# Glucocorticoid-Induced Metabolic Disturbances are Exacerbated in Obesity

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**Abstract**

Obesity and chronically elevated glucocorticoids result in similar co-morbidities, but the effect of a combination of these ailments on metabolic outcomes is unclear. Measures of glucose homeostasis and markers of hepatic lipid accumulation were assessed to determine whether obesity exaggerated the effects of dexamethasone-induced metabolic disturbances. The combination of obesity and glucocorticoids resulted in hepatic steatosis and synergistic impairments in insulin sensitivity and this was matched with synergistic elevations in markers of lipolysis. These findings suggest lipolysis may be a key player in glucocorticoid-induced insulin resistance and fatty liver in people with obesity.

## Introduction

Cushing's syndrome is an endocrine disorder that manifests in response to chronically elevated levels of glucocorticoids and is often associated with changes in adipose mass and distribution (1), fatty liver (2,3), impaired glucose tolerance and type 2 diabetes (4,5). Although prevalence of Cushing's syndrome is rare, it is estimated that at any given time 1-3% of the US, UK and Danish populations are prescribed exogenous corticosteroids, which may increase their risk for developing some of the same metabolic complications observed in Cushing's syndrome (6–9).

Similarly to Cushing's syndrome, obesity is often accompanied by a multitude of metabolic complications, such as insulin resistance (10–12) and NAFLD (13,14) and is becoming a worldwide epidemic. Comparing the high rates of medically prescribed corticosteroids with the prevalence of overweight and obesity in developed countries, it is likely that the combination of obesity and glucocorticoid excess is present in many individuals; though, to our knowledge, this has not been studied.

Given the similar co-morbidities associated with obesity and chronically elevated glucocorticoids, we hypothesized that the combinations of these two conditions would lead to worse metabolic outcomes than either of them alone. This is supported by studies in rats showing that corticosterone and high-fat diets combine to cause worsened insulin resistance and non-alcoholic fatty liver disease (NAFLD; (15,16)). However, the underlying mechanisms leading to these outcomes remain unclear.

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There is an array of physiological changes that occur as a result of elevated glucocorticoids including decreased lean mass (17–19), increased fat mass (18,20,21) and increased lipolysis (22–24), all of which have been associated with decreased insulin sensitivity (25–27). Likewise, obesity, insulin resistance and increased fatty acid flux are associated with NAFLD (13,28–30). It has also been demonstrated that inhibition of lipolysis promotes insulin sensitivity (25,31–33). Recent tissue-specific knockouts of glucocorticoid signaling molecules have implicated adipose tissue as a central node linking glucocorticoid action and lipolysis to systemic insulin resistance and NAFLD (34–37).

Here, we show that chronically elevated glucocorticoids in the presence of obesity have synergistic effects on lipolysis, insulin resistance and fatty liver disease. Obese glucocorticoid-treated mice have reduced fat mass compared to all other groups, yet have hyperglycemia and severe insulin resistance; therefore, we speculate that lipolysis drives insulin resistance in obese animals.

## Methods

**Patient Recruitment and data collection**: Written informed consent was obtained and the study was approved by the internal review board of the University of Michigan Medical System. All procedures follow the Helsinki declaration. Patients undergoing transsphenoidal adenomectomy at the University of Michigan were recruited and data was collected as described in Hochberg et al. (18). Control patients were those that had non-secreting adenomas and Cushing's patients were those that had ACTH-secreting adenomas.

**Animal Procedures:** C57BL/6J adult male mice were purchased from the Jackson Laboratory at nine weeks of age. All animals were on a light dark cycle of 12/12hrs and housed at 22°C. Following a week of acclimation, chow-fed 12-week dexamethasone-treated mice were treated as described previously (18). Additional cohorts of mice used in these experiments either remained on a standard diet (normal chow diet; NCD; 5L0D LabDiet; 13% fat; 57% carbohydrate; 30% protein) or were provided a high fat diet (45% fat from lard; 35% carbohydrate mix of starch, maltodextrin and sucrose; 20% protein from casein; cat# D12451) for either eight or twelve weeks. Mice were group housed with four mice per cage and food consumption was measured weekly by weight reductions per cage and calculated to reflect estimated intake of each mouse per day in a given cage. Mice remained on their respective diets for the duration of the study. All mice were provided with access to food and water *ad libitum* throughout the study, unless otherwise noted. Mice were treated with an estimated dose of 1mg/kg per day of water-soluble dexamethasone (Sigma–Aldrich) based on mL consumed per week, treatment was provided in their drinking water (NCD n=12; HFD n=32), or they were given regular drinking water (control group; NCD n=12; HFD n=22) for three or six weeks, as indicated. For the six-week dexamethasone treatment and prior to being euthanized, 16 HFD-fed, dexamethasone-treated mice appeared ill and died or were euthanized and thus removed from all analyses once symptoms were noticed. Due to the immunosuppressive nature of dexamethasone, we suspect the illness was due to infection, though this was not confirmed. Animal body weight and composition was determined weekly using a digital scale and EchoMRI 2100, respectively. At the end of treatment, mice were fasted for 16h, dexamethasone water was not removed during this time, and

euthanized by cervical dislocation at ZT3 after isoflurane anesthesia. Immediately following euthanasia, mice were dissected and the right inguinal white adipose tissue (iWAT) and epididymal white adipose tissue (eWAT) depots were carefully removed and weighed adipose tissues, along with a section of the left lateral lobe of the liver were snap frozen in liquid nitrogen for later analysis. Small pieces of tissues were fixed in 10% phosphate-buffered formalin for histology. All animal procedures were approved by both the University of Tennessee Health Science Center and University of Michigan Institutional Animal Care and Use Committees.

