MASS SPECTROMETRY BASED LIPIDOMICS AS A TOOL IN THE SEARCH FOR BIOMARKERS AND MECHANISMS OF DISEASE

By

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A dissertation submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

in

Biochemistry

MONTANA STATE UNIVERSITY
Bozeman, MT

November 2016

DEDICATION

This dissertation is dedicated first to my beautiful wife Hannah, her constant love and support has made the entire process possible. I would like to recognize all of the deeply personal sacrifices she has made for me. I would also like to recognize the contribution of each of my children, Martin, Geoffrey and Gwendaline. Each of them has shown an understanding, beyond their years, of my personal desire to achieve something great and have given-up so much of their time with their father for this accomplishment. For these contributions I am truly indebted to each of them.

This dissertation is also dedicated to the memory of my grandfather, Martin. He instilled in me the desire to be great and never questioned my abilities. This has led me to never question my abilities.

For this, I thank you all.

ACKNOWLEDGEMENTS

I would like to thank my advisor Edward Dratz Ph.D. for is exceptional support. I have had an extraordinary opportunity to grow professionally and intellectually because of his support and guidance.

I would also like to specifically acknowledge the contributions of Brian Bothner Ph.D. and Valérie Copié Ph.D., for without their guidance my educational career would not have been as productive as it has been. I would like to thank the remainder of my committee for their guidance over the past several years. In addition I would like to recognize the contributions of the Bothner lab members, especially Monika for her expert guidance and willingness to help. I would also like to thank the members, both past and present, of the Dratz research group for their assistance.

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ABSTRACT

Lipidomics studies a highly diverse class of compounds insoluble in water and soluble in organic solvents. Lipids are a major component of cells and tissues, take part in a rich network of metabolic reactions, and are implicated in many disease mechanisms. Lipidomics complements genomics, proteomics and the more common metabolomic analysis of hydrophilic metabolites and can provide new insights into disease mechanisms. The problem approached in this thesis was to compare different methods of sample preparation for lipidomics and apply lipidomics to the study of two major health problems: Nonalcoholic Fatty Liver Disease (NAFLD) and Alzheimer's Disease (AD). Excessive dietary intake of sucrose and fructose, common in the Western Diet, increases deposition of triacylglycerides in the liver and leads to cognitive decline in experimental animals. NAFLD increases the risk of type 2 diabetes, obesity and AD.

The high diversity and hydrophobicity of lipids complicates their separation, detection and analysis. However, modern chromatography and mass spectrometry instrumentation and techniques are greatly improving the capability of lipidomic analysis. A lipid extraction protocol was optimized for reproducibility and yield, and was used to extract lipids from rat liver under sucrose stress in a model of human NAFLD and human brain cortex from Alzheimer's Disease (AD) compared to controls. The samples were analyzed using mass spectrometry.

The NALFD study did not yield the expected results, instead these data provided a foundation for designing future experiments in progress and to validate the methods used in the AD study. The AD studies showed that several phosphatidylcholine species are down regulated along with acetyl-CoA, which may be the source of low levels of the neurotransmitter acetylcholine in AD. Two different chromatography methods were used to seek a higher coverage of different lipids. Differences in the lipids in AD and controls were evident in the ω-6 and ω-3 fatty acids. The precursors of long ω-3s synthesis were increased while the products EPA and DHA were decreased. In a similar fashion, precursors to long ω-6s were found to be decreased, while the products were increased. This suggests that the ω-6 synthesis pathway may be outcompeting the ω-3 synthesis.

CHAPTER ONE

WHAT ARE LIPIDS AND HOW CAN LIPIDOMICS ANSWER METABOLOMIC QUESTIONS?

Lipidomics is Distinct from Other 'Omics' Fields of Study

Lipidomics is the Study of a Diverse Set of Bioactive Hydrophobic Molecules.

Lipids are a class of hydrophobic or amphipathic biological molecules which have a low solubility in water and a high solubility in nonpolar solvents [2, 3]. Lipidomics has gained a great deal of stimulus from the Lipid MAPS Consortium created in 2003 (http://www.lipidmaps.org/), with in excess of 18 million dollars in funding since that time. The purpose of Lipid MAPS is to identify all of the lipid species in mammalian cells and to quantify changes in these lipids in relation to disease. The ultimate goal of Lipid MAPS is to better understand the role of the lipidome in health and disease. To date, the Lipid MAPS Consortium has characterized and cataloged approximately 40,000 unique lipid species, which are curated in a freely accessible, online database of mass spectra and other properties (http://www.lipidmaps.org/data/structure/index.html).

Since lipid structures occur in such an enormous variety, detection and classification of complex lipids is greatly complicated. Many complex lipid species that can be detected do, or at least can, exist as biologically relevant structural isomers. For example, there are a great variety of double bond positions in the unsaturated and polyunsaturated fatty acid components of lipids. While Q-TOF instruments do give class specific identification and fatty-acyl length and double bond counts, this technology is

not capable of definitively identifying the specific location of those double bonds within the fatty acid; more current technology may have such capabilities. For example, ion mobility mass spectrometry, coupled to a recent advancement in chromatography, automated serial chromatography (ASC), explained in detail later, may be able to provide separation of complex lipid isomers prior to MS detection[4, 5].

Phospholipid species are divided into eight distinct classes, based on the identity of their polar head groups. Each of the lipid classes are divided further into several subclasses based on the connectivity of the acyl chains to the glycerol backbone and the acyl chain identity. Each of the lipid classes displays a unique set of physical and chemical properties. The distribution of lipid classes, subclasses and species varies a great deal among tissues and within tissues. Many complex lipids have several structural isomers that complicate mass spectrometry analysis and might not be resolved by mass spectrometric instruments commonly in use.

Lipids have complex, and in many cases, poorly understood roles in cell biology. The most widely understood biochemical roles of lipids are in forming the framework for membrane structure and as depots for energy storage. Excessive energy intake, such as high sugar or high glycemic carbohydrates, leads to an increased need to store energy. Complex lipids fill the energy storage need, primarily in the form of triacylglycerides[6-8]. In addition, the role of lipids as structural components of cellular membranes has been extensively studied. A large fraction of the plasma membrane and the membranes of the intercellular organelles are made of lipids. The lipid composition of membranes is widely thought to be responsible for controlling membrane fluidity, which can modulate

protein movement within the membrane. A third function of lipids is acting as potent signaling molecules[9, 10]. Lipids can be stored as components of membranes or in droplets in many tissues and their release by specific enzyme activity, control their availability for signaling[11]. Lipids, used as signaling molecules, are typically able to traverse lipid bilayers without specific transporters, possibly allowing for more rapid and localized signaling.

The Lipidomics Field has Arisen from an Unmet Need in other 'Omics' Fields.

There exists a gap in the understanding of many disease mechanisms since genomics, proteomics and metabolomics studies to date do not fully explain onset, symptoms or severity of many diseases[12, 13]. There is evidence that lipids play important signaling roles in normal health and disease states[10, 14, 15]. Indeed, lipids and their signaling mechanisms are increasingly being implicated in many of the world's most troublesome chronic and epidemic diseases such as nonalcoholic fatty liver, type 2 diabetes and Alzheimer's Disease.

Lipids have been shown to have isomer specific binding pockets in the active sites of some proteins[15, 16]. In addition, complex lipids may serve as carriers of precursor molecules that are required for signal transduction; for example phosphatidylcholine. The neurotransmitter acetylcholine is synthesized in vivo, in the brain and neuromuscular junctions, from the precursors acetyl-CoA and choline. Choline is a highly polar molecule that is poorly transported across the BBB. Choline can be carried as the head group of a phosphatidylcholine lipid species and can incorporate into the BBB. The major source of choline required for the synthesis of acetylcholine in the brain is supplied

to the brain as a family of phosphatidylcholines[17]. In other cases, lipid molecules have been shown to be carried on proteins such as carrier proteins like fatty acid binding protein FABP5, that are highly specific[18, 19], that may in some cases assist specific lipids to cross membranes.

It is clear that cellular lipidomes are interwoven with many types of metabolism and engage in highly complex interactions. Defects in the synthesis of either complex lipid molecules or lipid carrier proteins are often candidates to provide a more complete picture of the biochemistry underlying or accompanying many diseases[20]. A large amount of information about lipids present in systems of interest can be gathered using current lipidomic analytical technologies.

Mass Spectrometry Based Lipidomics

Advances in Mass Spectrometry are Driving Advancements in Lipidomics.

Technological and methodological advances in mass spectrometry have supported the development of increasingly more comprehensive and penetrating studies of lipids and their involvement in disease. The first mass spectrometer (MS), the "parabola spectrograph" was developed by JJ. Thomson in 1910 [21, 22]. Since that time several notable advancements in mass spectrometry have allowed lipidomics to become established and to progress at an accelerating rate. Electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) are soft ionization methods that minimize fragmentation of molecules, while ionizing them for MS analysis. ESI and MALDI are the most common ionization techniques used in lipidomics [21, 23]. The

quadrupole-time-of-flight (Q-TOF) mass analyzer, developed by William Stephens in 1946, allows detection and fragmentation of ions with a high degree of accuracy and resolution.

ESI coupled to liquid chromatography (LC) subjects analytes in solution to an electric potential conducted through a needle in the ion source. The electric potential causes charge to be transferred from the needle to the solvent drops, and electrostatic repulsion of the drops causes the solvent to form a fine droplet spray in what is called a Taylor Cone. The solvent is rapidly evaporated, assisted by dry, heated gas and multiple charges on the solvent droplets cause ejection of charged analyte ions from the solvent drops (as shown schematically in figure 1.1). A large fraction of the analytes are left as intact, charged molecular ions.

ESI is well suited for time-of-flight (TOF) mass analyzers. Ions are fed into the high vacuum of the mass analyzer through a series of electrostatic lenses that are used to focus and shape the ion beam. Ions are accelerated through the quadrupole by a DC voltage and at the same time, the trajectories of ions of interest are stabilized while traveling through the quadrupole by a radio frequency (RF) field, allowing specific ions to pass to the detector. The quadrupole also allows ions to be isolated and fragmented in the collision cell, if desired, to collect additional structural information by tandem mass spectrometry. The ions and their fragments are collected into "packets" and accelerated to the detector with high voltage pulses. The lighter ions have a shorter time of flight to the detector after the accelerating pulse and the heavier ions have a longer time of flight. In transit to the detector, most modern TOF instruments have a reflector, which increases

resolution by lengthening the flight tube, decreasing the 3-dimensional variation of the ion packets, which also increases sensitivity. The detector records the time that the ions arrive. The mass-to-charge ratio (m/z) of the molecules are computed from the time of flight data, and used to plot a chromatogram showing the ionizable mass features in the sample [21, 24].

MS based Lipidomics Has Several Challenges.

Liquid Chromatography Mass Spectroscopy (LCMS), ion mobility MS (IMS-MS) and MALDI are commonly used methods in the lipidomics field. Current technologies are capable of detecting and quantifying several thousand lipid species, some of which may be relevant to disease states. However lipidomic methodolgies are not without challenges in sample preparation, analyte separation, data collection and analysis.

Chromatography of lipids is complicated by several factors. Due to the hydrophobic nature of the lipids, the LC solvents used in chromatography must be largely hydrophobic and, in some cases, anhydrous. This poses several compatibility problems with both chromatography and mass spectrometry. Reverse Phase (RP) columns often have C-18 or shorter hydrocarbon chains affixed to and extending away from silica beads. The C-18 or other hydrocarbon chains and their extension into the mobile phase are crucial for efficient separation of the analytes. Most, if not all, RP columns require a small but significant amount of water to separate analytes properly. Very high concentrations of hydrophobic solvents drive water out of the columns. As a result, strongly hydrophobic solvents risk stationary phase collapse and may shorten the useful life of expensive RP columns through chemical drying of the column.

Lipids may aggregate into small clusters of the same or similar lipid species or may aggregate into larger, more structured micelles or inverted micelle structures, depending on the solvent environment at nano- to picomolar concentrations. Lipid aggregation can be finicky and is dependent on many factors including solvent polarity, lipid concentration, and lipid class among others. The aggregation phenomena can make the ionization of lipids both solvent and lipid species dependent. Aggregation of lipids into large supramolecular self-assembled structures of lipids is usually driven largely by the hydrophobic effect in polar solvents. Aggregated lipids can complicate MS analysis in several important ways; first, aggregated lipids may not effectively, or uniformly, ionize for detection. Second, aggregated lipids that may ionize could have mass to charge ratios (m/z) which correspond to the mass of a lipid multimer, rather than the monomer. Depending on solvent conditions, individual lipid species may aggregate at concentrations of 100pmol/µL in 2:1 $CHCl_3$/MeOH, 50pmol/µL 1:1 $CHCl_3$/MeOH and 10pmol/µL in 1:2 $CHCl_3$/MeOH[25].

Some of the organic solvents desired, due to constraints on analyte solubility or LC behavior, might not efficiently donate protons for the ionization process, significantly decreasing MS sensitivity. In addition, it may not be possible to dissolve solvent additives that favor ionization, such as ammonium acetate or ammonium fluoride[26], into lipid compatible solvents such as methanol or acetonitrile. Further, some of the desirable solvents for good separation are relatively viscous and their use tends to generate large column backpressure at relatively low LC flow rates, requiring specialized instrumentation and columns to handle the high column back pressures.

Chromatography and mass spectrometry instrumentation may need to be purged of traces of solvents from prior experiments with hydrophilic analytes to obtain stable lipid chromatography. In some solvent combinations, this purging is simple and quick, but in other cases, the purging is a laborious and time-consuming task. One approach is to dedicate instrumentation to the analysis of lipophilic samples, however this requires additional, expensive equipment. In addition, instrumentation reserved for lipidomic studies may need to be plumbed with specialized tubing and fittings and be equipped with specific solvent resistant seals. In addition, equipment used for lipid separations may have higher maintenance costs, possibly increaseing downtime for cleaning and component replacement and/or shorter useful lifespan, compared to similar equipment not used for lipid analysis.

Lipids are known to be powerful signaling molecules, involved in many aspects of metabolism, recently reviewed in part by Chen and Bazan[27] and separation of isomeric lipids may be critical to lipidomic analysis. Brown et al. has shown that some proteins have isomeric specific lipid binding pockets[9]. We have observed lipid isomers with different levels of expression in disease states with no apparent change in the expression of the larger group of isomers; where one isomeric compound has an increased expression and the other shows a decrease in expression. Such isomer specific changes would go unnoticed in cases where isomeric separation is not achieved. It is likely that one lipid isomer may be very influential in cellular signaling while other isomers may have no effect on cellular signaling. As technology in lipidomics continues to advance, isomer specific signaling is becoming more apparent.

Lipids can be efficiently separated using high-pressure liquid chromatography (HPLC) however, with some difficulty. In general, lipid species within classes can be well separated using RP C18 columns; in some cases a RP C8 column may be more efficient. In RP chromatography, a nonpolar stationary phase, usually a silica bead with a hydrocarbon acyl chain attached to it, is used. The mobile phase is most often a gradient of acidified water to acetonitrile or methanol, also with the acid modifier. The fatty acyls of the lipid analytes interact with the hydrophobic portion of the stationary phase as it is carried by the mobile phase through the column; the more hydrophobic acyl chains interact with the stationary chain more strongly and are retained on the column longer. Hydrophobic interaction liquid chromatography (HILIC) is efficient in separating lipid classes. HILIC uses a polar stationary phase and a predominantly non-polar mobile phase. Many HILIC columns use silica beads as the stationary phase and a mobile phase that consists of a gradient of organic solvent to water, both with acid modifiers. The polar head groups of the lipids interact with the polar stationary phase. The molecules with the more non-polar head groups are retained longer on the column.

HILIC and RP chromatography techniques have been combined for nearly complete isomeric lipid separation in either of two ways. First, the sample is fractionated into lipid classes using a RP separation in a first stage. The fractions can then be further separated through HILIC chromatography. Manual fractionation of the samples is somewhat difficult to reproduce, labor intensive and time consuming. A more attractive method is automated serial chromatography (ASC)[4, 5]. Separation of lipids using ASC requires two HPLC systems and a valve connecting them. The sample is first introduced

to the HILIC column of the ASC. Class specific separation of the sample is achieved as the sample is carried through the stationary phase of the HILIC column by the mobile phase. Using a valve and a sample loop fractions from HILIC are collected, stored and then injected into a RP column where further separation is carried out (figure 1.2). The timing of the valve can be controlled to collect smaller or larger fractions corresponding to the elution profiles of the lipid classes. This method resolves most of the drawbacks of manual fractionation as computer control is automated and is reproducible to within a fraction of a second.

Recent advancements in ion mobility spectroscopy MS (IMS-MS) have made commercial IMS-MS instrumentation available[28-30]. IMS-MS exploits the gas phase collisional cross section of molecules to achieve a prefractionation by gas phase mobility that depends on the shape of the molecules in the gas phase and can lead to a superior level of MS resolution of compounds. This separation technique is particularly appealing to lipidomics, as structural isomers of lipids are extremely common and most isomers have a somewhat different 3-dimensional shape and possibly a distinct function within the cell. Isomeric compounds have very similar polarity and hydrophobicity, making HPLC separation difficult and in addition, most isomers have the same exact mass. Thus, the largest differences observable in isomers is often the collisional cross section of the individual molecules. IMS-MS technology coupled to ASC, is likely to be a rapid and effective method for the separation and detection of lipid isomers.

Data sets obtained through mass spectrometry based lipidomics are often very large, involving several thousand mass features. This volume of data can be difficult to

manage, store, transfer and analyze. Advancements in computer and software technologies are making transfer, storage and management of large MS and MS/MS data sets easier and more convenient. The data, once analyzed and pared down to a manageable level, is a list of mass features that change significantly with biological conditions. The mass features can be searched in several lipid databases, LipidMaps (40,300 structures), Metlin (64,700 structures) and LipidBlast (212,516 in silico structures) for putative identifications [31-34]. Many lipid-based data bases allow automated batch searching of both MS and MS/MS data sets. The putative identifications obtained from these database searches must be validated manually with experimental tandem mass spectroscopy (MS/MS) data and ideally, with authentic standards. Many, but not all, of the lipid structures in Metlin and many more in LipidMaps databases have experimentally obtained MS/MS data. The LipidBlast database is an in silico MS/MS database, developed from theoretical fragmentation[33]. For the structures not catalogued with MS/MS data, predictions about fragmentation can be made and similar authentic standards might be available for purchase to collect such data for validation. In addition, confirming some of the putative identifications may require synthesis of unique compounds.

Lipids are relatively easily isolated and fragmented during MS/MS experiments if their concentrations are sufficiently high. Fragmentation of phospholipids usually follows a predictable fragmentation pattern[35]. In both positive and negative modes the polar head group fragments with relatively little collisional energy, often as low as 10 eV[35, 36]. In positive mode the PA and SM head groups produce prominent product ions

while the PA, PG, PE, PI and PS head groups are found through neutral loss[37]. In negative mode the PC and SM, as well as PS, give neutral losses while the PA, PE, PG and PI head groups are identified by product ions[37]. To identify the fatty acyl groups more fragmentation energy is needed, usually 20 to 40 eV. In negative mode, a product ion corresponding to each of the fatty acids is usually generated and in positive mode a neutral loss of fatty acid is typically observed.

Despite the above challenges, many research groups have reported successful studies of lipidomics, using mass spectrometry methods. Chromatography methods can be adapted to suit the available instrumentation and solvents can be modified or changed to accommodate solubility and other analysis issues to obtain satisfactory separations. Lipid isomers can usually be at least partially separated with good chromatography, ideally including ASC. Identification of complex lipids can be greatly assisted through MS/MS data and many, perhaps all, necessary standards can be synthesized synthetically. If synthesis of authentic standards is not feasible, in silico fragmentation may provide sufficient insight into lipid identifications.

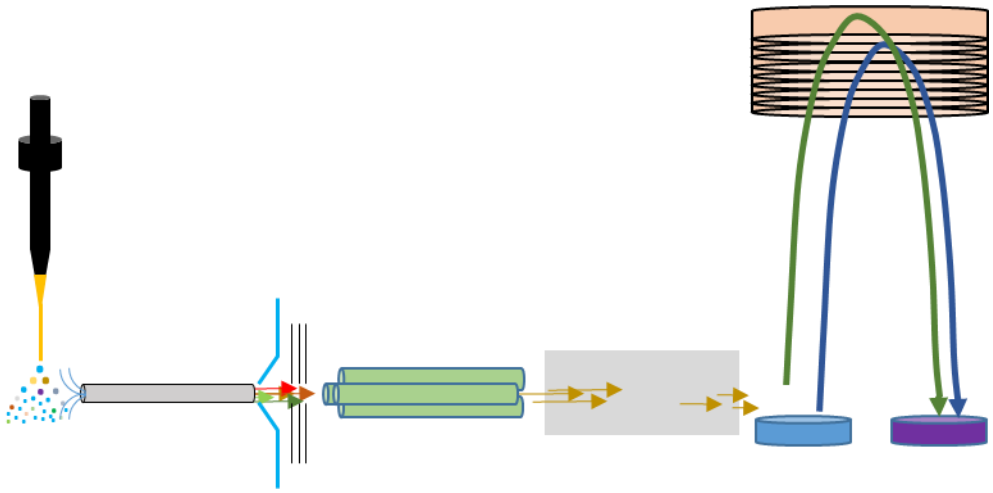

Figure 1.1. Schematic representation of a generic ESI-Q-TOF-MS instrument. Effluent from an LC is introduced to the source through a charged needle and is rapidly dried by heated gas. This rapid drying causes desolvation, fragmentation of drops and creates gas phase ions. The ions enter the MS instrument through a capillary and the beam of ions is focused through a narrow passage comprised of a skimmer and focusing lenses. The quadrupole mass filter allows only ions of a specific mass or range of masses to pass. For MS/MS analysis, precursor ions are isolated in the quadruple and fragmented in the collision cell. The product ions are transmitted to the pulser and accelerated into the flight tube. Most modern time-of-flight instruments have a vertical flight tube with a reflectron that allows ions that start in the flight tube at a lower position on the pulser (blue arrow) to "catch-up" to ions that are at a higher position (green arrow). Finally, the ions are detected by the detector.

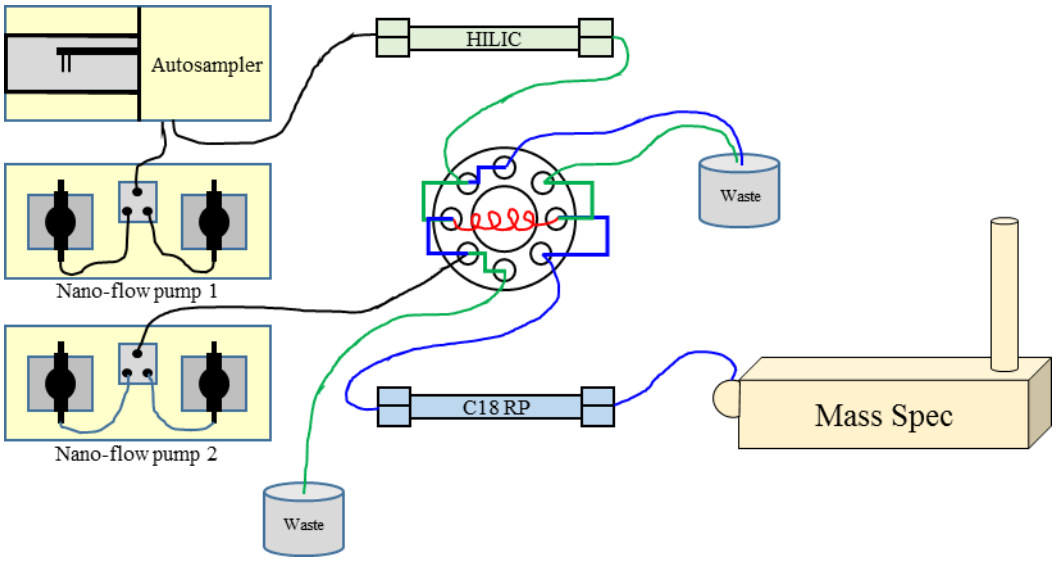

Figure 1.2. Automated serial chromatography (ASC) allows near complete isomeric separation of complex lipid species. Samples are introduced to the system sequentially via an autosampler. With the valve in position 1 (green lines) the first nano-flow pump uses mobile phase to carry the sample through a HILIC column to achieve class-specific separation of complex lipid analytes, and pump 2 is flowing to waste. The specific class of lipid is collected in a sample loop (red coil in valve) and the valve is switched to position 2. In position 2, pump 2 is able to collect the sample from the sample loop and carry it through a RP C18 column to achieve lipid class species-specific separation. The make-up of the mobile phase in pump 2 may or may not be the same as it is in pump 1. Following RP C18 separation the analytes are introduced to the mass spectrometer. Employing an ion-mobility mass spectrometer would exploit the collisional cross section of the analytes and can give more complete isomeric-specific separation and detection of analytes.

