**Gene Expression Signature in Adipose Tissue of Acromegaly Patients**

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**SHORT TITLE:** Analysis of Acromegalic Adipose Tissue

**KEYWORDS:** Acromegaly, Lipolysis, Insulin Resistance, Growth Hormone

**WORD COUNT:** 4602

**DISCLOSURE STATEMENT:** The authors have nothing to disclose

**Abstract:**

To study the effect of chronic excess growth hormone on adipose tissue we performed RNA sequencing in adipose tissue biopsies from patients with acromegaly (n=9) or non-functioning pituitary adenomas (n=11). The patients underwent clinical and metabolic profiling including assessment of HOMA-IR. Explants of adipose tissue were assayed *ex vivo* for lipolysis and ceramide levels. Patients with acromegaly had higher glucose, higher insulin levels and higher HOMA-IR score. We observed several previously reported transcriptional changes (*IGF1*, *IGFBP3*, *CISH, SOCS2)* that induced by GH in liver but are also induced in adipose tissue. We also identified several novel transcriptional changes, some of which may be important for GH/IGF responses (*PTPN3* and *PTPN4*) and the effects of acromegaly on growth and proliferation. Several differentially expressed transcripts may be important in GH-induced metabolic changes. Specifically, induction of *LPL*, *ABHD5*, and *NRIP1* can contribute to enhanced lipolysis and may explain the elevated adipose tissue lipolysis in acromegaly patients. Higher expression of *TCF7L2* and the fatty acid desaturases *FADS1, FADS2* and *SCD* could contribute to insulin resistance. Ceramides were not different between the two groups. In summary, we have identified the acromegaly gene expression signature in human adipose tissue. The significance of altered expression of specific transcripts will enhance our understanding of the metabolic and proliferative changes associated with acromegaly.

**Introduction**

Acromegaly, i.e. excessive growth hormone (GH) production secondary to a pituitary adenoma, is a rare condition with an annual incidence of 3 patients per million (Holdaway & Rajasoorya 1999). The excess GH has important metabolic effects; the two most significant effects of GH on metabolism in adipose tissue are insulin resistance and lipolysis (Vijayakumar *et al.* 2010). Insulin resistance, presenting as diabetes or impaired glucose tolerance, is found in most acromegalic patients (Ezzat *et al.* 1994), and contributes to the enhanced morbidity (Colao *et al.* 2000).

There are not many studies addressing the effect of GH specifically on the subcutanous adipose tissue. Induction of STAT5 tyrosine phosphorylation and IGF1 mRNA expression has been detected in human subcutaneous adiopse tissue biopsies taken after acute GH administration (Jørgensen *et al.* 2006). Subcutaneous adipocytes extracted from acromegalic patients are insulin resistant *ex* vivo, and after a glucose tolerance test there was 50% less insulin binding to its receptor and markedly decreased insulin-related antilipolytic activity (Bolinder *et al.* 1986). *In vivo* measurement in humans detected GH-induced lipolysis in subcutanous adipose tissue (Gravhølt *et al.* 1999). Pharmacologic inhibition of lipolysis reduced GH-induced insulin resistance, suggesting that some of this resistance is dependent on higher abundance of free fatty acids (Nielsen *et al.* 2001). Microarray of gene expression has been published for subcutaneous adipose tissue biopsies before and after one year of GH treatment in GH deficient patients (Khalfallah *et al.* 2001).

New techniques of transcriptomics open an opportunity to get a full picture of RNA expression in tissue and there is very little information on adipose tissue RNA expression in acromegaly patients. To study the effects of excess GH, we used unbiased RNA sequencing in adipose tissue from acromegaly patients and controls. We found a distinctive pattern of changes in many transcripts that are highly associated with acromegaly. Many of these alterations may contribute to the metabolic effect of GH and reveal novel mechanisms of GH-induced insulin resistance and lipolysis in adipose tissue.