**Insulin Tolerance Tests and Hyperinsulinemic Euglycemic Clamp Experiments:** Insulin responsiveness was assessed via an insulin tolerance test (ITT). Following a six hour fast, mice were given an intraperitoneal (IP) injection of insulin (Humulin R, Lilly, Indianapolis, IN, USA) as described in figure legends. Blood was collected from a tail cut and blood glucose was determined using a One Touch Ultra Glucometer (Lifescan) prior to and every 15 minutes post injection. For the hyperinsulinemic euglycemic clamp experiments, C57BL/6J adult (70d) male mice were fed HFD for eight weeks and treated with dexamethasone in their drinking water for three weeks or regular drinking water. Animals were anesthetized with an IP injection of sodium pentobarbital (5060 mg/kg). Indwelling catheters were inserted into the right jugular vein and the right carotid artery, respectively. The free ends of catheters were tunneled subcutaneously and exteriorized at the back of the neck via a stainless-steel tubing connector (coated with medical silicone) that was fixed subcutaneously upon closure of the incision. Animals with healthy appearance, normal activity, and weight regain to or above 90% of their pre-surgery levels were used for the study. Experiments were carried out in conscious and unrestrained animals using techniques described previously (38–40). Briefly, the primed (1.0 Ci)-continuous infusion (0.05 Ci/min and increased to 0.1 Ci/min at t = 0) of [3-$^{3}$H] glucose (50 Ci/ml in saline) was started at t = -120min. After a five hour fast, the insulin clamp was initiated at t = 0, with a prime-continuous infusion (40 mU/kg bolus, followed by 8.0 mU/kg/min) of human insulin (Novo Nordisk). Euglycemia (120˜130 mg/dL) was maintained during the clamp by measuring blood glucose every 10 min and infusing 50% glucose at variable rates, accordingly. Blood samples were collected from the right carotid artery at t = 80, 90, 100, and 120 min for determination of glucose specific activity. Blood insulin concentrations were determined from samples taken at t = -10 and 120 min. A bolus injection of [1-$^{14}$C]-2-deoxyglucose ([$^{14}$C]2DG; PerkinElmer) (10 Ci) was given at t = 120 min. Blood samples were taken at 2, 5, 10, 15, and 25 min after the injection for determination of plasma [$^{14}$C]2DG radioactivity. At the end of the experiment, animals were anesthetized with an intravenous injection of sodium pentobarbital and tissues were collected and immediately frozen in liquid nitrogen for later analysis of tissue [1-$^{14}$C]-2-deoxyglucose phosphate ([$^{14}$C]2DGP) radioactivity. Blood glucose was measured using an Accu-Chek glucometer (Roche, Germany). Plasma insulin was measured using the Linco rat/mouse insulin ELISA kits. For determination of plasma radioactivity of [3-$^{3}$H]glucose and [1-$^{14}$C]2DG, plasma samples were deproteinized with $ZnSO_4$ and $Ba(OH)_2$ and counted using a Liquid Scintillation Counter (Beckman Coulter LS6500 Multi-purpose Scintillation Counter). Glucose turnover rate, hepatic glucose production and tissue glucose uptake were calculated as described elsewhere (39–41).

**Serum Glycerol and Fatty Acid Determination:** Following 11 weeks of dexamethasone treatment, 21-week-old *ad libitum* chow fed C57BL/6J male mice

were anesthetized with isoflurane and blood was collected into heparin-coated capillary tubes via retro orbital bleed both prior to and 15 minutes following intraperitoneal injection of 10mg/kg isoproterenol (Sigma-Aldrich) in Dulbecco's phosphate-buffered saline (Thermo Fisher). Serum from these mice, as well as from a cohort of 28-week old mice on either HFD or chow, six-weeks post-dexamethasone treatment was collected following an overnight fast. Glycerol was assessed via Serum Triglyceride Determination Kit (Sigma-Aldrich) and fatty acids were quantified using the HR Series NEFA-HR(2) kit (Wako Diagnostics), in accordance with manufacturer's guidelines.

**Cell culture:** 3T3-L1 fibroblasts (pre-adipocytes) were cultured in 10% newborn calf serum, 4.5 g/L Dulbecco's Modification of Eagle's Medium (DMEM) with 1% penicillin, streptomycin and glutamine (PSG), until confluence. Cells were switched to a differentiation cocktail at two days post confluence (250nM dexamethasone, 500M 3-isobutyl-1-methylxanthine and 1g/mL insulin in 10% fetal bovine serum (FBS), in high glucose DMEM with 1% PSG) for four days (42). Media was replaced with differentiation medium containing only insulin for an additional three days. For the following three days, cells remained in FBS media with no additional treatment. To assess markers of lipolysis, cells remained in FBS media and were treated with ethanol (vehicle) or 250nM dexamethasone for five days before lysing.

**Assessment of Triglyceride Content in Cells and Tissue:** 3T3-L1 cells were grown and treated as described above. At the end of the treatment period, cells were lysed in homogenization buffer (50 mM Tris pH 8, 5 mM EDTA, 30 mM Mannitol, protease inhibitor) and subjected to three freeze thaw cycles with liquid nitrogen, thawed at room temperature. Frozen liver tissue was homogenized with 5mm stainless steel pellets in this buffer using a TissueLyser II (Qiagen). Lipids were extracted using KOH and a chloroform to methanol (2:1) extraction. Triglyceride content was assessed using the Serum Triglyceride Determination Kit (Sigma) and absorbance was detected as described in (43).

**Histology:** Tissues were fixed in 10% phosphate-buffered formalin for 24 hours and then stored in 70% ethanol until further processing. Tissues were dehydrated, embedded in paraffin and sent to the University of Michigan Comprehensive Cancer Center Tissue Core where they were processed and stained with hematoxylin and eosin (H&E) to assess cell morphology.

