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 beenno 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 induction, 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

## Insulin Tolerance Test

## 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 (13). 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) (14). The organic layer of the extract was dried under nitrogen gas and reconstituted in 100 uL of 60:40 acetonitrile: isopropanol. 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. There was a trend for a higher body weight (p=0.22), body mass index (BMI) (p=0.22) and abdominal circumference (p=0.3). Cushing's disease patients were younger than their controls (p<0.001) and had smaller tumors (p<0.01). Three out of the 5 Cushing's disease patients had diabetes while 1 of the 11 controls had diabetes (P=0.03).

## 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 1A), an effect that was primarily in lean body mass (Figure 1B). This is consistent with the effects of glucococorticoids on muscle atrophy that has been previously reported.. After approximately 4 weeks, we observed an elevation in both total fat mass, and percent adiposity in the dexamethasone treated mice (Figure 1C). Throughout the study, we did not detect any differences in food intake between the groups (Figure 1D). 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 1E). Upon sacrifice after 12 weeks of dexamethasone treatment, adipose tissue was dissected and weighed. As shown in Figure 1F, 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 2A).

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.

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 2B-c). 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 2D). Another mechanism for modulation of glucocorticoid signaling is through the enzymatic activities of 11-HSD1/2 which control the local concentrations of cortisol in adipose tissues. We observed… (Figure 2E). Together these data suggest ….

## 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). We evaluated several of these genes in subcutaneous adipose tissue from dexamethasone treated mice, and observed elevations for XXXXXXXX. (Figure 3D)

## Enzymes involved in fatty acid catabolism include

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 explants from Cushing’s patients we observed XXXXX (Figure 3E). 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 upregulated 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 did observe 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 perlipin 4 by glucocorticoids has been reported in CRF-Tg+ mice (PMID 21187916)

e AACSL4,ACAT2,ALDH9A1, Acetyl-Coenzyme A acyltransferase 2 (ACAA2),ACOX1,EHHADH,ACSL3,ECHS1,ALDH3A2,ADH6,ACADL,ADH4,ACADSB,ACADM,CPT2,ACOX3,HADHA,ECI2,ACADS carnitine/acylcarnitine translocase (SLC25A20) which is important in transport of fatty acids for oxidation was induced 1.3 fold (p=0.004) The peroxisomal fatty acid beta oxydation enzyme hydroxysteroid (17-beta) dehydrogenase 4 (HSD17B4) was induced 1.4 fold (q=0.013)

which catalyzes the last step of the mitochondrial fatty acid beta-oxidation was induced 1.3 fold (p=0.005)

## Genes controlling gluconeogenesis and glycolysis

Several glucose metabolism genes, and specifically glycolysis genes were expressed at higher levels in Cushing's disease patients. These included Fructose-bisphosphate aldolase A and C (ALDOA and ALDOC), Both Lactate dehydrogenase chain genes, LDHA and LDHB,HK3,DLAT,FBP1, Enolase 1 (ENO1), Acetyl-coenzyme A synthetase (ACSS2),ALDH9A1, , Aldose 1-epimerase (GALM), Pyruvate dehydrogenase E1 component beta subunit (PDHB) ,PGM1, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ALDH3B1,ENO2,DLD,PGAM1,ALDH3A2,ADH6

Glucose-6-phosphate dehydrogenase (G6PD)

TIGAR , Fructose-2,6-bisphosphatase (C12orf5) that directs glycolysis products to the hexose phosphate shunt which leads to synthesis of NADPH required for fatty acid synthesis was induced 2.1 fold (q=0.005)

TCA cycle genes

The TCA cycle pathway was expressed at higher levles (q\*), to be continued

Isocitrate dehydrogenase (IDH1),DLAT,IDH2,ACLY, aconitase 1 (ACO1 q=0.07) , Malate dehydrogenase (MDH1 q=0.13)),PC,SUCLG1,OGDH, Pyruvate dehydrogenase E1 component subunit beta, mitochondrial (PDHB),DLD,FH,CS,ACO2,SDHC,PDHA1,SUCLA2

## Glycogen Synthetic Genes in Adipose Tissue Are Upregulated by Elevated Glucocorticoids

Glycogen synthase 2 (liver) (GYS2) was strongly induced 3.8 fold (P=0.004)

UGP2 which creates the glycogen precursor UDP-glucose was induced 1.3 fold (p=0.01)

glycogen branching enzyme (GBE1) was induced 1.6 fold (p=0.027)

PYGB

Steroid biosynthesis enzymes \*?)

