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Omega 3 Rich Diet Modulates Energy Metabolism via GPR120-Nrf2 crosstalk in a Novel Antioxidant Mouse Model

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Abstract

With obesity rates reaching epidemic proportions, more studies concentrated on reducing the risk and treating this epidemic are vital. Redox stress is an important metabolic regulator involved in the pathophysiology of cardiovascular disease, Type 2 diabetes, and obesity. Oxygen and nitrogen-derived free radicals alter glucose and lipid homeostasis in key metabolic tissues, leading to increases in risk of developing metabolic syndrome. Oxidants derived from dietary fat differ in their metabolic regulation, with numerous studies showing benefits from a high omega 3 rich diet compared to the frequently consumed “western diet” rich in saturated fat. Omega 3 (OM3) fatty acids improve lipid profile, lower inflammation, and ameliorate insulin resistance, possibly through maintaining redox homeostasis. This study is based on the hypothesis that altering endogenous antioxidant production and/or increasing OM3 rich diet consumption will improve energy metabolism and maintain insulin sensitivity. We tested the comparative metabolic effects of a diet rich in saturated fat (HFD) and an omega 3-enriched diet (OM3) in the newly developed ‘stress-less’ mice model that overexpresses the endogenous antioxidant catalase. Eight weeks of dietary intervention showed that mice overexpressing endogenous catalase compared to their wild-type controls when fed an OM3 enriched diet, in contrast to HFD, activated GPR120-Nrf2 crosstalk to maintain balanced energy metabolism, normal circadian rhythm, and insulin sensitivity. These findings suggest that redox regulation of GPR120/FFAR4 might be an important target in reducing risk of metabolic syndrome and associated diseases.

Graphical Abstract:

Redox regulation of GPR120-Nrf2 cross-talk in an OM3 fed catalase overexpressing mice:

Overexpression of catalase in the Bob-Cat mice model coupled with an enriched diet of OM3 fatty acids was shown to be metabolically beneficial. Energy homeostasis seen in this model was the result of induction of the GPR120/FFAR4, which by interacting with Nrf2 pathway (redox-sensitive) in adipose tissue resulted in redox balance, improved insulin sensitivity, anti-inflammation, enhanced circadian rhythm, decreased body weight and healthy fat mass.

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Keywords

Diet-induced Obesity; Redox Stress; Adipose Tissue; Catalase

1. Introduction:

The overwhelming prevalence of diet-induced obesity (DIO) and insulin resistance (IR) is strongly associated with the increased morbidity and mortality related to metabolic syndrome [1]. This is of great concern in the United States of America where the obesity rates are rising and is currently approximately 39.8%, with over 93.3 million adults affected, and there is still a lack of understanding of its etiology [2]. Redox regulation is key for systemic metabolic homeostasis. Furthermore, redox stress is an important mediator of metabolic changes seen in obesity and its comorbidities which comprise the metabolic syndrome [3]. Oxygen and nitrogen-derived free radicals alter glucose and lipid homeostasis in key metabolic tissues such as adipose, liver, brain, and skeletal muscle. During conditions of high redox stress, the body naturally attempts to compensate by increasing the production of endogenous antioxidants (including superoxide dismutase-SOD, catalase etc.) to counteract the excess free radicals that could damage signaling pathways necessary for energy production. It is believed that an increased level of reactive oxygen species (ROS) plays a key role in IR, since human and rodent models of IR are typically characterized by an imbalance in ROS compared to antioxidants/reducing agents [4, 5]. However, recent studies have also shown the importance of maintaining adequate ROS production for intracellular signaling [6]. Furthermore, the concept of reductive stress, an imbalance in the oxidative state where the ratio of oxidized to reduced molecules is too low [7–9] is also shown to be associated with an altered metabolic state such as hyperglycemia [10] or IR [11]. Therefore, a balance between free radicals and antioxidants is key in maintenance of tissue function and systemic metabolic homeostasis.

In addition to redox stress, nutritional intake plays a key role in modulating energy metabolism. DIO animal models are commonly used to study altered metabolic changes consequential to fat storage within various fat pads. In general, diets containing > 40% high-fat lard, milk, and butter promote excess lipid accumulation in adipose tissue leading to adipocyte hyperplasia and hypertrophy, alterations in adipokine secretion, hypoxia, and elevated circulating free fatty acids (FFA) in less than 8 weeks of ad libitum diet intervention [12, 13]. Furthermore, inflammatory pathways within the adipose tissue are activated as a consequence of excess lipid accumulation, which in turn drives a pro-inflammatory state provoking IR and inflammation in other metabolic tissues including liver, skeletal muscle, and pancreatic β -cells [14, 15]. The severity of the consequences to a high-fat diet is dependent on the composition, length, and degree of fatty acid saturation [16, 17]. Contrary to the negative effects seen in diets with high levels of saturated fat (lard, milk fat, and butter), high-fat diets predominately composed of omega 3 (OM3) polyunsaturated fatty acids (PUFA) have been shown to have beneficial effects on metabolic function [18–21]. In general, diets comprised of fish oil, which is high in OM3, lower systemic IR [22], decrease fasting triglyceride [23] and cholesterol levels [24, 25], and reduce inflammation [22]. These beneficial outcomes are in contrast to diets with high levels of saturated fats [16, 23]. Further

understanding of the possible mechanisms by which OM3 fatty acids promote metabolic health came when Olefsky's group discovered that GPR120/FFAR4, a free fatty acid receptor (highly expressed in adipose tissue) for which long chain omega 3 fatty acids are ligands, improved adipose tissue function and energy metabolism by its insulin sensitizing and anti-inflammatory effects [26–28]. OM3 fatty acids also alter the balance of reductive and oxidative species, and are additionally critical in glucose and lipid metabolism [26]. Furthermore, alterations in redox homeostasis through increased intake of OM3 fatty acids have been linked to activation of the nuclear factor E2-related factor 2 (Nrf2) pathway [29]. Nrf2 is a transcription factor, key in regulating redox homeostasis [30] by inducing the transcription of endogenous antioxidants including catalase, glutathione transferase, heme oxygenase (HO-1), and NAD(P)H: Quinone Oxidoreductase 1 [31–33]. These studies pointed to the plausible mechanisms by which varying dietary fat composition can influence metabolic homeostasis by modulating redox stress.

In our previous studies investigating dietary or exercise interventions in atherosclerotic mice models, we observed that increased redox stress or inflammation led to an increased antioxidant response by the tissues affected by the insult (for example vasculature). Our results showed that in most instances, the major endogenous antioxidant upregulated in response to the insults was, catalase [34, 35]. Catalase is a major antioxidant, endogenously produced by various tissues, to neutralize excess hydrogen peroxide (H_2O_2) produced by dismutation of superoxide, yielding water and oxygen [36]. In addition to our studies, numerous other studies have shown that catalase (mouse) overexpression for example, targeted to mitochondria (mCAT) in mice provided evidence of being an anti-cancer agent by delaying the progression of transgenic oncogene and syngeneic tumors [37], while overexpression of catalase (human) in mitochondria showed improvements in skeletal muscle function in aged rodents vs their WT littermates [38]. In the context of cardiovascular disease, restoration of catalase activity in the vascular aortic wall profoundly reduced inflammatory markers and prevented abdominal aortic aneurisms through modulation of matrix metalloproteinase activity [39]. On the other hand, negative metabolic consequences occur in systems devoid of catalase. Within the context of DIO, a catalase knockout rodent model had exacerbated IR, amplified oxidative stress, and accelerated macrophage infiltration in epididymal white adipose tissue [40] indicating catalase is a key antioxidant vital for glucose homeostasis and adipose tissue function. More recently, Heit *et al* showed mice devoid of catalase developed an obese, pre-diabetic phenotype, further showing the importance of antioxidant catalase in metabolic regulation [41]. These evidences support catalase as an ideal antioxidant for investigating the effects of redox balance in obesity and its associated comorbidities due to its vital role in metabolic homeostasis in both humans and rodent models. The findings discussed in these studies led us to generate a mouse overexpressing catalase which will serve as a good model to study redox regulation of metabolic diseases. We hence generated the “Bob-Cat” stress-less mice model, a hybrid between catalase transgenic mice [Tg(CAT)[±]] [42] and leptin-deficient, obese mice (heterozygous JAX 000632, B6.Cg-Lep^{ob}/J). We have earlier shown that this novel mouse model had lower redox stress and improved adipose function compared to the Ob/Ob phenotype (JAX 000632, B6.Cg-Lep^{ob}/J) and expressed both human and mouse catalase [43].

We hypothesized that modulating redox stress by altering endogenous antioxidant content (overexpression of catalase) and/or via dietary intervention will improve energy metabolism, adipose tissue function, and overall glucose and lipid homeostasis. To better understand the interplay between redox regulation and dietary intervention in improving energy balance and maintaining insulin sensitivity, we compared the redox effects of a high-fat lard diet (HFD) and a high-fat omega 3-enriched diet (OM3) in a catalase-overexpressing ‘stress-less’ mice model [43]. In this model, we previously showed the ubiquitous overexpression of catalase altered anthropometric parameters, overall energy metabolism, as well as adipose tissue function in both male and female mice compared to WT controls [43]. These characteristics make this mice model an excellent method for studying dietary effects of high fat lard and fish oil diets on metabolic homeostasis of both male and females, now critical for all clinical trials. Our study showed that OM3 enriched diet, in contrast to the HFD intervention activated the GPR120-Nrf2 cross-talk to maintain balanced energy metabolism, normal circadian rhythm and insulin sensitivity in mice overexpressing catalase compared to the WT controls. Therefore, increasing endogenous antioxidant production in combination with an OM3 rich diet will maintain energy balance, improve adipose tissue function, and lower risk of obesity and its comorbidities.

2. Methods

2.1 Mouse Model and Diets

A successful breeding colony of both catalase transgenic [Tg(CAT)[±]] and the ‘stress-less’ Bob-Cat mice, which ubiquitously expresses both human and mouse catalase, has been established in our facility [43]. The care and use of animals was performed according to protocols approved by Marshall University IACUC. To ensure relevance to human clinical studies conducted on obese adults, it was necessary to use fully developed mice (thus being between 12–24 weeks of age which correlates with a 20–30 year old human [44]) and a 45% high fat diet. According to the Center for Disease Control (CDC database derived from NHANES studies), obese individuals typically consume a 45% fat diet (the “Western Diet” [45]. Therefore, sixteen week old [Tg(CAT)[±]], Bob-Cat, and their control C57Bl6/WT mice (n = 8/group/sex) were maintained on a 12h light/dark cycle and fed ad libitum either normal chow-NC (Lab Diet-5001, St. Louis, MO), High-Fat Lard-HFD diet (Envigo TD06415, Somerset, NJ) containing 45 kcal% Lard, or Omega-3 rich-OM3 diet (Envigo TD130700, Somerset, NJ) containing 45 kcal% of Menhaden Fish Oil, for 8 weeks (Supplementary Table 1). Both male and females were used due to the differences in overall regulation of energy homeostasis and metabolism of essential fatty acids (such as OM3) between the two sexes [46, 47]. In order to investigate chronic effect of the dietary intervention, we conducted an 8 week feeding study. Animal body weights and food consumption were recorded weekly. Energy consumed per diet group was determined by dividing the grams of chow consumed by kcal per grams (g) of chow.

2.2 Fat and lean body mass-ECHO-MRI

Body composition (fat and lean mass) was determined using magnetic resonance imaging, ECHO-MRI (Houston, TX) according to manufacturers’ recommendations. Each mouse was individually placed within the MRI machine and three or more measurements were taken.

