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Dietary Omega-3 Fatty Acids Reduce Adiposity and Alter Glucocorticoid-Associated Transcripts in Epididymal White Adipose Tissue of C57BL/6 Male Mice Raised on a High Fat Diet

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Abstract

Background: Synthetic Glucocorticoids (GC) are widely used for their immunosuppressive capability, however chronic use is associated with adverse metabolic effects that are exacerbated when GC is combined with a high fat diet. Omega (n)-3 PUFA supplementation has been shown to rescue GC-induced hyperlipidemia, hyperinsulinemia and inflammation.

Objective: To evaluate the effect of n-3 PUFAs during chronic GC treatment to prevent metabolic dysfunction.

Methods: 3-week old C57BL/6 male mice were weaned onto a high fat diet that would result in obesity and metabolic dysfunction with long-term consumption. After 3 weeks, experimental mice were switched to a high fat diet rich in n-3 PUFAs. Concomitantly, half of each dietary group received GC (40 mg/m^2/day) in the form of oral prednisolone. All animals remained on their respective diets/treatments for an additional 4 weeks. Weight gain, glucose homeostasis, fatty liver and inflammation were measured, and epididymal white adipose tissue (eWAT) transcriptome analysed.

Results: Switching to a diet high in n-3 PUFAs during a 4-week GC protocol did not cause in significant changes in weight gain or glucose homeostasis but resulted in significant decreased eWAT accumulation and macrophage infiltration independent from GC therapy. However, eWAT transcriptome analysis demonstrate GC upregulation of the acute phase proteins Orm1 and Orm2 are limited with an n-3 PUFA diet.

Conclusions: Chronic GC therapy in combination with a Western type-high fat diet results in eWAT accumulation and inflammation. A diet rich in n-3 PUFAs protects against adiposity and early transcriptional alterations involved in metabolic derangement during GC therapy.

Keywords

Polyunsaturated fatty acid, Glucocorticoids, Epididymal white adipose tissue, Inflammation

Introduction

Glucocorticoids (cortisol in humans, corticosterone in rodents) are naturally produced steroid hormones that regulate both the immune system and energy metabolism. Synthetic Glucocorticoids (GC), such as prednisolone

and dexamethasone are widely used for the treatment of many inflammatory conditions [1, 2] and are also a critical component of pediatric Acute Lymphoblastic Leukaemia (ALL) treatment where they act as a cytotoxic agent [3]. Unfortunately, chronic administration of GC are associated with many adverse metabolic side effects that include glucose intolerance [4], type 2 diabetes, skeletal muscle wasting [5], Non-Alcoholic Fatty Liver Disease (NAFLD), lipodystrophy and visceral adipose tissue accumulation [6, 7]. The *in vivo* effects of GC are complex as they influence the transcription of many target genes in various tissues. Within adipose tissue, GC are required for adipocyte differentiation, induction of lipogenic genes, and the regulation of adipose endocrine function [8] and in humans GC are often associated with excessive weight gain as seen in Cushing's disease [9, 10], a phenotype shared by children undergoing GC therapy as a part of pediatric ALL treatment [11-16]. Animal studies clearly demonstrate that an increase in GC results in increased fat deposition [17, 18] and studies using genetically targeted adipose tissue-specific increases in GC result in hyperphagia and increased visceral adiposity, with animals consuming a high fat diet being more sensitive [19].

Many of the side effects of GC treatment resemble the phenotype associated with high fat feeding and obesity. Studies in various rodent models demonstrate combining GC with a high fat diet increases fatty liver development and visceral adiposity, as well as resulting in hyperglycemia and hyperinsulinemia [20-22]. Treatment with GC also increases body fat mass and can induce long-lasting changes in body composition that are much more pronounced when combined with a high fat diet [22, 23]. The Western diet, high in saturated fat and n-6 PUFAs, might be particularly detrimental when combined with GC as this fatty acid profile results in an increased risk for obesity and adipose tissue inflammation [24, 25].

N-3 fatty acids, such as Eicosaentaenoic acid (EPA) and Docosahexaenoic acid (DHA) have been reported to improve obesity-associated metabolic dysfunction including insulin resistance [26], inflammation [27], dyslipidemia, and NAFLD [28]. Although the effect of n-3 PUFA supplementation on GC-induced metabolic dysfunction is poorly understood, dietary n-3 fatty acids have been shown to attenuate dyslipidemia induced by high dose GC [29], and rescue offspring from adipocyte inflammation induced by in utero GC exposure when given to mothers prenatally [30].

The objective of this study was therefore to determine if increased amounts of n-3 PUFAs *vs* n-6 PUFAs can reduce the development of metabolic dysfunction and alter adipose tissue transcription during GC treatment.

Methods

Experimental animals

C57BL/6 male mice (n = 40) were obtained from breeder pairs purchased from Envigo Laboratories Inc. (Pratville, AL), and bred and housed in a USDA approved animal facility at the University of Memphis. All mice were weaned at 3 weeks of age onto a purified 45% high fat diet (HF-n6; Research diets, Inc., New Brunswick, NJ) 45% fat, 35% carbohydrate and 20% protein; diet composition is reported in table 1). This diet is similar to the 45% high fat diet (D12451, Research diets, Inc., New Brunswick) that is readily used to generate a diet-induced obesity model [31-35], with the addition of tert-Butylhydroquinone (tBHQ) to be equivalent to the experimental n-3 PUFA rich diet. At 5 weeks of age, mice were separated into individual cages and conditioned to daily pureed sweet potato consumption (0.2-0.3 g). This was done

Table 1: Composition of high fat diets[1].

