Impact of a Western Diet on Genes Associated with Cholesterol Levels and Obesity

in *Mus musculus*

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April 2, 2012



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A Senior Comprehensive Project in Partial Fulfillment of the Requirements for a Bachelor of Science Degree from Allegheny College

I hereby acknowledge and pledge o fulfill my responsibilities as defined in the Honor Code and to maintain the integrity of both myself, and the College community as a whole.

_____
Pledge

Approval by the Senior Thesis Committee:

_____
Dr. Donmoyer

_____
Dr. Webb

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Impact of a Western Diet on Genes Associated with Cholesterol Levels and Obesity in *Mus musculus.*

Abstract

Each year in the United States, more people die from heart disease than any other cause of death. Obesity, while a growing problem in developed nations around the world, is far more prevalent in the United States than any other country. One notable contributor to the rise of both of these diseases is the typical American diet, or Western diet. Obese patients experience a greater incidence of heart disease than non-obese in part due to unhealthy cholesterol levels. The liver produces proteins, which aid in the production of cholesterol; adipose tissue, or fat, synthesize proteins called adipokines that help to regulate appetite. This study examined the regulation of two genes associated with cholesterol levels, Niemann-Pick C1-like 1 (NPC1L1) and 3-Hydroxy-3-methylglutaryl Coenzyme-A reductase (HMGCR), and two adipokines that regulate appetite, Adiponectin and Leptin. Relative mRNA abundance of these four genes was compared in liver and adipose tissue taken from mice fed either a Western or control diet. It was hypothesized that expression of Leptin would be increased in Western Diet treatment group while the expression of Adiponectin, NPC1L1, and HMGCR would decrease within the same group. Results showed that HMGCR mRNA levels decreased significantly while Leptin mRNA levels increased significantly in response to a Western diet. NPC1L1 and Adiponectin mRNA levels increased slightly in response to a Western diet and were not significantly more than the control group. All mice fed the Western diet gained weight at a significantly faster rate than mice fed the control diet. This study confirmed that a Western style diet can alter the expression of genes associated with cholesterol levels and appetite, and increase body weight.

Introduction

Heart disease is the leading killer of people in the United States. It is responsible for 26 percent of all deaths each year (Heron *et al*., 2009). Research has suggested that a Western style diet puts an individual at a 1.35 times greater risk for myocardial infarction, even after adjustments for risk factors such as age, sex, education, smoking, physical activity, and body mass index (Iqbal *et al*., 2008). A Western diet is characterized by an elevated intake of eggs, high fat foods and dairy products, sugary drinks, red meats, refined grains, and sugary desserts. This diet is considered "Western" because it is generally consumed in more developed countries and primarily associated with the United States. This diet has a greater abundance of man-made trans fatty acids (trans fats), saturated fats, cholesterol, and sodium than most diets consumed around the world.

The relationship between the consumption of saturated fats and unhealthy cholesterol levels has been extensively documented. While a diet containing saturated fats increases both bad (LDL) and good (HDL) cholesterol, the increase in LDL is much greater, which in turn increases the LDL to HDL ratio (Lichtenstein, *et al*., 2006). This ratio is crucial as HDL cholesterol helps the body remove excess LDL cholesterol from the blood. By raising this ratio, excess LDL cholesterol greatly increases the risk of heart disease (Manninen, *et al*., 1992).

Furthermore, previous research recommends replacing saturated fats with polyunsaturated fats to lower the plasma concentration of LDL cholesterol and the LDL:HDL ratio. Replacing saturated fat with polyunsaturated fat is also associated with a lower risk of coronary heart disease (CHD), which as previously stated is the leading

cause of death in the United States, where this Western diet is so prevalent (Katan, *et al.*, 2010).

The research of Hu and Willet (2002) suggest that there are 3 major dietary changes that can lessen the risk of CHD. First, Hu and Willet recommend eliminating saturated and trans-fats from one's diet in favor of non-hydrogenated unsaturated fats. Also, the addition of omega-3 fatty acids from fish, fish oil supplements, or plants is linked to a lower incidence of cardiovascular disease. Lastly, all diets should include large amounts of vegetables, fruits, nuts, whole grains, and fish in order to promote a healthy cardiovascular system (Fung, *et al.*, 2001b).

The purpose of this study was to more closely examine the effects of a Western diet high in fats on specific gene expression in mouse liver and adipose tissue. I focused on four genes that are associated with cholesterol and obesity. Niemann-Pick C1-like 1 (NPC1L1) and 3-Hydroxy-3-methylglutaryl Coenzyme-A reductase (HMGCR), both associated with cholesterol, are expressed in liver tissue. Leptin and Adiponectin are adipokines associated with obesity and are both expressed in adipose tissue.

β-Actin was used as a marker of general expression level because it is one of the most widely used reference genes for qRT-PCR (Turabelidze, *et al.*, 2010). Also, β-Actin was used in a similar study that also investigated differential gene expression after administration of a high-fat diet (Schonau-Taylor, 2011).

The first gene examined in liver tissue, which is related to cholesterol levels, was NPC1L1. NPC1L1 is mainly recognized as an apical surface protein in absorptive enterocytes (intestinal cells), however it is also expressed in the liver where its function is not completely understood. It is suggested that hepatic NPC1L1 acts to prevent

excessive biliary loss of cholesterol (Temel, *et al*., 2007). The work of Miettinen and Gylling (2000) suggests that a high-fat and cholesterol diet increases the secretion of biliary cholesterol.