Changes in cell ceramide and glucosylceramide have been also shown to be important *in vitro* and in obesity and glucocorticoid-induced insulin resistance (Adams *et al.* 2004; Aerts *et al.* 2007; Holland *et al.* 2007). To assess whether ceramides are important in GH-induced insulin resistance we also measured ceramides in the same tissue samples.

**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 a cohort undergoing transsphenoidal adenomectomy at the University of Michigan Medical Center for acromegaly or non-functioning pituitary adenoma over a 12 month period. All but one patient were newly diagnosed, none had previous surgery and only one previously diagnosed patient had a history of treatment with a somatostatin analog. Exclusion criteria were age <18 years old, 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 is utilized to seal the surgical field upon completion of the procedure. A total of 500 mg of this fat graft was used for the study. ~200 mg were utilized for ex vivo lipolysis assay, ~300 mg was snap frozen in liquid nitrogen and stored at -80 degrees for RNA preparation and ceramide analysis.

***Ex vivo* lipolysis**

25 mg pieces of adipose tissue were pre-incubated for 15 minutes in KRBH buffer (sigma) at 37°C and then incubated for 1 hour at 37°C in 300 μl KRBH in the presence or absence of isoproterenol 30nM in duplicate. Glycerol was assayed in supernatants using a glycerol assay kit (sigma) as instructed by manufacturer.

**Ceramide Determinations**

Ceramide analysis of tissue samples was performed by liquid chromatography-triple quadrupole mass spectrometry (LC-QQQ) 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) (Bligh & Dyer 1959). 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 *et al.* 2009) 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 acromegaly patients), which had clinical data but no RNAseq data. These data are available through the Gene Expression Omnibus (GSE57803).

For re-analysis of the dataset reported by Huo et al (Huo *et al.* 2006), we downloaded these data from the Gene Expression Omnibus (GSE2120) and compared the 48h treated control data to the 48h GH treated data. Analysis was performed using limma (version 3.18.7; (Smyth 2005)), with all p-values adjusted by the method of Benjamini-Hochberg (Benjamini & Hochberg 1995).

**Statistics**

All statistical tests were performed using the R package (version 3.0.2,(R Development Core Team & R Core Team 2011)). To correct for multiple hypotheses testing, p-values were adjusted by the method of Benjamini and Hochberg (Benjamini & Hochberg 1995) and referred to in this manuscript as q-values. Statistical significance was set at p/q<0.05 for most comparasons except for GSEA analysis in which a p<0.25 was used.

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 acromegaly patients. Normality assumption was checked by Shapiro-Wilk test. Wilcoxon rank sum test was 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 (Fox & Weisberg 2011)). Correlation coefficients were calculated by Pearson's product-moment.

We used Gene Set Enrichment Analysis (GSEA v2.0.13 (Mootha *et al.* 2003; Subramanian *et al.* 2005)) to determine whether our rank-ordered gene list for the comparison of acromegaly vs control patients is enriched in genes from gene ontology, KEGG, transcription factor or microRNA target gene sets. The gene list was ranked based on log based 2 fold change and the statistical significance of the enrichment score was determined by performing 1000 phenotype permutations. Other settings for GSEA preranked were left by the software default. To test for enrichment of genes identified in the Huo *et al.* dataset (Huo *et al.* 2006), we used Fisher’s exact test.

**Results and Discussion**

**Patient characteristics**

Clinical and metabolic measurements were obtained for 9 acromegaly patients and 11 controls. Patient characteristics are shown in Table 1. There was no statistically significant difference in body mass index (BMI), abdominal circumference or weight. Acromegaly patients were younger (p=0.011) and taller than their controls (p=0.036).

**Acromegaly patients were more insulin resistant and had higher lipolysis**

Acromegaly patients had elevated fasting glucose levels (p=0.013) and higher fasted insulin (p=0.012, Figure 1A-B). When combined, we observed higher HOMA-IR scores in the acromegalic patients than in the controls (p=0.001, Figure 1C), reflecting a significant decrease in insulin sensitivity in the acromegaly patients, consistent with previous clinical findings (Ezzat *et al.* 1994).

