**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 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.

* qPCR

Cells and tissues were lysed in TRIzol 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 (1). mRNA expression level was normalized to *Actb* (Table 1).

* Western blotting
* Isoproterenol test
* Stats

**Results**

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

Our group has previously published data (2) 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. However, it is not possible to determine when these individuals developed this disease and what their weight status was prior to their diagnosis.

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**