The second gene examined in association with cholesterol levels was HMGCR. HMGCR catalyzes the conversion of HMG-CoA to mevalonate, the rate-limiting step in cholesterol synthesis (Endo, 1992). Studies have shown a decrease in HMGCR protein levels in subjects who eat high-fat diets (Gibbons and Pullinger, 1986). In other clinical studies, NPC1L1 and HMGCR proteins have been the targets of therapies for achieving reduced LDL cholesterol concentrations (Gouni-Berthold *et al*., 2008). Drugs that inhibit HMGCR, also known as Statins, have been shown to decrease the development and even stimulate the regression of atherosclerosis. Statins competitively inhibit HMGCR activity, which in turn prevents the production of exogenous cholesterol (Stancu and Sima, 2001). A different drug, Ezetimibe, targets the absorptive NPC1L1 proteins in the intestines but may also act on NPC1L1 proteins in liver tissue. It is known that in the intestine, ezetimibe prevents NPC1L1 from incorporating into clathrin-coated vesicles and thus inhibits cholesterol uptake (Ge *et al.,* 2008). Recent work suggests that ezetimibe also leads to improved hepatic insulin sensitivity and lipid accumulation. These are regarded as key factors in the development of Non-Alcoholic Fatty Liver Disease (NAFLD), which can progress to liver failure (Ahmed and Byrne, 2010).

One adipokine that was examined in this study was Leptin. Leptin is a hormone secreted by adipose tissue that binds with receptors in the hypothalamus, enhancing metabolic rate and signaling satiety. This interaction counteracts the actions of

neuropeptide Y, which is a hormone secreted by the hypothalamus to signal hunger (Sikka *et al.* 2010). Leptin levels in blood have been shown to be dramatically increased in obese humans (Friedman and Halaas, 1998). Leptin concentration has a significant positive correlation with a Western diet and increases the risk of obesity in mammals (Fung *et al*., 2001a).

Another protein that is commonly associated with obesity is Adiponectin. Adiponectin is an anti-inflammatory adipokine that regulates energy metabolism and insulin sensitivity. Diminished concentrations have been observed in patients with obesity, vascular disorders such as atherosclerosis, and even type 2 diabetes mellitus (Fasshauer *et al*., 2004). Research supports the negative correlation between obesity and Adiponectin levels (Arita *et al*., 1999).

This study compared the expression of NPC1L1, HMGCR, Leptin and Adiponectin between mice fed a western diet and those fed a standard mouse chow. To determine relative mRNA levels of each gene, quantitative real time-polymerase chain reaction (qRT-PCR) was used. It was hypothesized that gene expression for Leptin would be increased in the Western Diet treatment group while the expression of Adiponectin, NPC1L1, and HMGCR would decrease within the same group.

Materials and Methods

Mice

Seven 8-10 week-old and five 3-4 week-old female C57BL/6J mice were used during this study and randomly split into two equal groups (n=6). Mice were weighed at

the beginning of the experiment and once per week throughout the remainder of the feeding schedule. All mice were kept in a 12-hour light and dark cycle.

Diet

The Western diet group was fed a mouse chow diet that mimics the typical high fat "Western" diet of many Americans. In this diet, 39.9% of the total calories came from fat, 16% from protein, and 44.1% from carbohydrate. The chow was obtained from LabDiet (Richmond, IN), refrigerated at 2°C, and administered *ad libitum* for 15 weeks.

The control group was fed a standard mouse chow diet referred to as a control diet. In this diet, 14.03% of the total calories came from fat, 25.97% from protein, and 60% from carbohydrate. This chow was also obtained from LabDiet and administered *ad libitum* for 15 weeks but was stored at room temperature. All mice were given drinking water throughout the duration of the experiment.

Tissue Collection

Mice from both groups were euthanized following Animal Review Committee (ARC) approved methods – two minutes CO_2 inhalation followed by cervical dislocation. To expose the abdominal cavity, mice were cut ventrally exposing the liver and adipose tissue. The median lobe of the liver and 2-4 forceps pinches of fat, from deposits in the lower abdomen, were harvested from each mouse. All tissues were harvested using forceps and placed in separate Falcon tubes containing 5ml TriZol (Invitrogen, Carlsbad, CA) to inhibit RNase activity; tubes were flash frozen with dry ice. Samples were then immediately homogenized.

Homogenization

Tissues were homogenized using a Brinkmann Polytron PT-MR 3000 homogenizer at 12,500 rpm for 1 minute. The homogenizer was sterilized with Diethylpyrocarbonate (DEPC) treated water, followed by three washes of RNase free water. For samples in which RNA extraction was done at a later date, the homogenized tissue was separated into small microcentrifuge tubes and stored in a -70°C freezer.

RNA Isolation and Quantification

Total RNA was isolated by following the manufacturer's instructions (Appendix A) for the RNeasy Universal Kit (Qiagen, Austin, TX), collected in 60µL and stored at -70°C. The concentration of RNA was measured in ng/µl using a Nanodrop spectrophotometer, and the ratio of the absorbances (260/280 nm) indicated the quality of the RNA.

Complementary DNA (cDNA) Synthesis

cDNA was synthesized from isolated RNA using the iScript cDNA Synthesis Kit from Bio-Rad (Hercules, CA). The manufacturer's protocol was followed (Appendix B). *c*DNA was immediately stored at -20°C.

Mouse Oligonucleotide Primers

Primer sets for Leptin, Adiponectin, and β-Actin were designed and provided by Hannah Schonau-Taylor. Forward (F) and reverse (R) sequences for Niemann-Pick C1-like1 (NPC1L1) used in a previous study (Duval, *et al*., 2006), were ordered from

Eurofins (Huntsville, AL). HMGCR gene identification number and sequence was accessed using the NCBI database (NCBI GenBank, 2011). The forward and reverse sequences were designed using the Primer Design Tool from Operon (Eurofins MWG Operon, 2012). The design parameters were as follows: PCR primer length of 18-25 nucleotides, GC content of 50-65%, melt temperature range 50-80°C, and length of PCR product was 120-160 nucleotides. The lyophilized forward and reverse primers were reconstituted by adding sterile water in order to bring the primer concentration to 10μM. The forward and reverse sequences for each gene were:

Table 1. Primer Sequences Used

	Forward Primer (5' – 3')	**Reverse Primer (5' – 3')**
Leptin	GTTTTTCCATCACCCCTGCACCC	AGCACCAGACAGAGCTGAGCAC
Adiponectin	GGCAGCAGCTTTCGTCCACTTC	AAGTCCCTCCTCCACCCCTCAG
NPC1L1	GCAAGGTGATCAGGAGGTGA	ATCCTCATCCTGGGCTTTGC
HMGCR	TAGGAGGCTACAACGCCCAT	CCACCCACCGTTCCTATCTC
β-Actin	CGTCTTCCCCTCCATCG	CTCGTTAATGTCACGCAC

