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Effect of a Dietary Intervention on Glucocorticoid-Induced Metabolic Syndrome

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EFFECT OF A DIETARY INTERVENTION ON GLUCOCORTICOID-INDUCED METABOLIC SYNDROME

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DEDICATION

First, I would like to dedicate this master's thesis dissertation to my father, Edward Lee Beech, for infusing within me the importance of hard work and higher education; my beloved mother, Winifred Ann Beech, for her continuous guidance and support of my education and adventurous endeavors throughout life; and my sister, Sara Beech, for her continuous love and support.

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ABSTRACT

This study aimed to determine if a dietary intervention of fish oil could reduce the risk of metabolic syndrome phenotype associated with glucocorticoids (GC), given as part of the acute lymphocytic leukemia (ALL) treatment. Weaned C57BL/6 male mice were assigned either a standard rodent chow or Western-type diets containing 45% calories from fat consisting of lard (high omega-6/low omega-3 fatty acids). At six weeks of age, GC treatment was given daily for 28 days to half the mice in each diet group. Mice on the lard diet either remained on this diet or were switched to an isocaloric diet containing 45% fat from fish oil (high omega-3/low omega-6 fatty acids). Our data showed that the fish oil diet (high in omega-3 fatty acids) reduced body mass gain, fatty liver development, and glucose tolerance when used in combination with high dose GC therapy. The data suggests that consuming more omega-3 fatty acids than omega-6 fatty acids might reduce the metabolic syndrome phenotype associated with ALL treatment.

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LIST OF SYMBOLS AND/OR ABBREVIATIONS

°C	Degrees Celsius
^{14}C	Carbon 14 Isotope
^{3}H	Hydrogen 3 Isotope
AA	Arachidonic Acid
ALA	Alpha Linolenic Acid
ALL	Acute Lymphoblastic Leukemia
ALT	Alanine Aminotransferase
ATMs	Adipose Tissue Associated Macrophages
cm	centimeters
CO_2	Carbon Dioxide
DHA	Docasahexaenoic Acid
EPA	Eicosapentaenoic Acid
g	grams
g/kg	grams per kilogram
G6Pase	Glucose-6-Phosphatase
GCs	Glucocorticoids
GLUT	Glucose Transporter
GRs	Glucocorticoid Receptors
GTT	Glucose Tolerance Test
HDL	High-Density Lipoprotein
HFD	High Fat Diet
HFL n-6	High Fat Lard Omega 6 Fatty Acid

HFO n-3	High Fat Fish Oil Omega 3 Fatty Acid
IL-1	Interleukin 1
IL-1β	Interleukin 1β
IL-6	Interleukin 6
IL-8	Interleukin 8
KCl	Potassium Chloride
KH_2PO_4	Monopotassium Phosphate
LA	Linoleic Acid
$mg/m^2/day$	milligrams per meter squared per day
$MgSO_4$	Magnesium Sulfate
mm	millimeters
mmol	millimoles
mmol/L	millimoles per liter
mOsm	milliOsmoles
n-3	Omega-3 Polyunsaturated Fatty Acids
n-6	Omega-6 Polyunsaturated Fatty Acids
NaCl	Sodium Chloride
NAFLD	Non-Alcoholic Fatty Liver Disease
$NaHCO_3$	Sodium Bicarbonate
NASH	Nonalcoholic Steatohepatitis
nm	nanomoles
PEPCK	Phosphoenolpyruvate Carboxykinase
rpm	rate per minute

SGLT	Sodium-Dependent Glucose Co-Transporter
SREBP	Sterol Regulatory Element Binding Protein
TNF- α	Tumor Necrosis Factor Alpha
um^2	micrometers squared
USDA	U.S. Department of Agriculture
VLDL-C	Very Low Density Lipoprotein Cholesterol

BACKGROUND

Pediatric acute lymphoblastic leukemia (ALL) constitutes 30% of all childhood malignancies in the United States, making it the most common form of childhood cancer.[1] This cancer originates with abnormal white blood cell (lymphoblast) accumulation in the bone marrow and blood.[1,2] The current event-free survival rate is now 75%-85% for patients diagnosed with pediatric ALL.[2] However, despite a very successful cancer treatment, these survivors have multiple long term negative health consequences that are associated with an increase in body mass. These detrimental side effects resemble metabolic syndrome and comprise of specific symptoms of abdominal obesity, impaired glucose tolerance, high blood pressure, elevated triglyceride levels, low high-density lipoprotein (HDL) levels, and recently added, non-alcoholic fatty liver disease (NAFLD).

The standard treatment protocol for ALL includes multiple chemotherapeutic agents and high dose glucocorticoids. During the induction phase (first 28 days of therapy) patients receive Vincristine, Daunorubicin, PEG-Asparaginase, Cyclophosphamide, Cytarabine, and Mercaptopurine along with approximately 40 mg/m^2/day of synthetic glucocorticoids, specifically Prednisone.[3]

It is well known that chronic glucocorticoid treatment alters metabolic programs favoring weight gain.[4] During ALL treatment most weight gain occurs during the induction phase of treatment during which time high doses of the synthetic glucocorticoid, Prednisone/Prednisolone, is administered. This suggests that the treatment contributes to the metabolic syndrome phenotype associated with ALL therapy.[5]

Glucocorticoids (GCs) are stress hormones naturally secreted by the adrenal cortex to help decrease inflammation and control energy metabolism. As stated above, certain GCs such as Prednisone/Prednisolone are part of the chemotherapy treatment protocol of pediatric ALL.[6] Prednisone/Prednisolone is typically used for its anti-inflammatory and cytolytic effect on thymocytes and therefore, is essential in treatment because of the dramatic increase in immature thymocytes as well as chemotherapy-induced inflammation seen during pediatric ALL.[7] Synthetic GCs are shown to cause massive cell death, or apoptosis, in lymphoid malignant cells by transcriptionally regulating and altering a variety of different genes.[8] GCs bind to ligand-activated zinc finger transcription factors called glucocorticoid receptors (GRs) found in the cytosol of the cell. After binding to GRs, GCs move to the nucleus and function as a DNA sequence-specific transcriptional regulator of certain GC-responsive target genes.[9] These target genes are shown to help control hepatic energy metabolism, especially protein and sugar homeostasis.[4]

Sugar homeostasis is the balance of insulin and glucagon to maintain steady ranges of blood glucose levels. When blood glucose is high, insulin is secreted by the pancreatic beta cells to facilitate the transport of glucose into the cells for energy. During a fasted state or prolonged exercise, glucagon from the alpha cells of the pancreas and natural GCs are released to simulate catabolic reactions in the body by antagonizing anabolic insulin actions.[4] However, a chronic increase of natural or synthetic GCs can alter certain processes causing an increase in anti-inflammatory molecules and gluconeogenic enzyme levels such as an increase in phosphoenolpyruvate carboxykinase (PEPCK). An increase in these gluconeogenic enzyme levels causes the body to use non-

carbohydrate precursors to make glucose, which causes an increase in blood glucose levels. Other alterations caused by GCs can lead to the inhibition of glucose uptake into muscle and adipose tissue as well as increased adipose tissue lipolysis to generate fatty acids as an energy source for muscles. These alterations promote a further rise in blood glucose levels and eventually insulin resistance or type 2 Diabetes Mellitus.[4] Free Fatty acids have been shown to be elevated in obese individuals due to enlarged adipose tissue that releases more free fatty acids. Free fatty acids can cause an increase in blood glucose by inhibiting insulin's anti-lipolytic action, leading to an increase in the rate of free fatty acids into circulation. [10] Furthermore, an increase in free fatty acids can increase the secretion of glucagon, and therefore, gluconeogenesis and high levels of glucose in the blood. [11]

Although acute exposure of GCs increases lipolysis in adipose tissue, chronic use of GCs can lead to an increase in total mass of adipose tissue, especially in conjunction with a high fat diet (HFD) that is low in omega-3 polyunsaturated fatty acids (n-3).[12,13] Increases in adipose tissue may be caused by the redistribution of fat from peripheral to central and visceral depots leading to further deregulation of the metabolism. Most pediatric patients with ALL, as well as patients with Cushing's syndrome, have fat redistribution as a common phenotype. The shared feature between these two types of patients is chronically increased GCs of either natural or synthetic origins, with the naturally increased GC levels occurring due to irregular hormonal and metabolic processes and stress responses.[4,14]

Changes in fat distribution are not the only issues with chronic exposure to GCs. Although GCs are known for their anti-inflammatory activity, long-term use tends to

promote other inflammatory issues that stimulates steatosis of the liver.[15] Chronically elevated GC levels alter the regulation of liver processes which can cause increased triglyceride synthesis, decreased fatty acid oxidation, and increased accumulation of lipids, leading to fatty liver.[16]

Long-term deregulation of hepatic processes is detrimental as the liver is responsible for the control of glucose and lipid homeostasis. Untreated liver disease can result in liver failure and even death.[17] Non-alcoholic fatty liver disease (NAFLD) is a chronic inflammatory condition that can range from simple steatosis, also called simple fatty liver, to nonalcoholic steatohepatitis (NASH). NASH can later progress into more advanced inflammatory stages such as fibrosis, cirrhosis, hepatic apoptosis, and hepatocellular carcinoma.[18] Fatty liver results from the accumulation of triglycerides in the cells due to an increased uptake of free fatty acids and *de novo* liponeogenesis in the hepatocytes while also exhibiting a decreased secretion of very low density lipoprotein cholesterol (VLDL-C) out of the liver.[19] GCs alter the transcriptional regulation of many different metabolic genes and enzymes such as glucose-6-phosphatase (G6Pase) and PEPCK. These alterations have an immense effect on how macronutrients are metabolized and stored in the body. However, the entire effect of GC treatment in concurrence with a HFD on hepatic inflammation and fibrosis remains unclear. There is some evidence that a HFD of about 60% of calories in conjunction with GCs rapidly induces the development of fatty liver disease. In addition to the production of fatty liver disease, there is lipid spillover from central adipose tissue, which rapidly promotes insulin deregulation leading to the diabetic phenotype and hepatic insulin resistance.[15]

Conversely, studies have found positive effects of a HFD that is high in n-3 fatty acids on improved liver outcomes such as a decrease in hepatic steatosis, inflammation and necrosis.[20] Omega-3 fatty acids have been shown to be safe and effective for the treatment of fatty liver disease by improving hypertriglyceridemia and down regulating leptin and resistin levels and upregulating adiponectin expression.[20,21] In turn, increased adiponectin levels are associated with decreased liver inflammation and fibrosis as well as decreased hepatic and systematic insulin resistance.[22]

Insulin resistance, also known as Type 2 Diabetes Mellitus, can be a consequence of chronic liver disease, especially in the presence of hyperlipidemia. Insulin lowers blood glucose and inhibits lipolysis. Therefore, hyperlipidemia in combination with insulin resistance favors an increase in fat mass and lipolysis, causing elevated levels of free fatty acids and therefore, a further decrease in the effectiveness of insulin signals. The reduced insulin signaling causes hyperglycemia, which leads to elevated hepatic glucose, up regulation of *de novo* liponeogenesis, and a further increase in free fatty acids.[23] The rise of free fatty acids causes an escalation of oxidative stress, fatty liver disease, and increase in adipose tissue. Insulin resistance and the accumulation of adipokines, such as leptin and the pro-inflammatory cytokines, Tumor Necrosis Factor alpha (TNF-α) and interleukin 6 (IL-6), exacerbate the oxidative stress and perpetuate the liver inflammation leading to further liver damage.[24]

TNF-α is released by adipose tissue associated macrophages (ATMs).[25] Previous work demonstrated that an increase in GCs causes adipocyte hypertrophy and thereby increases pro-inflammatory TNF-α secretion.[26] TNF-α is an inhibitor of the GLUT4 insulin signal through its influence on the tyrosine kinase activity of the insulin receptor,

thus causing a prevention of glucose uptake in the cells.[25] Therefore, GCs may cause insulin resistance as a result of the increased release of TNF-α from hypertrophied adipocytes. This accounts for increased TNF-α serum levels that are present during obesity and hyperinsulinemia.[27]

