. The remaining two discordant groups were the mBMI<BMI group (whose mBMI is less than their true BMI, mBMI residuals < -2) and the mBMI>BMI group (whose mBMI is greater than true BMI, mBMI residuals > 2). The distributions of BMI (Fig 2, left panel) and mBMI (Fig 2, right panel) for the 5 groups are shown.

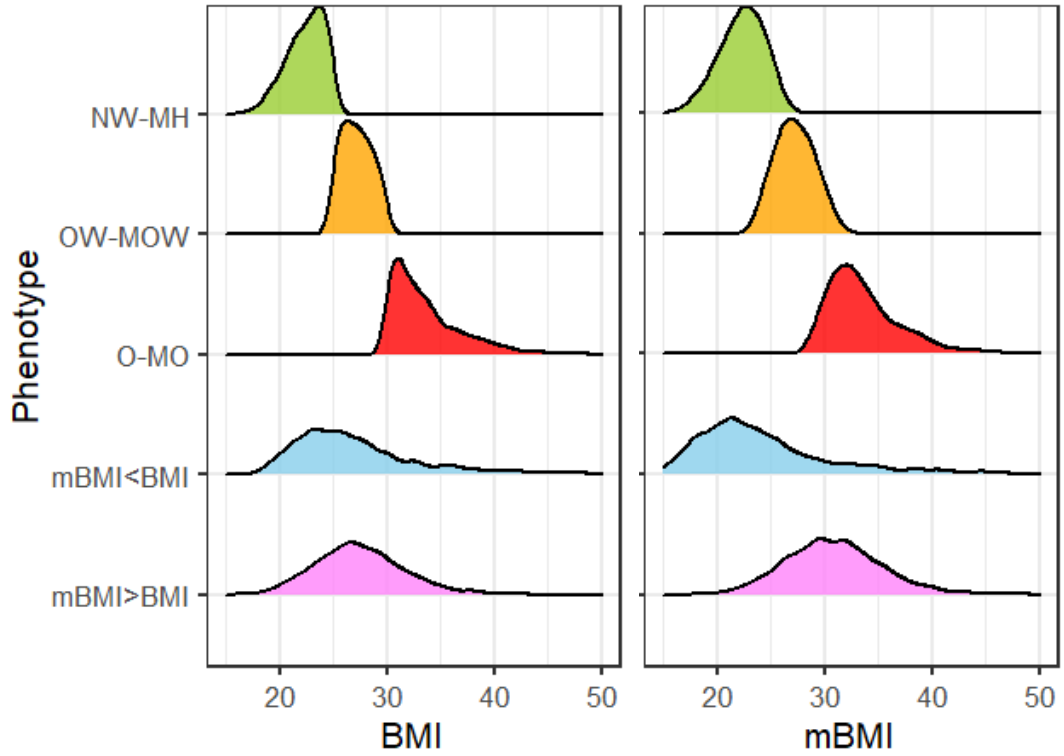

Fig 2. The distribution of BMI and mBMI across obesity groups. Density histograms of BMI (left panel) and mBMI (right panel) are depicted for each group.

The OW-MOW group constitute the largest proportion (25.0%) followed by the NW-MH (24.1%). The two discordant groups (mBMI>BMI & mBMI<BMI) make up 38.2% of the total population. These groups had a comparable median BMI and mean age, while their mBMI score was substantially different. The median (IQR) mBMI for the group whose mBMI>BMI was 30.7 and 22.7 for those with mBMI<BMI (Table 2).

Table 2. The characteristics of subjects across different obesity groups

Group	n (%)	BMI, median (IQR)	mBMI, median (IQR)	Age (year), mean, SD	% Women
NW-MH	2495 (24.1)	22.7 (2.7)	22.5 (3.1)	48.3 (14.8)	62.9
OW-MOW	2584 (25.0)	27.1 (2.4)	27.2 (2.7)	52.4 (14.2)	42.1
O-MO	1312 (12.7)	32.6 (3.9)	32.9 (4.0)	52.0 (13.0)	54.5
mBMI<BMI	1970 (19.1)	25.9 (6.9)	22.7 (6.7)	50.8 (14.2)	60.2
mBMI>BMI	1978 (19.1)	27.2 (5.4)	30.7 (5.9)	53.8 (14.0)	57.3

Cardiometabolic traits associate with having mBMI different from BMI

The five different groups of obesity appear to display distinct characteristics (Fig 3). We performed one way ANOVA with Tukey's test for post-hoc analysis. P-values adjusted for multiple comparisons were computed. Unless indicated, all pair-wise comparisons were statistically different. Non-significant pairs are indicated by bars connecting the two pairs. Individuals in the O-MO group (i.e., those with a combination of high BMI and high mBMI) are characterized by unfavourable lipoprotein profiles (high cholesterol, high triglycerides and low HDL-C) (Fig 3A), as well as being more insulin resistant, having higher FBG, 2h-PLG, HBA1 and high blood pressure compared to the NW-MH and OW-MOW groups (Fig 3B). Individuals with a higher mBMI than their BMI (i.e., the mBMI>BMI group) showed values of cardiometabolic traits that resemble those of O-MO group, while those in the mBMI<BMI group had profiles of cardiometabolic traits similar to that of NW-MH group (Fig 3A & Fig 3B). Moreover, despite having similar median BMIs, the mBMI>BMI groups have significantly different mBMI, cholesterol, triglycerides, HOMA-IR and 2h-PLG levels.

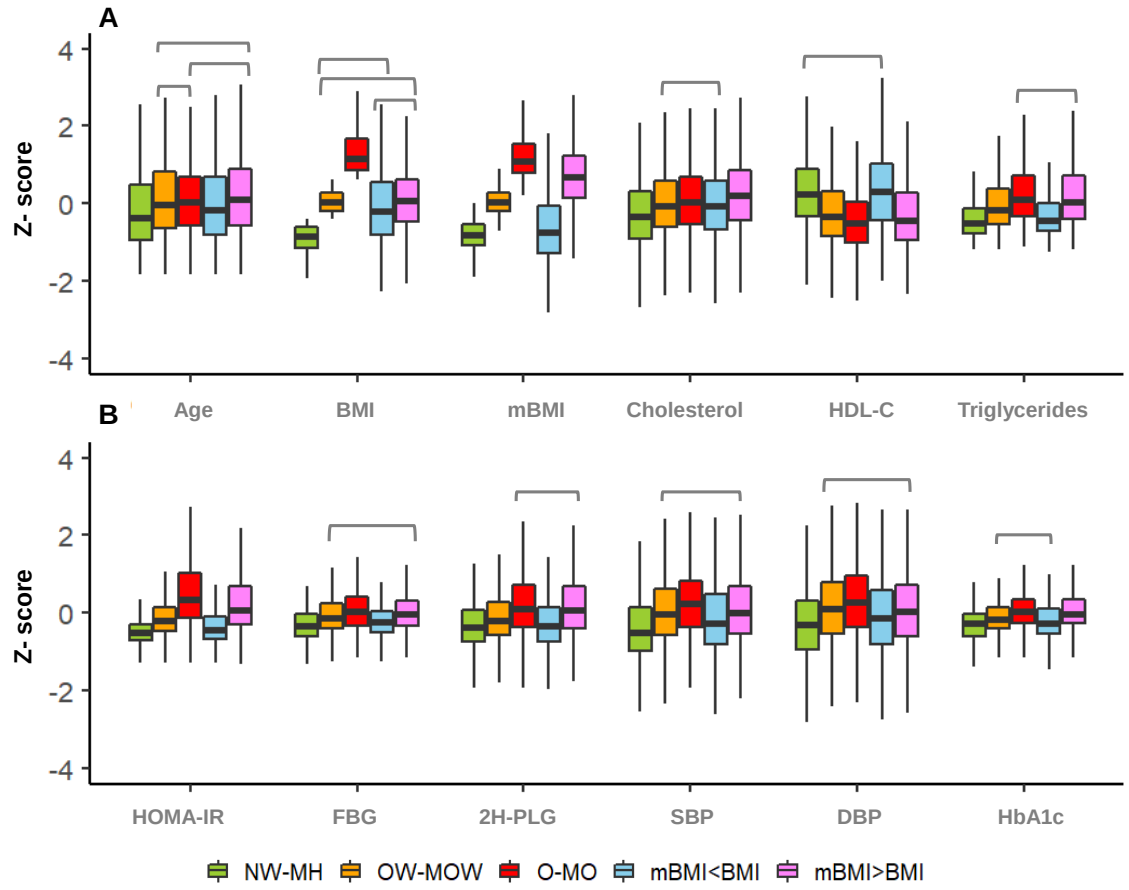

Fig 3. Cardiometabolic traits associated with different obesity phenotypes. *Z*-score values for each cardiometabolic trait were calculated as follows (*z* = x-mean)/SD to allow better comparison across groups. Box plots represent the distribution of *z*-scores of the respective cardiometabolic trait in each group. BMI, body mass index, HDL-C, high density cholesterol, HOMA-IR, homeostatic model assessment of insulin resistance, FBG, fasting blood glucose, 2H-PLG, 2 hour post load glucose, SBP, systolic blood pressure, DBP, diastolic blood pressure, HbA1C, haemoglobin A1c. Pairwise comparisons where adjusted p-value was >0.05 are show by solid bars connecting the two groups.

To assess the cardiometabolic risk between the two discordant groups, we performed a logistic regression analysis (with cardiometabolic features as predictors and the discordant groups as the outcome), adjusted for age, sex and BMI or age, sex and BMI and clinical lipids (total cholesterol, HDL-C and triglycerides). The metabolic traits differed substantially in their metabolic health risk profiles between the discordant groups despite these groups having a similar BMI. Lower HDL-C (odds ratio 95% CI = 0.4, 0.3–0.5), elevated triglycerides (odds ratio 95% CI = 2.3,

2.1–2.5), elevated 2h-PLG (odds ratio 95% CI = 1.7, 1.6–1.9) and high HOMA-IR (odds ratio 95% CI = 2.9, 2.5–3.3) were significantly associated with the mBMI>BMI group relative to mBMI<BMI group (Table 3). Except for FBG and HBA1C, all the associations remained significant after further adjustment for clinical lipids (although the effect size was reduced in most cases) (Table 3, right panel).

Table 3. Association of cardiometabolic risk factors with metabolic discordant groups.

Risk factors	mBMI>BMI relative to mBMI<BMI[1]		mBMI>BMI relative to mBMI<BMI[2]	
	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P
Cholesterol	1.3 (1.2–1.4)	2.24E-12	1.2 (1.1–1.3)	1.75E-07
HDL-C	0.4 (0.3–0.5)	1.16E-90	0.5 (0.4–0.6)	4.83E-55
Triglycerides	2.3 (2.1–2.5)	2.76E-63	1.5 (1.4–1.7)	4.47E-14
FBG	1.2 (1.1–1.3)	4.57E-02	1.1 (0.9–1.2)	4.45E-01
2h-PLG	1.7 (1.6–1.9)	1.41E-37	1.5 (1.4–1.7)	1.02E-21
HbA1c	1.2 (1.1–1.4)	2.32E-03	1.0 (0.9–1.1)	9.59E-01
HOMA-IR	2.9 (2.5–3.3)	7.30E-63	2.2 (1.9–2.5)	5.78E-33
SBP	1.3 (1.2–1.4)	1.56E-08	1.2 (1.1–1.3)	3.22E-06
DBP	1.2 (1.1–1.3)	1.21E-08	1.2 (1.1–1.3)	1.33E-04

[1]Logistic regression between discordant groups (mBMI>BMI relative to mBMI<BMI) and metabolic risk factors adjusted for age, sex and BMI

[2]Logistic regression between discordant groups (mBMI>BMI relative to mBMI<BMI) and metabolic risk factors adjusted for age, sex, BMI and clinical lipids (total cholesterol, HDL-C and triglycerides).

The findings observed in the AusDiab cohort were validated on the BHS cohort (except for the 2h-PLG and the HbA1C measures which were not available in the BHS cohort). Consequently, individuals in the mBMI>BMI group had a significantly elevated levels of triglycerides (odds ratio 95% CI = 2.0, 1.7– 2.4), HOMA-IR (odds ratio 95% CI = 2.1, 1.6–2.9) and cholesterol (odds ratio 95% CI = 1.4, 1.2–1.6) and lower HDL-C (odds ratio 95% CI = 0.7, 0.6–0.8) relative to those in the mBMI<BMI

group (Fig 4A). These associations remained significant after adjusting out for the effect of clinical lipids (Fig 4B).

