THE ROLE OF FLAXSEED IN THE EXPRESSION OF DNA METHYLTRANSFERASES, LEPTIN, AND PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR ALPHA IN AN OBESE ANIMAL MODEL

A Thesis
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

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In Partial Fulfillment of the Requirements
for the Degree of
MASTER OF SCIENCE

Major Department:
Health, Nutrition, and Exercise Sciences

April 2015

Fargo, North Dakota

North Dakota State University
Graduate School

Title

The role of flaxseed in the expression of DNA methyltransferases, leptin, and peroxisome proliferator-activated receptor alpha in an obese animal model

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MASTER OF SCIENCE

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ABSTRACT

Diet-induced obesity (DIO) has been shown to increase DNA methyltransferase (DNMT) expression and DNMT binding at obesity-associated genes. Natural compounds have the potential to reverse obesity-associated gene expression via the regulation of DNA methylation. The objective of this study was to determine the effect of the bioactive compounds of flaxseed on DNMT and obesity-associated gene expression. Sixty C57BL/6J male mice were randomly assigned into one of the following diet groups and fed for eight weeks: 45% kcal fat; 45% kcal fat, 10% whole flaxseed; 45% kcal fat, 6% defatted flaxseed; 45% kcal fat, 4% flaxseed oil; and 16% kcal fat. DNMT1, DNMT3a, DNMT3b, leptin, and peroxisome proliferator-activated receptor (PPAR)-α expression levels in muscle and adipose tissues were determined by real-time PCR.

The results indicate that bioactive compounds of flaxseed affect selected gene expression. Further research is needed to identify the specific mechanisms regulating leptin or PPAR-α expression during DIO development.

ACKNOWLEDGEMENTS

The completion of my thesis has been both a challenging and rewarding experience. I would first like to thank Dr. Rhee for her time and guidance throughout this process. She went above and beyond during every step of the process. Second, I would like to thank Dr. Reindl and Dr. Hall for their guidance as well as Shireen Chikara for assisting me with laboratory procedures. I would also like to thank my husband, Michael Evenocheck and my family; Mary, Patrick, and Michael Woodard for their support during this time.

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Flaxseed oil……..The fat component derived from flaxseed; rich in omega-3 fatty acid, alpha-linolenic acid (ALA) (Baranowski et al., 2012).

Defatted flaxseed…….The portion of flaxseed when the fat is removed. It contains lignans with antioxidant properties, including secoisolariciresinol diglucoside (SDG) (Wang et al., 2010). This also contains protein (Oomah & Mazza, 1993) and fiber (Qian, Cui, Wu, & Goff, 2012).

DNA methylation…… The addition of methyl groups to the promoter region of genes. Contributes to the expression or silencing of genes (Lavebratt, Almgren, & Ekström, 2012).

DNA methyltransferases (DNMTs)..The enzymes involved in DNA methylation. These include DNMT1, DMNT3a, and DNMT3b which are responsible for de novo methylation and play a role in gene expression (Lavebratt, Almgren, & Ekström, 2012).

Epigenetics…….. Heritable non-genetic cellular memory resulting in differently expressed physical characteristics or phenotypes without actual changes to DNA sequence (Riddihough, & Zahn, 2010). Epigenetic mechanisms include DNA methylation, histone modifications, and chromatin remodeling (Choi & Friso, 2010).

Phenotype…….. A product of the interaction between inherited allelic variation and environmental factors, which results in an observable characteristic and is accompanied by alterations in gene expression (Koza et al., 2006).

Gene expression…….....Messenger ribonucleic acid (mRNA) is reverse transcribed into complimentary deoxyribonucleic acid (cDNA) and used as a template in polymerase chain reaction (PCR) in which amplification is detected as fluorescence. The fluorescence is then quantified as gene expression (Bio-rad, 2015). Gene expression is presented as relative gene expression by fold change in expression, which is determined by comparison to an internal control gene (Schmitten & Livak, 2008).

Upregulation……Increased expression of a gene resulting from increased transcription (Chiba et al., 2004).

CHAPTER 1. INTRODUCTION

In the past decade, the prevalence of obesity has increased dramatically in the United States. This is a serious and costly issue. More than one-third of the adult population and 17% of children are obese (Centers for Disease Control and Prevention, 2012). In 2010, 69.2% of adults 20 years of age or older were considered overweight or obese. Obesity greatly increases the likelihood of developing many chronic diseases including heart disease, Type 2 diabetes, certain types of cancers, and stroke. In 2008, the estimated cost of obesity in the United States was 147 billion dollars (Centers for Disease Control and Prevention, 2012). Identifying the factors involved in the development and regulation of obesity may facilitate the development of a solution.

There has been speculation as to the cause of this dramatic increase in obesity. Nutrition has been a primary focus. Many have attempted to identify the role of diet in increased weight gain, and how to manipulate it to promote weight loss.

Whole flaxseed has been recognized as a potential aid in weight loss because of its rich source of health-promoting compounds such as omega-3 fatty acid and the antioxidant lignan, secoisolariciresinol diglucoside (SDG). The flaxseed hull contains the greatest amount (11.7–24.1g SDG/kg defatted flaxseed) of SDG (Zhang & Xu, 2007).Therefore, defatted flaxseed has been used in the studies to determine the activity of SDG in the body (Wang et al., 2010). SDG has shown beneficial effects in high-fat diet-fed obese mice in relation to bodyweight (Park & Velasquez, 2012; Fukumitsu et al., 2008). Flaxseed oil has been studied for its effect on health because of its high percentage of omega-3 fatty acid. Flaxseeds contain about 36-40% total fat, in which omega-3 fatty acid account for about 53.2% of total fat (Popa et al., 2012). Flaxseed oil reduced the size of adipocytes, resulting in better function of fat cells by reducing harmful

inflammatory markers such as monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α) within adipose cells (Baranowski et al., 2012).

Many other elements have been identified as factors in obesity development, including genetics. Epigenetics is a branch of genetics which shows promise in regards to obesity prevention and treatment. The methylation of deoxyribonucleic acids (DNAs) can dictate whether the gene is expressed or silenced. Epigenetic regulations in gene expression by dietary elements have been observed in breast cancer. Dietary compounds, such as epigallocatechin gallate (EGCG), genistein, witheferin A, curcumin, resveratrol, and guggulsterone, are effective in altering the methylation pattern of DNAs involved in tumor development by down-regulation of deoxyribonucleic acid methyltransferases (DNMTs) (Mirza et al., 2013). This same effect has not yet been observed in regards to flaxseed consumption, but could potentially regulate the expression of genes with a recognized role in obesity.

Both leptin and peroxisome proliferator-activated receptor alpha (PPAR-α) have a known role in obesity. PPAR-α increases energy expenditure (Lefebvre, Chinetti, Fruchart, & Staels, 2006), while leptin decreases energy intake (Mahan et al., 2012). If the expression of these genes could be influenced by diet, this mechanism could play a prospective role in obesity prevention.

Statement of the Problem

Obesity has become a problem in the United States. Introducing foods with properties that can alter the expression of genes involved in the development of obesity into the diet is a potential tool. This has been achieved in other conditions through epigenetic modulation by DNA methylation, but has not been fully established in obesity.

Purpose of the Study

The purpose of this study was to identify a relationship between the different health-promoting compounds of flaxseed, SDG and omega-3 fatty acid, and the expression of DNMTs, leptin, and PPAR-α in an obese mouse model. A second objective was to establish a relationship between expression of DNMTs relative to the expression of genes with a known role in obesity, such as leptin and PPAR-α. Finally, a correlation between gene expression and body weight in male C57BL/6J mice was also determined.

Research Questions

The followings are research questions this study aimed to answer:

1. Does dietary intervention affect the mRNA expression of DNMTs?
2. Does dietary intervention affect the mRNA expression of leptin or PPAR-α?
3. Does DNMT expression correlate with expression of leptin or PPAR-α?
4. Does leptin or PPAR-α expression correlate with bodyweight?

Hypothesis

The hypothesis for this study was that flaxseed supplementation would decrease expression of DNMTs, increase expression of leptin or PPAR-α, and decrease body weight in an obese animal model.

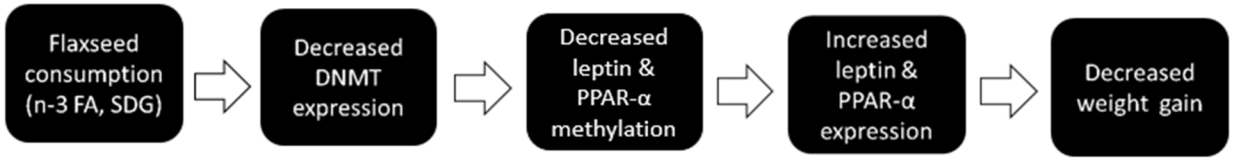

Figure 1. Schematic Representation of Hypothesis

Limitations

Possible limitations to this study involving the animals include the short duration of the feeding intervention, which may not have allowed the mice to gain sufficient weight. Studies with feeding interventions extended beyond eight weeks have shown significantly more weight gain in C57BL/6J mice (Lin, Thomas, Storlien, & Huang, 2000). This study was performed using an animal model and may not be generalizable to humans in regards to regulatory mechanisms involved in obesity and obesity reduction, which are different between species. This experiment was carried out in a controlled setting and may not accurately represent the events occurring in a natural environment.

Possible limitations to methodology include fluctuations and variations in gene expression detected by RT-qPCR, which may be influenced by operator variability (Bustin & Nolan, 2004). Another limitation to methodology was not including western blotting to confirm proteins identified by RT-qPCR.

Delimitations

To minimize limitations pertaining to the animals, all persons involved in this project were trained and qualified to perform all duties involving the mice. All necessary measures were taken to keep the animals healthy throughout the course of the study, and in the event of an issue the veterinarian was notified. A sample size of 12 mice in each group to achieve a power of 0.8 and an alpha error rate equal to 5% was used to achieve statistical significance. The diets represent a high-fat diet with 45% of calories coming from fat as well as a low-fat diet that has 16.4% of calories from fat to ensure diet-induced obesity by the mice.

To minimize errors related to the methodology, each gene target was run in duplicates to ensure results are directly related to difference in gene expression. A negative, water, and

endogenous control, 18S rRNA, was used during RT-qPCR analysis to minimize the effect of outside factors.

Assumptions

The following are assumptions in this research:

1. The mice will develop diet-induced obesity.
2. The mice consuming the high-fat diet supplemented with defatted flaxseed will show lower expression of DNMTs and higher expression of leptin and PPAR-α, and will have a lower body weight gain than the high-fat control group, as well as the mice fed with whole flaxseed or flaxseed oil supplemented diet.

CHAPTER 2. REVIEW OF LITERATURE

Obesity

The World Health Organization (WHO) defines obesity as a body mass index (BMI) of 30kg/m^2 or greater. Increased accumulation of fat mass in the body results when a person takes in more energy than is expended for an extended period of time (Centers for Disease Control and Prevention, 2012). Obesity is characterized by inflammation and adipocyte hypertrophy. The combination of these two can lead to the accumulation of harmful substances into adipose tissue and dysfunction of the cells (Baranowski et al., 2012). Obesity greatly increases the likelihood of developing many chronic diseases including heart disease, Type 2 diabetes, certain types of cancers, and stroke (Centers for Disease Control and Prevention, 2012). A study of black and white men and women in the U.S. found that increased body mass is associated with higher risk of mortality across age groups, and is increasing for younger age groups in particular (Masters et al., 2013). These results indicate that obesity-related mortality will only continue to grow.