	High fat n-6 diet (D10011203)	High fat n-3 diet (D05122102)
Macronutrients		
Protein, %kcal	20	20
Carbohydrate, %kcal	35	35
Fat, %kcal	45	45
Saturated, %kcal	31.6	31.5
Polyunsaturated, %kcal	32.9	46.7
Total, %kcal	100	100
Energy, kcal/g	4.72	4.72
Ingredients, g		
Casein, 80 Mesh	200	200
L-Cystine	3	3
Sucrose	172.8	172.8
Maltodextrin 10	100	100
Cellulose, BW200	50	50
Soybean Oil	25	25
Lard	177.5	-
Menhaden Oil (200 ppm tBHQ)	-	177.5
Tert-Butylhydroquinone (tBHQ)	0.0355	0.0355
Mineral Mix S10026	10	10
Vitamin Mix V10001	10	10
Choline Bitartrate	2	2
Cholesterol	0.58	-
C14:0, Myristic	2.1	14
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	-	6
C20:0, Arachidic	0.4	0.3
C20:4, Arachidonic, n6	0.5	-
C20:4, n3	-	3.1
C20:5, Eicosapentaenoic, n3	-	23.3
C22:5, Docosapentaenoic, n3	0.2	4.1
C22:5, n6	-	0.6
C22:6, Docosahexaenoic, n3	-	29

[1]Diets were formulated by Research Diets, Inc. (www.researchdiets.com). Original to this work.

as the sweet potato served as a vehicle for oral prednisolone administration. At 6 weeks of age, half of the mice (n = 20) were switched to a high fat diet (experimental diet) rich in n-3 PUFAs (HF-n3; Research diets, Inc., New Brunswick, NJ; 45% fat, 35% carbohydrate and 20% protein; diet composition reported in table 1). The diets are isocaloric (4.7 kcal/g), with 88% of the fat in the HF-n6 diet from lard, and 88% of the fat in the HF-n3 diet from menhaden oil. The remaining 12% of fat in both diets was provided by soybean oil. Fatty acid profiles for both high fat diets are given in table 1. Levels of saturated fat was similar, while the mono- and polyunsaturated fatty acid differed between the diets, resulting in a n6/n3 ratio of 13.1 (HF-n6) and 0.3 (HF-n3). In addition to diet change, both high fat diet groups (HF-n6 and HF-n3) were randomly divided with half of the mice in each group (n = 10) receiving GC in the form of oral prednisolone (People's Custom Pharmacy, Memphis, TN) at a dose of 40 mg/m^2/day added to the sweet potato (GCplus). Control groups received the vehicle alone (GCmin). The age of animals, and route and dose of prednisolone administration were chosen to resemble standard GC treatment (40 mg/m^2/day) in pediatric ALL patients during the induction phase of ALL therapy [15]. To calculate the mouse dose, body surface area was used as determined by Gargiulo et al., [36]. This resulted in a dose of 6 mg/kg/day and falls within the concentration range that has previously been shown to be effective in mice [37]. To confirm that this dose is sufficient a pilot study was performed to see if the dose is effective at reducing spleen weight as had previously been shown [38]. Spleen weight was reduced by administration of prednisolone with this dose and treatment time (P = 0.03). During the experiment, mice consumed water and food ad libitum. Body weight and food consumption were monitored twice a week, with food being replaced every 3-4 days. After 4 weeks on the respective diets and prednisolone treatment, mice were fasted overnight and euthanized by CO_2 inhalation. Whole blood was collected immediately prior to sacrifice via the facial vein. Tissues were harvested and immediately processed or snap frozen in liquid nitrogen and stored at -80 °C. All experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Experimental Animals and were approved by the University of Memphis Institutional Animal Care and Use Committee.

Glucose tolerance test

Two days prior to sacrifice, mice were subjected to an Intraperitoneal Glucose Tolerance Test (IPGTT). Mice were fasted for 6 h, after which baseline fasting blood glucose levels were measured from the tail vein using a OneTouch Ultra 2 Meter (Bayer Healthcare, Tarrytown, New York). Glucose (2 g/kg body weight) was administered by intraperitoneal injection and blood glucose levels were measured every 30 minutes for 90 minutes via the tail vein.