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CHAPTER TWO

INCREASED FRUCTOSE CONSUMPTION LEADS TO ABERRANT ENERGY METABOLISM AND INCREASED LIPID DEPOSITION IN LIVER

Energy Metabolism of the Liver

The disaccharide sucrose is composed of glucose and fructose. In the gut sucrose is hydrolyzed to glucose and fructose by a sucrase-isomaltase complex (figure 2.1) and many diets are high in fructose. Glucose can be metabolized in most tissues in the body, including the liver, but fructose is metabolized almost completely in the liver in humans. These two monosaccharides are metabolized by the liver in importantly different ways. Many of the intermediates of glucose and fructose metabolism have different metabolic fates[38]. The regulation of facilitated diffusion across membranes and into cells, the first catabolic steps[39], and the metabolic fates of glucose and fructose are different, as discussed below. Fructose is not an insulin secretagogue like glucose, fructose is not transported into cells by insulin sensitive pathways, and fructose can in fact lower circulating insulin[40, 41]. Excess glucose or fructose primarily drives the synthesis of fatty acids, by largely different pathways, and this process is of considerable importance in health and disease. High fructose, high glucose, high sucrose or highly glycemic diets lead to fatty liver, metabolic syndrome, and type 2 diabetes[42-45]. Evidence is also building that high dietary sugars contribute to Alzheimer's disease, which is increasingly being called Type 3 diabetes by many people in the field[46-48].

Glucose Metabolism in the Liver.

The descriptions of metabolism that follows are largely taken from well accepted sources [3, 8, 38], except where specifically referenced otherwise. Glucose, ingested through the diet, or generated by gluconeogenesis traverses membranes and enters cells through facilitated diffusion, primarily via GLUT2 in the liver. Glucose is catabolized by glycolysis in a similar step-wise manner in most tissues. Glycolysis begins in the liver with glucose phosphorylation by glucokinase to form glucose-6-phosphate, where a molecule of ATP provides the phosphate. Phosphorylation confines glucose to the cell, since glucose-6-phosphate cannot traverse the GLUT channels. Glucose-6-phosphate is isomerized to fructose-6-phosphate (F6P) catalyzed by phosphoglucose isomerase. Then, in the major regulatory step of glycolysis (more on this regulation later), phosphofructokinase-1 (PFK-1) catalyzes phosphorylation of F6P to form fructose-1,6-bisphosphate (F1,6BP), at the expense of a second molecule of ATP (figure 2.2). In the final reaction of the first half of glycolysis, aldolase catalyzes the splitting of the 6-carbon F1,6BP into two 3-carbon molecules; glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP). These two 3 carbon molecules have varied and interwoven fates in metabolism. G3P is most-often shuttled to the second stage of glycolysis, but can also be used in other important synthetic reactions. DHAP can be converted to a second molecule of G3P, through the action of triose phosphate isomerase, or can be used in the synthesis of complex lipids with the reduction of the ketone and addition of an acyl group at the sn1 position, to form a molecule of *acyl*-glycerol 3-phosphate (*acyl*G3P).

Up to this point, glycolysis has required the net investment/consumption of two molecules of ATP/glucose. G3P enters the ATP payoff stage of glycolysis, where glyceraldehyde-3-phosphate dehydrogenase catalyzes a coupled reduction and phosphorylation reaction to generate two high energy molecules; 1,3-bisphosphoglycerate (1,3-BPG) and a molecule of NADH. Phosphoglycerate kinase catalyzes the transfer of a phosphate from 1,3-BPG to ADP, yielding one ATP and one 3-phosphoglycerate (3PG) molecule, per molecule of 1,3-BPG. The following reaction is nearly energy neutral, and transfers a phosphate from the 3 position of 3PG to the 2 position of 2-phosphoglycerate (2PG), catalyzed by phosphoglycerate kinase. The second high energy intermediate of glycolysis, phosphoenolpyruvate (PEP), is formed from 2PG in a dehydration reaction, catalyzed by enolase. Pyruvate kinase catalyzes the conversion of PEP to pyruvate to generate ATP in the final reaction of the glycolytic pathway (figure 2.2). Each three carbon piece of glucose can yield two ATPs; thus glycolysis generates 2 ATP per glucose, after accounting for the two ATPs invested in the initial steps.

Fructose Metabolism in the Liver.

Like glucose, fructose consumed in the diet must be taken up by the tissues for catabolism. GLUT5 carries out the facilitated diffusion of fructose from the mircrovilli of the intestine to the blood and by GLUT 2, from the blood to the cytosol of the liver cells[49, 50]. There is no control of fructose uptake by insulin. Fructose is taken up much faster than glucose and the presence of glucose further stimulates fructose uptake, under most conditions[51, 52]. Once inside the cell fructose is very rapidly phosphorylated to

fructose-1-phosphate (F1P), in an ATP consuming reaction, catalyzed by fructokinase (figure 2.2). This reaction is so rapid it is thought to be largely responsible for the rapid decrease in cellular ATP levels that have long been known to occur with fructose consumption[53]. Aldolase B, different than the aldolase active in glycolysis (aldolase A) carries out the cleavage of F1P into DHAP and glyceraldehyde, which is metabolized into either an additional DHAP or G3P, through a multi-step process, which consumes additional ATP (figure 2.2). Glyceraldehyde can also be reduced to glycerol in a reaction catalyzed by alcohol dehydrogenase, with the oxidation of NADH and the glycerol can be used in the synthesis of triacylglycerols and other lipids. Glycerol can be converted to glycerol-3-phosphate with the use of an additional ATP, in a reaction catalyzed by glycerol kinase, further dropping cellular ATP levels. Then, glycerol-3-phosphate can be converted to DHAP by glycerolphosphate dehydrogenase.

Glyceraldehyde kinase catalyzes the phosphorylation of glyceraldehyde to G3P at the expense of a molecule of ATP. The rapid influx of fructose into the cell, followed by the rapid consumption of 2 ATP/fructose depletes cytosolic ATP and depletes inorganic phosphate levels in the cell and these changes strongly turns on glycolysis, as a side reaction, since the drop in ATP and the elevation of AMP (due to the activity of adenylate kinase) turns on PFK-1, which is a principal site of regulation of glycolysis (figure 2.2). Cytosolic ATP levels are slow to recover due to the drop in mitochondrial inorganic phosphate levels. Both G3P and DHAP generated from fructose can feed into glycolysis (figure 2.2). Triose phosphate isomerase can catalyzes the isomerization of DHAP to G3P, promoting glycolysis.

Regulation of Carbohydrate Metabolism.

Glycolysis is regulated at three reactions within the pathway at glucokinase, phosphofructokinase-1 (PFK1) and pyruvate kinase. The step catalyzed by glucokinase is regulated through the competitive inhibitor glucokinase regulatory protein (GKRP), that is also responsible for intercellular localization of glucokinase [54, 55]. The activity of GKRP is modulated by both fructose-6-phosphate, a positive effector, and fructose-1-phosphate, a negative effector[56, 57] (figure 2.2). Fructose is phosphorylated to F1P by fructokinase at a very rapid rate, which is likely faster than F1P can be metabolized, since fructose consumption leads to lipogenesis[58, 59]. Fructose consumed in the diet elevates F1P which inhibits GKRP (overcoming the F6P activation of GKRP) and accelerates glucokinase to drive the glycolysis pathway reactions forward[59, 60].

The major control point of glycolysis is the reaction catalyzed by PFK1. This reaction is the rate limiting control point of glycolysis, under most conditions. Importantly, PFK1 is activated by elevated levels of AMP and fructose-2,6-bisphosphate (F26BP). F26BP is the phosphorylated product of phosphofructokinase-2 (PFK2) from fructose-6-phosphate (F6P), which is a regulatory substance that influences the ratio between glycolysis and gluconeogenesis, and is not itself an intermediate in the metabolic pathway. PFK1 is also subject to feedback inhibition from products of glycolysis (phosphoenol pyruvate) and the TCA cycle (citrate).

Thus fructolysis results in the rapid consumption of cytosolic ATP, resulting in increased ADP and the production of AMP through the action of adenylate kinase, which accelerates PFK1 and glycolysis. Additionally, high levels of pyruvate produced by

accelerated glycolysis could feed into and accelerate the TCA cycle in the mitochondria and to produce high levels of citrate, which tends to turn off the TCA cycle and to be transported out of the mitochondria for use in fatty acid synthesis. The concentration of F6P would also increase due to the accelerated rate of glucokinase. The increased ratio of AMP/ATP in the cell, coupled to the increased concentration of F6P due to the metabolism of fructose, causes the regulation of PFK1 to heavily favor glycolysis.

Pyruvate kinase (PK) is also an important regulatory point of glycolysis. When fructose levels are high, the PK regulatory step becomes the main regulatory point of fructose/glucose metabolism, because the PFK1 regulation strongly favors glycolysis. Fructose metabolites enter glycolysis at the DHAP/G3P step, as detailed earlier. DHAP or G3P produced from fructose promote both glycolysis and gluconeogenesis, while favoring glycolysis[39, 61]. PK is allosterically activated by F1,6BP, produced by both glycolysis and fructolysis, and a high ratio of AMP/ATP, a byproduct of the rapid ATP consumption driven by fructose metabolism (figure 2.2). Pyruvate from glycolysis is transported to the mitochondrial matrix where it is converted into acetyl-CoA by the pyruvate dehydrogenase complex and PK is allosterically inhibited by acetyl-CoA[62].

Therefore, fructose consumption leads to the production of several regulatory effectors through the fructolysis process. The allosteric activator F1,6BP is produced by the reverse aldolase reaction of glycolysis as fructolysis can produce high levels of G3P and DHAP that can be shuttled to glycolysis, as a result of fructose consumption and metabolism. The levels of the powerful PFK1 regulator, AMP would likely increase from the adenylate kinase reaction, due to the rapid, initial step of ATP consumption in

the fructolysis pathway. Pyruvate is used to synthesize acetyl-CoA through the action of pyruvate dehydrogenase complex in the mitochondria. Acetyl-CoA is itself a powerful inhibitor of PK and acetyl-CoA is the precursor for the synthesis of fatty acids.

It is of great importance that the activating control of PK exerted by F1,6BP is stronger than that of the inhibitory control exerted by acetyl-CoA[63] (figure 2.2). In addition, inhibitory control of the TCA cycle by citrate would slow the activity of the TCA cycle. Nutritional conditions present in the high fructose Western Diet will tend to greatly elevate citrate and acetyl-CoA levels and thus promote FA production from excess acetyl-CoA and deposition as hepatic lipid droplets[44, 60, 64]. Therefore, the reaction catalyzed by pyruvate kinase would progress at an accelerated rate in the liver under conditions of high fructose consumption, generating higher amounts of pyruvate as indicated in Figure 2.2.

Pyruvate generated through glycolysis must first be transported from the cytosol to the mitochondrial matrix where it is converted into acetyl-CoA by the pyruvate dehydrogenase complex (PDH), before it can enter the citric acid cycle. The activity of PDH is regulated through feedback inhibition by its product acetyl-CoA conversion of acetyl-CoA in the mitochondria would drive PDH activity generating elevated concentrations of acetyl-CoA in the cytoplasm. Acetyl-CoA is a major branch point of metabolism. Acetyl-CoA can be converted to fatty acids, used in the synthesis of amino acids or burned for energy in the TCA cycle (figure 2.3). Before Acetyl-CoA can be converted to fatty acids it must first be converted to citrate through the TCA cycle,

exported from the mitochondria to the cytosol, and then converted back to acetyl-CoA and ultimately malonyl-CoA as input to FA synthesis.

Compartmentalization of energy metabolism plays a very large role in driving FA synthesis which occurs in the cytoplasm. While the level of ATP in the cytosol of the cell is depleted by fructose, this may not be the case in the mitochondria. Under conditions where carbon is flowing into pyruvate and citrate, and in the presence of sufficient oxygen, the TCA cycle is able to generate a great deal more ATP than glycolysis and, NADH from glycolysis is transported to the mitochondria to yield ATP.

While the TCA cycle does not respond directly to hormonal signals, it is sensitive to both allosteric regulation and substrate availability. The main regulatory points of the TCA cycle are the reactions catalyzed by citrate synthase (CS), isocitrate dehydrogenase (ID), and α-ketoglutarate dehydrogenase (αKD). The relatively high levels of ATP and NADH in the mitochondria would tend to turn off the TCA cycle at each of these points.

The regulation of the forward reaction catalyzed by citrate synthase (CS) is particularly relevant to the synthesis of fatty acids and complex lipids. The CS reaction is regulated by substrate availability as well as allosteric regulation. High levels of acetyl-CoA and low levels of Citrate strongly activate CS and, high levels of ATP and NADH inhibit the forward reaction of CS. Citrate can be siphoned off to fatty acid synthesis in the cytoplasm thus, promoting the CS reaction, even in the presences of high ATP and NADH. The reactions catalyzed by ID and α-KD are also inhibited by high levels of ATP and NADH but are not activated by low levels of citrate. Thus, as levels of acetyl-CoA rise with fructose consumption, citrate synthase catalyzes the conversion of acetyl-

CoA to citrate which is exported from the mitochondria activating CS and inhibiting the following steps of the TCA cycle. Therefore, high levels of citrate produced in the mitochondria are likely used in the synthesis of fatty acids.

Results of Excessive Fructose Consumption.

In summary, dietary fructose is converted to glycolytic intermediates that can enter glycolysis and also can greatly accelerate the metabolism of glucose in glycolysis. Fructose enters glycolysis mainly as DHAP, but can also enter the pathway as G3P, with the consumption of ATP (figure 2.2). All of these entry points are downstream of the rate-limiting PFK1 step of glycolysis. This, and fructose induced depletion of ATP will force glycolysis to run at a substantially increased rate, resulting in the production of increased amounts of PEP and pyruvate. Citrate builds-up from the upstream conversion of acetyl-CoA through the action of citrate synthase controlled by high levels of acetyl-CoA in the mitochondria. Then, due to high mitochondrial levels of ATP and NADH, citrate is exported from the mitochondria and used in the synthesis of fatty acids.

Complex Lipid Biosynthesis in the Liver.

Acetyl-CoA is a major branch point of metabolism (figure 2.3). Fatty acids are anabolized through a series of six reactions catalyzed by fatty acid synthase (FAS); these reactions are repeated several times, adding two carbons each cycle, culminating in the release of palmitic acid, with 16 carbons and no double bonds (figure 2.3). The free fatty acid is released from the FAS and may be esterified as an acyl-CoA to the sn-1 position of G3P. Subsequent reactions, not related to fatty acid synthesis, may esterify longer chain acyl-CoAs at the sn2 position and a phosphoryl-head group to the sn3 position[8].

It is hypothesized that the increased levels of acetyl-CoA, due to a greatly accelerated rate of glycolysis and other reactions from fructose consumption, leads to an increased rate of synthesis of malonyl-CoA. The increased levels of malonyl-CoA would likely lead to an increased rate of deposition of complex lipid species[42, 43, 65].

Non-Alcoholic Fatty Liver Disease is the
Excessive, Deposition of Lipid Droplets.

Often these lipid deposits are localized in small droplets in many regions of the liver however, the deposits can be quite significant and sometimes encompass entire lobes or other large regions of the liver. While fatty liver by itself is benign, current research suggests it is the first "hit" of a two hit mechanism[66] that may lead to much more severe comorbidities such as type 2 diabetes, obesity, cancer[67, 68] and possibly Alzheimer's Disease.

Excessive high fructose corn syrup is often identified as the most significant causal agent of NAFLD[40, 43, 64] and dietary intervention has been proposed as a method for treatment and prevention of NAFLD[69]. It has long been known that excess fructose consumption can contribute to hepatic lipid deposition (described previously). However, the specific mechanism by which NAFLD occurs remains unclear. A set of experiments is presented, using rats maintained on a high sucrose diet, to examine the lipidome present in the liver as a result of chronic, excessive fructose consumption to gain some understanding of the mechanisms underlying NAFLD onset and progression.

The Brains of Alzheimer's Disease Patients Exhibit Unique Lipidome Profiles.

Alzheimer's disease is characterized, in part, by significant atrophy (loss of tissue mass) of the brain[70-72] (figure 2.4). The brain has a very high lipid content and it is likely that a significant portion of the brain mass lost is lipid and that the lipid loss is not homogenous[36, 71, 73]. The human brain gray matter is approximately 36% lipid, myelin can be as high as 81% lipid and white matter roughly 49-66% lipid by dry mass[74]. The specific make-up of those lipids changes in response to normal aging, in a brain region specific manner[75]. Brain mass loss in AD patients is global but most greatly affects the Entorhinal Cortex, Hippocampus and Ventricle regions[76, 77].

The main roles of lipids are in cellular membrane structure, energy storage and hormonal activities. It is well known that lipids are the main structural component of all cellular membranes. In addition, the human brain is estimated to consume in excess of 20% of the body's total energy for function and maintenance[78, 79]. The brain is supplied metabolic energy from two well-accepted sources from elsewhere in the body, glucose stores and ketone bodies. Glucose, stored as glycogen primarily in the liver, is readily mobilized to maintain blood sugar levels and generate energy for export to other tissues. In an environment where glucose is difficult to obtain for metabolism, the brain will consume ketone bodies as an alternative fuel[80]. Ketone bodies are generated in the liver mitochondria from fatty acids and under some circumstances in the brain[81], as a source of energy.

Ketone body synthesis begins with the condensation of two molecules of acetyl-CoA in a reaction catalyzed by thiolase to form acetoacetyl-CoA. In the following

reaction, catalyzed by HMG-CoA synthase a third molecule of acetyl-CoA is added to Acetoacetyl-CoA to form β-hydroxy-β-methylglutaryl-CoA (HMG-CoA). In a subsequent cleavage reaction, catalyzed by HMG-CoA lyase, one acetyl-CoA molecule is released and a ketone body, acetoacetate, is generated. Dehydrogenation of acetoacetate by β-hydroxybutyrate dehydrogenase yields β-hydroxybutyrate, another ketone body. The release of a CO_2 molecule from acetoacetate generates acetone, the third ketone body, in a nonenzymatic process.

Acetyl-CoA used for ketone body synthesis is a product of carbohydrate metabolism, β-oxidation of fatty acids, or in some cases can be generated from amino acid metabolism. Classically accepted brain biochemistry asserts that the brain is incapable of mobilizing and utilizing fatty acids for energy production because fatty acids are not capable of traversing the blood brain barrier (BBB)[3, 38]. More current research is beginning to tell a much different story. Phospholipids are a diverse group of lipids and have been shown to be transported intercellularly between membranes[82]. One study by Pelerin et.al. has shown that fatty acid binding and transport proteins are expressed in rat brain tissues[83] and another study by Pan et.al. showed that fatty acid binding protein-5 facilitated transport of DHA across the BBB in cultured cells[18]. DHA is almost certainly not burned for fuel in the brain, but is essential for synaptic activity and proper brain function[84, 85]. In addition, Klosinski et.al. has shown that lipids from white matter are catabolized to ketone bodies to meet the energy demands in the brains of aging female rats[81]. Further, DHA supplementation has been shown to improve learning and memory function in age-related cognitive decline[86], and to oppose cognitive decline in fructose

fed rats possibly through a nutrigenomic mechanism[87]. Alzheimer's disease patients have a marked decrease in brain volume[72] which suggests changes in brain lipids. Therefore, it is hypothesized that AD brain samples will exhibit substantially different lipidome profiles when compared to controls.

Alzheimer's Disease patients have a marked decrease in the neurotransmitter Acetylcholine[88] and its nicotinic receptor[89-91]. The levels of acetylcholine are much more affected than are the levels of free choline in the Alzheimer's brain [88]. Acetylcholine in the neurons of the brain is synthesized from the essential precursors acetyl-CoA and choline[17]. Acetyl-CoA used in the synthesis of acetylcholine is sourced from pyruvate as a result of glycolysis[9]. The highly polar choline group is not capable of traversing the BBB and to date no effective transport protein has been found for such a purpose; the sole source of choline in the brain is from phosphatidylcholine and lysophosphatidylcholine species in the brain[92]. These phospholipids are likely capable of traversing the BBB through a flip-flop mechanism[93-95]. It is hypothesized that brain tissues of AD patients would exhibit decreased levels of various phosphatidylcholine species and acetyl CoA, precursors of acetylcholine synthesis, which we found to be true, as shown in Chapter 5.

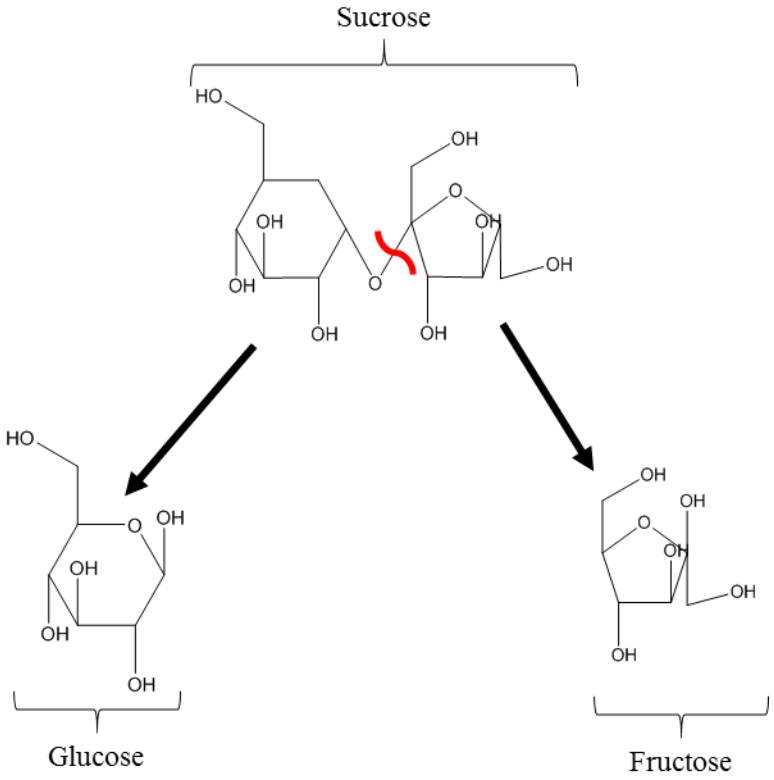

Figure 2.1. Ingested sucrose is rapidly hydrolyzed to the monosaccharides glucose and fructose in the gut. Approximately 40-50% of the fructose component of sucrose is converted to glucose in the liver.

