­The Cushing's disease adipose gene expression profile in human adipose tissue

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Abstract:

Glucocorticoids have major effects on adipose tissue metabolism To study tissue mRNA expression changes induced by chronic eleveated endogenous glucocorticoids we performed RNA sequencing patients with Cushing disease (n=5) compared to eleven patients with non functioning pituitary adenoma.

(n=11). We found higher expression of transcripts involved in several metabolic pathways, including…

# Introduction

Cushing’s Disease, or persistently high circulating levels of cortisol secondary to a pituitary adenoma, leads to a significant truncal obesity and diabetes (Cushing 1932). Obesity and diabetes are major factors in morbidity and mortality in Cushing’s syndrome 7-9. Cushing's Disease is very rare (incidence of 1.2–2.4 per million (Lindholm *et al.* 2001), but iatrogenic Cushing's syndrome, caused by chronic glucocorticoid treatment is very common and leads to a similar clinical manifestations.

Numerous studies have shown that glucocorticoids have profound effects on adipose tissue metabolism, including promotion of adipocyte differentiation 1 and induction of lipolysis and lipogenesis2, 3. Despite the widespread chronic glucocorticoid exposure, there have been no human *in vivo* studies on global gene expression changes in adipose tissue in response to long term exposure to glucocorticoids.

To study the effect of excess endogenous glucocorticoids on adipose tissue, we used RNA sequencing to study adipose tissue from Cushing's disease patients and controls with non-secreting adenomas. We found a distinctive pattern of changes in many transcripts that are highly associated with Cushing's disease. Many of these genes explain previously observed metabolic effects of excess glucocorticoids described *in vitro*, in animal models and in humans. These include enhanced fatty acid and triglyceride biosynthesis; protein degradation, activation of glycolysis and reductions in immune responses.

# Materials and 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 patients. Patients were recruited consecutively from those undergoing transsphenoidal adenomectomy at the University of Michigan for Cushing's disease or non-functioning 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) and insulin (Life Technologies) as instructed by the manufacturers.

## Subcutaneous fat biopsy

During the course of pituitary surgery a routine subcutaneous fat graft for sealing the surgical field is taken immediately after anasthesia, before glucocorticoid treatment. ~500 mg of this fat graft was used for the study. ~100 mg were utilized for ex vivo lipolysis assay, ~200 mg was snap frozen in liquid nitrogen and stored at -80 degrees for RNA preparation and ceramide analysis.

## Treatment of Animals with Dexamethasone

Twenty-four C57BL/6 adult male mice were purchased from Jax at 9 weeks of age. Following a one-week acclimation period, mice were either treated with dexamethasone (N=12) or used as controls (N=12). Water-soluble dexamethasone (Sigma) dissolved in double diluted water (ddH2O) was given at a calculated dose of 1mg/kg/day for a 30g mouse drinking x amount of water for 12 weeks; however, water was provided *ad libitum*. Controls were given regular ddH2O.

## Insulin Tolerance Test

## Grip Test

Grip strength was measured at baseline, 4, 8 and 12 weeks following treatment using a Chatillon digital force gauge (AMETEK). Mice were placed on the grid having all 4 paws in contact with the apparatus and slowly pulled backwards by the tail. Mice were given 5 trials with about 10 seconds rest in between trials. Strength was measured by peak torque (N).