Another component of weight management is the hormone leptin. This hormone is responsible for the regulation of energy balance through the suppression of appetite in order to reduce food intake. [28] Increased leptin, along with decreased adiponectin, has been shown to be connected with amplified severity of steatosis and fibrosis of the liver and increased occurrence of insulin resistance.[29] Previous animal studies have shown that n-3 treatment can cause a decrease in leptin and resistin levels in serum and adipose tissue, while increasing the adipokine, adiponectin, over time.[20] Adiponectin expression can be inhibited by both GCs and the pro-inflammatory cytokine TNF-α.[26] Excess TNF-α and leptin hormone has also been shown to impair insulin resistance by inducing insulin resistance in pancreatic β cells by stimulating the release of mediators that are toxic for these cells.[30,31]

These factors that cause insulin resistance and NAFLD can cause patients to experiences signs and symptoms of NAFLD such as malaise, fatigue, right upper quadrant pain, abdominal discomfort, hepatomegaly, jaundice, ascites, and indications of metabolic syndrome. However, most cases of NAFLD can appear as asymptomatic, and therefore, biomarkers are useful to assess liver status including aspartate- and alanine aminotransferase levels (ALT), total and direct bilirubin, fasting serum glucose, and lipid panel.[15]

While the liver and the pancreas are key regulatory organs of metabolism, other important and less studied organs are the small and large intestines. Former studies have shown that GCs may play a role in the increased intestinal uptake of glucose, which may contribute to the development of hyperglycemia in patients on chronic GC therapy.[32] There are a variety of sugar transporters in the intestine including GLUT2, GLUT5, GLUT7, GLUT9, and sodium-dependent glucose co-transporter (SGLT1).[33] The variety is indicative of adaptation of the intestinal sugar transport system depending on the different dietary proportions of macronutrients consumed.[34] Although there is limited evidence on how these transporters function during digestion, carbohydrates in the intestine require absorption by a two-step, two-membrane-transport process. The SGLT1 and the facilitative fructose transporter, GLUT5, in the brush border membrane lining the intestinal lumen[35] absorb monosaccharides, GLUT2 is a facilitative transporter of both glucose and fructose in the basolateral membrane of the small intestine and is activated by glucose transport through SGLT1.[36, 37]

During postnatal small intestinal development specific GCs have been shown to regulate cytokine gene expression. This suggests that cytokines may alter sugar absorption.[38] Some studies have shown also that Prednisone increased glucose absorption while other studies resulted in no effect.[39] An increase in glucose being transported to the liver could lead to increased fat accumulation in the liver, which can be another determinant of NAFLD.

Limited, but current research shows that the ratio of omega-6 fatty acids (n-6) to n-3 fatty acids may be important in preventing or reducing the metabolic syndrome

phenotypes such as obesity, NAFLD, dyslipidemia, and insulin resistance observed in these pediatric patients.[40–43]

Omega-3 fatty acids play a beneficial function in the reduction of pro-inflammatory cytokines such as TNF-α and IL-8, reducing the risk of liver disease and insulin resistance.[44] Omega-3 fatty acids may increase anti-inflammatory cytokines by incorporating n-3 fatty acids into cell membrane phospholipids, which interferes with the metabolism of linoleic acid (LA), the n-6 precursor to arachidonic acid (AA). Omega-3 and n-6 fatty acids are competitively metabolized by the same enzyme system, therefore, the PUFA that is most abundant will be preferentially metabolized. When n-3 fatty acids are most abundant, they will replace AA in the cell membrane and decrease the pro-inflammatory derivatives of AA. If more n-6 fatty acids are present, then the pro-inflammatory AA-derived eicosanoids will dominate over the anti-inflammatory n-3 derived eicosanoids. [45] When having a higher dietary intake of n-3 fatty acids, there is an increase in anti-inflammatory mediators and a decrease in the pro-inflammatory mediators, which will reduced inflammation and oxidative stress.[46]

Although considerable research has been devoted to proving the beneficial effects of GCs as part of the treatment therapy for ALL, few studies have addressed the role of nutrition, specifically fatty acids, in the reduction of detrimental side effects caused by chronic GC use.

Certain PUFAs have been advertised as a beneficial component in the diet that may aid in the reduction of inflammation as well as the decrease in the prevalence of atherosclerosis, obesity, and diabetes.[20] The two major types of essential PUFAs that demonstrate alterations in the body are n-3 and n-6 fatty acids.[47] Dietary n-3 fatty acids

are commonly found in the forms of essential α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docasahexaenoic acid (DHA) and are found in food sources such as fish, flaxseed oil, and seeds.[48,49] These specific forms have been shown to decrease inflammation by reducing the eicosanoids and other mediators derived from AA.[50] Eicosanoid products such as prostaglandins are derived from n-6 fatty acids. Therefore, the appropriate ratio between n-6 and n-3 fatty acids is critical to produce positive health effects.[51] Common types of dietary n-6 fatty acids are LA and AA, which are found in nutritional sources such as liquid corn, sunflower, olive, and safflower oils. The western-type diet characteristically contains low PUFAs with a higher amount of n-6 fatty acids than n-3 fatty acids. This high ratio of n-6 fatty acids:n-3 fatty acids leads to an increase in inflammation and issues regarding weight and metabolism.[52]

Obesity and metabolic disorders have increased over the past decade due to the increased consumption of a western-type diet, which is high in calories, saturated fat, and sugar and low in n-3 fatty acids. Pediatric patients undergoing treatment for ALL often consume foods high in saturated fat, n-6 fatty acids, and sugar before, during, and after treatment. Increased consumption of these types of foods contributes to the increase in visceral adipose tissue, NAFLD, insulin resistance, and glucose transporter deregulation, which are also phenotypes that ALL patients develop.[15] However, increasing consumption of n-3 fatty acids may be able to decrease GC induced health consequences including the redistribution and increase of adipose tissue, as well as the increase in the occurrence of inflammation, hypertension, hyperlipidemia, and liver disease.[12,53]

This information has led to the hypothesis that a western type diet containing high amounts of fat and carbohydrates will exacerbate the risk of developing metabolic

syndrome induced by GC treatment. A secondary hypothesis is that the replacement of lard (higher in n-6 fatty acids) with fish-derived fat (higher in n-3 fatty acids) will reduce the metabolic disorder phenotypes induced by chronic, long-term exposure to GC and ultimately reduce the risk for the development of obesity, type 2 diabetes, hypertension, dyslipidemia, and glucose intolerance.

MATERIALS AND METHODS

Experimental Animals

Newly weaned C57BL/6 male mice were obtained from breeder pairs (purchased from Harlan Laboratories, Inc., Indianapolis, IN) and housed in a USDA approved animal facility at the University of Memphis. Mice were weaned to a standard rodent chow diet (Chow) (6% kcal from fat) or a Western-type diet containing 45% of the calories from fat (HFL n-6) (45 kcal% fat containing predominantly lard, 41% carbohydrate, 20% sucrose, 9% corn starch, and 12% Maltodextrin 10; Research Diets, Inc., New Brunswick, NJ). At six weeks of age, mice were separated into individual cages. Mice on the chow diet remained on the diet, while mice on the HFL diet were randomly divided with half remaining on the original HFL n-6 and the remainder switched to a diet containing 45% kcal fat from Menhaden (fish) oil (HFO n-3) for an additional four weeks (Table 1A) (Research Diets, Inc.). All animals received food and water *ad libitum* and were maintained in individual cages with a 12-hour dark/12-hour light cycle. The HFL n-6 and HFO n-3 diets both contained 45% of kcals from fat. The HFL n-6 diet contains a greater ratio of n-6 to n-3 fatty acids than the HFO n-3 diet (Table 1B). The HFL n-6 diet also

contains a greater amount of Stearic, Oleic, and Linoleic fatty acids than the HFO n-3 diet. The HFO n-3 diet contains a greater composition of Myristic, Palmitoleic, Stearidonic, Eicosapentaenoic (EPA), Docosapentaenoic, and Docosagexaenoic (DHA), which are the n-3 fatty acids that have been shown to be beneficial in decreasing inflammation in the body and decrease the risk of cardiovascular and other metabolic issues.[59] Due to the increase in the n-3 fatty acid components compared to the n-6 fatty acid components in the HFO n-3 diet, the n-6 to n-3 fatty acid ratio is lower in the HFO n-3 diet. (Tables 1 A and B).

Mice further received daily doses of the GC Prednisolone or vehicle only for the final four weeks. Prednisolone was administered at 0.003 grams/mouse/day (40 mg/m^2/day) in a vehicle of 0.25g sweet potato. Control mice received 0.25g sweet potato only. The prednisolone dose given is based on the amount given to pediatric ALL patients during the induction phase of their therapy. Food intake and body mass were determined twice weekly. All experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Experimental animals and were approved by the University of Memphis Institutional Animal Care and Use Committee.

Table 1. Composition of Experimental Diets. HFL n-6 with 45% kcals/g from lard; HFO n-3 with 45% kcals/g from menhaden oil. (A) ingredients in HFL-n-6 and HFO n-3 diets; (B) fatty acid composition of HFL n-6 and HFO n-3 diets.
(A)

Ingredients	**HFL n-6**		**HFO n-3**	
	gm (%)	kcal (%)	gm (%)	kcal (%)
Casein, 80 Mesh	200	800	200	800
L-Cystine	3	12	3	12
Sucrose	172.8	691	172.8	691

Table 1. Continued

Ingredients	**HFL n-6**		**HFO n-3**	
	gm (%)	kcal (%)	gm (%)	kcal (%)
Corn Starch	72.8	291	72.8	291
Maltodextrin 10	100.0	400	100.0	400
Cellulose, BW200	50	0	50	0
Soybean Oil	25	225	25	225
Lard	177.5	1598	0	0
Menhaden Oil (200 ppm tBHQ)	0	0	177.5	1598
tBHQ	0.0355	0	0	0
Mineral Mix S10026	10	0	10	0
Vitamin Mix V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
Cholesterol	0.58	0	0	0

*BHQ: tertbutylhydroquinone

(B)

Ingredients (g)	**HFL n-6**	**HFO n-3**
Lard	177.5	0
Menhaden Oil, ARBP-F	0	177.5
Soybean Oil	25	25
Total	202.5	202.5
C14, Myristic	2.1	14.0
C16:1, Palmitoleic	2.5	17.7
C18, Stearic	19.8	6.6
C18:2, Linoleic	56.2	16.1
C18:3, Linolenic, n3	4.2	4.3
C18:4, Stearidonic, n3	0	6.0
C20, Arachidic	0.4	0.3
C20:4, Arachidonic, n6	0.5	0
C20:4, n3	0	3.1
C22:5, Docosapentaenoic, n3	0.2	4.1
C22:5, n6	0	0.6
C22:6, Docosahexaenoic, n3	0	29.0
Total	190.7	189.6
Saturated (g)	60.2	59.8
Saturated (%)	31.6	31.5
Monounsaturated (g)	67.7	41.3
Monounsaturated (%)	35.5	21.8
Polyunsaturated (g)	62.8	88.5
Polyunsaturated (%)	32.9	46.7
n6 (g)	57.0	17.9
n3 (g)	4.4	66.6
n6/n3 ratio	13.1	0.3

Histology

Small intestines were harvested and length measured. Liver and epididymal adipose tissues were harvested and weighed. A portion of each tissue was fixed in formalin (Fisher Scientific Co. LLC) embedded in paraffin and 4um sections were stained with hemotoxylin and eosin. The mean adipocyte area (average surface area of 10-15 randomly selected adipocytes from each mouse (n=6) were determined using Axiovision r4.8.2 software. Analysis and documentation were performed using an imager M2 microscope (Axiocam MRC, Zeiss).

Glucose Uptake

Glucose uptake was measured *in vitro* using the everted sleeve method.[60] Briefly, small intestines were harvested and immediately transferred into cold mammalian 290 mOsm Ringer solution (117 mmol NaCl, 4.7 mmol KCl, 1.2 mmol KH_2PO_4, 1.2 mmol $MgSO_4$ and 20 mmol $NaHCO_3$). 1.5 cm long segments were cut starting at the most proximal region of the intestine. The 1.5 cm segments were everted and secured onto the ends of a four mm-diameter stainless steel rods using surgical thread to secure the ends. Mounted tissues were immediately placed in ice-cold 290 mOsm aerated (95% oxygen and 5% CO_2) Ringer solution. Mounted tissue samples were incubated for five minutes in 37°C aerated Ringers solution before being positioned for two minutes over a stir bar rotating at ~1,200 rpm in a solution of aerated mammalian Ringers solution containing 50 mmol/L D-glucose and a tracer concentration of radiolabeled (^{14}C) D-glucose to determine D-glucose uptake by the tissues. Radiolabelled (^{3}H) L-glucose was added to the incubation solution to correct for D-glucose that is associated with the adherent fluid and passively absorbed.[60] At the end of two minutes, tissues were rinsed for 20 seconds

in cold mammalian ringers solution, removed from the rods, and weight was determined. The tissues were solubilized (Solvable, Perkin Elmer, Waltham, MA) at 55°C for four hours, scintillation fluid was added (Ultima Gold, Perkin Elmer, Waltham, MA), and disintegrations per minute were measured. (Tri-Carb 2900TR, Perkin Elmer, Waltham, MA). Calculated rates of glucose absorption were expressed as nmoles of D-glucose transported per minute per mg of tissue.