The primer sets were completed by combining 100μl of the forward and reverse primers and bringing the total volume to 1ml.

qRT-PCR

All tissues were tested with β-Actin while HMGCR and NPC1L1 were analyzed in liver tissue and Leptin and Adiponectin were analyzed in adipose tissue. mRNA was analyzed using qRT-PCR and the Step-One software (Applied Biosystems). The Applied Biosystems Step-One Thermal Cycler recorded the cycle threshold (C_t) value for each reaction. The qRT-PCR protocol devised by Dr. Ann Kleinschmidt of Allegheny College was followed (Appendix C). All samples were run in triplicate with Power SYBR Green, an intercalating dye that is 200X brighter when bound to double-stranded DNA, which allows for quantitative amplification. C_t's were determined by initially incubating samples at 95°C for 10 minutes. Then samples were incubated at 95°C for 15 seconds and 55° C for 1 minute for 40 cycles. The melt curve was produced by incubating samples at 95°C for 15 seconds, then at 60°C for 1 minute and finally at 95°C for 15 seconds.

C_t values were averaged for 3 wells of each target gene, unless there was an inconsistent value of at least 2 standard deviations away from others and then only two were averaged. Also, trials that produced an average C_t that was inconsistent with others in the respective treatment group were run again. The cycle threshold is the number of cycles required for the fluorescent signal to surpass the background fluorescence. Lower cycle thresholds indicate higher initial concentrations of mRNA. The average C_t from each target gene was subtracted from the average β-Actin C_t of the same sample, to indicate a relative amount of expression. β-Actin is used as a baseline of gene expression because it theoretically stays constant over a wide array of physiological conditions.

Statistical Analysis and Calculations

To test for the significance in change in weight and change in rate of weight gain between mice fed the Western diet and those fed a control diet, a repeated measures analysis of variance (ANOVA) was performed. Mice were divided into two groups, mature and adolescent, for this test because those that started the feeding when they were adolescents had a much lower initial body weight.

The average cycle threshold for each gene was subtracted from the average β-actin cycle threshold to determine ΔC_T. The change in change in cycle threshold ($\Delta\Delta C_T$) was determined by subtracting the average ΔC_T of each gene in the control group from the average ΔC_T of the same gene in the Western diet group. This $\Delta\Delta C_T$ was then converted into relative amounts using the formula $2^{(\Delta\Delta CT)}$. Standard deviation (SD) and standard error of the mean (SEM) were calculated for the C_t's of each target gene. Also, a two-sample t-test assuming equal variance was performed to determine P-values between the C_t's of each gene in the Western and control diet groups.

Results

Effects of Western Diet on Body Weight

To examine the effects of a high fat diet on mRNA levels of the specific genes selected, mice were either fed a western style diet, which is characterized by a higher percent of energy coming from fats, or a control diet of standard laboratory mouse chow. Mice were weighed before administering diets (week 0) and every week for 15 subsequent weeks. Western diet mice had dramatically increased amounts of abdominal adipose tissue (Figure 1).

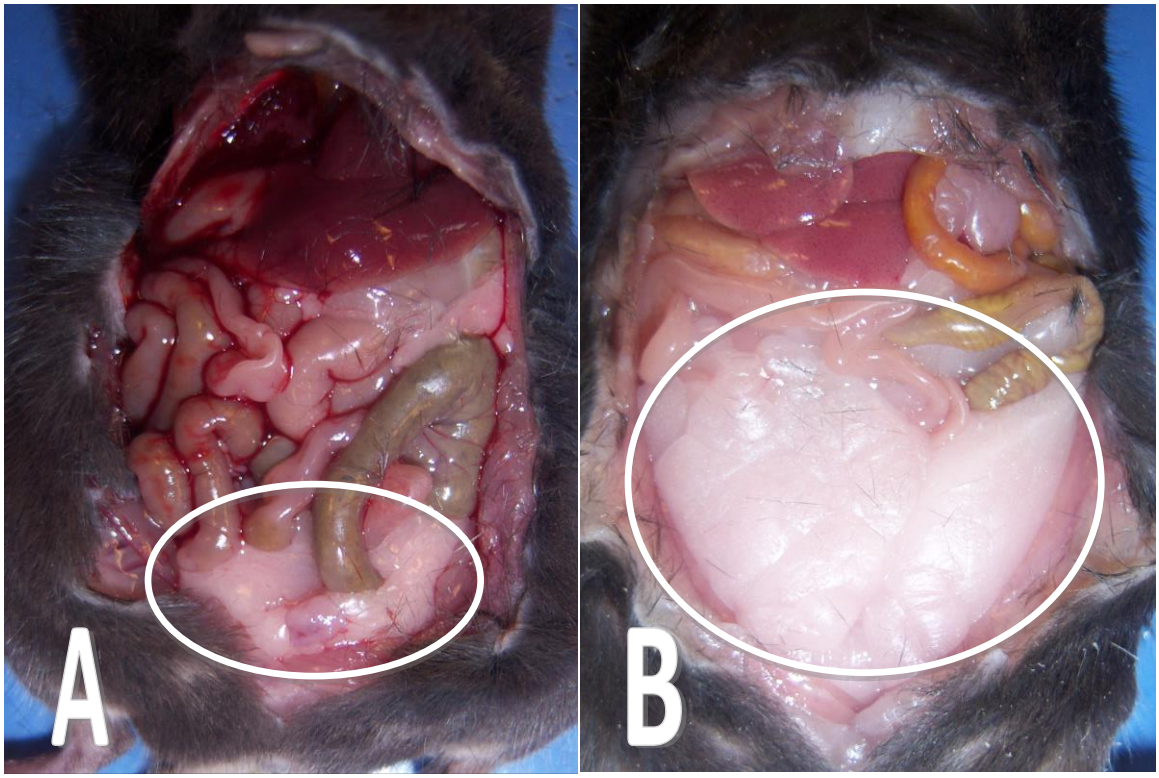


Figure 1. Increased Abdominal Adipose Tissue in Mice Fed Western Diet. Western and control diets were administered *ad libitum* for 15 weeks. Abdominal fat of mouse fed control diet (A) and Western diet (B).