Cell isolation and flow cytometry analysis

Immediately after sacrifice, bilateral eWAT pads were harvested, the testes and lymph nodes were removed before weight was recorded. A portion of eWAT (0.6 g) was cut into small pieces and digested in 2 mL of DMEM (HyClone, GE Healthcare Life Sciences, Logan, UT) containing 2 mg/mL type 2 collagenase (Worthington Laboratories, Lakewood, NJ) at 37 °C with continuous agitation (240 RPM) for 40 minutes. After digestion, samples were diluted with DMEM and filtered through a 40 µm nylon cell strainer and pelleted (500 × g for 10 minutes at 4 °C) to enrich fat-associated immune cells in the Stromal Vascular Fraction (SVF). Cells were then re-suspended in 500 µL PBS with 2% FBS, and viability was measured using trypan blue (Mediatech Inc., Manassas, VA) staining. Prior to antibody staining, Fc receptors were blocked with Trustain FcX (Biolegend, San Diego, CA) at room temperature for 10 minutes. LIVE/DEAD™ Fixable Aqua (Life Technologies, Eugene, OR) was used to exclude dead cells. The following fluorescently conjugated antibodies were used for cell identification: PE-Cy7 anti-CD11c (clone N418, BioLegend, San Diego, CA), Pacific Blue anti-F4/80 (clone BM8, BioLegend, San Diego, CA), APC-Cy7 anti-B220 (clone RA3-6B2, BioLegend, San Diego, CA), FITC anti-CD11b (clone M1/70, BioLegend, San Diego, CA). All samples were fixed overnight with Fixation/Permeabilization solution (eBioscience, Carlsbad, CA) at 4 °C and analysed using an LSR II Flow Cytometer (BD Biosciences, San Jose, CA) and BD FACSDIVA software (BD Biosciences, San Jose, CA). Data obtained were further analysed using FlowJo (FlowJo LLC., Ashland, OR). To determine macrophage infiltration in a lean control group, we used gender and age matched C57BL/6 mice (n = 7) that consumed a non-purified chow diet (Teklad global 2018, Envigo Laboratories Inc.; 18% fat, 58% carbohydrates, 24% protein; 3.1 kcal/g) which does not induce eWAT accumulation.

Histological analysis

A representative sample of liver and eWAT was fixed in 10% neutral buffered formalin solution (Fisher Scientific Co. LLC, Dallas, TX) for 48-72 h, dehydrated in a series of graded ethanol solutions, cleared with xylene, embedded in paraffin, and 5 µm sections stained with hematoxylin and eosin (H&E). Histological analysis and documentation were performed using an Imager M2 microscope (Axiocam MRC, Zeiss, Oberkochen, Germany). Individual adipocyte sizes were determined using Axiovision r4.8.2 software. Two to three fields at 20X magnification, with twenty to forty cells per field, were quantified for each mouse.

Cytokine analysis

Plasma and eWAT lysates were used for cytokine and adipokine analysis. Plasma was isolated from whole blood collected in EDTA tubes. eWAT lysates were prepared from snap frozen tissue samples by rinsing samples in PBS, cutting into 1-2 mm pieces, and homogenizing in PBS. An equal volume of Cell Lysis Buffer 2 (R&D Systems, Minneapolis, MN) was added, and the tissues were lysed at room temperature for 30 minutes with gentle agitation. Debris was removed by centrifugation. Plasma and eWAT lysates were diluted with 75 µL of Calibrator Diluent RD6-52 (R&D Systems, Minneapolis, MN) and processed according to the manufacturer's instructions.

Cytokine assays were performed according to R&D Magnetic Luminex Bead Assay instructions (R&D Systems, Minneapolis, MN). Assay plates were analysed using the Luminex® MAGPIX® Analyzer and Luminex® xPONENT® Multiplex Assay Acquisition and Analysis Software to distinguish bead colour and fluorescence intensity.

Transcriptional analysis of eWAT

Total RNA was extracted from 0.5 mg of snap frozen eWAT, using QIAzol Lysis Reagent (Qiagen, Hilden, Germany) and a hand-held homogenizer. Total RNA was purified using RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany). RNA integrity was determined by an Agilent Bioanalyzer2100, using Agilent RNA Nano Chips and RNA 6000 Nano Reagents (Agilent Technologies, Waldbronn, Germany). RNA quantity and purity were determined using NANO Drop 2000 spectrophotometer (Fisher Scientific Co. LLC, Dallas, TX) and isolated RNA was stored at -80 ˚C. RNA integrity was calculated using RIN (RNA integrity number). The mean RIN was 8.4, where all samples where above 8 except one sample in the HF-n6 GCmin group that had a RIN of 6.4.

Microarray analysis was performed on total RNA extracted from eWAT of mice fed the HF-n6 diet and treated with GC or vehicle alone (n = 4 per group). 100 ng of isolated total RNA was used to make labelled single stranded complimentary DNA according to Affymetrix protocol for GeneChip Whole Transcriptome Expression Arrays. Labelled DNA were hybridized to Affymetrix MoGene 2.0 ST arrays, washed and stained using Affymetrix GeneChip Fluidics Station 450. Arrays were scanned on the Affymetrix GeneChip Scanner 3000 7G system, and raw data processed with GeneChip Command Console and Expression Console software's (Affymetrix, Santa Clara, CA) for background correction and normalization, using the robust multiarray average method. Data analysis and statistical evaluations were performed in Partek Genomics Suite 6.6 software (Partek Inc., St. Louis, MO). Differentially expressed genes were identified as those with a false discovery rate $P < 0.05$ and a fold change difference of ≥ 2.0 relative to the experimental control (HF-n6 GCmin). The MGI 6.08 Informatics tool (The Jackson Laboratory, Bar Harbor, ME) was used to identify biological processes, molecular functions, and cell components of differentially expressed genes.

