

12-2-2014

The Effects of Dietary Macronutrient Composition on Whole Body Insulin Sensitivity through Modification of Cellular Lipid Composition

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The Effects of Dietary Macronutrient Composition on Whole Body Insulin Sensitivity through Modification of Cellular Lipid Composition

Daniel J. Freidenreich, PhD
University of Connecticut, 2014

Individuals with metabolic syndrome (n = 15) were fed two hypocaloric diets, ~500 kcal below energy requirements, of individually prepared foods for 21 days. The diets consisted of a low-carbohydrate diet (LCD) (% carbohydrate:fat:protein = 7:74:20) and a low-fat diet (LFD) (55:28:20) reflective of the current US dietary recommendations. The LCD diet resulted in a net weekly intake of 2.69 g of arachidonic acid (ARA) in comparison to the 1.27 g/wk consumed during the LFD. The LCD significantly increased plasma ARA and decreased dihomo-gamma-linolenic acid (DGLA) in all three circulating lipid fractions as well as buccal cells in comparison to the LFD. Correlational analysis of plasma lipids with HOMA2 scores showed plasma ARA to be positively associated with insulin sensitivity in all three lipoprotein fractions during both diet phases. Total buccal cell ARA did not correlate to measures of insulin sensitivity in either diet phase, but DGLA was negatively correlated to insulin sensitivity during the LCD. The highly significant and consistent increase in circulating ARA after feeding a low-carbohydrate diet could be a result of multiple factors including better preservation of the existing ARA pool, increased dietary intake, greater endogenous synthesis and greater release into the circulation from adipose triglyceride stores. Given the importance of ARA in cellular membranes as it relates to insulin sensitivity, these results indicate that a low-carbohydrate diet promotes a favorable circulating and membrane fatty acid composition.

**The Effects of Dietary Macronutrient Composition on Whole Body Insulin
Sensitivity through Modification of Cellular Lipid Composition**

Daniel J. Freidenreich

B.S., Rutgers University, 2006

M.A., University of Connecticut, 2009

A Dissertation

Submitted in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy
at the
University of Connecticut

2014

APPROVAL PAGE

Doctor of Philosophy Dissertation

The Effects of Dietary Macronutrient Composition on Whole Body Insulin Sensitivity
through Modification of Cellular Lipid Composition

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ACKNOWLEDGEMENTS

Dr. Volek: I can't thank you enough for all that you have done for me. You allowed me the freedom to explore my interests, particularly with this dissertation project, allowing me to begin my ventures in working with leukocytes. You listened to my often times extravagant ideas with an open mind and responded with a guiding hand to keep me in check and help me realize those ideas. I cannot verbalize how much I have grown as a scientist under your tutelage. I also admire your ability to fearlessly pursue your scientific interests, even though they often go against the grain. Anyone can be a follower, but few people have what it takes to forge new scientific frontiers as you do. Finally, I have always admired the respect, courtesy and equality you give not only to your fellow faculty, but to your students as well. In summary, you are the kind of mentor, scientist and individual I have been able to admire and strive to emulate during my time at UConn. I have enjoyed working with someone as enthusiastic about science as I am and I look forward to the additional time we will be working together during my post doctoral fellowship.

Dr. Kraemer: The one thing you taught me which I will never forget is the concept of a paradigm. How I arrive at my scientific theories has always been relatively random, but your idea of structuring the central theorem with a paradigm and consequently relating the rest of the supporting concepts to that paradigm has greatly helped me when formulating my ideas. It is a concept/tool I will be able to use for the duration of my scientific career. Thank you for teaching me this tool and for all the knowledge you have passed down about the scientific process and exercise science.

Dr. Maresh: I would like to thank you for being the guidance that brought together this department. Although the success of the department has hinged on the united efforts of all the faculty and students that have been a part of it, you helped to guide and shape it to become the success that it is. You are not only a great example of a leader, but of what it takes to be a professor. I have always been awed by how in every defense in our department (and I do mean every) you always have such pertinent points and knowledge. The expanse of knowledge that you retain is quite remarkable and something to aspire to. Thank you for being such a model individual.

Dr. Lee: Thank you for all your help setting up my cell experiments and allowing me to work in your lab as I developed my methods. You, Dr. Park and your students are always so willing to help others and always possess the appropriate knowledge to provide accurate guidance to those like myself with little experience in cell work. I have always been impressed by the cleanliness, organization and proficiency of your lab. One day I hope to be able to run a lab with a similar level of efficiency and practical knowledge.

Dr. Phinney: Although we have only met a couple of times, I greatly appreciate the foundation you have built in the areas of arachidonic acid metabolism and low carbohydrate research (and not just because of all the citations it provided me). The few times we met you demonstrated to me your open mindedness, breadth of knowledge and humility, all crucial factors I believe for a successful and respected scientist. Not only are these qualities that I admire but I also hope to emulate them in the future.

Dr. Smith: Thank you Dr. Smith for teaching me how to cryopreserve cells and for always being there to answer my random questions about working with cells, cell media and culture vessels. Your willingness to pass along your knowledge helped me set up cryopreservation facilities in my own department. So thank you for all your assistance.

Dr. Norris and Dr. Elaine Lee: I could not have performed my flow cytometry experiments without you both. Dr. Norris, you taught me the foundations of flow cytometry, helped me to design the template for my experiments and were always there for me to ask questions about my results and to help me move forward with my method development when I hit stumbling blocks. I appreciate your willingness to share your time (often more than you expected) and knowledge with me. Dr. Elaine Lee, you helped me finally understand fluorescence minus one which aided me in further developing my experimental template. I also appreciate the time you took to me how to use our flow cytometer, to understand compensation and how to interpret the graphical and numeric output of the flow cytometer. I felt so slow the first days we worked together, but with your time and patience I learned all I needed to move forward with my method development. So thank you for all your time and help even though I know you were helping so many other students and faculty at the same time.

Current and Former Volek Lab Mates: All the projects we worked on together from REPS to MEAL and FASTER were such expansive and arduous projects, but together we

managed to complete them all. The MEAL study, which my dissertation is derived from, was no small task and I most certainly could never have completed the project on my own. The fact that we completed MEAL with our small group is a testament to the hard work, capabilities and cooperation of our group. Thank you to all of you because I would not have completed my dissertation project without all of your help.

My Family: I would like to thank my parents and sisters for their love and support as I worked on my project and for their understanding of the long hours it took to complete my degrees here. I would also like to thank my parents for listening to me for hours (I don't even know how many) as I went on and on talking about the ideas I had for my project, all the new literature I kept finding and for being there during the frustrating times. You always knew what to say to encourage me to keep moving forward and not give up. I love you all.

Eunice: Finally I would like to thank my girlfriend, Eunice, for all her love and support. You always encouraged me to keep on moving forward even when things were difficult. Spending time with you always gave me the emotional boost I needed to keep working and reinvigorate my working capacity. Especially while I was writing my dissertation, you kept me on task, took care of me and allowed me to focus on my work so I could finish on time. You are always there for me and I am and always will be grateful for that.

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Definitions of Terms

Abbreviations

List of Abbreviations

Saturated Fatty Acid	SFA
Monounsaturated Fatty Acid	MUFA
Polyunsaturated Fatty Acid	PUFA
Linoleic acid	LA
Dihomo- γ -Linolenic acid	DGLA
Arachidonic acid	ARA
Hypercaloric high Carbohydrate Diet	HHCD
Hypercaloric High Fat Diet	HHFD
De novo Lipogenesis	DNL
Protein	PRO
Carbohydrate	CHO
Low Carbohydrate Diet	LCD
Low Fat Diet	LFD
Dihydroxacetone Phosphate	DHAP
Very low density lipoprotein	VLDL
Low Density Lipoprotein	LDL
small,dense Low Density Lipoprotein	sdLDL
Triglyceride	TG
Triglyceride Fatty Acid	TGFA
Cholesterol Ester	CE
Phospholipid	PL
Metabolic Syndrome	MetS
Blood Urea Nitrogen	BUN
Nuclear Factor Like 2	Nrf2
Kelch-like ECH-associated protein 1	Keap 1
Antioxidant Response Element	ARE
Glutamate-Cysteine Ligase	GCL
NAD(P)H:Quinone Oxidoreductase 1	NQO1
Forkhead Transcription Factor 3a	FOXO3a
Glutathione	GSH
Manganese Superoxide Dismutase	MnSOD

List of Abbreviations

Prostaglandin F Family	PGF
Insulin Receptor Substrate	IRS
Phosphoinositide 3-kinase	PI3K
Phosphoinositol 4,5-bisphosphate	PIP ₂
Phosphoinositol 3,4,5-trisphosphate	PIP ₃
Protein Kinase B	PKB/Akt
Mammalian target of rapamycin complex 2	MTORC2
Enzymes that hydrolyze guanosine triphosphate	GTPase
Glucose transporter	GLUT
Soluble NSF Attachment Protein Receptor	SNARE
Plasma Cell Differentiation Factor 1	PC-1
Type 2 Diabetes mellitus	T2DM
Slow Twitch	ST
Fast Twitch	FT
Tumor Necrosis Factor alpha	TNF- α
(6-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-6-Deoxyglucose)	6-NBDG

Fatty Acid Nomenclature

Fatty Acid Nomenclature	
Common Name	Numeric
Palmitic acid	(16:0)
Stearic acid (DMA)*	(18:0 DMA)
Stearic acid	(18:0)
Arachidic acid	(20:0)
Behenic acid	(22:0)
Lignoceric acid	(24:0)
Palmitoleic acid	(16:1n7)
Oleic acid	(18:1n9)
Gondoic acid	(20:1n9)
Eruric acid	(22:1n9)
Nervonic acid	(24:1n9)
Linoleic acid	(18:2n6)
Dihomo- γ -Linolenic acid (DGLA)	(20:3n6)
Arachidonic acid (ARA)	(20:4n6)
Adrenic acid	(22:4n6)
Osbond acid	(22:5n6)
Eicosadienoic acid	(20:2n6)
α -Linolenic acid	(18:3n3)
Eicosapentaenoic acid (EPA)	(20:5n3)
Clupanodonic acid	(22:5n3)
Docosahexaenoic acid (DHA)	(22:6n3)

*DMA stands for Dimethylacetyl

Chapter 1: Introduction

Overview

In the last 50 years the United States has seen a dramatic rise in obesity. In the early 1960's the prevalence of obesity among the US population was ~10%, however by 2010 this percentage increased up to ~36% indicating that greater than one-third of the US adult population had become obese (44-46). Obesity is often viewed from the perspective of energy imbalance manifesting when intake of calories exceeds the amount of calories being expended. But calories are not treated equally metabolically. The level of carbohydrate consumption can strongly direct processes such as oxidative disposal, de novo lipogenesis and adipose storage. Therefore the emphasis should be placed on the macronutrient composition of the diet (as opposed to energy balance) and how the ratio of carbohydrate to fat drives factors in the etiology of obesity and insulin resistance.

The greatest increase in the obesity epidemic occurred during the period of 1976 to 1999. Tracking of patterns and trends in food portion sizes during this time interval indicated a dramatic increase in food portion sizes, particularly in meals consumed at home and in fast food restaurants (115). The growth in portion sizes increased overall caloric intake from 1791 kcal/d in the late 1970's to 1985 kcal/d in the mid 1990's with the most dramatic portion of this increase occurring during the early to mid-90's (115). The increase in calories was derived from carbohydrate with little change in protein and fat (183). This net increase of 194 kcal/d, principally from carbohydrate, equates to an increase of ~20 lbs of body weight a year. During this same time interval there was also a dramatic increase in the consumption of high fructose corn syrup (HFCS) and during the early to mid-90's was the greatest rise in total sweetener consumption (refined sugar, HFCS and others) (65). Chronic hypercaloric consumption does not simply lead to increases in body mass, but also leads to inflammation, metabolic and cardiovascular disorders. Obese individuals can display elevated systolic and diastolic blood pressure,

elevated triglycerides, insulin, HOMA-IR, CRP and MCP-1 (87, 166, 169). As the obesity epidemic spread and a growing number of metabolic and cardiovascular risk factors started to become associated with it, a new classification known as Metabolic Syndrome (MetS) was developed. MetS is a cluster of metabolic and cardiovascular risk factors that can lead to cardiovascular disease and diabetes (62). These risk factors are specifically defined as abdominal obesity, hypertension, dyslipidemia and hyperglycemia which relate to the physiological measures of waist circumference, blood pressure, triglycerides, HDL-C and fasting plasma glucose, which are all linked with dietary carbohydrate intake ((173)). Data compiled from NHANES indicated that 56% of US adults had a waist circumference that would qualify them for MetS in 2009-2010 (127). The underlying causes of MetS are thought to be abdominal obesity and insulin resistance (62). Proper treatment of MetS to reverse the health risks and mortality associated with it (including obesity) require interventions aimed at reducing excess body mass and improving insulin sensitivity, which should include attention toward an appropriate level of dietary carbohydrate.

In comparison to a low-fat, high carbohydrate diet, a low carbohydrate, high fat diet leads to greater decreases in body weight, fasting glucose, insulin, HOMA-IR, triglycerides and improves HDL-C (174). A low carbohydrate diet also leads to greater improvements in circulating markers of inflammation such as TNF-alpha, IL-8, MCP-1, I-CAM and E-Selectin (49). Although a low fat, high carbohydrate diet can decrease body weight and improve some markers of inflammation, the overall benefits of a low carbohydrate diet in regards to the risk factors associated with MetS such as abdominal obesity, triglycerides, HDL-C, plasma glucose, insulin sensitivity and inflammation are greater. Although some of the mechanisms behind the greater weight loss with a low carbohydrate diet are understood (102), how this diet improves insulin sensitivity, one of the underlying causes of MetS, has been less studied.

Although the insulin signaling cascade has been well characterized, insulin resistance is a multifaceted and complex phenomenon. Much of the research has focused on the protein components of the insulin signaling cascade (172), however there is evidence that the

lipid composition of skeletal muscle membranes are highly associated with measures of insulin sensitivity (13, 121). The purpose of this dissertation project is to determine the affect of dietary macronutrient composition on cellular membrane composition, whole body insulin sensitivity and to determine the relationship between cellular lipids and insulin sensitivity.

Study Design

Individuals with MetS (n = 15) underwent 5 months of controlled hypocaloric feeding subdivided into seven, 3-wk diet phases. Subjects started with a guided free-living low carbohydrate phase prior to controlled feeding. Participants were then fed individually catered prepared meals containing progressively increasing quantities of CHO (47g, 82g, 131g, 179g, 250g and 344g of carbohydrate). Blood samples were collected following an overnight (10-12 h) fast at the end of each 3-wk diet phase.

Aims

Aim 1. To demonstrate that variations in carbohydrate intake will alter the composition of plasma lipids. My **working hypothesis** is that lower carbohydrate intake will increase polyunsaturated fats (PUFA), specifically arachidonate and DHA, decrease saturated fats (SFA), specifically laurate, myristate and palmitate, and decrease 16:1n7 which is associated with de novo lipogenesis (DNL), relative to a standard American diet.

Novelty. Although there were 7 diet phases, this dissertation will only focus on the diet phases with the lowest and highest carbohydrate intake. The controlled feeding allows for analysis of the fatty acid composition of the diet and to correlate these changes to those found in the serum across different quantities of carbohydrate feeding.

Aim 2. To demonstrate that the macronutrient composition of the diet will change the lipid composition of buccal cell membranes. Buccal cells can be obtained non-invasively and have demonstrated changes in lipid composition with changes in dietary fat intake (106).

Working Hypothesis #1: Fatty Acid Composition. A low carbohydrate diet will increase arachidonic acid (ARA), a fatty acid highly associated with insulin sensitivity and decrease di-homo-gamma linolenic acid (DGLA), an arachidonic acid precursor associated with insulin resistance.

Working Hypothesis #2: Blood vs Membrane Composition. The change in buccal cell membrane composition of arachidonic acid will mimic the change in plasma arachidonic acid.

Novelty. Buccal plasma membrane composition in individuals with MetS has never been assessed, nor the effects of carbohydrate intake on buccal membrane composition.

Aim 3. To observe correlations between changes in the lipid composition of buccal cell plasma membranes and whole body insulin sensitivity.

Working Hypothesis: The low carbohydrate intake will improve whole body insulin sensitivity. This change will be positively correlated to arachidonic acid and negatively correlated to DGLA as has been previously observed in skeletal muscle.

Novelty. Insulin sensitivity in individuals with MetS has not been previously correlated to the fatty acids of buccal cells, nor has the effect of carbohydrate intake on variations in the relationships between buccal cell fatty acids insulin sensitivity.

Chapter 2: Review of Literature

Introduction

Although obesity and metabolic syndrome have clearly characterized definitions, the mechanisms behind these metabolic disorders are less clear. Obesity is often explained as a mechanism behind insulin resistance and MetS is believed to be caused by abdominal obesity and insulin resistance. However, evidence indicates that obesity, insulin resistance, changes in circulating FFA and TG and even blood pressure (the factors associated with MetS) are all downstream effects of chronic hypercaloric intake. The importance of the macronutrient composition of the diet to the contribution of obesity and other metabolic dysfunctions as well as to the reversal of these metabolic alterations will be investigated below. Some of the known mechanisms of insulin resistance will be delineated and related back to the metabolic alterations induced by overfeeding. Many of these mechanisms are related to the proteins involved in the insulin signaling cascade, but there is evidence that cellular lipids may also impact insulin sensitivity. The concepts of cell membrane composition and organization and their relationship to insulin sensitivity will also be discussed as well as the efficacy of leukocytes as a model system for the study of insulin resistance at the level of the cell. A summary of these findings will be used to explain the rationale behind the paradigm of this project.

Definition of Obesity and Metabolic Syndrome

Obesity is defined as a body mass index of 30 or greater (21). MetS is defined by a cluster of clinical risk factors that predispose individuals to cardiovascular disease (CVD), type II diabetes mellitus (T2DM) and all-cause mortality (61, 127). These risk factors are commonly known as abdominal obesity, hypertension, dyslipidemia and hyperglycemia which are clinically defined as elevated waist circumference (≥ 102 cm for men and ≥ 88 cm for women), elevated blood pressure (systolic BP ≥ 130 mm Hg,

diastolic BP ≥ 85 mm Hg), elevated triglycerides (TG ≥ 150 mg/dL), low HDL-C (< 40 mg/dL for men, < 50 mg/dL for women) and elevated fasting plasma glucose (≥ 110 mg/dL) (61). The cause of both of these metabolic disorders is hypercaloric intake. The metabolic perturbations associated with hypercaloric intake are dependent on the macronutrient composition of the diet. Evidence to support the claim that hypercaloric intake precedes obesity, MetS, insulin resistance and the associated pro-inflammatory state comes from investigations of short-term overfeeding.