The National Health and Nutrition Examination Survey 2009-2010 reported that more than one-third (35.7%) of U.S. adults and 17% of children are classified as obese. In the United States, the prevalence of obesity increased greatly from 1990 to 2010. In 2010, as many as 13 states classified 30% or more of its population as obese (Centers for Disease Control and Prevention, 2012). The reason for this dramatic increase in obesity is widely investigated. Obesity is believed to be a combination of environment, genetics, decreased physical activity, and increased consumption of food and energy dense foods (Centers for Disease Control and Prevention, 2012). One of the many factors currently being researched as a contributor to obesity is epigenetics. Conversely, epigenetics may also be protective factor against the development of obesity.

Epigenetics

Epigenetics is a type of non-genetic cellular memory that can be affected by environmental factors. Epigenetic alterations affect gene expression without making changes to the deoxyribonucleic acid (DNA) sequence, and result in an alternative phenotype or observable characteristics. Epigenetic modifications are alterations to DNA that can be reversed, passed from one generation to the next, and maintained throughout replication and transcription (Riddihough & Zahn, 2010). Epigenetic mechanisms include DNA methylation, histone modification, and chromatin remodeling (Choi & Friso, 2010).

The epigenome refers to the changes in gene expression resulting from environmental factors (Genetics and Social Science, 2015). There are several ways in which the environment can affect the epigenome. For example, exercise may cause alternative gene expression in the brain as has been observed in mice (Able & Rissman, 2013). Diet also has been hypothesized to lead to abnormal gene expression resulting in cancer (Jaenisch & Bird, 2003). These are just a couple of studies that support the theory that environmental exposures can modify gene expression due to epigenetic modifications.

With the increased prevalence of obesity in recent years, epigenetic regulation of weight gain has attracted considerable attention. It is known that genes are differently expressed in the obese state when compared to a lean control (Baranova et al., 2005; Koza et al., 2006; Moraes et al., 2003), which makes obesity a plausible target for epigenetic modification. Diet and nutrition are environmental factors that have been researched as a potential target for this type of mechanism, which will be reviewed in the following section.

Nutrition

There is evidence to suggest that changes in the diet may have an effect on weight gain through epigenetic mechanisms. Specifically, diet composition and/or dietary compounds are proposed to mediate this effect. This has been observed in studies where differences in diet between groups lead to differential gene expression and ultimately improvement in obesity-related indicators.

Miller et al. (2008) used a diet-induced obesity (DIO) mouse model to identify the changes in gene expression due to diet composition, as well as gene expression following weight loss after DIO. There were four diet groups including a low-fat (10% fat), high-fat (60% fat), high-carbohydrate with low-fat liquid formula (1674kj/L, 10% fat), and a high-fat energy restricted diet, which was pair fed with the high-carbohydrate with low-fat liquid formula group. The energy-restricted group was given a quantity of food to match the bodyweight of the high-carbohydrate group as determined in a pilot study. After 13 weeks of experimental diet feeding, half of the high-fat group was switched to a low-fat diet for weight loss, while the other half continued the high-fat diet. When analyzing gene expression in the adipose tissue of the mice, the high-carbohydrate and the high-fat restricted group showed global differences in gene expression patterns, regardless of body weight. Due to mice of the same body weight having different gene expression patterns, the authors concluded that diet composition was responsible for changes in gene expression, and the possibility of epigenetic involvement. The high-fat, low-fat, and weight reduction group also showed three distinguishable gene expression patterns, further indicating the effects of diet on gene expression. Altered gene expression was maintained in the weight reduction group after maintained weight loss following DIO, which the authors concluded may also be due to epigenetic involvement (Miller, Becker, Prabhu, & Cooke, 2008).

Although this study doesn't explain the role of altered gene expression in development or reduction of obesity, it provides evidence that diet is an environmental factor that can alter gene expression.

As mentioned previously, reversibility of gene expression is a key component of epigenetic mechanisms. In order for diet to be an epigenetic regulator, it must produce changes in gene expression that are reversible. To study this, Wistar rats were fed either a standard diet (10.3% fat) or a high-fat-sucrose (HFS) diet (45% fat) in which half of the rats from the HFS diet were switched to the standard diet after 10 weeks. At the end of 20 weeks, the rats were sacrificed and the retroperitoneal adipocytes were analyzed for changes in gene expression. The results indicated that being switched to the standard diet following a HFS diet induced epigenetic changes including alterations in methylation of genes involved in obesity, specifically the promoter regions of leptin, and fatty acid synthase (Fasn). Examination of the promoter regions showed both hyper- (CpG sites 6-7 and 29-30) and hypomethylatoin (CpG site 15) of leptin and hypomethylation of Fasn (CpG site 1 and 5). These results denote that diet can reversibly modify gene expression (Uriarte, Paternain, Milagro, Martinez, & Campion, 2013).

Researches have shown that supplementation of certain compounds is protective against weight gain regardless of diet. For example, Pichiah et al. (2012) examined the effects of seabuckthorn extract (*Hippophae rhamnoides L*) on obesity through regulation of food intake and gene expression utilizing a male C57BL/6J obesity-prone mouse model. Eight mice were assigned to one of four dietary treatments including a normal diet, a high-fat diet control group, a high-fat diet supplemented with 500 mg/kg body weight seabuckthorn ethanolic extract, or a high-fat diet supplemented with 1000 mg/kg body weight seabuckthorn ethanolic extract for 13 weeks. At the end of the study, the mice supplemented with seabuckthorn extract did not gain

significant weight compared to the normal diet group. Although serum leptin was higher in all 3 high-fat diets, it was reduced in the seabuckthorn extract supplemented diets compared to the high-fat diet alone. The liver tissue was analyzed for expression of genes with a known role in obesity including PPAR-α, PPAR-γ, and carnitine palmitoyltransferase-1(CPT-1). PPAR-γ expression was higher in the group supplemented with 1000 mg/kg seabuckthorn compared to all other groups. PPAR-α and CPT-1 expression was significantly higher in the 1000 mg/kg seabuckthron group compared to the other high-fat groups. These results indicated supplementation of a dietary compound was successful in preventing obesity and was accompanied by an altered expression of genes when consuming a high-fat diet (Pichiah, Moon, Park, Moon, & Cha, 2012). It is of interest whether or not similar results can be observed through epigenetic regulation.

Sohair et al. (2015) performed a similar study focusing on non-alcoholic fatty liver disease in which mice were fed a standard diet or high-fat, high-sucrose diet (HFHS) and supplemented with one of three dietary compounds: quercetin, berberine, or o-coumaric acid. They found that the HFHS diet led to reduced PPAR-γ expression compared to the rats fed a standard diet. However, when consuming a HFHS diet supplemented with berberine, PPAR-γ expression was significantly upregulated compared to the HFHS diet alone. This is significant because of the function of PPAR-γ in metabolic disorders/obesity which is associated with improved insulin resistance and glucose tolerance (Ragab, Elghaffar, El-Metwally, Badr, Mahmound, & Omar, 2015).

Because obesity has been shown to be accompanied by changes in gene expression and diet is associated with reversible changes in gene expression, diet composition is a likely target for studying epigenetic modification. As discussed previously, specific compounds have been

identified that lead to less weight gain and/or improvement in obesity-related indicators despite consumption of a high-fat diet. Specific to the current study it is of interest whether or not the health promoting compounds present in flaxseed are capable of producing anti-obesity effects via epigenetic mechanisms.

Flaxseed

Flaxseed has attracted attention for its potential health benefits, which include a positive impact on weight loss. According to the United States Department of Agriculture (USDA), flaxseed contains fiber and has been studied for its possible role in cholesterol reduction (Nandi & Ghosh, 2015). Flaxseed is about 27% fiber and 18% protein per 100 grams (USDA, 2016). For the purpose of this study we focused on health benefits of flaxseed due to its omega-3 fatty acid (Baranowski et al., 2012) and antioxidant lignan content (Park & Velasquez, 2012). In addition to whole flaxseed, individual components of flaxseed have been shown to influence body weight.

Flaxseed contains omega-3 fatty acid in the form of α-linolenic acid (ALA) (Baranowski et al., 2012). Flaxseed oil has been observed to be beneficial in the obese state by improving adipocyte function without reducing body weight by reducing adipocyte size and decreasing the amount of inflammatory markers such as MCP-1 and tumor necrosis factor-α (TNF-α) in adipose tissue (Baranowski et al., 2012). Additionally, supplementation with flaxseed oil has also provided protective mechanisms that coincide with consuming a diet high in fats or carbohydrates by preventing weight gain, decreasing blood glucose levels, decreasing blood lipids and improving lipid profile (El-Shobaki, Mohamed, Abd-El-Azeem, & Hegazy, 2013). Although it is clear that flaxseed oil is beneficial in obesity, it is significant to know if it can influence gene expression to combat obesity.

Alpha-linolenic acid (ALA) from flaxseed has the potential to be involved in epigenetic regulation. Pregnant mice supplemented with flaxseed oil had significantly increased methylation on intron 1 of the fatty acid desaturase 2 (FADS2) gene compared to a control group that received soybean oil as the fat source. This was also observed in the promoter region of FADS2 in the flaxseed oil supplemented group compared to the control group, but this was only significant when the mice were first provided a diet that was deficient in ALA prior to pregnancy (Niculescu, Lupu, & Craciunescu, 2012). Due to flaxseed oil supplementation showing positive effects in obesity and having epigenetic potential, flaxseed oil is a plausible topic of study in epigenetic regulation of weight gain.

Secoisolariciresinol diglucoside (SDG) is the bioactive compound in flaxseed exhibiting antioxidant properties (Park & Velasquez, 2012). To study its effects in obesity, Park and Velasquez (2012) fed rats a high-fat/high-fructose diet supplemented with 0.02% SDG lignan-enriched flaxseed powder, which was determined to provide about 1.5 mg SDG lignan per day after calculating average daily food intake of the rats. Compared to rats fed a standard diet, those supplemented with SDG showed significantly less weight gain, visceral fat accumulation, plasma leptin concentration, and an improvement in lipid profile and systolic blood pressure. These results indicate when consuming a high-fat/high-fructose diet, supplementation with SDG can ameliorate obesity while exhibiting positive effects on blood pressure and lipid profiles (Park & Valasquez, 2012).

Tominaga et al. (2012) investigated the effects of a synthetic pure form of secoisolariciresinol, (-)-secoisolariciresinol ((-)-SECO),which is the metabolite of SDG. The study was carried out in a diet-induced obesity mouse model. The authors hypothesized that (-)-SECO would inhibit weight gain in DIO. Mice were fed either a high-fat or standard diet. Oral

administration of (-)-SECO was given at 0, 0.058, 0.58, and 5.8 mg/kg body weight. Body weight was reduced at all three doses of (-)-SECO, but was only statistically significant at 5.8 mg/kg body weight, which was reduced by 31% compared to a high-fat control group. This result was independent of food intake. In the liver tissue of the (-)-SECO treated mice, decreased expression of fatty acid synthase (Fasn) and sterol regulatory element-binding protein (SREBP)-1c,was observed. This finding is significant because decreased expression of these genes indicate decreased fat synthesis (Tominaga et al., 2012). Decreased body weight gain, and reduced expression of obesity associated genes with (-)-SECO supplementation may indicate the ability of flaxseed lignan to prevent weight gain in DIO by regulation of gene expression.