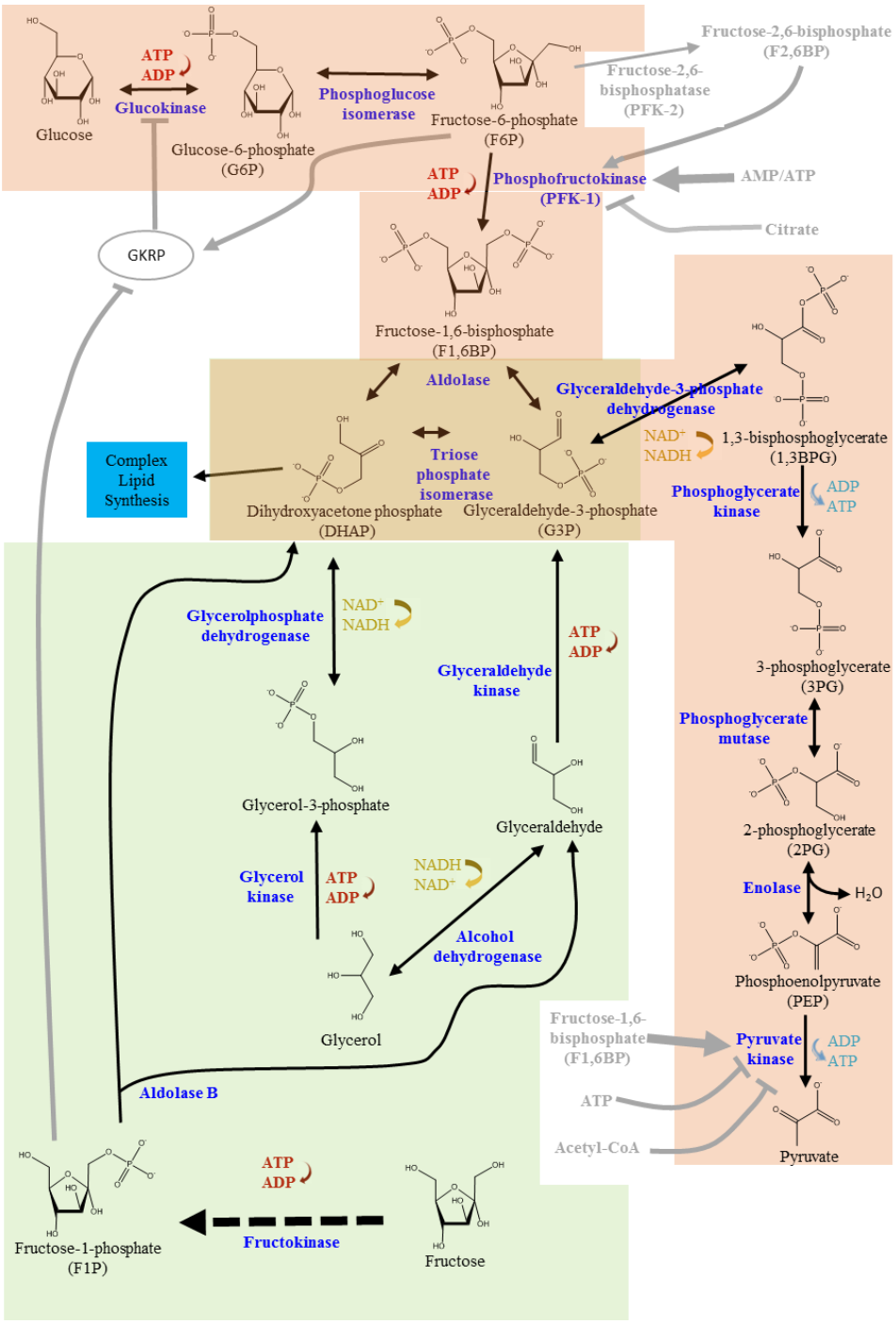

Figure 2.2 Glucose is metabolized in a highly controlled set of reactions known as glycolysis to control energy conversion and use. Ingested fructose perturbs the control of glycolysis, through the very rapid depletion of cellular ATP levels (dashed arrow). Several intermediates of fructose metabolism are potent regulators of glycolysis. The production of these regulators cause glycolysis to progress uncontrollably in the presence of fructose. The excess pyruvate produced is ultimately converted to fatty acids.

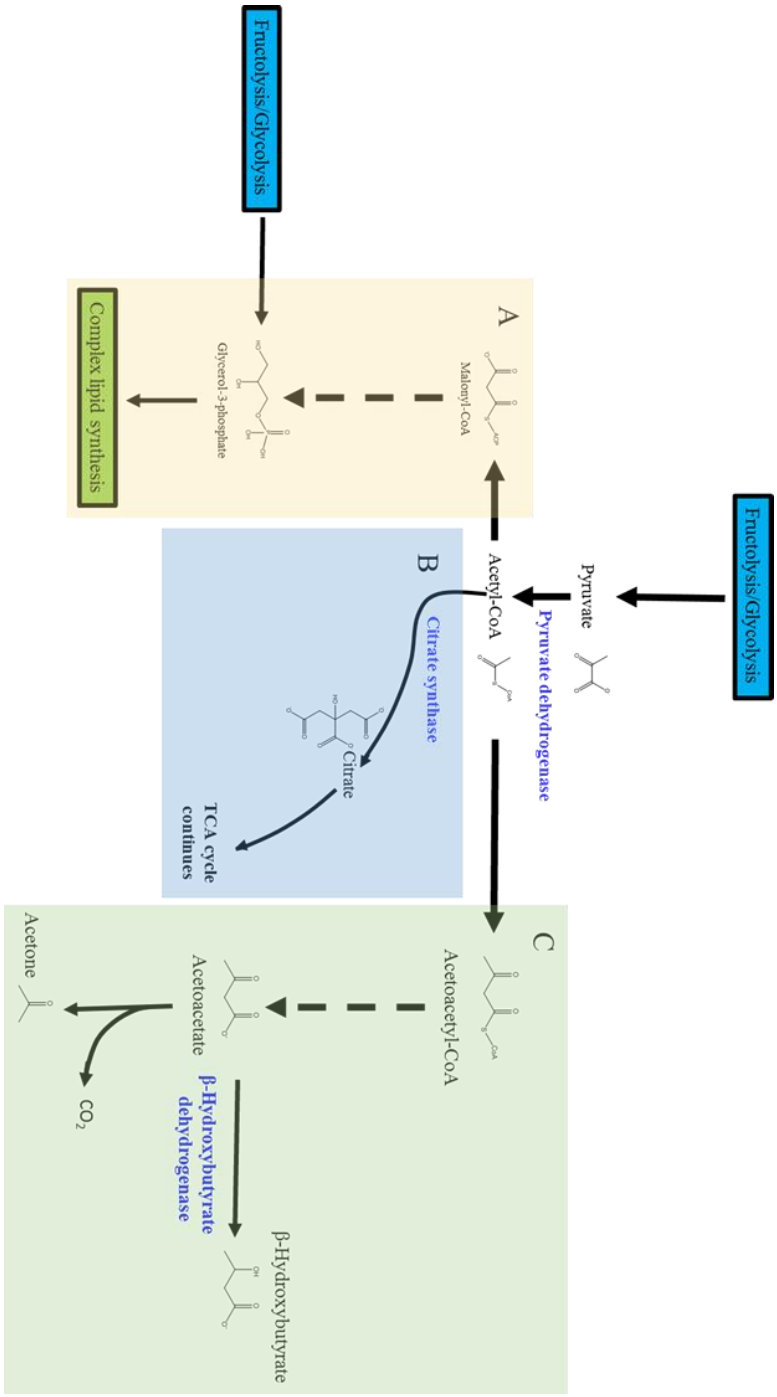

Figure 2.3. Acetyl-CoA is a major branch point of glucose and fructose metabolism. Acetyl-CoA can be shuttled to fatty acid synthesis (A), burned for energy in the TCA cycle (B) or used in the synthesis of ketone bodies (C). Under conditions of glucose metabolism most acetyl-CoA is shuttled to the TCA cycle (B) or to ketone synthesis (C). During times of high fructose consumption most acetyl-CoA is used in fatty acid synthesis (A) or in ketone body synthesis (C).

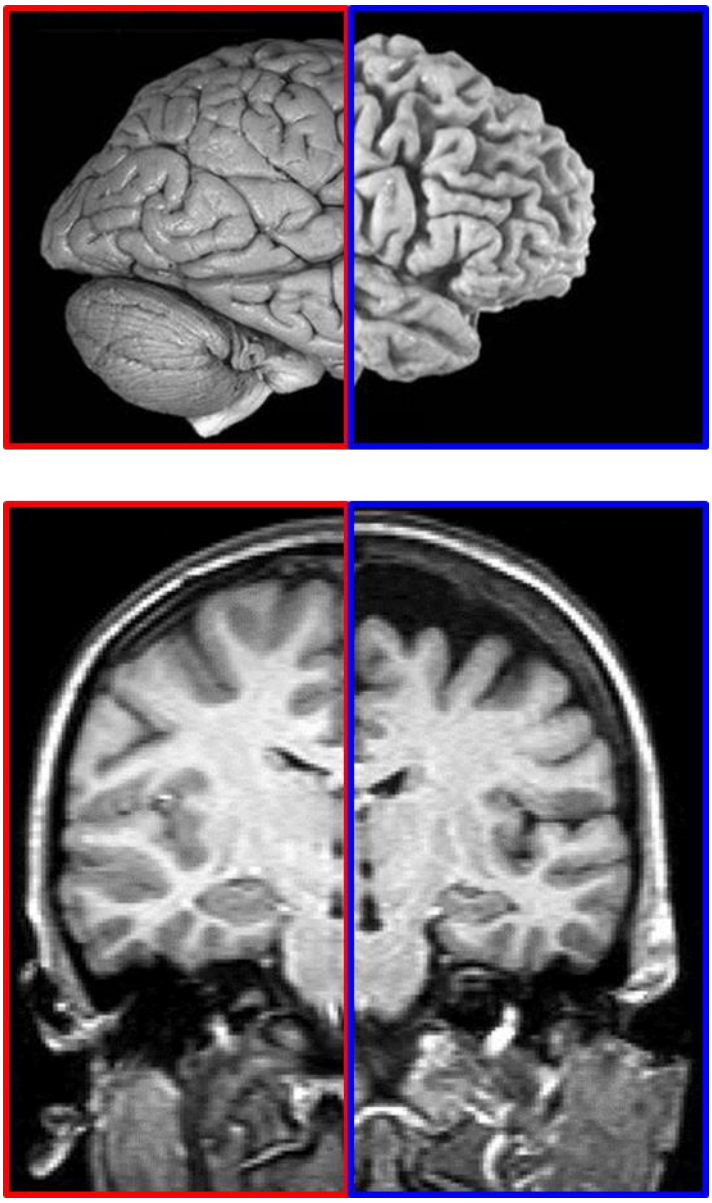

Figure 2.4. The normal ageing brain appears "full", without deep crevices and fills the inner space of the skull (bottom left). The brain of a patient with Alzheimer's disease exhibits noticible structural atrophy. In addition, it appears shrunk away from the inner walls of the skull (bottom right).

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CHAPTER THREE

A HIGHLY REPRODUCIBLE AND EFFICIENT LIPID EXTRACTION PROTOCOL ENHANCED USING 3D PRINTING OF CENTRIFUGE ADAPTERS FOR OPTIMUM GLASS VIALS

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

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Contributions: Assisted in sample preparation and data analysis.

Manuscript Information Page

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Lipids
Status of Manuscript:
____ Prepared for submission to a peer-reviewed journal
_X_ Officially submitted to a peer-review journal
____ Accepted by a peer-reviewed journal
____ Published in a peer-reviewed journal

Springer Link
Submitted to the journal Lipids for publishing October 28th 2016

Abstract

Four published lipid extraction techniques were compared, using 17 authentic internal standards. A modified lipid extraction protocol was developed with improved reproducibility, an attribute that is crucial for lipidomic comparisons of samples. The total lipid yield and lipid class specific extraction yields were compared for the 5 methods and the modified protocol produced the highest yield of complex lipids. In addition to yields, other factors such as throughput, minimizing solvent volumes, and laboratory equipment required were considered. Many investigators use dichloromethane instead of chloroform for lipid extraction, however, we found that this substitution decreases lipid yield. A very important consideration was the choice of glass vials and caps that matched the sample scale and volumes desired and minimized contamination from cap seals. There were no centrifuge adapters available for the optimum glass vials with inert cap liners that we desired to use for convenient sample sizes. Thus, we used 3D printing to create swinging bucket centrifuge adaptors for the optimum glass vials, which have performed flawlessly over hundreds of runs. We provide the .stl code used for 3D printing of the centrifuge adapters for the inexpensive glass vials that are ideal for lipid extraction at the desired scale. The .stl code can be modified for 3D printing of adapters for different centrifuge tube carriers or other vials, using widely available 3D printers. The reproducible, high throughput extraction technique reported has been tested on liver and brain tissue, as well as E. coli and MERSA cell pellets, with similar favorable results.

Introduction

The several 'omics' fields (e.g. genomics, epigenomics, transcriptomics, proteomics, metabolomics, and lipidomics) have enjoyed much study and a great deal of relevant information has emerged from this work. Most of the activity in metabolomics has investigated the water soluble metabolites, and lipidomics has not seen nearly the magnitude of study as these other omics fields, despite an increasing number of diseases being linked, at least in part, to lipid metabolism [67, 96-99]. Lipids are essential for many cellular functions, such as; signal transduction, lipid second messengers, control of lipid rafts, and hormone synthesis[100-102], in addition to membrane structure and energy storage. As the list of diseases linked to aberrant lipid metabolism grows, so too does the need for increased focus on analysis of the lipid molecules that may be involved. Lipids may be isolated from tissues, fluids and other biological materials through several published extraction techniques, involving partitioning into organic solvents. Much of the current infrastructure for metabolomics, nucleic acid and protein analysis in current labs has been designed to use plastic microcentrifuge tubes, which tend to contaminate the organic solvents needed for lipid extraction. Glass vials with solvent inert cap seals are essential for lipid analysis, but centrifuge adapters for suitable glass vials are often not available. We have used 3D printing to create custom centrifuge adaptors (figure 3.1) for the glass vials that we found optimum for different protocols. Furthermore, the optimum glass vials we selected are quite inexpensive and reliably survive centrifugation.

Many of the lipid extraction techniques in the literature rely principally on the early work of Folch[103] and Bligh and Dyer[104], with modifications seeking to increase

yield or lipid class specificity[105-107]. Several publications have used modifications of these methods, however, the publications often do not clearly describe what the modifications are. Here we describe a modified Bligh and Dyer protocol with detailed description of the modifications, which has been developed to optimize the reproducibility and recovery of complex lipids, while minimizing contamination from plasticizers, glue or other material in the cap liners.

Materials

Organic solvents (Chloroform, Methanol, and Dichloromethane) were Fisher Optima, LC/MS grade solvents, with the exception of Isopropanol. Several sources of isopropanol were tested and showed relatively large background signals on ESI LC-MS. The isopropanol with the lowest background was purchased from Alfa Aesar and was 99.7% pure HPLC grade. The formic acid and water was also Fisher Optima LC/MS grade. Nitrogen gas was supplied by boil off from a liquid nitrogen storage tank. Most lipid standards were purchased from Avanti Polar lipids. Arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenioic acid (DHA) standards were purchased from Cayman chemical. The glass 1-dram vials used for the bulk of the lipid extraction work (Kimble, P/N 60811B1) and the GC vials had PTFE faced rubber liners (P/N 14-812-49) and were purchased from Fisher Scientific. In situations where the liner would be punctured, crimp top Viton liners were used (P/N 03-378-343) with compatible glass vials (P/N 03-391-6). When necessary micro volume inserts were used (P/N 14-823-374) in the vials.

Tissues from Sprague Dawley rats, raised on different diets, were supplied by Dr. Jason Burkhead at University of Alaska, Anchorage. Rats were raised to 36 weeks and livers were harvested after cervical dislocation, followed by dissection. All animal husbandry and experimental procedures were reviewed and approved by the University of Alaska Anchorage Institutional Animal Care and Use Committee under OLAW Assurance #A3710–1. The liver and other rat tissues were flash frozen, shipped on dry ice and stored at -80C until extraction. A whole liver lobe from one animal was ground in a 55mm mortar with a pestle, in liquid nitrogen to fine, sand-like, consistency, to minimize variation of lipid content in different regions of the liver[108, 109]. Individual, 15 mg, portiions of this whole liver sample were used for each of the five lipid extraction techniques compared here.

Several authentic standards were obtained from Avanti Polar Lipids and were chosen to be different in mass from any naturally occurring lipid. The internal standards, shown in Table 3.1, were selected to be representative of common lipid classes, to run close to many of the naturally occurring lipids of interest on C-18 RP chromatography. The standard compounds used in this study were dissolved in 2:1 chloroform:methanol to create a mixed lipid standard solution. Aliquots of the lipid standard were dispensed with a Hamilton syringe into clean vials or mixed with the tissue extracts, dried under nitrogen and extracted, using each of the methods tested.

Four lipid extraction techniques from the literature[105-107, 110] were evaluated and compared to the hybrid method described here. Most currently used lipid extraction techniques have their origins in either the Bligh and Dyer (B&D)[104] or Folch[103] methods,

but with numerous modifications [103, 104]. Major differences between the two methods are that the B&D extraction is a consecutive, 2-step extraction starting with a single phase, while the Folch is a single step, two phase extraction. Furthermore, the final chloroform:methanol:water ratios differ between the two methods, 1:2:0.8 B&D and 8:4:3 Folch by volume. The solvent to sample ratio, is also significantly different, 4:1 in B&D and 20:1 in Folch by weight. The relatively large solvent to sample ratio of the Folch extraction make it technically difficult to perform in small scale glassware, commonly used in modern equipment, however Iverson et.al. have shown, that the unmodified B&D extraction underestimates the total lipid content of large samples with total lipid content above 2% [111].

Desirable glass vials and inert caps were selected suitable for the scale of the extractions and with minimal contamination from the caps, but no centrifuge adaptors were available for these vials. Therefore we designed and 3D printed of custom designed swinging bucket centrifuge adapters, using PTFE tubing as input filament (Newegg P/N 9SIV0S64AW1548) and an Ultimaker 2 3D-Printer. Measurements of the centrifuge buckets and the vials were conducted using a Vernier caliper. Both the length and width of the adapter insert was designed to with 1mm clearance within the bucket. The holes for the vials were designed to give 0.5mm clearance for the diameter of the vial and a depth equal to the height of the shoulder of the vial, leaving the entire cap and vial neck protruding from the adapter; and each swinging bucket carrier held 6-1 dram vials. Inserts were printed in pairs. Each pair of inserts was balanced to within 0.01 gram by

drilling a ¼-inch hole in the bottom of the heavier one to remove material. The balance hole was drilled in an area of the insert between vial wells.

Methods

Lipids were separated and detected using an Agilent 1290 UPLC system, coupled to an Agilent 6538 Q-ToF MS. The UPLC was equipped with a Zorbax eclipse XDB-C18 (2.1x150mm 1.8µm) RRHD column, supplied by Agilent. The mobile phase consisted of solvent system A, containing 0.1% formic acid and B, containing chloroform:methanol:isopropanol 1:2:4. A linear gradient of 65%B to 95%B was used to elute peaks over 12 minutes with a 95%B hold time of 2 minutes after the gradient. The flow rate was set to 300 µL/min. MS data was collected on an Agilent 6538 running in positive mode over a mass range of 150 m/z to 1700 m/z. MS capillary voltage was set to 3500 volts, drying gas to 4L/min, nebulizer at 45 psi, fragmentor at 45volts, and skimmer set to 500 volts. Data was collected from 1 minute to 15 minutes at a rate of 1Hz.

The four modified lipid extraction techniques taken from the literature were Want 2010[105], Wu 2008[106], Garner 2011[112], and Tajima 2013[107]. These were compared to each other, as well as to a hybrid method, that was assembled from aspects of those methods and described here. Prior to the extractions a single liver lobe sample was ground under liquid nitrogen to a fine powder in a mortar and pestle. The motor and pestle was rinsed with water, followed by methanol and finally with chloroform before immersing in liquid nitrogen. After the hybrid method was developed, it was also applied to brain tissue and pellets of bacteria without further modificaton. A 50µL aliquot of the mixed lipid

standard solution (Table 3.1) was added to a 1-dram vial using a Hamilton Syringe (P/N 1750) and dried under nitrogen and then a 15mg liver portion was added to the vial. Each extraction was done in triplicate.

The Want et al extraction protocol (CE3) was carried-out as described[105, 113], with minor changes. 15mg samples and 50µl standard aliquots were combined with 0.45 mL methanol/water (1:1) and homogenized in a 2mL all glass Dounce homogenizer. The homogenate was transferred to a 1-dram glass vial, with a PTFE liner in a phenolic cap, and centrifuged at 6800g for 15minutes in a Sorvall Legend Mach 1.6R centrifuge at 4°C, using the in-house 3D-printed glass vial adaptors described above (Figure 3.1). The original protocol called for the samples to be centrifuged for 10 minutes at 16,000g, however, this is beyond the capability of this centrifuge and frequently resulted in broken vials in another centrifuge. When the samples were centrifuged, at 6800g for 15 minutes, we obtained excellent results. The aqueous supernatant was collected after centrifugation using a Pasteur pipette and stored at -80°C. The pellet was combined with 0.45 mL dichloromethane:methanol (3:1) and transferred to the same Dounce homogenizer used previously and homogenized. The second homogenates were transferred to a 1-dram glass vial, using a Hamilton syringe. The original protocol transfers the homogenates to plastic Eppendorf tubes to be centrifuged; this is not desired, as components of the plastic tubes are dissolved by the DCM in significant quantities, which are readily detectable by MS. Supernatants were collected into amber, glass autosampler vials, using a Hamilton syringe and dried overnight in a fume hood (only this method required drying overnight,

extracts from other methods were dried under a gentle stream of pure nitrogen). Extracts were dissolved in 100µL methanol:water 1:1.

The Wu et al, stepwise extractions were performed as described [106]. 15 mg samples and 50µl standard aliquots were dissolved in 120 µl cold methanol and 30 µl cold water in a 5 ml homogenization tube with 0.5g CK14 soft tissue ceramic beads (VWR P/N 10144-496), shaken with a BioSpec mini bead beater for 20 seconds, and centrifuged at 6000xg to remove the beads from the sample. Great care must be exercised to remove all traces of ceramic beads from the sample as significant HPLC damage could occur from bead fragments. Alternatively, we found that a 2ml Dounce homogenizer produces comparable results to the bead beater. The sample was incubated on ice for 5 minutes and transferred to a 1-dram glass vial. 60 µl chloroform (4ml per g sample) was added to the homogenate and vortexed. The homogenate was incubated on ice for 10 minutes, and vortexed every 30 seconds for 5 seconds. The sample was centrifuged at 6800g for 15 minutes as before. The supernatant was removed into a clean 1-dram vial and 60 µl chloroform (4ml per gram sample) and 60 µl water (4ml per gram sample) was added; and the samples were vortexed and centrifuged as before. Each layer of the resulting biphasic mixture was removed to amber, glass GC vials. The upper, aqueous, layer was stored at -80°C and the lower, organic, layer was dried in a Savant SC110 Speedvac, set to low (no heat) temperature. Use of a Speedvac is not recommended as the organic solvents quickly evaporate under vacuum and tend to cause damage to the polycarbonate lid of the Speedvac; in addition, considerable sample may be lost. The samples were re-extracted as before and dried at room temperature under a

gentle stream of nitrogen gas. Organic extracts were resuspended in 100 µL chloroform:methanol (1:1).

