The Effects of Weight Loss Induced by a Melanocortin 4 Receptor Agonist on Plasma Fatty Acid Concentrations in Non-human Primates

by

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LIST OF ABBREVIATIONS

α-MSH	α-melanocyte-stimulating hormones
ACTH	Andrenocortropin
AA	Arachidonic acid
AgRP	Agouti-related peptide
ALA	Alpha-linolenic acid
ARC	Arcuate nucleus
BMI	Basal mass index
BMR	Body metabolic rate
CART	Cocaine and amphetamine-regulated transcript
COX	Cyclooxygenase
DASHA	Dietary approaches to stop hypertension
DHA	Docosahexaenoic acid
DGLA	Dihomogamma linolenic acid
EPA	Eicosapentaenoic acid
LA	Linoleic acid
LCD	Low calorie diet
LH	Lateral hypothalamus
LOX	Lipoxygenase
LRb	Leptin-receptor
MC	Melanocortin
MC4R	Melanocortin 4 receptor

NPY	Neuropeptide Y
PC	Pro-hormone convertases
PGE_2	Prostaglandin series 2
PGE_3	Prostaglandin series 3
POMC	Proooiomelanocortin neuron
PVN	Paraventrivular nucleus
STEDMAN	Study of the effect of diet on metabolism and nutrition
VLCD	Very low calorie diet
VMH	Ventromedial nucleus of the hypothalamus

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ABSTRACT:

Obesity is one of the most prevalent and challenging clinical conditions in society today. It is estimated that 61% of people living in the United States are overweight or clinically obese (1, 2). Obesity is also associated with diseases known to have high inflammatory responses, such as dyslipidemia, and stroke. Elevated circulating omega-3 fatty acid concentrations are associated with decreased markers of inflammation. Previous studies found that increasing omega-3 fatty acid intake in obese subjects during weight loss reduced markers of inflammation and increased insulin sensitivity (3-5). It is possible that weight loss itself alters plasma fatty acid concentrations. Melanocortin 4 receptors (MC4R), located at the end of proopiomelanocortin neurons (POMC), play a role in decreasing food intake and increasing energy expenditure. This study investigated the effect of weight loss induced by a MC4R agonist on plasma fatty acid concentrations in monkeys.

Hypothesis

1. a) Polyunsaturated omega-3 fatty acid concentrations (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) in plasma will be higher after weight loss compared to baseline concentrations.

   b) Polyunsaturated omega-3 fatty acid concentrations (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) in plasma will be lower after weight regain compared to concentrations after weight loss.

2. a) Saturated fatty acid concentrations (myristic acid, palmitic acid, and stearic acid) and polyunsaturated omega-6 fatty acid concentrations (arachidonic acid (AA)) in plasma

will be lower after weight loss induced by a MC4R agonist compared to baseline concentrations.

b) Saturated fatty acid concentrations (myristic acid, palmitic acid, and stearic acid) and polyunsaturated omega-6 fatty acid concentrations (arachidonic acid (AA)) in plasma will be higher after weight regain compared to concentrations after weight loss.

3. a) Insulin sensitivity after weight loss will be higher compared to baseline concentrations and the magnitude of the improvement of insulin sensitivity will be negatively correlated with the decrease in saturated fatty acid concentrations (myristic acid, palmitic acid, and stearic acid) in plasma after weight loss induced by a MC4R agonist.

b) Insulin sensitivity after weight regain will be lower compared to sensitivity after weight loss induced by a MC4R agonist and the magnitude of deterioration of insulin sensitivity will be negatively correlated with the increase in saturated fatty acid concentrations (myristic acid, palmitic acid, and stearic acid) in plasma after weight regain.

Specific aim

To measure plasma fatty acid concentrations and to estimate insulin sensitivity using HOMA ratio in obese non human primates before and after weight loss induced by MC4R agonist treatment and again after MC4R agonist treatment is removed and weight is regained.

Methodology

12 obese rhesus macaques were enrolled in an open-label clinical trial. All monkeys were treated with a MC4R agonist for 8 weeks. Their diet was the same and consistent throughout the trial. Three blood samples were taken at different time points: 1) before the drug was administered at day 0, 2) after weight loss induced by the drug for eight weeks at day 56 and 3) after weight was regained when the drug was discontinued for four weeks at day 70. Plasma fatty acid concentrations were measured using gas chromatography-mass spectrometry (GC-MS). T-tests were used to measure differences in plasma fatty acid concentrations before and after weight loss and again after weight regain. All statistical analyses were performed using STATA version 10.0 and differences were considered significant at $p<0.05$.

Significance

This study explored the effect of a MC4R agonist that is under investigation as a novel weight loss drug. This drug may prove instrumental in the treatment of obesity and its co-morbid diseases.

CHAPTER ONE

BACKGROUND & SIGNIFICANCE

Thirty four percent of the people living in the United States are overweight (BMI of 25-29.9 kg/m^2) and 27% are clinically obese (BMI >30 kg/m^2) (1). Obesity is associated with a high risk of diabetes, hypertension, dyslipidemia, coronary heart disease, stroke, osteoarthritis, gallbladder disease, sleep apnea and certain types of cancer (1). Therefore obesity and its co-morbidities are very expensive medical problems. The economic cost of obesity related diseases is estimated to be 6% of all the national healthcare expenditures in the United States (2). Previous studies indentified that obesity is the result of a mixture of environmental and genetic factors. There are only a handful of monogenic causes of obesity that have been discovered. These are related to leptin, leptin receptors, pro-opiomelanocortin, and the melanocortin 4 receptor mutations (6).

The treatment for obesity is weight loss. In theory, weight loss follows a simple formula. To lose weight you have to be in a state of negative energy balance, which means the amount of energy consumed (food intake) must be less than the amount of energy expended (basal metabolic rate (BMR), and physical activity). The least intrusive weight loss methods and those most often recommended by health care providers are adjustments to eating patterns that reduce energy intake and to increase physical exercise. Other methods of losing weight include the use of drugs and supplements that suppress appetite or block fat or carbohydrate absorption. Finally, the most intrusive method for weight reduction is surgery. Bariatric surgery artificially reduces the size of the stomach, limiting the amount of food and thus the amount of energy that can be consumed. The

body has many mechanisms to maintain energy balance. The following overview outlines some of the endogenous mechanisms that control energy balance.

Endogenous regulation of energy balance

Energy balance is a complex process that is regulated by central and peripheral systems (1). Some of the effects of dysregulation of energy homeostasis are obesity and type II diabetes (1, 5 , 7). In today's developed societies, energy dense foods are easy to obtain. In addition, developed societies are characterized by having low energy expenditure because of stationary work environments and low physical activity.

Since the endogenous regulation of energy balance is so organized, altering any part in this complex system results in a "homeostasis-induced" physiological compensation (7). Consequently, one of the greatest challenges is to find an effective method to reduce body weight and to defeat natural homeostatic mechanisms to maintain weight without inducing negative side effects. A logical understanding of the regulation of food intake, appetite, lipid metabolism and energy expenditure is required to move toward successfully solving this problem.

Kennedy and his colleagues were the first to theorize that the brain controls energy balance (9). Their theory was centered around body fat. When the stability of body fat stores is threatened, the brain must receive a signal in proportion to the current level of adipose tissue (9). The arcuate nucleus (ARC) in the hypothalamus is the major region of the brain that is responsible for energy balance. There are other regions in the brain that are associated with the arcuate nucleus such as the lateral hypothalamic (LH), and the

paraventricular nucleus (PVN). All of these regions play a role in energy balance. The brain responds to multiple factors to regulate body weight; among these factors are insulin, leptin, glucose, glucocorticoids, and adiponectin (10). When the brain receives these signals, it either increases or decreases food intake and energy expenditure (8, 9, 10).

Leptin

Leptin is a protein hormone secreted from adipose tissue in proportion to fat mass and plasma triglycerides concentrations (11). Leptin influences energy expenditure through the hypothalamus, as evidenced by the high expression of leptin receptors (11). Hypothalamic leptin receptors are located in the arcuate nucleus on neuropeptide Y (NPY) and proopiomelanocortin (POMC) neurons. Leptin binds to leptin-receptors (LRb) and activates a second messenger, JAK-STAT3. Subsequently, neuropeptides are released and cause a decrease in food intake (11). A study by Ingalls and his colleagues showed that mice with leptin deficiency had early-onset obesity due to an increase in food intake and a decrease in energy expenditure (12). This is one piece of evidence suggesting leptin plays a key role in regulating appetite and energy expenditure via the melanocortin (MC) system.

Insulin

Insulin is a hormone that regulates the uptake of glucose from the blood stream into peripheral tissue and increases glycogen storage in the liver and muscle (11). Woods and Porte were the first to propose the role of insulin in energy regulation (13). Their study

demonstrated that the level of activity of insulin receptors in the hypothalamic arcuate nucleus is proportional to body fat in baboons. The administration of insulin directly into the brain results in a dose-dependent decrease in food intake and body weight (13). Insulin binds to its receptors in the arcuate nucleus and activates phosphatidylinositol-trisphosphate (PIP3) kinase (3, 4, 5), which causes the production of α-MSH; a peptide that helps reduce food intake by binding to its receptor at the lateral hypothalamic and paraventricular nucleus (11, 14, 15).

Fatty Acids

In the past, scientists thought that fatty acids were not able to cross the blood-brain barrier. However, more recent studies have proven that fatty acids can cross the blood-brain barrier and they may serve as messengers in the central nervous system rather than as nutrients or fuel (16). The roles of fatty acids in the brain have been described as "fatty acid sensing" (16). Both the arcuate nucleus and the lateral hypothalamus have neurons that are sensitive to fatty acids (16). The relationship between fatty acids and the arcuate nucleus and lateral hypothalamus can be explained by the resetting of sympathetic tone which leads to obesity and metabolic dysfunctions (16). Sympathetic tone is the amount of fatty acids over basal levels necessary to elicit a response and maintain homeostasis. High concentrations of triglycerides and fatty acids in the plasma lead to higher concentrations of fatty acids in the arcuate nucleus and lateral hypothalamus. When there are high concentrations of fatty acids in the arcuate nucleus and lateral hypothalamus, and the "fatty acid sensing" action is reduced. This leads to a failure to respond to excess fatty

acids by reducing food intake. The decrease in sympathetic tone can lead to obesity and metabolic dysfunction (16).

Melanocortin and energy expenditure

As previously discussed, the role of leptin and insulin in regulating energy expenditure is through entering the brain from the circulation and reducing energy intake by up-regulating neural pathways that are related to energy and food intake. Increasing data has identified the melanocortin system, located in the hypothalamus, as a significant regulator in energy balance. Multiple signals are integrated via the melanocortin system including leptin, insulin, and fatty acids among others.

The melanocortin system starts with dividing the pro-opiomelacortin (POMC) protein into seven peptide hormones by pro-hormone convertases (14,18). The peptide hormones are divided into two different classes of peptides, melanocortin peptides and neuropeptides. The melanocortin peptides include andrenocortropic hormone (ACTH), and α-melanocyte-stimulating hormones (MSH), β-MSH, and γ-MSH (14,18). The melanocortin hormones bind to five G-coupled melanocortin receptors (MCRs), which are members of the rhodopsin family of 7-transmembrane receptors (14, 19, 20). Table 1 illustrates the characteristics of the five members of the melanocortin receptor system, each with a differing affinity for specific melanocortins hormones (15, 19, 21).

Table 1: The characteristics of the G-coupled melanocortin receptors

	MC1R	MC2R	MC3R	MC4R	MC5R
Location	Melanoma cells, melanocytes, testis, pituitary, hair follicle, anti-inflammatory cells, skin glands, periaqueductal grey	Skin, Adrenal cortex, and adipocytes	Brain, heart, testis, placenta, gut.	Brain, adipose tissue	Adrenal gland. adipose tissue. kidney, leuocytes, lungs, lymph node, mammary gland, ovary, testis, uterus, brain, skeletal muscle, exocrine tissue
Purpose	Pigmentation, inflammation	Steroidgenesis	Energy homeostasis, sexual behavior, inflammation	Appetite regulation, energy homeostasis	Exocrine function
Agonist	α-MSH	ACTH	α,β, and γ MSH	ACTH, α and β MSH	ACTH, α-MSH

Pro-opiomelacortin (POMC) and neuropeptide Y neurons are connected to other hypothalamic sites such as the paraventricular nucleus and lateral hypothalamus. The release of α-MSH and cocaine-and-amphetamine-regulated transcript in the arcuate nucleus suppresses feeding while the release of neuropeptide Y and Agouti-related peptide increases feeding (11, 14). Leptin and insulin can stimulate pro-opiomelacortin and cocaine-and-amphetamine-regulated transcript at the arcuate nucleus. Leptin, insulin and fatty acids have an agonist effect on POMC, and antagonist effect on the NPY and AgRP (11, 14). Leptin and its receptor are located in one neuron POMC which is

connected to other neurons that express the melanocortin 4 receptor (MC4R). This neural trail then interacts with other brain centers to coordinate eating behavior and regulate basal metabolism and energy expenditure (14). Figure 1 shows the effect of leptin and insulin on NPY and POMC neurons.