Due to availability of mice, five mice began their feeding schedule at 23 days old (adolescent). Adolescent mice fed the Western diet were heavier than those in the control group; however, the difference between the two groups after 15 weeks was not significant. The rate of weight gain in Western diet group was significantly greater than the rate of weight gain in the control diet group (Figure 2).

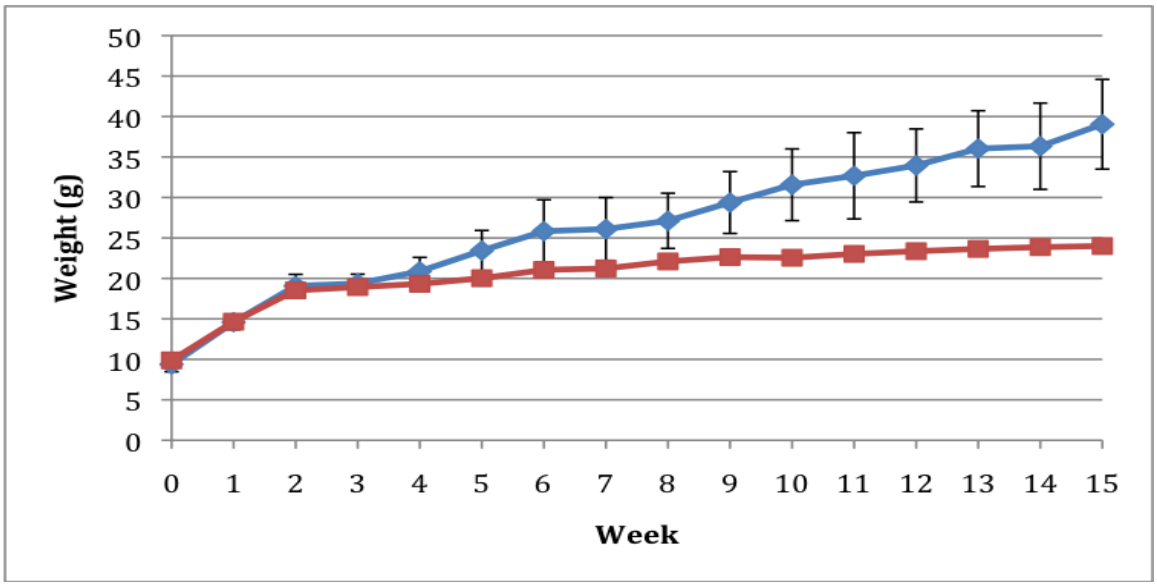


Figure 2. Western diet leads to more rapid weight gain than control diet in adolescent mice. Western and control diets were administered *ad libitum* for 15 weeks. Mice were weighed prior to administering diets (week 0) and once per week for the remainder of feeding schedule. Blue series represents average weight of mice eating western diet (n=2). Red series represents average weight of mice eating control diet (n=3). Error bars represent standard error of the mean. Error bars are too small to be seen in the red series. There was no significant difference in average weight between groups after the 15-week feeding (P=0.0897). There was a significant difference in rate of weight gain between two groups (P<0.001).

The seven mice that began their feeding schedule at 8-9 weeks old (mature) had significant increases in both body weight after 15 weeks and rate of weight gain (Figure 3).

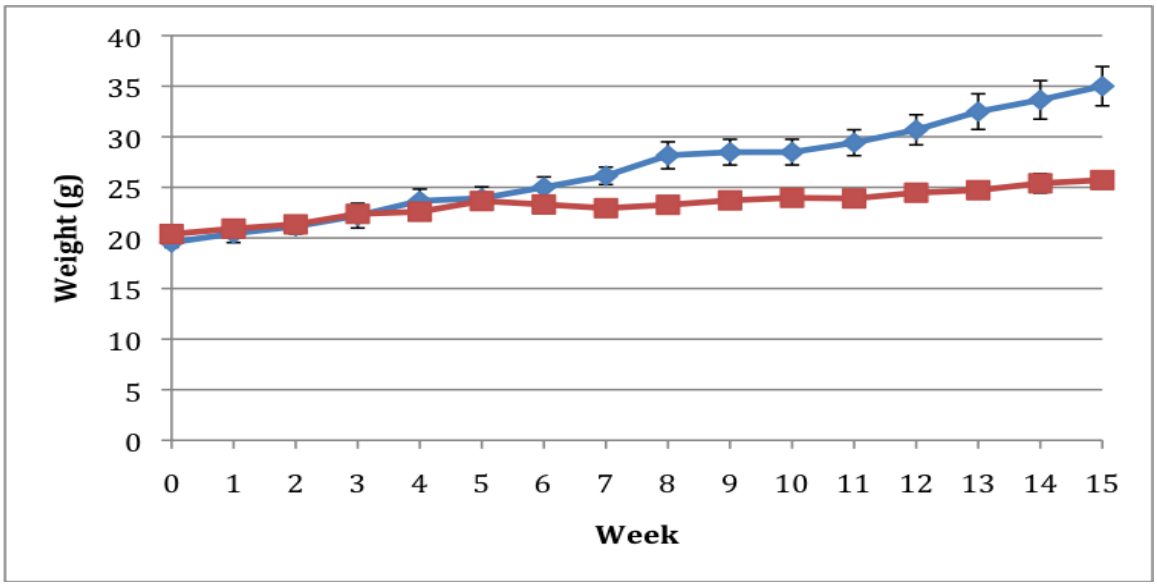


Figure 3. Western diet leads to significant weight gain and more rapid weight gain than control diet in mature mice. Western and control diets were administered *ad libitum* for 15 weeks. Mice were weighed prior to administering diets (week 0) and once per week for remainder of feeding schedule. Blue series represents average weight of mice eating western diet (n=4). Red series represents average weight of mice eating control diet (n=3). Error bars represent standard error of the mean. Error bars are too small to be seen in the red series. There was a significant increase seen in average weight between the two treatment groups after the 15-week feeding (P=0.0494). There was a significant difference in rate of weight gain between the groups (P<0.001).