## Ceramide determination

Ceramide analysis of tissue samples was performed by liquid chromatography-triple quadrupole mass spectrometry according to a modified version of the protocol reported in. Briefly, frozen tissue samples were pulverized under liquid nitrogen, then 20 mg portions were extracted using 1.6 mL of a 2:1:0.8 mixture of chloroform:methanol:water containing internal standards (50 ng each of C17 and C25 ceramide and C12 glucosylceramide per sample) The organic layer of the extract was dried under nitrogen gas and reconstituted in 100 uL of 60:40 acetonitrile: isopropanol (Bligh & Dyer 1959). The re-constituted extract was analyzed by electrospray ionization LC-MS/MS on an Agilent (Santa Clara, CA) 6410 triple quadrupole instrument operating in positive ion multiple reaction monitoring mode. The LC column used was a Waters (Milford, MA) Xbridge C18 2.5 µ, 50 mm x 2.1 mm i.d. Mobile phase A was 5mM ammonium acetate, adjusted to pH 9.9 with ammonium hydroxide; mobile phase B was 60:40 acetonitrile:isopropanol. The gradient consisted of a linear ramp from 50 to 100%B over 5 minutes, a 20 minute hold at 100%B, and re-equilibration at 50%B for 10 minutes. Injection volume was 25 µL. Ceramides and glucosylceramides were identified by retention time and by MS/MS fragmentation parameters, and were quantitated by peak area relative to the closest-matching internal standard using Agilent MassHunter Quantitative Analysis software.

## Transcriptomic Analysis

Total RNA was extracted from adipose tissue using the RNEasy kit (Qiagen) and its quality was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies). At the University of Michigan DNA Sequencing Core, cDNA libraries from polyA mRNA were prepared using TruSeq cDNA synthesis kit and sequenced using a HiSeq 2000 (Illumina). Samples were run on 2 lanes of a HiSeq 2000 (Illumina) generating 8 612 682 to 16 469 501 single-ended 50 bp reads per sample. These were aligned to the human genome (Enembl GRCh37.74, Genbank Assembly ID GCA\_000001405.14) using TopHat version 2.0.10 (Kim *et al.* 2013), Bowtie 2 version 2.1.0 (Langmead & Salzberg 2012) and Samtools version 0.1.18 . Reads were mapped to known genes using HTseq (Anders *et al.* 2014). Gene expression was analyzed using DESeq2 version 1.2.10 (Love *et al.* 2014). These subjects corresponded to the patients described in Table 1, with the exception of subjects 29 and 31 (both Cushing's disease patients), which had clinical data but no RNAseq data.

## Statistics

Descriptive statistics such as means and standard deviations were determined for clinical measurements. Student’s t-test was used to test the difference in means of these measurements between control and Cushing's disease patients. Normality assumption was checked via Shapiro-Wilk test. Wilcoxon rank sum tests were performed for HOMA-IR score, insulin levels and the 14:0, 16:0, 20:0 ceramides and the C16:0 glucosylceramide species as these data were not normally distributed.

Welch’s t-test was used for basal lipolysis since the equal variance assumption was rejected by Levene's test (car package version 2.0-19). Correlation coefficients were calculated by Pearson's product-moment. Statistical significance in this study was defined as a p/q-value of less than 0.05. All statistical tests were performed using the R package (version 3.0.2,(R Core Team 2013)). To correct for multiple hypotheses, p-values were adjusted by the method of Benjamini and Hochberg (Benjamini & Hochberg 1995).

We used Gene Set Enrichment Analysis (GSEA v2.0.13 (Subramanian *et al.* 2005; Clark & Ma’ayan 2011)) to determine whether our rank-ordered gene list for the comparison of Cushing's disease versus control patients is enriched in genes from gene ontology, KEGG, transcription factor or microRNA target gene sets. The gene list was ranked based on t-statistics and the statistical significance of the enrichment score was determined by performing 1000 phenotype permutation. Other settings for GSEA were left to the software defaults. All code and raw data from this study are available through the Gene Expression Omnibus (GSEXXXXX) and at http://bridgeslab.github.io/CushingAcromegalyStudy/

