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GLUCOCORTICOIDS INDUCE MOLECULAR AND PHENOTYPICAL CHANGES IN VISCERAL ADIPOSE TISSUE WHEN CONSUMING A HIGH FAT DIET

by

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Abstract

Visceral adipose tissue (VAT) physiology is negatively affected by chronic glucocorticoid (GC) usage, and is exacerbated by a "Western" diet. However, the impact of ω-3 supplementation into a "Western" diet, during chronic GC usage, remains unknown. Therefore, we determined the impact of both diets ("Western" vs. ω-3 supplemented) in conjunction with chronic GCs, on VAT physiology. Sixty-four male C57BL/6 mice (n=8-16/group) were subjected to 4-weeks of dietary intervention (high fat lard [HFL] vs. high fat fish oil [HFO], with or without prednisolone [40mg/kg/m^2] daily). We hypothesized that ω-3 supplementation would protect VAT physiology from chronic GC-induced negative effects. Overall, both HFO groups gained less body weight, displayed less VAT and smaller adipocytes, retained a greater percentage of M2-polarized macrophages, and exhibited beneficial alterations in gene expression as compared to both HFL groups. Our data indicate that VAT physiology is protected by an increase in dietary ω-3s, irrespective of GC usage.

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Chapter 1

Introduction

Background

Metabolic syndrome (MS) is a clustering of metabolic irregularities, and considered by some to develop directly from complications of obesity.[1,2] It is generally considered to have 3 etiologic categories: 1) obesity and disorders of adipose tissue, 2) insulin resistance, and 3) various independent molecular factors (ie; hepatic, immunologic, vascular).[2] However, as the development of MS is a complex process, other known contributing factors also include age, inflammatory status, and hormonal changes.[2] In spite of all the contributing factors that lead to MS, abdominal obesity and abnormal body fat distribution are central.[2]

There is sufficient evidence that obesity independently contributes to metabolic irregularities (ie; hypertension, hypercholesterolemia, hyperlipidemia, hyperglycemia) that drive the MS phenotype.[2] Recently, statistics from the Centers of Disease Control show that roughly 70% of adults age 20 years and over were classified as overweight (includes obesity) in 2013-2014.[3] Additionally, ~20% of adolescents age 12-19, ~17% of children age 6-11, and ~9% of children age 2-5 were classified as obese in 2013-2014.[3] Unfortunately, the underlying etiology of obesity results from various complex relationships between an individual's genetics, diet, and environment.

One such group that has an increased risk for developing MS and its associated long-term complications is the pediatric acute lymphoblastic leukemia (ALL) population.[4-6] ALL is a pediatric cancer of the bone marrow, and has a now ~90% event-free survival rate.[4,5] Throughout treatment these young patients receive an assortment of chemotherapeutics along with glucocorticoids (GC).[4,5] However, chronic exposure to GC is associated with various metabolic

irregularities, such as abdominal adiposity, hyperglycemia and insulin resistance (IR), and dyslipidemia among others.[7,8] These metabolic disturbances increase the risk of developing MS, diabetes, and cardiovascular disease for not only the pediatric ALL population, but also other chronic GC users.[5,9-14] More so, GC-induced side effects such as abdominal obesity are exacerbated by the consumption of a high fat "Western" type diet.[10,12,15,16] This project seeks to elucidate the relationship and response between visceral adipose tissue to chronic GC exposure and diet type, using C57BL/6 mice as a model. This particular strain of mice, is a well-known model for experiments mimicking human metabolic derangements that are observed in obesity.[17]

Adipose Tissue Biology

Adipose tissue is a multicellular, endocrine organ that influences the function of nearly all other organ systems, by producing a diverse set of signaling molecules.[18-20] It acts as a flexible caloric reservoir, responding to energetic and hormonal cues to either expand (fatty acid synthesis, lipogenesis) during energy excess or downsize (lipolysis, fatty acid oxidation) and release free fatty acids (FFA) during energy deficit.[18,21] This flexibility is protective, and prevents the excessive lipidation and resultant lipotoxicity of other cells.[18,21] Adipose tissue's ability to respond to changes in energy demand, reflect the individual adipocytes ability to adapt to nutrient intake.[18]

Adipose tissue endocrine function is the collective secretome of individual adipocytes and other resident cells, termed "adipokines".[18,22,23] Acting both locally and systemically, adipokines communicate whole body energy status, modulate energy intake and metabolism, immune processes, vasculogenesis, and matrix remodeling.[18,22,23] Two well-studied adipokines include leptin, which is best known for its satiety function, and adiponectin, known for inducing insulin sensitivity in metabolic tissues.[18] The secretome profile of adipose tissue however shifts

dynamically in response to energy status, serving as a window into its physiology.[18] This cohesive dialog links adipocyte biology and adipose tissue physiology to whole body homeostasis.[18]

Adipose tissue differs in regard to its anatomical location (subcutaneous adipose tissue [SAT], visceral adipose tissue [VAT], and intramuscular adipose tissue) and displays different structural organization, cellular type/size, and function.[23] SAT is classified as either superficial (sSAT) or deep (dSAT), and VAT is classified as omental, mesenteric, retroperitoneal, gonadal, or pericardial in humans.[23] While both SAT and VAT are important, attention is primarily on VAT as it is associated with metabolic dysfunction.[24]

Adipocytes, being the predominant cell type within adipose tissue, are key to maintaining energy homeostasis.[18] They store energy as triglycerides (TG) within their cellular lipid droplet without the development of lipotoxicity, and hydrolyze those TG into FFA for fatty acid (FA) oxidation.[18,21] The capacity of a mature adipocyte to carry out its normal functions (ie; lipolysis, lipogenesis, FA oxidation, FA synthesis) describes its metabolic status.[23] Interestingly, visceral adipocytes have a greater rate of lipolysis and FFA release than subcutaneous adipocytes, part of what makes subcutaneous adipocytes ideal for long-term energy storage.[23]

Adipose tissue can be further classified as brown adipose tissue (BAT) or white adipose tissue (WAT), which is based on the primary type of adipocyte within that tissue.[18,21,23] Adipocyte classification is based on a color spectrum of white, beige (or brite), and brown, due to their differences in mitochondrial density and cellular lipid content.[18,23] White adipocytes are the "classical" fat cells and make up the majority within WAT.[18] They are also positively associated with metabolic dysfunction, as visceral and subcutaneous regions of WAT expand during energy excess.[18,23] On the other hand brown adipocytes, which are central for

thermogenesis in mammals, make up the primary cell type within BAT.[18,23] Another distinct type of brown adipocyte is the beige adipocyte, which has a mixed phenotype and arises from WAT in response to cold or hormonal stimuli.[18] Of note, in human adults BAT is a much smaller percentage of total adipose tissue compared to WAT, but can be found in the thorax and abdominal region.[23]

Excess energy that is partitioned into adipocytes results in adipocyte hypertrophy and adipogenesis (hyperplasia), when the rate of lipogenesis is greater than lipolysis and FA oxidation.[18,19,25] Excessive expansion however can be healthy or unhealthy, as it puts stress on the adipocyte, as well as has chemical (ie; oxygen) and physical limitations (ie; extracellular matrix [ECM] remodeling).[18] As hypertrophy of the adipocyte occurs, the cell starts accumulating reactive oxygen species and toxic lipid species. [18,19,22] This leads to cellular stress, lipid spillover and insulin resistance. [18,21] If unresolved, the adipocyte will undergo apoptosis.[18]

Adipogenesis on the other hand, reflects the balance between pre-adipocyte proliferation/differentiation/apoptosis and turnover of mature adipocytes.[19] Stem cells which may undergo commitment to become pre-adipocytes, require the appropriate signaling and gene expression to differentiate into mature adipocytes.[26] Transcription factors required for this process include peroxisome proliferator gamma (PPARγ) and members of the CCAAT-enhancer-binding-proteins (C/EBP) family.[19,27] GC are one of the hormones required for pre-adipocyte differentiation, and induce key transcription factors required for the complete induction of a fully mature adipocyte.[16,28]

For healthy tissue expansion, that accommodates adipocyte hypertrophy and adipogenesis, the ECM must be modified and provide a flexible environment for growth.[18,21] For adipocytes and adipose tissue stroma, the ECM provides structural support and chemical signals

that help maintain their proper function (ie; direct mesenchymal stem cell lineage specification, proliferation, and differentiation).[18,21] Specific proteolytic enzymes that are key to modifying the ECM include fibrinolytic and matrix metalloproteinases.[21] Dysregulation of ECM synthesis and turnover leads to fibrosis within adipose tissue and its dysfunction, propagating metabolic dysfunction.[21]

Diet Composition and Obesity

A high fat diet (HFD) is defined as any diet that provides more than 30% of energy as fat. HFDs rich in saturated FAs and ω-6 polyunsaturated fatty acids (PUFAs), like the "Western" type diet, are consistently used to generate successful models of obesity in laboratory animals.[29,30,31] This has allowed the recreation of conditions associated with human obesity and MS, including weight gain, IR, hyperglycemia, dyslipidemia, hypertrophy of adipocytes, adipogenesis, altered adipokine levels, and altered local and systemic inflammatory markers.[29,30,31] Several studies have undertaken characterizing the effects of both a "Western" type diet and chronic GC usage in various tissues.[32,33] A limited number of studies have even examined the combined effects, albeit with varying percentages and types of dietary fat.[34-36] However, to date no studies have examined early onset changes in young animals, within the whole body or visceral adipose tissue, during both consumption of a "Western" type diet and chronic GC usage.

On the other hand, HFDs rich in ω-3's PUFAs (ie; α-linolenic acid [ALA], Docosahexaenoic Acid [DHA] and Eicosapentaenoic Acid [EPA]) have been identified as protective and do not induce the same metabolic effects as the "Western" type diet.[37,38] This is because they elicit various beneficial effects like: 1) reduce the amount of arachidonic acid (AA) in cell membranes, 2) increase FA oxidation, thereby reducing adiposity and immune cell

infiltration, 3) yield higher levels of adiponectin, with a resultant increase in insulin sensitivity, and 4) competitively displace AA from enzymatic action by cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome p-450 (CYP450) enzymes, thus reducing pro-inflammatory eicosanoid synthesis.[37,39,40] Furthermore, supplementation of ω-3's into a "Western" type diet raises the ω-3:ω-6 ratio, allowing for a more optimal balance.[38-42] This is described as beneficial for health, as ω-6 PUFAs (ie; linoleic acid and AA) promote pro-inflammatory processes.[38-40] Thus supplementation with ω-3 PUFAs should reduce the consequences of consuming a "Western" type diet, as well as help reduce the negative effects associated with chronic GC usage. The attenuation of negative effects elicited by GC, is in part due to the ability of ω-3's to increase FA oxidation and promote insulin sensitivity.

Adipose Tissue Lipid Metabolism: Esterification and Hydrolysis

Upon uptake of plasma FFA into the adipocyte, and a series of steps within the glycerophospholipid synthesis pathway, FFA get converted into phosphatidic acid.[18,22] From here, phosphatidic acid can be shunted into either the synthesis of various phospholipids or synthesis of TG, both represent separate arms of glycerolipid synthesis.[18,22] FFA that are esterified into TG (lipogenesis) get stored within the lipid droplet.[18,22] Lipogenesis primarily occurs in adipose tissue, but does also occur in the liver.[25] GC act synergistically with anabolic hormones, like insulin, to promote lipid storage and adipocyte hypertrophy.[16]

The cellular lipid droplet, once thought to be inert, is now known to play an active role in maintaining systemic energy balance.[18] In white adipocytes, the large generally unilocular lipid droplet occupies most of the cell, placing its borders near the endoplasmic reticulum (site for TG synthesis) and mitochondria (site for TG hydrolysis).[18] The lipid droplet membrane proteome, which includes structural proteins and metabolic enzymes specific to the adipocyte, is crucial for

metabolism.[18] During periods of low energy and/or hormonal stimulation, the adipocyte reduces its lipid stores through hydrolysis of TG.[18,22] This hydrolytic action is carried out in a stepwise process by various adipocyte specific lipases, that sequentially cleave one FA from the TG until three FAs and one glycerol are released.[22] Hydrolysis by hormone sensitive lipase (HSL) and possibly other non-HSL TG lipases are regulated by perilipin, a lipid droplet scaffolding protein that is enriched in white adipocytes.[18,22]

Adipose Tissue, Obesity, and Inflammation

Obesity, particularly visceral adiposity, creates a state of chronic low-grade sterile inflammation, which is considered central in the development of obesity-related metabolic dysfunction.[43,44] Inflammation is the body's protective response to infection (non-sterile) and cellular injury (sterile), that encompasses both the innate and adaptive arms of the immune system. However, it is intended only to resolve the insult and promote healing, not be sustained. The sterile inflammatory response begins with cellular recognition of injury, and release of chemical mediators from the site of injury (either from the injured cell, surrounding cells, or resident immune cells).[45] These released chemical mediators, which include cytokines, chemokines, and eicosanoids, recruit phagocytic cells to the site of injury.[45] These phagocytic cells function to contain the insult, as well as to recruit additional immune cells to assist in removing dead cells and initiate healing.[45] Adipose tissue hosts a wide variety of cells, that shift dynamically in response to stress and injury.

Adipose tissue generally consists of adipocytes, the stromal vascular fraction (SVF), and ECM.[19,20] Healthy adipose tissue SVF is composed of various cell types, which include: pre-adipocytes, fibroblasts, endothelial cells, erythrocytes, pericytes, stem cells, and quiescent immune cells (which produce interlukin-4 [IL-4], IL-13, IL-10, and IL-2).[19,20,43,46] However,

during obesity the microenvironment within adipose tissue changes resulting in: 1) increased adipocyte hypertrophy and stress, 2) increased reactive oxygen species and toxic lipids, 3) hypoxia, and 4) lipid spill over from adipocytes.[20] This leads to the activation and recruitment of immune cells, with a resultant production of pro-inflammatory mediators, e.g. tumor necrosis factor-α [TNF-α], IL-6, monocyte chemoattractant protein-1 [MCP-1], inducible nitric oxide synthase [iNOS], interferon-γ [INF-γ], and IL-1β, all of which affect adipocyte and adipose tissue function.[19,20,43,44,47] This immune milieu fluctuation reflects the delicate relationship between metabolism and immune system.[20]

Glucocorticoid Effects

Pharmaceutical GC have a widespread use within the pediatric and adult population both in the U.S. and abroad.[9,48,49] They are prescribed for a variety of pathologies due to their powerful immunosuppressive and anti-inflammatory effects.[4,5,9,49] Chronic usage of GC however, is associated with significant side effects due to their physiological requirement for life and ability to effect nearly all cells in the body.[1,9,48-52]

Acutely, GC alter various pathways that are important for both producing a stress response and initiating physiologic responses that follow a diurnal rhythm.[49,50] These different responses elicited by GC are dependent on many factors, including timing, duration, concentration, and location within the body. Basal GC levels are regulated by the master circadian clock in the superchiasmatic nucleus of the hypothalamus, creating a diurnal rhythm with peak concentrations in the morning and a gradual decline throughout the day.[49,50,52-55] In addition to basal levels, there is also daily variability resulting from pulsatile secretions in response to mental and physical status.[49,50,52,55]

Upon their release, GC shift metabolic processes towards catabolism and away from anabolism, in order to supply glucose to the body.[49-52,54,56] This occurs through multiple mechanisms including: gluconeogenesis and glycogenolysis, insulin resistance, and skeletal muscle and adipose tissue breakdown.[49-52,54,56] GC influence however, also spans the central nervous system, immune system, and cardiovascular system.[50,53,54] These affects however are transient and reactionary, with the intent of either overcoming a stressful stimuli or adjusting the body's activities towards the periodicity of day and night.[1,51,52,54,55]

The appropriate diurnal rhythmicity of GC release is imperative for maintaining homeostasis.[49,51,54,57] Disruption of this rhythmicity occurs during chronic exposure to GC, such as during chronic stress, Cushing's syndrome, shift work, or pharmacological treatment.[49,51,54,57] Phenotypic alterations induced by chronic exposure include abdominal obesity, hepatosteatosis, IR and hyperglycemia, myopathy, immunosuppression, and hypertension among others.[16,49,51,54,57] The development of abdominal obesity induced by GC represents lipodystrophy, seen as the preferential expansion of visceral adipose depots and reduction of subcutaneous depots.[16] This preference is attributed to a greater local concentration of GC, as well as a greater expression of the glucocorticoid receptor (GR) in visceral adipose depots.[16] Our hypothesis is that dietary supplementation of ω-3 PUFAs, in the form of fish oil, will rescue visceral adipose tissue function from the negative effects of chronic GC usage and a "Western" type diet. We expect that once GC are administered, metabolic dysfunction by way of specific parameters will follow and be exacerbated by diet type.

Study Aims

Aim 1: Determine phenotypical changes in visceral adipose tissue associated with diet and/or glucocorticoid treatment.

Aim 2: Determine transcriptional changes that are induced in visceral adipose tissue with glucocorticoid treatment when consuming a high fat lard diet, rich in ω-6 polyunsaturated fatty acids.

Chapter 2

Material and Methods

Animals and Experimental Setup

For this study, we used C57BL/6 male mice (n=64). Breeder pairs were obtained from Envigo Laboratories, Inc. (Indianapolis, IN), and were bred in the animal facility at the University of Memphis. Mice were housed in standard cages within a climate-controlled room (21˚C) on a 12:12-h light-dark cycle (lights turned on at 0800 hr.). Mice had access to food and water *ad libitum*. All housing and experimental procedures were in accordance with the 8th edition of the *Guide for the Care and Use of Laboratory Animals*, and approval by The University's Institutional Animal Care and Use Committee was obtained.