Median values of fat and lean mass readings were calculated per mouse and subsequently averaged per genotype and diet. Comparisons between groups were made by one or two-way ANOVA.

2.3 Metabolic Parameters (Comprehensive Laboratory Animal Monitoring System-CLAMS)

Changes in metabolic parameters in response to the dietary interventions were determined during the final week of the study using indirect calorimetry by measuring O₂ consumption (VO₂) and CO₂ production (VCO₂), respiratory exchange rate (RER), food intake (FI), Energy Expenditure (EE), as well as X-Ambulatory counts (XAMB, physical activity) using the CLAMS system (Columbus Instruments, Columbus, OH, USA). As recommended in the instruction manual, each mouse was placed individually in the metabolic cages and was supplied with a sufficient amount of their respective diet for the duration of the data collection (three consecutive days). Analyses were made using the data collected during the middle 48 hours of the 72-hour procedure, which is from approximately 0600 hours of the first day to 0600 hours of the third day. These time points allowed for both adequate time to acclimate to the CLAMS environment and provide accurate data for assessment of all measured parameters. Food intake (FI) was measured by CLAMS and energy intake was calculated by dividing the grams of food consumed by the kcal of energy per gram of each laboratory diet. RER is calculated as the ratio of carbon dioxide production and oxygen consumption. Carbohydrate (CHO) oxidation was calculated using the formula $((4.585 \times \text{VCO}_2) - (3.226 \times \text{VO}_2)) \times 4$, and similarly, fat oxidation was calculated using the formula $((1.695 \times \text{VO}_2) - (1.701 \times \text{VCO}_2)) \times 9$ as described by Peronnet et. al [48]. EE (heat production) was calculated as the Cal/h/lean mass (g) to account for the lean body weight. Average values of EE, RER, FI per day, as well as XAMB locomotor activity per day (counts movement made across the cage measured with infrared sensors) were determined for each mouse in all groups. Results were further broken down into light and dark cycles at 2 h time intervals for a total of 48 hours per mouse. One and two-way ANOVA was used to determine comparative changes between the various genotypes fed NC, HFD or OM3 diets.

2.4 Blood and Tissue Collection

At the end of 8 weeks, animals were fasted overnight and anesthetized using isoflurane immediately prior to cardiac puncture. Blood was collected in heparinized tubes, centrifuged, plasma separated, and stored. Tissues including adipose and liver were removed, weighed, and flash frozen in liquid nitrogen, followed by long-term storage at -80°C.

2.5 Circulating metabolic parameters

Whole blood was used to measure fasting glucose and ketone levels (Precision Xtra Glucometer) then centrifuged for 10 min. to separate the plasma and red blood cells. Thirty-five µL of plasma was placed on a Cholestech cassette and read on a LDX Cholestech system (Cholestech Corporation Hayward, CA) to determine Glucose, High Density Lipoprotein (HDL), Low Density Lipoprotein (LDL), and Total Cholesterol (TC) levels. If data collected fell outside the range (sensitivity of the assay), the less than (“<”) or (“>”)

was used to best represent the results. The remaining plasma was frozen at -80°C for further analysis of circulating markers.

Triglyceride (TG) levels were assessed in plasma using Triglyceride Colorimetric Assay Kit (Cayman Chemicals, Ann Arbor, MI). Plasma insulin was analyzed using an ultrasensitive mouse Insulin ELISA kit (Crystal Chem, Downers Grove, IL). The end point colorimetric assays were performed using a BioRad Benchmark Plus microplate reader according to manufacturer's instructions.

HOMA-IR is a surrogate measure of insulin resistance routinely used in research studies [49–51]. HOMA-IR was calculated using the formula: fasting insulin [$\mu\text{IU/ml}$] x fasting glucose [mmol/L]/22.5.

Circulating FGF-21 was assessed using Mouse and Rat FGF-21 ELISA (Biovendor, Modrice, Czech Republic) according to manufacturer's protocol. Absorbance was read using a BioRad Benchmark Plus microplate reader. Calculations were conducted in accordance with the best-fit line created from the standard curve of plotted absorbance values against the known concentrations of standards.

2.6 Adipose mRNA Expression

RNA was isolated from 100 mg of perigonadal adipose tissue using TRI Reagent according to the manufacturer's recommended protocol (Sigma, Saint Louis, MO). RNA concentration was determined using the NanoDrop 1000 (NanoDrop Technologies Inc., Thermo Scientific, Wilmington, DE, USA). Reverse transcription of total RNA ($1\text{ }\mu\text{g}$) was performed using iScriptTM cDNA Synthesis Kit (Bio-Rad, Hercules, CA). RT-qPCR was conducted using iQ SYBRTM Green Supermix (Bio-Rad, Hercules, CA). The mouse primers used in this study are provided in Supplementary Table 2. 18S and β -Actin primers were used as the housekeeping reference genes. All samples were analyzed in duplicate or triplicate in the Bio-Rad MyiQ or Bio-Rad CFX ConnectTM (Bio-Rad, Hercules, CA) instrument and a transcript was considered non-detectable when the Cq value was ≥ 40 . The mRNA level of the gene of interest for each group was normalized to that of the referenced control using the comparative Pfaffl Equation ($2^{-\Delta\text{CT}}$) [52] and expressed as fold change compared to the control C57Bl6/WT mice fed normal chow (WT NC) or WT within each dietary group.

2.7 Western Blot

Approximately 50 mg of perigonadal adipose tissue was homogenized in 100 μL of Radio-immunoprecipitation assay buffer (RIPA buffer) supplemented with protease inhibitor cocktail. Protein concentrations were determined by the Lowry Method [53]. For each sample, 50–60 μg protein, were prepared in loading buffer (90% Laemmli and 10% 2-mercaptoethanol) and boiled for 5 min. at 100°C . Samples were run using SDS-PAGE and separated on 12% Precast Gel (BioRad, Hercules, CA), at 100 V for 60–70 min. Electrophoretic transfer of the proteins onto a nitrocellulose membrane was performed at 100V for 60 min. on ice. Thermo Scientific Memcode Stain: Pierce MemCodeTM Reversible Protein Stain Kit (Thermo Fisher Scientific, Rockford, IL) was used as a loading control. Membranes were blocked with 1X Tris Buffered Saline (1X TBST), 0.05% Tween 20, pH 7.6, and 5% dry milk for one hour at room temperature. Blots were incubated overnight with

mouse GPR120 antibody (1:250 in 1X TBST and 5% dry milk) (Santa Cruz, Dallas, TX). After washing with 1X TBST, membranes were incubated with secondary anti-mouse IgG (1:3000 in 1X TBST and 5% dry milk) for 60 min. at room temperature. Membranes were washed and the immunocomplex was detected with Luminata™ Forte Western HRP (Millipore, Billerica MA). Densitometry of the bands was quantified using BioRad Image Lab Software (BioRad, Hercules, CA) and normalized to MemCode Stain of total protein.

2.8 Catalase Enzymatic Activity

Catalase activity was measured in perigonadal adipose tissue protein lysates using the method of Aebi [54]. Approximately 50 mg of adipose tissue was homogenized in 100 μ L of 50 mM KH_2PO_4 , 5 $\mu\text{g}/\mu\text{L}$ Aprotinin, and 2 μL of 0.1 M PMSF. Eight microliters of each homogenate was added to 1 mL of 25 mM of Hydrogen Peroxide (H_2O_2) and analyzed on a Shimadzu Spectrophotometer for one minute. The initial rate of disappearance of H_2O_2 was recorded for 1 min. at a wavelength of 240 nM. Each sample was measured in duplicate or triplicate. A standard curve was generated using 1–5 units of bovine catalase (Sigma, Saint Louis, MO). Specific activity of catalase was calculated based on the standard curve and total protein used based on the Lowry's Method [53].

2.9 Quantification of Oxidized Proteins

Carbonylated proteins are a hallmark of redox stress [55]. Oxidized proteins were measured in lysates of perigonadal adipose tissue by determining the presence of carbonylated proteins using the Protein Oxidation Detection OxyBlot kit (Millipore, Billerica, MA) in accordance with the manufacturer's instructions. The method is based on the principle that proteins modified by oxidative stress result in an addition of carbonyl groups to their side-chains. The carbonyl groups are detected after derivatization to 2,4-dinitrophenylhydrazine (DNP) by treating with 2,4-dinitrophenylhydrazine (DNPH). An antibody specific to DNP is then used to determine carbonylated proteins (relative oxidative stress levels) in each sample. Briefly, each lane was loaded with 20 μg of the derivatized protein and ran on a 12% BioRad Precast Gel. After gel electrophoresis, proteins were transferred to a nitrocellulose membrane at 100 V for 60 min. Equal loading and transfer efficiency was evaluated by use of the Pierce MemCode™ Reversible Protein Stain Kit (Thermo Fisher Scientific, Rockford, IL). Non-specific binding sites were then blocked for one hour with 1X Phosphate Buffered Saline (1X PBST) and Tween 20 and 10% Bovine Serum Albumin (BSA). A 1:500 dilution of primary antibody, Rabbit-Anti-DNP (Millipore OxyBlot Kit, Billerica, MA) was added and rocked overnight at 4°C, followed by washes with 1X PBST. Blots were conjugated with a 1:300 dilution of goat anti-rabbit IgG (Horseradish Peroxidase conjugated) for 1 h rocking at room temperature. Membranes were washed and the immunocomplex was detected with Luminata™ Forte Western HRP (Millipore, Burlington, MA). All images were acquired and analyzed by a BioRad ChemiDoc and Image Lab Software (BioRad, Hercules, CA). Oxidized proteins were expressed as the ratio of the optical density of dinitrophenylhydrazine (DNP) to total protein as determined by the Memcode stain.

2.10 Statistical Analysis

Data were statistically analyzed using one and two-way ANOVA followed by Bonferroni's multiple comparison tests using Version 7 of GraphPad Prism among all mice groups. Data

are presented as mean $\pm$ standard error of the mean (S.E.M.) unless fold change or percent of WT was provided. $p < 0.05$ was considered statistically significant. RT-qPCR gene expression was determined by use of the Pfaffl equation [52] and represented as fold change with significance denoted as differences in delta CT/genotype and diet. GPR120 protein and oxidized carbonyl protein analysis was represented as percentage of the WT control mouse group normalized by total protein quantified by memcode stain.

3. Results

3.1 Body weight and body composition

Sixteen week old male mice overexpressing (human and mouse) antioxidant catalase ([Tg(CAT)[±]] and Bob-Cat) [43] were fed either normal chow (NC), high-fat lard (45% kcal, HFD), or high-fat omega 3 enriched (45% kcal, OM3) diet, ad libitum for a period of 8 weeks. Changes in body weight and body composition (fat and lean mass ratio) in the catalase overexpressing mice were compared to the control wild type (WT) mice fed similar diets over the 8 week intervention. Body weight recorded each week and averaged per intervention group. As shown in Figure 1, all three genotypes maintained their body weights when fed NC over the 8 week period. However, WT and [Tg(CAT)[±]] mice consuming a high-fat lard diet (HFD) gained significantly more weight (8 and 10 fold, $p < 0.0001$) compared to their NC fed littermates (Figure 1A and 1B), but interestingly, Bob-Cat mice on HFD only gained approximately 5 fold increase in body weight (not significant) compared to their NC fed littermates. In contrast, when mice were fed OM3 (45% kcal menhaden fish oil) an increase in body weight was only observed in the WT mice group. In fact, there was a loss in body weight in both [Tg(CAT)[±]] (5 fold lower) and Bob-Cat (>1 fold lower) mice fed OM3 diet compared to their NC fed littermates. Even more interesting was the significant decrease in body weight observed in the [Tg(CAT)[±]] (>5 fold, $p < 0.0001$) and Bob-Cat (>2 fold, $p < 0.01$) mice fed OM3 diet compared to the WT mice fed the same diet. These dietary influences were observed in both the overall change in body weights (Figure 1A) as well as the average weekly changes in body weight measured throughout the study (Figure 1B), showing a general trend of increased body weight per week in the HFD and steady or decreased body weight in the OM3 fed groups among the mice overexpressing catalase. Two-way ANOVA showed a significant difference in body weight between genotype ($p < 0.05$) and diet ($p < 0.0001$) groups in addition to the observed interaction between the two ($p < 0.0001$) factors.