# Results

## Patient characteristics

Clinical and metabolic measurements were obtained for 5 Cushing's disease patients and 11 controls, who were admitted with non-secreting adenomas. Patient characteristics are shown in Table 1. Our Cushing’s patients were in general younger and had smaller tumors than the patients with non-secreting adenomas. In our cohort there was a trend towards elevated body weight (p=0.47), body mass index (BMI) (p=0.27) and abdominal circumference (p=0.07, Figure 1A), consistent with Cushing’s patients having elevated fat mass. We also detected a non-significant elevation in HOMA-IR score (2.6 Fold, p=0.67 by Wilcoxon test, Figure 1B), driven largely by increases in fasting insulin levels (p=0.30). Three out of the 5 Cushing's disease patients had diabetes while only 1 of the 11 controls had diabetes (p=0.03). These data are consistent with elevated glucose intolerance in patients with Cushing’s syndrome. We observed significant elevations in both ALT and AST in serum from Cushing’s patients. To evaluate lipolysis in explants from these patients we measured glycerol release from isolated subcutaneous adipose tissue and found a 3.1 fold elevation in glycerol release from these tissues (p=0.049 via Student’s *t*-test). These data support previous studies which implicate elevated lipolysis and higher rates of non-alcoholic fatty liver disease in Cushing’s patients.

## Dexamethasone Treatment of Mice As a Model of Cushing’s Syndrome

To validate the gene expression changes observed in human subjects, we treated C67BL/6J mice with dexamethasone in their drinking water to mimic the systemic effects of cortisol overproduction. These mice had an initial catabolic phase in which their body weight was rapidly reduced (Figure 2A), an effect that was primarily in lean body mass (Figure 2B). This is consistent with the effects of glucococorticoids on muscle atrophy that has been previously reported. After approximately 5 weeks, we observed an elevation in both total fat mass, and percent adiposity in the dexamethasone treated mice (Figure 2C-D). Throughout the study, we did not detect any differences in food intake between the groups (Figure 2E). To evaluate insulin sensitivity, we performed insulin tolerance tests on these mice at XXX weeks of age, and found that while they had reduced fasting glucose at this stage, they were resistant to insulin-induced reductions in blood glucose (Figure 2E). Upon sacrifice after 12 weeks of dexamethasone treatment, adipose tissue was dissected and weighed. As shown in Figure 2F, we observed elevated subcutaneous fat mass in these animals.

## Transcriptomic analysis of human adipose tissue from Cushing’s patients

To determine which genes and pathways are altered in adipose tissue in the human Cushing's disease subjects, we analyzed the transcriptome from subcutaneous adipose tissue mRNA from the 5 Cushing's disease patients and 11 controls. We identified 473 genes that had significantly different expression in Cushing's patients, of these 192 genes were expressed at a lower level and 281 at a higher level in the adipose tissue from the disease patients. These transcripts form a signature identifying transcriptional differences in adipose tissue in response to long-term exposure to glucocorticoids (Figure 3A).

To identify conserved pathways underlying these changes, gene set enrichment analysis was performed on these data. As summarized in Table 2, we detected enrichment of genes in the several categories involved in metabolism, including higher expression of gene sets involved in lipid biosynthesis, glucose metabolism, activation of amino acid degradation and protein degradation and reductions in protein synthesis . We also observed reduced for transcripts involved in immune function. These will be discussed below.

We next evaluated the levels of the glucocorticoid receptor (*NR3C1*) and the mineralcorticoid receptor (*NR3C2*) and observed no significant downregulation of these receptors at the mRNA level in Cushing’s patients (Figure 3B). Another potential mechanism for negative feedback of glucocorticoid signaling is through the enzymatic activities of 11-HSD1/2 which control the local concentrations of cortisol in adipose tissues. We observed a reduction in *HSD11B1* mRNA levels, potentially desensitizing adipose tissue to cortisol by reducing the conversion of cortisone to cortisol. Induction of leptin by glucocorticoids has been previously reported in human adipocytes (Halleux *et al.* 1998) and in human adipose tissue *in vivo* (Papaspyrou-Rao *et al.* 1997). We observed an increase in Leptin (*LEP*) but no significant changes in adiponectin mRNA levels (*ADIPOQ*, q=0.94; Figure 3C).

