**Introduction**

**Methods**

* Patient Recruitment

The study was approved by the institutional review board of the University of Michigan Medical System. Written informed consent was obtained from all of the patients. Patients were recruited consecutively from those undergoing a transsphenoidal adenomectomy at the University of Michigan for Cushing's disease or nonfunctioning pituitary adenoma over a 12-month period. Exclusion criteria were age <18, current hormone treatment including glucocorticoids, malignancy, inflammatory disease, diabetes type 1 and established pituitary hormone deficiencies. For each patient, a data sheet was completed including, age, sex, anthropometric measurements, diagnosis of hypertension, diabetes, results of blood tests and medications. Fasting blood samples were assayed for glucose (Siemens Advia 1800, Deerfield, IL, USA) and insulin (Life Technologies) as instructed by the manufacturers.

* Treatment of Animals with Dexamethasone

C57BL/6J adult male mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) at nine weeks of age. Following a one-week acclimation period, mice were treated as described previously (1) or were either kept on normal chow (NCD) or given high fat diet (45% fat; x carbs; x protein) for 12 weeks. Mice stayed on their respective diets and were treated with 1 mg/kg per day of dexamethasone (Sigma–Aldrich) in their drinking water (*n*=x) or used as controls (*n*=x) for six weeks. All animal procedures were approved by the University of Tennessee Health Science Center Institutional Animal Care and Use Committee. Animal body weight and composition was determined weekly using an echoMRI 2100. Food was weighed weekly, with food intake determined as the decrease in food weight per mouse per week per cage. All mice were provided with access to food and water *ad libitum* throughout the study. At the end of treatment, mice were fasted for 16 h and were sacrificed by cervical dislocation at ZT3 after isoflurane anesthesia. Following cervical dislocation, a sagittal incision was made along the medioventral surface of each mouse and the skin was carefully pulled back to expose the subcutaneous fat depots. The incision was extended along the anterior surface of each hind limb to allow careful dissection of the inguinal fat pads. A small incision was then made into the rectus abdominus muscle to expose the abdominal cavity. The epididymal fat pads were identified and carefully dissected out. The right fat pads from each mouse were weighed and snap frozen in liquid nitrogen for later analysis, along a section of the large lobe of the liver. Small pieces of these tissues, as well as the pancreas were placed in 10% formalin for histology.

* ITT

Insulin tolerance was assessed following five weeks of treatment (27 weeks of age). Following a 6-h fast, mice were given i.p. injections of insulin (Humulin R, Lilly, Indianapolis, IN, USA) at a concentration of 2.5 mU/g. Blood glucose was determined at 15-min intervals post-injection using a One Touch Ultra Glucometer (Lifescan).

* Clamp (get from metabolic phenotyping core?)
* Serum ALT-get from Hochberg paper
* Cell culture

3T3-L1 fibroblasts (pre-adipocytes) were cultured in 10% newborn calf serum, high glucose Dulbecco's Modification of Eagle's Medium (DMEM) with 1% pencilin, streptomycin and glutamine until confluence. A differentiation cocktail including 250nM dexamethasone, 3-isobutyl-1-methylxanthine and insulin in 10% fetal bovine serum, high glucose DMEM with 1% PSG at two days post confluence for four days. Media was then replaced including only insulin in the cocktail for an additional three days. The following three days cells remained in FBS media with no additional treatment. To assess lipolysis, cells either remained in FBS media or were treated with an additional dose of 250nM dexamethasone for five days before lysing.

* Liver and cells TG/TG assay
* Histology

The liver, IWAT, EWAT, BAT and pancreas were kept in 10% formalin for 24 hours and then stored in 70% ethanol until further processing. Following a series of wash steps, tissues were embedded in paraffin wax and sent to either the University of Michigan University of Michigan Comprehensive Cancer Center Tissue Core or the Unit for Laboratory Animal Medicine In-vivo Animal Core (University of Michigan, Ann Arbor) where they were processed and stained with H&E or trichrome to assess cell morphology/inflammation and collagen formation, respectively.

* Analysis of mRNA

Cells and tissues were lysed in TRIzol and RNA was extracted using the PureLink RNA mini kit (Life Technologies). cDNA was synthesized from 0.5-1g 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 was performed as previously described (2). mRNA expression level was normalized to *Actb* (Table 1).

* Protein 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 and 10 mM sodium pyrophosphate) on ice then centrifuged for 15 minutes at 13 000 RPM at 4°C. Clarified lysates were loaded on SDS-PAGE gels, transferred and blotted using antibodies raised against ATGL, HSL, pHSL, CGI-58, GAPDH, and ACTIN. Antibody complexes were detected by anti-mouse and anti-rabbit fluorescent conjugated antibodies and visualized using an Odyssey image scanner and blots were quantified using the Odyssey software version 2.1 (LiCOR).