Quantification of gene expression by RT-PCR was carried out using the Bio-Rad CFX96 Touch Real-Time PCR System PCR system. Complimentary DNA synthesized from 1 µg of isolated total RNA, by using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), was stored at -20 ˚C. Real-time PCR was conducted with PowerUP SYBR Green Master Mix (Life Technologies, Carlsbad, CA) and specific IDT primers (Integrated DNA Technologies, Coralville, IO) listed in table 2. PCR reaction conditions were as follows: 50 ˚C for 2 min, 95 ˚C for 2 min followed by 40 cycles of 95 ˚C for 15 s, 60 ˚C for 1 min and 72 ˚C for 15s min. Each gene was assessed in triplicate reactions and normalized to hydroxymethylbilane synthase (Hmbs) [39] and data obtained by comparative CT method (expression relative to the experimental control (HFn-6 GCmin)) using the CFX Manager Software v3.1 (Bio-Rad, Hercules, CA). The data discussed in this publication comply with MIAME (Minimum Information About a Microarray Experiment) requirements and have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE99064 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE99064).

Table 2: *Mus musculus* primers[1].

Gene	Sequence length	Forward primer	Reverse primer
Fads2	1508	CTCCCAAGATGC CG TAGAAAG	GCTCATCCCTATG TACTTCCAG
Isyna1	1850	CCAGACCACTGT GTG GTGAT	TGGAGCACCAAG GT GTTTGT
Orm1	779	CTGCTTCTTCTCCT GCTGAC	GACTGTCCCTCTATG CCAAA
Orm2	*774*	CTTTCTTG GTCTCCTTCTCCAG	AGAAGGCTGTCA CA CACG
Hmbs	1611	CGTGGGAAC CAGCTCTCTGA	GAGGCGGGTGTT GAGGTTTC

[1]Primers were formulated by Integrated DNA Technologies, Inc. (www.idtdna.com). Original to this work.

Statistical analysis

All data are presented as means ± SEM. Statistical analyses were performed with GraphPad Prism version 7 software, and statistical significance was established at $P < 0.05$. All data sets were tested for homogeneity of variance by using the D'Agostino-Pearson omnibus test. Non-parametric testing was chosen due to non-homogeneity of data sets and small sample sizes. Significance between two experimental groups was determined by using the Mann-Whitney test. Statistical analysis of body weight and blood glucose over time were compared using a repeated measures 2-way ANOVA. When an overall ANOVA was significant ($P < 0.05$), differences between treatment groups were assessed with Tukey's post hoc analysis ($P < 0.05$).

Results

Diet rich in n-3 PUFAs reduced body weight gain

All mice gained weight during the 4-week treatment period (Figure 1A). In the absence of GC, the HF-n6 group gained significantly more weight than the HF-n3 group, while GC treatment did not have a significant effect when consuming a particular diet (Table 3). Although not significant, the reduction in weight gain with GC administration consistent with GC-induced muscle atrophy [40, 41]. GC did not increase calorie consumption on either HF-n6 ($P = 0.95$) or HF-n3 ($P = 0.32$) and there was no significant loss of food consumption when switching to the HF-n3 diet ($P = 0.38$) (Table 3). Feed efficiency, calculated as weight gained per kilocalorie (kcal) consumed during the 4-week treatment period, was higher for GCmin mice consuming the HF-n6 diet compared to HF-n3

fed mice; whereas there was no difference with GC treatment (Table 3).

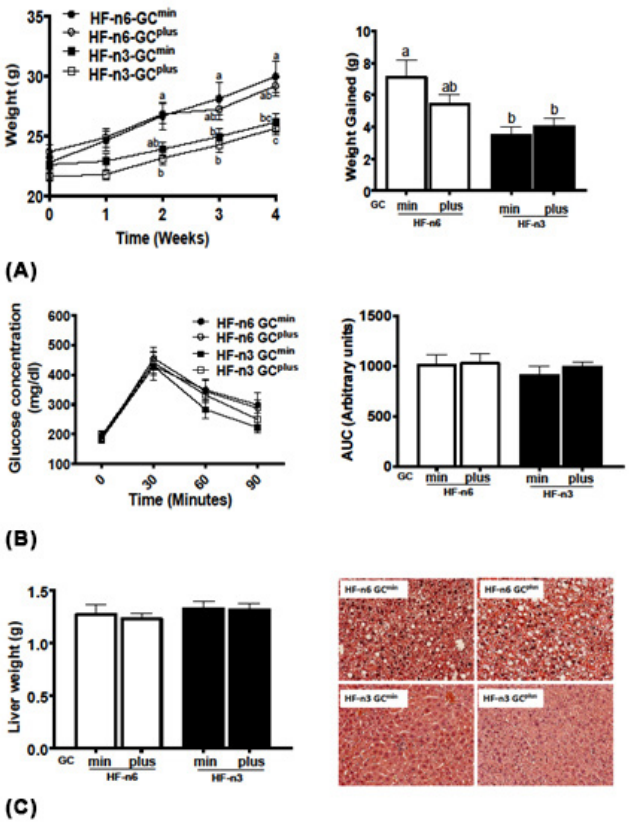

Figure 1: (A) Body weight in control (GCmin) or GC-treated (GCplus) C57Bl/6 male mice fed high fat diets (45% of energy from fat) either rich in n-6 PUFAs (HF-n6) or n-3 PUFAs (HF-n3) for 4 weeks. Left panel indicate weight over time and right panel indicate weight gained. Values are expressed as mean ± SEM, n = 10/group. Means without a common letter differ, P < 0.05, data analysed using 2-way ANOVA with repeated measures (left panel) and one-way ANOVA (right panel) followed by Tukey's post hoc analysis. **(B)** Intraperitoneal glucose tolerance test was performed on all groups after a 6 hour fast following their respective diet/treatment protocols for 4 weeks. Glucose clearance are showed (left panel) and area under the glucose curve (AUC) calculated (right panel). Values are mean ± SEM, n = 6-10 per group. **(C)** Liver weight (n = 10/group) and representative haematoxylin and eosin (H&E) stained liver tissue sections.