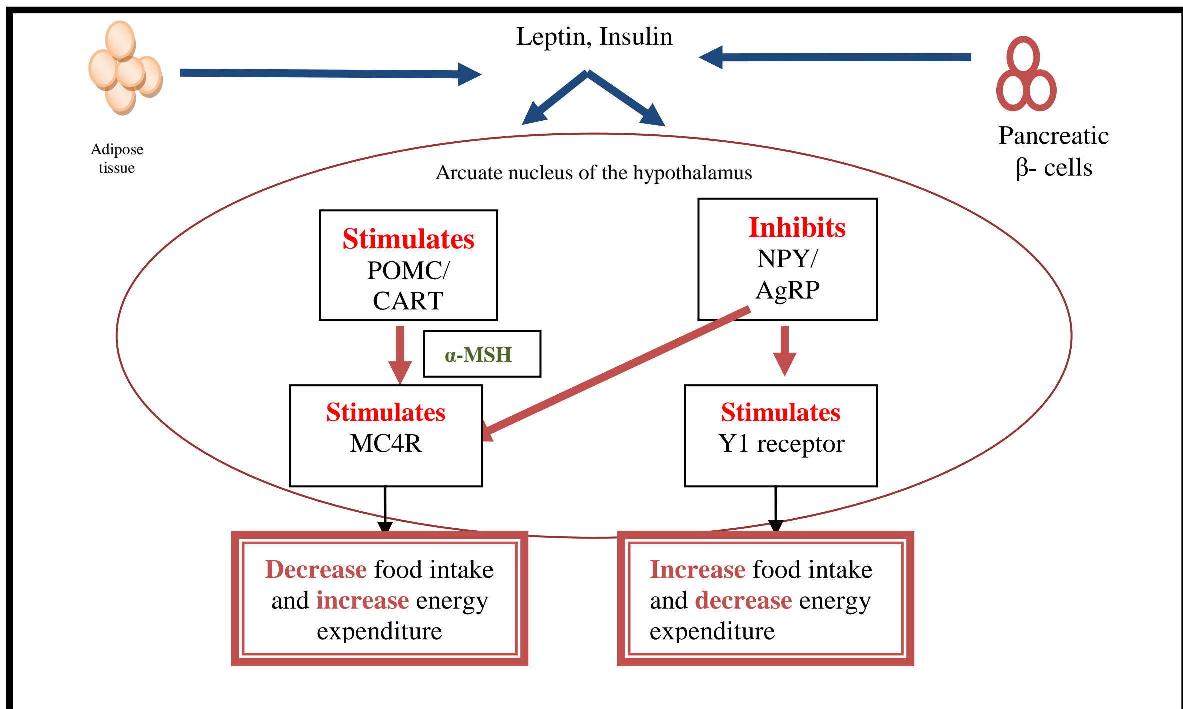


Figure 1: The actions of leptin and insulin in energy balance through the melanocortin system.

Melanocortin 4 receptor (MC4R)

The MC4R is a 322 amino acid protein whose gene is located on chromosome 18 at 18q21.3 (15). MC4R is highly expressed in the brain at the isocortex, olfactory region, hippocampus amygdal, cortex, corpus striatum, hypothalamus, and the dorsal horn of the spinal cord (15). Andrenocortropic hormone (ACTH) and α-MSH bind to MC4R and activate adenylate cyclase, resulting in an increased level of cAMP. cAMP in these neurons signals downstream to activate other second messangers to reduce appetite (15).

In 1998, MC4R was discovered to have a role in weight regulation (22). Research on animals after this discovery reported that MC4R knockout mice were hyperphagic and obese (20, 23-25). This finding suggests that disruptions in MC4R activity results in obesity.

A study conducted by Nijenhuis and his colleagues showed that individuals with MC4R mutations and obesity had lower levels of α-MSH, which suggests that α-MSH has a role in MC4R activity (26). In addition, AgRP has been shown to have an antagonist effect on MC4R by inhibiting α-MSH binding with MC4R resulting in increased food intake (11). Figure 2 illustrates the effect of AgRP and α-MSH on MC4R activity.

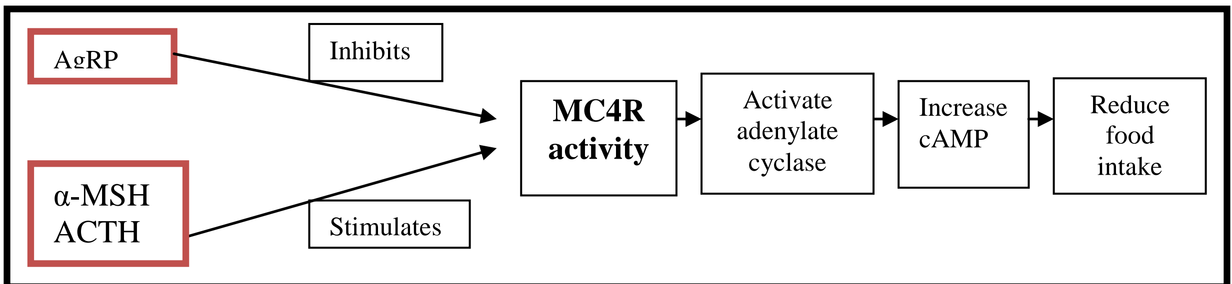


Figure 2: The effect of AgRP and α-MSH on MC4R activity

The goal of the proposed study is to investigate the effect of weight loss induced by a highly specific MC4R agonist on circulating fatty acid concentrations. Looking at Figure 2, treatment with a MC4R agonist is expected to reduce food intake. It is possible that an MC4R agonist also increases energy expenditure. The effects of this pharmaceutical approach to weight loss may change fatty acids concentrations differently than a reduction in food intake alone.

Weight loss and fatty acids composition

"Weight loss" refers to the reduction of total body mass. Weight loss involves loss of fat, muscle, and/or fluid. Preferential weight loss is the loss of fat mass while preventing the loss of muscle mass. In addition, some fatty acids are essential to the body and losing essential fatty acids may be problematic. In this section, the effect of weight loss on fatty acids composition is described.

Ramel and his colleagues (2008) measured plasma fatty acid composition as a molar ratio expressed as (weight %) in subjects on an energy-restricted diet supplemented with omega-3 fatty acids (4). Three hundred and twenty four overweight subjects enrolled in the study (138 males, 186 females) and 278 completed the study. The subjects were randomly allocated to one of four different diets; no seafood (controls), lean fish (cod fish 3 times/week), fatty fish (salmon 3 times/week), or fish oil capsules (6 capsules/day). The subjects followed their instructed diet for eight uninterrupted weeks. All diets were designed to have the same macronutrient composition (30% fat, 50% carbohydrate, and 20% protein) and to provide 30% less energy than their estimated energy requirements for weight maintenance estimated using the Harris Benedict equations (4).

The study showed that participants in each group lost weight, had higher circulating omega-3 fatty acid concentration, and were more insulin sensitive. Participants in the control group had the same results as participates in the other groups suggesting that weight loss with no additional omega-3 fatty acid consumption leads to a relative increase in plasma omega-3 fatty acids (DHA, EPA) concentrations. The study suggests

that weight loss alone has an effect on the fatty acid profile by selectively retaining or increasing the relative amount of omega-3 fatty acids.

Another study by Hlavaty et al (27), investigated the effect of weight loss on fatty acid profiles by using a low-calorie diet supplemented with omega-3 fatty acids. Forty women were assigned to a low-calorie diet (1313 kcal/day; 22.7% from protein, 28.7% from fat and 48.6% from carbohydrate) with or without omega-3 fatty acid supplementation (790 mg/day of omega-3 polyunsaturated fatty acid of which 620mg/day was EPA and DHA, for 3 weeks (27). The study reported plasma fatty acid concentrations in molar percentages. For the purpose of the proposed study, the focus will be only on the control group. The control group lost 4.5% of their initial weight compared to the experimental group who lost 2.7% of their initial weight. The relative total saturated fatty acid concentration decreased from 44.37 to 43.97 mol%. Lauric acid did not change but the relative myristic acid concentration decreased from 14.14 to 12.59 mol% and the relative palmitic acid concentration increased from 29.95 to 31.14 mol%. Total relative omega-3 fatty acid concentration decreased from 6.21 to 5.85 mol%. DHA increased from 4.10 to 4.17 mol% but EPA decreased from 1.17 to 0.78 mol%. Total relative omega-6 fatty acid concentrations increased from 38.19 to 38.82 mol%. Again the study demonstrates that weight loss alters fatty acids profile by selectively altering the proportions of circulating fatty acids.

These studies differ in that one reported an increase in omega-3 fatty acid concentration and the other reported a decrease in omega-3 fatty acid concentration with weight loss.

This difference may be related to the methods each study followed. These conflicting results raise the question of whether weight loss induces a selective loss of saturated fatty acids and omega-6 fatty acids with retention of omega-3 fatty acids?

Kunesova M and his colleagues (3) also investigated fatty acid profiles with weight loss. They measured the serum lipids in weight percent of two groups of obese women when they were on a very low calorie diet (VLCD) with omega-3 fatty acids supplements or placebo for four weeks. The VLCD consisted of 525 kcal/day, with 40 grams from protein, 9 grams from fat and 7 grams from carbohydrate. The study revealed that total saturated fatty acids in serum lipids (phospholipids and triglyceride) decreased after the consumption of a VLCD with or without omega-3 supplementation. In addition, omega-3 fatty acids (DHA and EPA) concentrations increased in the triglyceride fraction but EPA decreased in the phospholipids fraction in both groups. On the other hand, the omega-6 fatty acid, arachidonic acid (AA) increased in the phospholipid and triglyceride fractions. This study showed that VLCD and weight loss changed the relative plasma fatty acid concentration. These results suggest weight loss does induce a selective reduction of saturated fatty acids but the data about changes of omega-6 and omega-3 fatty acids remains unclear.

This study is designed to measure plasma fatty acid concentrations in obese non-human primates before and after weight loss induced by MC4R agonist treatment to specifically address the selective effects of weight loss on circulating fatty acid concentrations. This study differs from previous studies in that it will measure the actual concentrations of

fatty acids rather than expressing fatty acid concentrations as weight percent.

Dietary fat intake and plasma lipids

There is strong evidence suggesting that long chain polyunsaturated fatty acid (LCPUFA) composition in cell membranes is largely determined by diet (3). Raatz and his colleagues investigated the effect of high fat and low fat diets on plasma fatty acid percentages (21). Raatz reported plasma fatty acids percentages varied in response to total fat intake with a significantly greater total percentage of omega-6 fatty acids in plasma phospholipid and cholesteryl esters fractions after the consumption of a high fat diet. The low fat diet was associated with a significantly greater amount of omega-3 fatty acids in plasma phospholipids and cholesteryl esters (21). This difference was likely related to a decrease in competition for the enzymes of elongation and desaturation. A lower consumption of omega-6 fatty acids favors the enzymes for elongation and desaturation of available omega-3 fatty acids. This change may also be related to the loss of omega-6 fatty acids and the retention of omega-3 fatty acids which leads to an increase in the omega-3 fatty acid percentage and a decrease in omega-6 fatty acid percentage (21).

This suggests that dietary fat intake affects the composition of plasma lipids. In this study all the subjects consumed the same diet thereby removing the effects on differences in diet composition on weight loss and changes in fatty acid profiles.

The importance of long chain polyunsaturated fatty acids and essential fatty acids in the human body

Essential fatty acids cannot be synthesized endogenously and must be consumed as part of dietary fat, which accounts for 25-50% of the total energy content of most Western diets (28). Alpha-linolenic acid (ALA: omega-3) and linoleic acid (LA: omega-6) are the parent fatty acids from which more elongated and unsaturated fatty acids are derived. Good sources of LA are corn oil, cottonseed oil, soybean oil, safflower oil, canola oil, and olive oil (29). Good sources of ALA are green leafy vegetables and walnuts (29). The location of the double bond in the fatty acid molecule is the difference between these two classes of unsaturated fatty acids. Omega-6 fatty acids have at least two double bonds; the first double bond is at the 6th carbon from the methyl group end and the second double bond is at the 9th carbon from the methyl group end. Omega-3 fatty acids have at least three double bonds; the first double bond is at the 3rd carbon from methyl group end, second double bond is at the 6th carbon, and the third double bond is at the 9th carbon from the methyl group (28, 29). Longer-chain unsaturated omega-3 and omega-6 fatty acids are synthesized by and compete for the same enzymes; desaturases and elongases (29).

Omega-3 fatty acids

In omega-3 fatty acid metabolism, alpha-linolenic acid is converted to stearidonic acid by the enzyme delta-6-desaturase. For the enzyme to function properly, it requires sufficient B6, magnesium and zinc (28). Stearidonic acid is then converted to eicosatetraenoic acid by the enzyme elongase. Eicosatetraenoic acid is then converted to eicosapentaenoic acid

(EPA) by the enzyme delta-5-desaturase. The enzyme is dependent on vitamin C, niacin and zinc to function properly. A high intake of linoleic acid and trans-fatty acids block the enzymes required for linoleic acid and Alpha-linolenic acid metabolism (29). Figure 3 illustrates the series of metabolic steps of elongation and desaturation of omega-3 fatty acids (28). EPA and DHA are found preformed in the oils of fatty fish. As cited by Simpoulos, the health benefits of omega-3 fatty acids consumption was first recognized in a Greenland Eskimos study (29). In the study, it was observed that a high consumption of omega-3 fatty acids through seafood was correlated with low levels of heart diseases, diabetes, and lung diseases (29).

Omega-6 fatty acids

In omega-6 fatty acid metabolism, linoleic acid is converted into gamma linolenic acid by delta-6-desaturase enzyme. For the enzyme to function properly, it requires sufficient B6, magnesium and zinc. (28). Next is the conversion of gamma linolenic acid into dihomogamma linolenic acid (DGLA) by the enzyme elongase. DGLA is then converted by delta-5-desaturase to arachidonic acid (22). arachidonic acid is found preformed mainly in the phospholipids of grain-fed animals and eggs. Figure 4 shows the series of metabolic steps for the synthesis of arachidonic acid from linolenic acid (30).