Both the antioxidant and omega-3 fatty acid components of flaxseed have been shown to improve negative health indicators in obesity such as adipocyte function, inflammatory makers (Baranowski et al., 2012), blood pressure, and blood lipid profiles (Park & Velasquez, 2012). Secoisolariciresinol diglucoside and ALA also appear to potentially be influenced by epigenetic regulation. When discussing epigenetic regulation of gene expression, it is important to understand the mechanism by which substances work. There is evidence that nutritional components may affect the way DNA is transcribed, resulting in more or less weight gain.

DNA Methylation

As discussed earlier, DNA methylation is an epigenetic mechanism. Methylation is the addition of methyl groups to the DNA structure most often occurring at C-phosphate-G (CpG) dinucleotides. The group of enzymes responsible for altering the way DNA is methylated are called DNA methyltransferases (DNMTs). Three of interest in gene expression include DNMT1, DNMT3a, and DNMT3b. Deoxyribonucleic acid methyltranferase1 assures identical methylation patterns of genes in each cell division, and DNMT3a and DNMT3b are responsible for the

addition of methyl groups to genes (Lavebratt, Almgren, & Ekstrom, 2012). The role of other DNMTs such as DNMT2, is not as well understood (Schaefer et al., 2010). Addition of methyl groups to DNA prevents transcription of the gene due to limited availability for transcription factors (Shen et al., 2014). Conversely, less methylation of genes leads to increased expression of the gene. Deoxyribonucleic acid methylation has been found to repress the expression of tumor suppressor genes by hypermethylation (Rhee et al., 2002). We aim to determine whether a similar mechanism can be observed with the addition of health-promoting compounds to the diet.

Fang et al. (2003) have shown that EGCG a polyphenol found in green tea inhibits DNA methyltransferase activity and consequently, reactivates genes involved in tumor development (Fang et al., 2003). This is significant because it shows that consumption of a dietary compound, and more specifically a polyphenol, can lead to increased expression of genes by preventing DNMTs from adding methyl groups to genes. Flaxseed also contains polyphenols making it an appealing target for similar research.

Moleres et al. (2013) examined the DNA methylation response to weight loss of obese adolescents. Subjects were obese adolescents who participated in a 10-week intervention for weight loss. They were divided into two groups based on weight loss response; high and low responders. High responders lost greater than 1.1 BMI-standard deviation score (BMI-SDS) (n=12) and low responders lost less than 0.4 BMI-SDS (n=12). A methylation score of 27,578 CpG sites of 14,495 genes was calculated comparing baseline methylation of DNA, obtained through venous blood, to methylation after the intervention. Those with the highest methylation score had a greater BMI-SDS decrease than those with a lower methylation score. To confirm results, they analyzed seven CpG sites correlated to genes AQP9, DUSP22, HIPK3, TNNI3, TNNT1, F12, and PTPRG by matrix assisted laser desorption/ionization – time of flight

(MALDI-TOF) (n=107). This approach also revealed significant changes in DNA methylation between the two groups in five of the regions. AQP9 is involved in lipogenesis and is associated with increased weight gain. Therefore it is likely the that increased methylation score of the subjects who better responded to weight loss had reduced expression of this gene, resulting in more weight loss (Moleres et al., 2013). This work is evidence that genes involved in weight regulation are potential targets for the study of epigenetic modification by DNA methylation.

To examine the mechanism responsible for changing the methylation patterns and the resulting expression of a gene, Milagro et al. (2009) studied the methylation pattern of gene promoters. They used male Wistar rats to observe the effects of a high-fat diet on the methylation pattern of the leptin promoter obtained from retroperitoneal adipocytes. They were able to identify a region on the promoter that showed increased methylation in the animals fed the high-fat diet, which correlated with lower leptin levels in the blood. This is significant because it suggests that the methylation pattern of the promoter region may regulate the expression of leptin, a gene involved in energy metabolism, as well as the involvement of diet in epigenetic regulation of leptin (Milagro et al., 2009).

Fujiki et al. (2009) examined the relationship between the methylation pattern and expression of a gene involved in adipocyte regulation, PPAR-γ. They observed hypermethylation on the promoter of PPAR-γ in pre-adipocytes. During differentiation, it was de-methylated leading to increased mRNA expression. They also treated the cells with a DNA methylation inhibitor, which resulted in increased PPAR-γ expression in a dose dependent manner. They observed increased methylation and reduced expression of PPAR-γ in visceral adipose tissue of diabetic mice compared to wild type mice. The authors concluded that PPAR-γ is regulated by methylation of the promoter region and that DNA methylation and metabolic syndrome may be

related (Fujiki, Kano, Shiota, & Murata, 2009). This is evidence that increased methylation leads to gene silencing and this can occur in genes with a role in energy regulation. In our study, we aimed to identify the role of other genes involved in the development of obesity and subsequent regulation by DNA methylation.

Jacobsen et al. (2012) studied the effects of short-term high-fat overfeeding (HFO) on DNA methylation in human skeletal muscles. The participants consumed a control diet (35% fat, 50% carbohydrates, 15% protein) or a HFO diet (60% fat, 32.5% carbohydrates, 7.5% protein) providing 50% more calories than the control diet for five days followed by a washout period of six to eight weeks and then consumed the other diet. In the group that consumed the control diet first, they observed that following the HFO diet, DNA methylation was increased in 83% of CpG sites, most of which (98%) were hypomethylated in the control diet. They also observed a change in methylation of 24 genes associated with type 2 diabetes, such as cyclin-dependent kinase inhibitor 2A and 2B (CDKN), solute carrier family 30 (zinc transporter) member 8 (SLC30A8), and PPAR-γ, following the HFO diet. In the group that consumed the HFO diet first, they did not see a significant change in methylation. However, they did observe a trend towards reversibility, showing non-significant changes in methylation of genes. This was particularly observed in the genes that were most affected by the HFO diet in the participants in the control diet first group. They did not observe significant changes in gene expression overall in either group, although they did observe a trend towards increased expression of DNMT3a and DNMT1 ($p=0.08$ and $p=0.10$, respectively). The experimental diets were provided over a short duration, but the changes in DNA methylation and trend towards reversibility by the HFO provide evidence that the methylation pattern may influence gene expression during long term interventions (Jacobsen et al., 2012). This provides further evidence that diet affects the

methylation pattern of genes; however, the relationship between methylation and gene expression must be determined to establish if an epigenetic mechanism is involved. It also shows that muscle is a potential tissue to study in regards to epigenetic regulation.

Elango et al. (2009) studied the role of DNA methylation in *Apis mellifera,* otherwise known as the western honeybee. They observed that genes with low-CpG content were involved in basic biological processes whereas genes with high-CpG content were involved in developmental processes. The low-CpG content is indicative of DNA methylation due to deletion of CG rich regions over time. However, the authors hypothesized that genes with high-CpG content or genes involved in developmental processes are more subject to epigenetic regulation (Elango, Hunt, Goodisman, & Yi, 2009). Therefore, it is possible that genes with a high content of CpG rich regions, especially in the promoter region, or genes involved in developmental processes are likely targets for epigenetic regulation by DNA methylation.

It is clear that diet can act as an environmental factor to epigenetically regulate gene expression. In our study we chose genes known to be involved in obesity development.

O**bese Gene Expression**

Both peroxisome proliferator-activated receptors and leptin are involved in obesity development, and are therefore favorable targets for studying regulation of weight gain.

Peroxisome Proliferator-Activated Receptor Alpha

Peroxisome proliferator-activated receptors (PPARs) are transcription factors that regulate the activity of proteins involved in energy metabolism. Peroxisome proliferator-activated receptor alpha is one of the three isoforms, and is highly expressed in the liver and skeletal muscle, in addition to other tissues. In the liver, it induces the expression of enzymes/proteins that enable fatty acid oxidation such as fatty acid transport proteins, long-chain

acyl-CoA synthetase, and acyl-CoA oxidase. In the skeletal muscle in particular, PPAR-α induces expression and regulates activity of pyruvate dehydrogenase kinase 4 (PDK4) involved in energy metabolism (Ferre, 2004).

Guerre-Millo et al. (2000) examined the effects of a known PPAR-α activator, fenofibrate, on characteristics of metabolic syndrome. They observed that C57BL/6 mice fed a high-fat diet supplemented with fenofibrate (0.05%) gained less perirenal and epididymal fat than mice fed the same diet without supplementation, regardless of intake (Guerre-Millo et al., 2000). This demonstrated the importance of PPAR-α in obesity regulation, and suggests that an increase in this gene may help prevent weight gain due to a high-fat diet.

A similar study was conducted with male Wistar rats. A different PPAR-α agonist, WY14643, was used. Wistar rats fed a high-fat diet (59% fat, 21% protein, 20% carbohydrate), supplemented with WY14643(3 mg/kg body weight/day) had significantly less muscle and liver triglyceride accumulation and less visceral fat weight gain than rats fed the same diet without supplementation (Ye et al., 2001). This also shows the importance of PPAR-α in regards to weight regulation, and this mechanism is carried out in muscle tissue.

As discussed earlier, when a dietary element with health-promoting properties such as seabuckthorn is added to the diet, the expression of PPAR-α increased (Pichiah et al., 2012). The results of the study suggest the involvement of diet in the regulation of PPAR-α expression. It is plausible that another dietary compound, such as flaxseed, may be able to produce a similar effect.

Lee et al. (2012), studied the effects of *Adenophora triphylla* (A TREE) on obesity and PPAR-α expression in a DIO mouse model. A TREE was used because it has antioxidant activity. Mice were fed one of five diets including a normal control, a high-fat control, a high-fat

diet supplemented with 0.5% catechin, or a high-fat diet supplemented with 0.5% or 1% A TREE. Catechin was used as a control because it also acts as an antioxidant. Supplementation with catechin and A TREE was provided after five weeks of feeding a high-fat diet alone. Feeding was continued for an additional five weeks, resulting in a 10-week feeding time. They found that A TREE was not as strong of an antioxidant as catechin based on its electron donating capacity. However, A TREE caused a significant reduction in xanthine oxidase (XO) and thiobarbituric acid reactive substances (TBARS) and an increase in catalase (CAT) and superoxide dismutase (SOD) activity in vivo compared to the high-fat diet alone, indicating antioxidant capabilities. Following supplementation of A TREE and catechin, adipocyte size was reduced by 28.4% and 42.8% respectively, compared to the high-fat control group. In the A TREE groups, body weight gain was significantly less than the high-fat control group. In the liver tissue, PPAR-α expression was significantly increased in the A TREE group in a dose dependent manner compared to the high-fat control group. The authors concluded that A TREE produces anti-obesity effects and acts as an antioxidant (Lee, Lee, Lee, Kim, & Kim, 2013). This indicates that a compound with antioxidant capabilities correlates with both decreased body weight and increased expression of PPAR-α. It is plausible that other compounds with antioxidant capabilities, such as SDG, may have a similar effect on PPAR-α expression and weight gain in conjunction with a high-fat diet. However, it remains to be determined if a dietary compound such as flaxseed that also has antioxidant properties can alter PPAR-α expression by DNA methylation.