All mice were weaned at 3 weeks of age to a standard rodent chow (CH, n=16) [2018 Taklad Global 18% Protein] or a high fat lard diet (HFL, n=48) [D10011203] with 45% of the energy from fat (45% Kcal from lard-based fat, 41% carbohydrate, 20% sucrose, 9% cornstarch, 12% maltodextrin 10) with a ω-6:ω-3 ratio of ~13:1. All diets were purchased from Research Diets, Inc. (New Brunswick, NJ) in pelleted form. The composition for experimental diets are given in Table 1A and 1B. At 5-weeks of age, all mice were separated into individual cages and received 0.2-0.3g of sweet potatoes daily. After 1 week of entrainment, mice consuming the HFL diet were either maintained on this diet or switched to an isocaloric high fat diet with the majority of fat coming from Menhaden fish oil (HFO, n=24) [D05122102]. The HFO diet has a ω-6:ω-3 ratio of ~1:4. Mice weaned onto the CH diet remained on CH for the duration of the experiment as the control group. The HFL and HFO groups were further randomly divided with half receiving GC in the form of oral prednisolone (40mg/kg/m^2) daily, using sweet potatoes as

the vehicle (GC+). The control group received vehicle alone (GC-). Prednisolone (3mg/mL) [Rx#1144364] was purchased from People's Custom Pharmacy.

Glucocorticoid treatment lasted a total of 28 days (4 weeks). This treatment time and dose was based on the GC therapy given to pediatric patients undergoing treatment for ALL. Groups will be identified as follows: HFL GC+ (n=12), HFL GC- (n=12), HFO GC+ (n=12), HFO GC- (n=12), CH GC+ (n=8), CH GC- (n=8). At the end of GC therapy (mice 10-weeks of age) animals were euthanized by CO_2 inhalation. During the experimental time, animals were monitored for body weight, feeding habits and food consumption.

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

A

Ingredients	**HFL**		**HFO**	
	gm (%)	kcal (%)	gm (%)	kcal (%)
Protein	24	20	24	20
Carboyhydrate	41	35	41	35
Fat	24	45	24	45
Total		100		100
kcal/g	4.72		4.72	
Casein, 80 Mesh	200	800	200	800
L-Cystine	3	12	3	12
Sucrose	172.8	691	172.8	691
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	0.0355	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: tert-Butylhydroquinone

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

B

Ingredients (g)	**HFL**	**HFO**
Lard	177.5	0
Menhaden Oil, ARBP-F	0	177.5
Soybean Oil	25	25
Total	202.5	202.5
C14:0, Myristic	2.1	14.0
C16:1, Palmitoleic, n7	2.5	17.7
C18:0, Stearic	19.8	6.6
C18:2, Linoleic, n6	56.2	16.1
C18:3, Linolenic, n3	4.2	4.3
C18:4, Stearidonic, n3	0	6.0
C20:0, Arachidic	0.4	0.3
C20:4, Arachidonic, n6	0.5	0
C20:4, n3	0	3.1
C20:5, Eicosapentaenoic, n3	0	23.3
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
ω-6 (g)	57.0	17.9
ω-3 (g)	4.4	66.6
ω-6/ω-3 ratio	12.9:1 (~13:1)	1:3.7 (~1:4)

Glucose Tolerance Test

Two days prior to euthanasia (day 26 post GC treatment initiation) mice were subjected to a glucose tolerance test (GTT). Mice were fasted for 6 hours prior to baseline fasting blood glucose sampling via the tail vein. Glucose levels were determined by a handheld glucometer (Onetouch Ultra 2 Meter, Bayer Healthcare, Tarrytown, New York). Mice then received an intraperitoneal injection of glucose (2g/kg body weight) and blood was collected every 30 minutes for 90 minutes and glucose levels determined.

Tissue and Blood Collection

Immediately before euthanasia, blood was collected from the facial vein into EDTA-coated tubes for plasma isolation. After euthanasia, spleen and epididymal adipose tissue were harvested and immediately weighed. A portion of epididymal adipose tissue were snap frozen in liquid nitrogen for the use of transcript and protein analysis. Another portion of epididymal adipose tissue were fixed in 10% phosphate buffered formalin (Fischer Scientific Co. LLC) for histological analysis. A final portion of epididymal adipose tissue, approximately 0.5g, was placed into DMEM/High Glucose [4.0mM L-Glutamine, 4500 mg/L Glucose, sodium pyruvate] (HyClone, HyClone Laboratories, Inc., Logan, UT) for immune cell isolation. Whole spleens were harvested and placed into RPMI 1640 1x with L-glutamine solution (Corning, Mediatech, Inc., Manassas, VA) containing 2% FBS for cell isolation.

Histology and Adipocyte Size Determination

Fixed adipose tissue were imbedded in paraffin, sectioned to a thickness of 5μm and stained with Hematoxylin and Eosin (H&E). Representative areas were used to determine adipocyte size using an imager M2 microscope (Axiocam MRC, Zeiss, Oberkochen, Germany) and Axiovision r4.8.2 software. Images of adipose tissue slides were captured on 10x and 20x magnification. Adipocyte size was estimated using Axiovision r4.8.2 software by circling all whole adipocytes that were in complete view in the 20x magnification pictures taken. A minimum of 2 pictures per slide per mouse, and up to 3 pictures per slide per mouse, were used for obtaining adipocyte size.

Cytokine and Adipokine Analysis

Plasma and adipose tissue lysates were used for cytokine and adipokine measurements. Adipose tissue lysates were prepared from snap frozen tissue samples by rinsing samples in

1xPBS, cutting into 1-2mm pieces, homogenizing with Kinematica Polytron PT 10-35 (Brinkmann Instruments, Rexdale, Ont. Canada) in 1xPBS. Then an equal volume of Cell Lysis Buffer 2 (R&D Systems, Minneapolis, MN) was added and tissues were lysed at room temperature for 30 minutes with gentle agitation. Debris was removed by centrifugation.

Cytokine and adipokine assay was prepared according to the R&D Magnetic Luminex Bead Assay (R&D Systems, Minneapolis, MN) instructions and plated in duplicates, along with quality controls and standards. Samples were analyzed on the Luminex MAGPIX analyzer. Analytes included: TNF-α, IL-4, IL-10, IL-α, IL-1β, IL-6. Leptin. Data was then generated using the xPONENT program, build 4.2.1324.0, and exported to excel for further analysis.

Cell Isolation

Cells were isolated from whole spleen and epididymal adipose tissue. Spleens were homogenized in media (RPMI with 2% FBS) using a 40µm nylon sterile cell strainer (Fisherbrand, Fischer Scientific, CO LLC, Dallas, TX). Cells were pelleted and red blood cells lysed using Cell Lysis Buffer [155mM NH_4Cl + 12mM $NaHCO_3$ + 0.1mM EDTA]. After lysis, cells were washed with 1xPBS and 2%FBS, and then re-suspended in 3mL of 1xPBS with 2%FBS on ice for cell counting.

Adipose tissue was cut into small pieces in DMEM containing 2 mg/mL type II collagenase (Worthington, Lakewood, NJ), using ~2mLs per fat pad. Samples were incubated at 37˚C while shaking (240 RPM) for 40 minutes. Homogenate was then diluted with media and filtered through a 40µm nylon sterile cell strainer. Cells were then centrifuged at 500 RPM, 4˚C for 10 minutes and re-suspended in 500µL 1xPBS with 2% FBS on ice for cell counting.

For counting of cells, a 10µL aliquot of samples were mixed in a 1:1 ratio with 10µL Trypan Blue solution (Corning, Mediatech, Inc., Manassas, VA) and loaded onto a

hemocytometer (Marienfeld, Lauda-Königshofen, Germany) for counting. Cell counts per sample were then extrapolated by averaging 4 (5x5) squares per sample.

Flow Cytometry

Approximately 1-5x10^6 cells were used per sample for antibody staining. Cells were then incubated with fragment crystallizable block (Fc block) (FcX, Biolegend, San Diego, California) diluted in 1xPBS with 2% FBS [1:50], on a horizontal orbital plate shaker protected from light, and at room temperature for 10 minutes to decrease non-specific binding of antibodies. After blocking, cells were then incubated with specific antibodies at optimum concentrations (Table 2) in the presence of Live Dead Aqua (Life Technologies, Eugene, OR) for 30 minutes, on a plate shaker protected from light, and at room temperature. All samples were fixed using Fixation/Permeabilization solution (eBioscience, San Diego, California) overnight at 4˚C according to the manufacturer's instructions.

All samples were analyzed using a LSR II Flow Cytometer (BD Biosciences, San Jose, California) and BD FACSDIVA software (BD Biosciences, San Jose, CA). Data obtained was then further analyzed using FlowJo software (FlowJo LLC, Ashland, OR).

Table 2. Antibodies used for flow cytometry. Stain 1 for Lymphocytes and Myeloid cells, Stain 2 for Lymphocytes.

	Marker	Clone	Concentration	Conjugate	Source
Live/Dead	LDA		1:200	Pacific Orange	Life Technologies
Stain 1					
	CD11b	M1/70	1:100	FITC	BioLegend
	GR1	RB6-8C5	1:100	APC	BioLegend
	CD45R/B220	RA3-6B2	1:100	APC/Cy7	BioLegend
	F4/80	BM8	1:100	Pacific Blue	BioLegend
	CD11c	N418	1:100	PE/Cy7	BioLegend
	TCRβ-chain	H57-597	1:100	PE	BioLegend
Stain 2					
	CD3ε	145-2C11	1:100	PE/Cy7	BioLegend
	CD8a	GK1.5	1:100	APC	BioLegend
	CD4	53-6.7	1:100	APC/Cy7	BioLegend

Microarray

Epididymal fat pads from mice on the HFL GC+ (n=4) and HFL GC- (n=4) groups were used for microarray gene expression analyses. Adipose tissue aliquots of ~0.5mg were kept at -80˚C until ready for mRNA isolation. Total RNA was isolated using QIAzol Lysis Reagent (Qiagen, Hilden, Germany) and RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany). RNA integrity was determined by agarose gel electrophoresis, using Agilent RNA Nano Chips (Agilent Technologies, Waldbronn, Germany) and Agilent RNA 6000 Nano Reagents part 1 (Agilent Technologies, Waldbronn, Germany) on the Agilent Bioanalyzer (Agilent Technologies, Waldbronn, Germany). RNA quantity (OD-260) and purity (260/OD-280) were

determined using the NANO drop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

Eight mouse RNA samples were analyzed using Affymetrix MoGene 2.0 ST arrays, following the manufacturers protocol for GeneChip Whole Transcript PLUS Reagent kit, for expression arrays (Affymetrix, Santa Clara, CA). Microarrays were then run on the GeneChip Scanner 3000 7G system (Affymetrix, Santa Clara, CA), with .CEL files created by the onboard Affymetrix GeneChip Command Console Software (Affymetrix, Santa Clara, CA).

Data was exported into Partek Genomics Suite 6.6 software (Partek Incorporated, St. Louis, MO) for statistical analysis and gene cross reference in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Array data was normalized using robust multiarray average (RMA), and differentially expressed genes were identified as those with a p-value <0.05 and a fold change value $\geq \pm 1.5$ relative to controls (HFL GC-).

Data were also analyzed using DAVID Bioinformatics Resources 6.8 (National Institute of Allergy and Infectious Disease, National Institutes of Health) and MGI 6.08 (The Jackson Laboratory) for further pathway and gene ontology analysis. Identified Genes of interest, which met both statistical significance and fold change threshold, were then validated using quantitative reverse transcriptase-polymerase chain reaction (RT-PCR).

Reverse Transcriptase-Polymerase Chain Reaction

Specific genes of interest (Table 3) were validated by quantitative RT-PCR in HFL GC- (n=4), HFL GC+ (n=4), HFO GC- (n=4), HFO GC+ (n=4), CH GC- (n=3), and CH GC+ (n=3) groups. Total RNA was isolated from adipose tissue aliquots (~0.5mg) using QIAzol Lysis Reagent (Qiagen, Hilden, Germany) and RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany). RNA integrity was determined by agarose gel electrophoresis, using Agilent RNA

Nano Chips (Agilent Technologies, Waldbronn, Germany) and Agilent RNA 6000 Nano Reagents part 1 (Agilent Technologies, Waldbronn, Germany) on the Agilent Bioanalyzer (Agilent Technologies, Waldbronn, Germany). RNA quantity (OD-260) and purity (260/OD-280) were determined using the NANO drop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). cDNA was then synthesized from 1*u*g RNA using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) on the T100 Thermal Cycler (Bio-Rad, Hercules, CA).

Quantitative RT-PCR was performed using Bio-Rad CFX96 Touch real-time PCR system and PowerUp SYBR Green Master Mix (Life Technologies, Carlsbad, CA). Primers (Table 3) were purchased from Integrative DNA technologies (Coralville, IO). Murine HMBS served as the endogenous control.[58] Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method and normalized to HMBS on CFX Manager Software v3.1 (Bio-Rad, Hercules, CA).

Table 3. Primer designs for Mus musculus sequences

Gene	Sequence Length	Forward 5'→3'	Reverse 5'→3'
Orm1	779	CTGCTTCTTCTCCTGCTGAC	GACTGTCCCTCTATGCCAAA
Orm2	774	CTTTCTTGGTCTCCTTCTCCAG	AGAAGGCTGTCACACACG
Fads2	1508	CTCCCAAGATGCCGTAGAAAG	GCTCATCCCTATGTACTTCCAG
Isyna1	1850	CCAGACCACTGTGTGGTGAT	TGGAGCACCAAGGTGTTTGT
HMBS	1611	CGTGGGAACCAGCTCTCTGA	GAGGCGGGTGTTGAGGTTTC

Statistical Analysis

Statistical procedures used for analysis of data from: dietary intake, anthropometric measurements, adipocyte size, cytokine/adipokine assay, and immune cell populations were performed in GraphPad Prism version 6 and 7. Data are presented as means ± standard error of

the mean (SEM). Statistical significance between different experimental groups was determined using non-parametric Mann-Whitney test, due to small n values and unknown distribution of the data, and Welch's t-test. As well as repeated measures 2way ANOVA with Tukey's multiple comparisons test. P-values <0.05 were considered statistically significant.

Chapter 3

Results

A High Fat Lard Diet in Conjunction with Glucocorticoids Induce Metabolic Dysfunction Not Seen in a High Fat Fish Oil Diet

Mice exposed to the HFL diet, containing 45% kcal from lard (ω-6 PUFA), gained more weight overall during the intervention period than their CH and HFO fed counterparts (Figure 1a and 1b). Specifically, HFL GC- mice gained more weight in total than their isocaloric HFO fed counterparts (Figure 1a and 1b) despite no significant difference in food intake (Figure 2). On the other hand, HFO GC+ mice gained significantly more than their CH GC+ counterparts (Figure 1a), despite dietary differences in energy density [(HFO 45% fat, 4.72kcal/g) vs. (CH 6% fat, 3.2kcal/g)] (Figure 2).

GC administration caused a decrease in weight gain for groups consuming the HFL and CH diets only, albeit not significant (Figure 1a). This trend however was not seen with animals on the HFO diets, where the HFO GC+ group gained slightly more weight than their GC- counterparts. It is well documented that GC induce muscle atrophy in rodents.[7,8] This data therefore suggests that the ω-3 rich diet is protective against muscle atrophy induced by GC, however for this study we were unable to confirm this by way of body composition analysis.

Changes in weight between groups during the intervention period began at week 2, when mice were approximately 8 weeks old. Differences were maintained until sacrifice at week 4, when mice were approximately 10 weeks old. HFL groups had significantly greater increases in weight when compared to both CH and HFO counterparts, from week 2 onward (Figure 1b). At sacrifice, HFL fed mice weighed on average 6.38% more than HFO fed mice, and 11.91% more than CH fed mice.

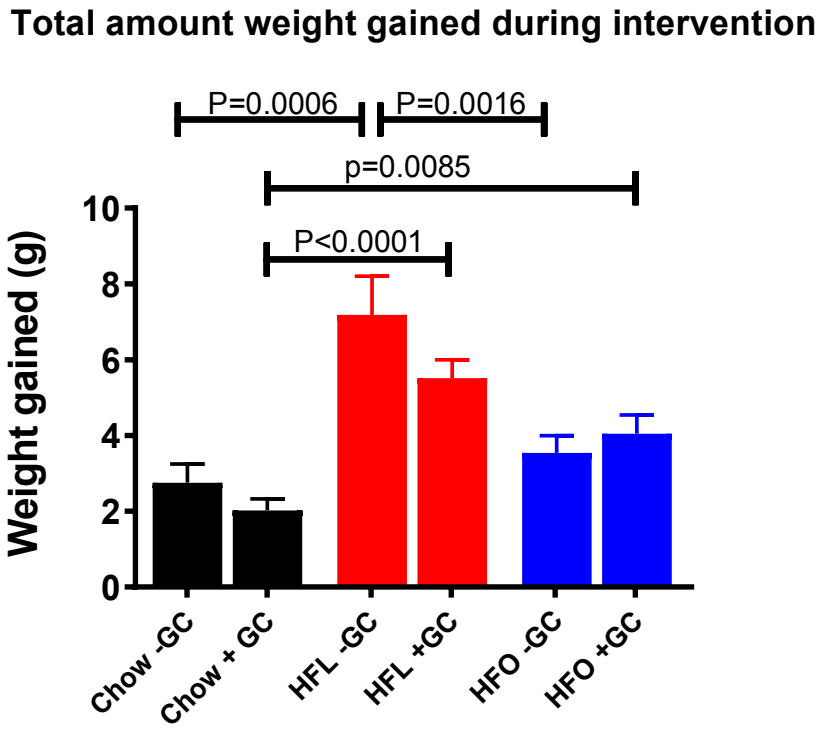

Figure 1a. Weight gained during the 4-week intervention. Groups: CH GC- and GC+ n=8, HFL GC- and GC+ n=10, HFO GC- and GC+ n=10. Total body weight was measured bi-weekly from the beginning to the end of intervention. Weight at the end of intervention was then subtracted from the weight at the beginning of intervention, to calculate the difference. Significant differences were observed between: CH GC- vs HFL GC-, CH GC+ vs HFL GC+, CH GC+ vs HFO GC+, and HFL GC- vs HFO GC- groups. Data analyzed using Mann-Whitney test. Results represent mean ± SEM.