Overfeeding/Hypercaloric Intake

Dissociation of Obesity and Insulin Resistance

In the US, increased insulin resistance is associated with excess body mass, typically the result of chronic hypercaloric intake (59). Two studies observed the effects of a mixed hypercaloric diet with an increase in body mass index within the normal range (BMI < 25). Both studies demonstrated increases in fasting insulin concentrations and an increase in the HOMA-IR (implying a decrease in insulin sensitivity) (14, 41).

Furthermore during an OGTT there was an increase in the AUC of insulin explained by a decrease in the rate of insulin clearance and an increase in insulin secretion (14, 41).

Similar increases in the AUC of insulin as a result of a decrease in insulin clearance and an increase in insulin secretion were observed after a test meal (260 kcal, 62% CHO, 32% FAT and 6% PRO) (41). Although these studies did observe statistically significant increases in body weight, these changes were within a normal BMI demonstrating the dissociation of decreased insulin sensitivity with obesity.

Dissociation of Inflammation and Insulin Resistance

Insulin resistance can also be dissociated from inflammation. Individuals fed a mixed hypercaloric diet for 3 days showed a weight gain of ~ 2.7 kg (< 1.0 kg/m²) and demonstrated increased fasting glucose and insulin with decreased insulin sensitivity (by hyperinsulinemic-euglycemic clamp) (160). Although a statistically significant increase in CRP was observed the post-hypercaloric feeding values were still within the normal clinical range. MCP-1 showed a statistically significant increase, but there were no changes in sICAM-1, the number of circulating leukocytes, leukocyte activation markers,

the number of subcutaneous adipose tissue macrophages or the M1/M2 macrophage ratio in subcutaneous adipose tissue. These results indicate that there were decreases in insulin sensitivity without significant increases in inflammation or weight gain. Together these studies demonstrate the dissociation of obesity and inflammation from increases in insulin resistance.

Macronutrient Composition and Metabolic Complications

The preceding paragraphs demonstrated that hypercaloric intake, prior to the onset of obesity and inflammation, begins the process of insulin resistance. However, it is the macronutrient composition of the hypercaloric diet that dictates the metabolic disturbances attributed to short-term overfeeding. Mixed hypercaloric diets by majority tend to demonstrate increases in fasting insulin and decreases in insulin sensitivity (14, 41, 120, 160). Hypercaloric high carbohydrate diets demonstrate increases in fasting insulin and triglycerides with decreases in serum FFA(4, 26, 36, 73, 154, 180). Hypercaloric high fat diets tend to show no change in fasting glucose, or fasting insulin, a decrease in serum FFA and decreases in triglycerides (4, 17, 36, 73, 154). Hypercaloric diets only show significant increases in body weight with interventions lasting greater than 1 week, after which they all demonstrate increases in body weight (4, 14, 26, 36, 41, 73). The metabolic perturbations associated with each diet are the physiological outcomes of the metabolic machinery attempting to adapt to hypercaloric intake. The etiology of the underlying mechanisms can explain the observed perturbations when CHO or fat are overfed.

Management of Excess CHO and Fat

Humans typically store approximately 500g (2000 kcal) of CHO as glycogen distributed in a 4:1 ratio between muscle and the liver (104). In contrast adipose tissue and skeletal muscle can store greater than 6 kg (60,000 kcal) of triglycerides, the storage form of fat (104). The capacity of the human body to store fat is much greater than its ability to store CHO. Excess fat can be managed through storage or oxidation, but limitations in CHO storage requires greater metabolic flexibility to manage excess CHO intake. Possible pathways to manage excess CHO are storage as glycogen, oxidation, conversion to fatty

acids (de novo lipogenesis, DNL) and fecal excretion. These pathways are interdependent.

Macronutrient Metabolism

CHO and fat overfeeding have very different effects on macronutrient oxidation and energy expenditure. Hypercaloric high CHO diets (HHCD) show increased CHO oxidation, decreased fat oxidation and an overall increase in total energy expenditure (TEE) (1, 3, 26, 73, 105, 141). Hypercaloric high fat diets (HHFD) tend to either not alter fat oxidation or increase it, while not changing CHO oxidation or TEE (73, 105, 141). These differences in energy metabolism occur due to the limitations of CHO storage. At the onset of a HHCD, disposal of excess CHO is dispersed between increased CHO oxidation and storage, with glucose carbons stored as either glycogen or fat through DNL. The greatest increase in glycogen storage takes place at the onset of the HHCD and although there are increases in both whole body and hepatic DNL, there is no net DNL (fat synthesis > fat oxidation) at this early stage (1, 3). Similarly although CHO oxidation is significantly increased, the increase in CHO oxidation is not yet enough to significantly increase REE. However, by the fourth day of an HHCD the RQ > 1.00 indicative of net DNL, CHO oxidation has increased to the point of significantly increasing REE and glycogen stores have stabilized, having increased to a total of ~800-1,000g (1, 3). Beyond this point CHO can no longer be stored as glycogen leading to either greater increases in CHO storage as fat through DNL or oxidation. Fecal excretion was assessed 21 days after either an HHCD or HHFD and showed that the HHCD in comparison to the HHFD had a 44% increase in dry weight, a 30% increase in loss of energy and greater losses in CHO and PRO (91). Since fecal excretions were only measured on a single day during the hypercaloric diets, the time course of these changes cannot be discerned. This exact time course of events may not be true for all HHCD and likely depends on the quantity of excess calories and the percentage of CHO in diet. However it does delineate that excess CHO are first stored as glycogen prior to major increases in both CHO oxidation and net DNL.

De Novo Lipogenesis

De novo lipogenesis is the synthesis of fatty acids from glucose derived carbons. Glucose is converted to acetate via glycolysis and subsequently synthesized into fatty acids via fatty acid synthase. Under eucaloric conditions whether high fat or high CHO, there is no net DNL and the absolute quantity of lipid produced was < 10g/dy (68, 76, 77).

Overfeeding shows dichotomous results. Five days of overfeeding fat (~1523 kcal or 50% EE extra) had no effect on hepatic DNL, but CHO overfeeding (~1827 kcal, 50% EE extra) increased hepatic DNL to a rate of < 5g/d with no net DNL (68, 141). In contrast, CHO overfeeding for 7 days (incremental increases in total kcal from 3600 to 5000) increased net whole body DNL producing 142 g/d (3). Another study which overfed CHO for 4 days (2.5x TEE total) demonstrated up to 170g/d of DNL (1). A third study which overfed CHO (~1816 kcal extra in lean subjects) for 4 days showed a significant increase in net DNL of ~28 g/d (109). The discrepancies in the quantity of DNL in the overfeeding literature likely arise from the percentage of CHO in the diet and the composition of CHO. Studies which observed an increase in net DNL, derived 75% or more of total calories from CHO, while the single study which did not show net DNL fed 70% of calories from CHO. The ratio of simple:complex CHO was not disclosed in most of these studies but simple CHO have a much stronger affect on DNL than complex CHO (76, 78). Although the quantity of DNL with CHO overfeeding is currently irreconcilable, its' increase is still important as it indicates an important shift in metabolism.

Fate of Glucose Carbons

DNL indicates the conversion of glucose to fatty acids, but under normal dietary conditions in healthy adults, the majority of glucose converted to lipid is in the form of glycerol. Glycerol is synthesized from glucose through entry into the glycolytic pathway until dihydroxyacetone phosphate (DHAP) is generated. DHAP is converted to glycerol by the subsequent actions of glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase. This process is referred to as the Kennedy pathway. Evidence that the majority of glucose carbons are converted to glycerol and not fatty acids under normal conditions has been demonstrated in vitro in adipose tissue. In vitro cultures of human

abdominal adipose tissue biopsied from healthy or non-diabetic subjects showed between 72-99% of labeled glucose was incorporated into glycerol and only about 1-28% was converted to fatty acids (53, 57, 60, 145, 146). In vivo evidence that glycerol is the dominant lipid generated by glucose is derived from studies that infuse labeled ^{14}C -glucose. In lean healthy individuals on a normal diet, greater than 90% of labeled glucose carbons were located in the glycerol moiety of isolated VLDL-TG with less than 6% in the VLDL-TGFA (8, 10). However, consumption of a HHCD alters the allocation of glucose carbons between glycerol and fatty acids. Two days of an HHCD decreased the appearance of labeled glucose carbons in VLDL-glycerol to ~28% and increased the quantity of labeled glucose carbons to ~72% in VLDL-TGFA (8). A potential mechanism for this switch between glycerol and fatty acid production might be the increase in plasma insulin associated with HHCD. The promoter region of the fatty acid synthase gene has an insulin response element (177). Adipose tissue segments from human biopsies incubated with varying concentrations of insulin and radiolabeled glucose demonstrated that the proportion of labeled glucose carbons increased in the fatty acid fraction (60). Notably, the increase in labeled glucose carbons in fatty acids occurred at physiological levels of insulin (60). Even if an HHCD does not increase DNL above lipid oxidation, it still has an important physiological role in explaining the modifications to lipid metabolism observed in obesity and MetS.

Effects of DNL on Serum Lipid Composition

Induction of DNL by HHCD plays an important role in regulating the plasma concentrations of FFA and TG as well as the lipid composition of serum lipids. Consumption of an HHCD for 4 days decreased total plasma fatty acids, but increased the concentrations of palmitate and palmitoleate and decreased the concentrations of oleate and linoleate (2). The decrease in total plasma FFA can be explained by a decrease in the net transport rate of FFA (lipolysis) while the clearance rate is increased (9). These changes in FFA cycling are observed in both HHCD and isocaloric high CHO diets (9). The increased rate of clearance may be due to an increased rate of re-esterification of plasma lipids. Hypercaloric intake of ~2500 kcal showed greater rates of re-esterification

than ~500 kcal of hypercaloric intake (67). It is plausible that the increased rate of re-esterification that contributes to decreased plasma FFA may also contribute to changes in plasma TG.

After 4 days of a HHCD total VLDL-TGFA increased nearly 10-fold and although all VLDL-TGFA increased, palmitate and palmitoleate increased to the greatest extent (12 and 20-fold respectively) (2). The increase in VLDL-TGFA was caused by an increase in the secretion rate of both de novo VLDL-TGFA and preformed VLDL-TGFA (those formed from sources other than DNL such as from plasma FFA) (2). Preformed VLDL-TGFA increased ~2 fold (212 ± 52 to 566 ± 157 $\mu\text{mol/kg/d}$) but de novo VLDL-TGFA increased ~45-fold (3.2 ± 1.1 to 150 ± 56 $\mu\text{mol/kg/d}$) (1). Therefore the increase in DNL on an HHCD dramatically increased the proportion of fatty acids produced by DNL in the blood indicating that de novo synthesis plays a controlling factor in serum lipid composition. DNL predominantly generates palmitate and through subsequent enzymatic actions of desaturation and elongation can generate palmitoleate, stearate and oleate (2). The incorporation of fatty acids into triglycerides is dependent upon activation of the fatty acid by the addition of acetyl-CoA (as demonstrated in adipose tissue) (53, 145). Palmitate, stearate and oleate show the greatest activation rates for incorporation into TG (palmitoleate was not assessed) (53, 145). A combination of increased de novo production and their greater propensity for activation and incorporation into triglycerides explains why these fatty acids along with palmitoleate and linoleate comprise greater than 97% of VLDL-TGFA (2).

The shift in glucose carbons from producing glycerol to fatty acids, while total triglycerides are increasing implies another source of glycerol production other than glucose. Glyceroneogenesis is the production of glycerol from non-carbohydrate sources. Although evidence for the effects of CHO on glyceroneogenesis are mixed, there are indications that a HHCD may increase glyceroneogenesis (118). As previously mentioned a HHCD induced an increase in the PRO content of fecal material, however nitrogen balance was retained by a decrease in urinary nitrogen excretion (91). Other investigations of HHCD also showed a decrease in urinary nitrogen excretion and BUN

(3, 179). Furthermore HHCD has been shown to increase the plasma concentrations of alanine, leucine, isoleucine, and valine while decreasing serine, threonine and asparagine, which with the exception of leucine, are all gluconeogenic amino acids (179). While the concentrations of amino acids with ketogenic potential such as lysine, tyrosine and phenylalanine showed no change in their concentrations (179). Given that the amino acids with the greatest change in concentration are gluconeogenic non-carbohydrate sources of carbon for glyceroneogenesis, there is a possibility that this alternate pathway to glycerol production contributes to the increase in serum triglycerides. In summary an HHCD increases DNL, decreases FFA, increases VLDL-TGFA and can modify the lipid composition of serum fatty acids both free and bound.

Changes in Metabolism Result in Altered Serum Lipids

Overfeeding models demonstrate the potential influence that diet has on serum lipid concentrations and composition. However, investigations of obesity and MetS have the potential to validate the precision with which the model fits the actual data. Obese individuals compared to healthy controls show increases in 16:0 and 16:1n7 in the TG fraction, 16:0 and 20:4n6 in the CE fraction and 18:0 in the PL fraction with 20:3n6 and 20:4n6 also tending to show increases in the PL fraction. In individuals with MetS in comparison to healthy controls have increased 16:1n7 and 20:3n6 with decreased 18:2n6 in the PL fraction, increased 16:1n7 and 18:3n6 in the CE fraction and potentially increases in 16:0, 16:1n7, 18:2n6 and 18:1n9 in the TG fraction, although not enough data was accumulated. Individuals with MetS in comparison to obese individuals have increased 16:1n7 and decreased 18:2n6 in the PL fraction, increased 16:1n7 in the CE fraction with no differences in the TG fraction, but again not enough data was accumulated on the TG fraction. These data on obesity and metabolic syndrome indicate that there are significant changes in lipid composition that mirror the model of overfeeding studies lending credence to the idea that hypercaloric intake can influence lipid composition and plays a role in metabolic alterations present in obesity and MetS.

Summary

In summary, the macronutrient composition of the diet is a major determinant in the progression towards insulin resistance. Short-term overfeeding studies indicate that diets high in carbohydrate lead to the onset of insulin resistance prior to significant weight gain that would classify an individual as obese. Therefore diets high in carbohydrate lead to signs of insulin resistance prior to the onset of other factors commonly associated with the progression towards MetS such as obesity and inflammation. Furthermore, modifications to lipid and CHO metabolism during HHCD demonstrate effects on serum lipid composition which mirror changes observed in individuals with obesity and MetS.

Eucaloric/Hypocaloric Intake

The Macronutrient Composition of Hypocaloric Diets Affects Metabolic Parameters

The combination of caloric excess and a high percentage of CHO lead to greater metabolic perturbations and signs of dysfunction than a diet with a high percentage of fat and similar caloric excess. Similarly, with hypocaloric diets, low carbohydrate, high fat diets (LCD) show greater improvements in metabolic parameters than low fat, high carbohydrate diets (LFD). Subjects with atherogenic dyslipidemia placed on either a hypocaloric LCD or LFD for 12 weeks showed decreases in total body mass, BMI, fat mass, percent body fat, abdominal fat, fasting insulin, HOMA and leptin, but only the LCD decreased fasting glucose (12%) (174). In comparison to the LFD, the LCD showed greater decreases in body mass (2x LFD), BMI, fat mass, abdominal fat, insulin (-49 v -17%), HOMA (-55 v -18%) and leptin (-42 v -18%) (174). LCD also shows greater improvements in triglycerides (-51 v -19%), HDL-C (+13 v -1%) and the ApoB/Apo A-1 ratio (-16 v +8%) (174). Regardless of a 3x greater intake of SFA on the LCD there was a greater decrease in TG-SFA on the LCD (-57 v -24%) (174). The LCD in contrast to the LFD showed a 31% decrease in palmitoleic acid which can be used as a marker of DNL due to its low concentration in both the diet and blood (2, 174). The mean LDL particle size was also increased promoting the less atherogenic pattern A LDL phenotype (174). An improved LDL phenotype has been commonly observed with diets

high in fat (38, 89). Both diets led to similar decreases in C- reactive protein (-23%), VEGF (-21%), P-Selectin (-21%), and V-CAM (-6%), but the LCD showed greater decreases in TNF- α (-32% v -12%), IL-8 (-33 v -4%), MCP-1 (-24 v -5%), E-Selectin (-34 v -14%) and I-CAM (-17 v -3%) (49). Overall a hypocaloric LCD improves body mass, body composition, glycaemia, plasma lipids and inflammation to a greater extent than a hypocaloric LFD.

Effects of Dietary Lipids on Serum Lipid Composition

There is not a direct relationship between fatty acid consumption and its concentration in the blood. Saturated fatty acids (SFA) are a prime example. LCD with 2-3x the dietary intake of SFA as comparative standard diets and LFDs respectively showed decreased SFA in the TG and CE fractions of serum lipids (48, 49). In contrast the n3 polyunsaturated fatty acids (PUFA), EPA and DHA, show increased plasma concentrations with increased dietary consumption. A eucaloric LCD enriched in MUFA and n3 PUFA demonstrated increased EPA and DHA in both the phospholipid and cholesterol ester fractions of serum lipids (48). Supplementation with EPA and DHA increases these PUFA in blood lipids (19, 34, 86, 161, 182). The effect of increased arachidonic acid (ARA) in the diet is not as straightforward as the n3 PUFA. Supplementation with ARA increases its concentration in the phospholipid and triglyceride fractions of blood lipids (56, 84, 90, 161). In two eucaloric LCD, one enriched in unsaturated fats (UFA) and other enriched in saturated fats with equal amounts of ARA in the diet, ARA increased in the TG, PL and CE fractions of blood lipids in the SFA diet, but showed no changes during the UFA diet (48). In addition, the UFA enriched diet showed decreased DGLA in the PL and CE fractions with an increase in DGLA in the TG fraction (48). Similar observations have been made with EPA and DHA supplementation, where concentrations of ARA in blood lipids are usually unaltered but ARA precursors such as DGLA and LA can show decreases in the PL fraction (19, 34, 86, 161, 182). These studies indicate that changes in ARA in blood lipids depends on the consumption of other lipids in the diet.

Consistent Changes in Serum Lipids Observed with a Hypocaloric LCD

In a hypocaloric LCD model there appears to be consistent changes in serum lipids. ARA increases while palmitate and myristate decrease resulting in decreased SFA and increased PUFA in the PL and CE fractions (49, 129, 174). The increase in AA has been attributed to a decrease in lipid peroxidation as demonstrated by a decrease in urinary 8-iso PGF_{2α} (48). Animal models utilizing ketogenic diets show a reduced production of ROS (83, 88, 157). Another observed change on a LCD is a decrease in 16:1n7 in all three serum lipid fractions, likely reflecting decreased DNL (49, 174).

