CLINICAL STUDY

Serum proteome changes in acromegalic patients following transsphenoidal surgery: novel biomarkers of disease activity

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Abstract

Context: Transsphenoidal adenomectomy is the primary treatment for acromegaly. However, assessment of the therapeutical outcome remains problematic since the existing biomarkers of disease activity frequently show discordant results.

Objective: To discover novel serum biomarkers of disease activity in acromegalic patients before and after surgery.

Design: Serum samples of eight newly diagnosed acromegaly patients before and after transsphenoidal surgery were analyzed for proteomic changes by two-dimensional gel electrophoresis. Protein spots displaying statistically significant changes, pre- versus post-surgery, were identified by mass spectrometry (MS), tandem MS (MS/MS), and western blot analysis.

Results: Six protein spots displaying decreased intensities after surgery were identified as transthyretin (two isoforms), haptoglobin α_2 , β -hemoglobin, and apolipoprotein A-1 (two isoforms). One protein spot, identified as complement C4B precursor, was increased after the surgery.

Conclusions: Seven serum protein spots were differentially expressed following surgery in acromegalic patients. The identified proteins represent potential novel biomarkers to assess the effectiveness of surgical treatment in acromegalic individuals. Future studies will validate the use of the identified proteins as biomarkers of disease activity after medical treatment of acromegaly.

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Introduction

GH is synthesized and released by somatotrophic cells in the anterior lobe of the pituitary gland (1). Abnormal GH secretion leads to impairments in growth and metabolic processes (2–4). Acromegaly is an endocrine disorder, usually the consequence of a GH producing pituitary adenoma (1), characterized by elevated serum levels of GH and insulin-like growth factor 1 (IGF1) (1, 5). If left untreated or poorly controlled, premature mortality ensues caused mainly from cardiovascular diseases (6–8). The primary treatment for acromegaly remains transsphenoidal surgery (9, 10). GH-secreting microadenomas are usually successfully removed by surgery, whereas the outcome is less favorable with larger tumors (11–13). Assessment of the surgical outcome is therefore important, but no ideal biomarker is currently

available (14). Moreover, discrepant results of serum GH and IGF1 levels, i.e. elevated GH and normal IGF1 or vice versa, are often observed (14–16). Thus, the absence of totally reliable biochemical markers of acromegaly makes the evaluation of the treatment outcome difficult.

In this study, experiments were performed to identify serum biomarkers associated with acromegaly before and after transsphenoidal adenomectomy. The serum samples were analyzed using a two-dimensional gel electrophoresis (2DE) proteomic approach and western blotting. Seven proteins displayed significant changes after surgery. These proteins represent biomarkers to evaluate the outcomes of surgical treatment of acromegalic patients. Moreover, results similar to ours reveal potential GH-responsive proteins that could be used to develop assays for the detection of GH in clinical and sporting (doping) scenarios.

Materials and methods

Subjects and serum samples (performed at Aarhus University Hospital, Aarhus, Denmark)

Eight acromegalic patients (three females and five males) were included in this study and were 26–71 years of age (mean age = 51 years). All patients presented with classic symptoms and signs of acromegaly including the presence of a pituitary adenoma visualized by magnetic resonance imaging. All patients were treated with surgery alone, i.e. none of the patients had received dopamine agonists, somatostatin analogs, or the GH receptor antagonist, pegvisomant, at any time point, and no patient had received radiation therapy. Serum samples were obtained before and 3–6 months after transsphenoidal surgery. The patients were hospitalized the day before and blood was drawn the following morning in the fasting state and during an oral load of glucose (75 g). After incubation for 30–60 min at room temperature, the samples were centrifuged at 3500 *g* for 10 min at 4 °C. Serum was removed and stored at –20 °C for an interval of 1–4 years.

All subjects gave a written informed consent before participating in the study, which was approved by The Central Denmark Region Committees on Biomedical Research Ethics (200401184) in adherence to the Declaration of Helsinki. The protocol was also approved by the Ohio University Institutional Review Board.

