TANDEM MASS SPECTROMETRY FOR IDENTIFICATION OF NOVEL LIPID BIOMARKERS OF OBESITY AND COLON POLYP RISK

By

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ABSTRACT

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Obesity is associated with numerous comorbidities including chronic low-grade inflammation, metabolic dysregulation, and increased risk of colon cancer. Colon polyps are clusters of cells that continue proliferating, leading to the development of colon adenomas which increase the risk of colon cancer. Obesity increases circulating *de novo* proinflammatory signaling molecules and growth factors, which influence polyp growth and survival, such as cytokines and oxygenated fatty acid (FA) metabolites referred to as oxylipids. FAs are sequestered into larger complex lipids such as phospholipids (PLs), and phospholipases liberate these esterified FAs for metabolism. Omega-3 (ω-3) and omega-6 (ω-6) polyunsaturated FAs (PUFAs) are of interest in obesity and cancer research, since PUFAs are substrates for oxygenating enzymes which produce oxylipids. There is a dearth of research using newly developed mass spectrometry (MS)-based lipidomic technologies for the characterization and determination of novel lipid biomarkers of altered metabolism and colon polyp risk. Using cutting-edge methods in tandem mass spectrometry (MS/MS), the plasma lipidome was profiled from 126 Caucasian males that were previously recruited for a cross-sectional study investigating biomarkers of colon polyps. Statistical models were adjusted for cofounding factors such as age and smoking. It was hypothesized that three distinct types of lipidomic markers would emerge from the data: lipids associated with only obesity, lipids associated with both obesity and colon polyps, and lipids associated with colon polyps independent of obesity (i.e., after adjusting statistical models for body mass index (BMI) and waist circumference (WC)). Obesity was associated with distinct PUFA

differences in plasma phospholipids (PPLs), such as decreases in linoleic (LA, ω-6), eicosapentaenoic (EPA, ω-3), and docosahexaenoic acid (DHA, ω-3) in lysophospholipids (LPLs) and ether linked phosphatidylcholines (PC); and increases in long chain ω-6s in diacyl PLs. Non-esterified plasma PUFA concentrations were similar across BMI and WC. Plasma vicinal diols, oxylipids formed through soluble epoxide hydrolase (sEH) metabolism, were inversely associated with obesity. The oxylipids 5-hydroxyeicosatetraenoic acid (HETE), a 5-lipoxygenase (5LOX) metabolite, and 11-HETE, which is formed from non-enzymatic oxygenation, were both increased in obesity. Specific concentration ranges of these HETEs and vicinal diols were determined to be associated with an increased likelihood of obesity. Results of lipidomic data modeled with colon polyps indicated that no specific PPL was associated with colon polyps in our population. However, PPL % total FA differences were highly associated with colon adenomas. The presence of colon adenomas was associated with lower levels of palmitic acid (PA) in PPL, even after adjusting statistical models for obesity. Similarly, colon polyp type (i.e., hyperplastic or adenoma) was significantly associated with increases in plasma non-esterified 5- and 11-HETE, even after adjusting models for obesity. Since lipids such as 5-HETE and LPLs are ligands for inflammation regulating pathways, therefore, future research should investigate whether these lipids influence colon polyp progression. Taken together, the results reported in this dissertation offer new insight into the underlying biology associated with altered metabolism in obesity and the presence of colon polyps, and the lipids reported herein may be useful as plasma biomarkers of altered metabolism and colon polyps.

Dedicated to those with learning disabilities who
were told higher education was not for them.

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KEY TO ABBREVIATIONS

AEA	Arachidonoyl ethanolamide
AG	Arachidonoyl glycerol
ALA	Alpha-linolenic acid
APC	Adenomatous polyposis coli
ARA/AA	Arachidonic acid
BH FDR	Benjamini-Hochberg false discovery rate
BHT	Butylated hydroxytoluene
BMI	Body mass index
CAE	Carcinoembryonic antigen
Cer	Ceramide
CI	Confidence interval
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
COX	Cyclooxygenase
CRA	Colorectal adenomas
CRC	Colorectal cancer
CYP	Cytochrome p450 enzymes
D5D	Delta-5-desaturase
D6D	Delta-6-desaturase
DGLA	Dihomo-gamma linolenic acid
DHA	Docosahexaenoic acid

DHEA	Docosahexaenoyl ethanolamide
DHET	Dihydroxyeicosatrienoic acid
DiHETE	Dihydroxy eicosatetraenoate
DiHODE	Dihydroxyoctadecaenoic acid
DPA	Docosapentaenoic acid
DTA	Docosatetraenoic acid
EAE	Enzyme activity estimates
EET	Epoxy eicosatrienoate
ELOVL-6	Elongation of very long chain fatty acid protein-6
EPA	Eicosapentaenoic
EpETE	Epoxy eicosatetraenoate
EpOME	Epoxy octadecaenoate
ER	Endoplasmic reticulum
ESI	Electrospray ionization
FA	Fatty acid
FADS	Fatty acid desaturase
FAME	Fatty acid methyl ester
FAP	Familial adenomatous polyposis
FAS	Fatty acid synthase
FFA	Free fatty acid
GC-FID	Gas chromatography flame ionization detection
HDoHE	Hydroxy docosahexaenoate
HDPA	Dihydroxydocosapentaenoic acid

HETE	Hydroxyeicosatetraenoic acid
HETrE	Hydroxyeicosatrienoic acid
HIF-1a	Hypoxia-inducible factor 1 alpha
HODE	Hydroxyoctadecadienoic acid
HOTrE	Hydroxyoctadecatrienoic acid
HPLC	High performance liquid chromatography
HR	Hazard ratio
IL	Interleukin
IP-10	Interferon-g inducible protein-10
LA	Linoleic acid
LCAT	Lecithin:cholesterol acyltransferase
LOX	Lipoxygenase
LPL	Lysophospholipid
LPLase	Lipoprotein lipase
LTB4	Leukotriene B4
LXA4	LipoxinA4
MCP-1	Monocyte-chemoattractant protein 1
MetS	Metabolic syndrome
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem Mass Spectrometry
MUFA	Monounsaturated fatty acids
NA	Nervonic acid

NEFA	Non-esterified FAs
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSAID	Non-steroidal anti-inflammatory drugs
OA	Oleic acid
ODE	Octadecanoid
OR	Odds Ratio
oxoETE	oxoeicosatetraenoate
oxoODE	oxooctadecadienoic acid
PA	Palmitic acid
PC	Phosphatidylcholines
Pc	Principal components
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PG	Prostaglandin
PGE2	Prostaglandin E2
PI	Phosphatidylinositol
PL	Phospholipids
PLA	Phospholipase A enzyme
POA	Palmitoleic acid
PPAR-g	Peroxisome proliferator-activated receptor gamma
PPL	Plasma phospholipid
PRR	Pattern recognition receptors
PS	Phosphatidylserine

PUFA	Polyunsaturated fatty acid
QC	Quality control
QToF	Quadrupole time-of-flight
RKHS	Reproducing kernel Hilbert spaces regressions
RR	Relative Risk
Rv	Resolvin
S1P	Sphingosine-1-phosphate
SA	Stearic acid
SatFA/SFA	Saturated fatty acid
SCD n-7	Ratio of palmitoleic to palmitic acid
SCD n-9	Ratio of oleic to stearic acid
SCD-1	Stearoyl-CoA desaturase-1
sEH	Soluble epoxide hydrolase
SM	Sphingomylein
SPE	Solid phase extraction
SVD	Singular value decomposition
T2D	Type-2 diabetes
TBX2	Thromboxane B2
TLR	Toll-like receptors
TNF-a	Tumor necrosis factor alpha
UPLC	Ultra performance liquid chromatography
WAT	White adipose tissue
WC	Waist Circumference

ω-3	Omega-3
ω-6	Omega-6

CHAPTER 1: INTRODUCTION, CHAPTER SUMMARIES, AND LITERATURE REVIEW

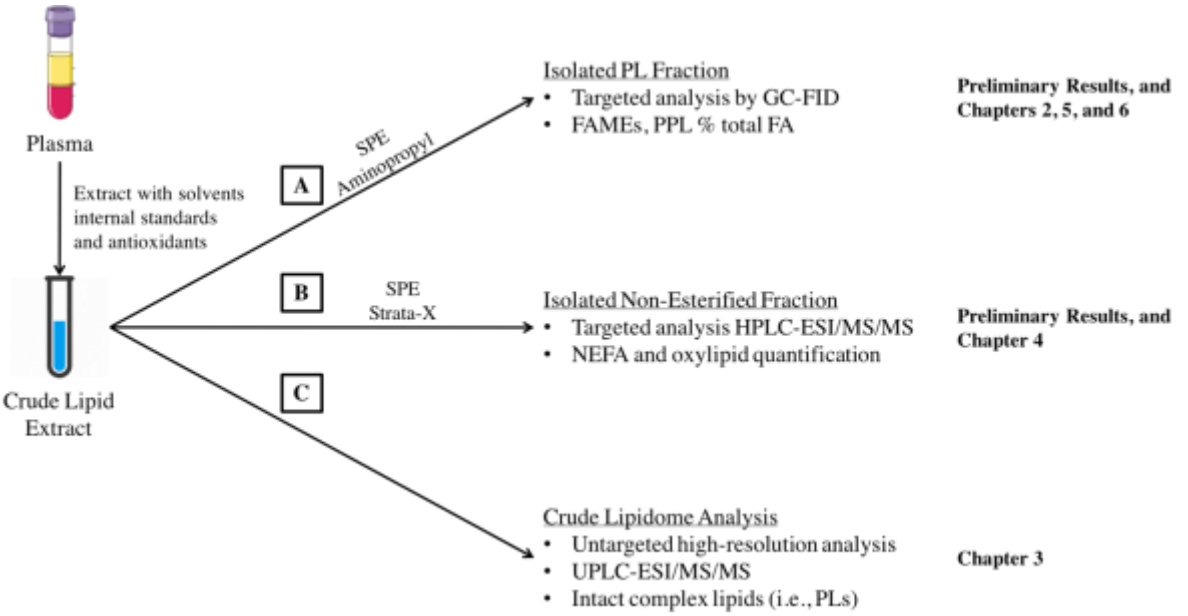
Introduction

Over one-third of adults and nearly 17% of adolescent Americans are defined as obese. Obesity is associated with numerous comorbidities including chronic low-grade inflammation, metabolic dysregulation, and increased risk of colon cancer. It is estimated that 15-30% of cancer deaths in the US are attributed to excess body weight [1]. The Fenton Lab has reported, in a population of 126 Caucasian males, that obesity, and serum adipokines, cytokines, and growth factors are associated with an increased likelihood of colon polyps [2-4]. Pickens et al. reported in this population that PPL % total FA differences are associated with obesity, and pooled obese plasma (n=10) had increased non-esterified proinflammatory oxylipids and decreased vicinal diols compared to pooled lean plasma (n=10) [5]. The observations with PPL % total FA differences in obesity were consistent with reports from recent meta-analyses [6, 7]. At the time, there was limited research into oxylipid profiles associated with obesity so there were no cross-study comparisons in humans. Since FAs are esterified in PLs and phospholipases liberate FAs to serve as substrates for oxylipid biosynthesis, there was a clear need to determine if the % FA differences in obesity occurred in specific PL species, and if non-esterified FA and oxylipid concentrations differed in the entire study population.

The research of this dissertation expands on the work of the Fenton lab, in particular Pickens et al. and Comstock et al., by further investigating the relationship between obesity, serum adipokines and cytokines, FAs esterified in PPLs, non-esterified plasma FAs and oxylipids, and

colon polyp risk. There were three analytical methods employed to generate lipidomic data from different lipid fractions, and these methods are outlined in Figure 1. Obesity is associated with altered serum cytokine levels, and these cytokines may activate pathways that inhibit or enhance FA desaturation or elongation [8]. Thus, the first step was to determine if PPL % total FA differences were associated with serum cytokines (Figure 1A). The remaining lipid analyses were conducted utilizing two cutting-edge methods in MS/MS: 1) Non-esterified FA and oxylipids were isolated using solid phase extraction and quantified using a sensitive targeted high performance liquid chromatography (HPLC)-electrospray ionization (ESI)-triple quadrupole MS/MS analysis (Figure 1B); 2) Intact PPLs and their esterified FAs were characterized using ultra performance liquid chromatography (UPLC)-ESI-high-resolution quadrupole time-of-flight (QToF) MS/MS (Figure 1C). The lipidomic data from MS/MS analyses were then modeled to determine lipid biomarkers associated with obesity and serum cytokines. Next, the PPL % total FA data and data from MS/MS analyses were modeled to assess colon polyp risk, and statistical models were adjusted for BMI and WC. This workflow was essential to determine which lipids and lipid metabolic pathways may be altered by obesity or serum cytokines, that could influence colon polyp risk or progression, and which lipids or lipid metabolic pathways could be altered independent of obesity or obesity-related factors, that may affect colon polyp risk. The results outlined in this dissertation provide a deeper understanding into the underlying biology of altered lipid metabolism associated with obesity and colon polyps, and offer profound implications for biomarker discovery and therapeutic drug targets.

Figure 1: Overview of lipid analyses employed in dissertation research.



A) Plasma lipids were extracted and PLs were isolated using aminopropyl solid phase extraction (SPE) columns. Isolated PPLs were methylated to form FAMEs and analyzed by gas chromatography-flame ionization detection (GC-FID). The process of chemical derivatization of FAs to form FAMEs, liberates FAs that are esterified into complex lipids (i.e., PLs). The data from the FAME analysis is used in the Preliminary Results, and Chapters 2, 5, and 6 of the dissertation. B) Plasma lipids were extracted and non-esterified FAs and oxylipids were isolated using Strata-X SPE columns. The isolated non-esterified fraction was analyzed using HPLC-ESI-MS/MS, allowing for absolute quantification of sensitive low abundant lipid metabolites. The data from the targeted non-esterified analysis is used in the Preliminary Results, and Chapter 3. C) Plasma lipids were extracted using a plasma crash to obtain crude lipid extracts which contained all lipid species. These crude lipid extracts were profiled using UPLC-ESI-QToF MS/MS in negative mode to investigate differences in individual PLs and determine FA esterified to each PL detected. The data from the crude lipidome analysis is used in Chapter 3.

Chapter Summaries

The remainder of Chapter 1 will be dedicated to a literature review, and addressing previous findings from the Fenton lab focusing on initial results published by Pickens et al. Collectively, Chapters 2-6 represent manuscripts for publication and dissemination in peer-reviewed scientific journals.

Building upon previous work from the Fenton lab, the relationship between serum cytokines, obesity, and PPL % total FA differences were investigated in Chapter 2. Specifically, increases in serum C-peptide were inversely associated with FA desaturase enzyme activity estimates (EAE), even after adjusting statistical models for BMI, WC, or proinflammatory cytokines. This observation implicated a potential unknown mechanism, where FA desaturase inhibition may occur through a C-peptide mediated pathway. This chapter has been published in *PLoS ONE* and can be found at https://www.ncbi.nlm.nih.gov/pubmed/27023786.

The next logical step was to determine if previously reported % FA differences occur in specific PL classes. In Chapter 3, a cutting-edge method in MS/MS was developed to profile the entire plasma phospholipidome, while simultaneously determining FAs esterified to each phospholipid detected. The relationship between phospholipidomic profiles, obesity, C-peptide, adipokines, and cytokines was assessed using high-dimensional statistical analyses. Over 1700 plasma lipids were identified and the responses were modeled with the lipidomic data by: 1) assessing the entire plasma phospholipidome concomitantly, and 2) assessing each of the 1700 lipids individually. This chapter has been submitted to *Nature Scientific Reports*. In Bayesian analyses, whole phospholipidomic profiles accounted for over 60% of the inter-individual variation of BMI, WC, and leptin in our study participants. Obesity was associated with specific

classes of PLs and PLs containing specific esterified FAs that agree with initial results published by Pickens et al.

It was still unclear whether differences in esterified PPL FAs (i.e., a decrease in PPL LA) would be reflective of non-esterified FAs (i.e., a decrease in non-esterified LA) in obesity. Chapter 4 focuses on the non-esterified lipidome and investigating obesity's association with non-esterified FAs and oxylipids. Chapter 4 has been submitted to *Metabolism Clinical and Experimental*. Overall, non-esterified FAs and PUFAs were not associated with obesity, however, certain classes of oxylipids were associated with obesity. Arachidonic acid (ARA)-derived 5- and 11-HETE were significantly increased in obesity, even after normalizing HETE concentrations to ARA. Vicinal diols (i.e., alcohols on adjacent carbon atoms)-derived from LA, EPA, and DHA were inversely associated with BMI and WC, even after normalizing vicinal diols to parent PUFA or precursor epoxide concentrations. Specific concentration ranges of HETEs and vicinal diols were determined to be associated with an increased likelihood of obesity. Taken together, these results suggest obesity is associated with altered metabolism through 5LOX (i.e., 5-HETE), reactive oxygen (i.e., 11-HETE), and sEH (i.e., vicinal diol) pathways.

After identifying lipids associated with obesity and serum cytokines, Chapter 5 shifts focus to investigating plasma lipid biomarkers associated with colon polyp risk. Chapter 5 determines associations with the presence of colon adenomas and percentages of saturated and monounsaturated FAs (MUFAs) in PPLs. All statistical models were adjusted for age, smoking, and BMI or WC. Elaidic acid, a trans FA, was elevated in those with adenomas. Individuals with PA percentages >30.25 were significantly less likely to have adenomas compared to those with PA percentages ≤ 28.12. Higher EAEs of stearoyl-CoA desaturase-1 (SCD-1) and elongation of very long chain fatty acid protein-6 (ELOVL-6) were associated with individuals being 1.5 times

more likely to have an adenoma. These results indicated altered metabolism of saturated FAs and MUFAs through desaturases and elongases, and a biological model was proposed to explain these observations. Chapter 5 was published in *Cancer Epidemiology, Biomarkers & Prevention* and can be found at https://www.ncbi.nlm.nih.gov/pubmed/26721667.

Chapter 6 investigates associations between colon polyps and PPL % total PUFAs. Some PUFAs are highly correlated to other PUFAs and obesity, thus, statistical models could not be adjusted for BMI or WC. Instead, a high-dimensional factor analysis was employed to cluster PUFAs into groups, in an unbiased manner. Increases in long chain ω-6 factor loadings significantly increased the likelihood of individuals having an adenoma. Increased PPL docosatetraenoic acid (DTA), an ω-6, was the only PUFA associated with hyperplastic polyps and adenomas. Chapter 7 was published in the *European Journal of Cancer Prevention* and is available at https://www.ncbi.nlm.nih.gov/pubmed/27768609.

Appendix A consists of supplementary information from Chapters 2-6. Appendix B outlines preliminary data associating polyp type with MS/MS data sets. Of the >1700 lipids in the plasma phospholipidome, none were individually associated with polyp type after false discovery correction. However, the entire plasma phospholipidome accounted for over 20% of the variation in polyp type, which indicates a general change of the lipid profile in different polyp types, rather than a specific change in levels of a specific lipid. In plasma oxylipid single marker regressions, ARA-derived oxylipids were significantly elevated in those with polyps. More specifically, 5- and 11-HETE were associated with polyp type even after normalization to ARA and adjustment of either BMI or WC. Entire oxylipid profiles only accounted for a marginal (< 9%) amount of variation associated with polyps.

Literature Review

Obesity

Obesity is a global epidemic affecting adults and children in both westernized and industrialized nations. According to the 2009-2010 National Center for Health and Statistics report, one-third of all adults and nearly 17% of adolescent Americans are defined as obese. Obesity prevalence among men and boys has significantly increased over the past decade, while women and girls have only seen minor increases in obesity [9]. The problem with obesity is not cosmetic, it is considered a risk factor for many common diseases and health disparities that plague society. The medical cost of obesity in America rose from $78.5 billion dollars in 1998 to estimated $147 billion dollars in 2008 [10]. In fact, morbidities associated with obesity are second only to smoking in the U.S. [11].

An obese individual is classified as having a BMI ≥30, while overweight individuals have a BMI of ≥25. BMI is calculated through dividing kilograms body weight by height in meters squared [9]. Obesity is a risk factor for heart disease, certain cancers, stroke, metabolic syndrome (MetS), insulin resistance, high blood pressure, and non-alcoholic fatty liver disease [12]. BMI is a quick and inexpensive tool used to assess health status and risk factors. However, it can be misleading and give false representation in individuals with unique body types (i.e. body builders). BMI is also frequently used in epidemiological studies to investigate correlations between body type and diseases.

In the US, it is estimated that 15-30% of cancer deaths are attributed to excessive body weight [1]. Body fat can be differentially distributed in regions throughout the body. Increased central adipose fat accumulation, deemed android obesity, can be assessed through measuring WC,

and increased WC is a risk factor for several diseases. Men with a WC >101cm have a 50% increased risk of developing colon cancer [13]. Excess visceral fat is also associated with increased levels of proinflammatory cytokines and disruptions in homeostatic adipokines. The expansion of white adipose tissue (WAT) results in adipokine dysregulation, adipocyte hypoxia, and induction of the M1 macrophage [14]. WAT is an endocrine organ responsible for regulating metabolism and secreting adiponectin, leptin, tumor necrosis factor alpha (TNF-α), interluekin-1 (IL-1), and IL-6 [15]. Adiponectin and leptin and have an inverse relationship in obese individuals. Leptin is ligand for the OBR receptor and activates signal transducing pathways such JAK, MAPK, and disrupts insulin pathways [13]. Increased insulin resistance, insulin overexpression, and low plasma adiponectin are associated with obesity [15, 16]. These inflammatory biological states are thought to be involved in cancer proliferation and several chronic diseases [17].

Overall obesity contributes to a variety of known inflammatory biological pathways that may initiate carcinogenesis. Aside from the biological changes associated with increased WAT, other underlying lifestyle factors may contribute to obesity's role in cancer such smoking or eating high fat processed foods. Some risk factors for obesity are genetic predispositions, lack of adequate amounts of physical activity, influence by environmental or socioeconomic factors, age, and consuming unhealthy diets [18]. This adds difficultly in making health claims such as obesity causes colon cancer, because biological, genetic, age, and lifestyle factors may interact in a manner that promotes an adequate environment for cancer development in obese individuals.

Inflammation

Inflammation is a normal biological response involving a complex network of cells, intra and extracellular stimuli, organs, and tissues to maintain homeostasis. There are two types of

inflammation: acute and chronic. Acute inflammation is triggered by numerous biochemical, environmental, and psychological factors, which elicit signaling cascades and induce activation or class switching of immune cells [17]. Chronic inflammation is a persistent inflammatory response implicated in several diseases (i.e. MetS and obesity) and several cancers. The inability to resolve inflammation is a characteristic of chronic inflammation, which leads to prolonged infiltration of immune cells, and tissue hyperplasia and destruction [19].

All immune cells are derived hematopoietic stem cells located in the bone marrow, which splits into two distinctive lineages known as the common lymphoid progenitor (CLP) and common myeloid progenitor (CMP). The CLP is the precursor for B cells and T cells, and both function as members of the adaptive immune response. The CMP is the precursor for innate immune response that is critical for activating cell mediated immunity, including cells such as the macrophage, dendritic cell, mast cell, and neutrophil [20-22]. Pattern recognition receptors (PRRs) present on innate immune cells, such as toll-like receptors (TLRs), recognize specific molecular patterns and signal an immune response [23]. Some TLRS are also receptors for endogenous biomolecules such as saturated non-esterified FAs (NEFAs). Activation of TLRS and other PRRs elicit downstream signaling cascades, activating proinflammatory gene transcription and suppression of anti-inflammatory genes.

There are numerous ligands, which elicit signaling or inhibit cascades either at the cell surface, in the cytoplasm, or in the cell nucleus. In some cases, ligand-receptor binding can activate inflammatory genes such as nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and TNF-α, resulting in the transcription of proinflammatory proteins. Similarly, peroxisome proliferator-activated receptor gamma (PPAR-γ) ligands are considered anti-inflammatory due to inhibition of NFκB activation. PPAR-γ binds to PPAR-γ-response elements

and either activates or represses transcription of genes [24]. For instance, PPAR-γ ligands increase mRNA expression and circulating concentrations of adiponectin, which is an anti-inflammatory adipokine inversely associated with BMI [25]. Recently, Mirzaei et al reported that obese individuals with increased concentrations of serum PPAR-γ ligands are at a higher risk for MetS [26]. Inflammatory cytokines stimulate adhesion molecule and chemokine expression on endothelial cells, allowing leukocyte infiltration into tissues. Chemokines signal leukocytes to cross endothelial barriers such as monocyte-chemoattractant protein 1 (MCP-1) for macrophages, IL-8 for neutrophils, [27] and interferon-γ inducible protein-10 (IP-10) and several other proteins specific for T helper 1 cells [28].

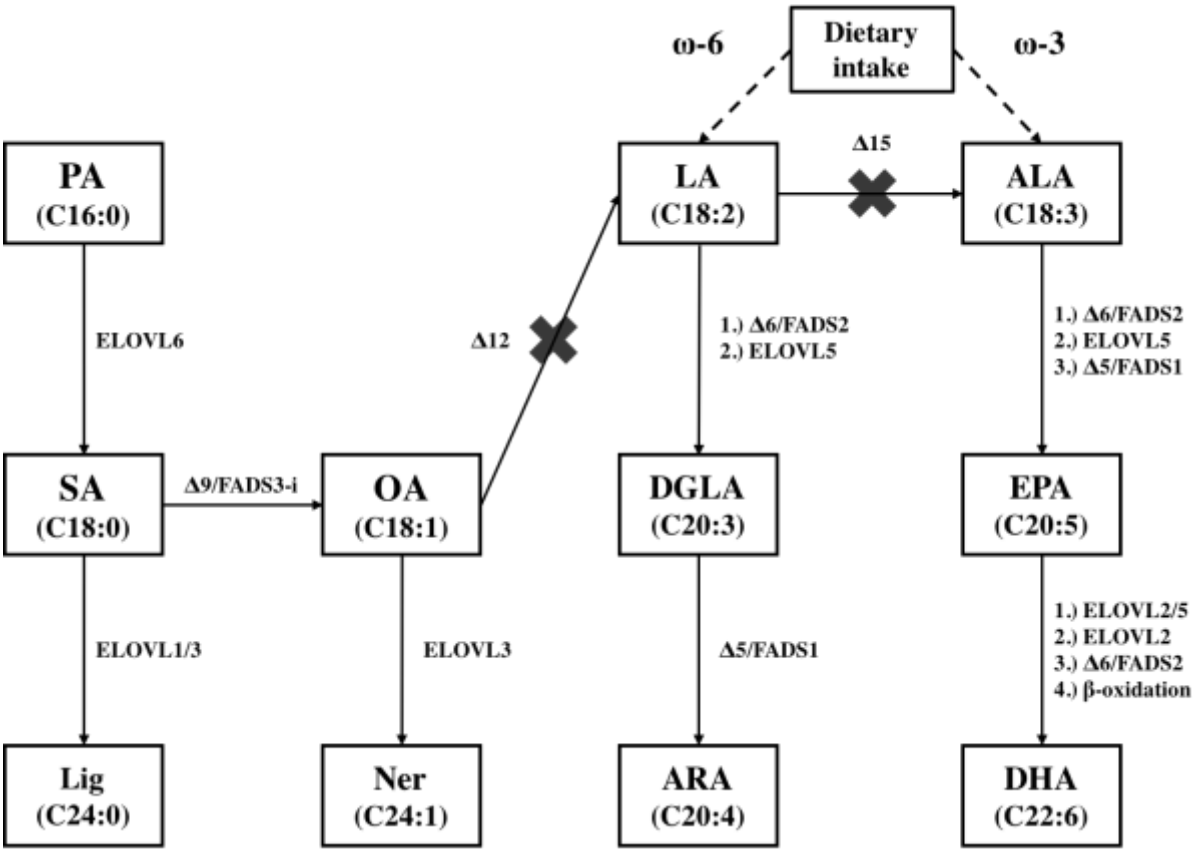
In obesity, the expansion of WAT produces hypoxic-like tissue microenvironments, which induces macrophage polarization [29]. Hypoxia-inducible factor 1 alpha (HIF-1α) is increased in obese adipose tissue and decreases after weight loss [30]. Pathways related to HIF-1α interact with the NFκB pathways, thus, adipocyte hypoxia can activate proinflammatory gene activation [31]. This adipocyte driven secretion of HIF-1α stimulates proinflammatory macrophage activation (i.e. M1 lineage) [32]. Each excess kilogram of human fat results in the accumulation of an estimated 20-30 million macrophages [33]. M1 macrophages are primarily responsible for secretion of TNF-α and MCP-1, resulting in recruitment of monocytes from the blood, which develop into macrophages, and increased adipocyte apoptosis that is characteristic of insulin resistance [34]. TNF-α is associated with decreased GLUT4-mediated uptake of glucose and is characteristic of insulin resistance [35]. TNF-α also activates neutral sphingomyleinase and induces ceramide formation [36]. Ceramides induce apoptosis of adipocyte-derived stem cells [37]. Adipocyte death results in the release of free fatty acids (FFAs), which can activate TLR mediated inflammatory response in macrophages. Much research has been conducted into the link

between hypoxic-like adipocytes, inflammation, altered lipid metabolism, cancer, and insulin resistance.

Lipids

Lipids are comprised of diverse groups of compounds including: FAs, sterols (i.e., cholesterol and cholesterol esters), glycerolipids, and glycerophospholipids and sphingomyelins (SMs). FAs are hydrocarbon-like molecules which contain a carboxylic acid group. There are several classifications of FA: 1) saturated FAs (SFA), if the carbon chain completely saturated with hydrogen atoms. 2) MUFA, if the FA chain contains one site of unsaturation. 3) PUFA, if the FA contains two or more sites of unsaturation. In humans, most FAs can be synthesized endogenously by proteins coded from ELOVL genes (i.e. ELOVL-1, -2, -5, and -6) and desaturation reactions involving proteins from fatty acid desaturase (FADS) genes: FADS1, or delta-5-desaturase (D5D); FADS2, or delta-6-desaturase (D6D); FADS3 isoforms, or SCD-1 and SCD-2 [38]. The exception to endogenous FA biosynthesis is the essential ω-6 precursor LA and ω-3 precursor alpha-linolenic acid (ALA). Specifically, humans cannot synthesize these two FAs due to a lack of delta-12- and delta-15-desaturase enzymes. In some organisms (i.e. algae) oleic acid (OA) is converted to LA by delta-12-desaturase, and then LA can be converted to ALA by delta-15-desaturase [39]. Thus, to acquire LA and ALA, humans must rely on dietary intake to obtain sufficient quantities of essential FAs. FA biosynthesis is outlined in Figure 2.

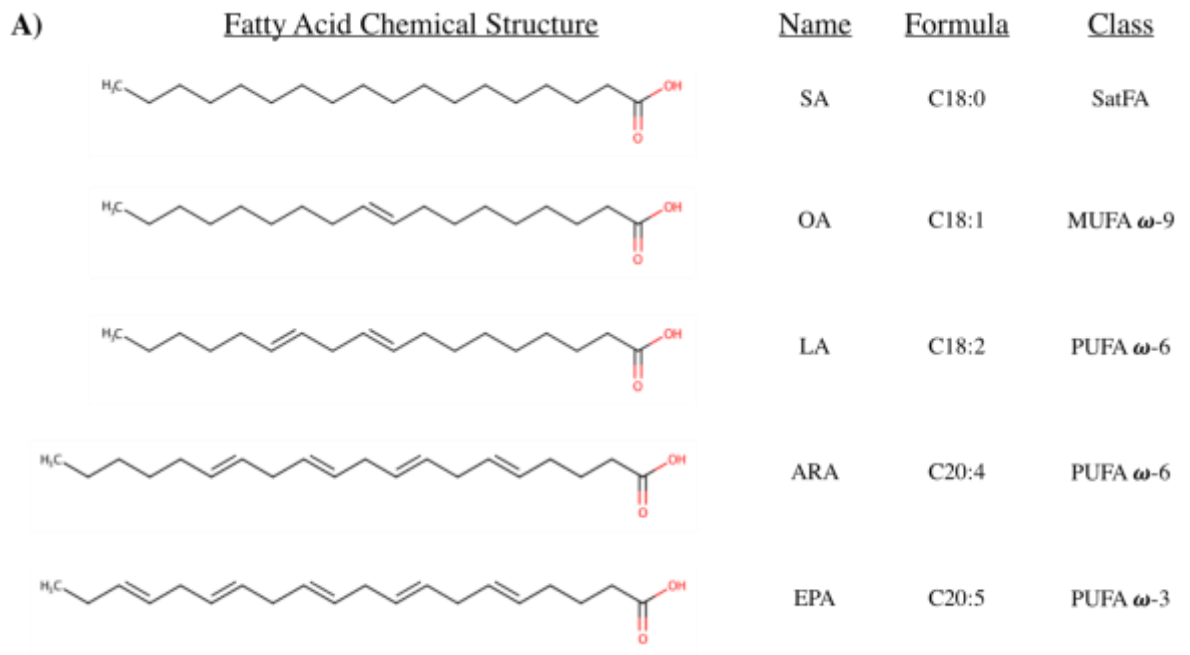
Figure 2: Fatty acid biosynthesis in humans.



Obesity is associated with Westernized dietary intake patterns [40], which alters the FA composition of PLs and PPLs [41]. Most studies evaluating the role of lipids in obesity have focused on dietary PUFA intake and supplementation, lipoprotein particle variation, serum di- and tri-glyceride composition, circulating saturated and unsaturated FFAs, or changes to ω-6/ω-3 ratio. An overview of chemical structural differences between SFAs, MUFAs, and PUFAs are displayed in Figure 3A. Circulating lipoproteins (i.e. HDL, LDL, and VLDL) contain FA sequestered in cholesterol esters, triglycerides, and in the PL membrane. In humans, lipases are involved in the hydrolysis of esterified FA. For instance, lipoprotein lipase (LPLase) releases esterified oxylipids from VLDL particles, which is speculated as a mechanism of delivering signaling oxylipids to peripheral tissues [42].

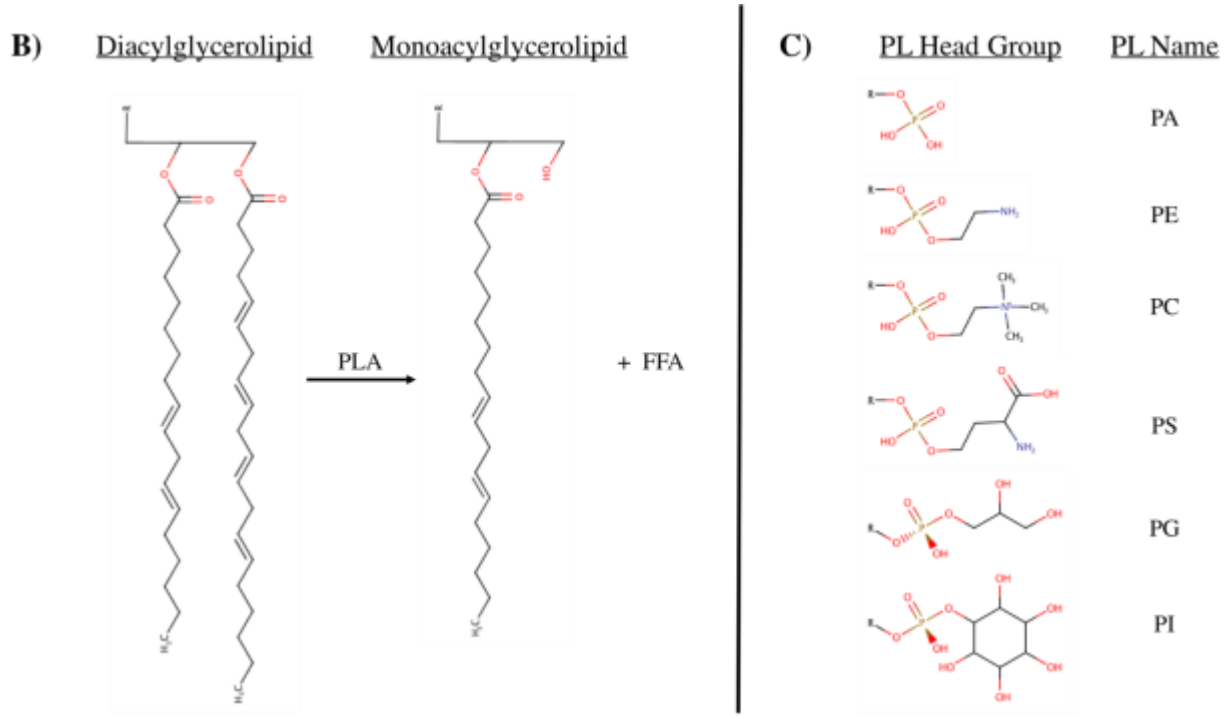
Typically, in glycerol-backboned PLs, saturated FAs are located at the sn-1 position of PLs and PUFAs are located at the sn-2 position. However, MUFAs and PUFAs can also occupy the sn-1 position of a PL. Figure 3B displays the structures of a PL containing LA and ARA. Phospholipase A1 (PLA1) cleaves FAs located at the sn-1 location and PLA2 cleaves FAs from the sn-2 location from PLs [43]. PLA is associated with generation of signaling molecules, from ARA (i.e. prostaglandins), but PLA also is involved in generation of FFA and LPLs (Figure 3B) [44]. PL classes are distinguished based on the phosphate linked head groups (Figure 3C), such as Phosphatidyl: Ethanolamine (PE), Choline (PC), Serine (PS), Glycerol (PG), and Inositol (PI), and sphingolipids such as SMs [45]. Liberation of FAs from SM produces sphingosine-1-phosphate (S1P), which is implicated in several cancers (i.e. colon cancer).

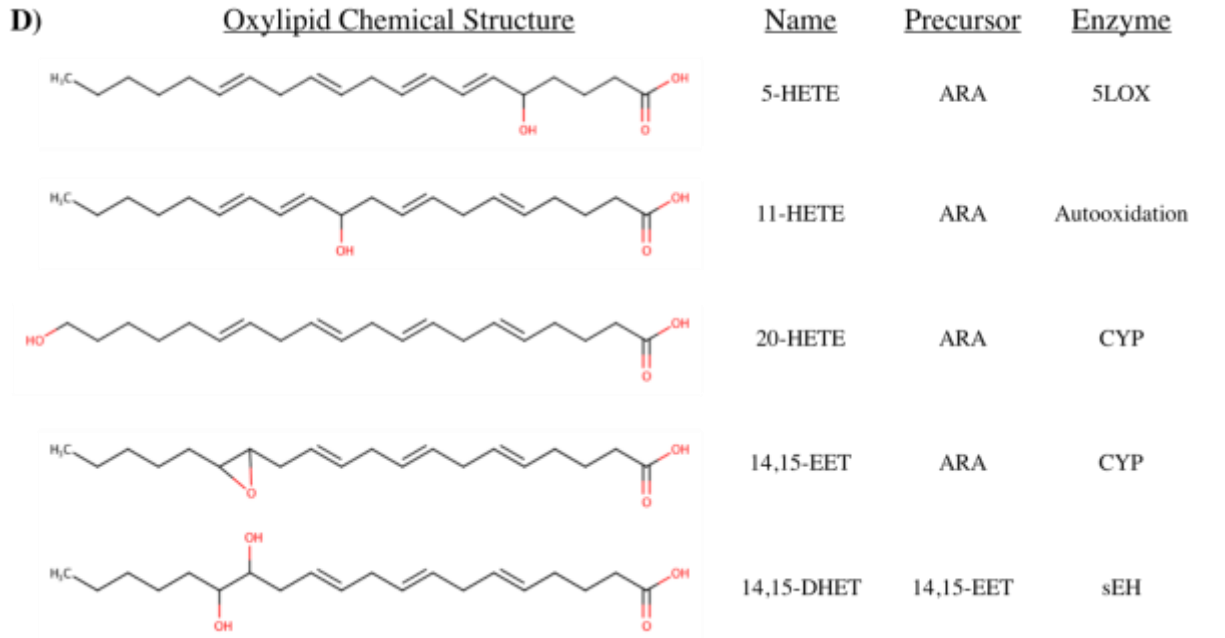
Figure 3: Chemical structures of fatty acids, phospholipids, and oxylipids.



(A) The chemical structures for a SFA, MUFA, and three PUFAs. These FAs, along with others, can be esterified sn-1 and sn-2 position of PLs. (B) The structure of a diacylPL with LA and ARA esterified. PLA enzymes will liberate a FA from diacylPLs, producing an LPL and FFA. (C) There are several PL head groups that could be attached the "R" position of the PL or LPL displayed in Figure 3B. Liberated FAs from PLs serve as substrates for oxylipid synthesis. For instance, the liberated ARA FFA from Figure 3B, would severe as a substrate for oxygenating enzymes and oxylipid biosynthesis. (D) Several ARA-derived oxylipids that could be formed through the oxygenation by 5LOX (i.e., 5-HETE), autooxidation (i.e., 11-HETE), CYP (i.e., 20-HETE), CYP epoxygenases (i.e., 14,15-EET), and an example of secondary ARA-derived oxylipid metabolism such as the hydrolysis of the epoxide 14,15-EET by sEH to produce the vicinal diol 14,15-DHET.

Figure 3 (cont'd)

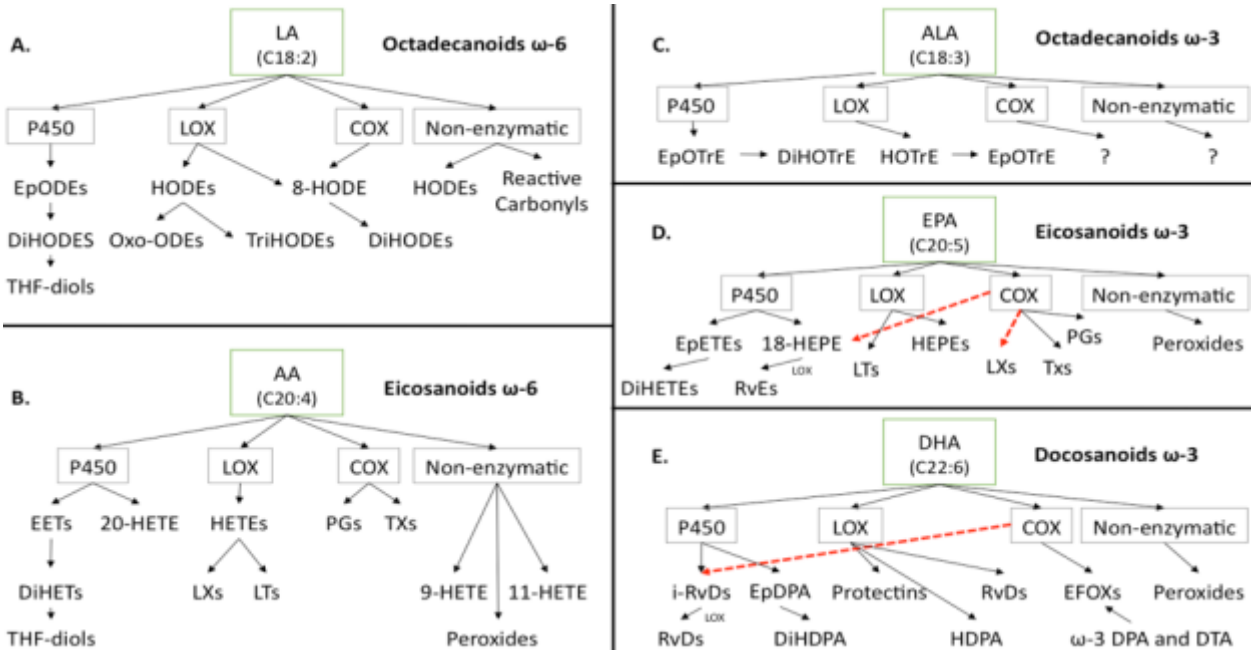




The lipidome, which includes NEFAs and oxylipids, influences production of inflammatory cytokines [35]. PUFAs compete as substrates for oxygenating enzymes such as cytochrome p450 enzymes (CYP) [46], cyclooxygenase (COX) [47], and LOX [48]. The competition between PUFAs and oxygenating enzymes are presented in Figure 4. Through these enzymatic oxygenations, PUFA oxylipid function is enhanced or reduced; thus regulating inflammation. ω-3 PUFAs are typically considered anti-inflammatory lipids, compared to ω-6 PUFAs which are considered more proinflammatory [49]. Therefore, complex lipids (i.e., PLs) may affect inflammation depending upon the presence of specific esterified FAs [35].

ω-3 PUFAs also impact the regulation of numerous genes. In particular, one important function of ω-3s is the activation of PPARs (i.e α and γ) that promote physiological responses such as FA β-oxidation, adipogenesis, and inhibition of NFκB signaling [50]. Interestingly, oxygenated EPA (E-series) and DHA (D-series) derived ω-3 resolvins (Rv), as seen in Figure 4D and 4E, exert their anti-inflammatory function in the pico-molar range (i.e., over 100 times more potent than non-oxygenated EPA and DHA) interacting as ligands for PPAR-γ, lipoxin A4, and GPR32 receptors [51]. Several ω-6 octadecanoids (ODEs) and eicosanoids exert their inflammatory like effects by inducing adhesion molecule expression and leukocyte adhesion to endothelial cells [52]. Despite ω-6 FA and oxylipid associations with proinflammation, some ω-6 ODEs and eicosanoids are potent pro-resolving molecules, such as the ω-6 oxylipids: 13-hydroxyoctadecadienoic acid (HODE), 15-HETE, PGJ2, and 15-deoxy-$\Delta^{12,14}$-PGJ2 which are all PPAR-γ activators [53, 54].

Figure 4: PUFA oxylipid enzymatic and non-enzymatic biosynthetic pathways, in humans.[a]



[a]Boxes are listed around initial oxygenating steps from respective PUFAs. Red-hashed arrows indicate specialized aspirin triggered oxylipids species. This image combines known Kegg's lipid metabolism pathways, with current literature reporting of known PUFA oxylipids; obtained from the following sources: [55-66].

Although some ω-3s and ω-6s have similar functions as ligands, the specific response elicited is dependent on the cell type. For instance, in colon tumor cells, DHA acts as an antagonist of PPARs. Certain cell types also can affect the transformation of ω-3 and ω-6 oxylipids to enhance or suppress their respective functions. For instance, the ω-6 ODE 13-oxooctadecadenoic acid (oxoODE) is a potent endogenous PPAR-γ activator in colon cells. The 13-oxoODE converting enzyme 13-HODE dehydrogenase, is highly expressed in colon epithelial cells, and researchers suggest increased 13-HODE dehydrogenase may aid inflammation control in the colon [67]. ARA-derived lipoxinA4 (LXA4) is also a potent pro-resolving lipid mediator capable of inhibiting leukocyte transmigration across epithelial cells, even though, as previously mentioned, several other ARA derived oxylipids stimulate transmigration [64]. The importance of the lipidome's function in exacerbating and controlling inflammation is a growing interest in chronic inflammatory diseases.

Lipidome biomarkers are attractive in chronic disease research since they may: indicate disease state, give insight into underlying biology associated with altered metabolism in diseases, and provide new compound classes and mechanisms for drug targeting. Several instances of lipids as disease biomarkers have already been reported. For instance, plasma S1P has been reported as a promising biomarker of ovarian cancer [68]. In one study, serum lipid analysis revealed correlations between pancreatitis and levels of several ARA and LA oxylipids [69]. Numerous studies have shown increased D6D and decreased D5D enzyme activity are linked to an increased risk of type-2 diabetes (reviewed in [6]). Increased activity of FADS3-i enzymes have been associated with MetS and insulin resistance [70]. The importance of lipidome biomarkers in disease identification will continue to progress with further breakthroughs in MS and characterization of lipidome profiles in disease states.

Colon cancer

The mechanism of colorectal cancer (CRC) progression is well understood. Colon epithelial cells are constantly sloughed off and replaced by new cells every 4-5 days [71]. Some individuals develop cell clusters called polyps, which continue proliferating and can develop into abnormal adenomas and eventually tumors [72]. Early identification with colonoscopies and removal of adenomas significantly reduces the risk of developing adenocarcinomas [73]. Factors such as genetics, age, smoking, ulcerative colitis, diet, and obesity have been shown to increase the risk of developing colon cancer [73, 74]. Colon cancer risk may also be associated to not just obesity, but also changes in weight and site of weight distribution. An epidemiological study into the Health Professionals Follow-Up Study of over 45,000 men found a BMI >22.5 associated to significant increased risk of colon cancer. Weight changes 2-4 years prior had an increased risk association, 1.14 hazard ratio (HR), for 10-pound incremental increases in weight [75]. A prospective cohort study from Cancer Prevention Study II investigated relative risk (RR) of BMI to CRC and found RRs 1.20 for overweight, 1.47 for obese, and 1.84 for severely obese individuals [76]. In this same population, obese individuals had an increased risk of developing all cancers; RR 1.09.

A growing body of evidence associates inflammation with epithelial cell transformation and the process of carcinogenesis [77, 78]. Various models describing the process leading to colon cancer begin with an initiating event, such as inherited or somatic mutations in the adenomatous polyposis coli (*APC*) tumor suppressor gene. If these initiated precancerous cells (Apc+/-) survive, they proliferate during the promotional phase of carcinogenesis, during which, further genetic damage can be incurred, such as mutations in the p53 or deletions in colon cancer genes [79].

In inflammation-associated colon cancer, such as cases associated with inflammatory bowel disease, non-genetic stimuli also encourage the survival and proliferation of initiated cells [78]. Excess adipose tissue may produce promotional signals that can act on colon epithelial cells and increase colon cancer risk associated with the obese state. How this may happen is not understood, but leptin, insulin-related factors, and proinflammatory cytokines may be responsible for elevated proliferative growth and in the carcinogenic process from colon polyps to tumors [80]. There is enough biological plausibility in animal models to suggest obesity, and obesity-associated adipokines and growth factors, increase colon adenoma formation.

The inflammatory mediators associated with colon cell carcinogenesis also alter lipid metabolism. Many of these lipid metabolites have functions involving increased colon adenoma proliferation and decreased apoptosis. For instance, proinflammatory ARA-derived PGE2 has been shown to promote colon tumor growth and suppress silencing genes through DNA methylation [81]. Non-steroidal anti-inflammatory drugs (NSAIDs) block COX-2 synthesis of PGE2. Interestingly, the long-term use of NSAIDs reduces the RR of CRC by 40% (reviewed in [82]). Specifically, daily use of the NSAID aspirin reduced polyp development in individuals with familial adenomatous polyposis (FAP) [83]; FAP patients have an increased risk of developing CRC, nearly 100% [81]. Recently, a specialized class of aspirin-triggered lipoxins and Rvs (Figure 4 D and E) was discovered, which are more potent anti-inflammatory molecules compared to their non-aspirin triggered counterparts, and are suggested to also contribute to the synergic effects observed between aspirin/NSAID use, PGE2, and colon cancer risk [84]. Altered enzyme expression in colon cancer cells also affects lipid metabolism. For example, the enzyme alpha-methylacyl-CoA racemase is upregulated in adenocarinomas and colon cancer cells, which increases beta-oxidation of branched chain FA and FA metabolites [85]. The sphingolipid

metabolite S1P is implicated in adenoma growth and survival. Recently, S1P was described as the missing link between chronic inflammation and colon cancer through the newly identified IL-6/STAT3/S1P receptor 1 positive feedback loop [86]. Increased concentration of S1P can result from increased S1P synthesis by S1P kinase, and S1P kinase activity is implicated in colon cancer carcinogenesis [87]. There is enough biological evidence to suggest metabolites of altered lipid metabolism in blood components may be indicative of colon cancer progression.

Currently the most accurate method of detecting CRC and colon polyps is through an invasive colonoscopy procedure. Early identification with colonoscopies and removal of adenomas significantly reduces the risk of developing adenocarcinomas [73]. The current recommendation from the American Cancer Society is individuals receive a colonoscopy every 10 years starting at age 50. Obese individuals have a 35% decreased probability of being recommended a colonoscopy by their physician [88]. Thus, blood-based detection of colon polyps is an attractive noninvasive alternative to colonoscopy procedures, would reduce healthcare costs, and potentially provide early clinical detection to decrease future risks of colon cancer development. For examples, serum carcinoembryonic antigen (CAE) is a frequently used prognostic proteome marker of CRC, despite having a low sensitivity, but combining serum CAE with total glycosylated sphingolipids (i.e., gangliosides) produced a 2-fold increase in early-stage CRC identification [89]. Although gangliosides are difficult to isolate and analyze, lipidome biomarkers appear promising to the future of blood based CRC detection, whether as standalone biomarkers or incorporated with proteome biomarkers to enhance sensitivity.