Subcutaneous adipose tissue chunks for lipolysis assay were available from 6 acromegaly patients and 9 controls. As expected by previous data on GH and lipolysis, the results suggested that acromegaly patients may have higher basal lipolysis (p=0.11), and higher lipolysis in the presence of isoproterenol (p=0.058) even though they did not achieve statistical significance (Figure 1D). These data are consistent with previous reports linking GH signaling with increased lipolysis (Moller *et al.* 2009).

**Transcriptomic Analysis**

To determine which genes are altered in adipose tissue in acromegaly subjects, we performed a transcriptomic analysis of subcutaneous adipose tissue mRNA from 7 acromegalic patients and 11 controls. We identified 671 genes that had significantly different expression in acromegaly, of these 241 genes were down-regulated and 431 were up-regulated in adipose tissue from the acromegalic patients. These transcripts form a signature identifying transcriptional differences in adipose tissue in response to long-term exposure to GH (Figure 2 and Supplementary Table 1).

Gene set enrichment analysis testing KEGG pathways (Ashburner *et al.* 2000; Young *et al.* 2010) showed enrichment of genes in the categories involved in metabolism, including fatty acid metabolism, biosynthesis of unsaturated fatty acids, valine leucine and isoleucine degradation, propanoate metabolism and citrate cycle (Supplementary Tables 2 and 3). We also examined the transcription factor networks that may underlie these changes in mRNA levels. We identified an up-regulation of several candidate transcription factors and microRNA’s (Supplementary Table 4) whose targets are significantly altered in acromegalic white adipose tissue. Notably among these are an up-regulation of GATA-1 and FOXO4 targets and a down-regulation of PPAR, NF-κB and SRF responsive genes.

Previous work by Huo *et al*. (Huo *et al.* 2006) examined an analogous *in vitro* system, where 3T3-F442A adipocytes were treated with GH for a variety of time points. The longest time point (48h) is potentially analogous to the chronic GH exposure that occurs in adipose tissue from acromegaly patients. We re-analyzed that data set, looking at only the 48h GH treatment time point. In our dataset we found 560 significantly different acromegaly genes out of a total of 22810 genes tested (or 2.5%). Out of these 560 genes, 266 have mouse homologs, and 310 probes in the Huo *et al* dataset. From these probes, 32 were significantly different in both datasets (or 9.3%). Therefore the genes from the acromegaly dataset are enriched in the growth hormone treated dataset (p=7.8 × 10-5). The genes that were significantly different in both datasets were *Bst1, Capn6, Ccng1, Cish, Elovl5, Fads1, Fasn, Fmo1, Gdf5, Ggct, Hmgcs1, Igf1, Igfbp3, Itpr2, Klf4, Mpdz, Phldb2, Pkd2, Pld1, Prlr, Pten, Ptger3, Rpa3, Scd2, Scp2, Sept4, Socs2, Wisp2* and *Wnt11.*

**Established GH responsive genes are up-regulated in adipose tissue from acromegaly patients**

Since acromegaly is caused by an overproduction of GH, we first analyzed known GH responsive genes. We found that expression of previously reported GH responsive genes, including *IGF1* (3 fold, q<10-5), and *IGFBP3* (2.3 fold, q<10-5) are elevated in acromegalic patients (Figure 3A). IGF-1 has been shown to be induced in adipocytes exposed to GH (Fleenor *et al.* 2006), while there were no previous reports regarding IGF-BP3 induction in adipose tissue. The confirmation of these previously reported acromegaly or GH-induced transcriptional changes strengthens our interpretation of other transcriptional changes. Neither the growth hormone receptor (GHR) nor the IGF-1 receptor (*IGF1R)* was significantly altered in acromegalic adipose tissue.