Mechanism of Arachidonate Preservation in a Low Carbohydrate Diet

As previously mentioned the increase in ARA is facilitated by a decrease in its oxidative degradation. Two potential pathways exist which can mediate the reduction in reactive oxygen species (ROS) production. The first is the Nrf2 pathway. Nrf2 is sequestered in the cytosol by Keap-1 (82). Keap-1 can be stimulated to release Nrf2 by oxidation of Cys151 and phosphorylation of Ser40 by PKC (116). Once Nrf2 is released, it translocates to the nucleus where it can bind the antioxidant response element (ARE). The ARE is a promoter for at least 2 endogenous antioxidants: the GCL which is responsible for GSH synthesis and NQO1 which regulates ubiquinones (111, 170, 171, 181). In rats fed ketogenic diets Nrf2 nuclear translocation was increased as well as the activity of NQO1 and GCL and GSH concentrations (83, 108). The second pathway is via FOXO3a. Low concentrations of β-hydroxybutyrate (1-2 mM) can lead to inhibition of HDAC 1 increasing histone acetylation and leading to the increased expression of genes downstream of the transcription factor FOXO3a (144). Protein products downstream of FOXO3a, MnSOD and catalase, were elevated in kidney tissue isolated from β-hydroxybutyrate treated rats (144). It therefore appears that β-hydroxybutyrate can reduce oxidative stress by increasing histone acetylation near genes downstream of FOXO3a, allowing for this transcription factor to bind to its now available promoter leading to the transcription and translation of MnSOD and catalase. Another gene regulated by FOXO3a, metallothionein 2, was also upregulated and may contribute to reductions in oxidative stress through metal ion sequestration (Fe, Cu) and quenching of free radicals such as superoxide and the hydroxyl radical (138). In comparison, individuals with MetS,

show increased oxidative stress, decreased antioxidant status and increased urinary 8-epi-PGF_{2α} (47, 52, 152, 166, 169).

The reduction in ROS generation is not the only mechanism for decreased lipid peroxidation. Small, dense LDL (sdLDL) are increased in individuals with MetS (79). SdLDL are more susceptible to peroxidation than larger, more dense LDL (31, 163). This seems to be due to a greater quantity of PUFA in the sdLDL and a lower amount of free cholesterol (31, 163). PUFA are the most readily peroxidizable lipids and free cholesterol seems to protect lipids from oxidation potentially by decreasing the permeability of the phospholipid monolayer to superoxide (163). LCDs have been shown to change the LDL particle distribution toward the less atherogenic pattern A by increasing mean LDL particle size (38, 89, 174). A LCD therefore leads to lower lipid peroxidation by increasing LDL particle size, decreasing the susceptibility of the LDL to peroxidation, and decreasing ROS generation.

Summary

Hypocaloric LCD improve metabolic parameters to a greater degree than a LFD. These improvements include, body mass, body composition, blood lipids and inflammation. Hypocaloric LCD also show consistent changes in blood lipid composition such as increased ARA and decreased SFA and palmitoleic acid. The increase in ARA is facilitated by a decrease in oxidative stress with consumption of a LCD. Overall, LCD have greater metabolic and health benefits on the risk factors associated with MetS than a comparative LFD.

Insulin Resistance

Insulin Signaling Pathway

The insulin receptor is a heterotetramer consisting of two ligand binding alpha subunits on the external membrane surface and two beta subunits inside the plasma membrane

which have tyrosine kinase activity (172). The insulin signaling cascade initiates with the binding of insulin to the alpha subunit of the insulin receptor. This leads to induction of the tyrosine kinase capabilities of the beta subunits leading to autophosphorylation. The insulin receptor substrate (IRS) temporarily binds to the insulin receptor leading to phosphorylation of several of its tyrosine residues. Once phosphorylated the IRS can now be bound by SH2 domain containing proteins (172). Phosphoinositide 3-kinase (PI3K) consists of two subunits, the p85 α regulatory subunit with an SH2 domain and the p110 subunit with catalytic activity. PI3K binds to the IRS docking protein through its p85 α subunit which leads to the activation of the p110 subunit. The p110 subunit then converts the plasma membrane lipid phosphoinositol 4,5-bisphosphate (PIP₂) to phosphoinositol 3,4,5-trisphosphate (PIP₃). Akt/PKB (protein kinase B) contains a PH domain (pleckstrin homology) which binds the newly created PIP₃ anchoring it to the plasma membrane (112, 172). The now anchored PKB can be activated by PDK1 (phosphoinositide-dependent protein kinase-1) which also co-localizes to PIP₃ by its own PH domain and mTORC2 (mammalian target of rapamycin complex 2) on threonine-308 and serine 473 respectively (112). PKB then acts on several small GTPases which induce the translocation of GLUT4 containing vesicles and SNARE regulatory proteins which mediate GLUT4 vesicle fusion to the plasma membrane (94).

Perturbations in Insulin Signaling

Modifications to proteins in the insulin signaling cascade have been a focus of research into the mechanisms of insulin resistance. These modifications are diverse, ranging from transcriptional regulation to post-translational modifications resulting in altered functionality or decreased protein content. Many parts of the insulin signaling cascade are affected from the insulin receptor to GLUT4.

Initiation of the insulin signaling cascade begins with the insulin receptor itself (insulin is the effector of this pathway). Studies of leukocytes from obese individuals stratified based on the level of obesity indicate increasing pathologies with increased obesity.

Individuals with a BMI under 40 show decreased numbers of both total and high-affinity insulin receptors but with a compensatory increase in receptor affinity and insulin secretion, allowing for the maintenance of insulin binding (12). Obese individuals with a BMI above 40 show decreased numbers of both total and high-affinity insulin receptors with a concomitant decrease in insulin receptor affinity and insulin secretion (12). These studies indicate a reduction in insulin receptor protein content associated with the level of obesity.

Plasma cell differentiation factor-1 (PC-1) is an inhibitor of tyrosine kinase activity (101). Fibroblasts from diabetic individuals have increased PC-1 protein expression and overexpression in cell culture decreases the tyrosine kinase activity of the insulin receptor (101). In non-obese, non-diabetic individuals, PC-1 content in both skeletal muscle and adipose tissue is negatively correlated with insulin sensitivity as assessed by the intravenous insulin tolerance test (50, 51). PC-1 also has a soluble form (sPC-1) in the plasma with similar molecular weight and enzyme activity to tissue bound PC-1 (Frittita 1999). sPC-1 is inversely related to skeletal muscle PC-1 content and its concentration in plasma negatively correlates with the waist/hip ratio and mean blood pressure (Frittita 1999). These studies indicate that PC-1 mediated inhibition of insulin receptor tyrosine kinase activity may play a role in insulin resistance in both skeletal muscle and adipose tissue.

There are over 50 serine/threonine residues on IRS-1 and IRS-2 (28). Phosphorylation of these residues leads to either reduced tyrosine phosphorylation of the IRS by the insulin receptor or to degradation of the IRS, both leading to decreased insulin mediated action (58, 123). The reduction in insulin receptor induced tyrosine phosphorylation of IRS is caused by reduced binding of IRS to the juxtamembrane region of the insulin receptor, thereby reducing the association of IRS with the insulin receptor resulting in reduced tyrosine phosphorylation of IRS (123). The specific residue that is phosphorylated dictates the fate of the IRS protein, with different serine kinases having varying affinities for each residue (58, 97, 100, 168). Many serine residues can be phosphorylated at one time, thereby affording intricate regulation to the activity of the IRS (100, 168).

Further downstream of the insulin receptor lies glucose transporter 4 (GLUT4), which is responsible for insulin mediated glucose uptake. GLUT4 receptor density is greater on slow twitch (ST) muscle fibers than fast twitch (FT) muscle fibers in lean healthy subjects (55). Compared to lean control counterparts, obese and obese individuals with T2DM demonstrated decreased GLUT4 protein expression in ST muscle fibers of 20% and 40% respectively (55). Other studies found no difference in GLUT4 expression of skeletal muscle from obese or insulin resistant individuals because they homogenized the muscle tissue prior to analysis instead of analyzing in tact cells by fiber type (54, 124). Obese individuals with T2DM have lower GLUT4 protein density in ST fibers than FT fibers in contrast to lean and obese non-insulin resistant counterparts (55). In addition, obesity and insulin resistance alter the ST/FT ratio (55). These modifications in GLUT4 expression likely explain the dichotomy observed in studies that homogenize muscle tissue.

Subcutaneous adipocytes from T2DM individuals have decreased IRS-1 protein expression and unchanged IRS-2 protein expression, leading to an increased role for IRS-2 as the substrate for tyrosine phosphorylation by the insulin receptor (136). However, IRS-2 required a greater insulin concentration than IRS-1 to bind and stimulate PI3K activity (136). Although serine phosphorylation of IRS-1 can lead to degradation of IRS-1 (58), modification of IRS-1 gene expression may also modulate IRS-1 protein levels. Adipose tissue from non-obese, non-diabetic subjects showed decreased IRS-1 protein expression predominantly in subjects with a family history of T2DM, but was present in subjects without any genetic pre-determinants as well (20). Subjects with low IRS-1 protein expression, reduced by 65% in comparison to controls, had significantly greater adipocyte cell size and a waist/hip ratio indicative of increased abdominal fat. These low IRS-1 individuals also had low GLUT4 protein expression and low mRNA expression of both IRS-1 (~50%) and GLUT4 (~60%) (20).

Decrements in IRS-1 protein and GLUT4 expression have been associated with obesity as explained above and increased adipocyte cell size associated with obesity has been

shown to increase circulating levels of the cytokine TNF- α (63). Exposure of 3T3-L1 adipocytes to TNF- α for 96h showed decreased protein expression of both IRS-1 and GLUT4 by 80% with minimal decreases to insulin receptor protein expression (155). However, the remaining protein content functions normally. The insulin receptor still autophosphorylated and GLUT4 still translocated from intracellular vesicles to the plasma membrane indicating that multiple simultaneous modifications to the insulin signaling pathway likely occur to result in the insulin resistant phenotype observed at the organismal level (155).

There are a multitude of proteins modified in the insulin signaling cascade with reduced insulin sensitivity all the way to insulin resistance. The interactions of these many protein modifications lead to a diverse set of mechanisms behind insulin resistance. However, regardless of the complexity of these modifications, perturbations to insulin signaling are not restricted to protein modifications. There is evidence that cellular lipids also play an important role in the regulation of insulin sensitivity.

The most commonly studied impact of cellular lipids on insulin resistance is through intracellular lipid accumulation (5, 7, 15, 16, 30, 110, 114, 151, 158, 162, 167, 178). Several studies indicate that intramyocellular lipids (IMCL) are elevated in obese, obese diabetic and diabetic individuals often without decrements in mitochondrial oxidative capacity (5, 7, 15, 16, 30, 110, 151, 158, 162, 178). However, it is not the accumulation of intracellular lipids that is of the utmost importance but the location of these lipids. Endurance trained athletes demonstrate increased IMCL but not insulin resistance (167). In fact endurance trained athletes show greater concentrations of intracellular lipids than overweight and overweight diabetic individuals (167). The major difference between normal healthy sedentary and endurance trained individuals and individuals with diabetes is the location of the IMCL (114). Endurance trained and healthy individuals show greater lipid accumulation around the intermyofibrillar mitochondria while diabetic individuals demonstrate greater localization of intracellular lipids around the subsarcolemmal mitochondria (114). However in T2DM or obese individuals, endurance training shifts the intracellular lipid localization from the subsarcolemmal mitochondria to

the intermyofibrillar mitochondria, and this transition is associated with an improvement in insulin sensitivity (diabetics only) regardless of a lack of change in total IMCL (33, 114). Therefore the localization of intracellular lipid accumulation is more important than the increase in intracellular lipids itself.

There is evidence of an emerging relationship between the lipid composition of the plasma membrane and insulin resistance. In skeletal muscle insulin sensitivity is positively associated with long chain PUFA (C20-22) (13, 121). Further investigation demonstrates that ARA has the strongest positive relationship to insulin sensitivity as assessed by euglycemic clamp of all the long chain PUFA and concomitantly the strongest inverse relationship with fasting insulin (13, 121). In contrast, DGLA, a precursor to ARA, has the strongest positive correlation to fasting insulin and the third strongest negative correlation to insulin sensitivity of all the fatty acids including palmitate, except palmitoleate and oleate (13, 121). The ARA/DGLA ratio had the overall greatest negative correlation to fasting insulin and the overall greatest positive correlation to insulin sensitivity, greater than that of ARA alone and of the percentage of total long chain PUFA (13). ARA also shows the strongest inverse relationships to percent body fat and BMI of any fatty acid while DGLA shows the strongest direct relationship of any fatty acid (121). The relationship of ARA to insulin sensitivity is best explained by the role of ARA in the organization of the plasma membrane.

Plasma Membrane Organization

Development of a Membrane Organization Model

In 1972 Singer and Nicholson proposed their model system for membranes which consisted of a fluid mosaic of alternating proteins and phospholipids which were seemingly unorganized (150). The concept of lipids being organized into domains with a functional impact was proposed not long after in 1982 (85). Lipid rafts were initially proposed in 1997 as lipid domains consisting of sphingolipids and cholesterol as the base of a lipid platform used to promote cell signaling (147). Nine years later in 2006 lipid

rafts were renamed to membrane rafts and officially defined as “small (10–200 nm), heterogeneous, highly dynamic, sterol and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions” (134).

Formation of Lipid Rafts

The formation of lipid rafts is due to lipid-lipid, lipid-protein and protein-protein interactions. Lipid-lipid interactions focus on the relationships between sphingolipids, cholesterol and other lipids. Sphingolipids have special properties which aid in both microdomain formation and tight packing with cholesterol. The sphingosine headgroup of sphingolipids contains an 18 carbon amino alcohol with a single unsaturation just beneath the head group (149). Sphingolipids contain only a single fatty acid moiety which is usually a saturated fatty acid with 16-24 carbons. The lack of unsaturated carbon bonds allows cholesterol to pack closely to sphingolipids. The amide linkage in sphingolipids may, although not finitely proven, bind to the 3-OH group of cholesterol enforcing their proximity. Finally, the sphingosine back bone contains an amido and hydroxyl group which both allow for the formation of hydrogen bonds either with water or other lipids, including other sphingolipids (148). The hydrogen bond forming potential not only allows for the bonding to various lipids but also to lateral stability allowing for domain formation. The interaction of cholesterol with other hydrocarbon chains forces them into elongated conformations, leading to thicker membrane regions which may promote segregation based on hydrophobic mismatch (98). The combined properties of sphingolipids and cholesterol lead to the formation of thick, dense and ordered domains (98, 148).

Lipid-protein interactions also play an important role in raft formation. Proteins inserted in the membranes have individual structures and as such are fitted to specific lipids (98). These lipids are often referred to as non-annular lipids and cannot be separated from the protein via detergent extraction (98). The acyl chains of these lipids fill in the grooves of the proteins (70). The specific lipid is determined in part by the protein length or the length of its transmembrane domain if it has one (98). Earlier it was mentioned that lipid

raft domains are dense and thick and so the thickness or length of a protein-lipid assembly may play a part in its allocation to a raft or non-raft fraction. Many membrane proteins also have sphingolipid binding domains which could facilitate their incorporation into lipid rafts (98). Protein-protein interactions facilitate the fusion of smaller lipid raft domains into larger signaling complexes (98). Smaller rafts can contain a signaling protein. When the signaling protein binds its ligand, a multimer can form. The formation of a multimer creates a cross-link between the proteins of two lipid rafts, facilitating coalescence of the lipid rafts.

Lipid Raft Composition

Lipid rafts are bilayer structures (span both the exofacial and cytofacial leaflets of the plasma membrane)(132). In comparison to the bulk plasma membrane, lipid rafts contain twice as much cholesterol, 30% more sphingomyelin, 3-4x increase in PS, decreased PI and PC, the same PE content and are enriched in PE plasmalogen species containing arachidonic acid (132). The greater concentration of PE plasmalogens in rafts leads to a 30% greater concentration of arachidonic acid in the raft fraction than the bulk membrane (132). Lipid rafts can be isolated by detergent or non-detergent methods. When rafts are isolated with cold 1% Triton X-100 mixed in a buffer, homogenized and isolated by flotation on a 5% to 30% linear sucrose density gradient, they are called detergent resistant membranes or DRMs(18). However, the use of different detergents such as NP-40, CHAPS, Brij 98, octylglucoside and Lubrol yield rafts with slightly different lipid compositions (39, 81, 137). Lipid rafts can also be isolated via non-detergent methods such as isotonic sucrose or mechanical lysis, followed by density gradient separation to yield plasma membrane only lipid rafts that closely resemble those found in the live cell (153). The difference in lipid composition between DRMs and lipid rafts is that the detergent method tends to selectively extract the exofacial leaflet lipids and exclude the cytofacial leaflet lipids. This makes the DRMs appear to have a greater concentration of saturated lipids and lower levels of glycerophospholipids such as anionic phospholipids, phosphatidylethanolamine and PE plasmalogens (132). This is because the cytofacial leaflet contains the anionic phospholipids as well as PE while the exofacial leaflet

contains cholesterol, sphingomyelin and glycosphingolipids (such as the gangliosides GM1,-2 and -3) (29, 42, 125, 133).

Lipid Rafts and Insulin Signaling

Tyrosine kinases are frequently found in lipid rafts (133). One tyrosine kinase, the insulin receptor, is constitutively located in caveolae in cells that have caveolae (165). In cells that lack caveolae, insulin receptors are recruited into rafts by insulin (165). Receptors that are constitutively located in or are recruited into lipid rafts are dependent on lipid raft integrity (133). Tyrosine phosphorylation occurs primarily in lipid rafts (99). Cholesterol depletion studies which disrupt lipid raft integrity have shown no change in the ability of insulin to bind to the insulin receptor (122), but show decreases in several downstream events such as IRS-1 phosphorylation (122, 165), activation of PKB/Akt (122), ATP citrate lyase phosphorylation (64), glucose uptake (64, 122) and glucose oxidation (92). There may or may not be a decrease in autophosphorylation of the tyrosine kinase (122, 165). (Cholesterol depletion is carried out using methyl- β -cyclodextrin, filipin or lovastatin.) Also within the tyrosine kinase cascade, at least half of the PIP₃ precursor PIP₂ is located within lipid rafts (72, 131). 1-stearoyl 2-arachidonoyl is the major molecular species of the phosphoinositides (25, 69, 71, 74). The importance of arachidonic acid to phosphoinositides may be that it provides a specific substrate for phosphoinositide modifying proteins including phosphoinositide kinases and phosphatases. It has been shown that phosphoinositide phosphatases show substrate specificity based on the head group and the fatty acid composition of the polyphosphoinositide (139). Also given the diverse actions of the various PI3K IA class, they may have substrate specificity as well. Although phosphatidylinositol, the precursor to the phosphoinositides is more resistant to loss of arachidonic acid than other phospholipids (66, 175), it is possible that decreases in membrane phosphatidylinositol ARA content, could affect the generation of molecular species of phosphoinositides, changing the preferences of its kinases and phosphatases, leading to modified insulin signaling.