The Fenton lab previously reported increased BMI category is associated with an increased likelihood of adenoma presence, compared to no polyps [2]. More specifically, obese individuals were more likely to have ≥3 polyps compared to lean individuals and also more likely to have a

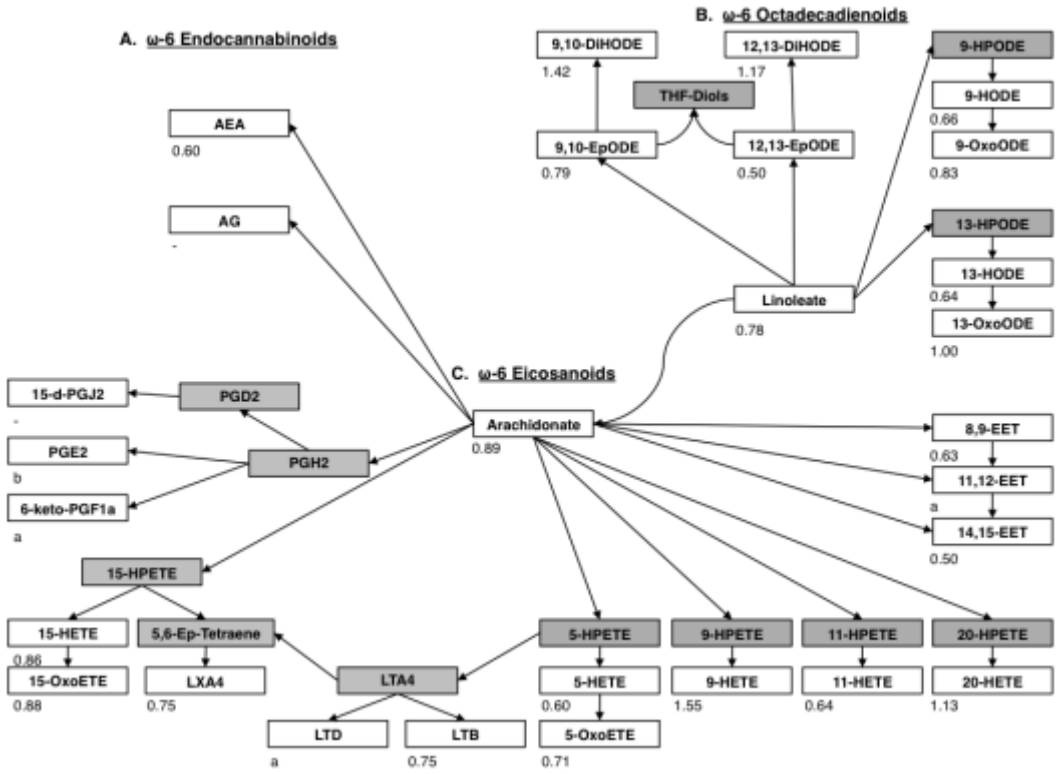
tubular adenoma than no polyps. The Fenton lab also reported proinflammatory adipokines IP-10 and TNF-α were associated with tubular adenoma presence [2]. Circulating insulin-related factors are implicated in the proliferative growth and carcinogenic process from colon polyps to tumors [80]. In addition, our lab reported associations with insulin-related serum factors and colon polyp number in the same population [4]. These factors included C-peptide (a marker of insulin production), soluble receptor for advanced glycation end products, and vascular endothelial growth factor. Together these data suggest serum and plasma markers exist, in our study population, which are indicative of colon polyp presence and number. These data are consistent with inflammation-associated CRC mechanisms, discussed above, in relation to pro-inflammatory cytokines and insulin-related factors. Therefore, in our study population, we expect lipidome markers indicative of colon polyp type should exist.

Preliminary Data

Prior results in our study population revealed associations between BMI, obesity, adipokines, insulin-related factors, and colon polyps. As mentioned, several ω-6 oxylipids are associated with an increased pro-inflammatory response and some ω-6 oxylipids are linked to colon cancer progression (i.e. PGE2). In order to identify preliminary differences between individuals with colon polyps and individuals without colon polyps, we investigated several ω-6 oxylipids and endocannabinoids using a targeted non-esterified FA and oxylipid HPLC-MS/MS method; as described by Pickens et al. [5]. This analysis was conducted on 2 plasma pools for comparing obese individuals with colon polyps to obese individuals without colon polyps (Figure 5). These results are displayed as fold changes between plasma of obese individuals with colon polyps to plasma of obese individuals without colon polyps.

In general, we observed a decrease in ω-6 FAs, endocannabinoids, and oxylipids (Figure 5). The exceptions were: in sEH end products 9,10- and 12,13-dihydroxyoctadecaenoic acid (DiHODE) (Figure 5B), PGE2 was only above the limit of detection in the obese pool with polyps, 20-HETE, and 9-HETE a marker of autooxidative stress (Figure 5C). As mentioned, proinflammatory PGE2 has been shown to promote colon tumor growth and suppress silencing genes through DNA methylation [81]. sEH enzymes convert "potent" endogenous epoxide anti-inflammatory mediators to "less potent" vicinal diol mediators, and SEH expression is increased in inflammation-induced colon carcinoma tissues [90]. Therefore, our preliminary evidence elucidates that altered lipid metabolism is detectable in the plasma lipidome.

Figure 5: Preliminary targeted data of pooled obese plasma from individuals with colon polyps (n=10) and pooled obese plasma from individuals without colon polyps (n=10).

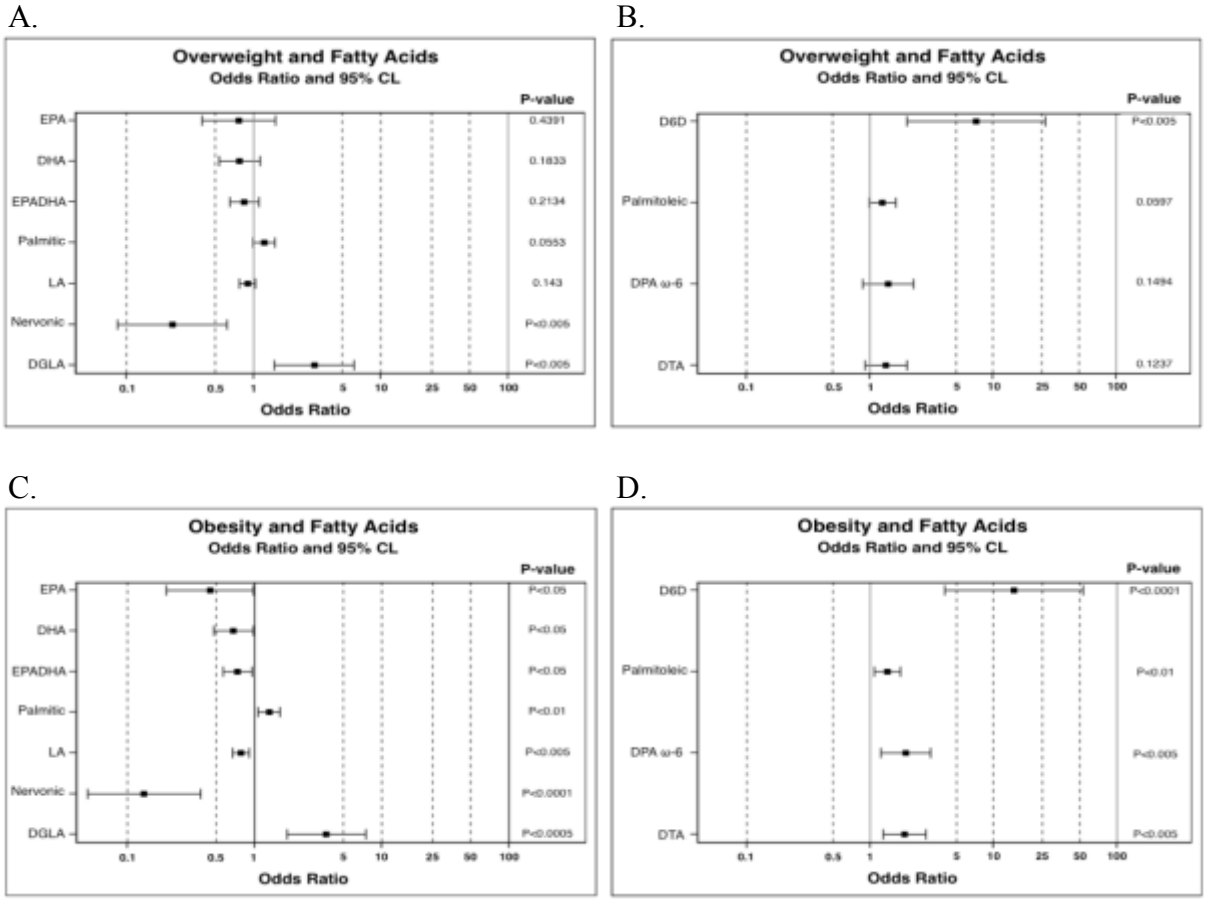


(A) ω-6 Endocannabinoids. (B) Linoleate derived ω-6 octadecadienoids. (C) Arachidonate derived ω-6 eicosanoids. (A, B, and C) Non-esterified plasma PUFAs and oxylipids are listed in boxes: colored white if measured and grey if not measured. Number listed in the lower left-hand corner represents fold change calculated as obese with colon polyps to obese without colon polyps for each analyte, where symbol – represents metabolites which were below limit of detection, symbol "a" represents metabolites below limit of detection in the obese no polyp plasma pool, and symbol "b" represent metabolites below limit of detection in the obese polyp plasma pool.

Previously, in this population, Pickens et al. investigated PPL FA differences associated with BMI [5]. This analysis was conducted through lipid extraction, PPL column isolation, methylation to FA methyl esters (FAMEs), and semiquantitative analysis using GC-FID (see Figure 1A), as previously described [5]. This FAME data set was later used in the publications outlined in Chapter 2, 5, and 6. Pickens et al. reported that specific PPL FAs were associated with obesity and increasing BMI [5]. In brief, we observed overweight participants were 0.23 (0.09-0.62) times less likely to have nervonic acid (NA) incorporated into PPLs and were 3.01 (1.46-6.18) times more likely to have dihomo-gamma linolenic acid (DGLA) incorporated into PPLs (Figure 6A).

Overweight participants were also 7.30 (2.00-26.60) times more likely to have increased D6D EAEs, calculated as the ratio of DGLA to LA (Figure 6B). Overweight individuals tended to have higher PA 1.21 (1.00-1.48) and palmitoleic (POA) 1.27 (0.99-1.63) acid in PPLs. Obese participants were less likely to have EPA 0.45 (0.20-0.99), DHA 0.68 (0.48-0.98), combined EPA and DHA 0.74 (0.57-0.96), LA 0.78 (0.68-0.91), and NA 0.14 (0.05-0.38) incorporation into PPLs (Figure 6C). Obese individuals were more likely to have PA 1.30 (1.07-1.58), DGLA 3.68 (1.81-7.46), POA 1.39 (1.09-1.77), DTA 1.90 (1.28-2.82), and docosapentaenoic acid (DPA) ω-6 1.95 (1.22-3.09) incorporation into PPLs. In obesity, individuals were 14.64 (4.01-53.40) times more likely to have elevated D6D EAEs (Figure 6D) and tended to be 0.76 (0.57-1.01, P-value 0.06, data not shown) times less likely to have elevated D5D EAE, calculated as the ratio of ARA to DGLA.

Figure 6: Plasma phospholipid fatty acid odds ratios by BMI category.[a]



[a]Odds ratio results from polytomous logistic regression compared to lean BMI as reference group. 1B and 1D: odds ratios have been calculated on the basis that there is a unit change of 0.1 for the respective beta coefficient for each given parameter.

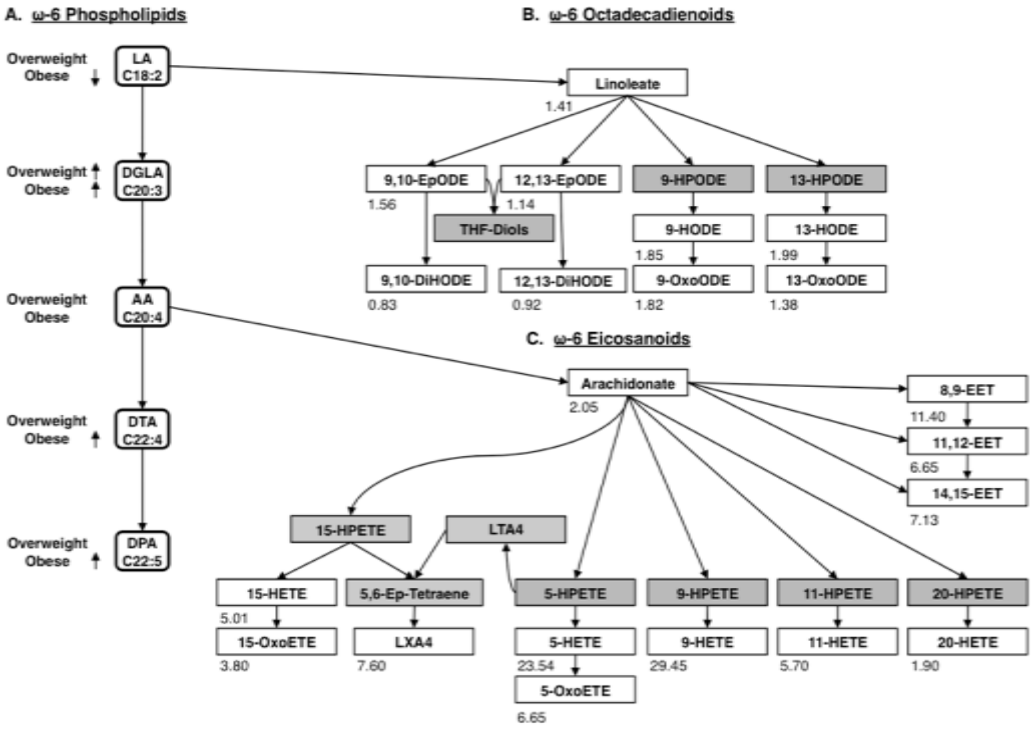
These data demonstrate specific PPL % total FA differences that are indicative of altered lipid metabolism. However, since our previous method analyzed PPL % total FAs as FAMEs, and this type of FAME analysis liberates esterified FA from PPLs (see Figure 1A), we were unable to determine if these esterified FAs differ in specific PL classes (i.e. PE, PC, SM, or LPL). Due to the observed increase in ω-6 and decrease in ω-3 PPL PUFAs, we investigated non-esterified plasma ω-6 and ω-3 oxylipids in pooled obese (n=10) and lean (n=10) plasma using the targeted HPLC-MS/MS method (Figure 1B) [5]. Combining data from PPL FA analysis, with fold changes between obese to lean plasma pools in our non-esterified plasma PUFAs and oxylipids analysis, is visualized in tandem, in Figures 7 and 8. In this Preliminary data section, to avoid overlap in FA names between the PPL and non-esterified plasma PUFAs, in Figures 7 and 8, we refer to all non-esterified plasma PUFAs unabbreviated and as their conjugate base ending with the suffix –ate.

When ω-6 FAs were analyzed, obese participants were less likely to have LA incorporated into their PPLs and more likely to have of DGLA, DTA, and DPA incorporated into PPLs (Figure 7A). Non-esterified plasma linoleate concentration was increased in the obese pool (Figure 7B). In obesity, increased 9,10- and 12,13-epoxyoctadecaenoate (EpOME) was observed along with a decrease in their downstream products 9,10- and 12,13-DiHODE. Also, 9- and 13- HODE were increased in the obese pool along with their respective ketone metabolites 9- and 13-oxoODE. ARA in PPLs did not differ across BMI categories, but there was an increase in ω-6 PPL FAs along with D6D EAEs, warranting investigation of non-esterified plasma arachidonate and oxylipids (Figure 7C). All monohydroxy products 5-, 9-, 11-, 15-, and 20-HETE had elevated concentrations in the obese pool, with largest fold increases in 5- and 9-HETE. Respective oxidized products 5-oxoeicosatetraenoate (oxoETE) and 15-oxoETE were also increased in the obese pool. Arachidonate-derived epoxides 8,9-, 11,12-, and 14,15-epoxyeicosatrienoate (EET)

were all increased. In the obese pool, the concentration of the arachidonate-derived Rv LXA4 also increased.

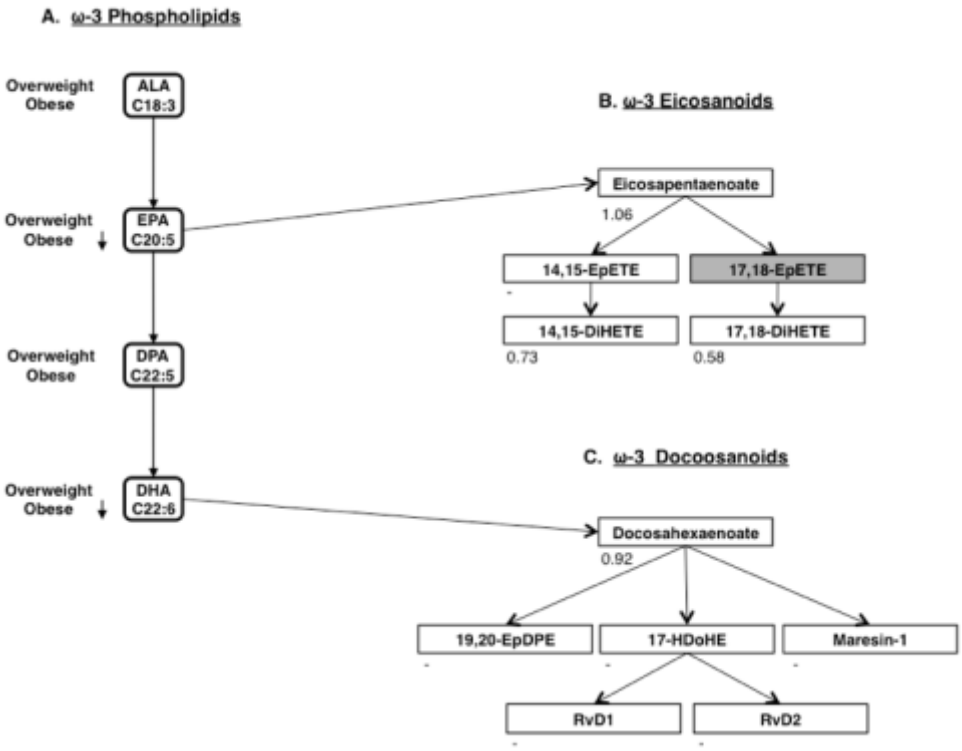
Our results also showed our obese participants had significantly decreased total ω-3 as well as EPA and DHA incorporation into PPLs, with no change in DPA ω-3 PPL incorporation (Figure 8). In obesity, non-esterified plasma eicosapentaenoate only slightly increased, and its epoxidated product 14,15-epoxyeicosatetraenoate (EpETE) was below LOD in both pools (Figure 8). Both ω-3 eicosanoids 14,15- and 17,18-dihydroxy eicosatetraenoate (DiHETE) decreased in obesity. Non-esterified plasma docosahexaenoate was slightly decreased in the obese pool (Figure 8). ω-3 docosanoids 19,20-epoxydocosapentaenoate (EpDPE), 17-hydroxydocosahexaenoate (HDoHE), Rv D1 (RvD1), and RvD2 were below LOD in both plasma pools. Maresin 1 was detectable in the lean pool, but it was below LOD in the obese pool so no fold change could be calculated.

Figure 7: ω-6 PPL % total FA (n=126), and targeted ω-6 non-esterified PUFA and oxylipid analysis of pooled obese plasma (n=10) and lean plasma (n=10).



(A) Visual representation of results from ω-6 PPL pathway, PPL analysis. (B) Linoleate derived ω-6 octadecadienoids. (C) Arachidonate derived ω-6 eicosanoids. (B and C) Non-esterified plasma PUFAs and oxylipids are listed in boxes: colored white if measured and grey if not measured. Number listed in the lower left-hand corner represents fold change calculated as obese to lean for each analyte, where a – represents metabolites which were below limit of detection.

Figure 8: ω-3 PPL % total FA (n=126), and targeted ω-3 non-esterified PUFA and oxylipid analysis of pooled obese plasma (n=10) and lean plasma (n=10).



(A) Visual representation of results from ω-3 PPL pathway, PPL analysis. (B) Eicosapentaenoate derived ω-3 eicosanoids. (C) Docosahexaenoate derived ω-3 docosanoids. (B and C) Non-esterified plasma PUFAs and oxylipids are listed in boxes: colored white if measured and grey if not measured. Number listed in the lower left-hand corner represents fold change calculated as obese to lean for each analyte, where a – represents metabolites which were below limit of detection. Maresin 1's fold change is designated with a -, since it was only above LOD in the lean pool.

CHAPTER 2: RELATIONSHIP BETWEEN BODY MASS INDEX, C-PEPTIDE, AND DELTA-5-DESATURASE ENZYME ACTIVITY ESTIMATES IN ADULT MALES

This chapter has been published in Pickens et al., relationship between body mass index, C-peptide, and delta-5-desaturase enzyme activity estimates in male adults. *PLoS One.* 2016 Mar 29; 11(3):e0149305

Abstract

Obesity, in particular abdominal obesity, alters the composition of plasma and tissue fatty acids (FAs), which contributes to inflammation and insulin resistance. FA metabolism is modulated by desaturases and may affect adipokine and insulin secretion. Therefore, we examined relationships between adipokines, a marker of insulin production, and plasma FA desaturase enzyme activity estimates (EAEs) in obesity. Plasma phospholipid (PPL) FAs were isolated from 126 males (ages 48 to 65 years), derivatized, and analyzed using gas chromatography. Delta-6 desaturase (D6D) and delta-5 desaturase (D5D) EAEs were calculated as the ratio of PPL 20:3/18:2 and 20:4/20:3, respectively. In body mass index (BMI) and waist circumference (WC) adjusted polytomous logistic regression analyses, PPL. FAs and FA desaturase EAEs were associated with C-peptide and adiponectin. Individuals with elevated D6D EAEs were less likely (OR 0.33) to have serum adiponectin concentrations > 5.37 μg/mL, compared with adiponectin concentrations 3.62 μg/mL. Individuals with increased D5D EAEs were less likely (OR 0.8) to have C-peptide concentrations 3.32 ng/mL, and > 1.80 and 3.29 ng/mL, compared with those with C-peptide 1.76 ng/mL. The proinflammatory cytokine tumor necrosis factor-α (TNF- α) was

positively associated with C-peptide, but TNF- α was not associated with the D5D EAE. C-peptide and adiponectin concentrations are associated with specific PPL FAs and FA desaturase EAEs. The relationship between C-peptide concentrations and D5D EAEs remained significant after adjusting for BMI, WC, and TNF-α. Thus, future research should investigate whether D5D inhibition may occur through a C-peptide mediated pathway.

Introduction

Obesity is a chronic disease affecting over one-third of US adults [91]. Obesity is associated with excess lipid storage in white adipose tissue (WAT), adipokine dysregulation, insulin resistance, and chronic low-grade inflammation [92]. Adipokines are adipose-derived cytokines, which have functions in regulating metabolism and inflammation. The expansion of WAT alters adipokine secretion and fatty acid (FA) metabolism, and also influences low-grade inflammation associated with insulin resistance and type-2 diabetes (T2D) [93]. In obesity, circulating concentrations of anti-inflammatory adipokines are lower (i.e. adiponectin) and pro-inflammatory adipokines (i.e. leptin) are elevated compared with lean individuals [94].

Adipokines are necessary for normal cellular function, but dysregulated adipokine secretion can have pathological effects. Leptin is important for regulating body fat [92], but in obesity, leptin concentrations are elevated and individuals can become "leptin resistant" resulting in increased weight gain (reviewed in detail [95]). Leptin and adiponectin concentrations have an inverse relationship in obese individuals. Adiponectin is an adipokine that increases FA oxidation and glucose utilization in tissues [96]. In obesity-associated insulin resistance, adiponectin concentrations are lower and adiponectin receptors are downregulated [97]. C-peptide, a protein

cleaved from pro-insulin, is inversely associated with adiponectin, and C-peptide is positively associated with leptin secretion [98]. While C-peptide is not an adipokine, it is used as a biomarker of insulin secretion which is altered in obesity [99]. Increases in several plasma FAs trigger inflammation, which contributes to insulin resistance and results in increased C-peptide concentrations. There is a relationship between FAs, obesity, adipokines, and insulin resistance, however, it is unknown whether adipokines are associated with specific FAs.

FAs are classified into 3 categories: saturated FAs (SFAs), monounsaturated FAs (MUFAs), and polyunsaturated FAs (PUFAs). PUFAs can be of the omega-3 (ω-3) or omega-6 (ω-6) family, and obese individuals tend to have lower blood concentrations of ω-3s and greater blood concentrations of ω-6s [100]. FAs such as PUFAs are obtained through dietary intake, or endogenously synthesized by elongating and desaturating enzymes. Obesity-associated inflammation may alter enzyme activity and this altered enzymatic expression can modify lipid metabolism [101]. For instance, obese individuals with insulin resistance have decreased expression of the enzyme delta-5-desaturase (D5D) in skeletal muscle [6]. Obesity is also associated with lipid changes such as increased plasma SFAs [102], in particular, palmitic acid (PA) and stearic acid (SA) [103]. Elevated circulating concentrations of SFAs can increase inflammation and affect secretion of pro-inflammatory cytokines [104], in particular, tumor necrosis factor-α (TNF-α) which impairs insulin receptor downstream signaling [105]. Because FAs may influence adipokine secretion and insulin resistance, determining associations between FAs and FA desaturase enzymes, adipokines, and markers of insulin production may lead to a better understanding of obesity-associated pathologies and lead to discovery of potential therapeutic targets.

Most studies investigating the role of lipids in obesity focus on altered dietary FA intake [102] or red blood cell FA concentrations [106]. Similarly, numerous studies investigate the association of obesity with adipokines or insulin resistance [93, 107]. However, there is limited information on specific plasma FA associations with adiponectin, leptin, and C-peptide after adjusting (i.e. statistically) for obesity. Determining whether changes in FAs and FA metabolism occur independent of obesity and abdominal adiposity may allude to unknown biological relationships between FAs and adipokine dysregulation. Therefore, in this study we examined the relationship between C-peptide, adiponectin, leptin, and plasma FAs and FA desaturase enzyme activity estimates (EAEs) in overweight adults.

Materials and Methods

Ethics statement

The study was approved by the Biomedical and Health Institutional Review Board of Michigan State University (IRB# 08-786). The Biomedical and Health Institutional Review Board is one of three IRB committees on the Michigan State University East Lansing campus. Michigan State University's IRBs were established to advance the goal of conducting research with diligence and integrity. The purpose of the committee is to protect the rights, welfare and privacy of human subjects who participate in research conducted by students and/or faculty affiliated with MSU. At the time of enrollment, written informed consent was obtained from each participant.

Study population

Male subjects (n=126, >96% Caucasian) 50-65 years of age were previously recruited from the Tri-County Gastroenterology P.C., Clinton, MI, as previously reported [2]. In brief, nurses at the GI center were responsible for recruitment, explaining the study, and obtaining consent. Patient exclusion was performed, as previously reported [2]. In brief, patients were excluded for: 1) current immunosuppressants or antibiotics, 2) allergic disorders such as eosinophilic or mast cell disorders, severe asthma, 3) severe co-morbidities like end stage renal disease or liver disease with cirrhosis, autoimmune illness, chronic hepatitis, other chronic infections, 4) diabetes. At time of enrollment, trained staff collected anthropometric measurements and venous blood of study participants. These measurements were used to calculate BMI (kg/m^2). Patients reported fasting before venous blood was collected, so lipid results will not be altered by dietary intake. Study participants were classified as lean (BMI <25), overweight (25≤ BMI <30), or obese (BMI ≥30). Serum and plasma fractions were separated from venous blood after collection, stored at -80°C. In addition, the samples were blinded and coded so there are no unique identifiers. Clinical metadata on subject co-morbidities, current medications, family history and tumor characteristics was also collected.

Serum adipokine and C-peptide analysis

Adipokines and C-peptide were analyzed using ELISA or multiplex cytokine kits as previously reported [4]. A commercially available leptin ELISA kit was performed per manufacturer's instructions (R&D Systems, DY398; Minneapolis, MN). C-peptide concentrations were measured as directed by the manufacturer (Calbiotech, Spring Valley, CA, REF; CP1795).

Total adiponectin measurements were performed following the manufacturer's instructions (Alpco Diagnostics, Salem, NH).

Plasma phospholipid extraction, isolation, and analysis

In brief, approximately 200 mg plasma per subject was weighed and extracted using a modified Rose and Oaklander extraction [108]. Phospholipids (PLs) were isolated using Isolute-XL ® SPE aminopropyl columns (500 mg; Bioatage, Charlotte, NC) as described by Agren et al [109]. Fatty acid methyl esters (FAMEs) were prepared as previously described [5]. Plasma PL (PPL) FAMEs were analyzed using HS-Omega-3 Index® methodology at OmegaQuant Analytics, LLC (Sioux Falls, SD) as previously described [110].

Statistical analyses

Frequencies, means, and standard deviations were calculated for descriptive analyses (Table 1). Each FA was expressed as a percentage of total PPL. FA enzyme activity estimates (EAE) were calculated as the ratio of product-to-substrate for delta-5-desaturase (D5D) and delta-6-desaturase (D6D) as follows: D5D = arachidonic (AA)/dihomo-γ-linolenic acid (DGLA); D6D = DGLA/linoleic acid (LA). The total PPL ω-3, herein referred to as total ω-3, was calculated as ∑ alpha-linolenic acid (ALA) + eicosapentaenoic acid (EPA) + docosapentaenoic acid ω-3 (DPAω-3) + docosahexaenoic acid (DHA); The total PPL ω-6, herein referred to as total ω-6, was calculated as ∑ LA + linoelaidic + DGLA + AA + docosatetraenoic acid (DTA) + DPAω-6; ω-6/ω-3 ratio was calculated as total ω-6/ total ω-3. Both Pearson and Spearman correlations were performed to correlate C-peptide, adipokines, and FAs and EAEs, but since several variables were non-normally distributed, Spearman correlations are presented (Table 2). Odds ratios (OR) and

95% confidence intervals were calculated using polytomous logistic regression models for categorical outcome data. Categories were defined by dividing the population into tertiles based on serum adipokine and C-peptide concentration. In all logistic regression models, adipokines and C-peptide were analyzed categorically as dependent variables, with the reference category defined as individuals in the first tertile; or the third of population with the lowest concentration leptin, adiponectin, and C-peptide, respectively. FAs were analyzed as continuous independent variables in logistic regression models. We have previously reported age is associated with several FAs and EAEs [5], therefore, all models were adjusted for age. Due to high correlation (>0.9, data not shown) between body mass index (BMI) and waist circumference (WC), these anthropometric measurements could not be analyzed in the same model. Instead, two additional models were run, the first with the addition of BMI and the second with the addition of WC. The odds ratios for ALA, DTA, DPA ω-6, and D6D EAEs were calculated on the basis that there is a unit change of 0.1 for the respective beta coefficient for each given parameter. Statistically significant when $p \leq 0.05$ and a statistical trend was defined as $0.05 < p \leq 0.09$. Statistical analyses were conducted using SAS version 9.3 (Cary, NC).

Table 1: Age, anthropometric, serum adipokines and insulin marker, and plasma phospholipid fatty acid percentages.[a]

	Overall
n	126
Age (years)	56.9 ± 4.7
Age range [b]	(48 - 65)
BMI (kg/m^2)	29.7 ± 5.2
BMI range [b]	(19.20 – 45.57)
WC (inches)	41.4 ± 6.1
WC range [b]	(29.75 – 57.50)
Leptin (ng/mL)	9.9 ± 10.5
Leptin range [b]	(0.31 - 49.47)
Adiponectin (μg/mL)	4.8 ± 2.3
Adiponectin range [b]	(1.02 – 13.20)
C-peptide (ng/mL)	2.9 ± 1.8
C-peptide range [b]	(0.72 – 9.90)
PA	29.2 ± 3.1
SA	14.5 ± 2.2
LGCA	1.6 ± 0.5
NA	0.4 ± 0.1
ALA	0.2 ± 0.4
EPA	0.8 ± 0.7
DHA	3.0 ± 1.3
DPAω-3	
total ω-3	4.9 ± 2.0
LA	19.6 ± 3.7
DGLA	2.7 ± 0.9
AA	10.3 ± 2.8
DTA	0.4 ± 0.1
DPAω-6	0.3 ± 0.1
total ω-6	34.1 ± 3.9
ω-6:ω-3	8.1 ± 3.0
D5D	4.1 ± 1.6
D6D	0.14 ± 0.05

[a]Values expressed as mean ± standard deviation unless otherwise noted. PPL measurements expressed as percentage of total.
[b]Values listed in parenthesis expressed as range of values corresponding to age, BMI, WC, leptin, adiponectin, and C-peptide concentrations, respectively

Table 2: Plasma phospholipid fatty acids and enzyme activity estimates are associated with serum adipokines and C-peptide.[a]

Fatty Acid/ EAE	Leptin	Adiponectin	C-peptide
PA	0.026	-0.221	-0.068
	0.7691	**0.0128**	0.4491
SA	0.313	-0.080	0.236
	0.0004	0.3745	**0.0077**
LGCA	-0.120	0.022	-0.175
	0.1816	0.8057	**0.0498**
NA	-0.354	0.130	-0.251
	<.0001	0.1456	**0.0046**
ALA	-0.221	0.118	0.0165
	0.0131	0.1894	0.8549
DHA	-0.114	0.027	0.007
	0.2039	0.7617	0.9423
EPA	-0.279	0.106	-0.111
	0.0016	0.2371	0.2152
DPAω-3	-0.172	**0.049**	-0.163
	0.0541	0.5854	*0.0688*
Total ω-3	-0.203	0.074	-0.351
	0.0225	0.4133	0.6967
LA	-0.185	0.284	-0.182
	0.0383	**0.0012**	**0.0412**
DGLA	0.432	-0.181	0.263
	<0.0001	**0.0430**	**0.0029**
AA	0.125	-0.022	-0.062
	0.1644	0.8113	0.4927
DTA	0.267	-0.192	0.050
	0.0025	**0.0314**	0.5789
DPAω-6	0.344	-0.223	0.222
	<0.0001	**0.0122**	**0.0125**
Total ω-6	0.033	0.184	-0.152
	0.7145	**0.0394**	*0.0895*
ω-6:ω-3 ratio	0.193	0.027	-0.013
	0.0307	0.7667	0.8878
D6D	0.422	-0.288	0.293
	<.0001	**0.0011**	**0.0009**
D5D	-0.275	0.143	-0.263
	0.0018	0.1104	**0.0029**

[a]Spearman correlations between fatty acids, leptin, adiponectin, and C-peptide. White rows represent corresponding correlation coefficient, gray rows directly underneath represent corresponding p-value. p-values bolded if $p \leq 0.05$ and italicized if $0.05 < p \leq 0.09$.

Results

Participant characteristics and FA concentrations are displayed in Table 1. Serum adiponectin concentrations were significantly associated with PPL FAs (Table 3 and 4). Lignoceric acid (LGCA) was the only FA significantly associated with serum adiponectin concentrations in the second tertile, > 3.64 and ≤ 5.37 μg/mL, when compared with individuals with adiponectin concentrations in the first tertile, ≤ 3.62 μg/mL (Table 3). For each unit increase in LGCA, individuals were approximately 3 times more likely to have serum adiponectin concentrations in the second tertile compared with the first tertile in all models. LGCA was not associated with serum adiponectin in the third tertile. Serum adiponectin concentrations in the third tertile, > 5.37 μg/mL, were significantly associated with several FAs across logistic regression models when compared with individuals with adiponectin concentrations in the first tertile, ≤ 3.62 μg/mL (Table 4). The only SFA associated with the third tertile of adiponectin was PA. For each unit increase in PA, individuals were approximately 0.80 times as likely to have serum adiponectin concentrations in the third tertile compared with the first tertile in all models (Table 4).

Table 3: The second tertile of serum adiponectin concentrations is significantly associated with PPL LGCA.[a]

	Model 1 Age		Model 2 Age + BMI		Model 3 Age + WC	
Fatty acid	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**
PA	0.99 (0.86, 1.12)	0.830	1.00 (0.87, 1.15)	0.981	1.00 (0.87, 1.14)	0.962
SA	0.95 (0.79, 1.15)	0.629	0.98 (0.80, 1.19)	0.800	0.98 (0.74, 1.19)	0.831
LGCA	3.21 (1.20, 8.56)	**0.020**	2.86 (1.06, 7.74)	**0.038**	2.92 (1.09, 7.82)	**0.033**
NA	0.90 (0.39, 2.04)	0.791	0.75 (0.32, 1.76)	0.507	0.74 (0.31, 1.76)	0.497
ALA [b]	1.01 (0.67, 1.54)	0.958	0.97 (0.64, 1.48)	0.890	0.98 (0.64, 1.49)	0.918
EPA	2.02 (0.91, 4.47)	*0.085*	1.84 (0.84, 4.00)	0.126	1.81 (0.82, 4.01)	0.142
DHA	1.23 (0.88, 1.72)	0.227	1.19 (0.85, 1.68)	0.312	1.18 (0.83, 1.67)	0.348
Total ω-3	1.19 (0.95, 1.50)	0.130	1.16 (0.92, 1.47)	0.213	1.16 (0.91, 1.47)	0.231
LA	1.06 (0.93, 1.20)	0.382	1.04 (0.91, 1.18)	0.579	1.04 (0.91, 1.18)	0.550
DGLA	0.85 (0.52, 1.41)	0.529	0.99 (0.58, 1.69)	0.963	0.96 (0.56, 1.65)	0.885
AA	0.97 (0.84, 1.13)	0.703	0.97 (0.83, 1.14)	0.716	0.98 (0.84, 1.14)	0.763
DTA [b]	0.30 (0.01, 6.55)	0.447	0.59 (0.02, 15.06)	0.750	0.47 (0.02, 12.23)	0.647
DPA ω-6 [b]	0.88 (0.63, 1.21)	0.418	0.95 (0.68, 1.34)	0.768	0.93 (0.66, 1.30)	0.658
Total ω-6	1.01 (0.91, 1.13)	0.806	1.01 (0.90, 1.12)	0.933	1.01 (0.91, 1.13)	0.864
ω-6:ω-3	0.98 (0.84, 1.13)	0.739	0.99 (0.85, 1.15)	0.871	1.00 (0.85, 1.16)	0.952
D5D	0.97 (0.73, 1.28)	0.819	0.91 (0.69, 1.21)	0.518	0.93 (0.70, 1.23)	0.591
D6D [b]	0.66 (0.30, 1.46)	0.305	0.82 (0.35, 1.93)	0.653	0.78 (0.33, 1.85)	0.577

[a]Models defined as: Adiponectin = fatty acid + independent variable(s) next to model number. Fatty acids expressed as percent of total phospholipids. P-values bolded if $p \leq 0.05$ and italicized if $0.05 < p \leq 0.09$.

[b]Odds ratios calculated on the basis that there is a unit change of 0.1 for the respective beta coefficient for each given parameter.

Table 4: The third tertile of serum adiponectin concentrations is significantly associated with PPL PA, LA, Total ω-6, and D6D EAE.[a]

	Model 1 Age		Model 2 Age + BMI		Model 3 Age + WC	
Fatty Acid	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**
PA	0.78 (0.65, 0.93)	**0.006**	0.82 (0.68, 0.99)	**0.037**	0.82 (0.68, 0.99)	**0.035**
SA	0.85 (0.69, 1.05)	0.129	0.90 (0.72, 1.11)	0.321	0.92 (0.74, 1.15)	0.461
LGCA	1.55 (0.57, 4.22)	0.388	1.24 (0.44, 3.53)	0.687	1.26 (0.44, 3.55)	0.668
NA	1.66 (0.75, 3.69)	0.213	1.18 (0.49, 2.84)	0.714	1.05 (0.43, 2.58)	0.911
ALA [b]	1.33 (0.92, 1.92)	0.13	1.25 (0.85, 1.84)	0.261	1.24 (0.84, 1.81)	0.281
EPA	1.60 (0.70, 3.64)	0.265	1.32 (0.57, 3.09)	0.519	1.13 (0.48, 2.69)	0.777
DHA	0.99 (0.69, 1.41)	0.949	0.91 (0.63, 1.33)	0.639	0.87 (0.59, 1.27)	0.463
Total ω-3	1.12 (0.89, 1.41)	0.349	1.03 (0.80, 1.33)	0.797	1.00 (0.77, 1.28)	0.979
LA	1.27 (1.10, 1.45)	**0.001**	1.20 (1.04, 1.39)	**0.011**	1.20 (1.04, 1.39)	**0.011**
DGLA	0.62 (0.36, 1.04)	*0.069*	0.84 (0.48, 1.48)	0.547	0.86 (0.48, 1.52)	0.598
AA	0.93 (0.80, 1.09)	0.361	0.95 (0.81, 1.12)	0.515	0.96 (0.82, 1.13)	0.649
DTA [b]	0.04 (0.001, 0.92)	**0.044**	0.16 (0.01, 5.20)	0.304	0.16 (0.01, 4.98)	0.294
DPA ω-6[b]	0.67 (0.46, 0.97)	**0.033**	0.80 (0.54, 1.18)	0.255	0.79 (0.54, 1.17)	0.243
Total ω-6	1.15 (1.01, 1.30)	**0.031**	1.13 (0.99, 1.29)	*0.070*	1.14 (1.01, 1.30)	**0.049**
ω-6:ω-3	1.05 (0.91, 1.21)	0.546	1.08 (0.92, 1.26)	0.34	1.11 (0.95, 1.29)	0.207
D5D	1.12 (0.86, 1.46)	0.395	1.02 (0.77, 1.34)	0.991	1.03 (0.78, 1.36)	0.827
D6D [b]	0.20 (0.08, 0.53)	**0.001**	0.33 (0.12, 0.90)	**0.031**	0.33 (0.12, 0.92)	**0.034**

[a]Models defined as: Adiponectin = fatty acid + independent variable(s) next to model number. Fatty acids expressed as percent of total phospholipids. P-values bolded if $p \leq 0.05$ and italicized if $0.05 < p \leq 0.09$.

[b]Odds ratios calculated on the basis that there is a unit change of 0.1 for the respective beta coefficient for each given parameter.

PPL ω-6 PUFAs were not associated with adiponectin concentrations in the second tertile (Table 3). However, PPL ω-6 PUFAs were significantly associated with adiponectin concentrations in the third tertile (Table 4). Most ω-6 PUFAs were inversely associated with the third adiponectin tertile when analyzed individually, except LA (Table 4). For each unit increase in LA, individuals were approximately 1.20 times more likely to have serum adiponectin concentrations in the third tertile compared with the first tertile across all models. Total ω-6 was positively associated with adiponectin (Table 2). For each unit increase in total ω-6, individuals were approximately 1.14 times more likely to have serum adiponectin concentrations in the third tertile rather than the first, in all models (Table 4). The D6D EAE was inversely associated with adiponectin (Table 4). Specifically, for each unit increase in D6D, individuals were 0.20 (0.08, 0.53) times as likely in the age-adjusted model, and 0.33 times as likely in the BMI- and WC-adjusted models, to have serum adiponectin concentrations in the third tertile compared with the first.

Serum C-peptide concentrations in the second tertile, > 1.80 and ≤ 3.29 ng/mL, were also significantly associated with FAs, compared with C-peptide concentrations in the first tertile, ≤ 1.76 ng/mL (Table 5). Generally, PPL PUFAs were significantly associated with C-peptide only after adjusting for BMI or WC (Table 5). For each unit increase in the ω-3 PPL ALA, individuals were 1.56 (1.01. 2.43) times more likely in the WC-adjusted model to have serum C-peptide concentrations in the second tertile compared with the first tertile. The ω-6s were also significantly associated with C-peptide only after adjusting for BMI and WC. For each unit increase in PPL AA, individuals were approximately 0.80 times as likely in the BMI- and WC-adjusted models, to have serum C-peptide concentrations in the second tertile compared with the first tertile. For each unit increase in PPL DTA, individuals were 0.70 (0.49, 0.99) times as likely in the BMI-adjusted

model, and 0.62 (0.42, 0.93) times as likely in the WC-adjusted model, to have serum C-peptide concentrations in the second tertile rather than the first. The D5D EAE was inversely associated with C-peptide across all logistic regression models. For each unit increase in D5D, individuals were approximately 0.69 times as likely to have serum C-peptide concentrations in the second tertile compared with the first tertile, across all models.

Serum C-peptide concentrations in the third tertile, ≥ 3.32 ng/mL, were also significantly associated with FAs, compared with C-peptide concentrations in the first tertile, ≤ 1.76 ng/mL, (Table 6). No PPL SFAs were associated with the third tertile of C-peptide. PPL ω-3 PUFAs were not associated with the third tertile of C-peptide. AA was the only ω-6 PUFA associated with C-peptide after adjusting for BMI and WC. For each unit increase in AA, individuals were approximately 0.80 times as likely in the BMI- and WC-adjusted models, to have serum C-peptide concentrations in the third tertile compared with the first tertile, respectively. The D5D EAE was inversely associated with C-peptide across all logistic regression models. For each unit increase in D5D, individuals were approximately 0.73 times as likely across all models, to have serum C-peptide concentrations in the third tertile compared with the first tertile.

Table 5: The second tertile of serum C-peptide concentrations is significantly associated with PPL AA, DTA, and D5D EAE.[a]

	Model 1 Age		Model 2 Age + BMI		Model 3 Age + WC	
Fatty Acid	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**
PA	1.11 (0.95, 1.29)	0.188	1.02 (0.87, 1.19)	0.791	1.01 (0.87, 1.18)	0.871
SA	1.25 (1.01, 1.55)	**0.043**	1.19 (0.96, 1.48)	0.116	1.14 (0.90, 1.42)	0.276
LGCA	0.51 (0.21, 1.26)	0.146	0.56 (0.21, 1.44)	0.227	0.54 (0.20, 1.44)	0.218
NA	0.36 (0.15, 0.83)	**0.017**	0.45 (0.17, 1.15)	0.095	0.56 (0.21, 1.49)	0.244
ALA [b]	1.19 (0.85, 1.67)	0.302	1.40 (0.94, 2.07)	0.099	1.56 (1.01, 2.43)	**0.048**
EPA	0.75 (0.38, 1.51)	0.866	0.92 (0.44, 1.92)	0.832	1.20 (0.55, 2.60)	0.645
DHA	0.90 (0.64, 1.27)	0.537	0.98 (0.69, 1.41)	0.922	1.04 (0.72, 1.51)	0.817
Total ω-3	0.97 (0.78, 1.20)	0.767	1.07 (0.85, 1.35)	0.549	1.14 (0.90, 1.45)	0.285
LA	0.96 (0.85, 1.08)	0.489	1.04 (0.91, 1.18)	0.576	1.05 (0.93, 1.20)	0.433
DGLA	1.62 (0.95, 2.76)	*0.078*	1.19 (0.67, 2.11)	0.550	1.07 (0.59, 1.95)	0.823
AA	0.87 (0.74, 1.02)	*0.085*	0.81 (0.67, 0.97)	**0.019**	0.77 (0.63, 0.93)	**0.006**
DTA [b]	0.90 (0.66, 1.23)	0.503	0.70 (0.49, 0.99)	**0.047**	0.62 (0.42, 0.93)	**0.020**
DPA ω-6 [b]	1.28 (0.88, 1.86)	0.189	1.08 (0.72, 1.61)	0.721	1.02 (0.68, 1.54)	0.919
Total ω-6	0.90 (0.80, 1.02)	0.102	0.91 (0.80, 1.04)	0.162	0.90 (0.78, 1.03)	0.113
ω-6:ω-3	1.02 (0.88, 1.17)	0.832	0.98 (0.85, 1.14)	0.818	0.95 (0.82, 1.11)	0.547
D5D	0.67 (0.50, 0.91)	**0.009**	0.70 (0.51, 0.94)	**0.019**	0.69 (0.51, 0.94)	**0.019**
D6D [b]	2.47 (1.02, 5.99)	**0.045**	1.32 (0.52, 3.39)	0.559	1.09 (0.41, 2.86)	0.869

[a]Models defined as: C-peptide = fatty acid + independent variable(s) next to model number. Fatty acids expressed as percent of total phospholipids. P-values bolded if $p \leq 0.05$ and italicized if $0.05 < p \leq 0.09$.

[b]Odds ratios calculated on the basis that there is a unit change of 0.1 for the respective beta coefficient for each given parameter.

Table 6: The third tertile of serum C-peptide concentrations are significantly associated with PPL AA and D5D EAE.[a]

	Model 1 Age		Model 2 Age + BMI		Model 3 Age + WC	
Fatty Acid	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**
PA	1.01 (0.86, 1.19)	0.887	0.90 (0.75, 1.08)	0.246	0.89 (0.74, 1.08)	0.233
SA	1.19 (0.95, 1.48)	0.124	1.10 (0.87, 1.40)	0.422	1.04 (0.82, 1.33)	0.742
LGCA	0.41 (0.16, 1.04)	*0.060*	0.49 (0.17, 1.40)	0.183	0.47 (0.16, 1.35)	0.163
NA	0.39 (0.17, 0.90)	**0.027**	0.56 (0.21, 1.47)	0.238	0.71 (0.26, 1.94)	0.507
ALA [b]	1.11 (0.79, 1.56)	0.566	1.33 (0.89, 1.99)	0.163	1.48 (0.95, 2.32)	*0.086*
EPA	0.95 (0.51, 1.76)	0.866	1.30 (0.64, 2.61)	0.466	1.85 (0.87, 3.96)	0.112
DHA	1.01 (0.73, 1.41)	0.937	1.17 (0.80, 1.70)	0.418	1.28 (0.87, 1.89)	0.211
Total ω-3	0.97 (0.78, 1.20)	0.773	1.14 (0.89, 1.46)	0.316	1.23 (0.95, 1.59)	0.117
LA	0.91 (0.81, 1.03)	0.132	1.01 (0.88, 1.17)	0.850	1.02 (0.89, 1.18)	0.736
DGLA	1.82 (1.06, 3.13)	**0.030**	1.14 (0.62, 2.09)	0.680	1.04 (0.55, 1.94)	0.912
AA	0.91 (0.77, 1.06)	0.209	0.82 (0.68, 0.99)	**0.044**	0.78 (0.64, 0.95)	**0.014**
DTA [b]	1.04 (0.77, 1.41)	0.804	0.73 (0.50, 1.06)	0.101	0.67 (0.44, 1.01)	*0.057*
DPA ω-6 [b]	1.54 (1.06, 2.23)	**0.024**	1.19 (0.78, 1.81)	0.432	1.15 (0.76, 1.76)	0.512
Total ω-6	0.89 (0.79, 1.01)	*0.065*	0.90 (0.79, 1.04)	0.153	0.88 (0.77, 1.02)	0.093
ω-6:ω-3	1.00 (0.87, 1.16)	0.966	0.95 (0.81, 1.12)	0.557	0.91 (0.77, 1.08)	0.285
D5D	0.69 (0.52, 0.93)	**0.015**	0.76 (0.56, 1.04)	*0.082*	0.75 (0.55, 1.02)	*0.068*
D6D [b]	3.16 (1.29, 7.71)	**0.012**	1.28 (0.47, 3.47)	0.625	1.09 (0.39, 3.01)	0.872

[a]Models defined as: C-peptide = fatty acid + independent variable(s) next to model number. Fatty acids expressed as percent of total phospholipids. P-values bolded if $p \leq 0.05$ and italicized if $0.05 < p \leq 0.09$.

[b]Odds ratios calculated on the basis that there is a unit change of 0.1 for the respective beta coefficient for each given parameter.

Discussion

In this cross-sectional study, we investigated associations between C-peptide, leptin, and adiponectin, and PPL FAs or EAEs in a population of 126 males (> 96% Caucasian) ages 48-65. Summaries of our most significant results for leptin, adiponectin, and C-peptide across models are provided in Table 6. Leptin was positively associated with PPL SA and the ω-6/ω-3 ratio (Table 7). However, significant associations between PPL FAs and leptin faded across tertiles, after adjusting for BMI and WC (Table 7). Elevated concentrations of adiponectin were positively associated with PPL LA, and elevated concentrations of adiponectin were inversely associated with the D6D EAE. C-peptide was inversely associated with PPL DTA and the D5D EAE (Table 7). Together these data indicate specific PPL FAs and EAEs are associated with adiponectin and C-peptide concentrations even after adjusting for BMI and WC. These EAEs are generally reflective of FA metabolism, however, they may not completely represent enzyme kinetics in tissues. Therefore, reported altered EAEs could be related to other factors such as diet. We did not directly collect or assess dietary intake in this study, however, we used PPL FAs in our analysis which are correlated with dietary fat intake [111-113]. In addition, we recognize that the generalizability of these observations is limited and should be verified in larger populations.