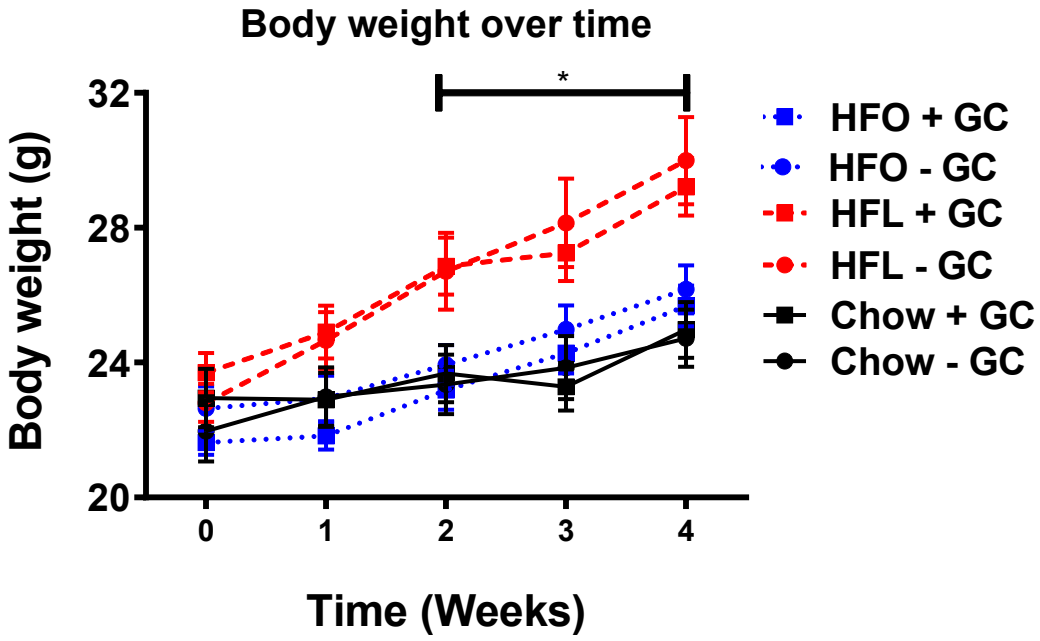

Figure 1b. Weight gained during the 4-week intervention. Groups: CH GC- and GC+ n=8, HFL GC- and GC+ n=10, and HFO GC- and GC+ n=10. Total body weight measured bi-weekly from beginning to end of intervention. Significance identified at: *week 2* [(CH GC- vs HFL GC+), (HFL GC- vs HFO GC+), (HFL GC+ vs HFO GC+)], *week 3* [(CH GC- vs HFL GC-), (CH GC- vs HFL GC+), (CH GC+ vs HFL GC-), (CH GC+ vs HFL GC+), (HFL GC- vs HFO GC+)], and *week 4* [(CH GC- vs HFL GC-), (CH GC- vs HFL GC+), (CH GC+ vs HFL GC-), (CH GC+ vs HFL GC+), (HFL GC- vs HFO GC-), (HFL GC- vs HFO GC+), (HFL GC+ vs HFO GC+)]. Data analyzed using repeated measures 2way ANOVA with Tukey's multiple comparisons test. Results represent mean ± SEM. *$p<0.05$

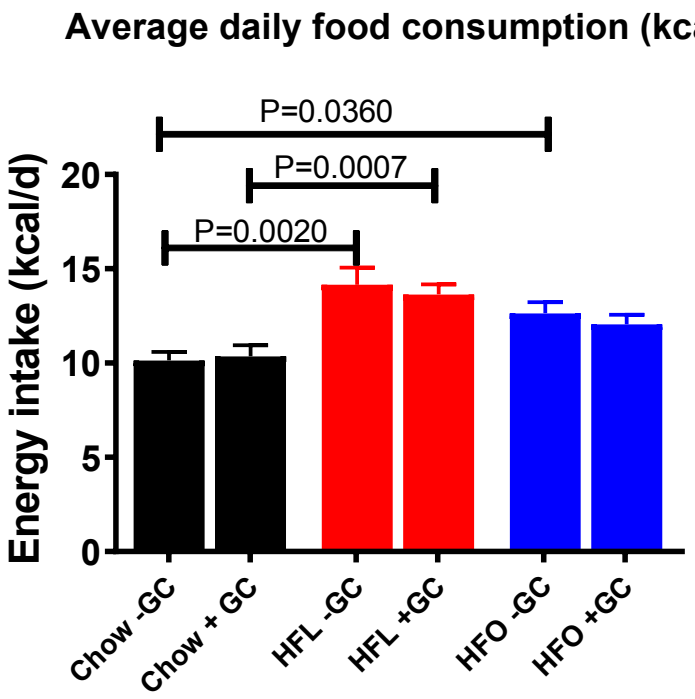

Figure 2. Average daily food consumption (kcal). Food consumption was measured twice weekly for all mice. Groups: CH GC- n=4, CH GC+ n=5, HFL GC- and GC+ n=10, HFO GC- and GC+ n=10. Average daily food consumption in kcals per mouse was obtained by multiplying the amount of food consumed in grams per day by the energy value for each diet [(4.72 kcal/g, HFL and HFO diets) and (3.2 kcal/g, CH diet)]. Both HFL groups took in significantly more kcals per day than their CH counterparts. Whereas only HFO GC- mice took in significantly more kcals per day than CH GC- mice. Data analyzed using Mann-Whitney test. Results represent mean ± SEM.

Consistent with the greater energy density of both HFL and HFO diets, compared to the standard rodent chow diet, both CH groups gained less weight (g) per kcal of food consumed (Figure 3). Interestingly, both HFO groups gained less weight (g) per kcal consumed than their isocaloric HFL counterparts (Figure 3). Specifically, HFL GC- mice gained significantly more weight (g) per kcal food consumed than HFO GC- mice (Figure 3). This suggests a greater energy efficiency within HFO groups, which is consistent with data showing fish oil based diets induce expression of transcripts involved in FA oxidation as compared to FA synthesis when consuming a high fat diet.[59,60]

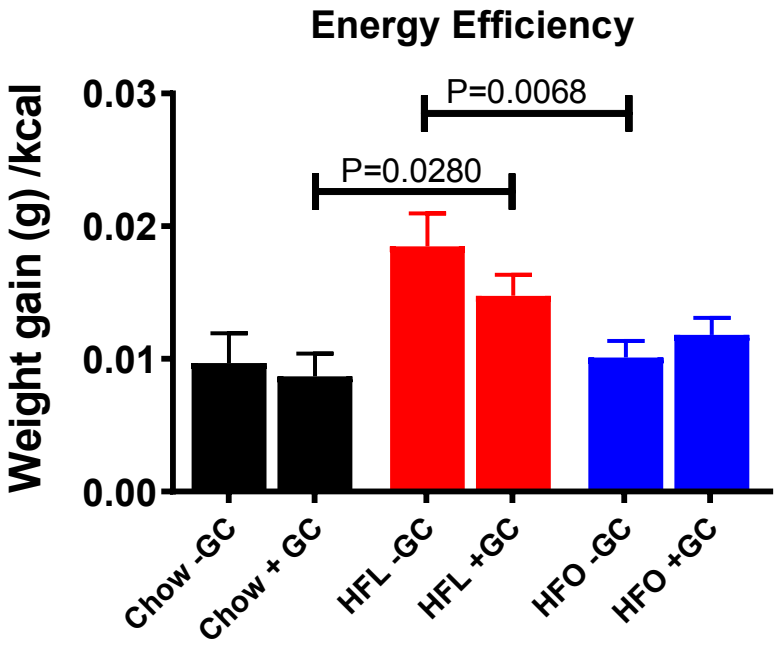

Figure 3. Energy efficiency: weight gained per kcal of food consumed. Groups: CH GC- n=4, CH GC+ n=5, HFL GC- and GC+ n=10, HFO GC- and GC+ n=10. Weight gained during 4 week intervention was divided by the amount of kcals consumed. Significant differences were observed between: CH GC+ vs HFL GC- and HFL GC- vs HFO GC- groups. Data analyzed using Mann-Whitney test. Results represent mean ± SEM.

Dysfunctional carbohydrate metabolism, which is demonstrated by insulin resistance and hyperglycemia, is a hallmark of diet-induced obesity and GC usage. At the end of the intervention period (week 10) and prior to sacrifice, fasting blood glucose levels were obtained from mice, and then a glucose tolerance test was performed. There was no statistical difference in fasting blood glucose between all groups (Figure 4). However, significant differences where observed in glucose clearance by both HFL and HFO groups, at time points 30 and 60 minutes compared to CH counterparts (Figure 4). In addition to the differences in glucose clearance, there was also a greater variability in blood glucose concentration within both HFL groups compared to all others (Figure 5).

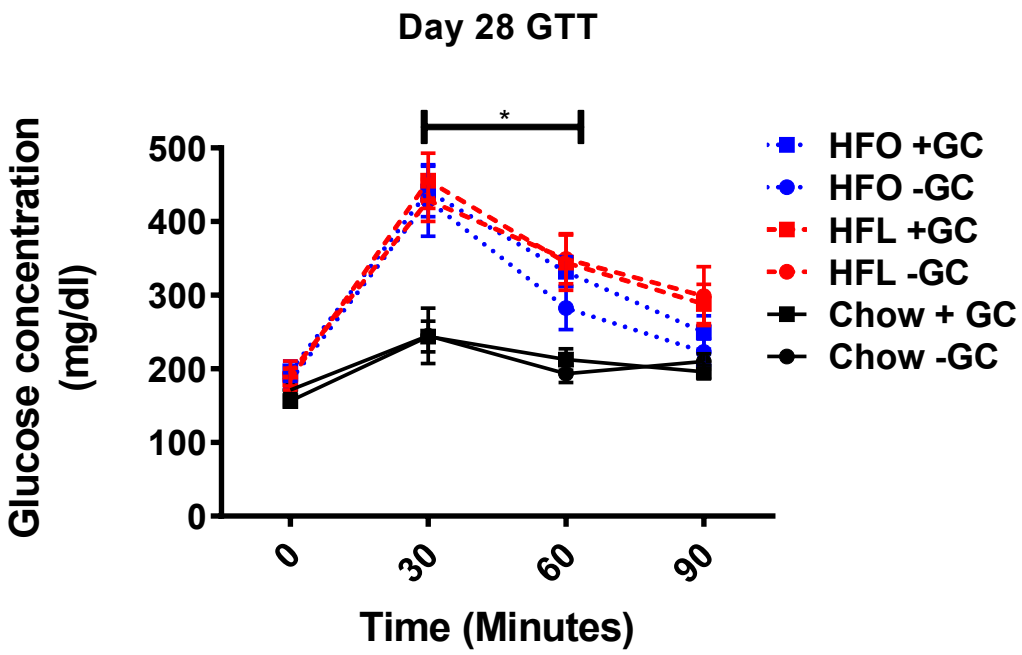

Figure 4. Glucose tolerance test over time. Blood glucose concentration was measured over a 90-minute period after IP injection with 2g glucose/kg body weight at week 10 (or day 28). Groups: CH GC- n=4, CH GC+ n=6, HFL GC- and GC+ n=10, HFO GC- n=6, HFO GC+ n=10. Both HFL and HFO mice showed significantly greater blood glucose concentrations in combination with slower glucose clearance, compared to CH mice at time points 30 and 60 mins. Data analyzed using repeated measures 2way ANOVA with Tukey's multiple comparisons test. Results represent mean ± SEM. *$p<0.05$

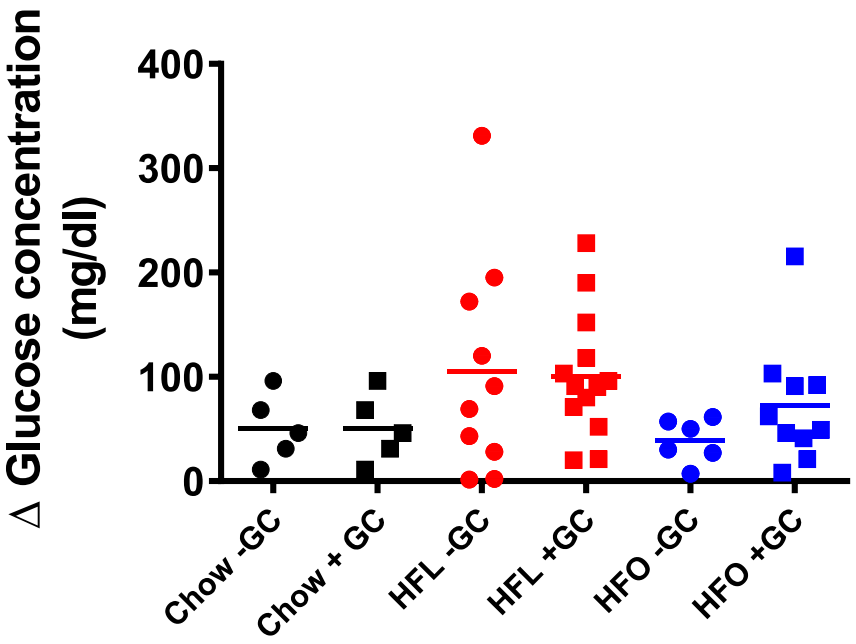

Figure 5. Difference in glucose concentration between fasting and 90-minute time points. The difference in blood glucose concentration, measured at week 10, between the initial fasting concentration and the 90-minute time point was calculated. Groups: CH GC- and GC+ n=5, HFL GC- n=10, HFL GC+ n=13, HFO GC- n=6, and HFO GC+ n=10. There was no statistical significance between groups, however both HFL groups show greater variability in blood glucose concentration compared to others. Data analyzed using Mann-Whitney test. Results represent mean ± SEM.

A High Fat Lard Diet in Conjunction with Glucocorticoids Induce Negative Visceral Adipose Tissue Alterations Not Seen in a High Fat Fish Oil Diet

At sacrifice the epididymal fat pads (left and right), which represent visceral adipose tissue, were harvested and weighed. Both HFL groups exhibited statistically greater amounts of visceral adipose tissue (g) compared to their CH and HFO counterparts (Figure 6). Specifically, HFL GC+ mice (mean=1.46g) had a 201.0% greater amount of visceral fat than CH GC+ (mean=0.49g) and a 69.6% greater amount than HFO GC+ (mean=0.86g) mice. Whereas, HFL GC- mice (mean=1.49g) had a 292.9% greater amount of visceral fat than CH GC- (mean=0.38g) and a 90.1% greater amount than HFO GC- (mean=0.79g) mice. On the contrary, HFO GC+ mice had a 77.4% greater amount of visceral fat than CH GC+ mice, and HFO GC-

had a 106.6% greater amount than CH GC- mice. Seeing as both HFL groups gained near identical amounts of visceral fat (Figure 6 and Figure 7), this further supports the idea that differences between HFL GC- and GC+ groups in overall weight gained (Figure 1a) are likely due to differences in skeletal muscle mass.

Additionally, the differences in adipose tissue mass (g) between groups was mirrored in percentage visceral fat of total body weight (Figure 7). These data suggest that the HFO diet protects against visceral adipose tissue accumulation, even in the presence of GC. This reduction in fat accumulation coincides with prior literature, which shows fish oil based diets increase the expression of transcripts involved in FA oxidation and decreases transcripts involved in FA synthesis.[59]

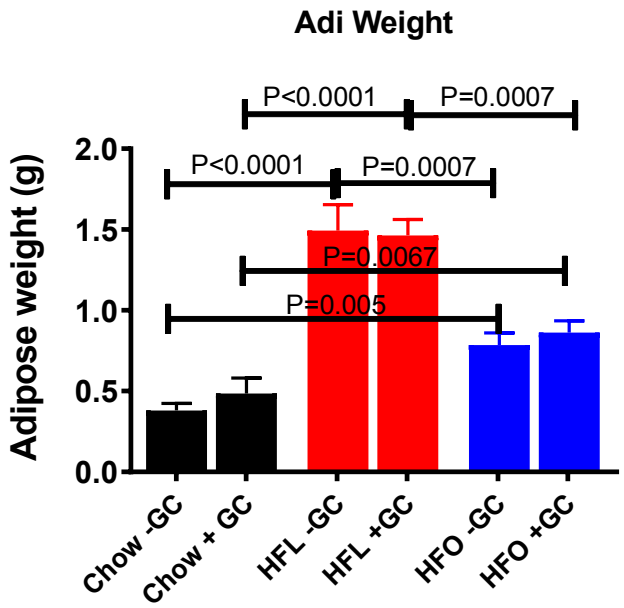

Figure 6. Visceral adipose tissue weight (g). Visceral adipose tissue, represented by the epididymal fat pad, was weighted at sacrifice. Groups: CH GC- and GC+ n=8, HFL GC- and GC+ n=10, HFO GC- and GC+ n=10. Adipose tissue (g) was significantly different between: HFL GC+ vs CH GC+, HFL GC- vs CH GC-, HFL GC+ vs HFO GC+, HFL GC- vs HFO GC-, CH GC+ vs HFO GC+, and CH GC- vs HFO GC- groups. Data analyzed using Mann-Whitney test. Results represent mean ± SEM.