* *In vivo* Lipolysis

Mice were briefly anesthetized with isoflurane and blood was taken via retro orbital bleed from X-week old fed mice at baseline and 15 minutes following an i.p. injection of 10mg/kg isoproterenol (Sigma-Aldrich) in PBS. Glycerol and free fatty acids were assessed via Serum Triglyceride Determination Kit (Sigma-Aldrich) and HR Series NEFA-HR(2) (Wako Diagnostics), respectively, in accordance with manufacturer’s guidelines.

* Stats

**Results**

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

Our group has previously published data (1) that illustrates different physiological and gene expression outcomes between those with Cushing’s disease (ACTH-secreting pituitary adenoma) and controls (non-secreting pituitary adenoma). More recently, we speculated that the conditions within the groups may vary according to obesity status. Here we have re-analyzed the data stratifying the Cushingoid and control groups by BMI, classifying these individuals as “Not obese” (BMI < 30) and “Obese” (BMI ≥ 30). The presence of Cushing’s in individuals with a high BMI leads to increased insulin resistance (measured by HOMA-IR score), above that of Cushing’s or obesity alone.

To further investigate if obesity status influences insulin sensitivity in the presence of high glucocorticoids we performed an insulin tolerance test (ITT) on lean (NCD) and diet-induced obese (HFD) mice that were untreated (Control) or treated with glucocorticoids (Dexamethasone; Figure 1A-B--schematic). All groups were given a relatively large dose of insulin (2.5 U/kg) to account for the known insulin resistance typically seen in diet-induced obese (cite). HFD-fed, dexamethasone-treated mice were significantly more resistant to insulin-stimulated glucose uptake when compared to all other groups. Though, it is important to note that the NCD-fed, dexamethasone treated animals still experienced some insulin resistance at this high dose. Additionally, HFD/dexamethasone exhibited fasting hyperglycemia, with a significant interaction between diet and drug (p=0.00009).

Clamp data

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

Obesity and chronic elevations in glucocorticoids have been associated with increased liver fat and even non-alcoholic fatty liver disease (NAFLD). We observed slight increases in plasma AST and ALT, which are liver enzymes associated with liver disease (cite?), in Cushing’s patients and obese controls; interestingly, levels were further elevated in obese Cushing’s patients, synergistically so in the case of ALT (Figure 2).

Since elevated liver enzymes are just one indicator of liver disease, they are not sufficient to lend a diagnosis, we studied this in our mouse model. HFD-fed, Dexamethasone treated mice had significantly elevated liver triglycerides when compared to all other groups (Figure 2). In support of this, H&E staining of hepatic tissue clearly depicts higher lipid levels in this group (Figure 2). Collagen/trichrome data…

Expression of genes involved hepatic *de novo* lipogenesis (*Srebf1* and *Fasn*) was assessed via qPCR (Figure 2). Both transcripts were highly elevated in response to HFD alone; however, levels of both these enzymes were reduced in HFD/dexamethasone livers. This finding indicates that lipid accumulation resulting from dexamethasone treatment is likely occurring via a different mechanism than transcriptional activation of *de novo* lipogenesis.

# Dexamethasone Causes Decreased Fat Mass in HFD-Fed Mice

Dexamethasone treatment lead to decreased body mass in both NCD and HFD groups (FIG 3). The reduced body mass was primarily due to lean mass loss. Surprisingly, there was also a loss in fat mass in the HFD-fed, dexamethasone treated group (Figs-- MRI and fat pad weights). There were no significant differences in food consumption.

Fat cell size/inflammation…

Dexamethasone Treatment Results in Increased Lipolysis

Lipolysis has previously been associated with insulin resistance, is a known cause of Non-Alcoholic Fatty Liver Disease (NAFLD; cite), and has been shown to increase with glucocorticoid treatment. We first assessed whether there was a direct effect of dexamethasone on adipocytes in culture (figure 4). 3T3-L1 fibroblasts were either kept in media alone (pre-adipocytes), differentiated (mature adipocytes) or treated with dexamethasone following differentiation (mature adipocytes +dexamethasone) over a 15-day period. Dexamethasone treatment following differentiation lead to decreased lipid content and increased glycerol release into the media, indicating increased lipolysis. To assess this further, we measured lipolytic enzyme mRNA and protein expression levels in these cells (figure 4). Expression of ATGL (encoded by *Pnpla2*) and HSL (encoded by *Lipe*) were enhanced following dexamethasone treatment.