Leptin

Leptin is another gene of interest because of its role in obesity. Leptin is a hormone that acts on the central nervous system to regulate energy intake and expenditure (Meiera &

Gressner, 2004). However, leptin administration results in different effects on intake in obese vs. non-obese animals. Using an animal model, Moraes et al. (2003) studied leptin in diet-induced obesity. Decreased food intake and fat stores were seen in lean control mice given leptin; although, it was not seen in the obese mice. The large amounts of leptin circulating in the plasma of the obese mice, without coinciding weight loss, indicated leptin resistance (Moraes et al., 2003).This is an example of leptin resistance, in which leptin no longer exhibits its function due to defects in the leptin receptor and signaling pathway (Crujeiras et al., 2015). Therefore, because increased leptin expression doesn't necessarily indicate increased leptin action due to the development of leptin resistance, increased leptin expression must not be considered without changes in body weight.

El-Haschimi et al. (2014) identified two possible mechanisms responsible for leptin resistance in an animal model of diet-induced obesity mice. One was the inability of peripheral leptin to activate signal transducers and activators of transcription (STATs) in the hypothalamus, and the second was a defect of intracellular signaling of leptin (El-Haschimi, Pierroz, Hileman, Bjørbæk, & Flier, 2000). When targeting leptin as a treatment for obesity, the objective is to increase the expression of leptin in the cell rather than increase the amount of leptin circulating in the blood.

In this study, we determined the impact of a high-fat diet on energy intake and leptin expression and correlation with weight gain. Milagro et al. (2009) studied the effect of a high-fat diet on the methylation pattern of the leptin promoter. They used male Wistar rats and assigned them to either a standard diet (16.6% protein, 73.1% carbohydrates, 10.3% fat) group or a cafeteria diet (9.3% protein, 31.5% carbohydrates, 59.2% fat) group and fed them for 11 weeks. The cafeteria diet group had increased circulating leptin levels and increased mRNA leptin levels

in retroperitoneal adipocytes. They also identified two CpG islands (-562 to -399 pb and -324 to -187 pb) that could be epigenetically regulated. One particular position, -443, was more highly methylated in the cafeteria diet group compared to the control. The extent of its methylation was correlated with lower circulating leptin and lower body weight in the cafeteria diet group. These results suggest that leptin can be epigenetically regulated by DNA methylation (Milagro et al., 2009). Due to leptin expression being altered by diet and accompanied by altered promoter methylation, it is possible that leptin expression may be epigenetically regulated by diet.

Boque et al. (2013) investigated the effects of apple polyphenols (AP) in conjunction with a high-fat sucrose (HFS) diet on weight regulation. Male Wistar rats were fed either a standard diet (20% protein, 67% carbohydrate, 13% fat), a HFS diet (20% protein, 35% carbohydrate, 45% fat), or a HFS diet supplemented with AP (0.70 g/kg rat). The rats were fed the HFS diet for 64 days and supplementation lasted for 56 days (Boque et al., 2012). When supplemented with AP, in the absence of differences in food intake, prevention of body weight and adiposity gain was observed in the rats. In epididymal adipocytes from the HFS diet group, leptin gene expression was up-regulated, but when supplemented with AP, leptin expression was observed to be the same as the rats on the standard diet. The increase in leptin expression is likely due to increased fat mass because adipose cells secrete leptin (Boque et al., 2013). The increased leptin expression with increased fat mass is counterintuitive due to the function of leptin in regulating energy intake and increasing energy expenditure. However, leptin resistance is developed in DIO (Moraes et al., 2003). Therefore, increased leptin expression does not mean increased leptin action and serum leptin needs to be measured to determine if leptin resistance is developed. The promoter region of leptin was also examined by Boque et al. (2013) to assess changes in the methylation pattern. It was found that CpG methylation (position -292 and -198)

increased in the HFS diet and was significantly reduced (position -198) with AP supplementation. There was a positive correlation between Lep_CpG22 methylation and leptin expression. The positive correlation observed was not expected because, typically, increased methylation leads to reduced expression; however, this could be due to where the methylation occurred (negative regulatory elements in the promoter). Leptin is a likely target for epigenetic modification through diet due to leptin expression correlating with the methylation pattern on the promoter region after supplementation, although the relationship is unclear (Boque et al., 2013).

Fan et al. (2011) used a DIO mouse model to examine leptin promoter methylation. Although not statistically significant, they found that addition of n-3 FA to the diet indicated a trend towards reduced leptin promoter methylation. They also observed reduced leptin expression in the adipose tissue, decreased body weight, and decreased energy intake of the n-3 FA diet groups than the n-3 FA deficient groups (Fan, Liu, Shen, Deckellbaum, & Qi, 2011). This provides evidence that n-3 FA supplementation in combination with a high-fat diet may prevent weight gain, and suggests a possible epigenetic involvement. Flaxseed also contains n-3 FA; warranting further investigation regarding a possible connection between flaxseed consumption and epigenetic regulation of leptin expression.

Epigenetics has become a field of great interest and shows promise as a target for obesity treatment. Flaxseed has shown many beneficial effects in the obese state and it remains to be seen if flaxseed modulates these effects by DNA methylation. More specifically if it can modulate the expression of PPAR-α and/or leptin.

Summary

Thus far literature shows a connection between diet composition and body weight. There is also evidence that the addition of certain compounds present in food can lead to less weight

gain despite consumption of a high-fat diet. Compounds present in food have been shown to influence gene expression leading to an alternatively expressed phenotype, especially in cancer studies. Current research is limited in regards to epigenetic regulation of weight gain. Flaxseed is an example of a food that has been shown to have beneficial effects on heath. Although, it is unclear what mechanism is responsible for producing these beneficial effects. In this study, we will examine the effects of flaxseed supplementation on body weight and obese gene expression. We will also look for the potential of epigenetic regulation by measuring DNA methyltransferases.

CHAPTER 3. METHODS

Obesity has become a prominent issue in the United States. Researchers have targeted many of the contributing factors, one of which is epigenetics, in hopes of identifying a solution to the obesity epidemic. Mirza et al. (2013) identified epigenetic mechanisms capable of silencing genes involved in tumor cell development by the introduction of dietary compounds. A similar approach of adding flaxseed to the diet could be effective against obesity by influencing epigenetic mechanisms of gene regulation in adipose and muscle tissues. The purpose of this study was to identify the effects of different health-promoting compounds of flaxseed on the expression of deoxyribonucleic acid methyltransferases (DNMTs), leptin, and peroxisome proliferator-activated receptor alpha (PPAR-α). This study also aimed to investigate a relationship between the expression of DNMTs and the expression of genes with a known role in obesity, including leptin and PPAR-α. Finally, the correlation between gene expression of leptin, PPAR-α, and body weight was determined. This study was based on a parent study which was approved by the North Dakota State University (NDSU) Institutional Animal Care and Use Committee (IACUC #A13019).

Research Design

A randomized control research design was used to compare gene expression of DMNTs, leptin, and PPAR-α by health-promoting components present in flaxseed added to the diet in an obese animal model.

Flaxseed

Whole flaxseed was obtained from three different suppliers and pooled prior to milling. Milling was completed such that all particles passed a 30 mesh screen. The samples was defatted using a large soxhlet unit using hexane for 16 hours. After evaporation of the hexane the oil was

packaged in a bottle and stored frozen until needed for diet formulation. The defatted flaxseed from the soxhlet was air dried in a fume hood 12 hours prior and stored frozen until diet formulation.

Animals and Diets

Five to six-week old male C57BL/6J mice (~20 g) were purchased from The Jackson Laboratory (Bar Harbor, Maine). The mice were selected because they are prone to diet-induced obesity when fed a high-fat diet (Tominaga et al., 2012). The animals were housed individually in plastic-bottom cages in the Animal Nutrition and Physiology Center at NDSU with a controlled 12-hour light: dark cycle. The room was kept at a temperature of 22-25°C and humidity of 42-55%.

After two days of acclimation, the mice were randomly assigned into five treatment groups (n=12). Fresh food and water were provided daily and mice had access to food *ad libitum*. The diet was formulated by a commercial company (TestDiet, St. Louis, MO) in a pellet form. All groups were fed a high-fat diet, with the exception of group five, which was fed a low-fat diet. The high-fat diet consisted of 18% of calories from protein, 45% of calories from fat, and 37% of calories from carbohydrates. The low-fat diet consisted of 19% of calories from protein, 16% from fat, and 65% from carbohydrates (TestDiet, St. Louis, MO). Group one served as a high-fat control. Group two was supplemented with 10% whole flaxseed. Group three was supplemented with 6% defatted flaxseed. Group four was supplemented with 4% flaxseed oil. Group five was the low-fat control and pair-fed to group two. The amount of each component of flaxseed was formulated to reflect the amount present in whole flaxseed. The amount of food consumed by the mice in group two was measured daily and the same amount was weighed and fed to group five the following day. Animals were weighed weekly and food intake was also

measured weekly for monitoring of intake, with the exception of group two which was measured daily. After eight weeks of the experimental diet feeding, the mice were sacrificed with carbon dioxide (CO2) gas and then exsanguinated by anterior cardiac puncture. Skeletal muscle and adipose tissues were harvested, weighed, frozen in liquid nitrogen, and immediately stored at -80°C.

Oxidation of Mouse Food

Peroxide values (PV) of the extracted oil was measured using AOCS Method Cd 8-53 (1998) as modified by Crowe and White (2001). Extracted oil (0.5 g) was weighed into a 25 mL Erlenmeyer flask and 3 mL of acetic acid:chloroform (3:2 v/v) and 50 µL of potassium iodide was added and mixed for 1 min. Next, 3 mL of distilled water was dispensed into the mixture, followed by the addition of 300 µL of 1% starch indicator solution. The oil samples was then titrated using either 0.001 N sodium thiosulfate or 0.01 N sodium thiosulfate until a clear color is obtained. Peroxide values were calculated using the equation: peroxide value = [((S-B) * N * 1000) / W], where S = volume of titrant for sample, B = volume of titrant for blank, N = normality of $Na_2S_2O_3$ (meq/ml), 1000 = unit conversion (g/kg), and W = sample mass (g). The peroxide values of each treatment will be measured in duplicate.

Gene Expression Measurement

Ribonucleic Acid (RNA) Extraction

Quantitative real-time polymerase chain reaction (RT-qPCR) was performed to quantify gene expression of DNMT1, DNMT3a, DNMT3b, leptin, and PPAR-α. Total ribonucleic acid (tRNA) was extracted from adipose and muscle tissues using TRI Reagent (Molecular Research Center, Cincinnati, OH). Five hundred microliters of TRI Reagent was added per 500 mg tissue for homogenization. Following homogenization of the tissues, 50 µl 1-bromo-3-chloropropane

(BCP) (Molecular Research Center, Cincinnati, OH) (1:10 ratio of BCP to TRI Reagent) was added and the samples were shaken vigorously for 30 seconds. They were then incubated at room temperature for two to three minutes and centrifuged at 12,000 x *g* for 15 minutes at 4°C (Allegra 21R centrifuge, Beckman Coulter). The supernatant containing the RNA was then be transferred into a new tube, 125 µl of isopropyl alcohol (IBI Scientific, Peosta, IA) and 125 µl high salt solution (0.8 M sodium citrate and 1.2 M NaCl) (Molecular Research Center, Cincinnati, OH) were added. The samples were centrifuged at 12,000 x *g* for 10 minutes at 4°C. Prior to centrifuging, the homogenized adipose tissue samples were held at room temperature for 10 minutes. After centrifuging, the supernatant was removed and discarded. The remaining RNA pellet was washed twice with 500 µl 75% ethanol (Decon Labs, King of Prussia, PA) and then centrifuged at 12,000 x *g* for 5 minutes at 4°C. The RNA pellet was dissolved in 75 µl RNase free water and then incubated at 55-60°C for 10 minutes. The RNA pellet was dissolved in water stored at -80°C for subsequent RNA quantification. Following quantification, the RNA was diluted to 200 ng/µl.

