DETECTION OF ACTH LEVELS IN PITUITARY TUMORS FROM PATIENTS WITH CUSHING’S DISEASE USING \textsuperscript{1}H MAGNETIC RESONANCE SPECTROSCOPY (MRS)

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DISCLOSURES

NO DISCLOSURES
Cushing’s disease

- Cushing’s disease is characterized by the overproduction of cortisol from adrenal cortex which is regulated by a pituitary-tumor secreting adrenocorticotropic hormone (ACTH).

- Sometimes, tumors other than pituitary can also produce excess ACTH and trigger the overproduction of cortisol, a condition referred to as Cushing’s Syndrome.

- A prompt diagnosis of Cushing’s disease or Cushing’s syndrome is necessary to find out the source of ACTH production. Bilateral inferior petrosal sinus sampling (BIPSS) is widely used to confirm Cushing’s disease.

- BIPSS is an invasive procedure, development of non-invasive techniques will be of immense value.

- In this study, we have analyzed surgically resected pituitary-tumor tissues from Cushing’s disease and other hormone-secreting pituitary-tumor patients to determine if $^1$H-MRS can be used in diagnosis of Cushing’s disease.
**Methods**

**Patients and Samples collection:** Fifteen pituitary tumors (LH/FSH = 10; PRL = 3; and ACTH = 2) were collected from pituitary adenoma patients undergoing transsphenoidal hypophysectomy at the Houston Methodist Hospital. Informed consent was obtained from each patient following an IRB protocol approved by the Houston Methodist Hospital and Research Institute. The diagnosis was based on detailed clinical, biochemical, radiological and histopathological characteristics of study participants.

**Tissue extraction:** Pre-weighed pituitary tumor tissue samples were extracted in methanol:chloroform solvent mixture (2:1, v/v, 4 °C). The tissue sample was left in contact with the solvent-mixture for 5 minutes (kept on ice) in tubes containing ZrO₂ beads. After 5 min., the tubes were spun at 4000 rpm for 3 minutes (3x1 min. duration, with cooling in between to avoid tissue warming) using a tissue homogenizer, Bead Bug (Benchmark Scientific, Edison, NJ, USA). Later, chloroform and Millipore water (1:1 ratio, v/v, 4 °C) were added to the tissue extract and the tubes were spun for another 2 minutes. Finally, the methanol and chloroform layers were separated by centrifugation (RCF = 10,000×g, 10 min., 4 °C) to obtain aqueous and lipid components. The solvents were dried in a CentriVap® vacuum concentrator (Labconco corporation, Kansas City, MO). The residue from the methanol layer was reconstituted in 180 µL D₂O containing 1.0 mM DSS (internal standard), and the pH of the solution was adjusted to 7.4 ± 0.05, and the residue from chloroform layer was re-dissolved in 180 µL CDCl₃ containing 1% tetramethyl silane (TMS) (internal standard).

**¹H MR spectroscopy (¹H-MRS):** One-dimensional (1D) proton (¹H) and two-dimensional (2D) ¹H-¹H total correlated spectroscopy (TOCSY) NMR spectra were acquired on a 600 MHz spectrometer. 2D TOCSY spectra were used to confirm the metabolites identified in the 1D ¹H-MRS spectra.
We compared the metabolite profiles (obtained using $^1$H-MRS) of LH/FSH, PRL and ACTH secreting (Cushing’s disease) pituitary-tumors.

Figure 1: Representative $^1$H-MRS spectra of methanol:chloroform extracts from LH/FSH, PRL, and ACTH secreting pituitary tumors showing metabolite profiles. The broad peaks observed in ACTH secreting tumor (Cushing’s disease) at 0.90 and 1.40 ppm indicate the presence of elevated levels of ACTH hormone (marked as peptide). Other metabolites of interest are lactate, myo-inositol (ml), glycine, scyllo-inositol (sl) and phosphoethanolamine (PE) which were decreased in ACTH compared to the LH/FSH secreting tumors. Abbreviations: BCAAs, branched chain amino acids; Ala, alanine; NAA, N-acetyl aspartate; Glu, glutamate; Asp, aspartate; PE, phosphoethanolamine; PC, phosphocholine; GPC, glycerophosphocholine; ml, myo-inositol; sl, scyllo-inositol; Tau, taurine; Gly, glycine; *: exogenous compound (solvent impurity).
Results: Comparison of metabolite profiles of LH/FSH, PRL and ACTH secreting tumors

Concentrations of metabolites in LH/FSH, PRL and ACTH (Cushing’s disease) secreting pituitary tumors