Table 7: Summarized logistic regression results of adipokines and C-peptide associations with plasma phospholipid fatty acids and enzymes activity estimates.[a]

PPL FA / EAE	Serum Parameter	Model 1 Age	Model 2 Age + BMI	Model 3 Age + WC
	Leptin			
	< 4.60 ng/mL	ref	ref	ref
SA	> 4.75 and ≤ 9.01 ng/mL	↑	↑	↑
	≥ 9.26 ng/mL	↑	↑	-
	Leptin			
	< 4.60 ng/mL	ref	ref	ref
ω-6/ω-3	> 4.75 and ≤ 9.01 ng/mL	↑	↑	-
	≥ 9.26 ng/mL	↑	-	-
	Adiponectin			
	≤ 3.62 µg/mL	ref	ref	ref
LA	> 3.64 and ≤ 5.37 µg/mL	-	-	-
	> 5.37 µg/mL	↑	↑	↑
	Adiponectin			
	≤ 3.62 µg/mL	ref	ref	ref
D6D	> 3.64 and ≤ 5.37 µg/mL	-	-	-
	> 5.37 µg/mL	↓	↓	↓
	C-peptide			
	≤ 1.76 ng/mL	ref	ref	ref
DTA	> 1.80 and ≤ 3.29 ng/mL	-	↓	↓
	≥ 3.32 ng/mL	-	-	↓
	C-peptide			
	≤ 1.76 ng/mL	ref	ref	ref
D5D	> 1.80 and ≤ 3.29 ng/mL	↓	↓	↓
	≥ 3.32 ng/mL	↓	↓	↓

[a]Serum parameters are bolded, followed by concentrations for tertile 1, 2, and 3 for each respective adipokine or C-peptide. Ref indicates reference category used in the logistic regression model. ↑ represents increased odds and ↓ represents decreased odds, compared with the reference category, and are only provided if the p-value ≤0.09. A – indicates no difference compared with the reference category.

Currently there are no clinically defined biological cutoffs for circulating adiponectin or C-peptide concentrations. However, previous researchers have used tertiles to investigate associations with C-peptide concentrations [114] and adiponectin [115] in their study populations. We report PPL FAs and EAE differences were specific to adiponectin tertile. Inflammation suppresses adiponectin expression and secretion [116]. ω-6 PUFAs are considered "pro-inflammatory like", especially AA derived eicosanoids. AA can be endogenously synthesized through elongation and desaturation of LA. Here we show PPL LA was positively associated with greater adiponectin concentrations (Table 4). Despite most ω-6 PUFAs having an inverse relationship with adiponectin when analyzed individually, total ω-6 PUFAs were positively associated with adiponectin (Table 2 and 4). LA is elongated and desaturated to form DGLA, which is dependent on the enzyme D6D. Inflammation is positively associated with the D6D enzyme, and inhibiting D6D reduces inflammation [117]. The D6D EAE is reflective of D6D enzyme activity in several tissues, and the D6D EAE is calculated as the ratio of DGLA/LA (reviewed in detail [6]). We have previously shown D6D EAE is highly associated with both BMI and WC [5]. In this study, the third tertile of adiponectin was inversely associated with the D6D EAE after controlling for BMI and WC. This result was expected since individuals with adiponectin concentrations in the third tertile would likely have less inflammation compared with those with adiponectin concentrations in the first tertile. Increased D6D activity may promote inflammation in a way, which contributes to inhibiting adiponectin expression and/or decreasing adiponectin concentrations. Future studies should investigate the effects of altered cellular D6D enzyme activity on adiponectin expression and secretion.

Elevated concentrations of PPL AA and D5D EAEs were inversely associated with the second and third tertile of C-peptide after adjusting for BMI and WC (Table 4 and 5). The D5D

EAE is calculated as the ratio of AA/DGLA (reviewed in detail [6]). Our D5D EAE results indicate as C-peptide concentrations increase there is a greater amount of DGLA relative to AA. However, DGLA was only associated with C-peptide in the age-adjusted model (Table 6). DGLA is considered to be more "anti-inflammatory like", compared with AA [118]. Decreased AA production would limit the substrate availability of AA for pro-inflammatory eicosanoid biosynthesis or elongation to form longer chain ω-6s (i.e. DTA). We report PPL DTA was inversely associated with C-peptide concentrations after adjusting for BMI and WC (Table 5 and 6). From our point of view, the PPL DTA observation strengthens our speculation that FA metabolism (i.e. D5D) is altered in individuals with elevated C-peptide concentrations.

In this study we identify new relationships between C-peptide and D5D EAEs. What is unique about our current study is we associate C-peptide with D5D EAEs in a population of obese adults. Previous research has shown D5D EAEs are inversely associated with obesity, insulin resistance, T2D, and metabolic syndrome (reviewed in detail [6]). Our patient exclusion criteria excluded individuals with T2D. We initially speculated that the inverse relationship with C-peptide and D5D EAEs was likely due to underlying obesity-associated inflammation, since inflammation (i.e. TNF-α) alters insulin downstream signaling [105]. However, C-peptide concentrations were inversely associated with D5D EAEs after adjusting for age and TNF-α (data not shown). We have previously reported inflammatory factors such as TNF-α are highly correlated to BMI [5]. Therefore, TNF-α could not be analyzed in BMI and WC adjusted models due to multicollinearity with these anthropometric measurements. We found that TNF-α was not correlated with D5D EAEs (p-value = 0.9, data not shown), but TNF-α was positively correlated with C-peptide ($p<0.001$, data not shown). This led us to speculate C-peptide could be a potential regulator of D5D enzyme activity or expression.

Insulin functions in regulating D5D and D6D enzyme expression (reviewed in detail [119]). For instance, in insulin-dependent (i.e. type-1-diabetes (T1D)) animal models, D5D and D6D mRNA increase after insulin injections [120]. However, desaturase enzymes are not altered in T2D models, thus, the results are not consistent with T1D models. Montanaro et al reported there are no alterations to D5D and D6D mRNA or EAEs in insulin-independent (i.e. T2D) animals compared with control animals [121]. There is an overwhelming amount of human data associating increased D6D EAEs and decreased D5D EAES with obesity, insulin resistance, T2D, and metabolic syndrome (reviewed in detail [6]). One difference between T1D and T2D models is the endogenous production of pro-insulin and, thus, C-peptide. The lack of C-peptide in T1D models may account for increases in D5D mRNA and EAEs that were observed after insulin injections. Recent findings suggest C-peptide is able to activate signaling pathways associated with FA metabolism, independent of insulin [8]. Therefore, greater concentrations of C-peptide could be suppressing D5D enzyme activity or expression, which would explain why numerous researchers report D5D EAEs are inversely associated with obesity, insulin resistance, T2D, and metabolic syndrome (reviewed in detail [6]). Thus, it is possible C-peptide may function in regulating D5D enzyme activity. Future studies should investigate whether D5D inhibition may occur through a C-peptide mediated pathway.

CHAPTER 3: OBESITY, ADIPOKINES, AND C-PEPTIDE ARE ASSOCIATED WITH DISTINCT PLASMA PHOSPHOLIPID PROFILES IN ADULT MALES, AN UNTARGETED LIPIDOMIC APPROACH

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Abstract

Obesity is associated with dysregulated lipid metabolism and adipokine secretion. Our group has previously reported obesity and adipokines are associated with % total fatty acid (FA) differences in plasma phospholipids. The objective of our current study was to identify in which complex lipid species (i.e., phosphatidylcholine, sphingolipids, etc) these FA differences occur. Plasma lipidomic profiling (n=126, >95% Caucasian, 48-65 years) was performed using chromatographic separation and high resolution tandem mass spectrometry. The responses used in the statistical analyses were body mass index (BMI), waist circumference (WC), serum adipokines, cytokines, and a glycemic marker. High-dimensional statistical analyses were performed, all models were adjusted for age and smoking, and p-values were adjusted for false discovery. In Bayesian models, the lipidomic profiles (over 1,700 lipids) accounted for >60% of the inter-individual variation of BMI, WC, and leptin in our population. Across statistical analyses, we report 51 individual plasma lipids were significantly associated with obesity. Obesity was inversely associated lysophospholipids and ether linked phosphatidylcholines. In addition, we identify several unreported lipids associated with obesity that are not present in lipid databases.

Taken together, these results provide new insights into the underlying biology associated with obesity and reveal new potential pathways for therapeutic targeting.

Introduction

Obesity is associated with insulin resistance, oxidative stress, chronic low-grade inflammation, and dysregulated secretion of adipose-derived cytokines (i.e., adipokines) [122]. These conditions are related to hypoxic adipose tissue microenvironments which can lead to an accumulation of proinflammatory immune cells such as M1 macrophages [34]. Obesity is also associated with altered lipid metabolism (reviewed in detail [6]) and many lipids function as signaling molecules involved in proinflammatory pathways. The chronic low-grade inflammation in obesity can alter insulin receptor downstream signaling leading to insulin resistance (reviewed in detail [123]), and insulin resistance can affect lipid storage and lipid metabolism [124].

The lipidome (i.e., the complete lipid profile) is comprised of complex lipid species such as phospholipids (PLs), lysoPLs (LPLs), sphingolipids, triacylglycerols, and cholesterol esters. These lipid species contain esterified fatty acids (FAs), for instance, there are two esterified FAs in PLs such as: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG), and phosphatidylinositol (PI), and there is one esterified FA in their respective LPLs: LPC, LPE, LPS, LPG, and LPI. FAs are endogenously synthesized except for the essential polyunsaturated FA (PUFAs) linoleic acid (LA, C18:2 omega-6 (ω-6)) and alpha-linolenic acid (ALA, C18:3 omega-3 (ω-3)). These essential PUFAs can only be obtained through dietary intake, and they serve as substrates for elongating and desaturating enzymes that produce long chain PUFAs. Obesity is associated with increased elongation and desaturation of FAs, which

can lead to higher levels of LA-derived long chain PUFAs such as dihomo-λ-linolenic acid (DGLA, C20:3 ω-6) and arachidonic acid (ARA, C20:4 ω-6) [6, 7]. Accumulations of ARA in combination with increased oxidative stress and increased proinflammatory oxygenating enzyme activity, results in increased proinflammatory oxylipid mediators that stimulate inflammation.

Despite FAs being directly involved in inflammatory signaling, the metabolism of complex lipids, in which FAs are esterified, can also stimulate proinflammation associated with obesity and insulin resistance. For instance, the metabolism of sphingomyelins (SM) produces ceramides (Cer) which induce apoptosis in adipose-derived stem cells [37], are described as antagonists of insulin signaling [35], and Cer levels are increased in obesity (reviewed in detail [125]). In addition, obese individuals have increased phospholipase A_2 (PLA_2) activity [126] and increased lecithin:cholesterol acyltransferase (LCAT) activity [127], which both increase production of LPLs. LPLs are ligands for G-protein coupled receptors (reviewed in detail [128]) and are ligands for class A scavenger receptors which induce polarization of the anti-inflammatory M2 macrophage lineage [129]. Thus, complex lipids and their esterified FAs function in regulating proinflammatory and anti-inflammatory responses related to obesity-associated pathology.

Currently, a majority of epidemiological studies investigate associations between obesity and FAs by conducting a FA methyl ester (FAME) analysis and analyzing FAs as % of total FAs (reviewed in detail [7]). These FAME analyses involve hydrolyzing FAs from their parent lipid (i.e., PLs, sphingolipids, etc), meaning researchers are unable to identify in which complex lipid species (i.e., LPLs and ceramides) FA differences may occur. Our lab has previously reported using a FAME analysis that obesity [5], adipokines, and the glycemic marker C-peptide [130] are associated with plasma PL FA differences. The objective of our current study was to investigate the traits obesity, adipokines, proinflammatory cytokines, and C-peptide, by using high-resolution

tandem mass spectrometry (MS/MS) to 1) identify specific plasma lipid species with FA differences associated with traits, and 2) assess the contribution of the entire plasma lipidome on the variation of traits using multidimensional statistical methods such as reproducing kernel Hilbert spaces (RKHS) regressions. We hypothesize that previously reported FA differences associated with obesity occur in specific lipid classes, and determining the lipid classes will provide new insights into the underlying pathology associated with obesity and could reveal new pathways for therapeutic targeting.

Materials and Methods

Study population

Data are further described elsewhere [2-5, 131, 132]. In brief, healthy and asymptomatic male subjects (n = 126, > 96% Caucasian) 48 to 65 years of age were enrolled between 2009 and 2011 in a cross-sectional study. Data are comprised of clinical metadata on individuals' co-morbidities, family history, and use of medications. Individuals with the following medical conditions were excluded: 1) cancer in the previous two years, 2) surgery in the previous two years, 3) type-1 and type-2 diabetes, and 4) autoimmune diseases. Immediately after enrollment, trained staff collected anthropometric measurements and venous blood of study participants. In brief, body mass index (BMI) was assessed by recording patient's height using a stadiometer and weight using digital platform scale. The plasma fraction was separated from whole blood by centrifugation and stored at -80°C until time of analysis. Smoking status was assessed as "ever smoked" or "never smoked". A previous complete description of this study can be found elsewhere [2]. The study

was approved by the Biomedical and Health Institutional Review Board of Michigan State University (IRB# 08-786).

Plasma preparation

A detailed list of chemicals, solvents, and internal standards used in plasma lipid extractions are presented in Appendix A. Plasma lipid extraction was performed following a Waters application note [133], but modified as specified. A quality control (QC) sample was made by pooling 10 μL plasma from all 126 samples, and this QC sample was extracted and processed alongside individual samples. Plasma samples were thawed on ice. A 50 μL aliquot of each plasma sample was transferred to a labeled microcentrifuge tube containing 200 μL of the extraction solution (2:1 v/v, 100 μg/mL BHT, 5 ng/μL PLs and 0.2 ng/μL Cer). These mixtures were then incubated on ice for 15 minutes, then vortexed for 30 s. After vortexing, protein crash was performed by centrifugation in an accuSpin™ Micro R centrifuge at 13,000 x g for 5 min at 4 °C (Fisher Scientific, Waltham, MA). Following centrifugation, each lower organic phase was collected and transferred to new labeled microcentrifuge tube, evaporated to dryness in a Savant SpeedVac (ThermoQuest, Holbrook, NY) for 3 h, with no applied heating, then stored under high-purity nitrogen at 4 °C for no longer than 1 day. Dried residues were re-dissolved in 500 μL HPLC-grade isopropanol:acetonitrile:water (2:1:1 v/v/v), then vortexed gently. Re-dissolved extracts were again centrifuged 13,000 x g for 5 min at 4 °C (Fisher Scientific). After centrifugation, 100 μL drawn from the top of the supernatant was transferred to an amber autosampler vial with glass insert, and the vials were purged with high-purity nitrogen and sealed.

Lipidomic profiling by UPLC-MS/MS analyses

Mass spectrometric analysis was accomplished following Waters application note [133], but modified as specified. In brief, reverse phase ultra-performance liquid chromatography (UPLC)-ESIneg-MS/MS was performed on a Waters ACQUITY UPLC (Waters, Milford, MA) coupled to a Waters Xevo G2-XS quadrupole time-of-flight mass spectrometer (Waters). Chromatographic separation was performed with an ACQUITY UPLC CSH C18 1.7 μm 2.1x100 mm column (Waters) held at 55°C. The autosampler (Waters**)** temperature was held at 10°C. The UPLC method was shortened to 15 min and the mobile phases and gradients, along with mass spectrometer settings are outlined in Table A1 and A2. Data acquisition was performed using MS^E in continuum mode with leucine enkephalin as lock mass for mass correction. The QC sample was injected after every 10th sample throughout the entire analysis.

Data alignment and processing of plasma lipids

Results from UPLC-MS^E analyses were imported into Progenesis QI v2.0 (Nonlinear Dynamics, Durham, NC**)**. Peaks were aligned using one of the QC samples as references. Peak picking was performed under normal conditions, collecting signals from 4802 ions. The Progenesis QI generated data set was subjected to relative mass defect filtering to narrow ions to those of potential lipids [134], and ions with relative mass defects < 350 and > 950 parts-per-million (ppm) were excluded from the data set. The mass defect filtered data set was then imported into R for multivariate statistical analyses. Since large -omic data sets often have 10-20% missing values [135, 136], 1126 ions with >20% missing values were excluded from the data set, the 10 IS were split from the data set, and peak areas for the remaining 1,745 ions were mean imputed. Next, due to a time of injection affect (details are presented in Figure A1), the data matrix peak areas were

normalized to the IS PC(8:0/8:0) signal. After IS normalization of data matrix, the coefficient of variation between QC injections was <5% for the most significant lipids outlined in this manuscript.

Statistical analyses

Our study was powered (0.8) to detect differences in plasma FAs, as previously described [131]. Statistical analysis of the data was performed using R v3.2.2 [137], and all R-code used in these analyses are publicly available at https://github.com/AustinPickens/Untargeted-Lipidomics. The responses used in the statistical analyses were the traits: BMI, waist circumference (WC), log transformed serum leptin, log transformed serum total adiponectin, log transformed serum c-peptide, log transformed serum interleukin-6 (IL-6), log transformed serum tumor necrosis factor-alpha (TNF- α), log transformed serum interferon gamma-induced protein-10 (IP-10), and serum monocyte chemoattractant protein-1 (MCP-1). The analysis consisted of two parts: (1) Single lipid regressions on each trait associating the abundance of the lipid in plasma with the responses, to identify individual plasma lipids significantly associated with each response; (2) High dimensional analyses including principal components (Pc), singular value decomposition (SVD), and a model regressing the responses in all lipids in plasma, to identify whether the inter-individual differences can be attributed to the lipidomic profile. P-value correction was performed using Benjamini-Hochberg false discovery rate (BH FDR) and Bonferroni [138, 139].

Single lipid regressions. Response variables include: BMI, WC, log transformed leptin, log transformed total adiponectin, log transformed c-peptide, log transformed IL-6, log transformed TNF- α, log transformed IP-10, and MCP-1. We herein refer to these responses as traits, and these

traits consist of phenotype y_i $(i = 1, \ldots, 126)$ indexed by individuals (i) and the set of predictors coming from the lipidomic data as well as clinical covariates including age of the participant (a_i) 56.9 ± 4.7 (mean ± s.d.), the status of smoking behavior (s_i) 25.40% ever smoked, 57.14% never smoked, 17.46% smoking unknown. In each regression only one of the lipids was included from the pareto standardized lipidomic data (l_{ij}). The statistical model was adjusted for each lipid, $j = (1, \ldots, 1745)$ as follows:

$$y_i = \beta_{0j} + a_i\beta_{1j} + s_i\beta_{2j} + l_{ij}\beta_{3j} + \varepsilon_{ij},$$

let β_{0j} be a general intercept, and $\beta_{1j}, \beta_{2j},$ and β_{3j} the corresponding regression coefficients and ε_{ij} a random residual following a IID normal distribution centered at zero. A total of 1,745 regressions, one per lipid, were adjusted.

High dimensional analyses. The lipidomic data is a high-dimension dataset with a higher number of parameters to estimate (p=1745) then observations (n=126); for example p>>n. Thus, the following analysis were performed: A) Pc derived from lipidomic data abundances, B) SVD derived from the lipids estimated effects obtained in the single marker regressions, and C) whole lipidome regressions, as described in [140-142].

A.) Pc derived from lipid abundances were evaluated by regressing BMI against Pc scores individually. The statistical model was adjusted for each Pc, $k = (1, \ldots, 10)$ as follows:

$$BMI_i = \beta_{0k} + a_i\beta_{1k} + s_i\beta_{2k} + Pc_{ik}\beta_{3k} + \varepsilon_{ik}$$

let BMI be the phenotype $(i = 1, \ldots, 126)$ indexed by individuals (i) and the set of predictors coming from the Pc scores derived from lipid abundances and let β_0 be a general intercept, and β_{1k}, β_{2k}, and β_{3k} the corresponding regression coefficients and ε_{ik} a random residual following a IID normal distribution centered at zero. A total of 10 regressions, one per Pc, were adjusted. Finally, Pc4 was regressed on the individual lipids, one at a time to identify the lipids driving Pc4. The statistical model adjusted for each lipid, j as follows:

$$Pc4_i = \beta_{0j} + l_{ij}\beta_{1j} + \varepsilon_{ij}$$

in this analysis, Pc4 loadings are derived from lipid abundances and the set of predictors coming from lipidomic data and let β_{0j} be a general intercept and β_{1j} the corresponding regression coefficients and ε_{ij} a random residual following a IID normal distribution centered at zero. A total of 1,745 regressions, one per lipid, were adjusted.

B) Finally, the SVD of the 1,745 estimated effects for each lipid on the nine responses were derived. The results displayed in Figure 3 are the first two vectors that spanned the rows based on the column space of the SVD displayed in a biplot representation [143].

C) Finally, the lipidome was fully accounted for in a whole genome regression analysis [141, 142]. The probabilistic model assumed used in each of the responses was as follows,

$$y_i = \beta_0 + a\beta_1 + s\beta_2 + \sum_{j=1}^{j=1745} x_{ij}\alpha_j + \varepsilon_i,$$

where $u_i = \sum_{j=1}^{j=1745} x_{ij}\alpha_j$ represents the total effect of the lipids on trait y_i, x_{ij} is the lipidomic abundance for participant i and lipid j, and α_j is the estimated effect. Assume that the responses

consist of the traits indexed by individuals (i) and the set of predictors coming from the lipidomic data. u_i follows a normal distribution centered zero with variance covariance $u_i \sim N(0, G\sigma_g^2)$, and G is an *nxn* matrix of distances to measure similarities between participants with respect to their lipid profiles, as previously described [144]. These analyses were conducted using the R-package BGLR [145], using a long Markov Chain of 200,000 iterations and 50,000 samples were discarded for burn in. Inference was done based on one of every 5 samples of the last 150,000. High dimensional regressions were adjusted to the lipidomic data as describe before for other omics [140, 146].

Structural identification of plasma lipids

Lipids in this study are presented in the form of Xretention time_mass-to-charge ratio (i.e., X1.17_564.3289 [1.17 mins, 564.3298*m/z*]). The structural of identification of plasma lipids was performed if the integrated peak areas for the ion: were significant in two or more models from single marker regression analyses of responses, or had radii ≥ 0.2 in SVD, or was one of the top ten lipids positively and inversely associated with Pc4, and the *m/z* exhibited a fragmentation pattern indicative of a free FA, LPL, or PL specie. Since the objective of this study was to identify free FA and PL differences related to obesity and obesity-associated adipokines, significant lipid ions that did not have fragment patterns matching free FA and PLs are listed as unknown, and their spectra are listed in Table A3. Plasma lipids meeting our criteria for identification that are not listed were too low in abundance to obtain a useful MS/MS spectrum and are listed as too low to identify (TLTI). Lipid structure identification was confirmed using Lipid Maps (http://lipidmaps.org) and Human Metabolome database (http://www.hmdb.ca), and manual

evaluation of MS/MS spectra. The "_" denotation is used between FAs in glyceroPL structures, since the FA position (i.e., either sn1 and sn2) were not determined.

Results

Participant characteristics

Median and range values of age, smoking, anthropometric, and serum adipokines, glycemic, and inflammatory markers of the overall population (n=126) and separated by BMI categories are presented in Table 8. In brief, lean and obese participants were older than overweight participants. Both BMI and WC increased with increasing BMI category. Serum leptin and C-peptide significantly increased across BMI category, and serum adiponectin was decreased in obese participants compared to lean and overweight participants. Serum TNF-α and IP-10 were significantly elevated in obese participants compared to overweight and lean participants, while MCP-1 was only elevated in obese participants compared to lean.

Lipidomic profiles explain a large proportion of inter-individual variation of obese traits

The lipidomic profiles accounted for an important proportion of the inter-individual variation of all responses (Table 9). For several traits (i.e., BMI, WC, and leptin) lipidomic profiles accounted for over 60% of the variation in the samples. Lipidomic profiles accounted for roughly 40% of the variation in total-adiponectin and C-peptide concentrations. For the inflammatory cytokines IL-6, TNF-α, IP-10, and MCP-1, lipidomic profiles accounted for about 25% of variation in the samples. The 95% confidence intervals for most responses was roughly 10-20% for each respective trait.

Table 8: Median [range] of the participant characteristics, in the sample, and divided by BMI category.

Parameter	Overall n=126[a]	Lean n=28[a]	Overweight n=46[a]	Obese n=52[a]	BH FDR p-value[b]
Age (years)	57.5 [48-65]	58 [50-65]^{A}	53.5 [48-65]^{B}	59 [50-65]^{A}	p< 0.005
Ever Smoked (% total)	31	25	24	27	-
BMI (kg/m^2)	29.1 [19.2-45.6]	23.6 [19.2-25]^{A}	28.1 [25.4-29.9]^{B}	33.7 [30-45.6]^{C}	p< 0.0001
WC (cm)	104.8 [75.6-146.1]	85.7 [76.2-101.6]^{A}	101.6 [82.5-113.7]^{B}	119.4 [75.6-146.1]^{C}	p< 0.0001
Leptin (ng/mL)	6.1 [0.3-49.5]	2.1 [0.3-6]^{A}	5.2 [0.7-10.2]^{B}	13.7 [3.8-49.5]^{C}	p< 0.0001
Total Adiponectin (μg/mL)	4.2 [1-13.2]	5.4 [1.9-13.2]^{A}	4.8 [2.2-8.5]^{A}	3.7 [1-8.5]^{B}	p< 0.005
C-peptide (ng/mL)	2.4 [0.7-9.9]	1.4 [0.7-5.1]^{A}	2.1 [0.9-8.1]^{B}	3.3 [1-9.9]^{C}	p< 0.0001
IL-6 (pg/mL)	1.6 [0-137]	0.2 [0-40.9]^{A}	1 [0-96.6]^{A}	3.4 [0-137]^{A}	-
TNF-α (pg/mL)	7.4 [1.6-67.1]	5.7 [2.8-26.2]^{A}	7.3 [1.6-24.1]^{A}	8.6 [1.6-67.1]^{B}	p< 0.0005
IP-10 (pg/mL)	306 [120-1192]	257.5 [120-557]^{A}	271 [145-1029]^{A}	378 [147-1192]^{B}	p< 0.0005
MCP-1 (pg/mL)	483 [85-1018]	428 [218-875]^{A}	488 [285-1018]^{AB}	511 [85-922]^{B}	p< 0.05

[a]Values expressed as median [range]. Cytokines quantified from serum.
[b]Kruskall Wallis one-way ANOVA was conducted across BMI categories along with Dunn's test for multiple comparison. p-values were adjusted using Benjamini-Hochberg false discovery rate (BH FDR) correction.
Body mass index (BMI), waist circumference (WC), tumor necrosis factor-alpha (TNF- α), interferon gamma-induced protein-10 (IP-10), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1).

Table 9: Percent of the inter-individual differences in response variables that can be attributed to lipidome profiles, posterior mean [95% Confidence region].[a]

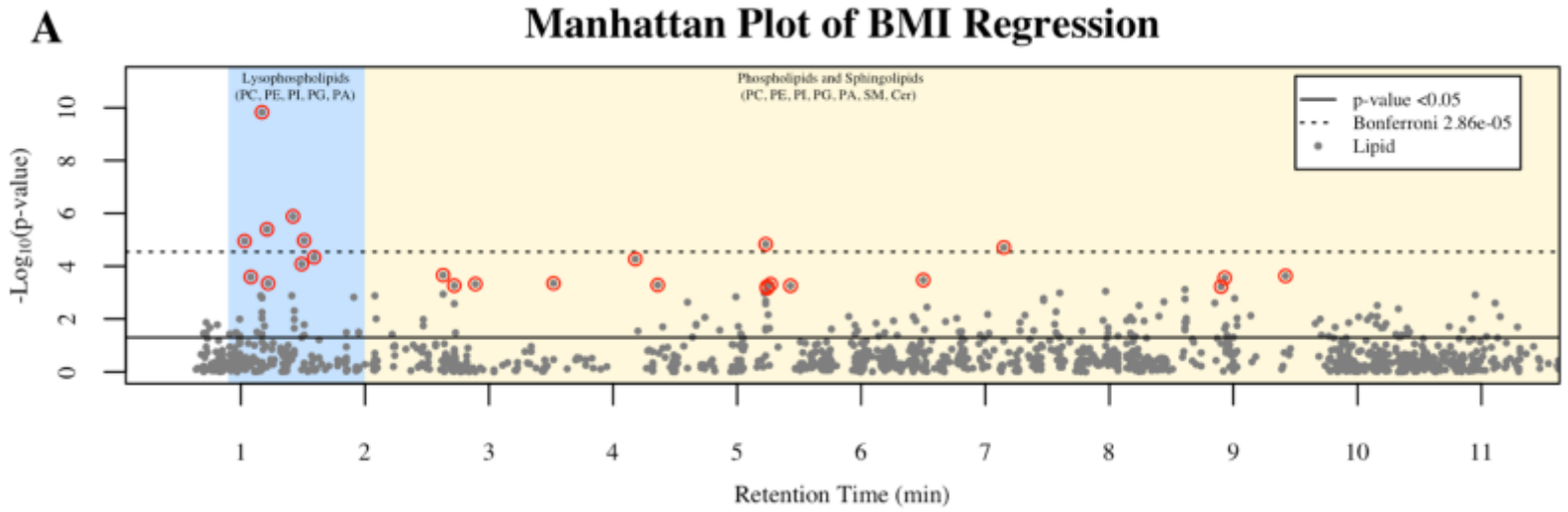
Parameter	$\sigma_u^2/\sigma_u^2+\sigma_\varepsilon^2$ % [95% CI]
BMI	64% ± [38%, 84%]
WC	69% ± [46%, 85%]
Leptin	76% ± [56%, 90%]
Total Adiponectin	42% ± [21%, 66%]
C-peptide	48% ± [23%, 73%]
IL-6	23% ± [10%, 43%]
TNF- α	28% ± [12%, 54%]
IP-10	23% ± [10%, 43%]
MCP-1	24% ± [11%, 45%]

[a]Results of responses modeled with entire phospholipidomic profiles using RKHS regressions.

Phospholipid classes and individual phospholipids are associated with obese traits

Results of age and smoking adjusted single lipid regressions are outlined in Figure 9 for the traits BMI, WC, leptin, total adiponectin, and C-peptide, and for each trait the primary id, estimated effects, and p-values of lipids with BH FDR p-values <0.05 are displayed in Table A4-8, respectively. Since no lipids were significantly associated with any response after 11.5 mins, the Manhattan plots were created to span from 0.5 (i.e., the start of MS/MS data collection) to 11.5 mins for clarity. In BMI regressions, 25 plasma lipids had BH FDR p-values <0.05 and LPLs accounted for over 70% of lipids with Bonferroni p-values <0.05 (Figure 9A). WC was significantly associated with 35 lipids below the BH FDR (Figure 9B). Leptin was significantly associated with 20 plasma lipids below the BH FDR (Figure 9C). In total adiponectin regressions there were 22 lipids below the BH FDR (Figure 9D). For WC, leptin, and total adiponectin, over 50% of lipids with Bonferroni p-values <0.05 were LPLs (Figure 9 B, C, and D, Table A5-7). C-peptide was significantly associated with 11 plasma lipids below the BH FDR (Figure 9E). Neither IL-6, IP-10, or MCP-1 had any lipid significant below the BH FDR (data not shown).

Figure 9: Manhattan plots of responses with 1,745 plasma lipids. X-axis represents the retention time of plasma lipids and Y-axis represents the minus log ten transformed p-value.[a]



[a]Manhattan plots of the –log10(p-value) for the traits: A) BMI, B) WC, C) Leptin, D) Total Adiponectin, and E) C-peptide. Single lipid regression models defined as: $Trait_i = Age_i + Smoking_i + Lipid_j$ where $i = (1,...,126)$ and $j = (1,...,1745)$. Each y-axis represents -log10(p-value) for each respective model, and each x-axis represent the retention time of the metabolite in minutes. Plasma lipid Benjamini-Hochberg false discovery corrected p-values <0.05 are circled in red. Body mass index (BMI), waist circumference (WC), tumor necrosis factor-alpha (TNF- α), interferon gamma-induced protein-10 (IP-10), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1).

Figure 9 (cont'd)

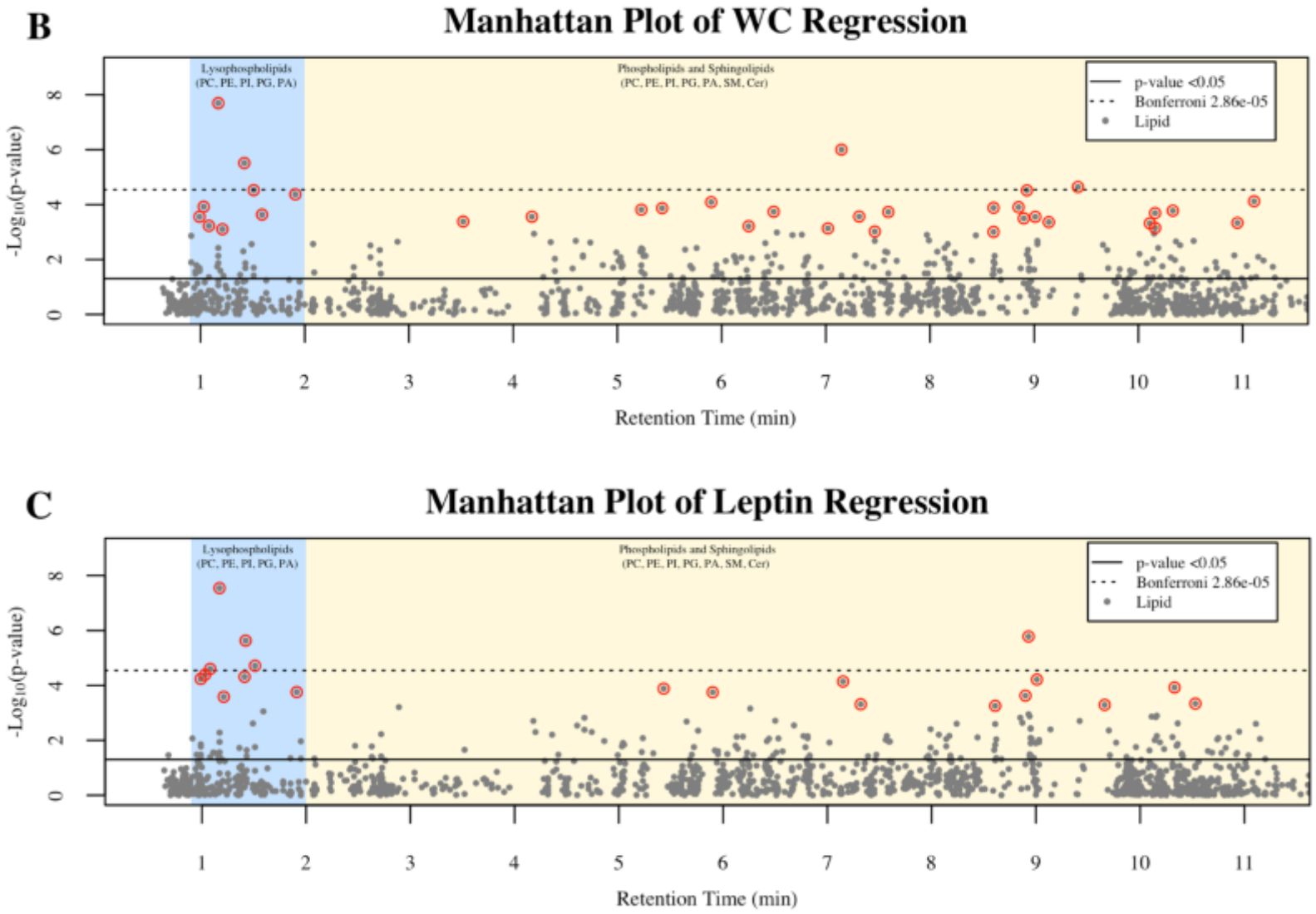
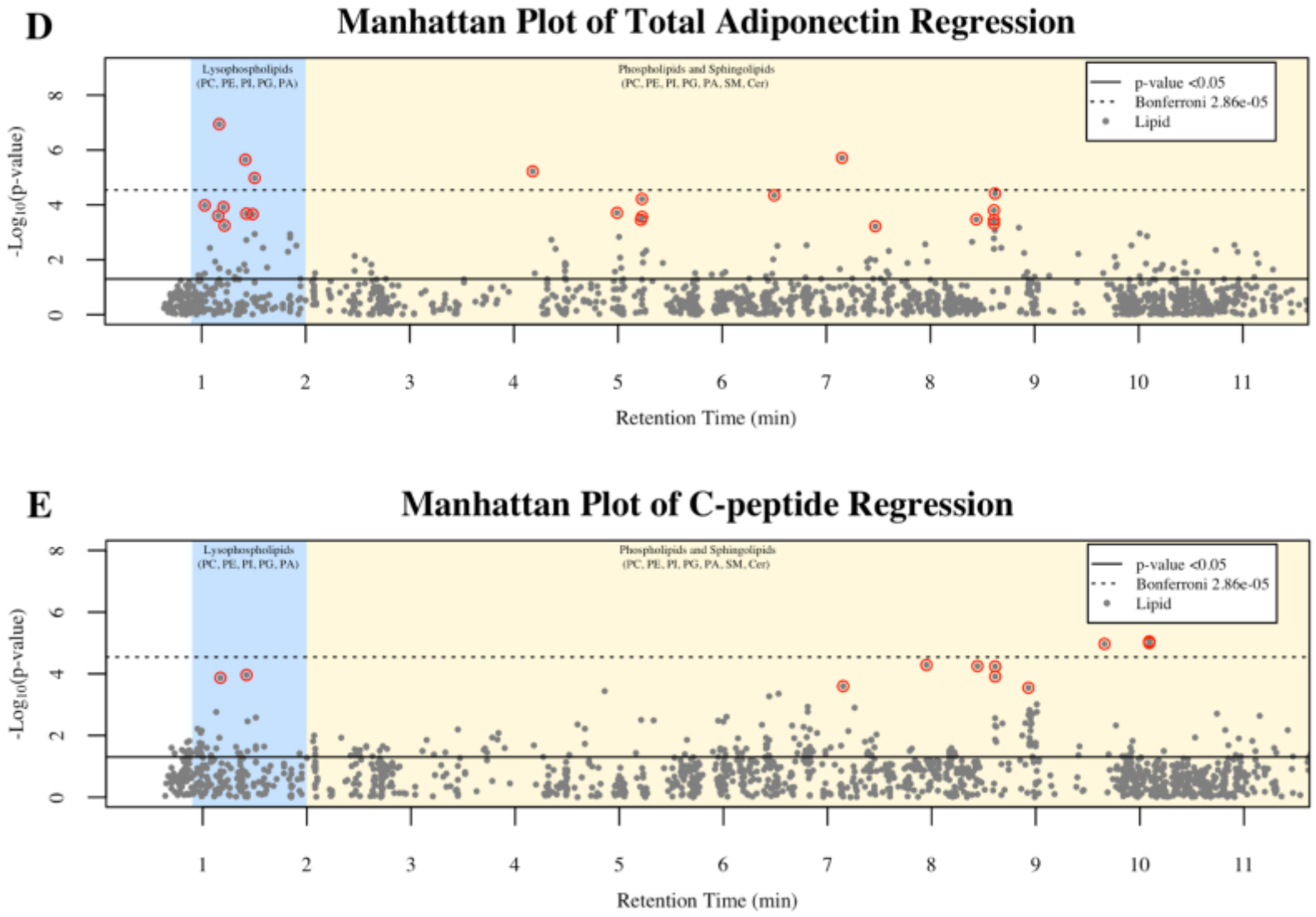
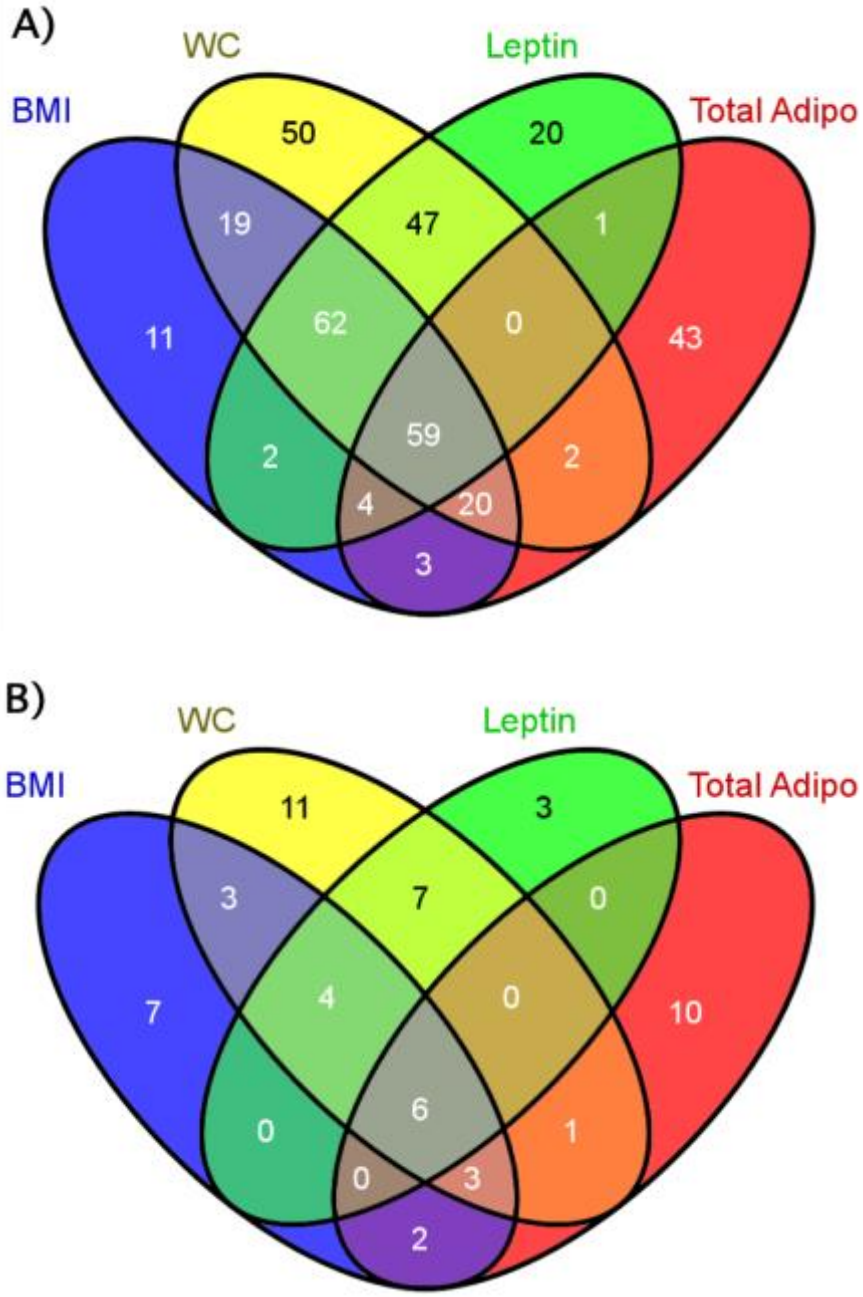


Figure 9 (cont'd)



Next, lipids significant across several traits from single lipid regression were inputted into the software Venny [147], to illustrate the lipids significant between several responses using Venn diagrams. Since Venny creates a Venn diagram for up to 4 traits, the anthropometrics BMI and WC, and the adipokines leptin and total adiponectin were selected for the illustration. Relationships between the traits and lipids with p-values <0.05 are presented in Figure 10A. There were numerous lipids with p-values <0.05 associated with traits (Figure 9 and 10A), for instance: 180 lipids with BMI, 259 lipids with WC, 195 lipids with leptin, and 132 lipids with total adiponectin. In total, there were 59 lipids associated between the four traits (Figure 10A). A Venn diagram of only lipids with BH FDR p-values <0.05 for the traits are presented in Figure 10B. There were 6 lipids with BH FDR p-values <0.05 associated between the four traits (Figure 10B), and the primary ids were: X1.17_564.3289, X1.42_566.3497, X1.21_476.2768, X1.51_592.3513, X1.03_562.3132, and X7.15_786.5626. A complete list of lipids significant in at least 2 or more single marker regression models are outlined in Table 10.

Figure 10: Venn diagrams of the lipids significantly associated with body mass index, waist circumference, leptin, and adiponectin in single lipid regressions.[a]

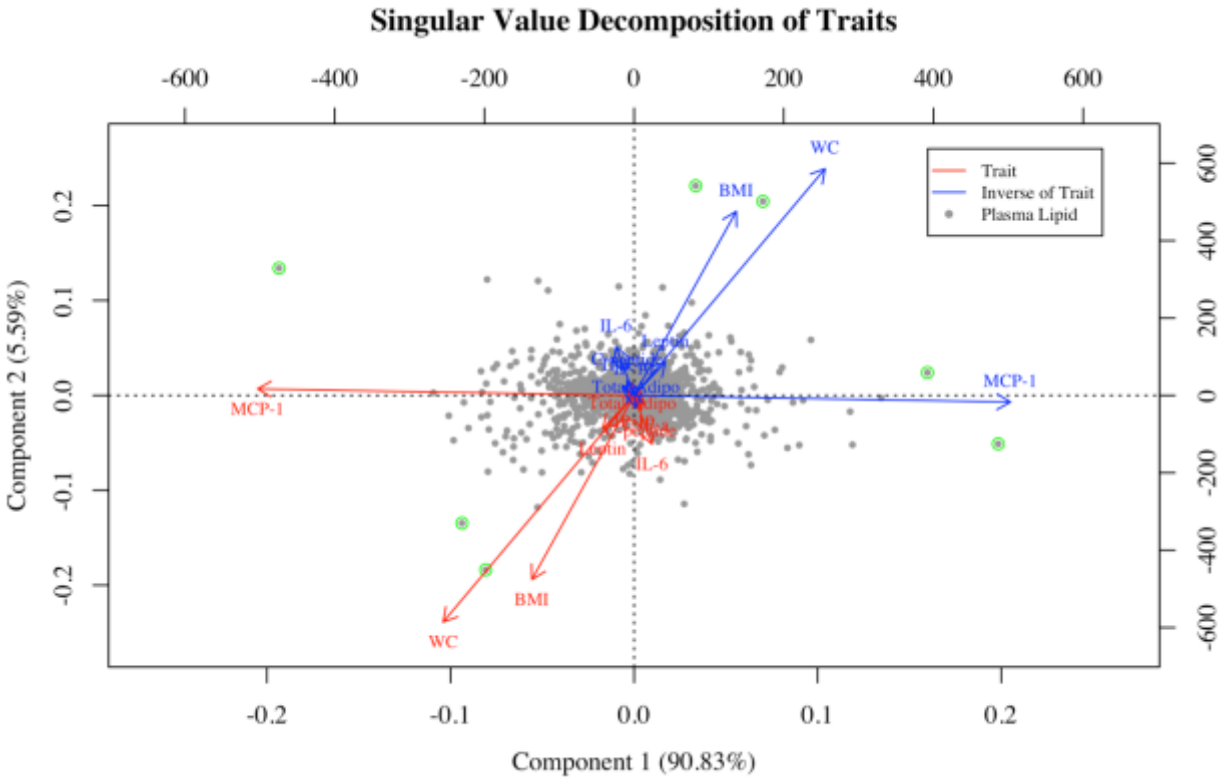


[a]Venn diagrams displaying the relationship of lipids significantly associated between the traits body mass index (BMI), waist circumference (WC), leptin, and total adiponectin (Total Adipo). A) Relationship of lipids with p-values <0.05 for the traits. B) Relationship of lipids with Benjamini-Hochberg p-values <0.05.

Biplot

A biplot analysis (Figure 11) was derived from the 1,745 beta coefficients from each respective lipid in single marker regressions and the 9 traits (i.e., BMI, WC, leptin, total adiponectin, C-peptide, IL-6, IP-10, and MCP-1). The vectors 1 and 2 from the SVD derived from the plasma lipid estimated effects, are represented with dots, and the loadings of the traits are represented with red arrows (in general, leftmost and bottom axes), and the inverse loadings of the traits are represented with blue arrows (in general, uppermost and right axes). Vectors 1 and 2 accounted for 96% of the variation in the lipidomics profiles. The orthogonal projection of the dots in the vectors are an approximation of the effect of each lipid for that trait. For instance, X12.43_934.7918 contributes to higher MCP-1 concentrations, while X8.17_830.6155 and 12.14_850.7682 both contribute to lower MCP-1 concentrations. The lipids X8.24_880.6145 and 3.52_421.3076 contributed to higher BMI, WC, and MCP-1 concentrations, and the lipids X1.51_592.3513 and X1.35_669.3989 contributed to lower BMI, WC, and MCP-1 concentrations. In addition, the score of MCP-1 in the first vector was scaled by a factor 25 (i.e., original score >12,000) to display it within the range of the other traits in the figure. The traits IL-6, leptin, total adiponectin, C-peptide, and IP-10 did not significantly load into vectors 1 and 2. A full list of lipids with radi > 0.1 in SVD are displayed in Table A9.

Figure 11: Biplot of the 1,745 estimated effects from single marker regressions and the trait loadings. Values of the effects are in red and the inverse of the values are in blue.[a]

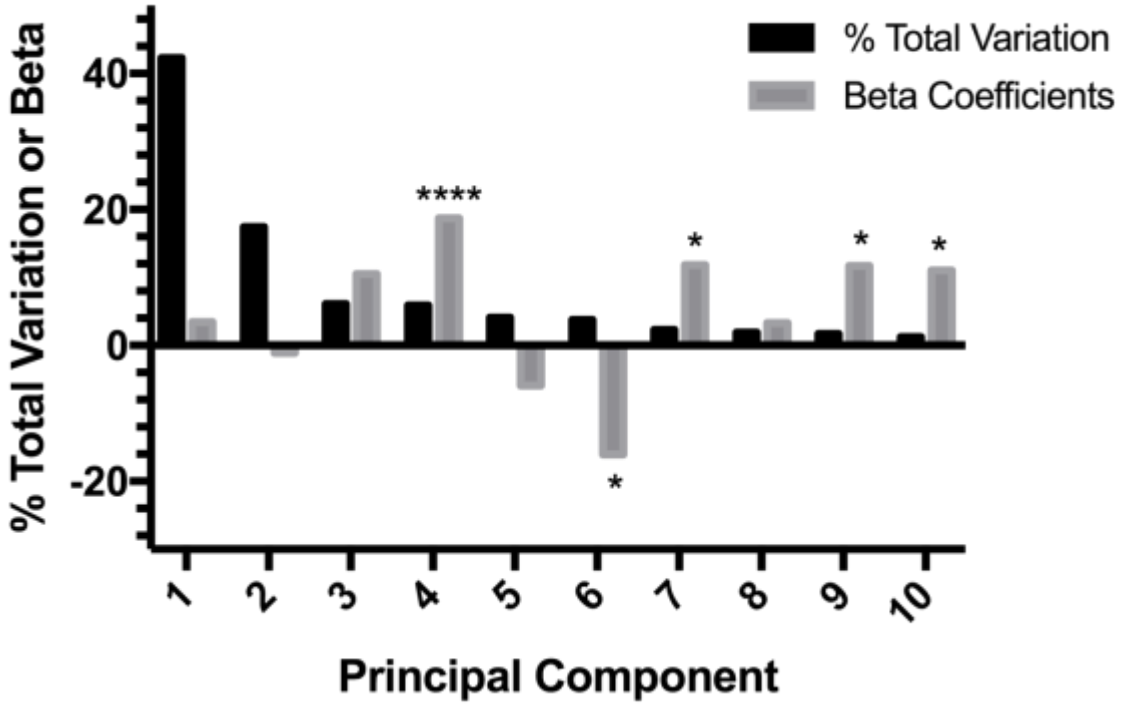


[a]Singular value decomposition modeled on the 1,745 regression beta coefficients from plasma lipids in age and smoking regression for each respective parameter. (•) represent the plasma lipid loading score into vectors 1 and 2. Plasma lipids are highlighted if radius > 0.15. Parameters are listed in red text with red arrows and the inverse of the parameters are listed in blue text with blue arrows. The score of MCP-1 in vector 1 is scaled by a factor 25 (i.e., original score >12,000). Body mass index (BMI), waist circumference (WC), tumor necrosis factor-alpha (TNF- α), interferon gamma-induced protein-10 (IP-10), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1).