GH, IGF1, and total haptoglobin measurements (performed at Aarhus University Hospital)

Serum GH was measured by a DELFIA assay (Perkin-Elmer, Turku, Finland) and serum IGF1 levels were determined by an in-house noncompetitive, time-resolved immunofluorometric assay. Both assays have been previously described (17). Total haptoglobin levels were determined by Cobas c-systems (Roche Diagnostics), in short, human haptoglobin was precipitated with a specific antiserum and the turbidity was estimated. The method had a detection range of 0.1–5.7 g/l (or 1.0–57 mmol/l) and had a reproducibility of 0.7–1.3 coefficient of variation %.

Sample preparation for proteomic analysis (performed at Ohio University, Athens, OH, USA)

Serum samples were shipped frozen on dry ice from Aarhus, Denmark to Athens, Ohio. Upon arrival, samples were stored frozen at –80 °C for 3 weeks until further analysis. In general, all proteomic procedures were performed as described previously (18–21). Briefly, serum protein concentrations were determined by the Bradford method. No significant difference in total protein concentration was found

between the samples obtained pre- and post-surgery ($P > 0.05$ in a paired *t*-test). Albumin depletion of the samples was performed using a ProteoPrep Blue Albumin and IgG Depletion kit (Sigma) following the manufacturer's instructions. After depletion, 0.3 mg of each sample was diluted in sample buffer containing 7 M urea, 2 M thiourea, 1% w/v SB 3–10, 3% w/v CHAPS, 0.25% v/v Bio-Lyte 3/10 ampholytes (Bio-Rad Laboratories, Inc.), and 1.5% v/v protease inhibitor cocktail (Sigma). Disulfide bonds were reduced by adding tributylphosphine. Following reduction, sulfhydryl groups were alkylated with iodoacetamide.

Two-dimensional gel electrophoresis (performed at Ohio University)

Samples were subjected to 2DE following procedures previously described (18–21). Serum was transferred to individual wells of an isoelectric focusing (IEF) tray (Bio-Rad) with 17 cm immobilized pH gradient (IPG) strips (pH 3–10 linear, Bio-Rad) and incubated for 2 h at room temperature. IEF was then performed in a PROTEAN IEF cell (Bio-Rad), where strips were rehydrated at 50 V for 12 h after which proteins were separated at 10 000 V for 60 000 V × h. Once IEF was complete, the IPG strips were removed and transferred to disposable trays containing 2 ml of equilibration buffer (6 M urea; 2% SDS; 375 mM Tris-HCl, pH 8.8; 20% glycerol). The samples were equilibrated with subtle shaking for 45 min. Next, 4.5 cm was cut from each end of the 17 cm IPG strips. The resulting 8 cm strips (~pH 5–8) were loaded on a 15% polyacrylamide gel for SDS-PAGE. Proteins were separated in a Mini-PROTEAN 3 cell (Bio-Rad) at 270 V × h. Following electrophoresis, the gels were stained using SYPRO Orange (Invitrogen). Images of stained gels were obtained using a PharoFX Plus Molecular Imager (Bio-Rad) with an excitation wavelength of 488 nm and emission detected at 605 nm. Protein spot intensities and matching were performed using the image analysis software PDQuest Advanced v. 8.0 (Bio-Rad). Spot intensities were normalized to total image density in each gel, which depended on the total protein content of each sample. Protein spots that were differentially expressed between the groups ($P < 0.05$) were excised from the gels and sent to ProteaBiosciences, Inc. (Morgantown, WV, USA) for identification by mass spectrometry (MS) and tandem MS (MS/MS) as described below.

MS analysis (performed at ProteaBiosciences, Inc.)

Protein spots displaying significant ($P < 0.05$) intensity changes pre- and post-treatment were identified by MS and MS/MS using matrix-assisted laser desorption/ionization–time of flight (MALDI–TOF) and MALDI–TOF–TOF. Acrylamide gel plugs containing individual spots were dehydrated and then rehydrated