**mRNA Extraction and Analysis:** Cells and tissues were lysed in TRIzol using the TissueLyser II, as decribed above, and RNA was extracted using a PureLink RNA kit (Life Technologies). cDNA was synthesized from 0.5-1g of RNA using the High Capacity Reverse Transcription Kit (Life Technologies). Primers, cDNA and Power SYBR Green PCR Master Mix (Life Technologies) were combined in accordance with the manufacturer's guidelines and quantitative real-time PCR (qPCR) was performed as previously described (44) using the QuantStudio 5 (Thermo Fisher Scientific). mRNA expression level was normalized to *Actb* and analyzed using the delta delta Ct method after evaluation of several reference genes. qPCR primer sequences are listed in Table 1.

**Protein Extraction and Analysis:** Cells and tissues were lysed in RIPA buffer (50 mM Tris, pH 7.4, 0.25% sodium deoxycholate, 1% NP40, 150 mM sodium chloride, 1 mM EDTA, 100 uM sodium orthovanadate, 5 mM sodium fluoride, 10 mM sodium pyrophosphate and 1x protease inhibitor), centrifuged at 14,000rpm for 10 minutes at 4°C. Clarified lysates diluted in loading buffer. Lysates were heated at 85-95°C and proteins were separated by SDS-PAGE (Life Technologies) and trans-

**Table 1:** Primers used for RT-qPCR analyses.

| Gene | Forward Sequence | Reverse Sequence |
|---|---|---|
| *Actb* | ATGTGGATCAGCAAGCAGGA | AAGGGTGTAAAACGCAGCTCA |
| *Fasn* | GGAGGTGGTGATAGCCGGTAT | TGGGTAATCCATAGAGCCCAG |
| *Pnpla2* | CCACTCACATCTACGGAGCC | GATGCAGAGGACCCAGGAAC |
| *Srebf1* | AGGCCATCGACTACATCCG | TCCATAGACACATCTGTGCCTC |

ferred onto nitrocellulose membranes overnight at room temperature. Membranes were blotted using anti-adipose triglyceride lipase (ATGL; 54 kDa; Cell Signaling Technologies; catalog #30A4). Antibody complexes were detected by anti-mouse and anti-rabbit fluorescent conjugated antibodies (Invitrogen) and visualized using an Odyssey CLx image scanner. Blots were quantified using Image Studio software version 5.2 (LiCOR) and normalized to Revert Total Protein Stain (LiCOR).

**Statistics**: All data are presented as mean +/- standard error of the mean. For animal studies, two-way ANOVA analyses were performed to test for significance of diet and dexamethasone treatment, as well as their interaction. Pairwise comparisons, normality and equal variance were tested using Shapiro-Wilk and Levene's tests, respectively. Pending those results, a Mann-Whitney, Welch's or Student's *t*-test were used. P-values below p=0.05 were considered significant. All statistical tests were performed using the R software package version 3.30. All raw data and analysis scripts are available at `https://github.com/BridgesLab/CushingAcromegalyStudy`.

# Results

## Dexamethasone-Induced Insulin Resistance is Worsened in the Presence of Obesity

We have previously published data suggesting differential gene expression in adipose tissue between those with Cushing's disease and controls depending on their obesity status (18). Based on this finding, we speculated that the glucocorticoid responses may vary according to obesity status. Here, we have re-analyzed the data stratifying the Cushingoid and control groups by BMI (Figure 1A), classifying participants as either "Not obese" (BMI $<$ 30) or "Obese" (BMI 30). We found no significant differences in BMI in the control group compared to the Cushing's group. However, a near-significant interaction between obesity status and Cushing's diagnosis for HOMA-IR score (p= 0.057; Figure 1B) was observed. Furthermore, we observed a modest (17%) increase in HOMA-IR score when comparing non-obese subjects with and without Cushing's disease, yet a 3.4-fold increase in the obese patients.

To investigate if obesity status influences insulin sensitivity in the presence of elevated glucocorticoids we performed an insulin tolerance test (ITT) on lean (NCD) and diet-induced obese (HFD) mice that were untreated (Water) or treated with glucocorticoids (Dexamethasone). HFD-fed, dexamethasone-treated mice were significantly more resistant to insulin-stimulated glucose disposal when compared to all other groups (Figure 1C). Additionally, HFD dexamethasone-treated mice exhibited dramatic fasting hyperglycemia, with a significant interaction between diet and drug