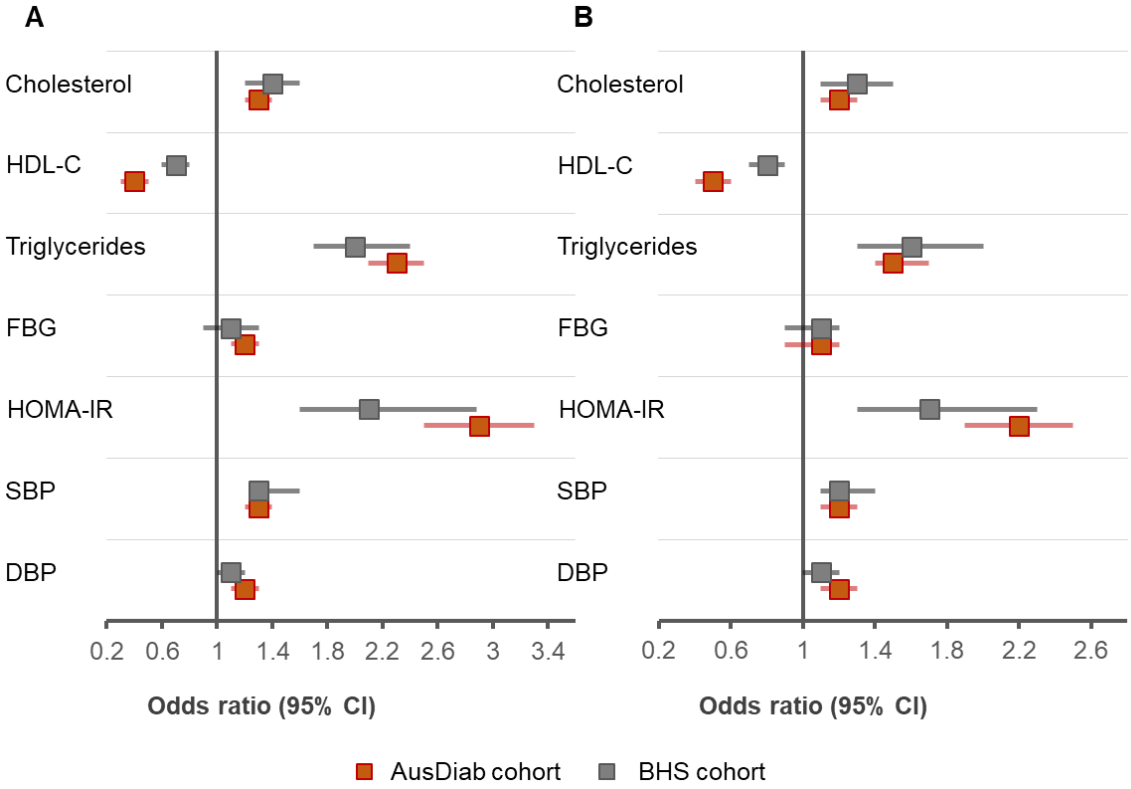

Fig 4. Association of cardiometabolic risk factors with metabolic discordant groups (mBMI>BMI relative to mBMI<BMI). A logistic regression analysis between the discordant groups as outcomes and metabolic traits as predictors was performed adjusting for age and sex (A) and age, sex and clinical lipids (B). Orange and dark squares represent results in the AusDiab the BHS cohorts respectively. The whiskers represent 95% confidence intervals.

The risk of diabetes and CVD significantly differ between metabolic discordant groups

In a subgroup analysis involving the discordant groups, individuals in the mBMI>BMI group relative to those in the mBMI<BMI group had greater odds of prevalent diabetes (odds ratio 95% CI = 3.2, 2.3 – 4.6). The risk of incident diabetes was just over two times higher among mBMI>BMI group relative to mBMI<BMI (odds ratio 95% CI = 2.3, 1.4–3.7) (Table 4). Comparing the risk of CVD between the metabolic discordant groups, we observed that those with mBMI>BMI had a significantly greater risk of prevalent and incident CVD compared to their counterparts with

mBMI<BMI. Individuals whose mBMI>BMI had 1.8 times higher odds of prevalent CVD (Table 4). A Cox proportional hazard regression against CVD events showed a differential risk of CVD between the discordant groups, adjusting for baseline age (time scale) and for sex, BMI, diabetes and smoking status (HR 95% CI = 1.8, 1.3–2.6) (Table 4). Additional adjustment for clinical lipids on top of baseline age, sex, BMI, smoking and diabetes status had a minimal effect on the associations with CVD outcomes. We validated the associations with CVD (particularly prevalent disease) in the BHS cohort (Table S5).

Table 4. The association between metabolic discordant groups and disease outcomes

Outcome	mBMI>BMI relative to mBMI<BMI[1]		mBMI>BMI relative to mBMI<BMI[2]	
	Odds ratio (95% CI)	**P**	**Odds ratio (95% CI)**	**P**
Prevalent diabetes	3.2 (2.3–4.6)	3.95E-11	2.5 (1.7–3.6)	9.69E-07
Incident diabetes	2.3 (1.4–3.7)	5.89E-04	1.7 (1.0–2.8)	5.25E-02
Prevalent CVD*	1.8 (1.3–2.7)	1.98E-03	1.8 (1.3–2.9)	2.57E-03
	Hazard ratio (95% CI)	**P**	**Hazard ratio (95% CI)**	**P**
Incident CVD*	1.8 (1.3–2.6)	8.98E-04	1.7 (1.2–2.4)	8.92E-03

[1]Logistic/Cox regression between cardiometabolic outcomes and metabolic discordant groups (mBMI>BMI relative to mBMI<BMI) adjusted for age, sex and BMI

[2]Logisti /Cox regression between cardiometabolic outcomes and metabolic discordant groups (mBMI>BMI relative to mBMI<BMI) adjusted for age, sex, BMI and clinical lipids (total cholesterol, HDL-C and triglycerides).

*The model is adjusted for diabetes status and smoking in addition to age, sex and BMI or age sex, BMI and clinical lipids

Metabolic BMI residuals predict cardiometabolic outcomes independent of BMI

The mBMI residuals calculated for each individual in the whole cohort showed significant associations with T2D (both prevalent and incident) and with CVD events. Akaike's information criterion (AIC) and Likelihood ratio test (LRT) were calculated to compare the two competing nested models (i.e., one containing mBMI residuals the

other without mBMI residuals). Using these approaches, we showed that models with mBMI residuals fit better in predicting cardiometabolic outcomes. E.g. Models with residuals show smaller AIC (AIC = 2563.1) compared to models without mBMI residuals to predict prevalent diabetes (AIC = 2652.4) and p-value for LRT of 2.6E-06. In predicting incident diabetes, the model with mBMI residuals fit significantly better (AIC = 1726.3) than the model without residuals (AIC = 1742.4) and p-value for LRT = 8.38E-06 (Table S6). In general, mBMI residuals were positively associated with higher risk of prevalent T2D (odds ratio 95% CI = 1.7, 1.5 – 1.9) after accounting for age, sex and BMI. The residuals also predicted incident diabetes (odds ratio 95% CI = 1.3, 1.2 – 1.5) (Table 5).

Table 5. The association of mBMI residuals, mBMI and BMI with disease outcomes

Outcome	**mBMI residual**[1]		**BMI**[2]		**mBMI**[3]	
	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P
Prevalent diabetes	1.7 (1.5–1.9)	6.48E-21	2.4 (2.1–2.6)	1.38E-55	2.6 (2.4–3.0)	5.11E-62
Incident diabetes	1.3 (1.2–1.5)	2.34E-05	1.8 (1.6–2.1)	2.46E-19	2.0 (1.7–2.2)	1.62E-21
Prevalent CVD[7]	1.3 (1.2–1.4)	5.53E-05	1.3 (1.1–1.5)	2.59E-04	1.4 (1.2–1.6)	1.74E-09
	Hazard ratio (95% CI)	P	Hazard ratio (95% CI)	P	Hazard ratio (95% CI)	P
Incident CVD[7]	1.2 (1.1–1.3)	3.13E-03	1.2 (1.0–1.3)	3.19E-02	1.2 (1.1–1.4)	1.03E-03
	mBMI residual[4]		**BMI**[5]		**mBMI**[6]	
	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P
Prevalent diabetes	1.6 (1.4–1.8)	3.78E-14	2.2 (1.9–2.5)	2.20E-37	2.3 (2.0–2.5)	3.36E-39
Incident diabetes	1.3 (1.1–1.5)	1.55E-03	1.7 (1.5–2.0)	1.12E-13	1.7 (1.5–2.0)	3.72E-13
Prevalent CVD[7]	1.3 (1.1–1.4)	1.40E-04	1.3 (1.1–1.5)	6.83E-04	1.4 (1.2–1.6)	1.80E-05
	Hazard ratio (95% CI)	P	Hazard ratio (95% CI)	P	Hazard ratio (95% CI)	P
Incident CVD[7]	1.1 (1.0–1.2)	6.15E-02	1.1 (0.9–1.2)	3.42E-01	1.1 (1.0–1.3)	1.01E-01

[1]Logistic/Cox regression between cardiometabolic outcomes and mBMI residuals adjusted for age, sex and BMI.
[2]Logistic/Cox regression between cardiometabolic outcomes and BMI adjusted for age, sex and mBMI residuals.
[3]Logistic/Cox regression between cardiometabolic outcomes and mBMI adjusted for age and sex.
[4, 5, 6] additionally adjusted for clinical lipids (total cholesterol, HDL-C and triglycerides) on top of age, sex and BMI/mBMI residuals
[7]the model includes diabetes status and smoking as covariates.

We also tested if mBMI residuals associate with prevalent CVD (such as history of heart attack and or stroke) and incident CVD after adjusting for age, sex, BMI, smoking status and diabetes status. Metabolic BMI residuals were positively

associated with prevalent CVD (odd ratio 95% CI = 1.3, 1.2–1.4) and higher risk of incident CVD event (HR 95% CI = 1.2, 1.1–1.3) (Table 5). Many of the associations of mBMI residuals with outcomes were independent of lipoprotein measures (Table 5). The odds ratios of BMI and mBMI associated with outcomes are also presented (Table 5). In the BHS validation cohort we found similar findings (particularly before accounting for clinical lipid measures) (Table S7).

The mBMI residuals associate with lipidomic profiles

In age and sex adjusted analyses, we found significant association of mBMI residuals with the plasma lipidome (480 out of 569 lipid species). While diacylglycerol and triacylglycerol species were particularly strongly associated in positive direction with mBMI residuals, most hexosylceramides, lyso and ether phospholipids were negatively associated (Fig 5A, Table S8). E.g. LPC(18:2)[sn1] decrease by 3.5% per unit increase in mBMI residual, p = 9.72E-147. Of the triacylglycerol species, TG(52:1)[NL-18:0] was the strongest predictor (8.7% increase per unit of mBMI residual, p= 5.53E-185) (Fig 5, Table S8). Further adjustment for clinical lipids (cholesterol, HDL-C and triglycerides resulted in weaker effect sizes but most associations remained significant except for few the triacylglycerol and diacylglycerol species (Fig 5B, Table S8).

Many associations appear to be dependent on fatty acyl chain composition. Lipids containing the 18:2 and odds or branched chain fatty acids were negatively associated while saturated species were positively associated with mBMI residuals. Considering phosphatidylcholine class, some of the contrasting associations of saturated and even chain fatty acids and odd chain fatty acids with mBMI residuals are shown in (Fig 5C). Finally, we report similar association of lipidome with BMI and mBMI residuals (Table S9). The effect sizes of lipids associated with BMI and mBMI residuals were highly correlated (Fig 5D). But it appears from the regression results that mBMI residuals have stronger effect sizes compared to BMI.

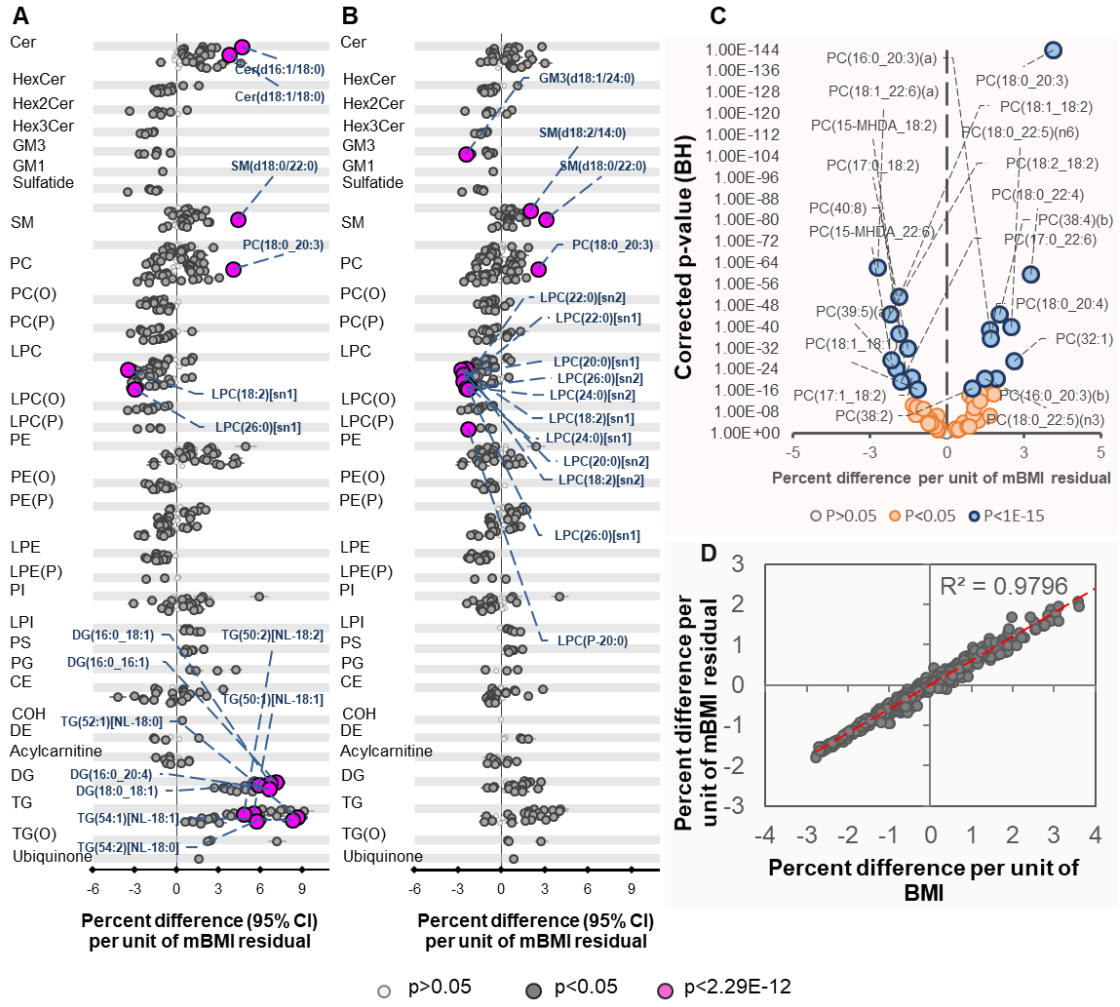

Fig. 5 Association of mBMI residuals with plasma lipidomic profile. Linear regression analysis between mBMI residuals and lipid species concentrations adjusting for age and sex (A) or age, sex, total cholesterol, HDL-C and triglycerides (B). Grey open circles show species (p>0.05), grey and purple closed circles show species with p<0.05 and p<2.29E-12 respectively, after correction for multiple comparisons using BH. The whiskers represent 95% confidence intervals. (C) Association of phospholipid fatty acids with mBMI residuals. (D) Correlation between effect sizes associated with BMI versus mBMI residuals.