Body composition (total fat and lean mass) were determined for each mice group by ECHO-MRI at baseline, 4 week, and 8 week time points (Figure 1C and 1D). Within the males, there was a significant difference among the diet groups in fat mass by the 8 week time point ($p < 0.0001$). When provided a HFD, mice gained a significant amount of fat mass compared to the same genotypes provided NC ($p < 0.05$), but overall there was a trend for the WT mice to accumulate more fat mass at a faster rate than mice overexpressing catalase (Figure 1C). However, when provided an OM3 rich diet, the WT group gained only a 3 fold increase in fat mass ($p < 0.05$) compared to the 8–10 fold increase when fed HFD. This gain in fat mass was even lower and not significantly different in [Tg(CAT)[±]] and Bob-Cat mice compared to NC fed littermates (1–2 g) when fed OM3 rich diet. Lean mass measurements revealed NC

fed WT mice trended to gain the largest amount of lean mass. HFD did not significantly alter the lean mass of any genotype, however, all male mice provided an OM3 diet gained lean mass from baseline to the 8 week time point (-0.5 g) with the exception of the [Tg(CAT)[±]] group (Figure 1D) which lost 2.2 g of lean mass. This loss in lean mass in the [Tg(CAT)[±]] was reflective of their loss in total body weight when fed OM3 diet. Bob-Cat mice on OM3 rich diet gained 1.67 g of lean mass at 4 weeks and then decreased almost back to baseline by the 8 week time point.

As seen in males, the female WT, [Tg(CAT)[±]], and Bob-Cat mice of each diet group also showed an overall significant difference in body mass with regard to diet ($p<0.0001$) (Figure 2). The NC fed mice had minimal changes in body weight over the 8 week period, while all genotypes provided HFD diet gained between 2.5 – 3.5 g of body weight (not significant). Additionally, similar to males, female Bob-Cat mice provided an OM3 diet lost a significant amount of weight compared to every other genotype provided HFD (Figure 2A). Weekly body weight changes (Figure 2B) showed that all mice fed an OM3 diet did not gain more than one gram of weight over the entire 8 week period.

Similar to males, ECHO-MRI showed that females on HFD, regardless of genotype, increased fat mass at a much faster rate and doubled the amount of fat gained in comparison to both their NC and OM3 fed littermates (Figure 2C). Mice provided an OM3 diet did not gain significant amounts of fat mass in comparison to the NC or OM3 fed WT groups. Interestingly, Bob-Cat females on OM3 diet had a $>9\%$ increase in lean mass in comparison to NC fed Bob-Cats and WT controls ($p<0.05$; Figure 2D) in spite of them losing the most body weight (Figure 2A).

3.2 Liver and adipose weights

In addition to a gain in visceral adiposity, another hallmark of the metabolic syndrome is the redistribution of ectopic fat in other metabolic tissues such as the liver. Therefore, visceral adipose tissue and liver weights were measured during tissue collection from all groups. In males, analysis by two-way ANOVA revealed a significant difference in visceral adipose tissue weight among diet groups ($p<0.0001$). A threefold increase in visceral adipose tissue was observed in all groups fed HFD in comparison to the NC fed WT group as displayed in Table 1. In contrast, mice provided OM3 diet did not have significantly larger visceral fat depots than NC fed mice groups. However, the [Tg(CAT)[±]] and Bob-Cat mice fed OM3 diet had much lower levels of visceral fat in comparison to their respective HFD fed groups in addition to a twofold lesser weight compared to the OM3 fed WT group. This data correlated with the observed body weight and fat mass (ECHO-MRI analysis) changes seen in these mice groups. In regard to the liver weight, analysis by two-way ANOVA showed a significant effect among the genotypes of male mice ($p<0.0001$). It was intriguing that the NC and HFD fed [Tg(CAT)[±]] groups had much larger livers ($>17\%$ increase, $p<0.001$) compared to NC WT group. However, Bob-Cats and all mice groups provided OM3 diet did not show significant differences in liver weight.

Female mice showed similar trends with the highest visceral adiposity seen among the HFD fed mice (Table 1). Significant increases in visceral adipose tissue was observed between [Tg(CAT)[±]] on HFD and WT mice on NC diet ($p<0.01$). OM3 fed female groups also did

not significantly differ in adipose tissue weight compared to their NC fed littermates. Interestingly, and contrary to male mice, females provided HFD had lower liver weights (no significance) and mice provided an OM3 diet showed a trend towards higher liver weight compared to all other diet groups.

3.3 Weekly food and energy consumption

Weekly food and energy consumption for each mice group was measured to determine if caloric intake was responsible for the observed differences seen in body weight or fat/lean mass. Twoway ANOVA showed an overall significant association between diet and genotype ($p < 0.0001$) in addition to a significant difference observed within the genotypes ($p < 0.0001$) and the various dietary interventions ($p < 0.0001$). As depicted in Figure 3A, a significant increase in average food intake was seen for NC fed [Tg(CAT)[±]] and Bob-Cat mice ($p < 0.0001$ and $p < 0.01$ respectively) compared to the NC fed WT group. Additionally, when provided a HFD or OM3 diet, all groups ate significantly less ($p < 0.01$) grams (g) of food in comparison to NC fed [Tg(CAT)[±]] and Bob-Cat mice. Figure 3B showed that as the study progressed, mice provided HFD or OM3 diet ate less chow per week. Due to differences in the total calories between the three diets (NC = 4.09 kcal/g compared to 4.6 kcal/g (HFD and OM3), (shown in Supplemental Table 1), the average kcal/g consumed was also calculated for each mice group to determine total energy intake. Two-way ANOVA showed a significant difference with regard to male mouse genotype ($p < 0.0001$) and diet group ($p < 0.0001$). As shown in Figure 3C, there was a general trend of lower energy intake when mice were fed HFD or OM3 diet in comparison to NC. A significant trend was observed when mice overexpressing catalase fed NC consumed more energy than all groups fed HFD or OM3 diet.

Weekly food intake among the female mice groups (Figure 3D–F) showed similar eating patterns to those observed in the male mice groups. There was a significant interaction between diet and genotype ($p = 0.0013$), genotype alone ($p < 0.001$), and diet alone ($p < 0.0001$). The largest quantity of chow consumed was by the groups provided NC regardless of the genotype (Figure 3D). A significant reduction in food intake was seen in all mice provided a HFD or OM3 diet ($p < 0.0001$) in comparison to the NC fed WT group. This trend was similar between males and females. Regarding energy intake, there was a significant interaction between the genotype and diet group ($p = 0.0017$), as well as between genotype alone ($p < 0.001$), and between diet groups ($p < 0.0001$) (Figure 3F). Female mice provided HFD or OM3 diet consumed less energy compared NC fed littermates. However, the decrease in food and energy intake did not correlate with the increase in body weight and adiposity seen in HFD diet fed animals compared to NC and OM3 diet fed littermates.

3.4 Blood lipid profile and ketones

Post-surgery, the lipid profile was measured on plasma samples collected from each mouse fasted at least 12 h prior to the surgery using an LDX Cholestech kit. As shown in Table 2, all mice groups fed HFD ($p < 0.001$) had significantly higher Total Cholesterol (TC) levels in comparison to the NC fed WT mice and all mouse groups on OM3 rich diet. In the groups fed HFD or OM3, the Bob-Cat mice had the highest levels of HDL followed by the HFD fed [Tg(CAT)[±]]. This is also true when comparing the OM3 Bob-Cat group to every group

provided NC. Two-way ANOVA analysis of plasma triglyceride (TG) levels showed there was a significant genotype ($p<0.0001$) and diet ($p<0.01$) interaction among the groups. Interestingly, male Bob-Cat mice, regardless of diet, had the highest levels of plasma TG. In fact, within each diet group, Bob-Cat male mice had 3 fold higher TG levels compared to the other two genotypes.

As shown in Table 2, female TC level was < 100 mg/dL for all mice groups except the HFD fed WT and Bob-Cat groups. The female HFD fed [Tg(CAT) $^{\pm}$] mice displayed much lower TC (<100 mg/dL) compared to the male mice (162.3 ± 9.16 mg/dL) fed the same diet. (It is to be noted, when individual mice readings fell outside the assay's range of sensitivity, the less than (" $<$ ") or (" $>$ ") was used to best represent the average results from each mice group.) Assessment of HDL revealed a significant diet interaction ($p<0.003$). Female mice trended to have a little lower HDL levels in comparison to males. However, consistent with males, highest levels of HDL was found in the [Tg(CAT) $^{\pm}$] HFD fed females. Another interesting trend observed was that within the OM3 diet fed groups, the Bob-Cat male and female groups had the highest levels of HDL. Data analysis of TG levels revealed a significant interaction between diet and genotype ($p=0.01$) within the female mice groups. There was an overall trend for HFD mice to have equal or increased levels of plasma TG in comparison to their respective genotypes on NC or OM3 diet. However, it was interesting that OM3 diet only showed a trend towards lower TG levels compared to NC and HFD in the WT group. There was also a gender effect where female WT and [Tg(CAT) $^{\pm}$] mice, regardless of diet, expressed higher levels of TG in comparison to the males. However, Bob-Cat females had lower levels of circulating TG in comparison to male Bob-Cats.

Ketone levels of NC fed [Tg(CAT) $^{\pm}$] mice were lower (not significant), but the Bob-cat group ($p<0.05$) were significantly higher than the NC fed WT male group. When mice were provided HFD, each genotype doubled its ketone level compared to their NC littermates with the exception of the HFD fed Bob-Cat mice, which showed the lowest levels within the HFD groups (3 fold decrease, $p<0.05$). OM3 diet feeding also increased plasma ketone levels (2 fold increase) in comparison to littermates provided NC. Specifically, the OM3 fed WT group ($p<0.01$) had significantly higher levels than the NC fed WT, and the OM3 Bob-Cat males having the highest levels of all groups [> 3 fold compared to the NC fed WT ($p<0.0001$) and ≈ 2 fold compared to the NC fed Bob-Cat group ($p<0.05$)].

A significant interaction between diet and genotype was seen in fasting ketone levels ($p<0.01$). Within females, the ketone levels showed a trend to be higher than males within each respective mice group with the exception of mice provided the OM3 diet.

3.5 Metabolic parameters using CLAMS

Metabolic parameters were determined at the end of the 8 week study using indirect calorimetry by measuring O_2 consumption (VO_2) and CO_2 production (VCO_2), Respiratory Exchange Rate (RER), Food Intake (FI), Energy Expenditure (EE), as well as X-Ambulatory counts (XAMB - physical activity) using the Comprehensive Laboratory Animal Monitoring System (CLAMS) (Columbus Instruments, Columbus, OH, USA).