There was a correlation between *IGF1* mRNA and levels of IGF-1 in serum in the acromegaly patients (R2=0.51, p=0.043; Figure 3C), reflecting that increased induction of *IGF1* in adipose tissue is similar in its extent to serum IGF1 induction. Serum IGF1 is primarily thought to be derived from liver tissue (Haluzik *et al.* 2003). Our data demonstrates that expression of the adipose tissue *IGF1* gene correlates well with that of serum IGF-1, suggesting that adipose tissue may also be a source of IGF-1 in acromegalic subjects.

**A novel negative feedback loop is induced by chronic exposure to high GH levels**

*SOCS2* AND *CISH,* both suppressors of cytokine signaling that have been shown to be important in down-regulating GH signaling, are up-regulated in acromegaly (1.7 and 3.3 fold respectively (q<0.0015 and q<10-5, Figure 3C). These have been shown to be induced in liver and muscle by GH (Clasen *et al.* 2013), and SOCS2 has also been reported to be induced in adipocytes by GH (Fleenor *et al.* 2006; Huo *et al.* 2006).

Among signaling pathways, MAP kinase signaling, insulin signaling (see below) and JAK-STAT signaling were down-regulated in the acromegaly patients including lower expression of ERK1 (*MAPK3*) and ERK5 (*MAPK7*) and a 4 fold lower expression of their downstream transcription factors *FOS, JUN, JUNB* (all three are ~2 fold lower in acromegaly patients, q<0.02, Supplementary Table 2 and Supplementary Figure 1A).

We observed induction of the tyrosine phosphatases, *PTPN3* (also called PTP-H1, 2.2 fold higher q<0.001) and *PTPN4* (1.6 fold higher, q<10-4) in acromegaly (Figure 3D). *PTPN3* has been reported to bind GH receptor *in vitro* in the presence of GH (Pasquali *et al.* 2003), and its overexpression reduces STAT5 signaling in response to GH (Pilecka *et al.* 2007). *Ptpn3* Knockout mice have excessive GH activity, as demonstrated by excessive growth accompanied by a strong induction of liver *IGF1* mRNA and serum IGF-1 (Pilecka *et al.* 2007). This is the first report of enhanced abundance of PTPN3 mRNA in response to GH exposure. The increased expression of *PTPN3* that we have observed in acromegaly suggests that this may be an additional negative feedback pathway induced by GH and reducing GH signaling.

**Genes controlling DNA replication, proliferation and apoptosis**

We observed a difference in expression of several different genes regulating cellular proliferation in acromegalic subjects. Broadly the KEGG category containing DNA replication was enriched in acromegalic white adipose tissue, (Supplementary Table 2). In addition to this, expression of Cyclin C (*CCNC;* 1.23 fold q<0.01), Cyclin E (*CCNE1;* 2.5 fold q<10-4*)*, which are important for transition from G1 to S, were increased in acromegalic patients, and the negative regulator, cyclin dependent kinase inhibitor B (*CDKN2B*) was decreased (0.6 fold q<0.01, Supplementary Figures 1B). Notably, *CDKN2B* is also a diabetes susceptibility gene identified repeatedly in GWAS studies (Saxena *et al.* 2007; Sladek *et al.* 2007).

Additional DNA replication genes that were induced were nucleosome assembly protein 1-like 1 (*NAP1L1,* 1.3 fold q<0.02) and origin recognition complex, subunit 2(*ORC2*, 1.6 fold q<0.002), which are important for DNA replication, and the antiapoptotic regulators *BAG4*(BCL2-associated athanogene 4. 1.7 fold q<10-4) and *CAPN6* (calpain 6, 2.4 fold q<0.001) were also induced (Supplementary Figures 1C). Together these implicate increased cell division, potentially of immune, vascular or pre-adipocyte cells in adipose tissue depots.