Overall Summary

High carbohydrate diets promote insulin resistance, with evidence of the onset of insulin resistance prior to weight gain and inflammation. In an attempt to dispose of the excess CHO consumed, de novo lipogenesis converts glucose carbons to free fatty acids altering the pool of circulating triglycerides by favoring the addition of palmitate, oleate and palmitoleate, potentially modifying other circulating fatty acids in hepatically-derived lipid fractions. Excessive carbohydrate intake also promotes increased inflammation and oxidative stress, potentially altering fatty acid composition through reactive oxygen species-mediated destruction of highly unsaturated fatty acids. This leads to an altered circulating lipid composition, modifying the lipid environment of the cells. The phospholipid and fatty acid composition of the membrane is important, with different fatty acids having varying effects on insulin sensitivity. Arachidonic acid not only has the greatest positive relationship to insulin sensitivity, but it is highest in concentration in lipid rafts, the plasma membrane site of insulin signaling. In contrast, the arachidonic acid precursor, dihomo-gamma linolenic acid (DGLA), shows one of the strongest negative relationships to insulin sensitivity. Therefore manipulation of dietary carbohydrate may modify the circulating lipid composition which may in turn alter insulin sensitivity by affecting membrane fatty acid composition. Low carbohydrate diets have emerged as an effective counter measure to MetS improving the circulating lipid profile, decreasing inflammation, decreasing body mass and improving insulin sensitivity. However, the mechanism(s) by which a low carbohydrate diet induce alterations in insulin sensitivity have not yet been identified. Therefore the purpose of this project is to determine how manipulating the carbohydrate and fat content of the diet affects circulating serum lipids, and how this might alter the plasma membrane composition of cells affecting insulin sensitivity in individuals with metabolic syndrome. In order to accomplish this goal, cheek cells will be used as a cellular model system to study the effects of the diet on membrane fatty acid composition and individuals with

MetS will be enrolled since individuals with this disorder are known to possess varying levels of insulin resistance.

Chapter 3: Methods

Study Participants

Individuals with metabolic syndrome were recruited as participants in this study. Potential subjects were screened for participation based on the criteria for metabolic syndrome which was defined as having three or more of the following: waist circumference (≥ 101.6 cm men, ≥ 88.9 cm women), blood pressure ($\geq 130/85$ mm Hg) or current use of antihypertensive medication, and fasting plasma glucose (≥ 100 mg/dL), triglycerides (≥ 150 mg/dL), and HDL-C (< 40 mg/dL men, < 50 mg/dL women). During the screening process subjects were excluded if they were diagnosed with Type I or II Diabetes, liver, kidney, or other metabolic or endocrine dysfunction or if they were trying to lose weight or had a body mass change of ± 3 kg in the previous 3 months. Fifteen overweight/obese men and women aged 30-66 were enrolled. Their characteristics are summarized in Table 1.1. The participants in this study were primarily sedentary, however those who were physically active were asked to maintain their current training regime (verified by activity logs) while the sedentary individuals were instructed not to engage in a new exercise regimen to limit the effects of physical activity on the dependent variables. The study was approved by the University of Connecticut Institutional Review Board and all participants signed an informed consent form.

Dietary Intervention

Baseline dietary information was obtained via 3-day food record logs and analyzed using nutrient analysis software. The menu/diet composition for each of the six feeding phases ranging in carbohydrate content from 47g to 346g/dy was generated using the same nutrient analysis software (Nutritionist Pro, Axxya Systems, Stafford, Texas). The total calories consumed during each diet phase were individualized based on the baseline resting metabolic rate data and an activity factor (1.2-1.5). Calories were reduced by 300 kcal/d from the determined needs of each subject to allow for gradual weight loss as a

motivation for participation in the ~5 month study. Saturated fat, monounsaturated fat and polyunsaturated fat were kept at 46%, 34% and 20% of total fat content respectively for every phase. Protein was maintained at 1.8 g/kg reference body weight according to the Metropolitan Height-Weight tables. Therefore total calories and protein content were maintained throughout each phase while the total calories derived from fat and carbohydrate were modified.

Dietary Analysis

Since the nutrient analysis software does not contain lipid data for the arachidonic acid content of foods the USDA National Nutrient Database for Standard Reference Release 26 was consulted. However, even this database only provides 20:4 undifferentiated polyunsaturated fatty acid content (20:4n3 and 20:4n6). Food descriptions in the nutrition software were matched as closely as possible to the USDA database to ascertain the content of undifferentiated 20-carbon polyunsaturated fatty acids (20:4UD). The specific arachidonic acid content of whole eggs, one of the primary dietary sources of ARA, was ascertained from an article assessing the accuracy of the lipid content of some commonly consumed foods in comparison to the USDA database (159). This article demonstrated a 60% increase in arachidonic acid content of whole cooked eggs in comparison to the USDA database. The arachidonic acid content of cooked wild Alaskan native sockeye salmon was not listed in the USDA database, so the uncooked value of this fish was used in the ARA calculations. There are plans to have cooked samples of this fish analyzed for lipid composition at a later date. Other animal sources of arachidonic acid could not be compared between the USDA database and the literature given the specificity, preparation and number of food items.

The total amount (in grams) of food items by category in the following categories were tallied: beef, egg, dairy, fish, poultry and pork. Vegetables and nuts were excluded from the analyses as they contain little to no arachidonic acid content. The tallied gram amounts for each food item in each category were used in combination with the 24UD values for the specific food items to determine the total 24UD content for each food category and the total overall 24UD intake for each week in phases I and VI of the diet

intervention study. 24UD consumption per week instead of per phase was determined since participation in each diet phase was not of uniform duration due to scheduling, illnesses or other unforeseen and uncontrollable circumstances. The duration of phase I and phase VI averaged 4.75 and 3.2 weeks respectively.

Fat Mass

Body composition was assessed by dual energy x-ray absorptiometry (DEXA) (Lunar Prodigy, GE Healthcare, Pittsburgh, PA) at the end of each diet phase in the morning after an overnight fast.

Blood and Buccal Cell Collection and Processing

Subjects arrived at the Human Performance Laboratory after a minimum 12hr fast (with the exception of water) and 24hr abstinence from exercise, caffeine, over the counter medications and alcohol. Subjects provided a urine sample prior to blood collection for the assessment of hydration status by urine specific gravity. An acceptable USG was under 1.025 and subjects above this cutoff were provided with water followed by a second USG assessment after a 30 min waiting period.

Blood samples were obtained from a vein in the antecubital fossa or lower arm after subjects sat in a reclined position for 15 min. Whole blood was collected into EDTA, serum and serum separator vacuum tubes using a butterfly needle. Plasma tubes were immediately spun in a benchtop centrifuge chilled to 4°C at 1500 xg for 15 min. Serum tubes were allowed to clot for 15 min at room temperature prior to centrifugation with the same settings as plasma samples. The serum separator tube was sent to a certified medical laboratory (Quest Diagnostics, Wallingford, CT) for assessment of glucose, total cholesterol (TC), HDL-C, TG, and calculated LDL-C concentrations using automated enzymatic procedures (Olympus America Inc., Melville, NY). The plasma and serum samples were aliquoted into cryostorage tubes and stored in an ultra-low freezer for batch analysis.

Subjects were provided with a cup of water and instructed to rinse their mouths for 15 seconds. Subjects were then provided two stiff bristle brushes (MasterAmp Buccal Swab Brush, Epicentre Biotechnologies, Madison WI). The subjects were instructed to rub the brush vigorously against the interior of the cheeks, one brush per cheek, while rotating the

brush head. The brush heads were then detached from the stems and placed in a screw cap cryostorage tube with 1 mL of water. The buccal samples were then stored in an ultra-low temperature freezer for batch analysis.

Analysis of Insulin

Insulin was assayed in duplicate using an ELISA kit (ALPCO, Salem NH). Intra- and inter-assay coefficients of variation (CV) were 5.3 and 7.2% respectively.

Analysis of Insulin Sensitivity

Insulin sensitivity was evaluated by the homeostasis model assessment 2 (HOMA2) (95). HOMA is an index model derived from fasting blood glucose and insulin concentrations (103). Both the insulin resistance (HOMA-IR) and insulin sensitivity (HOMA%S) indices were used and calculated using the downloadable excel sheet with built in macros (164).

Plasma and Buccal Cell Fatty Acid Analysis

Plasma and buccal cell lipids were analyzed as previously described by Lipid Technologies LLC (Austin, MN USA)(49). In summary the procedure used for the analysis was as follows. Lipids were extracted from samples by the method of Bligh-Dryer whereby samples were mixed with chloroform, methanol and water causing partitioning of lipids into the resulting chloroform layer (11). Lipid extracts were dried under a stream of nitrogen gas and resuspended in 50 μ L of chloroform. Lipid classes including but not limited to triglycerides, phospholipids and cholesterol esters were separated on commercial silica gel G plates (AnalTech, Newark, DE, USA). The chromatographic plates were developed in a solvent system consisting of petroleum ether (bp 30-60°C):diethyl ether:acetic acid (80:20:1, by volume). Subsequent to development, the silica gel plates were sprayed with a methanolic solution containing 0.5% 2,7-dichlorofluorescein which was then used to visualize the lipid classes under ultraviolet light. The lipid bands of interest were then scraped into Teflon lined screw cap tubes. The samples were then transesterified using 10% boron trifluoride in methanol (Supelco,

Bellefonte, PA, USA) in an 80°C water bath for 90 min. Resulting fatty acid methyl esters were extracted with water and petroleum ether and stored frozen until analysis by gas chromatography.

The fatty acid methyl ester composition of the lipid classes was analyzed by capillary gas chromatography. Methyl ester samples were dried under nitrogen gas and resuspended in hexane. The fatty acid methyl esters were separated and quantified using a Shimadzu capillary gas chromatograph (GC 17) using a 30 m Restek free fatty acid phase (FFAP) coating and EZChrom software. The instrument temperature was programmed from 190 to 240°C at 7°C/min with a final hold of 10 min resulting in the separation and measurement of fatty acid methyl esters ranging from 12:0 to 24:1. The detector temperature was 250°C. Helium was used as a carrier gas at a flow rate of 1.4 mL/min and a split ratio of 1:25. Chromatographic data was collected and analyzed by EZChrom software (Scientific Products, CA, USA). Fatty acids were identified by comparison to fatty acid standards and quantitating using peak area in comparison to internal standards. Resolution of individual peaks was distinguishable for peaks representing 0.05% of the total fatty acid methyl esters. Plasma fatty acid data is presented in relative terms (wt%).

Statistical Analysis

Dietary Intake. Normality testing was performed using the Kolmogorov-Smirnov test. The Wilcoxon signed rank test was performed on the dietary intake data. The alpha level was set at $p \leq 0.05$. Exact significance values were used for comparison to the alpha level.

Plasma Fatty Acid Analysis. Normal data was analyzed using the Student's paired T-test while non-normal data was analyzed using both the paired Student's T-test and the Wilcoxon signed rank test. Two-tailed Exact significance values were generated for the Wilcoxon signed rank test. Since the parametric and nonparametric analyses were in agreement, all statistical values were reported from the parametric tests. The alpha level was set at $p \leq 0.05$.

Buccal Cell Lipid Composition, Insulin Sensitivity And Fat Mass Loss. Data was analyzed using the paired Student's T-test for buccal cell fatty acids, HOMA2-IR and fat

mass loss. The Wilcoxon signed rank test was used to assess HOMA2%S as the data was not normal. The alpha level was set at $p \leq 0.05$.

Correlations. Pearson correlations were used to assess the relationships between insulin sensitivity scores and plasma and buccal cell fatty acids both within each diet phase and as the change between each phase. The alpha level was set at $p \leq 0.05$.

Chapter 4: Results

Subject Characteristics

A total of 15 participants were enrolled in this study consisting of 11 men and 4 women. The average age of the participants was 45.1 ± 10.2 . Waist circumference and blood pressure were the two most frequently occurring MetS qualifying characteristics present in 87 and 73% of participants respectively. Five subjects were taking medication to treat high blood pressure. The average BMI was $37.8 \pm 6.4 \text{ kg/m}^2$. All participants were considered obese based on BMI except for one participant who was overweight. A complete list of subject characteristics is listed in Table 1.1.

Dietary Analysis

The greatest amount of arachidonic acid was consumed during phase I at 2.69 g/wk in comparison to phase VI at 1.27 g/wk. This led to a daily decrease in arachidonic acid intake of 204 mg/dy during phase VI in comparison to phase I. During phase I significantly more fatty meats and whole eggs were consumed while during phase VI significantly more low-fat dairy was consumed. During phase I significantly greater amounts of arachidonic acid were consumed from beef, dairy, egg and pork. In phase I the greatest amount of arachidonate was derived from eggs (~56%) and pork (~15%), while during phase VI the greatest amount of arachidonate was derived from eggs (~38%) and poultry (~25%) (Figure 1.1 to 1.4). The sharp decline in total pork consumption between the diet phases led to the decrease in arachidonic acid derived from pork. Eggs were the greatest source of arachidonic acid in the diet during both phases with 239 mg/100g of ARA in egg. Although more dairy was consumed during phase VI, the substitution of reduced fat dairy products, mainly American cheese, cream cheese and the removal of sour cream, the only arachidonic acid containing dairy products, led to a greater amount of arachidonic acid derived from dairy during phase I. A complete nutrient analysis is provided in Table 1.2.

Fat Mass

Total fat mass loss during phase I was greater than during phase VI (-2.22 ± 1.49 kg vs -0.28 ± 1.53 kg, $P = 0.000$). Fat mass loss per week was also significantly greater during phase I than phase VI (-0.47 ± 0.31 kg vs -0.09 ± 0.48 kg, $P = 0.001$).

Plasma Lipids

Triglycerides. In the triglyceride omega-6 fraction 18:3n6, 20:2n6, 20:3n6 and 22:4n6 increased from phase I to phase VI while arachidonic acid (20:4n6) itself decreased (see Table 2.1). Of the remaining fatty acids in the triglyceride fraction 16:1n7 and 20:1n9 increased while 14:1n5, 18:1n9, 20:3n9, 18:2n6, 22:5n6 and all the measured n-3 PUFA 18:3n3, 20:5n3, 22:5n3 and 22:6n3 did not differ between phases.

Cholesterol Esters. In the cholesterol ester fraction 20:4n6 and its initial precursor, 18:2n6, decreased while desaturation and elongation intermediates in the arachidonic acid pathway 18:3n6 and 20:3n6 increased between phases I and VI (see Table 2.2). Of the n-3 PUFA 18:3n3 increased while its elongation and desaturation products 20:5n3 and 22:6n3 did not change. The MUFA 16:1n7 increased and 18:1n9 was unchanged.

Phospholipids. In the phospholipid fraction the omega-6 fatty acids changed as follows from phase I to phase VI: 18:2n6, 18:3n6 and 22:5n6 did not change, 20:2n6 and 20:3n6 increased while 20:4n6 and 22:4n6 decreased (see Table 2.3). The omega-3 fatty acids 20:5n3 and 22:6n3 did not change while 22:5n3 increased. Palmitoleic acid (16:1n7) did not change nor did any of the omega-9 fatty acids 20:1n9, 20:3n9, 22:1n9 and 24:1n9 with the exception of oleic acid, which tended to increase from phase I to phase VI ($P = 0.097$).

Plasma Fatty Acids and Insulin Sensitivity

HOMA2 Scores. The HOMA2-IR score increased from phase I to phase VI (1.20 ± 0.44 to 1.46 ± 0.62 , $P = 0.05$) indicating an increase in insulin resistance. However the change

in insulin sensitivity (HOMA2%S) was not significant (99.62 ± 14.66 to 83.97 ± 12.35 , $P = 0.249$).

HOMA2-IR Phase I. HOMA2-IR in phase I was correlated to the following fatty acids during phase I (see Table 3.1): TG16:1n7 and PL20:3n9 tended to be positively correlated ($P = 0.056$ and $P = 0.098$), PL24:1n9 tended to be negatively correlated, CE16:1n7 and the linoleic acid products PL 20:2n6 and 20:3n6 were significantly positively correlated while PL22:1n9 and ARA (20:4n6) in all 3 lipoprotein subfractions was significantly negatively correlated.

Insulin Sensitivity (HOMA2%S) Phase I. HOMA2%S in phase I was correlated to the following fatty acids during phase I (see Table 3.1): PL20:4n6 and PL24:1n9 tended to be positively correlated ($P = 0.095$ and $P = 0.051$), CE18:3n6, PL20:2n6, PL22:5n6 tended to be negatively correlated ($P = 0.052$, $P = 0.066$ and $P = 0.81$ respectively), TG20:4n6 and PL22:1n9 were significantly positively correlated while TG16:1n7, CE16:1n7 and PL20:3n6 were significantly negatively correlated. The differences between the measures of insulin resistance and insulin sensitivity were: CE 18:3n6 was a new tendency in HOMA%S only, PL20:2n6 and PL20:4n6 only tended in HOMA%S and CE20:4n6 and PL20:3n9 were not correlated to HOMA2%S. It is uncertain why these differences exist since HOMA2-IR is simply the inverse of HOMA2%S.

HOMA2-IR Phase VI. HOMA2-IR in phase VI was correlated to the following fatty acids during phase VI (see Table 3.1): TG22:4n6, CE20:4n6 and PL20:4n6 tended to be negatively correlated ($P = 0.057$, $P = 0.067$ and $P = 0.095$ respectively), TG20:4n6, TG20:5n3 and TG22:6n3 were significantly negatively correlated.