Discussion

Lipidomics and metabolomics studies show that BMI is strongly associated with dysregulation in lipid metabolism (8, 9, 25, 27). This had inspired several groups to investigate whether the metabolite signatures can be used to estimate BMI; the “mBMI” and if this presents a portable surrogate measure to phenotype obesity and

characterise cardiometabolic risk (8, 9). In the present study, we constructed a holistic lipidome based BMI score that represents the mBMI of an individual. The mBMI reflects the metabolic consequences of obesity and hence provides additional information over measured BMI for a reliable assessment of cardiometabolic risk. Given the complex nature of obesity, mBMI can also serve to characterize obesity. We here report key associations of mBMI residuals and metabolic discordant groups with cardiometabolic traits, diabetes and CVD.

Recently, using ridge regression, a mBMI score was constructed using 650 urinary metabolites that were measured by untargeted approach and this explained 49% of the variation in BMI (8). In another study, a 47% variability was noted when utilizing lipid species to predict BMI using Lasso model (9). In the present study, the lipidome (including 569 lipid species spanning across the sphingolipid, phospholipid and glycerolipid classes) explained 64.9% of BMI variability, implying that dysregulation in lipid metabolism could be largely driven by obesity. Compared to previous modelling studies, we report a higher proportion of BMI variance explained by the lipidome in the present study. While, previous studies used stringently associated metabolites to predict BMI, we here included all the measured lipids in model to determine how well the entire lipidome explains BMI, rather than focusing on those that are associated with BMI. Moreover, our BMI model was built based on targeted LC-MS/MS based lipidomics, while previous studies used non-targeted and shot gun lipidomics (8, 9). Therefore, the observed difference in the BMI variance being explained in our study and others could be related to the number of predictors being included in the model, population setting, experimental design and modelling approaches. Generally, models based on limited set of metabolites result in weaker variance in BMI being explained compared to models of richer metabolite profiles (8).

Despite its simplicity and convenience, BMI alone does not capture the myriad health consequences of heterogeneous obesity phenotypes (28). Many metabolic and clinical studies have uncovered that people with the same or similar BMI display a substantial difference in their metabolic health outcomes (29, 30). For instance, Ruderman *et al* have identified a subset of individuals whose BMI was within normal range but show features of cardiovascular risk such as insulin resistance, high

triglycerides and coronary heart disease (31, 32). There are also metabolically healthy people (with no known metabolic abnormalities) who are overweight or obese based on their BMI (33). Thus, relying on BMI alone as a marker for obesity and associated metabolic health consequences leads to unreliable risk assessment. Here, with the rich source of sample size (n=10,339) we have attempted to stratify individuals into different groups based on the disparity between mBMI and BMI. The groups with discordant mBMI while having a comparable BMI displayed distinct health risk profiles. Indeed, we recognize that there are many possible BMI-mBMI combinations for fine phenotyping of obesity in larger population cohorts.

Interestingly, individuals with a substantially lower mBMI compared to their actual BMI present with a more favourable metabolic health profile that resembles the normal weight and healthy metabolome group. E.g., the mBMI<BMI group displayed a lower triglyceride, higher HDL-C, lower HOMA-IR and lower 2h-PLG values. In contrary, those individuals whose mBMI is greater than BMI had cardiometabolic features similar to the obese-metabolically obese phenotype. These findings agree with the findings of Cirulli and colleagues who did similar analysis (8). Several prior studies had attempted to identify individuals who are normal weight on the basis of their weight and height but obese/overweight based on their metabolic profile (32, 34, 35). Inline to the existing data, we observed that, mBMI was highly correlated with features of dyslipidaemia. These findings furthermore, highlight a relatively stronger link between mBMI and metabolic health risk relative to measured BMI in a certain group of population.

Overweight and obesity are considered risk factor for diabetes (36-38). In most cases, the risk of diabetes increases with BMI. However, existing data have also shown high prevalence of diabetes among normal weight people (39, 40). This indicates that lean individuals could have metabolic disturbances responsible for the pathophysiology of T2D and these can be captured by making use of mBMI. In the current study, we noted that, compared to the measured BMI, mBMI was more strongly associated with the risk of diabetes (particularly with prevalent diabetes). Interestingly, the group whose mBMI is greater than their true BMI had 3 time higher odds of having diabetes, compared to the group whose mBMI was lower than true

BMI. But, we did not observe a statistically significant difference in the risk of incident diabetes between the discordant groups after adjusting out for clinical lipids presumably due to the small number of incident cases in these groups.

While BMI is an independent risk factor for CVD (41, 42), not every obese or overweight subject shows abnormal cardiovascular risk profiles. There is a remarkable metabolic heterogeneity in obesity, and hence the risk of CVD (43-45) that present a significant challenge for management. Thus, the BMI measure as a marker of metabolic risk particularly in CVD settings fall short in many ways. For instance, in Framingham CVD risk scores, BMI had never become a component of the discriminatory features in predicting CVD outcomes (46). Moreover, a significant portion of obese individuals (31.7%) have been shown to remain free of CVD for life (i.e., metabolically healthy) (47). Furthermore, a recent debate over obesity paradox (in which obesity is rather associated with reduced risk of CVD and or improved survival (48-50) arises partly due to the use of BMI as a single measure to assess CVD risk. In the present study, we found that, mBMI residuals predicted CVD risk independent of BMI and clinical lipids. This sheds light on the importance of metabolite BMI scores to improve upon the inherent pitfalls of BMI as a tool for CVD risk assessment.

Finally, we examined the relationship of mBMI residuals with the lipidomic profile. The majority of plasma lipid class/subclasses were significantly associated with mBMI residuals. While, lipids representative of glycosphingolipids and phospholipids were generally negatively associated, most ceramide, diacylglycerol and triacylglycerol species were positively associated. Previously, we have found ceramide, dihydroceramide and triacylglycerol species to be associated with insulin resistance in young adults (51). Indeed, most of the lipid classes/subclasses associated with mBMI residuals observed in the present study have been associated with T2D and CVD (27, 52-54) and BMI (11). The lipidome association with BMI and mBMI residuals are very similar, although the effect sizes for residuals appear to be stronger. This suggests, that the mBMI residuals reliably capture the metabolic dysregulation associated with disease outcomes.

The rich lipidomic data and large sample size being utilized are the major strengths of the present study. However, there are limitations to mention: 1) we modelled mBMI using ridge regression which like other linear models assumes the relationship between the outcome and predictors is always linear. 2) Lack of some outcome measures in the BHS validation cohort, thus the observed associations requires further replication. 3) While the mBMI was validated in an independent cohort, its association with actual BMI was weaker than in the development cohort. There could be several reasons for this, such as the presence of unwanted variation and biological/population differences between the cohorts. These factors were not examined in details in this manuscript.

In summary, our results suggest the potential of lipidomic datasets to construct mBMI score for better risk assessment and characterize cardiometabolic outcomes associated with metabolic complications. Indeed, the mBMI mirrors BMI related metabolic alterations and captures additional biological information over measured BMI. Furthermore, mBMI and or mBMI residuals helps stratify obesity into groups; the identification of different obesity phenotypes in large samples not only improves the assessment of disease risk but also provide new insights into the biology and mechanisms that lead disease outcomes.

Author Contributions

HBB extracted plasma samples, performed LC-MS/MS analysis, analysed the data and wrote the manuscript. GO & AATS provided statistical support. CG and KH developed LC-MS/MS methods and provided support for the LC-MS/MS analysis and statistical analysis. MC developed extraction protocols and extracted plasma samples. NM supported the LC-MS/MS experiment and data pre-processing and analysis. JES and DJM, coordinated the AusDiab data, interpreted results and revised the manuscript. PJM oversaw this work and revised the manuscript. PJM and DJM are the guarantors of this work and shall take the responsibility for the full access and integrity of the data. All authors have approved the final version of the manuscript.

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Chapter 7. Plasma lipidomics improves risk prediction for type 2 diabetes

Chapter 7 examines the entire AusDiab cohort (including the cross-sectional and longitudinal endpoints). Using both the cross-sectional and longitudinal data, we elucidated plasma lipid species associated with prevalent outcomes (diabetes and prediabetes) and incident diabetes. We further examine distinct lipidomic profiles associated with impaired glucose tolerance and impaired fasting blood glucose. Finally, we generated metabolic risk scores for glycemic measures and subsequently tested whether multivariate models with the inclusion of these scores improve upon to the traditional risk markers to predict incident type 2 diabetes in the whole population or among otherwise healthy individuals with normal fasting glucose (<6.1mmol/L) at baseline.

Chapter 7 has been prepared for submission to Nature Communications.

To facilitate a better flow between chapters within this thesis, the Supplementary Tables for this chapter has been presented separately (Appendix V).

Plasma lipidomics improves risk prediction for type 2 diabetes

Short title: Improved risk prediction for type 2 diabetes.

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Abstract

There is a global obesity epidemic; associated with this is a dramatic increase in the incidence of type 2 diabetes (T2D). Early detection of T2D or the identification of those at increased risk of T2D provides the opportunity for early intervention to prevent onset or progression of the disease. Here, utilizing a high-throughput lipidomic analysis, we systematically investigated the association of molecular lipids with prediabetes and diabetes in the Australian Obesity and Diabetes study cohort (AusDiab). Using metabolic risk score modelling, we were able to improve 5 year incident T2D risk prediction over risk prediction tools using conventional clinical risk factors.

Introduction

Diabetes is a major public health threat across the globe. Over 463 million prevalent cases of diabetes were reported in 2019 (1). An estimated 212.4 million people (50.0%) of all people remained undiagnosed and some 352 million had impaired glucose tolerance (IGT). In order to combat this public health threat, importance of identifying individuals at risk for T2D has been ranked as one of the top research priority towards diabetes prevention (2).

Existing diagnostic markers for prediabetes and diabetes are currently based on thresholds of fasting blood glucose (FBG) or 2-hour post glucose levels (2h-PLG), with the latter obtained from an oral glucose tolerance test (OGTT). Elevated levels of glucose initially lead to the prediabetic state (where elevated FBG is classified as impaired fasting glucose (IFG, 6.1 ≤ FBG ≤ 6.9 mmol/L) and elevated 2h-PLG is classified as IGT, 7.8 ≦ 2h-PLG ≦ 11.1mmol/L). The prediabetic state is considered heterogeneous, where both IFG and IGT can exist independently of each other (usually termed isolated IFG and isolated IGT respectively (3)) and reflect differential metabolic dysregulation. While IFG is characterised by raised hepatic glucose output and a defect in early insulin secretion, IGT is often seen as peripheral insulin resistance (3). These differences in dysregulated metabolic states results in relatively low co-prevalence and are coupled with disproportionate incidence levels in age groups and sexes, where IGT is more often seen in older individuals and

women (4). T2D is diagnosed when either fasting glucose ≥7.0 mmol/l or 2h-PLG ≥11.1 mmol/l.

As IGT can exist independently of IFG, it is often difficult to identify individuals with IGT within a population. The gold standard OGTT is an inconvenient test to apply, limiting its use as a screening tool unlike FBG. However, FBG does not correlate with 2h-PLG and is a poor marker of individuals who have IGT. This results in a large majority of individuals with isolated IGT, whom have a similar risk profile to develop T2D and its complications as people with IFG not being identified until later in the disease progression. Both measures have different relationships with the key complications of diabetes such as cardiovascular disease (CVD). Although, diabetes mellitus prevention program works better on IGT (5), there is no quick way to identify them.

Lipids are small structurally diverse biological molecules that are essential to life. Lipid species have been associated with both prediabetes and T2D (6-10). We have previously reported differing plasma lipid associations with FBG and 2h-PLG in a small subset of the Australian Diabetes, Obesity and Lifestyle (AusDiab). Study. As lipids associate differently with fasting and post load glucose, this raises the possibility of capturing individuals at risk of T2D without the need for difficult assays such as an OGTT. Metabolic scoring by using the plasma lipidome to estimate measures of glucose, particularly 2h-PLG, may be a novel method to obtain clinically relevant information.

We have recently refined our lipidomic methodology (11), offering greater granularity to a broader range of lipid classes, and have applied it to the entire AusDiab baseline cohort (12).The AusDiab is a longitudinal population based study (n = 11,247) which was designed to determine the prevalence of chronic disease in Australia, particularly diabetes, obesity and CVD. Here we report the relationship between the plasma lipidome, risk factors and outcomes of impaired glucose tolerance. We also report a series of multivariate models to capitalize on the unique lipidome signature to predict T2D from both the general population and in high-risk individuals not likely captured by traditional screening tools.

Material and methods

Participants and samples

The AusDiab is a prospective study examining the prevalence and risk factors of T2D, obesity and CVD in the Australian adult population (13). T2D was defined as fasting glucose measurement (FBG) of 7.0 mmol/L or greater or a 2-hour post load glucose measurement (2h-PLG) of 11.1 mmol/L or greater or on medication for diabetes. Prediabetes was defined as either impaired fasting glucose (FBG = 6.1 to 6.9 mmol/L), impaired glucose tolerance (2h-PLG = 7.8 to 11 mmol/L), or a combination of both. According to the current definition some proportion of individuals diagnosed as IGT overlap with IFG. Thus, we term individuals with only IGT but otherwise normal FBG levels as isolated IGT. Similarly, individuals with IFG but normal 2h-PLG were termed isolated IFG. We utilized all the AusDiab participants (n=10, 339) after excluding (n=908) ineligible participants either due insufficient amount of plasma samples (n=13), missing data (n=876) or technical issues during MS analysis (n=19).

Prevalent diabetes and prediabetes*:* At baseline there were 7,733 normal glucose tolerant individuals (NGT), 1920 subjects with prediabetes (613 IFG, 988 IGT and 319 with both) and 395 newly diagnosed T2D included in these analyses. There were 291 individuals with known T2D excluded from the analyses. Characteristics of the study cohort are described in Supplementary Table 1.