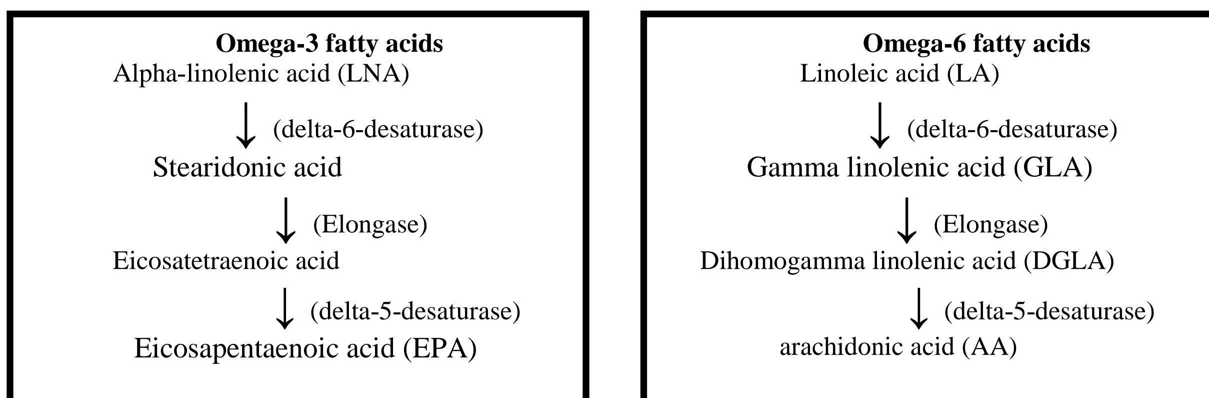


Figure 3 and Figure 4: the series of metabolic steps of omega-3 and omega-6 fatty acids

As cited by Simpoulos, these two classes of essential fatty acids have important opposing physiological functions as omega-6 fatty acids are considered to be pro-inflammatory and omega-3 fatty acids are considered to be anti-inflammatory (29). With the intake of fish or fish oil, the EPA and DHA from the diet replace the omega-6 fatty acids in the cell membrane, especially arachidonic acid (29, 30). This happens in most cell membranes, but especially in the membranes of platelets, erythrocytes, neutrophils, monocytes, and liver cells (29, 30).

Arachidonic acid and eicosapentaenoic acid are the starting point of eicosanoid production. Arachidonic acid (omega-6) derived eicosanoids are commonly pro-inflammatory and eicosapentaenoic acid (omega-3) derived eicosanoids are commonly anti-inflammatory (30) . Due to the high intake of omega-6 fatty acids in the Western diet, concentrations of eicosanoids from arachidonic acid, including prostaglandins, thromboxanes, and leukotrienes, are thought to be high in the human body compared to the eicosanoids from eicosapentaenoic acid (29, 30).

Two enzymes catalyze the conversion of essential fatty acids to eicosanoids: the first enzyme is cyclooxygenase (COX), which produces the prostaglandins. The second enzyme is lipoxygenase (LOX) or 5-lipoxygenase (5-LOX) which produces the leukotrienes. The body cannot store eicosanoids, but they are synthesized from fatty acids in the membrane of the cell when they are needed (29, 30).

Prostaglandins

The synthesis of prostaglands starts when a cell is stimulated by trauma, growth hormone or cytokines. Cytosolic phospholipase A (cPLA) at the cell membrane wall hydrolyzes fatty acids from the phospholipid membrane (30). Which fatty acids are hydrolyzed depends on the availability of arachidonic acid or eicosapentaenoic acid. If more arachidonic acid than eicosapentaenoic acid is incorporated into the phospholipid membrane then $cPLA_2$ at the cell membrane wall hydrolyzes arachidonic acid from the phospholipids (30). If more eicosapentaenoic acid than arachidonic acid is incorporated into phospholipids, $cPLA_3$ at the cell membrane wall hydrolyzes eicosapentaenoic acid from the phospholipids (30). The release of essential fatty acids is the “rate-determining” step for eicosanoid production (30). Another reaction happens, at the endoplasmic reticulum and nuclear membrane, cyclooxygenase (COX) produces prostaglands from arachidonic acid and eicosapentaenoic acid released through cPLA activities. There are two types of cyclooxygenase (COX) enzymes: COX-1 and COX-2. COX-1 is responsible for prostaglandin synthesis. When arachidonic acid is released, COX-1 forms prostaglandins of the 2 series (PGE_2) which have more pro-inflammatory response than prostaglandins of the 3 series (PGE_3) that are produced from eicosapentaenoic acid. The

second enzyme, COX-2, is responsible for inducing inflammatory responses such as constriction and dilation of the vascular smooth muscles, aggregation or disaggregation of platelets and regulates the inflammatory response (30).

Leukotrienes

Leukotrienes induce an inflammatory response in immune cells such as macrophages, leukocytes and mast cells. Examples of the various leukotrienes are LTA_4, LTA_5, LTB_4 and LTC_4. Leukotrienes use both autocrine and paracrine signaling to control the cell's response (29). Leukotrienes are produced in the body from arachidonic acid and eicosapentaenoic acid by the enzyme 5-LOX with the help of cPLA (30). When more arachidonic acid is released from the plasma membrane than eicosapentaenoic acid, 5-LOX alters arachidonic acid in conjunction with 5-lipoxygenase-activiting protein (FLAP) to release epoxide (LTA_4) (30). When more eicosapentaenoic acid is released from the membrane than arachidonic acid, 5-LOX and FLAP convert eicosapentaenoic acid to an epoxide (LTA_5) (30). LTA_4 and LTA_5 continue to form several different molecules depending of the cell type and inflammatory stimulus. For example LTA_4 is hydrolyzed to LTB_4 by leukotriene A_4 hydrolase (LTA_4H) (30). LTB_4 has a chemotactic effect on the migration of neutrophils to the injured tissue. LTC_4 is formed by conjugating LTA_4 with glutathione catalyzed by LTC_4 synthase (30). Leukotrienes are responsible for allergic reactions, sustaining an inflammatory state, and increasing vascular permeability (30).

Prostaglandins and leukotrienes are secreted to induce an inflammatory response. In small amounts, the eicosanoids from arachidonic acid are biologically active as a helpful

response to infection, but if it is produced in large amounts, they start to form thrombus and atheromas causing other inflammatory disorders (29). The amount and balance of essential fatty acids in a person's diet and subsequently the amount incorporated into phospholipids will affect the body's eicosanoid functions, which may result in an increased risk for some inflammatory diseases (30). The omega-6/omega-3 ratio in the diet is thought to play a role in maintaining a balance between normal response to infections and excess inflammation.

Omega-6/omega-3 Fatty Acid Concentration Ratio and inflammation

Recent research has emphasized the importance of the omega-6 to omega-3 fatty acid ratio in the diet regarding the development of several chronic inflammatory conditions. The optimal dietary omega-6/omega-3 ratio is estimated to be 2:1 to 3:1. However, most Western diets range between 10:1 to 20:1 in favor of omega-6 fatty acids (29). When the balance shifts towards excessive omega-6 fatty acid in the diet, more omega-6 fatty acids are incorporated into cell membranes. This leads to increased PGE_2 and LTA_4 formation and the risk of an inflammatory condition increases (28, 29, 31). Western diets are low in omega-3 fatty acids due to increased consumption of vegetable oils from corn, sunflower seeds, safflower seeds, cottonseed and soybeans (29, 31). Increasing levels of omega-3 fatty acids in the diet by adding canola, walnut and flax oils, and lowering the omega-6/omega-3 fatty acid ratio may exert inflammation suppressive effects by increasing the production of PGE_3 and LTA_5 (28, 29, 31).

Obesity is an inflammatory state and previous studies have shown that weight loss can

reduce markers of inflammation (4, 29, 31, 32). The degree to which markers of inflammation are reduced may be due to the changes in the fatty acid profile. The aim of this study is to investigate the effects of weight loss on circulating fatty acid concentrations.

Insulin sensitivity

Despite the fact that the relationship between obesity and lower insulin sensitivity has been known for 40 years, the mechanism behind how obesity influences insulin's action is unidentified (33). A study by Younsi and his colleagues (33) investigated the relationship between insulin sensitivity and erythrocyte phospholipid membrane composition in a negative energy state. The study included 45 female subjects (21 normal weight and 24 overweight) who were counseled to follow a hypo-caloric diet to lose weight for three months (33). Ten percent (~600 kcal reduction from the total caloric need) weight loss was the goal for study. The diet composition was 50% carbohydrate, 30% fat and 20% protein. The study reported that weight loss improved insulin sensitivity but there was no significant correlation with the change in erythrocyte sphingomyelin phospholipid classes (Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphateidy-linositol, and) and insulin sensitivity.

Numerous studies showed that weight gain induced by a high fat diet is associated with a decrease in insulin sensitivity (34, 35, 36, 38). Weight loss is the only treatment for obesity that improves insulin sensitivity (4, 35). On the other hand, subjects with normal body mass index can develop acute insulin resistance with consumption of a high fat

meal (35,36). The data suggest a relationship between fatty acids and insulin sensitivity.

Molar percentages (mol %) versus concentration (μmol/L) of fatty acid measurements

Many of the previous studies measured individual fatty acids as a ratio to the whole (weight %); which was reported as relative percentage of the calculated non weighted total area. The weight % method provides information about the relation between each fatty acid but does not provide a concentration for each fatty acid. The quantitative (μmol/L) method measures the actual concentration of each fatty acid (37). In the proposed study, the quantitative method will be used to measure plasma fatty acid profiles because we believe this method is more accurate in measuring changes over time.

CHAPTER TWO

SPECIFIC AIMS

The goal of this study is to investigate the effect of weight loss and weight regain on plasma fatty acid concentrations in monkeys.

Hypothesis

1. a) Polyunsaturated omega-3 fatty acid concentrations (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) in plasma will be higher after weight loss compared to baseline concentrations.

b) Polyunsaturated omega-3 fatty acid concentrations (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) in plasma will be lower after weight regain compared to concentrations after weight loss.

2. a) Saturated fatty acid concentrations (myristic acid, palmitic acid, and stearic acid) and polyunsaturated omega-6 fatty acid concentrations (arachidonic acid (AA)) in plasma will be lower after weight loss induced by a MC4R agonist compared to baseline concentrations.

b) Saturated fatty acid concentrations (myristic acid, palmitic acid, and stearic acid) and polyunsaturated omega-6 fatty acid concentrations (arachidonic acid (AA)) in plasma will be higher after weight regain compared to concentrations after weight loss.

3. a) Insulin sensitivity after weight loss will be higher compared to baseline concentrations and the magnitude of the improvement of insulin sensitivity will be negatively correlated with the decrease in saturated fatty acid concentrations (myristic acid, palmitic acid, and stearic acid) in plasma after weight loss induced by a MC4R

agonist.

b) Insulin sensitivity after weight regain will be lower compared to sensitivity after weight loss induced by a MC4R agonist and the magnitude of deterioration of insulin sensitivity will be negatively correlated with the increase in saturated fatty acid concentrations (myristic acid, palmitic acid, and stearic acid) in plasma after weight regain.

Specific aims

1. To measure plasma fatty acids concentrations in obese monkeys before weight loss and MC4R agonist treatment.
2. To measure plasma fatty acids concentrations in obese monkeys after weight loss and MC4R agonist treatment.
3. To measure plasma fatty acids concentrations in obese monkeys after weight regain when MC4R agonist therapy was discontinued.
4. To calculate insulin sensitivity by HOMA-IR using fasting glucose and fasting insulin concentrations in obese monkeys before and after weight loss induced by an MC4R agonist and again after weight regain.

CHAPTER THREE

METHODOLOGY

Study Design & Methods

This is a sub-analysis of a study conducted at the Oregon National Primate Research Center (ONPRC, Beaverton, Oregon). This open-label clinical study of 12 male rhesus monkeys measured the effect of a MC4R agonist on body weight. Weight was measured and blood samples were collected for plasma fatty acid, triglyceride, glucose, and insulin analysis at three time points throughout the study. The first weight measurement and blood sample was collected at baseline; before the MC4R agonist was administered. The second weight measurement and blood sample was collected after eight weeks of MC4R agonist administration. The third weight measurement and blood sample was collected four weeks after discontinuation of MC4R agonist administration. The primary purpose of the study was to measure the effect of weight loss on plasma fatty acid and triglyceride concentrations, and insulin sensitivity. The second are purpose of the study was to measure the effect of weight regain on plasma fatty acid and triglyceride concentrations, and insulin sensitivity.

Rhesus Monkeys (macaca mulatta)

Rhesus monkeys have been a mainstay of biomedical research in many areas of human physiology, immunology, and health, and they have also been used widely in psychological studies, especially of behavioral development, learning, and social adjustments. The human blood factor, Rh, is named for the rhesus monkey, because our

understanding of blood antigens was most clearly demonstrated in studies of these monkeys. Rhesus monkeys were used for the discovery, development, and testing of the polio vaccine. The Oregon national primate center preserves colonies of around 3,800 rhesus monkeys. Rhesus monkeys are originally from Asia starting from northern India to northern China. They were brought to the United States to Brown University in the 1950's and transferred to Oregon in 1964. Typical body weights range from 11 to 26.5 lb (5-12 kg) for adult male rhesus monkeys, and from 9 to 24 lb (4-11 kg) for adult females.

Animal Housing and Environmental Conditions

The monkeys were housed in the same room at the ONPRC but in individual stainless steel cages with a squeeze mechanism for ease of restraint. Individual housing allowed for accurate measurement of weight and food intake. ONPRC follows the space recommendations published in the National Research Council's Guide for the Care and Use of Laboratory Animals. The dimensions of a cage for an individual monkey that weighed between 10 kg and 15 kg, was 6.0 square feet for the floor area and 32 inches in height. The lights were turned on at 7 am and turned off at 7 pm. The monkeys were observed for 20 minutes three times a day by the staff of ONPRC: during the morning mealtime at 9:30 am, during the afternoon meal at 2:30 pm, and at 6:40 pm just before the dark cycle commenced.