To assess the effects of glucocorticoid-induced lipolysis *in vivo,* we measured the by-products of triglyceride breakdown, glycerol and free fatty acids in basal and stimulated conditions (figure 4). Stimulation of lipolysis was achieved via isoproterenol, a -adrenergic receptor agonist, or by a 16-hour fast. For isoproterenol stimulation of lipolysis fed mice were i.p. injected with 10 mg/kg isoproterenol and basal levels were determined prior to injections. Serum free fatty acids and glycerol were measured for each of these conditions. Dexamethasone treatment led to increases in glycerol and free fatty acids across all conditions.

qPCR lipolytic genes in these mice

These data show that glucocorticoids directly stimulate lipolysis in adipose tissue.

# Dexamethasone-Induced Lipolysis is increased in HFD-Fed Mice

To determine whether the effect of dexamethasone-induced in vivo lipolysis was exacerbated in the context of obesity we measured serum glycerol following a 16-hour fast (figure 5). Similarly, was elevated in dexamethasone treated animals and there was a significant interaction between drug and diet (p value).

We also quantified mRNA and protein expression of lipolytic enzymes, ATGL and HSL, in the iWAT of these mice. Consistent with the above findings, expression was elevated in the dexamethasone-treated groups and there was a significant interaction of drug and diet . These data show that glucocorticoid-stimulated lipolysis is augmented in the context of obesity.

**Discussion**

Chronic glucocorticoid treatment is known to cause, or at least be associated with, many co-morbidities such as increased fat mass, decreased muscle mass, insulin resistance and non-alcoholic fatty liver disease (NAFLD) in the lean setting, all of which can have a negative effect on metabolism. These side effects are similar those seen in obesity; however, the combination of chronically elevated glucocorticoids in the context of obesity has not assessed. Here we show that glucocorticoid-induced symptoms are exacerbated when paired with obesity.

Obese patients with Cushing’s disease were found to have a higher BMI than obese control patients and this was paired with increases in HOMA-IR score, indicating increased insulin resistance as well as increases in the liver enzyme ALT, a marker of liver disease. However, it is impossible to determine the physiological status of the patients before they got a tumor; therefore, we cannot discern whether obesity was present prior to tumor development or after tumor development, possibly as a result of the disease and contributing to the worsening of comorbidities. For this reason, we designed a mouse study to investigate whether being obese prior to glucocorticoid treatment leads to worsened outcomes.

HFD, dexamethasone-treated mice had hyperglycemia, which was not present in any of the other groups, as well as severe insulin resistance. Clamp data..

Significant elevations in liver fat accumulation was also seen in HFD-fed mice with even further increases in the HFD-fed, dexamethasone treated group, consistent with the elevated ALT levels seen in the obese Cushing’s patients.

To our surprise, the glucocorticoid treatment in obese mice led to an overall reduction in adipose, which was not depot-specific. Previous work from our lab shows increased fat mass following chronic dexamethasone treatment (1), which has also been reported by others using various glucocorticoids. Therefore, increased fat mass is not the culprit contributing to worse insulin resistance and increased liver fat when comparing HFD control mice to HFD dexamethasone-treated mice.

Lipolysis has been associated with decrease fat mass, fatty liver disease, insulin resistance and is known to be induced with glucocorticoids in both humans (1) and mice. We assessed *in vivo* lipolysis in lean mice given glucocorticoids for x weeks measuring serum glycerol and fatty acid levels in the basal (fed) and stimulated conditions. Stimulation of lipolysis was achieved by a 16 hour fast or by i.p. injections of isoproterenol, a known -adrenergic agonist, to fed mice. Dexamethasone treatment led to significant increases in both glycerol and fatty acid release in all conditions when compared to controls. Elevations in markers of lipolysis were even greater in obese, dexamethasone-treated mice in the fasted state.

There is some debate as to which genes glucocorticoids are acting on to promote lipolysis.

Downregulation of Pde3b (3) and upregulation of -adrenergic receptors and/or lipase transcripts have all been proposed as possible mechanisms.

End: Glucocorticoids are a commonly prescribed drug used to treat a multitude of health issues and are known to induce a variety of metabolic side effects; however, their actions in persons with obesity has not been studied to date. The data presented here shows that obesity does in fact pose a greater risk for the harmful co-morbidities associated with chronically elevated glucocorticoids and this should be considered when determining treatment options. More work is required in the area to assess whether blocking glucocorticoid/lipolytic action in the fat tissue would be beneficial to prevent or enhance recovery of the afore-mentioned glucocorticoid-induced comorbidities.