3.5.1 Food intake (CLAMS)—A three-day assessment of food intake (FI) (Figure 4A–B) was conducted using CLAMS, which allowed analysis of eating patterns and insight on circadian rhythm. All male mice on HFD in the WT and [Tg(CAT)[±]] mouse groups ate at a more constant rate throughout the 48 hour time period compared to mice provided NC and mice overexpressing catalase (fed OM3 diet) which ate more frequently through the dark cycle and less during the light cycle. Most intriguing, was the evidence that male Bob-Cat mice followed a similar eating pattern to the NC fed group (decreased food intake during light cycle and increased during the dark cycle) indicating that diet (NC or OM3) had not altered their circadian rhythm to the degree that was observed in other two genotypes fed HFD and the WT mice fed OM3.

As shown in Figure 4B, CLAMS analysis on female mice provided NC showed a normal eating pattern (higher consumption in the dark vs light cycle). Similar to what was seen in the male mice groups, the female Bob-Cats on HFD showed a normal eating pattern in contrast to the other two genotypes fed the same diet. However, when fed OM3 diet, the differences in the eating pattern were lost. In general, a more stable pattern of energy consumption occurred in mice with lower fat mass as shown previously in Figure 1 and 2.

3.5.2 Energy expenditure and physical activity (CLAMS)—In addition to FI, Energy Expenditure (EE) and physical activity (XAMB) were also analyzed using the CLAMS technology. As seen in Figure 5A, EE, calculated indirectly as Cal/h/g of lean body mass in each male mice group, did not significantly differ between genotypes when provided NC diet. The HFD fed groups had significantly higher levels of EE in the dark cycle ($p < 0.01$) compared to NC fed WT mice. However, during the light cycle, HFD [Tg(CAT)[±]] mice had much lower levels than both groups overexpressing catalase provided HFD. EE in all groups provided OM3 diet remained at intermediate levels to that seen in the NC and HFD fed groups, yet levels were significantly higher than the NC WT group ($p < 0.05$). Overall, differences between groups were much greater during the dark vs. light cycle. Analysis of the X-Ambulatory (XAMB) activity, depicted in Figure 5B, showed there was higher activity in the [Tg(CAT)[±]] and Bob-Cat mice compared to NC WT mice ($p < 0.05$). Interestingly, these were also the two groups where the greatest weekly food intake and energy intake (Figure 3A & C) was also observed. Likewise, when all genotypes were provided HFD or OM3 diet, the WT mice had the lowest activity levels, not reaching above 3000 counts per 2 hours, suggesting that the overexpression of catalase might have some influence on the activity levels observed in the male groups.

Overall, within female groups the analysis of EE showed less variation in circadian rhythm (Figure 5C) in comparison to that observed within male groups. Similar to males, females on HFD had higher levels of EE in comparison to the mice fed NC or OM3, with the WT mice fed HFD showing the highest levels during both the light and dark cycles ($p < 0.05$) and the [Tg(CAT)[±]] mice mostly during the dark cycle ($p < 0.01$). XAMB analysis among female groups on NC and OM3 diet showed a trend for Bob-Cat mice to have higher activity levels than WT or [Tg(CAT)[±]] mice. This may have accounted for the observed lower body weight in the Bob-cat mice shown earlier (Figure 2). Animals fed HFD had either similar or lower activity levels compared to NC fed WT mice with the exception of the female HFD fed

[Tg(CAT)[±]] mice which displayed significantly higher activity during the dark cycle (3 fold, $p < 0.01$).

3.5.3 Respiratory Exchange Ratio (CLAMS)—Using CLAMS, we also determined the Respiratory Exchange Ratio (RER) for each mice group at the end of the 8 week study. RER, an indication of which type of fuel (carbohydrate (CHO) or fat) is primarily being metabolized to supply energy demands, was lowest in the male NC fed WT mice group as well as in all HFD fed mice (Figure 6A). However, when groups overexpressing catalase were provided NC, the RER was significantly higher compared to the WT mice during both light ($p < 0.01$) and dark cycles ($p < 0.001$). When provided an OM3 enriched diet, the RER levels remained intermediate between the NC and HFD groups. As seen within the NC groups, the mice overexpressing catalase fed OM3 also had a higher RER in comparison to the OM3 fed WT groups. Since RER levels reflect the circadian patterns of food consumed, it also followed similar light and dark cycle pattern as seen with FI (Figure 5). The NC fed groups followed a normal circadian pattern but the HFD fed groups did not. It was interesting to note that Bob-Cat mice fed NC or OM3 diet followed a normal pattern which was not evident when these mice were fed HFD.

We further delineated the levels of CHO and fat oxidation based on the VO₂ and VCO₂ data derived from the CLAMS analysis. As seen in Figure 6B, the most significant differences seen in CHO oxidation of male groups were observed during the dark cycle. However, in comparison to the WT groups, mice overexpressing catalase provided NC had significantly higher levels of CHO oxidation ($p < 0.0001$) independent of the light or dark cycle. Conversely, when provided HFD, there were no differences between genotypes, but all mice groups had significantly lower levels of CHO oxidation than the NC fed WT groups during the dark cycle. Similar to that seen with the NC groups, OM3 diet also showed that mice overexpressing catalase had significantly higher levels of CHO oxidation compared to both the NC fed WT mice and OM3 fed WT mice independent of the time of day. Contrary to CHO oxidation, NC fed WT mice had significantly higher levels of fat oxidation ($p < 0.0001$) compared to every other group during the light cycle and significantly higher levels compared to mice overexpressing catalase ($p < 0.0001$) on NC diet in addition to OM3 diet during the dark cycle. Mice provided HFD showed a trend for having the highest levels of fat oxidation regardless of the genotype during the dark cycle (not significant). When fed an OM3 diet, it was interesting that the mice overexpressing catalase had significantly lower levels of fat oxidation in comparison to the WT mice provided OM3 diet during both light and dark cycles. However, in comparison to litter mates provided NC, both groups overexpressing antioxidant catalase had significantly lower levels of fat oxidation ($p < 0.01$) during the dark cycle.

When comparing the various diets, female mice followed similar trends in RER compared to what was seen in male mice (Figures 6 & 7). NC fed female diet groups had the typical circadian cycles. Although unlike male mice, female NC fed Bob-Cats showed a trend for having a lower RER than the NC fed WT females throughout the light and dark cycles. Within the female HFD groups, WT and [Tg(CAT)[±]] had a significantly lower RER during both light and dark cycles ($p < 0.01$), while Bob-Cats only showed a trend. However, it was interesting that the HFD fed Bob-Cats had a significantly higher RER compared to the HFD

fed WT group during the dark cycle ($p<0.05$). When provided an OM3 diet, Bob-Cat and WT groups had a significantly lower RER compared to NC fed WT mice ($p<0.01$), but [Tg(CAT)[±]] mice only had significantly lower levels ($p<0.05$) during the dark cycle. This was not seen in the male mice groups overexpressing catalase fed OM3 diet, where we observed higher levels of RER than the NC fed WT mice.

Calculations of CHO oxidation in females showed that in contrast to males, NC fed female mice overexpressing catalase had similar or significantly lower levels of CHO oxidation (NC fed Bob-Cat) compared to their WT littermates (Figure 7B). On the other hand, similar to males, when fed a HFD, the CHO oxidation in each mice group was significantly decreased in the dark cycle compared to NC fed WT mice and no significant differences were seen among the female genotypes fed HFD. Also, as observed in males, OM3 fed female [Tg(CAT)[±]] mice had significantly higher levels of CHO oxidation ($p<0.0001$) compared to both NC and OM3 fed WT mice, but levels did not significantly differ from NC fed WT mice. On the other hand, Bob-Cat females either had similar or significantly lower levels of CHO oxidation compared to their NC or OM3 fed WT control groups. With regard to fat oxidation, the same general trends occurred in both female and male diet groups with the exception that female fat oxidation trended to be much higher (kcal/h/g) than males on the same intervention. It is of special interest however, among the female groups, the NC and OM3 Bob-Cat mice groups trended to have the highest levels of fat oxidation regardless of the time of day. This may be an indication of why these groups trended to have one of the lowest body weight and fat mass compared to the other intervention groups (Figure 2).

3.6 Insulin Sensitivity

Both redox stress and dietary interventions (HFD and OM3) can modulate insulin sensitivity; hence we measured fasting levels of glucose and insulin and subsequently calculated the HOMAIR, an indirect measure of insulin sensitivity. As depicted in Figure 8A, a genotypic ($p<0.01$) and dietary effect ($p<0.0001$) was observed in glucose levels. There was a trend for lower glucose levels in mice fed OM3 diet compared to NC mice groups, and significantly lower levels in mice fed OM3 vs. HFD fed groups ($p<0.01$). Bob-Cat mice on OM3 diet had the lowest glucose levels in comparison to every other group. All genotypes on HFD had increased levels of glucose compared to every other group. Similarly, a significant genotypic ($p=0.0005$) interaction was seen in circulating insulin levels (Figure 8B). Interestingly, in spite of the large range within the group, the HFD fed [Tg(CAT)[±]] mice had the highest insulin levels compared to any other mice group ($p<0.05$). Calculated Homeostasis Model Assessment for Insulin Resistance (HOMA-IR) (Figure 8C), revealed a significant difference in genotype ($p<0.001$) and diet ($p<0.05$). As expected, due to the larger range in the insulin levels within this group, the [Tg(CAT)[±]] mice on HFD also had the highest calculated HOMA-IR ($p<0.01$). Interestingly, ketone levels showed no significant differences in the [Tg(CAT)[±]] male and WT mice fed NC where insulin signaling was optimal (Table 2). In fact, the ketone levels in the HFD fed WT male group were >2 fold higher than the levels in the [Tg(CAT)[±]] group on HFD, thus indicating insulin is repressing ketone body production. Thus, these mice may not be classified as insulin resistant or hyperinsulinemic [56]. In contrast, [Tg(CAT)[±]] mice on NC or OM3 had much lower insulin levels and calculated HOMA-IR.

In female mice, glucose levels showed no significant differences between groups as depicted in Figure 8D. However, when comparing diets, the [Tg(CAT)[±]] mice had the highest levels of glucose within each dietary group. Also, as observed in males, there was a trend for HFD fed mice of each genotype to have an overall increase and mice provided OM3 diet to have an overall equivalent or reduced plasma glucose level compared to littermates placed on NC, though none reached significance. An overall diet interaction was observed for insulin (Figure 8E) among female groups ($p < 0.001$). Contrary to the highest levels of insulin in the [Tg(CAT)[±]] males, the female mice had the lowest average insulin levels of all mice provided HFD. Additionally, in contrast to males, female ketones did not significantly differ in the female HFD fed [Tg(CAT)[±]] in comparison to any other female mice group (Table 2). Bob-Cat mice within each diet group showed the highest insulin levels among the genotypes, and was significantly higher ($p < 0.05$) compared to both the NC and HFD fed WT females. HOMA-IR calculations revealed an interaction between diet and genotype ($p < 0.05$) as well as a genotype interaction ($p < 0.0001$). In contrast to the males, the female [Tg(CAT)[±]] had the lowest calculated HOMA-IR and the HFD fed Bob-Cat groups had the highest, pointing to a possible sexual dimorphism in regard to insulin sensitivity within the 'stress-less' mice models.

