­The Cushing's disease adipose gene expression profile reveals effects of long term glucocorticoids on adipose tissue lipid, protein and glucose metabolism

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Abstract:

Glucocorticoids have major effects on adipose tissue metabolism. To study tissue mRNA expression changes induced by chronic elevated endogenous glucocorticoids, we performed RNA sequencing on subcutaneous adipose tissue from patients with Cushing’s disease (n=5) compared to patients with non-functioning pituitary adenomas (n=11). We found higher expression of transcripts involved in several metabolic pathways, including lipogenesis, proteolysis and glucose oxidation as well as decreased expression of transcripts involved in inflammation and protein synthesis. To further study this in a model system, we subjected mice to dexamethasone treatment for 12 weeks and analysed their inguinal (subcutaneous) fat pads, which led to similar findings. Additionally, mice treated with dexamethasone showed drastic decreases in lean body mass as well as increased fat mass, further supporting the human transcriptomic data. These data provide insight to transcriptional changes that may be responsible for the co-morbidities associated with chronic elevations of glucocorticoids.

# 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 disease (Ntali *et al.* 2015). Cushing's Disease is very rare with an 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 similar clinical manifestations.

Numerous studies have shown that glucocorticoids have profound effects on adipose tissue metabolism, including promotion of adipocyte differentiation (Hauner *et al.* 1987) and induction of lipolysis and lipogenesis (Divertie *et al.* 1991; Samra *et al.* 1998; Kršek *et al.* 2006; Campbell *et al.* 2011). 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 of adipose tissue biopsies 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 anesthesia, 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 The Jackson Laboratory at nine weeks of age. Following a one-week acclimation period, mice were either treated with 1 mg/kg/day of dexamethasone (Sigma-Aldrich) in their drinking water (N=12) or used as controls (N=12). All animal procedures were approved by the University of Tennessee Health Science Center Institutional Animal Care and Use Committee. Animals were weighed weekly, with body composition determined 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 maintained on a standard rodent diet throughout the study.

## Insulin Tolerance Test

Insulin tolerance was measured at 11 weeks of dexamethasone treatment (21 weeks of age). Following a six-hour fast, mice were given intraperitoneal injections of insulin (Humulin R, Lily) at a concentration of 1 mU/g. Blood glucose was determined with a One Touch Ultra Glucometer (Lifescan).

## 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 four paws in contact with the apparatus and slowly pulled backwards by the tail. Mice were given five trials with about 10 seconds rest in between trials. Strength was measured by the average peak torque (N) over the five trials.

## Quantitative Real-Time PCR

RNA was extracted with the PureLink RNA mini kit (Life Technologies). Synthesis of cDNA from 1 ug of RNA was performed using the High Capacity Reverse Transcription Kit (Life Technologies). cDNA and primers were added to Power SYBR Green PCR Master Mix in accordance with the manufacturer’s guidelines (Life Technologies) and subjected to quantitative real-time PCR as described (Lu et al., 2014). Primer sequences used are listed in Table 1. mRNA expression levels of all genes were normalized to *Actb* for adipose tissue and *Gapdh* for muscle tissue after confirming that these mRNAs are unaffected by dexamethasone treatment.

## 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 (Kasumov *et al.* 2010). 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 2, 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 to q-values by the method of Benjamini and Hochberg (Benjamini & Hochberg 1995). All data are presented as mean +/- standard error of the mean.

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 (MSigDB version 4.0). 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 GSEA results are in Supplementary Tables 2-3 and summarized in Table 3. All code and raw data from this study are available through the Gene Expression Omnibus 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 2. 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 and truncal obesity (Lamberts & Birkenhäger 1976).

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 via χ2 test). 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 (Kršek *et al.* 2006) and higher rates of non-alcoholic fatty liver disease in Cushing’s patients (Rockall *et al.* 2003).