Figure 2: A chart showing the concentrations of aqueous metabolites in LH/FSH, PRL, and ACTH secreting tumors. In ACTH secreting tumors, lactate, scylo-inositol (sl), glycine, and phosphoethanolamine (PE) were significantly decreased compared to LH/FSH-secreting tumors. Although myo-inositiol (ml) was also decreased, the change was not statistically significant. Between ACTH and PRL groups, NAA was relatively low in PRL, and the difference was statistically significant.
Results 3: Elevated levels of Tyrosine and Phenylalanine residues in pituitary tumors from patients with Cushing’s disease

$^1$H-MRS showing the elevated levels of ACTH in pituitary tumor of patients with Cushing’s disease

Figure 3: Representative $^1$H NMR spectra (aromatic region, 6.70 – 7.40 ppm) of tissue extracts from LH/FSH, PRL, and ACTH secreting tumors. ACTH-secreting tumors with Cushing’s disease showed the presence of elevated levels of phenylalanine and tyrosine residues, arising from ACTH hormone, as broad signals. These Cushing’s disease-specific MRS signatures can be used in quantifying the levels of ACTH in pituitary tumors.
Results 4: Comparison of lipid profiles of LH/FSH, PRL and ACTH secreting tumors

We also compared the lipid profiles of pituitary tumors secreting LH/FSH, PRL and ACTH (Cushing’s disease).

Figure 4: Representative $^1$H-MRS spectra of methanol:chloroform extracts from LH/FSH, PRL, and ACTH secreting pituitary tumors showing their lipid profiles. In ACTH secreting tumors, cholesterol was slightly elevated whereas GPE, CholinePLs, and PLs were decreased compared to the LH/FSH-secreting tumors. Abbreviations: Chol, cholesterol; PUFAs, poly-unsaturated fatty acids; GPE, glycerophosphocholine, SM, sphingomyelin; CholinePLs, choline-containing phospholipids; PLs, total phospholipids. *: residual H$_2$O in deuterated chloroform (NMR solvent).

Figure 5: A chart showing the concentrations of lipid components in ACTH along with LH/FSH and PRL secreting tumors. In ACTH secreting tumors, cholesterol is slightly elevated while GPE, choline containing PLs (CholinePLs), plasmalogens, and total phospholipids were found to be decreased. None of the above lipids showed statistically significant difference in comparing ACTH with LH/FSH or PRL secreting pituitary tumors. Since pituitary tumors are non-proliferating, there may not be much changes in membrane lipids.
Discussion

- We used ex vivo $^1$H MRS to characterize pituitary tumors from patients with LH/FSH, PRL, and ACTH secreting pituitary adenomas.
- In Figure 1 and 2, we observed that lactate and glycine were significantly decreased indicating a relatively weaker glycolytic flux in ACTH-secreting tumors compared to LH/FSH and PRL secreting tumors.
- We have also observed decrease in myo-inositiol (mI) and scylo-inositol (sI) levels in ACTH tumors compared to LH/FSH secreting tumors. mI and its phosphorylated compounds are major metabolites in brain and pituitary gland and play a major role in their cellular function. Moreover, myo-inositol serves as an osmolyte in the CNS, and its phosphorylated compounds play a pivotal role in diverse cellular functions such as DNA repair, nuclear RNA export and synaptic membrane trafficking [Fisher et al., J Neurochem, 2002]. Depletion of mI in pituitary tissue during tumorigenesis may be partly due to the changes in DNA repair pathways. In addition, decreased levels of scylo-inositol can be correlated to reduced levels of mI in pituitary tumors.
- Decrease in the levels of phosphoethanolamine (PE) also indicates it’s altered metabolism in tumors.
- Using NMR based metabolomics, we have demonstrated that elevated levels of ACTH hormone is detectable in tumor tissues from patients diagnosed with Cushing’s disease. Selective detection of broad proton signals at 0.90 and 1.40 ppm, and also in the region, 6.80 – 7.40 ppm (tyrosine and phenylalanine residues of ACTH peptide) in tumors of patients with Cushing’s disease clearly indicates the presence of elevated levels of ACTH hormone (Figure 3).
- This ex-vivo detection of elevated levels of ACTH in pituitary tumors from Cushing’s disease using $^1$H-MRS can be translated to in vivo diagnostics using MRI clinical scanner. Such an approach will be an alternative to the invasive BIPSS procedure currently used for the confirmation of Cushing’s disease.
• For the first time, we have shown that elevated levels of ACTH in patients with Cushing’s disease can be detected by $^1$H-MRS of tumor tissue extracts.

• This finding can lead to non-invasive diagnosis of Cushing’s disease using *in vivo* $^1$H-MRS, which may serve as an alternative to the invasive BIPSS procedure.

• We are recruiting more patients with Cushing’s disease in order to further validate our current findings.