3.7 Diet-redox stress interaction in adipose tissue

3.7.1 Redox status—In order to determine if differences in redox environment, due to dietary intervention, contributed to the observed metabolic changes in the 'stress-less' mice models, we measured signatures of adipose tissue redox stress: i.e. oxidized carbonyl groups, expression of Nrf2 (a transcription factor and key regulator of redox homeostasis), and the key target genes of Nrf2, catalase activity, and HO-1 (antioxidants transcribed upon activation of Nrf2). Oxidized carbonyl groups, measured using the OxyBlot protein oxidation kit (Millipore), interestingly showed that within male groups, there was no major induction of oxidized proteins in the NC or HFD fed groups (Supplementary Figure 1A). However, in mice fed an OM3 enriched diet, we saw higher levels of oxidized carbonyls, with the highest levels seen within the OM3 fed WT mice group ($p < 0.05$) followed by the OM3 fed Bob-Cat group (not significant). Within females (Supplementary Figure 1B), there was a trend for the mice overexpressing catalase to have lower levels of oxidized carbonyl groups in comparison to the WT littermates. The only exception was the Bob-Cat mice fed OM3 diet, which showed a significant increase ($p < 0.05$) in oxidized carbonyl groups.

When mRNA expression of Nrf2, a redox sensitive transcription factor was determined, the male Bob-Cat mice showed a gradual increase in Nrf2 induction when fed HFD (>4 fold) or OM3 (>9 fold) compared to its NC fed littermates and the other genotypes fed any diet. However, none reached statistical significance (Figure 9A). Similar trends of Nrf2 induction were also seen in the Bob-Cat female mice where the levels increased to >50 fold when fed HFD and >120 fold ($p < 0.05$) when fed OM3 diet vs. the WT group (Figure 9B).

Both antioxidant catalase and HO-1 are downstream targets activated by Nrf2. Therefore, catalase activity and expression of HO-1 was measured in the adipose tissue of each mice group (Figure 9C–F). Catalase activity was down-regulated in HFD and OM3 fed WT and [Tg(CAT)[±]] male mice. However, activity levels remained 10 fold higher (not significant) in

the Bob-Cat mice fed any diet, compared to the other two genotypes. Interestingly, the females expressed at least 5 fold increased catalase activity in all the genotypes compared to their male counterparts (Figure 9C–D). In addition, the mice overexpressing catalase trended to have higher levels of catalase activity compared to the WT mice. HO-1 mRNA expression was shown to be the highest in the male and female Bob-Cat mice groups (Figure 9F–G). Similar to the mRNA of its transcriptional activator, Nrf2, in males, the Bob-Cat mice showed a gradual increase in HO-1 induction when fed HFD (>3 fold) or OM3 (>15 fold, $p<0.05$) compared to the controls on the same respective diets. Similar trends were seen among the Bob-Cat female mice. An induction of HO-1 was observed in the Bob-Cat HFD group (>63 fold) and OM3 group (>208 fold) compared to WT controls fed the same diet. The induction of both catalase and HO-1 observed in the Bob-Cat male and female mice fed an OM3 diet provides evidence that Nrf2 had translocated to the nucleus and activated its downstream targets.

3.7.2 GPR120/FFAR4 expression—Our data thus far has shown that in general, mice overexpressing catalase fed OM3 diet in comparison to mice fed HFD or WT mice fed any of the diets, had lower body weight and fat mass, decreased energy consumption, and maintained normal glucose and insulin levels. However, sexual dimorphism was observed within some of the measured metabolic parameters. GPR120/FFAR4 is a lipid sensing, long chain fatty acid receptor highly expressed in adipose tissue and macrophages and is attributed to the beneficial anti-inflammatory and insulin sensitivity effects of OM3 diet [26]. Therefore, we investigated whether GPR120 was contributing to the alterations in metabolic parameters seen within the antioxidant overexpressing mice and to the observed sexual dimorphism. As shown in Figure 10A–C, the male [Tg(CAT)[±]] mice had a lower expression of GPR120 at baseline (NC fed animals) however the levels increased 2–3 fold higher (not significant) than WT mice, when fed HFD or OM3 diets. In contrast, there was a gradual induction of GPR120 mRNA expression in Bob-Cat mice groups when fed NC to HFD (>12 fold induction, $p<0.01$) on HFD and over 108 fold ($p<0.01$) when fed OM3 diet (Figure 10A). Western blotting of the protein expression of GPR120 showed a genotypic effect (Figure 10B–C) among the male groups ($p=0.002$). It was intriguing that, regardless of the diet, Bob-Cat mice groups had the highest levels of GPR120 protein expression.

In females, the [Tg(CAT)[±]] group showed a slight decrease in GPR120 expression (Figure 10D) when fed HFD or OM3 ($p<0.05$), compared to the NC fed diet groups. In contrast, similar to the male mice, Bob-Cat female mice provided an OM3 diet had a significant increase ($p<0.05$) in GPR120 mRNA expression compared to WT mice. As indicated in Figure 10E–F, Western blotting showed that the GPR120 protein level in the adipose tissue only had a trend towards increased expression in NC fed female mice overexpressing catalase ([Tg(CAT)[±]] and Bob-Cat), whereas those fed HFD had a lower expression compared to their WT littermates. As seen in the males, OM3 fed Bob-Cat female mice also showed a significant increase ($p<0.05$) in GPR120 protein expression compared to NC fed WT mice.

3.6.3 Adipokine modulators of insulin sensitivity—Adipose tissue produces and secretes adipocytokines which play both an autocrine and paracrine role in metabolic

pathways involving insulin sensitivity as well as in inflammation [57]. Their levels can also be influenced by an OM3 rich diet [23, 58] and redox status of the adipose tissue [43, 57, 59–61]. We first measured adiponectin, which alleviates insulin resistance by stimulating lipid oxidation and anti-inflammatory processes [12]. As shown in Figure 11A, within male groups, we saw both a significant difference among the genotypes ($p<0.0001$) in addition to the diet ($p<0.05$). Bob-Cat mice provided a NC and OM3 rich diet had a >6 fold ($p<0.05$) and >19 fold ($p<0.001$) increase in adiponectin mRNA levels respectively, compared to the WT groups fed similar diets. All female Bob-Cat groups showed a trend to have the highest fold change (Figure 11B) in adiponectin expression compared to the WT mice groups fed the same diet, but similar to males, the most significant fold increase was within the OM3 fed Bob-Cat mice group (>82 fold increase, $p<0.01$).

Adiponectin works in concert with Fibroblast Growth Factor 21 (FGF-21) [62], both an adipokine and hepatokine, with paracrine effects on adipose tissue [63]. It was most recently identified to be a stress response hormone [64] in addition to its known beneficial function as a metabolic regulator of glucose and lipid homeostasis and insulin sensitivity [65]. Therefore, we investigated both the adipose mRNA expression and the plasma levels of FGF-21. Within the adipose tissue from male mice (Figure 11C), FGF-21 levels were not significantly different. However, there was a trend for mice overexpressing catalase on a NC diet to have a lower FGF-21 expression while littermates provided HFD or OM3 diet showed a >2 fold increase. The highest levels of FGF-21 within each diet group overexpressing catalase were within the HFD groups. Female mice showed similar results with the exception of the HFD fed [Tg(CAT)[±]] group where there was a lower expression of FGF-21 in comparison to the NC ($p<0.05$) and HFD (not significant) fed WT mouse group. However, the most significant finding was that the OM3 fed Bob-Cat female mice had a >60 fold increase ($p<0.01$) compared to the WT group fed the same diet (Figure 11D). With FGF-21 also being secreted by liver, we used an ELISA kit to measure circulating levels of FGF-21. Within the male mouse groups, mice overexpressing catalase had significantly lower levels ($p<0.05$) than the NC WT mice as seen in Figure 11E. However, when provided HFD, [Tg(CAT)[±]] had significantly higher levels ($p<0.05$) compared to every other mouse group provided HFD. Interestingly, Bob-Cat mice, regardless of the diet, showed a trend for having lower plasma FGF-21 levels than the WT mice of each respective diet group. Females circulating levels of FGF-21 were also measured (Figure 11F). Contrary to males, female mice overexpressing catalase provided NC diet did not have significantly lower levels of circulating FGF-21 than their WT littermates. In fact, the NC fed [Tg(CAT)[±]] had the highest levels of circulating FGF21, which did not correlate with the mRNA expression of FGF21 in the adipose tissue. Perhaps the liver was a major source of the circulating FGF21. It was also surprising to find that when provided either high-fat diet (HFD or OM3) intervention, female Bob-Cat mice trended to have higher levels of plasma FGF-21 compared to their WT littermates.

4. Discussion

Obesity and its comorbidities are characterized with increased levels of ROS that alter lipid and glucose homeostasis in key metabolic organs [3, 66, 67]. This leads to increased body weight and fat mass thus increasing risk for hyperglycemia, hyperlipidemia,

hyperleptinemia, systemic inflammation, and IR [3]. Both endogenous and exogenous antioxidant supplementation, known to mitigate the negative effects of redox stress, were shown to lower the levels of ROS in these metabolic diseases [40, 68–70]. Additionally, in both lean and obese rodent models, dietary interventions, such as those rich in OM3 fatty acids (including EPA and DHA) in contrast to saturated fatty acids (lard diet), also lowered body weight and fat mass, increased insulin sensitivity, and induced browning of white adipose tissue through anti-inflammatory actions [18, 23, 26, 71]. However, the mechanisms leading to these beneficial effects were not clearly defined. We recently showed that mice overexpressing catalase in a genetically obese background (Bob-Cat), in contrast to its wild-type littermates, significantly lowered redox stress ('stress-less' mice), improved energy metabolism, and altered the expression of key adipocytokines [43]. The overexpression of catalase in this mouse model with a genetic obese background was implicated to be the key factor responsible for these effects. Therefore, these mice presented characteristics of an effective model to study the interaction between redox regulation and dietary intervention, on adipose tissue function and glucose and lipid signaling in a "diet-induced obesity" model. In the present study eight weeks (chronic effect) of dietary intervention in the 'stress-less' mice model overexpressing catalase in comparison to their WT controls showed that the high-fat omega-3 enriched (OM3) diet, in contrast to the high-fat lard (HFD) diet, stabilized body weight and fat mass, maintained balanced energy metabolism and normal circadian rhythm, and sustained insulin sensitivity by regulating the GPR120-Nrf2 cross-talk.

Administration of OM3 diet, in contrast to the HFD fed animals, for 8 weeks showed that mice overexpressing catalase (male and female Bob-Cats), maintained or lowered body weight and fat mass, similar to that observed in NC fed mice groups, despite a lower food intake (total g and kcal/g consumed) of chow provided ad libitum. Decreased food intake when fed HFD in comparison to a normal rodent diet has been reported in previous studies as the result of a higher caloric and satiating diet [72]. These observations support the importance of the composition of a meal on fat accumulation and distribution during weight management/weight loss therapies in humans [73]. This is of particular interest in obese subjects, where an excessive accumulation of visceral fat mass elevates the risk of numerous health conditions including coronary heart disease, IR, osteoarthritis, and hypertension [74], which further contributes to the 8 year reduction in life expectancy in these individuals [75–77]. In contrast, the increased life expectancy observed in studies conducted in catalase overexpressing mice [78] may be attributed to the type of diet consumed.