## 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 glucocorticoids on muscle atrophy that has been previously reported (Pleasure *et al.* 1970). 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 21 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 3, we detected enrichment of genes in several categories involved in metabolism, including higher expression of gene sets involved in lipid biosynthesis, glucose metabolism, activation of amino acid degradation, protein degradation, and reductions in protein synthesis. We also observed reduced expression of transcripts involved in immune function. These will be discussed below.

We next evaluated the levels of the glucocorticoid receptor (*NR3C1*) and the mineralocorticoid 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 trend towards reductions in *HSD11B1* mRNA levels (24% reduced, q=0.49), 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 1.8 fold higher level of Leptin (*LEP*) expression, and a trend towards higher resistin (*RETN)* expression but no significant changes in adiponectin mRNA levels (*ADIPOQ*, q=0.94; Figure 3C).

## Lipogenesis 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. All the major genes involved in the synthesis of fatty acids were expressed at higher levels including *ACACA, FASN, AACSL4/5,ACSL1/3/4,* and *ELOVL1/5/6* (Figure 4A). Desaturation of fatty acids is an essential aspect of *de novo* fatty acid synthesis, and we also observed elevations in all fatty acid desaturases *SCD, FADS1, FADS2* and *HSD17B12* (Figure 4B). The triglyceride synthesis genes *GPAM*, *DGAT2*, *DGAT1*, *AGPAT2/3 ,GPD1,* and *LPIN1* , were also all upregulated in subcutaneous adipose tissue from Cushing’s patients (Figure 4C).

In spite of increased lipid deposition and elevations of lipogenesis 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 (Figure 1D). 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*) nor Adipose Triglyceride Lipase (*PNPLA2*) were significantly changed at the transcriptional level in subcutaneous adipose tissue from Cushing’s patients (Figure 4D). 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=0.056).

We also observed elevations in several genes that regulate steroid biogenesis, described in Figure 4E, 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 lipogenesis 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, Acss2, Acs1, Dgat, Agpat2, Dhcr7/24* and *Acaca1*. (Figure 4F). 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 in Cushing's patients

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, *HK3, FBP1, ALDOC, ENO1, IDH1, ME1 AND DLAT.* Upregulations in *Idh1* and *Me1* were also noted in mouse adipose tissue, along with other transcripts involved in glucose oxidation such as *Aco1, Ldhb* and *Mdh1* (Figure 5B).

The major glycogen synthesis transcripts were induced, including *GYS2, UGP2* and *GBE1*. This agrees with biochemical studies which implicate glucocorticoid treatment in elevated hepatic and adipose tissue glycogenesis (Engel & Scott 1951; Segal & Gonzalez Lopez 1963; Baqué *et al.* 1996). The relevance of this effect in adipose tissue has not yet been explored.The major glycogen synthesis transcripts were induced, including *GYS2, UGP2* and *GBE1*. This agrees with biochemical studies which implicate glucocorticoid treatment in elevated hepatic glycogenesis (Engel & Scott 1951; Segal & Gonzalez Lopez 1963; Baqué *et al.* 1996). The relevance of this effect in adipose tissue has not yet been explored.

## 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 proteasomal genes *Psmd1* and *8* (Figure 6B). Similar inductions of the proteasomal 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 proteasomal pathways (via KEGG, net enrichment score 1.76, q=0.01; Figure 6D), but also an induction of genes involved in amino acid catabolism (Figure 6E) and a general 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 (KEGG pathway, net enrichment score 1.84, 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 that 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. Broadly these changes reflect a shift towards more rapid conversion of glucose through glycolysis and the TCA cycle and shifting of glucose and protein metabolites towards lipogenic pathways in adipose tissue.

Cushing's disease patients have a significant change in fat distribution (Mayo-Smith *et al.* 1989), and higher lipogenesis, 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 lipogenesis genes in human and mouse subcutaneous adipose tissue. In addition to a shift towards lipid storage, we also observed elevated expression of glycogen synthesis genes in the Cushing's disease patients.