Apoptosis signal-regulating kinase 1 (*MAP3K5*) expression is higher in acromegaly (1.3 fold q<0.02), and there is also higher expression of its downstream substrates p38α (*MAPK14* 1.2 fold q<0.005), p38*δ*  (*MAPK13*, 2.3 fold q<10-4) and *MAPKAPK3* (1.4 fold q<0.03, Supplementary Figure 1D). The pathway is thought to regulate apoptosis in response to stress. The effect of GH on these transcripts has not been reported before, and they could account for the effects of enhanced cell proliferation and apoptosis in response to GH (Waters & Brooks 2012).

**Transcriptional changes regulating lipid metabolism and localization that may contribute to enhanced lipolysis**

To determine the potential causes of the increased lipolysis observed in Figure 1D, we examined the expression of human lipases in these adipose tissues. We observed no significant difference in expression of the three classical triglyceride lipolysis enzymes hormone sensitive lipase (*LIPE*), adipose triglyceride lipase (*PNPLA*) or monoglycerol lipase (*MGLL*; Figure 4A). Lipoprotein lipase (*LPL*), the lipase important for lipolysis of triglycerides in apolipoproteins, was significantly more highly expressed in acromegaly patients (1.9 fold, q<10 -3, Figure 4A). A strong induction of LPL expression in response to GH and absence of change in HSL was demonstrated before in a preadipocyte cell line (Padines-Figuères *et al.* 1990; Pradines-Figueres *et al.* 1990; Barcellini-Couget *et al.* 1994) and in adipose tissue biopsies from GH deficient patients after treatment with GH (Khalfallah *et al.* 2001). Notably, studies that addressed LPL enzymatic activity and not expression have found either no change or even a reduction in LPL activity in response to GH treatment of human adipocytes *in vitro* (Simsolo 1995; Richelsen *et al.* 2000).

Although neither Hormone Sensitive Lipase (*LIPE*) nor ATGL (*PNPLA2*) were altered, two direct regulators of HSL and ATGL activity in adipocytes, abhydrolase domain containing 5 (*ABHD5*, also called CGI58 (Lass *et al.* 2006)), and nuclear receptor interacting protein 1 (*NRIP* (also called RIP140 (Ho *et al.* 2011)) were expressed at higher levels in adipose tissue from acromegaly patients (2.1 fold q<10-3 and 1.6 fold, q<0.03 Figure 4B). CGI58 is an allosteric activator of lipolytic activity and RIP140 regulates it's activity, therefore these data suggest that their transcriptional up-regulation could contribute to the induction of lipolysis by GH. NRIP1 has also been proposed to be a transcription regulator of genes involved in lipid and glucose metabolism (Ho *et al.* 2011) and its induction could contribute to additional metabolic effects of GH including disrupted glucose metabolism.

We next examined the expression of G-protein coupled receptors that induce lipolysis. The β3 adrenergic (*ADRB3*) was more highly expressed in acromegaly patients compared to the controls (2.9 fold, q< 0.002). The 1 receptor was also more highly expressed in acromegalic adipose tissue though it did not reach statistical significance (1.4 fold, q=0.14; Figure 4B) suggesting a potential sensitization of these patients to adrenergic stimuli. The Calcitonin receptor like (*CALCRL*), which can function as either an adrenomedullin or CGRP receptor subunit, is higher in acromegaly (1.5 fold, q<0.05, Figure 4B). Activation of these receptors also activates cAMP-dependent lipolysis and may contribute to enhanced lipolysis in these patients (Linscheid *et al.* 2005; Go *et al.* 2007; Iemura-Inaba *et al.* 2008).

Angiopoietin like 4 (*ANGPTL4*), which has been recently shown to be induced in muscle in response to acute GH treatment, and suggested as a possible regulator of lipolysis (Clasen *et al.* 2013) was not observed to have significantly different expression between the acromegaly patients and the controls (q=0.77).

