**Recheck:**

**Capital letters, abreviations, commas, parenthesis**

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

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**Abstract:**

GH affects several molecular pathways regulating proliferation and metabolism. Objective: To study tissue transcriptional changes induced by GH, we took adipose tissue biopsies from patients with excess GH due to acromegaly (n=9) and controls with non functioning pituitary adenoma (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. Adipose tissue was analysed by RNAseq. Patients with acromegaly had a higher glucose, higher insulin levels and higher HOMA-IR score. . We observed several previously reported transcriptional changes (*IGF1*, *IGFBP3*.*CISH, SOCS2)* that are classically known to be induced by GH in liver but are also induced in adipose tissue. We identified several novel transcriptional changes, some of which may be important for GH signal regulation (*PTPN3* and *PTPN4*) and the effect of GH on growth and proliferation. Several transcripts could potentially be important in GH-induced metabolic changes. Specifically, induction of *LPL*, *ABHD5*, *ACVR1C* could contribute to enhanced lipolysis and may explain the s enhancement of adipose tissue lipolysis in acromegaly patients . Higher expression of *TCF7L2* and the fatty acid desaturases *FADS1, FADS2* and *SCD* and could contribute to insulin resistance. Expression of *HSD11B1* was reduced and *GR* was increased, predicting modified glucocorticoid activity in acromegaly. The significance of altered expression of specific transcripts will enhance our understanding of the metabolic and proliferative changes associated with acromegaly

**Introduction**

Acromegaly, excess growth hormone (GH) production secondary to a pituitary adenoma, Acromegaly is a rare condition with an annual incidence of 3 patients per million 1. The excess GH has important metabolic effects, . The two most significant effects of GH on metabolism in adipose tissue are insulin resistance and lipolysis (1). Insulin resistance, presenting as diabetes or impaired glucose tolerance, is found in most acromegalic patients (2), and contributes to the enhanced morbidity (3).

There are not many studies addressing the affect 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 treatment2. Subcutanous 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 decreaseed insulin-related antilipolytic activity 3. *In vivo* measurement in humans detected GH-induced lipolysis in subcutanous adipose tissue 4. Pharmacologic inhibition of lipolysis reduced GH-induced insulin resistance, suggesting that some of this resistance is dependent on higher abundance of free fatty acids 5. Microarray of gene expression has been published for subcutanous adipose tissue biopsies before and after one year of GH treatment in GH deficient patients 6.

New techniques of transcriptomics open an opporunity to get a full picture of RNA expression in tissue. and there is very little information on adipose tissue RNA expression in these patients. To study the effect of excess GH on adipose tissue, we used unbiased RNA sequencing to study 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 shown to be important in vitro and in obesity and glucocorticoid-induced insulin resistance7-9. To assess whether ceramides are important in GH-induced insulin resistance we also measured ceramides in the same tissue samples.

**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 acromegaly or non-functioning pituitary adenoma over a 12 month period. All but one patient were newly diagnosed, none had previous surgery and only the one previously diagnosed patient had a history of treatment with a somatostatin analog. 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 is utilized to seal the surgical field upon completion of the procedure. 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.

***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.

**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 (4), Bowtie 2 version 2.1.0 (5) and Samtools version 0.1.18 . Reads were mapped to known genes using HTseq (6). Gene expression was analyzed using DESeq2 version 1.2.10 (7) . 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.

For re-analysis of the dataset reported by Huo et al (8), 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; (9)), with all p-values adjusted by the method of Benjamini-Hochberg (10).

**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 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 (12)). Correlation coefficients were calculated by Pearson's product-moment. Statistical significance in this study was defined as a p-value of less than 0.05. All statistical tests were performed using the R package (version 3.0.2,(11)). To correct for multiple hypotheses testing problem, p-values for ceramide levels were adjusted by the method of Benjamini and Hochberg (10).

We used Gene Set Enrichment Analysis (GSEA v2.0.13) (Ref: PMID: 16199517 and PMID: 12808457) to determine whether our rank-ordered gene list for the comparison of acromegaly vs control patients is enriched in genes from gene ontology, KEGG, or transcription factor targets 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 by the software default. To test for enrichment of genes identified in the Huo *et al.* dataset (8), we used Fisher’s exact test..

**Results**

**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=0.01) 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 10.

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 1E. These data are consistent with previous reports linking GH signaling with increased lipolysis (14).

**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 downregulated and 431 were upregulated 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 (13,15) showed enrichment of genes in the gene ontology categories involved in metabolism, including fatty acid metabolism, biosynthesis of unsaturated fatty acids, valine leucine ane isoleucine degradation, propanoate metabolism and citrate cytle (Table 2).