The Garner et al lipid extractions were completed as described [112], with minor changes. 15mg samples and 50µl standard aliquots were combined with 0.45mL chloroform:methanol (1:2) and homogenized in a 2 ml Dounce homogenizer. The homogenates were transferred to 1-dram glass vials and the homogenizer was washed with 0.15mL chloroform, followed by 0.23mL water. The samples were vortexed for 30 seconds, followed by centrifugation at 6800g for 15 minutes. The exact centrifugal force was not specified in the Garner protocol. The organic layer was transferred to an amber, glass GC vial and dried under nitrogen; 0.25mL ice cold chloroform was added to the homogenate in the 1 dram vial and vortexed. The homogenate was centrifuged a second time as before. The organic layer was added to the previous, now dry, organic sample and dried under nitrogen. The aqueous layer was stored at -80°C. Samples were resuspended in 100µL chloroform:methanol (1:2).

The Tajima et al lipid extractions were carried out as described [107], with minor changes. 15mg samples and 50µl standard aliquots were combined with 1mL ice cold methanol in a 2ml Dounce homogenizer and homogenized on ice. The homogenates were transferred to 1-dram glass vials and the homogenizers were washed with 0.5mL chloroform followed by 0.4mL 20mM potassium phosphate (KPi) buffer. The washes were added to the sample to achieve a ratio of chloroform:methanol:KPi of 1:2:0.8. The samples were subjected to cycles of vortex for 15 seconds followed by incubation on ice for 45 seconds, for a total time of 5 minutes. 1mL chloroform and 1mL 20mM KPi pH

8.0 were added and the samples were vortexed and centrifuged at 4°C and 6800g for 15 minutes. The organic fractions were removed from the vials to amber, glass GC vials and dried under nitrogen. The aqueous fraction and the tissue debris were stored at -80°C separately. The organic fraction was resuspended in 100µL chloroform:methanol:isopropanol (1:2:4).

The lipid extraction method we describe here is as follows: 15mg samples containing the ground tissue and 50µl internal standards were combined with 0.3mL -20°C chloroform in a pre-chilled 1mL Dounce homogenizer and homogenized on ice. The samples were transferred to 1-dram glass vials and the homogenizers were washed with 0.3mL -20°C chloroform followed by 0.6mL -20°C methanol then 0.45mL ice cold 20mM KPi pH 8.0. The samples were allowed to incubate on ice for 5 minutes; vortexing 5 seconds, every 30 seconds. The samples were centrifuged at 4°C and 2000g for 2 minutes. The organic layers were removed with a Hamilton syringe to 1.8mL amber, glass GC vials. 0.5mL chloroform was added to the samples and the samples were vortexed and centrifuged as before. The second organic layer was combined with the previous organic layer and dried under nitrogen. Extreme care was used to not remove any of the aqueous layer. The dry organic layer was resuspended in 100µL chloroform:methanol (1:2).

In addition to extractions of the samples with standards, authentic standards alone were also analyzed. The standards were aliquoted into the extraction vials, dried under nitrogen and each of the extractions methods were carried out as described previously without further changes.

Results

Total lipid yield was determined by weight, using the standard samples with and without liver. The samples without liver were used to calculate the lipid mass recovered from the standard mixture. The mass of the standards extracted by each method was subtracted from the mass of the lipid recovered in the samples with liver plus standards. This allowed calculation of the percent total lipid yield from the wet tissue mass (Figure 3.2) by each method tested.

All of the complex lipid standards tested were detected by MS in either positive or negative mode, with some standards being detected in both positive and negative modes. The cardiolipin, was not detected in either mode, which was unexpected, as others have reported success detecting cardiolipins and we have been able to detect other cardiolipin species in unrelated biological samples. The integrated MS signal areas from the standards extracted with the liver samples and were compared to standards that were dried under nitrogen, and directly solubilized in chloroform:methanol 1:2, to determine percent yield per class in the complex mixtures, which were taken as 100% (Figure 3.3).

Discussion

Comparison of the extraction techniques, based on percent yield per lipid class, shown in Figure 3.3, shows that the Want protocol in general has high yields of many classes of lipids. The LPC class of lipids, which would be expected to have a low expression in most biological samples due to its toxicity, is detected in relatively very low concentrations, whereas the other choline containing species, SM and PC, are detected in relatively high amounts.

None of the lipid classes extracted by the Wu protocol give exceptionally high yields of lipid. However, lipid extraction following the Wu protocol is relatively consistent across nearly all classes of lipids, with the exception of PIP2 species. The Garner protocol has relatively high yields of SM, PE, PC and TAG species and relatively poor yields of cholesterol (Chol) and DAG species. The Tajima extraction gives relatively high yield of LPC, while giving a relatively low yield of PC and SM.

The protocol presented here shows relatively consistent lipid yield per class, when compared with other lipid extractions. As expected the yield of the LPC class of lipids is very low and is consistent with most other extraction protocols. The protocol presented here has relatively high yields of EPA, PG, Chol, PI, PE, DAG, and PS with lower yields of LPC, PC and SM (figure 3.3).

Other factors, in addition to yield, were also be considered. Extraction techniques requiring long centrifuge steps were not desired due to the time involved and the possibility of oxidation of sensitive lipids. The Want, Wu, Garner and Tajima protocols all require at least one 15-minute centrifuge run and in the Wu protocol, two are required. Our protocol requires two, 2-minute centrifuge runs. In addition to the duration of centrifuge run, the force of the run was also considered. Many of the protocols specify a very high force, above 10,000g. This is difficult to achieve as desirable sized glass tubes or vials are frequently not rated for these forces and when they are, the vessel often does not have a cap. Our protocol requires 2,000g and has been successfully performed as low as 500g. In addition to long centrifugation steps, the Want and Wu protocols call for long

sample drying steps. Lipids, especially when dried or largely dried, are susceptible to oxidation and long time periods of drying are not desired.

Equipment and supplies required for some of the extraction methods may not be commonly available. For example, a glass centrifuge tube capable of withstanding 16,000g, as in the Want and the Garner protocols, requires large capped centrifuge tubes capable of containing in excess of 15mL volume. These tubes were manufactured by one company and are now not available. In addition, currently available drawn glass 2mL vials used initially, do not correctly fit into available centrifuges and frequently break as a result.

The solvents involved were also considered. The Want protocol uses dichloromethane (DCM) in place of chloroform citing personal safety. While DCM is a slightly safer organic solvent to handle than chloroform, from a health standpoint, this is a relative consideration as both solvents pose similar handling hazards and DCM poses a higher risk of fire[114-116]. All of the organic solvents in these extractions should be handled with care and used in a laboratory fume hood. A critical look at the lipid class extraction data reveals that the Want protocol does not significantly increase the yield of any class of lipid above all other extraction methods. In addition, in the case of LPC, EPA and PIP2 the detection level is significantly decreased compared to other extraction techniques. The loss in yield attributed to using DCM as opposed to chloroform far outweighs the handling considerations here.

The solvent volumes required were also considered. Most of the protocols used relatively small microliter volumes, with the exception of the Garner protocol. The

Garner protocol is a modified Folch extraction and thus uses significantly higher volumes of solvents. The volumes are high enough that with samples in excess of 30mg, the required solvent volumes will not fit into the inexpensive 1-dram glass vials we have used successfully. In addition, larger volumes of chloroform and other organic solvents are hazardous to store and costly to dispose of.

Reproducibility was considered highly important, because comparison of different samples is at the core of lipidomics experiments. The Wu protocol had the highest average standard deviation across all lipid classes with 27.6%. The Want, Garner and Tajima protocol all had average standard deviations of approximately 13.5%. Our protocol had the lowest average standard deviation of 11.0%.

We developed a method to adapt existing laboratory equipment for the purpose of modern lipidomics experiments and describe a 3D printing method to create centrifuge adapters for the most desirable and inexpensive glass vials. We compared this method to several recently published extraction protocols for lipidomics. Our method has better reproducibility and higher throughput and can safely be performed in nearly any moderately equipped laboratory with currently available supplies, with the addition of 3D printed centrifuge adapters. In addition, yields from this protocol are, in many cases, higher than yields of other tested protocols, while using similar or smaller volumes of organic solvents. The higher reproducibility of our method is perhaps the most important characteristic for lipidomics experiments, seeking lipids and mass features that differ with health and disease or other experimental variables.

Figure 3.1. Centrifuge adaptors, designed to hold six optimal 1 dram vials, were created using 3-D printing technology. A non-conventional PTFE filament was used for the printing, as described in the text.

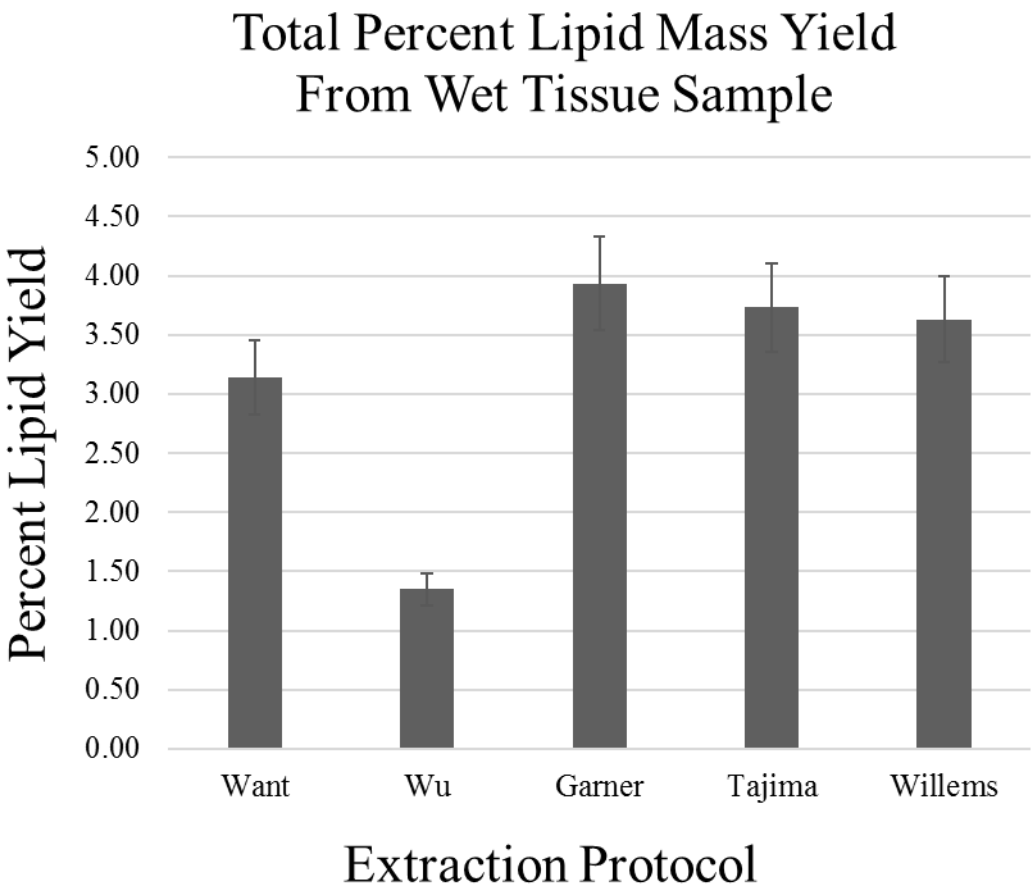

Figure 3.2. The percent lipid mass yield recovered from each of the extraction methods was calculated from the authentic standards and the mass of the frozen wet tissue samples. The standard aliquots were first dried under nitrogen in a weighed vial then the ground tissue samples were added to the vials. The vials with dried standards and samples were then weighed again to obtain the mass of the standards and samples. The standards and samples were then subjected to the extraction protocols. After drying the organic extracts, the vials were again weighed to obtain a mass of lipid recovered. The percent lipid yield reported here represents the dried lipid mass recovered as a percent of the wet tissue weight.

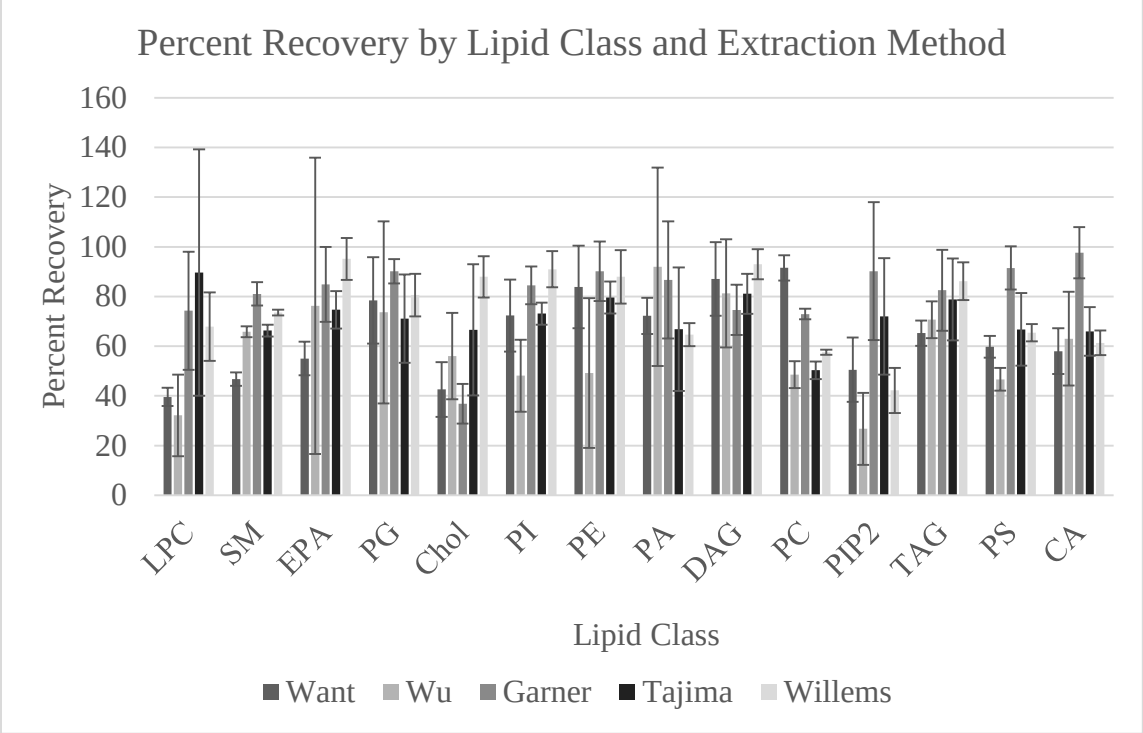

Figure 3.3. Lipid standards representative of each class of lipids were detected in each sample. The abundance of the standard detected in a standard mix without extraction, directly solubilized in solvent, was compared to the amount of standard recovered from each extraction after being dried and mixed with a liver sample. The values reported are for the same single lipid of each class, however, that single lipid is representative of the entire class of lipids to which it belongs.

Table 3.1. Authentic lipid standards were combined in the amounts shown to create a standard mix. The amounts of each added standard were based on chromatography data to obtain ionization signals similar to the rat liver samples being studied.

Class	Lipid Name	MW	(ng) in STD mix (ng)
PC	1-heneicosanoyl-2-(4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl)-sn-glycero-3-phosphocholine	876.24	10.3
PE	1-heptadecanoyl-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-sn-glycero-3-phosphoethanolamine	754.03	10.9
PG	1-heptadecanoyl-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-sn-glycero-3-phospho-(1'-rac-glycerol) (ammonium salt)	785.04	9.8
PS	1-dodecanoyl-2-tridecanoyl-sn-glycero-3-phospho-L-serine (ammonium salt)	637.78	11
PA	1-heptadecanoyl-2-(9Z-tetradecenoyl)-sn-glycero-3-phosphate (ammonium salt)	632.85	9.6
PI	1-heptadecanoyl-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-sn-glycero-3-phospho-(1'-myo-inositol) (ammonium salt)	890.13	10.7
LPC	1-(10Z-heptadecenoyl)-sn-glycero-3-phosphocholine	507.64	10.1
PIP2	1-heptadecanoyl-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-sn-glycero-3-phospho-(1'-myo-inositol-3',4'-bisphosphate) (ammonium salt)	1084.2	112
CA	1'-[1,2-dipentadecanoyl-sn-glycero-3-phospho],3'-[1-pentadecanoyl,2-(9Z-hexadecenoyl)-sn-glycero-3-phospho]-sn-glycerol (ammonium salt)	1343.8	107.9
SM	N-(dodecanoyl)-sphing-4-enine-1-phosphocholine	646.92	15.9
Chol	cholest-5-en-3ß-ol(d7)	393.7	497.2
MG	1-heptadecanoyl-rac-glycerol	344.54	1000
DG	1,3(d5)-di-(9Z-hexadecenoyl)-glycerol	569.91	1000
TG	1,3(d5)-dipentadecanoyl-2-(9Z-octadecenoyl)-glycerol	810.34	1000
FA	5Z,8Z,11Z,14Z-eicosatetraenoic acid	304.5	309.2
FA	Eicosapentaenoic acid	302.5	311.7
FA	Docosahexaenoic acid	328.5	296.7

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CHAPTER FOUR

LIPIDOMIC MARKERS OF NONALCOHOLIC FATTY LIVER DISEASE IN A RAT MODEL

Fatty Liver Disease: Complex Ailment with Common Biochemistry

Fatty liver disease is the abnormal hepatic fat deposition, mostly containing triacylglyceride (TAG) species, deposited as droplets that can either be localized or spread through most of the liver (figure 4.1). Fatty liver disease (FLD) has few if any outward symptoms and a range of possible causes rooted in diet and/or high alcohol consumption. Variants of FLD include alcoholic steatohepatitis (ASH), nonalcoholic steatohepatitis (NASH) and nonalcoholic fatty liver disease (NAFLD). Pathology of ASH and NASH are very similar. Both exhibit hepatic fat deposition, inflammation and eventual scaring of the liver. The differentiating factor of ASH and NASH is chronic, excessive alcohol use.

FLD in general, and more specifically, NAFLD has been shown to have a high incidence of comorbidity with decreased brain volume in Alzheimer's disease, type 2 diabetes, obesity, cancer, hypertension, dyslipidemia and increase liver post-transplant mortality [67, 68, 117-119]. Current estimates of the prevalence of NALFD and NASH in the US population are astonishingly high: 21.4% (32.5 million) and 19.0% (35.0 million) of adults being affected, respectively. Minorities are disproportionately more highly affected[120]. Given the high rates of comorbidity with several devastating diseases listed above and the alarmingly high prevalence in the US adult population, more studies as to

the cause, development of early diagnosis, and treatment of NALFD and NASH are needed.

Both NAFLD and NASH are often diagnosed by accident through incidental observation of the liver during an ultra sound (US), computerized tomography (CT) or magnetic resonance imaging (MRI) examination due to comorbid conditions or other unrelated ailments. NAFLD and NASH very rarely have any outward symptoms. Thus, these conditions can, and often do, go unnoticed for many years and are often found at autopsy. Given the prohibitively high costs of imaging techniques like US, CT and MRI; it is of substantial interest to develop a safe, cost effective, and accurate early warning test for NAFLD and NASH. Alerts for advanced liver disease can be recognized by elevated liver enzymes in the blood. The most common laboratory blood test uses an elevation in liver aminotransferase enzymes, but this does not provide a conclusive diagnosis[121, 122]. A more specific blood or blood plasma test could possibly provide earlier and more accurate warning, however, none are in development as far as we know.

High fructose, high sucrose, or high ethanol consumption all drive fatty liver disease in experimental animals. Ethanol metabolism and toxicity shares similarities with fructose metabolism and toxicity[123]. Dietary ethanol is oxidized by alcohol dehydrogenase to acetaldehyde, a toxic product. Acetaldehyde is oxidized by aldehyde dehydrogenase to form acetate. Acetate is transported to the mitochondria, where it is coupled to CoA by AcetylCoA ligase, is further catabolized, and may be ultimately exported from the mitochondria to the cytosol as citrate. Elevated levels of citrate in the cytosol promotes the rapid synthesis of malonyl-CoA, through the action of acetyl-CoA

carboxylase. Malonyl-CoA is an activated precursor to fatty acid synthesis and high Malonyl-CoA ultimately leads to lipid deposition in the liver. Chronic, excessive alcohol use is known to be the causative agent of ASH (figure 4.2).

Fructose metabolism leads to the same end products as ethanol metabolism, albeit through a different mechanism (chapter 2); thus, NAFLD and AFLD may be linked by converging pathways leading to a common causative agent (Figure 4.2). Dietary fructose is rapidly taken up by the liver and phosphorylated to fructose -1-phosphate by, with the rapid consumption of ATP. A drop in ATP and resulting increase in ADP and AMP turns on glycolysis. Fructose-1-phosphate is rapidly catabolized to DHAP and glyceraldehyde. Glyceraldehyde may be phosphorylated to glyceraldehyde-3-phosphate (GA3P) with an additional consumption of ATP. The high levels of DHAP and GA3P can take part in gluconeogenesis, increasing liver glucose levels that are not controlled by insulin. GA3P can also be converted to pyruvate and can lead to very high levels of pyruvate from both fructose and glucose catabolism. Pyruvate is transported into the mitochondria, converted to acetyl-CoA, and cause high levels of acetyl-CoA which can be ultimately exported from the mitochondria as citrate. As with ethanol metabolism, elevated citrate ultimately leads to fatty acid and TAG synthesis. The TAG's are packaged as droplets and deposited in the liver (figure 4.2).

Although the specific causes of NAFLD and NASH have not been fully established, chronic, excessive fructose consumption is known to induce NAFLD and NASH in experimental animals and is a leading hypothesis for the cause of hepatic fat accumulation among nonalcoholic humans [40, 42, 64].

The recognized symptoms of NAFLD and NASH are excessive hepatic fat deposition[44, 117], which is difficult or expensive to detect, and elevated aminotransferase[122] levels. Therefore, it is reasonable to examine liver tissue for aberrant hepatic lipid metabolism under fructose or sucrose stress with the ultimate goal of seeking evidence of aberrant liver metabolism in the blood. We developed a highly reproducible, modified Bligh and Dyer protocol and used it to extract the lipid content from the liver tissues of fructose stressed and control rats[124]. Ultra-performance liquid chromatography mass spectrometry (UPLC-ESI-MS) was employed to analyze the levels of the different lipid species in the liver extracts. Lipid species showing aberrant expression in treatment vs. control subjects may be mapped to biochemical pathways to reveal aberrations in metabolism[32, 34]. Using a rat model to test the impact of diet on NAFLD, male Wistar rats were fed one of four calorie controlled diets in the current study, in collaboration with Dr. Jason Burkhead at the University of Alaska, Anchorage. The stress diets contained 30% sucrose with and without a copper supplement, the control diets contained 10% sucrose with and without the copper supplement and used corn starch and dextrin to equalize the caloric content of the 30% and 10% sucrose diets (table 4.1). Pups were raised on a standard chow for 6 months before feeding the experimental stress and control diets for an additional 12 weeks. Rats were sacrificed at 36 weeks and perfused with an osmotically balanced salt solution to remove blood from the liver. The organs were harvested and flash frozen and the livers were sectioned and distributed for analysis.

Materials and Methods

A liver section from each animal was ground under liquid nitrogen using a mortar and pestle. Lipid extraction was performed in triplicate on 15mg portions of ground liver, using a Bligh and Dyer extraction modified to increase reproducibility[124]. All volumes of organic solvents were dispensed using Hamilton syringes equipped with a cemented needle and a stainless steel plunger (P/N 1725). The approximately 15mg portions of ground liver tissue were weighed, combined with 0.3mL -20°C chloroform in a pre-chilled Dounce homogenizer and homogenized on ice. The sample was transferred to a 1-dram glass vial with a PTFE lined rubber insert in a phenolic cap (Kimble, P/N 60811B1; Fisher). The homogenizer was washed in three steps with 0.3mL -20°C chloroform, followed by 0.6mL -20°C methanol, and 0.45mL ice cold 20mM KPi, pH 8.0. Washes were combined with the sample and incubated on ice for 5 minutes; vortexing for 5 seconds, every 30 seconds. The samples were centrifuged at 4°C and 2000g for 2 minutes. The organic layer was removed using a Hamilton syringe and transferred into a 1.8mL amber glass autosampler vial. 0.5mL of chloroform was added to the sample, briefly vortexed, and centrifuged as before. The organic layer was removed, combined with the first organic layer and dried under nitrogen. The dry organic layer was resuspended in 200ul chloroform:methanol (1:2) per 15mg of sample.