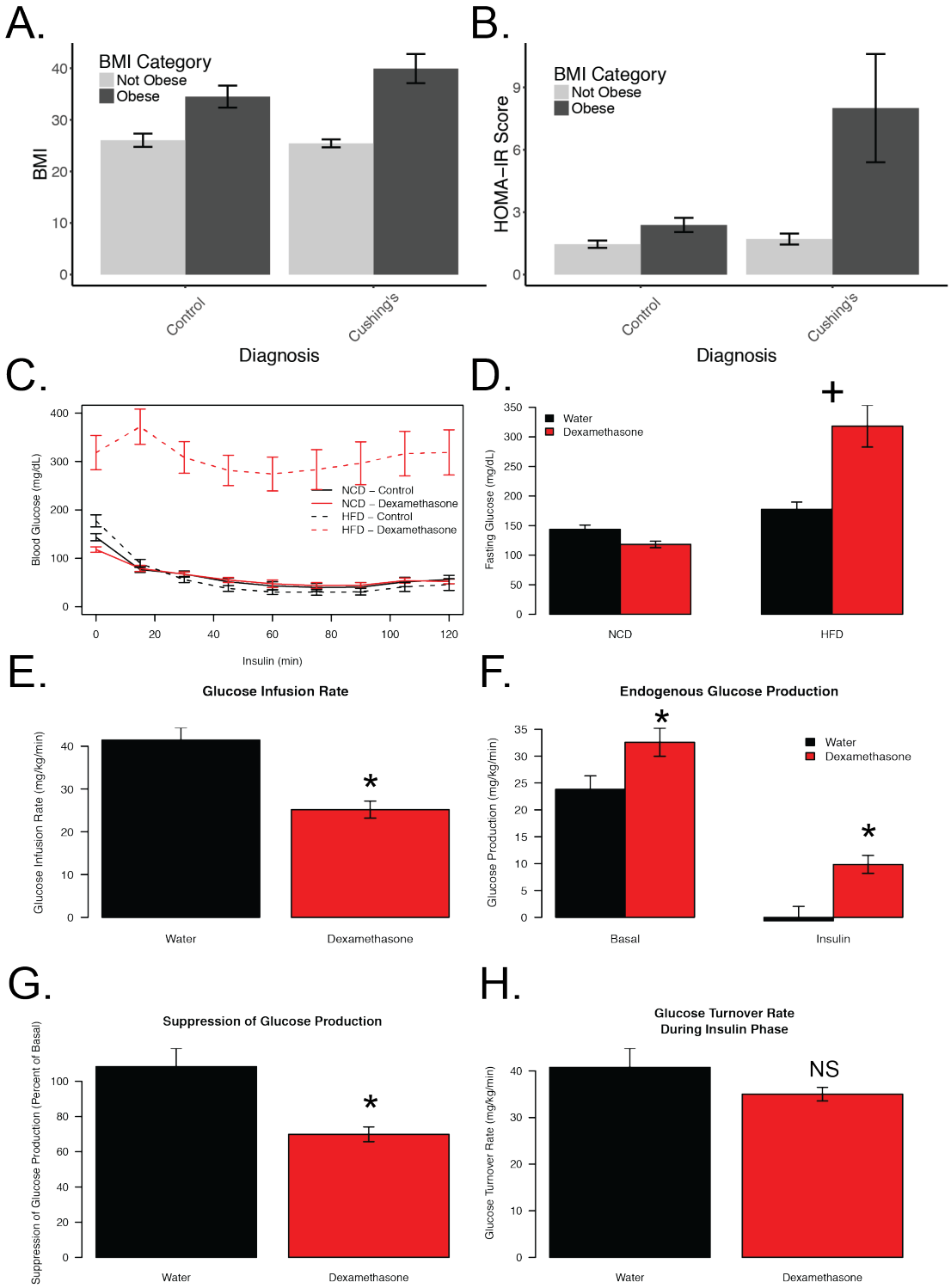


**Figure 1: Reductions in glucose handling are exacerbated in obese individuals with elevated glucocorticoids.**Cushings (non-obese n=3; obese n=5) and control (non-obese n=5; obese n=6) BMI (A) and HOMA-IR scores (B) stratified by obesity status. Mouse blood glucose levels during insulin tolerance test (C) and prior to insulin injection (basal; D). Insulin was given via i.p. injection at a concentration of 2.5mU/kg following five weeks of dexamethasone (NCD n=12; HFD n=12) or vehicle (NCD n=12; HFD n=12) treatment and 17 weeks of diet. Mouse glucose infusion rate (GIR; E) and endogenous glucose production (EGP; F) during euglycemic clamp following 3 weeks of dexamethasone (n=14) or vehicle (n=11) treatment and 11 weeks of HFD. For clamp experiments, insulin was infused at 8 mU/kg following a prime continuous infusion of 40mU/kg bolus. All mice were fasted for 5-6 hours prior to experiments. Asterisks in between two bars of the same condition indicate a significant interaction between diet and treatment. Centered asterisks indicated statistically significant treatment effect.

(p=0.00009; Figure 1D). While HFD animals had a 24% increase in fasting glucose when compared to NCD animals, in the presence of dexamethasone, HFD-fed animals had a 122% increase in fasting glucose relative to NCD controls not treated with dexamethasone. In the lean, NCD-fed animals, dexamethasone caused an 18% decrease in fasting glucose.

To evaluate glucose homeostasis in more detail we performed a hyperinsulinemic-euglycemic clamp in obese mice (11 weeks of HFD) treated with dexamethasone for the three weeks. This shorter HFD/dexamethasone exposure still caused dramatic insulin resistance, hyperglycemia and reductions in lean mass, but no differences in fat mass between the groups (Supplementary Figures 1A-D). Animals were clamped while conscious and glucose levels during the clamp as well as insulin turnover rate were similar between groups (Supplementary Figure 1E,F). During the hyperinsulinemic phase, the glucose infusion rate was 39% lower in obese dexamethasone-treated mice when compared to obese controls indicating insulin resistance at euglycemia (Figure 1E). Basal endogenous glucose production (EGP) was 37% higher in the dexamethasone- treated group (p=0.026). Moreover, in the control group, EGP was reduced to near zero by a high dose of insulin but only reduced 70% in the dexamethasone group (p=0.0091) resulting in glucose production being higher during the insulin phase in dexamethasone-treated mice (p=0.014) when compared to controls (Figure 1F-G). Glucose turnover was slightly decreased in the presence of insulin (p=0.141; Figure 1H). Despite these modest changes in glucose turnover, there were significant reductions in the obese, dexamethasone-treated animals in 2-deoxyglucose uptake in heart (34% reduced, p=0.0003) and gastrocnemius tissues (68% reduced; p=0.00002; Supplementary Figures 1G-H). These data suggest that increased glucose production and impaired suppression by insulin are the primary causes of insulin resistance and hyperglycemia in obese, dexamethasone-treated animals.

## HFD-Induced Liver Steatosis in Dexamethasone-Treated mice

Obesity and chronic elevations in glucocorticoids are associated with increased liver fat and NAFLD (2,13). We observe increases in plasma ALT, a liver enzyme associated with liver disease, in obese Cushing's patients (38% increase in non-obese subjects versus a 2.8 fold increase in obese subjects, p=0.13 for the interaction of disease and obesity status; Figure 2A). In our mouse model of HFD-fed, dexamethasone-treated mice, we observe drastically elevated liver triglycerides when compared to all other groups with a significant interaction of drug and diet (p=0.000068; Figure 2B). In support of this, H&E staining of hepatic tissue clearly depicts exacerbated lipid levels in the obese, dexamethasone-treated group when compared to obese controls and lean groups (Figure 2C).