Different BMIs display different lipidomic profiles

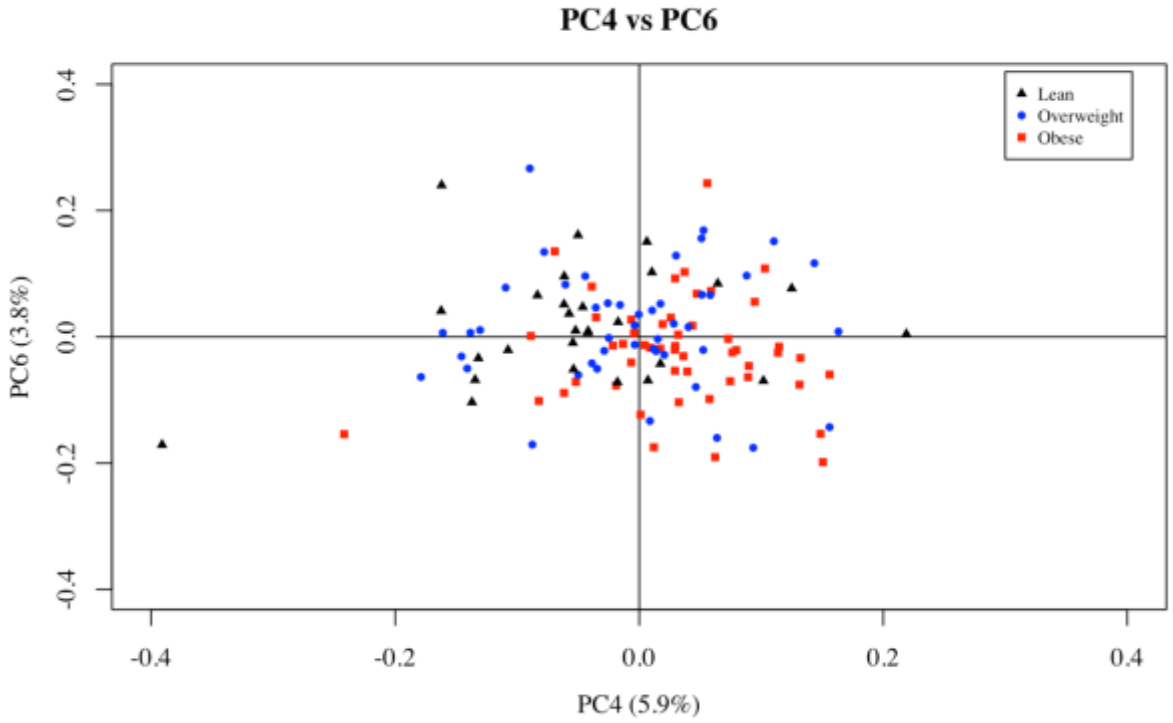
Next, BMI was regressed on Pc scores derived from the 1,745 lipid abundances (Figure 12). The first 10 Pc scores were analyzed individually and models were adjusted for age and smoking. The 4th principal component accounted for 5.9% of Eigen value percent total variation and was positively associated (estimated effects = 18.7) with BMI (Bonferroni $p<0.05$, data not shown). The 6th principal component was inversely associated with BMI, and principal components 7, 8, 9, and 10 were positively associated with BMI; estimated effects: -16.1, 11.9, 11.8, and 11.0, respectively. In order to determine which lipids were driving the 4th principal component scores, the 4th principal component scores were regressed on the 1,745 lipids individually, and the top 10 beta coefficients positively and inversely associated with the 4th principal component scores, and the lipids respective Bonferroni corrected p-values are presented in Table A10. The results from regressing BMI on Pc4 and Pc6 showed a positive and inverse association, respectively. As the loadings in Pc4 increase, BMI also increases, and while the loading in Pc6 increase, the BMI is decreases. When examining the relationship between Pc4 scores and BMI category, 73% of obese participants, 50% of overweight participants, and 29% of lean participants had Pc4 scores greater than zero (Figure 13). Similar results were observed with Pc6 scores and BMI category, such that 67 % of obese, 43% of overweight, and 39% of lean participants had Pc6 scores less than zero.

Figure 12: Principal component analysis derived from 1,745 lipid abundances. Individual analysis of first 10 principal components, includes Eigen value % of total variation of principal components, and BMI principal component scores regression.[a]



[a]X-axis represent the principal component, and Y-axis represent the percent total variation of principal components or beta coefficient, respectively. Only the first 10 principal components (Pc) are displayed. Black bars represent the percentage of the sum of all possible eigenvalues; the Eigen value % of total variation. Gray bars represent the beta coefficients from regressions; model: (BMI_i = Age_i + $Smoking_i$ + Pc_k) where *i=(1,...,126)* and *k=(1,..,10)*. * p-value <0.05 and **** p-value <0.0001. Body mass index (BMI).

Figure 13: Plot of principal component 4 vs 6 scores (n=126) derived from plasma lipidomic profiles. Patients data points are differentiated by symbol and colored based on BMI category.



Structural identification of primary ids

Finally, the primary ids significantly associated with traits across several models were selected for structural identification. The primary ids, lipid common name, and list of models where each respective primary id was significant, are outlined in Table 10. PL head groups were determined as follows: PCs were confirmed by loss 60.02 Da (methyl formate), PEs were confirmed by presence of 140.01 *m/z* (ethanolamine phosphate), PIs were confirmed by presence of 241.01 *m/z* (inositol phosphate-H_2O), and PSs were confirmed by neutral loss of 87.03 Da (loss of serine). Eleven LPLs were associated with traits from several lipid classes including LPC, LPE, and LPS. These LPLs identified included: LPC(17:0), (18:1), (18:2), (18:3), (20:1), (20:2), (20:5), and (22:6); LPE(18:1) and (18:2); and LPS(20:4). The primary id X1.35_669.3989 fell within the retention time range of LPLs but did not exhibit a fragmentation pattern indicative of PLs (Table A3) and was not available in lipid databases. The free FA palmitic acid was confirmed by 255.2331 *m/z* and matching the retention time (2.65 min) with a pure standard. The primary ids X4.18_794.5050, X5.43_1068.6601, and X10.11_1381.8480 did not have a fragment pattern indicative of PLs, although X5.43_1068.6601 did produce the m/z 1023.6949 indicative of formate (45.99 Da) loss.

Six PCs with ether linked chains were associated with several traits. These PCs with ether linked lipids included: PC(O-16:0_20:5), PC(O-16:1_18:2), PC(O-18:1_22:5), PC(O-16:0_18:2), PC(O-18:0_22:6), and PC(O-18:1_18:2). Ether linked chains in PCs were confirmed as follows: 466.3303 *m/z* for O-16:0, 464.3147 *m/z* for O-16:1, 494.3616 *m/z* for O-18:0, and 492.3460 *m/z* for O-18:1. In addition, there were several diacyl PLs associated with traits that contained FA chains ≥ C18:0 and FA chains indicative of ω-6s, including: PC(18:2_18:2), PC(18:0_18:2), PC(20:1_20:4), PE(18:0_20:4), PC(18:0_22:5), PC(18:0_20:3), and PE(18:0_20:3). Since

sphingolipids do not fragment well in negative mode, primary id X10.33_871.6904 was determined to be SM(d19:1/24:1) as follows: by confirmation of methyl formate loss of parent ion and presence of daughter ion 811.6074 *m/z* in negative mode, and the presence of 827.70 *m/z* (the SM(d19:1/24:1) $[M+H]^+$) at 10.33 min in positive mode, and the fragments 278.28 *m/z* (indicative of SM d19:1 [long chain base-$H_2O]^+$) and 336.37 *m/z* (indicative of SM with 24:1 FA in positive mode). A complete table of the primary ids, common name, molecular adducts, theoretical *m/z*, and mass error (<0.05 for all identified lipids) are presented in Table A11.

Table 10: Identification of plasma lipid biomarkers.[a]

Primary ID	Common name	Models where significant
X0.99_586.3136	LPC(20:5)	WC, leptin, Pc4
X1.03_562.3132	LPC(18:3)	BMI, WC, leptin, total adipo
X1.08_612.3280	LPC(22:6)	BMI, WC, leptin
X1.17_564.3289	LPC(18:2)	BMI, WC, leptin, total adipo, C-peptide
X1.21_476.2768	LPE(18:2)	BMI, WC, leptin, total adipo
X1.22_544.2648	LPS(20:4)	BMI, total adipo
X1.35_669.3989	Unknown	SVD
X1.42_566.3497	LPC(18:1)	BMI, WC, leptin, total adipo, C-peptide
X1.49_478.2927	LPE(18:1)	BMI, total adipo
X1.51_592.3513	LPC(20:2)	BMI, WC, leptin, total adipo, SVD
X1.59_554.3446	LPC(17:0)	BMI, WC
X1.91_594.3757	LPC(20:1)	WC, leptin
X2.65_255.2331	Palmitic acid	Pc4
X3.52_421.3076	TLTI	BMI, WC, SVD
X4.18_794.5050	Unknown	BMI, WC, total adipo
X4.37_881.5140	PI(16:0_22:6)	Pc4
X5.23_826.5592	PC(18:2_18:2)	BMI, WC, total adipo
X5.43_1068.6601	Unknown	BMI, WC, leptin
X5.90_810.5600	PC(O-16:0_20:5)	WC, leptin
X6.50_828.5729	PC(18:1_18:2)	BMI, WC, total adipo
X7.15_786.5626	PC(O-16:1_18:2)	BMI, WC, leptin, total adipo, C-peptide
X7.32_864.6056	PC(O-18:1_22:5)	WC, leptin
X7.47_788.5783	PC(O-16:0_18:2)	WC, total adipo

[a]The structural of identification of plasma lipids was performed based if *m/z*: was significant in two or more models from single marker regression analyses of responses, or had a radii ≥ 0.2 in singular value decomposition analysis, or was one of the top ten lipids positively and inversely associated with principal component 4. Plasma lipids meeting this criterion that were too low to identify (TLTI) are listed. Plasma lipid common names determined by lipid structure identification using Lipid Maps and Human Metabolome databases, and manual confirmation of mass spectra. The "_" between fatty acids in glycerophospholipid structures is used, since the position at the sn1 and sn2 position cannot be determined. Plasma lipids listed as unknown did not match a lipid library database search or exhibit a fragmentation pattern indicative of phospholipids in ms/ms analyses. Spectra of unknowns are listed in Table 19. Body mass index (BMI), waist circumference (WC), total adiponectin (total adipo), principal component 4 (Pc4) and singular value decomposition (SVD).

Table 10 (cont'd)

Primary ID	Common name	Models where significant
X8.17_830.6155	PC(18:0_18:2)	SVD
X8.23_950.6408	TLTI	Pc4
X8.24_880.6145	PC(20:1_20:4)	SVD
X8.44_716.5227	PE(16:0_18:1)	total adipo, C-peptide
X8.61_1038.473	TLTI	WC, C-peptide
X8.61_766.5363	PE(18:0_20:4)	WC, leptin, total adipo, C-peptide
X8.67_864.6048	PC(O-18:0_22:6)	Pc4
X8.90_880.6027	PC(18:0_22:5)	BMI, WC, leptin
X8.93_856.6036	PC(18:0_20:3)	BMI, WC, leptin, C-peptide
X9.01_1128.5388	TLTI	WC, leptin
X9.03_842.5446	TLTI	Pc4
X9.42_814.5927	PC(O-18:1_18:2)	BMI, WC
X9.45_1261.8069	TLTI	Pc4
X9.66_768.5494	PE(18:0_20:3)	leptin, C-peptide
X9.96_1034.5762	TLTI	Pc4
X10.11_1381.848	Unknown	WC, Pc4
X10.19_802.5695	TLTI	Pc4
X10.33_871.6904	SM(d19:1/24:1)	WC, leptin, Pc4
X11.20_1012.7670	TLTI	Pc4
X12.14_850.7682	TLTI	SVD
X12.43_934.7918	TLTI	SVD
X13.07_988.794	TLTI	Pc4
X13.10_920.7410	TLTI	Pc4
X13.11_919.7343	TLTI	Pc4
X14.31_881.7973	TLTI	Pc4
X14.33_869.3325	TLTI	Pc4
X14.35_1062.754	TLTI	Pc4
X14.38_880.7349	TLTI	Pc4

Discussion

This study characterized plasma PL profiles related to obesity and obesity-associated serum adipokines, cytokines, and the glycemic marker C-peptide. Specifically, we report that several classes of LPLs (i.e., LPC, LPE, and LPS) are inversely associated with BMI, WC, leptin, and C-peptide. Plasma ether linked PCs were also significantly decreased in obesity, including 16 and 18 carbon saturated and unsaturated ethers. On the other hand, total adiponectin, an anti-inflammatory adipokine inversely associated with obesity, was positively associated with LPLs and C18:2 containing PLs. Overall we report that C18:2 is lower in several lipid classes (i.e., LPLs, diacyl PCs, and ether linked PCs) in obese individuals. These data indicate that plasma PL FA differences occur in specific PL species, and since obesity is associated with altered lipid metabolism, our results provide insight into metabolic pathways that may be of interest for future research and therapeutic targeting.

In a recent randomized control trial, supplementation with ω-3 PUFAs increased ω-3s in LPLs and lowered ω-6s in LPLs, but overall LPLs were still lower in obese compared with normal and overweight individuals [148]. Several studies report obesity is inversely associated with LPLs. For instance, overweight and obese individuals have lower LPC(18:1) and (18:2) [149], and Rauschert et al. reported WC is inversely associated with LPC(18:1) and (18:2) [150]. What is unique about our study is that we report eleven LPLs are inversely associated with obesity, using an untargeted mass spectrometric approach. Our most significant result was the inverse relationship between LPC(18:2), and BMI, WC, leptin, and the glycemic marker C-peptide. Higher levels of LPC(18:2) were independently associated with a decreased risk of type-2 diabetes in a European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam study [151]. The

drastic decrease in C18:2 in our results is likely the ω-6 PUFA LA, and we previously reported in this study population, that median plasma PL LA levels are roughly 3% lower in obese individuals compared to lean [5].

After weight loss in obese individuals there is an increase in LPCs, in particular C18:2, suggesting the inverse association between LPLs and obesity could be related to diet or weight loss [152, 153]. In experimental models with high fat diets (HFD), there are rapid decreases in plasma LPC(18:1), (18:2), (20:1), and (20:5) [154]. Some studies suggest that increases in some LPLs such as LPC(18:0) are indicators of high fat diet induced obesity [155], and in obesity resistant mice, HFD increases plasma LPCs in particular LPC(18:0), (18:1), (18:2), (22:6) [156]. The decrease in plasma LPLs may result from an increased accumulation of LPLs in tissues (i.e., liver) [157], however, in some obese tissues such as skeletal muscle there are reported decreases in LPC(17:0), (18:2), and (20:2) [158]. In addition, LPCs are ligands for class A scavenger receptors that induce M2 macrophage polarization, thus, reducing M1 macrophage populations [129]. The expansion of white adipose tissue results in adipokine dysregulation, adipocyte hypoxia, and induction of the M1 macrophage [14]. Considering each excess kilogram of human fat results in the accumulation of an estimated 20-30 million macrophages [33], the decrease in LPCs could be related to an increase in obese adipose tissue macrophages.

Overall a majority of the significant plasma PL FAs differences in our results are PUFAs, in particular C18:2 which decreased in LPLs, ether linked PCs, and diacyl PCs. A recent meta-analysis reported that long chain PUFA profiles, in particular ω-6s, are dysregulated in overweight and obese individuals [7]. In obesity, decreases in LA are associated with increases in LA-derived DGLA by the enzyme delta-6-desaturase (D6D). Often researchers investigate the estimated enzyme activity (EAE) of desaturases through FA product to precursor ratios (i.e., D6D EAE =

ratio of DGLA/LA) (reviewed in detail [6]). We have previously reported in our study population that PL DGLA levels and D6D EAEs are increased in obese individuals, but there were no observed differences in ARA [5]. In our current study we report C20:3 (i.e., DGLA) is increased specifically in PC(18:0_20:3) and PE(18:0_20:3), and interestingly increases C20:4 (i.e., ARA) containing PLs such as PC(20:1_20:4) and PE(18:0_20:4). In addition, we have also reported in our study population that C-peptide concentrations are inversely associated with plasma PL delta-5-desaturase (D5D) EAEs (i.e., the ratio of ARA/DGLA) even after adjusting obesity (i.e., BMI and WC) [130]. In our current study, for each one unit change in PE(18:0_20:3), the average change in mean C-peptide concentrations was roughly 2.13 units (Table A8). It is likely that PLs such as PE(18:0_20:3) and PC(18:0_20:3) are important lipids contributing to the increases in PL DGLA levels that is observed in numerous obesity related studies (reviewed in detail [6, 7]).

Taken together, our results implicate several metabolic pathways that may be related to the altered lipid metabolism reported in obesity. Interestingly, enzymes responsible for increases in LPLs are elevated in obesity such as increased PLA_2 activity [126] and increased LCAT activity [127]. Therefore, one would expect since PLA_2 and LCAT activity are higher in obesity, LPLs would also be higher in plasma. However, this is not consistent with our results and the results of other studies [148-150, 152, 153]. The decrease in LPLs are most likely related to an increased LPL accumulation in obese tissues [157], uptake by macrophages [129], or due to an increased beta-oxidation of FAs (reviewed in detail [159]). Obesity is associated with increased oxidative stress and reactive oxygen species [160], and ether lipids serve as radical scavenging species (reviewed in detail [161]). In obesity there are increased circulating oxidized lipoproteins [162], and it is suggested that ether lipids act as plasma antioxidants preventing lipoprotein oxidation [163]. Therefore, our observed decrease in plasma ether linked PCs may result from the

degradation of ether lipids due to increased free radical savaging. We previously reported in our study population that non-enzymatic autooxidative produced oxylipids are increased in obese plasma [5]. It is also possible that decreased plasma ether linked PCs are due to decreased production in the peroxisome, since obesity is associated with ER stress which can alter peroxisome function (reviewed in detail [164]). To our knowledge, no research group has characterized the % total plasma PL FA levels in obesity (i.e., using a FAME analysis), and sought to identify specific PL species where these FA differences occur, using an untargeted mass spectrometric approach. Since a vast majority of epidemiological studies assess PL FA differences as % total FAs, our research provides insight to specific PL species that future studies should investigate for biomarkers of altered lipid metabolism and if these PL and FA differences affect proinflammation.

CHAPTER 4: SOLUBLE EPOXIDE HYDROLASE-DERIVED PLASMA OXYLIPIDS ARE INVERSELY ASSOCIATED WITH OBESITY

This chapter has been submitted to *Clinical and Experimental Metabolism* by Pickens et al. (2016).

Abstract

Background

Oxylipids are oxygenated polyunsaturated fatty acid (PUFA) metabolites that are responsible for the onset and resolution of the inflammatory response. Enzymatic oxygenation through the lipoxygenase (LOX) or cytochrome P450 (CYP) pathways can form oxylipids that have either pro-inflammatory or proresolving functions depending on the type of PUFA substrate and degree of metabolism. The objective of this study was to determine how PUFA substrates and their corresponding oxylipids are associated with obesity.

Methods

Plasma non-esterified FA and oxylipids were isolated from 123 Caucasian males using solid phase extraction and quantified using high performance liquid chromatography-tandem mass spectrometry. Statistical analyses included linear regressions, factor analysis, and polytomous logistic regressions, and the responses were body mass index (BMI) and waist circumference (WC). Models were adjusted for age and smoking, and p-values were corrected for false discovery per Benjamini-Hochberg and Bonferroni.

Results

We report that BMI and WC are positively associated with proinflammatory oxylipids derived from arachidonic acid (ARA), specifically 5- and 11-hydroxyeicosatetraenoic acid (HETE), even after normalization to ARA concentrations. Individuals with 5-HETE concentrations > 5.01 nM or 11-HETE concentrations and > 0.89 nM were over 5 times more likely to be obese compared to those with ≤ 1.86 nM and ≤ 0.39 nM, respectively. Our most significant result was that vicinal diols (i.e., alcohols on adjacent carbon atoms) from linoleic, eicosapentaenoic, and docosahexaenoic acid were inversely associated with obesity. Across all statistical tests, vicinal diols were inversely associated with obesity whether normalized to parent PUFA concentrations, normalized to precursor epoxides, or in factor analysis. Since vicinal diols are formed through soluble epoxide hydrolase (sEH) metabolism of CYP epoxygenated PUFAs, these results indicate obesity is likely associated with altered sEH and/or CYP epoxygenase metabolism. Taken together, our results indicate obesity is associated with specific oxylipids indicative of altered PUFA metabolism through several pathways (i.e., LOX, reactive oxygen species, sEH, and CYP epoxygenase), rather than attributed solely to altered dietary PUFA intake.

Introduction

Chronic low-grade inflammation is causally linked to the pathogenesis of obesity-associated metabolic diseases, such as insulin resistance, type-2 diabetes, cardiovascular disease and non-alcoholic fatty liver disease [17, 122, 165]. The caloric overload in obesity results in lipid accumulation in adipose tissue that triggers circulating monocyte recruitment into lipid-laden tissue [17]. Macrophage infiltration and accumulation in lipid-laden adipose tissue is a defining

feature of the chronic, low-grade inflammatory state in obesity [122]. In fact, each excess kilogram of human fat results in the accumulation of an estimated 20-30 million macrophages [33]. One primary mechanism through which macrophages regulate inflammation is through the production of potent bioactive lipid mediators referred to as oxylipids. Oxylipids are oxygenated fatty acid (FA) metabolites that are responsible for the onset and resolution of the inflammatory response [53, 54]. When the resolution of inflammation fails, it perpetuates a pro-oxidant environment and results in a positive feedback loop leading to the destruction of tissues [34, 35, 52, 165].

Oxylipids are potent bioactive lipid mediators that have essential roles in normal physiology and function. Oxylipids formed from polyunsaturated fatty acids (PUFAs) are important mediators of PUFA effects in the body. PUFA within membrane phospholipids serve as substrates for the biosynthesis of oxylipids through either enzymatic or non-enzymatic pathways [166]. Obesity is associated with westernized dietary intake patterns [40], which alters phospholipid FA composition of both plasma and cellular membranes [41]. There is enzymatic competition for elongation, desaturation, and oxygenation of several structurally similar omega-6 (ω-6) and omega-3 (ω-3) PUFAs. Accumulations of one PUFA family (i.e. ω-3) will offset production of the other (i.e. ω-6) and can impact oxylipid production. For instance, increased plasma ω-3s are associated with higher concentrations of plasma ω-3 oxylipids and a concomitant reduction in plasma ω-6s [167]. Oxylipids are endogenously synthesized by the enzymes cytochrome P450 (CYP) enzymes [46], cyclooxygenase (COX) [47], lipoxygenase (LOX) [48], and non-enzymatic pathways such as reactive oxygen species (ROS), especially during oxidative stress. PUFAs such as linoleic (LA), alpha-linolenic (ALA), arachidonic acid (ARA), dihomo-γ-linolenic acid (DGLA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) are substrates for these oxygenating enzymes and non-enzymatic reactions.

Obesity is associated with pro-oxidative stress and increased ROS [168]. ROS are formed as a normal end products of cellular metabolism and by phagocytic cells, such as macrophages during inflammation. CYP metabolism of PUFAs form abundant classes of oxylipids such as PUFA epoxides (i.e., formed by CYP2J2, CYP2C8, and CYP2C9) and omega-hydroxy PUFA (i.e., formed by CYP4F, CYP4A, and CYP2U). There are several CYP epoxygenases with preferential affinity to PUFA substrates. In particular, the epoxygenase CYP2J2 family are highly expressed in tissues affected by obesity-associated pathologies such as liver and skeletal muscle [169], adipose tissue [170], pancreas [171], and cells such as macrophages and monocytes [172, 173]. In general, PUFA epoxides are described as more "anti-inflammatory like" [174]. For instance, CYP2J2 activity and PUFA epoxides (i.e., ARA-derived epoxides) inhibit NF-κB, thus, reducing inflammatory responses [175]. However, some epoxides such as LA-derived epoxides produced by cells such as macrophages are commonly referred to as leukotoxins [176-178]. PUFA epoxides are further metabolized to form vicinal diols (i.e., alcohols on adjacent carbon atoms) by the enzyme soluble epoxide hydrolase (sEH), and vicinal diols are described as more "inflammatory like" [179].

Recently, we reported pooled obese plasma had lower concentrations of vicinal diols (the end products from sEH metabolism of PUFA epoxides), and higher concentrations of oxylipids associated with proinflammatory function including: 5-hydroxyeicsaotetraenoic acid (HETE) (5LOX product), 11-HETE (non-enzymatic product), 15-HETE (15LOX product), and 9,10- and 12,13-epoxyoctadecenoic acid (12,13-EpOME; CYP epoxygenase products) compared to pooled lean plasma [5]. Pooling is a useful method to assess preliminary differences, however, no statistical assessment of variation in the data can be performed. In our current study, plasma oxylipids were quantified using an expanded profile of 62 plasma oxylipids to determine oxylipid

classes and concentrations significantly associated body mass index (BMI) and waist circumference (WC) in 123 males. After false discovery correction, vicinal diols were inversely associated with both BMI and WC, and several HETEs were positively associated with both BMI and WC, even after normalization to plasma concentrations of parent PUFAs.

Materials and Methods

Study population

Data are further described elsewhere [2-5, 180, 181]. In brief, healthy and asymptomatic male subjects (n = 123, > 96% Caucasian) 48 to 65 years of age were enrolled between 2009 and 2011 in a cross-sectional study. Data are comprised of clinical metadata on individuals' co-morbidities, family history, and use of medications. Individuals with the following medical conditions were excluded: 1) cancer in the previous two years, 2) type-1 and type-2 diabetes, and 3) autoimmune diseases. Immediately after enrollment, trained staff collected anthropometric measurements and venous blood of study participants. The plasma fraction was separated from whole blood by centrifugation and stored at -80°C until time of analysis. Smoking status was assessed as "ever smoked" or "never smoked". A previous complete description of this study can be found elsewhere [2].

Chemicals and glassware preparation

All glassware used in the analysis was cleaned using a series of nanopure water, methanol, isopropanol, and hexane to remove any lipid contaminants prior to plasma extraction. The solvents used for extraction, isolation, and high performance liquid chromatography (HPLC) tandem mass

spectrometry (MS/MS) analysis were either HPLC- or LC/MS-grade. Quantitative standard curves and deuterium labeled internal standard mixes and were made as previously described [182].

Plasma extraction

Extraction and isolation of non-esterified PUFA and oxylipids was performed as previously described [5, 182], but modified as specified. Plasma was thawed on ice. Approximately 500 μL plasma was transferred to a conical tube containing 1.5 mL HPLC-grade methanol and butylated hydroxytoluene (BHT) (100 μg/mL BHT). Next, 15 μL deuterium labeled internal standard mix and 7.4 μL LC/MS-grade formic acid were added to the plasma and methanol. This solution was vortexed for 2 min, then centrifuged at 4700 x *g* for 20 min at 4°C. Supernatant was collected and transferred to a solution containing 95 μL formic acid and 95 mL methanol.

Solid-phase extraction and isolation

Solid-phase extraction was performed for each sample using Phenomenex Strata-X (60 mg/3 mL, Phenomenex, Torrane, CA), as previously described [5]. In brief, columns were conditioned with 4mL methanol BHT (100 μg/ mL BHT), followed by 4 mL HPLC-grade water. Extracts were loaded onto the columns, then washed with 20% methanol in water and were dried for 20 min under vacuum. Analytes were eluted with 4 mL HPLC-grade acetonitrile: methanol (1:1 v/v) and collected. Solvents were removed from collected elutions by evaporation using a Savant SpedVac (ThermoQuest, Holbrook, NY). These dried isolates of non-esterified PUFAs and oxylipids were stored under high-purity argon for no longer than 7 days. Dried residues were

reconstituted in 100 μL methanol, vortexed gently, then transferred to a microcentrifuge tube containing 50 μL water. The mixture was centrifuged at 14,000 × *g* for 10 min at 4°C, then 75 μL from the top of the supernatant was transferred to an amber auto-sampler vial with a low volume insert. The samples were capped under high-purity argon and stored a -20°C for no longer than 7 days.

LC/MS/MS analysis

Liquid chromatography separations were performed using an Ascentis Express C18 column (10 cm × 2.1 mm; 2.7 μm particles, Sigma-Aldrich, St. Louis, MO) maintained at 50 ˚C on a Waters ACQUITY UPLC system (Waters, Milford, MA). Mobile phase A was 0.1% formic acid in water and mobile phase B was acetonitrile. Analytes were eluted during a 15 min gradient using a flow rate of 0.3 mL/min. The mobile phase gradient began at 1% B, followed by a linear increase to 40% B at 2 min; then to 80% B at 8 min, and 99% B at 9 min, at which the composition was held until 13 min; then returned to 1% B until 15 min. The autosampler was cooled to 10 ˚C, and injection volume was set to 5 μL.

The column was connected to a Waters Xevo TQ-S triple quadrupole mass spectrometer (Waters, Milford, MA). Analytes were detected using electrospray ionization (ESI) with polarity switching between negative-ion mode (for oxylipids and PUFAs) and positive-ion mode (for endocannabinoids) using multiple reaction monitoring (MRM). To improve signal-to-noise ratios, the transition pairs were arranged into nine different data acquisition functions with durations spanning retention times of the target metabolites rather than over the entire data analysis period. The capillary voltage was set at 2.5 kV for negative ESI mode and 3.0 kV for positive mode. Additional source parameters in both modes were: source temperature 150 ˚C, desolvation

temperature 500 ˚C, desolvation gas flow rate 600 L/h, cone gas flow rate 150 L/h. MRM parameters including cone voltage, collision voltage, parent ion, and daughter ion were optimized by flow injection of pure standard for each individual compound. TargetLynx software was used for peak detection, integration and quantification.

Data merging and oxylipid exclusion

The LC/MS/MS analyses quantified 62 PUFAs and oxylipids. The full list of analytes in our study, along with parent and daughter ion, oxylipid precursor PUFA, and oxylipid class are outlined in Table A12. Since -omic data typically has a large proportion of missing values [135, 136], analytes with >50% missing values were excluded from the analysis. The remaining 32 analytes in the data matrix consisted of: LA and LA-derived 9-hydroxyoctadecadienoic acid (HODE), 13-HODE, 9-oxooctadecadienoic acid (oxoODE), 13-oxoODE, 9,10-EpOME, 12,13-EpOME, 9,10-dihydroxyoctadecadienoic acid (DiHOME), and 12,13-DiHOME; ALA-derived 13-hydroxyoctadecatrienoic acid (HOTrE); DGLA-derived 15-hydroxyeicosatrienoic acid (HETrE); ARA and ARA-derived 5-HETE, 11-HETE, 12-HETE, 15-HETE, 20-HETE, 14,15-epoxyeicosatrienoic acid (EET), 8,9-dihydroxyeicosatrienoic acid (DHET), 11,12-DHET, 14,15-DHET, arachidonoyl ethanolamide (AEA), arachidonoyl glycerol (AG), leukotriene B4 (LTB4), prostaglandin E2 (PGE2), thromboxane B2 (TBX2); EPA and EPA-derived 5,6-dihydroxyeicosatetraenoic acid (DiHETE), 17,18-DiHETE; and DHA and DHA-derived 19,20-dihydroxydocosapentaenoic acid (HDPA) and docosahexaenoyl ethanolamide (DHEA).

Statistical analyses

Our study was powered (0.8) to detect differences in plasma FAs, as previously described [180]. The responses used in the statistical analyses were the anthropometrics: BMI and WC (continuous variables), and BMI category (categorical variable). The median, Q1, and Q3 concentrations were calculated for each plasma PUFA and oxylipid, which are outlined in Table 11. The variables LA, ARA, and 12,13-DiHOME are reported as levels (i.e., peak area of compound/ peak area of internal standard) in Table A13, since a significant proportion of participant concentrations were above (i.e., 10 fold) the highest standard curve value. This was performed so the relationship between these three lipids could still be assessed with the responses. In this study, oxylipid totals and product to precursor ratios were calculated as follows: Total HODE = ∑ 9-HODE + 13-HODE; Total oxoODE = ∑ oxoODE + 13-oxoODE; Total EpOME = ∑ 9,10-EpOME + 12,13-EpOME; Total HETE = ∑ 5-HETE + 11-HETE + 12-HETE + 15-HETE + 20-HETE; Total DHET = ∑ 8,9-DHET + 11,12-DHET + 14,15-DHET; Total DiHETE = ∑ 5,6-DiHETE + 17,18-DiHETE; 9-oxo/HODE = 9-oxoODE/9-HODE; 13-oxo/HODE = 13-oxoODE/13-HODE; 9,10-Di/EpOME = 9,10-DiHOME/9,10-EpOME; 12,13-Di/EpOME = 12,13-DiHOME/12,13-EpOME; 14,15-DH/EET = 14,15-DHET/14,15-EET.

Statistical differences in PUFA and oxylipid concentrations (Table 11), and levels (Table A13), between BMI categories were determined using Kruskal Wallis non-parametric one-way ANOVA with Dunn's test for multiple comparison. Since a majority (>70%) of PUFAs and oxylipids were not normally distributed, the data matrix was log transformed for parametric analyses. For regressions, BMI and WC consist of phenotype y_i $(i = 1, \dots, 123)$ indexed by participants (i) and the set of predictors coming from oxylipid data as well as clinical covariates including age of the participant and the smoking status "ever smoked" or "never smoked". The

linear relationship between anthropometrics and plasma lipids was assessed by regressing BMI and WC on log transformed PUFAs, oxylipids, and oxylipid parent-to-precursor ratios individually (i.e., one at a time). Models were defined as:

$$y_i = age_i + smoking_i + log(lipid_{ij})$$

where $j = (1, \ldots, 44)$ represents the log transformed PUFAs and oxylipids, oxylipid totals, and oxylipid product-to-precursor ratios. Next, since many PUFAs and oxylipids are collinear and cannot be analyzed together in regression models, a factor analysis was employed to analyze PUFAs and oxylipids as groups.

The R package 'psych' [183] was used to generate biomarker patterns derived from the 32 PUFAs and oxylipids (i.e., excluded oxylipid totals and product-to-precursor ratios). Four factors were determined based on the scree plot [184] and Eigen values > 1 [185], as previously described [132]. The factor-loading matrix was then rotated using varimax orthogonal transformation. BMI and WC were regressed on the 4 factors individually (i.e., one at a time), with models defined as:

$$y_i = age_i + smoking_i + factor_{ik}$$

where $k = (1, \ldots, 4)$ represents the 4 factors derived from PUFAs and oxylipids. To determine whether differences in oxylipid concentrations were due to metabolism or altered PUFA availability (i.e., substrate levels), each participants oxylipid concentration was normalized to their concentration or level of each PUFA, respectively. BMI and WC were then regressed on the log transformed PUFA-normalized oxylipids individually (i.e, one at a time). Models were defined as:

$$y_i = age_i + smoking_i + log(lipid_{ic})$$

where $c = (1, \ldots, 33)$ represents log transformed PUFA normalized oxylipids and oxylipid totals.

Finally, for oxylipids significant across multiple statistical tests, the concentrations of these oxylipids were categorized into tertiles (referenced against the lowest category) for obese relative to lean, using polytomous logistic regressions as previously described [132]. The test for trend was carried out for the oxylipid of interest across tertiles, respectively. Polytomous logistic regression models were defined as:

$$BMI\ category_i = age_i + smoking_i + lipid_{ig}$$

where $g = (1, \ldots, 7)$ represents the oxylipids: 9,10-EpOME, 12,13-EpOME, 9,10-DiHOME, 5-HETE, 11-HETE, 17,18-DiHETE, and 19,20-HDPA as categorical variables.

In all statistical analyses, missing PUFA and oxylipid values were imputed as the $\sqrt{minimum\ value}$ for each respective PUFA and oxylipid. P-values were corrected for false discovery rate (FDR) according to Benjamini-Hochberg (BH) and Bonferroni [138, 139]. Statistical analyses of data were performed using R v3.2.2 [137], except polytomous logistic regressions which were performed using SAS version 9.4 (SAS, Cary, NC), as we have previously described [2-4, 180]. All statistical code used in analyses are publicly available at https://github.com/AustinPickens/Targeted-Lipidomics.

Results

Characteristics and oxylipid concentrations of study participants

Median, Q1, and Q3 values of age, smoking, anthropometric, and non-esterified PUFAs and oxylipid concentrations for the overall population (n=123) and separated by BMI category are presented in Table 11. Non-parametric ANOVAs were conducted for each variable in Table 11, across BMI categories, and BH FDR corrected p-values are presented. In brief, lean and obese participants were older that overweight participants. As expected, both BMI and WC increased with increasing BMI category. Concentrations of LA-derived oxylipids did not differ across BMI category (Table 11), however, 12,13-DiHOME and 12,13-Di/EpOME levels were decreased in obese individuals compared to both overweight and lean (Table A13). DGLA-derived 15-HETrE concentrations were significantly elevated in obese individuals compared to both overweight and lean (Table 11). ARA-derived 5-, 15-, and 20-HETE concentrations were higher in obese individuals compared to lean, and 11-HETE concentrations were elevated in obesity compared to both overweight and lean. 12-HETE concentrations were significantly higher in obese individuals compared to lean in the multiple comparison test, but the model p-value was not significant after p-value correction (BH FDR p=0.1141, data not shown). EPA-derived 17,18-DiHETE concentrations were decreased in obese individuals compared to both overweight and lean. DHA-derived 19,20-HDPA was significantly decreased in obese individuals compared to both overweight and lean, and also decreased in overweight individuals compared to lean (Table 11).

Table 11: Obesity is associated with increased plasma concentrations of monohydroxy arachidonic acid-derived oxylipids, and decreased plasma concentrations of eicosapentaenoic acid- and docosahexaenoic acid-derived vicinal diols (values expressed as median [Q1, Q3]).

Variable	Overall	Lean	Overweight	Obese	BH FDR p-value[b]
9-HODE	14.3 [9.5, 23.9]	17.1 [10.0, 30.9][A]	14.4 [9.8, 21.5][A]	13.5 [9.3, 25.1][A]	-
13- HODE	13.4 [8.6, 22.0]	17.4 [8.3, 25.7][A]	13.4 [9.0, 19.1][A]	10.8 [8.3, 22.6][A]	-
Total HODE	29.3 [19.1, 47.4]	38.6 [19.2, 52.4][A]	29.3 [19.2, 39.6][A]	24.2 [17.6, 51.9][A]	-
9- oxoODE	7.9 [5.1, 12.0]	7.7 [5.1, 11.4][A]	8.5 [5.8, 11.7][A]	8.3 [5.0, 12.2][A]	-
13- oxoODE	2.2 [1.4, 3.8]	2.2 [1.5, 3.3][A]	2.2 [1.3, 4.1][A]	2.3 [1.5, 3.8][A]	-
Total oxoODE[e]	11.0 [6.7, 15.7]	11.1 [7.3, 15.3][A]	11.0 [6.7, 16.4][A]	11.0 [6.7, 15.2][A]	-
9,10-EpOME	21.6 [15.0, 32.7]	19.8 [15.3, 32.1]][A]	19.8 [14.4, 34.2][A]	24.0 [15.9, 30.0][A]	-
12,13-EpOME	40.2 [27.0, 57.9]	40.2 [28.5, 76.5][A]	47.4 [25.2, 60.0][A]	37.8 [26.1, 52.8][A]	-
Total EpOME	64.2 [42.6, 94.2]	70.8 [41.7, 102.0][A]	65.4 [42.6, 100.2][A]	60.6 [42.6, 84.0][A]	-
9,10-DiHOME	8.4 [4.7, 16.4]	9.0 [5.7, 39.6][A]	10.8 [4.8, 17.4][A]	6.6 [4.1, 13.1][A]	-
13-HOTrE	2.0 [1.1, 3.9]	3.2 [1.5, 4.4][A]	1.8 [1.2, 3.0][A]	1.8 [1.1, 3.5][A]	-
15-HETrE	0.4 [0.2, 0.6]	0.2 [0.2, 0.5][A]	0.3 [0.2, 0.5][A]	0.5 [0.3, 0.7][B]	p≤0.05
5-HETE	2.8 [1.6, 9.5]	1.5 [0.8, 6.2][A]	2.4 [1.7, 5.0][AB]	4.5 [2.1, 12.0][B]	p≤0.05
11-HETE	0.7 [0.3, 1.3]	0.4 [0.2, 0.8][A]	0.5 [0.3, 0.9][A]	0.9 [0.5, 1.7][B]	p≤0.05
12-HETE	9.4 [6.0, 15.3]	7.5 [3.8, 14.1][A]	8.5 [6.2, 13.6][AB]	11.9 [6.7, 17.8][B]	-
15-HETE	0.8 [0.5, 1.2]	0.8 [0.4, 1.0][A]	0.6 [0.5, 1.1][AB]	1.0 [0.7, 1.5][B]	p≤0.05
20-HETE	0.8 [0.5, 1.2]	0.5 [0.3, 0.9][A]	0.7 [0.4, 1.0][AB]	0.9 [0.6, 1.3][B]	p≤0.05
Total HETE	19.0 [10.9, 32.8]	19.4 [8.9, 25.8][A]	16.5 [11.2, 22.7][A]	22.4 [11.3, 39.5][A]	-
14,15-EET	1.3 [1.0, 2.2]	1.2 [0.5, 1.8][A]	1.3 [1.0, 2.3][A]	1.9 [1.1, 2.2][A]	-
8,9-DHET	0.6 [0.4, 0.8]	0.6 [0.3, 0.8][A]	0.6 [0.5, 0.8][A]	0.5 [0.4, 0.9][A]	-
11,12-DHET	1.0 [0.8, 1.4]	1.0 [0.7, 1.5][A]	1.0 [0.8, 1.4][A]	1.1 [0.8, 1.4][A]	-
14,15-DHET	1.6 [1.3, 2.0]	1.5 [1.3, 2.3][A]	1.6 [1.2, 2.1][A]	1.6 [1.3, 1.9][A]	-
Total DHET	3.2 [2.5, 4.2]	3.0 [2.4, 4.8][A]	3.2 [2.6, 4.2][A]	3.2 [2.6, 4.0][A]	-
AEA	0.2 [0.2, 0.2]	0.2 [0.1, 0.2][A]	0.2 [0.1, 0.2][A]	0.2 [0.2, 0.2][A]	-
AG	1.4 [0.8, 2.0]	1.3 [0.8, 1.6][A]	1.6 [0.8, 1.9][A]	1.4 [0.9, 2.1][A]	-
LTB4	0.3 [0.2, 0.5]	0.2 [0.2, 0.4][A]	0.3 [0.2, 0.6][A]	0.3 [0.2, 0.5][A]	-
PGD2	4.4 [2.2, 10.3]	6.6 [3.3, 12.1][A]	4.1 [2.4, 13.4][A]	4.2 [2.0, 6.6][A]	-
TBX2	0.3 [0.2, 0.9]	0.3 [0.2, 0.6][A]	0.5 [0.2, 1.3][A]	0.3 [0.2, 0.8][A]	-
EPA[a]	0.2 [0.1, .3]	0.2 [0.1, 0.5][A]	0.2 [0.1, .4][A]	0.2 [0.1, 0.3][A]	-
5,6-DiHETE	5.0 [3.0, 7.3][A]	5.7 [3.5, 12.2][A]	5.3 [2.5, 7.2][A]	4.6 [3.2, 6.0][A]	-
17,18-DiHETE	8.5 [5.9, 14.3]	14.8 [9.8, 23.5][A]	8.0 [5.9, 14.6][A]	6.8 [5.0, 9.2][B]	p≤0.05
Total DiHETE	13.4 [9.5, 25.0]	23.1 [12.7, 37.5][A]	13.2 [9.0, 24.7][A]	10.9 [9.3, 18.6][A]	p≤0.05
DHA[a]	4.6 [3.3, 7.8]	5.6 [3.4, 8.3][A]	4.6 [3.2, 8.1][A]	4.5 [3.5, 7.3][A]	-
19,20-HDPA	2.8 [2.1, 4.4]	4.5 [3.1, 5.7][A]	3.3 [2.2, 5.0][B]	2.5 [1.6, 3.0][C]	p≤0.05
DHEA	0.5 [0.3,0.8]	0.6 [0.5,1.1][A]	0.5 [0.5,0.7][A]	0.5 [0.2, 0.8][A]	-

[a]Plasma non-esterified EPA and DHA are expressed as μM.
[b]Categorical analysis of BMI by Kruskal Wallis one-way ANOVA and Dunn's test for multiple comparison. P-values were corrected for false discovery rate (FDR) according to Benjamini-Hochberg (BH).

Obesity is associated with increased plasma HETEs and decreased vicinal diols

Next, BMI and WC were regressed on PUFAs, oxylipids, and oxylipid product-to-precursor ratios individually, to determine the estimated effects of each lipid for each anthropometric (Figure 14 and Table 12). Models were adjusted for age and smoking. P-values from single lipid regressions that were significant after BH FDR correction are circled, and p-values significant below Bonferroni cutoff ($p<0.0011$) are displayed above the hashed line in Manhattan plots (Figure 14). In general, the oxylipids significantly associated with BMI and WC after FDR correction consisted of LA-derived epoxides, ARA-derived HETEs, and LA-, EPA-, and DHA-derived vicinal diols. In fact, the majority of oxylipids significant below the Bonfferoni cutoff, across both BMI and WC models, were vicinal diols (Figure 14).

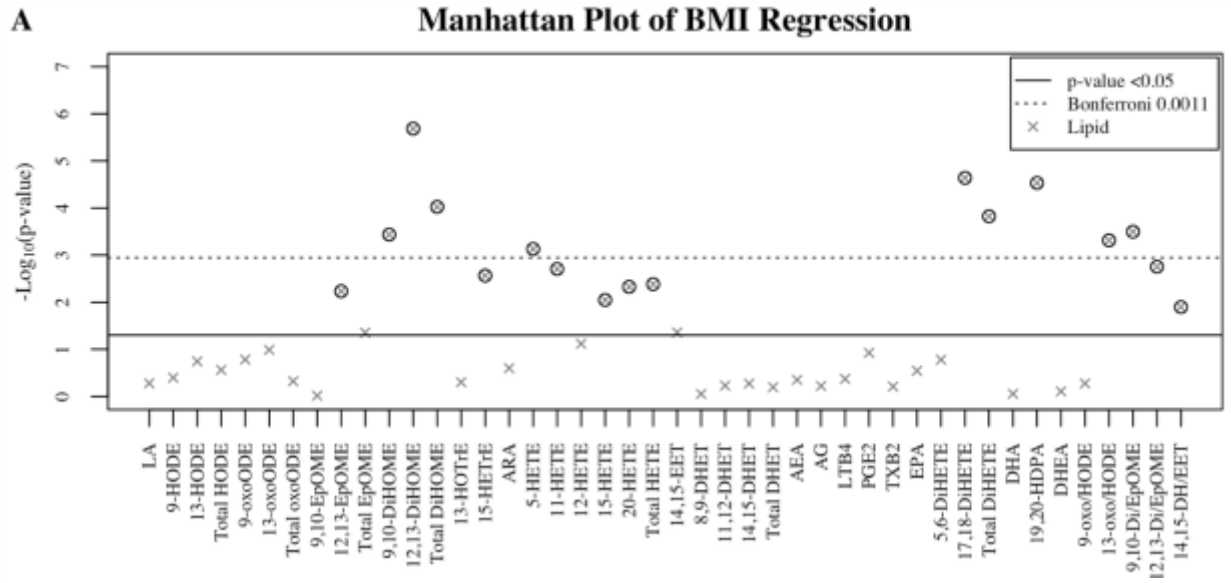
The estimated effects of oxylipids were similar between BMI and WC models (Table 12). For each unit increase in LA-derived 12,13-EpOME, Total EpOME, 9,10-DiHOME, 12,13-DiHOME, and Total DiHOME, the average BMI decreased by 2.11, 1.69, 1.70, 2.53, and 1.95 units, respectively (Table 12). DGLA- and ARA-derived oxylipids had a positive relationship with both BMI and WC. For each unit increase in 15-HETrE, and 5-, 11-, 12-, 15-, and 20-HETE, and 14,15-EET, the average BMI increased by 1.35, 1.54, 1.00, 1.60, 2.28, and 1.47 units, respectively (Table 12). EPA- and DHA-derived vicinal diols were inversely associated with BMI and WC. For each unit increase in 17,18-DiHETE and 19,20-HDPA, the average BMI decreased by 2.43 and 3.52 units respectively. To assess whether oxylipid differences were a result of altered metabolism, BMI and WC were regressed on oxylipid product-to-precursor ratios (Figure 14 and Table 12). Neither LA-derived 13-oxoODE nor 13-HODE were significantly associated with BMI or WC when analyzed individually, however, for each unit increase in the ratio of 13-oxoODE to 13-HODE (13-oxo/HODE), the average BMI increased by 2.63 units. The ratio of vicinal diols

normalized to their precursor epoxide had inverse associations with BMI and WC. Each unit increase in LA-derived 9,10-Di/EpOME, 12,13-Di/-EpOME, and ARA-derived 14,15-DH/EET were associated with BMI decreasing by roughly 2 units (Table 12).

HETE, octadecanoid, and ω-3 PUFA factors are associated with obesity

BMI and WC were regressed on 4 factors derived from PUFA and oxylipids, and models were adjusted for age and smoking. The factor loading matrix is presented in Table 13. The varimax rotated factor-loading matrix represents each factor's correlation with each individual lipid. Factors were named based on the majority of lipids with high correlations in each factor as follows: factor 1: HETE; factor 2: Octadecanoid; factor 3: ω-6 PUFA; and factor 4: ω-3 PUFA. HETE and Octadecanoid scores were associated more with WC compared to BMI (Table A14). Increased HETE scores tended to be associated with increased WC, while increased Octadecanoid scores tended to be associated with decreases in WC. ω-3 PUFA scores were significantly associated with both BMI and WC (Table A14). For each unit increase in ω-3 PUFA scores, the average BMI decreased by 1.71 units and the average WC decreased by 2.28 units, respectively.

Figure 14: Manhattan plots of body mass index and waist circumference regressed on non-esterified plasma polyunsaturated fatty acids, oxylipids, and oxylipid product to precursor ratios.[a]



[a]Manhattan plots of the –log(p-value) for body mass index (BMI) and waist circumference (WC) regressed on log transformed PUFAs, oxylipids, and oxylipid parent-to-precursor ratios individually (i.e., one at a time). Single non-esterified plasma fatty acid, oxylipid, and product to precursor ratio regression models are defined as: BMI_i or $WC_i = age_i + smoking_i + log(lipid_{ij})$, where $i = (1,...,123)$ and $j = (1,...,44)$. Each y-axis represents -log10(p-value) for each respective model, and x-axis represent the plasma non-esterified fatty acid, oxylipid, and oxylipid product-to-precursor ratios. Plasma lipids with Benjamini-Hochberg false discovery rate p-values ≤0.05 are circled.

Figure 14 (cont'd)

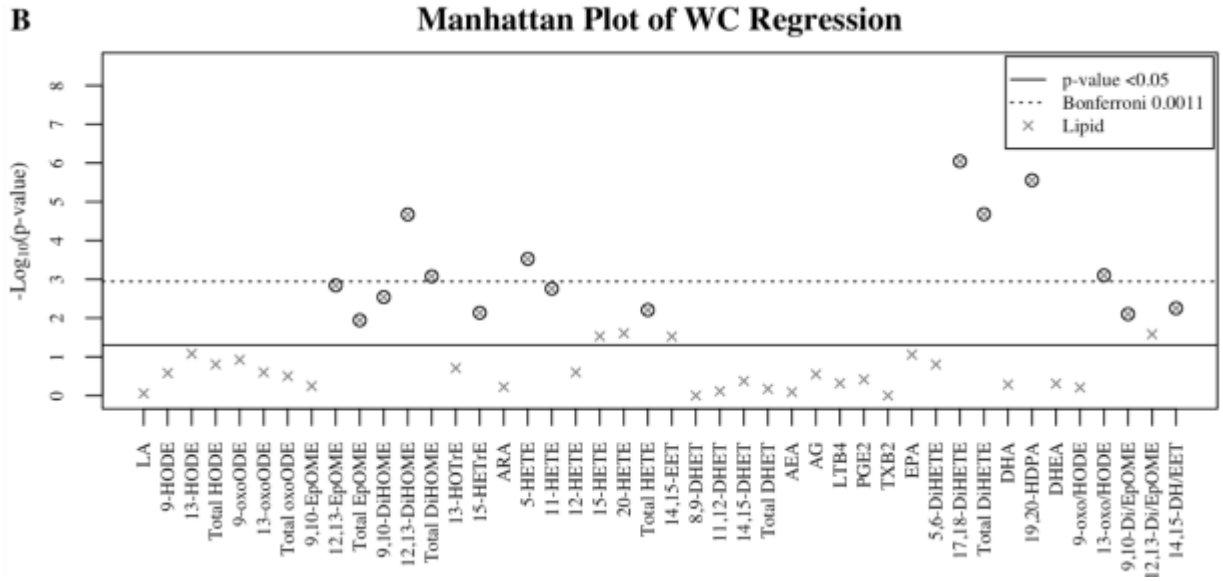


Table 12: Estimated effects of plasma non-esterified fatty acids and oxylipids from single lipid regression models.[a]

	Body Mass Index		*Waist Circumference*	
Oxylipid	**Beta**	**p-value**	**Beta**	**p-value**
LA	0.52	-	0.14	-
9-HODE	-0.59	-	-0.89	-
13- HODE	-0.91	-	-1.35	-
Total HODE	-0.76	-	-1.12	-
9-oxoODE	-0.97	-	-1.24	-
13-oxoODE	1.08	-	0.87	-
Total oxoODE	0.22	-	0.26	-
9,10-EpOME	0.04	-	-0.58	-
12,13-EpOME	-2.11	p≤0.01	-2.78	p≤0.005
Total EpOME	-1.69	p≤0.05	-2.42	p≤0.05
9,10-DiHOME	-1.70	p≤0.001	-1.64	p≤0.005
12,13-DiHOME	-2.53	p≤0.0001	-2.62	p≤0.0001
Total DiHOME	-1.95	p≤0.0001	-1.93	p≤0.0001
13-HOTrE	-0.35	-	-0.77	-
15-HETrE	1.94	p≤0.005	1.99	p≤0.01
ARA	0.89	-	0.47	-
5-HETE	1.35	p≤0.005	1.65	p≤0.005
11-HETE	1.54	p≤0.005	1.78	p≤0.005
12-HETE	1.00	*0.076*	0.75	-
15-HETE	1.60	p≤0.01	1.53	p≤0.05
20-HETE	2.28	p≤0.005	2.09	p≤0.05
Total HETE	0.54	-	0.66	-
14,15-EET	1.47	p≤0.05	1.82	p≤0.05
8,9-DHET	-0.15	-	0.00	-
11,12-DHET	-0.60	-	-0.37	-
14,15-DHET	-0.79	-	-1.16	-
Total DHET	-0.90	-	-1.47	-
AEA	0.97	-	0.35	-
AG	-0.39	-	-0.91	-

[a]Beta coefficients and p-values were determined by regressing body mass index (BMI) and waist circumference (WC) on log transformed PUFAs, oxylipids, and oxylipid parent-to-precursor ratios individually (i.e., one at a time). Single non-esterified plasma fatty acid, oxylipid, and product to precursor ratio regression models are defined as: BMI_i or $WC_i = age_i + smoking_i + \log(lipid_{ij})$, where $i = (1,...,123)$ and $j = (1,...,44)$.