## Lipogeneic Genes are Upregulated in Response to Elevated Glucocorticoids

Increased subcutaneous fat mass is a hallmark of Cushing’s syndrome, and could potentially be mediated through activation of adipogenesis or lipogenesis. Our transcriptomic data support the hypothesis that lipogenesis is activated in these tissues via transcriptional activation of fatty acid synthesis and triglyceride synthesis. Several genes involved in the synthesis of and desaturation of fatty acids were expressed at higher levels including *FASN, AACSL4/5,ACSL1/3/4,* and *ELOVL1/5/6*. (Figure 3A) Desaturation of fatty acids is an essential aspect of *de novo* fatty acid synthesis, and we also observed elevations in *SCD, FADS1, FADS2* and *HSD17B12* (Figure 3B).

Transcripts involved in triglyceride synthesis that were expressed at higher levels in Cushing's patients include mRNAs for the two main fatty acid synthesis enzymes Acetyl-Coenzyme A carboxylase (*ACACA*), Mitochondrial glycerol-3-phosphate acyltransferasel (*GPAM*), Diacylglycerol O-acyltransferase homolog 2 (*DGAT2*), *DGAT1*), *AGPAT2/3 ,GPD1,* and *LPIN1* , were all significantly upregulated in adipose tissue from Cushing’s patients (Figure 3C).

In spite of increased lipid deposition and elevations of lipogenic genes in Cushing’s patients adipose tissue, there have been several studies linking elevated glucocorticoids to increased lipolysis. In our patients, this was observed in *ex vivo* explants of subcutaneous adipose tissue. Among genes that may liberate fatty acids from triglycerides, Lipoprotein lipase (*LPL*) was induced 1.45 fold (q=0.055) but neither Hormone Sensitive Lipase (*LIPE*) or Adipose Triglyceride Lipase (*PNPLA2*) were significantly changed at the transcriptional level in subcutaneous adipose tissue from Cushing’s patients (Figure 3F). It is possible that insulin resistance due to glucocorticoids caused decreased repression of lipolysis leading to its upregulation, but our data supports an insulin-independent activation as well, since in our explants insulin was not present. We detected an elevation of Perilipin 4 (*PLIN4*) which is one of the proteins that coat intracellular lipid storage droplets (induced 1.45 fold, q=0.05). Induction of perilipin 4 by glucocorticoids has been reported previously in CRF trangenic mice ()

We also observed elevations in several genes that regulate steroid biogenesis, including several cytochrome P450 family members, steroid reductases (*SRD5A1*, *SRD5A3*) , Aldo-keto reductase family 1 member C1 (*AKR1C1*), steroid sulfatase (STS) , 7-dehydrocholesterol reductase (DHCR7), NAD(P) dependent steroid dehydrogenase-like (NSDHL) and HMG-CoA synthase (HMGCS1).

To examine whether lipogenic genes are activated in mice, we tested several of these genes in subcutaneous adipose tissue from dexamethasone treated mice, and observed elevations in *Fasn, Gpam, Gpd1, Acs1, Dgat, Agpat2* and *Acaca1*. (Figure 3F). Interestingly, we did not observe an elevation in the mouse isoform of *SCD*, but saw instead a reduction in *Scd1* mRNA.

## Genes controlling glucose oxidation are elevated

Several glucose metabolism genes, and specifically glycolysis and TCA cycle genes were expressed at higher levels in Cushing's disease patients (Figure 5). Strongly induced genes included,

Glycogen synthase 2 (*GYS2*) was strongly induced 3.8 fold (q=0.004)

*UGP2* which creates the glycogen precursor UDP-glucose was induced 1.3 fold (q=0.01) glycogen branching enzyme (*GBE1*) was induced 1.6 fold (q=0.027). All three glycogen phosphorylase enzymes were also elevated, suggesting that rapid glycogen turnover may occur in adipose tissue of Cushing’s patients.

## Genes that regulate protein catabolism are upregulated in adipose tissue.

We found two major pathways of protein homeostasis in response to glucocorticoids. In concert with reductions in lean body (including muscle) mass (Figure 2B), we observed substantial muscle weakness in mice treated with dexamethasone (Figure 6A). In a separate cohort, after one week of dexamethasone treatment skeletal muscle, mRNA levels of the E3 ligases (Atrogin-1 and MuRF1 were induced) as were the proteosomal genes *Psmd1* and *8* (Figure 6B). Similar inductions of the proteosomal genes were observed in subcutaneous adipose tissue from mice treated with dexamethasone for 12 weeks (Figure 6C).