Lean mass is representative of the muscle tissue mass equivalent of all the body parts containing water, excluding fat, bone minerals, and such substances which do not contribute to the NMR signal, such as hair, claws, etc (ECHO-MRI (Houston, TX) users' manual). It was interesting that in the Bob-Cat male groups, we observed an increase in lean mass at 4 weeks and decrease at 8 weeks independent of diet. Other studies have shown similar fluctuations in lean mass as the body begins to adapt to alterations in diet or food/caloric intake [79]. At the 4 week time point, the body likely had not completely adjusted to the diet, however at around the 8 week time point, a complete response to the dietary intervention had occurred. In addition to lean mass, other studies have also seen similar fluctuations in bone mass in density [80]. Nonetheless, it is unclear why only Bob-Cat male

mice behaved as such compared to the other genotypes. One can only speculate that it might be a response to the differences in redox regulation in these novel mice model.

CLAMS assessment of energy metabolism (energy intake vs. energy expenditure) showed differences between the genotypes tested on various diets. The three day CLAMS measurement of FI supported the earlier observation of lower weekly food intake by the HFD and OM3 fed groups despite the genotype [72]. In addition, it was observed that HFD and OM3 feeding altered the eating patterns (circadian rhythm) in WT mice compared to those fed NC. This is consistent with other studies that showed HFD feeding alters the quantity, time of day, and how much chow is consumed during each visit to the food hopper [81–83]. However, this diet effect (HFD or OM3) on eating patterns was not altered in either gender of the [Tg(CAT)[±]] and Bob-Cat mice, which followed similar circadian rhythm patterns of eating as that seen in mice provided NC diet. This observation suggested that catalase overexpression (i.e. redox balance) may be shifting the paradigm of a high-fat diet altering circadian rhythm and patterns of food intake. This speculation is further supported by our previously published observations where the secretion of key adipokines that modulate hypothalamic appetite regulation was altered in mice overexpressing catalase [43].

In addition to measurement of FI, the CLAMS analysis also provided insights into the differences in RER and EE in the various genotypes and diet interventions. We observed a significant increase in RER as well as CHO oxidation (hence lower fat oxidation) in the NC fed male mice overexpressing catalase compared to their WT groups fed NC. This might be attributed to the increased expression of human catalase gene, since other studies have shown changes in energy metabolism as a result of increased antioxidant catalase [41, 43] in addition to alterations in substrate utilization as a result of differences in genetics [84]. In contrast, all groups on a high-fat diet intervention (lard or fish oil), had a lower RER and levels of CHO oxidation (higher fat oxidation) which has been reported in numerous other studies as a result of higher fat % available to be oxidized from the HFD provided to the animals [85–87]. The ability of OM3 diet to lower the RER and increase fat oxidation within the male mice groups overexpressing catalase and female Bob-Cats vs the Bob-Cat mice fed NC may have contributed to the observed decrease in their body weights compared to their littermates that overexpress catalase fed NC or HFD. On the contrary, male mice overexpressing catalase provided an OM3 diet, had significantly lower fat oxidation compared to WT mice provided a NC diet. This may be a beneficial outcome from the intake of OM3 fatty acids termed “metabolic flexibility;” a newer concept describing the body’s ability to match fuel oxidation to fuel availability [88]. We believe this may have been acquired in our mouse model through intake of the OM3 diet during the 8 week study period. Another plausible reason for the lower levels of fat oxidation observed may be due to “altered metabolic partitioning” of fatty acids where there is a reduction in oxidation and increased re-esterification of particular fatty acids dependent on their structure [89]. This would also provide reasoning as to why the same effect was not seen in the HFD fed mice overexpressing catalase. In comparison to males, fat oxidation trended to be elevated in females (kcal/h/g) even though all were provided the same type of diet and the quantity consumed by females was not higher than what was consumed by males. Even more interesting was the opposite effects of CHO vs. fat oxidation seen in male and female Bob-

Cat mice groups provided an OM3 diet. We believe this was a result of sexual dimorphism [90].

All HFD fed groups in our study, having significantly higher levels of EE (heat production) is consistent with previous studies [91], where a significant increase in EE was observed within just one week of HFD feeding [81]. It is hypothesized that this phenomenon is due to the increased body mass and amount of oxygen necessary to facilitate normal cellular/tissue function in addition to higher energy required to catabolize the 45% high-fat diet vs. the 13.4% fat in the NC diet. However, the increase in EE of the HFD groups is not sufficient to create energy balance resulting in an accumulation of body weight and fat mass. Though calorically (45% fat) similar to the HFD, EE levels in OM3 fed mice remained intermediate to NC and HFD fed mice, yet EE levels were still significantly higher than the NC fed WT group. Previous studies have also shown an OM3 rich diet increases EE levels by enhancing thermogenesis via activating GPR120 [23, 92]. Male [Tg(CAT)[±]] and Bob-Cat mice also displayed significantly higher activity (XAMB) levels compared to WT mice fed NC or high-fat (HFD or OM3) diets. Furthermore, when provided HFD or OM3 diet, the WT mice had the lowest activity levels, not reaching above 3000 counts per two hours. These results indicate that overexpression of catalase might be increasing the activity levels within the male gender. Previous reports have suggested that in rodent models, a high caloric diet can decrease XAMB counts of physical activity by up to 28% [81]. It is also possible that the increased body weight and fat mass may have been a secondary factor in the hypo-activity. Interestingly, the effect was not as severe in groups overexpressing catalase; again an indication of the beneficial effect of antioxidant overexpression coupled with an OM3 diet. This is of great importance since evidences in human obesity studies have shown that HFD promotes a more sedentary, less physically active lifestyle in addition to alterations in sleep/wake cycles [93]. Alterations in redox balance (as seen within the catalase overexpressing mice), such as by OM3 dietary interventions, might improve metabolic imbalance and circadian rhythm abnormalities in obese humans.

Eight weeks of dietary intervention altered the circulating metabolic profile in the catalase overexpressing mice. As seen in other studies, and expected with increased fat mass, mice fed a HFD had the highest levels of TC [94]. The only exception was the female [Tg(CAT)[±]] mice fed HFD which had similar TC levels to all mouse groups fed NC and OM3 diet. This female group also had significantly higher levels of activity (XAMB counts) which might have influenced the lowering of cholesterol levels [95]. In contrast to the HFD, and as previously documented [24], mice provided an OM3 diet did not alter TC and the levels remained similar to that seen in NC fed mice groups. HDL levels were highest among the groups fed HFD in both genders, but among OM3 fed mice, there was a higher ratio of HDL:TC compared to the HFD groups. Furthermore, among the OM3 fed mice groups, Bob-Cat males and females had the highest levels of HDL making their HDL:TC ratio the highest among all mice groups thus showing the significance of the regulatory interaction between overexpression of antioxidant catalase and feeding OM3 diet on lipid profile. There have been previous studies conducted in the leptin deficient, Ob/Ob mice (parent group of the Bob-Cat mice model) showing increases in HDL level [96, 97]. It has been postulated that although typically in human obesity, HDL levels are substantially lower, the decrease in functional leptin in the obese, Ob/Ob mice may be playing a role in the higher HDL (being

the major lipoprotein in rodents) levels [97]. Since Bob-Cats are heterozygous for the Ob gene, it is plausible that leptin is playing a role in modulating HDL levels. Surprisingly, male Bob-Cats had the highest TG levels. A similar effect, although to a lower degree, was also seen in the NC and OM3 fed female Bob-Cats and the other female genotypes on similar diet. In general, the mice provided OM3 diet had lower plasma TG levels, which was expected as the result of a higher intake of OM3 fatty acids [16, 23]. Bob-Cat mice, compared to the other genotypes, also had increased levels of plasma ketones in all groups (except the HFD males), and those provided an OM3 diet had the highest ketone levels independent of gender. Generally, high ketone levels are classically associated with metabolic dysfunction and diabetes [98–101], but more recent studies have shown that lower carbohydrate diets provoking ketosis cause an inverse correlation between circulating ketones and plasma glucose levels, thus suggesting higher levels of ketones are associated with more favorable effects on glycemic control [102]. Other studies have now recognized ketones as imperative signaling molecules promoting metabolic function and regulating appetite [56, 103]. These studies further solidify the beneficial effects of OM3 diet coupled with antioxidant catalase overexpression on metabolic parameters.

Consistent with other studies on the metabolic effects of an OM3 enriched diet [26, 104, 105], irrespective of gender, the lowest glucose levels were seen in the Bob-Cat groups fed OM3 diet. Sexual dimorphism was observed in insulin levels. The highest average levels were in the HFD fed [Tg(CAT)[±]] males, while the HFD [Tg(CAT)[±]] females had the overall lowest levels of plasma insulin. The same results were reflected in the HOMA-IR. Furthermore, males in general had higher levels of insulin than females regardless of diet or genotype. This could be a direct effect of the increased visceral adipose tissue and liver weights in males compared to females [15]. Insulin levels are key in the metabolic function of the liver [106], so it was interesting that the males had an increase in liver weight, but not the females, as well as NC and HFD fed [Tg(CAT)[±]] mice groups compared to WT littermates. Despite increases in insulin, plasma ketone levels showed no significant differences in male HFD fed [Tg(CAT)[±]] mice compared to the WT mice fed HFD. In fact, ketone levels were >2 fold in the WT vs [Tg(CAT)[±]] HFD group indicating insulin is repressing ketone body production in the [Tg(CAT)[±]] mice [103, 107]. Nevertheless, most importantly, Bob-Cat mice fed OM3 rich diet maintained glucose and insulin homeostasis throughout the duration of the 8 week study. Mechanistically, this result was expected to occur in part by the production of OM3-derived inflammatory resolution mediators [29], the higher ketones generated, “metabolic flexibility [88],” and potential alterations in “metabolic partitioning” [89] (indicated by the CLAMS fat oxidation analysis) in the Bob-Cat mice. However, in addition to these effects, the major contributor to the favorable metabolic profile of the Bob-Cat mice fed OM3 diet is through the activation of its receptor, GPR120 [26, 108, 109].

GPR120 (FFAR4) is a long-chain fatty acid receptor highly expressed in adipose tissue [26] and activated by OM3 fatty acids [22, 26, 92, 110]. It plays beneficial roles in anti-inflammatory pathways in adipose tissue, food preference, glucose homeostasis, and insulin sensitivity, all of which are interrelated to regulate metabolic energy homeostasis in both physiological and pathophysiological conditions [111]. Though there are contradicting reports of GPR120 not required for these beneficial effects of OM3 fatty acids [112], the

support for its role in OM3 mediated effects stems from genetic studies performed in human. The human studies showed that mutations in GPR120 were associated with increased risk of obesity and IR [108]. GPR120 is also a novel risk factor for diet induced obesity (DIO) [26, 113]. For these reasons, it was most compelling that within the OM3 fed Bob-Cat mice groups, which overexpresses antioxidant catalase within an obese parent background, we observed the highest levels of both mRNA and protein expression of GPR120 in the perigonadal adipose tissue. Based on our measurements of redox stress markers, the OM3 fed Bob-Cat mice also had an increased level of oxidized carbonyl groups within the adipose tissue. Together, this might suggest that redox regulation is playing a role in the upregulation of GPR120 expression and beneficial outcomes of the OM3 diet within the antioxidant-overexpressing mice of both genders. Redox regulation of GPR120 has not been previously shown in prior studies.