MS and MS/MS data was collected with a UPLC-ESI-MS, using an Agilent 1290 UPLC system coupled to an Agilent 6538 Q-ToF MS. The UPLC was equipped with a Zorbax Eclipse XDB-C18 (2.1x150mm 1.8µm), rapid resolution high definition (RRHD) column (Agilent). The mobile phase consisted of solvent A: 0.1% formic acid in water

and B: chloroform:methanol:isopropanol 1:2:4. A linear gradient of 65%B to 95%B was used to elute peaks over 50 minutes with a 7 minute hold time at 95%B, followed by an equilibration time of 3 minutes at 65%B. The flow rate was set to 0.200 mL/min with an MS collection rate of 1Hz.

Data were analyzed using the automated, R-based XCMS program available from XCMSonline. Principle component analysis (PCA) was used to evaluate the differences in the amounts of each mass feature between samples. PCA plots were generated to visualize the multi-dimensional data. In addition, cloud plots were generated to simultaneously visualize fold-changes, p-values, and the direction of the changes (increase/decrease) in the levels of mass features that differed between samples.

Results

Examination of the PCA plots revealed little variation in technical replicates but significant variation among treatment groups. From this it was concluded, that the lipid extraction technique is reproducible, due to the tight clustering of technical replicates, but that an unknown factor was affecting the expression level of lipid species detected in the different treatment groups. A literature search revealed a plausible explanation for the sample heterogeneity; the spatial distribution of lipids in liver is far from homogenous, as revealed by matrix assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS)[109, 125] which shows relative levels of different lipids (figure 4.3). In this pilot experiment, random regions of the liver were used from different animals for lipid extraction. In addition to the heterogeneous distribution of lipids in liver tissues we noted a considerable difference in color between samples.

Four additional samples were available, that were each an entire lobe of liver. These were ground in liquid nitrogen to obtain samples which were much less heterogeneous, due to regional sampling of the tissue than in the first sample set. Portions (15mg) of the second set of ground samples were weighted in duplicate, extracted and the samples were analyzed as before.

Multivariate analysis, visualized in PCA plots, showed very good technical replication. In this case, the samples show slightly more variance in PC1 (25%) than the variance in PC2 (22%) (figure 4.4). The variation in PC2 appears to correlate with the relative intensity of the red color of the samples (figure 4.5). The variation of color in the samples is hypothesized to be due to differences in residual blood after the balanced salt solution perfusion used to remove blood from the animals. The variation in PC1 was hypothesized to be due to which lobe of liver was used; but as that data was not recorded, a supported conclusion cannot be drawn.

Method Validation

Statistical analyses were carried out on the original set of 12 liver sections to evaluate the utility of the data analysis process. Grouping samples by diet and using groups A and C (table 4.1), show 61 mass features with fold change of ≥ 1.5 and a p-value of ≤ 0.01 associated with the difference in sucrose intake without copper supplement as illustrated in the Cloud Plot in Figure 4.6. Many of these mass features were putatively identified with the use of the METLIN database. Mapping putatively identified mass features to biochemical pathways through XCMSonline's mummichog[126] feature revealed that pathways for synthesis of long chain fatty acids, complex lipids (phosphatidylcholine

and sphingomyelin) and intermediates in cholesterol synthesis were involved (figure 4.7). This supports the extraction and analysis techniques used as only lipid compounds were identified in a data base that is made up primarily of hydrophilic metabolites.

Summary

Several findings have come from this preliminary study that are informing the design of more refined studies, planned for the near future. Additionally, NIH grant proposals, based on this preliminary data, have been submitted to pursue such refined studies. The first consideration is that liver tissue is not spatially homogenous with respect to lipid composition. Global comparisons of the lipidomics of a treatment course of animals must control for this heterogeneity. Either the same section or same portion from each liver must be used or preferably, the entire liver from each animal should first be homogenized by grinding and then sampled. Perfusion techniques must be improved and standardized. A blood vessel dilation agent, such as sodium nitrite, should be used during profusion to ensure more efficient and reproducible perfusion.

Two additional considerations should be examined prior to further studies. The control diet that used Maltodextrin to balance caloric content inadvertently introduced significant amounts or iron to the control chow. The diets should be adjusted to eliminate this difference in the iron levels. In addition, the type of animals used for the animal model should be considered. The Wistar rat is an outbred genetically diverse model and a more inbred animal strain may be a better choice as nutrigenomic studies have revealed changes in brain gene networks in response to dietary intake of fructose[87] and it is possible that a similar effect may be observed in the liver.

Moving Forward with Minor Changes in Experimental Design.

Lipdiomic profiling was successfully used to demonstrate very low technical variation and to differentiate hepatic lipid samples from rats fed different diets. A diverse group of lipid classes was extracted from liver tissue, using a method consistent with high throughput experiments. The extracted lipids were detected via UPLC-ESI-MS and multivariate data analysis was able to detect significant differences in the lipid profiles of animals fed different diets.

High sensitivity measurements of the liver lipidome in an accepted animal model of NAFLD has revealed shortcomings with the tissue sampling used in this study. Future experimentation will use a more carefully controlled chow content to ensure the differences in the lipidomic measurements are reflective of the variation in the diets. Sucrose, rather than fructose, was used in this study. High fructose corn syrup added to drinking water should be used for carbohydrate stress, for a stronger effect on fatty liver pathology and to more closely replicate the western diet. The efficiency and reproducibility of the animal perfusion technique needs to be improved. Harvesting of the liver will be more closely controlled to account for the heterogeneous lipid distribution in the liver. With these minor adjustments, experimental data promises to reveal possible lipid biomarkers of NAFLD and perhaps NASH which could be sought in blood plasma. If such biomarkers could be found in plasma that could support the goal of developing an early diagnosis method for NAFLD that is efficient, rapid and inexpensive.

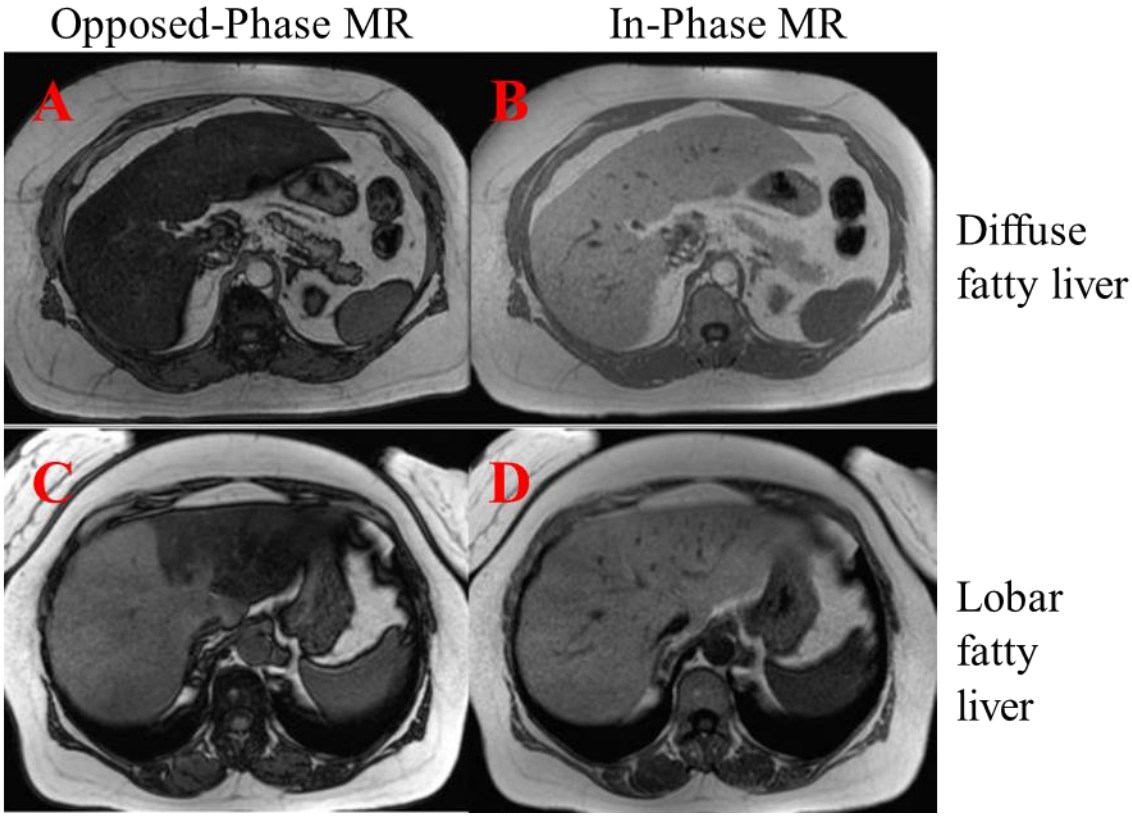

Figure 4.1. Opposed phase and In-phase MRI images show regions of the liver with large lipid deposits. The liver in panel A appears uniformly darker than the liver in panel B. The dark areas (the entire liver in this case) are the lipid deposits. In panel C, a large region of the liver is darkened, showing a lobe-specific regional deposition of lipid droplets.

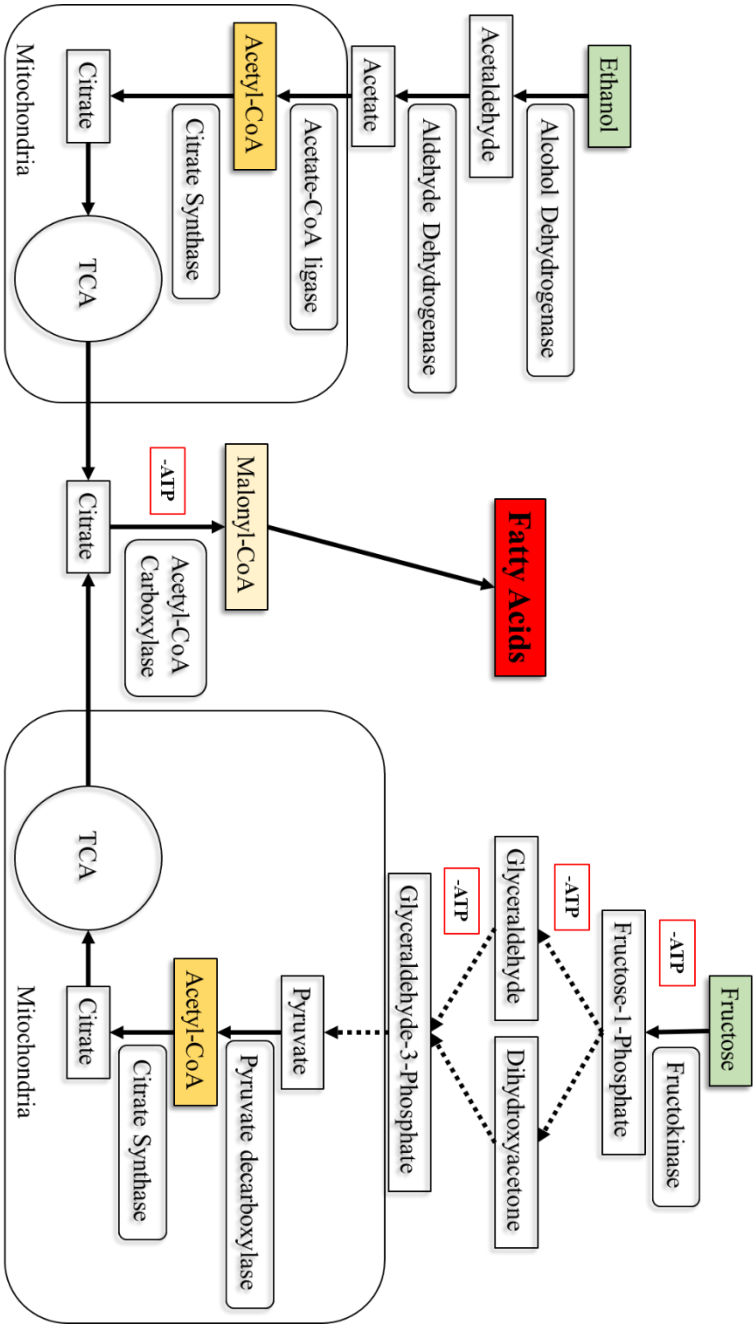

Figure 4.2. Fructose metabolism and ethanol metabolism, although through different mechanisms, yield the same products. Given the similarity of the end products it seems reasonable to investigate the deposition of lipids in NAFLD as a product of excessive fructose consumption because excessive alcohol consumption leads to AFLD.

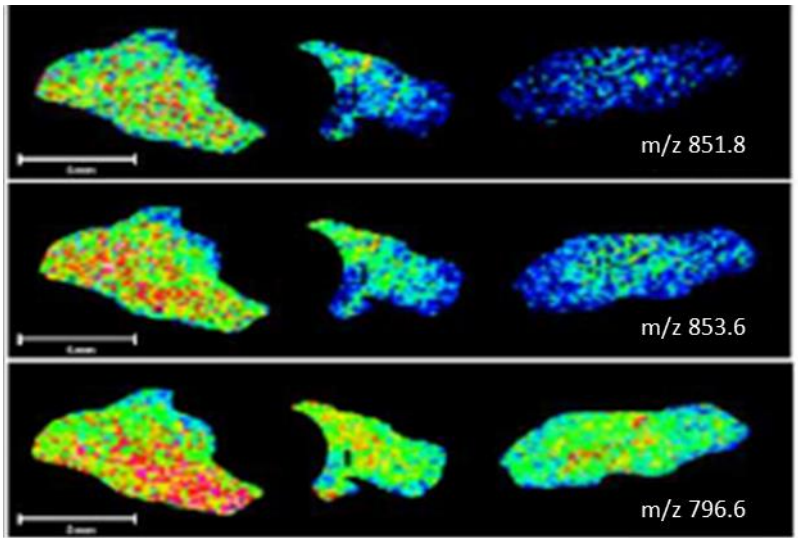

Figure 4.3. MALDI MS imaging of selected lipid masses show that the lipid location and lipid deposition in the liver is very heterogeneous. Park E-S, et al. 2014

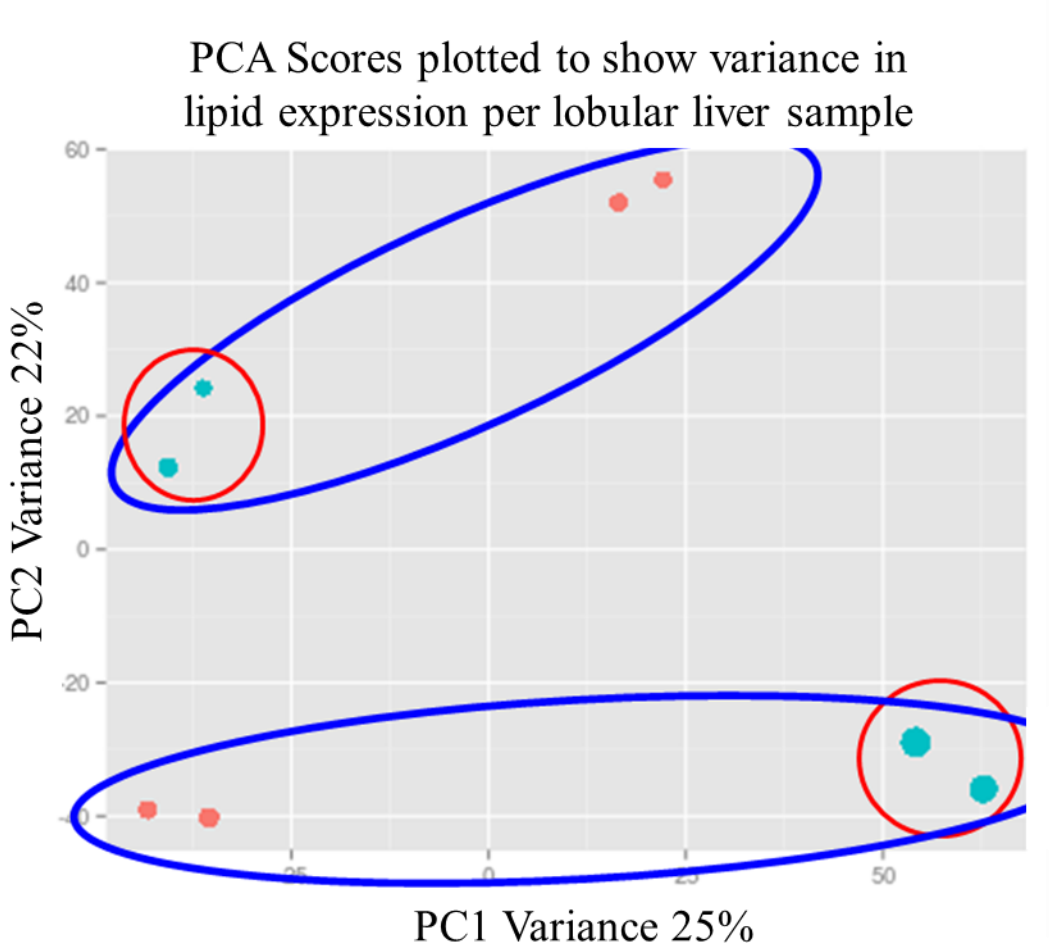

Figure 4.4. PCA analysis reveals replicate liver extractions (diets A, pink and C, blue) have relatively little variation in lipidome profiles while sample replicates have large variations. In addition, samples from separate treatment groups are more similar to each other than are the sample replicates.

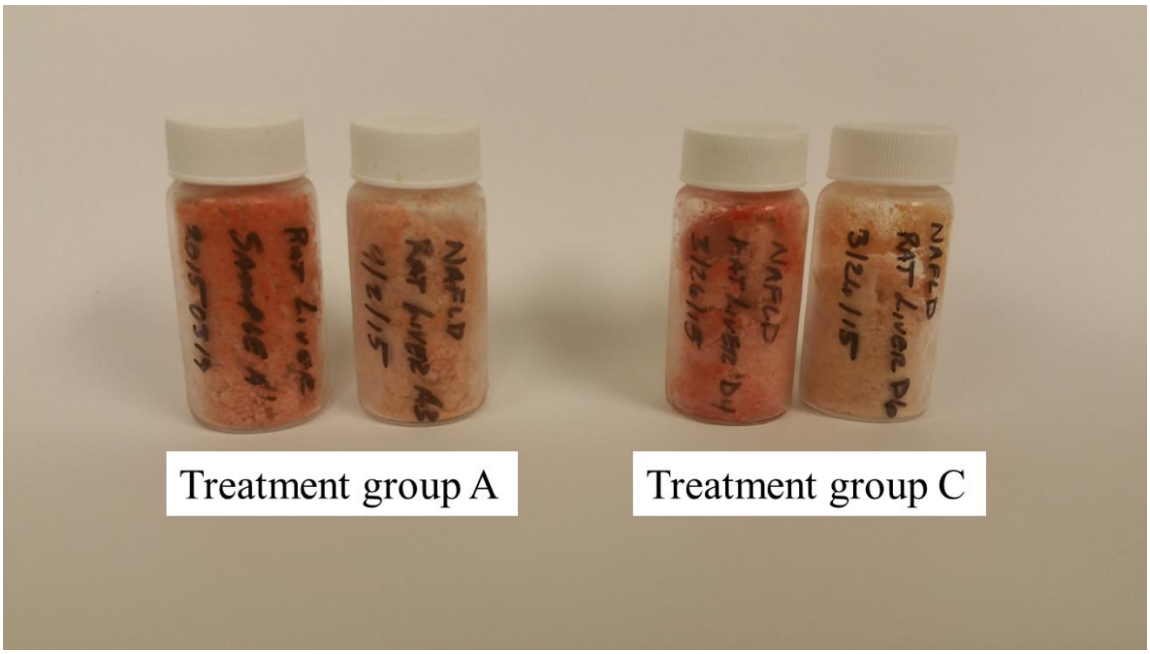

Figure 4.5. Visual analysis of the ground liver tissue shows a darker red color and a lighter red color in each of the treatment groups. In a PCA the two samples that show a darker red color grouped together as did the two lighter colored samples. This supports the conclusion that variations in the perfusion technique is responsible for a large amount of the variation found in the samples.

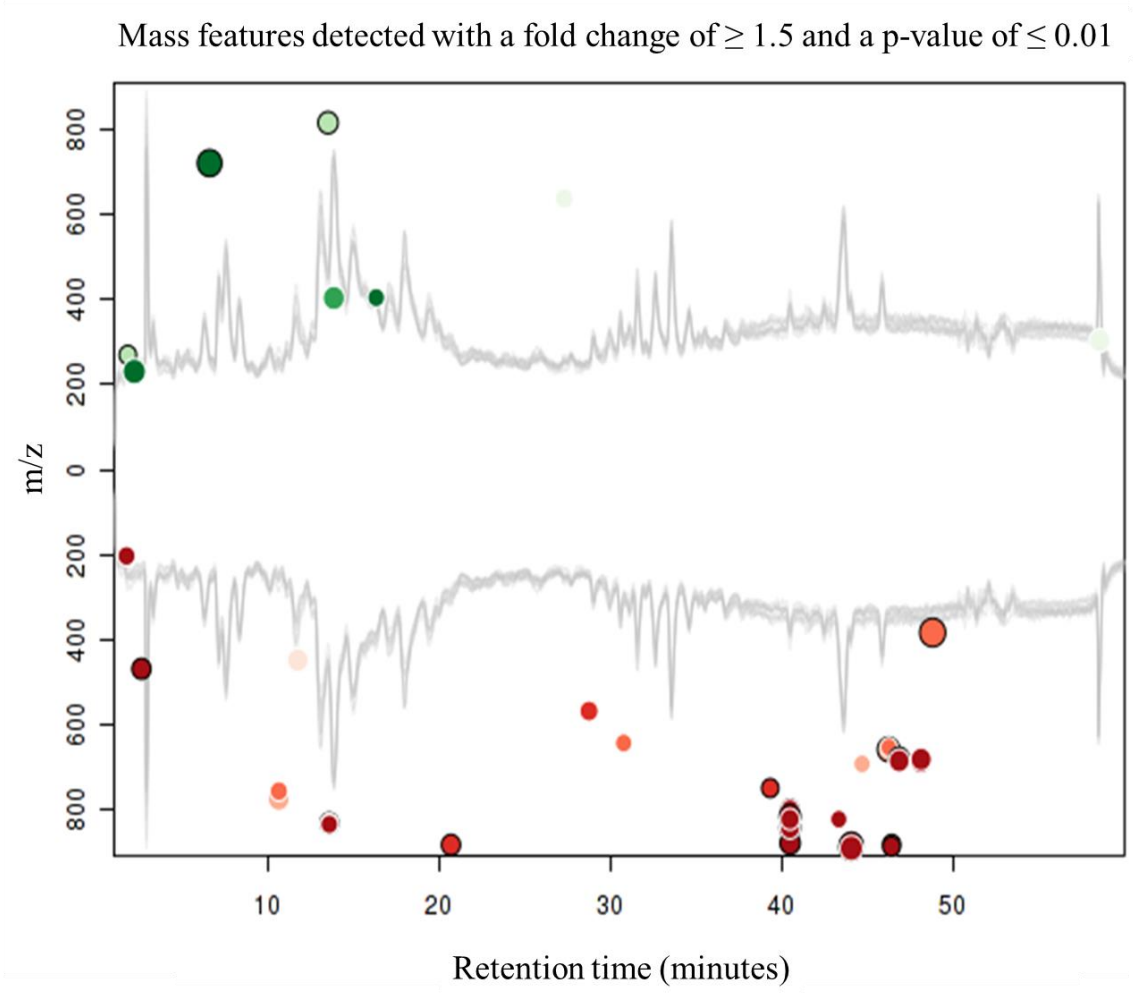

Figure 4.6. A representative cloud plot generated by XCMSonline, comparing diets A and C (table 4.1). The color of the spot represents the polarity of the change (up/down). The intensity of the color represents the p-value, the darker the color the lower the p-value. The diameter of the spot represents the magnitude of the fold change, the larger the diameter the larger the fold change.