Diet Composition

A high fat, pelleted, monkey chow was provided ad libitum to the monkeys before the study began and for the duration of the study. Pellets were approximately 1.25 cm in diameter by approximately 2.0 cm to 2.5 cm long. Each pellet provided 3.8 kcal/g and

consisted of 55.3 % carbohydrate, 16.1 % protein and 31.9 % fat (TestDiet, Richmond, IN). Pellet intake was measured by counting the numbers of pellets provide to each monkey and the number of pellets remaining after each eating session each day. The difference between these two values was equal to the number of pellets consumed by each monkey. The food was removed each day by 4.30 pm. Water was available throughout the day.

MC4R Agonist Delivery

Throughout the course of the study the MC4R agonist was administered in a continuous manner through an infusion pump (Alzet, Model 2ML2, Cupertino, CA) surgically inserted in the monkey's upper back on the first day of the study. This continuous administration system allowed for the drug concentration in the monkey's system to remain stable for the 8-week duration of the study.

Sedation Procedure for Insertion of the MC4R Agonist Delivery Pump, Weight Measured and Blood Sampling

Monkeys were sedated using telezol (0.1 mg/kg) or ketamine (10 mg/kg) depending on the type of surgery performed for the insertion of the drug delivery pump. Additional anesthesia was used to keep the animals sedated until the weight measurement and blood sampling procedures were completed. Technicians observed the monkeys throughout the procedure and until they recovered from sedation.

Weight Measurement

Weight was measured three times during the study to 0.1 kg with a Transcell TI-500 SL scale (Buffalo Grove, IL) while the monkeys were sedated. The first weight measurement was at baseline, before the MC4R agonist was administered. The second weight measurement was after eight weeks of MC4R agonist administration. The third weight measurement was four weeks after MC4R agonist administration was stopped.

Blood Sample Collection and Analysis

Fasting blood samples were obtained via saphenous vein collection at 8:00 am after a 15 ½ hour fast. The right hind leg was shaved and disinfected with alcohol. A 1 ml sample of whole blood was collected into tubes containing EDTA or heparin. A whole blood sample was removed immediately after collection. The remaining samples were placed on ice for 30-60 minutes and then spun in a Sorvall Legend centrifuge (Thermo Scientific, Waltham, MA) at 2500 rpm for 20 minutes. Plasma was collected and stored frozen at -80°C until ready for measurement.

Fasting Glucose and Insulin Measurements

Fasting blood glucose concentration was measured in whole blood immediately after collection with a glucometer (Onetouch Ultra Blood Glucose Monitor, LifeScan, Milpitas, CA, USA). Plasma was assayed for insulin concentration by the staff at ONPRC/OHSU Endocrine Services Laboratory using an Immulite 2000 (Siemens, Chicago, IL).

Insulin Sensitivity Calculation

Insulin sensitivity was calculated using the homeostatic model assessment for insulin resistance (HOMA-IR) by the following formula:

HOMA-IR = (Fasting Glucose (mg/dL) x Fasting Insulin uU/mL))/405 (constant).

Triglyceride Measurement

EDTA plasma was sent to the clinical pathology lab at Oregon Health & Science University (Portland, Oregon) and analyzed for total triglyceride concentration. Triglycerides in the plasma samples were hydrolyzed to glycerol and free fatty acids by lipase. Glycerol was measured by a horseradish peroxidase coupled colormetric assay using the multi-calibrator Synchron system (Beckman Coulter, Fullerton, CA).

Plasma Fatty Acid Measurement

Danielle Podesta, a master's student at OHSU, wrote the method for the plasma fatty acid analysis. Plasma samples for fatty acid analysis were prepared in Dr. Melanie Gillingham's laboratory and analyzed in the Bioanalytical Shared Resource Pharmacokinetics Core Laboratory at OHSU using the method of Lagerstedt et al (37). Glass reaction tubes were rinsed with 0.1N hydrochloric acid (HCL) and hexane and then left to dry. An internal standard was prepared that contained one µg of $d3C_{10}$, two µg of $d3C_{14}$, 20 µg of $d3C_{16}$, 20 µg of $d3C_{18}$, 10 µg of $d3C_{20}$, and 2 µg of $d3C_{22}$ (Cambridge Isotope Laboratories, Andora, MA) per tube. The mixture of internal standards was diluted with 2 $CHCl_3$: 1 MeOH to yield a total volume of 5 ml. A stock solution of one mg butylated hydroxytoluene (BHT) in one ml of 2 chloroform:1 Methanol (MeOH) was

prepared. 100 µl of BHT and 200 µl of the internal standard mixture were added to each sample tube and the tubes were dried with nitrogen.

Fatty Acid Standard Curve Preparation

To prepare the standard curve, free fatty acid stock solutions (Nu-Chek Prep, INC, Elysian, MN) were sonicated for 15 minutes. The standard curve mixture included 5 µg/ml of C8:0, 5 µg/ml of C10:0, 50 µg/ml of C14:0, 5 µg/ml of C14:1, 500 µg/ml of C16:0, 50 µg/ml of C16:1, 150 µg/ml of C18:0, 500 µg/ml of C18:1, 500 µg/ml of C18:2, 5 µg/ml of C18:3, 150 µg/ml of C20:4, 50 µg/ml of C20:5, and 50 µg/ml of C22:6. The fatty acid standard curve stock solution was mixed in a volumetric flask to a final volume of one ml with of 2 $CHCl_3$:1 MeOH. A blank tube was prepared with 100 µl of 2 $CHCl_3$:1 MeOH. Known volumes of the standard curve mixture were added to prepared tubes as follows: tube 1, 300 µl; tube 2, 200 µl; tube 3, 100 µl; tube 4, 50 µl; tube 5, 25 µl; and tube 6, 10 µl.

25 µl of plasma was added to glass tubes without the standard curve mixture. A mixture of 90:10 acetonitrile (MeCN) : 6 N HCL was prepared and two ml was added to each tube. This mixture was mixed twice for 30 seconds and the glass tubes were capped tightly and placed into a 100˚C oven for 45 minutes. The tubes were then cooled to room temperature. Meanwhile, a 90:10 mixture of methanol (MeOH): sodium hydroxide (NaOH) was prepared and two ml was placed into each tube. Each tube was mixed twice for 30 seconds and capped tightly and placed in a 100˚C oven. After 45 minutes the tubes were cooled to room temperature, 350 µl 6 N HCL and 2 ml of hexane were added to

each tube, and mixed twice for 30 seconds and spun at 2100 RPM for 10 minutes. The hexane layer in each tube was transferred to a clean conical 13 x 100 mm tube (prewashed with HCL and hexane). Then, all tubes were dried under nitrogen. A 90:10 mixture of MeCN: pentaflorabenzene (PFB) was prepared and 50 µl of this mixture was placed in each tube. Then, 10 µl of triethylamine was added to each tube and mixed twice for 30 seconds. The tubes were allowed to stand at room temperature for 30 minutes and then 150 µl of 0.1 N HCL and 1 ml of hexane were added to each tube and mixed twice for 30 seconds. Tubes were spun at 600 rpm for 10 minutes. The top layer of hexane in each tube was transferred to a clean 13 x 100 round bottom tube and allowed to dry under nitrogen. Once the tubes were dried, 1 ml of hexane was added to each tube and mixed. Gas chromatography vials were then filled with 250 µl of the mixture from each tube and loaded into a DSQ II Single Quadrupole gas chromatographer/mass spectrometer (GCMS) (Hewlett-Packard 6890 with 5973 mass selective detector, Palo Alto, CA). The GCMS was programmed to operate in the negative ion chemical ionization mode with methane as the reagent gas. Fatty acid PFB-esters were separated on a DB-5mx capillary column (30 m x 0.25 mm x 0.25 µm film thickness; ThermoFisher Scientific, Inc., Waltham, MA) with helium as the carrier gas.

The fatty acids analyzed included: C14:0, C14:1, C16:0, C16:1, C18:0, C18:1, C18:2, C18:3, C20:4, C20:5 and C22:6, and were detected using selected ion monitoring for the corresponding free fatty acid resulting from the loss of PFB. Each fatty acid was matched to the deuterated internal standard closest in the retention time. Peak area ratios of known amounts of standard fatty acids and the internal standards were used to generate

calibration curves to quantify fatty acids in the plasma samples using Xcalibur software (ThermoFisher Scientific, Inc., Waltham, MA). Individual fatty acid peaks were compared to internal standards of known concentration and identified by molecular mass and retention time. The sums of saturated (C8:0, C10:0, C14:0, C16:0, C18:0), monounsaturated (C14:1, C16:1, C18:1), polyunsaturated (C18:2, C18:3, C20:4, C20:5, C22:6), omega-3 fatty acids (C18:3, C20:5, C22:6) and omega-6 fatty acids (C18:2, C20:4) were calculated for each sample at each time point by adding together absolute concentrations of respective individual fatty acids within each summed group. Fatty acid concentrations were reported in µmol/L.

Statistical Analysis

T-tests and correlation analyses were conducted with weight loss and weight regain as variables of interest (outcomes) and each plasma fatty acid concentration at each time point as the predictor variables. T-test analyses were also conducted with insulin sensitivity and plasma fatty acid concentrations at each time point as predictor variables. Weight loss and weight regain were analyzed separately for their correlations with plasma fatty acid concentrations.

A p-value of < 0.05 was considered significant for all performed tests. Data were analyzed using Stata, version 10.0 (College Station, TX). The statistical tests that were used in the study included: T-tests to determine the significance of differences in weight (kg) between baseline and week eight, baseline and week 12 and week eight and week 12 and to determine the significance of differences in plasma fatty acid concentrations at

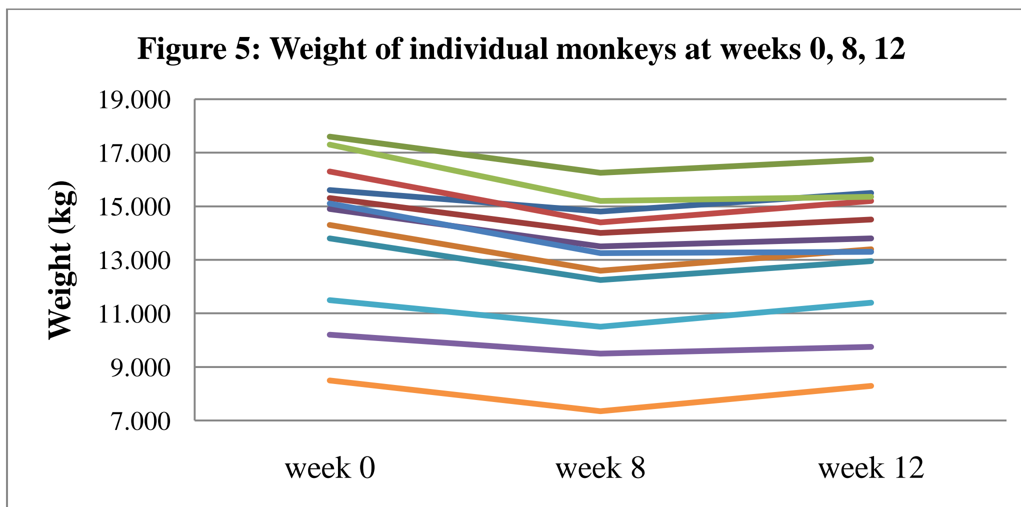
each time point. Spearman correlations were performed between the amount of weight lost at week eight and the amount of weight regained at week 12 and each plasma fatty acid concentration; and the sum of omega-3 fatty acid concentrations (α-linolenic acid, DHA, EPA), omega-6 fatty acid concentrations (linoleic acid, and AA), monounsaturated fatty acid concentrations (myristoleic acid, palmitoleic acid, and oleic acid), and saturated fatty acid concentrations (myristic acid, palmitic acid, and stearic acid). Spearman correlations were also performed to determine the relationship between insulin sensitivity and plasma saturated fatty acid (myristic acid, palmitic acid, and stearic acid) and omega-6 fatty acid concentrations (linoleic acid, and AA) at week eight (after weight loss) and week 12 (after weight regain).

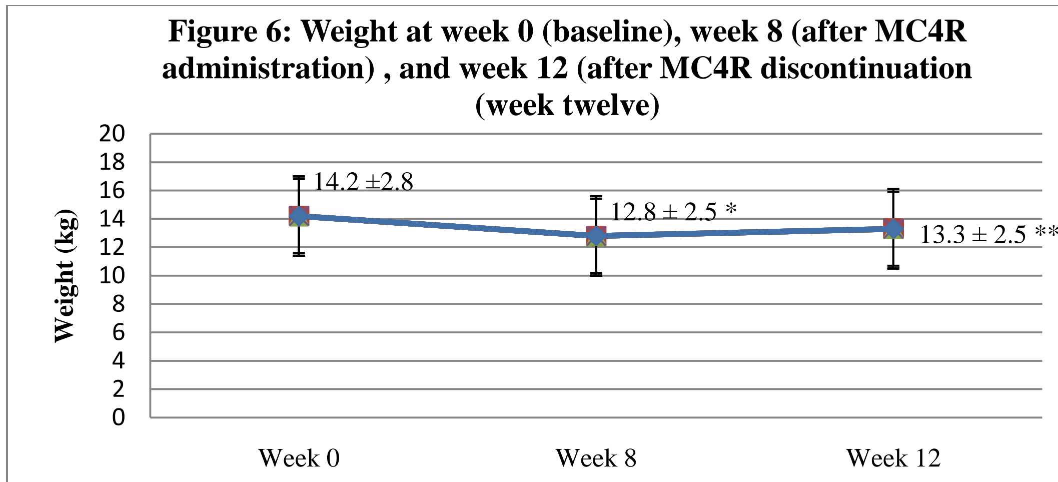
CHAPTER FOUR

Results

Weight

The monkeys lost an average of 9.9% of baseline weight and a weight loss rate of 0.06 ± 0.04 kg per week. The monkeys regained an average of 0.55 ± 0.30 kg (95% CI: 0.36 - 0.74 kg) during the four weeks after the MC4R agonist was discontinued. The average weight at week 12 was 13.4 ± 2.5 kg. This represented an average rate of weight gain of 0.14 ± 0.07 kg per week (p = 0.001) and an average increase of 4.3% of body weight.of their baseline weight after the administration of MC4R agonist at week eight and gain an average of 4.3% after the discontinuation of the MC4R agonist at week 12. The monkeys weighed 14.2 ± 2.8 kg at baseline and 12.8 ± 2.5 kg at week eight (p = 0.001). All monkeys lost weight during the eight weeks of MC4R agonist administration. The range of weight loss was (0.8 – 2.1 kg) (Figure 5). The average weight loss was 1.4 ± 0.44 kg (95% CI: 1.12-1.68 kg) after eight weeks of MC4R agonist administration compared to baseline weight (Figure 6).