Previous work by Huo *et al*. (8) 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.

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=7.8615 × 10-5). The genes which 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, Wnt11.*

**Established GH responsive genes are upregulated 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*,and *IGFBP3* are elevated in acromegalic patients (Figure 3A-B). IGF-1 has been shown to be induced in adipocytes exposed to GH (16), while there were no previous reports regarding IGF-BP3 induction in adipose tissue. The confirmation of these previously reported acromegaly or GH dependent transcriptional changes strengthens our interpretation of other transcriptional changes.

Within the acromegalic subjects, 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 (17). Our data demonstrates 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 suppresors of cytokine signaling known to be important in downregulating GH signaling, are upregulated in acromegaly (figure XXX ). These have been shown to be induced in liver and muscle 11 by GH, and SOCS2 has also been reported to be induced in adipocytes by GH 12.

ERK1 (*MAPK3*) and ERK5 (*MAPK7*) which are signaling substrates downstream to GH-induced activation of MAP kinase pathway were expressed at lower levels in the acromegaly patients, again reflecting downregulation of GH signaling.

We observed induction of the tyrosine phosphatases, *PTPN3* (also called PTP-H1) and *PTPN4* in acromegaly (Supplementary Figure 1A-B). *PTPN3* has been reported to bind GH receptor *in vitro* in the presence of GH (18), and its overexpression reduces STAT5 signaling in response to GH (19). *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 (19). 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. Cyclin C (*CCNC*), Cyclin E (*CCNE1)*, which are important for transfer from G1 to S, were increased in acromegalic patients, and cyclin dependent kinase inhibitor B (*CDKN2B*) was decreased (Supplementary Figures 1C-D). Of note, *CDKN2B* is also a diabetes susceptibility gene identified repeatedly in GWAS studies (20,21). Nucleosome assembly protein 1-like 1 (*NAP1L1*) and origin recognition complex, subunit 2(*ORC2*), which are important for DNA replication, and the antiapoptotic regulators *BAG4*(BCL2-associated athanogene 4) and *CAPN6* (calpain 6) were also induced (Supplementary Figures 1E-H). Apoptosis signal-regulating kinase 1 (*MAP3K5*) expression is higher in acromegaly, and there is also higher expression of its downstream substrates p38α (*MAPK14*), p38*δ*  (*MAPK13,* \*p<10-5) and *MAPKAPK3* (Supplementary Figure 1I-J). 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.

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

To determine the potential causes of the increased lipolysis observed in Figure 1E, we examined the expression of human lipase in these adipose tissue lysates. 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 (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 (22) 13 14and in adipose tissue biopsies from GH deficient patients after treatment with GH (23). Notably, studies that addressed LPL enzymatic activity and not expression have found no change or even a reduction in LPL activity in response to GH treatment of human adipocytes *in vitro* (24,25).

Although neither Hormone Sensitive Lipase (*LIPE*) or ATGL (*PNPLA2*) were altered, a direct regulator of hormone sensitive lipase and adipose triglyceride lipase activity in adipocytes, abhydrolase domain containing 5 (*ABHD5*, also called CGI58, see Figure 4B) (26), was expressed at higher levels in adipose tissue from acromegaly patients. This suggests that this activator is a candidates for the induction of lipolysis by GH.

We also examined the expression of G-protein coupled receptors that induce lipolysis. The 3 adrenergic (*ADRB3*) and oxytocin receptors (*ACVR1C*), were more highly expressed in acromegaly patients compared to the controls, though at relatively low levels (Figure 4C-D).

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 (27) was not observed to be significantly different in transcript expression between the patients and the controls (p=0.77). As far as glycogen synthesis is concerned, expression of the glycogenolysis enzyme muscle glycogen phosphorylase (*PYGM*) was significantly higher in the acromegaly patients. The significance of glycogenolysis in adipose tissue, or changes in glycogen content in acromegalic adipose tissue have not been characterized.

**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 (p<10-5, Figure 4E ) in expression 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)*, a n activator of SCD, is also induced (p=0.001). SCD products and FADS2 mRNA have recently been shown to be induced by GH in mice 15, The change in expression of these enzymes could be possible link between acromegaly and insulin resistance. Activity of FADS1 and FADS2 is associated with metabolic syndrome 16 17.