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 proteasomal 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.

There are several limitations to our evaluation of insulin sensitivity in this study. One aspect is that two of the three patients with Cushing’s syndrome and diabetes were treated with antidiabetic medications. Secondly, it is possible that insulin resistance in these subjects/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 be 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 induce insulin resistance in subcutaneous 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, ARS and IHo provided funding. WFC and ALB recruited the patients and obtained clinical data. WFC supplied the biopsies and serum samples. IHo assayed the tissues for lipolysis and performed the serum measurements. QT, DB, IHa and IHo analyzed the RNAseq data. IHa generated the mouse data with assistance from EJS. This was analyzed by IHa, DB and QT. IHo and DB wrote the manuscript

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# References

Adams JM, Pratipanawatr T, Berria R, Wang E, DeFronzo RA, Sullards MC & Mandarino LJ 2004 Ceramide content is increased in skeletal muscle from obese insulin-resistant humans. *Diabetes* **53** 25–31.

Aerts JM, Ottenhoff R, Powlson AS, Grefhorst A, van Eijk M, Dubbelhuis PF, Aten J, Kuipers F, Serlie MJ, Wennekes T *et al.* 2007 Pharmacological inhibition of glucosylceramide synthase enhances insulin sensitivity. *Diabetes* **56** 1341–1349. (doi:10.2337/db06-1619)

Anders S, Pyl PT & Huber W 2014 HTSeq - A Python framework to work with high-throughput sequencing data. *Bioinformatics* 1–4. (doi:10.1093/bioinformatics/btu638)

Baqué S, Roca a, Guinovart JJ & Gómez-Foix a M 1996 Direct activating effects of dexamethasone on glycogen metabolizing enzymes in primary cultured rat hepatocytes. *European Journal of Biochemistry* **236** 772–777.

Beaufrere B, Horber FF, Schwenk WF, Marsh HM, Matthews D, Gerich JE & Haymond MW 1989 *Glucocorticosteroids Increase Leucine Oxidation and Impair Leucine Balance in Humans.* In *The American Journal of Physiology*, pp E712–E721.

Benjamini Y & Hochberg Y 1995 Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society. Series B* **57** 289–300.

Bligh EG & Dyer WJ 1959 A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37** 911–917.

Campbell JE, Peckett AJ, D’souza AM, Hawke TJ & Riddell MC 2011 Adipogenic and lipolytic effects of chronic glucocorticoid exposure. *American Journal of Physiology. Cell Physiology* **300** C198–C209. (doi:10.1152/ajpcell.00045.2010)

Clark NR & Ma’ayan A 2011 Introduction to statistical methods for analyzing large data sets: gene-set enrichment analysis. *Science Signaling* **4** tr4. (doi:10.1126/scisignal.2001966)

Cushing H 1932 The basophil adenomas of the pituitary body and their clinical manifestations. *Bulletin of the Johns Hopkins Hospital* **50** 157–158.

Dardevet D, Sornet C, Taillandier D, Savary I, Attaix D & Grizard J 1995 Sensitivity and protein turnover response to glucocorticoids are different in skeletal muscle from adult and old rats. Lack of regulation of the ubiquitin-proteasome proteolytic pathway in aging. *Journal of Clinical Investigation* **96** 2113–2119. (doi:10.1172/JCI118264)

Deng X, Elam MB, Wilcox HG, Cagen LM, Park E a, Raghow R, Patel D, Kumar P, Sheybani A & Russell JC 2004 Dietary olive oil and menhaden oil mitigate induction of lipogenesis in hyperinsulinemic corpulent JCR:LA-cp rats: microarray analysis of lipid-related gene expression. *Endocrinology* **145** 5847–5861. (doi:10.1210/en.2004-0371)

Dinarello C a & Kim S-H 2006 IL-32, a novel cytokine with a possible role in disease. *Annals of the Rheumatic Diseases* **65 Suppl 3** iii61–i64. (doi:10.1136/ard.2006.058511)

Divertie GD, Jensen MD & Miles JM 1991 Stimulation of lipolysis in humans by physiological hypercortisolemia. *Diabetes* **40** 1228–1232. (doi:10.2337/diabetes.40.10.1228)

Engel FL & Scott JL 1951 The role of hormones in adipose tissue glycogen synthesis in the rat; the adrenal cortex. *Endocrinology* **48** 56–69.