Table 12 (cont'd)

LTB4	0.52	-	0.53	-
PGD2	-0.69	-	-0.44	-
TBX2	0.28	-	0.00	-
EPA	-0.46	-	-0.83	*0.087*
5,6-DiHETE	-0.70	-	-0.82	-
17,18-DiHETE	-2.43	p≤0.0001	-3.19	p≤0.0001
Total DiHETE	-1.39	p≤0.005	-1.69	p≤0.001
DHA	0.11	-	-0.54	-
19,20-HDPA	-3.52	p≤0.0001	-4.48	p≤0.0001
DHEA	-0.23	-	-0.64	-
9-oxo/HODE	-0.51	-	-0.46	-
13-oxo/HODE	2.63	p≤0.0005	2.91	p≤0.001
9,10-Di/EpOME	-1.72	p≤0.0005	-1.47	p≤0.01
12,13-Di/EpOME	-2.03	p≤0.005	-1.67	p≤0.05
14,15-DH/EET	-1.89	p≤0.05	-2.40	p≤0.01

Table 13: Polyunsaturated fatty acids and oxylipids are highly correlated with factor-loading matrix.[a]

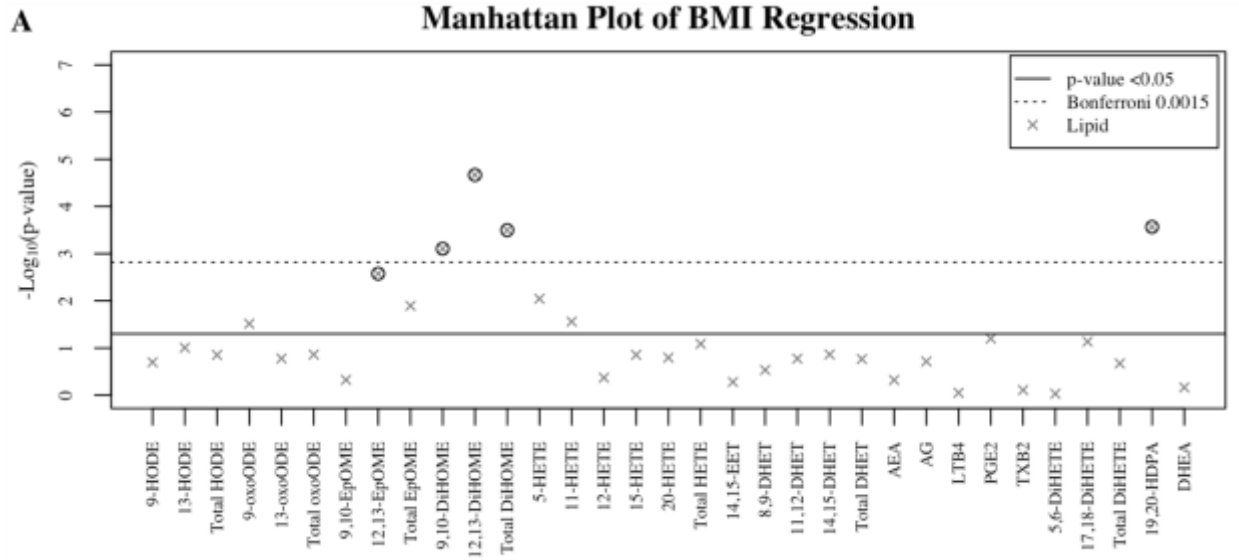
Lipids	HETEs	Octadecanoids	ω-6 PUFA	ω-3 PUFA
11-HETE	**0.82**	0.28	0.07	-0.18
15-HETE	**0.80**	0.25	0.10	-0.19
15-HETrE	**0.75**	0.33	0.09	-0.20
5-HETE	**0.74**	0.08	0.02	-0.12
8,9-DHET	**0.65**	-0.02	0.07	0.18
11,12-DHET	**0.63**	-0.14	0.21	0.44
14,15-DHET	**0.61**	-0.10	0.29	0.39
12-HETE	**0.61**	0.03	0.33	-0.07
14,15-EET	**0.58**	-0.08	-0.09	0.10
9-HODE	0.22	**0.88**	0.14	0.06
13-HODE	0.04	**0.88**	0.08	0.11
9,10-DiHOME	0.00	**0.79**	-0.03	0.03
12,13-DiHOME	-0.01	**0.78**	-0.11	0.19
12,13-EpOME	-0.19	**0.71**	0.25	0.29
13-HOTrE	0.16	**0.54**	-0.10	0.10
LA	0.03	0.02	**0.92**	0.05
ARA	0.03	0.01	**0.86**	0.00
9,10-EpOME	0.12	0.38	**0.70**	0.14
9-oxoODE	0.23	0.44	**0.65**	0.13
13-oxoODE	0.46	0.46	**0.59**	-0.11
AEA	0.00	-0.16	**0.56**	-0.05
20-HETE	0.41	-0.09	**0.55**	0.24
19,20-HDPA	0.09	0.18	0.01	**0.87**
17,18-DiHETE	-0.03	0.19	-0.16	**0.83**
5,6-DiHETE	0.30	0.06	-0.10	**0.74**
EPA	-0.05	0.09	0.32	**0.66**
DHA	0.08	0.00	0.48	**0.59**
DHEA	-0.17	0.16	-0.01	0.40
PGD2	-0.02	-0.09	-0.12	0.01
Ag	0.02	0.00	0.42	-0.01
TXB2	0.02	-0.23	0.32	0.05
LTB4	0.38	-0.03	-0.01	0.12

[a]Varimax rotated factor-loading matrix generated using the R package 'psych'. Factors were named based on the majority of lipids with high correlations in each factor, respectively. Numbers displayed under each factor represent each polyunsaturated fatty acid or oxylipid correlation with each factor, and correlations >0.5 are bolded.

PUFA normalized vicinal diols are inversely associated with obesity

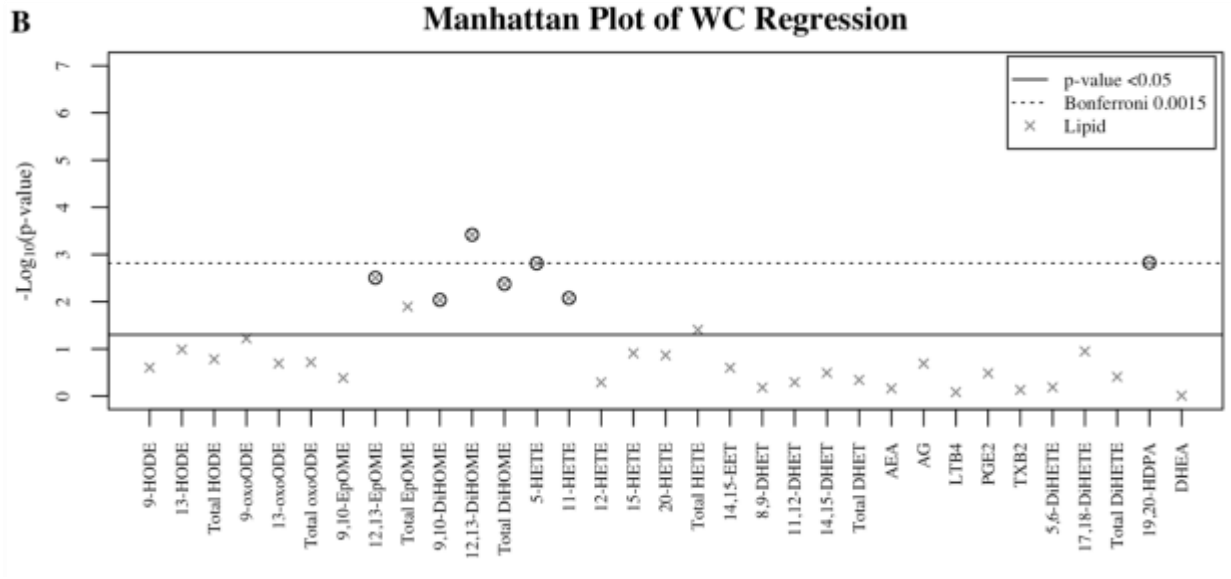
Given that PUFAs are obtained through dietary intake, and PUFAs serve as substrates for oxylipids, each oxylipid was then normalized to its parent PUFA. This was performed to assess whether our associations between anthropometrics and oxylipids were, in fact, due to altered levels of PUFA substrates or altered metabolic pathways affecting oxylipid production. Each patient's LA- and ARA-derived oxylipids were normalized to their LA and ARA levels, respectively, and each patient's EPA- and DHA-derived oxylipids were normalized to their EPA and DHA μM concentrations, respectively. BMI and WC were regressed on PUFA-normalized oxylipids, and models were adjusted for age and smoking. P-values from single regressions that were significant after BH FDR correction are circled, and p-values significant below Bonferroni cutoff ($p<0.0015$) are displayed above the hashed line in Manhattan plots (Figure 15). The estimated effects and p-values for BMI and WC regressions with PUFA-normalized oxylipids are presented in Table A15. The regression results for BMI and WC were similar. After normalization to LA, the oxylipids 12,13-EpOME, Total EpOME, 9,10-DiHOME, 12,13-DiHOME, and Total DiHOMEs were inversely associated with BMI and WC. ARA-derived oxylipids were positively associated with BMI and WC after normalization with ARA, in particular, 5-HETE and 11-HETE (Figure 15). 17,18-DiHETE normalized to EPA concentrations tended to be inversely associated with BMI. BMI and WC were both inversely associated with DHA normalized 19,20-HDPA concentrations.

Figure 15: Vicinal diols and hydroxyeicosatetraenoates normalized to parent polyunsaturated fatty acids are associated with obesity.[a]



[a]Manhattan plots of the –log10(p-value) for body mass index (BMI) and waist circumference (WC) regressed on log transformed PUFA-normalized oxylipids (i.e., 9,10-DiHOME/LA and 5-HETE/ARA) and oxylipid totals individually (i.e., one at a time). Single non-esterified plasma PUFA-normalized oxylipid models are defined as: BMI_i or $WC_i = age_i + smoking_i + \log(lipid_{ic})$, where $i = (1,...,123)$ and $c = (1,...,33)$. Each y-axis represents -log10(p-value) for each respective model, and x-axis represent the plasma non-esterified oxylipid or fatty acid. Plasma lipids with Benjamini-Hochberg false discovery rate p-values ≤0.05 are circled. Oxylipids totals calculated as follows: Total HODE = (∑9-HODE + 13-HODE)/LA; Total oxoODE = (∑ 9-oxoODE + 13-oxoODE)/LA; Total EpOME = (∑ 9,10-EpOME + 12,13-EpOME)/LA; Total HETE = (∑ 5-HETE + 11-HETE + 12-HETE + 15-HETE + 20-HETE)/ARA; Total DHET = (∑ 8,9-DHET + 11,12-DHET + 14,15-DHET)/ARA; Total DiHETE = (∑ 5,6-DiHETE + 17,18-DiHETE)/EPA.

Figure 15 (cont'd)



Obesity is associated with specific concentrations of oxylipids

Finally, since oxylipid concentration ranges are not well characterized in obesity, we separated several of our highly significant epoxides, HETEs, and vicinal diols into tertiles, to determine concentration ranges that are most likely to be associated with obesity. The response in polytomous logistic regressions was BMI category and models were adjusted for age and smoking. Categorical increases for 9,10-EpOME, 12,13-EpOME, and 9,10-DiHOME were not significantly associated with obesity compared to lean individuals (Table 14). However, for each tertile increase in 5-HETE, an individual was 2.57 times more likely to be obese rather than lean. Participants with 5-HETE concentrations either > 1.86 to ≤ 5.01nM or > 5.01 nM, were over 5 times more likely to be obese than those with 5-HETE concentrations ≤ 1.86 nM. Results for 11-HETE were similar to 5-HETE, for each tertile increase in 11-HETE, an individual was 2.4 times more likely to be obese rather than lean. Participants with 11-HETE concentrations > 0.89 nM were 5 times more likely to be obese than those with 11-HETE concentrations ≤ 0.39 nM. Vicinal diols derived from EPA and DHA were inversely associated with obesity. For each tertile increase in 17,18-DiHETE concentrations, an individual was 0.29 times as likely to be obese rather than lean. Participants with 17,18-DiHETE concentrations > 10.10 nM were roughly 0.09 times as likely to be obese than those with 17,18-DiHETE concentrations ≤ 5.59 nM. Similarly, for each tertile increase in 19,20-HDPA, an individual was 0.23 times as likely to be obese rather than lean. Patients with 19,20-HDPA concentrations > 3.77 nM were 0.05 times as likely to be obese than those with 19,20-HDPA concentrations ≤ 2.37 nM.

Table 14: Associations of non-esterified plasma oxylipids, as tertiles, with being obese relative to lean.

	Test for exposure	*Test for trend*
Plasma Concentration[a]	**OR[95% CI][b]**	**OR (p trend) [b]**
9,10-EpOME (nM)		1.21
≤ 16.2	1	(0.5185)
16.2 to ≤ 28.2	1.45 [0.46, 4.54]	
> 28.2	1.46 [0.45, 4.79]	
12,13-EpOME (nM)		0.79
≤ 29.6	1	(0.4211)
>29.6 to ≤51.9	1.11 [0.35, 3.51]	-
>51.9	0.61 [0.19, 1.93]	-
9,10-DiHOME (nM)		0.64
≤ 5.7	1	(0.1463)
>5.7 to ≤ 12.9	0.72 [0.26, 3.16]	
> 12.9	0.41 [0.13, 1.36]	
5-HETE (nM)		**2.57**
≤ 1.86	1	**(p≤0.005)**
>1.86 to ≤ 5.01	**7.28 [1.87, 28.36]**	
> 5.01	**5.30 [1.65, 17.05]**	
11-HETE (nM)		**2.40**
≤ 0.39	1	**(p≤0.01)**
> 0.39 to ≤ 0.89	*2.81 [0.86, 9.21]*	
> 0.89	**5.18 [1.53, 17.55]**	
17,18-DiHETE (nM)		**0.29**
≤ 5.59	1	**(p≤0.0005)**
> 5.59 to ≤ 10.10	0.68 [0.18, 2.60]	
> 10.10	**0.09 [0.02, 0.34]**	
19,20-HDPA (nM)		**0.23**
≤ 2.37	1	**(p≤0.0001)**
> 2.37 to ≤ 3.77	0.34 [0.08, 1.47]	
>3.77	**0.05 [0.01, 0.21]**	

[a]Plasma non-esterified oxylipids were separated into tertiles.
[b]Polytomous logistic regression was used to regress BMI category on oxylipid tertiles. All data is referenced against the lean BMI category. Both test for trend and test for exposure were adjusted for age and smoking. P-values bolded if p≤0.05 or italicized is p≤0.09 and >0.05.

Discussion

This study characterized non-esterified plasma PUFA and oxylipid concentrations associated with obesity. We report that BMI and WC are positively associated with monohydroxy ω-6 PUFAs derived from DGLA and ARA, specifically 15-HETrE, and 5-, 11-, 15-, and 20-HETE, respectively. In general, oxylipids derived from the ω-6 PUFA LA were inversely associated with obesity, especially epoxides and vicinal diols. In fact, our most significant result is that vicinal diols derived from LA and the ω-3 PUFAs EPA and DHA were inversely associated with obesity across several statistical tests, even after normalization to parent PUFA concentrations. Non-esterified plasma PUFAs did not differ with BMI or WC, and although we report the ω-3 PUFA factor was inversely associated with obesity, this is largely due to the fact that ω-3 vicinal diols were driving ω-3 PUFA factor-loading scores (Table 13). Taken together, our results indicate obesity is associated with specific oxylipids indicative of altered PUFA metabolism through several pathways, rather than attributed to solely altered dietary PUFA intake.

Typical American diets consist of a ω-6:ω-3 PUFA ratio of approximately 10:1 as a result of increased consumption of vegetable oil with a concomitant decrease in fish oil consumption [186]. In murine models, a high-fat diet (HFD) induces obesity-associated inflammation [187, 188], which mimics chronic low-grade inflammation and metabolic dysregulation in obese humans [189]. In obesity, CYP epoxygenase mRNA is decreased in adipose tissue [170] and sEH is increased in adipose tissue [190] and liver [191]. Increased sEH activity is associated with inflammation (i.e., TNF-α) in several chronic inflammatory diseases, and sEH is a target for pharmacological treatments (reviewed in detail [179]). One would expect that since sEH activity is higher in obesity there would be an increase in vicinal diols. However, here we show that vicinal

diols from several PUFAs are inversely associated in the plasma. This observation could be explained by shifts in the esterification of epoxides and diols into phospholipids, such as a mechanism of sequestration of diols, or our observation may reflect a depletion of epoxides and accumulation of diols in tissues that regulate metabolic processes dysregulated in obesity.

When sEH is inhibited, the production of diols decreases (reviewed in detail [192]), thus, inhibiting sEH to study epoxides leads to the effects of diols being underreported. For instance, it is known that inhibiting sEH increases epoxide esterification into phospholipids [193] and decreases inflammation in tissues [194]. A majority of research into vicinal diols has focused on those derived from ARA and LA (reviewed in detail [195]). We report ARA-derived DHETs are not significantly associated with BMI or WC when analyzed individually, however, the ratio of 14,15-DHET to 14,15-EET was inversely associated both BMI and WC. Unfortunately, 8,9-EET and 11,12-EET had >50% missing values, so these variables were excluded; thus, we could not calculate the ratios of all DHETs to EETs. To our knowledge, we are one of the first groups to report that plasma vicinal diol concentrations are decreased in obese humans. Future research should investigate whether changes in plasma vicinal diol concentrations could be used as markers of metabolic dysregulation or if decreases in plasma vicinal diols affect obesity-associated pathology.

Additionally, we report HETEs are elevated in obesity. Increases in 5- and 11-HETE were significantly associated with BMI and WC across all statistical tests. 5-HETE is a 5LOX product which is elevated in visceral obese adipose tissue [196]. In a recent randomized controlled trial, supplementation with fish oil did not reduce 5-HETE concentrations in overweight subjects, which suggests increased 5-HETE is due to altered metabolism rather than PUFA substrate availability [197]. 5-HETE increases TNF-α induced apoptosis of hepatocytes and inhibition of 5LOX

decreases hepatic macrophage infiltration of HFD obese mice [198]. It is possible that increases in proinflammatory 5-HETE contributes to pathological changes in obese tissues that promote oxidative stress and increase ROS, which can affect PUFA peroxidation. Interestingly, 11-HETE is a non-enzymatically ARA-derived oxylipid which is a marker of lipid peroxidation [199]. Increased 11-HETE is described as a biomarker ranging from coronary events [200] to cancers [201], however, to our knowledge there is no evidence associating 11-HETE with obesity in humans. Since obesity is associated with coronary heart disease and cancers, it is unsurprising that 11-HETE may be a biomarker of pathology associated with obesity. We report individuals with 11-HETE concentrations >0.89nM were over 5 times more likely to be obese compared to those with ≤ 0.39 nM. Taken together, our results may have profound implications for novel biomarkers of alerted metabolism in obesity if: the significant plasma oxylipids reported in this study directly impact inflammation in obesity; or if plasma concentrations of oxylipids are able to distinguish obese individuals with low-grade inflammation in larger more diverse sample sizes.

CHAPTER 5: ALTERED SATURATED AND MONOSATURATED PLASMA PHOSPHOLIPID FATTY ACID PROFILES IN ADULT MALES WITH COLON ADENOMAS

This chapter has been published in *Cancer Epidemiol Biomarker Prev.* Pickens et al., Altered saturated and monosaturated plasma phospholipid fatty acid profiles in adult males with colon adenomas. 2016 Mar;25(3):498-506

Abstract

Background

Altered lipid metabolism and plasma fatty acid (FA) levels are associated with colorectal cancer (CRC). Obesity and elevated waist circumference (WC) increase the likelihood of developing precancerous colon adenomas.

Methods

Venous blood was collected from 126 males, ages 48 to 65 years, who received routine colonoscopies. Plasma phospholipid (PPL) FAs were isolated, derivatized, and then analyzed using gas chromatography. Odds ratios (ORs) and 95% confidence intervals were determined using polytomous logistic regression after adjusting for confounding factors (i.e. age, smoking, WC, and BMI).

Results

PPL palmitic acid (PA) was inversely correlated with the presence of colon adenomas ($p = 0.01$). For each unit increase in palmitoleic acid (OR: 3.75, $p = 0.04$) or elaidic acid (OR: 2.92, $p = 0.04$) an individual was more likely to have adenomas relative to no colon polyps. Higher enzyme activity estimates (EAEs) of stearoyl-CoA desaturase-1 (SCD-1, $p = 0.02$) and elongation of very long chain-6 (Elovl-6, $p = 0.03$) were associated with an individual being approximately 1.5 times more likely to have an adenoma compared to no polyps.

Conclusions

PPL FAs and EAEs, which have previously been associated with CRC, are significantly different in those with adenomas when compared to those without polyps. PPL PA, elaidic acid, and SCD-1 and Elovl-6 EAEs are associated with adenomas independent of BMI and WC.

Impact

PPL PA, elaidic acid, and SCD-1 and Elovl-6 EAEs are associated with adenomas even after adjusting for obesity-related risk factors and may function as novel biomarkers of early CRC risk.

Introduction

Colorectal cancer (CRC) is the third most prevalent cancer among men and women in the US [202]. Risk factors for CRC include obesity, waist circumference (WC), age, smoking, physical inactivity, inflammatory bowel disease, and a family history of CRC or adenomas [203]. As much

as 70% of the risk of developing CRC has been attributed to modifiable risk factors, including diet [204]. Consequently, dietary intake of varying amounts of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) has been an area of active research in the pathology and prevention of CRC.

Several specific FAs are associated with CRC. For example, a higher erythrocyte oleic acid (OA) to stearic acid (SA) ratio has been associated with CRC [205]. Also, CRC is associated with higher levels of plasma phospholipid (PPL) SFAs, in particular palmitic acid (PA) [206]. Among PUFAs, dietary consumption of greater amounts of ω-3 PUFAs and lesser amounts of ω-6 PUFAs is typically associated with a decreased risk for developing CRC [207]. Diets higher in MUFAs and lower in SFAs also potentially prevent CRC [208]. Blood FAs associated with CRC may originate from dietary intake as well as from endogenous synthesis though lipid metabolism.

Altered lipid metabolism also is suspected to play a role in colon carcinogenesis during the transformation of colorectal polyps to CRC [208-210]. Dietary SFAs can be desaturated and elongated through the action of various enzymes. Stearoyl-CoA desaturase-1 (SCD-1) and elongation of very long chain fatty acid protein-6 (Elovl-6) are the rate-limiting enzymes controlling metabolic shifts towards production of long chain MUFAs. Upregulation of SCD-1, the desaturase responsible for converting PA and SA into MUFAs, has been linked to CRC [210]. MUFAs influence cellular apoptosis and are believed to play a role in the mutagenesis of tumors in several types of cancer, including CRC [209, 211]. However, FAs and the enzymes that regulate the endogenous production of long chain MUFAs have not been sufficiently investigated in relation to precancerous colon adenomas. Additionally, the complex mechanisms by which dietary FAs and lipid metabolism influence the development of CRC continue to be investigated.

The formation of adenomas precedes the onset of CRC, with removal of adenomas significantly decreasing the risk of developing CRC [212]. Determining the levels of specific PPL FAs associated with the presence of adenomas could lead to the identification of blood-based biomarkers useful for early CRC screening, increasing opportunities for preventative interventions. PPLs are reflective of endogenous and exogenous sources of FAs and have been used to measure CRC risk in relation to FA intake [206, 213]. Limitations in accurately measuring dietary intake combined with the need to assess endogenous lipid synthesis dictate that the direct analysis of PPLs is necessary in order to accurately determine the association between plasma FAs and colon carcinogenesis. Therefore, in this study we sought to identify specific PPL levels of SFAs, cis-MUFAs, and trans-MUFAs associated with the presence of colorectal adenomas

Materials and Methods

Study population and clinical parameters

Healthy male subjects (n = 126, > 96% Caucasian) 48 to 65 years of age were enrolled as previously reported [2]. Individuals were excluded for medical conditions associated with increased CRC risk [2]. Immediately after enrollment, trained staff collected anthropometric measurements and venous blood of study participants [2]. Smoking status was assessed as “ever smoked” or “never smoked”. Each individual received a full colonoscopy as previously described [4]. Serum and plasma fractions were separated from blood and stored at -80° C.

Plasma phospholipid extraction, isolation and analysis

In brief, approximately 200 mg plasma per subject was weighed and extracted using a modified Rose and Oaklander extraction [108]. PPLs were isolated using Isolute-XL ® SPE aminopropyl columns (500 mg; Bioatage, Charlotte, NC) as described by Agren et al [109]. Fatty acid methyl esters (FAMEs) were prepared as previously described [5, 214]. PPL FAMEs were analyzed using HS-Omega-3 Index® methodology at OmegaQuant Analytics, LLC (Sioux Falls, SD) as previously described [110]. The coefficient of variation for PPL extraction, isolation, and PPL FA analysis is less than 7% for the eleven FAs presented.

Statistical analyses

Frequencies, means, and standard deviations were calculated for descriptive analyses (Table 15). Each FA was expressed as a percentage of total PPL. Means were obtained for the PL FAs (Figure 16). PPL FA enzyme activity estimates (EAE) were calculated as the ratio of product-to-substrate. SCD-1 EAE was calculated in two ways [215]: SCD n-7 index (SCD n-7) = palmitoleic (POA) / PA, and SCD n-9 index (SCD n-9) = OA / SA. A variation of the Elovl-6 EAE was calculated as Elovl-6 = $\sum$ [SA + OA] / PA [216, 217]. The total PPL SFA, cis-MUFA, trans-MUFA were calculated as follows: total PPL SFA was calculated as $\sum$ PA + SA + arachidic + behenic + lignoceric; total PPL cis-MUFA was calculated as $\sum$ POA + eicosaenoic + nervonic (NA); total PPL trans-MUFA was calculated as $\sum$ palmitelaidic + elaidic. Spearman correlations were performed since several variables were not normally distributed. These correlations, presented in Table 16, were conducted using only the 106 individuals that had adenomas or no polyps.

Multiple imputation (seed = 20121119, imputations = 7) was used to impute all missing smoking data [218]. The factors—smoking, PA, SA, arachidic, behenic, lignoceric, POA, OA, NA, palmitelaidic, elaidic—were used in the imputation algorithm of missing values. Eicosenoic acid was removed from the imputation algorithm due to a high correlation with elaidic acid.

The Wilcoxon-Mann-Whitney test was performed to compare the PPL FA composition of participants with adenomas to that of those with no polyps. Polytomous logistic regression models for categorical outcome data were used to determine odds ratios (OR) and 95% confidence intervals (CI) for the likelihood of having an adenoma relative to no polyps. Categories were defined as polyp type: 1) Individuals with no colon polyps, and 2) Individuals with ≥ 1 adenoma. Individuals with polyps not classified as adenomas were excluded from statistical analyses. In all polytomous logistic regression models, polyp type was analyzed categorically as the dependent variable with the reference category defined as individuals with no colon polyps. The odds ratios for Elovl-6, SCD n-7, and palmitelaidic acid have been calculated on the basis that there is a unit change of 0.01 for the respective beta coefficient for each given parameter. All models were adjusted for age and smoking status except where noted.

Due to high correlation (> 0.9, data not shown) between BMI and WC, these anthropometric measurements could not be analyzed in the same model. Two additional models were run, the first with the addition of BMI and the second with the addition of WC. These models are referred to as model 2 and model 3 respectively (Table 17). FAs were analyzed as continuous (Table 16 and 17, and Figure 16) and categorical independent variables (Figure 17). FAs were categorized into tertiles (with lowest tertile as reference) for adenomas relative to no polyps. Test for trend was carried out across tertiles for the FAs of interest. Because smoking data was imputed, multiple imputation analyze (Proc MI ANALYZE) was used to determine the results from analysis

of the imputed datasets. P-values were considered statistically significant if $p \leq 0.05$ and a statistical trend if $0.05 < p \leq 0.09$. Statistical analyses were conducted using SAS version 9.3 (Cary, NC).

Results

Participant characteristics are displayed in Table 15. As previously reported [2], 37 (29.4%) participants had adenomas while 69 (54.8%) had no polyps. Seventeen (13.5%) participants had ≥ 3 polyps including at least one adenoma. Both BMI and WC increased with polyp type, as previously reported [2]. The PPL FA proportions are presented in Figure 16. PPL PA was significantly lower and total SFAs tended (p=0.0684) to be lower in those with adenomas compared to those without polyps. The PPL cis-MUFA POA was significantly higher in individuals with adenomas, while PPL cis-MUFA NA was significantly lower in the adenoma group compared to those with no colon polyps. The percentage of total trans-MUFAs in PPLs did not differ between the groups.

Elongating and desaturating EAEs were positively associated with polyp type. SFAs are enzymatically desaturated to form cis-MUFAs. Both SFAs and cis-MUFAs can be enzymatically elongated to form longer chain products. SCD n-7, SCD n-9, and Elovl-6 EAEs are non-invasive methods to assess FA metabolism [219], calculated as the FA product-to-precursor ratio for respective EAE. We observed SCD n-7 was significantly elevated (p=0.0163) in those with adenomas compared to those with no colon polyps. However, SCD n-9 did not differ (p=0.5868) between individuals with no polyps and those with adenomas. Elovl-6 was significantly elevated (0.0105) in those with adenomas compared to those with no colon polyps.

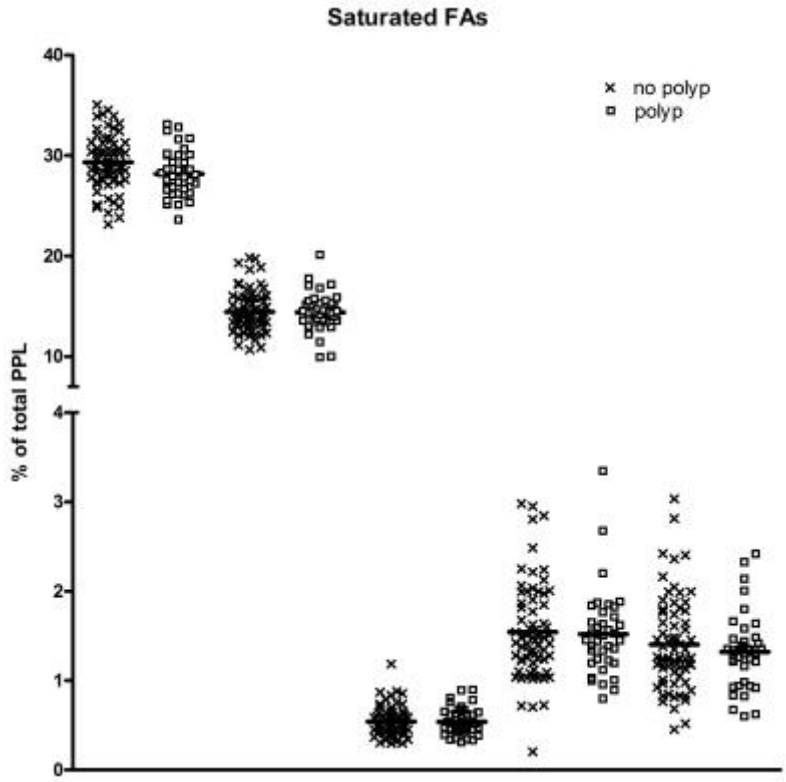
Table 15: Characteristics of study population.[a]

	Overall	No Polyp	Hyperplastic	Adenoma
	n = 126	n = 69	n = 20	n = 37
Age (years)	57 ± 5	57 ± 5	57 ± 4	57 ± 5
Ever Smoked (% total) [b]	31	15	4	12
BMI (kg/m^2)	30 ± 5	28 ± 4	29 ± 5	32 ± 6
WC (inches)	41 ± 6	40 ± 6	42 ± 4	44 ± 6

[a]Participants (n = 126) were male, > 96% Caucasian; Values expressed as mean ± standard deviation.
[b]Data missing for 22 participants.
BMI: body mass index; WC: waist circumference.

Figure 16: Fatty acid content of plasma phospholipids.

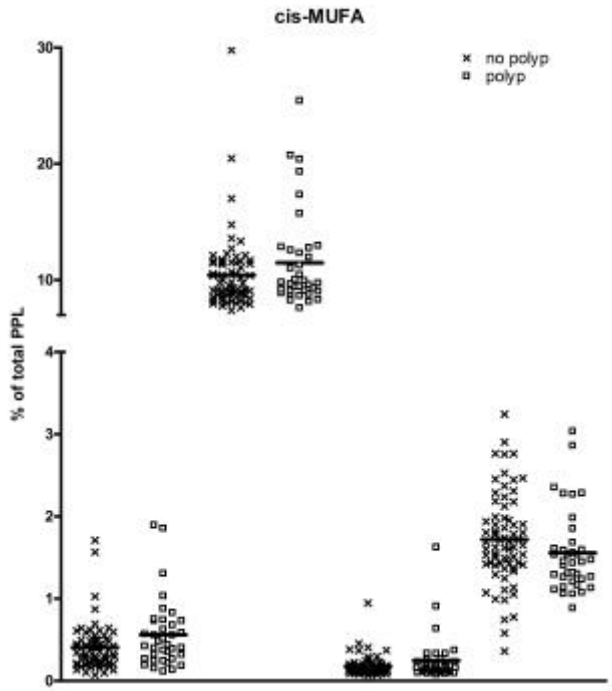
A.



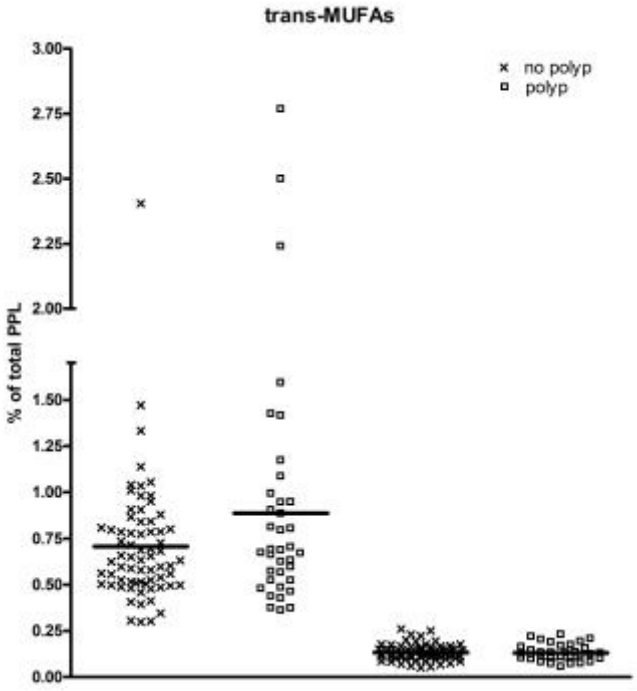
(A) Saturated FAs (SFAs), (B) Cis-monounsaturated FAs (MUFAs), and (C) Trans-MUFAs. The symbol "X" represents PPL FA levels of individuals with no polyps and "□" represents PPL FA levels of individuals with adenomas. The solid lines indicate the mean. FAs are expressed as a percent of total PPL FAs. A "*" indicates $p \leq 0.05$, calculated by Wilcoxon-Mann-Whitney nonparametric U-test. NA, nervonic acid; OA, oleic acid; PA, palmitic acid; POA, palmitoleic acid; SA, stearic acid.

Figure 16 (cont'd)

B.



C.



Several PPL FAs measured were significantly correlated with polyp type and with other SFAs and MUFAs (Table 16). Polyp type was not correlated with PPL palmitelaidic, elaidic, or total trans-MUFA. Polyp type was inversely correlated with PPL PA and NA (Table 16). Also, polyp type was positively correlated with PPL POA, SCD n-7, and Elovl-6. Colon polyps and several PPL FAs were correlated with confounding factors such as age, smoking status, BMI, and WC (data not shown). Polytomous logistic regression was performed to determine which PPL FAs and EAEs were significantly associated with adenomas after adjusting for these confounding factors (Table 17). Model 1 included PPL FA, and was adjusted for age and smoking. To account for the potential contribution of BMI or visceral adiposity (WC) to the likelihood of having an adenoma, two additional models were tested. Model 2 included PPL FA and was adjusted for BMI, in addition to age and smoking. Model 3 included PPL FA and was adjusted for WC, in addition to age and smoking.

The odds that an individual whose PPL contained high levels of PA would have an adenoma were significantly lower than those of an individual whose PPL contained low levels of PA. This was consistent across all three models (Table 17). Specifically, for each unit increase in PPL PA individuals were 0.83 (95% CI: 0.70 - 0.98) times as likely in model 1, 0.72 (95% CI: 0.58 - 0.89) times as likely in model 2, and 0.76 (95% CI: 0.62 - 0.92) times as likely in model 3 to have adenomas rather than no colon polyps. PPL SA, arachidic, behenic, and lignoceric acid showed no association with adenomas in these 3 models. However, for each unit increase in total PPL SFAs, individuals tended to be 0.90 (95% CI: 0.80 - 1.01) times as likely to have adenomas in model 1, and individuals were 0.84 (95% CI: 0.73 - 0.96) and 0.85 (95% CI: 0.74 - 0.97) times as likely to have adenomas compared to no colon polyps when adjusted for BMI or WC, respectively.

Table 16: Spearman correlation between fatty acids and polyp type.[a]

	PA	SA	Arachidic	Behenic	Lignoceric	Total SFA [b]	POA	OA	Eicosenoic	NA	Total Cis-MUFA	Palmitelaidic	Elaidic	Total Trans-MUFA	SCD n-7	SCD n-9	Elovl-6
Polyp type	**-0.245**	0.042	0.003	-0.033	-0.051	*-0.185*	**0.195**	0.124	0.094	**-0.205**	0.102	-0.033	0.127	0.110	**0.228**	0.048	**0.255**
	0.011	0.665	0.973	0.740	0.603	*0.058*	**0.045**	0.207	0.340	**0.035**	0.296	0.738	0.195	0.260	**0.019**	0.624	**0.008**
PA		0.023	-0.005	-0.042	-0.035	**0.752**	**0.337**	-0.041	**-0.352**	-0.238	-0.087	-0.083	**-0.193**	*-0.190*	**0.216**	-0.044	**-0.768**
		0.816	0.957	0.672	0.718	**<.0001**	**0.0004**	0.675	**0.0002**	0.014	0.378	0.400	**0.047**	*0.051*	**0.026**	0.655	**<.0001**
SA			0.078	0.150	-0.053	**0.551**	**-0.277**	**-0.526**	-0.149	**-0.346**	**-0.584**	-0.028	*0.180*	0.156	**-0.282**	**-0.783**	**0.209**
			0.428	0.125	0.587	**<.0001**	**0.004**	**<.0001**	0.127	**0.0003**	**<.0001**	0.772	*0.065*	0.109	**0.003**	**<.0001**	**0.031**
Arachidic				**0.549**	**0.423**	**0.247**	-0.085	-0.047	0.161	**0.453**	0.064	-0.046	0.014	0.011	-0.095	-0.079	0.079
				<.0001	**<.0001**	**0.011**	0.386	0.630	0.099	**<.0001**	0.516	0.640	0.888	0.915	0.331	0.424	0.422
Behenic					**0.666**	**0.310**	**-0.199**	**-0.225**	0.117	**0.239**	-0.156	0.011	0.094	0.092	**-0.192**	**-0.236**	-0.010
					<.0001	**0.001**	**0.041**	**0.021**	0.231	**0.013**	0.111	0.914	0.340	0.348	**0.048**	**0.015**	0.918
Lignoceric						**0.192**	-0.083	-0.008	0.157	**0.274**	0.025	-0.032	-0.008	-0.015	-0.073	0.005	-0.007
						0.049	0.400	0.932	0.109	**0.005**	0.800	0.746	0.938	0.878	0.454	0.958	0.947
Total SFA							0.094	**-0.305**	**-0.260**	**-0.274**	**-0.347**	-0.110	-0.061	-0.075	0.001	**-0.457**	**-0.437**
							0.338	**0.002**	**0.007**	**0.004**	**0.0003**	0.262	0.531	0.445	0.991	**<.0001**	**<.0001**
POA								**0.634**	-0.088	**-0.239**	**0.598**	*-0.175*	**-0.275**	**-0.273**	**0.988**	**0.572**	-0.037
								<.0001	0.368	**0.014**	**<.0001**	*0.072*	**0.004**	**0.005**	**<.0001**	**<.0001**	0.704
OA									**0.227**	0.010	**0.946**	-0.005	*-0.166*	-0.149	**0.661**	**0.924**	**0.341**
									0.019	0.917	**<.0001**	0.957	*0.089*	0.127	**<.0001**	**<.0001**	**0.0003**
Eicosenoic										**0.255**	**0.336**	-0.151	**0.191**	*0.166*	-0.044	**0.205**	**0.424**
										0.008	**0.0004**	0.122	**0.050**	*0.089*	0.657	**0.035**	**<.0001**
NA											**0.244**	0.028	-0.074	-0.062	**-0.210**	0.155	0.016
											0.012	0.777	0.451	0.528	**0.031**	0.112	0.874
Total Cis-MUFA												-0.050	*-0.167*	-0.152	**0.626**	**0.918**	**0.330**
												0.614	*0.086*	0.120	**<.0001**	**<.0001**	**0.001**
Palmitelaidic													**0.338**	**0.463**	*-0.173*	0.003	-0.016
													0.0004	**<.0001**	*0.076*	0.977	0.868
Elaidic														0.987	**-0.259**	**-0.208**	0.128
														<.0001	**0.007**	**0.033**	0.192
Total Trans-MUFA															**-0.257**	*-0.188*	0.113
															0.008	*0.054*	0.250
SCD n-7																**0.592**	0.066
																<.0001	0.503
SCD n-9																	0.159
																	0.103

[a]Correlations were conducted using only the 106 individuals that had no polyps or adenomas. Numbers in gray rows indicate spearman correlation coefficient, and numbers listed directly below, in white rows, indicate corresponding p-value. P-values bolded if significant ($p \leq 0.05$) and italicized if $0.05 > p \leq 0.09$. EAE, enzyme activity estimate; NA, nervonic acid; OA, oleic acid; PA, palmitic acid; SA, stearic acid; SFA, saturated fatty acids; SCD n-7, stearoyl-CoA desaturase n-7 EAE; SCDn-9, stearoyl-CoA desaturase n-9 EAE. Elovl-6; elongation of very long chain fatty acids-6 EAE.

Table 17: Association of fatty acids and enzyme activity estimates, as continuous variables, with having adenomas relative to no colon polyps.[a]

	Model 1		Model 2		Model 3	
Variable	**OR (95%CI)**	**P-value**	**OR (95%CI)**	**P-value**	**OR (95%CI)**	**P-value**
C16:0 Palmitic (PA)	0.830 (0.701, 0.982)	**0.0303**	0.718 (0.582, 0.886)	**0.0020**	0.756 (0.623, 0.917)	**0.0045**
C18:0 Stearic (SA)	0.973 (0.806, 1.174)	0.7737	0.917 (0.748, 1.123)	0.4008	0.914 (0.747, 1.119)	0.3851
C20:0 Arachidic	0.901 (0.058, 13.943)	0.9403	1.053 (0.058, 19.211)	0.9721	0.978 (0.059, 16.274)	0.9877
C21:0 Behenic	0.923 (0.400, 2.131)	0.8504	0.916 (0.383, 2.196)	0.8449	0.936 (0.400, 2.188)	0.8778
C24:0 Lignoceric	0.732 (0.306, 1.754)	0.4842	0.925 (0.366, 2.335)	0.8686	0.848 (0.345, 2.085	0.7192
Total SFA[b]	0.902 (0.803, 1.013)	*0.0811*	0.837 (0.730, 0.960)	**0.0112**	0.847 (0.742, 0.967)	**0.0144**
C16:1 Palmitoleic (POA)	3.750 (1.079, 13.036)	**0.0376**	2.442 (0.679, 8.783)	0.1716	2.652 (0.739, 9.519)	0.1347
C18:1 Oleic (OA)	1.097 (0.981, 1.226)	0.1048	1.106 (0.983, 1.245)	0.0932	1.103 (0.981, 1.239)	0.1002
C20:1 Eicosenoic	1.020 (0.995, 1.045)	0.1161	1.023 (0.997, 1.050)	*0.0794*	1.024 (0.998, 1.051)	*0.0741*
C24:1 Nervonic (NA)	0.568 (0.260, 1.243)	0.1570	0.743 (0.327, 1.690)	0.4792	0.745 (0.329, 1.686)	0.4794
Total Cis-MUFA[c]	1.088 (0.981, 1.206)	0.1103	1.099 (0.985, 1.226)	0.0901	1.100 (0.987, 1.226)	*0.0864*
C16:1 Palmitelaidic[d]	0.982 (0.894, 1.078)	0.7017	1.013 (0.916, 1.119)	0.8073	1.006 (0.912, 1.108)	0.9116
C18:1 Elaidic	2.915 (1.030, 8.246)	**0.0438**	3.111 (1.031, 9.388)	**0.0440**	3.224 (1.060, 9.801)	**0.0391**
Total Trans-MUFA[e]	2.708 (1.000, 7.337)	*0.0501*	2.990 (1.029, 8.687)	**0.0441**	3.066 (1.050, 8.955)	**0.0405**
SCD n-7[d,f]	1.538 (1.068, 2.215)	**0.0207**	1.383 (0.960, 1.992)	*0.0819*	1.410 (0.977, 2.035)	*0.0664*
SCD n-9[g]	2.229 (0.724, 6.864)	0.1623	2.846 (0.850, 9.534)	*0.0899*	2.739 (0.822, 9.132)	0.1010
Elovl-6[d,h]	1.358 (1.039, 1.775)	**0.0250**	1.467 (1.090, 1.973)	**0.0114**	1.405 (1.059, 1.865)	**0.0184**

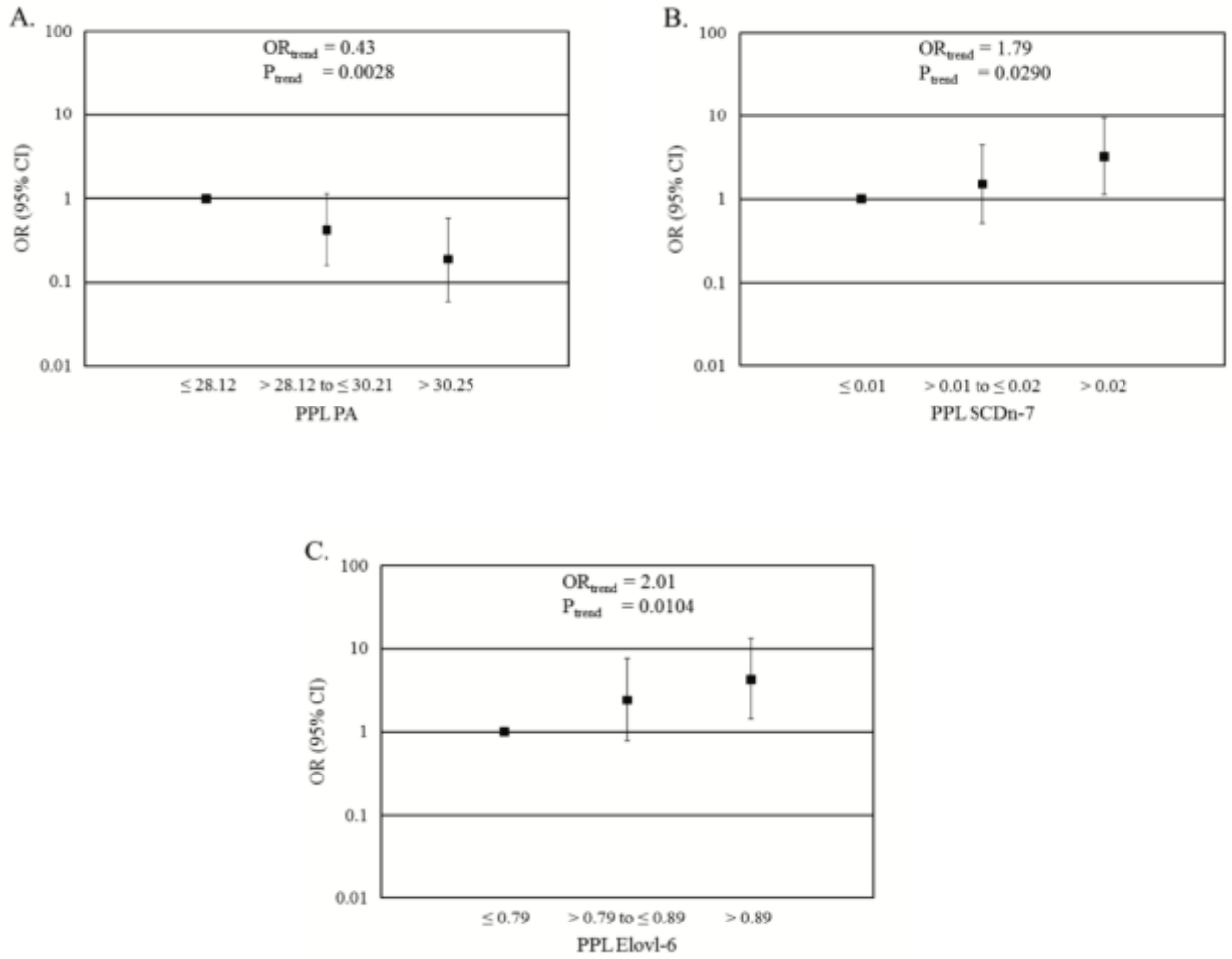
[a]Models defined as: Model 1: adenoma = fatty acid + age + smoking. Model 2: adenoma = fatty acid + age + smoking + BMI. Model 3: adenoma = fatty acid + age + smoking + waist circumference. Fatty acids expressed as percent of total phospholipids. P-values bolded if significant ($p \leq 0.05$) and italicized if $0.05 > p \leq 0.09$. EAE, enzyme activity estimate; MUFA, monounsaturated fatty acid; SFA, saturated fatty acids; SCDn-7, stearoyl-CoA desaturase n-7 EAE; SCDn-9, stearoyl-CoA desaturase n-9 EAE; Elovl-6, elongation of very long chain fatty acids-6 EAE. [b] Total SFA calculated as the $\sum$ PA+ SA + arachidic + behenic + lignoceric. [c] Total Cis-MUFA calculated as the $\sum$ POA+ OA + eicosenoic + NA. [d] Odds ratios for Palmitoelaidic, SCD n-7, and ELOVL-6 have been calculated on the basis that there is a unit change of 0.01 for the respective beta coefficient for each given parameter. [e] Trans-MUFA calculated as the $\sum$ palmitelaidic + elaidic. [f] SCDn-7 calculated as the ratio of POA / PA. [g] SCD n-9 calculated as the ratio of OA / SA. [h] Elovl-6 calculated as the ratio of $\sum$ [SA+ OA] / PA.

Some MUFAs were significantly associated with the presence of adenomas (Table 17). In model 1, for each unit increase in PPL POA, an individual was 3.75 (95% CI: 1.08 - 13.04) times more likely to have an adenoma compared to no colon polyps, but there were no significant associations after adjusting for BMI (model 2) or WC (model 3). PPL elaidic acid, a C18:1 trans-MUFA, was highly associated with an increased likelihood of adenoma presence in all 3 models analyzed. Specifically, for each unit increase in PPL elaidic acid individuals were 2.92 (95% CI: 1.03 - 8.25) times more likely in model 1, 3.11 (95% CI: 1.03 - 9.39) times more likely in model 2, and 3.22 (95% CI: 1.06 - 9.80) times more likely in model 3, to have adenomas relative to no colon polyps (Table 17). PPL palmitelaidic acid, a C16:1 trans-MUFA, was not significantly associated with adenomas. For each unit increase in PPL total trans-MUFA, calculated as the $\sum$ elaidic + palmitelaidic, individuals tended be 2.71 (95% CI: 1.00 - 7.34) times more likely to have adenomas in model 1, and individuals were 2.99 (95% CI: 1.03 - 8.69) and 3.07 (95% CI: 1.05 - 8.96) times more likely to have adenomas rather than no colon polyps in model 2 and model 3 respectively (Table 17).

Each unit increase in SCD n-7 was associated with individuals being 1.54 (95% CI: 1.07 – 2.22) times more likely to have adenomas than no polyps, and individuals with high SCD n-7 tended to be 1.38 (95% CI: 0.96 – 1.99) and 1.41 (95% CI: 0.98 – 2.04) times more likely to have adenomas rather than no polyps in models 2 and 3, respectively (Table 17). Unit increases in Elovl-6 were associated with adenomas in all 3 models analyzed. Specifically, for each unit increase in Elovl-6, individuals were 1.36 (95% CI: 1.04 - 1.78) times more likely in model 1, 1.47 (95% CI: 1.09 - 1.97) times more likely in model 2, and 1.41 (95% CI: 1.06 - 1.87) times more likely in model 3 to have adenomas relative to no colon polyps (Table 17).