Incident diabetes*:* Participants who attended baseline and follow up studies (n=5,572) were evaluated for incident diabetes after 5 years of follow up from 1999/2000. Individuals with known diabetes at baseline were excluded. Based on the WHO criteria described above, 218 subjects were diagnosed with T2DM during the 5 year follow-up period (Table 1) and 5,354 did not have diabetes (controls).

Plasma lipid extraction and lipidomics

Lipids from plasma were extracted using 1-butanol:methanol (1:1, v/v) method (BUME) containing the relevant internal standards as described previously (14). In brief, 10µL of plasma was mixed with 100µL of butanol:methanol (1:1) with 10mM ammonium formate and a set of internal standards. 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 being transferred into sample vials with glass inserts for analysis. The entire extraction process including pipetting of the 10 µL plasma, transferring this to 100 µL BUME solution was assisted by the liquid handling robot (Hamilton, INC).

Plasma lipids were measured using positive ionization mode with dynamic scheduled multiple reaction monitoring (MRM) on an Agilent 6490 QQQ mass spectrometer with an Agilent 1290 series reverse-phase HPLC system and a ZORBAX eclipse plus C18 column (2.1x100mm 1.8µm, Agilent) with the thermostat set at 45°C. Details of this method is described in our previous work (11) but adapted to a dual column set up (total run time, 14 minutes). The peaks of each lipid were integrated using MassHunter (Agilent).

Plasma quality control (PQC, plasma from a pooled set of 10 healthy individuals) were included at 1 PQC per 20 plasma samples as well as standard reference samples from the national institute of standards and technology (NIST1950) were incorporated into the analysis to assess potential variations in extraction process and instrument performances. Technical quality control samples (TQC) pooled from PQC extracts were included 1 per every 20^{th} sample to provide a measure of technical variation from the mass spectrometer. TQCs were used to monitor changes in peak area, peak width and retention time to determine the performance of the LC-MS/MS instrument and eventually to align for differences in the analytical performances across batches.

Naming convention of lipid species

The nomenclature of lipid species used here follows the guidelines established by the Lipid MAPS Consortium and the shorthand notation (15-17) and expanded upon by Liebisch et al [10]. Where it is impossible to fully characterize the fatty acid content and double bond position of glycerophospholipids, which typically contain two fatty acid chains the lipid is expressed as the sum composition of carbon atoms and double bonds (e.g. PC(38:6). In cases where the acyl chain composition is known, the naming convention reflects the detailed characterisation of the lipid; e.g.

PC(38:6) will be expressed as PC(16:0_22:6). Still PC(16:0_22:6) lacks further structural details such as the sn1 and sn2 positions. It will become PC(16:0/22:6) where the sn positions are known). This applies to several other lipid classes and subclasses. Lipid species with (a) and (b) designations represent species that are separated chromatographically but incompletely characterised. E.g., in PC(P-17:0/20:4) (a) and PC(P-17:0/20:4) (b) (a) and (b) indicate the elution order. Triglyceride species are monitored as a single neutral loss and the sum composition with their neutral loss experiment. e.g. TG(56:2) [NL-18:2]) refers to a triacylglycerol species with a total of 56 carbons and two double bonds accompanied with the 18:2 acyl chain as a neutral loss.

Statistical analysis

Statistical analyses were performed in R (3.4.1). Batch effects were corrected using median centring for PQC. Lipid data was $\log_{10}$ transformed, mean centred and scaled to its standard deviation prior to analysis. Logistic regression models were used to examine the association of each lipid species (as a predictor) with diabetes or prediabetes (as an outcome). Models were adjusted for age, sex, BMI. We also explored associations further adjusted for total cholesterol, HDL cholesterol and clinical triglycerides. We excluded any samples with missing clinical variables in each analysis. P-values were corrected for multiple comparisons using the Benjamini and Hochberg FDR correction (18).

Lipid model development

The lipid data was scaled to the standard deviation and centred to the mean prior to model development. We then generated a series of metabolic risk scores by using all the individual lipid species (747) in ridge regression models to predict different clinical measurements amenable to T2D risk (fasting glucose, 2h-PLG and HbA1c). The scores were generated using all non-diabetic individuals and are collectively termed “metabolic scores”. This resulted in three additional metabolic scores for each individual (metabolic FBG= mFBG, metabolic PLG=mPLG and metabolic HbA1c= mHbA1c).

Subsequently, we examined the added benefit of these scores in identifying individuals likely to develop T2D. We limited the analysis to incident diabetes over the 5-year follow-up. Base reference models were comprised of either AUSDRISK alone (19) or AUSDRISK with conventional glycaemic screening measurements (FBG and HbA1c). The benefit of adding the metabolic scores to the base model was assessed by comparing the Area under the Curve (AUC, specificity/sensitivity) and the net reclassification index (NRI) relative to the base models. This was done in both the entire non-diabetic population and also in a subset with normal fasting glucose ($\leq$ 6.1mmol/L).

Results

Lipids are highly associated with prevalent T2D

We examined the relationship between plasma lipids in the AusDiab cohort and prevalent type 2 diabetes. We utilised newly diagnosed T2D at baseline (not existing T2D, where treatment is already underway at the time of blood collection). Cross sectional analysis with logistic regression between NGT (n=7,733) and newly diagnosed T2D (n=395) adjusting for age, sex and BMI identified 572 lipids (including TG and TG species monitored as SIMs) out of 747 measured species associated with disease (after correction for multiple comparisons). A summary of the results and key associations are presented in Figure 1a. The strongest positive associations were seen in sphingolipid (predominately deoxyceramide, ceramide and dihydroceramide) and glycerolipid (diacylglycerol, triacylglycerol species). Conversely, lysophospholipids, including species of lysophosphatidylcholine, lysoalkylphosphatidylcholine and lysoalkenylphosphatidylcholine were negatively associated with T2D as were other alkyl and alkenyl subclasses (Supplementary Table 2).

T2D is often coupled with dyslipidaemia characterised by elevated triglyceride and lowered HDL-C. To observe lipid associations independent of bulk changes driven by differences in circulating lipoproteins, we further adjusted for clinical lipids (total cholesterol, HDL-C and triglycerides). In this analysis, 463 species were significantly associated after correction for multiple comparisons (Figure 1b, Supplementary Table 2).

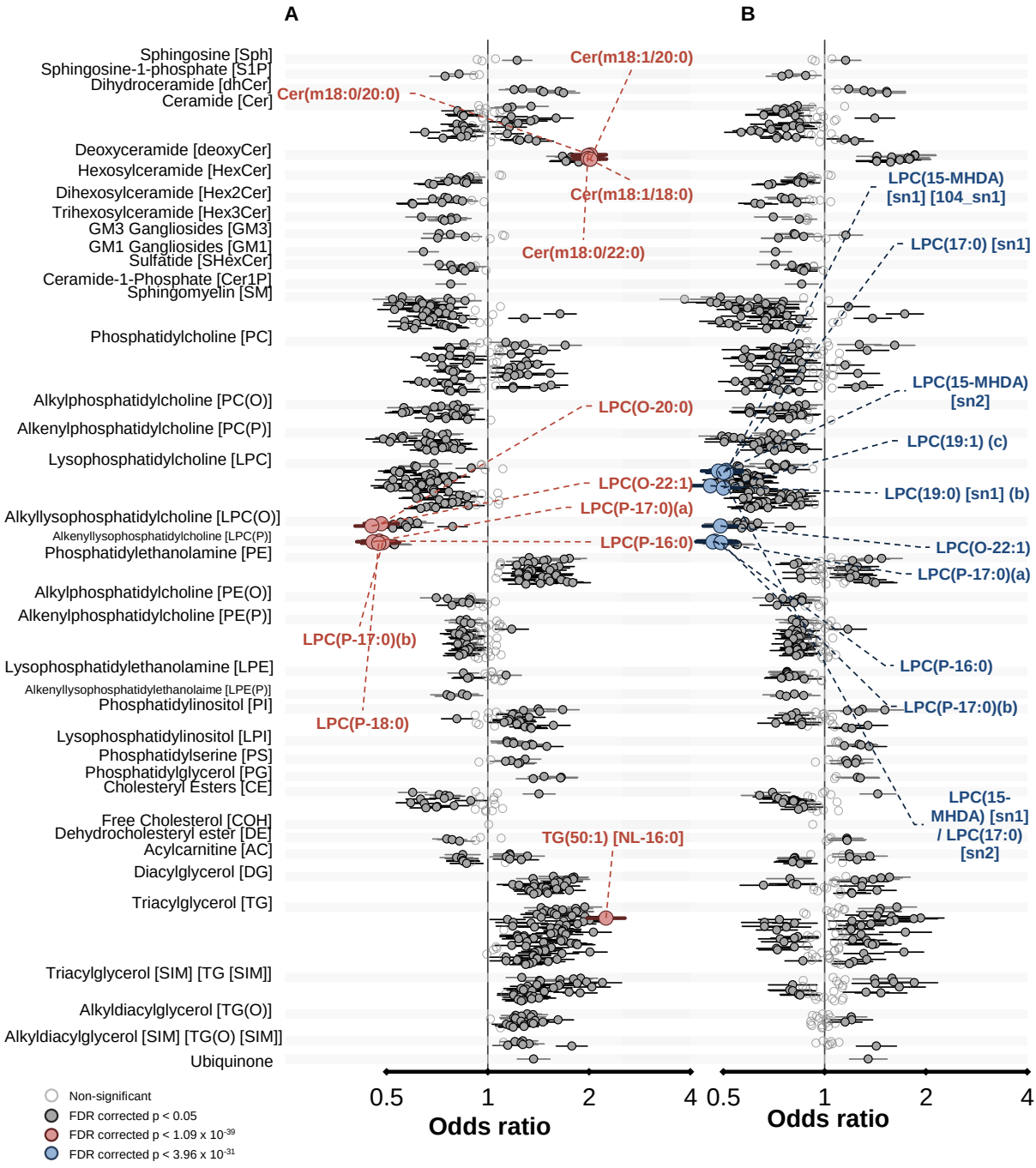

Figure 1 – Associations of individual lipid species with prevalent T2D. Logistic regression of lipid species against T2D (n = 395 cases versus 7,733 NGT controls) was performed adjusting (A) for age, sex, and BMI and (B) age, sex, BMI, total cholesterol, HDL-C and triglycerides. Associations are presented as forest plots; each circle represents the odds ratio for a lipid species, whiskers are the 95% confidence intervals. Open circles, not significant after FDR correction; grey circles,

significant after FDR correction; red and blue circles, top 10 species from each analysis (corrected p-value < 1.09×10^{-39} and 3.96×10^{-31} respectively).

The prediabetic lipidome presents with a similar signature to T2D.

We examined the associations of the baseline lipidomic profile with prediabetes (n = 1920 vs 7,733 NGT controls). We observed a similar, but slightly weaker, association profile with prediabetes compared to T2D (i.e. lower odds ratio, Figure 2). Lipid species containing an 18:2 fatty acid were particularly negatively associated with prediabetes once adjusted for clinical covariates. Detailed results can be found in Supplementary Table 3.

Figure 2 – Associations of individual lipid species with prediabetes. Logistic regression of lipid species against prediabetes (n = 1920 cases versus 7,733 NGT controls) was performed adjusting (A) for age, sex, and BMI and (B) age, sex, BMI, total cholesterol, HDL-C and triglycerides. Associations are presented as forest plots; each circle represents the odds ratio for a lipid species, whiskers are the 95% confidence intervals. Open circles, not significant after FDR correction; grey, significant after FDR correction; light blue and orange circles, top 10 species from each analysis (corrected p-value < 1.09×10^{-33} and 3.96×10^{-25} respectively).

There is heterogeneity in the lipidome of individuals with IFG and IGT.

We examined the association with isolated IGT (n = 988) and IFG (n = 613) relative to NGT (n = 7,733) using the AusDiab cohort. The lipid associations between NGT and IGT were much stronger than NGT and IFG. There were 128 lipid species that were associated with both IFG and IGT (15 species positive, 113 species negative). In contrast there were 263 species that were only associated with IGT and 86 species only associated with IFG (Fig. 3). In addition, 31 species (excluding SIMs) were positively associated with IFG but negatively associated with IGT while 44 species were negative with IFG and positive with IGT, (Supplementary Table 4).

Several associations were common between the two prediabetic states, these include the d18:2 sphingomyelins and several ether lipids including alkenylphosphatidylcholine species (Fig. 3). The negative associations with omega-6 fatty acids with IGT were similar to those with T2D (Fig. 3, Supplementary Table 4), with glycerophospholipid species esterified with 18:2 associated with IGT but not IFG. Deoxyceramide species were positively associated with only IGT. Similarly, the long chain acylcarnitines contrasting associations (negatively associated with IFG (Supplementary Table 4) and positively associated with IGT. Most of the branched and odd-chain lysoglycerophospholipids were negatively associated with IFG and IGT as with prediabetes and T2D.