Insulin Sensitivity (HOMA2%S) Phase VI. HOMA2%S in phase VI was correlated to the following fatty acids during phase VI (see Table 3.1): TG22:4n6, TG20:5n3 and TG22:6n3 tended to be positively correlated ($P = 0.053$, $P = 0.081$ and $P = 0.083$ respectively), PL20:1n9 and ARA (20:4n6) in all three lipoprotein subfractions were significantly positively correlated. The differences between HOMA2-IR and HOMA2%S

were as follows: CE 20:4n6 became significantly correlated instead of a tendency, TG and PL 20:4n6 were newly significantly related and TG 20:5n3 and TG 22:6n3 were no longer correlated with HOMA2%S.

Overall. Arachidonic acid in all three fractions was related to measures of insulin sensitivity, indicating that it was positively associated with insulin sensitivity. No other fatty acids had a consistent association with measures of insulin sensitivity in both phases.

Buccal Cell Fatty Acids

In cheek cell total lipids the omega-6 PUFA DGLA (20:3n6) increased while ARA (20:4n6) decreased from phase I to phase VI. Of the omega-3 PUFA, α -linolenic acid (18:3n3) and EPA (20:5n3) both increased. Of the MUFA palmitoleic acid (16:1n7) increased, while oleic acid (18:1n9) and nervonic acid (24:1n9) decreased from phase I to phase VI. A summary of all the changes in buccal cell fatty acids is provided in Table 2.4.

Buccal Cell Fatty Acids and Insulin Sensitivity

HOMA2-IR in phase I was significantly positively correlated to 20:3n6 and 22:1n9, significantly negatively correlated to 24:1n9 and the ARA:DGLA ratio and tended to be significantly negatively correlated to 18:1n9. HOMA2%S in phase I tended to be positively correlated 14:1n5, 18:1n9, and 24:0, tended to be negatively correlated to 16:1n7 and 22:1n9 and was significantly positively correlated to 24:1n9 and the ARA:DGLA ratio. HOMA2%S in phase VI tended to be positively correlated to 14:1n5 and 22:6n3 and was significantly positively correlated to 20:2n6. A summary of all the meaningful correlations between measures of insulin sensitivity and buccal cell fatty acids is presented in Table 3.2.

Correlations of the Changes Between Phases for All Lipids and Measures of Insulin Sensitivity

The change in HOMA2-IR between phases I and VI tended to be positively correlated to the change in buccal cell 22:4n6, phospholipid 18:2n6 and 22:1n9, tended to be negatively correlated to buccal cell 22:1n9 and phospholipid 22:6n3 and was significantly negatively correlated to phospholipid 22:5n3. The change in HOMA2%S between phases I and VI tended to be positively correlated to the change in buccal cell 24:0 and phospholipid 20:4n6, tended to be negatively correlated to buccal cell 20:5n3 and phospholipid 22:1n9 and was significantly negatively correlated to buccal cell 22:4n6 and cholesteryl ester 22:6n3. There were no significant correlations between the changes in HOMA scores and the changes in plasma triglyceride fatty acids. A summary of all the correlations between the changes in HOMA scores and all lipids can be found in Table 3.3.

Chapter 5: Discussion

Dietary Intake

Total arachidonic acid intake during phase I was 2.69 g/wk (385 mg/d) while during phase VI it was 1.27 g/wk (181 mg/d). This amounted to a weekly difference of 1.42 g/wk or 204 mg/d less arachidonic acid consumption during phase VI. During phase I arachidonic acid was primarily derived from egg followed by pork, poultry, beef, fish and dairy. The combined contribution of pork, poultry, beef, fish and dairy consumption to the total arachidonic acid consumed was less than the contribution of egg. During phase VI arachidonic acid was again primarily derived from egg, followed by poultry, beef, fish, pork and dairy. However, during phase VI, the amount of arachidonic acid derived from egg did not exceed the total amount of arachidonic acid consumed from all the other food sources combined. The amount of arachidonic acid from egg decreased dramatically by about 1.012 g/wk or 68%. The total amount of arachidonic acid consumed from fish and poultry did not change between phases. Although the amount of arachidonic acid intake from beef, dairy and pork decreased during phase VI, their contribution to the difference in arachidonic acid consumed between phases was negligible in comparison to the loss from decreased egg consumption. The reduction in egg consumption was primarily driven by the replacement of eggs for breakfast by carbohydrate rich foods such as waffles, cereals and oatmeal and dairy products such as yogurt.

Plasma Fatty Acids

ARA was greater and DGLA was lower in all 3 lipoprotein fractions in phase I in comparison to phase VI. These inverse changes between ARA and its immediate precursor, DGLA, agree with the findings of previous low carbohydrate diet studies (49, 129). ARA supplementation studies also find increased ARA in all 3 lipoprotein fractions, but DGLA did not significantly decrease in any fraction. Instead linoleic acid decreased in the CE and PL fractions while oleate and linoleic acid both non-significantly declined in the TG fraction (84, 113). This is an important distinction between the results

in this study and our prior work (49, 129) where circulating DGLA significantly and consistently decreased across all subjects consuming a very low-carbohydrate diet, and the results of ARA supplementation studies where DGLA is not altered (84, 113). In low carbohydrate diets the changes in 5acid in the three fractions vary, likely depending on the different diets employed in the studies (49, 129). In this study linoleic acid decreased in the CE fraction but did not change in either the TG or PL fractions. Therefore a disparity exists between increased ARA intake as either a supplement or as part of the food in the diet. When ARA is increased through selected food consumption, the increase in ARA is paralleled by a decrease in DGLA, but when ARA is increased through supplementation it inhibits incorporation of linoleic acid into all the lipoprotein subfractions. The difference in linoleic acid incorporation into lipoproteins between ARA supplementation and low carbohydrate diet studies may be due to differences in linoleic acid intake. Supplementation with ARA capsules only increases the consumption of ARA, but whole foods can provide ARA as well as other fatty acids, such as linoleic acid. Eggs for example are not only a good source of ARA but also of the essential fatty acid linoleic acid. An alternative explanation for the decrease in linoleic acid with supplementation of high levels of ARA is competitive absorption. Linoleic acid absorption has been shown to be decreased when competing with higher levels of ARA and α -linolenic acid in rat intestines (23, 24, 135).

Augmentation of serum phospholipid ARA is consistently observed during low carbohydrate diets. Although there are several potential mechanisms by which ARA is increased on a low carbohydrate diet, the composition and caloric intake of the diet affects the contribution of each mechanism. It has been previously postulated that the increase in plasma ARA content with consumption of a low carbohydrate diet is facilitated by a reduction in oxidative stress, leading to a decline in the peroxidation of ARA and better preservation of the existing ARA pool (48). This controlled low carbohydrate diet feeding study observed a decrease in urinary isoprostane PGF₂ α , a peroxidation product of ARA after 6 weeks (48). Further evidence of decreased oxidative stress during a LCD has been observed in animal studies. In the hippocampal mitochondria of rats fed a ketogenic diet, observations of increased glutathione, increased uncoupling

protein expression and decreased hydrogen peroxide production have all been observed (83, 157). Increased Nrf2 activation and its downstream products HO-1 and NQO1 have also been observed in the livers of rats after 3 weeks on a ketogenic diet as well as a decrease in mitochondrial hydrogen peroxide production (108). However, liver concentrations of glutathione were decreased for the duration of the ketogenic diet (108). In addition, 4-HNE, a lipid peroxidation product of linoleic and arachidonic acids, was initially increased on the diet and although it decreased thereafter, it was still elevated to a non-significant degree above the control diet group (108).

Ketogenic diets initially demonstrate increased oxidative stress (107, 108). However, through activation of the Nrf2 pathway and ketone body induced effects on mitochondrial superoxide dismutase, antioxidant defenses are augmented, leading to reduced oxidative stress (107, 144). However, the time course of these adaptation has been speculated to be about 3 weeks (107). But there is evidence that it may be a longer duration before these adaptations take place. Rats fed a ketogenic diet for 4 weeks showed no difference in liver 4-HNE in comparison to a standard chow diet, but they showed a dramatic increase in 4-HNE catabolism indicating that HNE disposal was increased (96). In addition, liver concentrations of ascorbic acid and the GSH/GSSR ratio were reduced in comparison to a standard diet (96). However, despite the increase in lipid peroxidation and oxidative stress, liver concentrations of linoleic acid and arachidonic acid were elevated in the ketogenic diet group in comparison to the standard diet group (96). The increase in linoleic acid is likely due to the nearly 3.5 fold increase in linoleic acid intake during the ketogenic diet, but no group received any arachidonic acid in their diets. Therefore, despite increased lipid peroxidation and no arachidonic acid in the diet, liver arachidonic acid still increased on the ketogenic diet. Therefore it seems likely that after consumption of a ketogenic diet for a greater duration than 3-4 weeks, the increase in antioxidant capacity could facilitate a decrease in lipid peroxidation, augmenting the ARA pool, but for shorter durations the adaptations may not be complete. In this study the controlled feeding ketogenic diet was 3 weeks in duration, but it was preceded by 3 weeks of a free-living ketogenic diet leading to a duration of 6 weeks on a ketogenic diet. So it is possible by this point that the antioxidant adaptations to the low carbohydrate diet could have

contributed to the increase in the ARA pool. However, since no measurements of lipid peroxidation products or their catabolic metabolites have been assessed yet, it is uncertain what the contribution of decreased oxidative stress would be.

A greater increase in dietary arachidonic acid intake may also contribute to the rise in serum phospholipid ARA observed during low-carbohydrate diets. In this study, an estimated additional 204 mg/d of ARA was consumed in phase I vs phase VI. Since this is the first low carbohydrate diet study to control and quantitate dietary intake in both the low carbohydrate and standard diet phases comparisons to previous low carbohydrate literature are difficult, but a higher level of arachidonic acid intake in another controlled low carbohydrate diet feeding study was also associated with an increase in serum PL ARA (48). Supplementation with ARA capsules have also shown increased serum PL ARA (84, 113). Further evidence to support the contribution of dietary ARA to the ARA pool is observed in mice lacking delta-6-desaturase and are therefore unable to endogenously produce ARA from linoleic acid. These mice demonstrate very low levels of tissue ARA in comparison to wild type littermates (156). However, supplementation of their standard diet with arachidonic acid shows improvements to normal levels of ARA in the tested tissue of brain and skin (156).

A third possible mechanism to explain the rise in circulating ARA is release from adipose tissue. Adipose tissue triglycerides contain between 0.17 and 0.20 percent ARA (43, 119). Therefore the loss of fat mass or the overall greater flux of fatty acid cycling could manifest in release of ARA into the serum PL ARA pool. Fat mass loss totaled 2.22 kg and 0.28 kg during phases I and VI respectively. Hypothetically, that would have led to the release of 5-fold greater amounts of ARA during phase I and VI respectively (i.e., approximately 123 mg/d and 23 mg/d, respectively).

Finally, changes in endogenous synthesis of ARA from its precursor linoleic acid, derived from either dietary or adipose tissue stores, may have also contributed to the observed differences in serum PL ARA, but differences in ARA synthesis were not assessed in this study. However, the lack of change in serum PL mead acid, a product of

oleic acid produced by the same pathway as ARA synthesis, did not significantly change between phases, potentially indicating a lack of change in endogenous synthesis.

The phospholipid fraction of the lipoprotein particle may be the preferred site of ARA for transport through the circulation for a couple of reasons. Normal rats injected with radiolabeled ARA, linoleic and α -linolenic acids into the portal veins had their livers excised (32). Analysis of the liver homogenate showed that the greatest amount of radiolabel in the liver phospholipids was from ARA (32). Chylomicron remnants with radiolabeled ARA incubated with HepG2 cells showed that ARA was predominantly incorporated into phospholipids (22). Together these studies indicate that the liver preferentially packs ingested ARA into phospholipids, potentially for repackaging into lipoproteins. LDL modified with radiolabeled ARA prepackaged in phospholipids, transfer their radiolabeled ARA to platelets and erythrocytes which can be used for eicosanoid production (37, 40, 80). Lastly, animal work demonstrating that radiolabeled ARA fed to rats showed the greatest amount of radioactivity in the phospholipid fraction of lymph lipids (27). These data demonstrate the preferential packaging of ingested lipids in the liver into phospholipids and the transfer of these phospholipid concentrated with ARA to cells and platelets through lipoproteins.

Many plasma fatty acids showed moderate to strong relationships with insulin resistance in either phase I or phase VI, but the majority of fatty acids did not maintain these relationships with insulin resistance between both phases. The majority of these fatty acids were long chain polyunsaturated fats (greater than 20 carbons). The only fatty acid that showed a consistent and strong relationship in both diet phases in the plasma was ARA in the TG fraction, which was inversely correlated to insulin resistance. ARA in the CE and PL pools were inversely related to insulin resistance during phase I, but were reduced to tending toward significance in phase VI. Clupanodonic acid was the only plasma fatty acid that correlated with the change in insulin resistance between diet phases.

In summary, the highly significant and consistent increase in circulating ARA after feeding a low-carbohydrate diet could be a result of multiple factors including better preservation of the existing ARA pool, increased dietary intake, and greater release into the circulation from adipose triglyceride stores.

Buccal Cell Lipids

Analysis of total cellular lipids from buccal cells indicated that ARA was significantly lower while DGLA was significantly greater during phase VI than phase I. These changes in cellular lipids mirrored the changes in the plasma phospholipid fraction of the lipoproteins. The buccal cell MUFA palmitoleic acid increased from phase I to VI while oleic acid and nervonic acid decreased, potentially to balance the increase in palmitoleic acid. The omega-3 fatty acids α -linolenic acid and EPA increased from phase I to phase VI. These changes in MUFA and omega-3 PUFA were not observed in the plasma phospholipid fraction of the lipoproteins. Although palmitoleic acid did increase in the CE and TG fractions no changes were observed in oleic acid, or EPA and α -linolenic acid decreased in the CE fraction in opposition to the increase observed in the buccal cells. The similar response of plasma phospholipids by buccal cell lipids may be due to the strong tendency for ARA to partition into phospholipids. Although buccal cell palmitoleic acid did not mirror plasma phospholipids the increase in the CE and TG fractions may have still contributed to its increase in the buccal cells since total cellular lipids were analyzed. The changes in the other fatty acids could have been compensatory changes to balance the total MUFA and PUFA of the cells. The majority of cellular lipids are in the phospholipid fraction and in this fraction of cells there is a necessity to balance the saturation:unsaturation index likely due to an attempt to maintain cellular membrane fluidity which is important for the maintenance of cellular functions (6, 75, 117, 126, 142, 143).

There were no significant relationships between the change in buccal cell lipids and the change in HOMA scores between phases I and VI that were consistent between the measures of insulin sensitivity. There were also no consistent correlations between buccal

cell lipids and measures of insulin sensitivity in both diet phases. DGLA showed a significant positive relationship while the ARA:DGLA showed a significant negative relationship to HOMA-IR during phase I. These correlations are consistent with previously established relationships between muscle phospholipids and insulin sensitivity as assessed by the hyperinsulinemic-euglycemic clamp (13, 121). However, the strong positive correlation between insulin sensitivity and muscle phospholipid ARA was not observed in the buccal cells. This could have potentially arisen from buccal cell total lipids and not only the phospholipid fraction being assessed in this study. It may also be related to the physiologically important role of muscle as a primary organ in the disposal of plasma glucose. The heterogeneity of changes in both the HOMA scores and the cellular lipids between subjects may have played a role. A fourth factor that could have affected this relationship is the study population. The relationships between insulin sensitivity and skeletal muscle fatty acids were performed in healthy, normally insulin sensitive individuals while participants in this study had MetS. To the knowledge of this author, no papers comparing the fatty acid composition of buccal cells or skeletal muscle in healthy vs MetS individuals has been performed. Therefore it is unknown if the relationships between fatty acids and insulin sensitivity would change. Another significant difference is the use of the hyperinsulinemic-euglycemic clamp to assess insulin sensitivity in comparison to the use of HOMA scores. Although HOMA scores and the hyperinsulinemic-euglycemic clamp have a high degree of correlation for the estimation of insulin resistance their means of assessing insulin resistance are still different (direct vs indirect index) (176).

Limitations

The USDA database lists a value of 0.080 g/100g for the ARA content of raw native Alaskan sockeye salmon which is likely to underestimate the cooked value. Therefore incorporating this value into the total arachidonic acid intake calculation results in a lower actual arachidonic acid intake. However, currently the USDA database does not list a cooked value for this particular type of fish. There are plans to have the fatty acid composition of the cooked fish assessed in the future. However, the corrected value for salmon will not have a great effect on the total arachidonic acid intake and the large

relative difference between the low-carbohydrate and high-carbohydrate diet phases since salmon consumption did not differ between diet phases. Correlations between buccal cell lipids and HOMA scores are also limited in comparison to previous studies utilizing muscle phospholipids as total cellular lipids, not solely the phospholipid fraction was assessed in this study.

Conclusion

In conclusion this study demonstrates that a low carbohydrate diet, in comparison to a diet homologous to a standard Western diet, increases plasma and cellular ARA and decreases the proportion of the ARA precursor DGLA in both fractions. This study also demonstrates the significant positive relationship between plasma arachidonic acid and insulin sensitivity. Although plasma arachidonate was correlated to insulin sensitivity, buccal cell arachidonate was not which contrasts the findings observed in skeletal muscle.

Further Research

In order to further explore the relationship between cellular lipid composition and insulin sensitivity leukocytes were isolated from subjects following each diet. These leukocytes were cryopreserved and analyzed for total cellular lipid composition. In addition, an assay was developed using a fluorescent glucose analogue, 6-NBDG, in order to determine differences in insulin mediated glucose uptake in leukocyte subset populations. The purpose of this effort was to determine differences in insulin sensitivity at a cellular level between the diet phases. The analysis of leukocyte lipids and the development of the glucose analogue assay are discussed and the results provided in appendix II.