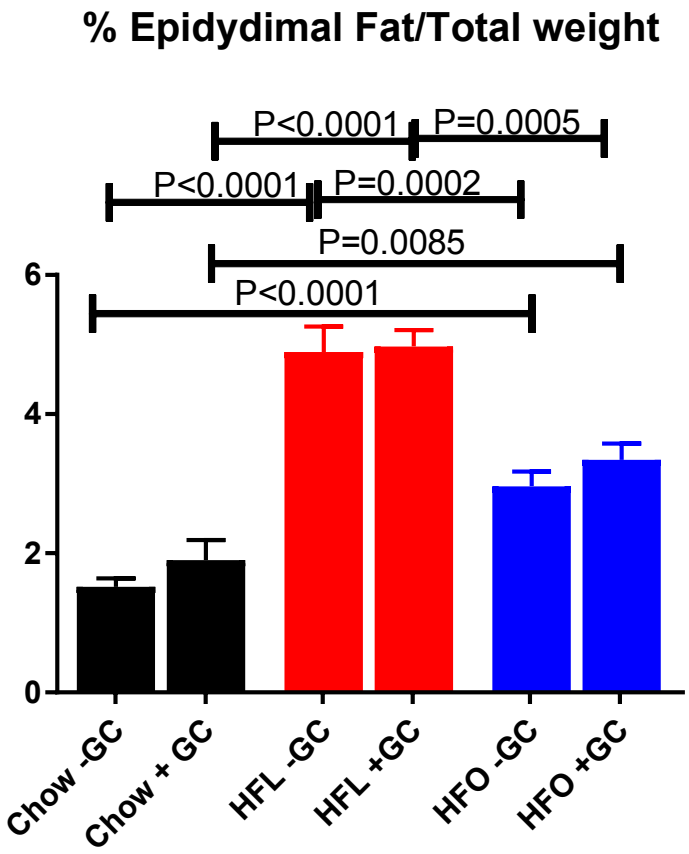

Figure 7. Percent visceral fat of total body weight. Visceral adipose tissue, represented by the epididymal fat pads, was weighted at sacrifice. The percent visceral fat of total body weight was calculated by, dividing the amount of visceral adipose tissue (g) by the total body weight (g) and moving the decimal place two spaces to the right. Groups: CH GC- and GC+ n=8, HFL GC- and CG+ n=10, HFO GC- and GC+ n=10. Percentage of visceral fat out of total body weight was significantly different between: HFL GC+ vs CH GC+, HFL GC- vs CH GC-, HFL GC+ vs HFO GC+, HFL GC- vs HFO GC-, CH GC+ vs HFO GC+, and CH GC- vs HFO GC- groups. Data analyzed using Mann-Whitney test. Results represent mean ± SEM.

In addition to the overall increase in visceral adipose tissue mass for mice fed the HFL diet, these mice also displayed significantly larger visceral adipocytes (mean=5220.5μm^2), as measured by area, compared to CH (mean=2406.5μm^2) and HFO (mean=3500.5μm^2) counterparts (Figures 8a and 8b). Adipocyte hypertrophy occurs during energy excess to an extent, and then leads to hyperplasia of adipose tissue.[18,19,25] Hyperplasia is a protective

mechanism, as hypertrophy of adipocytes cause cellular stress which can lead to insulin resistance and cell death if unresolved.[18,19,21,22] HFO fed mice exhibited smaller adipocytes compared to HFL counterparts (Figure 8a and 8b), with a difference in area of 1720μm^2, supporting the notion that fish oil based diets promote oxidative pathways, decreasing the burden of storage on cells.[59]

Additionally, larger differences in adipocyte size are observed between HFL GC+ and GC- groups compared to counterparts (Figure 8a and 8b), which suggests that GC may exacerbate adipocyte hypertrophy in the presence of a HFL diet. This greater difference in size within groups is not recapitulated in HFO or CH fed mice (Figure 8a and 8b), suggesting that HFO and CH diet types protect against adipocyte hypertrophy while in the presence of GC. As the only difference between HFO and HFL diet types is the primary source of fat (Table 1), this supports the idea of beneficial vs harmful lipid species regarding cell physiology.

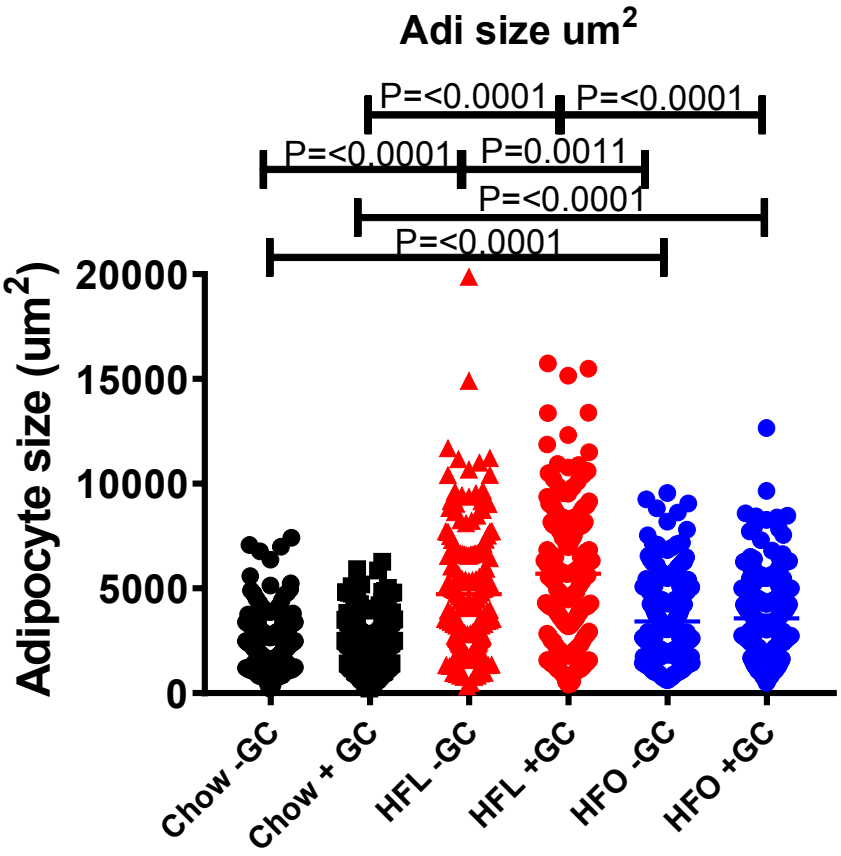

Figure 8a. Adipocyte size. Random images of visceral adipose tissue histology were selected for adipocyte size analysis. The area of all adipocytes within the plain of view per slide per mouse were determined and averaged for individual mouse values. Groups: CH GC- n=202, CH GC+ n=226, HFL GC- n=178, HFL GC+ n=182, HFO GC- n=221, HFO GC+ n=230. Significant differences are present between all experimental groups. Data analyzed using Mann-Whitney test. Results represent mean ± SEM.

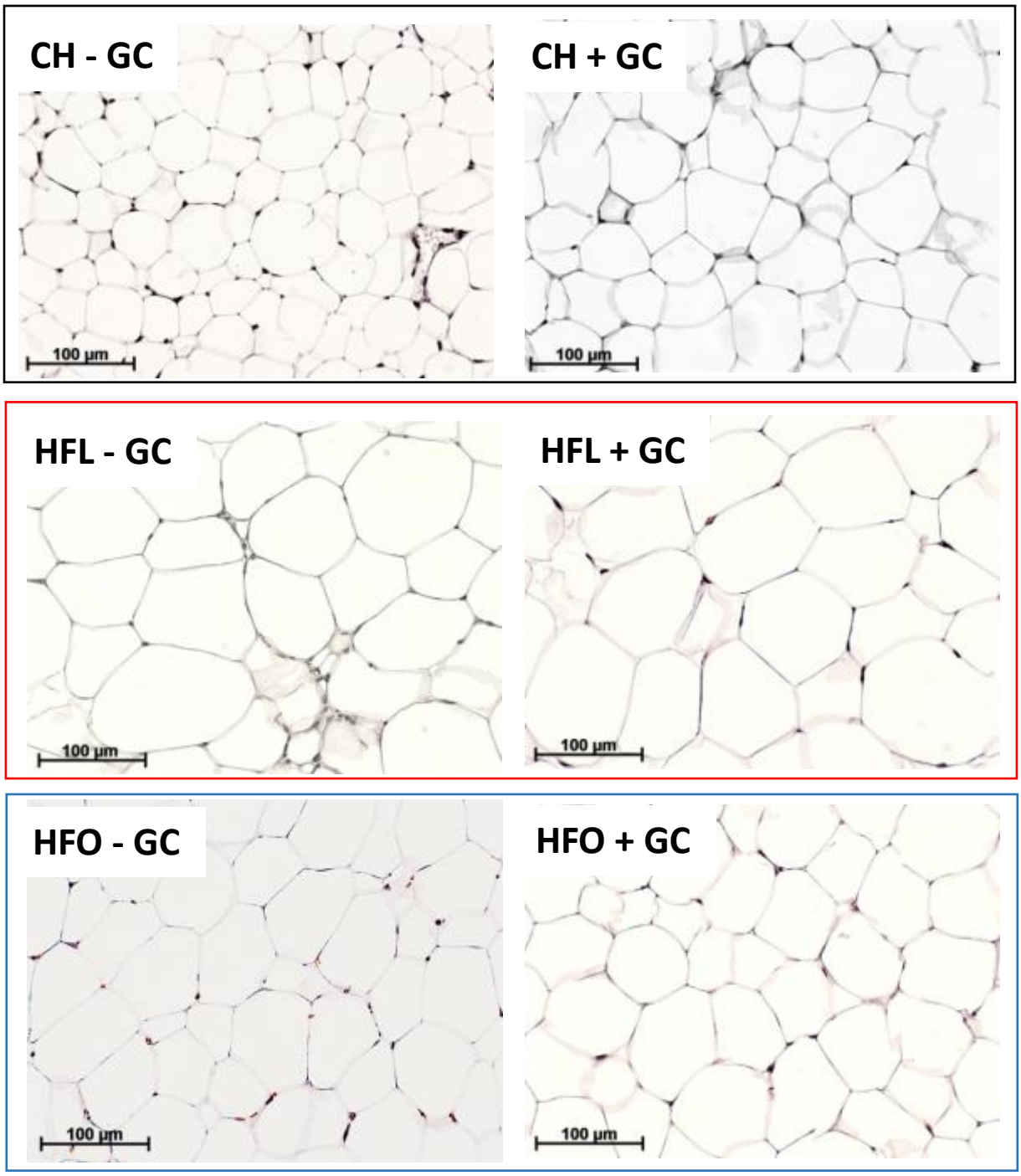

Figure 8b. Adipocyte area. Representative images of visceral adipose tissue demonstrate the hypertrophy that occurs with high fat diet consumption and GC usage. All pictures taken using 20X magnification.

To further characterize the differences in adipocytes by size, individual cells were plotted in a histogram for frequency and distribution analysis (Figure 9). Groups from largest to smallest mean adipocyte size are: HFL GC+ [mean= 5713μm^2], HFL GC- [mean= 4728μm^2], HFO GC+ [mean= 3572 μm^2], HFO GC- [mean= 3429μm^2], CH GC- [mean=2462μm^2], CH GC+ [mean=2351μm^2] (Figure 9). This view again describes a trend of adipocyte hypertrophy by both diet type and presence of GC. Coinciding with Figures 8a and 8b, larger differences in adipocyte size between HFL GC+ and GC- groups (δ=985μm^2) are observed (Figure 9), whereas smaller differences are seen between HFO GC+ and GC- (δ=143μm^2), and CH GC- and GC+ (δ=111μm^2].

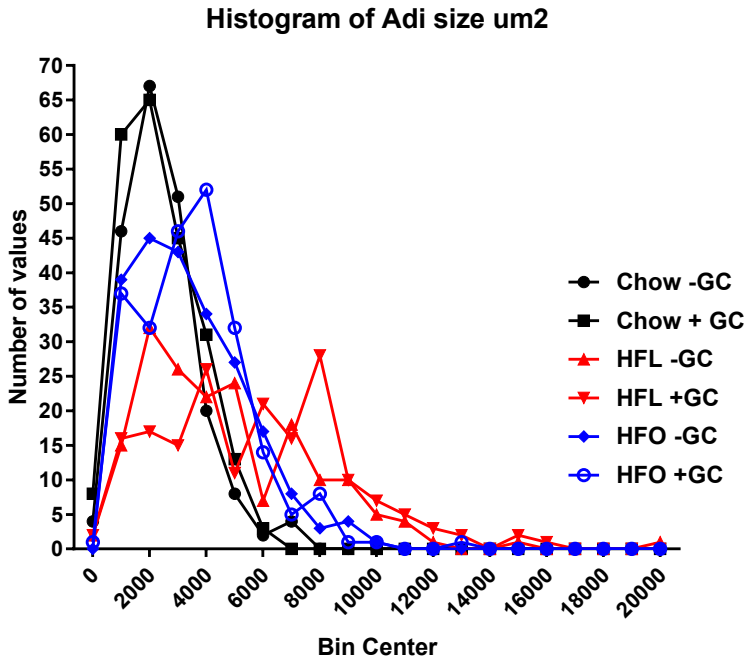

Figure 9. Distribution of adipocyte size. Individual adipocytes, chosen at random from adipose tissue histology, were plotted for frequency and distribution analysis. Groups: CH GC- n=202, CH GC+ n=226, HFL GC- n=178, HFL GC+ n=182, HFO GC- n=221, HFO GC+ n=230. Mean adipocyte size for HFL GC- (4728μm^2), HFL GC+ (5713μm^2), HFO GC- (3429μm^2), HFO GC+ (3572 μm^2), CH GC- (2462μm^2), and CH GC+ (2351μm^2) were obtained from descriptive statistics generated of plotted data.

Dietary Lipids Alter Select Cytokine and Adipokine Levels

The adipokine leptin, synthesized and secreted by adipose tissue, increases systemically during obesity to help regulate feeding behavior and act as a pro-inflammatory immune modulator.[61] In concordance with this, we identified significantly higher plasma leptin levels in HFL mice compared to HFO and CH counterparts (Figure 10). The observed increase in plasma leptin levels positively correlate to increases in adipose tissue mass (Figure 6), a well described trend in the literature. As leptin levels are decreased in HFO mice (compared to HFL mice), this suggests that fish oil based diets protect against hyperleptinemia, which likely results from an overall reduction in adipose tissue mass.

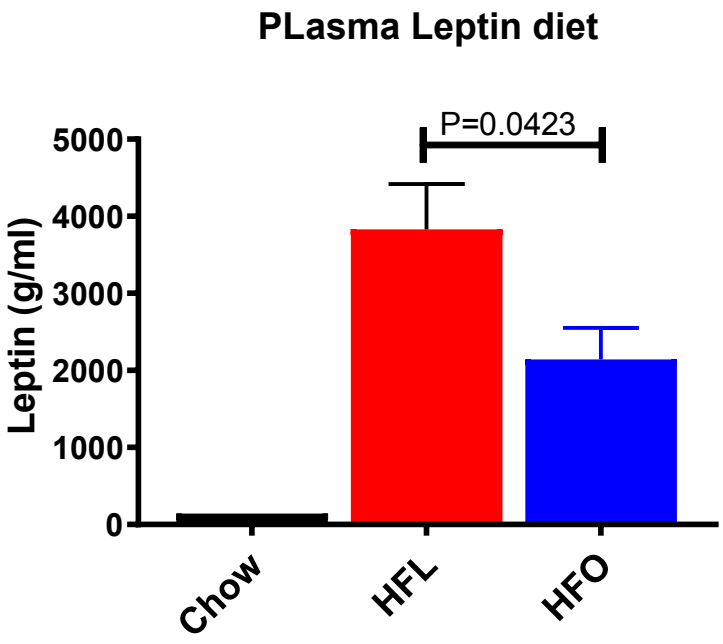

Figure 10. Plasma leptin levels. Plasma samples were obtained immediately prior to sacrifice at week 10. Groups: CH n=1, HFL n=13, HFO n=11. Leptin concentration was obtained using a multiplex magnetic bead assay. HFL mice showed increased circulating plasma leptin compared to their HFO counterparts. CH mice had little circulating plasma leptin. Only HFL vs HFO data was analyzed using Mann-Whitney 2-tailed test. Results represent mean ± SEM.