Table 3: Effect of diet and GC treatment on caloric intake and weight gain.

	HF-n6 GC^{min}	HF-n6 GC^{plus}	HF-n3 GC^{min}	HF-n3 GC^{plus}
Food intake (g/day)	3.00 ± 0.2	2.89 ± 0.0	2.67 ± 0.1	2.55 ± 0.1
Caloric intake (kcal/day)	14.16 ± 0.9	13.64 ± 0.5	12.64 ± 0.6	12.06 ± 0.5
Body weight gain (g)	7.18 ± 1.0^{a}	5.52 ± 0.5^{ab}	3.54 ± 0.5^{b}	4.05 ± 0.5^{b}
Feed efficiency (g/kcal)	18.48 ± 2.5^{a}	14.76 ± 1.6^{ab}	10.12 ± 1.2^{b}	11.81 ± 1.3^{b}

Values presented as mean ± SEM (n = 10). Feed efficiency = total weight gained / total amount of calories consumed during 4 weeks. Means without a common letter differ, P < 0.05, One-way ANOVA.

HF-n3 diet + GC did not alter glucose clearance but reduced liver fat accumulation

GC and obesity are highly associated with altered glucose homeostasis and diabetes and are considered risk factors for the development of NAFLD. At the end of the 4-week GC treatment there was no differences in fasting blood glucose levels between the 4 groups (HF-n6 GCmin, 193.4 ± 17.0; HF-n6 GCplus, 186.2 ± 14.9; HF-n3 GCmin, 184.3 ± 10.7; HF-n3 GCplus, 196.9 ± 13.6; P ≥ 0.7). There was also no difference in glucose clearance after an IPGTT (Figure 1B, left panel) as seen from area under the curve (AUC, P ≥ 0.4; Figure 1B, right panel). Additionally, while there was no difference in liver weight between any of the groups (P ≥ 0.2; Figure 1C, right panel), haematoxylin and eosin stained liver sections showed increased fat deposition in all mice consuming the HF-n6 diet (Figure 1C, left panel).

A diet high in n-3 PUFA reduces adiposity and adipose tissue associated inflammation

Consistent with the increase in weight, groups consuming the HF-n6 diet accumulated significantly more eWAT than the HF-n3 groups, independent of GC treatment

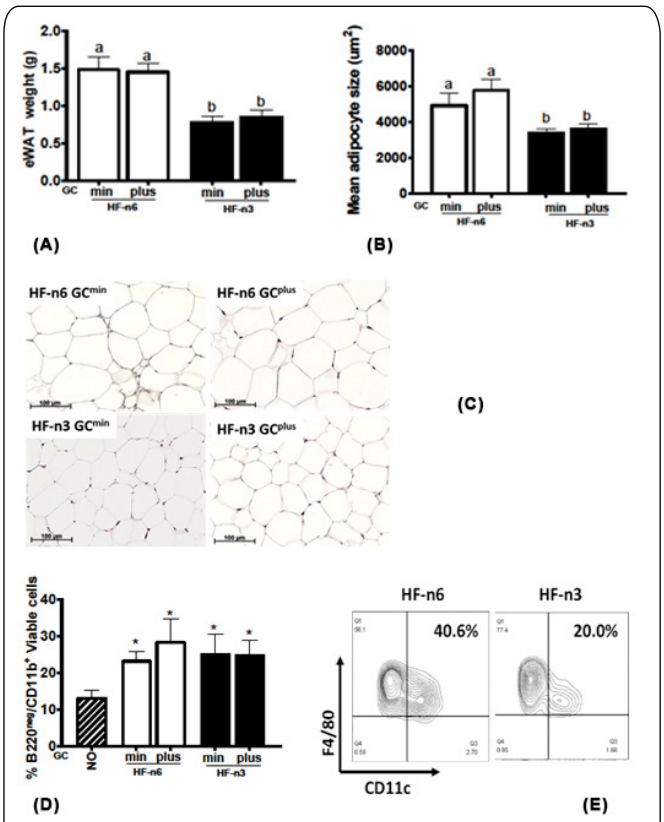

Figure 2: Epididymal white adipose tissue (eWAT) weight **(A)** and adipocyte size **(B)** were measured for each group after 4 weeks on their respective diet/treatment protocols. Values are means±SEM, n=10. Means without a common letter differ, P<0.05. Adipocyte size were measured using light microscopy (2-3 sections were quantified per mouse n= 6-9). Representative haematoxylin and eosin (H&E) stained adipose tissue sections. Scale bar=100 μm**(C)**. The percentage B220negCD11b+ cells in 0.6g of eWAT as measured by flow cytometry**(D)**. Values are means ± SEM, n=5-8. *P<0.05compared to non-obesity induced diet (NO). Representative flow cytometry plots of F4/80 and CD11c staining of gated LDAnedB220negCD11b+ cells **(E)**.