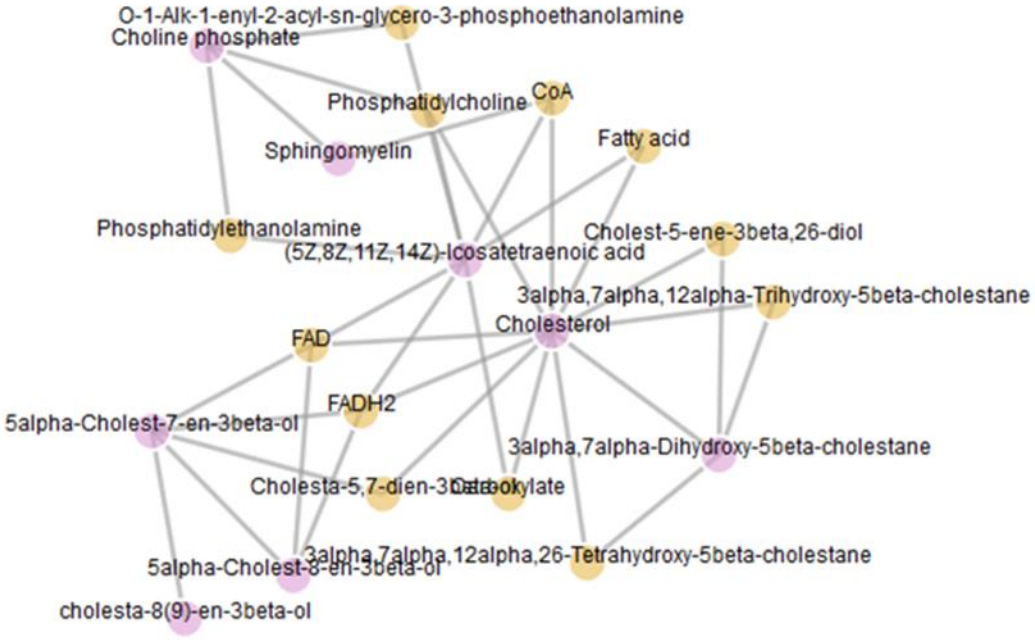

Figure 4.7. Pathway mapping by XCMSonline mummichog using the putative ID based on m/z and weighted by other features detected in the same pathway, shows perturbations of the metabolic activity network in the NAFLD rat model comparing diets A and C. The yellow spots signify metabolites which were not detected, but serve as connections between metabolites that were observed to change (purple spots). Importantly, only hydrophobic metabolites were detected in the samples.

Table 4.1. Diets used in each of the treatment groups. Diets were designed to feed animals high and low levels of fructose as sucrose and to also supplement each of those diets with copper.

Animal Group:	**A**	**B**	**C**	**D**
Casein	149.1	149.1	149.1	149.1
Sucrose	310.9	310.6	100	100
Maltose Dextrin	0	0	155.9	155.9
Corn Starch	410	410	465	464.7
Cellulose	40	40	40	40
Salt mix, 76, Cu-def	35	35	35	35
Vit. Mix, 76A	10	10	10	10
Dl-Methionine	3	3	3	3
Choline Bitartrate	2	2	2	2
Cu carbonate	0	0.22	0	0.22
Corn Oil	40	40	40	40
FD&C red	0	0.08	0	0.08
	1000	1000	1000	1000
Cu by ICP-MS	0.3	90	0.22	100
Fe by ICP-MS	40	40	119	149

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CHAPTER FIVE

LIPIDOMIC ANALYSIS OF HUMAN BRAIN CORTEX IN ALZHEIMER'S DISEASE REVEALS ABERRANT LEVELS OF ACETYLCHOLINE PRECURSOR SPECIES

Contribution of Authors and Co-Authors

Manuscript in Chapter 5

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Contributions: Planned experiments and contributed to study design. Provided feedback on manuscript and figures.

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Contributions: Assisted in data analysis, writing and editing manuscript.

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Contributions: Assisted in sample preparation and data analysis.

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Lipids
Status of Manuscript:
_X_ Prepared for submission to a peer-reviewed journal
____ Officially submitted to a peer-review journal
____ Accepted by a peer-reviewed journal
____ Published in a peer-reviewed journal

Springer Link
Submitted to American Journal of Alzheimer's Disease and Other Dementias for publishing November 12th 2016

LIPIDOMIC ANALYSIS OF HUMAN BRAIN CORTEX IN ALZHEIMER'S DISEASE REVEALABERRANT LEVELS OF ACETYLCHOLINE PRECURSOR SPECIES

Abstract

An improved, highly reproducible lipid extraction technique and lipidomic analysis was applied to human cerebral cortex brain from Alzheimer's disease patients (AD) and age matched controls. Lipid components were compared with principle component analysis, which identified 17 lipid species with expression levels capable of distinguishing AD from control samples. Several of the species identified were phosphatidylcholines and were decreased in AD compared to controls. In addition, the level of acetyl-CoA was significantly decreased. Both choline and acetyl-CoA are essential precursors of the neurotransmitter acetylcholine. Lower levels of acetylcholine have been reported in AD and treatments that raise acetyl choline have some beneficial effect. These changes were independent of APOE genotype, gender, age at time of death, and post mortem interval. It is possible that as acetylcholine levels drop, neurite outgrowth may be decreased and as a result the neurons shrink, leading to increased neurodegeneration.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by cerebral structural atrophy, the presence of amyloid-β plaques (Aβ), tau neurofibrillary tangles, and clinically presenting with progressive loss of cognitive function [127]. Factors implicated as possible causes of AD include oxidative stress [96, 128], neuroinflammation

[129], impaired acetylcholine metabolism [130], altered lipid levels and lipid metabolism [45, 131, 132]. Definitive diagnosis of Alzheimer's disease is currently limited to post mortem brain pathology, but imaging of brain amyloid deposits strongly correlates with AD status[133]. Alzheimer's disease occurs in two distinct subtypes, familial and sporadic Alzheimer's disease. The majority of Alzheimer's disease patients (90-95%) manifest symptoms of the disease after the age of 65 and are sporadic while the remaining 5-10% are familial that often has an earlier onset [134-136]. Altered lipid metabolism has been reported to significantly impact the pathophysiology of AD[16, 99, 137]. The brains of AD patients display markedly decreased levels of both phospholipids and sphingolipids, accompanied by an increase in ceramides [138-141], when compared to controls. The cleavage of APP by γ-secretase occurs in membrane lipid rafts[99, 142]. Variation in membrane levels of sphingomyelin and cholesterol have been found to affect γ-secretase activity[143], while ceramides promote Aβ production through the stabilization of the β-site of BACE-1[144]. In addition, Aβ peptides are hydrophobic and aggregation of membrane lipids, such as the ganglioside GM1, can increase the tendency of Aβ to form aggregates [145].

The most significant genetic risk factors for AD are the occurrence of three isoforms of Apolipoprotein E (APOE); ε-2, ε-3 and ε-4, whereas the ε-2 isoform confers lower risk and the ε-4 confers a higher risk[146]. APOE is a scaffold protein, responsible for cholesterol and phospholipid trafficking in the brain, and that also affects Aβ deposition[131, 147]. The ε-4 APOE isoform has a decreased binding affinity for $A\beta_{40/42}$ as well as other hydrophobic compounds and confers a substantial increase in the risk for all types of AD[146, 148]. On the other hand, ε-2 has a higher binding affinity for $A\beta_{40/42}$ lipids

and other hydrophobic compounds. If a person has two copies of ε-2, there is a substantial decrease in the risk of AD [131, 149]. Alternatively, there is a dose dependent increase in the risk of developing AD with each e4 allele that a person carries. Altered APOE function can lead to changes in the levels of $A\beta_{40/42}$, detectable in the CSF [150, 151]. Here we investigate whether changes can be detected in lipid species of the brain tissue in Alzheimer's Disease compared to controls and if the lipid changes correlate with APOE status.

Low levels of acetylcholine in the brain have been implicated in AD[138, 152]. Several FDA approved drugs for the treatment of AD inhibit acetylcholinesterase in the synaptic cleft, thereby increasing the availability of acetylcholine for neuronal transmission [153-155]. The short-term efficacy of these drugs in moderating cognitive decline suggests that there is a decrease in the supply of acetylcholine to the synaptic cleft in Alzheimer's Disease. Inhibiting acetylcholine esterase does not offer long-term relief of the symptoms of memory loss[156, 157]. The hypothesis that there is an insufficient supply of acetylcholine in AD brain that depresses cognitive function, is supported by reports of decreased acetylcholine in the brains of AD patients [158, 159].

The goal of this study was to identify lipid species in brain that change significantly between AD patients and aged matched controls. This was accomplished through application of a highly reproducible lipid extraction technique, developed in our laboratory[124], separation by UPLC, and identification of relevant mass features through ESI-Q-TOF MS.

Materials and Methods

Solvents were purchased from Fisher Scientific and were Optima LC/MS grade, with the exception of the isopropanol, which was purchased from Alfa Aesar (P/N 22906). Nitrogen gas was supplied from the boil off of an in-house liquid nitrogen storage tank. Glass vials (P/N 60811B1, 03-391), micro-vial inserts (P/N 14-823-374) and inert caps (P/N 14-812-49 and 03-378-343) were purchased from Fisher Scientific. Twelve Human cerebral cortex brain tissue samples were shipped on dry ice from the Victorian Brain Bank, Melbourne AU. Six of the samples were age-matched controls that did not meet the criteria for inclusion in AD diagnosis (NIA Reagan criteria). The other six samples met the standard criteria for AD diagnosis[160]. Mass spectral data collection was obtained using an Agilent 1290 UPLC system coupled to an Agilent 6538 ESI-Q-TOF, operating in positive mode.

A modified Bligh and Dyer lipid extraction method employing 17 isotopic internals standards, described previously[124], was used to extract a wide range of lipid classes from the brain tissues. Sections of cerebral cortex, containing 30-50mg of tissue were ground in a mortar and pestle, under liquid nitrogen. 50µL aliquots of a lipid standard mixture were dried under liquid nitrogen in 1-dram vials and 3 mg of ground tissue sample was added to each vial. Triplicate 3mg portions of each sample of ground tissue were analyzed and the remaining ground samples were stored at -80°C. A 0.3mL aliquot of -20°C chloroform was added to each vial. The samples with the standards were transferred to 1 mL Dounce homogenizers, homogenized on ice for 1 minute, and transferred back to the same 1-dram vial. The homogenizer was rinsed with 0.3mL -20°C

chloroform, followed by 0.6mL -20°C methanol, and then with 0.45mL 4°C 20mM KPi pH 8.0 and all the washes were added to the sample. The samples were incubated on ice and vortexed for 5 seconds, every 30 seconds for 5 minutes. Triplicate samples were interleaved so all were votexed in the same batch. The samples were centrifuged in a Sorvall Legend Mach 1.6R centrifuge at 4°C and 2000g for 2 minutes in 3D-printed centrifuge bucket adapters made to fit 6 of the 1-dram vials[124]. The organic layer was removed, using a Hamilton syringe, and placed in an amber autosampler vial, which was immediately capped, leaving a very small, 15-20µL, drop of organic layer behind. 0.5mL of -20°C chloroform was added to each vial, using a Hamilton syringe and the samples were vortexed and centrifuged as before. The organic layer was removed being very careful to not remove any of the aqueous layer. The organic fractions of each sample were combined in the same autosampler vial and dried under nitrogen. The samples were resuspended in 100µL/3mg (solvent/sample) -20°C 1:2 chloroform:methanol. Resuspended samples were placed in clean autosampler vials with Viton inserts and crimp-on caps.

Chromatography was carried out using an Agilent Eclipse Plus C8 RRHD 1.8µm 2.1x100mm column. The mobile phase solvent A was 0.1% formic acid and solvent B was 70/30 AcN/isopropanol with 0.1% formic acid. Sample injection volume was 2µL, the flow rate was 0.600mL/min with a gradient of 75%-99% B over 15 minutes, and the column compartment temperature was 60°C. It was necessary to run a sample free, solvent injection blank in between each analytical run to eliminate sample carryover.

Using an instrument for lipidomics, which is routinely used for the analysis of polar compounds, requires that the instrument be "preconditioned" with 4-6 gradient cycles before reproducible chromatography of hydrophobic compounds can be obtained. Some authors advocate the use of highly nonpolar solvents such as chloroform [107] or high levels of isopropanol for the best lipid chromatography, but we have found that the added resolution is frequently not worth the downtime of the instrument required for sufficient preconditioning. The solvents used in this study produce comparable chromatographic results with only one hour of preconditioning, running 4 blank solvent gradients to stabilize the column. In addition, highly nonpolar solvents, especially chloroform, are corrosive to seals and increase instrument maintenance requirements.

Mass spectrometry data was collected, operating the Agilent 6538 Q-TOF in positive ionization mode, monitoring a mass range of 150 m/z to 1700 m/z. The capillary voltage was 3500 volts, drying gas was 4L/min, the nebulizer was set to 45 psi, the fragmentor at 45volts, and the skimmer was set to 500 volts. Data was collected from 1 minute to 15 minutes at a rate of 1Hz.

Data analysis

Lipid mass feature intensities were normalized across samples using the total ion chromatogram (TIC) intensity. Analysis of mass feature intensity data was conducted using XCMS online[161] and with the R statistical computing platform. Data were visualized in a cloud plot, generated by XCMS online as well as PCA plots generated using R. PCA plots were used to evaluate the feasibility of using different lipid species to separate the AD and control and to seek possible correlation between; the APOE

genotype, post mortem interval, age at time of death, and gender. Lipid identifications were made through a combination of chromatographic retention time (RT), m/z values, and MS/MS data, using the LipidMaps, Metlin and NIST databases.

Results

Cerebral cortex brain section samples were analyzed from six AD and six control subjects. The AD patients had a mean age of 82.4 years (Std,11.5) of 11.5 YOA, the controls had a mean age of 74.3 years (Std of 5.4) (table 5.1). Apolipoprotein E (APOE) status was mixed, as shown in table 5.1.

XCMS online data analysis was carried out using the default "UPLC/UHD Q-TOF" parameters in XCMS, changing the fold change cut-off from 1.5 to 2. This resulted in 26 mass features with highly significant changes in levels ($p<0.01$) between AD and controls, as illustrated in a cloud plot (Figure 5.1). Isotope deconvolution left 17 unique mass features. Of the 17 mass features detected, 16 have a decreased level and 1 is increased in AD. Fifteen of the seventeen mass features have been putatively identified, as shown in Table 5.2.

MS/MS data was collected on the mass features that had sufficient intensity to be fragmented. Those mass features were 785.5837, 799.5993, and 812.6119 m/z and each of these fragmented to lose a 184.0747 m/z positive ion, which is consistent with the loss of phosphocholine[36]. The remainder of the mass features were putatively identified, based on their m/z values and in the case of acetyl-CoA the m/z and retention time (Table 5.2). Authentic standards will be required to conclusively identify these mass features.

Discussion

Analysis of the loadings from the PCA revealed 26 highly significant mass features with a fold change of ≥ 2.0. Isotope deconvolution led to 17 mass features that showed a significant change is expression levels between AD and Controls. Putative identification of 14 of 17 mass features was made based on mass and retention times. The mass features of interest had a significant decrease in expression in AD versus controls, with the exception of one mass feature at 1348.2383m/z. To identify the mass features, m/z values were used to search both the Metlin and the LipidMaps databases. The similarity of the retention times of the class specific internal standards were also used to support the identifications. Three of the mass features were of sufficient intensity to collect MS/MS data that was used to support the identification of those mass features. All of the mass features fragmented were phosphatidylcholine (PC) species. In addition to the PC species detected, a singly charged 2-molecule aggregate of acetyl-CoA was found to be significantly down regulated in AD patients (Table 5.2).

Principle component analysis was conducted on the MS data to reduce data complexity. PCA based on the 17 putatively identified mass features of the AD versus control samples reveals clustering of the AD samples that separate from the control samples. AD samples show a tighter clustering in both PC1 and PC2 than do the control samples, indicating that the lipid profiles of these 17 lipids of the AD patients have less variation than the lipid expression profiles of these 17 lipids in the control samples. Disease status clustering of the samples supports the use of these 17 lipid species as characteristic of AD. To evaluate the influence of APOE status, post mortem interval,

age at time of death, and gender on the PCA separations, the status of these measures were separately overlaid on the data points of the PCA plot (figure 5.2). For each measure, at least one representative was found in the AD and control groupings. This suggests the 17 lipids identified in this study are specific to AD and independent of gender, age, postmortem interval or APOE status.

The study subjects had a diverse group of APOE genotype status. The most common genotype was ε3/ε3 with five subjects, followed by four subjects with ε3/ε4 status and one each of ε2/ε4, ε2/ε3, and ε4/ε4. We were surprised to find no clear distinction in the data based on APOE genotype status, given the apparent role of APOE in the trafficking of lipids into and out of the cells of the brain[162, 163].

Alzheimer's disease is characterized by a loss of cognitive function. The loss of cognitive function appears to be at least partially due to a decrease in the levels of the neurotransmitter acetylcholine [17, 152, 164]. Acetylcholine synthesis is catalyzed by the action of choline acetyltransferase from acetyl-CoA and choline precursors (scheme 1) and the rate of acetylcholine synthesis is dependent on the bioavailability of acetyl-CoA and free choline [165]. In addition the reaction is close to equilibrium within the neurites of nerve cells [165, 166], and this makes the detectable steady state levels of acetylcholine in the brain very low.

Scheme 1.

$$Choline + AcetylCoA \rightleftharpoons \text{Acetylcholine} + \text{CoA}$$

Acetyl-CoA is also used in the liver for the synthesis of ketone bodies, which are water soluble and carried to the brain in the blood stream, without the need of

transporters. The ketone bodies meet the energy needs of the brain when glucose levels are low[3]. β-oxidation of lipids generates acetyl-CoA which can be converted to ketone bodies. A recent study has shown that astrocytes of the female rat brain catabolize lipids in the myelin sheath of neurons to make ketone bodies that fuel the activity of the neurons[81]. We hypothesize that in the environment of the glucose starved AD brain (frequently being called Type 3 diabetes)[46] the acetyl-CoA being generated may be rapidly converted to ketone bodies, such that the bioavailable levels of choline and acetylcholine are greatly decreased.

The brain's primary source of choline is through the action of phospholipase C on phosphatidylcholine (PC) species, although brain tissue can take-up small amounts of choline from the plasma [92]. A significant decrease in PC lipid species would presumably lead to a decrease in the availability of choline from the PC by phospholipiase C cleavage and thus a decrease in the bioavailability of choline in the neurons. A highly significant 2-3 fold decrease in the level of acetyl-CoA and a ≥ 2.0 fold decrease in the detection of several PC species was observed in this study. A significant decrease in acetyl-CoA and phosphatidylcholine precursors, that we find in the brain, would presumably lead to a decrease in the synthesis and bioavailability of acetylcholine in the neurons.

Summary

We have found a decrease in both acetylcholine precursors, choline containing phospholipid species and acetyl-CoA in the brains of AD patients compared to controls. These findings suggests that the decrease of acetylcholine driven neurotransmission in the brains of AD patients may be due to a decrease in the synthesis of the

neurotransmitter acetylcholine due at least in part to a decrease in the availability of essential precursors.

Table 5.2. Human cerebral cortex brain sections were taken from 6 Alzheimer's disease patients (AD) and 6 age matched controls. The samples were randomized and tracked only by case numbers until the study was completed. The AD samples had a higher average age (82YOA) than the control samples (74YOA) and the post mortem interval (PMI) of the AD samples was lower than for the controls (32 hours and 38 hours respectively). The APOE genotype was not controlled and was mixed amungst the samples.

Case ID	Age	Gender	Sample Mass	PMI (hours)	Diagnosis	APOE genotype
06/993	80.7	Female	294.43	59	Control	ε3/ε3
06/144	69.4	Male	230.56	24	Control	ε3/ε4
08/334	102.7	Female	145.67	69.5	AD	ε3/ε3
05/317	73.6	Male	72.04	49	Control	ε2/ε3
07/835	85.3	Female	166.32	60	AD	ε3/ε4
08/007	89.4	Male	195.79	13	AD	ε4/ε4
08/026	67.3	Female	58.43	24	Control	ε3/ε3
07/295	82.0	Male	134.8	29	Control	ε3/ε3
07/757	70.0	Male	288.93	9	AD	ε2/ε4
08/312	72.1	Male	271.86	13	AD	ε3/ε4
07/635	72.6	Male	159.74	42.5	Control	ε3/ε3
09/006	74.6	Male	244.95	30	AD	ε3/ε4

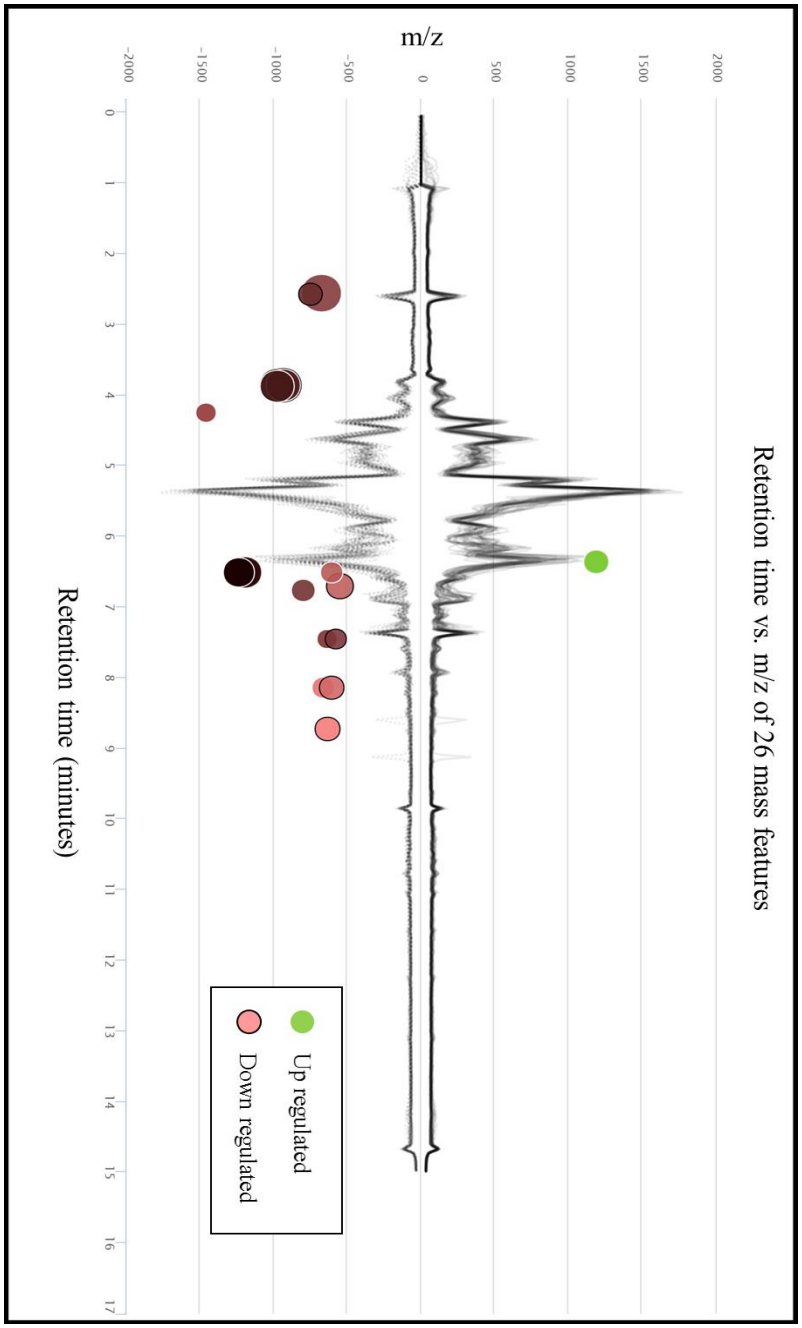

Figure 5.1. Metabolomic cloud plot generated by XCMSonline shows 26 mass features with differential expression in AD compared to control. One mass feature has an increase in expression while 25 have a decrease in expression. The size of the circle corresponds to the p-value (smaller circle, more significant p-value) and the intensity of the color corresponds to the fold change (darker color, higher fold change).