As obesity and consumption of a high fat diet are known to cause systemic and local inflammation, plasma and select tissue cytokine levels were measured. Pro- and anti-inflammatory cytokines assayed for include: TNF-α, IL-1β, IL-1α, IL-4, IL-10, and IL-6. Surprisingly, we only detected significant differences in IL-6, between HFO and HFL fed mice within adipose tissue (Figure 11). According to the literature, this observed increase in IL-6 within HFO fed mice would generally be interpreted as a sign of greater adipose tissue inflammation, which contradicts the anti-inflammatory nature of ω-3s. However, as IL-6 can function as both a pro- and anti-inflammatory cytokine, and recent data suggest its role in maintaining insulin sensitivity during a high fat diet challenge, we don't interpret this as a sign of inflammation.[62]

No other significant differences were observed for the additional cytokines (Data not shown). The absence of major differences in systemic and tissue cytokines within these mice, suggest that they have not yet reached a pro-inflammatory status. This may be a result of their young age (6 weeks-old to 10 weeks-old) during the intervention period, or the result of opposing anti-inflammatory actions by GC.

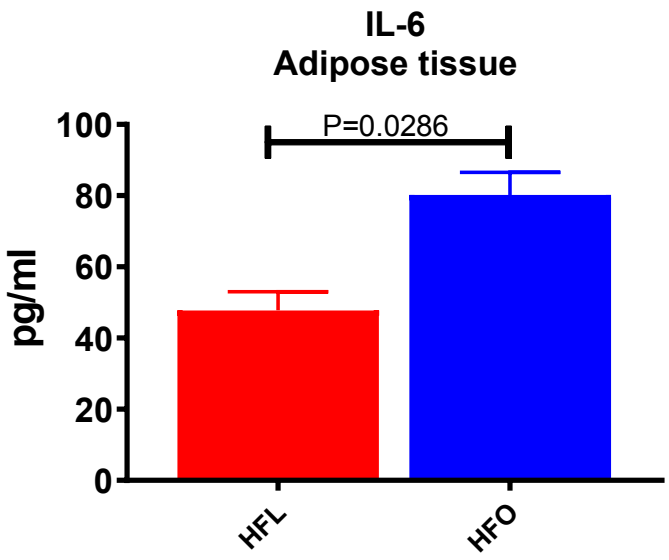

Figure 11. Visceral adipose tissue IL-6 levels. Adipose tissue IL-6 levels were determined from sample lysates collected at sacrifice (week 10). Groups: HFO n=4, HFL n=4. The concentration of IL-6 was obtained using a multiplex magnetic bead assay. HFO fed mice had significantly higher levels of adipose tissue IL-6 compared to HFL fed mice. Data analyzed using Mann-Whitney 2-tailed test. Results represent mean ± SEM.

Dietary Lipids Alter Visceral Adipose Tissue Immune Cell Populations

As the adipose tissue immune cell milieu is purported to change during a high fat dietary challenge, isolated immune cells were phenotyped (using stains from Table 2) in order to characterize resident cell populations.[20] We observed a greater infiltration of $LDA^{-}/B220^{-}/CD11b^{+}$ cells into adipose tissue of mice fed the high fat diet types (Figure 12, Top), which can represent macrophages, monocytes, or neutrophils.[63,64] Additionally, HFL fed mice had a significantly greater percentage of these cells compared to CH counterparts (Figure 12, Bottom). Slight, but not significant, differences were seen between HFO and HFL diet types (Figure 12, Bottom).

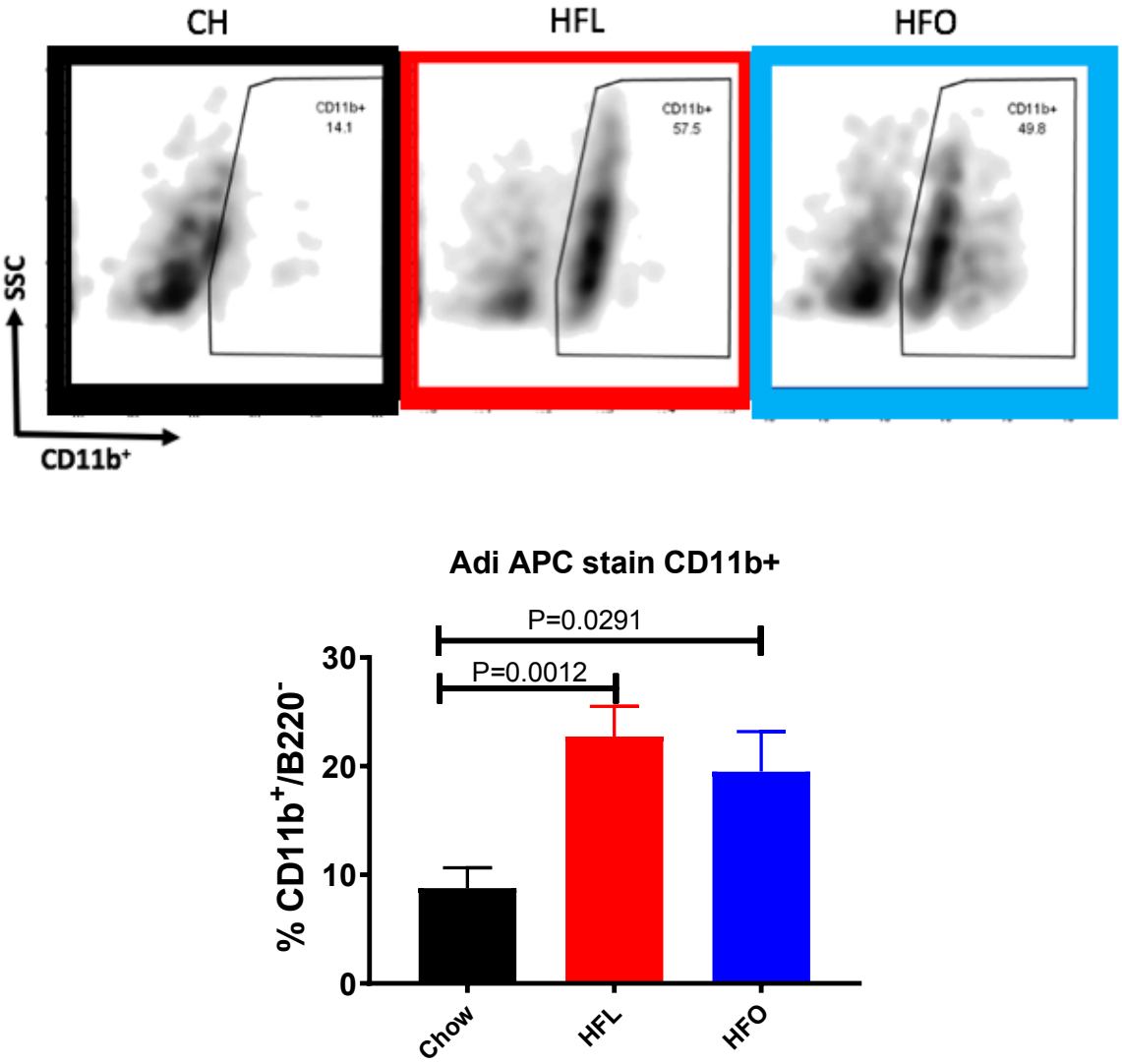

Figure 12. High fat diet increases visceral adipose tissue B220$^-$/CD11b$^+$ cell infiltration. Adipose tissue immune cells were harvested immediately after sacrifice. Cells were then stained for specific populations (Table 2). Top) FACS data analyzed in FlowJo, gated on LDA$^-$/B220$^-$/CD11b$^+$, represented on $\log_{10}$ scale. Both HFO and HFL mice exhibit an increased percentage of CD11b$^+$ cells in adipose tissue, as compared to CH fed mice. This immune cell population represents adipose tissue: macrophages, monocytes, neutrophils. Bottom) Groups: CH n=7, HFL n=7, HFO n=7. Data analyzed using Mann-Whitney 2-tailed test. Results represent mean ± SEM.

From these LDA$^-$/B220$^-$/CD11b$^+$ cells (Figure 12), further gaiting by F4/80$^+$ and CD11c$^+$ revealed two distinct and diet-specific macrophage populations (Figure 13, Top).[63,64] First, we observed a significantly greater percentage of F4/80$^+$ and CD11c$^-$ cells, representing alternatively

activated M2 macrophages, in HFO fed mice (Figure 13, Bottom Left). M2 macrophages are resident anti-inflammatory cells, that have been shown to undergo phenotypic switching to pro-inflammatory classically activated "M1" macrophages during obesity.[63,64] However, the greater presence of M2 macrophages in HFO mouse adipose tissue suggests a healthier environment, compared to HFL adipose tissue.

Second, we observed a significantly greater percentage of $F4/80^+$ and $CD11c^+$ cells, representing classically activated M1 macrophages, in HFL fed mice (Figure 13, Bottom Right). Accumulation of M1 polarized cells within adipose tissue, is pivotal to the development of adipose tissue inflammation and insulin resistance.[63,64] M1 macrophages have shown to infiltrate adipose tissue in response to cytokine and chemokine release, as well as FA spill-over, both of which occur in stressed adipocytes.

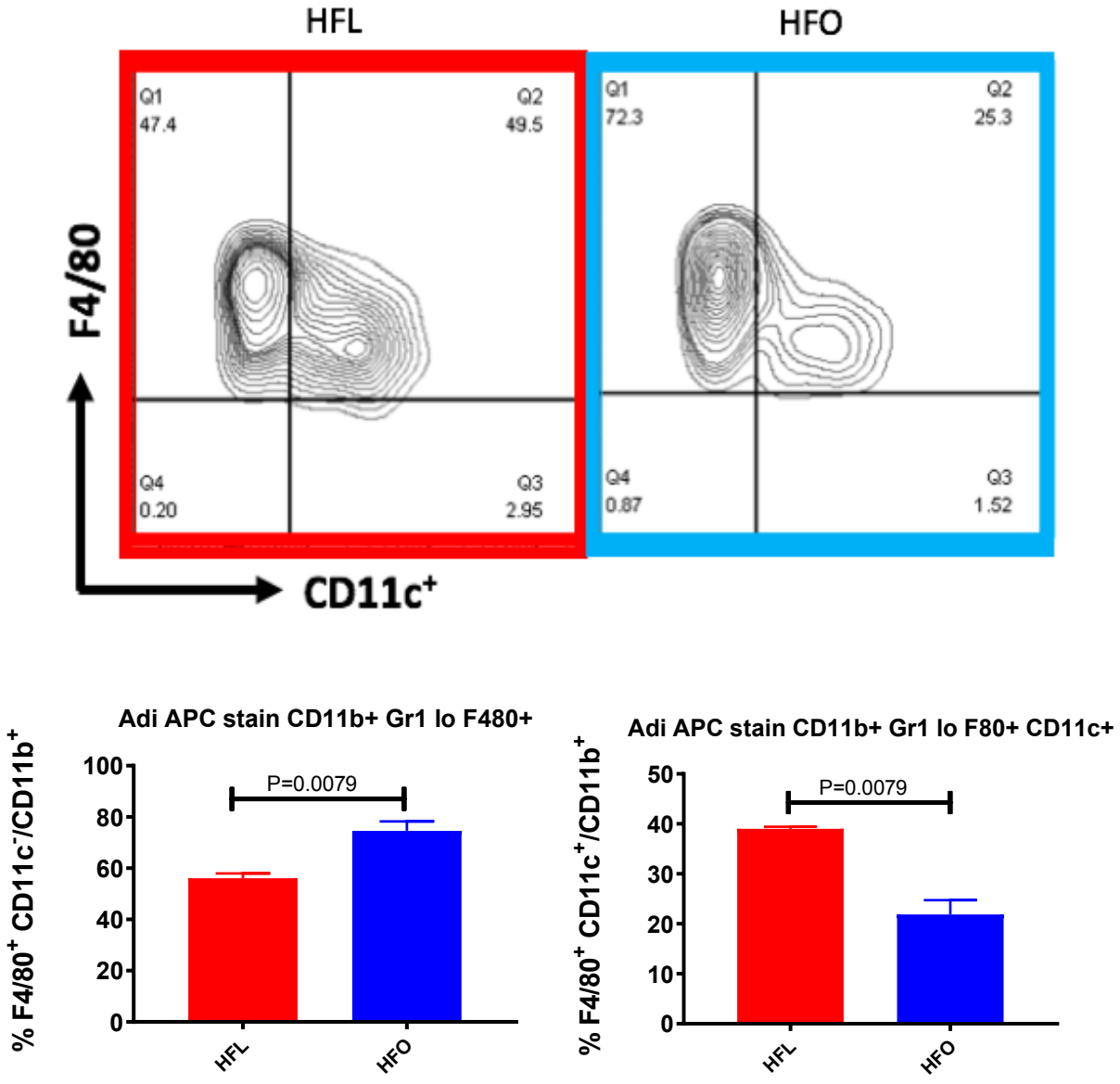

Figure 13. Diet specific macrophage populations. Adipose tissue immune cells were harvested immediately after sacrifice. Cells were then stained for specific populations (Table 2).
Top) FACS data analyzed in FlowJo, gated on $LDA^-/B220^-/CD11b^+/ GR1^{Lo}/F480^+/CD11c^+$, represented on log_{10} scale.
Bottom Left) Groups: HFL n=5, HFO n=5. HFO mice exhibited a greater percentage of $F4/80^+$ and $CD11c^-$ cells compared to HFL mice. This immune cell population represents adipose tissue alternatively activated "M2" macrophages.
Bottom Right) Groups: HFL n=5, HFO n=5. HFL fed mice exhibited a greater percentage of $F4/80^+$ and $CD11c^+$ cells compared to HFO fed mice. This immune cell population represents adipose tissue classical activated "M1" macrophages.
Data quantification represented in graphs analyzed using Mann-Whitney 2-tailed test. Results represent mean ± SEM.

Spleen lymphocyte populations are well known to decrease during GC usage, due to their cytolytic effects on immature thymocytes.[65] However, less is known about the impact of diet type. Thus, spleen cells where phenotyped (using stains from Table 2) based on diet. Cells were gated first by $LDA^-/CD3^+$, and then whether they were $CD4^+$ or $CD8^+$ (Figure 14, Top). No major differences were seen in the percentage of $CD4^+$ cells, however slight differences were observed in the amount of $CD8^+$ cells (Figure 14, Top). HFO fed mice had a 4% reduction in $CD8^+$ cells compared to HFL fed mice, and a 5% reduction compared to CH mice (Figure 14, Top). $CD8^+$ cells are a critical subpopulation of lymphocytes that include both cytotoxic T-cells, which mediate tumor and viral suppression, and a group of suppressor T-cells, which dampen certain immune responses.

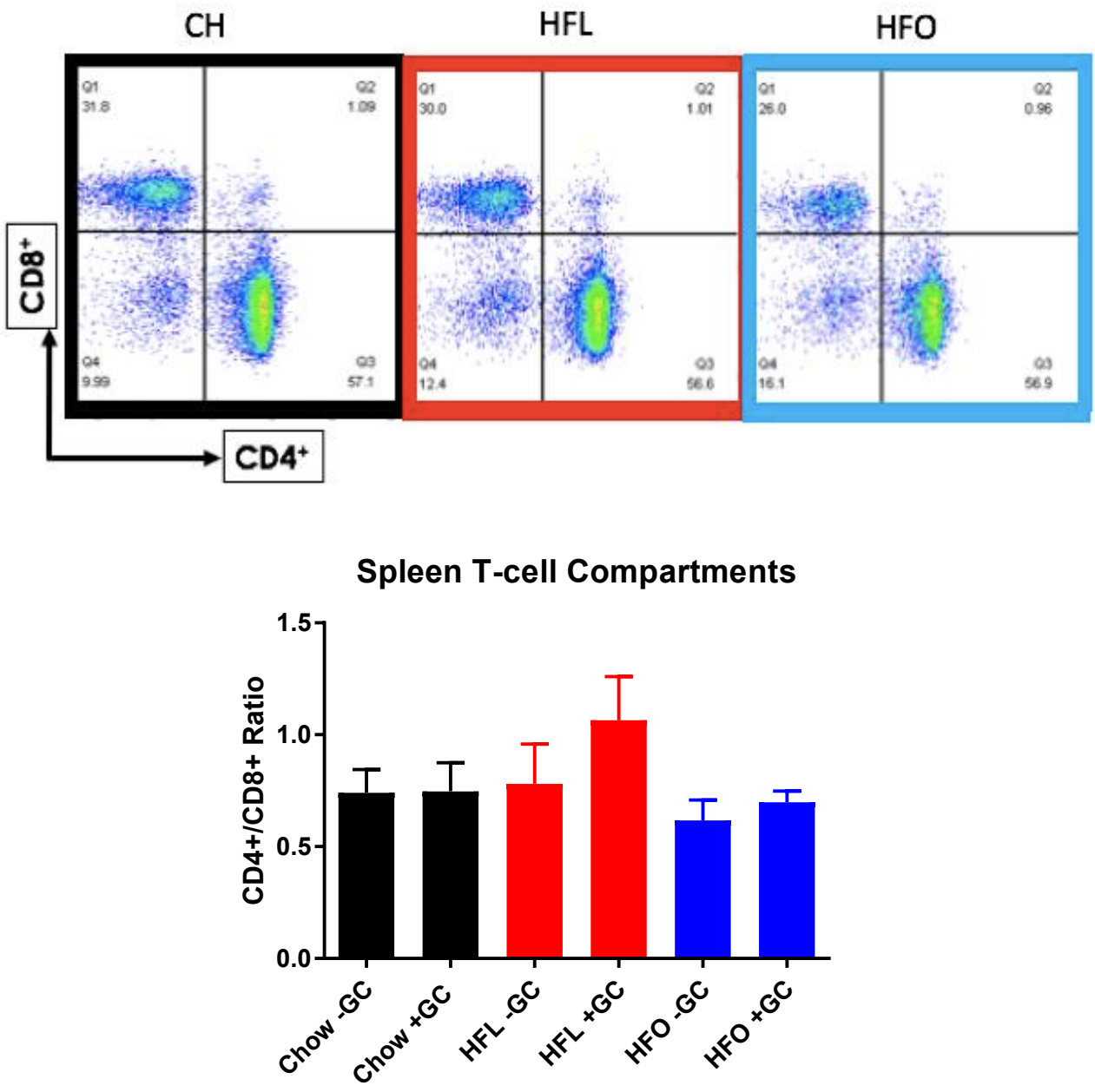

Figure 14. Diet Specific Spleen T-cell Populations. Spleen immune cells were harvested immediately after sacrifice. Cells were then stained for specific populations (Table 2). Top) FACS data analyzed in FlowJo, gated on $LDA^{-}/CD3^{+}/CD4^{+}$, represented on log_{10} scale. Bottom) Groups: CH GC- n=5, CH GC+ n=4, HFL GC- n=6, HFL GC+ n=9, HFO GC- n=6, HFO GC+ n=10 No significant differences were observed between groups. These immune cell populations represent spleen $CD4^{+}$ and $CD8^{+}$ cells. Data quantification represented in graphs analyzed using Mann-Whitney 2-tailed test. Results represent mean ± SEM.