RNA Extraction and Purification

RNA concentrations and absorbance ratios (260/280) were determined for each sample, an A indicates an adolescent mouse and an M indicates a mature mouse (Table 1). All RNA concentrations of liver tissues were very high (above 1000 ng/µl) with 260/280 values around 1.90, indicating a non-degraded and uncontaminated sample (Table 1). RNA concentrations of adipose tissue ranged from low to moderate levels (81-225 ng/µl) with all 260/280 values above 2.0, indicating a non-degraded and uncontaminated sample (Table 1).

Table 1. RNA Concentrations and 260/280 Ratios for Individual Mice

Diet	Mouse	Tissue	RNA Concentration (ng/µl)	Absorbance Ratio (260/280)
Western	1^{A}	Liver	1149.6	2.03
		Adipose	193.0	2.06
	2^{A}	Liver	3403.3	1.85
		Adipose	210.3	2.07
	3^{M}	Liver	1929.4	1.99
		Adipose	212.9	2.08
	4^{M}	Liver	2274.5	2.01
		Adipose	81.0	2.21
	5^{M}	Liver	2364.3	2.00
		Adipose	225.4	2.28
	6^{M}	Liver	3636.9	1.90
		Adipose	114.2	2.28
Control	7^{M}	Liver	2478.6	1.94
		Adipose	113.9	2.14
	8^{M}	Liver	3403.6	1.86
		Adipose	111.7	2.02
	9^{M}	Liver	3174.4	1.91
		Adipose	100.8	2.04
	10^{A}	Liver	3416.7	1.85
		Adipose	204.4	2.04
	11^{A}	Liver	3272.1	1.88
		Adipose	205.9	2.09
	12^{A}	Liver	1348.3	2.00
		Adipose	161.4	2.10

Gene Expression

The mRNA levels of each target gene were measured by cycle threshold (C_t) values via qRT-PCR. Each target was run in triplicate and then averaged to get the average C_t for each gene (Table 2). The average C_t for HMGCR in mice 1, 2, and 3 were all equivalent; to confirm this finding, the individual values of each well in triplicate can be found in Appendix D. Also, values with asterisks indicate sample that seemed

suspicious and were re-run in triplicate. For each value the re-run produced consistent results, so the original values were used to determine average C_t (Table 2).

Table 2. Average Cycle Threshold Values of Target Genes in Each Mouse

		Cycle Threshold					
Diet	Mouse	Liver β-Actin	HMGCR	NPC1L1	Adipose β-Actin	Adiponectin	Leptin
Western	1[A]	22.96	26.93	35.92	27.66*	32.66*	27.97*
	2[A]	21.3	26.93	35.92	23.48	29.31	25.96
	3[M]	22.98	26.93	35.25	23.32	28.3	25.95
	4[M]	21.29	27.6	35.91	25.98	31.67	28.96
	5[M]	23.96	27.59	36.6	24.91	30.98	24.93
	6[M]	22.29	27.94	36.25	25.03	27.97	25.98
Control	7[M]	21.28	23.24	37.17	27.66	35	33.33
	8[M]	22.95	23.61	35.23	28.01	31.65	31.33
	9[M]	24.63	23.92	36.24	25.96	32.65	30.62
	10[A]	22.96	24.95	37.28	27.35	34	31.62
	11[A]	22.97	23.92	36.58	26.64	33.33	31.97
	12[A]	21.96	26.93	34.59	24.96*	27.95*	29.94*

Relative cycle threshold (ΔC_t) was determined for each gene of interest by subtracting the mean cycle threshold for β-Actin of the same tissue from the mean cycle threshold of the gene of interest. Mean β-Actin cycle threshold in liver tissue was used for HMGCR and NPC1L1 while mean β-Actin cycle threshold in adipose tissue was used for Adiponectin and Leptin genes (Table 3). These values indicate the relative change in expression levels of the target genes in the treatment groups by using β-Actin to assess a level of general expression. As previously stated under most physiological conditions expression of β-Actin should be relatively constant. The mean change in cycle threshold values were calculated for each gene in both treatment groups; with which the standard

deviation (SD), standard error of the mean (SEM) and P-Values were calculated (Table 4). Testing the significance of the ΔC_t for each gene was considered to be a multiple comparisons test, so Bonferroni Correction was used to set α equal to 0.0125 in order to determine statistical significance.

Table 3. Change in Cycle Threshold (ΔC_t) of Target Genes

Diet	Mouse	HMGCR	NPC1L1	Adiponectin	Leptin
Western	1	3.97	12.96	5.00	0.31
	2	5.63	14.62	5.84	2.49
	3	3.95	12.27	4.98	2.63
	4	6.31	14.62	5.69	2.98
	5	3.63	12.64	6.07	0.02
	6	5.65	13.96	2.94	0.95
Control	7	1.96	15.89	7.34	5.67
	8	0.66	12.28	3.64	3.32
	9	-0.71	11.61	6.69	4.66
	10	1.99	14.32	6.65	4.27
	11	0.95	13.61	6.69	5.33
	12	4.97	12.63	2.99	4.98

All ΔC_t values in the Western diet treatment group seemed to be very consistent; the highest SEM was 0.53 (Table 4). The ΔC_t values in the control group were also fairly consistent; the highest SEM was 0.78 (Table 4).