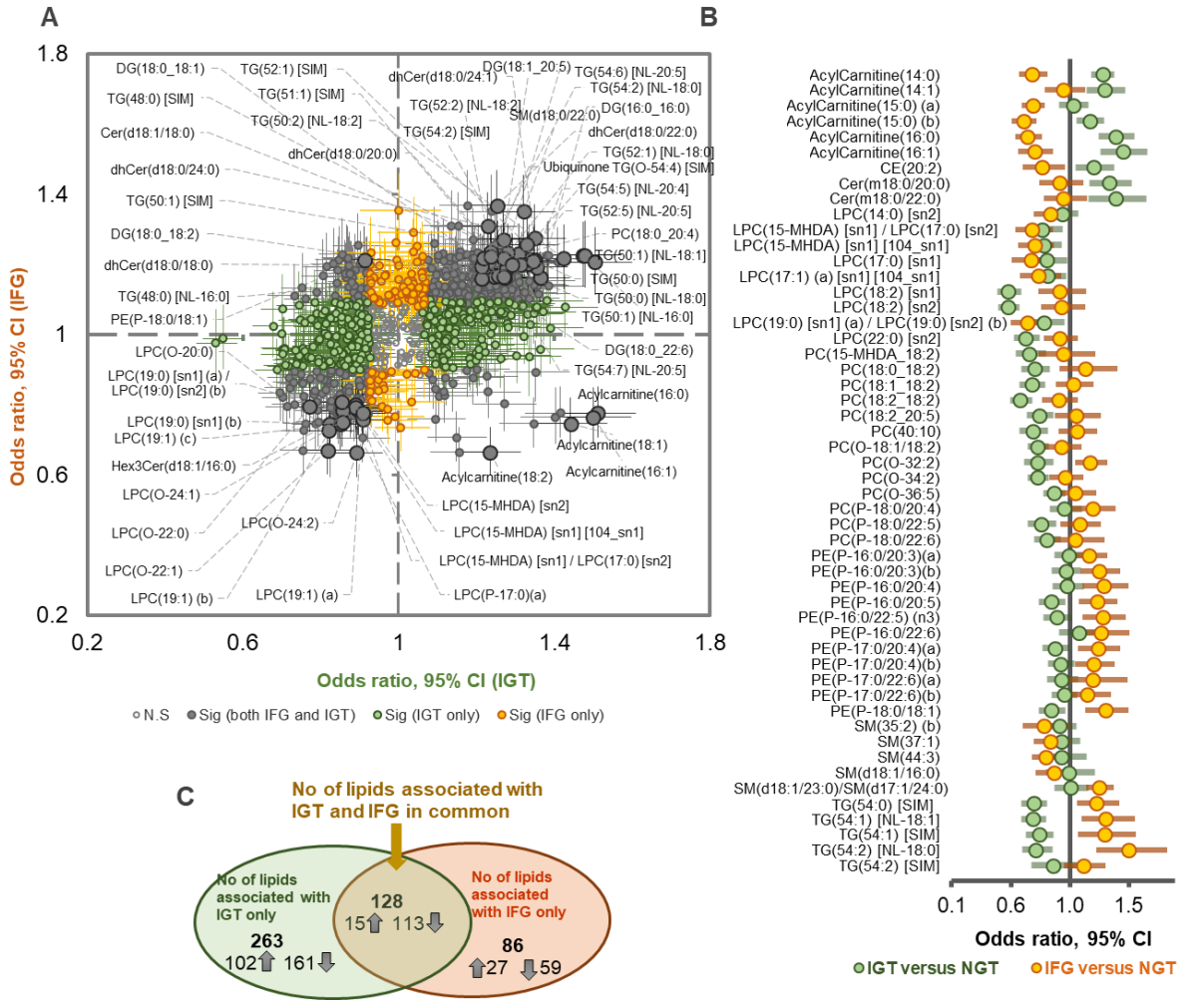

Figure 3 – Heterogeneous associations of lipid species with different pre-diabetic states. (A) Scatter plot of odds ratios between two different analysis examining associations with prediabetes, either IGT (corrected p < 0.05, green) or IFG (corrected *p* < 0.05, orange). Lipids that were common with IGT and IFG are highlighted in dark grey (with large circles showing the most significant species). (B) Selected lipids (based on smallest p-values) showing contrasting associations with IFG and IGT. (C) Venn diagram showing the number of unique and overlapped associations between IFG and IGT.

The mean adjusted concentrations for selected lipid species across the different glucose tolerance states are shown in Fig 4. These further reflect the metabolic heterogeneity associated with the pathogenesis of diabetes.

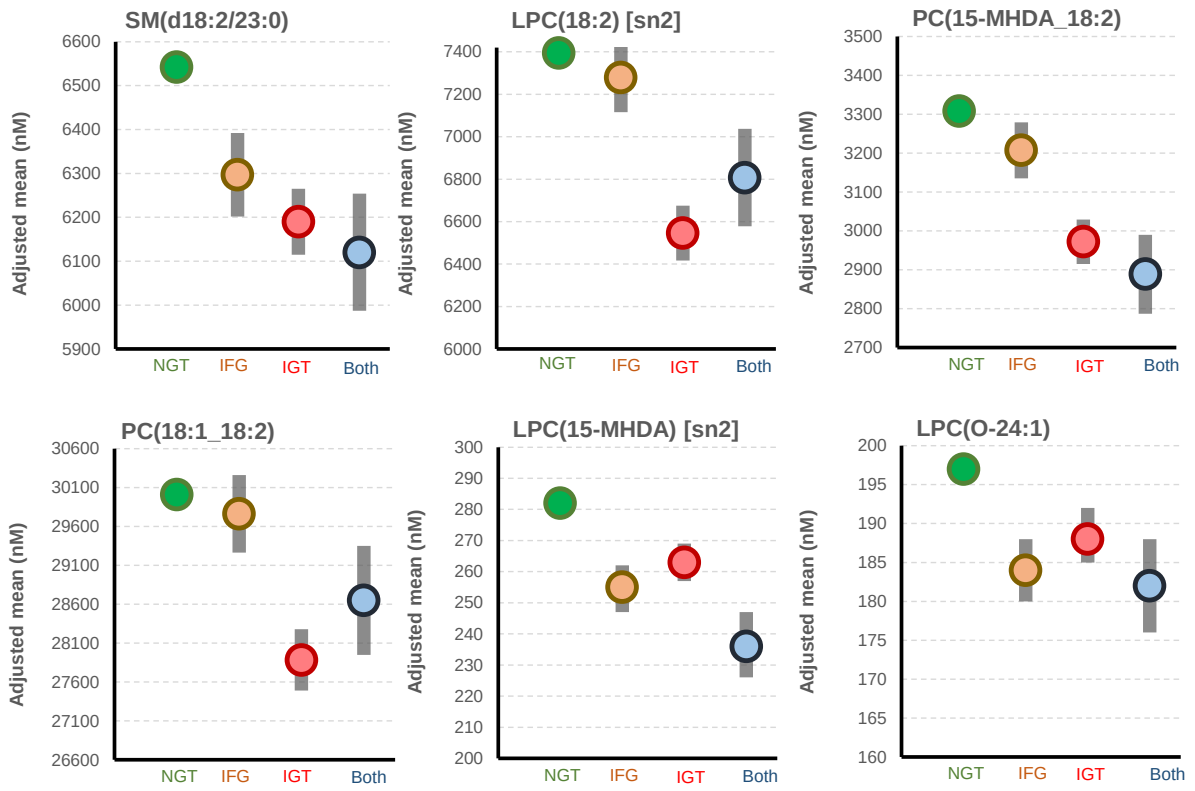

Fig. 4 Adjusted concentrations between the 4 different diagnostic groups of glucose impairment. ANCOVA analysis was performed adjusting for covariates; age, sex, BMI, total cholesterol, HDL-C and triglycerides. The mean adjusted concentration in nM are depicted on y-axis and groups on x-axis. Whiskers represent 95% confidence intervals.

Lipids are associated with future onset of T2D

Next we sought to determine whether lipid species at baseline were associated with future onset of T2D. In models adjusted for age, sex and BMI only, the associations of lipid species with incident T2D were similar to those observed for prevalent T2D, where many of the glycerolipid species were positively associated.

In addition, ceramide species with a d16:1, d18:1 or d20:1 sphingoid base were positively associated with incident T2D, including Cer(d18:1/18:0), odds ratio 1.3 (95% CI, 1.12-1.51, corrected p-value 1.75E-03) and Cer(d20:1/22:0), odds ratio 1.38 (95% CI, 1.19-1.60, corrected p-value 1.16E-04). However, most species with a d18:2 and d19:1 sphingoid base were not associated (Fig. 5a). Upon adjustment for clinical lipids, most of the of ceramide species with d18:2 and d17:1 displayed negative associations; e.g., Cer(d18:2/24:1), odds ratio 0.75 (95% CI, 0.64-0.88,

corrected p-value 3.66E-03) (Fig. 5b, Supplementary Table 5). Similar findings to this were observed in analyses with prevalent T2D (Supplementary Table 2).

Most sphingomyelin species were negatively associated with incident T2D, typically this effect was driven by species with a d17:1 or d18:2 base. Due to the nature of sphingomyelins, many of these species are not routinely separated when measured, i.e. SM(d18:1/24:1) and SM(d18:2/24:0). While the majority of sphingomyelin species measured in this study were separated, several others were not fully characterized i.e. SM(d18:1/18:0) and SM(d16:1/20:0). Sphingomyelins that were fully resolved without a d18:1 base were typically negatively associated with incident T2D, while those with a d18:1 or d18:0 were not significant except SM(18:0/22:0) which was positively associated with incident T2D odds ratio 1.52 (95% CI, 1.32-1.76, corrected p-value = 1.29E-07) (Fig. 5, Supplementary Table 5). In addition to the sphingoid based dependent associations, we observed strong positive associations with species of sphingolipids that arise from an atypical sphingolipid biosynthetic pathway, the deoxysphingolipids (synthesised from the condensation of alanine instead of serine with palmitoyl-CoA). We observed all 11 species of deoxyceramides to be strongly associated with the 5 year risk of T2D when adjusted for age, sex and BMI and 7 of the 11 remained significant after adjusting for clinical lipids (Fig. 5, Supplementary Table 5).

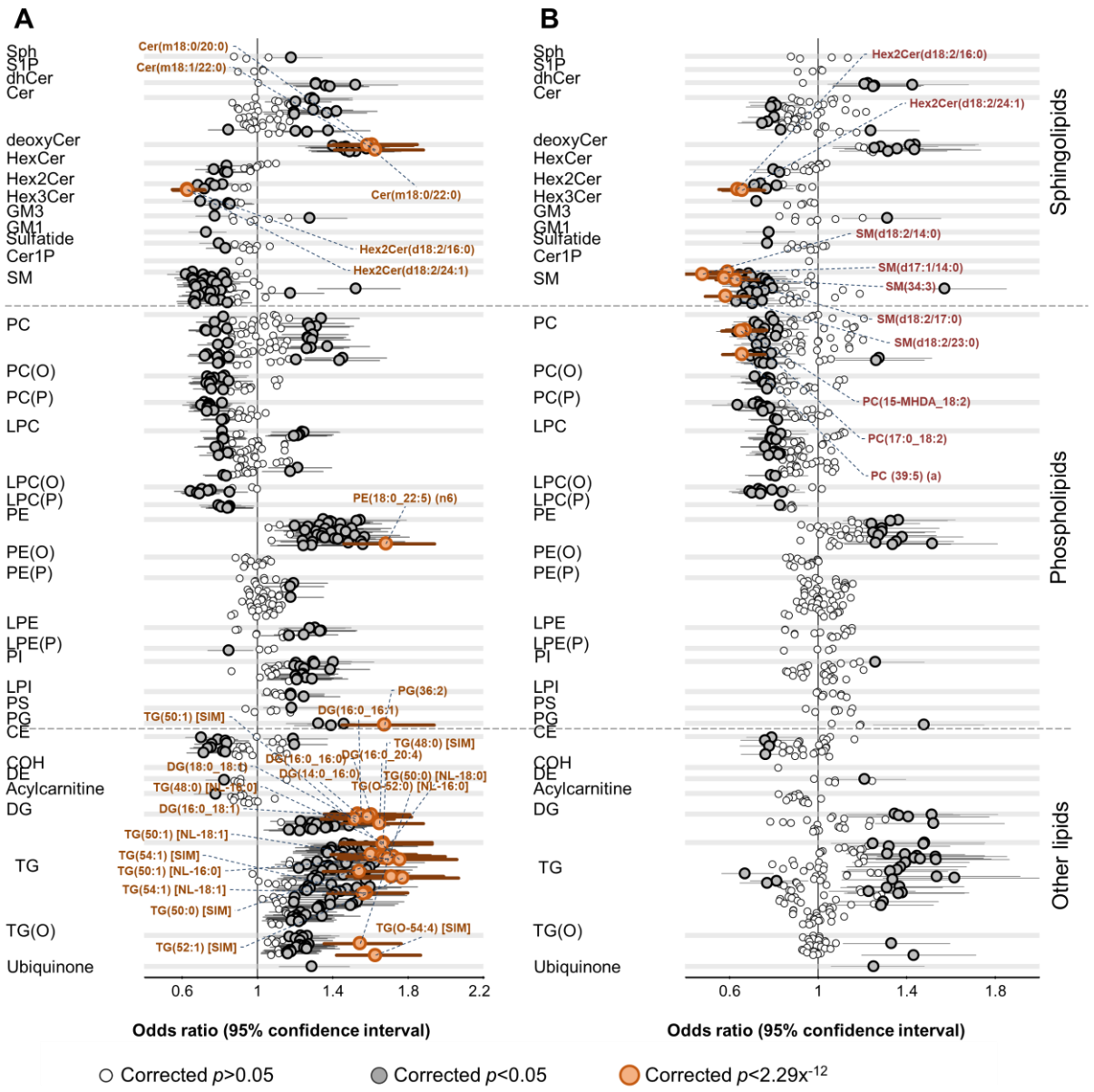

Figure 5– Associations of individual lipid species with incident T2D. Logistic regression of lipid species against incident T2D (n = 218 cases versus 5,354 NGT controls) was performed adjusting (A) for age, sex, and BMI and (B) age, sex, BMI, total cholesterol, HDL-C and triglycerides. Associations are presented as forest plots. Each circle represents the odds ratio for a lipid species, whiskers are the 95% confidence intervals. Open circles, not significant after FDR correction; grey, significant after FDR correction; orange, most significant species from each analysis (corrected p-value < 2.29 x 10^{-12}).

Generation of a metabolic score and improved risk prediction for type 2 diabetes

We next sought to determine the benefit in adding plasma lipids into existing risk scores for identifying individuals at risk of T2D. We utilised the AUSDRISK score as the base model and examined the benefit in adding conventional glucose measures (FBG and HbA1c) followed by the addition of metabolic scores generated with the lipidomics data.