Galton DJ & Wilson JP 1972 Lipogenesis in adipose tissue of patients with obesity and Cushing’s disease. *Clinical Science* **43** 17P.

Gathercole LL, Bujalska IJ, Stewart PM & Tomlinson JW 2007 Glucocorticoid modulation of insulin signaling in human subcutaneous adipose tissue. *The Journal of Clinical Endocrinology and Metabolism* **92** 4332–4339. (doi:10.1210/jc.2007-1399)

Geer EB, Islam J & Buettner C 2014 Mechanisms of glucocorticoid-induced insulin resistance: Focus on adipose tissue function and lipid metabolism. *Endocrinology and Metabolism Clinics of North America* **43** 75–102. (doi:10.1016/j.ecl.2013.10.005)

Halleux CM, Servais I, Reul BA, Detry R & Brichard SM 1998 Multihormonal control of ob gene expression and leptin secretion from cultured human visceral adipose tissue: Increased responsiveness to glucocorticoids in obesity. *Journal of Clinical Endocrinology and Metabolism* **83** 902–910. (doi:10.1210/jc.83.3.902)

Harris C, Roohk DJ, Fitch M, Boudignon BM, Halloran BP & Hellerstein MK 2013 Large increases in adipose triacylglycerol flux in Cushingoid CRH-Tg mice are explained by futile cycling. *American Journal of Physiology. Endocrinology and Metabolism* **304** E282–E293. (doi:10.1152/ajpendo.00154.2012)

Hauner H, Schmid P & Pfeiffer EF 1987 Glucocorticoids and insulin promote the differentiation of human adipocyte precursor cells into fat cells. *Journal of Clinical Endocrinology and Metabolism* **64** 832–835. (doi:10.1210/jcem-64-4-832)

Hazlehurst JM, Gathercole LL, Nasiri M, Armstrong MJ, Borrows S, Yu J, Wagenmakers AJM, Stewart PM & Tomlinson JW 2013 Glucocorticoids fail to cause insulin resistance in human subcutaneous adipose tissue in vivo. *The Journal of Clinical Endocrinology and Metabolism* **98** 1631–1640. (doi:10.1210/jc.2012-3523)

Holland WL, Brozinick JT, Wang L-PP, Hawkins ED, Sargent KM, Liu Y, Narra K, Hoehn KL, Knotts T a., Siesky A *et al.* 2007 Inhibition of ceramide synthesis ameliorates glucocorticoid-, saturated-fat-, and obesity-induced insulin resistance. *Cell Metabolism* **5** 167–179. (doi:10.1016/j.cmet.2007.01.002)

Kasumov T, Huang H, Chung Y-M, Zhang R, McCullough AJ & Kirwan JP 2010 Quantification of ceramide species in biological samples by liquid chromatography electrospray ionization tandem mass spectrometry. *Analytical Biochemistry* **401** 154–161. (doi:10.1016/j.ab.2010.02.023)

Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R & Salzberg SL 2013 TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biology* **14** R36. (doi:10.1186/gb-2013-14-4-r36)

Kršek M, Rosická M, Nedvídková J, Kvasničková H, Hána V, Marek J, Haluzík M, Lai EW & Pacák K 2006 Increased lipolysis of subcutaneous abdominal adipose tissue and altered noradrenergic activity in patients with Cushing’s syndrome: An in-vivo microdialysis study. *Physiological Research* **55** 421–428.