Metabolic Parameters

Metabolic parameters were determined on days 14 and day 27 after the diet switch and start of Prednisolone treatment. Fasting blood glucose levels were measured after mice were fasted for six hours. Glucose was administered at a concentration of 1g/kg of animal weight via an intraperitoneal injection. Blood samples from the tail vein were collected every 30 minutes for a 90 minutes period. Blood glucose was determined using a glucose meter (Onetouch Ultra 2 Meter).

Lymphoid Parameters

To determine Prednisolone efficacy, spleens were harvested and weighed. Splenocytes were isolated and counted using a hemacytometer and trypan blue was used for dead cell exclusion.

Liver Enzymes

Liver enzymes were measured using an Alanine Transaminase (ALT) Activity Assay kit (Abcam ab105134). Activity was determined according to manufacturer's instructions. Samples were diluted 1:5 in the assay buffer and colorimetric change was determined after 60-minute incubation and reading at 570nm using a microplate reader (Synergy 2, BioTek).

Statistical Analyses

Statistical significance was determined using the Mann-Whitney *U* test and. $p < 0.05$ was considered significant.

RESULTS

Diet Containing Fat From Menhaden Oil Reduced Body Mass Gain and Adiposity Independent of Glucocorticoid Treatment. Mice given a diet containing 45% fat from fish oil (Menhaden oil; major fatty acids EPA and DHA) had reduced body mass gain over a 28 day period as compared to mice fed an isocaloric diet containing 45% fat from lard, resulting in a significantly reduced body mass at the end of the experimental period (Figures 1 and 2). The glucocorticoid treatment (Prednisolone) did not significantly increase body mass gain, regardless of diet treatment.

Mice consuming the diet containing lard averaged an 18% increase in food consumed in the presence of the glucocorticoids compared to the fish oil diet (Figure 3). Weight gained per gram of food consumed demonstrated that mice on a fish oil diet gain 30% less weight (without glucocorticoids) and 32% less weight (with glucocorticoids) compared to mice consuming the lard diet (Figure 4).

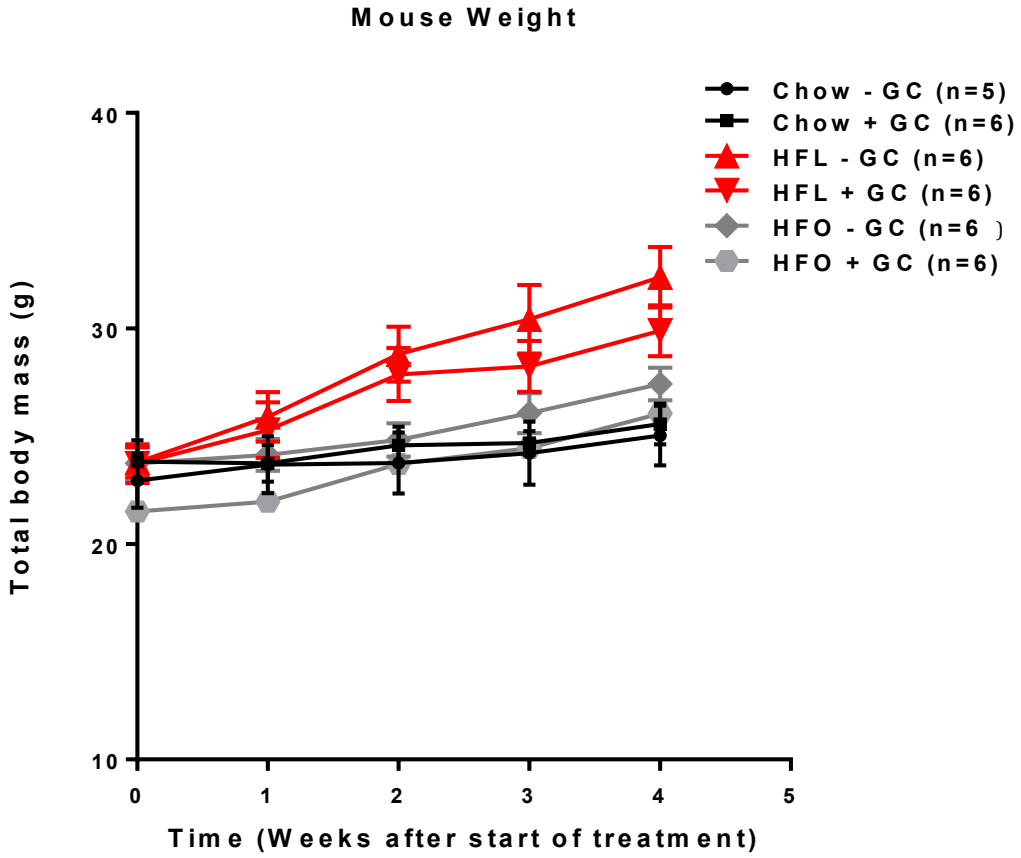


Figure 1. Weight Gain. Weight (grams) was measured weekly over the 28 day period that the mice received GCs while on a Chow, HFL n-6, and HFO n-3 diet (n= 5-6 mice for each group).

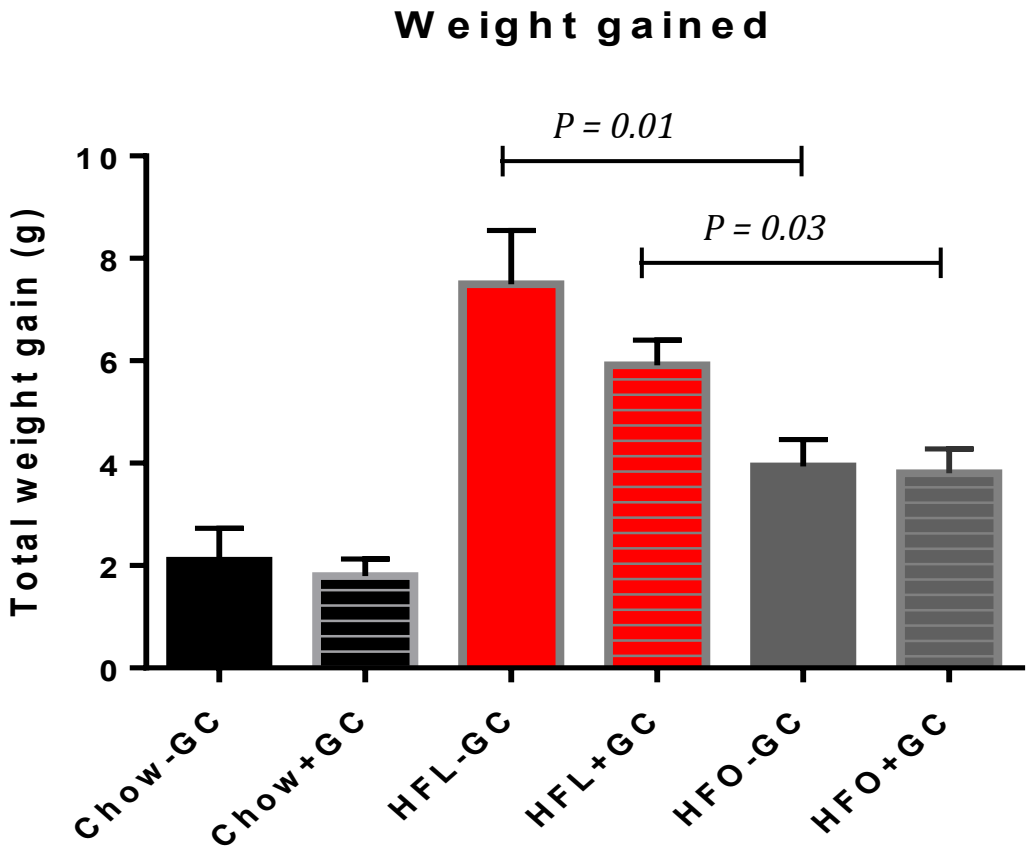


Figure 2. Total Weight Gain. Total weight gained was determined at the end of 28 days for each group. Total weight gained did not differ significantly between the Chow and the HFO n-3 diet, but there is a significant increase in total weight gain between the HFL n-6 diet and the HFO n-3 diet (P=0.01 in the absence of GC and P=0.03, in the presence GC) (n=6-9 mice per group).

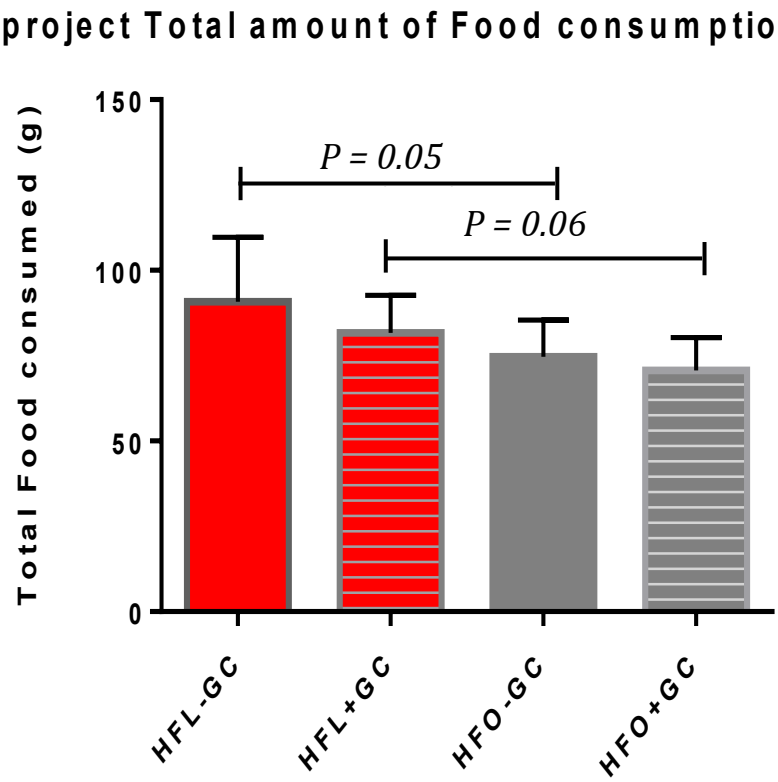


Figure 3. Total Food Consumption. Total food consumption was determined by weekly. In the presence of GC there is not a significant difference in food consumption, but the consumption of HFL n-6 diet did lead to a significant difference. (P=0.05, n=7-9 mice per group)**.**

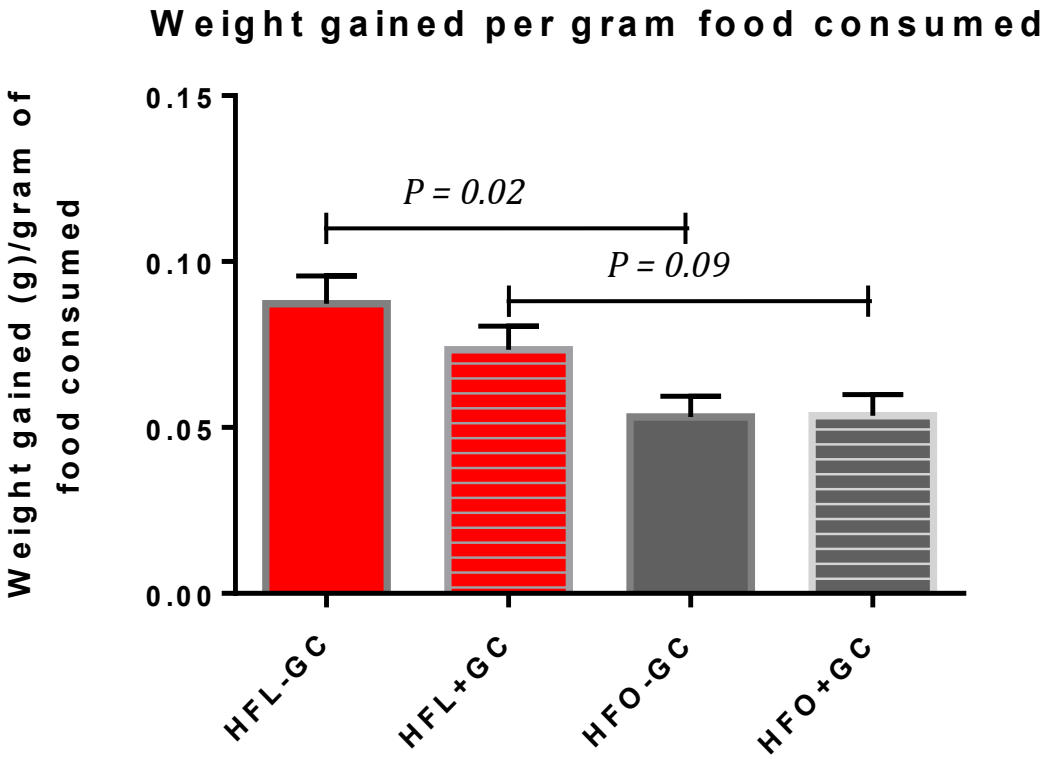


Figure 4. Weight Gained per Gram. GC treatment did not increase the weight gained per volume of food. There is a significant difference in the amount of weight gained per volume of food consumed (p=0.02, n=7-9 mice per group) of food consumed in mice on HFL n-6 or HFO n-3diets with and without GCs.