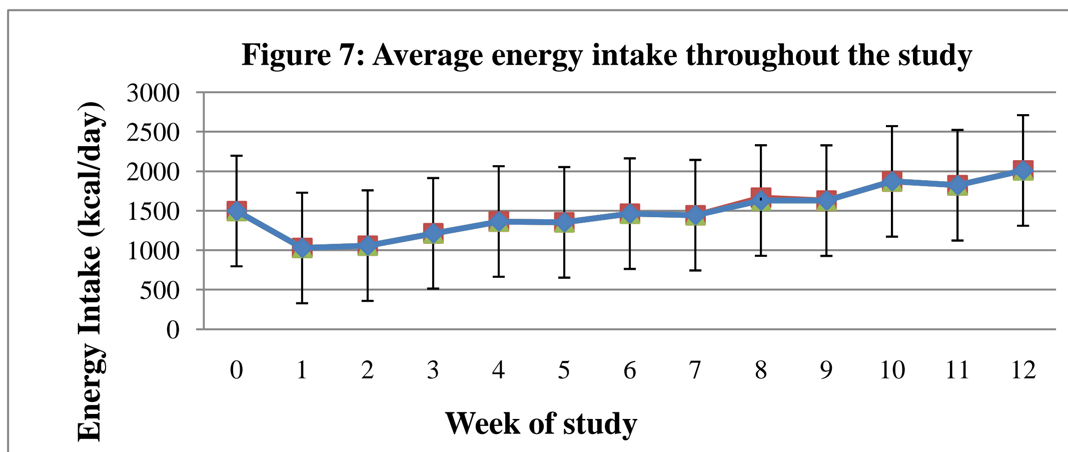




Mean ± SD
*** p-value =0.0001 for the difference in weight at week 0 and week eight.**
**** p-value=0.0001 for the difference in weight at week eight and week 12.**

Food Intake

The monkeys consumed an average of 1495 ± 842 kcal/day at baseline (Figure 7). During the first three weeks of MC4R agonist treatment, the monkeys consumed less energy, on average, than at baseline. During the last three weeks of MC4R agonist treatment (weeks 5 – 8), the monkeys consumed more energy, on average, than at baseline (1628 ± 640 kcal/day). During the four weeks after the MC4R agonist was discontinuated (weeks 9 – 12), the monkeys consumed an average of 2009 ± 630 kcal/day.



Mean ± SD

Plasma Fatty Acid Concentrations

Plasma Polyunsaturated Fatty Acid Concentrations

Table 2 summarizes the average concentrations of omega 3, α-linolenic acid, DHA, and EPA at week 0, 8 and 12. There was no significant difference in the average polyunsaturated fatty acid concentration at week 0, baseline and week 8, after weight loss [$p = 0.15$, 95% CI: -2527,-439 (μmol/L)]. Nor were there significant differences between the total polyunsaturated fatty acid concentrations at week 8 (after weight loss) and week 12 (after weight regain) [$p = 0.55$, 95% CI: -1975, 1116 (μmol/L)]. The average concentrations of total omega-3 fatty acids [$p = 0.48$, 95% CI: -169, 85 (μmol/L)], α-linolenic acid [$p = 0.11$, 95% CI: -97, 10 (μmol/L)], EPA [$p = 0.87$, 95% CI: -55, 48 (μmol/L)], and DHA [$p = 0.73$, 95% CI: -26, 37 (μmol/L)] were similar before (week 0) and after weight loss (week 8). In addition, the average concentrations of total omega-3 [$p = 0.21$, 95% CI: -36, 148 (μmol/L)], α-linolenic acid [$p = 0.32$, 95% CI: -25, 70 (μmol/L)], EPA [$p = 0.43$, 95% CI: -6.9, 47.3 (μmol/L)], and DHA [$p = 0.13$, 95% CI: -23, 50 (μmol/L)] were similar before (week 8) and after weight regain (week 12).

Table 2 also summarizes the average fasting plasma concentrations of total omega-6, linoleic acid and arachidonic acid at week 0, 8 and 12. There were no significant differences between the total omega-6 [$p = 0.15$, 95% CI: -2445, 440 (μmol/L)], linoleic acid [$p = 0.19$, 95% CI: -2288, 525 (μmol/L)] and arachidonic acid [$p = 0.73$, 95% CI: -881, 639 (μmol/L)] between week 0 and week 8. In addition, there were no significant differences between the average fasting plasma concentrations of total omega-6 [$p = 0.49$, 95% CI: -1999, 1028 (μmol/L)], linoleic acid [$p = 0.35$, 95% CI: -2242,

872(μmol/L)] and arachidonic acid [p = 0.29, 95% CI: -197, 596 (μmol/L)] at week 8 and week 12. There was no significant difference in the omega-6/omega 3 fatty acid ratio at baseline (week 0) and after weight loss (week 8) [p = 0.5, 95% CI: -4.4, 2.3] or between week 8 and week 12 (after weight regain) [p = 0.1, 95% CI: -5.67, 0.65].

Table 2: Fasting plasma polyunsaturated fatty acid concentrations at week 0 (baseline), week 8 (after weight loss) and week 12 (after weight regain):

Fatty acid concentrations (μmol/L)	Week 0 (Baseline)	Week 8 (Weight Loss)	Week 12 (Weight Regain)
Polyunsaturated Fatty Acids	4284 ± 1660 (2352 - 7102)	5328 ± 2393 (1626 - 9627)	5757 ± 2137 (2492 - 10726)
Omega 3 Fatty Acids	407 ± 160 (294 - 803)	449 ± 160 (255 - 754)	392 ± 61 (306 - 509)
α- linolenic acid (C18:3)	77 ± 46 (35 - 187)	120 ± 78 (19 - 298)	97 ± 41 (22 - 163)
EPA[1] (C20:5)	142 ± 66 (85 - 307)	146 ± 56 (84 - 259)	132 ± 27 (92 - 183)
DHA[2] (C22:6)	187 ± 53 (152 - 308)	182 ± 50 (151 - 322)	162 ± 9 (152 - 184)
Omega 6 Fatty Acids	38767 ± 1566 (2054 - 6517)	4879 ± 2294 (1370 - 8924)	5364 ± 2093 (2186 - 10290)
linoleic acid (C18:2)	3092 ± 1344 (1737 - 6138)	3974 ± 1859 (1248 - 7829)	4659 ± 1987 (2001 - 9560)
AA[3] (C20:4)	784 ± 978 (233 - 3731)	905 ± 709 (122 - 2340)	706 ± 53 (152 - 308)
omega-6/omega-3 ratio	10 ± 3 (5 - 19)	11 ± 4 (5 - 17)	13 ± 4 (7 - 23)

* mean ± SD (range)
[1] EPA: eicosapentaenoic acid [2] DHA: Docosahexaenoic acid. [3]AA: Arachidonic acid

Plasma Monounsaturated Fatty Acid Concentrations

Table 3 summarizes the average monounsaturated fatty acid concentrations at weeks 0, 8 and 12. There were no significant differences in concentrations of total monounsaturated fatty acid [p = 0.47, 95% CI: -4611,2286], oleic acid [p = 0.45, 95% CI: -4589, 2197] palmitoleic acid [p = 0.54, 95% CI: -84, 151], and myristolic acid [p = 0.43, 95% CI: -0.54, 1.2] between week 0 (baseline) and week 8 (weight loss). In addition, there were no

significant differences in concentrations of total monounsaturated fatty acids concentrations [p = 0.25, 95% CI: -988, 3394], oleic acid [p = 0.18, 95% CI: -722, 3427], palmitoleic acid [p = 0.08, 95% CI: -319, 26], and myristolic acid [p = 0.27, 95% CI: -7.6, 2.3] at week 8 (weight loss) and week 12 (weight regain).

Table 3: Fasting plasma monounsaturated fatty acid concentrations at weeks 0, 8 and 12

Plasma fatty acid concentrations (μmol/L)	Week 0 (Baseline)	Week 8 (Weight loss)	Week 12 (Weight regain)
Monounsaturated fatty acids	4594 ± 3983 (2039 - 15938)	5757 ± 4690 (2205 - 18701)	4553 ± 2371 (1076 - 10298)
Myristolic acid (C14:1)	11 ± 0.81 (10 - 13)	11 ± 0.79 (10 - 12)	13 ± 8 (10 - 38)
Pamitoleic acid (C16:1)	356 ± 169 (105 - 712)	322 ± 190 (89 - 734)	469 ± 288 (106 - 1029)
Oleic acid (C18:1)	4227 ± 3972 (1853 - 15563)	5423 ± 4594 (1531 - 17955)	4071 ± 2385 (1239 - 9976)

* mean ± SD (range).

Plasma Saturated Fatty Acid Concentrations

Table 4 summarizes the saturated fatty acid concentrations at weeks 0, 8 and 12. There are no significant differences in average concentrations of total saturated fatty acid [p = 0.60, 95% CI: -1218, 2000], palmitic acid [p = 0.72, 95% CI: -1150, 1612], or stearic acid [p = 0.84, 95% CI: -447, 538] between week 0 and week 8. On the other hand, there was a significant difference in the average myristic acid concentration between week 0 and week 8 [p = 0.003, 95% CI: 46, 182]. There are also significant differences in

average concentrations of total saturated fatty acid [p = 0.01, 95% CI: -2306, -267], stearic acid [p = 0.02, 95% CI: -1550, -140], and myristic acid [p <0.001, 95% CI: -401, -154] between week 8, after weight loss, and week 12, after weight regain. There was no significant difference in palmitic acid concentration between week 8 and week 12 [p = 0.72, 95% CI: -1165, 838].

Table 4: Fasting plasma saturated fatty acid concentrations at weeks 0, 8 and 12

Plasma fatty acid concentrations (µmol/L)	Week 0 (Baseline)*	Week 8 (Weight Loss)*	Week 12 (Weight Regain)*
Saturated fatty acids	6477 ± 2050 (4026 - 11557)	6086 ± 2255 (3124 - 11596)	7373 ± 1934[2] (3679 - 10013)
Myristic acid (C14:0)	375 ± 123 (160 - 615)	261 ± 183[1] (6 - 640)	539 ± 262[1] (48 - 808)
Palmitic acid (C16:0)	3183 ± 1535 (1881 - 7202)	2952 ± 1572 (1746 - 7659)	3116 ± 781 (1801 - 4448)
Stearic acid (C18:0)	2917 ± 778 (1751 - 4274)	2872 ± 887 (1372 - 3863)	3717 ± 1345[2] (1169 - 5779)

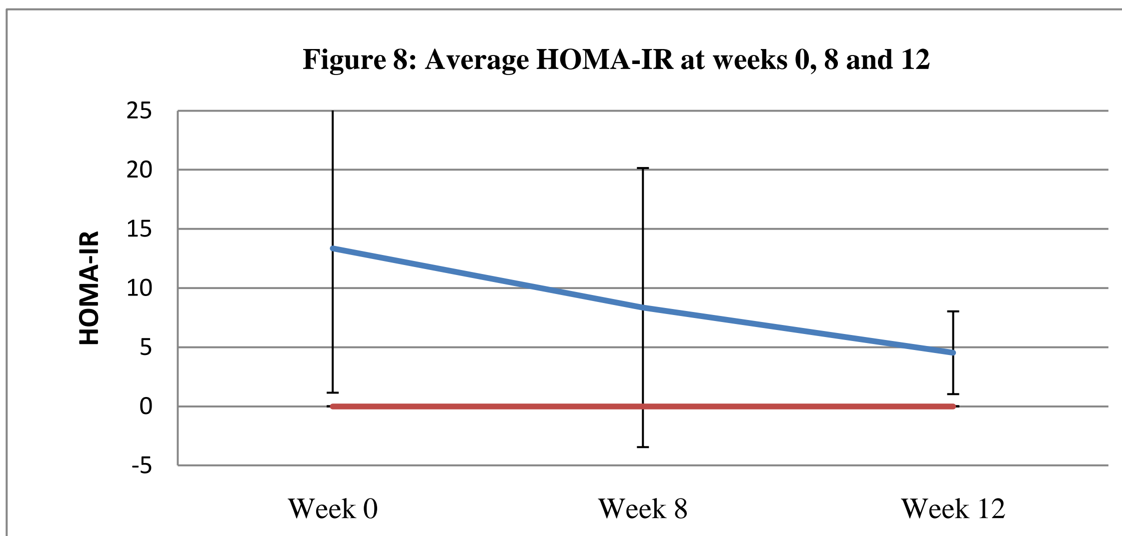
* mean ± SD (range)
[1] p-value < 0.001
[2] p-value < 0.02

Triglyceride Concentrations

The average triglyceride concentration was 57 ± 26 mg/dl at week 0, 28 ± 14 mg/dl at week 8, and 61 ± 37 mg/dl at week 12. Plasma triglyceride concentrations were significantly different between week 0 and week 8 [p = 0.003] and between week 8 and week 12 [p = 0.007].