(Figure 2A). Interestingly, GC treatment did not further increase eWAT weight in the HF-n6 (P = 0.9) or the HF-n3 diet (P = 0.5) (Figure 2A). The increase in eWAT weight was concomitant with mice consuming the HF-n6 diet having significantly larger adipocytes (Figure 2B and 2C). To determine if the adipocyte hypertrophy is associated with adipose tissue-associated inflammation, the presence of $CD11b^{+}F4/80^{+}CD11c^{+}$ eWAT macrophages was determined by cell isolation and flow cytometry. All high fat diet groups, irrespective of diet composition or GC therapy, accumulated eWAT $B220^{neg}CD11b^{+}$ cells ($P < 0.05$ *vs* gender and age matched control group that consumed a chow diet with little adipose tissue accumulation) with no difference between diet/treatment groups ($P \geq 0.3$) (Figure 2D). $F4/80^{+}$ macrophages were phenotyped for CD11c expression, a marker increased in adipose tissue of obese mice [42]. Animals consuming the HF-n6 diet, irrespective of GC treatment, had an increased $F4/80^{+}CD11c^{+}$ macrophages (HF-n6 *vs* HF-n3, 50.58% ± 0.8 *vs* 27.72% ± 2.9, $P < 0.01$, Figure 2E). Therefore, despite adipose tissue accumulation of macrophages with the HF-n3 diet, these cells did not express CD11c, a marker for classically activated macrophages. Interestingly, while there was no difference in plasma Interleukin (IL)-6 between groups (HF-n6 GC^{min}, 16.53 pg/ml ± 1.6; HF-n6 GC^{plus}, 15.1 pg/ml ± 1.1; HF-n3 GC^{min}, 14.9 g/ml ± 1.0, HF-n3 GC^{plus}, 15.43 pg/ml ± 1.3; $P > 0.5$, n = 3-6), there was an increase in adipose tissue specific IL-6 within animals consuming the HF-n3 diet, irrespective of GC treatment (HF-n6 *vs* HF-n3, 47.82 pg/ml ± 5.3 *vs* 80.17 pg/ml ± 6.3, P = 0.04, n = 4).

Transcriptional changes induced by GC treatment and diet

To further determine the effect of GC on eWAT, we analysed the transcriptomes of eWAT harvested from animals consuming the HF-n6 diet. RNA was isolated from 4 animals in the HF-n6 GCmin and HF-n6 GCplus groups, and then hybridized onto Affymetrix GeneChip Mouse Gene 2.0 ST arrays. Of the 41,345 total analysed genes, 3,112 were found to be significantly different relative to HF-n6 GCmin, with a false discovery rate P-value of < 0.05. Applying a stringency of > 2.0-fold change, while maintaining significance, we identified 58 transcripts that were differentially regulated by GC (Table 4). Of these, 20 genes (34.5%) were up-regulated and 38 (65.5%) were down-regulated by more than 2-fold. Classification of genes by their assigned ontologies revealed that the significant and predominant GC up-regulated (fold change ≥ 2.0) biological processes included: regulation of immune system processes, microtubule polymerization, regulation of G-protein coupled receptors, protein signalling pathways, acute-phase responses, and cellular responses to hormone stimulus. Whereas, significant GC down-regulated (fold change ≥ -2.0) processes included cell adhesion.

Four transcripts where further validated by Real-Time PCR in the HF-n6 GCmin and HF-n6 GCplus groups (Figure 3A). Genes chosen to be verified based on their involvement in lipid metabolism included Fads2, Isyna1, Orm1 and Orm2. Significant differences in relative expression was confirmed for Orm2, Fads2 and Isyna1- whereas, Orm1 was not significantly increased (P = 0.17, Figure 3A). Orm1

Table 4: Significantly altered transcripts from microarray[1].

Gene Symbol	Fold Change	*P*-value
Igkv15-103	-5.62152	0.00295871
Fads2	-4.1868	0.0344062
Isyna1	-4.1407	0.0396276
Slc16a2	-3.47202	0.0365477
Ngfrap1	-3.31542	0.0205543
Ncam1	-3.31107	0.0447343
Cnn1	-3.27052	0.0224935
Cyp2c29	-3.08092	0.0333264
Tat	-3.05592	0.0215643
Cdh3	-3.04589	0.0465308
Gabra4	-2.98103	0.00437535
Perp	-2.89987	0.0242788
Atp1b1	-2.69608	0.00234065
Tdo2	-2.55267	0.00465765
Saa1	-2.50861	0.0071927
Gadd45g	-2.36481	0.0461875
Xk	-2.36175	0.0132148
Tc2n	-2.30871	0.00719604
Wbp5	-2.26173	0.000725999
Fzd6	-2.25952	0.0493557
Baiap2|1	-2.24192	0.0175665
Fgfr2	-2.23424	0.00579348
Mmp7	-2.21776	0.0292445
Ptprf	-2.17483	0.0458902
Cpxm2	-2.15429	0.00926866
Cfi	-2.15025	0.0250528
Apobec3	-2.13661	0.0128495
Kcnip3	-2.09492	0.0373839
Krt8	-2.08657	0.0237106
Fkbp11	-2.07908	0.0360737
Prr15	-2.06956	0.0157844
Pdlim3	-2.06411	0.0117906
Ano1	-2.06336	0.0059965
Fam134b	-2.06004	0.0494315
Cyp39a1	-2.03985	0.00849954
Hyls1	-2.00243	0.0216092
Id3	2.00794	0.0422774
Rgs7	2.01133	0.0264399
Oxtr	2.01967	0.0447653
Orm1	2.0276	0.0383055
Rgs1	2.03062	0.00279752
Tppp	2.03974	0.0304489
Irs3	2.06418	0.0125488
Cryab	2.1818	0.0125332
Duoxa1	2.19882	0.0325163
Gfpt2	2.21176	0.039496
Prr32	2.25922	0.01336
Fos	2.30652	0.0114627