A High Fat Lard Diet in Conjunction with Glucocorticoids Alters Gene Expression in Visceral Adipose Tissue

Microarray analysis of visceral adipose tissue gene expression was used to further examine GC action in driving observed phenotypes when consuming a HFL diet. To identify candidate genes, we used the Affymetrix GeneChip Mouse Gene 2.0 ST array, a whole-transcript array, to determine differences in transcriptome profiles between HFL GC+ (n=4) and HFL GC- (n=4) groups. Using Partek Genomics Suit 6.6 software, we identified a total of 3,112 visceral adipose tissue transcripts, with a significant p-value of <0.05 for GC+.

In order to identify the impact of GC during consumption of a HFL diet on regulation of gene expression, we next identified HFL GC+ induced genes using a false discovery rate (FDR) with a significance set at 0.05 and a stringency of $\pm$1.5-fold change. In doing so, we identified 250 regulated transcripts that were differentially expressed compared to the HFL GC- control. These genes were further divided into either down-regulated 52% (130) or up-regulated 48% (120).

To further classify the nature of GC responsive gene activation in HFL fed mice, we examined the functional gene ontologies of the GC-responsive genes (n=250) in visceral adipose tissue using DAVID Bioinformatics Resource 6.8. Table 4a and 4b summarize the main significant ontologies of up- and down-regulated genes induced by GC in visceral adipose tissue of HFL fed mice.

GC-induced up-regulated visceral adipose tissue gene expression related to transport of molecules, response to cAMP, and positive regulation of ERK1 and ERK2 cascade (Table 4a). Suggesting primary alterations within cellular signaling, as well as activities regarding the cell cycle and cellular differentiation. Whereas, down-regulated gene expression related to lipid and

fatty acid metabolism, cell adhesion, and positive regulation of gene expression (Table 4b), unsurprisingly indicating alterations in lipid synthesis and degradation.

Table 4. Gene ontology. Functional classification of 120 GC-upregulated genes (p=<0.05, fold change = >±1.5) using DAVID Bioinformatics Resource 6.8 for analysis and pathway enrichment in Biological Processes. Relevant processes listed in order of significance.
A

Term	GO Number	Gene count	% Enriched	P-value	Adjusted P-value
response to cAMP	GO:0051591	4	3.4	2.70E-03	8.10E-01
response to cold	GO:0009409	3	2.5	2.00E-02	1.00E+00
cellular response to hormone stimulus	GO:0032870	3	2.5	2.70E-02	1.00E+00
amyloid precursor protein catabolic process	GO:0042987	2	1.7	3.20E-02	9.90E-01
regulation of immune system process	GO:0002682	2	1.7	3.20E-02	9.90E-01
microtubule polymerization	GO:0046785	2	1.7	6.30E-02	9.90E-01
transport	GO:0006810	16	13.6	6.60E-02	9.90E-01
response to organic cyclic compound	GO:0014070	3	2.5	7.00E-02	9.90E-01
cellular response to fluid shear stress	GO:0071498	2	1.7	7.80E-02	9.90E-01
inactivation of MAPK activity	GO:0000188	2	1.7	7.80E-02	9.90E-01
positive regulation of ERK1 and ERK2 cascade	GO:0070374	4	3.4	8.20E-02	9.80E-01
wound healing	GO:0042060	3	2.5	9.20E-02	9.90E-01

Table 4. Gene ontology. Functional classification of 130 GC-downregulated genes (p=<0.05, fold change = >±1.5) using DAVID Bioinformatics Resource 6.8 for analysis and pathway enrichment in Biological Processes. Relevant processes listed in order of significance.
B

Term	GO Number	Gene count	% Enriched	P-value	Adjusted P-value
lipid metabolic process	GO:0006629	8	6.8	1.40E-02	1.00E+00
cell adhesion	GO:0007155	8	6.8	1.80E-02	1.00E+00
negative regulation of cell projection organization	GO:0031345	2	1.7	2.20E-02	1.00E+00
amino acid transport	GO:0006865	3	2.5	2.30E-02	9.90E-01
positive regulation of canonical Wnt signaling pathway	GO:0090263	3	2.5	5.30E-02	1.00E+00
fatty acid catabolic process	GO:0009062	2	1.7	5.40E-02	1.00E+00
fatty acid metabolic process	GO:0006631	4	3.4	5.60E-02	1.00E+00
mesodermal cell differentiation	GO:0048333	2	1.7	5.90E-02	9.90E-01
regulation of insulin receptor signaling pathway	GO:0046626	2	1.7	6.40E-02	9.90E-01
positive regulation of gene expression	GO:0010628	6	5.1	7.00E-02	9.90E-01
lipoprotein transport	GO:0042953	2	1.7	8.50E-02	9.90E-01
canonical Wnt signaling pathway	GO:0060070	3	2.5	8.70E-02	9.90E-01
long-chain fatty acid metabolic process	GO:0001676	2	1.7	9.50E-02	9.90E-01

To more stringently examine the impact of GC during consumption of a HFL diet on regulation of gene expression, we further identified GC-induced genes from the 250-gene pool using a stringency of ±2.0-fold change. In doing so, we identified 58 regulated transcripts that were differentially expressed compared to the HFL GC- control. These genes were additionally divided into either down- 65.5%(38) or up-regulated 34.5%(20) groupings.

To further classify the nature of these 58 GC responsive genes, we examined functional gene ontologies using MGI 6.08 informatics tool. GC-induced up-regulated visceral adipose tissue gene expression (20, 34.4%) related to *regulation of gene expression* [Id3, Cryab, Fos, Peg10], *apoptosis* [Id3, Cryab, Peg10, Fgf13], *cell cycle* [Id3, Oxtr, Cryab, Fgf13], *cell differentiation* [Peg10], *signaling and signal transduction* [Rgs7, Oxtr, Rgs1, Fos, Dock8], *cell stress* [Oxtr, Cryab, Duoxa1, Fos, HSPb7], *immunological processes* [Orm1, Rgs1, Duoxa1, Dock8, Orm2], *transport* [Orm1, Duoxa1, Gabrr2, Slc22a4, Orm2, Fgf13], *microtubule polymerization and depolarization* [Tppp, Cryab, Fgf13], *carbohydrate metabolism* [Irs3, Gfpt2, Lctl], *lipid metabolism* [Irs3, Slc22a4], and *protein metabolism* [Duoxa1, Gfpt2] (Table 5). Whereas, down-regulated visceral adipose tissue gene expression (38, 65.5%) related to *lipid metabolism* [Fads2, Isyna1, Saa1, Cyp39a1], *transport* [Slc16a2, Gabra4, Tbc1d8, Atp1b1, Kcnip3, Ano1], *oxidation-reduction processes* [Fads2, Cyp2c29, Tdo2, Cyp39a1], *apoptosis* [Ngfrap1, Perp, Gadd45g, Fgfr2, Kcnip3, Krt8], *cell adhesion* [Ncam1, Cnn1, Cdh3, Perp, Atp1b1, Ptprf], *signaling and signal transduction* [Ncam1, Cdh3, Tbc1d8, Fzd6, Fgfr2, Ptprf, Ano1], *cell stress* [Ncam1, Tat, Gadd45g, Fgfr2, Rcan1, Fkbp11, Ano1], *protein metabolism* [Tat, Perp, Tdo2, Xk, Mmp7, Cpxm2, Cfi], *regulation of gene expression* [Cdh3, Tc2n, Tceal9, Kcnip3], *carbohydrate metabolism* [Cdh3, Baiap2l1], *immunological processes* [Perp, Saa1, Gadd45g, Rcan1, Cfi, Apobec3, Krt8], *cell differentiation* [Cdh3, Gadd45g, Fzd6, Fgfr2, Krt8], *cell cycle* [Gadd45g, Xk, Fgfr2, Mmp7], *actin organization* [Baiap2l1, Pdlim3],and *ECM remodeling* [Mmp7, Cpxm2] (Table 5).

Table 5. Altered transcripts in visceral adipose tissue. Relative expression levels of top GC-regulated transcripts (n=58) in visceral adipose tissue from mice fed a HFL diet, using Partek Genomics Suite 6.6 software. All genes listed met significance criteria using a FDR with significance set at 0.05.

Gene Symbol	Fold Change	Gene Symbol	Fold Change	Gene Symbol	Fold Change
Igkv15-103	-5.62152	Fzd6	-2.25952	Id3	2.00794
Fads2	-4.1868	Baiap2l1	-2.24192	Rgs7	2.01133
Isyna1	-4.1407	Fgfr2	-2.23424	Oxtr	2.01967
Slc16a2	-3.47202	Mmp7	-2.21776	Orm1	2.0276
Ngfrap1	-3.31542	Rcn1	-2.18259	Rgs1	2.03062
Ncam1	-3.31107	Ptprf	-2.17483	Tppp	2.03974
Cnn1	-3.27052	Cpxm2	-2.15429	Irs3	2.06418
Cyp2c29	-3.08092	Cfi	-2.15025	Cryab	2.1818
Tat	-3.05592	Apobec3	-2.13661	Duoxa1	2.19882
Cdh3	-3.04589	Kcnip3	-2.09492	Gfpt2	2.21176
Gabra4	-2.98103	Krt8	-2.08657	Prr32	2.25922
Tbc1d8	-2.90514	Fkbp11	-2.07908	Fos	2.30652
Perp	-2.89987	Prr15	-2.06956	Dock8	2.36998
Atp1b1	-2.69608	Pdlim3	-2.06411	Gabrr2	2.39803
Tdo2	-2.55267	Ano1	-2.06336	Peg10	2.39946
Saa1	-2.50861	Fam134b	-2.06004	Slc22a4	2.44775
Gadd45g	-2.36481	Cyp39a1	-2.03985	Lctl	2.77248
Xk	-2.36175	Hyls1	-2.00243	Fgf13	2.77714
Tc2n	-2.30871			Hspb7	3.02118
Wbp5	-2.26173			Orm2	3.81752

The combination of a HFL diet, mimicking the "Western" type diet, and chronic GC treatment promoted the largest changes in adipose tissue accumulation, adipocyte hypertrophy, and early immune alterations. Thus, we were particularly interested in transcripts associated with lipid metabolism and immune processes.

Transcripts of interest that are involved in lipid metabolism include Fads2 and Isyna1, both of which were down-regulated in mice receiving GC. Fatty acid desaturase 2 (Fads2) is the rate limiting fatty acid desaturase (within a series of steps), in converting the essential PUFAs ALA [18:3n-3] and linoleic acid (LA) [18:2n-6] into their corresponding long-chain counterparts: EPA [20:5n-3], DHA [22:6n-3], and AA [20:4n-6] within the endoplasmic reticulum.[66,67] PUFAs are incorporated into the diacylglycerol-backbone of phospholipids within the plasma membrane and subcellular membranes.[67] Here they act as structural determinants, contributing to the hydrophobic scaffold of various integral membrane proteins, and function as docking sites of protein domains in cellular transport and cellular signaling.[67] Fads2 expression is shown to be negatively regulated by EPA and AA *in vitro*.[66] Alterations in Fads2 expression and activity perturbs cellular PUFA content, which impacts various processes like: membrane signaling and transport, eicosanoid production and signaling, and gene expression.[66] Knock-out rodent models for Fads2 show: resistance to obesity, altered lipogenesis, and male and female sterility.[67] More so, as PUFAs play numerous roles in adipocyte processes (ie; gene expression, adipokine secretion, macrophage recruitment, insulin signaling, and lipid droplet formation), perturbations in their cellular concentrations, via alterations in Fads2 activity, lead to alterations in adipocyte function.[66] Interestingly, prior research by Barber E, et al. (2013) show that ω-3 PUFAs (EPA, DHA, DPA) suppress lipid storage in adipocyte lipid droplets *in vitro*, partly by increasing lipolysis and decreasing expression of proteins related to lipid droplet formation.[68]

On the other hand, inositol-3-phosphate synthase 1 (Isyna1) is the rate limiting enzyme that catalyzes the first step in biosynthesis of all inositol containing compounds.[69,70] Isyna1 converts glucose-6-phosphate into *myo*-inositol 3-phosphate, which can be dephosphorylated to yield free *myo*-inositol.[69,70] *Myo*-inositol is a critical component of membrane phospholipids and precursor for the phosphoinositide signaling pathway, as it can be phosphorylated at multiple positions to yield various inositol phosphates and phosphoinositols.[69,70] *Myo*-inositol and its derivatives have an established role in insulin signaling.[71] Perturbation of their synthesis leads to disruption in insulin signaling, which effects not only carbohydrate metabolism but also lipid synthesis and degradation.[72] The anti-lipolytic effects of insulin are primarily attributed to inhibition of hormone sensitive lipase through activation of cAMP-specific phosphodiesterase's. More so, synthesized *m*yo-inositol can be converted downstream into phosphatidic acid, the critical precursor molecule for diacylglycerol and triacylglycerol synthesis.[70] However, the contribution of inositol to triacylglycerol synthesis is unknown.

Transcripts of interest that are involved in immune processes include Orm1 and Orm2, both of which were up-regulated in mice receiving GC. Acute phase proteins orosomucoid-1 (Orm1) and Orm2, are abundant within plasma and are generally produced by the liver. Their expression is induced by stressful conditions, like inflammation and obesity, where they act as immunomodulators.[73] Their immunomodulatory role consists of inhibiting mitogen-induced proliferation of lymphocytes and aggregation of platelets, as well as chemotaxis, superoxide generation, and neutrophil aggregation.[73] Interestingly, Orms fall under the lipocalin family of proteins, which possess a pocket for lipid binding.[73] As such, they have been shown to interact with several lipid molecules, including fatty acids.[73] Using a high fat diet-induced obesity model, Lee YS, et al. (2010) showed that both Orm1 and Orm2 proteins are significantly induced within

epididymal adipose tissue in response to both metabolic and inflammatory signals.[73] Acting to suppress inflammation and aid in maintaining energy homeostasis, suggesting their role in coordinating metabolic homeostasis within adipose tissue.[73]

Taken together, these various GC-regulated genes provide novel insights into early transcriptional changes occurring within visceral adipose tissue while consuming a HFL diet, which mimics the "Western" type diet. As well as shedding light on potential mechanisms, still poorly understood, that drive visceral adiposity and metabolic dysfunction. Furthermore, they yield targets for future investigation.

A High Fat Fish Oil Diet Differentially Alters Gene Expression in Visceral Adipose Tissue Compared to a High Fat Lard Diet

Comparative analysis of relative gene expression changes within visceral adipose tissue was measured using quantitative RT-PCR between: HFO GC+, HFO GC-, HFL GC+, HFL GC-, CH GC+, and CH GC- mice. This was done in part to validate selected genes from microarray analysis within HFL GC+ and GC- mice, as well as examine differences by diet type. Genes assessed include: FADS2, ISYNA1, ORM1, and ORM2, with HMBS serving as the endogenous control.

Microarray results (using genes ISYNA1, FADS2, ORM1, ORM2) were validated within HFL GC- and GC+ mice, normalized to the endogenous control HMBS (Figure 15). This revealed consistent GC-induced down-regulation of both ISYNA1 and FADS2 genes in the HFL diet type. As well as consistent, GC-induced up-regulation of both ORM1 and ORM2 genes.