We used qPCR to measure the expression of genes involved in hepatic *de novo* lipogenesis, *Srebf1* and *Fasn*, in liver lysates (Figure 2D). We observed a significant effect of diet and drug on *Fasn* expression (p=0.014), and although both transcripts were somewhat elevated in response to HFD alone, no synergism in expression levels was observed with dexamethasone. This finding indicates that lipid accumulation in response to dexamethasone treatment is likely occurring via mechanisms other than accelerated glucocorticoid-dependent activation of *de novo* lipogenesis.

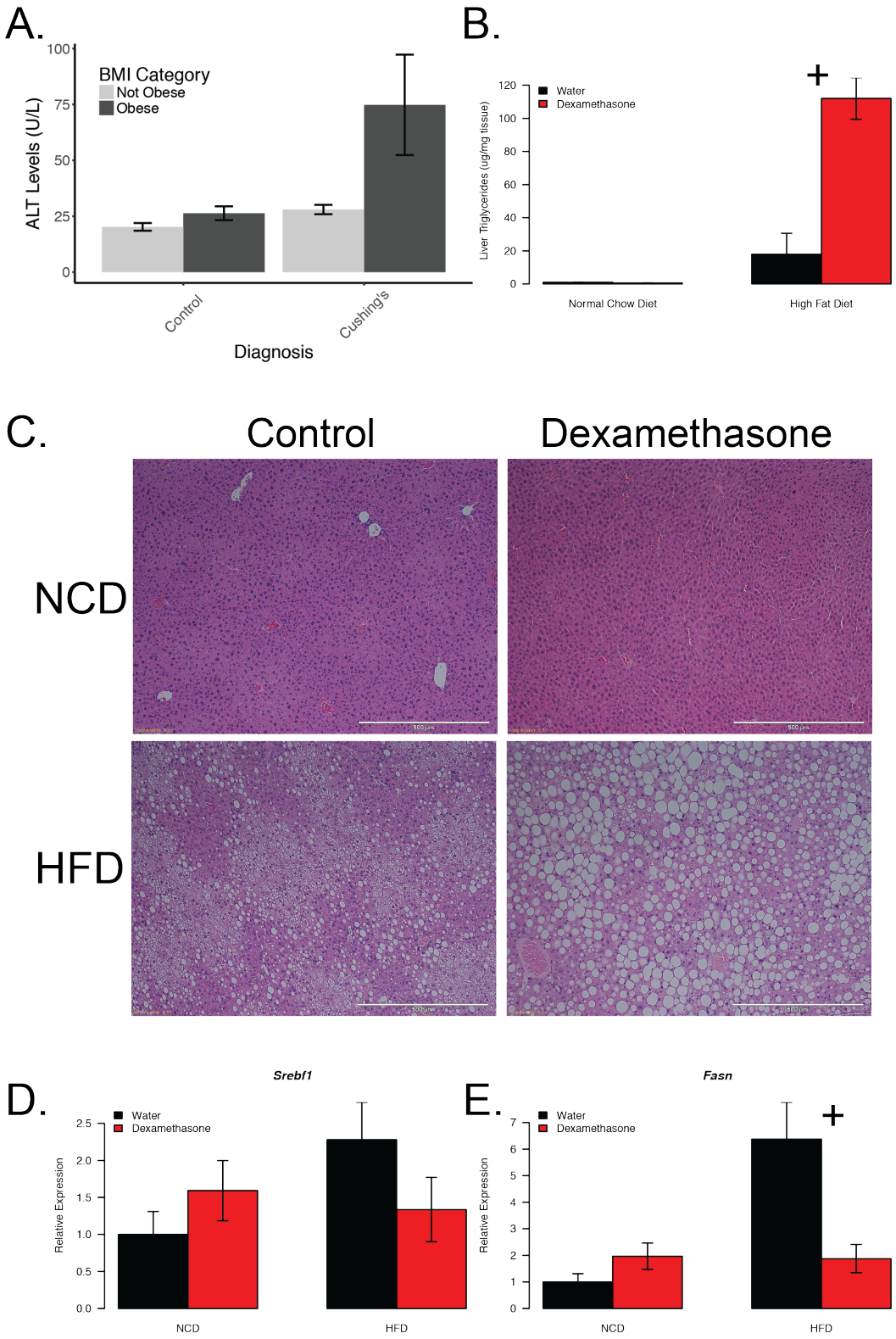




**Figure 2: Increased glucocorticoids lead to greater severity of hepatic steatosis in obese mice.** Patient ALT levels (A). Mouse hepatic triglyceride levels (B) and Hematoxylin and Eosin stained liver sections (C) and qPCR of hepatic de novo lipogenic transcripts (D, E). Mice were euthanized at 28 weeks of age following six weeks of dexamethasone (NCD n=7; HFD n=5) or vehicle (NCD n=6; HFD n=9) treatment and 18 weeks of diet. Liver stains are representative samples from each group. Asterisks indicate a significant interaction between diet and treatment.

## Dexamethasone Causes Decreased Fat Mass in Obese Mice

To understand the how dexamethasone effects body composition in these animals, we measured fat mass via EchoMRI. We observed reductions in fat mass in the HFD-fed dexamethasone-treated group (Figure 3A-B). These reductions do not appear to be depot-specific, as we observe reductions in both iWAT (65% reduced) and eWAT mass (59% reduced) at the end of the study in the HFD-fed animals treated with dexamethasone (Figure 3C). There were no significant reductions in fat mass, either by MRI or gross tissue weights of iWAT or eWAT depots in response to dexamethasone treatment in the chow-fed groups (Figure 3B-C). To determine if changes in body composition could be explained by altered caloric consumption (Figure 3D), we compared food intake among the groups. Surprisingly, we found that the dexamethasone-treated HFD-fed animals ate slightly more food, even though they lost substantial fat mass throughout the study (11% increase, p=0.032). These data suggest that the weight loss in obese animals provided dexamethasone is not due to reductions in food intake.