In adipose tissue from Cushing’s patients, we observed inductions of both the proteosomal pathways (KEGG ID XXXX, q=; Figure 6D), but also an induction of genes involved in amino acid catabolism (Figure 6E) and a global downregulation of ribosomal genes (Figure 6F). Together these data support the hypothesis that protein catabolism and reductions of protein synthesis also occur in adipose tissue in response to glucocorticoid exposure.

## Genes involved in proximal insulin signaling are unchanged in adipose tissue from Cushing’s Patients.

As described in Figures 1B and 2F, we observed insulin resistance in concert with elevated glucocorticoid levels in both mice and humans. Several mechanisms have been proposed to link glucocorticoids to insulin sensitivity including elevated lipolysis. As shown in Figure 7A, There was a slightly higher expression of insulin pathway transcripts including *FOXO1*, insulin receptor (*INSR*), *IRS1*, *IRS2* and p85 regulatory subunit of phosphoinositide-3-kinase (*PIK3R1*), consistent with previous studies (Gathercole *et al.* 2007; Tomlinson *et al.* 2010; Hazlehurst *et al.* 2013). The insulin pathway was generally expressed at significantly higher levels in the Cushing's disease patients compared to controls (q=0.006). These data do not support transcriptional downregulation of proximal insulin signaling genes as mediating insulin resistance in subcutaneous adipose tissue.

Changes in cell ceramide and glucosylceramide have been suggested to be important *in vitro* and in obesity and glucocorticoid-induced insulin resistance in skeletal muscle (Adams *et al.* 2004; Aerts *et al.* 2007; Holland *et al.* 2007). To test biochemically whether ceramides may play a role in the Cushing's disease associated insulin resistance in adipose tissue, we took a lipidomics approach to analyze ceramide species from the adipose tissue explants of the same patients. We observed no statistically significant changes in any ceramide species (Figure 7B, q>0.25).

## Inflammation

Several pathways involved in immune function were downregulated in adipose tissue from Cushing’s patients. This is consistent with the effects of cortisol in suppressing immune function generally. Adipose tissue leukocyte infiltration both relies on an intact immune system and also responds to changes in adiposity (Lumeng & Saltiel 2011). Among immune genes, we detected reductions in several genes that form the class II major histocompatibility complex, notably *HLA-DPB2, HLA-DRA, HLA-DRB9* and *HLADQA1*. (Figure 7C). These genes normally present antigens for T-cell recruitment. Consistent with this, we observed reductions in the mRNA of *IL32*, a hormone secreted by Natural Killer and T lymphocytes (Dinarello & Kim 2006). We also observed a downregulation in transcripts that are interferon gamma dependent. Together these data support the hypothesis that the decreased T-cell activation observed with cortisol signaling also impacts adipose tissue.

## Modifying Effect of Obesity on Glucocorticoid Responsiveness

In our small cohort of Cushing’s subjects, we examined whether some of the dramatic transcriptional changes we observed were modified by the obesity status of the patients. We were surprised to note that many genes which had strongly elevated transcripts in non-obese Cushing’s patients had largely blunted effects in obese Cushing’s patients. Some examples of this include *FASN, PSMD8* and *IDH8* (Figure 8A-C). Among genes that were more strongly induced in obese patients, most of these genes are involved in lysosomal function, including the cathepsins (*CTSB*, *CTSD*, *CTSZ*), *LAPTM5* and *LIPA* (Figure 8D). Although the small number of obese and non-obese Cushing’s patients in our study makes these observations quite preliminary, it is suggestive of both a general reduction of glucocorticoid sensitivity in obese subjects but also potentially an underappreciated role of lysosomes in obese patients with elevated cortisol levels.

# Discussion

In this study we have described a transcriptional signature in adipose tissue from subjects with Cushing's disease and verified several of these changes using a mouse model of glucocorticoid treatment. We have identified several pathways that are significantly changed in response to chronic glucocorticoid exposure.