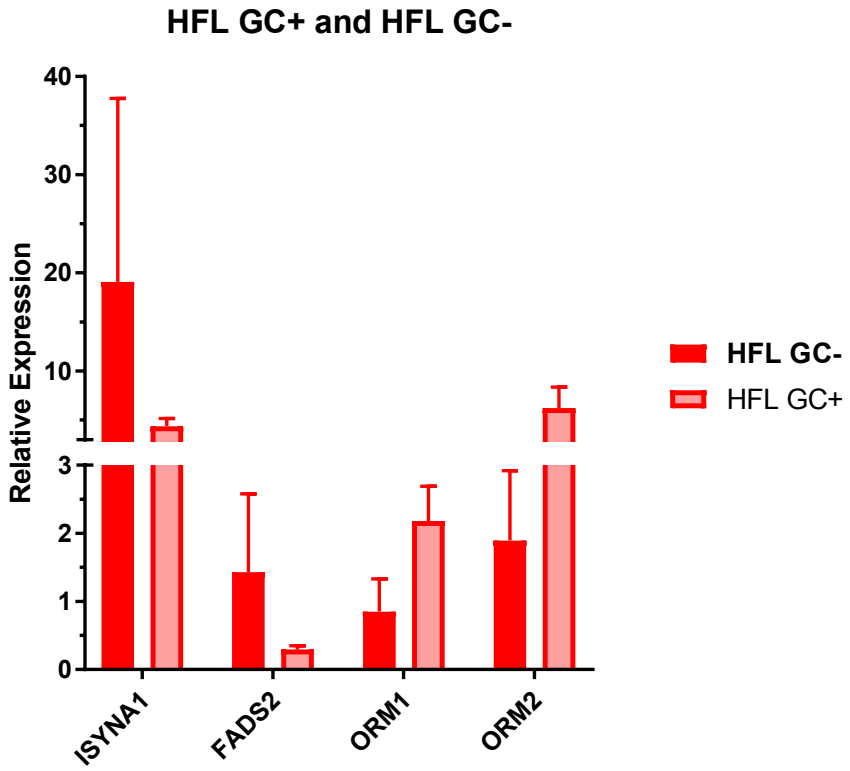

Figure 15. Validation of microarray results. Genes chosen for validation include: ISYNA1, FADS2, ORM1, and ORM2. Groups used include HFL GC+ (n=4) and HFL GC- (n=4). Data represents the relative expression of genes normalized to HMBS endogenous control gene. Data analyzed using Welch's test. Results represent mean ± SEM.

Next, we wanted to determine diet and GC-induced differences among our experimental groups, using the above stated genes from validation. We observed a decrease in FADS2 relative expression among all diet groups receiving GC (Figure 16). Minimal prior research has looked at FADS2 expression in conjunction with GC treatment, and of the few studies that have, all report GC-induced increases within subcutaneous depots.[74,75] Additionally, our data shows a diet specific trend in FADS2 expression, with a greater relative expression in both CH GC- and HFL GC- groups compared to the HFO GC- group (Figure 16). This agrees with prior research, which shows negative feedback regulation on FADS2 by its downstream metabolites (ie; EPA, AA).[66] As well as coincides with our HFL diet composition, which contains a greater amount of LA (in

comparison to its downstream product AA) compared to our HFO diet composition, which contains a greater amount of EPA and DHA (the downstream products of ALA).

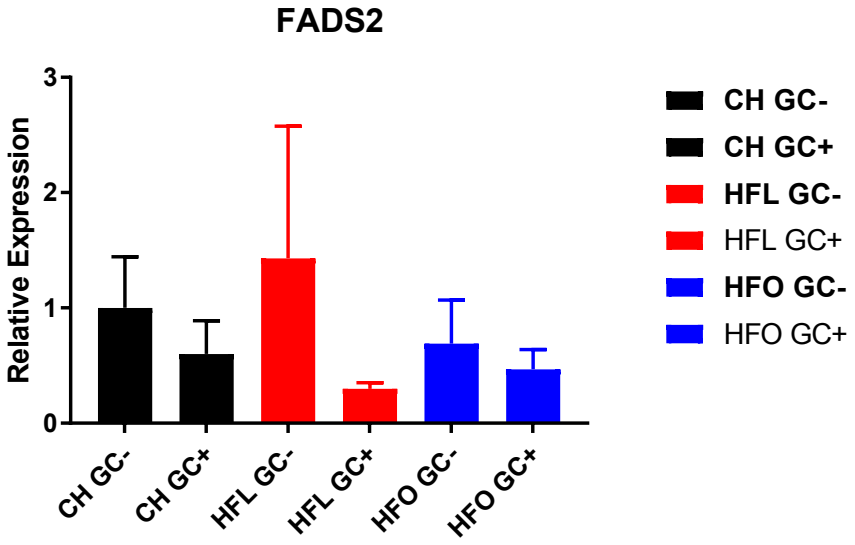

Figure 16. FADS2 expression among experimental groups. Groups used include: CH GC+ & GC- (n=3), HFL GC+ & GC- (n=4), and HFO GC+ & GC- (n=4). Data represents the relative expression of FADS2 normalized to HMBS endogenous control gene. Data analyzed using Welch's test. Results represent mean ± SEM.

We also observed a GC-induced down-regulation of ISYNA1 within only the HFL GC+ group compared to the HFL GC- group (Figure 17). More so, the HFL GC- group was the only group to exhibit a dramatic increase in ISYNA1 expression, as compared to CH GC- and HFO GC- groups (Figure 17). This suggests a HFL-diet specific increase in ISYNA1. Unfortunately, only one study to date has explored ISYNA1 expression in regard to adipocyte function, using 3T3-L1 cells.[76] Kim S, et al. (2007) showed that ISYNA1 expression is increased during early adipogenesis, 2 days following incubation with differentiation cocktail.[76] Additionally, no studies to date have reported ISYNA1 expression being regulated by GC.

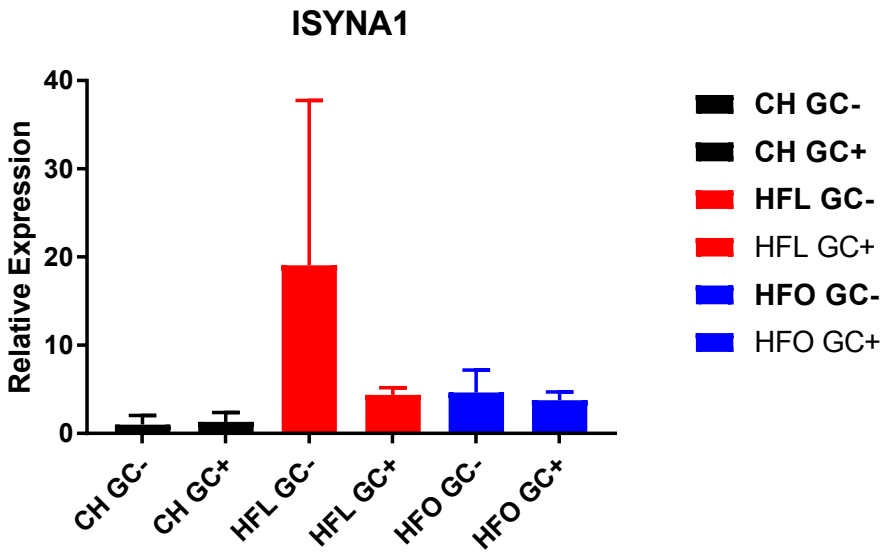

Figure 17. ISYNA1 expression among experimental groups. Groups used include: CH GC+ & GC- (n=3), HFL GC+ & GC- (n=4), and HFO GC+ & GC- (n=4). Data represents the relative expression of ISYNA1 normalized to HMBS endogenous control gene. Data analyzed using Welch's test. Results represent mean ± SEM.

Lastly, we observed a GC specific increase in ORM1 and ORM2 expression, however only within mice fed the HFL diet type (Figure 18). Suggesting a diet and GC specific alteration in early stress sensors of visceral adipose tissue. Interestingly, both ORM1 and ORM2 genes have been previously shown to contain GC responsive elements within their proximal promotor regions.[77] Furthermore, both HFO groups exhibited no relative increase in ORM1 and ORM2 expression compared to both CH mice and HFL GC- mice (Figure 18). Suggesting that a high fat diet alone does not immediately increase the expression of these acute phase proteins, but rather the combination of both a HFL diet and chronic GC treatment can induce their expression. Further supporting the role of ORM1 and ORM2 as early signals of cellular stress and inflammation.

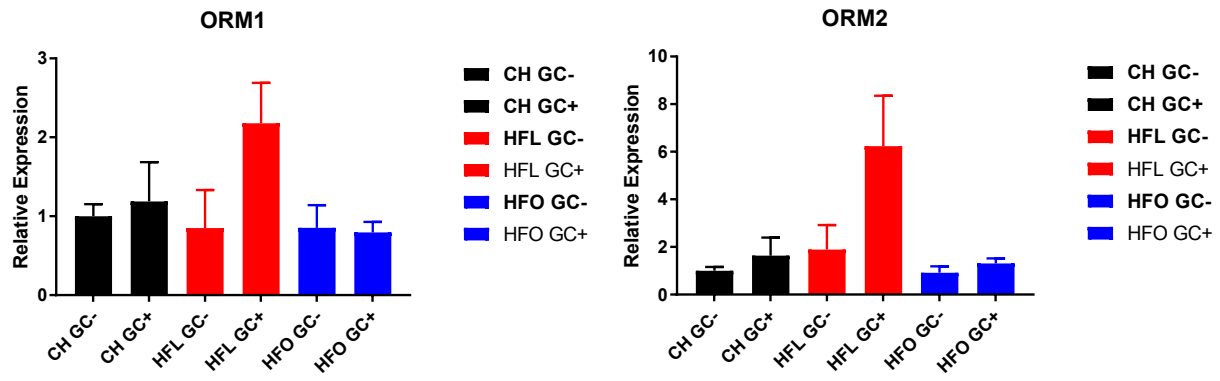

Figure 18. ORM1 and ORM2 expression among experimental groups. Groups used include: CH GC+ & GC- (n=3), HFL GC+ & GC- (n=4), and HFO GC+ & GC- (n=4). Data represents the relative expression of ORM1 and ORM2 normalized to HMBS endogenous control gene. Data analyzed using Welch's test. Results represent mean ± SEM.

Chapter 4

Discussion, Conclusions, and Recommendations

Discussion

With the rise in obesity over the past decades, researchers have focused on understanding the relationships between diet composition, metabolic dysfunction, and chronic illness. As such, we have developed a greater understanding of how dietary components (ie; types of fat and proteins, etc.) impact cellular and tissue physiology in various systems. Even further, we know now that metabolism and metabolic byproducts are intricately linked to immune function, behavior, reproduction, and gut health. Unfortunately, much of this prior research has solely focused on carbohydrates and proteins, leaving dietary lipids out of the picture. However, recent research has shown that lipids play critical functions throughout the body as bioactive signaling molecules, either directly or through enzymatic activity.

In the US, a typical "Western" diet consists of approximately 51% kcals from carbohydrates (primarily simple), 16% kcals from protein (primarily red meats), and 33% kcals from fat (primarily saturated and ω-6 fats).[78,79] High fat diets are considered any diet where $\geq$30% of kcals come from fat. Although the amount of fat is important, more recent emphasis is being placed on the source of fat (ie; saturated vs. unsaturated lipids). Saturated fats (ie; palmitate), which contain no double bonds in their hydrocarbon chain, are well known to cause cellular stress and metabolic dysfunction.[79] This is in part due to their structural nature, as they can pack tightly in cell membranes (modulating membrane fluidity and cell signaling), and act as ligands for toll like receptors on leukocytes. Limiting their percentage in the diet has shown great improvements in metabolic parameters, like insulin sensitivity and weight gain.

On the other hand, unsaturated fats which consist of monounsaturated (ie; ω-9) and polyunsaturated (ie; ω-3 and ω-6) fats, contain different amounts of double bonds at various locations in their hydrocarbon chain, appearing kinked in structure.[79] Their incorporation into the diet is generally associated with better health outcomes and metabolic function. Although as we will discuss, ω-6 fats especially in greater proportion to ω-3 fats, such as in the "Western" diet, can lead to significant metabolic dysfunction.[79] This optimal ratio between ω-6 and ω-3 fats, which has been at the forefront of recent research to improve health outcomes, can be achieved in one of two ways: 1) by altering the diet in such a way that there are less ω-6's and more ω-3's, or 2) by supplementing ω-3's directly into a diet high in ω-6's.

MS is a clustering of metabolic irregularities, considered by some to develop directly from complications of obesity and disorders of adipose tissue. MS increases the risk for developing chronic diseases, such as type 2 diabetes and cardiovascular disease, later in life.[1,2] One group shown to be at an increased risk for developing MS, is the pediatric acute lymphoblastic leukemia population.[4-6] This is due in part to their treatment regiment, as they receive an assortment of chemotherapeutics and GC over a 2-2.5 year period.[4,5] Chronic exposure to GC is associated with various metabolic irregularities, such as abdominal adiposity and insulin resistance, and has shown to be exacerbated by a high fat "Western" diet.[7,8,10,12,15,16]

No studies to date have examined the effects of dietary supplementation with ω-3 PUFAs into a high fat diet while receiving chronic GC treatment, in regard to measures of visceral adipose tissue in a younger animal model. Past research has focused on older rodent models that consume high fat diets (generally containing ~60% of kcals from fat, which is not physiologically relevant) and generally for much longer experimental durations, either with or without GC (acutely or chronically). Other studies have looked at the positive effects of ω-3

supplementation, either into a model using GC or using high fat diets singularly. One exception to this, is a study by Mark PJ et al. (2014) where they looked at the ability of ω-3 PUFAs, supplemented at birth into the diets of offspring from GC treated mothers, to rescue progeny from the reprogramming effects of GC.[80] They found that postnatal dietary supplementation with ω-3's limited the adverse fetal programing, brought on by GC treatment in mothers, on adipose tissue in offspring.[80]

Based on this information, we hypothesized that a high fat "Western" type diet would generate metabolic dysfunction, visceral adipose tissue expansion, and a pro-inflammatory milieu, such that it would drive the MS phenotype. We further hypothesized that these dysfunctions could be rescued by a fish oil based diet, rich in ω-3 PUFAs, possibly through its anti-inflammatory and lipid oxidative actions. This study focused on dissecting the relationship between specific dietary lipids and chronic GC treatment, in the context of metabolic function, immune parameters, and adipocyte morphological alterations, and gene expression in a young C57BL/6 murine model.

A High Fat Fish Oil Diet Reduces the Development of Metabolic Dysfunction Even in the Presence of Glucocorticoids Compared to a High Fat Lard Diet

Consistent with and extending previous research, our results show that a HFL diet (~13:1, ω-6:ω-3), which mimics the "Western" diet, is broadly deleterious. Six-week-old male mice in the HFL GC- group gained more weight in total over the 4-week intervention period than their isocaloric ω-3 rich HFO GC- (~1:4, ω-6:ω-3) counterparts. The observed differences in weight gain, occurred despite near equivalent food intake. Our observations coincide with the literature, which shows that ω-3 PUFAs stimulate fatty acid oxidation in part through activation of peroxisome proliferator-activated receptors (PPARs).[79,80]

Interestingly, HFL GC+ mice gained less weight overall, despite identical food consumption, than their HFL GC- counterparts. We attributed this to a loss of lean muscle mass, as muscle atrophy is a well-known consequence of GC usage. On the contrary, HFO GC+ mice did not exhibit a decrease in total weight gain compared to their HFO GC- counterparts, which suggests that the HFO diet type is protective against GC-induced muscle loss. Prior research into the effects of ω-3's on skeletal muscle health in both human and rodent models, show that they have intrinsic anabolic/anti-catabolic properties in skeletal muscle.[81] However, only one study to date has looked directly at the effects of ω-3 supplementation on GC-induced muscle atrophy. Fappi A, et al. (2014) supplemented 10-12 week-old male *Wistar* rats, fed a standard rodent diet, with a commercial ω-3 supplement for 40 days.[82] At day 30, rats started receiving subcutaneous dexmethasone for a total of 10 days.[82] Our preliminary data however, seem to contradict the findings of Fappi A, et al. (2014), who report that ω-3 supplementation does not protect against GC-induced muscle atrophy in gastrocnemius and tibialis anterior (type 2B fast twitch glycolytic muscle fibers).[82] Discrepancies between findings may be attributed to differences in diet as they used only a standard rodent chow, differences in rodent metabolism of GC, or the differences in GC potency as they used dexamethasone.

These significant differences in weight gained during the intervention period, began at week 2 when mice were approximately 8 weeks old and were maintained until sacrifice, when mice were approximately 10 weeks old. In total, HFL fed mice weighed on average 6.38% more than HFO fed mice. Specifically, HFL GC- mice weighed on average 8.2% more than HFO GC- mice, and HFL GC+ mice weighed on average 4.5% more than HFO GC+ mice. However, despite both diets being isocaloric and near identical food consumption for all groups, both HFL

groups gained more weight in grams per kcal of food consumed than their HFO counterparts. This suggests a greater energy efficiency in both HFO groups compared to HFL groups.[59,60,79]

In addition to weight gain, dysfunctional carbohydrate metabolism, seen as hyperglycemia and insulin resistance, is another defining hallmark associated with obesity and GC usage.[81] A reduction in glucose clearance can result from the inhibition of insulin signaling within cells, which has been shown to be induced by inflammatory cytokine signaling (ie; TNF-α and IL-6). To explore this, we subjected mice at 10 weeks (prior to sacrifice) to a glucose tolerance test. Surprisingly, these data revealed no differences in fasting glucose or glucose clearance between either HFL or HFO groups. Although a little disappointing, this may be attributed to the young age of mice during the intervention period and/or a lack of inflammation. In fact, much of the prior research studying the effects of high fat diets and GC usage are in older mice and/or for longer durations. However, despite the lack of differences in glucose clearance, we did observe a greater variation in blood glucose levels at the final 90-minute reading for both HFL groups that was not recapitulated in either HFO groups.