Appendix I

Subjects Characteristics, MetS Qualifiers and Experimental Diet Analysis

Table 1.1: Baseline Characteristics of the Participants

Baseline Participant Characteristics	
Characteristic	Value
Age (yrs)	45.13 \pm 10.19
Height (cm)	169.54 \pm 9.42
Weight (kg)	108.05 \pm 15.83
BMI (kg/m ²)	37.81 \pm 6.41
Waist (cm)	111.37 \pm 13.23
Systolic BP (mm Hg)	124.83 \pm 8.98
Diastolic BP (mm Hg)	81.51 \pm 8.66
Total Cholesterol (mg/dL)	189.13 \pm 28.23
HDL (mg/dL)	40.73 \pm 8.34
LDL (mg/dL)	127.36 \pm 37.98
TAG (mg/dL)	155.43 \pm 97.46
Glucose (mg/dL)	103.87 \pm 17.01
Values are means \pm SD	

Table 1.2: Nutritional Analysis of the Experimental Diets

Nutrient	Phase I	Phase VI
Energy (kcal/d)	2533.09 \pm 329.187	2486.54 \pm 334.49
Protein (%)	20.50 \pm 2.012	19.80 \pm 1.70
Protein (g)	128.36 \pm 6.814	122.10 \pm 11.47
Carbohydrate (%)	7.44 \pm 0.827	56.11 \pm 5.13
Carbohydrate (g)	46.52 \pm 1.865	345.58 \pm 28.52
Fiber (g)	15.46 \pm 0.675	34.69 \pm 4.93
Fat (%)	73.37 \pm 2.857	27.42 \pm 5.71
Fat (g)	207.43 \pm 33.935	77.52 \pm 26.03
SFA (%)	29.53 \pm 1.329	11.10 \pm 2.66
SFA (g)	83.48 \pm 13.736	31.48 \pm 11.39
MUFA (%)	26.80 \pm 1.860	8.10 \pm 1.83
MUFA (g)	75.96 \pm 14.115	22.95 \pm 8.04
Oleate (g)	70.60 \pm 13.410	20.09 \pm 7.33
PUFA (%)	11.96 \pm 0.879	5.17 \pm 1.29
PUFA (g)	33.62 \pm 4.654	14.66 \pm 5.54
n-3 PUFA (%)	1.22 \pm 0.204	0.58 \pm 0.14
n-6 PUFA (%)	10.74 \pm 0.711	4.59 \pm 1.17
18:2n6 (g)	26.42 \pm 4.085	10.56 \pm 4.30
18:3n3 (g)	2.92 \pm 0.625	1.23 \pm 0.51
20:4n6 (g)	2.69 \pm 0.320	1.27 \pm 0.57
20:5n3 (g)	0.16 \pm 0.073	0.12 \pm 0.04
22:6n3 (g)	0.33 \pm 0.116	0.28 \pm 0.07

Values are mean percentages \pm SD. Diets were analyzed using Nutritionist Pro, Axxya Systems, Stafford, Texas

Lipid Values for Plasma Lipoprotein Fractions and Buccal Cells

Table 2.1: Plasma Triglyceride Fatty Acids in Phase I and Phase VI

Plasma Triglycerides

Fatty Acid Name	Structure	Phase I	Phase VI	<i>p</i> -value
Myristoleic acid	(14:1n5)	0.25 ± 0.52	0.17 ± 0.16	0.601
Palmitoleic acid	(16:1n7)	2.63 ± 0.58	3.67 ± 0.94	0.000
Oleic acid	(18:1n9)	37.00 ± 2.77	35.57 ± 2.48	0.147
Eicosenoic acid	(20:1n9)	0.23 ± 0.08	0.29 ± 0.06	0.004
Mead acid	(20:3n9)	0.10 ± 0.01	0.08 ± 0.04	0.249
Linoleic acid	(18:2n6)	21.39 ± 3.07	21.09 ± 3.95	0.755
γ -Linolenic acid	(18:3n6)	0.43 ± 0.13	0.55 ± 0.12	0.024
Eicosadienoic acid	(20:2n6)	0.22 ± 0.09	0.30 ± 0.09	0.005
Dihomo- γ -Linolenic acid (DGLA)	(20:3n6)	0.25 ± 0.10	0.43 ± 0.08	0.000
Arachidonic acid (ARA)	(20:4n6)	1.77 ± 0.46	1.46 ± 0.34	0.002
Adrenic acid	(22:4n6)	0.18 ± 0.04	0.20 ± 0.04	0.028
Osbond acid	(22:5n6)	0.47 ± 0.36	0.41 ± 0.29	0.605
α -Linolenic acid	(18:3n3)	1.05 ± 0.47	1.15 ± 0.36	0.339
Eicosapentaenoic acid (EPA)	(20:5n3)	0.20 ± 0.08	0.22 ± 0.07	0.364
Clupanodonic acid	(22:5n3)	0.32 ± 0.12	0.33 ± 0.10	0.889
Docosahexaenoic acid (DHA)	(22:6n3)	0.73 ± 0.17	0.65 ± 0.27	0.378

Values are means ± SD

Units: wt %

Table 2.2: Plasma Cholesteryl Ester Fatty Acids in Phase I and Phase VI

Plasma Cholesteryl Esters

Fatty Acid Name	Structure	Phase I	Phase VI	p-value
<i>Palmitoleic acid</i>	(16:1n7)	1.69 ± 0.58	2.59 ± 0.85	0.000
<i>Oleic acid</i>	(18:1n9)	16.03 ± 1.55	16.07 ± 1.24	0.928
<i>Linoleic acid</i>	(18:2n6)	54.87 ± 4.47	52.68 ± 4.27	0.010
<i>γ-Linolenic acid</i>	(18:3n6)	0.48 ± 0.21	0.95 ± 0.37	0.000
<i>Dihomo-γ-Linolenic acid (DGLA)</i>	(20:3n6)	0.53 ± 0.22	0.92 ± 0.23	0.000
<i>Arachidonic acid (ARA)</i>	(20:4n6)	9.04 ± 1.35	7.13 ± 1.19	0.000
<i>α-Linolenic acid</i>	(18:3n3)	0.33 ± 0.08	0.51 ± 0.13	0.000
<i>Eicosapentaenoic acid (EPA)</i>	(20:5n3)	0.59 ± 0.22	0.67 ± 0.16	0.232
<i>Docosahexaenoic acid (DHA)</i>	(22:6n3)	0.49 ± 0.09	0.52 ± 0.25	0.609

Values are means ± SD

Units: wt %

Table 2.3: Plasma Phospholipid Fatty Acids in Phase I and Phase VI

Plasma Phospholipids

Fatty Acid Name	Structure	Phase I	Phase VI	p-value
<i>Palmitoleic acid</i>	(16:1n7)	0.62 ± 0.32	0.69 ± 0.20	0.311
<i>Oleic acid</i>	(18:1n9)	8.25 ± 0.60	8.68 ± 0.77	0.097
<i>Eicosenoic acid</i>	(20:1n9)	0.15 ± 0.04	0.16 ± 0.04	0.375
<i>Mead acid</i>	(20:3n9)	0.12 ± 0.05	0.15 ± 0.05	0.108
<i>Erucic acid</i>	(22:1n9)	0.52 ± 0.61	0.42 ± 0.55	0.205
<i>Nervonic acid</i>	(24:1n9)	1.26 ± 0.38	1.24 ± 0.36	0.713
<i>Linoleic acid</i>	(18:2n6)	21.05 ± 2.02	21.05 ± 2.57	0.989
<i>γ-Linolenic acid</i>	(18:3n6)	0.14 ± 0.09	0.13 ± 0.05	0.857
<i>Eicosadienoic acid</i>	(20:2n6)	0.27 ± 0.10	0.40 ± 0.11	0.003
<i>Dihomo-γ-Linolenic acid (DGLA)</i>	(20:3n6)	2.26 ± 0.79	3.61 ± 0.89	0.000
<i>Arachidonic acid (ARA)</i>	(20:4n6)	13.24 ± 1.73	10.88 ± 1.46	0.000
<i>Adrenic acid</i>	(22:4n6)	0.36 ± 0.08	0.41 ± 0.08	0.027
<i>Osbond acid</i>	(22:5n6)	0.30 ± 0.09	0.31 ± 0.16	0.876
<i>Eicosapentaenoic acid (EPA)</i>	(20:5n3)	0.69 ± 0.26	0.67 ± 0.14	0.734
<i>Clupanodonic acid</i>	(22:5n3)	0.69 ± 0.15	0.82 ± 0.17	0.001
<i>Docosahexaenoic acid (DHA)</i>	(22:6n3)	3.16 ± 0.52	3.08 ± 0.65	0.506

Values are means ± SD

Units: wt %

Table 2.4: Buccal Cell Fatty Acids in Phase I and Phase VI

Buccal Cell				
Fatty Acid Name	Structure	Phase I	Phase VI	p-value
<i>Myristic acid</i>	(14:0)	1.47 ± 0.39	1.55 ± 0.96	0.698
<i>Myristoleic acid</i>	(14:1n5)	0.53 ± 0.19	0.63 ± 0.26	0.230
<i>Pentadecylic acid</i>	(15:0)	0.44 ± 0.10	0.40 ± 0.14	0.150
<i>Palmitic acid</i>	(16:0)	14.40 ± 1.04	14.69 ± 1.80	0.485
<i>Palmitoleic acid</i>	(16:1n7)	5.87 ± 1.30	6.63 ± 0.95	0.014
<i>Margaric acid</i>	(17:0)	1.03 ± 0.11	0.98 ± 0.14	0.149
<i>Heptadecanoleic acid</i>	(17:1)	1.22 ± 0.46	1.36 ± 0.42	0.092
<i>Stearic acid</i>	(18:0)	13.18 ± 0.95	13.11 ± 1.03	0.829
<i>Oleic acid</i>	(18:1n9)	29.86 ± 2.99	27.91 ± 1.99	0.015
<i>Linoleic acid</i>	(18:2n6)	17.65 ± 1.95	17.36 ± 2.24	0.589
<i>γ-Linolenic acid</i>	(18:3n6)	0.23 ± 0.17	0.18 ± 0.07	0.291
<i>α-Linolenic acid</i>	(18:3n3)	0.17 ± 0.07	0.25 ± 0.11	0.033
<i>Arachidic acid</i>	(20:0)	0.67 ± 0.12	0.68 ± 0.12	0.880
<i>Eicosadienoic acid</i>	(20:2n6)	0.24 ± 0.05	0.25 ± 0.04	0.227
<i>Dihomo-γ-Linolenic acid (DGLA)</i>	(20:3n6)	0.91 ± 0.25	1.24 ± 0.29	0.000
<i>Arachidonic acid (ARA)</i>	(20:4n6)	3.66 ± 0.72	2.95 ± 0.61	0.000
<i>Eicosapentaenoic acid (EPA)</i>	(20:5n3)	0.19 ± 0.15	0.38 ± 0.40	0.034
<i>Behenic acid</i>	(22:0)	1.02 ± 0.18	1.00 ± 0.10	0.760
<i>Eruric acid</i>	(22:1n9)	0.70 ± 0.48	0.99 ± 0.86	0.185
<i>Adrenic acid</i>	(22:4n6)	0.44 ± 0.20	0.52 ± 0.42	0.438
<i>Docosahexaenoic acid (DHA)</i>	(22:6n3)	0.72 ± 0.25	0.69 ± 0.21	0.531
<i>Lignoceric acid</i>	(24:0)	1.95 ± 0.22	2.19 ± 0.40	0.139
<i>Nervonic acid</i>	(24:1n9)	0.79 ± 0.92	0.32 ± 0.31	0.031

Values are means ± SD

Units: wt %

Correlations Between Lipids and Indicators of Insulin Sensitivity

Table 3.1: Correlations Between Plasma Lipids and HOMA Scores

Correlations Between Plasma Fatty Acids and Indices of Insulin Sensitivity				
Fatty Acid	Phase I		Phase VI	
	HOMA2-IR	HOMA2%S	HOMA2-IR	HOMA2%S
TG16:1n7	0.542 †	-0.607 *		
TG20:4n6	-0.674 *	0.688 **	-0.664 *	0.757 **
TG22:4n6			-0.540 †	0.546 †
TG20:5n3			-0.685 **	0.501 †
TG22:6n3			-0.622 *	0.498 †
CE16:1n7	0.617 *	-0.634 *		
CE18:3n6		-0.549 †		
CE20:4n6	-0.567 *		-0.522 †	0.606 *
PL20:1n9				0.608 *
PL20:3n9	0.524 †			
PL22:1n9	-0.719 **	0.788 **		
PL24:1n9	-0.485 †	0.551 †		
PL20:2n6	0.634 *	-0.524 †		
PL20:3n6	0.650 *	-0.636 *		
PL20:4n6	-0.615 *	0.483 †	-0.482 †	0.618 *
PL22:5n6		-0.502 †		
TG: Triglyceride		* indicates $p \leq 0.05$		
CE: Choleteryl Ester		** indicates $p \leq 0.01$		
PL: Phospholipid		† indicates $0.05 < p \leq 0.1$		

Table 3.2: Correlations Between Buccal Cell Lipids and HOMA Scores

Correlations Between Buccal Cell Fatty Acids and Indices of Insulin Sensitivity				
Fatty Acid	Phase I		Phase VI	
	HOMA2-IR	HOMA2%S	HOMA2-IR	HOMA2%S
14:1n5		0.517 †		0.569 †
16:1n7		-0.506 †		
18:1n9	-0.54 †	0.504 †		
20:2n6				0.631 *
20:3n6	0.582 *	-0.564 *		
22:1n9	0.574 *	-0.55 †		
22:6n3				0.545 †
24:0		0.563 †		
24:1n9	-0.626 *	0.679 *		
<i>ARA:DGLA</i>	-0.612 *	0.688 **		

* indicates $p \leq 0.05$

** indicates $p \leq 0.01$

† indicates $0.05 < p \leq 0.1$

Table 3.3: Correlations Between the Change Values of HOMA Scores and the Fatty Acids of All Lipid Fractions

Correlations Between Changes in HOMA Scores and Fatty Acids of Different Lipid Fractions		
	HOMA2-IR	HOMA2%S
BC20:5n3		-0.48 †
BC22:1n9	-0.496 †	
BC22:4n6	0.476 †	-0.898 **
BC24:0		0.621 †
CE22:6n3		-0.852 **
PL18:2n6	0.525 †	
PL20:4n6		0.538 †
PL22:5n3	-0.653 *	
PL22:6n3	-0.494 †	
PL22:1n9	0.506 †	-0.557 †
* indicates $p \leq 0.05$ BC: Buccal Cell Fatty Acids ** indicates $p \leq 0.001$ CE: Cholesteryl Ester Fatty Acids † indicates $0.05 < p \leq 0.1$ PL: Phospholipid Fatty Acids		