with acetonitrile and 50 mM ammonium bicarbonate respectively. Proteins were then reduced with 250 mM dithiothreitol for 60 min at 55 °C, followed by alkylation with 650 mM iodoacetamide for 60 min at room temperature in the dark. Digestion was performed with 500 ng trypsin in 50 mM ammonium bicarbonate buffer overnight. Extraction of peptides was performed using 5% formic acid in 50% acetonitrile (dehydration), followed by rehydration with 50 mM ammonium bicarbonate. The procedure was performed three times per sample. The recovered peptides were then lyophilized, reconstituted in 10 mM acetic acid, and re-lyophilized to yield a purified protein digest extract. For MS and MS/MS analyses, the protein digest solution was loaded onto a C₁₈ ProteaTip by aspirating and expelling the sample 5–10 times within the sample vial. The bound sample was washed twice with 0.1% TFA/2% acetonitrile solution by aspirating and expelling 20 µl of the wash solution 5–10 times. The sample was spotted directly onto an MALDI target that was prespotted with 0.6 µl MALDI matrix (CHCA) using 1 µl of an elution solution (0.1% TFA/90% acetonitrile). Mass spectra were acquired on an ABI 4800 MALDI TOF/TOF analyzer. MS spectra were acquired in Reflector Positive Ion mode. Peptide masses were acquired for the range 850–4000 kDa. MS spectra were summed from 400 laser shots. Internal calibration was performed using a minimum of three trypsin autolysis peaks. For MS/MS, spectra were acquired until at least four peaks in the MS/MS spectra achieved a signal-to-noise (S/N) ratio equal to 70.

Database correlation analysis search parameters (performed at ProteaBiosciences, Inc.)

Protein identification from MS and MS/MS data used the following criteria: program for MS/MS data processing: ProteinPilot 3.0; search engine: Mascot (Matrix Science Inc., Boston, MA, USA); sample type: gel samples; digestion enzyme: trypsin; species: human; database: NCBIInr; search engine: type of search: combined MS and MS/MS; mass values: monoisotopic; protein mass: unrestricted; peptide mass tolerance: ± 0.3 –1 kDa; maximum missed cleavages: 1; variable modifications: carbamidomethyl (C); exclusion mass list: 1151.8, 1358.9, 1795.1, 2211.4, 2225.4, 2283.

Manual protein database searching with MS and MS/MS-generated peak lists (performed at Ohio University)

Protein identities were further verified by using the MS and MS/MS data obtained and the online software named Mascot. These procedures have been described previously (18–21). Search parameters included the following: MS: database: NCBIInr; taxonomy: *Homo sapiens*; enzyme: trypsin; missed cleavages allowed: 1; fixed modifications: none; protein mass: not specified;

peptide tolerance: ± 1.2 kDa; mass values: MH⁺; monoisotopic/average: monoisotopic; MS/MS: database: NCBIInr; taxonomy: *H. sapiens*; enzyme: trypsin; missed cleavages allowed: 1; fixed modifications: none; quantitation: none; peptide tolerance: ± 1.2 kDa; MS/MS tolerance: ± 0.6 kDa; peptide charge: 1⁺; monoisotopic/average: monoisotopic; precursor *m/z*: not specified; instrument: MALDI–TOF–TOF. Variable modifications that were included in separate and combined submissions for both MS and MS/MS were acetyl (K), carbamidomethyl (C), deamidated (NQ), oxidation (M), phospho (ST), phospho (Y), sulfo (S), sulfo (T), and sulfo (Y). The general criteria used for assessment of protein identity were a minimum significant match of two MS/MS fragment scores (22).

Western blot analysis (performed at Ohio University)

Western blot analyses were performed to confirm the identity of haptoglobin, apolipoprotein A-1 (apoA-1), and transthyretin. For one-dimensional (D) gel electrophoresis immunoblots, 0.05 mg of each sample was loaded onto a SDS-PAGE gel and transferred to PVDF membrane (Millipore, Billerica, MA, USA). For the 2D western blots, 0.3 mg of each sample was subjected to 2DE and transferred to a PVDF membrane. Membranes were then blocked in 5% nonfat dry milk and probed for 2 h with primary antibody. Antibodies against haptoglobin (mouse MAB anti-haptoglobin of human origin, 1:5000 dilution), apoA-1 (mouse MAB anti-apoA-1 of human origin, 1:500), and transthyretin (rabbit polyclonal antibody anti-prealbumin of human origin 1:500) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein bands and protein isoforms were identified with HRP-conjugated secondary antibody (1:5000 dilution) and Pierce ECL western blotting substrate (Thermo Scientific, Rockford, IL, USA). The resulting blots were scanned using a Pharos FX Plus Imaging System (Bio-Rad) and subjected to image analysis using Quantity One Quantification Program Software (Bio-Rad).