In contrast to the lipolytic phenotype of acromegalic patients, several fatty acid synthesis genes were expressed at significantly higher levels in acromegaly patients (Figure 4C). These include *ACSS2* (1.5 fold, q<0.03) and *ACSS3* (1.5 fold, q<0.03) which catalyze the activation of acetate for use in lipid synthesis . We also observed elevations in Acetoacetyl Co-A synthase (*AACS;* 1.75 fold, q<0.05), Acetyl Co-A carboxylse (*ACACA*, 1.62 q<0.02), and Acyl-CoA synthetase long-chain family member 3 *ACSL3* (1.3 fold, q<0.03)*,* whichconvert long-chain fatty acids into fatty acyl-CoA esters. Finally we observed an elevation in fatty acid synthase *FASN* (2.2 fold, q<0.02) in the acromegaly patients. By pathway analysis, both fatty acid metabolism and unsaturated fatty acid biosynthetic pathways were up-regulated in the acromegaly patients (see Supplementary Table 2 and below). This up-regulation may represent compensation by the adipose tissue due to enhanced lipid breakdown and oxidation in this and other tissues in acromegaly patients.

With respect to glycogen metabolism, expression of *UGP2* (1.4 fold, q<0.02) and expression of muscle glycogen phosphorylase (*PYGM*) (2.3 fold, q<0.0002) were higher in the acromegaly patients (Supplementary Figure 1E). The significance of glycogen in adipose tissue, or changes in glycogen content in acromegalic adipose tissue have not been characterized, but these findings would predict that the rates of glycogenolysis in acromegalic white adipose tissue would be elevated.

**Transcripts altered in acromegaly that may contribute to insulin resistance**

One of the most pronounced differences between the acromegaly patients and the controls was a ~3 fold higher expression (q<1x10-5, Figure 4D) of the three fatty acid desaturases - stearoyl-CoA desaturase (*SCD*, delta-9-desaturase), fatty acid desturase 1 (*FADS1*, delta-5-desaturase) and fatty acid desturase 2 (*FADS2*, delta-6-desaturase). Cytochrome b5 type A (*CYB5A)*, an activator of SCD, is also induced (1.7 fold, q=0.001). *SCD* products and *FADS2* mRNA have recently been shown to be induced by GH in mice (Oberbauer *et al.* 2011) The change in expression of these enzymes could be a possible link between acromegaly and insulin resistance due to an elevation of unsaturated fatty acids, as activity of *FADS1* and *FADS2* are associated with metabolic syndrome (Kröger & Schulze 2012; Mayneris-Perxachs *et al.* 2013).

We observed no decrease in expression of canonical transcripts important for insulin signaling and response to insulin in adipocytes, including insulin receptor (*INSR*), *IRS1, IRS2*, *AKT1-3*, or GLUT4 (*SLC2A4* ; see Supplementary Figure 2A). This indicates that the observed insulin resistance is not caused by mRNA decreases in these genes. In fact, the KEGG category containing insulin signaling genes was generally up-regulated in these tissues (Supplementary Table 2). AKT1 was significantly higher (1.3 fold, q<0.002) and the remainder of these genes trended to be more highly expressed in the adipose tissue from the acromegalic and insulin resistant patients, potentially underlying a transcriptional up-regulation that compensates for an alternative insulin resistance mechanism.

One previously identified candidate is the phosphoinositide-3-kinase, regulatory subunit 1 (*PIK3R1*, also called p85α), which was induced by GH in mouse adipose tissue and thought to contribute to GH-induced insulin resistance (del Rincon *et al.* 2007). In our study it's expression was not different in the acromegaly patients (q=0.95).

The cytokine modulators *STAT6* and the pro-inflammatory protein kinase IKKβ (*IKBKB*) are expressed at lower levels (Supplementary Table B). Furthermore, the pro-inflammatory cytokines *IL1B*, *IL6* and *MCP1* and the pro-inflammatory protein kinase *IKBKE* all trend towards lower expression, and genes from several KEGG pathways involved in inflammation were expressed at lower levels in the acromegaly cohort (Supplementary Table 2 and Supplementary Figure 2B). These data support the hypothesis that insulin resistance in these patients is not due to enhanced inflammatory signaling.