Glucocorticoid Treatment Reduces Spleen Size Independent of Diet.

Glucocorticoids have become part of the standard treatment for childhood ALL due to its cytolytic effects on thymocytes. Concomitantly with this decrease in immune cells is a reduction in immune organs size, including spleens.[7] To confirm GC efficacy spleen weight and spleenocyte number were determined. There was a reduction is spleen size compared to normal spleen sizes for the chow diet (22% reduction), lard diet (24% reduction) and fish oil diet (15% reduction; Figure 5). This was also true for splenocyte

number (Figure 6). Interestingly there was a significant difference in spleen sizes with the fish oil diet as compared to the chow or lard diet, where the HFO n-3 diets demonstrated larger spleen sizes, while the HFL n-6 diet show increased cell death independent of glucocorticoids.

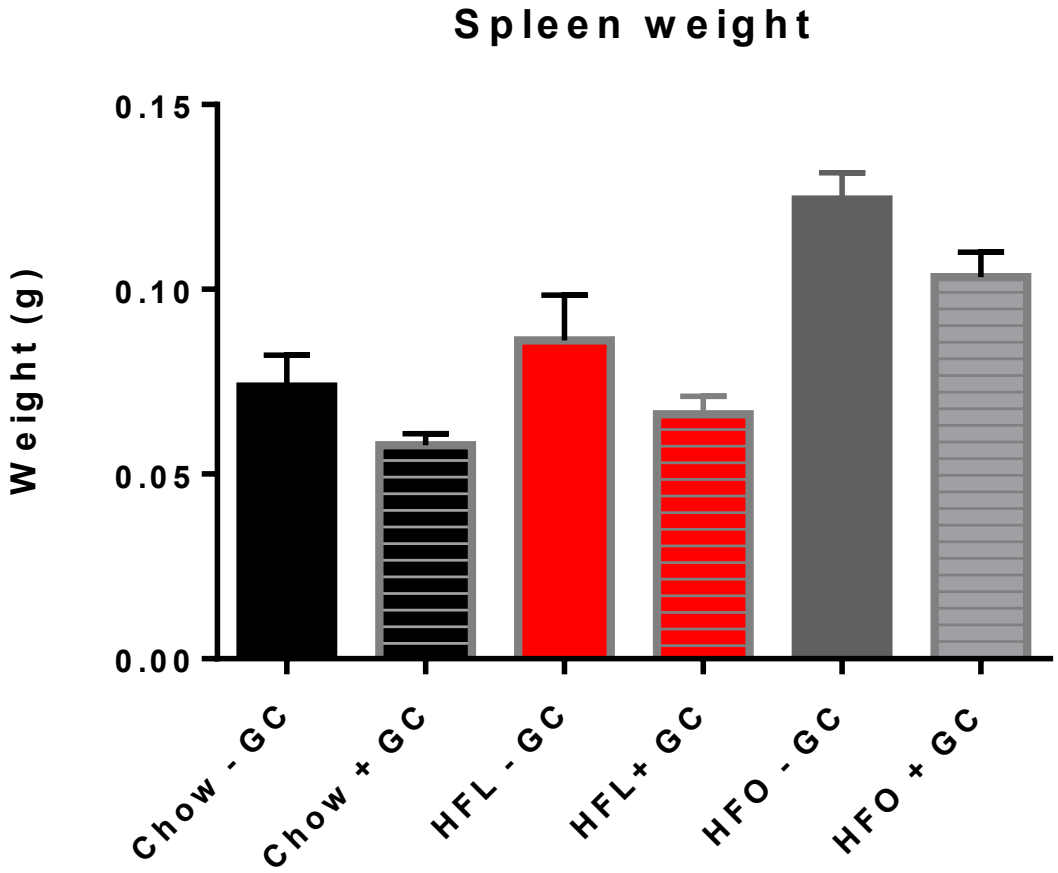


Figure 5. Spleen Weight. Spleens were harvested and weight determined. There was no significant difference between groups, but the spleen exposed always tended to be smaller (N=3-6 mice per group).

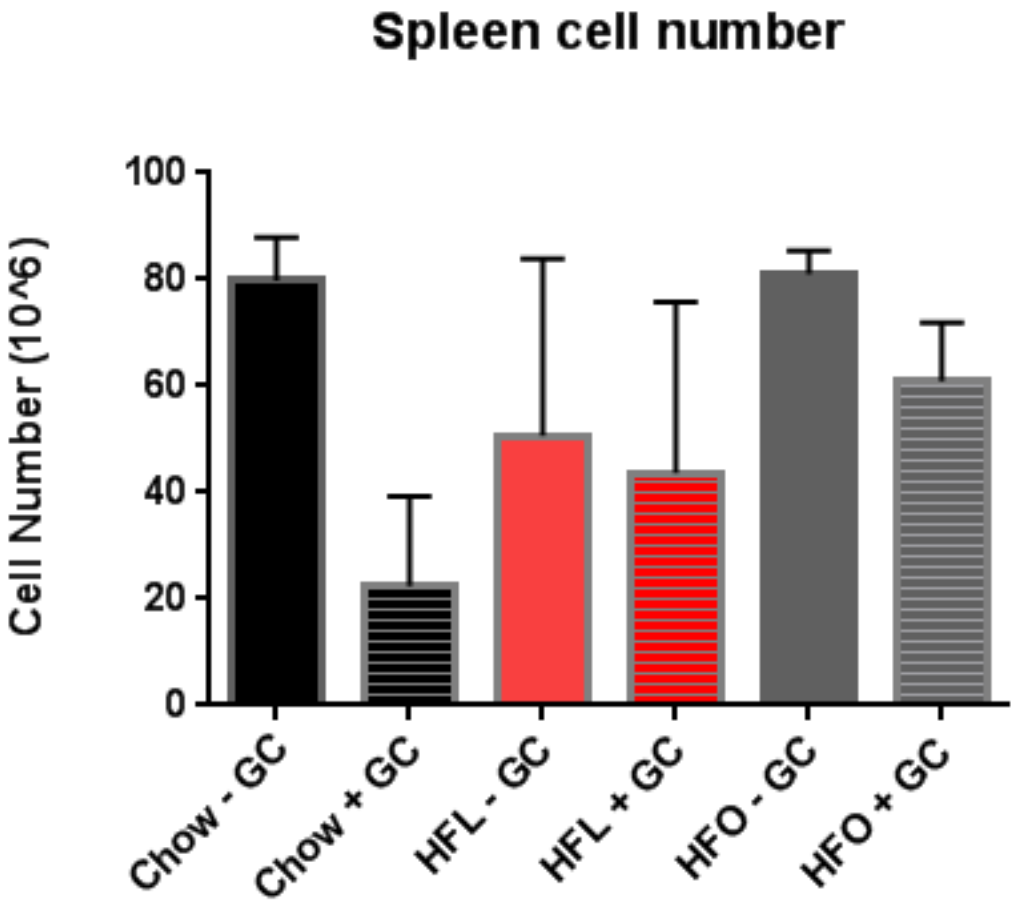


Figure 6. Splenocyte Numbers. Spleens were harvested and splenocytes isolated and counted using a hemacytometer and Trypan Blue exclusion dye. The P value for Chow and HFO n-3 with or without GC is 0.057. (n= 3-4 mice per group)

No Difference In Intestinal Glucose Uptake was Observed.

One successful experiment using the sleeve method to measure intestinal glucose absorption showed no difference in intestinal glucose uptake between mice on lard and fish oil diet (Data not shown). However, the small intestine lengths of mice fed the HFL n-6 diet were significantly shorter than those of the chow and HFO n-3 groups (Figure 7).