We observed no difference in expression of canonical transcripts important for insulin signaling and response to insulin in adipocytes, including insulin receptor (*INSR*), *IRS1, IRS2*, *AKT2-3*, or *SLC2A4* (GLUT4; see Supplementary Figure 2A-G). This indicates that the observed insulin resistance is not caused by transcriptional changes in these genes. AKT1 was 1.3 fold higher (p=0.001) and the restof these genes trended to be more highly expressed in the adipose tissue from the present study's insulin resistant patients, potentially underlying an upregulation that compensates for an alternative insulin resistant 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 (28) and thought to contribute to GH-induced insulin resistance (28). In our study it's expression was not different in the acromegaly patients(p=0.95). The cytokine modulators *STAT4* and *STAT6* and the proinflammatory protein kinase IKKβ (*IKBKB*) are expressed at lower levels (p=3x10-5,, p=0.009 and p=0.013 respectively), the pro-inflammatory cytokines *IL1B*, *IL6* and *MCP1* and the proinflammatory protein kinase *IKBKE* all trend towards lower expression, and genes from several KEGG inflammatory pathways trended (p=0.2) to be expressed at lower levels in the acromegaly cohort (data not shown). The JNK and PKC family members were upregulated by more than 15% between controls and acromegaly patients. 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 analyse ceramide species from the adipose tissue explants of these patients. Elevated ceramides have been proposed to mediate insulin resistance by several models 7-9. We observed no statistically significant changes in any ceramide species (Figure XXX, adjusted p-values all >0.25), indicating that ceramide elevations are not likely causative of insulin resistance in acromegalic white adipose tissue. We did however, detect modest elevations of C16:0, C18:0 and C24:0 ceramide species in acromegalic patients.

*TCF7L2*, a transcription factor regulating many metabolism genes known as a diabetes susceptibility gene (33) is upregulated in the acromegaly patients (Figure 4F). Mice with liver specific knockout of TCF7L2 are hypoglycemic, while transgenic mice overexpressing liver TCF7L2 are hyperglycemic. TCF7L2 in subcutaneous fat is higher and expression of splice isoforms is reduced in subcutaneous fat and in liver following bariatric surgery. Higher expression of TCF7L2 could also also be linked to insulin resistance in acromegaly.

**Glucocorticoid regulation**

11βHydroxysteroid dehydrogenase 1 (*HSD11B1*), the enzyme that activates cortisone to cortisol, was reduced 4 fold in acromegaly patients (Supplementary Figure 2H). The downregulation of expression and activity of this enzyme by GH/IGF1 has been confirmed both *in vitro* (34), in GH deficient patients treated with GH (35) and in acromegaly patients (36,37). In addition, we found higher expression of the glucocorticoid receptor (*NR3C1* p=10-5) and the mineralocorticoid receptor (*NR3C2*, p=0.046)\_ in acromegaly patients (Supplementary Figure 2I). Glucocorticoid receptor expression is repressed by cortisol, so the higher expression is expected given the reduced local cortisol production.

**Summary**

In this study we have described a transcriptional signature in adipose tissue from subjects with acromegaly. We identified 103 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 agrees 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.

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**Table Legends:**

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

**Table 2:** **Gene set enrichment of transcripts from acromegalic white adipose tissue.** Gene ontology biological process categories enriched by in transcripts that differ between acromegaly and control subjects. Type is the GO or KEGG classification of the term. DE indicates the number of differentially expressed genes in this category, Total is the total number of genes in that category. Lists were filtered to remove redundant categories.

**Figure Legends**

**Figure 1: Acromegalic patients tended to have reduced insulin sensitivity and 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) Plot of each subject’s BMI compared with the natural logarithm of their HOMA-IR score. A best fit line was drawn for each subject pool. E) *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.

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

**Figure 3: Previously reported GH targets are differentially expressed in acromegaly subjects.** A) mRNA Expression of A) *IGF1* and B) *IGFBP3* transcript levels in adipose tissue from control and acromegalic (Acro.) patients. Only the highest expressing transcript is shown for each gene. C) Comparason between *IGF1* mRNA and IGF-1 serum levels in patients with acromegaly Asterisks indicate p<0.05. Barplots are presented as mean +/- standard error of the mean.

**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 insulin sensitivity (A-B) or lipid metabolism (C-E). C) Lipases in human tissue were examined, only lipases with >100 FPKM are shown. Asterisks indicate p<0.05. Data indicates mean +/- standard error of the mean.

**Supplementary Data**

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

**Supplementary Figure 1: Expression changes of selected other transcripts.** mRNA Expression profile. Data indicates mean +/- standard error of the mean. Asterisk indicates p<0.05

**Supplementary Figure 2: Expression changes of selected insulin signal transduction genes.** mRNA Expression profile of genes involved in insulin signaling. Data indicates mean +/- standard error of the mean.

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