Table 4. Mean Change in Cycle Threshold (ΔC_t) of Target Genes

Diet		HMGCR	NPC1L1	Adiponectin	Leptin
Western	Mean Change in Cycle Thresholds (ΔCt)	4.70	13.29	4.94	1.38
	Standard Deviation (SD)	1.14	1.03	1.14	1.29
	Standard Error of Mean (SEM)	0.46	0.42	0.47	0.53
Control	Mean Change in Cycle Thresholds (ΔCt)	0.97	13.54	6.20	4.65
	Standard Deviation (SD)	1.91	1.56	1.85	0.84
	Standard Error of Mean (SEM)	0.78	0.64	0.76	0.34
	P Value	0.0053	0.8762	0.5278	0.0005

The difference between the mean changes in cycle threshold ($\Delta\Delta C_t$) was calculated for each gene by subtracting the mean change in cycle threshold of each gene in the control diet from that of the western diet (Table 5). This value compares difference in expression between the two treatment groups (Table 5). Relative quantification (RQ) values were then determined by raising 2 to the power of $\Delta\Delta C_t$ ($2^{\Delta\Delta Ct}$), which gives the relative fold increase or decrease of each gene in the Western diet group compared to the control group (Table 5).

Table 5. The Difference in ΔC_t Values Between Treatments ($\Delta\Delta C_t$) and Relative Quantification of Target Genes

	HMGCR	NPC1L1	Adiponectin	Leptin
ΔΔCt	-3.73	0.25	1.27	3.27
Relative Quantification (RQ)	0.08	1.19	2.41	9.65

HMGCR

A significant decreases in HMGCR expression was seen in the western diet group (P=0.0053) (Table 4) with approximately one-tenth of the expression in the Western diet group compared to that of the control diet (Table 5).

NPC1L1

Expression of NPC1L1 remained relatively constant with a mean ΔC_t of 13.29 in the Western diet group and 13.54 in the control diet group (P=0.8762) (Table 4).

Adiponectin

The expression of Adiponectin in mice of the western diet group was about twice (RQ = 2.41) that of control diet group (Table 5) yet no significant different was found (P=0.5278) (Table 4).

Leptin

Expression of leptin in mice of the western diet treatment was about 10 times (RQ = 9.65) of what it was in the control diet treatment group (Table 5). This increase in expression was statistically significant (P=0.0005) (Table 4).

Discussion

The purpose of this study was to compare the expression of NPC1L1, HMGCR, Leptin and Adiponectin between mice fed a western diet and those fed a standard mouse chow.

Effects of Western Diet on Body Weight

Body weight for each mouse was recorded before feeding started and once a week for the remainder of the feeding schedule. Initial body weights were similar within each group of mice, mature and adolescent. Upon visual observation there was a substantial increase in the amount of body fat between the mice fed the Western diet and those fed a control diet (Figure 1). Furthermore, the rate of weight gain for all mice fed the Western diet was significantly greater than that of all mice fed the control diet (Figures 2 and 3). At the conclusion of the feeding schedule, mice that began the diet when they were 8-9 weeks old had a significant difference in average body weight when

compared to mature mice fed the control diet (Figure 3). Although the difference in body weight of adolescent mice (3 weeks old) was not significant (Figure 2), with a p-value of 0.0897, the statistics were influenced by a small number of mice. The adolescent group being fed the Western diet (n=2) was half of the mature mice group being fed the Western diet (n=4). This was done because of limited availability of age-matched female mice. Only female mice were used due to availability of mice. These results are consistent with previous work, which suggests that significant differences in the weights of mice fed a high fat diet and mice fed a standard diet occur around week 12 of feeding (Winzell and Ahren, 2004).

Due to the significant differences in rate of weight gain and body weight between the Western diet group and the control diet group, it can be concluded that this high fat diet is much more effective at increasing body compared to the protocol of Schomau-Taylor (2011). In the specific study, the "high fat" diet consisted of the addition of $1/8^{th}$ cup of corn oil to standard mouse chow and was administered for 12 weeks. Also, instead of normal drinking water, this study used 5% sucrose drinking water. There was no significant difference in average body weights between the high fat and control diet groups at any point in that study.

Effects of Western Diet on Gene Expression

The genes NPC1L1, HMGCR, Leptin and Adiponectin all play crucial roles in human health. Heart disease, the leading cause of death in the United States, is related to NPC1L1 and HMGCR. The incidence of obesity in America has risen dramatically in the past couple decades and is closely related to heart disease. Leptin and Adiponectin

are genes commonly studied in obese subjects. Altering expression levels of any of these four genes can lead to extremely detrimental effects on human health.

HMGCR is expressed in the liver and catalyzes the rate-limiting step in cholesterol synthesis. A significant decrease in HMGCR mRNA abundance was found between the Western and control diet groups (Table 4). These results were expected and support the hypothesis. This is because as the body ingests more cholesterol in the diet, there is less of a need for the body to synthesize cholesterol on its own.

NPC1L1 is expressed in the liver and is suggested to prevent excessive biliary loss of cholesterol (Temel, *et al*., 2007). Expression of NPC1L1 was very similar in the Western diet group compared to the control group. The difference in expression between the groups was not significant and did not support the hypothesis of a decrease in expression in the Western diet group. Based on the work of Miettinen and Gylling (2000), which suggests that a high-fat and cholesterol diet increases the secretion of biliary cholesterol, it was hypothesized that a high fat diet may lead to a decrease in NPC1L1 expression because its hepatic function was assumed to prevent biliary cholesterol secretion. So if there is less NPC1L1 mRNA, the ability to prevent biliary cholesterol secretion would be lost. It is possible that NPC1L1 expression and excessive biliary cholesterol secretion in response to a high fat diet are not related and that other mechanisms are responsible for this response.

Leptin is a hormone secreted by adipose tissue that binds with Leptin receptors in the hypothalamus. This interactions enhances metabolic rate and signals the body to stop eating. This study suggests that eating a Western diet for 15 weeks can significantly increase the amount of Leptin mRNA (Table 4). This increase in Leptin

mRNA was expected. This study also demonstrated that this model of a high fat diet is more effective at increasing Leptin mRNA levels than the diet used by Schonau-Taylor (2011). In her study Leptin levels only increased 1.61-fold in the high fat diet group, compared to a 9.65-fold increase in the Western diet group of the current study.