Lamberts SW & Birkenhäger JC 1976 Body composition in Cushing’s disease. *The Journal of Clinical Endocrinology and Metabolism* **42** 864–868.

Langmead B & Salzberg S 2012 Fast gapped-read alignment with Bowtie 2. *Nature Methods* **9** 357–360. (doi:10.1038/nmeth.1923)

Lindholm J, Juul S, Jørgensen JOL, Astrup J, Bjerre P, Feldt-Rasmussen U, Hagen C, Jørgensen J, Kosteljanetz M, Kristensen L *et al.* 2001 Incidence and late prognosis of Cushing’s syndrome: A population-based study. *Journal of Clinical Endocrinology and Metabolism* **86** 117–123. (doi:10.1210/jc.86.1.117)

Long W, Wei L & Barrett EJ 2001 Dexamethasone inhibits the stimulation of muscle protein synthesis and PHAS-I and p70 S6-kinase phosphorylation. *American Journal of Physiology. Endocrinology and Metabolism* **280** E570–E575.

Love MI, Huber W & Anders S 2014 *Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data with DESeq2*. (doi:10.1101/002832)

Lumeng CN & Saltiel AR 2011 Inflammatory links between obesity and metabolic disease. *The Journal of Clinical Investigation* **121** 2111–2117. (doi:10.1172/JCI57132)

Mayo-Smith W, Hayes CW, Biller BM, Klibanski A, Rosenthal H & Rosenthal DI 1989 Body fat distribution measured with CT: correlations in healthy subjects, patients with anorexia nervosa, and patients with Cushing syndrome. *Radiology* **170** 515–518.

Menconi M, Fareed M, O’Neal P, Poylin V, Wei W & Hasselgren P-O 2007 Role of glucocorticoids in the molecular regulation of muscle wasting. *Critical Care Medicine* **35** S602–S608. (doi:10.1097/01.CCM.0000279194.11328.77)

Ntali G, Grossman A & Karavitaki N 2015 Clinical and biochemical manifestations of Cushing’s. *Pituitary*. (doi:10.1007/s11102-014-0631-4)

Papaspyrou-Rao S, Schneider SH, Petersen RN & Fried SK 1997 Dexamethasone increases leptin expression in humans in vivo. *Journal of Clinical Endocrinology and Metabolism* **82** 1635–1637.

Pleasure DE, Walsh GO & Engel WK 1970 Atrophy of skeletal muscle in patients with Cushing’s syndrome. *Archives of Neurology* **22** 118–125. (doi:10.1001/archneur.1970.00480200024002)

Price SR, England BK, Bailey JL, Van Vreede K & Mitch WE 1994 Acidosis and glucocorticoids concomitantly increase ubiquitin and proteasome subunit mRNAs in rat muscle. *The American Journal of Physiology* **267** C955–C960.

R Core Team 2013 R: A Language and Environment for Statistical Computing.

Rockall a., Sohaib S, Evans D, Kaltsas G, Isidori a., Monson J, Besser G, Grossman a. & Reznek R 2003 Hepatic steatosis in Cushing’s syndrome: a radiological assessment using computed tomography. *European Journal of Endocrinology* **149** 543–548. (doi:10.1530/eje.0.1490543)

Roohk DJ, Mascharak S, Khambatta C, Leung H, Hellerstein M & Harris C 2013 Dexamethasone-mediated changes in adipose triacylglycerol metabolism are exaggerated, not diminished, in the absence of a functional GR dimerization domain. *Endocrinology* **154** 1528–1539. (doi:10.1210/en.2011-1047)

Samra JS, Clark ML, Humphreys SM, Macdonald IA, Bannister PA & Frayn KN 1998 Effects of physiological hypercortisolemia on the regulation of lipolysis in subcutaneous adipose tissue. *Journal of Clinical Endocrinology and Metabolism* **83** 626–631. (doi:10.1210/jc.83.2.626)