Starting with scores generated using the AUSDRISK (Model 1), we observed a cross validated AUC of 0.763 (95% CI, 0.756 – 0.769, Table 1). Addition of FBG and HbA1c with the AUSDRISK score (Model 2), resulted in an AUC of 0.839 (0.832 – 0.846) corresponding to NRI's of 0.407 and 0.467 for events and non-events respectively (Table 1). We then tested Model 3, where mFBG, mPLG and mHbA1c was added to model 2. This resulted in a further improvement with an average AUC of 0.872 (0.867 – 0.878) corresponding to NRI's of 0.207 and 0.229 for events and non-events over Model 2 (Table 1).

We subsequently explored the added benefit of the metabolic scores in those individuals with normal fasting glucose only (FBG is < 6.1 mmol/L). We report that Model 1 (AUSDRISK alone) had an average AUC of 0.721, Model 2 (Model 1 + FBG + HbA1c) had an average AUC of 0.777 and Model 3 (Model 2 with metabolic scores) was 0.821 (Table 1).

Table 1 – Results of multivariate modelling using plasma lipid species to predict incident T2D.

Outcome	Model	AUC	NRI Continuous^	NRI event^	NRI non-event^
Incident T2D over 5 years (All)	**Model 1 –** AUSDRISK	0.763 (0.756 – 0.769)	-	-	-
	Model 2 – Model 1 + FBG + HbA1c	0.839 (0.832 – 0.846)	0.874 (0.849 – 0.900)	0.407 (0.180 – 0.434)	0.467 (0.461 – 0.473)
	Model 3 – Model 2 + Metabolic Scores*	0.872 (0.867 – 0.878)	0.436 (0.408 – 0.464)	0.207 (0.177 – 0.237)	0.229 (0.222 – 0.236)
Incident T2D over 5 years (<6.1 FBG only)	**Model 1 –** AUSDRISK	0.721 (0.711 – 0.732)	-	-	-
	Model 2 – Model 1 + FBG + HbA1c	0.777 (0.768 – 0.787)	0.874 (0.849 – 0.900)	0.407 (0.180 – 0.434)	0.467 (0.461 – 0.473)
	Model 3 – Model 2 + Metabolic Scores*	0.821 (0.813 – 0.829)	0.460 (0.423 – 0.497)	0.203 (0.164 – 0.243)	0.256 (0.249 – 0.264)

^ *Net reclassification index is calculated relative to the previous model*
* *Metabolic scores comprise of metabolic FBG, metabolic 2h-PLG and metabolic HbA1c.*

Discussion

In the present study, we have provided detailed characterisation the lipidomic signatures associated with both prevalent and incident T2D and prediabetes, including isolated IGT and IFG, using the very large AusDiab cohort. The lipidomic profile of associations with IGT was distinct from that of IFG. Characterizing the metabolic differences across the spectrum of glucose impairment states may enable the identification of specific pathways guiding the way to personalized medicine. We show that metabolic scores derived from the lipidomic dataset provided a meaningful improvement upon tradition clinical risk factors to predict the onset of T2D. The key findings describing the cross-sectional associations and multivariate modelling approaches are discussed below.

Negative association of odd and branched chain fatty acids with prediabetes and type 2 diabetes

We have previously described these lipid species containing odd and branched chain fatty acids (11). The *de novo* biosynthesis of odd chain fatty acids such as 15:0 and 17:0 has been shown to occur in mammalian system, using tracer experiments (carbon-13 labelled isoleucine and valine) (20). Recently, it was further

demonstrated that mmBCFAs are *de novo* synthesized via mitochondrial BCAA catabolism and much of the pool was independent of the contribution from microbiota (21). These findings provide compelling evidence that odd or mmBCFAs, are endogenously synthesized in mammals from the breakdown of BCAAs. While the catabolism of the BCAA occurs in the mitochondria, the products of this are then exported to the cytoplasm and used in the synthesis of odd and branched chain fatty acids.

Impaired BCAA catabolism (associated with elevated BCAAs) has been implicated in development of metabolic diseases (22). In humans, it has been repeatedly reported that elevated levels of BCAAs are associated with insulin resistance and T2D (23-26). However, only few studies have examined the possible relationship between the catabolic products of BCAAs (such as the methylpropanyl-CoA and methylbutryl CoA which is subsequently used in the synthesis of mmBCFA) in insulin resistance and T2D. One study found a strong positive correlation between adipose tissue mmBCFA and skeletal muscle insulin sensitivity (27). Examining serum odd- and branched-chain fatty acids Mika *et al.* identified significantly lower levels of odd chain fatty acids (OCFAs) and BCFAs among people with excessive weight, and also a negative correlation of serum content of *iso*-branched fatty acids with serum insulin (28). Moreover, a remarkably reduced level of mmBCFAs in obese animals have been reported (21).

A potential casual association between BCAA and T2D has been established using Mendelian randomisation examining the genes linked with BCAA levels in 16,596 individuals (29). Genes, particularly *PPM1K*, an activator of BCKD, were strongly associated with BCAA levels and T2D. These genes are likely involved in the regulation of downstream products of BCAAs, such as the mmBCFAs. Indeed, the likely cause of the negative association between odd and mmBCFAs is impaired catabolism of BCAAs. Whether defects in this unique and newly reported pathway has any causal effect on T2D pathogenesis needs to be investigated further.

Sphingolipid species containing the d18:2 base are negatively associated with prediabetes and type 2 diabetes

Our current chromatographic conditions allows the separation of multiple sphingolipid isomers (11). Owing to initial assumptions that serine palmitoyltransferase (SPT) was specific to palmitoyl-CoA, the resulting downstream sphingolipids have been typically reported as d18:1 bases. Evidence now demonstrates that the catalytic activity of serine palmitoyltransferase is not limited to palmitic acid (30) and further work has highlighted SPTLC3 as the predominate driver of plasma concentrations of odd and branched isoforms of sphingoid bases. A recent work by Karsai *et al* have shown that that only 57% of the sphingoid base in human plasma are sphingosine (d18:1), followed by d18:2 (21%), d16:1 (5%), and d17:1 (4.2%) (31). Our results indicate that almost all sphingolipids (typically ceramides and sphingomyelin) containing the d18:2 sphingoid base were associated with lower odds of both prediabetes and T2D. Ceramides have been shown to be associated with T2D (32-34) and several mechanism have been proposed (35-37). However, none of the existing studies have explored if the association of sphingolipids with disease is dependent on sphingoid base composition.

Here, we show that many ceramides with the d18:1 and d20:1 sphingoid bases, arising from palmitate and stearate respectively, were increased in prevalent T2D and prediabetes. In contrast, many d18:2 ceramides presented with negative associations. Sphingomyelin in general as a class were strongly negatively associated with diabetes (prevalent and incident) and prediabetes (with d18:2 species showing stronger associations). Although, our current methodology is unable to identify the position of the second double bond on the sphingoid base, a recent study by Karsai *et al* has demonstrated fatty acid desaturase 3 (FADS3) to be responsible for introducing the second double bond at the Δ14Z position forming d18:2 from d18:1 (31). This suggests a down regulation of FADS3 in T2D.

Deoxyceramide species are strongly associated with type 2 diabetes and prediabetes

Serine is not the only substrate for SPT, alanine and glycine incorporation has also been reported, leading to deoxyceramides (38), a subclass of sphingolipids lacking a hydroxyl group (-OH) at the carbon 1 position of classical ceramide species (39). Due to this structural deficit, deoxysphingolipids are unable to be phosphorylated to

SM nor glycosylated to glycosphingolipids, instead they accumulate in cells causing toxicity to insulin producing pancreatic β-cells (40) and other cell types (41). Accumulating evidence shows a positive association of the deoxysphingolipid subclass with the risk of T2D (40, 42, 43). The cytotoxic behaviour has been reported. Previously, we have reported a positive association between deoxyceramide species with age and BMI (12). In the present study, we observed the same species associate with prediabetes and T2D, even after controlling for the effect of age, sex, BMI, and clinical lipid measures. Whether there are causal relationships between deoxysphingolipids and cardiometabolic disorders such as T2D remains to be elucidated.

Heterogeneous lipidomic profiles associated with different prediabetic states

The two distinct diagnostic groups of prediabetes, IFG and IGT, have heterogeneous aetiologies. The distinction of these groups has implications for the management of people with diabetes (44). While, people with IFG often have hepatic insulin resistance with normal skeletal muscle insulin sensitivity, IGT is predominantly characterised by moderate to severe muscle and peripheral insulin resistance and normal to mild hepatic insulin resistance (45, 46). Generally, isolated IGT is associated with high triglyceride levels and cardiovascular risk, whereas isolate IFG had considerably weaker associations (47-49). Given these difference, it is also likely that these two groups are accompanied by distinct metabolic traits. There has been a limited attempt to explore the metabolic differences between IFG and IGT. Generally, we found that the plasma lipidome alteration are more pronounced in IGT (with many lipid associations) while fewer lipids associate with IFG.

It has been consistently reported in literature that there is strong negative association between levels of plasma linoleic acid (18:2) and T2D (50-52). Here, we show that the negative association of linoleic acid is largely driven by IGT, with almost no association seen with IFG. This suggests a differential role of the omega-6 fatty acid in the pathophysiology of IGT versus IFG. Linoleic acid is an essential fatty acid completely derived from diet. High intake of linoleic acid has been shown to be associated with lower risk of T2D and improved glycaemic control or insulin

sensitivity (53, 54). Potentially any benefits of linoleic acid may be limited to individuals developing T2D from IGT and not individuals who are predominately IFG.

Impaired mitochondrial function and fatty acid beta oxidation (FAO) has been proposed to lead to the progression and development of insulin resistance (55, 56). Previously, Koves et al. have identified lipid induced mitochondrial stress accompanied with increases in specific acylcarnitine species (such as 16:0 and 18:1) linked to skeletal muscle insulin resistance (likely due to shuttling of these fatty acids into the mitochondria) among high fat fed mice (56). Incomplete FAO has also been reported in people with muscle insulin resistance (57) with high intramyocellular fatty acid content likely due to dysregulation of FAO (58). In the present study, we show that IGT drives increased long chain acylcarnitine species, particularly those that arise from partial oxidation (16:1 and 14:1). Together with the negative associations observed with BCFAs (where the association was much stronger in IGT), this suggests mitochondrial dysfunction leading to IGT. Unlike IGT, the IFG appears to be negatively associated with many acylcarnitine species, indicating that a rise in fasting glucose, without a mitochondrial dysfunction or muscle insulin resistance would rather result in complete FAO without accumulation of these intermediary metabolites.

Metabolic modelling and approaches to risk prediction

Metabolic risk scores generated from lipidomic data contain information about metabolic dysregulation of lipid metabolism and hence we hypothesized that these scores will improve upon traditional risk factors to predict the risk of developing T2D. Due to the impracticability of an OGTT test and that 2h-PLG is a stronger predictor of all-cause mortality, the generation of a metabolic score for 2h-PLG using fasted plasma samples is an attractive solution to improve risk assessment for T2D. Conventional multivariate models (typically a combination of anthropometric measures, family history, clinical lipids and fasting blood glucose) capture some aspects of lifestyle and to a lesser extent genetic predisposition. Glucose measurements or HbA1c rely on detecting the early changes (i.e. insulin resistance) leading to T2D and so have a more restricted predictive capability. However, the inclusion of a metabolite profile to traditional markers has been shown to improve

model performance in several studies (60-62). Recent studies in using polygenetic risk scores (63, 64) observed little benefit when fasting glucose is included in these models. Our approach demonstrates not only improvements over a multivariate model that includes fasting glucose, but HbA1c as well.

One key benefit of the generation of metabolic scores, rather than modelling the outcome directly, is the increased power from modelling for a continuous variable across larger sample sizes. Modelling against the outcome directly would utilise all lipids to try to capture a signature from a small number of samples in this instance (n < 300). A further advantage is that the lipidomic models for the glucose measures likely capture a combination of genetic and environmental effects driving metabolic dysregulation that is in turn driving the progression towards T2D. In contrast the glucose measures themselves are likely a reflection of these processes and so have less predictive capacity. Consequently, this approach resulted in marked improvement to AUC and NRI over conventional clinical models.

Strengths and limitations

We were able to characterise many more lipid species in this data set, improving upon our original 259 species (34) to over 700 in this large population-based sample. With improved separation, additional structural detail can be inferred from fragmentation experiments performed on the same chromatography. While this approach also has its limitations, it provides improved detail to aid in interpretation of results.

Despite the large sample size, and high coverage of the plasma lipidome, the results presented here are from a single cohort. An external validation cohort, particularly from another demographic, would enable us to confirm the added benefit of plasma lipids in identifying individuals at risk of future T2D onset. Moreover, the Ausdiab suffers from the normal healthy volunteer bias (i.e where people who participated on average are likely to be healthier than people who don't). While we identified quite striking associations between the plasma lipidome and T2D, the role of lipids in T2D still remains to be fully elucidated. Identifying whether any of the lipids associated

with T2D fall within causal pathways and whether modulation of these species are able to prevent the onset of T2D and its complications needs further investigation.

Conclusion

Type 2 diabetes is a complex and heterogeneous disease. Early intervention has been demonstrated to reduce the burden of disease complications. Using a combination of a well phenotyped population-based cohort with comprehensive lipidomics profiling, we were able to map out the lipidome associated with prediabetes and T2D. The identification of different biological pathways between IFG and IGT better defines the dysregulation of lipid metabolism associated with the disease aetiology, potentially offering more specific treatment approaches. Lastly, we demonstrate that the lipidome obtained from fasting plasma, provides improvements in identifying individuals at risk of future T2D onset over conventional measures.

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Chapter 8. General discussion

Preface

The major objective of my PhD thesis was to perform a comprehensive targeted lipidomic analysis using state-of-the-art LC-MS/MS technology and examine the relationship between the plasma lipidome and cardiometabolic risk factors (age, sex, BMI, insulin resistance) and type 2 diabetes. We generated a comprehensive plasma lipidomic dataset in a large population-based cohort with well-phenotyped metabolic risk factors and disease outcomes. This was supplemented with two additional datasets: a smaller healthy cohort and a second population-based cohort that provided validation of our findings. The lipidomic datasets served to (i) to define dysregulation of lipid metabolism associated with health risk at the population level and (ii) act as a resource for future studies. Below, the major findings, main strengths, implications, limitations of this thesis and future directions of the work are discussed.