Table 5.3. A total of 26 mass features were identified as having a significantly different expression in AD samples when compared to control samples. Isotope deconvolution of the data left 17 mass features to be identified. Putative identifications were made on 16 masses using exact mass and retention time data. Fragmentation MS was attempted on all of the mass features however, only 3 were of sufficient intensity for successful fragmentation. Acetyl-CoA was identified as a singly charged dimer.

MS^2	fold	p-value	Up/down	Actual m/z	Lipid maps	LM m/z	LM ppm	Metlin Database ID	Metlin m/z	Metlin ppm
No	2.0	7.23E-07	DOWN	1635.2048	Not Found	---------	---------	Not Found	---------	---------
No	2.3	1.98E-07	DOWN	1619.1600	Acetyl-	1619.2589	61.1	Not Found	---------	---------
No	2.0	1.28E-06	DOWN	1616.1521	PC(38:5)	1616.1629	6.7	Not Found	---------	---------
No	2.1	7.90E-04	DOWN	1458.1635	Not Found	---------	---------	Not Found	---------	---------
No	2.1	1.44E-03	UP	1348.2383	Not Found	---------	---------	Not Found	---------	---------
No	2.9	1.55E-13	DOWN	1235.8356	Not Found	---------	---------	Ganglioside (41:0)	1235.8356	0.0
No	3.5	2.08E-06	DOWN	975.6608	PI(44:2)	975.6896	29.5	PI(44:2)	975.6896	29.5
Yes	2.1	2.34E-04	DOWN	812.6119	PC(38:3)	812.6164	5.5	PC(36:0)	812.6140	2.6
Yes	2.3	5.04E-05	DOWN	799.5993	Not Found	---------	---------	PC(36:4)	799.5960	4.1
Yes	2.2	9.88E-06	DOWN	785.5837	Not Found	---------	---------	PC(35:4)	785.5803	4.3
No	2.3	5.25E-03	DOWN	664.6671	Not Found	---------	---------	Cer(43:1)	664.6602	10.4
No	2.1	4.00E-04	DOWN	636.6359	Cer(41:1)	636.6289	11.0	Cer(41:1)	636.6289	11.0
No	2.0	2.63E-03	DOWN	608.6050	Not Found	---------	---------	Cer(39:1)	608.5976	12.2
No	2.2	9.07E-04	DOWN	606.5889	Cer(40:1)	606.6183	48.5	Cer(39:2)	606.5820	11.3
No	2.1	8.46E-05	DOWN	578.5831	Cer(38:1)	578.5870	6.8	Linoleyl arachhidate	578.5871	7.0
No	2.4	3.81E-04	DOWN	550.5523	Cer(36:1)	550.5557	6.1	Stearyl linoleate	550.5558	6.3
No	2.1	1.59E-04	DOWN	338.3394	Not Found	---------	---------	13-Docosenamide	338.3417	6.7

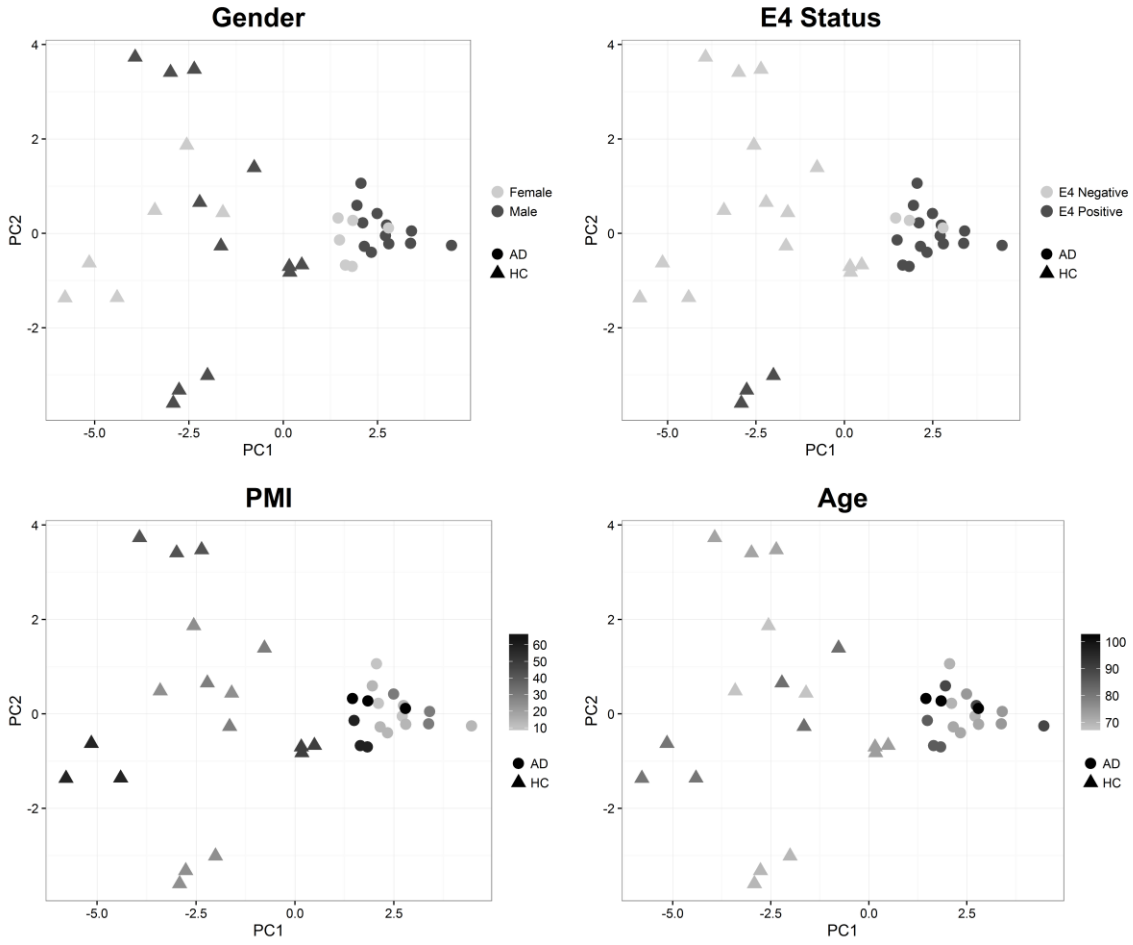

Figure 5.2. Principle component analysis (PCA) plots were generated using the 17 compounds identified as changing in AD compared to controls to reduce the complexity of the data. The AD and control samples each cluster together in this analysis. Gender, E4 status, post mortem interval (PMI) and age at time of death have been overlaied on the data points. In each metric, it can be seen that there is at least one sample in both the AD and the control group suggesting, these metrics do not affect the clustering of the AD and control samples.

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CHAPTER SIX

LIPIDOMICS REVEALS ABERRENT METABOLISM OF LIPID MOLECULES IN ALZHEIMER'S DISEASE CEREBRAL CORTEX

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Manuscript Information Page

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Lipids
Status of Manuscript:
_X_ Prepared for submission to a peer-reviewed journal
____ Officially submitted to a peer-review journal
____ Accepted by a peer-reviewed journal
____ Published in a peer-reviewed journal

LIPIDOMICS REVEALS ABERRENT METABOLISM OF LIPID MOLECULES IN ALZHEIMER'S DISEASE CEREBRAL CORTEX

Introduction

Alzheimer's Disease (AD) is characterized by a long period of mild, nonspecific symptoms, followed by progressive decline in both cognition and memory. The symptoms eventually affect all aspects of a person's life and the AD patient will eventually require constant care. The care required by patients with AD and AD-like dementias exert a substantial economic burden to both caregivers and society. These costs, per person with dementia, were estimated to be approximately $56,000/year in 2010[167].

A significant amount of AD research has focused on seeking early diagnosis and prevention of AD, which is not currently feasible. Furthermore, treatment of the disease in the later stages has shown little efficacy [168, 169]. The presymptomatic first stage of Alzheimer's disease is widely thought have the highest likelihood for effective intervention, however this has not yet been demonstrated [169-171]. The early stages of AD are difficult to diagnose, as clinical features are not yet apparent. There is evidence that a ketogenic diet and supplementation with the omega-3 fatty acid DHA administered during early symptomatic stages may be beneficial in slowing or decreasing AD pathology [172-175], but this approach has not yet been widely adopted in medical practice or nursing care.

AD is accompanied by a significant atrophy of the brain[70, 77]. The atrophy of the brain disproportionately affects the hippocampus, which is crucial for learning and

memory[71]. The brain has a high and heterogeneous lipid complement[74]. The white matter of the brain has the highest lipid content[74] and also shows significant atrophy in AD[77]. A recent mechanistic study of white matter atrophy in the aging female rat brain has shown an increase of fatty acids, fatty acid metabolism, and ketone bodies in the brain, which may be a response to a decline observed in plasma ketone bodies in AD [81]. It is hypothesized that catabolism of the myelin sheath of the neurons leads to production of ketone bodies in the brain which are used for energy metabolism by the neurons[81]. A separate study looking at a mouse model of Huntington's disease neurodegeneration, found that feeding a ketogenic diet significantly slowed the onset of symptoms compared to those fed a standard chow[176]. Both studies concluded that lipid metabolism is a potential target for therapeutic treatment.

Lipid metabolism in the liver and muscle are well understood[3], however, much less is known about lipid metabolism of the brain. This is a striking lack of knowledge in lipid metabolism, as the brain has the second highest lipid content of any tissue in the human body, second only to the adipose tissue[177]. In addition the lipid content of the various regions of the brain are not homogenous[74]. The lack of knowledge in brain lipid metabolism may be traced to the dogma that lipids have traditionally been seen as purely energy storage molecules or the structural component of cell membranes[3, 38]. In addition, it has been long assumed that the brain is not capable of energy metabolism beyond burning glucose and ketone bodies that are produced in the liver[3].

Advancements in lipid research have shown that lipids are also powerful signaling molecules that are involved in many aspects of metabolism. Lipid rafts on the cell

surface, for example, are involved in cellular signaling and have been shown to respond to stimuli by changing both size and protein composition on the cell surface; thus affecting cellular signaling[178]. The lipid sphingosine-1-phosphate (S1P), for example, has been shown to have roles in both inter and intra-cellular signaling, that is important in such functions as vascular maturation, cellular development and movement[179]. S1P is highly controlled at low levels, similar to other signaling molecules[179]. Other complex lipids have been shown to have highly conserved binding pockets on proteins[9]. Taken together, these examples support the idea of lipids as highly specific signaling molecules. Thus, it is highly desirable to characterize the lipidome as it changes in disease states. In a prior study we found that phosphatidylcholines and acetyl-CoA both decreased in AD cerebral cortex, compared to controls[180]. We inferred that this was the reason that the neurotransmitter acetylcholine, that must be synthesized from choline and acetyl-CoA, decreases in AD. In the present study we alter the chromatography and MS ionization methods used, compared to the prior study[180], to seek to investigate changes in a broader range of lipids in the lipidome of the human cerebral cortex in AD and age matched control brains.

Materials and Methods

Twelve human cerebral cortex brain sections, six AD and six controls, were shipped on dry ice from the Mental Health Research Institute, Melbourne AU to Montana State University, Bozeman, MT USA. Lipid extraction was achieved with a modified Bligh and Dyer protocol, described previously[124]. Briefly, triplicate 15mg aliquots of brain tissue, that had been ground in liquid nitrogen, were homogenized in 0.3mL -20°C

chloroform, in a Dounce homogenizer. The homogenates were collected in 1-dram glass vials with a dried mixture of 17 lipid internal standards and the homogenizer was rinsed with 0.6mL -20°C methanol and 0.45mL 4°C 20mM potassium phosphate buffer pH 8.0. The washes were added to the samples in the 1-dram vials. The samples were incubated on ice for 5 minutes, vortexed for 5 seconds, every 30 seconds. Samples were centrifuged at 2000xg for 2 minutes. The organic layer was collected and transferred to a new, amber, glass GC vial and dried under nitrogen; at the same time, 0.5mL -20°C chloroform was added to the samples remaining in the 1-dram vial and incubated as before. The samples in the vials were centrifuged a second time at 2000xg for 5 minutes and the organic layer was added to the previous, now dry, organic layer. The combined samples were dried under nitrogen and solubilized in 250µL/15mg (solvent/wet weight sample) -20°C 1:2 chloroform:methanol. Resuspended samples were sealed with a crimp-top cap with a Viton seal.

Chromatography and mass spectrometry were achieved with an Agilent 1290 UPLC equipped with an Agilent Eclipse Plus C8 RRHD 1. 8µm 2.1x100mm column coupled to an Agilent 6538 Q-TOF instrument. The HPLC was operated using a mobile phase solvent A of 10mM ammonium acetate pH 5.0 and solvent B of 70/30 AcN/isopropanol with 10mM ammonium acetate. The solvent gradient was 75%-99%B over 15 minutes and the column compartment was maintained at 60°C. After each run, a no-injection blank run was used to minimize sample carryover. Mass spectral data was collected at a rate of 1 Hz over the mass range of 150 m/z to 1700 m/z. MS/MS data was collected over the range of 100 m/z to 1700 m/z. For positive mode MS, drying gas was

set to 300°C, 4 L/min and 45 psi. For negative mode MS, drying gas was set to 350°C and 8 l/min at 55 psi. Capillary voltage was set at 3500V. In the prior AD lipidomics study a C18 column was used, solvent A was 0.1% formic acid and only positive ionization was used.

Data Analysis

The AD and controls samples were compared, based on the relative peak intensity of the mass features, using XCMSonline[161]. The data were analyzed, using the built in "UPLC/UHD Q-TOF" parameters of XCMSonline, changing the fold change cut-off from 1.5 to 2 with the p-value cut-off remaining at 0.05. Mass features with sufficient intensity for fragmentation and significant differences, based on a p-value of 0.01 between AD and controls, were selected for fragmentation, using the "Diff report" output of XCMSonline.

The MS/MS data, collected in both positive and negative modes, was used to identify mass features using MSConvert[181] and MS2Analyzer[182] platforms. MSconvert was used to extract MS/MS data from Agilent files. For the positive mode data, MS level and peak picking was set to 2; the threshold was set to count 40. For extraction of the negative mode data the MS level and peak picking levels were set to 2 and the threshold was set to absolute 5. Data was exported in MGF format.

Peak searching was accomplished with the aid of MS2Analyzer[182]. Peaks in both positive and negative ionization mode were searched for ions and/or neutral losses that correspond to polar head groups. Positive mode data was searched for neutral losses that corresponded to fatty acyls and negative mode data was searched for ions that

corresponded to fatty acyls. In all cases, the m/z window was set to +/- 0.05. Following automated searching, identifications were validated manually.

Results

A total of 161 positive and 60 negative mass features were found to differ significantly between AD and controls. Of these 70 mass features could be identified as complex lipid species from one of several lipid classes through MS/MS fragmentation. Of the 70 mass features that could be identified, 40 were detected in positive mode MS and 30 detected in negative mode. The lipid classes identified in positive mode were sphingomyelins (SM), cholesterol esters (CE), phosphatidylcholines (PC) and plasmalogens (PC-O). Those identified in negative mode were phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylglycerol (PG). Mass features detected and identified are reported in tables 6.1 and 6.2.

The mass spec intensities of the identified lipid species were summed by class and are presented graphically in figure 6.1. While SM, PC, CE, PI and PS species show a decreased level of expression and the plasmalogens show an increase in AD compared to controls, only the PS species differences are significant (p-value 0.0044). This suggests that although individual species of a class of lipids are changed in AD, compared to controls, as seen in table 6.1 and 6.2, other species of the same class appear to be able to compensate for those changes.

Several notable lipid species were detected as fatty acyls esterified to complex lipid species (table 6.1 and 6.2). The ω-6 fatty acid linoleic acid was found to be decreased and the highly pro-inflammatory arachidonic acid (AA), as well as the AA

elongation product 22:4 ω-6, were found to significantly increased in several lipid species in AD compared to controls. In addition, the DHA precursors α-linolenic acid (ALA), 24:5 ω-3, and 24:6 ω-3 were increased while the anti-inflammatory eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) were decreased in AD, when compared to controls. The MS intensities of these lipids were pooled by fatty acyl grouping of either pro-inflammatory or DHA precursor species to evaluate the possibility of significant differences between the two treatment groups, as discussed below.

Discussion

The altered chromatography method used in the present study, compared to our prior lipidomic study of AD and control brains[180], reveals a different pattern than before. The lipid species that were found to be dysregulated in the cerebral cortex of the AD brain were not all from the same class of lipids. In the present study there appears to be a dysregulation of lipid species from most lipid classes in the AD brain compared to controls. In addition, the dysregulation of the lipids was not all increased nor all decreased in AD compared to controls in the present study. This suggests that in the present study as one particular lipid species within a class is increased in expression another is decreased, perhaps an attempt of the cell to compensate for abnormalities in lipid production. This is supported by grouping the dysregulated lipids by class (figure 6.1).

The synthesis of different classes of phospholipids is interwoven and begins with the synthesis of phosphatidic acid (PA)[1]. Diacylglycerol species generated from PA are important precursors in the synthesis of PC, PE and PS species (figure 6.2). Either

choline or ethanolamine is esterified to the sn-3 position of PA to generate PC and PE species respectively, through a series of kinase and transferase reactions. PE can be converted to either PC[183] or PS and PC and can converted to PS and then to PE (figure 6.2)[1].

Regulation of Lipid Classes

By pooling the lipid species intensity data by class we found that nearly every class of lipid has a decrease in expression (figure 6.1). However, only the PS class of lipids is significantly down regulated with a p-value of 0.0044. In addition, the SM class of lipids nearly passes the Pearson's test for significance with a p-value of 0.074 (table 6.3). The finding of several lipid species with perturbations and relatively few lipid classes with net perturbations supports the hypothesis that compensatory mechanisms may exist in the brain to maintain phospholipid levels at the class level[1, 184].

It is not known if SM can be converted directly to PS, but it does not seem likely. On the other hand, choline used in the synthesis of SM lipids in the liver is supplied by PC lipids via a transferase reaction[185, 186] and these reactions have been shown to occur in the rat brain[187, 188]. Therefore, it may be that the individual PC species that are seen to have a significant decrease in expression is because of conversion to SM and that the cell may be compensating for the decrease by increasing the synthesis of other PC species.

In addition, it would be of interest to know if the PE class of lipids is increased in the AD brain at a level consistent with the level of decrease of PS. Unfortunately, we did not detect any PE species in our cortex samples under the present chromatography conditions, however, there is evidence in the literature that PE levels in the brains of AD

patients are decreased rather than increased[189, 190]. This continues to be of great interest because the synthesis of PE is interwoven and may be dependent on the concentrations of PC and PS species.

Acyl Chain Dysregulation

Complex lipids with fatty acyls that are either pro-inflammatory or anti-inflammatory were grouped and the relative expression of the groups were analyzed. The species of anti-inflammatory fatty acyls detected as a group show a decrease in level of expression with a p-value of 0.095. The pro-inflammatory species also shows a decrease in expression with a p-value of 0.095. While these groupings do not strictly pass the Pearson's test they are still of interest.

The fatty acyls EPA and DHA, the two most active anti-inflammatory fatty acyls, were detected in decreased levels in AD versus control samples, esterified mainly to choline containing lipids. In addition to EPA, three other DHA precursors were found to have an altered expression in AD, ALA, 24:5 ω-3 and 24:6 ω-3 and all three showed an increased level of expression in AD compared to controls (figure 6.3). This suggests that ALA may have a difficult time entering the DHA synthesis pathway and that when ALA does enter the pathway it may be "stalled" in the Sprecher pathway as 24:5 ω-3 and 24:6 ω-3. The Δ6 desaturase is involved in DHA synthesis at both of these points in the pathway suggesting there may be a decrease in the activity of the Δ6 desaturase in the synthesis of DHA as illustrated in figure 6.5.

The overall net decrease seen in pro-inflammatory fatty acyl species is in stark contrast to the increase seen in the most powerful pro-inflammatory AA and its

elongation product 22:4 ω-6. Significant increases were observed in AA and 22:4 ω-6. This is in agreement with the hypothesis that chronic neuroinflammation plays a role in the pathogenesis of AD[129, 191]. Phospholipase A_2 that hydrolyzes the ester bond at the sn-2 position of phospholipids[192] to release the polyunsaturated fatty acid present there is involved in signaling an inflammatory response. The decrease seen in the pro-inflammatory fatty acyls may be due to the significant decrease seen in linoleic acid containing species (figure 6.5).

Linoleic acid is the essential fatty acid precursor of AA synthesis. Two scenarios may be considered; first, the AD patients may have been consuming foods that contained too much linoleic acid, which is quite likely [193, 194]. The other scenario is that the downstream synthesis of AA is accelerated due to an increased use of the fatty acid in the synthesis and breakdown of complex lipids. The data in this study cannot differentiate between these two possibilities.

Taken together, the changes seen in the ω-6 and ω-3 synthesis pathways suggest that as the synthesis DHA is hindered the synthesis of AA may be accelerated. This is consistent with the biochemistry of the two pathways. Both the Δ6 and the Δ5 reactions of the pro-inflammatory and anti-inflammatory pathways are competing reactions as illustrated in figure 6.5. Thus it makes sense that as the de novo synthesis of AA in the liver from essential precursor linoleic acid increases then the de novo synthesis of EPA and DHA in the liver from the precursor ALA would be decreased (figure 6.5).

Conclusions

AD patients exhibit aberrant expression of many individual species of complex lipids. In addition, the species of complex lipids span many lipid classes. When pooled into classes of lipids, the larger classes, with the exception of the PS and possibly the SM, do not show significant differences in their expression in AD when compared to HC brain samples with the chromatography used in this study. The expression of the acyl chains esterified to phospholipids is also altered. Specifically, pro-inflammatory and anti-inflammatory fatty acyls were considered. Alterations in both pro-inflammatory and anti-inflammatory fatty acyls were observed, pointing to a decrease in anti-inflammatory response and an increase in pro-inflammatory responses. In future studies it will be desirable to utilize a wider range of chromatographic and extraction conditions to seek a complete coverage of the lipids present.

Table 6.1. Lipids identified through MS/MS data analysis with an increase in expression in AD compared to controls. Identifications were based on known fragmentation patterns of complex lipids in the data bases listed in methods and were manually curated. Gray boxes are lipid species that did not have sufficient intensity to identify the acyl chains.