Insulin Sensitivity

Insulin sensitivity estimated by homeostatic model assessment of insulin resistance (HOMA-IR) was 13 ± 11 at week 0, 8 ± 11 at week 8, and 4 ± 2 at week 12. There was a significant increase in insulin sensitivity as indicated by a decrease in HOMA-IR from week 0 to week 8 [p = 0.03, 95% CI: 0.59-9]. On the other hand, there was no significant difference in insulin sensitivity between week 8 and week 12 [p = 0.27, 95% CI: -3 - 11] (Figure 8). There was a much smaller standard deviation at week 12 than at the other time points. This observation maybe explained by the results of two monkeys who had unexpected increases in HOMA-IR with weight loss and then a decrease in HOMA-IR with weight regain. A third monkey had a very high HOMA-IR level of 42 at week 0.



*p-value = 0.03 for the difference from week 0 and week 8.

The HOMA-IR level in this monkey increased to 44 after weight loss at week 8 and then decreased significantly to 5 after weight regain at week 12. This increase in HOMA-IR at weight 8 was due to an increase in fasting insulin concentration after weight loss. The decrease in HOMA-IR at week 12 was due to the significant decrease in fasting insulin

after weight regain. The fasting glucose concentration also decreased in this monkey.

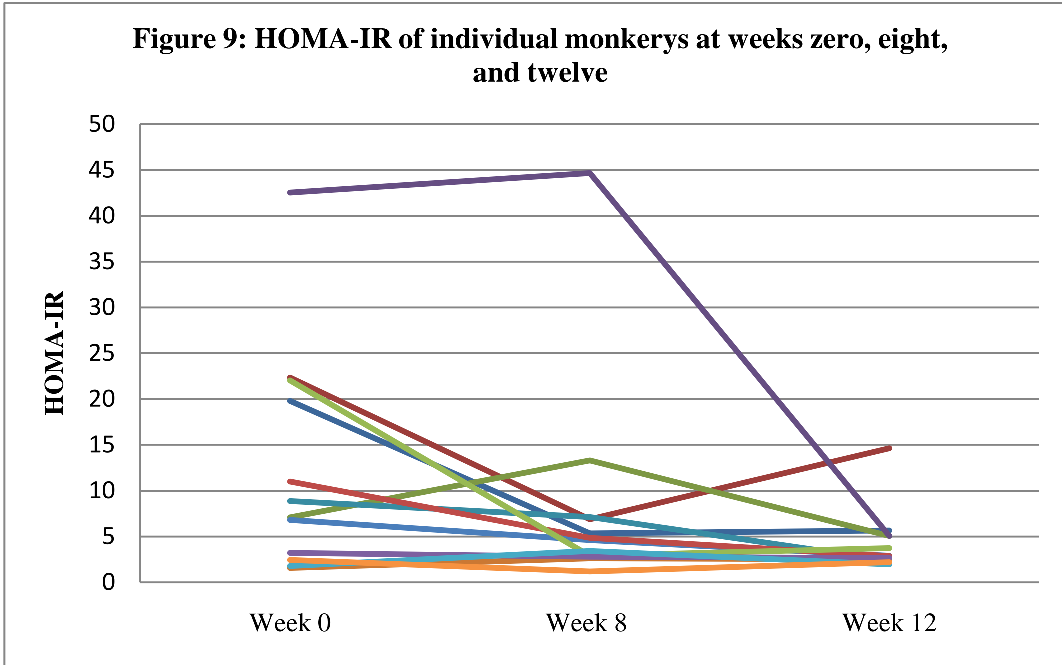


Figure 9: HOMA-IR of individual monkerys at weeks zero, eight, and twelve

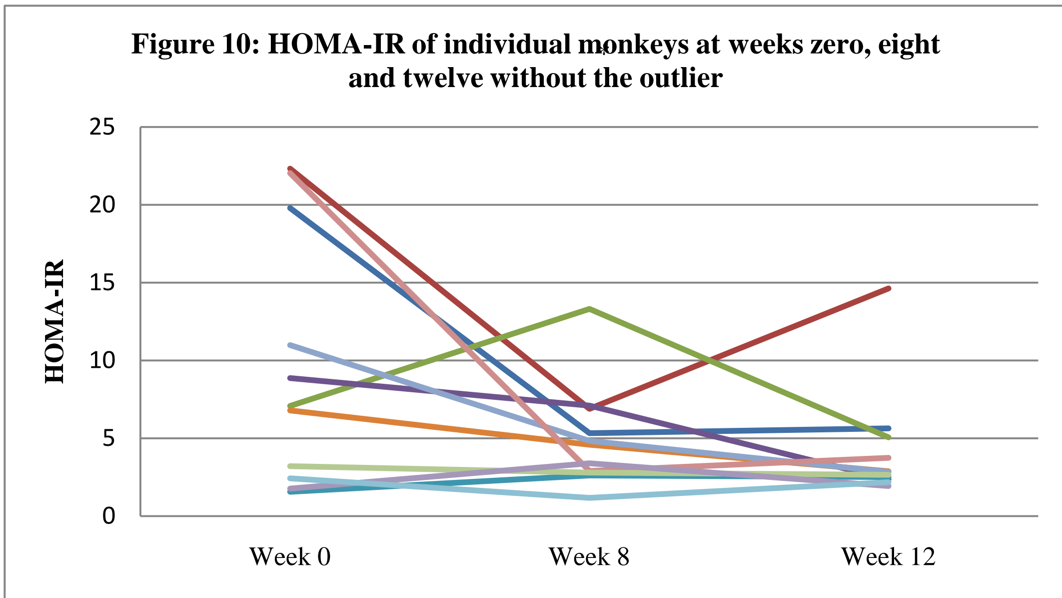
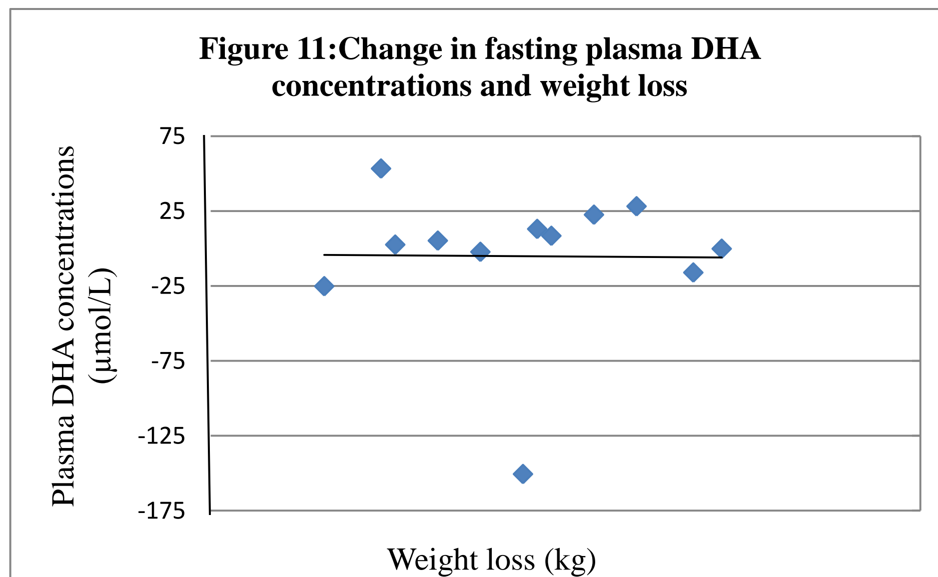


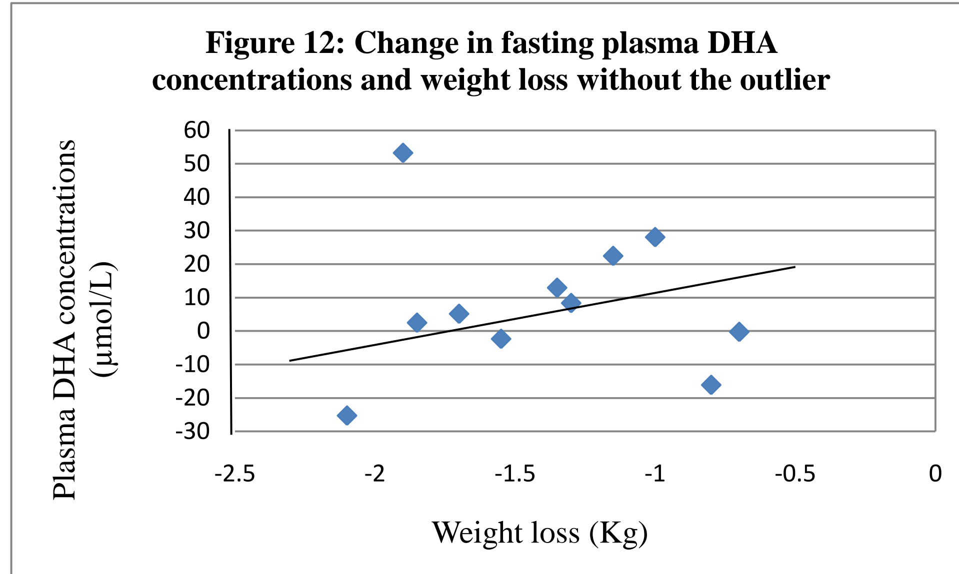
Figure 10: HOMA-IR of individual monkeys at weeks zero, eight and twelve without the outlier

Correlational Analysis

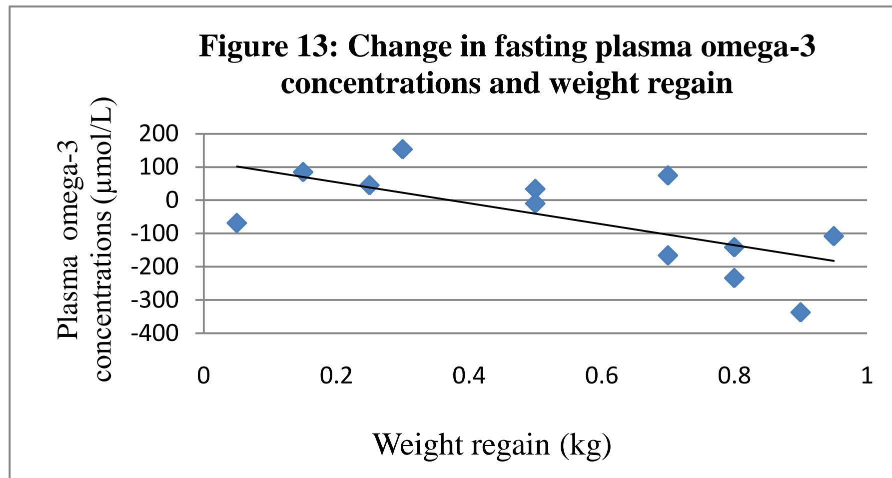
Change in Fasting Plasma Polyunsaturated Fatty Acid Concentrations and Change in Weight

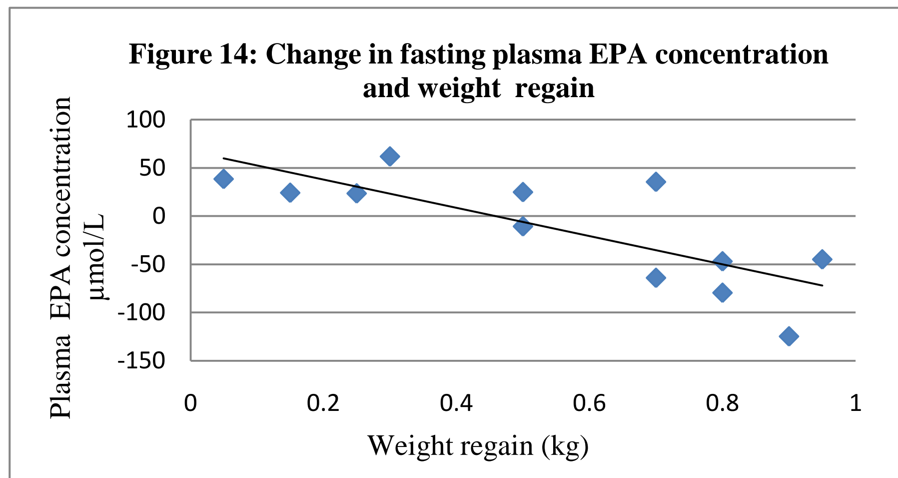
There was no correlation between the change in fasting plasma total polyunsaturated fatty acid concentration and change in weight after weight loss (Spearman -0.09, $p = 0.76$). When fatty acid concentrations were considered individually, there was a weak correlation between the change in plasma DHA concentration (Spearman -0.63, $p = 0.03$) and change in weight after weight loss when one outlier (-150.64 µmol/L) was removed (Figure 11 and 12). This correlation suggests that with greater weight loss, fasting plasma DHA concentration increases. There were no significant correlations between change in weight and EPA (Spearman -0.16, $p = 0.62$), α-linoleic acid (Spearman 0.24, $p = 0.44$), arachidonic acid (Spearman 0.22, $p = 0.48$), linoleic acid (Spearman -0.33, $p = 0.29$), omega-6 (Spearman -0.13, $p = 0.68$) and omega-3 fatty acid concentration (Spearman -0.02, $p = 0.95$).





There were no significant correlations between the changes in fasting plasma total polyunsaturated fatty acid concentrations (Spearman -0.42, p = 0.17), or omega-6 concentration (Spearman -0.41, p = 0.18) and weight regain. On the other hand, there was a weak correlation between the change in fasting plasma omega-3 concentration and weight regain (Spearman -0.67, p = 0.02) (Figure 13). This correlation suggests that greater weight regain is associated with lower fasting plasma omega-3 concentration. Additional studies are needed to confirm this finding. When investigating the change in each fatty acid concentration individually, there was a correlation between the change in fasting plasma EPA concentration (Spearman -0.72, p = 0.007) and weight regain (Figure 14). On the other hand, there was no correlation between fasting plasma DHA (Spearman -0.46, p = 0.12), α-linoleic (Spearman -0.43, p = 0.15), arachidonic acid (Spearman -0.20, p =0.53), or linoleic acid concentrations (Spearman -0.42, p = 0.17) and weight regain.