A High Fat Fish Oil Diet Reduces the Expansion of Visceral Adipose Tissue and Adipocyte Hypertrophy Even in the Presence of Glucocorticoids Compared to a High Fat Lard Diet

Adipose tissue is a flexible caloric reservoir, responding to energetic and hormonal cues to either expand (fatty acid synthesis, lipogenesis) during energy excess or downsize (lipolysis, fatty acid oxidation) and release free fatty acids during energy deficit.[18,21] This flexible nature of adipose tissue, protects other organs from lipidation and lipotoxicity. To explore the impact of diet type and GC usage on visceral adipose tissue expansion, we extracted both left and right epididymal fat pads from mice. Unsurprisingly, both HFL groups exhibited greater amounts of visceral adipose tissue in grams compared to their HFO counterparts. More so, as both HFL

groups gained near identical amounts of visceral fat, this further supports the idea that differences between overall weight in HFL GC- and GC+ groups is likely due to differences in skeletal muscle mass. These data support the notion that the HFO diet type protects against visceral adipose tissue accumulation, and more importantly irrespective of GC treatment.

Adipocyte hypertrophy which occurs during energy excess, represents a balance between lipogenesis and lipolysis.[18,19,25] Excessive hypertrophy of adipocytes however, leads to an increase in reactive oxygen species, endoplasmic reticulum stress, and lipid spillover.[18] Interestingly, it has previously been shown that stem cells in the stromal vascular fraction of visceral adipose tissue have a lower potential for adipogenesis or proliferation compared to stem cells within subcutaneous adipose tissue. To explore the impact of diet type and GC usage on visceral adipocyte hypertrophy, we measured the area of adipocytes from each experimental group. Both HFL GC+ (mean= 5713μm^2) and GC- (mean= 4728μm^2) groups had significantly larger adipocytes compared to their HFO GC+ (mean= 3572 μm^2) and GC- (mean= 3429μm^2) counterparts. Supporting the notion that fish oil based diets promote oxidative pathways and reduce the burden of lipid storage on adipocytes.[59] Moreover, differences were noted between HFL GC+ and GC- mice (δ=985μm^2), with those receiving GC having slightly larger adipocytes. However, this trend was not as drastically observed between HFO GC+ and GC- mice (δ=143μm^2), suggesting that GC may exacerbate adipocyte hypertrophy in the presence of a HFL diet.

This idea of GC-induced hypertrophy however poses an interesting conundrum, as GC have a well-known role in adipogenesis, which should reduce overall hypertrophy of individual adipocytes by creating more adipocytes. With this in mind, we pose the idea that GC may regulate some aspect upstream of the adipogenesis pathway, which might explain why

hypertrophy of adipocytes is occurring more so in GC treated groups as compared to non-GC treated groups. In line with this, we identified a significant decrease in the expression of ISYNA1 within HFL CG+ treated mice. Recent data by Jeffery et al, (2015) show that activation of adipogenic precursors within adipose tissue is dependent on the phosphoinositide 3-kinase (PI3K)-AKT2 pathway, which would be perturbed by a reduction in ISYNA1.[84]

A High Fat Fish Oil Diet Positively Modulates Immune Parameters Compared to a High Fat Lard Diet

To determine if the increase in weight generated an increase in systemic and tissue cytokine secretion, specifically TNF-α, IL-4, IL-10, IL-α, IL-1β, IL-6, we used a bead-based multiplex assay to determine protein levels. Surprisingly, the only observed difference between dietary groups was in IL-6 levels within adipose tissue. Where HFO fed mice had a greater amount of IL-6 than their HFL counterparts. This was quite shocking, as literature describes increased levels of IL-6 being associated with greater adipose tissue inflammation and resultant insulin resistance, which contradicts the anti-inflammatory nature of ω-3s. However recent findings utilizing an IL-6$^{-/-}$ KO model, suggest that IL-6 specifically during a high fat diet challenge is needed to maintain insulin sensitivity, although the underlying mechanisms are not yet understood.[62]

The lack of other differences in systemic and tissue cytokines levels between HFL and HFO groups, suggests that these mice have not yet reached a pro-inflammatory state. This may again be attributed to a couple of reasons 1) young age during the intervention period, 2) short time frame of high fat feeding, and 3) the use of prednisolone which is not as bioactive as dexamethasone, used more commonly in studies. It might also be, that as this time-point the GC are still acting in an anti-inflammatory manor and GC-resistance has not yet occurred. However,

when we assayed for the adipokine leptin in both plasma and tissue lysates, we observed significantly higher plasma levels in HFL fed mice compared with HFO counterparts. Our observation agrees with prior research, as increases in leptin are positively associated with increases in adipose tissue mass. Leptin, which is synthesized and secreted by adipose tissue, increases systemically during obesity to help regulate feeding behavior and act as a pro-inflammatory immune modulator.[61] This suggests that fish oil based diet protects against hyperleptinemia, which likely results from an overall reduction in adipose tissue mass.

An additional aspect for determining immune changes in response to diet type and GC usage, is by phenotyping immune cell populations using flow cytometry.[20] In adipose tissue, we observed a greater infiltration of $LDA^-/B220^-/CD11b^+$ cells for all mice fed the high fat diet types, which can represent macrophages, monocytes, or neutrophils.[63,64] Slight, but not significant, differences were seen between HFO and HFL groups. From this $CD11b^+$ cell population, we gated cells further by $F4/80^+$ and $CD11c^+$. This revealed two distinct macrophage populations based on the presence of CD11c (this marker would indicate pro-inflammatory M1-type macrophages). The fraction of $CD11c^+$ cells were increased with the HFL diet (with no difference between GC- and GC+) compared to the HFO diet. This suggests that even though there is an increase in macrophage infiltration into visceral adipose tissue with the HFO diet, the cells are M2-polarized which have an immunosuppressive function. This is consistent with the literature.[63,64]

M2 macrophages are resident anti-inflammatory cells, that have been shown to undergo phenotypic switching to pro-inflammatory classically activated "M1" macrophages during obesity.[63,64] Accumulation of M1 polarized cells within adipose tissue, is pivotal to the development of adipose tissue inflammation and insulin resistance.[63,64] M1 macrophages are

known to infiltrate adipose tissue in response to cytokine and chemokine release, as well as FA spill-over, both of which occur in stressed adipocytes. Together these data provide a clear difference between immune cell populations (M1 vs M2 macrophages) within visceral adipose tissue, that is dependent on the type of fat (ω-6 vs ω-3) present within the diet. Furthermore, the greater fraction of M2-polarized macrophages within visceral adipose tissue of HFO fed mice, suggests a healthier environment not see in adipose tissue of HFL fed mice.

Finally, we characterized T-cell compartments within spleens of HFL and HFO fed mice, as little is known regarding the effect of diet type on splenocyte populations. Cells were gated initially by $LDA^-/CD3^+$, and then whether they were $CD4^+$ or $CD8^+$. No major differences were observed in the percentage of $CD4^+$ cells between all experimental groups. However, slight differences were observed in the amount of $CD8^+$ cells, with HFO fed mice having a 4% reduction in total $CD8^+$ cells compared to HFL fed mice. This coincides with prior research, although limited, which show that ω-3 fatty acids can suppress $CD8^+$ T-cell activation and proliferation via elevated levels of myeloid-derived suppresser cells.[85] $CD8^+$ cells are a critical subpopulation of lymphocytes that include both cytotoxic T-cells, which mediate tumor and viral suppression, and a group of suppressor T-cells, which dampen certain immune responses. Interestingly, an increased presence of $CD8^+$ T-cells in response to a high fat diet has been shown to increase the risk for atherosclerosis, a key component in the development of cardiovascular disease.[86], We also observed a trend in greater percentage of $CD8^+$ cells within both high fat diet types receiving GC, not observed within the standard rodent chow diet type. Again however, there was still an overall decrease in $CD8^+$ cells with HFO fed mice compared to both HFL and CH counterparts.

A High Fat Lard Diet in Conjunction with Glucocorticoids Modulates Visceral Adipose Tissue Gene Expression

Microarray analysis of visceral adipose tissue gene expression was used to further examine GC action in driving observed phenotypes. Using the Affymetrix GeneChip Mouse Gene 2.0 ST array, we determined differences in transcriptome profiles between HFL GC+ and HFL GC- groups. In total, we identified 3,112 visceral adipose tissue transcripts that were significantly different in HFL GC+ mice compared to HFL GC- mice. Of these transcripts, 250 GC-regulated genes were differently expressed by $\pm$1.5 relative to GC- controls. Using greater stringency in relative expression, we identified 58 GC-regulated genes that were differentially expressed by $\pm$2.0. Within this group, 65.5%(38) were down-regulated in response to GC and 34.5%(20) were up-regulated.

In classifying these GC-regulated genes, using functional gene ontologies, we identified several interesting processes, both up- and down-regulated, in response to chronic GC treatment during consumption of a HFL diet. Up-regulated genes related to the regulation of gene expression, apoptosis, cell cycle, signaling and signal transduction, cell stress, and immunologic processes. Whereas down-regulated genes related to: lipid metabolism, oxidation-reduction activity, apoptosis, cell adhesion, signaling and signal transduction, cell stress, cell differentiation, immunologic processes, and regulation of gene expression.

As the combination of a HFL diet and chronic GC treatment promoted the largest changes in adipose tissue accumulation, adipocyte hypertrophy, and early immune alterations we further analyzed transcripts involved in those processes. Down-regulated transcript Fads2, encodes the rate limiting desaturase necessary for converting essential PUFAs, ALA and LA, into their corresponding long-chain counterparts; EPA, DHA, and AA.[66,67] Alterations in Fads2

activity perturbs cellular PUFA composition and impacts various processes like membrane signaling, eicosanoid production, and gene expression.[66] As PUFAs play critical roles within many adipocyte activities, disruption in their production leads to alterations in adipocyte and adipose tissue function.[66]

Down-regulated transcript Isyna1, encodes the rate limiting enzyme necessary for biosynthesis of all inositol containing compounds.[69,70] It converts glucose-6-phosphate into *myo*-inositol 3-phosphate, which can be dephosphorylated to yield free *myo*-inositol.[69,70] *Myo*-inositol is a critical component of membrane phospholipids, and precursor for the phosphoinositide signaling pathway which functions in insulin signaling.[71] Additionally, *m*yo-inositol can be converted through a series of steps into phosphatidic acid, the critical precursor molecule for diacylglycerol and triacylglycerol synthesis.[70]

Whereas up-regulated transcripts Orm1 and Om2, encode acute phase proteins that function as early immunomodulators within adipose tissue.[73] Induction of their expression occurs during inflammation and obesity. Orm1 and Orm2 proteins act to suppress excess inflammation and maintain energy homeostasis, playing a role in the coordination of metabolic homeostasis within adipose tissue.[73] Together these GC-regulated genes shed new insight into early transcriptional changes occurring within visceral adipose tissue while consuming a HFL "Western" type diet.

A High Fat Fish Oil Diet, Irrespective of Glucocorticoid Treatment, Differentially Alters Transcript Levels Compared to a High Fat Lard Diet

Comparative analysis of relative gene expression changes within visceral adipose tissue were measured using quantitative RT-PCR between both HFO and HFL groups to identify diet

and GC specific differences. Relative gene expression changes for FADS2, ISYNA1, ORM1 and ORM2 additionally validated our microarray results between HFL GC+ and GC- mice.

We identified a GC-induced reduction in relative expression of the FADS2 gene across both HFO and HFL groups. Unfortunately, minimal research has looked at FADS2 expression in conjunction with GC treatment. Of the few studies that have explored this relationship, all report GC-induced increases within SAT depots.[74,75] This data is therefore novel in that it is the first time that GC down-regulation of FADS2 expression within visceral adipose tissue has been demonstrated. Additionally, in agreement with prior literature which shows FADS2 undergoes negative feedback inhibition from LA and ALA metabolites, AA and EPA-respectively, our data shows a diet specific trend in FADS2 expression. A greater relative expression was seen within HFL fed mice as compared to HFO fed mice, most likely due to the higher amount of EPA and DHA (metabolites of ALA) within the HFO diet. Whereas the HFL diet contained primarily LA and very little of its metabolite AA.

We also observed a GC-induced down-regulation of ISYNA1, however only within the HFL GC+ group as compared to the HFL GC- group. To date, no studies have shown ISYNA1 undergoes regulation by GC, more so if this gene contains GC responsive elements. Interestingly, HFL GC- mice were the only group to exhibit a dramatic increase in ISYNA1 relative expression. Suggesting a HFL diet specific increase in ISYNA1 expression. Alas, only one study to date has explored ISYNA1 expression within adipocytes, using 3T3-L1 cells.[76] Kim S, et al. (2007) showed ISYNA1 expression to be increased during early adipogenesis, 2 days following incubation with the differentiation cocktail, in 3T3-L1 cells.[76]

Finally, we observed a GC specific increase in ORM1 and ORM2 relative expression within mice fed the HFL diet only. Suggesting a diet and GC specific alteration in early stress

sensors within visceral adipose tissue. Additional minor differences in ORM2 expression were noted between HFL GC- and HFO GC- groups, with the HFO GC- group having a lower relative expression. Interestingly, ORM1 and ORM2 genes are shown to contain GC responsive elements within their proximal promotor regions.[77] More so, as both HFO groups exhibited a lower relative expression of ORM1 and ORM2, compared to HFL counterparts, this suggests that a high fat diet alone does not immediately increase the expression of these acute phase proteins. Rather the combination of both a HFL diet, rich in ω-6s, and chronic GC treatment, is needed to induce their expression.

Conclusions

In summary, this study examined the effects of ω-3 and ω-6 dietary lipids with and without GC in young male C57BL/6 mice. Measures for metabolic function, adipose tissue morphology, immune cell characterization, cytokine/adipokine profiles, and visceral adipose tissue gene expression were analyzed. The fish oil based diet broadly protected against overall weight gain, visceral adipose tissue accumulation and adipocyte hypertrophy even in the presence of GC. Additionally, the fish oil based diet retained a greater percentage of resident anti-inflammatory M2-polarized macrophages, whereas the lard based diet accumulated a greater percentage of pro-inflammatory M1-polarized macrophages. Transcriptome analysis revealed that GC, in conjunction with a HFL diet, down-regulate transcripts involved in lipid metabolism whilst up-regulating immunomodulators within visceral adipose tissue. Finally, differences in gene expression between HFO GC+ & GC- treated mice and HFL GC+ & GC- treated mice depict lipid specific and GC-specific regulation of critical transcripts involved in energy and cell homeostasis. These results support the supplementation of ω-3 PUFAs into a high fat lard diet,

even in the presence of chronic GC treatment, at an early age. Directly benefiting metabolic and immune parameters, as well as beneficially regulating gene expression in young mice.

Recommendations for Future Research

The novel findings presented here, highlight the need for further investigation on many fronts. First, investigation into the effects of fish oil based diets on visceral adipose tissue whole transcriptome is needed to better delineate differences underlying diet specific drivers of observed phenotypes. Second, further investigation into the potential protective nature of fish oil based diets on GC-induced muscle atrophy is needed, as skeletal muscle is a major buffer of blood glucose and a primary site for fatty acid oxidation. Third, further study into the role of visceral adipose tissue IL-6 expression should be explored, as its function may be context specific such as in skeletal muscle. Fourth, the impact of GC on the regulation of adipose tissue stem cell populations should be at the forefront of investigation. As GC have a well-known role in adipogenesis, we were surprised to see that GC induced hypertrophy of visceral adipocytes more so than driving the formation of new adipocytes, and thus offsetting the burden of lipid storage. This is likely a depot specific phenomena, as chronic GC usage is known to reduce subcutaneous depots.

Larger experimental groups would also provide greater statistical power, and allow for a better understanding of association between various factors where effect size is small. As well as a more focused approach toward understanding the many underlying biomolecular pathways responsible for phenotypic changes identified and discussed here. Additionally, future studies should look into altering the ω-3:ω-6 PUFA ratio, to identify the optimal dosage of ω-3 PUFA supplementation in humans.

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Appendices

IACUC Approval
IACUC PROTOCOL
FOR USE OF LIVE VERTEBRATES FOR RESEARCH, TEACHING OR DEMONSTRATION
UNIVERSITY OF MEMPHIS

IACUC Protocol # 0749 Date Submitted to IACUC 7/21/14

Dates Protocol will be in effect: from 9/2/14 to 9/1/17
(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

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):

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

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 Pharmacology), 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

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.

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.)

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)**

List search words for the literature search:

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[2]	Weight[2]	Where Housed (Bldg./Rm#)
Hooded Wistar rats	45	2 months	male	250-350 gm	Psychology Bld./422I
C57BL/6 mice	60	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.
C57/BL6 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	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 ☐

If yes, please explain in box below.

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

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.

H. 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 ☐

If yes, what was the result (indicate in box below).

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

THE UNIVERSITY OF MEMPHIS

IACUC PROTOCOL ACTION FORM

To:	Marie van der Merwe
From	Institutional Animal Care and Use Committee
Subject	Animal Research Protocol
Date	September 2, 2014

The Institutional Animal Care and Use Committee (IACUC) has taken the following action concerning your Animal Research Protocol No. **0749** Effect of a dietary intervention on glucocorticoid-induced metabolic syndrome

☒ Your protocol is approved for the following period:

From: September 2, 2014 To: September 1, 2017

☐ Your protocol is not approved for the following reasons (see attached memo).

☐ Your protocol is renewed without changes for the following period:

From: To:

☐ Your protocol is renewed with the changes described in your IACUC Animal Research Protocol Update/Amendment Memorandum dated for the following period:

From: To:

☐ Your protocol is not renewed and the animals have been properly disposed of as described in your IACUC Animal Research Protocol Update/Amendment Memorandum dated

Amy L. de Jongh Curry, PhD, Interim Chair of the IACUC

Dr. Karyl Buddington, University Veterinarian
And Director of the Animal Care Facilities