Segal HL & Gonzalez Lopez C 1963 Early Effects of Glucocorticoids on Precursor Incorporation into Glycogen. *Nature* **200** 143–144. (doi:10.1038/200143a0)

Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES *et al.* 2005 Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences of the United States of America* **102** 15545–15550. (doi:10.1073/pnas.0506580102)

Tomlinson JJ, Boudreau A, Wu D, Abdou Salem H, Carrigan A, Gagnon A, Mears AJ, Sorisky A, Atlas E & Haché RJG 2010 Insulin sensitization of human preadipocytes through glucocorticoid hormone induction of forkhead transcription factors. *Molecular Endocrinology (Baltimore, Md.)* **24** 104–113. (doi:10.1210/me.2009-0091)

Wing SS & Goldberg AL 1993 Glucocorticoids activate the ATP-ubiquitin-dependent proteolytic system in skeletal muscle during fasting. *The American Journal of Physiology* **264** E668–E676.

# 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:** **Dexamethasone treatment results in decreased lean mass and increased fat mass in mice.** Weekly body weight (A), lean mass (B), fat mass (C) and percent fat (D) from control (black) and dexamethasone (red) treated mice. E) Average food consumption per mouse per day. F) Insulin tolerance test. Following a 6 hour fast, insulin (1 mU/g) was administered via IP injection and blood glucose was measured at baseline, and the indicated minutes post injection. G) Fat pad weights following 12 weeks of treatment.

**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 triglyceride 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 elevated glucocorticoids.** Schematic of A) glycolysis and B) 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:** **Increased glucocorticoids are associated with increased protein degradation and decreased strength.** A) Mouse grip strength (N) assessed at baseline, 4, 8 and 12 weeks of dexamethasone treatment. Muscle atrogene (B) and proteasomal transcript expression changes in gastrocnemius muscles from mice following 1 week of dexamethasone treatment. C) Proteosomal mRNA levels from subcutaneous adipose tissue of mice treated with dexamethasone for 12 weeks. Proteasomal (D) and protein catabolism (E) transcript expression changes in subcutaneous adipose tissue from Cushing’s and control subjects. F) Heatmap of differentially expressed ribosomal transcripts in Cushing’s and control subjects.

**Figure 7: Expression of insulin signaling transcripts, ceramides and inflammatory transcripts in control vs. Cushing’s subjects.** A) Insulin signaling transcript expression levels. B) Ceramide levels. C) MHC complex transcript expression levels.

**Figure 8: Transcript expression changes in Cushing’s are less robust after adjusting for obesity.** Differentially expressed *FASN* (A), *PSMD8* (B), *IDH1* (C), and lysosomal (D) transcripts in non-obese and obese Cushing’s subjects.