Reverse Transcription

A reverse transcription polymerase chain reaction into complimentary deoxyribonucleic acid (cDNA) was completed using 5X iScript Reverse Transcription Supermix (Bio-rad, Hercules, CA) on a PCR thermal cycler (Applied Biosystems 2720 thermal cycler, Applied Biosystems, Foster City, CA). Each reaction had a volume of 20 µl consisting of 4 µl 5X iScript Reverse Transcription Supermix, RNA template, and a variable volume of RNase free water, so that the amount of the RNA template was 1 ng/µl in a total volume of 20 µl per reaction.

Q*uantitative PCR*

Quantitative PCR was performed using SYBR Select Master Mix (Life Technologies, Carlsbad, CA) on a real-time PCR system (ABI 7500 Fast, Applied Biosystems, Foster City, CA) according to standard procedure. Total volume of each well was 10 µl, with 5 µl SYBR Select Master Mix, 1 µl each of forward and reverse primers (Table 1), and 3 µl of the cDNA template. Expression of DNMT1, DNMT3a, DNMT3b, leptin, and PPAR-α was s measured. 18sRNA expression was also measured and served as an internal control. The primer sequences were designed using Primer Express Software 2.0 (Applied Biosystems, Foster City, CA) and synthesized by Integrated DNA Technologies (IDT) (Coraville, IA), and are shown below (Table 1). The fold change in expression of the DNMTs, leptin, and PPAR-α was calculated using 2-ΔΔCT method [(CT gene of interest – CT internal control) sample A – (CT gene of interest – CT internal control) sample B)] (Schmitten & Livak, 2008), where sample 'A' is the amplification detected by PCR from control or high-fat control/low-fat control and 'B' is the amplification detected by PCR from the experimental diet.

Table 1. Primer Sequences Used for Quantitative PCR

Gene	Primer Combination	
DNMT1	Forward	5'-TGT GGA TGA ACC CCA GAT GTT -3'
	Reverse	5'- GTG GAG TCG TAG ATG GAC AGT TTC T -3'
DNMT3a	Forward	5'- GGC TGG CAG GTT GGA AAA -3'
	Reverse	5'- CTG CAA TTA CCT TGG CTT TCT TC -3'
DNMT3b	Forward	5'- CCA AAA GGA GGC CCA TTA GAG -3'
	Reverse	5' CCC GTT GCA ATT CCA TCA A -3'
Leptin	Forward	5'- AGC CTG CCT TCC CAA AAT GT -3'
	Reverse	5'- CGG AGA TTC TCC AGG TCA TTG -3'
PPAR-α	Forward	5'- AGA TCG GCC TGG CCT TCT AA -3'
	Reverse	5'- CCC TCC TGC AAC TTC TCA ATG -3'
18S rRNA	Forward	5'- CGC CGC TAG AGG TGA AAT TCT -3'
	Reverse	5'- CTT TCG CTC TGG TCC GTC TT -3'

Statistical Analysis

Statistical analysis was performed using SAS software (version 9.3, Cary, NC, USA). Differences in gene expression among diet groups were analyzed with a general linear model (GLM). The relationship among gene expression levels and weight gain and obesity-associated gene expression was calculated by Pearson correlation coefficient. A Type I error rate (alpha level) of 0.05 was used to assess significance. Peroxide value data was analyzed by analysis of variance (ANOVA) with a p-value of 0.05.

CHAPTER 4. RESULTS

Food Intake

Food intake among groups was significantly different ($p = 0.01$). Food intake was significantly lower in the high-fat flaxseed oil (HF+FO) diet group compared to the low-fat control (LFC) ($p = 0.04$) and high-fat whole flaxseed (HF+WF) ($p = 0.017$) diet groups (Table 2). No other significant differences in food intake were observed between groups.

Table 2. Average Weekly Food Intake throughout 8 Weeks of Diet Treatment

Diet Group	Average Weekly Food Intake (g)
High-Fat Control	25.23 ± 1.68
High-Fat Defatted Flaxseed	21.68 ± 0.44
Low-Fat Control	27.00 ± 1.13
High-Fat Flaxseed Oil	20.25 ± 0.22 [ce]
High-Fat Whole Flaxseed	27.53 ± 1.16

[1] Data are presented as mean ± standard error, $p < 0.05$
[2] [c] Significantly decreased compared to low-fat control group (pair-fed to high-fat whole flaxseed diet group), [e] significantly decreased compared to high-fat whole flaxseed group (high-fat + 10% whole flaxseed)
[3]All high-fat diets are 45% kcal from fat. Low-fat diet is 16% kcal from fat

Oxidation of Mouse Food

The food from HF+WF group was significantly less oxidized ($p < 0.05$) than the food from the high-fat control (HFC) group (Table 3). There were no other significant differences in oxidation between diet groups.

Table 3. Peroxide Value of Mouse Food

Diet Group	Peroxide Value (meq /kg fat)
High-Fat Control	6.8
High-Fat Defatted Flaxseed	3.2
Low-Fat Control	3.0
High-Fat Flaxseed Oil	3.2
High-Fat Whole Flaxseed	1.9 [a]

[1] Data are presented as mean, $p < 0.05$
[2] [a] Significantly lower compared to high-fat control
[3]All high-fat diets are 45% kcal from fat. Low-fat diet is 16% kcal from fat

Effect of Diet on Weight Gain

Prior to diet treatment there was a significant difference in body weight among diet groups ($p = 0.0310$). The high-fat defatted flaxseed (HF+DF) group weighted significantly less than the HF+WF group at the beginning of the study. After eight weeks of dietary treatment, weight gain was significantly different among groups ($p < 0.001$). HFC ($p = 0.0087$), HF+DF ($p < 0.001$), LFC ($p < 0.001$), and HF+FO ($p < 0.001$) diet groups had significantly less weight gain than the HF+WF group. The LFC group gained significantly less weight than the HF+DF ($p < 0.001$), HF+FO ($p = 0.0014$), and the HFC ($p < 0.001$) groups. The HF+FO group gained significantly less than the HFC ($p = 0.0079$) (Table 4).

Table 4. Pre-Weight, Post-Weight, and Weight Gain throughout 8 Weeks of Diet Treatment

Diet Group	Pre-Weight (g)	Post-Weight (g)	Weight Gain (g)
High-Fat Control	19.88 ± 0.38	33.80 ± 0.98	13.92 ± 0.83 [e]
High-Fat + Defatted Flaxseed	19.81 ± 0.47 [e]	31.55 ± 1.09	11.56 ± 0.69 [e]
Low-Fat Control	19.61 ± 0.65	23.72 ± 0.72	4.33 ± 0.52 [ebad]
High-Fat + Flaxseed Oil	19.93 ± 0.37	29.62 ± 0.62	9.54 ± 0.62 [ea]
High-Fat + Whole Flaxseed	21.10 ± 0.31	39.27 ± 1.54	18.17 ± 1.34

[1]Data are presented as mean ± standard error, $p < 0.05$

[2] [e]Significantly lower compared to high-fat whole flaxseed, [b]significantly lower compared to high-fat defatted flaxseed, [a]significantly lower compared to the high-fat control, [d]significantly lower compared to high-fat flaxseed oil

[3]All high-fat diets are 45% kcal from fat. Low-fat diet is 16% kcal from fat

Effect of Diet Treatment on Gene Expression

Muscle Tissue

After eight weeks of dietary treatment, there were differences in gene expression among diet groups in the muscle tissue ($p = 0.01$). The expression of DNMT3b mRNA was reduced by 21.26 fold ($p = 0.0092$), and PPAR-α mRNA was reduced by 17.47 fold ($p = 0.03$) in the HF+WF diet group compared to HFC group. The expression of DNMT3b mRNA was significantly reduced in the HF+WF group compared to HF+DF ($p = 0.0392$), LFC ($p = 0.0386$),

and HF+FO (p = 0.0144) groups. The expression of PPAR-α mRNA was significantly reduced in the HF+WF group compared to the HF+FO diet group (p= 0.0416) (Table 5).

Table 5. Fold Change in Gene Expression in Muscle Tissue after 8 Weeks of Dietary Treatment

Diet Group	DNMT1	DNMT3a	DNMT3b	Leptin	PPAR-α
HF+DF	-2.26 ± 1.79	-2.03 ± 1.12	-4.47 ± 3.48	-4.15 ± 3.66	-4.07 ± 3.65
LFC	-3.06 ± 1.93	-3.30 ± 2.11	-4.41 ± 3.03	-4.95 ± 3.75	-4.11 ± 3.33
HF+FO	-0.84 ± 0.22	-1.26 ± 0.39	-1.30 ± 0.74	-1.20 ± 0.51	-0.88 ± 0.27
HF+WF	-11.89 ± 10.22	-2.19 ± 1.43	-21.26 ± 19.11 [abcd]	-14.58 ± 13.63	-17.47 ± 15.55 [ad]

[1]Data are presented as mean ± standard error, n=12; Samples were analyzed in duplicate and the average of the two samples was used for statistical analysis.
[2a]Significantly decreased compared to HFC, [b] significantly decreased compared to HF+DF, [c] significantly decreased compared to LFC, [d] significantly decreased compared to HF+FO, [e] significantly decreased compared to HF+WF, $p < 0.05$.
[3]Results were normalized to 18S rRNA mRNA levels and expressed as fold change. The fold change values were calculated as a relative change in comparison to the high-fat control (expression equals 1).
[4]DNMT: DNA methyltransferase, PPAR-a: peroxisome proliferator-activated receptor alpha, HF: High-fat diet, DF: defatted flaxseed (6%), LFC: low-fat control, FO: flaxseed oil (4% flaxseed oil), WF: whole flaxseed (10%).
[5]All high-fat diets are 45% kcal from fat. Low-fat diet is 16% kcal from fat.

Adipose Tissue

In the adipose tissue, there were also differences in gene expression among diet groups (p = 0.0001). The expression of DNMT1 mRNA was reduced by 10.32 fold in the HF+FO diet group compared to HFC group (p < 0.001). Leptin mRNA expression was reduced by 12.27 fold in the LFC diet (p < 0.001), 7.06 fold due to HF+FO diet (p = 0.0037), and 4.96 fold in the HF+WF diet (p = 0.0403), compared to HFC diet. Following treatment with the LFC and HF+FO diet, a significant change in gene expression was observed (p < 0.001 and p = 0.0002 respectively). The expression of DNMT1 mRNA was significantly reduced in the HF+FO group compared to the LFC (p = 0.0010) and HF+WF (p = 0.0012) groups. Leptin mRNA expression was significantly reduced in the LFC group compared to the HF+DF (p < 0.001), HF+FO (p = 0.0314), and HF+WF groups (p = 0.0026) (Table 6).