## Dexamethasone Treatment Results in Increased Lipolysis

One potential mechanism that could explain reduced adiposity, increased insulin resistance and NAFLD is accelerated adipocyte lipolysis. Lipolysis has previously been associated with insulin resistance (25,31), is known to be elevated in patients with NAFLD(28), and has been shown to increase with glucocorticoid treatment (18,22–24). To assess whether dexamethasone was directly affecting the lipid content in adipose tissue, we measured markers of adipocyte lipolysis in cultured adipocytes. 3T3-L1 fibroblasts were undifferentiated (pre-adipocytes), differentiated and treated with vehicle (mature adipocytes) or dexamethasone following differentiation (mature adipocytes +dexamethasone) over a 15-day period. Dexamethasone treatment following differentiation led to decreased lipid content (52.4% reduction, p=0.005) and a 71% increase in the amount of glycerol in the media (p=0.001), suggesting increased lipolysis (Figure 4B). In order to identify a potential GR-dependent lipolytic target, we evaluated the levels of ATGL, the rate limiting enzyme in lipolysis. Expression of ATGL (encoded by the *Pnpla2* gene) was enhanced following dexamethasone treatment in 3T3-L1 cells at both the transcript (2.7 fold, p=0.002; Figure 4C) and protein (4.2 fold, p=0.025; Figure 4D-E) levels. These data show that glucocorticoids elevate both ATGL levels and metabolites of lipolysis in cultured adipocytes.

To measure the effects of glucocorticoid-induced lipolysis *in vivo,* we quantified glycerol levels in animals chronically exposed to dexamethasone in basal and stimulated conditions (Figure 4E). Stimulation of lipolysis was achieved via isoproterenol, a -adrenergic receptor agonist, or by a 16-hour fast. Twelve weeks of dexamethasone treatment led to significant increases in glycerol in the fed (2.9 fold), fasted (1.5 fold) and isoproterenol-stimulated (1.4 fold, $p<0.05$ for all pairwise comparisons) conditions, indicating that dexamethasone enhances basal and stimulated lipolysis *in vivo* in chow-fed mice, as has been previously reported (45). Consistent with these findings, mRNA analysis from iWAT of these mice showed an upregulation of *Pnpla2* transcripts in the dexamethasone-treated mice compared to controls (2.1 fold, p=0.016; Figure 4F).

Since the HFD-fed, dexamethasone-treated mice have more severe insulin resistance and hepatic lipid accumulation than the chow fed mice, we quantified serum

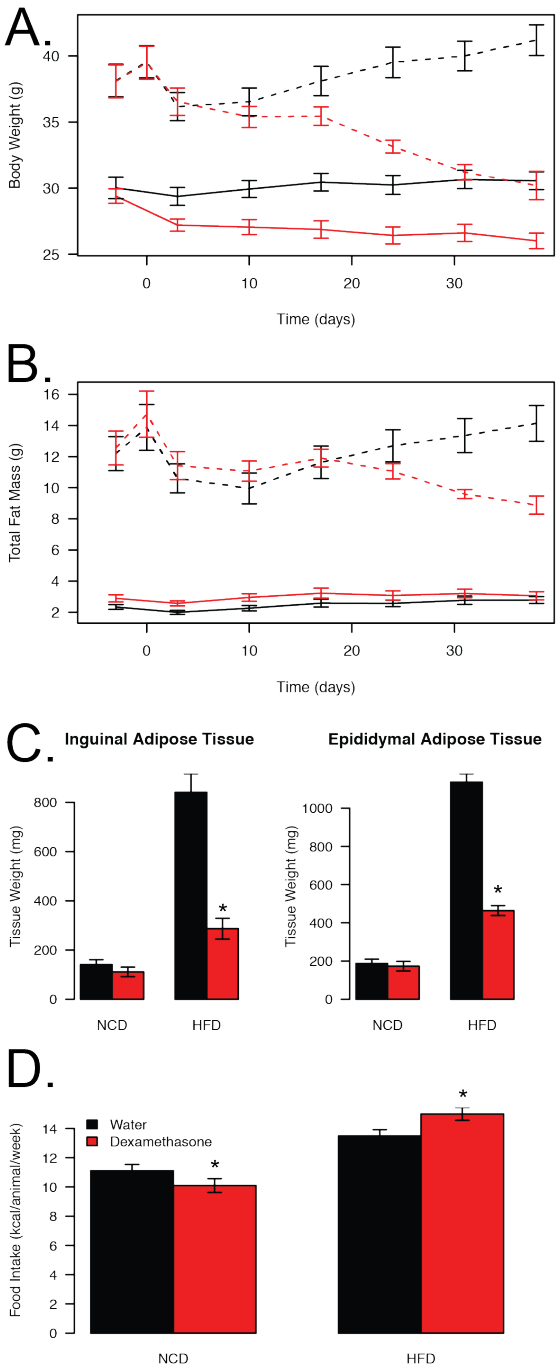


**Figure 3: Dexamethasone treatment reduces fat mass in obese mice.** Weekly total body mass (A) and fat mass (B) measures via EchoMRI in mice over the course of treatment (solid lines represent NCD mice and dashed lines represent HFD mice). Adipose tissue weights in 16 hour fasted mice following euthanasia (C). Mice were euthanized at 28 weeks of age following six weeks of dexamethasone (NCD n=8; HFD n=12) or vehicle (NCD n=8; HFD n=22) treatment and 18 weeks of diet. Food consumption measured weekly over the course of treatment (D). Asterisks indicate a statistically significant treatment effect.