Statistical analysis

All protein spot intensities identified by 2DE were analyzed for normality (the Shapiro–Wilk test) and variance (homogeneity test for two dependent samples) (23). Protein spots displaying normal distributions and equal variances were compared between the two groups using a two-tailed paired *t*-test (corresponding *P* values are reported; see significant spots A, B, and D). The nonparametric Wilcoxon signed-rank test was used to analyze the remaining data (*P* values are reported; see spots C, E, F, and G). GH and IGF1 levels between the groups were also analyzed using a two-tailed paired *t*-test. All tests were performed using SPSS v. 14.0 (IBM Corporation, Somers, NY, USA). The levels of significance were set at *P* < 0.05.

Results

GH and IGF1 values pre- and post-surgery

Both nadir serum GH concentrations during the oral glucose load and total IGF1 levels decreased significantly ($P < 0.05$) after surgery (GH: 5.29 ± 2.14 $\mu\text{g/l}$ (pre) versus 0.62 ± 0.21 $\mu\text{g/l}$ (post); IGF1: 637 ± 124 $\mu\text{g/l}$ (pre) versus 242 ± 41 $\mu\text{g/l}$ (post)). Results are presented as mean and s.e.m. (Fig. 1A and B). The serum GH and IGF1 levels for each patient pre- and post-treatment are shown in Fig. 1C and D. The decline in IGF1 expressed as SDSs was also highly significant: 12.8 ± 3.4 (pre) versus 2.9 ± 1.2 (post). GH levels decreased markedly in seven patients following the surgery. In contrast, patient 2 (Fig. 1C) showed only a modest reduction in serum GH levels. Serum levels of IGF1 were normalized in five patients following the surgical treatment (Fig. 1D). Patient 1 presented a decrease in GH (1.49 $\mu\text{g/l}$ (pre) versus 0.53 $\mu\text{g/l}$ (post)) but no reduction in IGF1 levels (220 $\mu\text{g/l}$ (pre) versus 211 $\mu\text{g/l}$ (post)). Patients 6 and 7 showed a pronounced reduction in GH serum levels following the surgery, whereas the decrease in IGF1 failed to reach the normal range.

Proteome changes post-surgery

The proteomic profiles of all serum samples were evaluated in each subject at pre- and post-treatment time points. Protein profiles on all gels were reproducible and presented similar spot patterns (Fig. 2). A total of 150 protein spots were detected in each gel. Protein spot intensities were analyzed for significant changes

between pre- and post-surgical samples. Seven protein spots were significantly altered ($P < 0.05$); six decreased and one increased post-surgery (Fig. 2).

Spot intensity changes observed in each individual subject pre- and post-surgery

Figure 3 (I) shows the average intensity for protein spots A–G before and after the surgery. Protein spots A–F were significantly decreased ($P < 0.05$) following transphenoidal surgery while protein spot G increased ($P < 0.05$). Spot intensity values pre- and post-surgery showed similar expression patterns (decrease/increase) in all subjects (Fig. 3 (II)), suggesting positive associations between the expression of these specific proteins and the outcome of the surgical procedure.

Protein identities

The protein identities of spots A–G (Fig. 2) were determined using MS and MS/MS (Table 1). Among these proteins, two located at ~ 15 kDa (A and B) were identified as isoforms of transthyretin (Fig. 4 (I)). Both were downregulated (spot A, $P = 0.02$ and spot B, $P = 0.01$) (Fig. 4 (II)) after surgery. Protein spots C–F also significantly decreased (Fig. 4 (II)) in the post-surgery serum samples. Spots C ($P = 0.04$) and D ($P = 0.02$) were identified as haptoglobin α_2 (~ 20 kDa) and β -hemoglobin (~ 12 kDa) respectively (Fig. 4 (I)). Spots E ($P = 0.03$) and F ($P = 0.03$) located at ~ 10 kDa were identified as two isoforms of apoA-1 (Fig. 4 (I and II)). Protein spot G (~ 35 kDa) ($P = 0.04$) was identified as complement C4B precursor and was found to be significantly increased after the treatment (Fig. 4 (I and II)).