Table 6. Fold Change in Gene Expression in Adipose Tissue after 8 Weeks of Dietary Treatment

Diet Group	DNMT1	DNMT3a	DNMT3b	Leptin	PPAR-α
HF+DF	-5.76 ± 2.20	-2.75 ± 1.46	-1.35 ± 0.24	-2.01 ± 0.65	-0.88 ± 0.32
LFC	-2.30 ± 0.68	-5.71 ± 1.45	-0.32 ± 0.11	-12.27 ± 3.94 [abde]	-0.32 ± 0.10
HF+FO	-10.32 ±6.06 [ace]	-5.08 ± 2.45	-1.42 ± 0.64	-7.06 ± 2.63 [ab]	-1.42 ± -.70
HF+WF	-2.46 ±0.40	-0.99 ± 0.29	-0.87 ± 0.17	-4.96 ± 3.24 [a]	-0.58 ± 0.11

[1]Data are presented as mean ± standard error, n=12; Samples were analyzed in duplicate and the average of the two samples was used for statistical analysis.
[2a]Significantly decreased compared to HFC, [b] significantly decreased compared to HF+DF, [c] significantly decreased compared to LFC, [d] significantly decreased compared to HF+FO, [e] significantly decreased compared to HF+WF, $p < 0.05$.
[3]Results were normalized to 18sRNA mRNA levels and expressed as fold change. The fold change values were calculated as a relative change in comparison to the high-fat control (expression equals 1).
[4]DNMT: DNA methyltransferase, PPAR-a: peroxisome proliferator-activated receptor alpha, HF: High-fat diet, DF: defatted flaxseed (6%), LFC: low-fat control, FO: flaxseed oil (4% flaxseed oil), WF: whole flaxseed (10%).
[5]All high-fat diets are 45% kcal from fat. Low-fat diet is 16% kcal from fat.

Correlation between DNMT, PPAR-α, and Leptin Expression

In each diet group gene expression in both muscle and fat tissues was correlated to one another. Weight gains were also correlated to leptin and PPAR-α expression for each diet group and tissue.

Muscle Tissue

In the muscle tissue, there was a positive correlation between PPAR-α and leptin ($p < 0.001$, $r = 1.00$), DNMT1 ($p < 0.0001$, $r = 1.00$), DNMT3a ($p < 0.0001$, $r = 1.00$) or DNMT3b mRNA expression ($p < 0.0001$, $r = 0.99$) in the HF+DF group. There was also a positive correlation between leptin and DNMT1 ($p < 0.0001$, $r = 1.00$), DNMT3a ($p < 0.0001$, $r = 1.00$), or D NMT3b mRNA expression ($p < 0.0001$, $r = 0.99$) in the HF+DF group. A positive correlation was also observed between DNMT1 and DNMT3a ($p < 0.0001$, $r = 0.99$) or DNMT3b ($p < 0.0001$, $r = 0.99$) and between DNMT3a and DNMT3b mRNA expression ($p < 0.0001$, $r = 0.97$) (Table 7).

Table 7. Correlation between Gene Expression for HF+DF in Muscle Tissue

	DNMT1	DNMT3a	DNMT3b	PPAR-α	Leptin
DNMT1	---	0.99**	0.99**	1.00**	1.00**
DNMT3a		---	0.97**	1.00**	1.00**
DNMT3b			---	0.99**	0.99**
PPAR-α				---	1.00**
Leptin					---

[1]Data are presented as Pearson correlation coefficients
[2]*p<0.05 ** p<0.0001
[3]HF+DF (high-fat diet [45% kcal from fat] + 6 defatted flaxseed)

In the LFC group, there was a positive correlation between PPAR-α and leptin ($p < 0.0001$, $r = 1.00$), DNMT1 ($p < 0.0001$, $r = 0.99$), DNMT3a ($p < 0.0001$, $r = 0.99$), or DNMT3b mRNA expression ($p < 0.0001$, $r = 0.99$). There was also a positive correlation between leptin and DNMT1 ($p < 0.0001$, $r = 1.00$), DNMT3a ($p < 0.0001$, $r = 0.98$), or DNMT3b mRNA expression ($p < 0.0001$, $r = 1.00$) in the LFC group. A positive correlation between DNMT1 and DNMT3a ($p < 0.0001$, $r = 0.96$), or DNMT3b ($p < 0.001$, $r = 1.00$) and between DNMT3a and DNMT3b mRNA expression ($p < 0.0001$, $r = 0.97$) was observed (Table 8).

Table 8. Correlation between Gene Expression for LFC in Muscle Tissue

	DNMT1	DNMT3a	DNMT3b	PPAR-α	Leptin
DNMT1	---	0.96**	1.00**	0.99**	1.00**
DNMT3a		---	0.97**	0.99**	0.98**
DNMT3b			---	0.99**	1.00**
PPAR-α				---	1.00**
Leptin					---

[1]Data are presented as Pearson correlation coefficients
[2]*p<0.05 **p<0.0001
[3]LFC (Low-fat control [16% kcal from fat], pair-fed to HF+FO group)

In the HF+FO group, there was a positive correlation between PPAR-α and leptin ($p < 0.0001$, $r = 0.97$), DNMT1 ($p < 0.0001$, $r = 0.89$), or DNMT3b mRNA expression ($p < 0.0001$, $r = 0.94$). There was a positive correlation between leptin and DNMT1 ($p < 0.0001$, $r = 0.91$) or DNMT3b mRNA expression ($p < 0.0001$, $r = 0.98$) in the HF+FO group. No significant correlation existed between DNMT1 and DNMT3a ($p > 0.05$, $r = 0.08$) or DNMT3a and

DNMT3b mRNA expression ($p > 0.05$, $r = -0.06$). However a significant positive correlation was observed between DNMT1 and DNMT3b mRNA expression ($p < 0.001$, $r = 0.91$) (Table 9).

Table 9. Correlation between Gene Expression for HF+FO in Muscle Tissue

	DNMT1	DNMT3a	DNMT3b	PPAR-α	Leptin
DNMT1	---	0.08	0.91**	0.89**	0.91**
DNMT3a		---	-0.06	0.24	0.07
DNMT3b			---	0.94**	0.98**
PPAR-α				---	0.97**
Leptin					---

[1]Data are presented as Pearson correlation coefficients
[2]*p<0.05 **p<0.0001
[3]HF+DF (high-fat diet [45% kcal from fat] + 4% flaxseed oil)

In the HF+WF diet group, PPAR-α mRNA expression was positively correlated with leptin ($p < 0.001$, $r = 1.00$), DNMT1 ($p < 0.0001$, $r = 1.00$), DNMT3a ($p < 0.0001$, $r = 0.99$), or DNMT3b mRNA expression ($p < 0.0001$, $r = 1.00$). There was also a positive correlation between leptin and DNMT1 ($p < 0.0001$, $r = 1.00$), DNMT3a ($p < 0.0001$, $r = 0.99$), or DNMT3b mRNA expression ($p < 0.0001$, $r = 1.00$) in the HF+WF group. A positive correlation between DNMT1 and DNMT3a ($p < 0.0001$, $r = 0.99$), or DNMT3b ($p < 0.001$, $r = 1.00$) and between DNMT3a and DNMT3b mRNA expression ($p < 0.0001$, $r = 0.99$) was observed (Table 10).

Table 10. Correlation between Gene Expression for HF+WF in Muscle Tissue

	DNMT1	DNMT3a	DNMT3b	PPAR-α	Leptin
DNMT1	---	0.99**	1.00**	1.00***	1.00**
DNMT3a		---	0.99**	0.99**	0.99**
DNMT3b			---	1.00**	1.00**
PPAR-α				---	1.00**
Leptin					---

[1]Data are presented as Pearson correlation coefficients
[2]*p<0.05 **p<0.0001
[3]HF+DF (high-fat diet [45% kcal from fat] + 10% whole flaxseed)

Adipose Tissue

In the adipose tissue, there was a positive correlation between PPAR-α and leptin ($p = 0.0002$, $r = 0.88$), DNMT1 ($p < 0.0001$, $r = 0.90$), or DNMT3a mRNA expression ($p < 0.0001$, r

= 0.93) in the HF+DF treatment group. There was also a positive correlation between leptin and DNMT1 ($p < 0.0001$, $r = 0.92$), or DNMT3a mRNA expression ($p < 0.0001$, $r = 0.96$). A significant correlation was not observed between leptin and DNMT3b mRNA expression ($p > 0.05$, $r = 0.25$). A positive correlation between DNMT1 and DNMT3a ($p < 0.0001$, $r = 0.97$) was observed. No significant correlation was observed between DNMT1 and DNMT3b ($p > 0.05$, $r = 0.25$) or DNMT3a and DNMT3b mRNA expression ($p > 0.05$, $r = 0.22$) (Table 11).

Table 11. Correlation between Gene Expression for HF+DF in Adipose Tissue

	DNMT1	DNMT3a	DNMT3b	PPAR-α	Leptin
DNMT1	---	0.97**	0.25	0.90**	0.92**
DNMT3a		---	0.22	0.93**	0.96**
DNMT3b			---	0.5	0.25
PPAR-α				---	0.88*
Leptin					---

[1]Data are presented as Pearson correlation coefficients
[2]*p<0.05 **p<0.0001
[3]HF+DF (high-fat diet [45% kcal from fat] + 6% defatted flaxseed)

In the LFC group, there was a positive correlation between PPAR-α and leptin ($p = 0.0037$, $r = 0.77$), DNMT1 ($p = 0.0001$, $r = 0.89$), or DNMT3b mRNA expression ($p < 0.0001$, $r = 0.94$) as well as between leptin and DNMT1 ($p < 0.0001$, $r = 0.92$), DNMT3a ($p = 0.01$, $r = 0.69$), or DNMT3b mRNA expression ($p < 0.05$, $r = 77$). A positive correlation was observed between DNMT1 and DNMT3a ($p < 0.05$, $r = 0.64$) or DNMT3b ($p < 0.05$, $r = 0.74$). No significant correlation was observed between DNMT3a and DNMT3b mRNA expression ($p > 0.05$, $r = 0.31$) (Table 12).

Table 12. Correlation between Gene Expression for LFC in Adipose Tissue

	DNMT1	DNMT3a	DNMT3b	PPAR-α	Leptin
DNMT1	---	0.64*	0.74*	0.89**	0.92**
DNMT3a		---	0.31	0.49	0.69*
DNMT3b			---	0.94**	0.61*
PPAR-α				---	0.77*
Leptin					---

[1]Data are presented as Pearson correlation coefficients
[2]*p<0.05 **p<0.0001
[3]LFC (Low-fat control [16% kcal from fat], pair-fed to HF+FO group)

In the HF+FO treatment group there was a positive correlation between PPAR-α and DNMT1 ($p < 0.0001$, $r = 0.99$), DNMT3a ($p = 0.0013$, $r = 0.81$), or DNMT3b mRNA expression ($p < 0.0001$, $r = 0.99$). In the HF+FO group, there was also a positive correlation between leptin and DNMT1 ($p = 0.02$, $r = 0.65$), DNMT3a ($p = 0.0004$, $r = 0.86$), or DNMT3b ($p = 0.02$, $r = 0.58$). A positive correlation was observed between DNMT1 and DNMT3a ($p < 0.05$, $r = 0.84$) or DNMT3b ($p < 0.001$, $r = 0.98$) and between DNMT3a and DNMT3b mRNA expression ($p < 0.05$, $r = 0.77$) (Table 13).