**Table 1: Primer sequences used for qPCR analyses.**

|  |  |  |
| --- | --- | --- |
| **Gene** | **Forward Sequence** | **Reverse Sequence** |
| *Acaca* | GCTAAACCAGCACTCCCGAT | GTATCTGAGCTGACGGAGGC |
| *Aco1* | AACACCAGCAATCCATCCGT | GGTGACCACTCCACTTCCAG |
| *Acsl1* | GCCTCACTGCCCTTTTCTGA | GCAGAATTCATCTGTGCCATCC |
| *Acss2* | CGTTCTGTGGAGGAGCCAC | GGCATGCGGTTTTCCAGTAA |
| *Actb* | ATGTGGATCAGCAAGCAGGA | AAGGGTGTAAAACGCAGCTCA |
| *Agpat2* | CGTGTATGGCCTTCGCTTTG | TCCATGAGACCCATCATGTCC |
| *Dgat2* | AACACGCCCAAGAAAGGTGG | GTAGTCTCGGAAGTAGCGCC |
| *Dhcr7* | ATGGCTTCGAAATCCCAGCA | GAACCAGTCCACTTCCCAGG |
| *Dhcr24* | AGCTCCAGGACATCATCCCT | TACAGCTTGCGTAGCGTCTC |
| *Fasn* | GGAGGTGGTGATAGCCGGTAT | TGGGTAATCCATAGAGCCCAG |
| *Fbxo32* | CTTCTCGACTGCCATCCTGG | GTTCTTTTGGGCGATGCCAC |
| *Gpam* |  |  |
| *Gpd1* | GTGAGACGACCATCGGCTG | TTGGGTGTCTGCATCAGGT |
| *Idh1* | CTCAGAGCTCTCTTGGACCGA | CATCTCCTTGCATCTCCACCA |
| *Ldhb* | AAAGGCTACACCAACTGGGC | GCCGTACATTCCCTTCACCA |
| *Mdh1* | GGAACCCCAGAGGGAGAGTT | TGGGGAGGCCTTCAACAAAC |
| *Me1* | GGACCCGCATCTCAACAAG | TCGAAGTCAGAGTTCAGTCGTT |
| *Psmd1* | TGCCAATCATGGTGGTGACA | ACACATCCTGACGTGCAGTT |
| *Psmd8* | ACGAGTGGAACCGGAAGAAC | CCGTGGTTGGCAGGAAATTG |
| *Rplp0* | GAAACTGCTGCCTCACATCCG | GCTGGCACAGTGACCTCACACG |
| *Rplp13a* | GCGGATGAATACCAACCCCT | CCTGGCCTCTCTTGGTCTTG |
| *Scd1* | CACTCGCCTACACCAACGG | GAACTGGAGATCTCTTGGAGCA |
| *Trim63* | GAGGGCCATTGACTTTGGGA | TTTACCCTCTGTGGTCACGC |

**Table 2: Clinical characteristics of Cushing’s and Control patients.** Data represents mean +/- standard error.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Cushing's disease (n=5) | Controls (n=11) | p-value |
| Height (cm) | 166 | 169 | 0.47 |
| Weight (kg) | 91 | 89 | 0.89 |
| BMI | 33 | 30 | 0.52 |
| Abdominal circumference (cm) | 112.4 | 100.65 | 0.16 |
| Tumor size (mm) | 0.95 | 1.96 | 0.01 |
| Age (years) | 39.8 | 63.4 | 0.0003 |

**Table 3: Summarized gene set enrichment analysis of pathways.** Selected pathway enriched in subcutaneous adipose tissue from Cushing’s patients via GSEA analysis. NES is the net enrichment score, asterisk indicates q<0.25. For a complete list see Supplementary Tables 2-3.

|  |  |  |
| --- | --- | --- |
| Pathway | Dataset | NES |
| M\_PHASE\_OF\_MITOTIC\_CELL\_CYCLE | Gene Ontology | 2.60\* |
| KEGG\_CITRATE\_CYCLE\_TCA\_CYCLE | KEGG | 2.41\* |
| KEGG\_BIOSYNTHESIS\_OF\_UNSATURATED\_FATTY\_ACIDS | KEGG | 2.41\* |
| REACTOME\_TRIGLYCERIDE\_BIOSYNTHESIS | Reactome | 2.24\* |
| PYRUVATE\_METABOLISM | Gene Ontology | 2.24\* |
| KEGG\_VALINE\_LEUCONE\_AND\_ISOLEUCINE\_DEGRADATION | KEGG | 2.16\* |
| STEROID\_BIOSYNTHETIC\_PROCESS | Gene Ontology | 2.11\* |
| KEGG\_STARCH\_AND\_SUCROSE\_METABOLISM | KEGG | 2.08\* |
| PROTEASOME\_COMPLEX | Gene Ontology | 1.78\* |
| KEGG\_ALLOGRAFT\_REJECTION | KEGG | -1.87\* |
| KEGG\_BASAL\_CELL\_CARCINOMA | KEGG | -1.86\* |
| KEGG\_RIBOSOME | KEGG | -2.33\* |

# 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 and KEGG 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 3: 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.