Total haptoglobin levels and western blot analyses pre- and post-surgery

Total haptoglobin serum levels were quantified (1.08 ± 0.24 g/l (pre); 1.28 ± 0.19 g/l (post)). No statistically significant differences were found following transphenoidal surgery (Fig. 5A). In agreement with these results, no differences in total haptoglobin levels were found by western blotting (Fig. 5B). In addition, no differences in apoA-1 and transthyretin were found by immunoblotting (Fig. 5B).

2D western blotting to confirm identities of transthyretin, haptoglobin, and apoA-1

Serum samples were subjected to 2D western blotting analysis to confirm the MS identifications of haptoglobin, apoA-1, and transthyretin. As shown in Fig. 6, western results are consistent with the MS results. Transthyretin isoforms were identified in a pI range of ~ 5.5 – 7.2 at two different MWs (~ 15 and ~ 35 kDa) (Fig. 6 I and II (1)).

Figure 1 Mean $\pm$ s.e.m. serum levels of nadir GH during the oral glucose load (A) and IGF1 (B) at pre-treatment (black) (5.29 ± 2.14 ; 0.62 ± 0.21) and post-treatment (white) (637 ± 124 ; 242 ± 41). * $P < 0.05$. The P value is derived from two-tailed paired t -test. For statistics, please see text. GH (C) and IGF1 (D) serum levels in individual subjects (1–8) pre-treatment (black) and post-treatment (white).

Figure 2 2DE gels showing the spot patterns. A total of 150 spots were analyzed. Images belong to patients 1–8 (see labels) and correspond to the pre- and post-surgery treatment. Seven spots displayed significant intensity changes post-treatment (A–G; dashed (---) squares). These protein spots were used for mass spectrometry identification.

Two of these isoforms correspond to protein spots A (MW ~15 kDa, pI ~6.0) and B (MW ~15 kDa, pI ~6.4) identified as transthyretin by MS. Haptoglobin $\alpha 2$ isoforms were identified at MW of ~20 kDa and a pI range of ~5.7–7.5 (Fig. 6 I and II (2)). One of the identified isoforms corresponds to spot C (MW ~20 kDa, pI ~6.2) identified as haptoglobin by MS. Finally, apoA-1 isoforms were located at a MW of ~28 kDa and pI ranging from ~5.2 to 7.8 (Fig. 6 I and II (3)). Protein spots E and F identified as apoA-1 by MS were not detected by western blotting techniques (Fig. 6 I (4)).

Discussion

This study focused on the analysis of the serum proteome of acromegalic patients before and after successful transsphenoidal adenomectomy. Although measurement of GH and IGF1 levels in acromegaly

is the standard assay for assessment of disease activity, they are not perfect (16, 24–30). Additional factors such as age, gender, body composition, and nutritional status may interfere with the results (31, 32). As an example, in this study we found that the serum GH levels were significantly reduced in seven of the eight patients following surgery, whereas only five patients showed decrease in IGF1. These findings in this small number of patients supports the observation from larger studies that discordant values of GH and IGF1 often occur (16, 24–30). It is also noteworthy that, despite the observation that quality of life (QoL) is impaired in active acromegaly and improves with therapy, there is not complete agreement between traditional biomarkers and QoL in acromegaly (33). Therefore, additional biomarkers for acromegalic patients would be beneficial in order to provide rapid and reliable readouts of disease activity after surgery.

Figure 3 (I) Plots showing intensity values (mean $\pm$ S.E.M.) before and after surgery for protein spots (A–G). (II) Spot intensity changes (intensity after treatment minus intensity before treatment) in individual subjects (1–8). Letters above each panel correspond to the spot shown in each graph.

Proteomics employing 2-D gel electrophoresis is a powerful technique that provides an atlas of proteins, that may show changes in protein levels and the presence of different protein isoforms or post-translational modifications (PTM) (34). Moreover, the use of fluorescent stains such as SYPRO Red, Ruby, and Orange (used in our study) improves the sensitivity of the technique, allowing the detection of individual protein spots at the nanogram level (4–8 ng) (21). We therefore have used a 2-D gel proteomic platform for the discovery of potential new serum biomarkers of acromegaly. We found seven proteins whose levels were significantly different ($P < 0.05$) after surgery in all patients. The protein spots were further analyzed by MS to reveal their identity.