Table 13. Correlation between Gene Expression for HF+FO in Adipose Tissue

	DNMT1	DNMT3a	DNMT3b	PPAR-α	Leptin
DNMT1	---	0.84*	0.98**	0.99**	0.65*
DNMT3a		---	0.77*	0.81*	0.86*
DNMT3b			---	0.99**	0.58*
PPAR-α				---	0.65*
Leptin					---

[1]Data are presented as Pearson correlation coefficients
[2]*p<0.05 **p<0.0001
[3]HF+DF (high-fat diet [45% kcal from fat] + 4% flaxseed oil)

In the HF+WF treatment group, there were no significant correlations observed between PPAR-α and DNMT1 ($p > 0.05$, $r = 0.08$), DNMT3a ($p > 0.05$, $r = -0.46$), or DNMT3b mRNA expression ($p > 0.05$, $r = 0.49$). No significant correlation was observed between leptin and DNMT1 ($p > 0.05$, $r = -0.21$), DNMT3a ($p > 0.05$, $r = 0.09$), or DNMT3b mRNA expression ($p > 0.05$, $r = -0.36$). No significant correlations were observed between DNMT1 and DNMT3a (p

> 0.05, r = 0.19) or DNMT3b mRNA expression (p > 0.05, r = 0.15). A significant negative correlation was observed between DNMT3a and DNMT3b mRNA expression (p < 0.05, r = -0.64) (Table 14).

Table 14. Correlation between Gene Expression for HF+WF in Adipose Tissue

	DNMT1	DNMT3a	DNMT3b	PPAR-α	Leptin
DNMT1	---	0.19	0.15	0.08	-0.21
DNMT3a		---	-0.64*	-0.46	0.09
DNMT3b			---	0.49	-0.36
PPAR-α				---	-0.29
Leptin					---

[1]Data are presented as Pearson correlation coefficients
[2]*p<0.05 **p<0.0001
[3]HF+DF (high-fat diet [45% kcal from fat] + 10% whole flaxseed)

Correlation between Gene Expression and Body Weight

Weight gain was correlated to leptin and PPAR-α expression for each diet group and tissue.

In the muscle tissue, no correlation was observed between weight gain and PPAR-α or leptin expression for any of the diet groups (Table 15).

Table 15. Correlation between PPAR-α mRNA or Leptin mRNA Expression and Weight Gain in Diet Treatment Groups in Muscle Tissue

Diet Group	PPAR-α	Leptin
HF+DF Weight Gain	-0.27	-0.28
LFC Weight Gain	0.41	0.41
HF+FO Weight Gain	-0.28	-0.26
HF+WF Weight Gain	0.54	0.55

[1]Data are presented as Pearson correlation coefficients
[2]PPAR-a: peroxisome proliferator-activated receptor alpha, HF: High-fat diet, DF: defatted flaxseed (6%), LFC: low-fat control, FO: flaxseed oil (4% flaxseed oil), WF: whole flaxseed (10%).
[3]All high-fat diets are 45% kcal from fat. Low-fat diet is 16% kcal from fat.

There was also no correlation found in the adipose tissue (Table 16).

Table 16. Correlation between PPAR-α mRNA or Leptin mRNA Expression and Weight Gain in Diet Treatment Groups in Adipose Tissue

Diet Group	PPAR-α	Leptin
HF+DF Weight Gain	-0.03	-0.1
LFC Weight Gain	-0.19	0.17
HF+FO Weight Gain	-0.38	-0.29
HF+WF Weight Gain	-0.23	-0.08

[1]Data are presented as Pearson correlation coefficients

[2]PPAR-a: peroxisome proliferator-activated receptor alpha, HF: High-fat diet, DF: defatted flaxseed (6%), LFC: low-fat control, FO: flaxseed oil (4% flaxseed oil), WF: whole flaxseed (10%).

[3]All high-fat diets are 45% kcal from fat. Low-fat diet is 16% kcal from fat.

CHAPTER 5. DISCUSSION

Obesity has reached epidemic proportions in the United States with more than one-third (35.7%) of U.S. adults and 17% of children classified as obese (Centers for Disease Control and Prevention, 2012). Many interventions have been geared towards prevention of obesity. One avenue researchers have explored is epigenetic regulation through DNA methylation. DNA methylation can cause genes to be more highly expressed or silenced through access of transcription factors to genes (Shen et al., 2014). Compounds in the diet have been found to influence gene methylation. Cancer researchers have identified a compound found in green tea, EGCG, to cause decreased activity of the enzymes responsible for adding methyl groups to genes, DNA methyltransferases (DNMTs), leading to reactivation of tumor suppressor genes (Fang et al., 2003). Flaxseed contains both omega-3 fatty acid and an antioxidant lignan, secoisolariciresinol diglucoside (SDG), which has been found to improve obesity-related indicators (Baranowski et al., 2012; El-Shobaki, Mohamed, Abd-El-Azeem, & Hegazy, 2013) and prevent weight gain despite consumption of a high-fat diet (Park & Velasquez, 2012). Likely gene targets for studying prevention of obesity may include those with a known role in obesity. For example, leptin which regulates energy intake and expenditure (Meiera & Gressner, 2004) and PPAR-α which regulates the activity of proteins involved in energy metabolism (Ferre, 2004) are two potential gene targets for this type of research.

In the present study, the effects of the health promoting compounds present in flaxseed on DNMT expression were investigated. As well as the relationships between DNMT and obesity-associated gene expression, and obesity-associated gene expression and body weight were determined. If negative correlations exist, further study would be warranted regarding flaxseed and prevention of weight gain through epigenetic regulation by DNA methylation.

Prior to dietary treatment, the HF+WF group had the highest body weight and was significantly greater than the HF+DF diet group. The HF+WF group also had the greatest average weekly food intake which was significantly greater than the HF+FO group. The LFC group also had significantly greater food intake than the HF+FO. After eight weeks of dietary treatment, the HF+WF group gained significantly more weight than all other diet groups. The LFC group had significantly less weight gain among diet groups, which is to be expected due to low-fat content of the diet. The average percent difference in weight gain between the HF+WF and the LFC is 49.3%, likely indicating the development of DIO by the HF+WF group. Compared to the LFC group the HFC, HF+DF, and the HF+FO diet groups gained 35.1%, 28.4%, and 22.1% more weight, respectively. It is possible that the mice did not fully achieve DIO due to the short duration of the study, which lasted eight weeks. Lin et al. (2000) observed that there are three stages of developing DIO in C57BL/6J mice. They reported stage two occurs at eight weeks of feeding and is accompanied by reduced food intake, increased leptin secretion, and maintained leptin sensitivity. At 19 weeks, stage three, the mice had increased food intake and reduced leptin sensitivity (Lin, Thomas, Storlien, & Huang, 2000). In the current study, the mice may not have fully reached DIO and thus the final body weights may not reflect actual differences in weight due to dietary treatment.

The HF+WF group gained significantly more weight than all other diet groups including the HFC group, which contradicts results from previous studies in which supplementation with n-3 FA or SDG was protective against weight gain when consuming a high-fat diet (El-Shobaki, S. mohamed, Abd-El-Azeem, & Hegazy, 2013; Park & Valasquez, 2012; Tominaga et al., 2012). However, whole flaxseed also contains fiber and carbohydrates, which has shown to increase short chain fatty acid fermentation in the colon with possible effects on lipid metabolism and

ultimately weight regulation (Conterno, Fava, Viola, & Tuoh, 2011). Although current research is inconclusive as to the exact relationship between gut environment and weight regulation, it is possible that a similar mechanism is responsible for the significant weight gain observed in the HF+WF group. This explanation would not be fitting for the HF+DF diet which has similar fiber and carbohydrate content because these mice gained less weight than the HF+WF group. Both the HF+DF and the HF+FO group gained significantly less weight than the HF+WF, which may indicate the ability of each component of flaxseed, the n-3 FA and the SDG, to protect against weight gain separately rather than in combination.

Additionally, the HF+FO group also consumed significantly less food than the HF+WF and LFC group, which raises the possibility that a high-fat diet accompanied by flaxseed oil intake may be protective against weight gain through regulation of food intake. Similar results have been seen in a DIO mice model where mice were fed a high-fat diet that was either n-3 FA rich or n-3 FA deficient. The mice that were in the n-3 FA containing group consumed significantly less food than the n-3 FA deficient group (Fan, Liu, Shen, Deckellbaum, & Qi, 2011).

Another possible explanation as to why the HF+FO group consumed significantly less food may be due to oxidation of the foods, as rancid fats cause foods to have an undesirable aroma and taste. To minimize the possibility of this happening fresh food was provided daily. We tested the peroxide value on samples of the food from each group and found that the HF+FO food was not significantly more oxidized than any of the other groups. Surprisingly, the HFC was the most oxidized, which was only significant compared to the HF+WF, and was not reflected in food intake.

In contrast, although the HF+DF gained significantly less weight than the HF+WF group, they did not consume significantly less food. This provides evidence that supplementation of

defatted flaxseed may have prevented weight gain by a mechanism other than through regulating intake, such as epigenetic regulation. Similar results have been found by Boque et al. (2013), when feeding rats a high-fat diet supplemented with apple polyphenols, which possess antioxidant properties. Supplementation with apple polyphenols in combination with a high-fat sucrose diet, prevented weight gain without decreased food intake. The authors speculated that this compound regulates gene expression through epigenetic regulation by DNA methylation (Boque et al., 2013). Due to the HF+DF group gaining significantly less weight than the HF+WF group without significantly less food intake, it is possible that SDG, with its antioxidant properties, is capable of preventing weight gain through epigenetic regulation as has been demonstrated with other antioxidants.

Unexpectedly, DNMT3b was significantly lower in the muscle tissue of the HF+WF group, which gained significantly more weight than all other groups. We would expect that DNMT3b, as well as DNMT1 and DNMT3a would negatively correlate with obesity-associated gene expression of PPAR-α or leptin because, in theory, reduced DNMT expression would indicate decreased methylation of genes allowing for increased gene expression. However, all significant correlations observed between DNMT expression and PPAR-α or leptin expression were positive in all treatment groups of both muscle and adipose tissue. A link between promoter methylation and gene expression has been well established. Fujiki et al. (2009) demonstrated that PPAR-γ expression is reduced when its promoter is hypermethylated, and when treated with a DNA methylation inhibitor its expression is increased in a dose dependent manner (Fujiki, Kano, Shiota, & Murata, 2009). In contrast, Yang et al. (2014) reported a positive correlation between DNA methylation and gene expression when the gene was methylated on the body region rather than the promoter region (Yang et al., 2014). Boque et al. (2013) and Ngo et al. (1996) also

observed a positive correlation between DNA methylation and gene expression. They concluded that the positive correlation may be due to DNA methylation occurring on negative regulatory elements, which could increase transcription on the gene (Boque et al., 2013; Ngo, Gourdji, & Laverriere, 1996). In the present study, analysis of DNA methylation was not performed. It is possible that PPAR-α and leptin were methylated on the body region rather than the promoter region resulting in a positive correlation. However, further research is needed to identify the relationship between DNA methylation and gene expression.