The Contribution of Food Sources to Total Arachidonic Acid Consumption

Figure 1.1: Average Arachidonic Acid Intake (%) Phase I

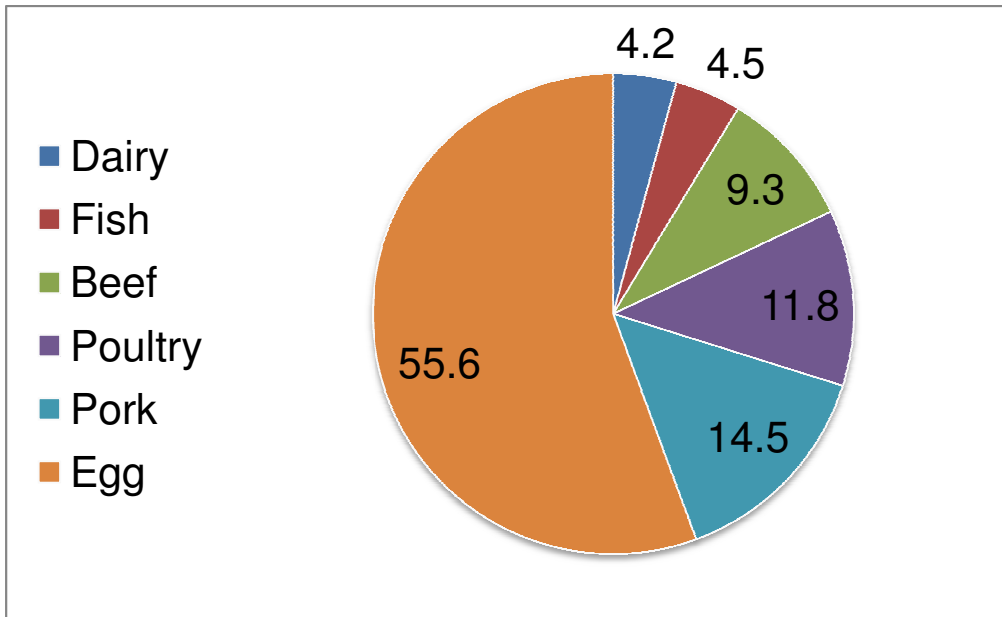


Figure 1.2: Average Arachidonic Acid Intake (grams) Phase I

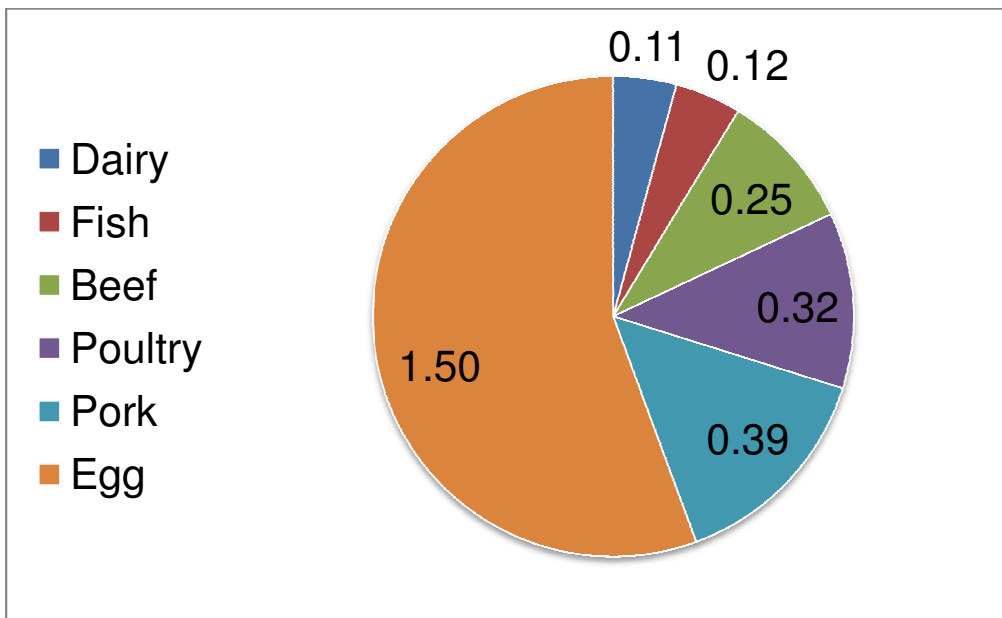


Figure 1.3: Average Arachidonic Acid Intake (%) Phase VI

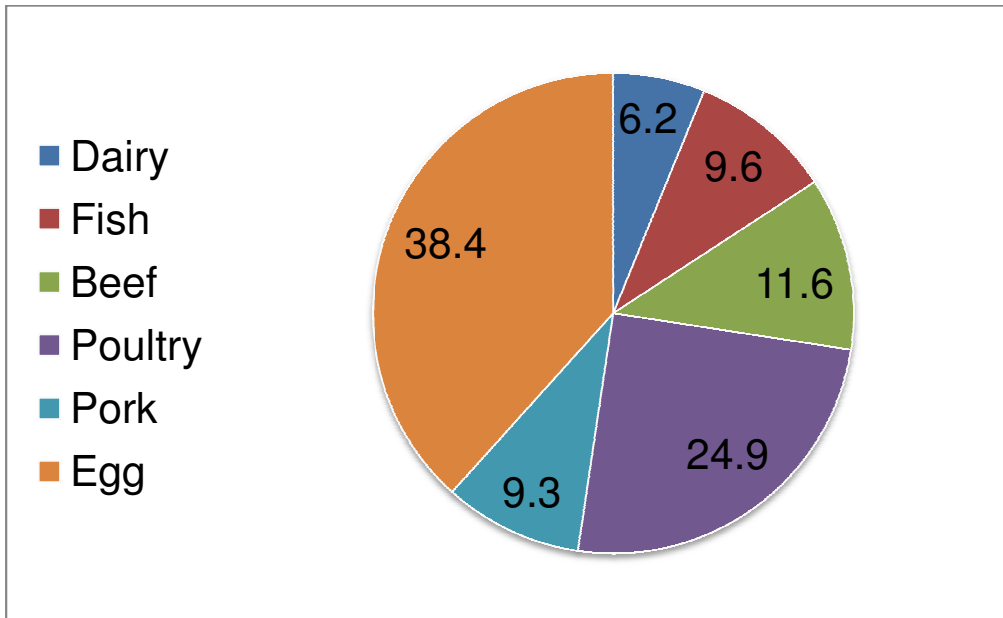
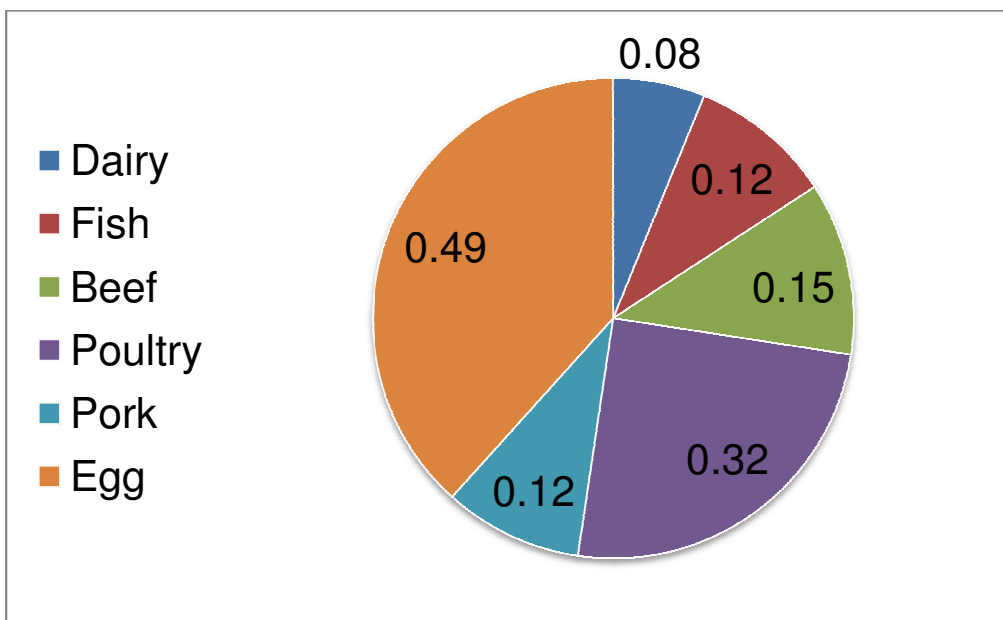