Mass Feature	Ionization Polarity	Head Group	Acyl chains	Change in intensity AD vs controls
775.6320	Positive	PC	(20:0/18:0)	66403.33
814.6819	Positive	PC	(20:4)	34389.70
896.7634	Positive	PC	(18:2/26:2)	10050.01
928.5836	Positive	SM	(28:1/18:1)	9179.98
1388.2305	Negative	PG		7898.63
844.7188	Positive	PC	(18:0/20:0)	6186.00
816.6879	Positive	SM	(18:1)	5786.16
1392.2126	Negative	PI		5340.12
1391.2166	Negative	PI		4904.80
739.5640	Positive	SM	(18:3)	4758.13
627.5298	Positive	SM	(18:2)	4082.84
870.7477	Positive	PC	(18:2/24:1)	4000.10
882.5198	Positive	PC	(22:4)	3664.01
902.8015	Positive	PC-O		3173.59
687.5381	Positive	PC	(18:1)	2868.38
894.7474	Positive	PC		2847.33
1393.2144	Negative	PG		2683.54
1366.1893	Negative	PI		2019.33
883.5234	Positive	PC		1907.52
1323.1462	Negative	PI		1883.34
930.5899	Positive	PC-O	(O-24:0/22:0)	1869.66
1322.1735	Negative	PG		1583.45
822.5232	Positive	SM	(18:1/24:5)	1533.63
902.7212	Positive	PC	(24:0/20:1)	1163.22
886.7618	Positive	PC	(24:1)	1141.93
1297.1281	Negative	PG		1079.79
1296.1235	Negative	PG		1031.38
908.6440	Positive	PC		892.06
1374.2169	Negative	PG		804.51
1375.2179	Negative	PS		786.23
884.7631	Positive	PC	(20:4/24:6)	609.97
1105.9525	Negative	PS		435.42
1252.1041	Negative	PI		435.30

Table 6.2 Lipids identified through MS/MS data analysis with an increase in expression in AD compared to controls. Identifications were based on known fragmentation patterns of complex lipids and manually curated. Gray boxes are lipid species that did not have sufficient intensity to identify the acyl chain species.

Mass Feature	Ionization Polarity	Head Group	Acyl chains	Change in intensity AD vs controls
399.3230	Negative	PA	(16:0)	-391.74
1175.7808	Negative	PS		-452.93
980.6047	Negative	PI		-538.38
940.5912	Positive	PC	(20:5)	-559.74
915.6002	Positive	PC	(22:5/24:3)	-729.78
887.6293	Positive	PC	(18:2)	-760.67
1535.0973	Negative	PG		-794.96
1534.0930	Negative	PG		-817.06
798.5381	Negative	PI	(18:1/14:0)	-1034.50
876.5422	Positive	PC	(18:2)	-1141.66
886.6251	Positive	PC	(18:2)	-1267.69
979.6019	Negative	PI	(20:1/16:0)	-1284.83
817.5717	Positive	PC	(24:0/14:2)	-1350.67
797.5359	Negative	PA	(22:4)	-1684.07
600.5631	Positive	CE	18:1/22:3	-1877.84
978.5978	Negative	PI	(26:7/18:1)	-2083.69
662.6510	Positive	SM	(18:0)	-2607.18
871.6307	Negative	PS	(24:3/22:6)	-2744.94
743.5369	Positive	SM	(18:1)	-2822.83
606.6138	Positive	SM	(18:1/18:1)	-3043.71
856.6389	Positive	SM	(18:0/26:2)	-3720.55
976.6614	Positive	PC	(22:0)	-4223.54
798.5959	Positive	SM	(18:0/22:3)	-4683.05
796.5785	Negative	PA	(22:4)	-5283.37
858.5947	Positive	PC	(24:2)	-5660.08
856.5782	Positive	SM	(18:0/26:2)	-5661.86
396.3487	Negative	PI		-8880.40
639.5690	Negative	PS		-9429.54
815.6852	Positive	SM	(18:1)	-10231.90
795.5759	Negative	PI		-18316.33
750.5951	Positive	PC	**(18:1/16:6)	-23323.05
798.5860	Positive	SM	(18:0/22:3)	-32643.72
794.5726	Negative	PS		-35505.83
636.6357	Positive	CE	18:1/22:2	-89628.30
797.5830	Positive	SM	(18:2/20:5)	-126312.90
796.5801	Positive	PC	(18:0)	-262604.63

Table 6.3 Average intensity of identified lipids by class. P-values were calculated using the data from individual species in the classes, not pooled data.

Lipid Class	HC	AD	p-value
Cer	185982.1	92212.6	0.1125
PG	51901.3	50101.6	0.6496
SM	72724.2	53401.1	0.0739
PC	96485.4	80570.1	0.2137
PC-O	6216.6	9312.3	0.8628
PI	84753.8	69877.8	0.3613
PS	121719.2	75086.5	0.0044

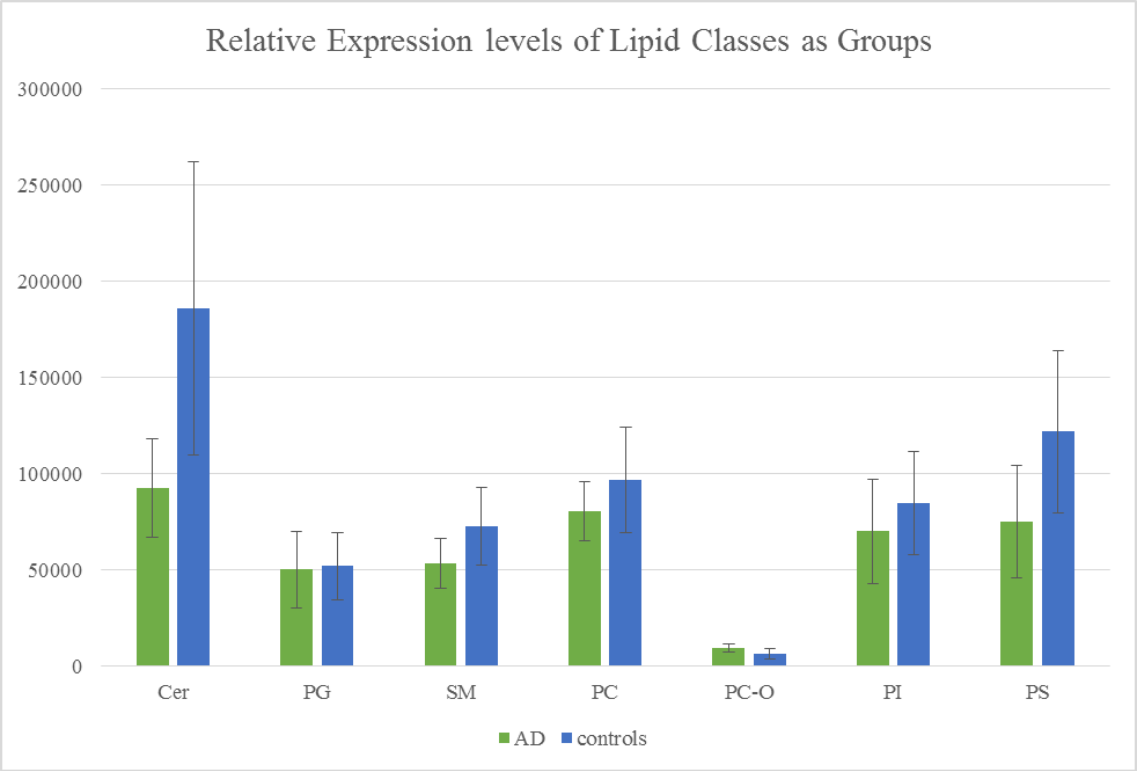

Figure 6.1 Class specific expression of lipids in AD and controls. Although a decrease in expression is apparent for all but the PC-O classes of lipids, only the decrease seen in the PS class is statistically significant.

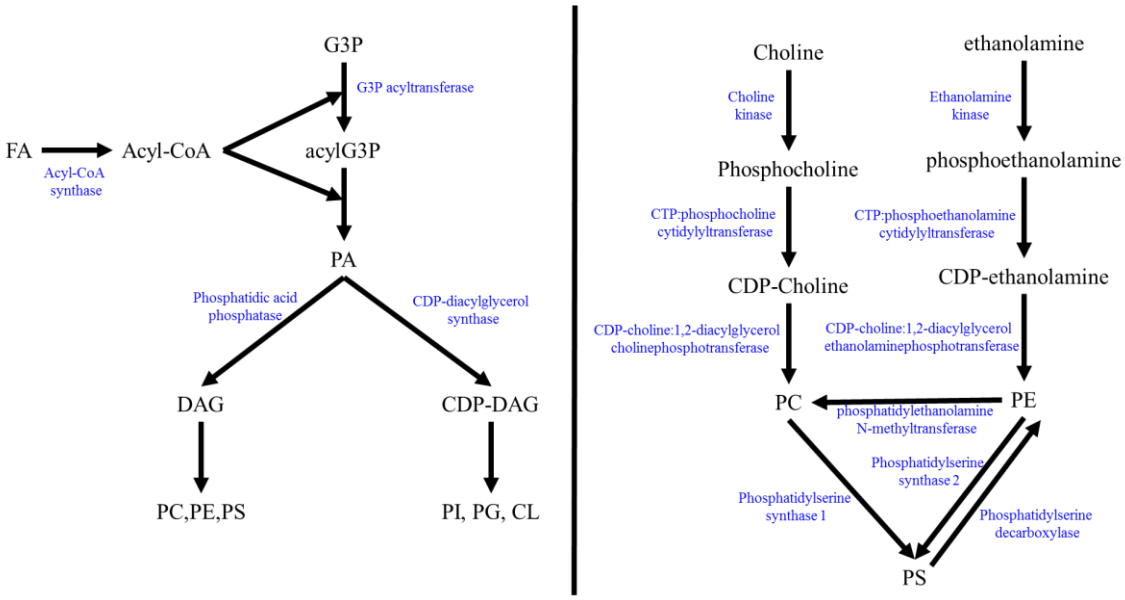

Figure 6.2. Synthesis of complex lipids follows well-known metabolic pathways. PC, PE and PS lipid classes come from the same diacylglycerol precursors. PE can be converted to PC and PS species through enzyme catalyzed head group exchange reactions and PC can be converted to PS through a similar exchange reaction. Taken from[1].

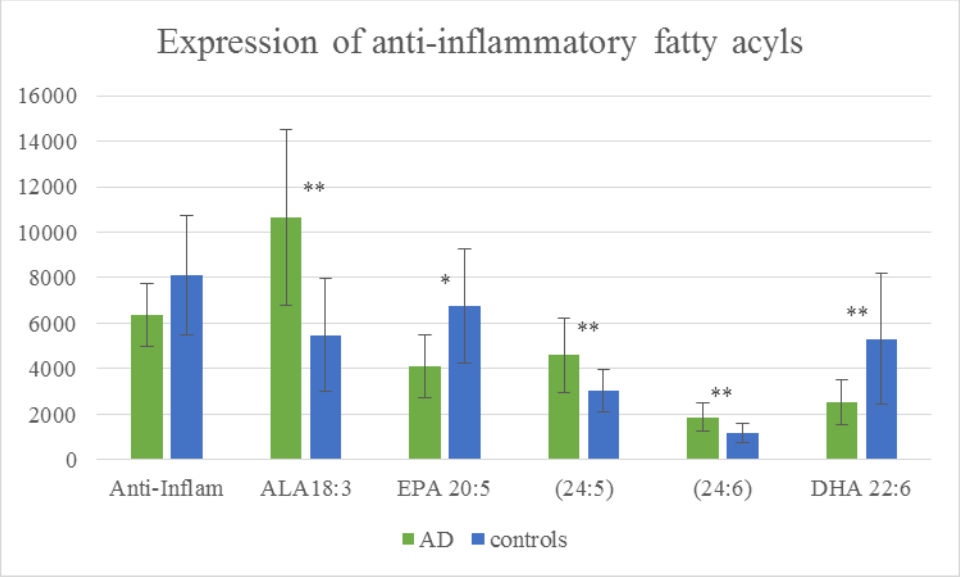

Figure 6.3. Anti-inflammatory fatty acyls as a group show a decrease in expression in AD compared to controls. Species upstream of desaturase reactions show an increase in expression while species downstream of the desaturase reactions show a decrease in expression as further illustrated in figure 6.5. * denotes a significant change (p-value ≥ 0.05) **denotes a highly significant change (p-value ≥ 0.01).

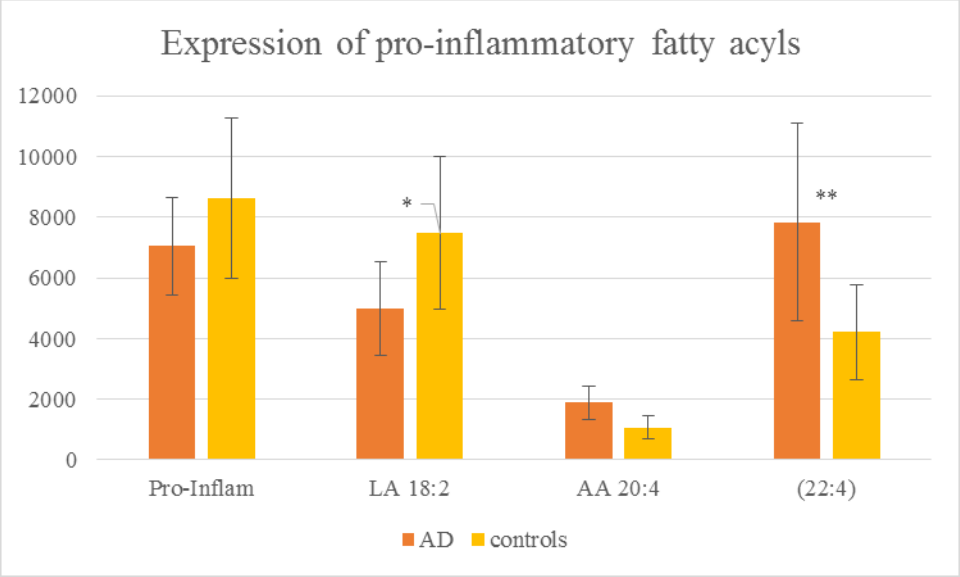

Figure 6.4. Pro-inflammatory fatty acyls show a decrease in expression in AD versus controls. The precursor linoleic acid shows a decrease in expression while the downstream desaturase product AA shows an increase in expression. * denotes a significant change (p-value ≥ 0.05) **denotes a highly significant change (p-value ≥ 0.01).

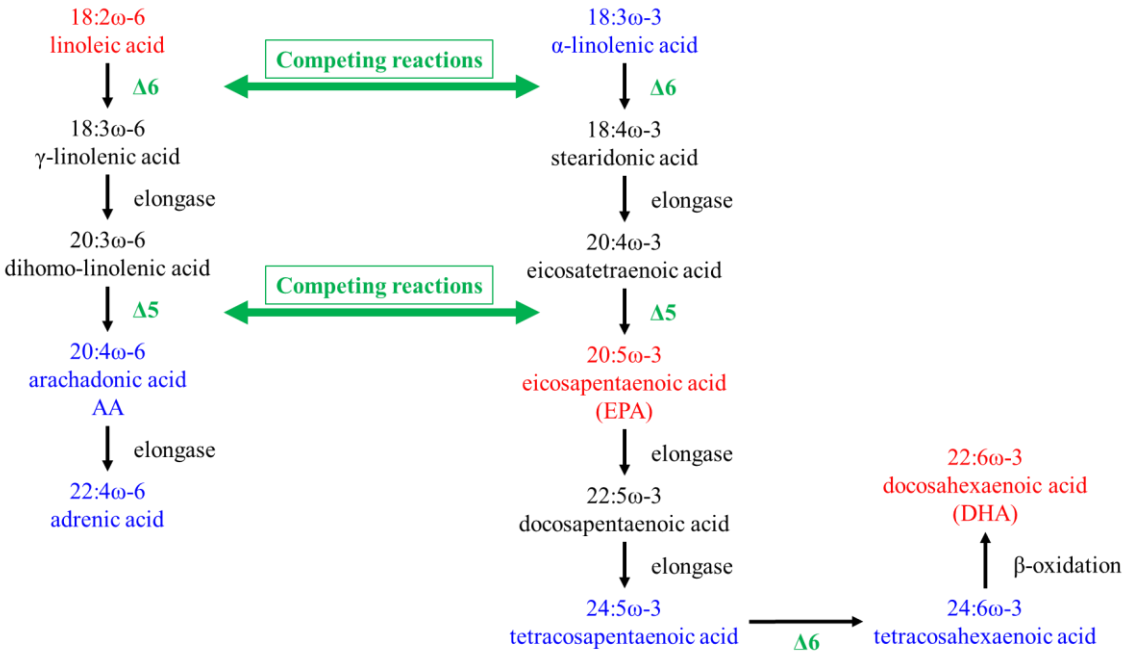

Figure 6.5. ω-6 and ω-3 fatty acid desaturation reactions in the liver compete for the same desaturase enzymes. Fatty acyls used as precursors in the desaturase reactions in the synthesis of DHA were found to be increased (blue), while products (red) were found to be decreased. In the synthesis of AA the precursor was found to be decreased (red) and AA was found to be increased (blue). This suggest that the competition for the desaturase enzymes may favor the ω-6 synthetic pathway in the AD patients, compared to controls.

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CHAPTER SEVEN

FUTURE DIRECTIONS IN LIPIDOMIC INVESTIGATIONS OF NAFLD AND AD: THE CONTINUING NEED FOR BIOMARKERS

Fructose intake has increased as the use of high fructose corn syrup (HFCS) as an added sweetener has increased greatly in the United States[195]and the world. Excessive fructose consumption has been shown to lead to lipogenesis[196], hepatic lipid deposition[42, 64, 197], and cognitive decline in animals[87], possibly related to epigenetic changes[198]. Chronic, excessive fructose intake causes an excessive rate of carbohydrate metabolism as reviewed in chapter 1. This excessive rate of carbohydrate metabolism leads to the deposition of complex lipids in the liver[117] and likely in other tissues as well. Then, at least in the liver, the lipid deposits make the tissues more susceptible to injury through a secondary mechanism such as oxidative stress[66]that leads to fibrosis and cirrhosis[199]. In addition, several of the chronic and growing diseases plaguing modern society have been linked to fructose/sucrose consumption and dyslipidemia[41, 42, 200-202]. Nonalcoholic Fatty Liver, Type 2 Diabetes and Alzheimer's Disease are major and growing problems in the US and the world, they are linked to imbalanced diets and all lack effective early warning biomarkers. This thesis has laid a foundation for a more informed and detailed pursuit of early warning markers of these diseases.

Dyslipidemia associated with NAFLD, T2D and AD can be investigated through lipidomic analysis. Lipidomics is a set of investigative tools used to assay the lipidome, as it relates to life processes. Lipidomics techniques are both qualitative and quantitative and appear to be excellent tools well suited to investigate disease mechanisms. Lipids

themselves are increasingly gaining recognition as powerful signaling molecules in cellular processes and disease[10, 143]. Classes of lipids and, in some cases, specific species of lipids can be traced to metabolic pathways and perturbations in lipid metabolic pathways may be able to aid in the understanding of disease progression.

Mechanisms of Non-alcoholic Fatty Liver Disease

Nonalcoholic fatty liver disease (NAFLD) is a lipid dysregulation disease characterized by excessive hepatic lipid deposits[203] that is thought to have a very high incidence in the US[120, 204]. Most patients affected by NAFLD do not display any outward symptoms[203] and it appears that a very large fraction of those afflicted have not been diagnosed[205]. The high incidence and low diagnosis is very significant since NAFLD is a large risk factor for Type 2 diabetes[206]. While the onset of NAFLD is not well understood, much of the research in the field suggests there is likely to be a "two hit" mechanism to disease onset. The first of the two hits is the deposition of lipid droplets in the liver, that increase the vulnerability of the liver to a secondary, poorly characterized, liver insult. Oxidative stress is the most widely accepted probable second hit in NAFLD[66]. Work with fructose fed rats that develop fatty liver, fibrosis and cognitive decline shows that the rats avoid significant liver and brain pathology when fed the omega-3 fatty acid DHA along with the fructose. Thus, feeding rats DHA avoids the second hit to the liver (and also avoids cognitive decline) but the rats still show the first hit of fatty liver and no obvious pathology[87].

The NAFLD study outlined in this work seeks to find lipid-based disease mechanisms present in the liver for NAFLD. Lipids that could serve as biomarkers

capable of differentiating individuals with NAFLD from those with normal liver pathology were not identified; however, the lipid extraction, data collection and data analysis methods were validated and the studies in this thesis point the way to improved experimental designs for future experiments, as described below.

A new, currently ongoing NAFLD study has been redesigned, based on the foundational work presented in this thesis, taking into account liver heterogeneity, balancing of dietary factors, and improved perfusion of the animals prior to tissue harvest. NIH grant proposals have also been submitted to support the redesigned experiments. The new studies should have a greatly increased capability to find mechanistic clues and biomarkers in liver tissues that also may be present in blood plasma. If such blood biomarkers could be found they would make a blood test for NAFLD safe, inexpensive and ideally, routine.

Lipidomics of Alzheimer's Disease

Alzheimer's Disease is characterized, in part, by the significant loss of brain volume and the brain has a very high lipid content[74]. However, AD currently can only definitively be diagnosed at autopsy, through the presence of plaques and tangles in brain tissues[207]. Like NAFLD, the mechanism of onset and progression of AD are poorly understood. In contrast to NAFLD, AD does not have a relatively effective diagnosis strategy. Early evidence for AD can be detected with brain scans, but these are very expensive, not widely available, and are rarely used for screening[208-210]. Diagnosis of AD requires clinical recognition of neurocognitive decline, which is only clear rather late in the disease. AD interferes with independence in everyday activities in conjunction with a

gradual and progressive decline in memory and learning abilities. In addition, the cognitive decline must not be better explained by some other ailment[211].

We carried out two studies that show aberrant lipid metabolism in AD patients[124, 180], when compared to controls. In the first study, we were able to show, through PCA, that advanced stage AD patients could be differentiated from control patients by the relative expression of 17 lipids in a small cohort of 12 participants. In addition, several species of phosphatidylcholine were shown to have a decreased level of expression and the level of acetyl-CoA is also decreased. This suggests the rate of the synthesis pathway of the neurotransmitter acetylcholine is reduced, due to a decline in the essential precursors choline and acetyl-CoA. Several of the drugs that slow the progression of AD inhibit acetylcholine esterase and AD patients have been reported to have lower acetyl choline in the brain[88, 212].

The second study, sought to investigate a wider range of lipids with different chromatography and an additional ionization method. The second study gave a somewhat different view of the complex lipids present and showed that the expression profiles of specific lipid species but only the PS class of lipids was altered in AD patients compared to controls. This suggests that while specific lipids may be dysregulated the cell is able to try to compensate for the aberrant production of one lipid molecule by adjusting the production of similar molecules, within the same class of lipids.

Analysis of the fatty acyls esterified to phospholipids showed that DHA and many DHA precursors have a lowered level of expression. The levels of the ALA precursor to longer ω-3s and 24:5 ω-3 were increased while the levels of EPA and DHA were

decreased. Taken together, these findings suggest that de novo synthesis of DHA, in the liver followed by transport to the brain, from the essential precursor ALA may be stalled/slowed at the Δ 5 and Δ6 desaturase reactions. It has previously been shown that the level of the rate limiting enzyme in liver for DHA synthesis in humans is decreased in parallel with the degree of cognitive decline[213]. The enzyme carrying out the rate-limiting step in DHA synthesis is lowered ($p < 0.048$)], and the levels of the DHA precursor were elevated, indicating that DHA synthesis is impaired. In addition, we found that the competing Δ 5 and Δ6 reactions of arachidonic acid synthesis have a similar and opposite effect on the lipidome. AD patients are known to have high levels of neuroinflamation[129] and DHA supplementation is beneficial in at least some cases of AD[214, 215]. Future studies of lipidomics in AD are planned due to the availability of additional samples. These future studies will be able to measure a larger number of subjects and will include three different extraction methods and three different chromatographic methods to seek to more thoroughly explore the lipidome in AD.

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