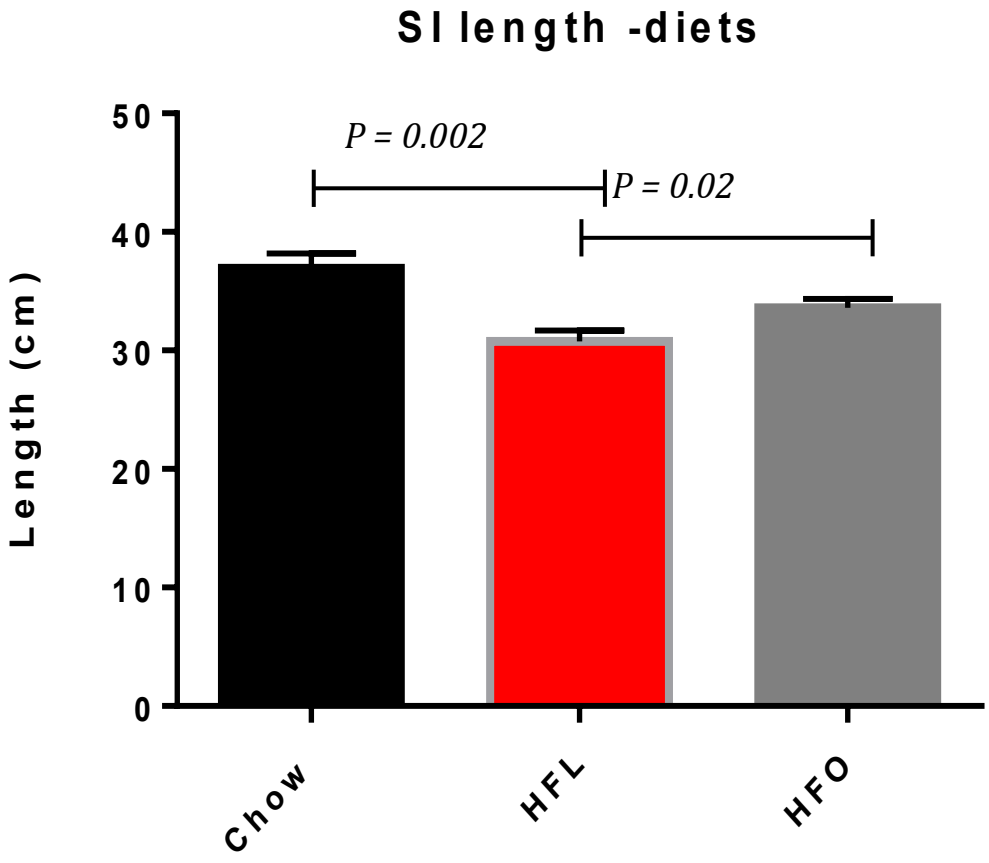


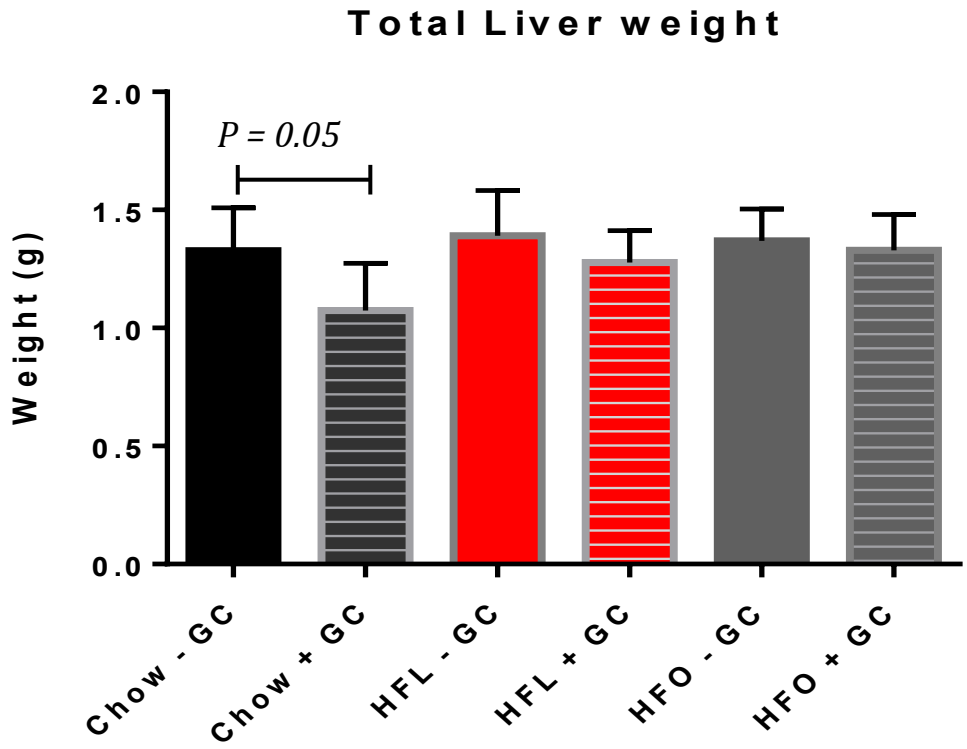
Figure 7. Small Intestine Lengths. Lengths measured in cm of mice on chow, HFL n-6, and HFO n-3 diets.

Liver and histology

Figure 8A demonstrates a significant decrease in liver weight with glucocorticoid treatment when the animals were on a standard chow diet (*P = 0.05*). No differences were observed in liver weight between untreated chow diet and the two high fat diets. Despite the fact that mice on the high fat diets have similar liver weight independent of glucocorticoid treatment, there is a dramatic fat deposition in the liver tissue of mice on

the lard diet by histologic examination. (Hematoxylin-eosin staining; Figure 8B) This fat accumulation is completely absent in the presence of the fish oil.

A



B

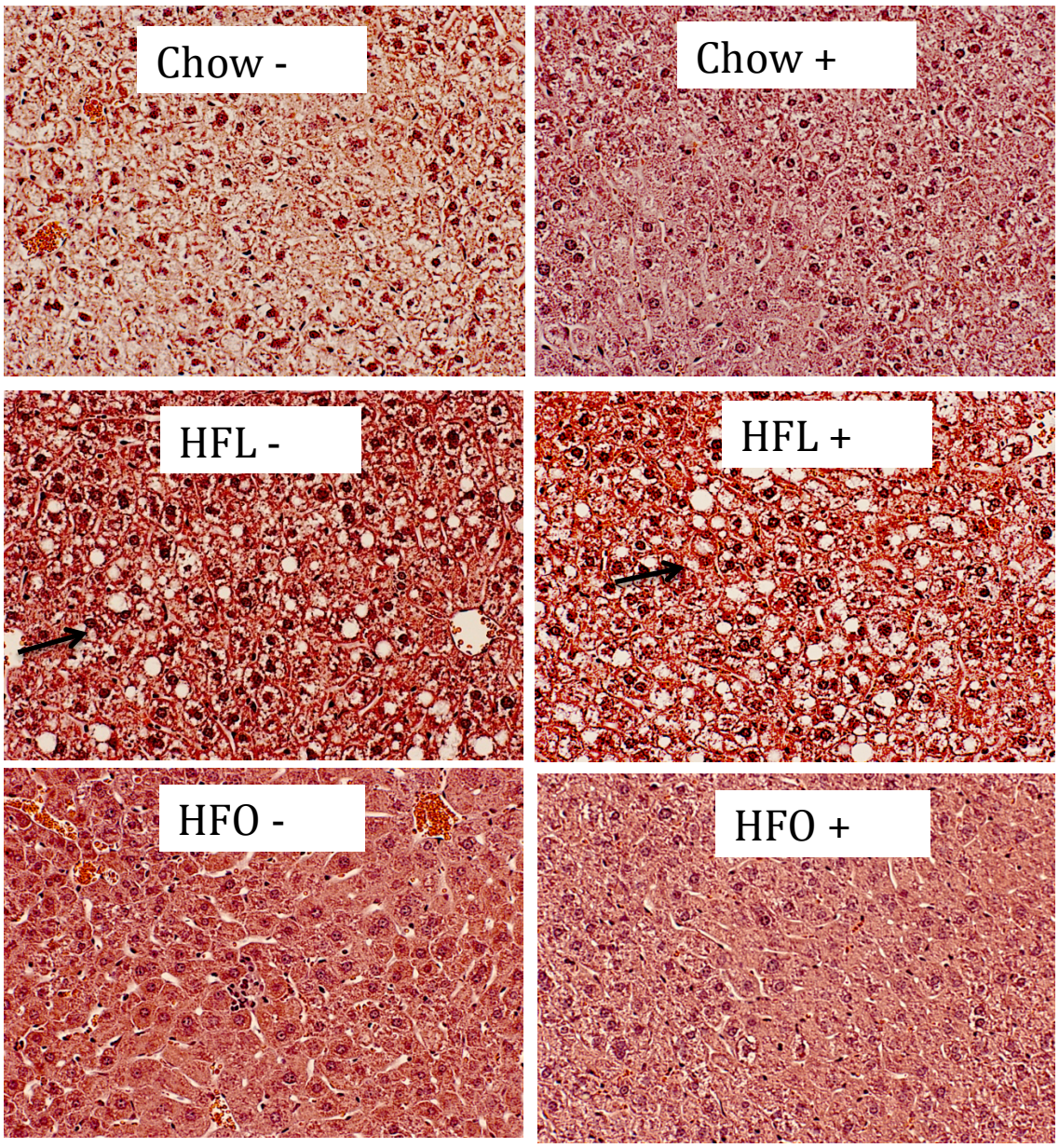


Figure 8. Liver Weight Morphology and Histology. (A) Livers were harvested and weighed; there was no increase in liver weight with the high fat diets, but GCs caused a decrease in liver weight when on a Chow diet. (B) Histology revealed an increase in fatty deposits in the liver (fatty deposit indicated by arrow) when on a HFL n-6 diet, but it is resolved when mice are switched to a HFO n-3 diet. (20x magnification)

Plasma Analysis of Liver Parameters was Not Significant.

Plasma Alanine Aminotransferase (ALT) activity was higher in the GC groups for both the HFL n-6 and HFO n-3 diets. However, the differences in plasma ALT activity were not significant when comparing the six groups (Figure 9).

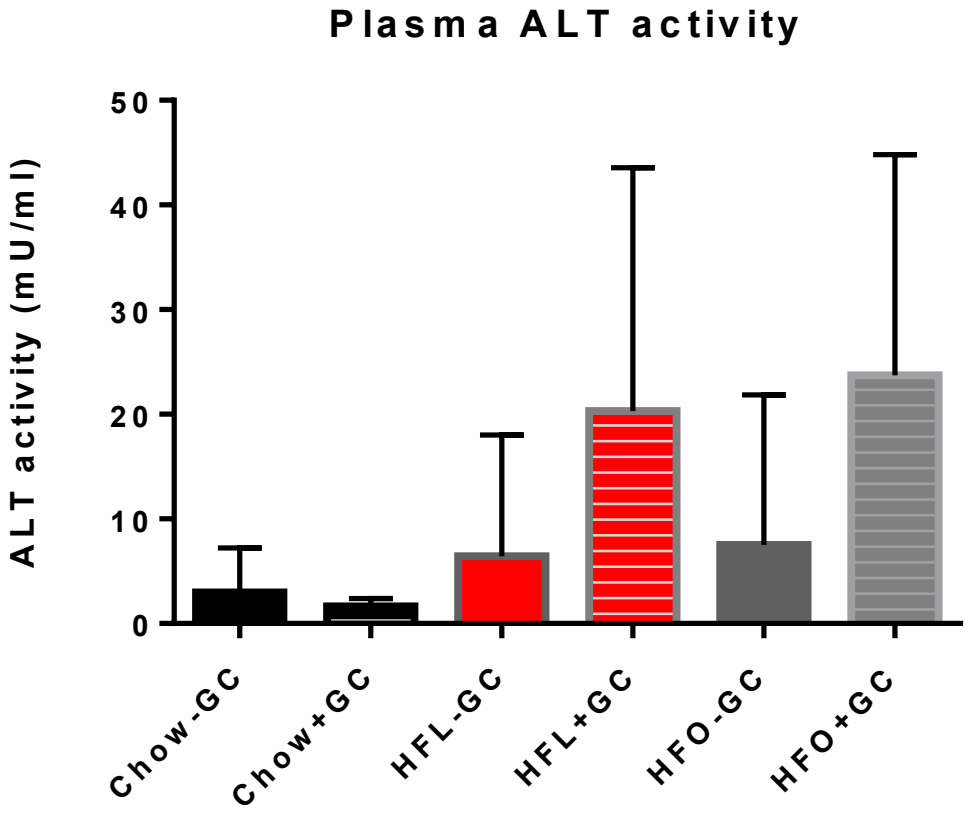
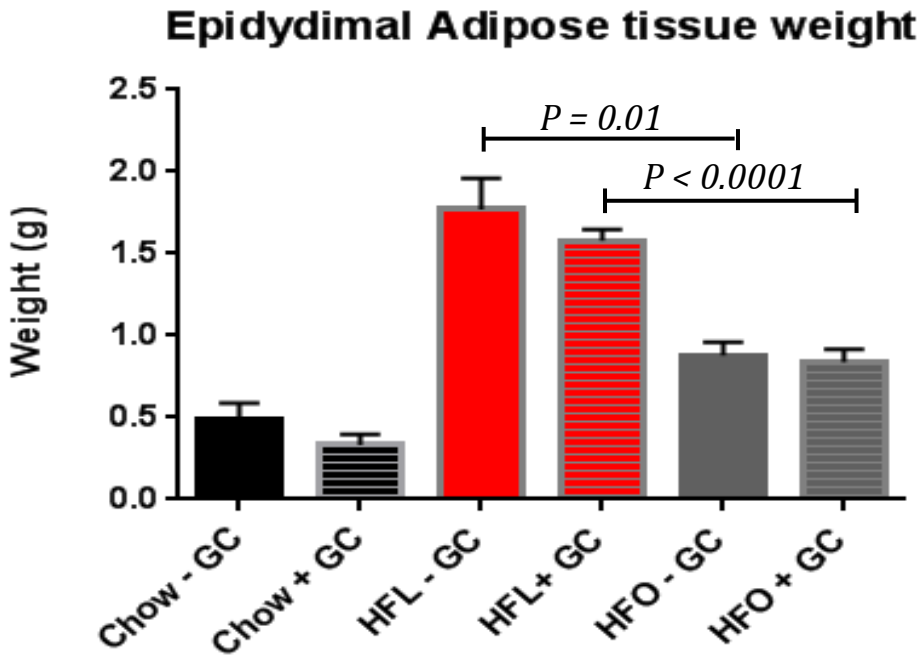


Figure 9. Liver Enzyme Activity. Plasma ALT activity levels were determined and there were no significant differences between groups (n= 3 mice).

Adipose Tissue Characteristics and Histology Were Observed for Differences in Weight and Size.

The weight of the epididymal fat pads reflected the overall weight of the mouse with the lard diet causing a significant increase in fat deposition as compared to the fish oil diet ($P = 0.01$ without glucocorticoid treatment and $P < 0.0001$ with glucocorticoids treatment; Figure 10A). As with body mass, glucocorticoids did not increase adipose tissue weight. However, histologically, there was a significant increase in adipocyte size in the presence of glucocorticoids only with the lard diet ($P = 0.0001$; Figure 10B and 10C).