To test biochemically whether ceramides may play a role in the acromegaly associated insulin resistance, we took a lipidomics approach to analyze ceramide and glucosylceramide species from the adipose tissue explants of these patients. Elevated ceramides have been proposed to mediate insulin resistance by several models (Chavez *et al.* 2003; Adams *et al.* 2004; Aerts *et al.* 2007; Holland *et al.* 2007). We observed a modest down-regulation of the mRNA levels of glycosylsphingolipid metabolic genes in our data (normalized enrichment score = -0.86 q=0.71). We then measured ceramide species from 7 acromegaly patients and 11 control patients directly and observed no statistically significant changes in any ceramide species (Supplementary Figure 3, q-values all >0.25). We did however, detect modest elevations of C16:0, C18:0 and C24:0 ceramide species in WAT from acromegalic patients. We therefore do not have strong evidence to support the hypothesis that ceramide elevations are causative of insulin resistance in acromegalic white adipose tissue.

*TCF7L2*, a transcription factor regulating many metabolism genes as well as a diabetes susceptibility gene (Ip *et al.* 2012) is up-regulated in the acromegaly patients (1.5 fold, q<0.0002, Figure 4E). Mice with liver specific knockout of *Tcf7l2* are hypoglycemic, while transgenic mice overexpressing liver *Tcf7l2* are hyperglycemic (Boj *et al.* 2012). *TCF7L2* in subcutaneous fat is higher and expression of splice isoforms is reduced in subcutaneous fat and in liver following bariatric surgery (Hindle *et al.* 2009). Higher expression of TCF7L2 could also therefore be linked to insulin resistance in acromegaly.

**Glucocorticoid regulation**

11βHydroxysteroid dehydrogenase 1 (*HSD11B1*), the enzyme that activates transformation of cortisone to cortisol, was reduced 4 fold in acromegaly patients (q<0.002. Figure 2F). The down-regulation of expression and activity of this enzyme by GH/IGF1 has been confirmed both *in vitro* (Napolitano *et al.* 1998), in GH deficient patients treated with GH (Paulsen *et al.* 2006) and in acromegaly patients (Moore *et al.* 1999; Frajese *et al.* 2004). In addition, we found higher expression of the glucocorticoid receptor (*NR3C1,* 1.5 fold q<10-5) and the mineralocorticoid receptor (*NR3C2*, 1.3 fold q<0.05) in acromegaly patients (Figure 2F). Glucocorticoid receptor expression is repressed by cortisol (Kalinyak *et al.* 1987), so the higher expression may be due to the reduced local cortisol levels caused by lowered *HSD11B1*.

**Summary**

In this study we have described a transcriptional signature in adipose tissue from subjects with acromegaly. We identified 671 adipose tissue genes altered in acromegaly. Some of these genes may be direct targets of increased GH or IGF-1 signaling in adipose tissue, whereas others may be secondary adaptations to this condition.

The fact that the patients consistently had a relatively uniform change of expression of these genes suggests that we are able to draw valid conclusions about adipose tissue in acromegalic patients even from this small cohort. Furthermore, as mentioned throughout, our data agree with several previous studies in animal models and patients. The confirmation of these previously reported GH-dependent transcriptional changes strengthens our interpretation of other transcriptional changes.

These data provide a variety of novel transcriptional changes that may be causative of the co-morbidities associated with acromegaly. 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 GH in adipose tissue.

**Declaration of interest**

The authors have no conflicts to declare.

**Funding**

This work was supported by Motor City Golf Classic (MCGC) Grant # G010640. This work utilized Metabolomics Core Services supported by grant U24 DK097153 of NIH Common Fund to the University of Michigan.

**Acknowledgements**

We thank Charlotte Gunden, Elizabeth Walkowiak and Eric Vasbinder for their valuable help in the study. We would also like to thank Solomon S. Solomon (UTHSC) and the members of the Bridges and Reiter laboratories (UTHSC) for helpful suggestions.