Aldo-keto reductase family 1 member C1 (AKR1C1) ,CYP1A1,CYP7B1,UGT2B4,HSD17B12,CYP17A1,CYP19A1,SRD5A3,SRD5A1,HSD17B2,CYP1B1,STS

7-dehydrocholesterol reductase (DHCR7) that catalyzes the conversion of 7-dehydrocholesterol to cholesterol was induced 1.6 fold (p=0.002)

NAD(P) dependent steroid dehydrogenase-like (NSDHL) which is involved in cholesterol biosynthesiswas induced 1.5 fold (p=0.0003)

HMG-CoA synthase (HMGCS1) which is important in cholesterol biosynthesis is induced 1.3 fold (p=0.016)

7 beta-hydroxysteroid dehydrogenase was induced 1.4 fold (p=0.004)

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

KEGG reactome lysosome pathway LIPA,LAPTM5,ACP5,GM2A,ATP6V0D2,cathepsin B (CTSB q=0.1)),CTSS,GLB1,SLC11A1, Clathrin heavy chain (CLTC),ARSB, Lysosome membrane protein 2 (SCARB2),DNASE2B, Non-specific phospholipase C (NPC1),MAN2B1,AGA,ATP6V1H, Sortilin (SORT1),ASAH1,PPT1,IGF2R,GNS,CD68,CTSD,PSAP,AP3B1,ACP2,ABCA2,PLA2G15,CTSZ,LGMN,AP3B2,AP1B1,AP1S1,GALC,SLC11A2,ARSG,SMPD1

KEGG\_VALINE\_LEUCINE\_AND\_ISOLEUCINE\_DEGRADATION: Acyl-coenzyme A oxidase (AOX1), Branched-chain-amino-acid aminotransferase (BCAT1),IL4I1, Succinyl-CoA:3-ketoacid-coenzyme A transferase (ACAT2), Succinyl-CoA:3-ketoacid-coenzyme A transferase (OXCT1), Hydroxymethylglutaryl-CoA synthase (HMGCS1),ALDH9A1,MCCC1, 3-ketoacyl-CoA thiolase (ACAA2), Enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase – EHHADH (p=0.056) - one of the four enzymes of the peroxisomal beta-oxidation pathway

EHHADH,DLD,ECHS1,ALDH3A2,HIBCH,DBT,ACADSB,ACADM,BCKDHB,IVD,HADHA,PCCB

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

As described in Figures XXX and YYY, 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 senstivitiy including XXXX..

Changes in cell ceramide and glucosylceramide have been shown to be important in vitro and in obesity and glucocorticoid-induced insulin resistance (10–12). To test biochemically whether ceramides may play a role in the Cushing's disease associated insulin resistance, we took a lipidomics approach to analyze ceramide species from the adipose tissue explants of the same patients. To evaluate elevated ceramamides as a potential mechanism, ceramides and glucosylceramides were determined using liquid chromatography-triple quadrupole mass spectrometry. We observed no statistically significant changes in any ceramide species (Figure XX, q>0.25).

## Inflammation

## Modifying Effect of Obesity on Glucocorticoid Responsiveness

# 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 interestingly, a 1972 paper found 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 the protesome and the amino acid degradation pathways in adipose tissue, suggesting that a similar induction occurs in adipose tissue in Cushing's disease. 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 18434349). Our results recapitulate the full effect of glucocorticoids on induction of expression of the key triglyceride synthesis enzymes.

Althought Cushing's disease patients had a significantly higher incidence of diabetes, adipose tissue ceramides were not different between the groups. The biopsies were subcutaneous adipose tissue and not visceral and our cohort is quite small and possibly underpowered to unequivocally refute the ceramide-mediated glucocorticoid-induced insulin resistance hypothesis. Still, even taking into account these limitatins our results do not support the ceramide hypothesis.

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.

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# Table Legends:

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

# Figure Legends

Figure 1:

Differential expression of genes in subcutanous adipose tissue from subjects with Cushing's disease compared to controls. Heatmap of the differentially expressed genes in white adipose tissue. In where several transcripts derived from a single were identified as differentially expressed, only the most significant was shown. Individual values are colored as the log fold change for a particular gene in a particular subject compared to the average expression of that gene across all cohorts, with brown indicating less expression and green indicating more expression (designated in the key as Row Z-score). The bar across the top indicates the subject’s diagnosis, red for Cushing's disease and blue for controls.

Table 2: Summarized gene set enrichment analysis of KEGG 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.

Figure: Expression changes of selected genes,. mRNA Expression profile of genes involved

Asterisks indicate p<0.05. Data indicates mean +/- standard error of the mean.

# 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)