Cushing's disease patients have a significant change in fat distribution (Mayo-Smith *et al.* 1989), and higher lipogensis, as measured by conversion of glucose to neutral lipid in *ex vivo* subcutaneous adipose tissue from Cushing's patients compared to obese controls (Galton & Wilson 1972). Higher triglyceride synthesis has also been found in animal models of Cushing's disease, including CRH overproducing mice, which also have elevated glucocorticoid levels (Harris *et al.* 2013) and dexamethasone treated mice (Roohk *et al.* 2013). These findings are consistent with our observed elevations of lipogenic genes in human and mouse subcutaneous adipose tissue.

Muscle wasting is a well recognized adverse event of excess glucocorticoids caused by both increased muscle proteolysis and decreased protein synthesis (Deng *et al.* 2004; Menconi *et al.* 2007). Exposure of rats to glucocorticoids activates the muscle ubiquitin-proteosome system (Wing & Goldberg 1993; Price *et al.* 1994) and increased muscle expression of proteases (cathepsins B and D, calpain) components of the ubiquitin-proteasome pathway (Dardevet *et al.* 1995) along inhibition of muscle protein synthesis (Long *et al.* 2001). A study in healthy humans also found that prednisone increases leucine oxidation supporting our observation of elevated amino acid catabolic genes (Beaufrere *et al.* 1989). We found a significant higher expression of both proteosomal and the amino acid degradation pathways in adipose tissue, suggesting that a similar induction occurs in adipose tissue in Cushing's disease. We also observe elevations in lysosomal genes, though these changes appear to be restricted to obese Cushing’s patients. The metabolic relevance of activated proteolysis in adipose tissue has not been widely explored and warrants further study.

Several studies have characterized the effect of glucocorticoids on triglyceride lipogenesis and have found increased expression of transcripts involved in triglyceride synthesis (reviewed in Macfarlane *et al.* 2008). Our results recapitulate the observed effect of glucocorticoids on induction of expression of the key triglyceride synthesis enzymes.

There are several limitations to our evaluation of insulin sensitivity in this study. One aspect is that patients with Cushing’s syndrome may be on insulin sensitizing medication. Secondly, it is possible that insulin resistance in these patients/mice are mainly due to muscle or liver insulin resistance and that adipose tissue may respond to insulin in a relatively normal fashion. Glucocorticoid-induced insulin resistance is thought to me mostly secondary to the increase in free fatty acids caused by the induction of lipolysis (Geer *et al.* 2014). Results from a recent study suggest that glucocorticoids do not induced insulin resistance in subcutanous adipose tissue *in vivo* in healthy subjects (Hazlehurst *et al.* 2013), suggesting that peripheral insulin resistance may not occur in adipocytes and that whole-body insulin resistance may primarily occur in muscle and liver tissues. This is consistent with our observations of a lack of changes in proximal insulin signaling transcripts in adipose tissues (Figure 7A) and a lack of elevated ceramides in our subcutaneous adipose tissue lysates (Figure 7B).

These data provide a variety of novel transcriptional changes that may be causative of the co-morbidities associated with Cushing's disease. Further studies in animals and cells using knockout or overexpression of specific transcripts may verify which of the changes is crucial in metabolic effects of glucocorticoids in adipose tissue.

# Declaration of interest

The authors have no conflict of interest.

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# Author Contributions

IHo conceived of the study, and DB and IHo. provided funding. QT, DB, IHa and IHo analysed the RNAseq data. IHa generated the mouse data with assistance from EJS. This was analysed by IHa, DB and QT. IHo and DB wrote the manuscript.

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# Figure Legends

**Figure 1: Metabolic characteristics of Cushing’s patients in our study.** A) Morphometric data from control (non-secreting adeoma) and Cushing’s subjects. B) HOMA-IR score, fasting insulin and fasting blood glucose from subjects. C) Liver enzymes from subjects D) Glycerol release from isolated subcutaneous adipose tissue. Asterisks indicates p<0.05.