A



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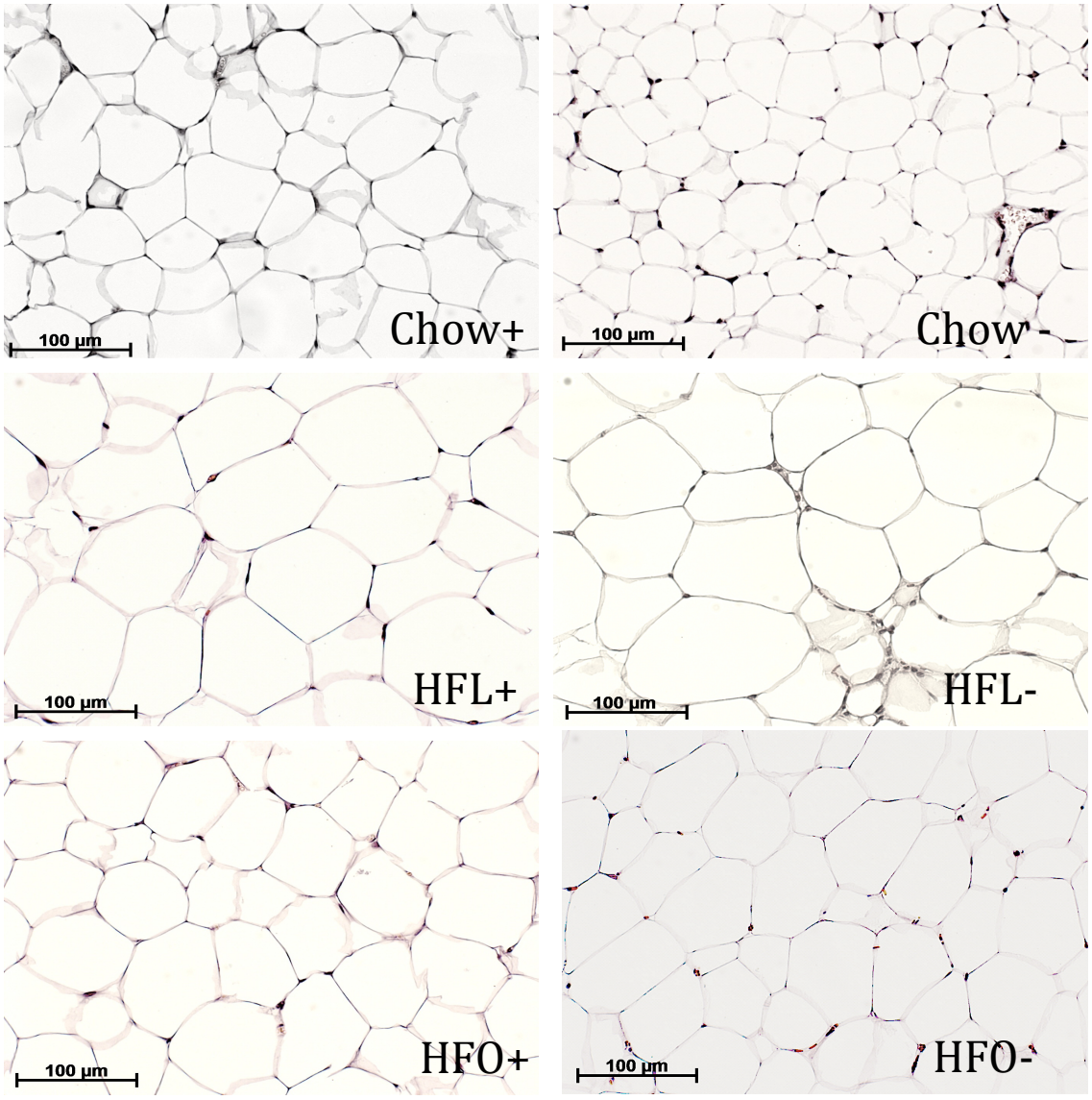
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C

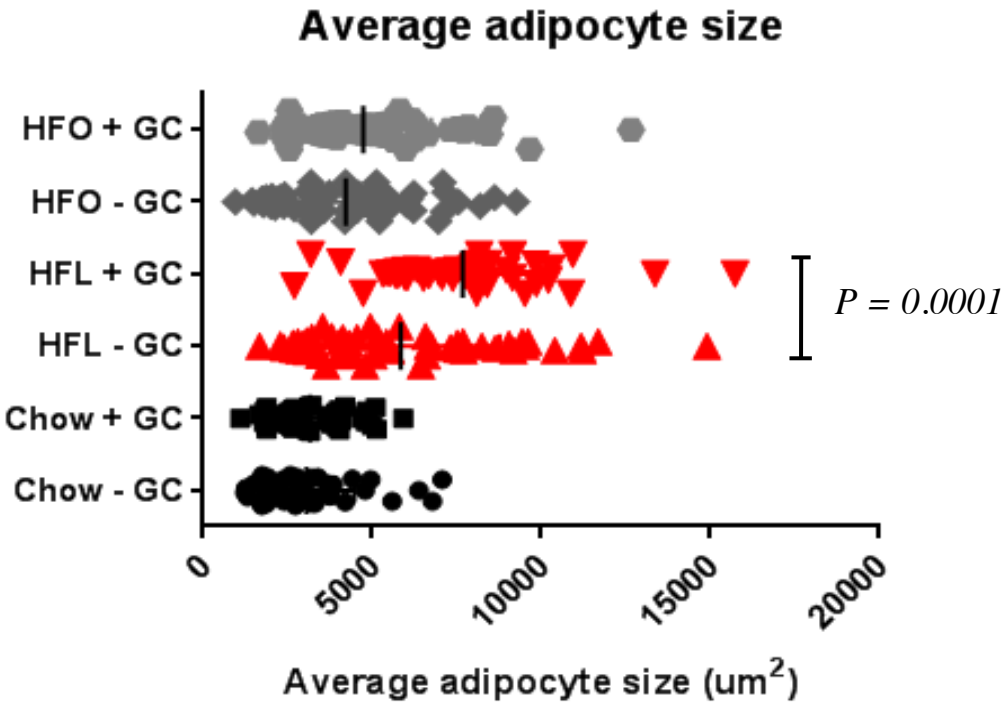


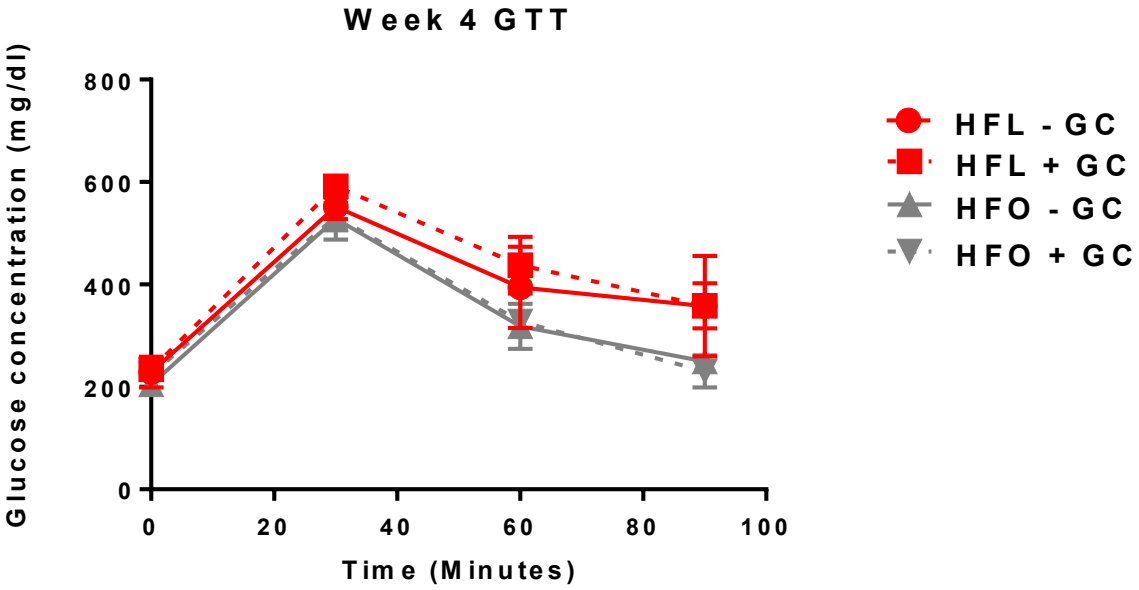
Figure 10. Adipose Tissue Weight and Morphology. (A) Epididymal fat pads were harvested and weighed. Mice on HFL n-6 had significantly larger fat pads, independent of GC treatment (p=0.0001). (B) Adipocyte morphology (20X magnification) demonstrate that the HFL diet increased the adipocyte size as compared to the Chow and HFO diet and that GCs exacerbate this phenotype significantly when on the HFL diet (p=0.0001).

Plasma Blood Glucose Levels Were Higher in HFL n-6 Diet Mice than HFO n-3 Diet Mice.

The impact of 40 mg/m^2/day of GC administration on glucose tolerance in correlation with the two different HFDs was assessed with an intraperitoneal glucose tolerance test (GTT). Our results show that a HFL n-6 diet caused a greater increase in blood glucose levels compared to the mice on a HFO n-3 diet. (Figure 11 A and B). The blood glucose levels of the HFL n-6 group with GC administration appeared to be higher

compared to the HFL n-6 groups without GCs. Figure 11 A and B demonstrates that the HFO n-3 diets showed similar blood glucose uptake throughout the course of the GTT regardless of the presence or absence of a GC. Glucose uptake of the HFO n-3 diet group had similar levels to the chow diet group (data not shown).

A



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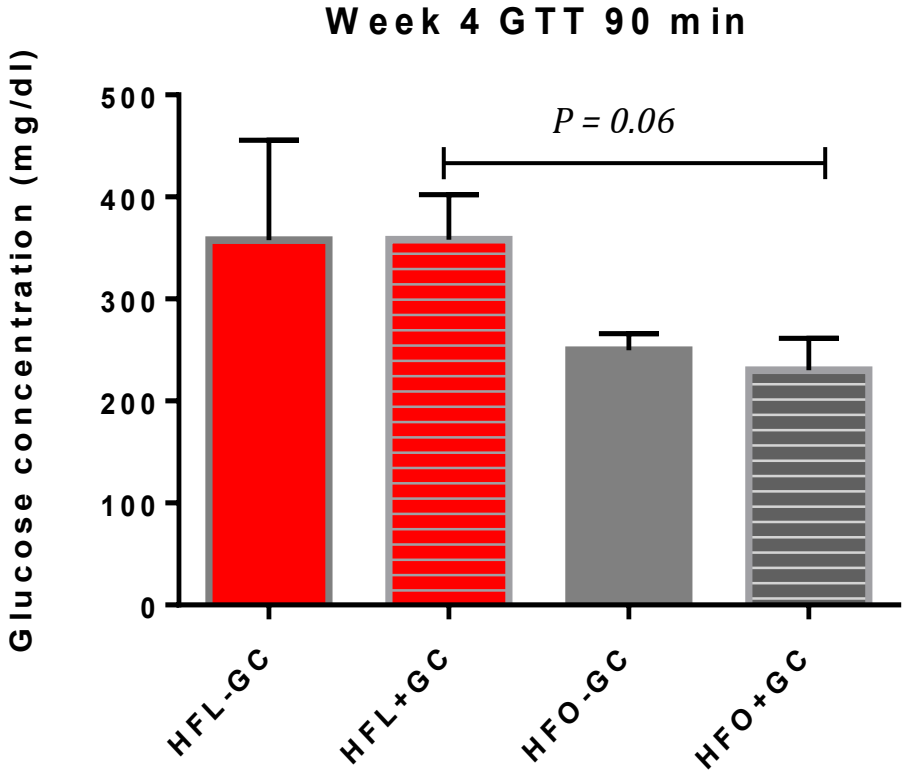

Figure 11. Glucose Tolerance test. (A) Blood glucose concentration (mg/dl) was measured over 90 minutes. **(**B) At 90 minute after glucose (1g/kg) administration, glucose levels were increases with the HFL n-6 diet and trended trend towards glucose intolerance (p=0.06) (n=3-5 mice per group).