Adiponectin is an adipokine that regulates metabolic rate and insulin sensitivity. The average level of Adiponectin mRNA in the Western diet group was 2.5 times greater than Adiponectin levels in the control diet group. These data do not support the hypothesis and disagree with previous literature. Arita *et al*. (1999) found a negative correlation between Adiponectin levels and obesity in humans. This inconsistency may be explained by the amount of time the mice in this study were considered overweight or obese. The mice in this study may not have been at a weight that is considered unhealthy for the entire duration of the experiment. Therefore, the body may not have had enough time to respond and adjust the expression of Adiponectin in response to being chronically obese.

Limitations and Follow-up Studies

The three limitations in this experiment were time, the age of mice and number of mice per treatment. While all mice were subjected to 15 weeks of either the control or Western diet, the longer mice can be subjected to the diet the better it models an unhealthy lifestyle. The Western diet is a lifelong habit of many people in the United States. All mice were not the same age when they were introduced to the diets because of limited subject availability. Either starting with mice of all the same age or using a larger sample size would be beneficial and give the data more statistical power.

Further investigation of hepatic NPC1L1 function is needed to determine if there is a relationship between mRNA level and diseases such as heart disease and obesity. Knowing more about hepatic NPC1L1 may also lead to the production and/or improvement of medications used to control cholesterol levels.

Lastly, there is some controversy over using β-Actin as a reference gene in various tissues. Many studies have noted that using β-Actin as a control negatively affects the accuracy of the results (Selvey *et al.,* 2001). Finding a more suitable reference gene for mouse liver and adipose tissue would make the data more reliable.

Implications

The relationship between a Western diet and heart disease and obesity has become common knowledge. Considering that more Americans die each year from heart disease than any other cause, understanding how our dietary habits can contribute to mortality is of vital importance. Compounded with the fact that obesity can be a precursor to heart disease, in addition to the other symptoms it brings about, it seems that a Western-style diet could be increasing our health risks.

Conclusion

This study illustrates that mice fed a Western diet can lead to a significant increase in Leptin mRNA abundance and a significant decrease in HMGCR mRNA abundance. Therefore, a healthy, well-balanced diet may help to regulate gene expression and help to control cholesterol levels and prevent obesity. This confirms the need to abandon the Western style diet that plagues our society today.

References

Ahmed MH and Byrne CD. (2010) Ezetimibe as a potential treatment for non-alcoholic fatty liver disease: is the intestine a modulator of hepatic insulin sensitivity and hepatic fat accumulation? Drug Discov Today 15(15-16) 590-595

Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, *et al*. (1999) Paradoxical Decrease of an Adipose-Specific Protein, Adiponectin, in Obesity. Biochem Biophys Res Comm 257(1) 79-83

Duval C, Touche V, Tailleux A, Fruchart JC, Flevet C, *et al*. (2006) Niemann–Pick C1 like 1 gene expression is down-regulated by LXR activators in the intestine. Biochem Biophys Res Comm 340(2006) 1259-1263

Endo A. (1992) The Discovery and Development of HMG-CoA Reductase Inhibitors. Journal of Lipid Research (33) 1569-1582

Eurofins MWG Operon. (2012). qPCR Probe Design Service. http://www.operon.com/tools/oligo-design-tools.aspx. Accesed 2011 Dec 8

Fasshauer M, Paschke R, & Stumvoll M. (2004). Adiponectin, Obesity, and Cardiovascular Disease. Biochimie 86(2004) 779–784

Friedman JM and Halaas JL. (1998) Leptin and the Regulation of Body Weight in Mammals. Nature (395) 763-770

Fung, TT, Rimm EB, Spiegelman D, Rifai N, Tofler GH, *et al*. (2001a) Association between dietary patterns and plasma biomarkers of obesity and cardiovascular disease risk. Amer Jour Clin Nutr (73) 61–67

Fung TT, Willet WC, Stampfer MJ, Manson JE, Hu FB (2001b) Dietary Patterns and the Risk of Coronary Heart Disease in Women. Arch Internal Med 161(15) 1857-1862

Ge L, Wang J, Qi W, Miao HH, Cao J, Qu YX, Li BL, Song BL. (2008) The cholesterol absorption inhibitor ezetimibe acts by blocking the sterol-induced internalization of NPC1L1. Cell Metab 7(6) 508- 519

Gibbons GF and Pullinger CR, (1986). Diurnal variations in the effects of an unsaturated-fat-containing diet on fatty acid and cholesterol synthesis in rat hepatocytes. Biochem Jour 1986 November 1; 239(3): 617–623

Gouni-Berthold I, Berthold HK, Gylling H, Hallikainen M, Giannakidou E, *et al*. (2008) Effects of ezetimibe and/or simvastatin on LDL receptor protein expression and on LDL receptor and HMG-CoA reductase gene expression: A randomized trial in healthy men. Atherosclerosis 198(2008) 198–207

Heron M, Hoyert DL, Murphy SL, Xu J, Kochanek KD, Tejada-Vera B. (2009) Deaths: Final Data for 2006. National Vital Statistics Reports 57(14)

Hu FB and Willet WC. (2002) Optimal Diets for Prevention of Coronary Heart Disease. Jour Amer Med Assoc 288(20), 2569-2578

Iqbal R, Anand S, Ounpuu S, Islam S, Zhang X, *et al*. (2008) Dietary Patterns and the Risk of Acute Myocardial Infarction in 52 Countries. Circulation 118:1929-1937

Katan B, Brouwer I.A, Clarke R, Geleijnse JM, Mensick RP. (2010) Saturated Fats and Heart Disease. Amer Jour Clin Nutr 92(2) 459-460

Lichtenstein AH, Appel LJ, Brands M, Carnethon M, Daniels S, *et al*. (2006) American Heart Association Scientific Statement: Diet and Lifestyle Recommendations Revision 2006. Circulation (14) 82-96