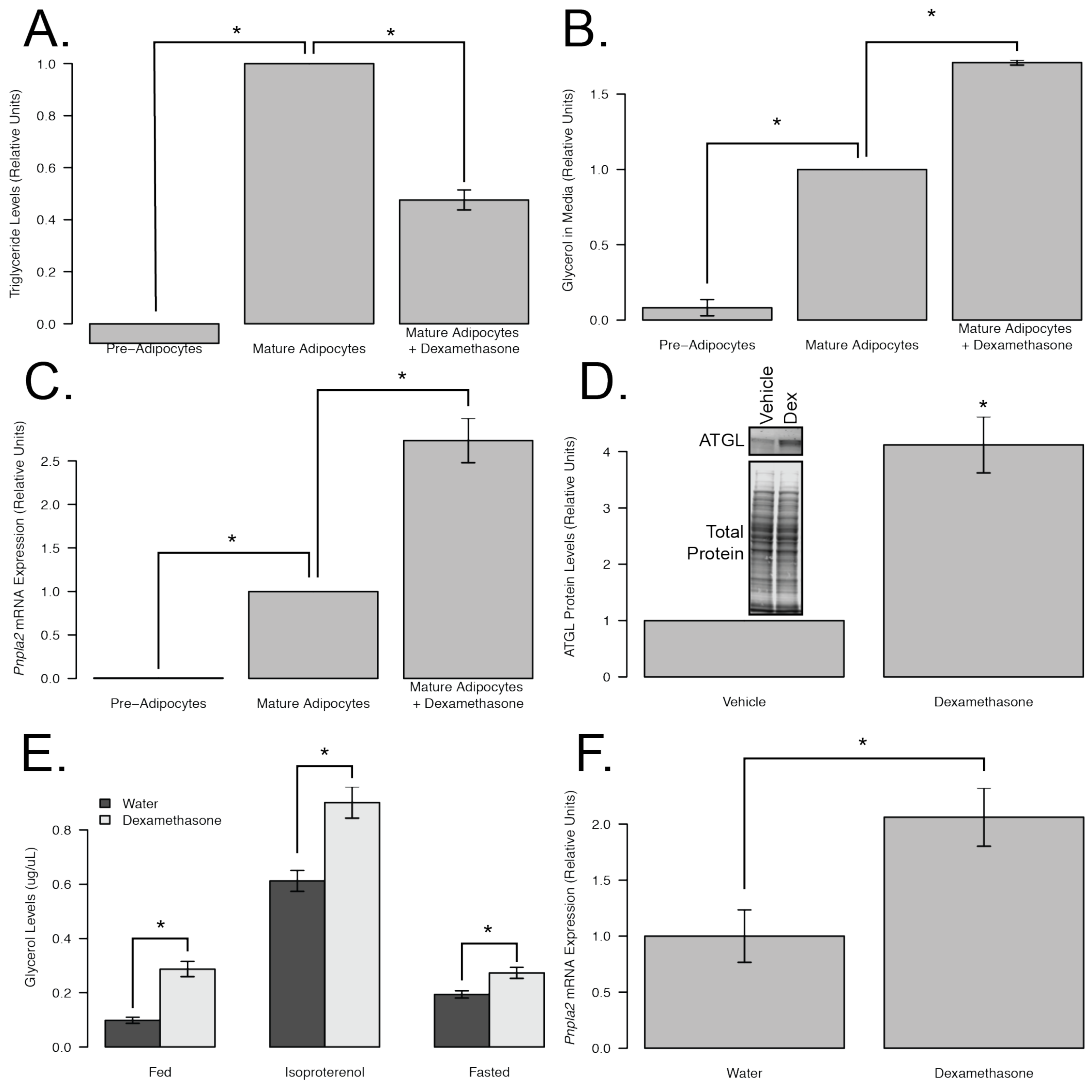


**Figure 4: Dexamethasone treatment induces lipolysis in vivo and in vitro.** Triglyceride levels (A), glycerol released in media (B), qPCR of lipolytic transcripts (C), and western blot of ATGL (D) from non-differentiated (pre-adipocytes; n=2) or differentiated 3T3-L1 mouse adipocytes (mature adipocytes) following five days of dexamethasone (n=3) or vehicle treatment (n=3). Serum fatty acid and glycerol levels at basal (fed) and following stimulation (10mg/kg isoproterenol or 16hr fast; E) and qPCR of IWAT lipolytic transcripts (F) in 22-week-old, 12-week dexamethasone- (basal and isoproterenol n=7; fasted serum and qPCR n=4) or vehicle- (basal and isoproterenol n=12; fasted serum and qPCR n=11) treated, chow-fed mice with the exception of isoproterenol-stimulated glycerol, which was performed one week prior to euthanasia. Asterisks indicated statistically significant treatment effect.

glycerol concentrations following a 16-hour fast (Figure 5A). We observed a nearly two-fold increase in serum glycerol levels by 6 weeks of dexamethasone treatment in the HFD-fed animals, compared with only a 18% increase in chow-fed mice. There was a significant interaction between dexamethasone exposure and diet (p=0.017) on glycerol levels. We then asked if the increase in lipolytic metabolites was suppressed by insulin during the hyperinsulinemic euglycemic clamp in the obese mice (Figure 5B). Consistent with our previous results, there was a 40% elevation in serum basal non-esterified fatty acids (NEFA's) in response to 3 weeks of dexamethasone treatment (p=0.004). During the insulin phase, dexamethasone treatment attenuated the ability of insulin to suppress serum NEFA levels with insulin leading to a 71% reduction in controls compared to only a 48% reduction in dexamethasone-treated mice (p=0.058). These findings suggest that dexamethasone elevates lipolysis in the obese setting and likely attenuates the suppressive effects of insulin.

We quantified mRNA and protein expression of ATGL in the iWAT of these mice (5C-E). Consistent with the hypothesis that ATGL activation could drive increased lipolysis in HFD and dexamethasone-treated mice, expression of ATGL was elevated in both dexamethasone-treated groups, with a significant synergistic effect of dexamethasone and obesity at both the transcript (p=0.02 for the interaction) and protein (p=0.043 for the interaction) levels. These data support the hypothesis that glucocorticoid-stimulated lipolysis is augmented in the context of obesity, potentially via increased transactivation of *Pnpla2*/ATGL.