Change in Fasting Plasma Monounsaturated Fatty Acid Concentration and Change in Weight

There were no correlations between changes in fasting plasma total monounsaturated fatty acid concentrations and weight loss (Spearman 0.26, p = 0.40) or plasma oleic acid (Spearman 0.26, p = 0.40), plasma palmitoleic acid (Spearman 0.07, p = 0.83), or plasma myristoleic acid concentrations (Spearman 0.42, p = 0.17) and weight loss. Nor were there any correlations between the changes in fasting plasma total monounsaturated fatty

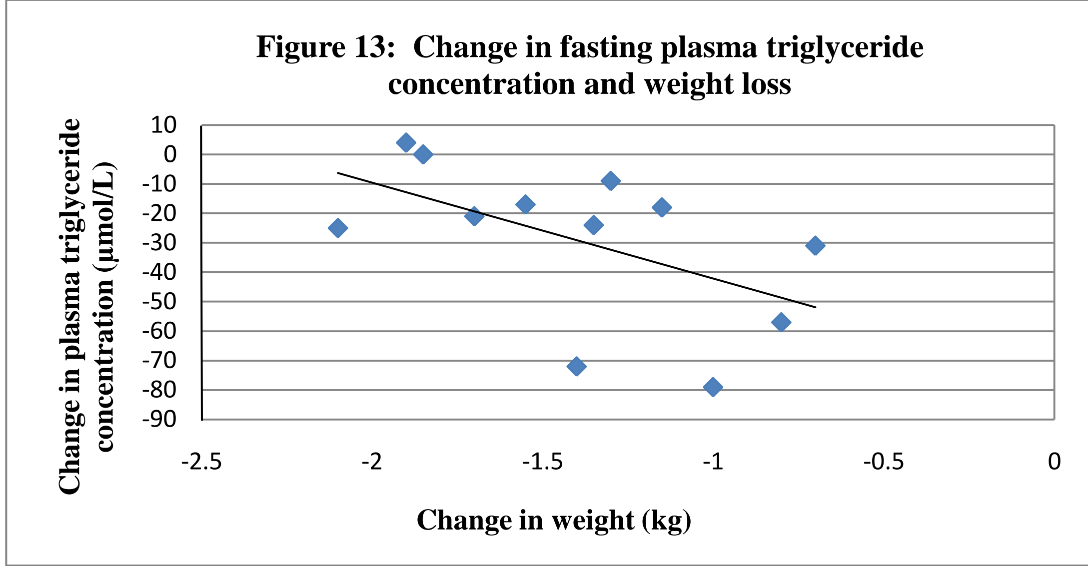
acid concentrations and weight regain (Spearman -0.40, p = 0.19) or plasma oleic acid (Spearman -0.42, p = 0.17) plasma palmiloleic acid (Spearman -0.003, p =0.99), or plasma myristoleic acid concentrations (Spearman 0.10, p =0.74) and weight regain.

Changes in Fasting Plasma Saturated Fatty Acid Concentrations and Change in Weight

There were no correlations between changes in fasting plasma total saturated fatty acid concentrations and weight loss (Spearman 0.32, p =0.31) or plasma myristic acid (Spearman 0.05, p =0.87), plasma palmitic acid (Spearman 0.16, p =0.60), plasma stearic acid concentrations (Spearman 0.14, p =0.66), and weight loss. Nor were there any correlations between change in fasting plasma total saturated fatty acid concentrations and weight regain (Spearman -0.29, p= 0.34), or plasma myristic acid (Spearman -0.17, p =0.60), plasma palmitic acid (Spearman -0.32, p =0.32), or plasma stearic acid concentrations (Spearman -0.26, p =0.41) and weight regain.

Change in Fasting Plasma Triglycerides and Change in Weight

There was a trend towards a negative correlation between the change in plasma triglyceride concentration and weight loss (Spearman -0.52, p =0.08) (Figure 13). This trend suggests that triglyceride concentrations decrease more with greater weight loss. In addition, there was no correlation between the change in plasma triglyceride concentration and weight regain (Spearman 0.06, p =0.85).



Insulin Sensitivity and Change in Weight

There was no correlation between the change in insulin sensitivity and weight loss (Spearman 0.06, p =0.84) nor was there a correlation between the change in insulin sensitivity and weight regain (Spearman 0.04, p =0.90). Furthermore, there was no correlation between the change in insulin sensitivity and the change in plasma saturated fatty acids or omega-6 fatty acid concentrations after weight loss or weight regain.

Table 5: Correlations between the change in fasting plasma fatty acid concentrations and weight loss and weight regain

Variables	Weight loss (week 8) Correlation	p-value	Weight regain (week 12) correlation	p-value
Polyunsaturated fatty acids	-0.09	0.76	-0.42	0.17
Omega 3	-0.02	0.94	0.67	0.02
α- linolenic acid (C18:3)	0.24	0.44	-0.43	0.15
EPA[1] (C20:5)	-0.16	0.62	-0.72	0.007
DHA[2] (C22:6)	-0.63	0.03	-0.46	0.12
Omega 6	-0.13	0.68	-0.41	0.18
linoleic acid (C18:2)	-0.33	0.29	-0.42	0.17
AA[3] (C20:4)	0.22	0.48	-0.20	0.17
Omega-6/omega-3 ratio	-0.17	0.59	-0.05	0.88
Saturated fatty acids	0.32	0.31	-0.29	0.34
Myristic acid (C14:0)	0.05	0.87	-0.17	0.6
Palmitic acid (C16:0)	0.16	0.6	-0.32	0.31
Stearic acid (C18:0)	0.14	0.66	-0.26	0.41
Monounsaturated fatty acids	0.26	0.40	-0.40	0.19
Palmitoleic acid (C16:1)	0.07	0.83	-0.003	0.99
Myristolic acid (C14:1)	0.42	0.17	0.10	0.74
Oleic acid (C18:1)	0.26	0.40	-0.42	0.17
Triglyceride	-0.52	0.08	0.06	0.85
Insulin Sensitivity (HOMA-IR)	0.06	0.84	0.04	0.90

[1] EPA: Eicosapentaenoic acid
[2] DHA: Docosahexaenoic acid
[3] AA: arachidonic acid.

Percent Change Between Baseline and Weight Loss Periods

Table 6 summarizes the average percent change of fasting plasma total polyunsaturated, omega-3, omega-6, monounsaturated, and saturated fatty acids from baseline (week 8), and from weight loss (week 12). There was a 37.8 ± 89.4% increase in the total polyunsaturated fatty acid concentration after weight loss which is not considered clinically relevant [95% CI: -19,95].There was 23.2 ± 52% increase in total polyunsaturated fatty acid concentrations after weight regain which is not considered clinically relevant [95% CI: -10,56]. There was 2.7 ± 37% decrease in the total saturated fatty acid concentration after weight loss which is not considered clinically relevant [95% CI: -26,21].There was 27 ± 31% increase in total saturated fatty acid concentrations after weight regain which is not considered clinically relevant [95% CI: 7,47]. There was 58 ± 109% increase in the total monounsaturated fatty acid concentration after weight loss which is not considered clinically relevant [95% CI: -12,127].There was 3.8 ± 59% increase in total monounsaturated fatty acid concentrations after weight regain which is not considered clinically relevant [95% CI: -33,41].

In most of the fatty acids, the percent change with weight loss and weight regain was not clinically significant. Thus, we conclude that in this particular model, weight loss and weight regain did not result in clinically relevant changes in fasting plasma fatty acid concentrations.

Table 6: Percent change in fasting plasma fatty acid concentrations after weight loss and weight regain

Fatty acid concentrations (%)	Percent change from baseline (week 8)	Percent change from weight loss (week 12)
	Mean ± SD (95% CI)	Mean ± SD (95% CI)
Polyunsaturated Fatty Acids	38 ± 89 (-19 , 95)	23 ± 52 (-10 , 56)
Omega 3	18 ± 45 (-10 , 47)	-3.6 ± 29 (-23 , 14)
α-linolenic acid (C18:3)	87 ± 145 (-5 , 179)	23 ± 106 (-44 , 90)
EPA[1] (C20:5)	14 ± 51 (-18 , 47)	0.87 ± 36 (-22 , 23)
DHA[2] (C22:6)	-0.31 ± 18 (-12 , 11)	-8 ± 14 (-16 , 1)
Omega 6	41 ± 98 (-21 , 104)	27 ± 57 (-9 , 63)
linoleic acid (C18:2)	51 ± 111 (-20 , 121)	36 ± 65 (-5 , 77)
AA[3] (C10:4)	96 ± 186 (-21 , 214)	13 ± 65 (-29 , 54)
Saturated fatty acids	-3 ± 37 (-26 , 21)	27 ± 31(7 , 47)
Myristic acid (C14:0)	-37 ± 36 (-59 , -14)	225 ± 237 (74 , 375)
Palmitic acid (C16:0)	7 ± 77 (-42 , 56)	18 ± 42 (-8 , 45)
Stearic acid (C18:0)	0.22 ± 28 (-18 , 18)	33 ± 46 (4 , 62)
Monounsaturated fatty acids	58 ± 109 (-12 , 127)	4 ± 59 (-33 , 41)
Pamitoleic acid (C16:1)	-2 ± 70 (-46 , 43)	55 ± 68 (12 , 99)
Myristoleic acid (C14:1)	-2 ± 12 (-10 , 5)	25 ± 73 (-21 , 71)
Oleic acid (C18:1)	65 ± 119 (-10 , 141)	0.05 ± 60 (-38 , 38)

* mean ± SD

[1] EPA: eicosapentaenoic acid 2. DHA: Docosahexaenoic acid 3. AA: Arachidonic acid

In summary, the monkeys lost weight after 8 weeks of MC4R agonist and gained weight after the MC4R agonist was discontinued. There were no significant differences in fasting plasma polyunsaturated, monounsaturated, and saturated fatty acid concentrations at baseline, after weight loss and after weight regain except for myristic acid. There was a significant difference in plasma triglycerides concentrations between baseline, after weight loss, and after weight regain. There was a significant difference in HOMA-IR at baseline and after weight loss which indicated an increase in insulin sensitivity. On the other hand, there was no significant differences in HOMA-IR after weight loss and after

weight regain. There was no correlation between weight change and change in fasting plasma polyunsaturated, monounsaturated, and saturated fatty acid concentrations. There was a weak negative correlation between weight loss and change in plasma DHA concentration which suggests that with greater weight loss, plasma DHA concentrations increase. Also, there was weak positive correlation between change in total omega-3 and weight regain which suggests that with greater weight regain, total plasma omega-3 concentrations increase. The strongest negative correlation was between weight regain and change in fasting plasma EPA concentration, which suggest that with greater weight regain, plasma EPA concentration decreased.

CHAPTER FIVE

Discussion

The objective of this study was to investigate the effect of weight change on fatty acid concentrations and insulin sensitivity. This study provided an exclusive opportunity to gain new insights into physiological changes occurring both during weight loss and weight regain. In this analysis, we reported eight weeks of weight loss induced by a MC4R agonist followed by four weeks of weight regain in 12 monkeys. During the first three weeks of administering a MC4R agonist, the monkeys ate 33% less than they ate at baseline. During the last four weeks of the weight loss period, the monkey's food intake was similar to the baseline intake. The monkey's diet caused weight gain and a decrease in insulin sensitivity in nine of the monkeys (75%); three monkeys did not develop obesity or insulin resistance and as result they considered diet resistant). The monkeys lost 9.9% of their initial weight. The results were consistent with previous studies in which weight change alone does not affect the fatty acid profiles (3,4,27,40). One of the key points of the study was that the monkeys consumed the same diet composition throughout the study, although the quantity consumed varied as a result of the effects of the MC4R agonist. There was no significant change in fatty acid profiles throughout the study. A high fat monkey chow (31.9% calories from fat) was provided to the monkeys before the study began. Administration of a MC4R agonist changed energy balance, but based on our results, we conclude that the drug had no major effect on the fatty acid profiles. Our data suggests change in diet composition is the driving factor for altering fasting fatty acid profiles. Moreover, there was no significant difference in calorie intake between the changes in diet resistant monkeys and the diet sensitive monkeys. During the

8 weeks of weight loss induced by MC4R agonist intervention, the only significant change in fatty acid concentrations was a decrease in the myristic acid concentration.

The Study of the Effects of Diet on Metabolism and Nutrition (STEDMAN) Project, analyzed plasma fatty acids during weight loss in humans. There were 27 subjects (median age 51, BMI: 32.6 kg/m^2) enrolled in a 12 month experiment. The experiment included six months of weight loss intervention, followed by six months of follow-up observations (38). During the first six months, all subjects were provided with a strong behavioral intervention and diet education by dieticians and specialists. The behavioral intervention consisted of 20 behavior change therapy sessions focused on decreasing caloric intake, and increasing energy expenditure through physical activity, and practicing the healthy eating habits of the Dietary Approaches to Stop Hypertension (DASH diet). After weight loss, the subjects were randomly selected to be in three different groups while continuing to lose weight. The first group was followed by a personal consultant by phone; the second group took part in an interactive website; the third group was used as the control group. The STEDMAN Project was part of a blinded trial of the Weight Loss Maintenance Study which was still in progress. Therefore, the STEDMAN Project researchers were blinded to subject group assignment at the time of this report. During the first six months of the experiment, the subjects lost an average of 6.3% of their initial weight, an average of 13.9 lbs ($p < 0.0001$) (38). During the weight loss intervention, positive dietary habits were practiced. For instance, fat intake was lower, and carbohydrate intake was higher. In addition, there were some changes in total fatty acid profiles, such as increases in saturated fatty acids concentrations especially in

palmitic acid and stearic acid. There was also an increase in the monounsaturated fatty acid, palmitoleic acid, concentrations after weight loss. On the other hand, there were no significant changes in polyunsaturated fatty acids concentration after weight loss and weight regain. The STEDMAN report did not provide any information about if the subjects successfully followed the DASH intervention. If the subjects did in fact follow the diet recommendations, the data supports the conclusion that changes in diet can lead to changes in the fatty acid profiles. The STEDMAN Project reported a decrease in free fatty acid concentrations and an increase in plasma saturated fatty acid concentrations after weight loss. In our study, there was no increase in saturated fatty acid concentrations and free fatty acids were not measured. On the other hand, there was a similarity between STEDMAN Project and our study in reporting no significant change in polyunsaturated fatty acid concentrations.