Furthermore, Nuclear factor erythroid-2-related factor 2 (Nrf2), is a redox sensitive transcription factor activated by long chain fatty acids (including EPA and DHA), phenolic antioxidants, and imbalances in redox stress [29, 114]. Raising levels of Nrf2 by endogenous production of electrophilic products or pharmacological agents has been shown to prevent or act as therapies for type 2 diabetes, metabolic syndrome, obesity, and cardiovascular disease through activating anti-inflammatory pathways [30, 115–118] in addition to lowering body weight and fat mass [119]. Specifically, in the Bob-Cat mice groups, when fed a diet high in OM3 fatty acids, there were higher levels of adipose tissue Nrf2 mRNA expression. We speculate this to have occurred as a result of the synergistic effect of antioxidant overexpression and consumption of OM3 rich diet. Studies concentrated on the beneficial effects of polyunsaturated fatty acids (primarily EPA and DHA) have shown that their oxidized derivatives regulate the redox environment by covalently and reversibly reacting with nucleophilic residues on target proteins [29, 120]. These reactions trigger the activation of cytoprotective pathways, including the Nrf2 antioxidant response [121]. Nrf2 activation subsequently causes an upregulation of phase II enzymes/antioxidants thus balancing oxidant: antioxidant ratios in addition to suppressing the NF- κ B proinflammatory pathway [29]. Both antioxidant catalase and HO-1 are two of the key antioxidants upregulated in response to induction of Nrf2 [31, 32, 122]. In our model, the Bob-Cat mice group fed OM3 diet had the highest levels of catalase activity in addition to mRNA expression of HO-1, providing further evidence of Nrf2 induction and subsequent activation of the antioxidant and cytoprotective response.

With the administration of OM3 fatty acids, it has previously been shown that the activation of GPR120 is linked to the secretion and circulating levels of the adipokine adiponectin [123] promoting anti-inflammation (downregulation of NF- κ B) and insulin sensitivity [124]. Interestingly, Nrf2 also decreases inflammation through the same pathway as GPR120 [29, 125], but no previous study has shown the cross-talk between GPR-120 and Nrf2. In the Bob-Cat mice fed OM3 diet, we saw a significant increase in adiponectin mRNA expression within the adipose tissue. The combined results of high expression of GPR-120, Nrf2 (and its downstream signaling-activation of catalase and HO-1) provide evidences for a potential cross-talk between activation of GPR120 and Nrf2 synergistically decreasing inflammation within the adipose tissue as well as modulating whole body metabolism in the Bob-Cat mice. Although Nrf2 has been studied in depth in the brain [126, 127] and heart [31, 122],

because of its cytoprotective and anti-inflammatory benefits, we provide evidence for a similar role in adipose tissue. Furthermore, as observed from our current findings from our novel mice model fed OM3 diet, we believe there is an interaction between Nrf2, GPR120, and adiponectin which could potentially give rise to new therapies in obesity, if its induction could aid in mediating energy homeostasis through adipokine expression and secretion.

Another metabolic regulator that is induced by both GPR120 and Nrf2 is FGF21 [92, 128]. In the current study, male mice overexpressing catalase trended to have increased FGF-21 mRNA expression compared to WT diet group controls, but the highest expression was in the HFD groups. Similar elevations in FGF-21 have been shown in studies investigating obese humans and rodent models [64, 129, 130]. Contrary to males, in female mice, the highest mRNA expression was within the OM3 fed groups, and mainly in Bob-Cat mice. Additionally, we observed plasma levels in female [Tg(CAT)[±]] and Bob-Cat mice fed HFD had opposite effects compared to males, yet, the FGF-21 levels were similarly increased when fed OM3 diet. However, there are conflicting reports on the regulation of FGF-21 by redox stress/Nrf2 activation [128, 131–133] or whether increased FGF-21 is actually metabolically beneficial.

It is of special importance to discuss the sexual dimorphism observed in the results of this study within the Bob-Cat mice groups. With gender differences in sex hormones (i.e. estrogen vs. testosterone), distribution of fat pads, and the role of epigenetics, it is essential to study both male and female genders to fully define energy-related metabolic signaling pathways [46, 47]. Additionally, to our knowledge, no study has investigated the gender differences of supplementation of OM3 fatty acids in relation to redox homeostasis, making the findings of the sexual dimorphism in the ‘stress-less’ model truly novel. Figure 12 provides a schematic overview of the sexual dimorphism observed within the Bob-Cat mice fed an OM3 enriched diet related to GPR120-Nrf2 cross talk. As seen in humans [90], we also observed higher serum TG levels in the Bob-Cat mice fed OM3 diet. Furthermore, males had significantly higher levels of serum TG when provided an OM3 diet compared to their WT littermates provided the same diet. Female Bob-Cat mice, in contrast, trended to have lower levels compared to the female WT control group. This is of importance since clinical trials show that in comparison to women, men have significantly lower levels of plasma total lipids/phospholipids of α -linoleic acid (ALA) and DHA in addition to less potent metabolic effects of the OM3 fatty acids EPA and DHA in decreasing risk for IR [134]. Plasma analysis of FGF-21 showed that Bob-Cat males have lower levels compared to WT controls and the opposite was seen within females. This is most intriguing due to recent studies showing that FGF-21 lowers TG levels within both human and rodent models [135]. Additionally, CLAMS analysis revealed that RER and CHO/fat oxidation in females trended to be higher compared to males regardless of the diet or genotype. In particular, we saw significant gender differences within the Bob-Cat mice. When fed OM3 diet, Bob-Cat males had much higher levels of RER/CHO oxidation and lower levels of fat oxidation compared to the WT mice group. However, in females, Bob-Cats fed OM3 diet had lower RER/CHO oxidation and higher levels of fat oxidation. Previous reports have shown that males and females differ in how they process polyunsaturated fatty acids [134] and that females retain higher levels of PUFA in circulation [136]. Additionally, the differences in oxidized substrates could also potentially play a role in modulating the circulating TG and FGF-21

levels providing reasoning for why we saw differences between the genders of the Bob-Cat mice..

Overall, this study provides compelling evidence that overexpression of catalase coupled with an enriched diet of OM3 fatty acids are metabolically beneficial. This combination was shown to increase adipose tissue expression of the GPR120/FFAR4, which by interacting with the Nrf2 pathway, resulted in decreased body weight and fat mass, enhanced or maintained circadian rhythm, anti-inflammation and insulin sensitivity, and regulation of key adipokines compared to HFD fed mice (**Graphical Abstract**). In fact, to our knowledge, this is the first study to provide evidence that GPR120 expression may be modulated by redox status in addition to providing the evidence of the GPR120-Nrf2 cross-talk mechanism. With the beneficial outcomes seen within the ‘stress-less’ Bob-Cat mice model provided an OM3 diet, we believe that this model is an excellent tool to further study adipose tissue function, crosstalk with other metabolic tissues, and metabolic signaling pathways involving energy homeostasis in both male and female mice. Also, in addition to obesity, inflammation in adipose tissue has been linked to a number of types of carcinogenesis [137] and cardiovascular events. [138–140]. Thus, using the ‘stress-less’ mice as a novel model, future studies may be conducted to look at the combination of antioxidant overexpression and other therapies for diseases of metabolic syndrome as well as lowering the risk and progression of the metabolic syndrome-associated cancers and CVD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

OM3	High-fat Omega 3-Enriched Diet
HFD	High-fat Lard Diet
DIO	Diet-Induced Obesity
ROS	Reactive Oxygen Species
mCAT	Mitochondrial Catalase
HO-1	Heme Oxygenase
[Tg(CAT)[±]]	Catalase Transgenic mice
NC	Normal Chow
CLAMS	Comprehensive Laboratory Animal Monitoring System

VO2	O2 Consumption
VCO2	CO2 Production
RER	Respiratory Exchange Rate
FI	Food Intake
EE	Energy Expenditure
XAMB	physical activity, X-Ambulatory Counts
CHO	Carbohydrate Oxidation
1X TBST	1X Tris Buffered Saline
DNP	Dinitrophenylhydrazone
DNPH	Dinitrophenyldiazine

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Highlights:

- Catalase overexpression coupled with an OM3 diet activates adipose tissue GPR120
- GPR120-Nrf2 crosstalk resulted in regulation of energy metabolism in adipose tissue
- Sexual dimorphism observed in redox regulation in response to OM3 diet
- Useful model for studying redox regulation in metabolic syndrome and cancer

Figure 1: Body weight and body composition (fat/lean mass) in male mice overexpressing catalase:

Body weight change (initial and final body weight) and body composition (fat/lean mass) determined by ECHO-MRI: **A.** Changes in whole body weight over 8 weeks; **B.** Overall weekly body weight measurements; **C.** Fat Mass (g) and; **D.** Lean Mass (g) of WT, [Tg(CAT)[±]], and Bob-Cat male mice fed NC, HFD, and OM3 diets (n = 6/group). Mice overexpressing catalase on an OM3 diet in contrast to the HFD fed animals either had no change or lost body weight and fat mass compared to NC fed mice groups. One-way and two-way ANOVA was performed on GraphPad Prism 7. Data is represented as mean ± S.E.M. Letters indicate significant p values, a=p<0.05, b=p<0.01, c=p<0.001, d=p<0.0001; symbols represent significant differences between genotypes * = compared to WT; an additional 'h' represents comparison to HFD fed WT.

Figure 2: Body weight and body composition (fat/lean mass) in female mice overexpressing catalase:

Body weight change (initial and final body weight) and body composition (fat/lean mass) determined by ECHO-MRI: **A.** Changes in whole body weight over 8 weeks; **B.** Overall weekly body weight measurements; **C.** Fat Mass (g) and; **D.** Lean Mass (g) of WT, [Tg(CAT) $\pm$], and Bob-Cat female mice fed NC, HFD, and OM3 diets (n = 4/group). Mice overexpressing catalase on an OM3 diet in contrast to the HFD fed animals either had no change or lost body weight and fat mass compared to NC fed mice groups. One-way and two-way ANOVA was performed on GraphPad Prism 7. Data is represented as mean $\pm$ S.E.M. Letters indicate significant p values, a= $p<0.05$, c= $p<0.001$; symbols represent significant differences between genotypes * = compared to WT; an additional 'o' represents comparison to OM3 fed WT.

Figure 3: Weekly food and energy consumption in male and female mice overexpressing catalase: Weekly food and energy consumption measured in wildtype and catalase overexpressing mice for 8 weeks: Male, **A.** average weekly food consumption for 8 weeks; **B.** weekly food consumption for 8 weeks, and **C.** calculated average energy consumption per mouse (n = 6/ group). Female, **D.** average of overall food consumption for 8 weeks; **E.** weekly food consumption for 8 weeks, and; **F.** calculated average energy consumption per mouse (n = 4/ group). WT, [Tg(CAT)]⁺, and Bob-Cat mice fed NC, HFD, and OM3 diet were analyzed for each gender. One-way and two-way ANOVA was performed on GraphPad Prism 7. Data is represented as mean $\pm$ S.E.M. Letters indicate significant p values: a=p<0.05, b=p<0.01, c=p<0.001, d=p<0.0001; symbols represent significant differences between genotypes * = compared to WT; an additional 'h' represents comparison to HFD fed WT.

Food Intake (CLAMS)

Figure 4: CLAMS analysis of food intake over a 48 hour period in male and female mice overexpressing catalase:

Light and dark cycle analysis of CLAMS measurements for 48 hours are provided: Male, **A.** and Female, **B.** Food Intake as average grams/2h for 48 hours by WT, [Tg(CAT)[±]], and Bob-Cat mice fed NC, HFD, and OM3 diet (n = 3/group). One-way and two-way ANOVA was performed on GraphPad Prism 7. Data is represented as mean ± S.E.M. Letters indicate significant p values: a=p<0.05, b=p<0.01, c=p<0.001, d=p<0.0001; symbols represent significant differences between genotypes * = compared to WT; an additional 'o' represents comparison to OM3 fed WT.