## Discussion

Chronic glucocorticoid elevations are associated with many co-morbidities such as increased fat mass (18,20,21), decreased muscle mass (17–19), insulin resistance (4,5) and NAFLD (2,3). These adverse effects are similar to those seen in obesity; however, the combination of chronically elevated glucocorticoids in the context of pre-existing obesity has not been assessed. Here, we show that the effects of glucocorticoid-induced insulin resistance and NAFLD are exacerbated when paired with obesity.

We found that obese patients with Cushing's disease have higher waist circumference (data not shown), indicative of central adiposity, and have a tendency for increases in HOMA-IR score, suggesting increased insulin resistance. Additionally, we observed increases in the liver enzyme ALT, a marker of liver disease. In line with these findings, increases in central adiposity, as is frequently observed in people with obesity, has been associated with enhanced fatty acid flux (i.e. lipolysis) when compared to lower body fat stores (46), which is thought to contribute to insulin resistance and fatty liver (25,47).

There are two limitations to these interpretations: one is the small sample size, and two, that it is not possible to determine the physiological status of Cushing's patients before they developed a tumor; therefore, we could not discern whether obesity was present prior to or after development of Cushing's disease. To address the question of whether the obese state modulates the effects of glucocorticoid excess, we designed mouse studies that assess the metabolic outcomes frequently associated with both obesity and exposure to elevated glucocorticoid concentrations.

We found that obese, dexamethasone-treated mice exhibited hyperglycemia and severe insulin resistance when compared to obese control mice. This was primarily

due to increased endogenous glucose production in these animals. The combination of HFD and dexamethasone also led to significant elevations in liver fat, consistent with a trend towards elevated ALT levels seen in the obese Cushing's patients. Cushing's disease is often paired with increased fat mass, which has been proposed to contribute to fatty liver (48,49). Indeed, obesity is a known risk factor of NAFLD (13,14). Previous work from our lab shows increased fat mass following 12 weeks of dexamethasone treatment (18) in chow-fed mice, in accordance with what others have reported (50). However, to our surprise, the glucocorticoid treatment in obese mice led to an overall reduction in adiposity. Therefore, when comparing obese control mice to obese dexamethasone-treated mice, increased fat mass is not the major mechanism behind the observed exacerbations in insulin resistance and increased liver fat.

Lipolysis has been linked to increased gluconeogenesis by several studies (51–54). One potential mechanism is that the increased flux of fatty acids, oxidized in the liver to acetyl-CoA, activate pyruvate carboxylase and gluconeogenesis (53,54). Glucocorticoids are known to stimulate lipolysis (18,22–24), possibly as a way to promote gluconeogenesis to maintain blood glucose levels, a key function of these hormones. Lipolysis has been implicated in insulin resistance (25,31) and NAFLD (28) and there is evidence that it is enhanced in the obese state (55). We found synergistic elevations in glycerol, indicative of enhanced lipolysis, as well as in hepatic fat accumulation in the HFD-fed, dexamethasone-treated mice, but no data supporting enhanced hepatic *de novo* lipogenesis. These findings suggest that lipolysis may drive enhanced hepatic lipid accumulation in these mice.

There is some debate as to which genes glucocorticoids are acting on to promote lipolysis. Downregulation of *Pde3b* (56) and upregulation of -adrenergic receptors (57) and ATGL transcripts (35,58,59) have been proposed as possible mechanisms. We assessed all of the previously proposed targets (data not shown) and found ATGL, the rate limiting enzyme for adipose triglyceride lipolysis, to be synergistically activated by obesity and glucocorticoid-treatment. These findings bear a resemblance to elevations in glycerol levels in obese, dexamethasone-treated mice when compared to diet or glucocorticoids alone. The mechanisms by which obesity and glucocorticoids synergize to activate ATGL expression are not clear at this time, nor are the relative contributions of other glucocorticoid receptor-dependent targets.

Further research is needed to determine whether the insulin resistance observed is due to obesity or the high fat content of the diet. We evaluated glucocorticoid treatment in obesity; however, Riddell and colleagues have reported similar findings when giving HFD and glucocorticoids in concert to rats, prior to the onset of obesity (15,16,60). Further studies are needed to determine whether diet or obesity status or both are the source of this elevated metabolic risk to glucocorticoids, and whether these mechanisms are similar.

In summary, glucocorticoids are commonly prescribed drugs used to treat a multitude of health issues, but are known to induce a variety of adverse metabolic effects. Their actions in persons with obesity are not yet clear, even though there is a significant number of obese individuals routinely taking prescription glucocorticoids. The data presented here show that the obese state exacerbates several co-morbidities associated with chronically elevated glucocorticoids. These effects should be considered by physicians when determining glucocorticoid treatment options for patients with obesity. Future studies will determine whether blocking glucocorticoid and/or lipolytic action in the fat tissue is beneficial for preventing or enhancing recovery

from glucocorticoid-induced metabolic disturbances.

## Acknowledgements

This study was supported by funds from NIH Grant R01-DK107535 (DB). This study also utilized the University of Michigan Metabolism, Bariatric Surgery and Behavior Core (U2C-DK110768), the Michigan Nutrition Obesity Research Center (P30-DK089503) and the University of Michigan Comprehensive Cancer Center Core (P30-CA062203). Erin Stephenson is partially supported by funding from Le Bonheur Children's Hospital, the Children's Foundation Research Institute and the Le Bonheur Associate Board. We would like to thank Jennifer DelProposto and Carey Lumeng for assistance with imaging liver sections, and Melanie Schmitt for assistance with glucose clamp studies. We would like to thank the other members of the Bridges laboratory, Thurl Harris (University of Virginia) and Edwards Park (UTHSC) for insights on this work.

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