Spots A and B were identified as isoforms of transthyretin. Serum transthyretin is a protein involved

in retinol metabolism and in the transport of thyroid hormones in blood (35, 36). Normal serum values for transthyretin are between 15.7 and 29.6 mg/dl (37). Four isoforms of transthyretin (MW $\sim$ 13.8–35.39 kDa, pI $\sim$ 5.02–5.52) have been detected in human plasma by 2DE (SWISS-2 D PAGE; <http://ca.expasy.org/swiss-2dpage/viewer>). Recent reports have identified additional isoforms of this protein in mouse serum at different pIs and MWs to those previously recorded in the databases (38–40). One possible explanation for these results is the presence of PTM that may lead to shifts in pI and/or MW of a given protein (19). Thus, the differences in mass and/or charge observed in our samples may be due to PTMs, e.g. glycosylation or phosphorylations.

To further support our MS results, conventional 1D and 2D western blots were performed. No significant differences in total transthyretin levels were found by 1D immunoblots. A possible explanation is that although 1D western blotting is a sensitive technique (picogram levels of detection) it only provides information on the 'total' level of protein, and not on the differences in expression of particular isoforms of the target protein. On the other hand, 2D immunoblots showed the presence of several transthyretin isoforms at two different MWs of $\sim$ 15 and $\sim$ 35 kDa in a pI range of $\sim$ 5.5–7.2. The 2D pattern of transthyretin isoforms was very similar following western blotting compared to 2DE gels. Two of these transthyretin isoforms corresponded to protein spots A and B (spot A MW $\sim$ 15 kDa, pI $\sim$ 6.0; spot B MW $\sim$ 15, pI $\sim$ 6.4), confirming the identity assigned by MS.

Protein spots C and D were identified as haptoglobin α 2 and β -hemoglobin respectively. Haptoglobin (HP) is an inflammation-inducible plasma protein, which in normal conditions is present in human serum in the range of 60–270 mg/dl (41). The HP molecule contains two different chains: β (heavy, 40 kDa) and α (light α 1, 8.9 kDa; α 2, 16 kDa). In the mature protein, the α and β chains are connected by disulfide bridges (β - α - α - β) (42). Two alleles (denoted 1 and 2) exist for the HP α gene in humans (43, 44). Therefore, three possible phenotypes results from these two alleles: HP 1-1, HP 2-2, and HP 2-1 (43, 44). The β chain is present in all HP phenotypes, and it is always identical. Thus, HP variations are due to the presence of different α chains. Twenty-four isoforms (MW $\sim$ 11.86–44.46 kDa, pI $\sim$ 4.81–6.07) of HP have been identified by 2DE in human plasma. In addition, studies have shown that HP α 2 chain expression is increased in patients with neck and head cancer (45). In our results we show that the expression of one isoform of HP α 2 (MW $\sim$ 20 kDa, pI $\sim$ 6.2) was markedly downregulated after surgery. Interestingly, no significant changes in the total concentration of HP were detected by a conventional ELISA assay; furthermore, the levels pre- and post-surgery were within normal ranges (1.08 ± 0.24 g/l (pre); 1.28 ± 0.19 g/l (post)). Supporting these results,

Table 1 Mass spectrometry of protein spots that changed significantly after treatment.

Spot	Gel (pI/MW)	Identity match	Accession no.	% Of change ^a	MS score ^b	Max sequence coverage (%)	Matched fragments	MS/MS score ^c	Max sequence coverage (%)	Matched fragments
A	6.0/15	Transthyretin	P02766	−20.60	172	95	13/40	767	87	9/52
B	6.4/15	Transthyretin	P02766	−27.04	150	87	10/26	314	67	6/35
C	6.2/20	Haptoglobin $\alpha 2$	P00738	−39.92	103	39	10/47	394	22	6/59
D	7.8/12	β -Hemoglobin	Q14484	−22.67	66	58	8/31	64	6	6/31
E	5.0/10	ApoA-1	P02647	−33.65	158	48	14/38	170	24	5/52
F	5.4/10	ApoA-1	P02647	−28.24	160	51	15/39	292	26	6/53
G	7.5/35	Complement C4B precursor ^d	P0C0L	52.38				77	1	2/27

^aPercentage (%) of change was calculated dividing the post-treatment mean intensities over the pre-treatment mean values.

^bA minimum MS score > 64 was considered significant.

^cA minimum of two significant MS/MS peptide fragments was considered to assign an ID for a spot.