Figure 2:

**Figure 3: Differentially expressed transcripts in subcutaneous adipose tissue from Cushing’s subjects.** A) Heatmap of genes with significant differential expression. The bar on the top indicates control subjects (non-secreting adenoma; black) and Cushing’s subjects (red). B) Genes involved in cortisol signaling. C) Leptin and Adiponectin mRNA levels. Asterisks indicate q<0.05.

**Figure 4: Elevated glucocorticoids result in elevated fatty acid and tryglyceride synthesis genes.** A) Fatty acid synthesis genes in Cushing’s and control patients. B) Fatty acid desaturases in Cushing’s patients. C) Triglyceride synthesis genes. D) Lipolysis genes. E) Steroid biogenesis genes. D) Evaluation of lipogenic genes in mouse subcutaneous adipose tissue. Asterisks indicate q<0.05.

**Figure 5: Glycolysis and glucose oxidation genes are upregulated with eleated glucocorticoids.** A) Schematic of glycolysis and the TCA cycle, colored by gene expression changes in subcutaneous adipose tissue from Cushing’s subjects. B) qPCR analysis of selected glucose oxidation genes from mouse subcutaneous adipose tissue after 12 weeks of dexamethasone treatment. Asterisks indicate q<0.05.

Figure 6:

Figure 7:

Figure 8:

# Table Legends:

Table 1: Clinical characteristics. Data represents mean +/- standard error.

Table 2: Summarized gene set enrichment analysis of pathways. Size is the total size of the KEGG category, NES is the normalized enrichment score, NOM p-value is the raw p-value and FDR q-value is corrected for multiple observations. Gene details lists the specific genes which led to the enrichment of this category in our data. A negative enrichment score indicates down-regulation of the category in Cushing's disease. For a complete list see Supplementary Tables 2-5.

# Supplementary Data

Supplementary Table 1: Expression changes between control and Cushing's disease subjects. Calculated expression, and expression changes for each gene are shown along with raw, and adjusted p-values and the fold change.

Supplementary Table 2: Gene set enrichment analysis of gene ontology enrichment categories. Size is the total size of the GO category, NES is the normalized enrichment score, NOM p-value is the raw p-value and FDR q-value is corrected for multiple observations. Gene details lists the specific genes which led to the enrichment of this category in our data. A negative enrichment score indicates down-regulation of the category in Cushing's disease.

Supplementary Table 2: Gene set enrichment analysis of transcription factor and miRNA pathways. These categories indicate that target genes regulated by these factors are altered in Cushing's disease white adipose tissue. Size is the total size of the category, NES is the normalized enrichment score, NOM p-value is the raw p-value and FDR q-value is corrected for multiple observations. Gene details lists the specific genes which led to the enrichment of this category in our data. A negative enrichment score indicates down-regulation of the category in Cushing's disease.

Table \*Patient characteristics

non functioning adenoma Cushing's disease

Table \* Ceramides and glucosylceramide measurements

p value non functioning adenoma Cushing's disease

0.44 0.30±0.009 0.32±0.024 Cer C14 (ng/mg)

0.56 0.70±0.032 0.65±0.064 Cer C18:1 (ng/mg)

0.58 3.4±0.355 3.09±0.415 Cer C16 (ng/mg)

0.36 0.43±0.012 0.47±0.043 Cer C18 (ng/mg)

0.78 0.57±0.032 0.56±0.011 Cer C20 (ng/mg)

0.96 7165±1260 7072±1274 Cer C22 (area)

0.30 7723±2073 5122±1277 Cer C24:1 (area)

0.56 4.26±0.578 6.77±3.79 Cer C24 (ng/mg)

0.75 0.32±0.028 0.30±0.05 Glu-Cer C16 (ng/mg)

0.49 0.38±0.013 0.36±0.014 Glu-Cer C18 (ng/mg)

0.43 0.18±0.0042 0.19±0.0059 Glu-Cer C18:1 (ng/mg)