Next, we separated several of our highly significant FAs and EAEs into tertiles, providing insight into the specific PPL FA ranges that were most likely to be associated with the presence of adenomas (Figure 17). For each tertile increase in PPL PA, individuals were 0.43 (95% CI: 0.25 - 0.75) times as likely to have adenomas rather than no colon polyps (Figure 17A). Categorical increases for POA, total SFA, elaidic, and total trans-MUFA showed no significant association with adenomas. However, for each tertile increase in SCD n-7, the calculated ratio of POA / PA, an individual was 1.79 (95% CI: 1.06 – 3.03) times more likely to have at least one adenoma rather than no polyps (Figure 17B). The association of Elovl-6 with colon adenomas was similar to the association of SCD n-7 with colon adenomas. For each tertile increase in Elovl-6, individuals were 2.01 (95% CI: 1.18 – 3.42) times more likely to have an adenoma rather than no polyps (Figure 17C).

Figure 17: Associations of plasma phospholipid fatty acid and enzyme activity estimates, as tertiles, with having adenomas relative to no colon polyps.



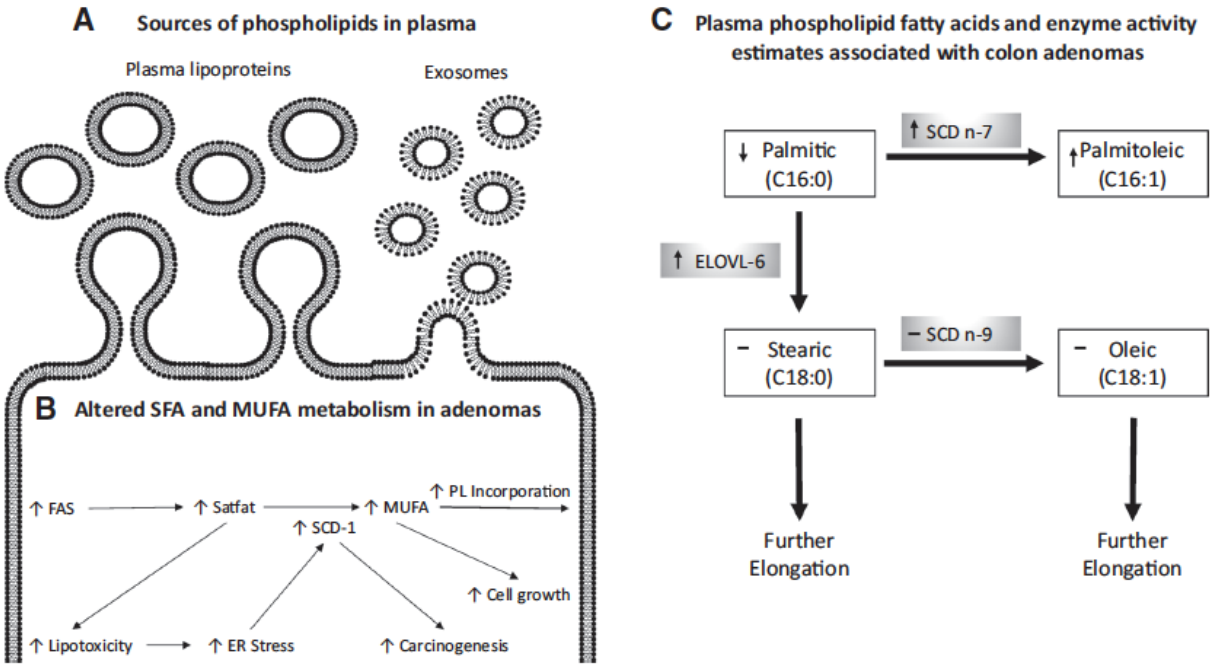
(A) Palmitic acid (PA), (B) ELOVL-6, and (C) SCDn-7. The symbol "■" represent the odds ratio and error bars indicate lower and upper confidence intervals, respectively. Both test for exposure and test for trend models adjusted for age and smoking. PA is expressed as a percent of total PPL FAs. Elovl-6, elongation of very long chain fatty acids-6 EAE, PA, palmitic acid; SCDn-7, stearoyl-CoA desaturase n-7 EAE.

Discussion

This study characterized PPL FA profiles associated with the presence of adenomas in adult males. Specifically, we report adenomas are positively associated with PPL elaidic, POA, total trans-MUFAs, as well as SCD n-7 and Elovl-6 EAEs. PPL PA was inversely associated with the presence of adenomas. These data indicate specific PPL FAs and EAEs are associated with adenomas even after adjusting for obesity, smoking, age, and elevated WC, which are factors known to increase CRC risk [203].

The PPL FA compartment is an ideal location for biomarker identification. Not only is the PPL FA compartment easily accessible to clinicians through a blood draw or simple blood spot using cards treated to prevent oxidation, but the PPL FA compartment also contains PL from sources such as plasma lipoproteins [220] and plasma microvesicle exosomes [221] (Figure 18A). Since PLs are endogenously synthesized, proportional differences in PPL FAs likely reflect cellular FA metabolism [222] (Figure 18B). If cellular FA metabolism is changed during the formation of adenomas, then new FA metabolites would be detectable in the PPL fraction. However, PL FA proportions in individuals also may reflect dietary FA intake [223], in addition to altered lipid metabolism associated [223] with colon carcinogenesis [209].

Figure 18: Relationship between cellular fatty acid metabolic pathways and observed associations of FAs and enzyme activity estimates with colorectal adenomas.



(A) Lipoproteins and exosomes are the most abundant sources of plasma phospholipid (PPL) FAs. (B) Increased fatty acid synthase (FAS) increases intracellular saturated fatty acids (SFAs). These SFAs have lipotoxic effects causing stearoyl-COA desaturase-1 (SCD-1) expression to increase. Higher concentrations of SFAs and expression SCD-1, increase monounsaturated FA (MUFA) production leading to increased PL MUFA incorporation and cellular enlargement. (C) Visual representation of PA metabolic pathway. PPL FAs appear in white boxes and EAEs appear in gray boxes. The arrow in each box indicates the direction of the association between the substrate and the likelihood of having an adenoma compared to having no colon polyps. A "–" indicates no observed association. Elovl-6, elongation of very long chain fatty acids-6 EAE; ER, endoplasmic reticulum; SCDn-7, stearoyl-CoA desaturase n-7 EAE; SCDn-9, stearoyl-CoA desaturase n-9 EAE.

The ability to easily measure changes in cellular fatty acid metabolism is important in the identification of biomarkers of colorectal polyp formation because colon adenomas are associated with changes in FA metabolism. For instance, colon adenomas are positively associated with fatty acid synthase (FAS) expression [224], which increases SFA synthesis, in particular PA synthesis [225]. Endogenous FA synthesis occurs in the smooth ER, where the enzymes ELOVL-6 and SCD-1 enzymes are located [226]. Elevated intracellular concentrations of SFAs are associated with increased lipotoxicity and endoplasmic reticulum (ER) stress [227-229]. The positive association of cellular stress responses and carcinogenesis are well documented (reviewed in detail [230]). Thus, our observation that higher SCD n-7 and Elovl-6 EAEs are associated with the presence of adenomas may be indicative of a cellular stress response to the process of carcinogenesis.

Aside from cellular stress, FA metabolism also increases during mitogenesis. Mitogenic factors associated with adenomas increase SCD-1 expression [231], which in turn increases *de novo* production of MUFAs such as POA [232]. In order for cell division to occur, cells must double their membrane FA content [233]. In particular, there is an increased demand for MUFA incorporation into PL membranes [209, 232]. Therefore, changes in FA metabolism (i.e. FAS, Elovl-6, and SCD-1) associated with increased cellular proliferation (i.e. adenomas), may be detectable by identifying specific proportions of PPL FAs and EAEs. Higher plasma SCD EAEs are associated with an increased risk of several cancers [234-236]. What remains unclear, is whether the resulting metabolites specifically participate in the process of carcinogenesis or if they are merely a by-product of the metabolism of abnormal cells. The visual representation of the PA pathway in Figure 18C incorporates results from our logistic regressions that demonstrate significant associations between the presence of adenomas and the PPL FAs and EAEs associated

with PA metabolism. Taken together, our data suggests the observed associations are likely the result of altered desaturation and elongation of PA during carcinogenesis.

PA is desaturated by SCD-1 to form the cis-MUFA POA. We used two separate estimates of SCD-1 activity, SCD n-7 and SCD n-9. We observed SCD n-7 EAE was positively associated with adenomas, and there was no association of SCD n-9 with adenomas. An increase in the proportion of plasma POA is positively associated with risk of future all-cause cancer mortality [237]. Higher levels of PPL POA are indicative of increased *de novo* synthesis, because dietary POA is rapidly oxidized after absorption resulting in negligible effects of dietary POA on the lipid profile [41]. Plasma SCD n-7 EAE positively correlates with SCD-1 enzyme activity measured in biopsied tissues, but SCD n-9 EAE does not [238]. Aside from PA, no other SFAs analyzed (SA, arachidic, behenic, or lignoceric) had significant associations with adenomas. We speculate the inverse association between PPL PA and adenomas reflects underlying changes in PA metabolism such as increased desaturation.

Our cross-sectional study was conducted in a population of males (n=126, > 96% Caucasian, ages 48-65) to identify associations between colon polyps and PPL FAs or EAEs. We recognize that the generalizability of these observations is limited. Therefore studies need to be conducted prospectively in larger, more diverse populations. In addition, we report PPL FA-based EAEs of Elovl-6 and SCD n-7 are associated with adenomas. These EAEs have yet to be extensively validated and may not fully represent enzyme kinetics in adenomas. Thus, reported differences in EAEs could be related to other factors (i.e. diet, preferential FA uptake, etc.) rather than enzyme activities as we did not directly collect or assess dietary intake in this study. To our knowledge, no research group has sought to establish a preliminary range of PPL FA or EAE levels

associated with colorectal adenomas. Our research suggests specific levels of PPL FAs and EAEs may be useful as novel biomarkers of colon carcinogenesis.

CHAPTER 6: LONG CHAIN ω-6 PLASMA PHOSPHOLIPID POYUNSATURATED FATTY ACIDS AND ASSOCIATION WITH COLON ADENOMAS, IN ADULT MALES, A CROSS-SECTIONAL STUDY

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Abstract

Objective

Dietary lipid intake can be associated with increased risk of colorectal cancer (CRC) depending on its composition. Carcinogenesis alters lipid metabolism to facilitate cell growth and survival. For instance, metabolites of polyunsaturated fatty acids (PUFAs) are associated with increasing colon cell proliferation. Also, pre-cancerous colon lesions (i.e., adenomas) increase risk of CRC. In this study we investigated associations between plasma PUFAs and the number of colon polyps and polyp type (i.e., hyperplastic and adenoma).

Methods

Healthy male subjects (n=126) ages 48-65 were recruited prior to a routine colonoscopy screening. Plasma phospholipid (PPL) PUFAs were isolated by solid phase extraction and methylated. FAMEs were analyzed by gas chromatography. Factor analysis was used to cluster

PUFAs into groups, then generated factors and individual PUFAs were analyzed using polytomous logistic regression.

Results

In our age- and smoking-adjusted polytomous logistic regression, for each unit increase in PPL docosatetraenoic acid (DTA), individuals were 1.43 (1.00, 2.06) and 1.33 (0.99, 1.80) times more likely to have hyperplastic polyps and adenomas rather than no polyps, respectively. In our factor analysis, high PPL ω-6 PUFA and trans-FA loading scores were associated with increased odds of adenoma presence rather than no polyps.

Conclusion

Increases in long-chain PPL ω-6 PUFAs are associated with an increased risk of adenomas. As relative levels of DTA increase in PPLs, individuals had increased odds of having hyperplastic polyps and adenomas. Elevated conversion of ω-6 PUFAs to longer-chain ω-6s such as DTA, may indicate altered PUFA metabolism at the tissue level.

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-related death in the US and the fourth most common cancer worldwide [239]. Factors associated with increased CRC risk include diet, age, smoking, and colon polyps (5). In particular, dietary lipid intake is suggested to play a major role in colorectal carcinogenesis [240], and westernized dietary patterns are associated with an increased risk of CRC [241]. For instance, westernized diets are higher in

dietary trans-fats [112, 242], and trans-fat intake is positively associated with both colon adenomas [243] and CRC [244]. However, several studies report low-fat diets do not significantly decreases CRC risk [245], including a randomized control intervention study [246]. Recently Habermann et al reported an interaction between dietary fat intake, CRC risk, and SNPs in genes involved in polyunsaturated fatty acid (PUFA) metabolism [247]. Increased intake of certain PUFAs are associated with increased risk of CRC [248], but the results of humans studies on PUFA intake and CRC risk are often inconsistent (reviewed in detail [249]). Few researchers have investigated associations between specific blood PUFA levels and pre-cancerous lesions such as colon adenomas [248, 250]. Instead most studies investigate associations between PUFAs and CRC related to different fat source intakes, which influence cell growth and survival [251]. Although the exact mechanism by which dietary fat intake and CRC development is not well understood, the metabolites of dietary PUFAs may influence CRC progression [252, 253].

PUFAs are classified as either omega-3 (ω-3) or omega-6 (ω-6). In general, ω-3 PUFAs are considered more "anti-inflammatory-like" while ω-6 PUFAs are considered more "proinflammatory-like" [5]. Neither ω-3 nor ω-6 essential PUFAs are synthesized by humans, thus they are only obtained through dietary intake. In clinical trials, ω-3 supplementation reduced CRC proliferation and the risk of colorectal adenomas (CRA) [254]. Additionally, ω-3 PUFA intake reduces risk of both CRA and CRC in animal models [255, 256]. The mechanism through which ω-3 PUFAs decrease CRC risk involves several signaling pathways (reviewed in detail, [253]). In particular, ω-3 PUFAs can inhibit delta-6-desaturase (D6D) and cyclooxygenase (COX) [253], reduce tissue arachidonic acid (ARA) [252], and reduce ARA eicosanoid biosynthesis [257].

In contrast to ω -3 PUFAs, ω-6 PUFAs such as linoleic acid (LA) [258], di-homo-gamma linolenic acid (DGLA) [259], and ARA [248] may act as tumor promoters. ω-6 PUFAs influence

production of pro-inflammatory cytokines and prostaglandins (PGs) [260]. Their biosynthesis begins with the desaturation of LA to form DGLA by delta-6 desaturase (D6D), and DGLA is then desaturated by delta-5 desaturase (D5D) to form ARA [257]. ARA is the substrate for enzymatic oxygenation for conversion to PGs (e.g., PGE2). PGE2 in particular may contribute to the growth of intestinal cancer cells (reviewed in detail, [261]). Inhibiting ARA enzymatic transformation by COX and D6D activity can decrease the risk of CRA and CRC development (reviewed in detail, [262]). While there is inconsistent evidence that diet is associated with CRC risk, the associations between CRA and individual PUFAs are not well studied. The purpose of this study was to determine association between plasma levels of individual PUFAs, adenomas, and the number of colon polyps present.

Materials and Methods

Study population and parameters

The study was approved by the Biomedical and Health Institutional Review Board of Michigan State University (IRB# 08-786). Healthy male subjects (n = 126, > 96% Caucasian) 48 to 65 years of age were enrolled between August 2009 and February 2011, as previously described [2]. In brief, individuals were recruited prior to undergoing a routine colonoscopy screening. These patients were reported as asymptomatic and clinical metadata on each individual's co-morbidities, family history, and medications were recorded. Individuals were excluded for medical conditions associated with increased CRC risk, as previously described [2]. In brief, the exclusion criteria included: 1.) inflammatory bowel diseases, 2.) cancer in the previous two years, 3.) familial adenomatous polyposis, 4.) surgery in the previous two years, 5.) type-1 and type-2 diabetes, and

6.) several autoimmune diseases. Immediately after enrollment, trained staff collected anthropometric measurements and venous blood of study participants, as previously described [2]. In brief, BMI was assessed by recording patient's height using a stadiometer and weight using digital platform scale. The plasma fraction was separated from whole blood and stored at -80°C. Smoking status was assessed as "ever smoked" or "never smoked". Each individual received a full colonoscopy as previously described [4]. In brief, polyps were identified and removed by a gastroenterologist, and classified by a pathologist.

Plasma phospholipid extraction, isolation and analysis

Approximately 200mg plasma per subject were weighed and extracted using a modified Rose and Oaklander extraction [108]. PLs were isolated using Isolute-XL® SPE aminopropyl columns (500 mg; Bioatage, Charlotte, NC) as described by Agren et al [109]. Fatty acid methyl esters (FAMEs) were prepared as previously described [214, 263]. PPL FAMEs were analyzed using HS-Omega-3 Index® methodology at OmegaQuant Analytics, LLC (Sioux Falls, SD) as previously described [110].

Statistical analyses

This study and manuscript are a secondary analysis of a previously recruited cross-sectional study. Our study was powered (0.8) to detect differences in several PPL FAs, which includes palmitic acid (previously reported [180]), through the package "pwr" [264] in R v 3.3.0 [137]; using functions: pwr.norm.test and pwr.anova.test. We applied an effect size of 0.5 (determined using PPL FA data in our population) and used the following parameters:

pwr.anova.test(k=3, f=0.5, sig.level=0.05, power=0.8); and pwr.norm.test(d=0.5, sig.level=0.05, power=0.8, alternative='greater').

Frequencies, medians, and standard deviations were calculated for descriptive analyses (Table 18). Each PUFA was expressed as a percentage of total PPL, to allow for cross-study comparison of our results, since in a vast majority of epidemiological studies (i.e., outside of Japan) also analyze their data as a percent of total FA (i.e., whole plasma, PPL, plasma cholesterol esters, and red blood cell or whole blood) [265-269]. Median and quartile values were obtained for the PPLs (Table 19 and 20). PPL PUFA enzyme activity estimates (EAE) were calculated as the ratio of product/substrate. The following variables were calculated and used in our analyses: D5D EAE was calculated as the ratio of ARA/DGLA; D6D EAE was calculated as the ratio of DGLA/LA; EPA+DHA, herein referred to as EPADHA, was calculated as $\sum$ EPA+DHA.

In all statistical tests, PUFAs were analyzed as continuous independent variables, and polyps as categorical dependent variables. Categories were defined as: Polyp type: 1) individuals with no colon polyps, 2) individuals with $\geq$ 1 hyperplastic polyp and $<$ 1 adenoma, and 3.) individuals with $\geq$ 1 adenoma; Polyp number: 1) individuals with no colon polyps, 2) individuals with 1 colon polyp, 3) individuals with 2 colon polyps, and 4) individuals with $\geq$ 3 colon polyps. Statistical differences in PUFA levels, between polyp type (Table 19) and polyp number (Table 20) were determined using Kruskal-Wallis one-way analysis of variance. Spearman correlations were performed to evaluate the relationship between PUFAs and colon polyps (Table 21).

Many PUFAs are collinear and cannot be analyzed together as independent variables in regression models, therefore, we employed a factor analysis to analyze PUFAs as groups. FA biomarker patterns were generated using factor analysis (Proc Factor, SAS), as previously described [2]. In brief, four factors were determined based on the scree plot [184] and eigenvalues

> 1 [185]. The FAME analysis conducted by OmegaQuant Analytics, LLC routinely quantifies 24 FAs that are reported in numerous epidemiological studies and are associated with dietary intake and FA metabolism. Our lab has previously reported saturated and monounsaturated FAs are associated with colon adenomas [180]. In our current study we report the following PUFAs: LA, linoelaidic, γ-linolenic, DGLA, ARA, DTA, DPA ω-6, ALA, EPA DPA ω-3, and DHA. However, to reduce bias in the factor analysis, all 24 FAs analyzed were included in factor generation. Next, FAs were removed from the factor analysis only when a FA's correlations were < 0.3, in the factor-loading matrix, across the four factors (Table 22A). Proc Factor assigns each person a factor loading score for each of the four factors from the data. The Proc Factor option Varimax was specified for orthogonal transformation of factor loading matrix.

Polytomous logistic regression models for categorical outcome data were used to determine odds ratios (OR) and 95% confidence intervals for polyp type (Table 22, 23, and 24) and polyp number (Table A16). In all logistic regression models, polyp type (Table 23 and 24) and polyp number (Table A16) were referenced against individuals with no colon polyps. Factor loading scores (Table 22) and PPL PUFAs and EAEs were analyzed as continuous independent variables in logistic regression models. All models were adjusted for age and smoking status except where noted. The logistic regression models were defined as: 1.) Polyp type = age + smoking + FAs, EAEs, or factor loading scores; or 2.) Polyp number = age + smoking + FAs, EAEs, or factor loading scores. Age was selected as a confounder since age is a risk factor for polyps and colorectal cancer risk (reviewed in detail [270]), and smoking was selected as a confounder since smoking is associated with colorectal adenomas [271-274]. BMI, waist circumference (WC), and serum adipokines and cytokines were not included as confounders in our models, because we have previously shown BMI and WC were highly correlated to several PPL PUFAs [263], and we have

also shown PPL PUFAs are highly associated with adipokines and cytokines and both BMI and WC [130]. Thus, mutual adjustment of collinear factors such as BMI, WC, and adipokines or cytokines could lead to unreliable and unstable estimates of regression coefficients.

There was approximately 17% missing smoking data due to incomplete data in clinical records and, therefore, missing smoking data was imputed in regression analyses. Multiple imputation (Proc MI, seed = 20121119, imputations = 7) was used to impute all missing smoking data [218]. The multiple imputation procedure applied was Markov Chain Monte Carlo method using multiple chained equations. Because imputation was used, multiple imputation analysis (Proc MI ANALYZE) was used to determine the results from analysis of the imputed datasets. In all logistic regression analyses, the odds ratios for linoelaidic, DTA, C22:5 DPA ω-6, and D6D, were calculated on the basis that there is a unit change of 0.1 of the respective beta coefficient for each given parameter. P-values were considered statistically significant if $p \leq 0.05$ and a statistical trend if $0.05 < p \leq 0.09$. Statistical analyses, excluding power analyses (R version 3.3.0), were conducted using software SAS version 9.4 (SAS, Cary, NC).

Results

Participant and polyp characteristics

The median age was 58 years and 31% of the participants ever smoked (Table 18). As previously reported [2], 37 (29.4%) participants had adenomas, 20 (15.9%) had hyperplastic polyps, while 69 (54.8%) had no polyps (Table 18A). Both BMI and WC increased with polyp type (Table 18A) and polyp number (Table 18B), as previously reported [2]. As the number of colon polyps increased, so did the percentage of subjects having at least one adenoma (Table 18B).

Table 18: Characteristics of study population.[a]

A) Characteristics of individuals by polyp type

Parameter	Overall	No Polyps	Any Polyps	Hyperplastic	Adenoma
	n=126	n=69	n=57	n=20	n=37
Age (years)	58 (53,61)	57 (53,61)	58 (53,60)	58 (53,60)	58 (53,60)
Smoker (% total)	31	15	15	4	12
BMI (kg/m2)	29 (26,33)	28 (24,31)	31 (28,34)	28 (26,32)	32 (29,36)
WC (in)	41 (37,46)	40 (36,45)	42 (40,47)	41 (38,45)	43 (40,49)

B) Characteristics of individuals by polyp number categories

Parameter	Overall	1 Polyp	2 Polyps	≥ 3 Polyps
	n=126	n=23	n=11	n=23
Age (years)	58 (53,61)	58 (53,60)	58(52,61)	57 (52,60)
Smoker (% total)	31	4	4	8
BMI (kg/m2)	29 (26,33)	30 (26,34)	29 (25,32)	32 (28,37)
WC (in)	41 (37,46)	41 (38,45)	43 (40,48)	45 (40,47)
Hyperplastic (%)	-	43.48	36.36	26.09
Adenoma (%)	-	56.52	63.64	73.91

[a]Values expressed as Median (Q1,Q3). BMI: body mass index; WC: waist circumference.

PPL PUFA proportions and ANOVA results

DTA and linoelaidic were the only ω-6 PUFAs associated with polyp type categories (Table 19). PPL DTA differed significantly across polyp type ($p<0.05$). There tended to be a difference in PPL linoelaidic ($p=0.064$) and the D6D EAE ($p=0.089$) across polyp type. PPL ω-3s were not associated with polyp type or polyp number. Several PPL ω-6s were associated with polyp number (Table 20). There was a significant difference in PPL LA across polyp number categories ($p<0.05$). PPL DTA tended to differ across polyp number ($p=0.073$).

Polyps are significantly correlated with PPL PUFAs and EAEs

Polyp number was significantly correlated with PPL linoelaidic, DTA, and D6D EAEs (Table 21). PPL ARA tended to be correlated with polyp number ($p=0.079$). Polyp type was correlated to similar PPL PUFAs and EAEs which were correlated to polyp number. Specifically, polyp type was significantly correlated with PPL linoelaidic, DTA, and the D6D EAE. Polyp type was not associated with ARA. However, polyp type tended to be inversely correlated with LA ($p=0.061$).

Table 19: Polyp type is associated with the percentage of ω-6 plasma phospholipid polyunsaturated fatty acids.[a]

Variable	No Polyp	Hyperplastic	Adenoma	p-value
C18:2-c LA	19.83 (17.79, 22.79)	18.86 (15.77, 21.42)	18.95 (16.76, 21.19)	0.111
C18:2-t Linoelaidic	0.23 (0.18, 0.29)	0.28 (0.23, 0.39)	0.30 (0.21, 0.40)	*0.064*
C20:3 DGLA	2.56 (1.96, 3.21)	2.58 (2.39, 3.31)	2.88 (2.16, 3.45)	0.484
C20:4 ARA	9.82 (8.64, 10.99)	11.60 (9.48, 12.72)	10.65 (9.17, 12.68)	0.106
C22:4 DTA	0.34 (0.22, 0.40)	0.41 (0.35, 0.50)	0.37 (0.30, 0.50)	**0.031**
C22:5 DPAω-6	0.21 (0.14, 0.34)	0.29 (0.19, 0.33)	0.26 (0.17, 0.32)	0.277
C20:5 EPA	0.63 (0.45, 1.07)	0.52 (0.38, 0.81)	0.63 (0.45, 0.76)	0.228
C22:5 DPAω-3	0.83 (0.65, 0.96)	0.84 (0.67, 1.08)	0.84 (0.62, 1.02)	0.831
C22:6 DHA	2.65 (2.15, 3.56)	2.86 (1.96, 3.72)	2.72 (2.13, 3.40)	0.915
EPADHA [b]	3.32 (2.74, 4.65)	3.40 (2.41, 4.55)	3.26 (2.63, 4.15)	0.740
D5D [c]	3.74 (2.77, 4.71)	4.56 (3.14, 5.23)	3.41 (2.76, 5.16)	0.534
D6D [d]	0.13 (0.10, 0.18)	0.15 (0.11, 0.19)	0.15 (0.12, 0.18)	*0.089*

[a]Fatty acids expressed as percent of total phospholipids. Values expressed as Median (Q1, Q3). Wilcoxon one-way nonparametric ANOVA. p-values bolded if $p \leq 0.05$ and italicized if $0.05 < p \leq 0.09$. ARA: arachidonic acid; D5D: delta-5-desaturase estimated enzyme activity; D6D: delta-6-desaturase estimated enzyme activity; DGLA, Dihomo-γ-linolenic acid; DHA: docosahexaenoic acid; DPA, docosapentaenoic acid; DTA: docosatetraenoic acid; EPA: eicosapentaenoic acid; LA: linoleic acid.
[b]EPADHA was calculated as $\sum$ EPA+DHA.
[c]D5D EAE was calculated as the ratio of ARA/DGLA.
[d]D6D EAE was calculated as the ratio of DGLA/LA.

Table 20: Polyp number is associated with the percentage of ω-6 plasma phospholipid polyunsaturated fatty acids.[a]

Variable	No Polyp	1 Polyp	2 Polyps	≥ 3 polyps	p-value
C18:2-c LA	19.83 (17.79, 22.79)	17.53 (16.07, 20.68)	19.16 (17.42, 19.64)	20.30 (16.83, 23.51)	**0.035**
C18:2-t Linoelaidic	0.23 (0.18, 0.29)	0.28 (0.19, 0.34)	0.33 (0.23, 0.52)	0.30 (0.19, 0.45)	*0.099*
C20:3 DGLA	2.56 (1.96, 3.21)	2.61 (2.27, 3.49)	2.53 (2.16, 2.95)	3.06 (2.15, 3.53)	0.512
C20:4 ARA	9.82 (8.64, 10.99)	10.90 (7.96, 13.95)	10.16 (9.03, 14.29)	10.35 (9.27, 12.51)	0.251
C22:4 DTA	0.34 (0.22, 0.40)	0.40 (0.30, 0.47)	0.39 (0.34, 0.52)	0.38 (0.27, 0.50)	*0.073*
C22:5 DPAω-6	0.21 (0.14, 0.34)	0.28 (0.24, 0.34)	0.25 (0.16, 0.31)	0.26 (0.16, 0.33)	0.322
C20:5 EPA	0.63 (0.45, 1.07)	0.54 (0.43, 0.88)	0.63 (0.42, 1.01)	0.59 (0.35, 0.76)	0.510
C22:5 DPAω-3	0.83 (0.65, 0.96)	0.84 (0.62, 1.06)	0.83 (0.54, 1.12)	0.84 (0.66, 1.03)	0.997
C22:6 DHA	2.65 (2.15, 3.56)	2.74 (2.07, 4.10)	2.75 (2.13, 3.24)	2.72 (2.04, 3.40)	0.890
EPADHA [b]	3.32 (2.74, 4.65)	3.39 (2.53, 4.77)	3.43 (3.16, 3.96)	3.26 (2.42, 4.15)	0.806
D5D [c]	3.74 (2.77, 4.71)	3.94 (2.76, 5.35)	3.50 (3.40, 5.71)	3.17 (2.67, 4.93)	0.761
D6D [d]	0.13 (0.10, 0.18)	0.15 (0.11, 021)	0.13 (0.11, 0.18)	0.15 (0.11, 0.18)	0.177

[a]Fatty acids expressed as percent of total phospholipids. Values expressed as Median (Q1,Q3). Wilcoxin one-way nonparametric ANOVA. p-values bolded if $p \leq 0.05$ and italicized if $0.05 < p \leq 0.09$. ARA: arachidonic acid; D5D: delta-5-desaturase estimated enzyme activity; D6D: delta-6-desaturase estimated enzyme activity; DGLA, Dihomo-γ-linolenic acid; DHA: docosahexaenoic acid; DPA, docosapentaenoic acid; DTA: docosatetraenoic acid; EPA: eicosapentaenoic acid; LA: linoleic acid.
[b]EPADHA was calculated as $\sum$ EPA+DHA.
[c]D5D EAE was calculated as the ratio of ARA/DGLA.
[d]D6D EAE was calculated as the ratio of DGLA/LA.

Table 21: Spearman correlation between polyunsaturated fatty acids and polyps.[a]

	LA	Linoelaidic	DGLA	ARA	DTA	DPAω-6	EPA	DPAω-3	DHA	EPADHA[b]	D5D[c]	D6D[d]
Polyp number	-0.119	0.210	0.113	0.157	0.205	0.108	-0.122	0.012	-0.049	-0.065	-0.003	0.185
	0.184	**0.019**	0.206	*0.079*	**0.021**	0.229	0.173	0.890	0.589	0.467	0.977	**0.039**
Polyp type	-0.167	0.204	0.107	0.151	0.200	0.124	-0.102	-0.002	-0.030	-0.049	-0.009	0.190
	0.061	**0.022**	0.234	0.092	**0.025**	0.167	0.257	0.979	0.738	0.587	0.918	**0.033**
Linoelaidic			-0.068	-0.122	-0.022	-0.011	-0.082	-0.193	-0.124	-0.090	-0.029	0.026
			0.451	0.174	0.808	0.907	0.360	**0.030**	0.166	0.318	0.749	0.769
DGLA				0.108	0.404	0.495	-0.226	0.135	0.039	-0.030	-0.710	0.859
				0.230	**<0.0001**	**<0.0001**	**0.011**	0.132	0.666	0.738	**<.0001**	**<.0001**
ARA					0.547	0.363	0.000	0.223	0.108	0.076	0.569	0.323
					<0.0001	**<0.0001**	0.996	**0.012**	0.228	0.398	**<.0001**	**0.001**
DTA						0.688	-0.468	0.146	-0.336	-0.405	0.029	0.517
						<0.0001	**<0.0001**	0.104	**0.001**	**<0.0001**	0.751	**<.0001**
DPAω-6							-0.510	-0.075	-0.232	-0.316	-0.182	0.526
							<0.0001	0.405	**0.009**	**0.001**	**0.042**	**<.0001**
EPA								0.464	0.598	0.758	0.191	-0.162
								<0.0001	**<0.0001**	**<0.0001**	**0.032**	*0.069*
DPAω-3									0.378	0.409	0.023	0.189
									<0.0001	**<0.0001**	0.795	**0.034**
DHA										0.964	0.075	0.112
										<0.0001	0.401	0.213
EPADHA											0.102	0.046
											0.256	0.612
D5D												-0.476
												<.0001

[a]Numbers in gray rows indicate Spearman correlation coefficient, and numbers listed directly below, in white lines, indicate corresponding p-value. P-values bolded if significant ($p \leq 0.05$) and italicized if $0.05 > p \leq 0.09$ ARA: arachidonic acid; D5D: delta-5-desaturase estimated enzyme activity; D6D: delta-6-desaturase estimated enzyme activity; DGLA, Dihomo-γ-linolenic acid; DHA: docosahexaenoic acid; DPA, docosapentaenoic acid; DTA: docosatetraenoic acid; EPA: eicosapentaenoic acid; LA: linoleic acid.
[b]EPADHA was calculated as $\sum$ EPA+DHA.
[c]D5D EAE was calculated as the ratio of ARA/DGLA.
[d]D6D EAE was calculated as the ratio of DGLA/LA.

ω-6 PUFA and trans-FA factors are associated with adenoma presence

A factor analysis was performed to predict how FAs clustered into factors that may be predictive of polyp number or polyp type (Table 22). The factor loading matrix is presented in Table 22A. The factor loadings indicate each factor's correlation with each individual FA. Four factors were named as, factor 1: ω-3 PUFA, factor 2: ω-6 PUFA, factor 3: trans-FA, factor 4: very long (VLC) SFAs. We excluded the logistic regression results from factor 4 loading scores since we have recently reported saturated FAs are associated with colon polyps [180]. In our polyp number logistic regressions, polyp number was not significantly associated with ω-3 PUFA, ω-6 PUFA, or trans-FA factors (data not shown). In our polyp type logistic regressions, hyperplastic polyps were not significantly associated with ω-3 PUFA, ω-6 PUFA, or trans-FA loading scores (Table 22B). However, ω-6 PUFA and trans-FA factor loading scores were significantly associated with adenomas, compared to the no-polyps reference group (Table 22B). A high ω-6 PUFA score was associated with increased odds of having an adenoma (OR: 1.60, 95%CI: 1.04-2.46) rather than no polyps. A high trans-FA score was also associated with increased odds of having an adenoma (OR: 1.57, 95%CI: 1.00-2.44) compared to the no-polyps reference group.

Table 22: Adenomas are associated with ω-6 and trans fatty acid factors.

A: PUFAs are highly correlated with factor loading matrix.[a]

Fatty acids	ω-3 PUFA	ω-6 PUFA	Trans-FA	VLC SFA
EPADHA	**0.88**	-0.29	-0.05	-0.04
DHA	**0.70**	-0.24	-0.04	0.01
EPA	**0.61**	-0.52	-0.10	-0.11
Nervonic	**0.66**	-0.08	0.13	0.46
DPA3	**0.48**	0.02	-0.17	-0.05
Docosatetraenoic	-0.14	**0.86**	0.00	0.09
DPA6	-0.12	**0.79**	0.02	-0.03
ARA	0.14	**0.65**	0.01	0.07
DGLA	-0.02	**0.60**	-0.17	-0.08
Linoelaidic	-0.04	-0.10	**0.34**	0.04
Eicosenoic	0.14	-0.15	**0.85**	0.06
Elaidic	-0.11	0.11	**0.81**	-0.05
Behenic	-0.21	-0.07	-0.04	**0.83**
Lignoceric	0.04	0.09	-0.11	**0.78**
Arachidic	0.01	0.05	0.18	**0.78**
Stearic	-0.57	-0.01	0.00	0.18
Palmitic	-0.60	-0.15	-0.20	-0.07
Variance explained by each factor [b]	2.35	2.55	2.45	2.22

B: ω-6 and trans fatty acid factors are significantly associated with adenomas[c]

Polyp type	Factor	p-value	OR (95% CI)
Adenomas	ω-3 PUFA	0.882	1.05 (0.63, 1.53)
Hyperplastic	ω-3 PUFA	0.234	0.77 (0.48, 1.25)
Adenomas	ω-6 PUFA	**0.034**	1.60 (1.04, 2.46)
Hyperplastic	ω-6 PUFA	0.090	1.53 (0.93, 2.72)
Adenomas	Trans-FA	**0.048**	1.57 (1.00, 2.44)
Hyperplastic	Trans-FA	0.800	1.09 (0.56, 2.11)

[a]Varimax rotated factor loading matrix generated by (Proc Factor, SAS v 9.4). Numbers listed under each factor correspond with each respective FA correlation with each factors. ARA: arachidonic acid; DGLA, Dihomo-γ-linolenic acid; DHA: docosahexaenoic acid; DPA, docosapentaenoic acid; DTA: docosatetraenoic acid; EPA: eicosapentaenoic acid.
[b]Total variance explained by each factor, generated from communality estimates (Proc Factor, SAS).
[c]Model defined as: Polyp type = factor loadings.

Polyps are associated with specific PUFAs and EAE in age adjusted logistic regressions

Colon polyps and several PPL PUFAs and EAEs were correlated with confounding factors such as smoking (data not shown). Polytomous logistic regressions were used to adjust for age, and to determine which PUFAs and EAEs were significantly associated with polyp type (Table 23 and 24) and polyp number (Table A16). In our polyp type models, hyperplastic polyps were significantly associated with ω-6 PUFAs (Table 23). For each unit increase in LA, individuals were 0.86 (0.74, 0.99) times as likely to have a hyperplastic polyp compared to the no-polyps reference group. For each unit increase in DTA, individuals were 1.43 (1.00, 2.06) times as likely to have a hyperplastic polyp compared to the no-polyps reference group. PPL EPA was the only ω-3 associated with polyps. For each unit increase in EPA, individuals tended to be 0.27 (0.06, 1.12) times as likely to have a hyperplastic polyp compared to the no-polyps reference group (Table 23). Adenomas were associated with ω-6 PUFAs in logistic regression analyses (Table 24). For each unit increase in linoelaidic and DTA, individuals tended to be 1.17 (0.98, 1.39) and 1.33 (0.99, 1.80) times more likely to have an adenoma compared to the no-polyps reference group, respectively.

Table 23: Hyperplastic polyps are associated with ω-6 polyunsaturated fatty acids.[a,b]

Variable	Hyperplastic (OR 95% CI)	p-value
C18:2-c LA	0.86 (0.74,0.99)	**0.038**
C18:2-t Linoelaidic [c]	1.06 (0.83,1.34)	0.646
C20:3 DGLA	1.08 (0.59,1.96)	0.812
C20:4 ARA	1.17 (0.97,1.41)	0.095
C22:4 DTA [c]	1.43 (1.00,2.06)	*0.052*
C22:5 DPA [c]	1.26 (0.87,1.83)	0.225
C20:5 EPA	0.27 (0.06,1.12)	*0.072*
C22:5 DPA	1.35 (0.40,4.61)	0.628
C22:6 DHA	0.96 (0.64,1.42)	0.821
EPADHA [d]	0.87 (0.64, 1.19)	0.385
D5D [e]	1.10 (0.82,1.48)	0.522
D6D [c,f]	1.96 (0.77,5.02)	0.161

[a]Fatty acids expressed as percent of total phospholipids. P-values bolded if $p \leq 0.05$ and italicized if $0.05 < p \leq 0.09$. ARA: arachidonic acid; D5D: delta-5-desaturase estimated enzyme activity; D6D: delta-6-desaturase estimated enzyme activity; DGLA, Dihomo-γ-linolenic acid; DHA: docosahexaenoic acid; DPA, docosapentaenoic acid; DTA: docosatetraenoic acid; EPA: eicosapentaenoic acid; LA: linoleic acid.
[b]Model defined as: Polyp type = fatty acid + age + smoking.
[c]Odds ratios have been calculated on the basis that there is a unit change of 0.1 for the respective beta coefficient for each given parameter.
[d]EPADHA was calculated as ∑ EPA+DHA.
[e]D5D EAE was calculated as the ratio of ARA/DGLA.
[f]D6D EAE was calculated as the ratio of DGLA.

Table 24: Adenomas are associated with ω-6 polyunsaturated fatty acids.[a,b]

Variable	Adenoma (OR 95% CI)	p-value
C18:2-c LA	0.91 (0.81,1.02)	0.121
C18:2-t Linoelaidic [c]	1.17 (0.98,1.39)	*0.075*
C20:3 DGLA	1.24 (0.77,2.01)	0.376
C20:4 ARA	1.11 (0.95,1.29)	0.190
C22:4 DTA [c]	1.33 (0.99,1.80)	*0.062*
C22:5 DPA [c]	1.20 (0.87,1.64)	0.266
C20:5 EPA	0.70 (0.35,1.39)	0.310
C22:5 DPA	1.48 (0.55,3.99)	0.436
C22:6 DHA	0.97 (0.70,1.33)	0.836
EPADHA [d]	0.94 (0.74,1.19)	0.589
D5D [e]	0.99 (0.77,1.28)	0.945
D6D [c,f]	1.90 (0.88,4.14)	0.104

[a]Fatty acids expressed as percent of total phospholipids. P-values bolded if $p \leq 0.05$ and italicized if $0.05 < p \leq 0.09$. ARA: arachidonic acid; D5D: delta-5-desaturase estimated enzyme activity; D6D: delta-6-desaturase estimated enzyme activity; DGLA, Dihomo-γ-linolenic acid; DHA: docosahexaenoic acid; DPA, docosapentaenoic acid; DTA: docosatetraenoic acid; EPA: eicosapentaenoic acid; LA: linoleic acid.
[b]Model defined as: Polyp type = fatty acid + age + smoking.
[c]Odds ratios have been calculated on the basis that there is a unit change of 0.01 for the respective beta coefficient for each given parameter.
[d]EPADHA was calculated as ∑ EPA+DHA.
[e]D5D EAE was calculated as the ratio of ARA/DGLA.
[f]D6D EAE was calculated as the ratio of DGLA/LA.

Associations with polyp number and PPL PUFAs and EAEs are shown in Table A16. LA and DTA were the only PPL ω-6 PUFAs significantly associated with polyp number. For each unit increase in PPL LA, individuals were 0.83 (0.72, 0.95) times as likely to have 1 polyp rather than no polyps. For each unit increase in PPL DTA, individuals were 1.43 (1.02, 2.03) times more likely to have 1 polyp, and tended to be 1.53 (0.95, 2.44) times more likely to have 2 polyps compared to the no-polyps reference group. For each unit increase in PPL DPAω-6 and linoelaidic, individuals tended to be 1.39 (0.98, 1.98) times more likely to have 1 polyp, and tended to be 1.22 (0.99, 1.51) times more likely to have 2 polyps rather than no polyps, respectively. PPL ω-3s were not associated with polyp numbers. The D6D EAE was significantly associated with having 1 polyp. For each unit increase in D6D, individuals were 2.45 (1.00, 5.96) times more likely to have 1 polyp compared to the no-polyps reference group.

Discussion

The purpose of this study was to identify specific PPL PUFAs and EAEs associated with colon polyp number and polyp type. We report that PPL ω-6 PUFA and trans-FA factor scores were positively associated with adenoma presence. When analyzed individually, ω-6 PUFA DTA was associated with hyperplasic polyps and adenomas in age- and smoking-adjusted logistic regressions. Polyp number was not associated with any PPL PUFA. Taken together, these data indicate that certain PPL PUFAs are associated with the presence of hyperplastic polyps and adenomas, but not with the total number of colon polyps. Our results suggest PPL ω-6 PUFAs, in particular DTA, may indicate altered FA metabolism associated with having polyps.

PUFAs are substrates for elongating, desaturating, and oxygenating enzymes. Altered levels of PUFA substrates (i.e., through dietary intake) can alter levels of downstream PUFA metabolites [167], which may promote tumor growth (reviewed in detail, [275]). Previous research has shown accumulations of LA and longer chain ω-6s (e.g., DGLA and ARA) in colon adenomas [276, 277]. In addition, adenocarcinomas have higher concentrations of DTA [276]. Despite DTA being a downstream metabolite of ARA, few studies investigate DTA effects on colon polyps. Instead, most studies investigating ω-6 PUFA associations with polyps or CRC risk focus on ARA oxygenated metabolites [252, 253].

PPL ω-6 PUFAs may be related to an increased risk of CRC [250, 278], and altered FA metabolism at the tissue level may be detectable in plasma [93, 238, 279-281]. The PPL FA compartment is ideal for biomarker discovery since it contains PL from sources such as plasma lipoproteins [220] and plasma microvesicle exosomes [221] which can be secreted from CRC cells [282]. If cellular PUFA metabolism is changed during CRC pathogenesis, then changes in PUFA metabolites could be detectable in the PPLs. What remains unclear is whether differences in ω-6 PUFAs detected in plasma are produced by polyps or other tissues such as the liver. At the time this study was conducted exosome isolation was not possible, but methods and technologies are now available to perform such analyses. Analysis of exosomes could allow researchers to determine whether changes in PPL ω-6 PUFAs originate from colon polyps or other tissues. This distinction may lead to a better understanding of how diet may influence adenoma risk related to ω-6 metabolism and how a diet rich in ω-6s may contribute to CRC risk.

This cross-sectional study was conducted in a population of males (n=126, > 96% Caucasian, ages 48-65) to identify associations between colon polyps and PPL FAs. We recognize that the generalizability of these observations is limited, and we acknowledge it is possible some

associations reported in this study could be the result of chance due to the number of comparisons relative to our sample size. Therefore, studies need to be conducted in larger, more diverse populations. We also acknowledge the second category of polyp number was underpowered in this study, but was included in analyses for reporting consistency, since we have several studies associating polyp number with serum and plasma biomarkers in this population [2-4, 180]. In addition, using EAEs and ω-6 PUFA factor loading scores, we report PPL ω-6 PUFA metabolism is altered in individuals with adenomas. These EAEs have yet to be extensively validated and may not fully represent enzyme kinetics. Thus, reported differences in ω-6 PUFAs could be related to other factors (e.g., diet) rather than altered ω-6 metabolism. We did not directly collect or assess dietary and alcohol intake in this study. Our lab and others have previously reported that red blood cell PL and PPL PUFAs reflect dietary PUFA intake [110, 283, 284], and our lab has also shown PPL PUFA levels are a good proxy of PL PUFA levels in the colon [110], however, we acknowledge that depending on the lipid compartment (i.e., RBC or plasma) or PUFA there could be variation. We acknowledge other relevant confounding factors were not included in our statistical analyses (see section Statistical Analyses for detailed description) due to issues of multicollinearity with PPL PUFAs and confounders.

Our research suggests PPL DTA is associated with both hyperplastic polyps and adenomas. Since our study is cross-sectional, our suggestion that elevated PPL DTA is associated with increased colon polyp risk, assumes the observed levels of DTA reflect longer-term DTA levels (i.e., years proceeding). To our knowledge, there is no evidence suggesting long-term exposure to elevated PPL DTA increases the risk of colon polyps. There are strong implications for diet recommendations and cancer prevention strategies if: 1.) increased PPL DTA is due to the altered

FA metabolism in polyps or organs such as the liver, and if 2.) DTA and DTA metabolites influence polyp formation or progression.

APPENDICES

APPENDIX A: Supplemental information

Chemicals and solvents used in high-resolution MS/MS analysis.

Chemicals and solvents

High performance liquid chromatography-grade (HPLC) solvents were used for the extraction and resuspension of all samples analyzed. The following solvents were used in the analysis: HPLC-grade methanol (Lot#: 0000118827, J.T. Baker, Phillipsburg, NJ), HPLC-grade chloroform (Lot#: 55296, Omnisolv, Charlotte, NC), HPLC-grade water (Lot#: 0000063121, J.T. Baker), HPLC-grade isopropanol (Lot#: SHBC6903V, Sigma-Aldrich, St. Louis, MO), and HPLC-grade acetonitrile (Lot #: 53324, EMD chemicals, Gibbstown, NJ).

Internal standards for each lipid class were selected based on commercial availability at time of analysis. The internal standards used in this study are as follows: PE(16:0-d_{31}/18:1) [Product#:110921; Lot#: LM160D31-181PE-33], PG-d_5(16:0/18:1) [Product#: 110919; Lot#:LM160-181PGD5-10], PS(16:0-d_{31}/18:1) [Product#: 110922; Lot#: LM160D31-181PS-25], LysoPE(13:0) [Product#: 110696; Lot#: LM130LPE-10], PC(24:0/24:0) [Product#: 110929; Lot#: LM240PC-28], Cer(d18:1/25:0) [Product#: LM-2225; Lot#: LM5-139D] (Avanti Polar Lipids, Alabaster, AL), and PC(8:0/8:0) [Product#: 10009874; Lot#181547-14] (Cayman Chemical, Ann Arbor, MI). The antioxidant butylated hydroxytoluene (BHT) (Sigma-Aldrich) was present in all solvents at a concentration of 0.1%.

Internal standards and extraction solution

A mixture of internal standard (IS) containing PE(16:0-d_{31}/18:1), PG-d_5(16:0/18:1), PS(16:0-d_{31}/18:1), LysoPE(13:0), PC(24:0/24:0), Cer(d18:1/25:0) (Avanti Polar Lipids), and PC(8:0/8:0) (Cayman Chemical) was prepared. This IS mixture was added to an extraction solution composed of HPLC-grade chloroform:methanol (2:1 v/v, Omnisolv, J.T. Baker) with 0.1% BHT (100 μg/μL, Sigma-Aldrich). The final concentration of IS in the extraction solution was 5 ng/μL for all PLs and 0.2 ng/μL for Cer.

Progenesis QI data importation

Raw UPLC-MS/MS data files were imported into Progenesis QI for data alignment and processing. The following adducts were selected based on the sample matrix (i.e., chloride), mobile phases (i.e., formate), and contaminants (i.e., trifluoroactetate and nitrate) that may have been present in either the samples or the mass analyzer at time of analysis. The following adducts were included for deconvolution of M, 2M, and 3M *m/zs*: M-H, M+formate-H, M-methylformate-H, M+Cl-H, M+trifluoroacetate-H, M-methyltrifluoroacetate-H, and M+nitrate-H. After peaking picking and deconvolution by Progenesis QI, a total of 4802 m/zs were identified. The data was first exported to EZ-Info v3.0 (Umetrics, San Jose, CA), then to Excel (Microsoft, Redmond, WA) for relative mass defect filtering.

Table A1: Chromatographic analysis parameters for high-resolution MS/MS analysis.[a]

UPLC Parameters

Parameter	Setting
Run Time:	15.00 min
Solvent A:	Acetonitrile/water (60:40) + 10 mM ammonium formate
Solvent B:	Isopropanol/acetonitrile (90:10) + 10mM ammonium formate
Column Temperature:	55.0 °C
Injection Volume (µL):	10.00
Equilibration Time:	0.1 min

UPLC Gradient Table

Time (min)	Flow Rate	%A	%B	Curve
Initial	0.400	60	40	Initial
1.50	0.400	57	43	6
1.60	0.400	50	50	1
9.00	0.400	57	43	6
9.08	0.400	30	70	1
13.50	0.400	1	99	6
13.60	0.400	60	40	6
15.00	0.400	60	40	1

[a]The instrument used in plasma lipid separation was a Waters ACQUITY UPLC using ACQUITY UPLC CSH C18 1.7 µm 2.1x100mm column (Waters, Milford, MA). The column was washed prior to analysis for 1.5 h with 50% dichloromethane and 50% 2-propanol, followed by 1.5 h with 100% 2-propanol. The UPLC method used in this analysis performed was based on the Waters application note: Issac et al. Lipid Separation using UPLC with Charged Surface Hybrid Technology. The UPLC method was modified to shorten the run time to 15 min.

Table A2: Mass spectrometric analysis parameters for high-resolution MS/MS analysis.[a]

Mass Spectrometer Source and Optics Settings

Parameter	Setting
Analyzer	Sensitivity mode
Capillary (kV)	1
Sampling Cone (kV)	10
Source Temperature (°C)	110
Source Offset	80
Desolvation Temperature (°C)	350
Cone Gas Flow (L/Hr)	25
LM Resolution	4.7
HM Resolution	15

[a]The MS/MS analysis of plasma lipids was performed using a Waters Xevo G2-XS quadrupole time-of-flight mass spectrometer (Waters). Data acquisition was performed using MS^E in continuum mode with lock mass application. Source parameters are as follows: Three MS functions were used to obtain MS/MS spectra and correct for mass drift: Function1 was used to obtain a parent ion spectra of lipids; Function 2 was used to obtain a fragmentation spectra of parent lipid ions; and Function 3 was used to measure leucine enkephalin as lock mass for mass correction.