Figure 5: CLAMS analysis of energy expenditure and total activity over a 48 hour period in male and female mice overexpressing catalase:

Light and dark cycle CLAMS analysis of Energy Expenditure (EE) and X-Ambulatory Movement (XAMB) per 2h over a 48 hour time period are provided: Male, **A.** Metabolic EE averaged as Cal/h/Lean(g) body mass and; **B.** XAMB Counts per 2h for 48 hours for WT, [Tg(CAT)]⁺, and Bob-Cat mice fed NC, HFD, and OM3 diet (n 4/group). **C&D.** represent data of female mice of each genotype and diet group (n 3/group). One-way and two-way ANOVA were performed on GraphPad Prism 7. Data is represented as mean $\pm$ S.E.M. Letters indicate significant p values: a=p<0.05, b=p<0.01, c=p<0.001, d=p<0.0001; symbols represent significant differences between genotypes * = compared to WT; an additional 'h' represents comparison to HFD fed WT.

Figure 6: CLAMS analysis of RER and substrate oxidation over a 48 hour period in male mice overexpressing catalase:

Light and dark cycle CLAMS analysis of average Respiratory Exchange Ratio (RER) and calculated CHO and Fat Oxidation per 2h over a 48 hour time interval: **A.** RER (CO₂ emission/O₂ consumption); **B.** CHO Oxidation, and; **C.** Fat Oxidation for WT, [Tg(CAT)[±]], and Bob-Cat mice fed NC, HFD, and OM3 diet (n = 4/group). One-way and two-way ANOVA was performed on GraphPad Prism 7. Data is represented as mean ± S.E.M. Letters indicate significant p values: a=p<0.05, b=p<0.01, c=p<0.001, d=p<0.0001; symbols represent significant differences between genotypes * = compared to WT; an additional 'o' represents comparison to OM3 fed WT.

Figure 7: CLAMS analysis of RER and substrate oxidation over a 48 hour period in female mice overexpressing catalase:

Light and dark cycle CLAMS analysis of average Respiratory Exchange Ratio (RER), and calculated CHO and Fat Oxidation for 48 hours: **A.** RER (CO_2 emission/ O_2 consumption); **B.** CHO Oxidation, and; **C.** Fat Oxidation for WT, [Tg(CAT) $^\pm$], and Bob-Cat mice fed NC, HFD, and OM3 diet (n = 3/group). One-way and two-way ANOVA was performed on GraphPad Prism 7. Data is represented as mean $\pm$ S.E.M. Letters indicate significant p values: a=p<0.05, b=p<0.01, c=p<0.001, d=p<0.0001; symbols represent significant differences between genotypes * = compared to WT; an additional h represents comparison to HFD fed WT while an additional 'o' represents comparison to OM3 fed WT.

Figure 8: Insulin Sensitivity in male and female mice overexpressing catalase:

Quantification of fasting blood glucose (glucometer), plasma insulin (ELISA), and calculated HOMA-IR: Male, **A.** Glucose; **B.** Insulin, and; **C.** HOMA-IR in WT, [Tg(CAT)[±]], and Bob-Cat mice fed NC, HFD, and OM3 diet (n = 5/group). Graphs **D-F.** show data collected from female mice of each genotype and diet group (n = 4). One-way and two-way ANOVA was performed on GraphPad Prism 7. Data is represented as mean ± S.E.M. Letters indicate significant p values, a=p<0.05, b=p<0.01.

Figure 9: NRF2 signaling in adipose tissue of male and female mice overexpressing catalase: Redox regulation was evaluated in adipose tissue by measuring mRNA expression of Nrf2 (transcription factor and redox regulator), and its downstream antioxidant targets, catalase and heme oxygenase-1 (HO-1): Male, **A.** and Female, **B.** Nrf2 mRNA expression level in the perigonadal adipose tissue measured by RT-qPCR. mRNA expression is depicted as fold change compared to WT mice fed the same diet by ddCT method; Male, **C.** and Female, **D.** Catalase enzymatic specific activity (U/mg protein) was measured using Aebi method [54]. Data for Catalase activity is represented as mean $\pm$ S.E.M. of WT, [Tg(CAT) $^{\pm}$], and Bob-Cat mice fed NC, HFD, and OM3 diet (n = 3/group); Male, **E.** and Female, **F.** HO-1 mRNA expression level in the perigonadal adipose tissue measured by RT-qPCR. mRNA expression is depicted as fold change compared to WT mice fed the same diet by ddCT method. One-way and two-way ANOVA was performed on GraphPad Prism 7. Letters indicate significant p values, a=p<0.05, b=p<0.01; symbols represent significant differences between genotypes * = compared to WT; an additional 'h' or 'o' represents comparison to HFD or OM3 fed WT mice respectively.

Figure 10: GPR120 mRNA and protein expression in adipose tissue of male and female mice overexpressing catalase:

Quantitative PCR and Western blot of GPR120 (FFAR4) in perigonadal adipose tissue at the end of 8 weeks of dietary intervention: **Male, A.** and **Female, C.** GPR120 mRNA expression measured by RT-qPCR in the adipose tissue from WT, [Tg(CAT)⁺], and Bob-Cat mice fed NC, HFD, and OM3 diet depicted as fold change compared to WT mice fed the same diet by Pfaffl CT method (n = 4 and 3/group respectively). **Male, B.** and **Female, D.** Western Blot of GPR120 protein in adipose tissue, shown as % densitometric ratio of Anti-GPR120 and total protein (memcode stain) per genotype to % ratio observed in WT mice fed similar diet (n = 4 and 3/group respectively). Oneway and two-way ANOVA was performed on GraphPad Prism 7. Letters indicate significant p values: a=p<0.05, b=p<0.01; symbols represent significant differences between genotypes * = compared to WT; an additional 'o' represents comparison to OM3 fed WT.

Figure 11: Adipose tissue mRNA expression and circulating levels of key adipokines determined in male and female mice overexpressing catalase:

Adipose tissue specific mRNA expression levels of key adipokines were measured by RT-qPCR: Male, **A.** and **C.** and Female, **B.** and **D.** of Adiponectin (n 4/group) and FGF-21 (n 4/group) respectively. Data depicted as fold change compared to WT mice fed the same respective diet using the Pfaffl CT method. Male, **E.** and Female, **F.** Circulating FGF-21 was measured using a mouse FGF21 ELISA kit according to manufacturer's protocol (n 3/group). All analysis were measured in WT, [Tg(CAT)⁺], and Bob-Cat groups fed NC, HFD, and OM3 diet. One-way and two-way ANOVA was performed on GraphPad Prism 7. Data for serum FGF-21 levels are represented as mean $\pm$ S.E.M. Letters indicate significant p values: a=p<0.05, b=p<0.01, c=p<0.001, d=p<0.0001; symbols represent significant differences between genotypes * = compared to WT; an additional 'o' represents comparison to OM3 fed WT.

Figure 12: Sexual dimorphism observed in an OM3 fed 'stress-less' mouse model: Schematic representation of the sexual dimorphism observed in the 'stress-less' mice model; Bob-Cat mice fed a diet enriched with OM3 fatty acids. These mice, when fed OM3 enriched diet, showed significant differences in circulating markers and CLAMS analysis of substrate oxidation. However, these differences did not impact the GPR120/Nrf2 crosstalk and downstream effects within this mice model. Significance to WT mice fed NC and OM3 depicted as increase: +, ↑ while decrease: −, ↓ respectively. p values are represented as $p < 0.01$ ++, ↑↑, $p < 0.0001$ +++, ↑↑↑ and likewise for a significant decrease.

Adipose tissue and liver weights

[illegible]

Adipose tissue and liver weights of each mouse group: Visceral adipose tissue and liver weights (g) were measured during tissue collection, at the end of 8 weeks of dietary intervention, from all male (n 6/group) and female (n 4/group) mice groups. Data are represented as mean $\pm$ S.E.M. Letters indicate significant p values, a=p<0.05, b=p<0.001, c=p<0.0001; symbols represent significant differences between genotypes * = compared to WT; an additional 'h' represents comparison to HFD fed WT.

Table 2:

Circulating levels of metabolic parameters

<u>Male</u>									
	NC			HFD			OM3		
Parameter	WT	[Tg(CAT) [±]]	Bob-Cat	WT	[Tg(CAT) [±]]	Bob-Cat	WT	[Tg(CAT) [±]]	Bob-Cat
TC (mg/dL)	<100	<100	<100	134.6 ± 7.98	162.3 ± 9.16 d [*] ;d ^{*h}	144 ± 10.8 b [*]	<100	<100	<100
HDL (mg/dL)	55.3 ± 4.30	65.2 ± 1.62	55.83 ± 2.15	>82.9 ± 6.51 d [*]	>93.3 ± 6.36 d [*]	>91.2 ± 3.61 c [*]	66.6 ± 3.50	61.5 ± 3.35	>77.89 ± 4.78
TG (mg/dL)	60.7 ± 5.48	81.7 ± 5.68	225.7 ± 25.0 d [*]	73.2 ± 4.87	79.5 ± 3.66	353.4 ± 24.87 d [*] ;d ^{*h}	61.2 ± 2.85	41.5 ± 3.36	223.2 ± 9.40 d [*] ; d ^{*o}
Ketone (mmol/L)	0.55 ± 0.28	0.38 ± 0.08	1.18 ± 0.07 b [*]	1.25 ± 0.14	0.56 ± 0.08	0.35 ± 0.43 a ^{*h}	1.6 ± 0.23 b [*]	1.01 ± 0.13	2.1 ± 0.11 d [*] a ^{*o}
<u>Female</u>									
TC (mg/dL)	<100	<100	<100	125 ± 0.38	<100	117.5 ± 4.79	<100	101	<100
HDL (mg/dL)	48.3 ± 5.48	55.83 ± 2.15	40.7 ± 3.98	>61.9 ± 5.67	>81.0 ± 5.41	>69.4 ± 7.84	>64.3 ± 4.29	62.1 ± 3.71	>75.2 ± 4.45
TG (mg/dL)	234.1 ± 34.3	197.7 ± 10.3	164.8 ± 10.9	220.6 ± 21.2	195.5 ± 7.8	254.4 ± 15.5	134.9 ± 9.29	190.4 ± 17.3	194.1 ± 3.0
Ketone (mmol/L)	1.35 ± 0.39	0.98 ± 0.12	1.48 ± 0.14	1.67 ± 0.30	1.0 ± 0.08	0.65 ± 0.56	0.9	1.2 ± 0.23	1.93 ± 0.10

Circulating levels of metabolic parameters in each mouse group: Lipid Profile of Total Cholesterol (TC) and High Density Lipoprotein (HDL) determined using the Cholestech kit, Triglyceride levels (TG) using the Triglyceride Colorimetric Assay Kit in plasma of Male (n 6/group) and Female (n 4/group) mice. Ketone levels using a glucometer in plasma of Male (n 4/group) and Female (n=2–9/group) mice. Measurements were performed in WT, [Tg(CAT)[±]], and Bob-Cat mice fed NC, HFD, and OM3 diets for 8 weeks. One-way and two-way ANOVA was performed on GraphPad Prism 7. Data is represented as mean ± S.E.M. Letters indicate significant p values, a= p<0.05, b= p<0.01, c= p<0.001, d= p<0.0001; symbols represent significant differences between genotypes * = compared to WT; an additional 'h' or 'o' represents significant differences between HFD and OM3 fed WT mice respectively.