^dSpot G was identified as complement C4B precursor based on the protein score obtained by ProteinPilot.

1D western blots showed no significant differences in total HP levels. On the other hand, several isoforms of HP $\alpha 2$ (MW $\sim$ 20 kDa, pI $\sim$ 5.7–7.5) were identified by 2D western blotting. One of the isoforms corresponded to protein spot C (MW $\sim$ 20 kDa, pI $\sim$ 6.2).

Protein spot D was identified as β -hemoglobin. Hemoglobin is the principal component of the erythrocytes. Hemoglobin is a molecule composed of four globular protein subunits and four prosthetic heme groups. Its main function is to transport O₂ from the lungs to the tissues (41). In normal physiological conditions, hemoglobin levels in serum range from 0.5 to 5.0 mg/dl (41). Previous studies have shown that total hemoglobin concentration has been positively correlated with IGF1 and IGFBP3 levels (46–48). However, these studies relate to heterotetrameric hemoglobin in erythrocytes (49) but not to the expression of free hemoglobin (released from the erythrocytes). Two isoforms of β -hemoglobin (MW $\sim$ 10.5 kDa, pI $\sim$ 6.88–7.05) are reported in the SWISS-2DPAGE database. Serum-free hemoglobin (α and β chains) has also been identified as a potential

biomarker for ovarian and prostate cancer (50, 51). In addition, a recent report by Chung *et al.* (49) reported hemoglobin α -chain as a biomarker of GH in serum. Moreover, serum proteomic studies by Sackmann-Sala *et al.* (19) identified an isoform of β -hemoglobin at a pI $\sim$ 8.0 with MW of $\sim$ 12 that changed due to increased GH/IGF1 action. In this study, we found that expression of one isoform of β -hemoglobin (MW $\sim$ 12 kDa, pI $\sim$ 7.8) was decreased in the serum samples collected after the surgery. We were initially concerned with the result since the presence of hemoglobin might be due to red blood cell contamination of the serum. However, this is unlikely since all samples were handled consistently, and none of the samples revealed signs of hemolysis. Moreover, we observed that only one isoform of free hemoglobin was differentially expressed following the pituitary surgery. No changes in additional isoforms were detected. This may indicate that the differential expression of β -hemoglobin (spot D) is a specific effect associated with the surgical treatment and/or GH action and not the result of hemoglobin contamination from red blood cells.

Figure 4 (I) Representative 2D serum gel. The locations of the seven protein spots displaying significant changes in intensity after transsphenoidal surgery are labeled A–G (--- dashed squares). (II) Representative 3D view of protein spots A–G displaying intensities pre-treatment (left) and post-treatment (right). The images were generated using the 3D Viewer tool of PDQuest software version 8.0, which converts the spot intensity data to topographical peaks and valleys. For each protein spot, left and right images belong to the same subject.

Figure 5 (A) Mean $\pm$ S.E.M. serum levels of serum levels of haptoglobin pre-treatment (white) ($1.08 \text{ g/l} \pm 0.24$) and post-treatment (black) ($1.28 \text{ g/l} \pm 0.19$). (B) Western blots of haptoglobin, apoA-1, and transthyretin in serum samples. Representative images of three different patients (ID: 1, 2, and 3) pre-surgery (lanes 1, 3, and 5) and post-surgery (lanes 2, 4, and 6). Equal amounts of total protein were loaded (0.05 mg) and separated by SDS-PAGE.