Dock8	2.36998	0.00802956
Gabrr2	2.39803	0.0297285
Peg10	2.39946	0.00270072
Slc22a4	2.44775	0.0153112
Lctl	2.77248	0.0435607
Fgf13	2.77714	0.0401206
Hspb7	3.02118	0.0391145
Orm2	3.81752	0.0489368

[1]Affymetrix GeneChip Mouse Gene 2.0 ST arrays (Applied Biosystems™) purchased from ThermoFisher Scientific (www.thermofisher.com). Original to this work.

and Orm2 are known acute phase proteins mainly synthesized by the liver and controlled by GC, IL-1, IL-6 and TNFα in humans and mice [43]. GC-induced expression of Orm1 and Orm2 has not been previously demonstrated in eWAT. Orm2 expression was significantly induced with GC, while both Fads2 and Isyna1 expression were decreased (Figure 3B, 3C and 3D). Fatty acid desaturase 2 (Fads2) is of particular interest as it regulates both n-3 and n-6 PUFA metabolism, and had previously been shown to be regulated by dietary fatty acid composition [44, 45].

We also investigated the effect of the n-3 rich diet on the expression of Orm1, Orm2, Fads2 and Isyna1, with and without GC. Despite a significant difference in eWAT weight and adipocyte size between the HF-n6 and HF-n3 dietary groups, there was no difference in the expression of Orm1 in the absence of GC (Figure 3A). Orm2 was significantly reduced with the HF-n3 diet, even in the absence of GC, but similar to Orm1, was not induced in the presence of GC (Figure 3B).

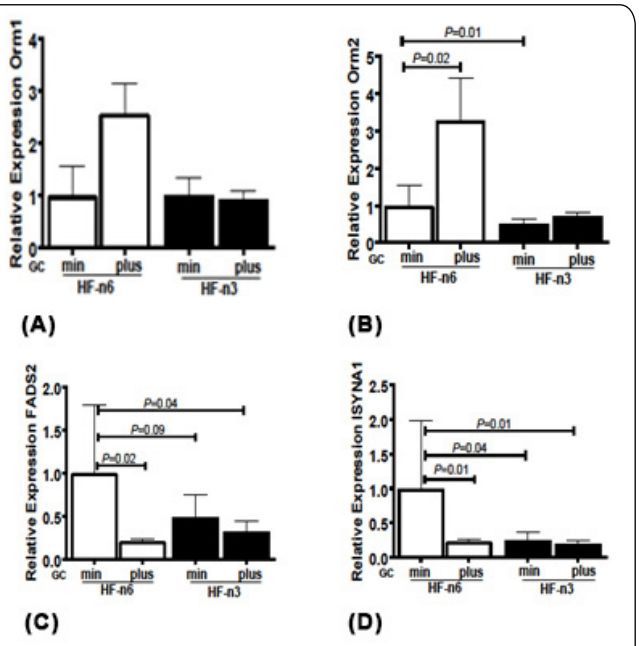

Figure 3: 4 candidate genes were selected from transcriptome analysis and diet/GC treatment effect on expression determined in eWAT. Relative expression of Orm1 **(A)**, Orm2 **(B)**, Fads2 **(C)** and Isyna1 **(D)** using hmbs as a reference gene. Values are means±SEM, n=4, P<0.05, HF-n6 GCmin as control for analysis.

Fads2 and Isyna1 were reduced by the HF-n3 diet, irrespective of GC treatment, suggesting that it is the HF-n6 diet, or the consequences of weight gain, that is inducing the expression of these transcript (Figure 3C and 3D).

Discussion

Chronic GC use is associated with co-morbidities that include fat accumulation, loss of muscle mass, insulin resistance, and NAFLD. Many of these adverse effects resemble phenotypes associated with obesity, and recent studies demonstrate that GC-induced metabolic dysfunctions are exacerbated with high fat feeding and obesity [46]. Current dietary protocols to prevent or reduce obesity have not only focused on reducing energy density, but also on manipulating the composition of macronutrients within the diet. Such modifications to diet composition, even without alterations to caloric intake, have proven successful and several studies report improvements to health parameters [47] and protection against the development of metabolic irregularities [30, 48]. Most notably, supplementation of n-3 PUFAs, either in the form of α-linolenic acid or its down-stream metabolites DHA and EPA, have been shown to provide a range of benefits in various disease models [49-51]. The advantages of increasing dietary consumption of essential n-3 PUFAs are suggested to result from maintenance of insulin secretion and sensitivity [52], promoting an anti-inflammatory environment [53], and modulating gene expression [26].