DISCUSSION

Patients undergoing ALL treatment (which includes high dose GCs) have an increased risk of developing metabolic syndrome later in life.[14,61,62] Pediatric survivors typically demonstrate signs of obesity, insulin resistance, dyslipidemia, and hypertension in remission from ALL indicating that GCs may have long term side effects even after therapy.[14,62] These side effects may be due to GCs main role in decreasing inflammation by suppressing the immune system and controlling energy metabolism, especially hepatic energy metabolism.

GCs have a role in ALL treatment due to their anti-inflammatory and cytolytic effects.[8] As GCs reduce immune cells, there is a concomitant reduction in the mass of immune organs. The significant decrease in spleen weights and spleen cells confirmed that GCs had a similar effect on immune cells and associated organs during the 28-day treatment.

Due to the unquestionable evidence of dietary intervention on human health, we focused our efforts on the effects of a diet high in n-6 fatty acids from lard compared to a diet high in n-3 fatty acids from fish oil. Our results mirrored data from a previous study reporting that animals consuming a diet high in n-3 fatty acids had less total adipose tissue, intra-abdominal fat, and less insulin resistance than a animals consuming a high fat lard diet. [63] The mice in the present study showed that the HFL n-6 diet promoted more overall weight gain, adipose tissue gain, more food consumption, and more weight gain per gram food than the mice on the HFO n-3 diet. This is in accordance with published work demonstrating that a diet high in EPA and DHA upregulate fatty acid

oxidation suggesting that the n-3 fatty acids may be protective against adipose tissue gain. It is now well established that n-3 fatty acids in fish oil increases fatty oxidation while the n-6 fatty acids from lard induces the expression of transcription factors that regulate lipid homeostasis such as Sterol Regulatory Element Binding Protein (SREBP), which promotes fatty acid synthesis.[64] The result of the weight gained by the mice demonstrates that the type of diet has a greater effect on weight gain than the presence of GCs.

Histological evaluation of the epididymal fat showed that the adipocytes from the HFL n-6 diet were larger in diameter compared to both the HFO n-3 and Chow diets. The adipocytes on the chow and HFO n-3 diet were similar in size, indicating that the n-6 fatty acids in the lard diet may alter physiological mechanisms that increase the size of the adipocytes. Moreover, the administration of glucocorticoids caused a significant increase in adipocyte size, but only when mice were exposed to the n-6 lard diet.

Long-term use of GCs can cause NAFLD due to alterations in hepatic metabolism.[4] NAFLD results from a combination of insulin resistance and an increase in the uptake of free fatty acids and *de novo* liponeogeneisis in the hepatocytes leading to an accumulation of stored fat in the liver.[4] Although there were no histological differences in fatty deposition in the liver between the HFL n-6 with and without GCs, ALT enzymes were increased in the HFD groups with GCs, albeit not significantly. This may indicate that the GCs could be causing liver damage, however, liver damage indicated by elevated plasma ALT levels and fibrosis, typically does not develop with less than four weeks of high fat feeding.[65–67] A longer time on the HFD may be needed to see more true liver damage as indicated by increased ALT enzymes. The histological results of the livers

show that the HFL n-6 diet caused more fat accumulation than the chow or HFO n-3 diets, which could further develop into more liver damage with a longer study period on the HFD indicated by high ALT enzyme levels and liver fibrosis. Therefore, the HFO n-3 diet may be protective and decrease the risk of NAFLD.

Similar to other findings where hyperglycemia was seen in a HFD in conjunction with a GCs, the data from the GTT's throughout our study demonstrates that the HFL n-6 diet rapidly induced a type 2 diabetic phenotype after four weeks on the diet.[15] This was characterized by glucose intolerance as measured by an intraperitoneal glucose tolerance test over 90 minutes. Due to the research that shows that insulin resistance could lead to NAFLD, a longer study period could show more data that demonstrates the development of NAFLD[68].

Fatty acids in the liver can alter glucose metabolism, therefore we evaluated possible alterations in intestinal uptake of glucose by the inverted sleeve method to determine if there is an alteration in glucose uptake in the presence of the different fatty acid composition in the diet. [69] Due to controversial research regarding the fluctuation of glucose transporters in the small intestines, we measured rates of glucose uptake by the small intestines of mice fed the HFL n-6 diet and the HFO n-3 diet. The data suggested that glucose uptake of the small intestine is not affected by the type of diet consumed during the seven weeks on the diet. However, there was a significant decrease in intestinal length of mice consuming the HFL n-6 diet as compared to the mice consuming a chow and HFO n-3 diet. However, the small intestines in both HFD groups showed to have similar glucose absorption rates, indicating that the number of transporters was most likely the same in the proximal areas of the intestines. However, the differences in the

intestinal lengths could possibly indicate that chow and HFO n-3 diet mice could have more glucose uptake capacity than the HFL n-6 diet mice. Further understanding of why a diet high in n-6 fatty acids could cause a decrease in intestinal size and yet, still advance to obesity and insulin resistance will need to be rendered.

CONCLUSION

This study demonstrates that a high fat diet higher in n-6 fatty acids can lead to detrimental side effects, especially in combination with GC therapy. Here we show that a diet high in n-6 fatty acids increases total body and adipose tissue weight, per volume of food consumed. In addition, the data shows that a HFL n-6 diet in the presence of 40 mg/m^2/day prednisolone increases fatty liver development and insulin resistance. However, the replacement of a HFL n-6 diet with a HFO n-3 diet will reduce the metabolic disorder phenotypes, specifically hyperglycemia, obesity, and liver disease to similar phenotypes of animals consuming a chow diet during glucocorticoid treatment.

The results suggests that a diet high in n-6 fatty acids may be beneficial for pediatric ALL patients undergoing GC treatment and might decrease the risk of developing symptoms associated with metabolism after their treatment.

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APPENDIX

A. IACUC Approval

IACUC PROTOCOL

FOR USE OF LIVE VERTEBRATES FOR RESEARCH, TEACHING OR DEMONSTRATION

UNIVERSITY OF MEMPHIS

IACUC Protocol # Date Submitted to IACUC 7/21/14

Dates Protocol will be in effect: from 8/19/14 to 5/31/16

(not to exceed three years including two yearly renewals)

Is this protocol related to an external grant or contract application? Yes ☐ No X

If yes, complete the following:

Agency. Date Submitted

Grant #

University account for Animal Care Facility per diem charge: _____

If the protocol is not related to an external grant or contract application, complete the following:

University account for Animal Care Facility per diem charge: 211700

Effect of a dietary intervention on glucocorticoid-induced metabolic syndrome.

Project Title: (If project relates to a grant or contract application, give that title; if the project is related to a class, give the course name and number):

I. Personnel

Investigator/Instructor: Marie van der Merwe

Department: Health and Sport Sciences

Academic Rank: Assistant Professor

Campus phone**:** 901 678 3476 Emergency phone: 901 406 7458

Attending Veterinarian: Karyl Buddington

Phone: 901 678 2359 Emergency phone: 901 258 1232

List all individuals that will handle animals using this protocol and their level of expertise (e.g. relevant qualifications). If the protocol applies to a class then so specify.

Marie van der Merwe – PhD (Molecular Pharmacoly), Postdoctoral Fellowship (Bone Marrow Transplantation): More than 10 years of experience using mice as a research model.

Simone Godwin – BA (Anthropology), Animal husbandry internship at Duke Lemur Center (Durham), Primate conservation and rehabilitation internship at Alouatta Sanctuary (Panama)

LeeAnna Beech – B.S (Dietetics), A.S (Science), Animal trainer certification at Dolphin Quest (O'ahu)

If additional personnel become involved in handling animals used in this protocol, it is the responsibility of the principal investigator to notify the Animal Care Facility in writing before they start.

If applicable, has the investigator/instructor and all personnel listed above

received the appropriate vaccinations (tetanus, rabies)? Yes ☐ No ☐

Is it necessary for personnel listed on this protocol to be tested for TB? Yes ☐ No X

If you have questions about the kind of vaccination or about TB, call the Animal Care Facility at 678 2034.

All U of M personnel involved in this protocol must complete the animal care and use training program (satisfactory completion of, or concurrent registration in Biol 7006/8006), or have completed a comparable training program at another institution before animals can be procured or before the experiments/teaching or demonstration. In submitting this protocol, I, as Principal Investigator/Instructor accept the responsibility for compliance with this requirement.

In addition, the Principal Investigator/Instructor must be willing to provide appropriate supervision for all persons working on this protocol. In the case of a class, the Instructor must be responsible for training any students in classes involved prior to using animals.

II. Project Description

A. Summary **(Enter a brief description below of your project, using lay terminology)**:

Acute lymphoblastic leukemia (ALL) treatment increases the risk for pediatric patients to develop obesity and symptoms similar to metabolic syndrome including type-2 diabetes and dyslipidemia. The treatment includes many chemotherapies and also high doses of glucocorticoids. The long term use of glucocorticoids has been associated with the increases in weight and also symptoms similar to metabolic syndrome. The goal of this experiment is to determine if dietary interventions can reduce risk and long term consequences associated with the use of glucocorticoids.

We will use a mouse model (C57BL/6) that will receive a 45% fat (lard) diet, similar to a Western diet. Control mice will be on a "normal" 10% fat diet. As an intervention, the fat source will be altered to a 45% fish oil diet (omega-3 fatty acids). Omega-3 fatty acids have been shown to reduce inflammation and decrease risk of development of glucose intolerance. While on the various diets, mice will be given the glucocorticoid, prednisone at a dose of $40mg/m^2$/day for 28 days. Prednisone will be administered orally using a vehicle on a daily basis. 28 days reflect the induction phase of ALL treatment during which time the patients receive high doses of prednisone and experience a dramatic increase in weight.

Eating habits (amount of food consumed) and weights of mice will be monitored twice a week. After two weeks (14 days), half of each group of mice will be anaesthetized to perform a DEXA scan (whole body fat composition) Blood will be drawn to determine glucose and insulin levels. A glucose tolerance test will be performed by administering glucose and then measure glucose levels in blood at various time points. The mice will be sacrificed to harvest brain, adrenal glands, liver, spleen, mesenteric lymph nodes and adipose tissue for analysis. The remainder of the mice will go through the same process at 28 day after the start of glucocorticoid treatment.

B. Describe IN DETAIL the procedures you will follow. You may do this in either of two ways **(Check one of the options below and follow the associated instructions)**:

1. Accompanying documentation (**include documentation in box below**).

X OR

2. By reference to previously published work (**provide a complete bibliographic citation in the box below, and describe any variations from the published technique**). ☐

Mice: C57BL/6 male mice will be used for this study and obtained from breeder pairs that are bred and housed at the animal facility on the University of Memphis campus.

Diets and treatments: All of the mice will be weaned at 3 weeks of age to the western diet with 45% lard and 41% carbohydrate (20% sucrose, 9% corn starch and 12% Maltodextrin 10). For Specific Aim 1 the mice will remain on this diet. For Specific Aim 2 the lard component will be replaced using fish oil at the start of glucocorticoid treatment to provide the long chain omega-3 fatty acids. The diets will be purchased from Research Diets, which has experience in producing the western diet for rodent studies.

Glucocorticoid therapy: To resemble the age group of ALL patients treated at St Jude, mice will be 6 weeks of age at the start of glucocorticoid therapy. Mice of this age are comparable to a 4 year old child. The glucocorticoid treatment will last for 28 days. Prednisone (40 mg/m2/day) will be orally administered on a daily basis by adding it to a vehicle of canned sweet potato or other canned fruits or vegetables. If the vehicle is not sufficient to mask the taste of prednisone, we might use prednisolone that is bioequivalent to prednisone, but is more palatable. This might only be needed for the first couple of doses as the increase of appetite drivel by the glucocorticoid might drive the consumption of the drug in vehicle. If prednisolone is not efficiently consumed, we will administer the drug by gavage. Again, this might only be required for the first couple of days until their appetite increases.