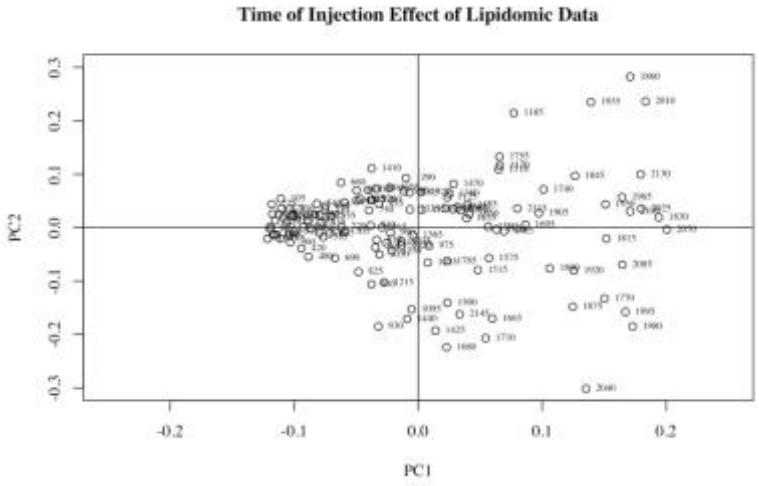
Mass Analyzer Settings and Collison Energies	
Function 1: TOF Parent Ion Function	
Survey Start Time (min)	0.0
Survey End Time (min)	15.0
Survey Ion Mode	ES Mode
Survey Polarity	Negative
[PARENT MS SURVEY]	
Survey Start Mass	60
Survey End Mass	1800
Parent Survey High CE (V)	30.0
Parent Survey Low CE (V)	10.0
Function 2: TOF Fragmentation Function	
Survey Start Time (min)	0.0

Table A2 (cont'd)

Survey End Time (min)	15.0
Survey Ion Mode	ES Mode
Survey Polarity	Negative
[PARENT MS SURVEY]	
Survey Start Mass	60
Survey End Mass	1800
Parent Survey High CE (V)	90
Parent Survey Low CE (V)	20
Function 3: TOF Lockmass Function	
Survey Start Time (min)	0.0
Survey End Time (min)	15.0
Survey Ion Mode	ES Mode
Survey Polarity	Negative
[PARENT MS SURVEY]	
Survey Start Mass	60.0
Survey End Mass	1800.0
Parent Survey High CE (V)	30
Parent Survey Low CE (V)	10

Figure A1: Time of injection effect and data matrix normalization.[a]

A)



[a]After initial data processing of mass defect filtered dataset, we investigated batch and time of injection effects in the data. The variable time of injection was created by multiplying the samples file number by 15 mins, thus, yielding the samples time of injection relative to the to the first injection of the analysis. We observed a time of injection effect after plotting principal components (Pc) 1 and 2. There were distinct clusters of samples based on their time of injection (mins) during the mass spectrometric analysis. In addition, the Pc 1 and 2 score were significantly associated with time of injection in regression analyses. A) Principal components derived from patient (n=126) lipidomic profiles. Number listed next to samples indicate the samples time of injection in mins relative to the first injection of analysis. Samples injected earlier in the analysis clustered tightly together, while samples injected later in the analysis drifted. Next, to further examine the time of injection effect, we plotted the range of intensities for each plasma lipid in the 126 samples. B) Figure displays time of injection and the range of plasma lipid intensities for each sample (n=126) analyzed. As time of injection increased, so did the range of metabolite intensities, indicating ion suppression throughout analysis. We determined the time of injection effect was due to ion suppression by trifluoroacetate contamination in the mass analyzer. Since a majority of plasma phospholipids are PCs, we normalized our entire data matrix by the IS PC(8:0/8:0). IS normalization of the data matrix removed the time of injection effect on plasma lipids. C)
Principal components derived from patient (n=126) IS normalized lipidomic profiles. Number listed next to samples indicate the samples time of injection in minutes. Normalization of the data matrix with IS PC(8:0/8:0) removed the time of injection effect. Next, to further examine if normalizing the data matrix removed the time of injection effect, we plotted the range of intensities for each plasma lipid in the 126 samples. D) Above figure displays time of injection and the range of plasma lipid intensities for each sample (n=126) analyzed. After IS normalization of the data matrix, the time of injection effect was removed.

Figure A1 (cont'd)

B)

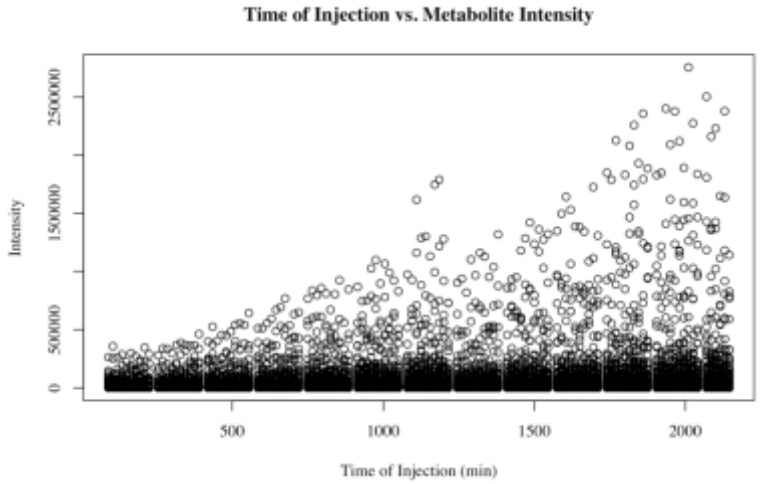
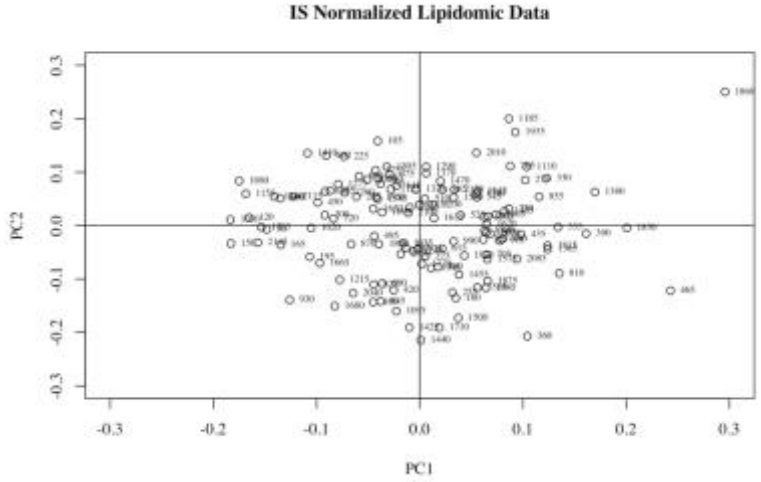


Figure A1 (cont'd)

C)



D)

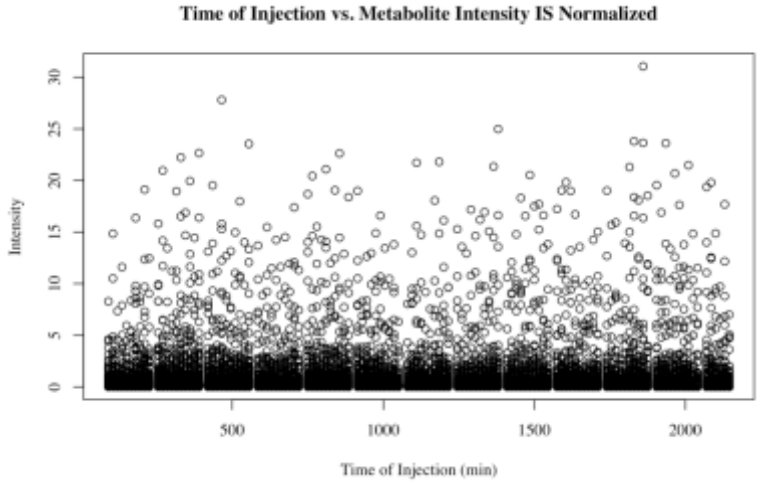


Table A3: Tables displaying MS/MS spectrum of primary ids (i.e., retention time_*m/z*) generated by Progenesis QI that were significantly associated with responses, but were not listed in lipidomics databases and libraries.[a]

Primary ID: X1.35_669.3989

Measured *m/z* (Da)	% base peak
174.9469	29.32
193.1514	5.68
218.0910	9.22
231.1625	25.90
257.1484	27.50
275.1544	48.08
277.1760	9.09
395.2210	100.00
396.2443	15.57
669.3997	52.81
670.4040	16.69
671.3848	6.04

Primary ID: X4.18_794.5050

Measured *m/z* (Da)	% base peak
540.2896	1.21
568.2869	0.92
794.5059	100.00
795.5094	46.56
796.5105	18.17
797.5401	3.34

Primary ID: X5.43_1068.6601

Measured *m/z* (Da)	% base peak
221.0662	4.70
536.5089	2.45
698.5816	7.19
1022.6674	100.00
1023.6949	61.47
1024.6909	21.31
1025.7070	2.86
1068.7111	3.03

[a]All MS/MS data were performed on samples with the most abundant parent ion(s) of interest, respectively, using the same chromatography column, solvent gradient, and mass analyzer setting. However, the collision energy used for parent ions MS/MS was increased to a ramp of 20-80V. Spectrum lists display all m/zs with >250 counts, from MS/MS of parent ions.

Table A3 (cont'd)

Primary ID: 10.11_1381.8480

Measured *m/z* (Da)	% base peak
755.6339	2.65
1335.8094	100.00
1336.8085	82.18
1337.8382	31.18
1381.8666	8.81
1382.8512	14.69

Table A4: Table of lipids significantly associated with BMI with FDR p-values <0.05. Beta coefficients, p-values, Benjamini-Hochberg FDR p-values, and Bonferroni p-values listed.

Primary ID	Beta	p-value	FDR p-value	Bonferroni p-value
X1.17_564.3289	-5.25	1.48E-10	2.59E-07	2.59E-07
X1.42_566.3497	-10.78	1.30E-06	1.14E-03	2.28E-03
X1.21_476.2768	-14.67	4.03E-06	2.35E-03	7.04E-03
X1.51_592.3513	-115.61	1.06E-05	3.92E-03	1.86E-02
X1.03_562.3132	-44.59	1.12E-05	3.92E-03	1.96E-02
X5.23_826.5592	-7.70	1.47E-05	4.28E-03	2.57E-02
X7.15_786.5626	-6.28	1.95E-05	4.87E-03	3.41E-02
X1.59_554.3446	-23.44	4.62E-05	1.01E-02	
X4.18_794.5050	-32.49	5.40E-05	1.05E-02	
X1.49_478.2927	-17.91	8.28E-05	1.44E-02	
X2.63_393.2768	14.21	2.19E-04	3.39E-02	
X9.42_814.5927	-11.67	2.33E-04	3.39E-02	
X1.08_612.3280	-13.31	2.56E-04	3.44E-02	
X8.93_856.6036	3.98	2.76E-04	3.45E-02	
X6.50_828.5729	-1.80	3.35E-04	3.90E-02	
X3.52_421.3076	85.75	4.46E-04	4.34E-02	
X1.22_544.2648	-36.54	4.50E-04	4.34E-02	
X2.89_493.3353	-33.94	4.72E-04	4.34E-02	
X5.27_1031.496	-56.39	4.80E-04	4.34E-02	
X4.36_825.5432	-29.25	5.16E-04	4.34E-02	
X2.72_639.5552	11.27	5.42E-04	4.34E-02	
X5.43_1068.6601	-28.22	5.51E-04	4.34E-02	
X8.90_880.6027	7.18	5.96E-04	4.34E-02	
X5.24_1108.4552	-21.48	5.97E-04	4.34E-02	
X5.24_952.4993	-16.66	6.86E-04	4.78E-02	

Table A5: Table of lipids significantly associated with waist circumference with FDR p-values <0.05. Beta coefficients, p-values, Benjamini-Hochberg FDR p-values, and Bonferroni p-values listed.

Primary ID	Beta	p-value	FDR p-value	Bonferroni p-value
X1.17_564.3289	-5.36	2.02E-08	3.52E-05	3.52E-05
X7.15_786.5626	-8.14	9.95E-07	8.68E-04	1.74E-03
X1.42_566.3497	-11.92	3.06E-06	1.78E-03	5.35E-03
X9.42_814.5927	-15.22	2.29E-05	8.85E-03	3.99E-02
X1.51_592.3513	-125.77	2.99E-05	8.85E-03	
X8.93_856.6036	5.17	3.04E-05	8.85E-03	
X1.91_594.3757	-67.46	4.25E-05	1.06E-02	
X11.11_1012.7635	-44.18	7.56E-05	1.58E-02	
X5.90_810.5600	-17.97	8.15E-05	1.58E-02	
X1.03_562.3132	-45.07	1.19E-04	1.81E-02	
X8.85_788.5789	-13.63	1.24E-04	1.81E-02	
X8.61_766.5363	4.13	1.31E-04	1.81E-02	
X5.43_1068.6601	-35.46	1.35E-04	1.81E-02	
X5.23_826.5592	-7.76	1.53E-04	1.91E-02	
X10.33_871.6904	-44.98	1.67E-04	1.92E-02	
X6.50_828.5729	-2.15	1.82E-04	1.92E-02	
X7.60_814.5916	-16.59	1.87E-04	1.92E-02	
X10.16_816.6098	-32.01	2.03E-04	1.97E-02	
X1.59_554.3446	-24.37	2.32E-04	2.12E-02	
X7.32_864.6056	-24.59	2.72E-04	2.12E-02	
X0.99_586.3136	-24.35	2.77E-04	2.12E-02	
X4.18_794.5050	-33.65	2.78E-04	2.12E-02	
X9.01_1128.5388	41.17	2.79E-04	2.12E-02	
X8.90_880.6027	8.58	3.18E-04	2.31E-02	
X3.52_421.3076	98.52	4.15E-04	2.90E-02	
X9.14_790.5935	-10.65	4.36E-04	2.93E-02	
X10.95_650.6069	24.14	4.62E-04	2.99E-02	
X10.11_1381.848	-42.51	4.80E-04	2.99E-02	
X1.08_612.3280	-14.34	5.93E-04	3.55E-02	
X6.26_834.5625	-16.98	6.11E-04	3.55E-02	
X10.16_826.6359	-30.64	7.08E-04	3.98E-02	
X7.02_772.5264	-10.17	7.36E-04	4.02E-02	
X1.21_476.2768	-12.46	7.90E-04	4.18E-02	
X7.47_788.5783	-9.13	9.57E-04	4.91E-02	
X8.61_1038.473	32.37	9.91E-04	4.94E-02	

Table A6: Table of lipids significantly associated with serum leptin with FDR p-values <0.05. Beta coefficients, p-values, Benjamini-Hochberg FDR p-values, and Bonferroni p-values listed.

Primary ID	Beta	p-value	FDR p-value	Bonferroni p-value
X1.17_564.3289	-0.92	2.85E-08	4.98E-05	4.98E-05
X8.93_856.6036	1.02	1.65E-06	1.36E-03	2.88E-03
X1.42_566.3497	-2.10	2.34E-06	1.36E-03	4.08E-03
X1.51_592.3513	-22.38	1.92E-05	8.39E-03	3.36E-02
X1.08_612.3280	-3.03	2.50E-05	8.74E-03	4.37E-02
X1.03_562.3132	-8.33	4.10E-05	1.19E-02	
X1.41_452.2762	-6.39	4.85E-05	1.19E-02	
X0.99_586.3136	-4.66	5.80E-05	1.19E-02	
X9.01_1128.5388	7.86	6.13E-05	1.19E-02	
X7.15_786.5626	-1.17	7.19E-05	1.25E-02	
X10.33_871.6904	-8.00	1.19E-04	1.89E-02	
X5.43_1068.6601	-6.19	1.31E-04	1.90E-02	
X1.91_594.3757	-10.83	1.75E-04	2.22E-02	
X5.90_810.5600	-2.99	1.78E-04	2.22E-02	
X8.90_880.6027	1.52	2.36E-04	2.75E-02	
X1.21_476.2768	-2.35	2.60E-04	2.84E-02	
X10.53_854.6676	-2.10	4.62E-04	4.70E-02	
X7.32_864.6056	-4.11	4.89E-04	4.70E-02	
X9.66_768.5494	3.00	5.11E-04	4.70E-02	
X8.61_766.5363	0.65	5.56E-04	4.85E-02	

Table A7: Table of lipids significantly associated with serum total adiponectin with FDR p-values <0.05. Beta coefficients, p-values, Benjamini-Hochberg FDR p-values, and Bonferroni p-values listed.

Primary ID	Beta	p-value	FDR p-value	Bonferroni p-value
X1.17_564.3289	0.39	1.14E-07	1.98E-04	1.98E-04
X7.15_786.5626	0.61	1.93E-06	1.31E-03	3.36E-03
X1.42_566.3497	0.93	2.26E-06	1.31E-03	3.94E-03
X4.18_794.5050	3.17	5.94E-06	2.59E-03	1.04E-02
X1.51_592.3513	10.15	1.05E-05	3.66E-03	1.83E-02
X8.62_886.4824	-4.53	3.83E-05	1.11E-02	
X6.50_828.5729	0.18	4.49E-05	1.12E-02	
X5.23_826.5592	0.63	6.07E-05	1.32E-02	
X1.03_562.3132	3.49	1.04E-04	2.01E-02	
X1.21_476.2768	1.09	1.21E-04	2.11E-02	
X8.61_912.4667	-2.37	1.58E-04	2.50E-02	
X4.99_859.5280	1.27	1.94E-04	2.71E-02	
X1.43_702.3160	2.36	2.08E-04	2.71E-02	
X1.49_478.2927	1.48	2.18E-04	2.71E-02	
X1.16_700.3013	1.79	2.50E-04	2.91E-02	
X5.23_1098.4881	1.59	2.73E-04	2.97E-02	
X8.44_716.5227	-1.42	3.36E-04	3.26E-02	
X5.22_1030.5052	2.71	3.52E-04	3.26E-02	
X8.61_980.4565	-2.94	3.55E-04	3.26E-02	
X8.61_896.4927	-2.09	4.81E-04	4.19E-02	
X1.22_544.2648	3.15	5.66E-04	4.70E-02	
X7.47_788.5783	0.73	6.02E-04	4.77E-02	

Table A8: Table of lipids significantly associated with serum C-peptide with FDR p-values <0.05. Beta coefficients, p-values, Benjamini-Hochberg FDR p-values, and Bonferroni p-values listed.

Primary ID	Beta	p-value	FDR p-value	Bonferroni p-value
X10.09_880.5267	2.95	8.88E-06	6.25E-03	1.55E-02
X10.09_744.5522	1.04	1.01E-05	6.25E-03	1.76E-02
X9.66_768.5494	2.14	1.07E-05	6.25E-03	1.87E-02
X7.95_790.5381	1.01	5.18E-05	1.68E-02	
X8.44_716.5227	2.05	5.61E-05	1.68E-02	
X8.61_1038.473	3.89	5.76E-05	1.68E-02	
X1.42_566.3497	-1.00	1.10E-04	2.61E-02	
X8.61_766.5363	0.41	1.23E-04	2.61E-02	
X1.17_564.3289	-0.38	1.34E-04	2.61E-02	
X7.15_786.5626	-0.62	2.51E-04	4.37E-02	
X8.93_856.6036	0.45	2.83E-04	4.49E-02	

Table A9: Table of primary IDs with Radii >0.1 from SVD biplot.

Primary ID	Vector 1	Vector 2	Radius
X12.43_934.7918	-0.19	0.13	0.24
X8.17_830.6155	0.03	0.22	0.22
X1.51_592.3513	0.07	0.20	0.22
X12.14_850.7682	0.20	-0.05	0.20
X8.24_880.6145	-0.08	-0.18	0.20
X3.52_421.3076	-0.09	-0.13	0.16
X1.35_669.3989	0.16	0.02	0.16
X10.21_1048.6206	-0.08	0.12	0.15
X3.45_269.1316	0.13	0.00	0.13
X5.27_1031.496	-0.05	0.12	0.13
X12.88_922.7355	0.12	-0.05	0.13
X8.28_814.6742	-0.05	-0.12	0.13
X5.23_964.5107	-0.05	0.11	0.12
X1.57_466.3290	0.12	-0.02	0.12
X13.87_920.717	0.03	-0.11	0.12
X1.2_488.2839	-0.01	0.11	0.12
X9.03_842.5446	0.02	0.11	0.11
X14.35_1062.754	-0.08	-0.08	0.11
X10.38_1017.6967	0.10	0.06	0.11
X9.96_1034.5762	-0.11	0.00	0.11
X0.99_963.3915	-0.10	-0.05	0.11
X1.93_419.3020	0.09	-0.05	0.10
X8.87_1060.5423	-0.10	-0.02	0.10
X1.91_594.3757	0.03	0.10	0.10
X3.52_459.3291	0.08	-0.06	0.10

Table A10: Beta coefficients and Bonferroni corrected p-values calculated from the regression of principal component 4 scores (n=126) with 1,745 plasma lipid metabolites.

Positive Betas

Metabolite	Beta	Bonferroni p-value
X13.10_920.7410	3.54	3.63E-05
X8.23_950.6408	3.53	4.02E-04
X13.07_988.7940	3.36	2.29E-02
X9.96_1034.5762	2.46	2.48E-06
X14.31_881.7973	2.38	3.74E-04
X13.11_919.7343	2.26	5.00E-04
X14.38_880.7349	2.19	4.18E-05
X14.33_869.3325	2.05	1.64E-08
X14.35_1062.754	1.99	4.74E-05
X2.65_255.2331	1.98	1.59E-06

Negative Betas

Metabolite	Beta	Bonferroni p-value
X9.03_842.5446	-1.76	6.52E-05
X11.20_1012.7670	-1.25	7.79E-08
X1.91_594.3757	-1.17	1.56E-03
X8.67_864.6048	-1.00	2.63E-02
X10.11_1381.848	-0.92	1.83E-04
X10.33_871.6904	-0.73	4.82E-02
X10.19_802.5695	-0.71	9.17E-08
X4.37_881.5140	-0.69	1.06E-07
X9.45_1261.8069	-0.66	4.26E-04
X0.99_586.3136	-0.65	4.09E-09

Table A11: Complete list of primary ids, common name, molecular ion, theoretical *m/z*, and mass error of lipids identified.[a]

Primary ID	Common name	Molecular Ion (ESI-neg)	Theoretical *m/z* (Da)	Mass error (Da)
X0.99_586.3136	LPC(20:5)	$[M+formate]^-$	586.3151	0.0015
X1.03_562.3132	LPC(18:3)	$[M+formate]^-$	562.3151	0.0019
X1.08_612.3280	LPC(22:6)	$[M+formate]^-$	612.3307	0.0027
X1.17_564.3289	LPC(18:2)	$[M+formate]^-$	564.3307	0.0018
X1.21_476.2768	LPE(18:2)	$[M-H]^-$	476.2783	0.0015
X1.22_544.2648	LPS(20:4)	$[M-H]^-$	544.2681	0.0033
X1.35_669.3989	Unknown	-		
X1.42_566.3497	LPC(18:1)	$[M+formate]^-$	566.3464	0.0033
X1.49_478.2927	LPE(18:1)	$[M-H]^-$	478.2939	0.0012
X1.51_592.3513	LPC(20:2)	$[M+formate]^-$	592.362	0.0107
X1.59_554.3446	LPC(17:0)	$[M+formate]^-$	554.3464	0.0018
X1.91_594.3757	LPC(20:1)	$[M+formate]^-$	594.3777	0.002
X2.65_255.2331	Palmitic acid	$[M-H]^-$	255.233	0.0001
X3.52_421.3076	TLTI	-		
X4.18_794.5050	Unknown	-		
X4.37_881.5140	PI(16:0_22:6)	$[M-H]^-$	881.5186	0.0046
X5.23_826.5592	PC(18:2_18:2)	$[M+formate]^-$	826.5604	0.0012
X5.43_1068.6601	Unknown	-		
X5.90_810.5600	PC(O-16:0_20:5)	$[M+formate]^-$		
X6.50_828.5729	PC(18:1_18:2)	$[M+formate]^-$	828.576	0.0031
X7.15_786.5626	PC(O-16:1_18:2)	$[M+formate]^-$	786.5655	0.0029
X7.32_864.6056	PC(O-18:1_22:5)	$[M+formate]^-$		
X7.47_788.5783	PC(O-16:0_18:2)	$[M+formate]^-$		
X8.17_830.6155	PC(18:0_18:2)	$[M+formate]^-$	830.5917	0.0238
X8.23_950.6408	TLTI	-		
X8.24_880.6145	PC(20:1_20:4)	$[M+formate]^-$		
X8.44_716.5227	PE(16:0_18:1)	$[M-H]^-$	716.5236	0.0009
X8.61_1038.473	TLTI	-		
X8.61_766.5363	PE(18:0_20:4)	$[M-H]^-$	766.5392	0.0029
X8.67_864.6048	PC(O-18:0_22:6)	$[M+formate]^-$	864.6124	0.0076

[a]The structural of identification of plasma lipids was performed based if *m/z*: was significant in two or more models from single marker regression analyses of responses, or had a radii ≥ 0.2 in singular value decomposition analysis, or was one of the top ten lipids positively and inversely associated with principal component 4. Plasma lipids meeting this criterion that were too low to identify (TLTI) are listed. Plasma lipid common names determined by lipid structure identification using Lipid Maps and Human Metabolome databases, and manual confirmation of mass spectra. The "_" between fatty acids in glycerophospholipid structures is used, since the position at the sn1 and sn2 position cannot be determined. Plasma lipids listed as unknown did not match a lipid library database search or exhibit a fragmentation pattern indicative of phospholipids in ms/ms analyses.

Table A11 (cont'd)

Primary ID	Common name	Molecular Ion (ESI-neg)	Theoretical *m/z* (Da)	Mass error (Da)
X8.90_880.6027	PC(18:0_22:5)	$[M+formate]^-$		
X8.93_856.6036	PC(18:0_20:3)	$[M+formate]^-$	856.6073	0.0037
X9.01_1128.5388	TLTI	-		
X9.03_842.5446	TLTI	-		
X9.42_814.5927	PC(O-18:1_18:2)	$[M+formate]^-$	814.5968	0.0041
X9.45_1261.8069	TLTI	-		
X9.66_768.5494	PE(18:0_20:3)	$[M-H]^-$	768.5549	0.0055
X9.96_1034.5762	TLTI	-		
X10.11_1381.8480	Unknown	-		
X10.19_802.5695	TLTI	-		
X10.33_871.6904	SM(d19:1/24:1)	$[M+formate]^-$	871.691	0.0006
X11.20_1012.7670	TLTI	-		
X12.14_850.7682	TLTI	-		
X12.43_934.7918	TLTI	-		
X13.07_988.7940	TLTI	-		
X13.10_920.7410	TLTI	-		
X13.11_919.7343	TLTI	-		
X14.31_881.7973	TLTI	-		
X14.33_869.3325	TLTI	-		
X14.35_1062.754	TLTI	-		
X14.38_880.7349	TLTI	-		

Table A12: Polyunsaturated fatty acid and oxylipids analyzed in this study and their respective optimized multiple reaction monitoring parameters.[a]

Metabolite	Parent > Daughter ion (*m/z*)	Precursor	Class
LA	279.2>59		PUFA
9-HODE	295.2>171	LA	Alcohol
13-HODE	295.2>195	LA	Alcohol
13-HOTrE	293.6>195	ALA	Alcohol
9-oxoODE	293.2>185	LA	Ketone
13-oxoODE	293.2>113	LA	Ketone
9,10-EpOME	295.2>171	LA	Epoxide
12,13-EpOME	295.2>195	LA	Epoxide
9,10-DiHOME	313.2>201	LA	Vicinal diol
12,13-DiHOME	312.2>183	LA	Vicinal diol
15-HETrE	321.1>221	DGLA	Alcohol
ARA	303.2>259		PUFA
5-HETE	319.2>115	ARA	Alcohol
9-HETE	319.2>151	ARA	Alcohol
11-HETE	319.2>167	ARA	Alcohol
12-HETE	319.1>179	ARA	Alcohol
12-HHTrE	279.2>179	ARA	Alcohol
15-HETE	319.2>219	ARA	Alcohol
20 HETE	319.2>245	ARA	Alcohol
5-oxoETE	317.2>203	ARA	Ketone
12-oxoETE	317.8>153	ARA	Ketone
15-oxoETE	317.2>113	ARA	Ketone
8,9-EET	319.2>115	ARA	Epoxide
11,12-EET	319.2>179	ARA	Epoxide
14,15-EET	319.2>219	ARA	Epoxide
8,9-DHET	337.2>127	ARA	Vicinal diol
11,12-DHET	337.2>167	ARA	Vicinal diol
14,15 DHET	337.2>207	ARA	Vicinal diol
5(S),6(R)-Lipoxin A_4	351.2>271	ARA	Lipoxin
LTB4	335.2>195	ARA	Leukotriene
TXB2	369.2>169	ARA	Thromboxane

[a]LA = linoleic acid; HODE = hydroxyoctadecadienoic acid; HOTrE = hydroxyoctadecatrienoic acid; oxoODE = oxooctadecadienoic acid; EpOME = epoxyoctadecenoic acid; DiHOME = dihydroxyoctadecadienoic acid; HETrE = hydroxyeicosatrienoic acid; ARA = arachidonic acid; HETE = hydroxyeicsaotetraenoic acid; HHTrE = hydroxyheptadecatrienoic acid; oxoETE = oxoeicosatetraenoic acid; EET= epoxyeicosatrienoic acid; DHET = dihydroxyeicosatrienoic acid; LTB4 = leukotriene B4; TXB2 = thromboxane B2; PG = prostaglandin; iPF = isoprostane; AG = arachidonoyl glycerol; AEA = arachidonoyl ethanolamide; EPA = eicosapentaenoic acid; HEPE = hydroxyeicosapentaenoic acid; EpETE = epoxyeicosatetratenoic acid; DiHETE = dihydroxyeicosatetraenoic acid; DHA = docosahexaenoic acid; EpDPE = epoxydocosapentaenoic acid; DiHDPA= dihydroxydocosapentaenoic acid; HDoHE = hydroxydocosahexaenoic acid; DiHDoHE = dihydroxydocosahexaenoic acid; DHEA = docosahexaenoyl ethanolamide; IS = deuterium labeled internal standard; DGLA = dihomo-gamma-linolenic acid; ALA = alpha-linolenic acid.

Table A12 (cont'd)

15-d-$\Delta^{12,14}$-PGJ2	315.2>271	ARA	Prostaglandin
2,3-dinor-8-iso PGF2α	325.1>237	ARA	Prostaglandin
5-iPF2α-VI	353.9>115	ARA	Prostaglandin
6-keto PGF1α	369.3>163	ARA	Prostaglandin
8-iso PGF2α	353.2>193	ARA	Prostaglandin
8-iso-15-keto PGE2	349.1>287	ARA	Prostaglandin
8-iso-15 PGF2α	353.1>193	ARA	Prostaglandin
8-iso-PGA1	335.2>235	ARA	Prostaglandin
8-iso-PGE2	351.1>271	ARA	Prostaglandin
PGE2	351.2>271	ARA	Prostaglandin
AG	379.2>287	ARA	Endocannabinoid
AEA	348.2>62	ARA	Endocannabinoid
EPA	301.2>257		PUFA
15-HEPE	317.1>219	EPA	Alcohol
8,9-EpETE	317.2>255	EPA	Epoxide
14,15-EpETE	317.2>207	EPA	Epoxide
5,6-DiHETE	335.2>145	EPA	Vicinal diol
5(S),15(S)-DiHETE	335.1>173	EPA	diol
14,15-DiHETE	335.2>207	EPA	Vicinal diol
17,18-DiHETE	335.2>247	EPA	Vicinal diol
DHA	327.2>283		PUFA
19,20-EpDPE	343.2>299	DHA	Epoxide
19,20-DiHDPA	361.2>273	DHA	Vicinal diol
17-HDoHE	343.2>281	DHA	Alcohol
10(S),17(S)-DiHDoHE	359.2>153	DHA	Protectin
7(S),17(S)-HiDPA	343.2>281	DHA	Protectin
Resolvin D1	375.2>141	DHA	Resolvin
Resolvin D2	375.2>175	DHA	Resolvin
7-Maresin1	359.2>177	DHA	Resolvin
DHEA	372.3>62	DHA	Endocannabinoid
ARA_*d8*	311.2>267	IS	PUFA
5(S)-HETE_*d*8	327.2>116	IS	Alcohol
15(S)-HETE_*d*8	327.2>226	IS	Alcohol
8,9 EET_*d*11	330.3>268	IS	Epoxide
8,9-DHET_*d*11	348.3>127	IS	Vicinal diol
PGE2_*d*9	360.2>280	IS	Prostaglandin
2-AG_*d*8	387.3>294	IS	Endocannabinoid
AEA_*d*8	356.3>63	IS	Endocannabinoid

Table A13: Obesity is associated with decreased linoleic acid-derived vicinal diols and the ratio of vicinal diols to precursor epoxides (values expressed as median [Q1,Q3]).

Variable[a]	Overall	Lean	Overweight	Obese	BH FDR p-value[b]
LA	0.7 [0.4, 1.0]	0.5 [0.4, 1.0]^{A}	0.7 [0.4, 1.2]^{A}	0.7 [0.5, 1.0]^{A}	-
12,13-DiHOME	3.3 [2.1, 5.7]	4.2 [1, 10.8]^{A}	4.8 [2.63, 6.17]^{A}	2.7 [1.6, 3.9]^{B}	$p \leq 0.05$
Total DiHOME	12.0 [7.1, 23.2]	13.9 [8.7, 53.0]^{A}	16.7 [7.8, 24.9]^{A}	9.8 [6.2, 18.6]^{B}	*0.061*
ARA	14.9 [10.4, 21.4]	13.5 [9.6, 17.3]^{A}	14.6 [10.1, 22.9]^{A}	16.0 [12.7, 22.3]^{A}	-
9-oxo/HODE	0.5 [0.4, 0.7]	0.5 [0.3, 0.6]^{A}	0.6 [0.5, 0.8]^{A}	0.5 [0.3, 0.6]^{A}	-
13-oxo/HODE	0.3 [0.1, 0.2]	0.1 [0.1, 0.2]^{A}	0.2 [0.1, 0.2]^{AB}	0.2 [0.1, 0.2]^{B}	*0.083*
9,10-Di/EpOME	0.4 [0.2, 0.9]	0.5 [0.3, 1.3]^{A}	0.5 [0.3, 1.0]^{AB}	0.3 [0.2, 0.7]^{B}	*0.065*
12,13-Di/EpOME	0.1 [0.1, 0.1]	0.1 [0.1, 0.1]^{A}	0.1 [0.1, 0.2]^{A}	0.1 [0.0, 0.1]^{B}	$p \leq 0.05$
14,15-DH/EET	1.3 [0.9, 1.8]	1.5 [1.3, 2.7]^{A}	1.3 [1.0, 1.9]^{A}	1.1 [0.9, 1.5]^{A}	-

[a]Plasma non-esterified polyunsaturated fatty acids, oxylipids, and product-to-precursor ratios are presented. LA, 12,13-DiHOME, and ARA values are presented as levels (i.e., peak area of compound/ peak area of internal standard), since a majority of participants concentrations were greater (i.e., >10 fold) than the highest standard curve value. Total DiHOME is calculated as the sum of 9,10-DiHOME nM and 12,13-DiHOME levels. Product-to-precursor ratios are calculated as 9-oxo/HODE = 9-oxoODE/9-HODE; 13-oxo/HODE = 13-oxoODE/13-HODE; 9,10-Di/EpOME = 9,10-DiHOME/9,10-EpOME; 12,13-Di/EpOME = 12,13-DiHOME/12,13-EpOME; 14,15-DH/EET = 14,15-DHET/14,15-EET.

[b]Categorical analysis of BMI by Kruskal Wallis one-way ANOVA and Dunn's test for multiple comparison. P-values were corrected for false discovery rate (FDR) according to Benjamini-Hochberg (BH). P-values italicized if ≤ 0.09 and >0.05.

Table A14: Higher HETE factor loadings were associated with being obese, and lower octadecanoid and ω-3 factor loadings were inversely associated with obesity.[a]

	Body Mass Index		*Waist Circumference*	
Factor	**Beta**	**p-value**	**Beta**	**p-value**
HETE	0.81	0.094	0.97	0.079
Octadecanoid	-0.77	-	-1.02	0.071
ω-6 PUFA	0.40	-	0.02	-
ω-3 PUFA	-1.71	p<0.0005	-2.28	p<0.0001

[a]Factors were derived from PUFAs and oxylipids. Body mass index and waist circumference were regressed on the factors (i.e., one at a time). Models were defined as: (BMI_i or WC_i = Age_i + $Smoking_i$ + $Factor_{ik}$) where $i=(1,...,123)$ represents study participants and k=(1,..,4) represents the factors derived from PUFAs and oxylipids.

Table A15: PUFA-normalized hydroxyeicosatetreanoates and vicinal diols are associated with body mass index and waist circumference.[a]

	Body Mass Index				*Waist Circumference*			
Oxylipid	**Beta**	**p-value**	**BH FDR p-value**	**Bonferroni p-value**	**Beta**	**p-value**	**BH FDR p-value**	**Bonferroni p-value**
9-HODE	-0.82	-	-	-	-0.85	-	-	-
13-HODE	-0.99	-	-	-	-1.12	-	-	-
Total HODE	-0.92	-	-	-	-0.99	-	-	-
13-HOTrE	-0.41	-	-	-	-0.60	-	-	-
9-oxoODE	-1.66	p≤0.05	-	-	-1.66	*0.0602*	-	-
13-oxoODE	1.14	-	-	-	1.21	-	-	-
Total oxoODE	-1.26	-	-	-	-1.28	-	-	-
9,10-EpOME	-0.71	-	-	-	-0.93	-	-	-
12,13-EpOME	-2.04	p≤0.005	p≤0.05	-	-2.30	p≤0.005	p≤0.05	-
Total EpOME	-1.93	p≤0.05	-	-	-2.21	p≤0.05	*0.0540*	-
9,10-DiHOME	-1.37	p≤0.001	p≤0.01	p≤0.05	-1.23	p≤0.01	p≤0.05	-
12,13-DiHOME	-1.88	p≤0.0001	p≤0.001	p≤0.001	-1.83	p≤0.0005	p≤0.05	p≤0.05
Total DiHOME	-1.51	p≤0.0005	p≤0.005	p≤0.05	-1.39	p≤0.005	p≤0.05	-
5-HETE	1.01	p≤0.01	*0.0512*	-	1.39	p≤0.005	p≤0.05	*0.0522*
11-HETE	1.06	p≤0.05	-	-	1.44	p≤0.01	p≤0.05	-
12-HETE	0.39	-	-	-	0.37	-	-	-
15-HETE	0.81	-	-	-	0.96	-	-	-
20-HETE	1.02	-	-	-	1.25	-	-	-
Total HETE	0.92	*0.0818*	-	-	1.25	p≤0.05	-	-
14,15-EET	0.32	-	-	-	0.67	-	-	-
8,9-DHET	-0.68	-	-	-	-0.33	-	-	-
11,12-DHET	-0.96	-	-	-	-0.53	-	-	-
14,15-DHET	-1.11	-	-	-	-0.85	-	-	-
Total DHET	-1.00	-	-	-	-0.63	-	-	-
AEA	-0.58	-	-	-	-0.37	-	-	-
AG	-0.77	-	-	-	-0.86	-	-	-
LTB4	-0.08	-	-	-	0.14	-	-	-
PGD2	-0.68	*0.0632*	-	-	-0.42	-	-	-
TBX2	-0.14	-	-	-	-0.19	-	-	-
5,6-DiHETE	-0.03	-	-	-	0.20	-	-	-
17,18-DiHETE	-0.73	*0.0733*	-	-	-0.74	-	-	-
Total DiHETE	-0.55	-	-	-	-0.43	-	-	-
19,20-HDPA	-2.60	p≤0.0005	p≤0.01	p≤0.01	-2.62	p≤0.005	p≤0.05	*0.0505*
DHEA	-0.30	-	-	-	0.02	-	-	-

[a]Beta coefficients and p-values were determined by regressions body mass index (BMI) and waist circumferences (WC) on log transformed plasma PUFA-normalized oxylipids individually (i.e., one at a time). Single lipid regression models were defined as: BMI_i or $WC_i = age_i + smoking_i + \log(lipid_{ic})$, where $i = (1,...,123)$ and $c = (1,...,33)$. Oxylipids totals calculated as follows: Total HODE = (∑9-HODE + 13-HODE)/LA; Total oxoODE = (∑ 9-oxoODE + 13-oxoODE)/LA; Total EpOME = (∑ 9,10-EpOME + 12,13-EpOME)/LA; Total HETE = (∑ 5-HETE + 11-HETE + 12-HETE + 15-HETE + 20-HETE)/ARA; Total DHET = (∑ 8,9-DHET + 11,12-DHET + 14,15-DHET)/ARA; Total DiHETE = (∑ 5,6-DiHETE + 17,18-DiHETE)/EPA.

Table A16: The presence of one colon polyp is associated linoleic and docosatetraenoic acid.[a,b]

PUFA	1 Polyp (OR 95% CI)	p-value	2 Polyps (OR 95% CI)	p-value	≥ 3 polyps (OR 95% CI)	p-value
C18:2-c LA	0.83 (0.72,0.95)	**0.010**	0.85 (0.71,1.02)	0.096	0.98 (0.85,1.12)	0.771
C18:2-t Linoelaidic [c]	1.02 (0.79,1.31)	0.893	1.22 (0.99,1.51)	*0.063*	1.18 (0.97,1.42)	0.091
C20:3 DGLA	1.15 (0.65,2.02)	0.630	0.96 (0.44,2.08)	0.922	1.34 (0.76,2.36)	0.315
C20:4 ARA	1.16 (0.97,1.38)	0.098	1.20 (0.95,1.52)	0.121	1.06 (0.89,1.27)	0.509
C22:4 DTA [c]	1.43 (1.02,2.03)	**0.040**	1.53 (0.95,2.44)	*0.078*	1.23 (0.86,1.77)	0.255
C22:5 DPA [c]	1.39 (0.98,1.98)	*0.064*	1.04 (0.61,1.75)	0.890	1.13 (0.77,1.64)	0.542
C20:5 EPA	0.40 (0.13,1.23)	0.109	0.78 (0.27,2.23)	0.641	0.61 (0.25,1.51)	0.285
C22:5 DPA	1.05 (0.25,4.41)	0.948	1.18 (0.22,6.44)	0.847	1.87 (0.64,5.48)	0.252
C22:6 DHA	0.97 (0.67,1.41)	0.891	1.10 (0.68,1.79)	0.688	0.88 (0.59,1.31)	0.529
EPADHA [d]	0.90 (0.68, 1.19)	0.462	1.02 (0.72, 1.45)	0.917	0.87 (0.64, 1.18)	0.382
D5D [e]	1.06 (0.79,1.41)	0.696	1.20 (0.84,1.72)	0.311	0.91 (0.66,1.25)	0.565
D6D [c,f]	2.45 (1.00,5.96)	**0.049**	1.46 (0.43,5.01)	0.545	1.68 (0.67,4.22)	0.265

[a]Fatty acids expressed as percent of total phospholipids. P-values bolded if $p \leq 0.05$ and italicized if $0.05 < p \leq 0.09$. ARA: arachidonic acid; D5D: delta-5-desaturase estimated enzyme activity; D6D: delta-6-desaturase estimated enzyme activity; DGLA, Dihomo-γ-linolenic acid; DHA: docosahexaenoic acid; DPA, docosapentaenoic acid; DTA: docosatetraenoic acid; EPA: eicosapentaenoic acid; LA: linoleic acid.

[b]Models defined as: Polyp number = fatty acid+age+smoking.

[c]Odds ratios have been calculated on the basis that there is a unit change of 0.01 for the respective beta coefficient for each given parameter.

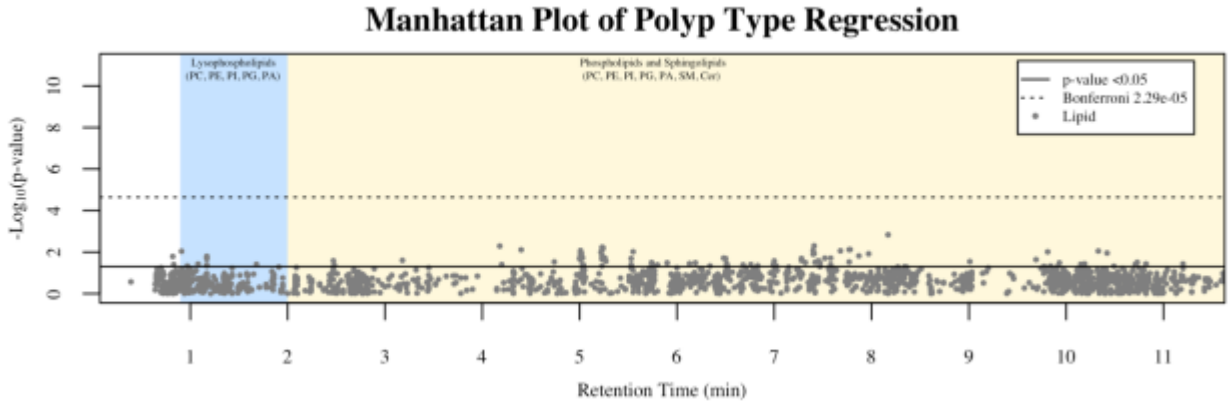
[d]EPADHA was calculated as ∑ EPA+DHA.

[e]D5D EAE was calculated as the ratio of ARA/DGLA.

[f]D6D EAE was calculated as the ratio of DGLA/LA.

APPENDIX B: Colon polyps are associated with lipidomic profiles

Figure B1: Individual plasma phospholipids are not associated with polyp type after false discovery correction.[a]



[a]Manhattan plots of the –log(p-value) for polyp type (i.e., no polyp, hyperplastic, or adenoma. Single lipid regression models defined as: Polyp Type$_i$ = Age$_i$ + Smoking$_i$ + Lipid$_j$ where i = (1,...,122) and j = (1,...,2813). Y-axis represents -log(p-value) and x-axis represent the retention time of the metabolite in minutes. Plasma lipid Benjamini-Hochberg false discovery corrected p-values <0.05 are circled in red. Lipids with <40% missing values were mean imputed in the data set displayed.

Table B1: The ARA-derived oxylipid 5- and 11-HETE are positively associated with polyp type, even after adjusting for BMI.[a]

	Age + Smoking				*Age + Smoking + BMI*			
	Beta	**P-value**	**BH FDR p-value**	**Bonferroni p-value**	**Beta**	**P-value**	**BH FDR p-value**	**Bonferroni p-value**
LA	-0.07	-			-0.09	-		
ARA	-0.09	-			-0.14	-		
EPA	-0.11	-			-0.07	-		
DHA	-0.11	-			-0.11	-		
AEA	0.01	-			0.04	-		
9-HODE	-0.06	-			0.04	-		
13- HODE	-0.08	-			0.04	-		
9-oxoODE	-0.01	-			0.16	-		
13-oxoODE	0.05	-			0.07	-		
5-HETE	0.27	p<0.0001	p<0.005	p<0.005	0.23	p<0.0005	p<0.01	p<0.01
11-HETE	0.31	p<0.0005	p<0.005	p<0.005	0.27	p<0.0005	p<0.01	p<0.01
12-HETE	0.18	0.05333			0.15	p<0.05		
13-HOTrE	0.06				0.09	0.1083		
15-HETE	0.20	p<0.05			0.15	p<0.05		
15-HETrE	0.24	p<0.005	p<0.05	0.07510	0.20	p<0.005	p<0.05	
20-HETE	0.07	-			0.07			
9,10-EpOME	-0.02	-			0.12	-		
12,13-EpOME	-0.16	-			0.04	-		
9,10-DiHOME	-0.07	-			0.05	-		
12,13-DiHOME	-0.06	-			0.11	-		
8,9-DHET	0.18	-			0.19	p<0.05		
11,12-DHET	0.21	-			0.21	p<0.05		
14,15-DHET	0.15	-			0.21	0.0726		
5,6-DiHETE	0.02	-			0.10	-		
17,18-DiHETE	-0.25	p<0.05			0.03	-		
19,20-HDPA	-0.24	-			0.09	-		
LTB4	0.13	-			0.12	-		
2-Ag	-0.15	-			-0.02	-		
TXB2	0.08	-			0.10	-		

[a]Non-esterified lipids were regressed on polyp type. Polyp type (i.e., no polyp, hyperplastic, or adenoma. Single lipid regression models defined as: $\text{Polyp Type}_i = \text{Age}_i + \text{Smoking}_i + \log(\text{Lipid}_j)$, where $i = (1,...,122)$ and $j = (1,...,30)$. Lipids with <40% missing values were min imputed in the results displayed.

Table B2: The entire plasma phospholipid explains a sizeable proportion of variation in polyp type.[a]

	σ_u^2 [95% Confidence Interval]
Plasma phospholipidome	0.22 [0.08,0.53]
Non-esterified lipids	0.08 [0.03,0.21]

[a]Polyp type was regressed on the entire plasma phospholipidome, and then non-esterified lipid data set. Polyp type consists of the phenotype y_i $(i = 1, \dots, 122)$ indexed by individuals (i) and the set of predictors coming from the lipidomic data as well as clinical covariates including age of the participant (a_i), and the status of smoking behavior (s_i). The following 2 equations were employed for each data set, in which lipids with <40% missing values were imputed as the min value for each lipid in each data set.

Plasma phospholipidome equation:

$$y_i = \beta_0 + age\beta_1 + smoking\beta_2 + BMI\beta_3 + WC\beta_4 + \sum_{j=1}^{j=2813} x_{ij}\alpha_j + \varepsilon_i$$

Non-esterified lipid equation:

$$y_i = \beta_0 + age\beta_1 + smoking\beta_2 + BMI\beta_3 + WC\beta_4 + \sum_{o=1}^{o=30} x_{io}\alpha_o + \varepsilon_i$$

In each respective equation,
$u_i = \sum_{j=1}^{j=2813} x_{ij}\alpha_j$ and $u_i = \sum_{o=1}^{o=30} x_{io}\alpha_o$
represents the total effect of the lipids on trait y_i, x_{ij} is the phospholipidomic abundance for participant *i* and lipid *j*, and α_j is the estimated effect, and x_{io} is the non-esterified lipid abundance for participant *i* and lipid *o*, and α_o is the estimated effect.

BIBLIOGRAPHY

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1. Hursting, S.D. and S.M. Dunlap, *Obesity, metabolic dysregulation, and cancer: a growing concern and an inflammatory (and microenvironmental) issue.* Ann N Y Acad Sci, 2012. **1271**: p. 82-7.

2. Comstock, S.S., et al., *Adipokines and obesity are associated with colorectal polyps in adult males: a cross-sectional study.* PLoS One, 2014. **9**(1): p. e85939.

3. Comstock, S.S., et al., *Cross-sectional analysis of obesity and serum analytes in males identifies sRAGE as a novel biomarker inversely associated with diverticulosis.* PLoS One, 2014. **9**(4): p. e95232.

4. Comstock, S.S., et al., *Association of insulin-related serum factors with colorectal polyp number and type in adult males.* Cancer Epidemiol Biomarkers Prev, 2014. **23**(9): p. 1843-51.

5. Pickens, C.A., et al., *Plasma phospholipids, non-esterified plasma polyunsaturated fatty acids and oxylipids are associated with BMI.* Prostaglandins Leukot Essent Fatty Acids, 2015. **95**: p. 31-40.

6. Kroger, J. and M.B. Schulze, *Recent insights into the relation of Delta5 desaturase and Delta6 desaturase activity to the development of type 2 diabetes.* Curr Opin Lipidol, 2012. **23**(1): p. 4-10.

7. Fekete, K., et al., *Long-chain polyunsaturated fatty acid status in obesity: a systematic review and meta-analysis.* Obes Rev, 2015. **16**(6): p. 488-97.

8. Kitamura, T., et al., *Proinsulin C-peptide activates cAMP response element-binding proteins through the p38 mitogen-activated protein kinase pathway in mouse lung capillary endothelial cells.* Biochem J, 2002. **366**(Pt 3): p. 737-44.

9. Ogden, C.L., et al., *Prevalence of obesity in the United States, 2009-2010.* NCHS Data Brief, 2012(82): p. 1-8.

10. Finkelstein, E.A., et al., *Annual medical spending attributable to obesity: payer-and service-specific estimates.* Health Aff (Millwood), 2009. **28**(5): p. w822-31.

11. Wellman, N.S. and B. Friedberg, *Causes and consequences of adult obesity: health, social and economic impacts in the United States.* Asia Pac J Clin Nutr, 2002. **11 Suppl 8**: p. S705-9.

12. Staff, M.C. *Obesity*. 6/7/2013 [cited 2013 July 22]; Available from: http://www.mayoclinic.com/health/obesity/DS00314/DSECTION=complications.

13. Matafome, P., et al., *COMMON mechanisms of dysfunctional adipose tissue and obesity-related cancer.* Diabetes Metab Res Rev, 2013.