Spots E and F correspond to two isoforms of apoA-1, a major component of high-density lipoproteins (HDL) in human plasma and promoter of cholesterol efflux from the tissues to the liver (52). The apoA-1 concentration in normal human plasma ranges between 90 and 130 mg/dl (41). Nine isoforms of apoA-1 (MW ~ 7.49 – 23.45 kDa , pI ~ 4.79 – 7.27) have been reported. We have identified two isoforms of apoA-1 (MW $\sim 10 \text{ kDa}$, pI ~ 5.0 and MW $\sim 10 \text{ kDa}$, pI ~ 5.4), which were significantly decreased post-surgery. Studies have shown that GH plays a role in the modulation of lipid metabolism in humans (53, 54). However, the effects of GH on cholesterol metabolism are still controversial. Some clinical studies on GH-deficient children showed no significant effects on the levels of total apoA-1 and HDL cholesterol after GH replacement treatment (55, 56), while others revealed decreases in apoA-1 serum levels (56, 57). In addition, normalization of GH serum levels in acromegalic patients after surgical and/or pharmacological treatment has been associated with increases in circulating apoA-1 (58). In this study, no significant difference in total apoA-1 levels was found by 1D western blots. As previously discussed, these results do not contradict previous observations, given that the changes in the levels of the two identified isoforms of apoA-1 occurred in response to the pituitary surgery, but not necessarily reflect the correlation between GH activity and apoA-1 levels. Interestingly, several isoforms of apoA-1 were identified by 2D western blots at a MW $\sim 28 \text{ kDa}$ and a pI range of 5.2–7.8; however, apoA-1 isoforms at lower MWs were not detected by this technique. A possible explanation is that post-translational modifications associated with the apoA-1 isoforms identified by MS may prevent the interactions between the antibody and its apoA-1 epitope. This is an important limitation of immuno-based techniques versus 2DE and MS.

Finally, spot G was identified as complement C4B precursor (MW $\sim 35 \text{ kDa}$, pI ~ 7.5). To date, two isoforms (MW ~ 31.73 – 31.94 kDa , pI ~ 6.41 – 6.54) of

this protein have been identified by 2DE. C4B precursor undergoes proteolytic cleavages to produce the mature form of the protein, C4 (reference values in blood: 15–50 mg/dl) (59, 60). In its activated form, C4 is a subunit of the C3 and C5 convertases, the enzymatic complexes that activate C3 and C5 of the complement activation pathway (61, 62). Therefore, production of C4 in excess could lead to overactivation of the complement pathways and the inflammatory response (61). Studies in humans and animal models have demonstrated the effect of GH/IGF1 in the modulation of the immune response (63–65). A recent study showed that GH levels influence the expression of mannan-binding lectin (MBL), a plasma protein involved in the initiation of the complement cascade (66). The same study showed that MBL levels were reduced in acromegaly patients treated with octreotide or pegvisomant (66). Therefore, increased presence of C4B precursor in the serum of acromegalic patients following surgery may indicate reduced formation of activated C4, and perhaps decreased activation of the complement pathway. Studies are needed to evaluate the effects of surgical and/or pharmacological treatment in the immune response of acromegalic patients.

Figure 6 Isoforms observed by 2D western blots; (A) 150 μg of total protein from serum was resolved in the first dimension in a linear pH range of 3–17 and in 15% acrylamide in the second dimension. (I) Representative 2D gel showing the location of protein spots (A–C, E, and F) (solid squares). Dashed (---) squares show the MW and pI range of the different protein isoforms identified by 2D western blots. Protein spots A and B were identified as transthyretin (1) (MW $\sim 15 \text{ kDa}$, pI ~ 6.0 ; MW $\sim 15 \text{ kDa}$, pI ~ 6.4). Protein spot C was identified as haptoglobin $\alpha 2$ (2) (MW $\sim 20 \text{ kDa}$, pI ~ 6.2). Isoforms of apoA-1 (3) were identified at a MW $\sim 28 \text{ kDa}$ and a pI range of ~ 5.2 – 7.8 . Protein spots E and F (4) (MW $\sim 10 \text{ kDa}$, pI ~ 5.0 ; MW $\sim 10 \text{ kDa}$, pI ~ 5.4) identified by MS as apoA-1 (see Table 1) were not detected by western blotting. Molecular weight markers are indicated to the left of the panels and pI markers are shown on the top. (II) Representative 2D western blots revealing (1) transthyretin, (2) haptoglobin $\alpha 2$ and (3) apoA-1. The identified protein isoforms (--- dashed squares) and the localization of protein spots A–C (solid squares) are shown.

In summary, the results presented here reveal potential biomarkers of disease activity after treatment by surgery. Further studies using serum samples from acromegalic patients undergoing different therapies (e.g. somatostatin analogs or GH receptor antagonists) as well as from surgical patients who did not achieve GH/IGF1 normalization would be beneficial. In addition, studies to investigate whether variations in the protein levels of the identified isoforms are directly associated to GH activity or other factors (e.g. cardiovascular and metabolic changes associated to decreased GH) are needed to further support our results.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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