Figure 1.4: Average Arachidonic Acid Intake (g) Phase VI



Supplemental Graphical Representation of Plasma and Cellular Lipids

Figure 2.1: Plasma Triglyceride Fatty Acids

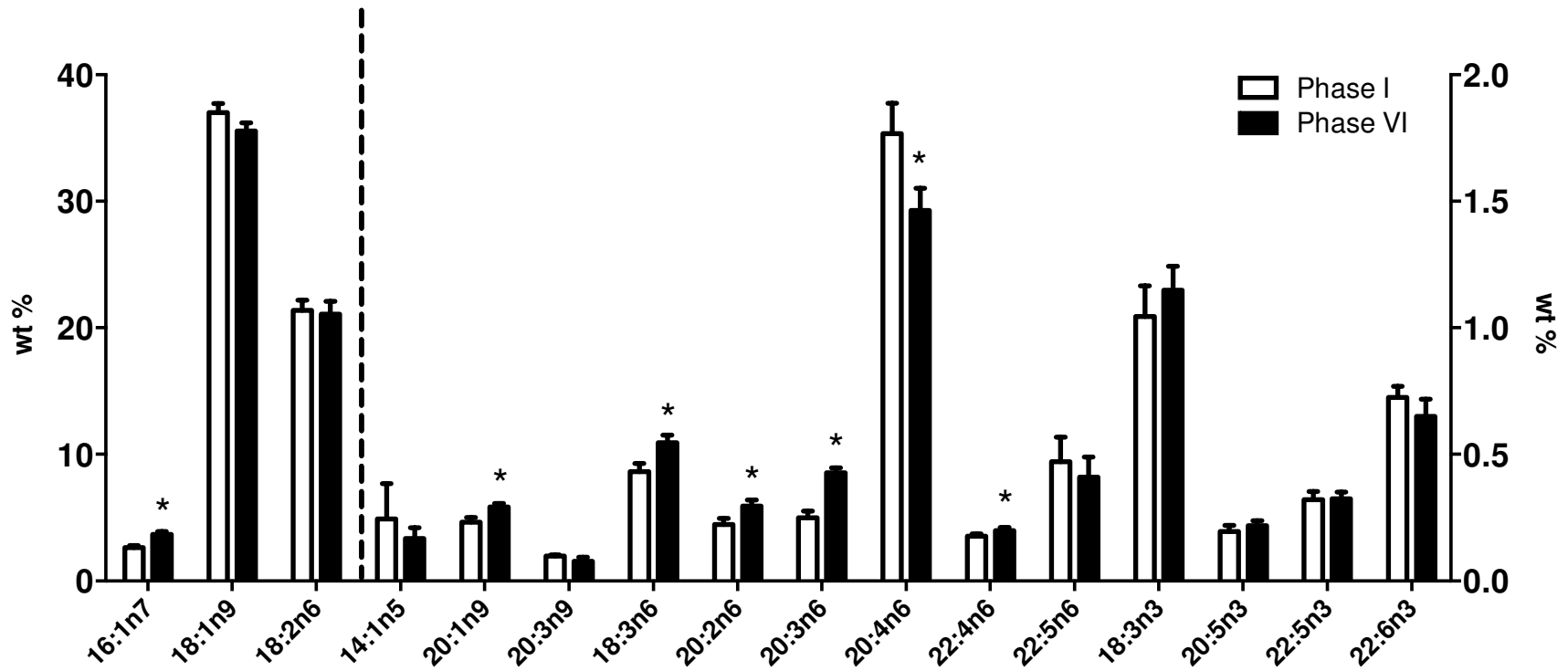


Figure 2.2: Plasma Cholesterol Ester Fatty Acids

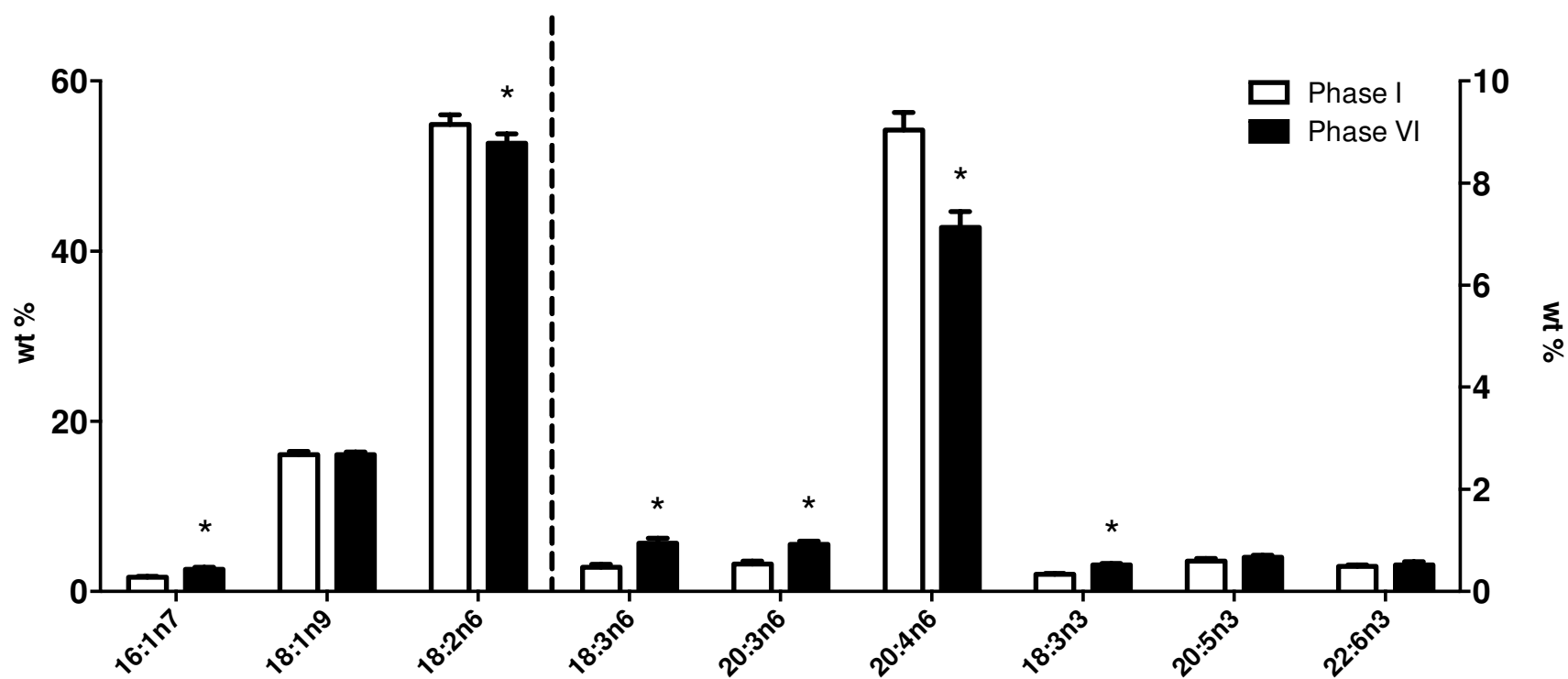


Figure 2.3: Plasma Phospholipid Fatty Acids

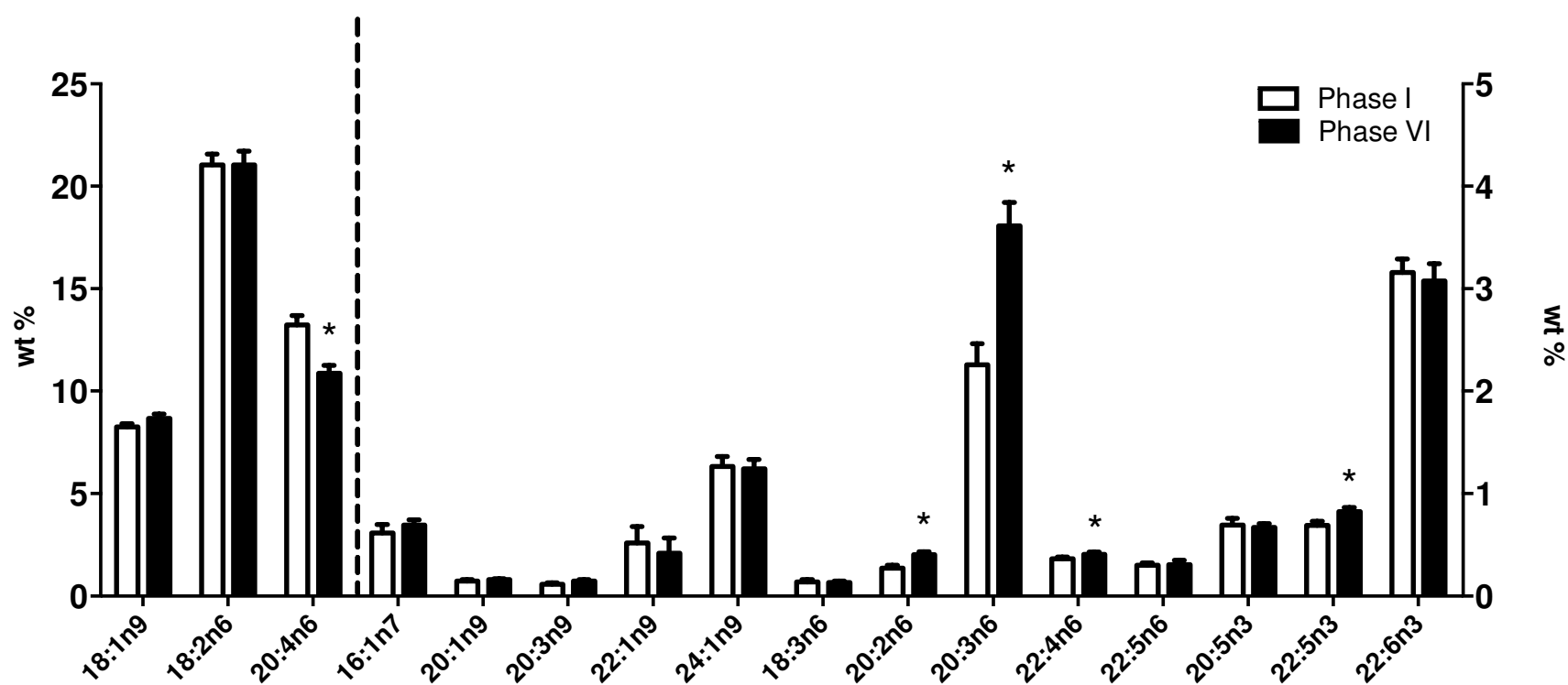


Figure 2.4: Buccal Cell Fatty Acids

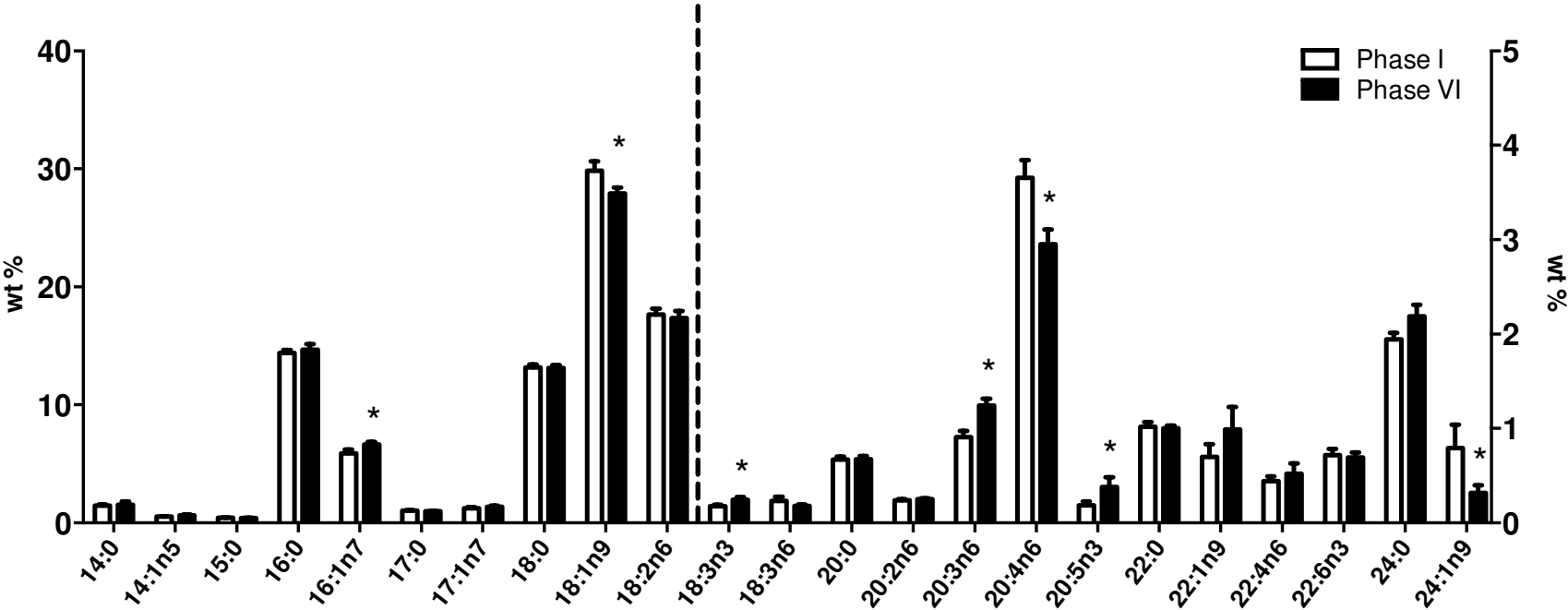


Figure 3.1: The Relationship Between TG ARA and HOMA-IR During Phase I

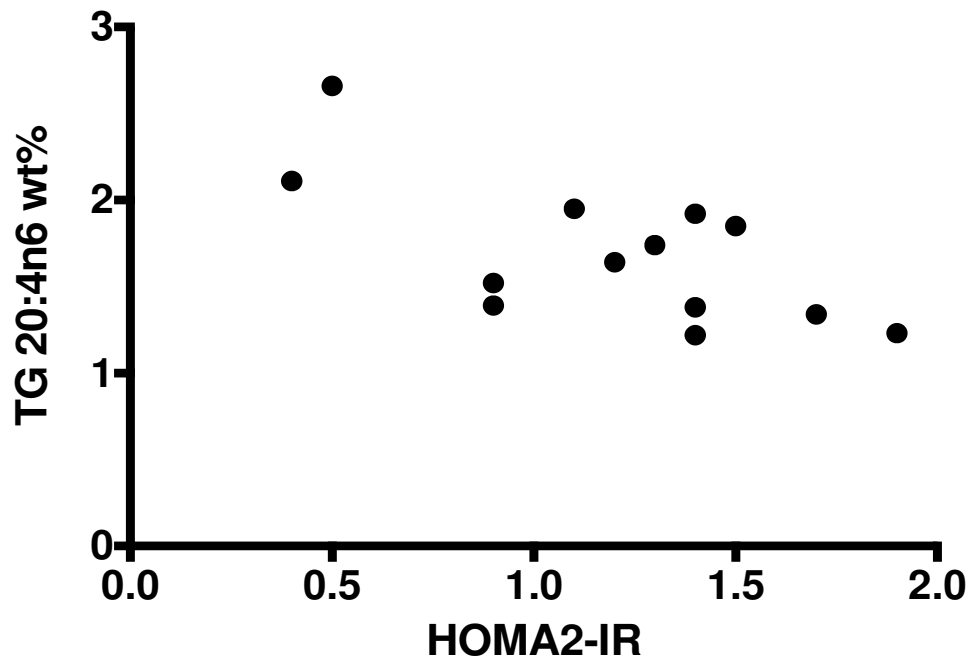
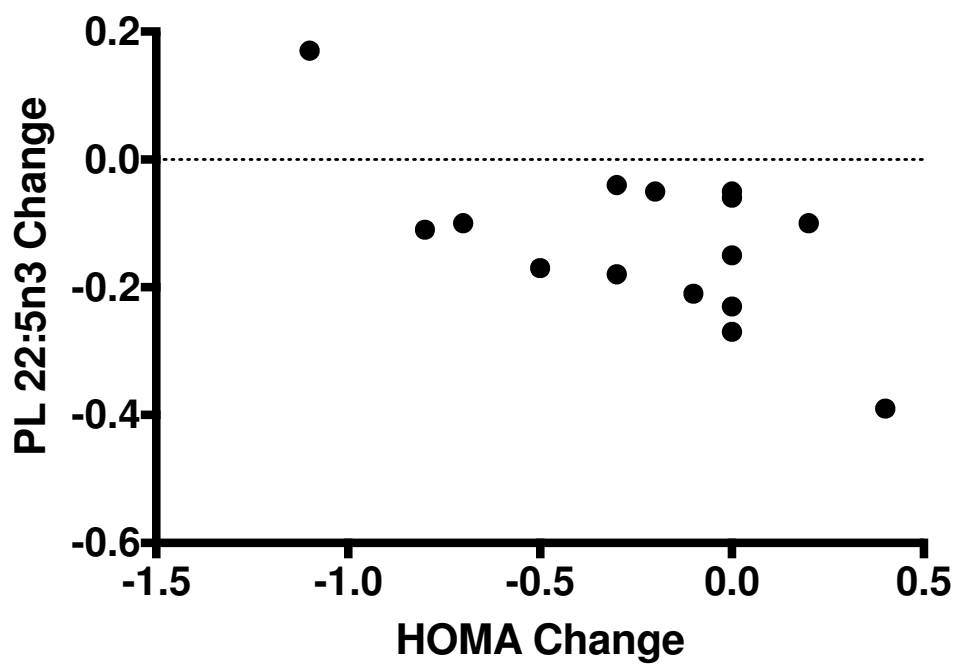


Figure 3.2: The Relationship between the Change in HOMA and the Change in Clupanodonic acid (Phase I minus Phase VI)



Appendix II

Isolation and Cryopreservation of Leukocytes

Total leukocytes were isolated from 20-30 mL of whole blood collected in EDTA tubes using the red blood cell lysis technique. Samples were processed within 3 hours of collection. Briefly, a 1x concentration of BD PharmLyse (Becton Dickinson Biosciences, San Jose, CA) is added to an aliquot of blood in a 1:10 blood:lysis buffer ratio. Samples are vortexed for 12 seconds followed by a 15 minute standing period to allow for lysis of erythrocytes. Samples are then centrifuged at 200 rcf for 10 minutes to pellet the leukocytes. The supernatant is removed and the pellet is resuspended in PBS-FBS (10% FBS v/v) in a 1:8 blood:wash buffer ratio which is centrifuged at 200 rcf for 10 minutes. The supernatant is removed followed by resuspension of the pellet in a total of 16 mL of RPMI. A 50 μ L sample is taken from the final cell suspension mixed in a 1:1 ratio with trypan blue then diluted with a volume of RPMI sufficient to determine cell viability and count under a brightfield microscope using a hemocytometer under a 40x objective (128). After the cell count is obtained the cell suspension is centrifuged at 200 rcf for 10 minutes prior to the addition of cyromedia (70% RPMI v/v, 20% FBS v/v and 10% DMSO v/v). Enough cyromedia was added to dilute the cells to 5×10^6 cells/mL. Cell suspensions were kept in 2 mL screw cap cryogenic vials and brought down to -80°C at a rate of $1^{\circ}\text{C}/\text{minute}$ using Biocision Cool Cell chambers. The next day (at least 6-8 hrs after placement in the deep freezer) cryovials were transferred to a liquid nitrogen freezer for long term preservation.

Leukocyte Lipid Composition

The leukocytes were prepared for lipid extraction as follows. Cryovials of leukocytes were thawed for 2 to 2.5 min in a 37°C . The 2 mL of thawed cells and cryomedia suspension were added to a 50 mL polypropylene centrifuge tube containing 160 μ L DNase I (10 mg/mL), 480 μ L of autologous serum and 13.36 mL of PBS. The cell suspension was incubated in the dark for 20 minutes at room temperature followed by centrifugation at 200 rcf for 10 min at room temperature. The cells were then

resuspended in PBS, transferred to a 13 x 100 borosilicate glass tube, centrifuged again at the same settings followed by resuspension in 2 mL of 4:1 methanol:hexane plus 50 µg/mL BHT supplemented with 17:0 TG as an internal standard. Total leukocyte lipids were then extracted by the method of Lepage and Roy (93). Briefly, the newly suspended sample is vortexed, placed on an ice bath followed by the addition of 200 µL of acetyl chloride. The tube is then sparged with nitrogen while incubated for 1hr in a water bath at 100°C. The tube is then cooled in an ice bath, followed by the addition of 6% K₂CO₃, then centrifuged at 4000 rpm for 2 min. The upper hexane layer is then removed for analysis. The methylated fatty acids solvated in hexane were analyzed by gas chromatography (Shimadzu GC-2010 Plus) equipped with a flame ionization detector and a DB-FFAP capillary column (15m x 0.1mm i.d. ,0.1 µm film thickness) (Agilent Technologies). The temperature program was as follows: the initially set temperature was 150°C with a 0.25 min hold; ramp: 35C/min to 200C, 8C/min to 225C with a 3.2 min hold, and then 80C/min to 245C with a 2.75 min hold. Fatty acid data are presented in relative terms (weight percent).

6-NBDG Leukocyte Uptake Assay: Method Development

Flow cytometric analysis of the fluorescent glucose analogue, 6-NBDG (Life Technologies, Carlsbad, CA), has not been previously performed in cryopreserved leukocytes and therefore required method optimization. The initial factor assessed was the type of media. RPMI (without glucose) (Life Technologies, Carlsbad, CA) and PBS were compared, with RPMI strictly assessed in a 5% CO₂ incubator kept at 37°C, while PBS was tested in atmospheric conditions, at both room temperature and 37°C. PBS was tested in the dark to simulate the light condition in the CO₂ incubator. The scatter and density plots of these media conditions were compared to that of freshly isolated cells. A pre-incubation period was tested to determine if cells were more metabolically active, determined by 6-NBDG uptake, immediately after thawing or given 2hr to acclimate post-thaw. 6-NBDG uptake was tested at various concentrations from 0 to 40 µM. During this testing propidium iodide was used to distinguish the uptake of 6-NBDG by live and dead cells. It was discovered that dead cells took up quite a high proportion of 6-NBDG

in comparison to live cells and so a dead cell removal kit was tested (Miltenyi Biotec, San Diego, CA). Comparison of the results with and without the removal of dead cells showed minimal alterations to the scatterplot, but it was observed that the dead and live cells were proportionately removed, leading to an overall decrease in total cells. This was likely due to the fact that the dead cell removal kit was suited for the removal of apoptotic cells which can be removed by Annexin V. However, the dead cells present under these testing conditions were likely necrotic cells which do not bear the Annexin V ligand on their cell surface. Therefore testing was continued without removing the dead cells. The plastic material for the incubation periods (pre-incubation and testing conditions) was also tested. Tissue culture treated plastic was compared to polypropylene. This was assessed using T-75 tissue culture treated flasks (BD Biosciences, San Jose, CA) and 50 mL polypropylene centrifuge tubes (Fisher Scientific, Pittsburgh, PA). In order to maximize cell viability and yield after thawing the presence of 3% autologous serum and DNase I (Roche Diagnostics, Indianapolis, IN) were tested and the scatterplots compared. Initial staining with the leukocyte phenotypic surface marker, CD3 (EMD Millipore, Temecula, CA), led to the discovery of extensive non-specific binding. 4% methanol, 4% paraformaldehyde, 10 and 20% FBS (MP Biomedicals, Santa Ana, CA) and 10 and 20% pooled human serum (not heat inactivated) were compared for effectiveness. Insulin (Sigma-Aldrich, St. Louis, MO) concentrations from 0 to 100 mIU/L were tested to determine which insulin concentration yielded the greatest difference in 6-NBDG uptake from the basal (no insulin) condition. The duration of incubation with insulin was also tested after an optimal insulin concentration was determined. Finally the collection settings on the flow cytometer including flow rate, the forward and side scatter thresholds and the number of events collected were assessed.

6-NBDG Leukocyte Uptake Assay: Method Development Results

Comparison of media types, RPMI and PBS, showed that PBS at room temperature demonstrated the scatter plot and density plot closest to freshly isolated cells. It must be noted that although the PBS condition was assessed immediately after the initial 2hr pre-incubation period, the RPMI condition was assessed after the insulin uptake assay. The

significance of this point is that the insulin uptake assay, at the time, was carried out using tissue culture treated plastic, which affects the final scatter plot as will be described below. The reason for this treatment difference was diminishing supplies of RPMI and the fact that the PBS treated cell scatter plot closely resembled the freshly isolated cell scatter plot. The use of PBS simplified the analysis process since a 5% CO₂ incubator was not required. An interesting observation was that both conditions carried out at 37°C showed diminished concentrations of neutrophils and monocytes.

The material of the cell culture container for both the pre-incubation period and the insulin uptake assay was assessed. Polystyrene tissue culture treated T-75 flasks were compared against polypropylene 50 mL centrifuge tubes. The cells were pre-incubated in RPMI for 2hr followed by the insulin uptake assay in tissue culture treated 12 well plates. As can be seen, more cells clung to the tissue culture treated plastic than the polypropylene container. Subsequent experiments were performed using only polypropylene 50 mL conical tubes for the pre-incubation period and 12x75 mm polypropylene culture tubes for the glucose uptake assay.

The metabolic activity of the cells post thaw was in question as this assay has not been previously performed in frozen cells. Therefore the metabolic activity of the cells was assessed immediately after thawing or after a pre-incubation period, allowing the cells to sit in media undisturbed for 2hrs. Metabolic activity was assessed by quantifying the mean fluorescence intensity (MFI) of concentrations of 6-NBDG from 10 to 40 µM in the presence of 100 mIU/L of insulin for 1hr. This particular concentration of insulin was chosen as it had been previously shown to induce maximal glucose analogue uptake in leukocytes (35, 130). The MFI was always greater after a 2hr incubation period indicating increased metabolic activity. However, as the concentration of NBDG was increased, the difference in the MFI diminished, especially at 40 µM.

In order to determine the optimal concentration of 6-NBDG for the assay, cells were incubated with concentrations of 6-NBDG ranging from 10 to 40 µM in the presence of 100 mIU/L of insulin. Propidium iodide was added to the cells just prior to reading on the flow cytometer in order to distinguish the uptake of 6-NBDG by live and dead cells. Uptake of 6-NBDG in live and dead cells showed the same pattern with increasing concentrations of 6-NBDG, but the uptake by dead cells at each concentration exceeded

that of live cells. Regardless, 30 μM was chosen as the optimal concentration as 40 μM broke the trend in linearity of 6-NBDG uptake by the cells indicating that it was or was close to a saturating concentration. An interesting observation here was that not only the MFI of 6-NBDG uptake increased with increasing concentration, but so did the number of live cells which internalized it.

After determining the optimal 6-NBDG concentration, insulin doses from 0 to 100 mIU/L were assessed to determine the concentration of insulin that maximized the difference in the MFI of NBDG uptake in comparison to the absence of insulin. This difference would be defined as the insulin mediated uptake of glucose. This value was determined to be 60 mIU/L. The duration of incubation was tested and indicated that there was no difference between 15 minutes and 60 minutes for 6-NBDG uptake under the optimal conditions. This was expected based on previous studies using NBDG to assess insulin stimulated glucose uptake in leukocytes (35). The initial use of the 1hr incubation period during optimization of the NBDG and insulin concentrations was to ensure maximal uptake had been achieved.

In order to maximize the number of viable cells the addition of 3% autologous serum was tested as a media supplement. Autologous serum decreased the number of dead cells and cell debris. Although it did not drastically alter the scatter plot, its effects can be observed in the density plot. Autologous serum did improve cell viability, but cell yield was still small. Clumps formed in the media after the 2hr pre-incubation period and in an attempt to prevent the clumping of cells, DNase was used to treat the cells during the 2hr pre-incubation period at a concentration of 0.1 mg/mL. DNase treatment drastically increased total cell yield and became a part of the pre-incubation media.

After having determined the cell culture containers, the composition of the pre-incubation media, and the optimal conditions for the glucose uptake assay, quantitation of glucose uptake by cell type was first tested using the cell surface marker CD3, which stains for T cells. The staining showed a great deal of non-specific binding. To find an optimal blocking agent to prevent the non-specific binding of phenotypic antibodies 4% methanol, 4% paraformaldehyde, 10 and 20% FBS and 10 and 20% human serum (not heat inactivated) were compared. The best results were achieved using either 10 or 20% FBS as no discernible difference between these two treatments were observed.

Paraformaldehyde and methanol had no effect, in other words, fixation prior to staining did not block non-specific binding. In addition, methanol was not a good candidate as it fixes and permeabilizes cells, which may affect the planned quantitation of extracellular GLUT4. Although human serum at both concentrations did also effectively prevent non-specific binding, it reduced the total cell yield, removing it as a viable option.

All of these experiments were performed on cells taken from an individual who was not a study participant. These cells were not cryopreserved as long as the subject samples. Test samples were only frozen for two weeks up to two months in comparison to subject samples that were frozen for close to two years. The realization that this assay would not work in actual subject cells came after conditions were optimized and the assay was tested on actual subject samples. The assay results did not replicate what was observed using test subject cells. 6-NBDG uptake did not differ between the insulin and the non-insulin treatment conditions. Further testing on the test subject samples revealed that the results of the assay varied with time frozen. Cells tested after 1 month of cryopreservation had a response to insulin, but cells frozen for 2 months did not. Further analysis of the insulin stimulated uptake of cells that had been frozen for 1 month or less revealed that although the cells did respond to insulin it was not in the predicted pattern. Although 60 mIU/L of insulin did elicit a response, it was not much greater than 30 mIU/L although it would be expected to be nearly double as observed in NBDG uptake tested in freshly isolated lymphocytes when comparing 25 v 50 mIU/L of insulin (35). Furthermore, cells tested only 1 day apart did not show the same insulin stimulated NBDG uptake at the optimal concentration of 60 mIU/L. initially it was thought that this was due to a difference in the concentration of cells incubated in the assay. However, even after manipulating the cell concentrations, the assay results were not replicable. Therefore, even in newly frozen cells, insulin mediated glucose uptake is perturbed. This indicated that cryopreserved cells have an abnormal response to insulin signaling. Therefore, since all the cell samples had been cryopreserved, changes in insulin mediated glucose uptake could not be observed.

Table 4.1: Leukocyte Fatty Acid Composition**Leukocyte Lipids**

Fatty Acid Name	Structure	Phase I	Phase VI	p-value
<i>Palmitic acid</i>	(16:0)	21.92 ± 8.01	21.99 ± 8.26	0.908
<i>Palmitic acid (DMA)</i>	(16:0 DMA)	27.49 ± 10.06	28.38 ± 15.25	0.759
<i>Palmitoleic acid</i>	(16:1n7)	0.13 ± 0.03	0.12 ± 0.04	0.314
<i>Stearic acid (DMA)</i>	(18:0 DMA)	2.43 ± 0.58	2.37 ± 0.98	0.791
<i>Stearic acid</i>	(18:0)	9.88 ± 1.63	10.03 ± 2.96	0.835
<i>Arachidic acid</i>	(20:0)	0.21 ± 0.05	0.19 ± 0.07	0.440
<i>Eicosadienoic acid</i>	(20:2n6)	0.31 ± 0.11	0.35 ± 0.20	0.346
<i>Behenic acid</i>	(22:0)	0.56 ± 0.16	0.56 ± 0.23	0.967
<i>Lignoceric acid</i>	(24:0)	0.75 ± 0.21	0.84 ± 0.33	0.301
<i>Oleic acid</i>	(18:1n9)	10.80 ± 2.44	9.97 ± 3.86	0.381
<i>Gondoic acid</i>	(20:1n9)	0.29 ± 0.10	0.22 ± 0.11	0.036
<i>Eruric acid</i>	(22:1n9)	0.11 ± 0.04	0.14 ± 0.09	0.210
<i>Nervonic acid</i>	(24:1n9)	1.97 ± 0.55	1.99 ± 0.79	0.933
<i>Linoleic acid</i>	(18:2n6)	5.25 ± 3.59	4.94 ± 2.10	0.760
<i>Dihomo-γ-Linolenic acid (DGLA)</i>	(20:3n6)	0.70 ± 0.18	0.97 ± 0.37	0.012
<i>Arachidonic acid (ARA)</i>	(20:4n6)	7.24 ± 1.50	6.89 ± 2.62	0.606
<i>Adrenic acid</i>	(22:4n6)	1.32 ± 0.35	1.22 ± 0.54	0.407
<i>Osbond acid</i>	(22:5n6)	0.14 ± 0.07	0.12 ± 0.06	0.465
<i>α-Linoleic acid</i>	(18:3n3)	0.07 ± 0.08	0.04 ± 0.03	0.251
<i>Eicosapentaenoic acid (EPA)</i>	(20:5n3)	0.21 ± 0.05	0.23 ± 0.12	0.359
<i>Clupanodonic acid</i>	(22:5n3)	0.73 ± 0.17	0.84 ± 0.37	0.332
<i>Docosahexaenoic acid (DHA)</i>	(22:6n3)	0.67 ± 0.23	0.71 ± 0.29	0.518
<i>Unknown</i>		6.70 ± 2.97	6.83 ± 3.97	0.948

Values are means ± SD

Units: wt %

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