In the STEDMAN Project, insulin sensitivity improved significantly (p 0.007) with weight loss as measured by a decrease in HOMA-IR. Insulin sensitivity did not change with weight regain. This improvement suggests that the STEDMAN project's subjects were at least mildly insulin resistant in the beginning of the study and insulin sensitivity improved after the weight loss intervention. In our study, there were similar results. We observed a significant decrease in HOMA-IR after weight loss but no significant change after weight regain.

For several years, the monkeys followed the high fat diet and nine of monkeys became obese and insulin resistant. The monkeys continued on the high fat diet during MC4R

agonist treatment which could affect insulin sensitivity. Previous studies suggest that saturated fatty acids effect insulin sensitivity through changing the phospholipids membrane composition and fluidity, potentially altering the glucose transporter (GLUT4) activity (46). In our study, there were no significant changes in total plasma saturated fatty acid concentrations but there was a significant change in insulin sensitivity. There was no correlation between saturated fatty acid and insulin sensitivity in our study.

Several studies demonstrate obese subjects who lose weight improve their insulin sensitivity (41, 42, 43, 44). The improvement in insulin sensitivity occurs regardless of whether the subject consumes a high fat or a high carbohydrate weight loss diet (47). However, the mechanism behind the relationship between weight loss and insulin sensitivity is still unclear. According to Schenk et al (2009), the decrease in the mobilization of free fatty acids due to weight loss plays a significant role in the improvement in insulin sensitivity (45). We did not measure free fatty acids in our study. It is possible that the amount of mobilization of free fatty acids was the same before and after weight loss.

Another study done by Elizondo A, el at (2008), used bariatric surgery as the weight loss method, and followed subjects for three months post-surgery. Fourteen subjects enrolled in the study. The experimental group was obese subjects (BMI: 45.4 ± 2.2 kg/m^2) who underwent subtotal gastrectomy with a gastro-jejunal anastomosis in roux en-Y. The control group was made up of seven non-obese subjects (BMI of 22.4 ± 0.6 kg/m^2) who underwent anti-reflux surgery. Diet information was not provided for either group. The

subjects most likely followed a variable diet after the surgery. The experimental group lost an average of 21% of their initial weight and decreased their BMI by an average of 60% ($p < 0.05$) (39). In addition, there was a significant change in insulin sensitivity. However, the change in fatty acid profiles after the weight loss induced by bariatric surgery could be combined with a significant change in diet composition and changes in gut hormones such as ghrelin. Fatty acid profiles changed in the experimental group three months after the surgery. Total plasma polyunsaturated fatty acids, and plasma omega-3 fatty acid increased by 22, and 29% respectively. There was a decrease in the omega-6/omega-3 fatty acid ratio by 51%. There is the possibility that the changes in the fatty acid profile were due to the changes in dietary composition as well as the weight loss.

Ramel and his colleagues (2008) measured plasma fatty acids as a molar ratio (weight %) in subjects on four different energy-restricted diets; three were supplemented with omega-3 fatty acids and one was the control group (4). There were 278 subjects randomly allocated to four different diets. The diets varied in the amounts of omega-3 fatty acids. The subjects followed their instructed diet for eight uninterrupted weeks. The diets each consisted of 30% fat, 50% carbohydrate, and 20% total protein. The subjects were counseled how to follow a diet that was 30% less energy than their estimated energy requirements for weight maintenance (4). Participants in all four groups lost weight (-5.2±3.2 kg) and had increased circulating omega-3 fatty acid percent after weight loss. The weight loss groups supplemented with omega-3 fatty acids had higher levels of omega-3 compared to controls. Insulin sensitivity improved with weight loss in all groups.

Kunesova M and his colleagues (3) investigated fatty acid profiles (weight percent) with weight loss in two groups (an experimental group with a very low calorie diet, supplemented with omega-3 fatty acid concentrations and a control group on a very low calorie diet supplemented with a placebo) for three weeks. The first week, the outpatient baseline week, subjects maintained their weight. During the following three weeks, the inpatient weight loss intervention phase, the subjects received a controlled consistent diet. Total saturated fatty acids in serum lipids (phospholipids and triglyceride) decreased after the consumption of a very low calorie diet supplemented with omega-3 fatty acids. Omega-3 fatty acids (DHA and EPA) increased in the triglyceride fraction but EPA decreased in the phospholipids fraction. The omega-6 fatty acid, arachidonic acid increased in the phospholipid and triglyceride fractions. Moreover, the omega-3 supplemented group had a higher level of omega-3 compared to the control group. Furthermore, the study showed that changes in different sources of fatty acid, triglycerides or phospholipids, can impact the total plasma fatty acid profiles during weight loss. We did not separate plasma triglycerides and phospholipids in our study. It is possible weight loss differentially effects fatty acid composition in triglycerides and phospholipids. Kunesova and his colleagues measured the concentrations of plasma fatty acid profile broken down to triglycerides and phospholipids and noted the same differences. In our study, we reported the total plasma fatty acid profiles without differentiating if it came from triglycerides, phospholipids or cholesterol esters. Previous studies have shown that each fatty acid had a different concentration in each part of the plasma fatty acid profile. For instance, linoleic acid and oleic acid concentrations are

different in cholesterol esters compared to phospholipids (53,54).

An additional study, done by Hlavaty et al (27), investigated the effects of weight loss on fatty acid profiles by using a low-calorie diet supplemented with omega-3 fatty acids. The subjects were assigned randomly to a low-calorie diet with or without omega-3 fatty acid supplementation for three weeks. As with the previous trial; the group supplemented with omega-3 group had an increase in plasma omega-3 fatty acid concentrations compared to the control group. This suggests that omega 3 supplementation increased plasma omega-3 fatty acid weight percent greater than weight loss alone. In this study, the diet was consistent which may explain the non significant change in plasma omega-3 fatty acid concentrations observed among unsupplemented controls.

In a randomized controlled study conducted by Pieterse, et al (2005), these same findings were observed. In this study, 49 subjects were assigned to two different groups. The experimental group followed a calorically-restricted diet with monounsaturated fatty acids (avocado) and the control group restricted calories with no change in fat composition for six weeks (40). The diet for the experimental group included 200 grams of avocado per day for six weeks. Subjects followed one of two separate seven day menu plans. The diets were explained by a dietician. There was no significant difference in plasma fatty acid profiles between groups except for the oleic acid concentrations. Oleic acid concentration was higher in the group that consumed avocado. The study concluded that monounsaturated fatty acid supplementation but not weight loss increased plasma oleic acid concentrations. In this study, diet composition did not change and there was no

significant change in plasma fatty acid profiles. This is similar to our findings.

All of these studies investigated the effect of weight loss with or without dietary fat modification on plasma fatty acids. These studies reported a greater affect on fatty acid profiles when weight loss is coupled with dietary modification. In our study, the monkeys had the same high fat diet throughout the study. It is possible we observed no change in plasma fatty acid profiles because the diet was not modified during the course of the study. The monkeys decreased their food intake when the MC4R agonist was administered during the weight loss phase. The monkeys then returned to their normal amount of food intake at the end of the weight loss phase. In addition, the monkeys were hyperphagic after the weight loss phase, and actually ate more than what they were eating before the MC4R agonist started. This rebound hyperphasia may be due to physiologic signals to promote weight regain.

Another possible reason for our findings is investigating plasma fatty acids at only three time points (at baseline, after weight loss and weight regain) may not be enough to detect the effect of weight change on fatty acid profiles. According to the food intake data, the monkeys started to decrease their food intake after the administration of the MC4R agonist. Examining the plasma fatty acid profile during caloric restriction may give more details about the effect of weight change on the plasma fatty acids. Also during weight loss, postprandial blood samples may show some of the effect of weight change on postprandial fatty acid processing. The peak postprandial sample at baseline may differ from the peak postprandial sample after weight loss and be more indication of the effect

of weight change on plasma fatty acid profiles.

Our study and the studies described here, measured the change of fatty acid concentrations in plasma. Plasma fatty acids are a good biomarker to detect the effect of recent changes in dietary fat intake on whole body fatty acid concentrations. Katan et al, investigated different biomarkers including adipose tissue, and erythrocyte fatty acid profiles to detect the effect of dietary fatty acids on the body. The study concluded that the best way to measure the effect of changes in dietary fat intake was by measuring the plasma fatty acid profile (48). However, if the diet was consistent throughout the weight loss intervention, adipose tissue could be a better biomarker for changes in whole body fat composition than plasma. Perhaps analysis of adipose biopsies would have shown a difference in our monkeys with weight loss. The fatty acid composition of adipose tissue is a good long term (approximate time of six months to two years) biomarker of dietary fatty acids, due to the slow turnover time in adipose fatty acids (49, 50). Our monkeys were on the high fat diet for several years, which makes a sample of adipose tissue a good measurement. There are correlations between the dietary polyunsaturated omega-3, omega-6 and saturated fatty acid levels in adipose tissue (52). On the other hand, there is a weak correlation between dietary monosaturated fatty acids and monosaturated fatty acids in adipose tissue (52). Eight weeks of weight loss in the context of a monkey's lifespan is considered a long term weight loss compared to humans.

We did observe small non-significant increases in polyunsaturated fatty acid concentrations with weight loss, a small decrease in saturated fatty acid concentrations

with weight loss except for myristic acid concentrations, and a small increase in saturated fatty acid concentrations with weight regain. In addition, there was a correlation between weight loss and an increase in DHA concentrations which supports our hypothesis, and there was a correlation between weight regain and decrease in EPA. It is possible we did not observe significant differences because the study was underpowered with the small sample size, the small weight loss percentage of less than 10%, and the limited number of samples.

To summarize, in our study there were no significant changes in plasma fatty acid concentrations with weight change. There was no correlation between weight change and plasma fatty acid concentrations. Also, we observed improvement in insulin sensitivity as measured by HOMA-IR with weight loss but this change in insulin sensitivity was not correlated with change in plasma fatty acid concentrations. Our study was well controlled and provided a consistent diet that focused on the effect of the changes in weight on plasma fatty acid concentrations. In conclusion, our data suggests the change in plasma fatty acid concentrations are primarily mediated by changes in dietary fat intake rather than the change in total body weight.

CHAPTER SIX

STRENGTH AND LIMITATIONS

The study used a novel drug, a MC4R agonist, with promising results. The study also was well controlled including a constant diet, monkeys as subjects and quantitative analysis of the (μmol/L) fatty acids. The constant diet throughout the study and between monkeys removed the effects of differences in diet composition on weight loss and changes in fatty acid concentration. The fatty acid pool changes depended on the intake of fatty acids from the diet and from mobilization of endogenous fatty acid stores. In this study, the monkey's diet was constant and thus there was no change in the type of exogenous fatty acids consumed at any time point or between monkeys. There was a difference in the amount of fat consumed and endogenous fatty acid mobilized based on the fact that the monkeys lost weight. The concentration (μmol/L) method was used to measure plasma fatty acid profiles because this method was proposed to be more accurate in measuring changes in the fatty acid profile.

This study was conducted on monkeys; animal research should be generalized to humans with caution. In addition, with a sample size of only 12 monkeys, this study might be under powered and result in a risk of type II error (β error). β error is the error of failing to reject the null hypothesis when it is in fact false. In other words, this is the error of failing to detect a difference when in truth, there is a difference.

Moreover, after the MC4R agonist was administered, the monkeys ate 33% less than they ate at baseline and lost 9.9% of their initial body weight. For this reason, having more

than three blood samples collected would be more beneficial in examining the effect of weight change during active weight loss on plasma fatty acid profiles. Currently, animals are fed twice a day, at 9:30 am and again at 2:30 pm. The food is removed daily at 4.30 pm. The blood samples were collect in the morning before the monkeys were fed, providing an overnight fast. The idea of adding more blood samples after a meal is theoretically simple, however practically a lot more difficult. We need to sedate the animals to take blood, and sedating an animal twice a day is not acceptable. Ideally, the animals will be on a swivel-tether system, and have a continuous catheter placed for sampling. Blood sampling would then be possible as many times as the study needed without multiple sedations. On the other hand, this method is very difficult and very expensive.

SUMMARY

This study investigated the effect of weight loss, induced by a MC4R agonist, on plasma fatty acid profiles. Weight loss, in many cases, is known to lower the risk of many chronic diseases associated with obesity. The mechanism behind how weight loss lowers the risk of chronic disease can be looked at in many ways. One indication of how weight loss may lower disease risk is with a decrease omega-6/omega-3 fatty acid ratio. In our study, the omega-6/omega-3 fatty acid ratio didn't change with weight loss.

In conclusion, we attempted to answer our hypothesis does weight loss induce a selective reduction of saturated fatty acid and omega-6 fatty acids with retention of omega-3 fatty acids? Our data suggests that weight change alone with no change in diet composition

does not significantly affect fatty acid profiles. Diet composition appears to be the driving factor in changes of plasma fatty acid profiles.

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