Reduced expression of DNMT1 in the HF+FO group was observed in the adipose tissue. This group gained significantly less weight than the HFC and HF+WF groups, but a positive correlation was also observed between DNMT1 and PPAR-α expression. Again, this could potentially be due to the previously mentioned mechanism.

Kamei et al., (2009) studied a model of transgenic mice overexpressing DNMT3a and found that DNMT3a expression was increased in the adipose tissue but not the skeletal muscle or liver tissue (Kamei et al., 2009). This may explain why DNMT3a expression was not increased in muscle tissue. However, DNMT3a was also not more highly expressed in the adipose tissue of any diet group. Although Kamei et al. (2009) concluded DNMT3a may play a role in obese adipose tissue, there were several differences in study design that may account for the differences observed in DNMT3a expression. Most notably was the intentional overexpression of DNMT3a (Kamei et al., 2009). Further research is needed to determine the role of DNMT3a in the tissues of obese mice.

It is also possible that the compounds present in flaxseed are not capable of regulating DNMT expression. Compounds such as epigallocatechin gallate (EGCG) have been found to demethylate genes to increase expression by decreasing DNMT1 expression, while others such

as curcumin have not exhibited this effect (Mirza et al., 2013). An alternative explanation for the flaxseed being unable to regulate obesity-associated gene expression may be due to their inability to activate DNMTs, with the exception of the HF+FO group in which DNMT1 was significantly reduced compared to HFC group. If DNMTs were activated they may be able to add methyl groups to genes, therefore regulating gene expression. It has been reported that compounds such as EGCG interact with DNMT1 at its catalytic center to initiate activity. Specifically, it has been proposed that these compounds contain a gallic/pyrogallic acid moiety that enables them to initiate DNMT activity (Li & Tollefsbol, 2010). The possibility exists that the compounds present in flaxseed are not capable of interacting with DNMT catalytic site, and thus not capable of epigenetic modification by DNA methylation. However, DNMT activity has not been measured in this study so this relationship remains inconclusive.

As expected, in the muscle tissue, PPAR-α mRNA expression was significantly reduced in the HF+WF group, which gained the most weight, although decreased compared to all groups, this was only significant in comparison with the HFC and HF+FO groups. This supports previous reports that PPAR-α increases energy expenditure (Ferre, 2004), and is decreased with increased weight gain (Guerre-Millo et al., 2000). This same result was not observed in the adipose tissue, but it could be due to tissue dependent expression of PPAR-α. Peroxisome proliferator-activated receptor alpha is more highly expressed in the liver and muscle tissues compared to adipose tissue (Ferre, 2004), which may have prevented a significant difference in expression in the adipose tissue.

Significantly less leptin mRNA expression occurred in the adipose tissue of the LFC group compared to all other groups. This was expected because the LFC group had the least weight gain; therefore, leptin expression is reflective of fat stores rather than ability to regulate weight in

DIO due to development of leptin resistance, which has been previously reported (Boque et al., 2013; Moraes et al., 2003). Both the HF+FO and the HF+WF groups had significantly less leptin mRNA expression in the adipose tissue compared to the HFC group. The HF+FO group gained the least weight of all three of the high-fat groups; however, this was only significant in comparison to the HFC or HF+WF groups. This is consistent with previous reports that supplementation with n-3 FA decreases leptin mRNA expression in adipose tissue. Fan et al. (2011) fed mice a low-fat control diet or one of four high-fat diets. The high-fat diets were supplemented with soybean oil, fish oil, a mixture of soybean and fish oil (1:1 ratio), which contained n-3 FA, or sunflower oil which was n-3 FA deficient. Body weight, food intake, and leptin expression were lower in all three of the n-3 FA diets compared to the n-3 FA deficient diet. Plasma leptin levels were decreased in all three n-3 FA diets, but this was only significant between the fish oil n-3 FA diet and the n-3 FA deficient diet (Fan et al., 2011). Together, the reduced leptin mRNA expression and decreased plasma leptin indicate maintained leptin sensitivity, which may help explain why the HF+FO diet group had least weight gain and reduced leptin expression compared to HFC. While not significant, Fan et al. (2011) also observed a decreased methylation in many of the leptin promoter CpG sites in the low-fat control groups compared to the high-fat diet groups. A reduced methylation level was also observed in the high-fat n-3 FA containing diets compared to the n-3 FA deficient groups, although also not statistically significant (Fan et al., 2011). The decrease in promoter methylation observed in both the high-fat n-3 FA containing diet and the low-fat control diet suggest a possible relationship between DNA methylation, weight regulation, and n-3 FA supplementation.

In contrast, the HF+WF group gained the most weight of all three high-fat diets. The reduced leptin mRNA expression and highest weight gain is counterintuitive. This may be explained by

the short duration of the feeding intervention, in which there was not sufficient time to allow body weight to reflect leptin action. This may be overshadowed by the increased intake associated with fiber and carbohydrate content of whole flaxseed as mentioned earlier, which resulted in weight gain not observed in the HF+FO group. Further research is needed to determine the role of flaxseed in leptin and weight regulation. Leptin mRNA expression was not significantly influenced in the muscle tissue which may be due to leptin being primarily secreted in the adipose tissue (Meiera & Gressner, 2004).

Leptin expression, through supplementation of n-3 FA, may not be solely regulated by DNA methylation, but may instead be regulated by other epigenetic mechanisms. When mice were fed a high-fat diet and supplemented with n-3 FA from fish oil, plasma leptin concentration and leptin expression in adipose tissue was significantly lower compared to the high-fat diet group without n-3 FA supplementation. Addition of n-3 FA did not affect methylation of CpG sites of the leptin promoter. However, it did reduce DNMT1 and MBD2 binding at promoter, but DNMT3a and DNMT3b were not affected. Also in the low-fat control group, there was a negative correlation between leptin promoter methylation and leptin expression. However, it was reversed in the mice fed a high-fat diet without n-3 FA supplementation, showing a positive correlation between leptin promoter methylation and leptin expression. The authors concluded the positive correlation observed in the obese mice may indicate an altered regulation of leptin expression in the obese state. Although the mechanism behind this is unknown, it is a possible explanation as to why leptin is increased in the obese state (Shen et al., 2014). Further investigation of this mechanism may help determine how to epigenetically target leptin in regards to weight regulation. Though other studies indicate that leptin may be regulated by DNA methylation of its promoter regions (Milagro et al., 2009), which is also possible through

supplementation of compounds in the diet (Boque et al., 2013); however, it is unclear if n-3 FA are capable of regulating leptin expression through DNA methylation.

Unexpectedly, in the current study no correlation was observed between weight gain and PPAR-α or leptin expression in any of the diet groups. This may also be due to the short length of the study in which weight did not yet reflect gene expression. It is possible that if feeding had continued to 19 weeks, as reported previously to be the final stage of DIO development (Lin et al., 2000), weight may have reflected gene expression as expected. The lack of correlation between leptin expression and bodyweight may also be explained by the development of leptin resistance. Lin et al. (2000) observed plasma leptin to be increased by 223% at eight weeks during DIO, which may explain leptin resistance (Lin et al., 2000). However, in this study serum leptin was not measured; therefore, development of leptin resistance as evidenced by increased serum leptin cannot be determined. Peroxisome proliferator-activated receptor alpha expression may not have been correlated with body weight due to it not being activated, as PPAR-α activity is regulated by ligands produced in the body (Ferre, 2004). However, PPAR-α activity was not measured in this study, so the relationship between body weight and PPAR-α expression also remains elusive.

Limitations to this study include the short duration of the feeding intervention which may not have allowed mice to fully develop DIO (Lin et al., 2000), as mentioned above. This study was performed using an animal model and may not be generalizable to humans in regards to regulatory mechanisms involved in obesity and obesity reduction, which are different between species. This experiment was carried out in a controlled setting and may not accurately represent the events occurring in a natural environment. Another limitation is not measuring serum leptin. If serum leptin would have been measured, it could have been identified if leptin resistance was

developed as increased plasma leptin is indicative of leptin resistance. This could potentially explain why the HF+WF and the HF+FO groups had significantly less leptin expression while simultaneously having significant differences in weight gain. The activity of PPAR-α determined by its ability to induce expression of genes involved in lipid catabolism, may have provided insight as to whether PPAR-α expression accurately reflected its function in obesity (Ferre, 2004). Measurement of DNMT activity, determined by the ability of the health-promoting compounds in flaxseed to initiate DNMT activity by interacting with its catalytic center, may have provided further insight into the role of diet in DNMT activation (Li & Tollefsbol, 2010). Another limitation to methodology is not including western blotting to confirm proteins identified by RT-qPCR. The methylation profile of leptin and PPAR-α was not measured in this study, limiting data to correlational reports between DNMT and leptin or PPAR-α expression.

Future research should focus on determining the ability of flaxseed to affect the concentration and/or activity of DNMTs as well as the mechanism responsible. Furthermore, studies should aim to identify if leptin and PPAR-α are epigenetically regulated, specifically by DNA methylation. As discussed previously, we did not look at the methylation profile of each gene and were unable to determine the relationship between gene methylation and its resulting expression. In future studies, plasma leptin should be considered to differentiate between increased leptin action and increased leptin expression resulting in leptin resistance.

In conclusion, the results indicate that supplementation with flaxseed affected selected gene expression. However, this was a preliminary study to observe if there was a possible relationship between DNMT and obesity-associated gene expression as a factor of flaxseed supplementation. Further research is needed to identify the specific mechanisms regulating leptin or PPAR-α expression during DIO development.

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NDSU NORTH DAKOTA STATE UNIVERSITY

November 26, 2012

Dr. Yeong Rhee
Health Nutrition & Exercise Science
EML

Re: IACUC Approval of Protocol, **#A13019 "The role of flaxseed and buckwheat In control of obesity"** Category C

Research Team: Y. Rhee, M. Pulkrabek, K. Hert, T. Heck

Approval Date:	**November 26, 2012**
Current Approval Period:	**November 26, 2012 to November 26, 2015**
Next Update Report Due:	**October 1, 2013**

The referenced protocol *(received: November 1, 2012)* has been reviewed by the NDSU Institutional Animal Care and Use Committee and has IACUC approval as of the date indicated above.

The IACUC requests that you keep a copy of this protocol on file at the location or facility where the animals will be housed. During the course of this project, if you plan any significant changes in the protocol, a Change in Protocol Form outlining the proposed changes must be submitted to the IACUC, and IACUC approval granted, before implementation of the changes. A report and renewal of the project is also required on an annual basis. A reminder will be sent to you about a month before the report due date.

Please feel free to consult with NDSU's Attending Veterinarian, to ask questions or discuss any animal-related needs or concerns throughout the duration your project. The IACUC chair is also available if you have questions regarding animal welfare or university requirements.

NDSU has an Animal Welfare Assurance on file with the Public Health Service's Office of Laboratory Animal Welfare (OLAW). The assurance number is **A3244-01**, last renewed on May 25, 2010. NDSU is also registered with the U.S. Department of Agriculture as an Animal Research Facility under the registration number **45-R-002**.

Thank you for your cooperation with NDSU IACUC procedures.

NDSU IACUC

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