14. Weisberg, S.P., et al., *Obesity is associated with macrophage accumulation in adipose tissue.* J Clin Invest, 2003. **112**(12): p. 1796-808.

15. Bardou, M., A.N. Barkun, and M. Martel, *Obesity and colorectal cancer.* Gut, 2013.

16. Vucenik, I. and J.P. Stains, *Obesity and cancer risk: evidence, mechanisms, and recommendations.* Ann N Y Acad Sci, 2012. **1271**: p. 37-43.

17. Ramos-Nino, M.E., *The role of chronic inflammation in obesity-associated cancers.* ISRN Oncol, 2013. **2013**: p. 697521.

18. Marti, A., et al., *Genes, lifestyles and obesity.* Int J Obes Relat Metab Disord, 2004. **28 Suppl 3**: p. S29-36.

19. Akbar, A.N. and M. Salmon, *Cellular environments and apoptosis: tissue microenvironments control activated T-cell death.* Immunol Today, 1997. **18**(2): p. 72-6.

20. Murphy, K., *Janeway's Immunobiology*. 8 ed. 2011, New York and Abingdon: Garland Science.

21. Delves, P.J. and I.M. Roitt, *The immune system. First of two parts.* N Engl J Med, 2000. **343**(1): p. 37-49.

22. Delves, P.J. and I.M. Roitt, *The immune system. Second of two parts.* N Engl J Med, 2000. **343**(2): p. 108-17.

23. Imanishi, T., et al., *Cutting edge: TLR2 directly triggers Th1 effector functions.* J Immunol, 2007. **178**(11): p. 6715-9.

24. Wall, R., et al., *Fatty acids from fish: the anti-inflammatory potential of long-chain omega-3 fatty acids.* Nutr Rev, 2010. **68**(5): p. 280-9.

25. Maeda, N., et al., *PPARgamma ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein.* Diabetes, 2001. **50**(9): p. 2094-9.

26. Mirzaei, K., et al., *Crosstalk between circulating peroxisome proliferator-activated receptor gamma, adipokines and metabolic syndrome in obese subjects.* Diabetol Metab Syndr, 2013. **5**(1): p. 79.

27. Surmi, B.K. and A.H. Hasty, *Macrophage infiltration into adipose tissue: initiation, propagation and remodeling.* Future Lipidol, 2008. **3**(5): p. 545-556.

28. Clark-Lewis, I., et al., *Structure-function relationship between the human chemokine receptor CXCR3 and its ligands.* J Biol Chem, 2003. **278**(1): p. 289-95.

29. Yu, M., et al., *MyD88-dependent interplay between myeloid and endothelial cells in the initiation and progression of obesity-associated inflammatory diseases.* J Exp Med, 2014. **211**(5): p. 887-907.

30. Koeppen, M., T. Eckle, and H.K. Eltzschig, *The hypoxia-inflammation link and potential drug targets.* Curr Opin Anaesthesiol, 2011. **24**(4): p. 363-9.

31. Eltzschig, H.K. and P. Carmeliet, *Hypoxia and inflammation.* N Engl J Med, 2011. **364**(7): p. 656-65.

32. Rius, J., et al., *NF-kappaB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1alpha.* Nature, 2008. **453**(7196): p. 807-11.

33. Lumeng, C.N. and A.R. Saltiel, *Inflammatory links between obesity and metabolic disease.* J Clin Invest, 2011. **121**(6): p. 2111-7.

34. Ouchi, N., et al., *Adipokines in inflammation and metabolic disease.* Nat Rev Immunol, 2011. **11**(2): p. 85-97.

35. Holland, W.L., et al., *Lipid mediators of insulin resistance.* Nutr Rev, 2007. **65**(6 Pt 2): p. S39-46.

36. Straczkowski, M., et al., *Relationship between insulin sensitivity and sphingomyelin signaling pathway in human skeletal muscle.* Diabetes, 2004. **53**(5): p. 1215-21.

37. Park, J.Y., et al., *Ceramide induces apoptosis via caspase-dependent and caspase-independent pathways in mesenchymal stem cells derived from human adipose tissue.* Arch Toxicol, 2011. **85**(9): p. 1057-65.

38. Nakamura, M.T. and T.Y. Nara, *Structure, function, and dietary regulation of delta6, delta5, and delta9 desaturases.* Annu Rev Nutr, 2004. **24**: p. 345-76.

39. Chi, X., et al., *Fatty acid biosynthesis in eukaryotic photosynthetic microalgae: identification of a microsomal delta 12 desaturase in Chlamydomonas reinhardtii.* J Microbiol, 2008. **46**(2): p. 189-201.

40. Ramsden, C.E., et al., *Lowering dietary linoleic acid reduces bioactive oxidized linoleic acid metabolites in humans.* Prostaglandins Leukot Essent Fatty Acids, 2012. **87**(4-5): p. 135-41.

41. Zong, G., et al., *Associations of erythrocyte palmitoleic acid with adipokines, inflammatory markers, and the metabolic syndrome in middle-aged and older Chinese.* Am J Clin Nutr, 2012. **96**(5): p. 970-6.

42. Shearer, G.C. and J.W. Newman, *Lipoprotein lipase releases esterified oxylipins from very low-density lipoproteins.* Prostaglandins Leukot Essent Fatty Acids, 2008. **79**(6): p. 215-22.

43. Balsinde, J., M.V. Winstead, and E.A. Dennis, *Phospholipase A(2) regulation of arachidonic acid mobilization.* FEBS Lett, 2002. **531**(1): p. 2-6.

44. Brown, W.J., K. Chambers, and A. Doody, *Phospholipase A2 (PLA2) enzymes in membrane trafficking: mediators of membrane shape and function.* Traffic, 2003. **4**(4): p. 214-21.

45. Han, X. and R.W. Gross, *Shotgun lipidomics: electrospray ionization mass spectrometric analysis and quantitation of cellular lipidomes directly from crude extracts of biological samples.* Mass Spectrom Rev, 2005. **24**(3): p. 367-412.

46. Arnold, C., et al., *Arachidonic acid-metabolizing cytochrome P450 enzymes are targets of {omega}-3 fatty acids.* J Biol Chem, 2010. **285**(43): p. 32720-33.

47. Smith, W.L., D.L. DeWitt, and R.M. Garavito, *Cyclooxygenases: structural, cellular, and molecular biology.* Annu Rev Biochem, 2000. **69**: p. 145-82.

48. Mabalirajan, U., A. Agrawal, and B. Ghosh, *15-Lipoxygenase eicosanoids are the putative ligands for vanilloid receptors and peroxisome proliferator-activated receptors (PPARs).* Proc Natl Acad Sci U S A, 2012. **109**(1): p. E1; author reply E2.

49. Simopoulos, A.P., *Omega-3 fatty acids in inflammation and autoimmune diseases.* J Am Coll Nutr, 2002. **21**(6): p. 495-505.

50. Draper, E., et al., *Omega-3 fatty acids attenuate dendritic cell function via NF-kappaB independent of PPARgamma.* J Nutr Biochem, 2011. **22**(8): p. 784-90.

51. Liao, Z., et al., *Resolvin D1 attenuates inflammation in lipopolysaccharide-induced acute lung injury through a process involving the PPARgamma/NF-kappaB pathway.* Respir Res, 2012. **13**: p. 110.

52. Chen, W., et al., *Dyslipidemia, but not hyperglycemia, induces inflammatory adhesion molecules in human retinal vascular endothelial cells.* Invest Ophthalmol Vis Sci, 2003. **44**(11): p. 5016-22.

53. Bell-Parikh, L.C., et al., *Biosynthesis of 15-deoxy-delta12,14-PGJ2 and the ligation of PPARgamma.* J Clin Invest, 2003. **112**(6): p. 945-55.

54. Ide, T., et al., *Activation of nuclear receptors by prostaglandins.* Thromb Res, 2003. **110**(5-6): p. 311-5.

55. Groeger, A.L., et al., *Cyclooxygenase-2 generates anti-inflammatory mediators from omega-3 fatty acids.* Nat Chem Biol, 2010. **6**(6): p. 433-41.

56. Hoffmann, I., et al., *Novel insights into cyclooxygenases, linoleate diol synthases, and lipoxygenases from deuterium kinetic isotope effects and oxidation of substrate analogs.* Biochim Biophys Acta, 2012. **1821**(12): p. 1508-17.

57. Yin, H., et al., *Identification of novel autoxidation products of the omega-3 fatty acid eicosapentaenoic acid in vitro and in vivo.* J Biol Chem, 2007. **282**(41): p. 29890-901.

58. Pilkington, S.M., et al., *Impact of EPA ingestion on COX- and LOX-mediated eicosanoid synthesis in skin with and without a pro-inflammatory UVR challenge--report of a randomised controlled study in humans.* Mol Nutr Food Res, 2014. **58**(3): p. 580-90.

59. Bishop-Bailey, D., et al., *Lipid-metabolizing CYPs in the regulation and dysregulation of metabolism.* Annu Rev Nutr, 2014. **34**: p. 261-79.

60. Spickett, C.M., *The lipid peroxidation product 4-hydroxy-2-nonenal: Advances in chemistry and analysis.* Redox Biol, 2013. **1**(1): p. 145-152.

61. Braden, G.A., et al., *Dietary fish oil accelerates the response to coronary thrombolysis with tissue-type plasminogen activator. Evidence for a modest platelet inhibitory effect in vivo.* Circulation, 1990. **82**(1): p. 178-87.

62. Chiang, N., et al., *Aspirin-triggered 15-epi-lipoxin A4 (ATL) generation by human leukocytes and murine peritonitis exudates: development of a specific 15-epi-LXA4 ELISA.* J Pharmacol Exp Ther, 1998. **287**(2): p. 779-90.

63. Dalli, J. and C.N. Serhan, *Specific lipid mediator signatures of human phagocytes: microparticles stimulate macrophage efferocytosis and pro-resolving mediators.* Blood, 2012. **120**(15): p. e60-72.

64. Oh, S.F., et al., *Pro-resolving actions and stereoselective biosynthesis of 18S E-series resolvins in human leukocytes and murine inflammation.* J Clin Invest, 2011. **121**(2): p. 569-81.

65. Serhan, C.N., et al., *Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals.* J Exp Med, 2002. **196**(8): p. 1025-37.

66. Wada, K., et al., *Leukotriene B4 and lipoxin A4 are regulatory signals for neural stem cell proliferation and differentiation.* FASEB J, 2006. **20**(11): p. 1785-92.

67. Altmann, R., et al., *13-Oxo-ODE is an endogenous ligand for PPARgamma in human colonic epithelial cells.* Biochem Pharmacol, 2007. **74**(4): p. 612-22.

68. Sutphen, R., et al., *Lysophospholipids are potential biomarkers of ovarian cancer.* Cancer Epidemiol Biomarkers Prev, 2004. **13**(7): p. 1185-91.

69. Stevens, T., et al., *Lipidomic profiling of serum and pancreatic fluid in chronic pancreatitis.* Pancreas, 2012. **41**(4): p. 518-22.

70. Bjermo, H. and U. Riserus, *Role of hepatic desaturases in obesity-related metabolic disorders.* Curr Opin Clin Nutr Metab Care, 2010. **13**(6): p. 703-8.

71. Haegebarth, A. and H. Clevers, *Wnt signaling, lgr5, and stem cells in the intestine and skin.* Am J Pathol, 2009. **174**(3): p. 715-21.

72. Lan, F., et al., *IL-23/IL-23R: potential mediator of intestinal tumor progression from adenomatous polyps to colorectal carcinoma.* Int J Colorectal Dis, 2011. **26**(12): p. 1511-8.

73. Reynolds, S., *Colonoscopy Reduces Risk of Death from Colorectal Cancer in High-Risk Patients*, in *NCI Cancer Bulletin*. 2012.

74. Staff, M.C. *Colon Cancer*. 2011 [cited 2013 April 8]; Available from: http://www.mayoclinic.com/health/colon-cancer/DS00035.

75. Thygesen, L.C., et al., *Prospective weight change and colon cancer risk in male US health professionals.* Int J Cancer, 2008. **123**(5): p. 1160-5.

76. Calle, E.E., et al., *Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults.* N Engl J Med, 2003. **348**(17): p. 1625-38.

77. Clevers, H., *At the crossroads of inflammation and cancer.* Cell, 2004. **118**(6): p. 671-4.

78. Balkwill, F. and L.M. Coussens, *Cancer: an inflammatory link.* Nature, 2004. **431**(7007): p. 405-6.

79. Fearon, E.R. and B. Vogelstein, *A genetic model for colorectal tumorigenesis.* Cell, 1990. **61**(5): p. 759-67.

80. Gravaghi, C., et al., *Obesity enhances gastrointestinal tumorigenesis in Apc-mutant mice.* Int J Obes (Lond), 2008. **32**(11): p. 1716-9.

81. Dubois, R.N., *Role of inflammation and inflammatory mediators in colorectal cancer.* Trans Am Clin Climatol Assoc, 2014. **125**: p. 358-73.

82. Berkel, H., et al., *Nonsteroidal antiinflammatory drugs and colorectal cancer.* Epidemiol Rev, 1996. **18**(2): p. 205-17.

83. Burn, J., et al., *A randomized placebo-controlled prevention trial of aspirin and/or resistant starch in young people with familial adenomatous polyposis.* Cancer Prev Res (Phila), 2011. **4**(5): p. 655-65.

84. Janakiram, N.B. and C.V. Rao, *Role of lipoxins and resolvins as anti-inflammatory and proresolving mediators in colon cancer.* Curr Mol Med, 2009. **9**(5): p. 565-79.

85. Marx, A., et al., *AMACR expression in colorectal cancer is associated with left-sided tumor localization.* Virchows Arch, 2008. **453**(3): p. 243-8.

86. Pyne, N.J. and S. Pyne, *Sphingosine 1-phosphate is a missing link between chronic inflammation and colon cancer.* Cancer Cell, 2013. **23**(1): p. 5-7.

87. Kawamori, T., et al., *Role for sphingosine kinase 1 in colon carcinogenesis.* FASEB J, 2009. **23**(2): p. 405-14.

88. Felsen, C.B., et al., *Colorectal cancer screening among primary care patients: does risk affect screening behavior?* J Community Health, 2011. **36**(4): p. 605-11.

89. Perez, C.A., et al., *Serum total gangliosides and TA90-IC levels: novel immunologic markers in colorectal cancer.* Cancer J, 2002. **8**(1): p. 55-61.

90. Norwood, S., et al., *Epoxyeicosatrienoic acids and soluble epoxide hydrolase: potential therapeutic targets for inflammation and its induced carcinogenesis.* Am J Transl Res, 2010. **2**(4): p. 447-57.

91. Ogden, C.L., et al., *Prevalence of childhood and adult obesity in the United States, 2011-2012.* JAMA, 2014. **311**(8): p. 806-14.

92. Makki, K., P. Froguel, and I. Wolowczuk, *Adipose tissue in obesity-related inflammation and insulin resistance: cells, cytokines, and chemokines.* ISRN Inflamm, 2013. **2013**: p. 139239.

93. Warensjo, E., et al., *Associations between estimated fatty acid desaturase activities in serum lipids and adipose tissue in humans: links to obesity and insulin resistance.* Lipids Health Dis, 2009. **8**: p. 37.

94. Considine, R.V., et al., *Serum immunoreactive-leptin concentrations in normal-weight and obese humans.* N Engl J Med, 1996. **334**(5): p. 292-5.

95. Myers, M.G., Jr., et al., *Obesity and leptin resistance: distinguishing cause from effect.* Trends Endocrinol Metab, 2010. **21**(11): p. 643-51.

96. Staiger, H., et al., *Expression of adiponectin receptor mRNA in human skeletal muscle cells is related to in vivo parameters of glucose and lipid metabolism.* Diabetes, 2004. **53**(9): p. 2195-201.

97. Weyer, C., et al., *Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia.* J Clin Endocrinol Metab, 2001. **86**(5): p. 1930-5.

98. Pham, M.N., et al., *Serum adipokines as biomarkers of beta-cell function in patients with type 1 diabetes: positive association with leptin and resistin and negative association with adiponectin.* Diabetes Metab Res Rev, 2013. **29**(2): p. 166-70.

99. Horwitz, D.L., et al., *Proinsulin, insulin, and C-peptide concentrations in human portal and peripheral blood.* J Clin Invest, 1975. **55**(6): p. 1278-83.

100. Kang, J.X. and K.H. Weylandt, *Modulation of inflammatory cytokines by omega-3 fatty acids.* Subcell Biochem, 2008. **49**: p. 133-43.

101. Tunstall, R.J., et al., *Exercise training increases lipid metabolism gene expression in human skeletal muscle.* Am J Physiol Endocrinol Metab, 2002. **283**(1): p. E66-72.

102. Phillips, C.M., et al., *High dietary saturated fat intake accentuates obesity risk associated with the fat mass and obesity-associated gene in adults.* J Nutr, 2012. **142**(5): p. 824-31.

103. Perreault, M., et al., *A distinct fatty acid profile underlies the reduced inflammatory state of metabolically healthy obese individuals.* PLoS One, 2014. **9**(2): p. e88539.

104. Boden, G., *Free fatty acids (FFA), a link between obesity and insulin resistance.* Front Biosci, 1998. **3**: p. d169-75.

105. Nieto-Vazquez, I., et al., *Insulin resistance associated to obesity: the link TNF-alpha.* Arch Physiol Biochem, 2008. **114**(3): p. 183-94.

106. Min, Y., et al., *Relationship between red cell membrane fatty acids and adipokines in individuals with varying insulin sensitivity.* Eur J Clin Nutr, 2011. **65**(6): p. 690-5.

107. Antuna-Puente, B., et al., *Adipokines: the missing link between insulin resistance and obesity.* Diabetes Metab, 2008. **34**(1): p. 2-11.

108. Rose, H.G. and M. Oklander, *Improved Procedure for the Extraction of Lipids from Human Erythrocytes.* J Lipid Res, 1965. **6**: p. 428-31.

109. Agren, J.J., A. Julkunen, and I. Penttila, *Rapid separation of serum lipids for fatty acid analysis by a single aminopropyl column.* J Lipid Res, 1992. **33**(12): p. 1871-6.

110. Gurzell, E.A., et al., *Is the omega-3 index a valid marker of intestinal membrane phospholipid EPA+DHA content?* Prostaglandins Leukot Essent Fatty Acids, 2014.

111. Matthan, N.R., et al., *Plasma phospholipid fatty acid biomarkers of dietary fat quality and endogenous metabolism predict coronary heart disease risk: a nested case-control study within the Women's Health Initiative observational study.* J Am Heart Assoc, 2014. **3**(4).

112. Thiebaut, A.C., et al., *Correlation between serum phospholipid fatty acids and dietary intakes assessed a few years earlier.* Nutr Cancer, 2009. **61**(4): p. 500-9.

113. Saadatian-Elahi, M., et al., *Plasma phospholipid fatty acid profiles and their association with food intakes: results from a cross-sectional study within the European Prospective Investigation into Cancer and Nutrition.* Am J Clin Nutr, 2009. **89**(1): p. 331-46.

114. Kurotani, K., et al., *High levels of stearic acid, palmitoleic acid, and dihomo-gamma-linolenic acid and low levels of linoleic acid in serum cholesterol ester are associated with high insulin resistance.* Nutr Res, 2012. **32**(9): p. 669-675 e3.

115. Wang, J., et al., *Adiponectin and metabolic syndrome in middle-aged and elderly Chinese.* Obesity (Silver Spring), 2008. **16**(1): p. 172-8.

116. Ouchi, N. and K. Walsh, *Adiponectin as an anti-inflammatory factor.* Clin Chim Acta, 2007. **380**(1-2): p. 24-30.

117. Obukowicz, M.G., et al., *Novel, selective delta6 or delta5 fatty acid desaturase inhibitors as antiinflammatory agents in mice.* J Pharmacol Exp Ther, 1998. **287**(1): p. 157-66.

118. Funk, C.D., *Prostaglandins and leukotrienes: advances in eicosanoid biology.* Science, 2001. **294**(5548): p. 1871-5.

119. Brenner, R.R., *Hormonal modulation of delta6 and delta5 desaturases: case of diabetes.* Prostaglandins Leukot Essent Fatty Acids, 2003. **68**(2): p. 151-62.

120. Poisson, J.P., *Comparative in vivo and in vitro study of the influence of experimental diabetes on rat liver linoleic acid delta 6- and delta 5-desaturation.* Enzyme, 1985. **34**(1): p. 1-14.

121. Montanaro, M.A., et al., *Hepatic delta9, delta6, and delta5 desaturations in non-insulin-dependent diabetes mellitus eSS rats.* Lipids, 2003. **38**(8): p. 827-32.

122. Emanuela, F., et al., *Inflammation as a Link between Obesity and Metabolic Syndrome.* J Nutr Metab, 2012. **2012**: p. 476380.

123. Shoelson, S.E., J. Lee, and A.B. Goldfine, *Inflammation and insulin resistance.* J Clin Invest, 2006. **116**(7): p. 1793-801.

124. van Diepen, J.A., et al., *Interactions between inflammation and lipid metabolism: relevance for efficacy of anti-inflammatory drugs in the treatment of atherosclerosis.* Atherosclerosis, 2013. **228**(2): p. 306-15.

125. Aburasayn, H., R. Al Batran, and J.R. Ussher, *Targeting ceramide metabolism in obesity.* Am J Physiol Endocrinol Metab, 2016. **311**(2): p. E423-35.

126. Garces, F., et al., *High plasma phospholipase A2 activity, inflammation markers, and LDL alterations in obesity with or without type 2 diabetes.* Obesity (Silver Spring), 2010. **18**(10): p. 2023-9.

127. Sutherland, W.H., et al., *Lecithin:cholesterol acyltransferase activity, plasma and lipoprotein lipids and obesity in men and women.* Atherosclerosis, 1979. **34**(3): p. 319-27.

128. Xu, Y., *Sphingosylphosphorylcholine and lysophosphatidylcholine: G protein-coupled receptors and receptor-mediated signal transduction.* Biochim Biophys Acta, 2002. **1582**(1-3): p. 81-8.

129. Zhu, X., et al., *Deletion of class A scavenger receptor deteriorates obesity-induced insulin resistance in adipose tissue.* Diabetes, 2014. **63**(2): p. 562-77.

130. Pickens, C.A., K.H. Matsuo, and J.I. Fenton, *Relationship between Body Mass Index, C-Peptide, and Delta-5-Desaturase Enzyme Activity Estimates in Adult Males.* PLoS One, 2016. **11**(3): p. e0149305.

131. Pickens, C.A., et al., *Altered Saturated and Monounsaturated Plasma Phospholipid Fatty Acid Profiles in Adult Males with Colon Adenomas.* Cancer Epidemiol Biomarkers Prev, 2016. **25**(3): p. 498-506.

132. Pickens, C.A., M.F. Albuquerque Pereira, and J.I. Fenton, *Long-chain omega-6 plasma phospholipid polyunsaturated fatty acids and association with colon adenomas in adult men: a cross-sectional study.* Eur J Cancer Prev, 2016.

133. Issac, G., S. McDonald, and G. Astarita, *Lipid Separation: using UPLC with Charged Surface Hybrid Technology*. 2011, Waters Corporation: Milford, MA, USA.

134. Ekanayaka, E.A., M.D. Celiz, and A.D. Jones, *Relative mass defect filtering of mass spectra: a path to discovery of plant specialized metabolites.* Plant Physiol, 2015. **167**(4): p. 1221-32.

135. Gromski, P.S., et al., *Influence of missing values substitutes on multivariate analysis of metabolomics data.* Metabolites, 2014. **4**(2): p. 433-52.

136. Armitage, E.G., et al., *Missing value imputation strategies for metabolomics data.* Electrophoresis, 2015. **36**(24): p. 3050-60.

137. Team, R.C. *R: A language and environment for statistical computing*. 2015; Available from: http://www.r-project.org/.

138. Benjamini, Y. and Y. Hochberg, *Controlling the false discovery rate: a practical and powerful approach to multiple testing.* Journal of the Royal Statistical Society B, 1995. **57**: p. 289-300.

139. Bland, J.M. and D.G. Altman, *Multiple significance tests: the Bonferroni method.* BMJ, 1995. **310**(6973): p. 170.

140. Vazquez, A.I., et al., *Multi-Omic Prediction of Disease Risk and Progression Using Bayesian Generalized Additive Models.* Genetics, 2016.

141. de los Campos, G., D. Gianola, and D.B. Allison, *Predicting genetic predisposition in humans: the promise of whole-genome markers.* Nat Rev Genet, 2010. **11**(12): p. 880-6.

142. Vazquez, A.I., et al., *Increased Proportion of Variance Explained and Prediction Accuracy of Survival of Breast Cancer Patients with Use of Whole-Genome Multiomic Profiles.* Genetics, 2016. **203**(3): p. 1425-38.

143. Greenacre, M., J., *Biplots: the joy of singular value decomposition.* Wiley Interdisciplinary Reviews: Computational Statistics, 2012. **4**(4): p. 399-406.

144. VanRaden, P.M., *Efficient methods to compute genomic predictions.* J Dairy Sci, 2008. **91**(11): p. 4414-23.

145. Perez, P. and G. de los Campos, *Genome-wide regression and prediction with the BGLR statistical package.* Genetics, 2014. **198**(2): p. 483-95.

146. Vazquez, A.I., et al., *Integration of Multi-Layer Omic Data for Prediction of Disease Risk in Humans.* WCGALP, 2014: p. 1-6.

147. C., O.J. *An interactive tool for comparing lists with Venn Diagrams*. 2007 [cited 2016 October 28th]; Available from: http://bioinfogp.cnb.csic.es/tools/venny/index.html.

148. Del Bas, J.M., et al., *Impairment of lysophospholipid metabolism in obesity: altered plasma profile and desensitization to the modulatory properties of n-3 polyunsaturated fatty acids in a randomized controlled trial.* Am J Clin Nutr, 2016. **104**(2): p. 266-79.

149. Kim, J.Y., et al., *Metabolic profiling of plasma in overweight/obese and lean men using ultra performance liquid chromatography and Q-TOF mass spectrometry (UPLC-Q-TOF MS).* J Proteome Res, 2010. **9**(9): p. 4368-75.

150. Rauschert, S., et al., *Lipidomics Reveals Associations of Phospholipids With Obesity and Insulin Resistance in Young Adults.* J Clin Endocrinol Metab, 2016. **101**(3): p. 871-9.

151. Floegel, A., et al., *Identification of serum metabolites associated with risk of type 2 diabetes using a targeted metabolomic approach.* Diabetes, 2013. **62**(2): p. 639-48.

152. Reinehr, T., et al., *Changes in the serum metabolite profile in obese children with weight loss.* Eur J Nutr, 2015. **54**(2): p. 173-81.

153. Graessler, J., et al., *Lipidomic profiling before and after Roux-en-Y gastric bypass in obese patients with diabetes.* Pharmacogenomics J, 2014. **14**(3): p. 201-7.

154. Barber, M.N., et al., *Plasma lysophosphatidylcholine levels are reduced in obesity and type 2 diabetes.* PLoS One, 2012. **7**(7): p. e41456.

155. Li, F., et al., *Lipidomics reveals a link between CYP1B1 and SCD1 in promoting obesity.* J Proteome Res, 2014. **13**(5): p. 2679-87.

156. Kim, H.Y., et al., *Lysophospholipid profile in serum and liver by high-fat diet and tumor induction in obesity-resistant BALB/c mice.* Nutrition, 2014. **30**(11-12): p. 1433-41.

157. Kim, H.J., et al., *Metabolomic analysis of livers and serum from high-fat diet induced obese mice.* J Proteome Res, 2011. **10**(2): p. 722-31.

158. Tonks, K.T., et al., *Skeletal muscle and plasma lipidomic signatures of insulin resistance and overweight/obesity in humans.* Obesity (Silver Spring), 2016. **24**(4): p. 908-16.

159. Zhang, L., et al., *Role of fatty acid uptake and fatty acid beta-oxidation in mediating insulin resistance in heart and skeletal muscle.* Biochim Biophys Acta, 2010. **1801**(1): p. 1-22.

160. Savini, I., et al., *Obesity-associated oxidative stress: strategies finalized to improve redox state.* Int J Mol Sci, 2013. **14**(5): p. 10497-538.

161. Wallner, S. and G. Schmitz, *Plasmalogens the neglected regulatory and scavenging lipid species.* Chem Phys Lipids, 2011. **164**(6): p. 573-89.

162. Weinbrenner, T., et al., *Circulating oxidized LDL is associated with increased waist circumference independent of body mass index in men and women.* Am J Clin Nutr, 2006. **83**(1): p. 30-5; quiz 181-2.

163. Maeba, R., et al., *Plasmalogens in human serum positively correlate with high- density lipoprotein and decrease with aging.* J Atheroscler Thromb, 2007. **14**(1): p. 12-8.

164. Lodhi, I.J. and C.F. Semenkovich, *Peroxisomes: a nexus for lipid metabolism and cellular signaling.* Cell Metab, 2014. **19**(3): p. 380-92.

165. Stapleton, P.A., et al., *Obesity and vascular dysfunction.* Pathophysiology, 2008. **15**(2): p. 79-89.

166. Volpe, C.M. and J.A. Nogueira-Machado, *The dual role of free fatty acid signaling in inflammation and therapeutics.* Recent Pat Endocr Metab Immune Drug Discov, 2013. **7**(3): p. 189-97.

167. Keenan, A.H., et al., *Basal omega-3 fatty acid status affects fatty acid and oxylipin responses to high-dose n3-HUFA in healthy volunteers.* J Lipid Res, 2012. **53**(8): p. 1662-9.

168. Furukawa, S., et al., *Increased oxidative stress in obesity and its impact on metabolic syndrome.* J Clin Invest, 2004. **114**(12): p. 1752-61.

169. Askari, A., et al., *Roles of the epoxygenase CYP2J2 in the endothelium.* Prostaglandins Other Lipid Mediat, 2013. **107**: p. 56-63.

170. Wamberg, L., et al., *Expression of vitamin D-metabolizing enzymes in human adipose tissue -- the effect of obesity and diet-induced weight loss.* Int J Obes (Lond), 2013. **37**(5): p. 651-7.

171. Zeldin, D.C., et al., *Predominant expression of an arachidonate epoxygenase in islets of Langerhans cells in human and rat pancreas.* Endocrinology, 1997. **138**(3): p. 1338-46.

172. Bystrom, J., et al., *Inducible CYP2J2 and its product 11,12-EET promotes bacterial phagocytosis: a role for CYP2J2 deficiency in the pathogenesis of Crohn's disease?* PLoS One, 2013. **8**(9): p. e75107.

173. Bystrom, J., et al., *Endogenous epoxygenases are modulators of monocyte/macrophage activity.* PLoS One, 2011. **6**(10): p. e26591.

174. Berger, J. and D.E. Moller, *The mechanisms of action of PPARs.* Annu Rev Med, 2002. **53**: p. 409-35.

175. Thomson, S.J., A. Askari, and D. Bishop-Bailey, *Anti-inflammatory effects of epoxyeicosatrienoic acids.* Int J Vasc Med, 2012. **2012**: p. 605101.

176. Zhang, W., et al., *Immunohistochemical dynamics of leukotoxin (9,10-epoxy-12-octadecenoic acid) in lungs of rats.* Int J Legal Med, 1995. **107**(4): p. 174-8.

177. Hayakawa, M., et al., *Neutrophils biosynthesize leukotoxin, 9, 10-epoxy-12-octadecenoate.* Biochem Biophys Res Commun, 1986. **137**(1): p. 424-30.

178. Ozawa, T., et al., *Neutrophil microsomes biosynthesize linoleate epoxide (9,10-epoxy-12-octadecenoate), a biological active substance.* Biochem Biophys Res Commun, 1988. **152**(3): p. 1310-8.

179. Morisseau, C. and B.D. Hammock, *Impact of soluble epoxide hydrolase and epoxyeicosanoids on human health.* Annu Rev Pharmacol Toxicol, 2013. **53**: p. 37-58.

180. Pickens, C.A., et al., *Altered saturated and monounsaturated plasma phospholipid fatty acid profiles in adult males with colon adenomas* Cancer Epidemiol Biomarkers Prev, 2015. **In press**.

181. Pickens, C.A., et al., *Obesity is Associated with Changes in Plasma Oxylipids*, in *The FASEB Journal*. 2015: Experimental Biology. p. 389.1.

182. Mavangira, V., et al., *Polyunsaturated fatty acids influence differential biosynthesis of oxylipids and other lipid mediators during bovine coliform mastitis.* J Dairy Sci, 2015. **98**(9): p. 6202-15.

183. Revelle, W. *psych: Procedures for Psychological, Psychometric, and Personality Research*. 2016; Available from: https://cran.r-project.org/package=psych.

184. Ledesma, R.D., P. Valero-Mora, and G. Macbeth, *The Scree Test and the Number of Factors: a Dynamic Graphics Approach.* Span J Psychol, 2015. **18**: p. E11.

185. Kaiser, H.F., *The Application of Electronic Computers to Factor Analysis.* Educational and Psychological Measurement, 1960. **20**(1): p. 141-151.

186. Blasbalg, T.L., et al., *Changes in consumption of omega-3 and omega-6 fatty acids in the United States during the 20th century.* The American Journal of Clinical Nutrition. **93**(5): p. 950-962.

187. Shaul, M.E., et al., *Dynamic, M2-like remodeling phenotypes of CD11c+ adipose tissue macrophages during high-fat diet--induced obesity in mice.* Diabetes, 2010. **59**(5): p. 1171-81.

188. Kayanoki, Y., et al., *The protective role of glutathione peroxidase in apoptosis induced by reactive oxygen species.* J Biochem (Tokyo), 1996. **119**(4): p. 817-22.

189. Gregor, M.F. and G.S. Hotamisligil, *Inflammatory mechanisms in obesity.* Annu Rev Immunol, 2011. **29**: p. 415-45.

190. De Taeye, B.M., et al., *Expression and regulation of soluble epoxide hydrolase in adipose tissue.* Obesity (Silver Spring), 2010. **18**(3): p. 489-98.

191. Bettaieb, A., et al., *Soluble epoxide hydrolase deficiency or inhibition attenuates diet-induced endoplasmic reticulum stress in liver and adipose tissue.* J Biol Chem, 2013. **288**(20): p. 14189-99.

192. Zhang, G., S. Kodani, and B.D. Hammock, *Stabilized epoxygenated fatty acids regulate inflammation, pain, angiogenesis and cancer.* Prog Lipid Res, 2014. **53**: p. 108-23.

193. Weintraub, N.L., et al., *Epoxide hydrolases regulate epoxyeicosatrienoic acid incorporation into coronary endothelial phospholipids.* Am J Physiol, 1999. **277**(5 Pt 2): p. H2098-108.

194. Li, N., et al., *Beneficial effects of soluble epoxide hydrolase inhibitors in myocardial infarction model: Insight gained using metabolomic approaches.* J Mol Cell Cardiol, 2009. **47**(6): p. 835-45.

195. Bishop-Bailey, D., et al., *Lipid-Metabolizing CYPs in the Regulation and Dysregulation of Metabolism.* Annu Rev Nutr, 2014.

196. Chakrabarti, S.K., et al., *Evidence for activation of inflammatory lipoxygenase pathways in visceral adipose tissue of obese Zucker rats.* Am J Physiol Endocrinol Metab, 2011. **300**(1): p. E175-87.

197. Nielsen, M.S., et al., *The effect of low-dose marine n-3 fatty acids on the biosynthesis of pro-inflammatory 5-lipoxygenase pathway metabolites in overweight subjects: a randomized controlled trial.* Prostaglandins Leukot Essent Fatty Acids, 2012. **87**(1): p. 43-8.

198. Martinez-Clemente, M., et al., *5-lipoxygenase deficiency reduces hepatic inflammation and tumor necrosis factor alpha-induced hepatocyte damage in hyperlipidemia-prone ApoE-null mice.* Hepatology, 2010. **51**(3): p. 817-27.

199. Guido, D.M., R. McKenna, and W.R. Mathews, *Quantitation of hydroperoxy-eicosatetraenoic acids and hydroxy-eicosatetraenoic acids as indicators of lipid peroxidation using gas chromatography-mass spectrometry.* Anal Biochem, 1993. **209**(1): p. 123-9.

200. Zu, L., et al., *Relationship between metabolites of arachidonic acid and prognosis in patients with acute coronary syndrome.* Thromb Res, 2016. **144**: p. 192-201.

201. Liu, J., et al., *Serum free fatty acid biomarkers of lung cancer.* Chest, 2014. **146**(3): p. 670-9.

202. Group, U.S.C.S.W. *United States Cancer Statistics: 1999–2011 Incidence and Mortality Web-based Report*. 2014 [cited 2015 May 29]; Available from: http://www.cdc.gov/uscs.

203. Haggar, F.A. and R.P. Boushey, *Colorectal cancer epidemiology: incidence, mortality, survival, and risk factors.* Clin Colon Rectal Surg, 2009. **22**(4): p. 191-7.

204. Willett, W.C., *Diet and cancer: an evolving picture.* JAMA, 2005. **293**(2): p. 233-4.

205. Kelly, S.B., et al., *Erythrocyte stearic acid desaturation in patients with colorectal carcinoma.* Dis Colon Rectum, 1990. **33**(12): p. 1026-30.

206. Hodge, A.M., et al., *Dietary and biomarker estimates of fatty acids and risk of colorectal cancer.* Int J Cancer, 2015.

207. Das, S., L.R. Martinez, and S. Ray, *Phospholipid remodeling and eicosanoid signaling in colon cancer cells.* Indian J Biochem Biophys, 2014. **51**(6): p. 512-9.

208. Bamia, C., et al., *Mediterranean diet and colorectal cancer risk: results from a European cohort.* Eur J Epidemiol, 2013. **28**(4): p. 317-28.

209. Igal, R.A., *Stearoyl-CoA desaturase-1: a novel key player in the mechanisms of cell proliferation, programmed cell death and transformation to cancer.* Carcinogenesis, 2010. **31**(9): p. 1509-15.

210. Liu, X., M.S. Strable, and J.M. Ntambi, *Stearoyl CoA desaturase 1: role in cellular inflammation and stress.* Adv Nutr, 2011. **2**(1): p. 15-22.

211. Ntambi, J.M. and M. Miyazaki, *Regulation of stearoyl-CoA desaturases and role in metabolism.* Prog Lipid Res, 2004. **43**(2): p. 91-104.

212. Zauber, A.G., et al., *Colonoscopic polypectomy and long-term prevention of colorectal-cancer deaths.* N Engl J Med, 2012. **366**(8): p. 687-96.

213. Arab, L., *Biomarkers of fat and fatty acid intake.* J Nutr, 2003. **133 Suppl 3**: p. 925S-932S.

214. Burdge, G.C., et al., *A method for separation of phosphatidylcholine, triacylglycerol, non-esterified fatty acids and cholesterol esters from plasma by solid-phase extraction.* Br J Nutr, 2000. **84**(5): p. 781-7.

215. Vessby, B., et al., *Indices of fatty acid desaturase activity in healthy human subjects: effects of different types of dietary fat.* Br J Nutr, 2013. **110**(5): p. 871-9.

216. Shimamura, K., et al., *5,5-Dimethyl-3-(5-methyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)-1-phenyl-3-(trifluoromethyl)-3,5,6,7-tetrahydro-1H-indole-2,4-dione, a potent inhibitor for mammalian elongase of long-chain fatty acids family 6: examination of its potential utility as a pharmacological tool.* J Pharmacol Exp Ther, 2009. **330**(1): p. 249-56.

217. Zarrouk, A., et al., *Fatty acid profiles in demented patients: identification of hexacosanoic acid (C26:0) as a blood lipid biomarker of dementia.* J Alzheimers Dis, 2015. **44**(4): p. 1349-59.

218. Yang, Y., *Multiple Imputation Using SAS Software.* Journal of Statistical Software 45, 2011.

219. Cormier, H., et al., *Effects of FADS and ELOVL polymorphisms on indexes of desaturase and elongase activities: results from a pre-post fish oil supplementation.* Genes Nutr, 2014. **9**(6): p. 437.

220. Khaw, K.T., et al., *Plasma phospholipid fatty acid concentration and incident coronary heart disease in men and women: the EPIC-Norfolk prospective study.* PLoS Med, 2012. **9**(7): p. e1001255.

221. Hosseini-Beheshti, E., et al., *Exosomes as biomarker enriched microvesicles: characterization of exosomal proteins derived from a panel of prostate cell lines with distinct AR phenotypes.* Mol Cell Proteomics, 2012. **11**(10): p. 863-85.

222. Swinnen, J.V., et al., *Selective activation of the fatty acid synthesis pathway in human prostate cancer.* Int J Cancer, 2000. **88**(2): p. 176-9.

223. Harris, W.S., et al., *Comparison of the effects of fish and fish-oil capsules on the n 3 fatty acid content of blood cells and plasma phospholipids.* Am J Clin Nutr, 2007. **86**(6): p. 1621-5.

224. Rashid, A., et al., *Elevated expression of fatty acid synthase and fatty acid synthetic activity in colorectal neoplasia.* Am J Pathol, 1997. **150**(1): p. 201-8.

225. Visca, P., et al., *Immunohistochemical expression of fatty acid synthase, apoptotic-regulating genes, proliferating factors, and ras protein product in colorectal adenomas, carcinomas, and adjacent nonneoplastic mucosa.* Clin Cancer Res, 1999. **5**(12): p. 4111-8.

226. Enoch, H.G., A. Catala, and P. Strittmatter, *Mechanism of rat liver microsomal stearyl-CoA desaturase. Studies of the substrate specificity, enzyme-substrate interactions, and the function of lipid.* J Biol Chem, 1976. **251**(16): p. 5095-103.

227. Weigert, C., et al., *Palmitate, but not unsaturated fatty acids, induces the expression of interleukin-6 in human myotubes through proteasome-dependent activation of nuclear factor-kappaB.* J Biol Chem, 2004. **279**(23): p. 23942-52.

228. Staiger, H., et al., *Palmitate-induced interleukin-6 expression in human coronary artery endothelial cells.* Diabetes, 2004. **53**(12): p. 3209-16.

229. Borradaile, N.M., et al., *Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death.* J Lipid Res, 2006. **47**(12): p. 2726-37.

230. Fulda, S., *Evasion of apoptosis as a cellular stress response in cancer.* Int J Cell Biol, 2010. **2010**: p. 370835.

231. Samuel, W., et al., *Transforming growth factor-beta regulates stearoyl coenzyme A desaturase expression through a Smad signaling pathway.* J Biol Chem, 2002. **277**(1): p. 59-66.

232. Simonsen, N.R., et al., *Tissue stores of individual monounsaturated fatty acids and breast cancer: the EURAMIC study. European Community Multicenter Study on Antioxidants, Myocardial Infarction, and Breast Cancer.* Am J Clin Nutr, 1998. **68**(1): p. 134-41.

233. Jackowski, S., *Cell cycle regulation of membrane phospholipid metabolism.* J Biol Chem, 1996. **271**(34): p. 20219-22.

234. Chajes, V., V. Joulin, and F. Clavel-Chapelon, *The fatty acid desaturation index of blood lipids, as a biomarker of hepatic stearoyl-CoA desaturase expression, is a predictive factor of breast cancer risk.* Curr Opin Lipidol, 2011. **22**(1): p. 6-10.

235. Chajes, V., et al., *Plasma phospholipid fatty acid concentrations and risk of gastric adenocarcinomas in the European Prospective Investigation into Cancer and Nutrition (EPIC-EURGAST).* Am J Clin Nutr, 2011. **94**(5): p. 1304-13.

236. Macasek, J., et al., *Plasma fatty acid composition in patients with pancreatic cancer: correlations to clinical parameters.* Nutr Cancer, 2012. **64**(7): p. 946-55.

237. Byberg, L., et al., *Cancer death is related to high palmitoleic acid in serum and to polymorphisms in the SCD-1 gene in healthy Swedish men.* Am J Clin Nutr, 2014. **99**(3): p. 551-8.

238. Hodson, L. and B.A. Fielding, *Stearoyl-CoA desaturase: rogue or innocent bystander?* Prog Lipid Res, 2013. **52**(1): p. 15-42.

239. Shim, Y.J., et al., *Phospholipid fatty acid patterns in the mucosa of human colorectal adenomas and carcinomas.* Nutrition Research, 2005. **25**(3): p. 261-269.

240. Methy, N., et al., *Dietary fatty acids and recurrence of colorectal adenomas in a European intervention trial.* Nutr Cancer, 2008. **60**(5): p. 560-7.

241. Yusof, A.S., Z.M. Isa, and S.A. Shah, *Dietary patterns and risk of colorectal cancer: a systematic review of cohort studies (2000-2011).* Asian Pac J Cancer Prev, 2012. **13**(9): p. 4713-7.

242. Trattner, S., et al., *Fatty acid composition of Swedish bakery products, with emphasis on trans-fatty acids.* Food Chem, 2015. **175**: p. 423-30.

243. Vinikoor, L.C., et al., *Consumption of trans-fatty acid and its association with colorectal adenomas.* Am J Epidemiol, 2008. **168**(3): p. 289-97.

244. Valenzuela, A. and N. Morgado, *Trans fatty acid isomers in human health and in the food industry.* Biol Res, 1999. **32**(4): p. 273-87.

245. Williams, C.D., et al., *Associations of red meat, fat, and protein intake with distal colorectal cancer risk.* Nutr Cancer, 2010. **62**(6): p. 701-9.

246. Beresford, S.A., et al., *Low-fat dietary pattern and risk of colorectal cancer: the Women's Health Initiative Randomized Controlled Dietary Modification Trial.* JAMA, 2006. **295**(6): p. 643-54.

247. Habermann, N., et al., *PTGS1, PTGS2, ALOX5, ALOX12, ALOX15, and FLAP SNPs: interaction with fatty acids in colon cancer and rectal cancer.* Genes Nutr, 2013. **8**(1): p. 115-26.

248. Murff, H.J., et al., *Dietary intake of PUFAs and colorectal polyp risk.* Am J Clin Nutr, 2012. **95**(3): p. 703-12.

249. Dommels, Y.E., et al., *Dietary n-6 and n-3 polyunsaturated fatty acids and colorectal carcinogenesis: results from cultured colon cells, animal models and human studies.* Environ Toxicol Pharmacol, 2002. **11**(3-4): p. 297-308.

250. Pot, G.K., et al., *Opposing associations of serum n-3 and n-6 polyunsaturated fatty acids with colorectal adenoma risk: an endoscopy-based case-control study.* Int J Cancer, 2008. **123**(8): p. 1974-7.

251. Yang, K., et al., *Expression profile of polyunsaturated fatty acids in colorectal cancer.* World J Gastroenterol, 2015. **21**(8): p. 2405-12.

252. Larsson, S.C., et al., *Dietary long-chain n-3 fatty acids for the prevention of cancer: a review of potential mechanisms.* Am J Clin Nutr, 2004. **79**(6): p. 935-45.

253. McEntee, M.F. and J. Whelan, *Dietary polyunsaturated fatty acids and colorectal neoplasia.* Biomed Pharmacother, 2002. **56**(8): p. 380-7.

254. Oh, K., et al., *<Dietary Marine n3 Fatty Acids in Relation to Risk of Distal.pdf>*. 2005.

255. Fini, L., et al., *Highly purified eicosapentaenoic acid as free fatty acids strongly suppresses polyps in Apc(Min/+) mice.* Clin Cancer Res, 2010. **16**(23): p. 5703-11.

256. Mandir, N. and R.A. Goodlad, *Conjugated linoleic acids differentially alter polyp number and diameter in the Apc(min/+) mouse model of intestinal cancer.* Cell Prolif, 2008. **41**(2): p. 279-91.

257. John E. Kinsella, K.S.B., and Jay W. Whelan, *Dietary unsaturated fatty acids in nteractions and possible needs in relation to eicosanoid synthesis.* J. Nutr. Biochem., 1990. **1**.

258. McEntee, J.W.a.M.F., *Dietary (n-6) PUFA and Intestinal Tumorigenesis.* The Journal of Nutrition, 2004.

259. Giuliani, A., et al., *Adipose tissue fatty acid composition and colon cancer: a case-control study.* Eur J Nutr, 2014. **53**(4): p. 1029-37.

260. Tokudome, S., et al., *Dietary n-3/long-chain n-3 polyunsaturated fatty acids for prevention of sporadic colorectal tumors: a randomized controlled trial in polypectomized participants.* Prostaglandins Leukot Essent Fatty Acids, 2015. **94**: p. 1-11.

261. Susanna C Larsson, M.K., Magnus Ingelman-Sundberg, and Alicja Wolk, *Dietary long-chain n3 fatty acids for the prevention of cancer mechanisms.* Am J Clin Nutr 2004, 2004. **79**: p. 935–45.

262. Whelan, M.F.M.a.J., *Dietary polyunsaturated fatty acids and colorectal neoplasia.* Biomed Pharmacother 2002. **56**: p. 380–387.

263. Pickens, C.A., et al., *Plasma phospholipids, non-esterified plasma polyunsaturated fatty acids and oxylipids are associated with BMI.* Prostaglandins Leukot Essent Fatty Acids, 2014.

264. Champely, S. *pwr: Basic Functions for Power Analysis.* 2015; Available from: http://cran.r-project.org/package=pwr.

265. Bigornia, S.J., et al., *The Omega-3 Index Is Inversely Associated with Depressive Symptoms among Individuals with Elevated Oxidative Stress Biomarkers.* J Nutr, 2016. **146**(4): p. 758-66.

266. Baack, M.L., et al., *What is the relationship between gestational age and docosahexaenoic acid (DHA) and arachidonic acid (ARA) levels?* Prostaglandins Leukot Essent Fatty Acids, 2015. **100**: p. 5-11.

267. Newman, J.W., et al., *Effect of omega-3 fatty acid ethyl esters on the oxylipin composition of lipoproteins in hypertriglyceridemic, statin-treated subjects.* PLoS One, 2014. **9**(11): p. e111471.

268. Wu, J.H., et al., *Plasma phospholipid omega-3 fatty acids and incidence of postoperative atrial fibrillation in the OPERA trial.* J Am Heart Assoc, 2013. **2**(5): p. e000397.

269. Harris, W.S., et al., *Erythrocyte omega-3 fatty acids increase and linoleic acid decreases with age: observations from 160,000 patients.* Prostaglandins Leukot Essent Fatty Acids, 2013. **88**(4): p. 257-63.

270. Grahn, S.W. and M.G. Varma, *Factors that increase risk of colon polyps.* Clin Colon Rectal Surg, 2008. **21**(4): p. 247-55.

271. Peipins, L.A. and R.S. Sandler, *Epidemiology of colorectal adenomas.* Epidemiol Rev, 1994. **16**(2): p. 273-97.

272. Kikendall, J.W., et al., *Cigarettes and alcohol as independent risk factors for colonic adenomas.* Gastroenterology, 1989. **97**(3): p. 660-4.

273. Zahm, S.H., P. Cocco, and A. Blair, *Tobacco smoking as a risk factor for colon polyps.* Am J Public Health, 1991. **81**(7): p. 846-9.

274. Monnet, E., et al., *Cigarette smoking and the risk of colorectal adenoma in men.* Scand J Gastroenterol, 1991. **26**(7): p. 758-62.

275. Nakanishi, M. and D.W. Rosenberg, *Multifaceted roles of PGE2 in inflammation and cancer.* Semin Immunopathol, 2013. **35**(2): p. 123-37.

276. Fernandez-Banares, F., et al., *Changes of the mucosal n3 and n6 fatty acid status occur early in the colorectal adenoma-carcinoma sequence.* Gut, 1996. **38**(2): p. 254-9.

277. Berstad, P., et al., *Fatty acids in habitual diet, plasma phospholipids, and tumour and normal colonic biopsies in young colorectal cancer patients.* J Oncol, 2012. **2012**: p. 254801.

278. Azrad, M., C. Turgeon, and W. Demark-Wahnefried, *Current evidence linking polyunsaturated Fatty acids with cancer risk and progression.* Front Oncol, 2013. **3**: p. 224.

279. Mathias, R.A., et al., *FADS genetic variants and omega-6 polyunsaturated fatty acid metabolism in a homogeneous island population.* J Lipid Res, 2010. **51**(9): p. 2766-74.

280. Bokor, S., et al., *Single nucleotide polymorphisms in the FADS gene cluster are associated with delta-5 and delta-6 desaturase activities estimated by serum fatty acid ratios.* J Lipid Res, 2010. **51**(8): p. 2325-33.

281. Suhre, K., et al., *Human metabolic individuality in biomedical and pharmaceutical research.* Nature, 2011. **477**(7362): p. 54-60.

282. Matsumura, T., et al., *Exosomal microRNA in serum is a novel biomarker of recurrence in human colorectal cancer.* Br J Cancer, 2015. **113**(2): p. 275-81.

283. Harris, W.S., et al., *Omega-3 fatty acids in cardiac biopsies from heart transplantation patients: correlation with erythrocytes and response to supplementation.* Circulation, 2004. **110**(12): p. 1645-9.

284. Stark, K.D., et al., *Global survey of the omega-3 fatty acids, docosahexaenoic acid and eicosapentaenoic acid in the blood stream of healthy adults.* Prog Lipid Res, 2016.