Overview of the major findings

The central hypotheses of this project was that the comprehensive examination of the human plasma lipidome is useful to identify new markers associated with cardiometabolic risk factors and outcomes. In chapter 3 of this thesis, we examined at the relationship between lipid species and common cardiometabolic risk factors including age, sex, BMI using a comprehensive plasma lipidomic dataset in two large population-based cohorts. We also explored the effect of smoking and menopause associated with perturbation in lipid metabolism and the role of lipid ratios in dissecting enzymatic pathways associated with cardiometabolic risk. In chapter 4, the association of the plasma lipidome with IR and the response to an OGTT was evaluated in healthy young adults. In the fifth and sixth chapters, we explored the lipidomic profiles of obesity to gain insight into the metabolic drivers of obesity and provide metabolic measures of obesity that are shown to be better predictors of disease risk. Finally, in the seventh chapter we elucidated that the changes in the plasma lipidomic profile precede the clinical onset of T2D and are useful to predict five year incident T2D.

Sex-differences in lipid metabolism

We initially hypothesized that lipidomics would provide important insight into the dysregulation of lipid metabolism associated with cardiometabolic disease and the interactions with age and sex. The large population-based samples utilized in the present studies enabled us to perform sex-stratified analyses and reliably define sex-related differences in lipid metabolism. Thus, one of the major finding of this thesis was the characterization of novel sex-specific lipidomic signatures of age and BMI which were validated on an independent cohort (chapter 3). We found that the plasma lipidome is highly sex-specific as published in chapter 3. Having identified the strong association of lipid metabolism with sex, we subsequently performed sex-specific analyses in assessing the association of lipid species with obesity (chapter 3), insulin resistance (chapter 4) and changes in obesity (chapter 5).

The difference between the sexes is one of the fundamental factors responsible for biological variation (1). Therefore, it is not surprising as men and women differ in several key aspects of metabolism in addition to their sex hormones. Sex differences in glucose and energy metabolism (2-4), the transcriptome (5) and the microbiome (6) are well recognized. Such sex-disparity is often overlooked in biomedical research including metabolomics studies. Until recently, many clinical and preclinical studies have been conducted in males only; while extrapolating the findings from one sex to the whole population (7). The potential consequence of this is a failure to discover sex-specific physiological processes which in turn leads to missing opportunities in identifying novel sex-specific disease control and therapeutic strategies in the era of precision medicine. There are several potential mechanisms leading to sex differences in lipid metabolism. These include, but are not limited to:

1) Differences in enzymatic activities. For instance, in the sphingolipid biosynthetic pathway, FADS3 was recently discovered to be responsible for introducing a double bond at omega-4 position of sphingoid bases (i.e, converting the d18:1 to d18:2 sphingoid bases) (8). The expression of FADS3 was significantly higher in women, thus, sex-differences in sphingolipid levels, particularly those related to the d18:2 species are likely driven by the action of this enzyme (8). In support of this, we have observed higher d18:2 to d18:1 ratios for all sphingolipids in

women with a significant p-gain. In chapter 3 of this thesis we have also highlighted the importance of using lipid ratios in these association studies to dissect lipid metabolic pathways and identify enzyme activities. Using the p-gain statistic, computed as described previously (9), we report that the ratio between two lipid concentrations carries more information than the two corresponding metabolite concentrations alone. Metabolite ratios can represent a proxy measure for enzyme activity. Lipid pairs provide superior information compared to individual lipids in the association with a given outcome (9, 10).

2) Influence of sex hormones. One of the reasons for the exclusion of females in clinical and preclinical studies has been the belief that there is a significant variability in biological processes in females attributable to differences in sex hormones (11). Failure to include both sexes in clinical studies potentially leads to ignorance of the role of sex-hormones in disease pathophysiology. Several mechanisms exist by which hormones can drive sex differences in metabolic regulation. For instance, sexual dimorphism in estrogen signalling partly explains difference in physiological processes in adipose tissue, liver and muscle (12). Estrogen promotes insulin sensitivity and reduces the risk of T2D in premenopausal women compared to men (13). Moreover, sex difference in triglyceride metabolism is well recognized and is partly mediated by estrogen signalling (14). Estrogen has also been shown to induce Phosphatidylethanolamine N-methyltransferase (PEMT) leading to a shift in the PC acyl species (15). Of note female mice relative male mice have been shown to generate higher phosphatidylcholine via the PEMT pathway (16). Earlier studies have also highlighted that there is a link between estrogen signalling and peroxisome proliferation (17, 18) which may explain some of the sex-related differences in lipid biosynthesis. Estrogen also upregulates the hepatic synthesis of long-chain polyunsaturated fatty acids (LCPUFAs) via ELOVL5 regulation (19). Isotope labelling studies with U-(13)C α-linolenic acid (ALA) has revealed a substantial increase in the conversion rate of ALA to n-3 (omega-3) LC-PUFAs in women than in men (20); foetal demand for lipids during pregnancy appear to be met by these adaptations. Moreover, sex hormones may affect important mediators of lipoprotein metabolism such as Apolipoprotein E (apoE) and lipoprotein lipase (LPL). Upon the

overexpression of these genes, sex-specific response to high cholesterol diet and atherosclerosis have been demonstrated (21, 22).

3) Gut microbiota. The human gut microbiome is a complex and dynamic totality of microbial biomass consisting 10–100 trillion microorganisms in the gastrointestinal tract (GIT) (23). Recent reports uncover a substantial difference in gut microbiome between male and female mainly in mice (24, 25) and this in turn influences sex dimorphism in metabolism and metabolic health (26). While, the microbiome influences several metabolic processes including obesity, glucose homeostasis and lipid metabolism (25, 26), the mechanism of sex-specific microbiome composition and how this might modify lipid metabolism is not well known. Baars *et al.* showed that, the microbiome modulated bile acid metabolism in male and female differently (26) suggesting that the microbiome contributes to sex-disparity in lipid metabolism. One of the potential mechanisms for the variation of gut microbiome between sexes have been suggested to be the difference in obesity and fat distribution between male and female. Recently, Min *et al.* have demonstrated sex-specificity in the association of microbiome composition and fat distribution (25) suggesting that there is a complex interplay between the microbiome and gender which potentially alters susceptibility to cardiometabolic diseases in male and female.

4) Difference in muscle mass and fat distribution. In general, men have a significantly higher skeletal muscle mass than women (27), while women have a significantly greater percent fat mass (28). Fat distribution and fatty acid kinetics in men and women contribute to sex-specificity in substrate metabolism (29). Importantly, these sex-related differences modify susceptibility to cardiometabolic disorders in men and women. For instance, in age and BMI matched analyses, men tend to display a higher visceral adipose tissue and hence are at higher risk to cardiovascular disease relative to women (28).

5) **Difference in dietary intake between men and women**

One of the considerable sex-specific differences constitutes dietary intake. For instance, women compared to men tend to eat more sugar and total fat and carbohydrate (that exceeded dietary recommendations). In contrast, men have been

shown to achieve the minimum recommended intake amount for polyunsaturated fat and total carbohydrate (30). Evidence from earlier study also shows that 16:1n-7 is a biomarker of long term consumption of carbohydrate (e.g. low fat/high carbohydrate diets) (31). Thus, during lipogenesis, the palmitate flux and substrate mass action could be responsible for some of the shifts in lipid species. Understanding the sex-specific dietary preferences and the outcome on lipid metabolism can potentially lead to new insight into the differential impact of diet on health in women and men.

In summary, the key factors driving sex-specific lipid metabolism are related not only to sex hormones but to the microbiota, enzyme activity, body composition, and diet and life style factors. Understanding the interplay between these factors and sex-specific lipid metabolism can potentially lead to new insight into the mechanisms of disease.

Common pathways perturbed in obesity, IR and T2D

We identified many common pathways altered in same direction in obesity (chapter 3), IR (chapter 4) and type 2 diabetes (chapter 7). These common pathways span several lipid classes/subclasses within the categories: sphingolipids, phospholipids and glycerolipids. As it was hypothesized initially, a strong perturbation in the lipidome was found in hyperinsulinemia and insulin resistance among young adults with no known diabetes. We report in chapter 3, simple sphingolipids such as dihydroceramide were increased with BMI in adults. The same trend was observed in IR among young adults (chapter 4) and T2D (chapter 7). SM and complex glycosphingolipids generally show strong negative association with diabetes (both prevalent and incident) in older adults (chapter 7), but interestingly did not show significant associations with measures of insulin resistance in young healthy people (chapter 4). These differences could be due to differences in sample size, age composition and health status of participants. The study on the association of lipids and IR (chapter 4) was based on young and apparently healthy adults (18-34 years old) with a relatively smaller sample size (n=241) compared older adults (>=25 years, n=10, 339) in the AusDiab. Thus, it is likely that SM and complex sphingolipids such as hexosylceramides and gangliosides could be markers of glucose dysregulation (such as in prediabetes) and established T2D rather than insulin resistance per se, as we did not observe significant changes in these

metabolites among young adults. However, the possible age effect should also be considered.

Glycerolipids (triacylglycerol and diacylglycerol species) were also found to be independent markers of several outcomes (obesity, IR and T2D). We have consistently observed the positive association of glycerolipids, independent of their acyl chain component, with all the three cardiometabolic conditions namely: obesity (chapter 3), insulin resistance (chapter 4) and T2D (chapter 7) particularly before accounting for clinical lipids. However, most of these associations were significantly attenuated upon adjusting for clinical measures of total cholesterol, triglyceride and HDL cholesterol levels. This is partly due to the fact that diacylglycerol and triacylglycerol classes are closely correlated with clinical triglycerides. Still, there were many triacylglycerol species that remain significantly associated with obesity, IR and T2D after adjusting for clinical lipid/triglyceride levels suggesting the importance of the fatty acid composition of the TG species when investigating the underlying associations with health and disease. Of note, the positive association of TG species containing saturated and monounsaturated fatty acids with BMI (chapter 3) and T2D (chapter 7) was evident after controlling for clinical triglycerides.

The specific mechanisms related to an increase in plasma triacylglycerol levels in obesity, IR and T2D remain unclear. However, the increase in free fatty acids (FFAs) is likely to be a major contributor. Interleukin 6 (IL-6) and TNF-α observed in obesity and insulin resistance are strongly associated with elevated FFAs (33). IL-6, aside from regulating immune functions, has been shown to stimulate secretion of triglycerides in the liver subsequently inducing hypertriglyceridemia (34). Similarly, in metabolic conditions, TNF-α stimulates lipolysis resulting in elevated circulating FFAs (35). The rise in circulating FFAs means that more can enter into cells and get re-esterified raising triacylglycerol levels (36). We also observed an increase in phosphatidylethanolamine (PE), with insulin resistance, BMI and T2D before controlling for clinical lipids. The associations with measures of IR such as HOMA-IR and the iAUC were lost when adjusting for clinical measures of cholesterol, triglyceride and HDL cholesterol levels. However, many PE and other phospholipid species remain associated with both BMI and T2D independent of the clinical

measures of cholesterol, HDL-C and triglycerides. These, associations also highlight the fatty acid composition dependence.

Phospholipid species containing the methyl-branched chain and odd chain fatty acids (OCFAs) such as 15-methylhexadecanoic acid (15-MHDA) were negatively associated with BMI (chapter 3) and T2D (chapter 7). A similar finding (negative association with odd and branched chain fatty acids with BMI) has been reported previously in a smaller set of the AusDiab cohort (37). It is generally proposed that, the decrease in branched and odd chain fatty acids in plasma is related to an increase in branched chain amino acid (BCAA) levels due to reduced mitochondrial catabolism. Increase in plasma BCAA levels during IR, obesity (38-40) and T2D (40-42) have been consistently reported. BCAAs constitute leucine, isoleucine, and valine that are essential amino acids derived from proteinaceous foods. Many enzymes exist in the mitochondria that are able to catabolise BCAAs (43). These include mitochondrial branched-chain amino-transferase (mBCAT) which is responsible for the conversion of BCAAs to branched-chain alpha-ketoacids (BCKAs). The branched-chain alpha-ketoacid dehydrogenase (BCKDH) then catalyses oxidative decarboxylation of branched alpha-keto acids to form odd/branched chain fatty acids endogenously. While even chain fatty acids arise from the *de novo* lipogenesis (with acetyl-CoA as a starting material), the source of OCFAs other than ruminant fats or dairy was until recently unclear (44, 45). Although, considered to be biomarkers of dietary intake (45-47), the OCFAs such as 15:0 and 17:0 could be derived from endogenous biosynthetic pathway either through the mitochondrial catabolism of BCAAs; i.e from valine and isoleucine derived propionyl-CoA (48) or via peroxisomal α-oxidation, particularly for the 17:0 (49). Thus, a mitochondrial dysfunction associated with impaired BCAA catabolism could be linked to lower levels of odd or branched chain fatty acids observed in obesity and T2D. Indeed, a study has shown that hypoxia associated with metabolic conditions such as obesity suppresses mitochondrial BCAA catabolism and subsequently leads to substantially lower levels of mmBCFAs (50). Understanding the potential endogenous biosynthetic pathways for OCFAs and mmBCAAs and the connection between BCAA metabolism and lipid metabolism leads to better characterization of the metabolic basis of disease pathogenesis.