To determine the effects of n-3 PUFA supplementation on models of metabolic dysfunction, many preclinical rodent experiments have studied the effects elicited during consumption of a high fat diet and/or drug course in older animals [29, 53-55]. However, there are no models to date that have determined the role of dietary fatty acids, specifically n-6 and n-3 PUFA, on GC-induced adipose tissue alterations in young mice after a relatively short-term high fat feeding in combination with GC treatment. We specifically focused on a pre-pubertal population in order to translate the results to the paediatric ALL population, a group that is treated with GC and is at increased risk of developing obesity and metabolic dysfunction [16, 56-59]. Our study design resembled the induction phase of ALL treatment in the dose and time frame of GC administration.

Here we show that after 4 weeks of GC treatment, there were no significant differences in weight gain or food consumption, although in the absence of GC, differences were detected between the different dietary groups. Interestingly, the HF-n3 fed mice accumulated less eWAT. There was no change in fasting glucose or a significant difference in glucose clearance after IPGTT, demonstrating that these animals have been not exposed to the high fat diet for a sufficient time to induce overt metabolic dysfunction. Although liver weights were comparable, liver fat accumulation was visible histologically in all groups consuming the n-6 PUFA rich diet. As we did not quantify liver triglycerides we do not know if there is an increase in fat accumulation with GC treatment. Harvey et al. demonstrated that after 18 weeks of a 45% high

fat diet (similar to the n-6 PUFA diet used in this study) with 6 weeks of GC (dexamethasone) exposure, the GC treatment resulted in increased liver triglyceride [46]. The mice fed the n-3 PUFA rich diet however did not have any evidence of fat accumulation even in the presence of GC. This result is consistent with previous studies demonstrating that n-3 PUFAs can mitigate hepatic steatosis through GPR120 and the suppression of SREBP-1c [60] and ACC [61], in addition to increasing CTP1, ACOX1 and PPARα protein levels [62]. These n-3 PUFA-induced changes improve hepatic lipid metabolism and reduce hepatic de novo lipogenesis.

Consistent with the short timeframe of drug and diet exposure not resulting in overt metabolic dysfunction, there was also no evidence of increased systemic inflammatory molecules. Adipose tissue associated macrophages play a key role in obesity-induced inflammation. This heterogeneous population of cells has the plasticity of being polarized by environmental cues with M1 or "classically activated" polarization, evident by the expression of CD11c, resulting in a pro-inflammatory phenotype, while M2 macrophages are characterized by the expression of genes encoding anti-inflammatory proteins [63]. There is an accumulation of M1 macrophages in response to fatty acid spill over and cytokines released form stressed adipocytes resulting in an altered M1/M2 ratio which is a critical determinant for the induction of inflammation in adipose tissue [64]. In our flow cytometric analyses, we observed an increase in infiltration of macrophage for both dietary groups, with no effect of GC. However, the animals consuming the n-6 PUFA diet had a greater accumulation of $CD11c^{+}$ macrophages. Although no difference was detected for systemic IL-6 levels, epididymal adipose tissue specific IL-6 protein levels were increased in n-3 PUFA group. IL-6 is often cited as a pro-inflammatory molecule during obesity-induced inflammation, but it is known to induce the expression of Il4ra and cause M2 polarization of macrophages [65]. It has also been specifically demonstrated that IL-6 regulates M2 polarization of macrophages in diet-induced obesity [66]. In addition to IL-6, murine macrophage-like RAW264.7 cells have also been shown to become M2 polarized when exposed to the n-3 PUFA, DHA [67].

Microarray analysis of the n-6 PUFA dietary group demonstrated that GC treatment resulted in subtle changes in eWAT gene expression. Highly downregulated transcripts included Fads2 and Isyna1, both involved in fatty acids metabolism. Fads2 is especially interesting as it encodes the rate limiting enzyme needed for long chain PUFA metabolism and its downregulation would result in reduced down-stream metabolites, including arachidonic acid, DHA, and EPA. Up-regulated transcripts included Orm1 and Orm2, both involved in the acute phase response and shown to act as early immune modulators within adipose tissue- with the ability to respond to metabolic derangement and act to suppress inflammation in order to maintain energy homeostasis [68]. We also demonstrated that these transcripts were not upregulated by GC when on the n-3 PUFA diet, suggesting that it is the n-6 PUFA-induced hypertrophied adipocytes that are responsible for the induction of these acute phase proteins.

Conclusion

In conclusion, while undergoing chronic GC treatment, a diet providing n-3 PUFA can beneficially alter the eWAT environment such that it reduces the inflammatory potential and thereby the risk for development of metabolic dysfunction associated with obesity. These results demonstrate a reduction in eWAT accumulation and infiltration of "classically activated" macrophage with the n-3 PUFA rich diet. Transcriptional changes within the eWAT also demonstrate, that GC in the presence of the n-6 PUFA diet induce stress resulting in the release in acute phase proteins, attenuated with n-3 PUFA rich diet.

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Conflict of Interest

Authors do not have financial conflict of interest.

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