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**Figure Legends**

**Figure 1: Acromegalic patients have reduced insulin sensitivity and trend for higher lipolytic activity than their controls.** A) Fasting blood glucose levels. B) Fasting insulin levels. C) HOMA-IR score from Control or Acromegaly subjects. D) *ex vivo* lipolysis as measured by glycerol release from excised white adipose tissue from control or acromegaly patients left untreated (Basal) or after stimulation with 30 nM isoproterenol (Iso). Data is presented as mean +/- standard error of the mean. Asterisk indicates q<0.05.

**Figure 2: Differential expression of genes in white adipose tissue from subjects with acromegaly compared to controls.** A) Heatmap of the differentially expressed genes in white adipose tissue. 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 acromegaly and blue for controls.

**Figure 3: GH targets are differentially expressed in acromegaly subjects.** A) mRNA Expression of *IGF1* and *IGFBP3* transcript levels in adipose tissue from control and acromegalic patients (B) Comparason between *IGF1* mRNA and IGF-1 serum levels in patients with acromegaly (C) Expression of mRNA for suppressors of growth hormone signaling (D) and Expression of tyrosine phosphatases associated with growth hormone signaling. Asterisks indicate q<0.05. Barplots are presented as mean +/- standard error of the mean. mRNA Expression is in units of RPKM (reads per kilobase per million reads).

**Figure 4: Expression changes of selected genes, potentially responsible for lipolytic or insulin sensitivity alterations in acromegaly patients.** mRNA Expression profile of genes potentially involved in lipid catabolism (A), regulation of lipolysis (B), fatty acid synthesis (C), fatty acid desaturation (D), *TCF7L2* (E), and glucocorticoid signaling (F). Asterisks indicate q<0.05. Data indicates mean +/- standard error of the mean. mRNA Expression is in units of RPKM (reads per kilobase per million reads).

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

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **Control (n=11)** | | **Acromegaly (n=9)** | | **p** |
| Age (years) | 63.4 | +/- 2.7 | 48.3 | +/- 4.9 | 0.011 |
| Height (cm) | 170.0 | +/- 2.4 | 180.1 | +/- 4.0 | 0.036 |
| Weight (kg) | 89.4 | +/- 6.7 | 103.9 | +/- 9.3 | 0.21 |
| BMI (kg/m2) | 30.7 | +/- 1.8 | 31.7 | +/- 2.1 | 0.69 |
| Abdominal Circumference (cm) | 100.7 | +/- 4.6 | 104.9 | +/- 6.3 | 0.59 |

**Supplementary Data**

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

**Supplementary Table 2:** 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 acromegaly.

**Supplementary Table 3:** 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 acromegaly.

**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 acromegalic 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 acromegaly.

**Supplementary Figure 1: Expression changes of selected other transcripts.** mRNA Expression profile of differentially expressed genes involved in A) MAPK Signaling, B) Cell cycle control, C) DNA synthesis and apoptosis D) ASK-1 Signaling and E) Glycogen metabolism. Asterisks indicate q<0.05. Barplots are presented as mean +/- standard error of the mean. mRNA Expression is in units of RPKM (reads per kilobase per million reads).

**Supplementary Figure 2: Expression changes of selected insulin signal transduction genes.** mRNA Expression profile of genes involved in insulin signaling (A) and inflammation (B). Asterisks indicate q<0.05. Barplots are presented as mean +/- standard error of the mean. mRNA Expression is in units of RPKM (reads per kilobase per million reads).

**Supplementary Figure 3: Ceramide levels in adipose tissue from control and acronegalic patients.** Ceramide (Cer) or glucosylceramide levels (GluCer) were determined as described in the methods and materials section. The number indicates the fatty acid species associated with each lipid. All values are normalized such that control values are equal to 1. Data indicates mean +/- standard error of the mean.

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