Experimental design:

For Specific Aim 1, 40 male mice will be divided into 2 experimental groups, with all mice fed the western diet with lard and with or without the addition of the glucocorticoid (GC) treatment (20/group). Half of each group - 10 mice – will only be treated for 14 days. This group will be anaesthetized (isoflurane) after a 10 h fast, a DEXA scan performed (anesthetized for 15 min) and blood collected via the saphenous vein in one of the back legs to determine fasting glucose and insulin levels and also determine cytokine levels. For the glucose tolerance test, these mice will be given a 1g glucose/kg body weight intraperitoneally and blood collected every 30 minutes for 90 minutes to measure glucose levels. Mice will then be euthanized (CO2 inhalation) to collect brain, adrenal glands, liver, spleen, mesenteric lymph nodes and adipose tissue for histology, biochemistry and immune cells harvest. This data will reveal if there is any early indication of the metabolic phenotype. The remainder of the mice will remain on the glucocorticoid treatment for another 14 days at which point this group will be anaesthetized (isoflurane) after a 10 h fast, a DEXA scan performed and blood collected via the saphenous vein in one of the back legs to determine fasting glucose and insulin levels and also determine cytokine levels. For the glucose tolerance test, these mice will be given a 1g glucose/kg body weight intraperitoneally and blood collected every 30 minutes for 90 minutes to measure glucose levels. Mice will then be euthanized (CO2 inhalation) to collect brain, adrenal glands, liver, spleen, mesenteric lymph nodes and adipose tissue for histology, biochemistry and immune cells harvest.

The same experimental approach will be used for Specific Aim 2, except the lard diet will be replaced by the fish oil based western diet.

Specific Aim	Western Diet type	Treatment	
1 (n = 40)	Lard	Plus Glucocorticoid	No Glucocorticoid
2 (n = 40)	Fish Oil	Plus Glucocorticoid	No Glucocorticoid

The goal of this experiment is to mimic the metabolic syndrome that is induced in the pediatric population treated for acute lymphoblastic leukemia. No restriction or dietary advice is currently given to these patients. We are interested in the kinetics of the onset of the syndrome and also what role the immune system plays is setting up the environment for the disorder.

The C57BL/6 diet induced obesity (dio) mouse model has been used previously to look at the effect of excess weight on various organ systems. As we are interested in the interaction between the immune system and other organs and what role it plays in the onset of metabolic phenotype, we cannot use isolated cell lines or model organisms such as yeast. Additionally, many reagents have been developed for the use of mouse tissues, especially antibodies that will be used to identify immune populations.

There will be 10 mice per group for each time point. This number should be sufficient to determine statistical significance. (There will also be 16 mice on regular chow to use as controls.)

C. Rationale for Involving Animals and the Appropriateness of Species and Number Used.

Indicate (**here**) briefly the short and/or long-term benefits (to humans and/or other animals) of this use of animals for research, teaching or demonstration. In addition, state briefly why living animals are required for this study, rather than some alternative model.

D. Do the procedures described in B above, have the potential to inflict more than momentary pain or distress (this does not include pain caused by injections or other minor procedures)? Yes ☐ No X

If yes, please address the following:

I have considered alternatives to procedures that might cause more than momentary or slight pain/distress, and I have not found such alternatives. As such, I have used one or more of the following methods and sources to search for such alternatives: **(check below each method used)**

☐ Agricola Data Base ☐ Medline Data Base ☐ CAB Abstracts

☐ TOXLINE ☐ BIOSIS ☐ Lab. Animal Sci. Journal

☐ Lab. Animals Journal ☐ Lab Animal ☐ Animal Welfare Info Center

☐ ATLA (Alternatives to Laboratory Animal Journal) ☐ Quick Biblio. Series

☐ Lab Animal Welfare Bibliography (QL55L27311988) ☐ "Benchmarks"

☐ "Alternatives to Animal Use in Research, Testing and Education"

☐ Current Contents

☐ CARL

☐ Direct contact with colleagues **(if selected, you MUST document this below)**

What is the length of time that the literature search covers

III. Animal Use

A. List all animal species to be used (example below).

Species Number[1]	Age[2]	Sex	Weight[2]	(Bldg./Rm#)
Hooded Wistar rats	45	2 months	male	250-350 gm	Psychology Bld./422I
C57BL/6 mice	96	2 months	Male	20-25 gm	Psychology Bld./422I

[1]Individuals using ectotherms need to only approximate numbers.
[2]Individuals using fish or other ectotherms need not answer this question.

Is any species threatened or endangered? Yes ☐ No X

B. Source of animals

X Commercial vendor (Source: Jackson Labs) Female mice will be purchased to breed with male mice available on the UM campus

X Bred at The University of Memphis

☐ Captured from wild

☐ Transferred from another study (IACUC) Protocol Number

☐ Donated (Source)

☐ Tennessee Wildlife Resources Agency

☐ Purchased and supplied by TMGC

Is the supplier a USDA approved source? Yes ☐ No ☐

If not, explain why:

☐ Animals are already in residence at U of M

C. Will surgery be conducted on animals? Yes ☐ No ☐

If yes, complete this section:

☐ Non Recovery Surgery ☐ Recovery Surgery

☐ Multiple Survival Surgery (**if the latter is checked, complete section F)**

Surgeon(s) (Name/Job/Title/Academic Rank Location of Surgery (Bldg. & Room #)

D. Will Anesthetic(s), Analgesic(s), or

Tranquilizing agents be administered? Yes X No ☐

If yes, complete this section (example below).

Species & Sex Agent Dose Route Performed by (Name/Title/Academic Rank)

male Hooded Wistar rats	sodium pentobarbitol	50 mg/kg	i.p.	Mr. Smith/Research Technician/B.Sc.
C57BL/6 mice	Isoflurane	2-4%	Inhalation	Marie van der Merwe/ Assistant Professor Simone Godwin/Masters Student LeeAnnaBeech/Masters Student

E. Will euthanasia be carried out? Yes X No ☐

If yes, complete this section (example below).

Species & Sex Agent Dose Route Performed by (Name/Title/Academic Rank)

male Hooded Wistar rats	sodium pentobarbitol	150 mg/kg	i.p.	Mr. Smith/Research Technician/B.Sc.
C57BL/6 mice	CO2	3L/min	Inhalation	Marie van der Merwe/ Assistant Professor Simone Godwin/Masters Student LeeAnnaBeech/Masters Student

If no, describe disposition of animal(s) at conclusion of this study in box below.

F. Will special housing, conditioning, diets or other conditions be required? Yes X No ☐

Mice will be on special high fat (either lard/fish oil) diets in addition to receiving daily oral glucocorticoids.

If yes, please explain in box below.

G. Will animals be removed from the U of M campus at any time? Yes ☐ No X

If yes, please indicate to where and for how long in box below.

G. If they are to be housed for more than 24 hours outside approved facilities at U of M, provide a scientific justification in box below

IV. Toxic and Hazardous Substances

A. Check off any of the following below that will be used in these experiments?

☐ Infectious agents (Fill out a, b)

☐ Radioisotopes (Fill out a, b, e)

☐ Toxic chemicals or carcinogens (Fill out a, b)

☐ Recombinant DNA (Fill out a)

☐ Experimental drugs (Fill out a)

☐ Malignant cells or hybridomas (Fill out a, c)

☐ Adjuvants (Fill out a)

☐ Controlled substances (Fill out a, d, e)

For each checked off category, answer the questions indicated below:

a. Identify the substance(s) and completely describe their use, including how will be injected or given to the animal(s):

b. Describe all procedures necessary for personnel and animal safety including biohazardous waste, carcass disposal and cage decontamination:

c. If transplantable tumors or hybridoma cells are to be injected into the animals, have the tissues/cells been tested for inadvertent contamination by viruses or mycoplasma? Yes ☐ No ☐

d. In the box below, provide a complete list of these substances, and if their use is not explicitly explained in the materials already provided, explain their use and role in the research.

Provide DEA license # covering the use of these substances:

To whom (or what entity) is the license issued?

e. Provide Radioisotope License Number:

To whom is the license issued?

V. Categories of Animal Experimentation Based Upon Level of Manipulation and Pain: (check off each category that is applicable to this application)

X A. Animals will be involved in teaching, research, experiments or tests involving no pain, distress, or use of pain-relieving drugs.

X B. Animals will be subject to mild stress only (e.g., food or water deprivation of less than 24 hours for use in behavioral studies such as operant conditioning; physical restraint for less than 30 minutes), and will not be subject to surgery, painful stimuli, or any of the other conditions described below. Procedures described in this protocol have the potential to inflict no more than momentary or slight pain or distress on the animal(s)----that is, no pain in excess of that caused by injections or other minor procedures such as blood sampling.

X C. Animals will have minor procedures performed, blood sampling, etc. while anesthetized.

X D. Live animals will be humanely killed without any treatments, manipulations, etc. but will be used to obtain tissue, cells, sera, etc.

☐ E. Live animals will have significant manipulations, surgery, etc. performed while anesthetized. The animals will be humanely killed at experiment termination without regaining consciousness.

☐ F. Live animals will receive a painful stimulus of short duration without anesthesia (behavior experiments with flight or avoidance reactions--e.g., shock/reward) resulting in a short-term traumatic response. Other examples in this category are, blood sampling, injections of adjuvants, or drugs, etc.

☐ G. Live animals will have significant manipulations performed, such as surgery, while anesthetized and allowed to recover. Such procedures cause post-anesthetic pain/discomfort resulting from the experiment protocol (e.g., chronic catheters. surgical wounds, implants) which cause a minimum of pain and/or distress. Also included are mild toxic drugs or chemicals, tumor implants (including hybridomas). tethered animals, short-termed physically restrained animals (up to 1 hour), mother/infant separations.

☐ H. Live animals will have significant manipulations or severe discomfort, etc. without benefit of anesthesia, analgesics or tranquilizers. Examples to be included in this category are: toxicity testing, radiation sickness, irritants, burns, trauma, biologic toxins, virulence challenge, prolonged: restrictions of food or water intake, cold exposure, physical restraint or drug addiction. All use of paralytic agents (curare-like drugs) must be included in this category. Describe any abnormal environmental conditions that may be imposed. Describe and justify the use of any physical restrain devices employed longer then 1 hour.

VI. Justifications for Category G Studies and Deviations from Standard Techniques

Describe in the box below any steps to be taken to monitor potential or overt pain and/or distress during the course of this study and how such pain or distress will be alleviated. Be as detailed as necessary to justify your procedure.

VII. Certifications

(By submitting this protocol, I am acknowledging that I comply with the certifications included in Section VII.) *(check one)*

X Animal Use for Research. I certify that the above statements are true and the protocol stands as the original or is essentially the same as found in the grant application or program/project. The IACUC will be notified of any changes in the proposed project, or personnel, relative to this application, prior to proceeding with any animal experimentation. I will not purchase animals nor proceed with animal experimentation until approval by the IACUC is granted.

Animal Use for Teaching/Demonstration. I certify that the information in this application is essentially the same as contained in the course outline and a copy of the laboratory exercises using animals is on file in the IACUC office. The IACUC will be notified of any changes in the proposed project, or personnel, relative to this application, prior to proceeding with any animal experimentation. I will not proceed with animal experimentation until approval by the IACUC is granted.

Estimate the cost of maintaining animals used in this protocol based on current per diem charge at University of Memphis.

Please specify cost per unit of time: $ 24/day (24c/cage/day)

Specify anticipated total costs for project duration: $ 1680

As supervisor of this project it is required that you inform your department chair concerning any animal per diem costs related to this project that are to be paid by the department.

By submitting this protocol, the Principal Investigator/Course Director indicates that the following have been considered:

1. Alternatives to use of animals.

2. Reduction of pain and stress in animals to the lowest level possible.

3. The proper needs of the animals with respect to housing and care.

4. The lowest number of animals used that will give the appropriate experimental results.

5. Use of the most primitive species that will give the appropriate experimental results.

6. Proper training of all personnel in the care and handling of the species used and in the procedures called for in this protocol before beginning the experiment/teaching or demonstration.

7. That this protocol is not an unnecessary repeat of results already in the literature or in the case of teaching/demonstrations, results that can be demonstrated using models or video material.

Principal Investigator/Course Director (Type Name) Marie van der Merwe

e-mail address mvndrmrw@memphis.edu

Date 7/21/14

Federal Law requires that members of the IACUC be given adequate time to read and review protocols including any changes or revisions in them.

The University of Memphis IACUC evaluates protocols on a continuous basis. Any protocols or modifications or renewals to any protocols to be considered at this time must be received by the Animal Care Facility no later than the end of the second week of the previous month.

Incomplete protocols will be returned to the principal investigator. We will not accept a FAXed protocol, renewal form or changes to a protocol.

E-mail the completed protocol to Dr. Guy Mittleman (Dept. Psychology):
mailto:<g.mittleman@mail.psyc.memphis.edu>?subject=RE: IACUC Application
January, 2008