Manninen V, Tenkanen L, Koskinen P, Huttunen JK, Manttari M, *et al*. (1992) Joint effects of serum triglyceride and LDL cholesterol and HDL cholesterol concentrations on coronary heart disease risk in the Helsinki Heart Study. Implications for treatment. Circulation (85) 37-45

Miettinen TA and Gylling H. (2000). Cholesterol Absorption Efficiency and Sterol Metabolism in Obesity. Atherosclerosis 153(1) 241-248

NCBI GenBank. (2011). Mus musculus 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGCR), mRNA. http://www.ncbi.nlm.nih.gov/nuccore/NM_008255.2. Accessed 2011 Dec 8

Schonau-Taylor H. (2011) Impact of a high fat diet on genes involved in insulin sensitivity and glucose metabolism. Allegheny College Senior Thesis

Selvey S, Thompson EW, Matthaei K, Lea RA, Irving MG, Griffiths LR. (2001) β-Actin – An Unsuitable Internal Control for RT-PCR. Moll and Cell Probes (15) 307-311

Sikka G, Yang R, Reid S, Benjo A, Koitabashi N, *et al*. (2010) Leptin is Essential in Maintaining Normal Vascular Compliance Independent of Body Weight. Intern Jour of Obes 34, 203-206

Stancu C, Sima A. (2001) Statins: Mechanism of Action and Effects. Jour Cell Mol Med 5(4): 278-387

Temel RE, Tang W, Ma Y, Rudel LL, Wilingham MC, *et al*., (2007) Hepatic Niemann-Pick C1–like 1 regulates biliary cholesterol concentration and is a target of ezetimibe. Jour Clin Invest 117(7): 1968–1978

Turabelidze A, Guo S, DiPietro LA. (2010) Importance of Housekeeping Gene Selection for Accurate Reverse Transcription-Quantitative Polymerase Chain Reaction in a Wound-Healing Model. Wound Repair and Regen 18(5) 460-466

Winzell M and Ahren B. (2004). The high-fat diet-fed mouse: a model for studying mechanisms and treatment of impaired glucose tolerance and type 2 diabetes. Diabetes 53:215-219

Appendix A: RNA Isolation Protocol using RNeasy Universal Kit (Qiagen)

1) Place tube with homogenate on bench top at room temperature for five minutes
2) Add 100 µl gDNA Eliminator Solution then vortex for 15 seconds
3) Add 180 µl Chloroform and vortex for 15 seconds
4) Let tube sit on bench top at room temperature for 2-3 minutes
5) Centrifuge at 12,000 rpm for 15 minutes at 4°C
6) Transfer the upper, aqueous phase (approximately 600 µl) to new microcentrifuge tube
7) Add one volume (approximately 600 µl) of 70% ethanol then vigorously vortex for 15 seconds
8) Transfer 700 µl of the sample to RNeasy Mini Spin Column, which is placed in a 2 ml collection tube
9) Centrifuge for 15 seconds at 8,000 rpm at room temperature
-Discard flow through
10) Repeat step 9 with remainder of sample
-Discard flow through
11) Add 700 µl of Buffer RWT to spin column
12) Centrifuge at 8,000 rpm for 15 seconds
-Discard flow through
13) Add 500 µl Buffer RPE to spin column
14) Centrifuge for 15 seconds at 8,000 rpm
-Discard flow through
15) Repeat steps 13 and 14
16) Centrifuge at full speed for one minute
17) Discard original 2 ml collection tube and place spin column in new 1.5 ml collection tube
18) Add 30µl RNase-free water to spin column
19) Centrifuge for one minute at 8,000 rpm
20) Repeat step 18 & 19 using new volume of RNase-free water
21) Store samples are kept at -70°C

Appendix B: cDNA Synthesis Protocol using iScript cDNA Synthesis Kit (Bio-Rad)

1) Thaw all components except iScript reverse transcriptase. Mix thoroughly and briefly centrifuge to collect contents at the bottom of the tube before using. Place components on ice.
2) Add the following components to a 0.2 ml PCR tube on ice:

Components	Volume
Nuclease-free water	Variable
5x iScript select reaction mix	4 µl
RNA sample (1 µg total RNA)	Variable
iScript reverse transcriptase (add last)	1 µl
Total	20 µl

Note: for multiple reactions, prepare a master mix with the above components, except RNA, and then dispense to each reaction.

3) Mix gently and incubate as follows:
For random-primed cDNA reactions, incubate for 5 min at 25°C, then 30 min at 42°C.
4) Incubate at 85°C for 5 min to heat-inactivate the reverse transcriptase.
5) Store cDNA product at –20°C to +4°C.

Appendix C: StepOne Real time-PCR Protocol
(Written by Dr. Kleinschmidt, Allegheny College)

Computer Set-up
1) Open Step One software
2) Click on Advanced Setup
3) Use Quantitation Method (ΔΔCT Comparative)
4) Select SYBR Green for reagents and include Melt Curve. Keep Ramp Speed Standard
5) Click on Plate Setup
6) Add all targets (genes or proteins being expressed)
7) Add all samples (tissue types)
8) Click on Assign Targets and Samples
9) Click Show in Wells to put each target and sample combination in three wells
10) Click on Run Method tab
11) Save to USB

Lab Set-up
1) For each well/reaction:
-10 µl 2X SYBR Green Master Mix
-9 µl of Rnase free water
-0.5 µl of RT (cDNA) reaction
2) A primer mix also needs to be made for each primer or target including
- 100 µl Forward Primer
- 100 µl Reverse Primer
- 800 µl RNase-free water
3) Add 0.5 µl of appropriate primer mix to each well/reaction
4) Contents in tubes must match the layout made in the computer setup

qRT-PCR
1) Start and Run qRT-PCR ABI 7500 instrument
2) Collect Results onto USB drive
3) Analyze Data using StepOne software

Appendix D: Cycle Threshold Values of HMGCR in Mice 1^{A}, 2^{M}, and 3^{M}

Mouse	HMGCR Cycle Threshold		
1^{A}	26.93	26.92	26.95
2^{A}	26.93	26.91	26.94
3^{M}	26.92	26.92	26.94