The tight associations observed between the lipidome and obesity (both with cross-sectional and longitudinal measures) has inspired us to further investigate the metabolic phenotyping of BMI. Consequently, in chapter 6, we derived a metabolic BMI score from the lipidomic dataset and this informed about the metabolic drivers of cardiometabolic risk. We report that the metabolic BMI residuals explained the risk of diabetes and CVD independent of the measured BMI (chapter 6). Genetics have never shown the expected level of correlation with BMI variability (explains <3% of BMI variability) (51). Only few genetic defects such as mutations in the melanocortin 4 receptor (MC4R) has been linked to extreme obese phenotype (52). However, such gene defects are rare and therefore, unlikely to be useful in obesity risk assessment in the general population. Instead, the metabolite readouts seem to carry better information about BMI compared to the genetic profile. This suggests the potential use of metabolic BMI to assess obesity and the associated metabolic complications. Indeed, the metabolic BMI residuals tend to predict diabetes and CVD independent of measured BMI. One of the potential explanation for this could be the fact that the mBMI score essentially captures metabolic dysregulation associated with cardiometabolic disorders and hence carries additional information upon BMI. These observations provide an important insight into the role of mBMI for characterization of cardiometabolic risk in large population cohorts. Of particular interest, individuals with the same BMI but having a significantly different mBMI were identified. As such, individuals having higher mBMI than BMI compared to those having lower mBMI than BMI were found to have higher risk of diabetes and CVD. In line with our initial hypothesis, multivariate models including metabolic scores derived from lipid species showed improvements in the AUC, sensitivity and specificity upon the existing risk models in predicting the 5-year risk of incident T2D (chapter 7). These models have the potential to distinguish individuals who are at higher risk of progressing to T2D independent of the commonly used measures such as FBG, PLG and HbA1c both in the high risk population and among individuals with baseline fasting blood glucose of <6.1mmol/L. The implications further to the lipidomic profiling would provide insight into the metabolic drivers and consequences of obesity and provide metabolic measures of obesity that will be better predictors of disease.

Implications of the findings

The results in this thesis have several implications which span clinical medicine and public health. Firstly, the profound age and sex-dependent alteration in the plasma lipidome as reported in chapter 3 has implications for future large epidemiological studies: 1) The age and sex specific patterns may be important and this may lead to better phenotyping for precision medicine; 2) Revisit study designs in large scale metabolomics studies separately for men and women and or older and younger populations. Although, many age and sex-specific association studies have been documented (53-56), these had been predominantly based on small set of metabolite/lipid classes/subclasses or species. 3) Highlight the importance of age and sex disparity in the dysregulation of lipid metabolism and associated metabolic diseases. Consequently, the findings call for sex-stratification during biomarker discovery and establishing reference materials and selecting individuals for clinical trials. Indeed, evidence shows that the burden of metabolic disorders such as obesity, diabetes and CVD differ significantly between men and women. Secondly, as outlined in chapter 4, altered lipid metabolism occurs not only among those with established disease but among apparently healthy young adults with hyperinsulinemia and IR. This warrants intervention in young individuals. Detection of such metabolic perturbations in young people enables timely intervention to prevent lipid induced metabolic complications such as T2D and CVD in later life.

Thirdly, the findings in chapter 5 showed a strong relationship between baseline lipid metabolism and risk of gaining WC or BMI over 5 years, suggesting that individuals with a specific metabolic phenotype at baseline might be at greater risk of increasing their WC or BMI. Consequently, groups of individuals can be targeted for intervention to prevent future WC or weight gain and associated metabolic consequences. The results in chapter 6 indicate that the lipidome can be utilized to derive a metabolic BMI score; a measure that mirrors the metabolic dysregulation associated with health risk. Metabolic BMI gives insight into the metabolic health status of individuals independent of the measured BMI. Thus, it provides additional information upon BMI in order to identify individuals at greater risk of metabolic diseases and stratify people into clinically relevant phenotypes for personalized intervention. The findings in Chapter 7 showed a significant heterogeneity in the lipidome of IFG and IGT which

has implications for the stratification and diagnosis of different subtypes of glucose impairments for personalized management of diabetes. Finally, the models including metabolic scores derived from fasting samples substantially improved upon FBG and HBbA1C to predict the onset of T2D and these findings have implications for screening people who are at high risk of future T2D without the need to perform technically demanding tests such as the OGTT.

Major strengths of this thesis

This thesis has several strengths. Firstly, we utilized the largest population-based cohorts in Australia: the AusDiab study (n=10,339) as a discovery and the BHS (n=4,492) as a validation cohort. Both cohorts are rich sources of well phenotyped outcomes and clinical covariates (chapter 3, 5, 6, 7). Consequently, we have performed a comprehensive plasma lipidomic analysis to define and understand lipid biology and metabolism at a population level including sex-stratified analyses with a reasonably high statistical power. Secondly, we used high-throughput LC-MS/MS methodology measuring over 700 lipid species. This improved lipidome coverage (compared to smaller set of lipids reported in literature) enabled us to identify new species associated with health and disease which have not been previously reported. Thirdly, the longitudinal study design of the AusDiab study allowed for the assessment of the temporal relationship between baseline lipidomic profile and changes in obesity during five year follow up (chapter 5) and incident type 2 diabetes (Chapter 7).

Potential limitations of the present studies

Despite the unprecedented advances in separation techniques and analytical platforms such as mass spectrometry, the current lipidomic studies suffer from several limitations that need to be overcome. One of the limitations in high coverage lipidomic methodology is the potential of isomeric overlaps which could affect associations with some outcomes. To overcome this, either the chromatographic gradient can be improved or different adducts or ionization systems could be adopted to selectively monitor these isomers, although this would likely impact on the coverage and high-throughput nature of the analyses. The other limitation is related to the source of plasma lipids. The plasma lipidome reflects a highly dynamic

and concerted effect of multi-organ system involved in the synthesis, breakdown, storage and distribution of lipids. While the bulk of lipids measured by mass spectrometry are derived from the lipoprotein particles, the origin for many atypical lipid species (for instance those of odd or branched chain fatty acids) is unclear. Last but not the least, the targeted lipidomic approach used here has a limited potential to discover new compounds, although it is ideal for understanding and validation of existing lipid biomarkers.

Lipidomic studies are largely hypothesis generating. The exact mechanisms and causal pathways leading to the observed alterations in lipidomic profiles associated with cardiometabolic risk factors and diseases outcomes need to be determined in *in-vivo* or *in-vitro* models. However, the validity of the cross-sectional associations observed in the AusDiab cohort is tested on independent cohort such as the BHS. Moreover, the use of lipid pairs such as lipid ratios rather than a single lipid, provided information that is a proxy for mechanistic studies. Lipid ratios can be used as a surrogate for enzymatic activity, and hence identify specific enzyme activity responsible for changes in lipids associated with disease risk. We have also examined whether baseline lipid levels predict longitudinal measurements such as changes in WC or BMI or incident type 2 diabetes. Although, these might not necessary reflect a casual pathway. Last but not the least, the AusDiab suffers from selection bias and the participants may be healthy and more educated that the general population.

Despite these limitations, the robust high-throughput targeted lipidomic approach (measuring 747 lipid species) on large population-based cohorts (with a size of 10,339 people in the AusDiab and 4,492 in the BHS) represents the largest study in the lipidomics field. Such large cohort studies offered statistical power and are crucial to identify and validate candidate biomarkers with confidence.

Future directions

The findings in this project raise interesting future possibilities for "*–omics"* research. While the lipidomic methodology used in this project was sensitive enough to separate a wide range of molecular lipid species, it is still open to further

improvements. Of note, the current HPLC column (Agilent, 100mm x 2.1mm, 1.8m C18) could be replaced by a longer column to ensure a greater resolution. The mobile phase flow rates and or solvent composition and run time can also be modified and tested if these result in better separation of molecular isomers. Longer run time compared to shorter run time (keeping all other conditions constant) yields cleaner peaks and detailed structural features contributing to the overall data quality, although this might not be suitable for large sample sizes.

Combining both targeted and untargeted lipidomic approaches in future studies could offer opportunities for both the discovery of novel compounds and determination of their relative concentration in biological samples to facilitate breakthroughs in understanding the role of lipids in disease and health. Untargeted lipidomics focuses on all the possible detectable lipids in including those previously unknown. But targeted lipidomics involves measurement of known/ previously defined lipids. Merging these two approaches is achievable (57). It is also possible to combine the lipidomics with metabolomics so that a wider range of metabolites including the polar and non-polar lipids can be analysed (58).

The identification of the strong association of the lipidome with disease outcomes and risk factors in this thesis is a key step for future studies to further dissect the direct mechanisms leading to the alteration of lipid levels in health and disease. Moreover, the results of the present studies inform future studies about the potential application of large scale lipidomic analysis to identify lipid biomarkers for diagnosis, prognosis and prediction of disease risk. In future, it is also possible that lipids associated with disease outcomes could be targeted for therapeutic benefit to prevent the progression and or onset of metabolic diseases.

Conclusions

High-throughput lipidomic analysis of large epidemiological cohorts offers an opportunity to identify novel lipidomic fingerprints in health and disease but also shape our understanding of the biology of lipid metabolism at a population setting. Utilizing two large population-based cohorts, we identified lipid signatures of age, and obesity that differ by sex: findings signifying the need to define sex-specificity in

the dysregulation of lipid metabolism associated with metabolic disorders. Further to this, the lipid alteration associated with IR seen among young adults without overt clinical outcomes suggest that metabolic perturbations occur prior to the onset of disease outcomes such as T2D. By extending our studies to longitudinal outcomes, we report plasma lipidomic signatures associated with changes in WC and in BMI over 5 years. These findings provide insight into the baseline metabolic phenotype associated with risk of increasing WC and or BMI.

Having observed a profound perturbation of lipidome in obesity, we derived a metabolic BMI (mBMI) score using the lipidomic dataset. The mBMI mirrors the dysregulation in lipid metabolism associated with health risk. Using this score, we identified individuals having comparable BMIs but different mBMI displayed different metabolic health risk profile. This approach is important for phenotyping risk in large cohorts, selecting individuals for clinical trials and has implications for personalized medicine. Finally, we extend the metabolic score concept to show that models containing metabolic scores directly derived from lipid species show improvements upon traditional risk factors to predict the onset of T2D. These findings not only assist in identifying those individuals at a higher risk of developing diabetes for targeted intervention, but also shed light on dysregulation in lipid metabolism associated with the pathogenesis of T2D.

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Appendix

Appendix I. Chapter 3. Supplementary Tables and Data

Chapter 3. S1 Data. Numerical data underlying figures and supplemental figures.
https://doi.org/10.1371/journal.pbio.3000870.s037

Chapter 3. S1 Table. The correlation structure between all lipid species in the whole cohort (men and women combined).
https://doi.org/10.1371/journal.pbio.3000870.s020

Chapter 3. S2 Table. The correlation structure between all lipid species in men.
https://doi.org/10.1371/journal.pbio.3000870.s021

Chapter 3. S3 Table. The correlation structure between all lipid species in women.
https://doi.org/10.1371/journal.pbio.3000870.s022

Chapter 3. S4 Table. Differences in the Pearson's correlations (men relative to women) for all lipid species.
https://doi.org/10.1371/journal.pbio.3000870.s023

Chapter 3. S5 Table. Validation of the association between sex and lipid species.
https://doi.org/10.1371/journal.pbio.3000870.s024

Chapter 3. S6 Table. Characteristics of women participants.
https://doi.org/10.1371/journal.pbio.3000870.s025

Chapter 3. S7 Table. Validation of the association of BMI with lipid species.
https://doi.org/10.1371/journal.pbio.3000870.s026

Chapter 3. S8 Table. Association of BMI with lipid concentration ratios.
https://doi.org/10.1371/journal.pbio.3000870.s027

Chapter 3. S9 Table. Association of plasma phospholipid fatty acid composition with BMI, age, or sex.
https://doi.org/10.1371/journal.pbio.3000870.s028

Chapter 3. S10 Table. Association of age with lipid concentration ratios.
https://doi.org/10.1371/journal.pbio.3000870.s029

Chapter 3. S11 Table. Association of sex with lipid concentration ratios.
https://doi.org/10.1371/journal.pbio.3000870.s030

Chapter 3. S12 Table. Association of sphingolipid ratios with BMI, age, or sex.
https://doi.org/10.1371/journal.pbio.3000870.s031

Chapter 3. S13 Table. Association of the d182:1/d181 sphingoid base ratios with BMI, age, or sex.
https://doi.org/10.1371/journal.pbio.3000870.s032

Chapter 3. S14 Table. Association of the PE(P)/PE(O) ratios with BMI, age, or sex.
https://doi.org/10.1371/journal.pbio.3000870.s033

Chapter 3. S15 Table. SwissLipids identifiers for lipid species.
https://doi.org/10.1371/journal.pbio.3000870.s034
Chapter 3. S16 Table. Characteristics of study participants.
https://doi.org/10.1371/journal.pbio.3000870.s035
Chapter 3. S17 Table. MRM transitions and conditions for examined lipid species.
MRM, multiple reaction monitoring.
https://doi.org/10.1371/journal.pbio.3000870.s036
Chapter 3. S1 Data. Numerical data underlying figures and supplemental figures.
https://doi.org/10.1371/journal.pbio.3000870.s037

Appendix II. Chapter 4. Supplementary Tables
https://datadryad.org/stash/share/xayYZ35LbOpmNCcLru9G5ivm4E0v4hAjjpWtjwOGS3I

Appendix III. Chapter 5. Supplementary Tables

Chapter 5. Table S1. MRM transitions and conditions for examined lipid species

Chapter 5. Table S2: Lipid categories, classes/subclasses and number of lipid species measured in this study

Chapter 5. Table S3: Baseline lipidomic predictors of changes in waist circumference

Chapter 5 Table S4: Baseline lipidomic predictors of changes in waist circumference among waist gainers /sable over 5 years (excluding those subjects who lost over 5% WC from baseline)

Chapter 5. Table S5: Lipidomic signatures associated with baseline waist circumference

Chapter 5. Table S6: Baseline lipidomic predictors of changes in BMI

Chapter 5. Table S7: Lipidomic signatures associated with baseline BMI

Chapter 5. Table S8: Sex-specific associations of baseline lipidomic profile with changes in WC

Chapter 5. Table S9: Sex-specific associations of baseline lipidomic profile with changes in BMI

Appendix IV. Chapter 6. Supplementary Tables

Chapter 6. Table S1: The baseline characteristics of CVD events and controls in the AusDiab and BHS cohort studies

Chapter 6. Table S2: The baseline characteristics of subjects with history of CVD and controls in the AusDiab and BHS cohort studies

Chapter 6. Table S3: MRM transitions and conditions for examined lipid species