
**Short title:** Proteomics in obese cerebrospinal fluid.

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ABSTRACT

Body weight control is tightly regulated in the hypothalamus. The inaccessibility of human brain tissue can be partially solved by using cerebrospinal fluid (CSF) as a tool for assessing the central nervous system’s production of orexigen and anorexigen factors. The present study investigates by means of proteomic analysis the differentially displayed proteins in human CSF from obese and non-obese subjects. We designed a case-control study conducted in a reference hospital where eight obese (cases) and eight non-obese (controls) women with idiopathic intracranial hypertension were included. Intracranial hypertension was normalized through the placement of a ventriculo- or lumboperitoneal shunt in the 12 months prior to their inclusion in the study. Isotope coded protein label (for proteins > than 10 kDa) and label-free liquid chromatography (for proteins <10 kDa) associated to mass spectrometry analysis were used. Eighteen differentially expressed proteins were identified. Many of them fall into three main groups: inflammation (osteonpontin, fibrinogen gamma and beta chain, alpha 1 acid glycoprotein 2 and haptoglobin) neuroendocrine mediators (neurosecretory protein VGF, neuroendocrine protein 7B2, chromogranin-A and chromogranin B), and brain plasticity (testican-1, isoform 10 of fibronectin, galectin-3 binding protein and metalloproteinase inhibitor type 2). The differential production of osteopontin, neurosecretory protein VGF, chromogranin-
A, and fibrinogen gamma chain was further confirmed by either enzyme-linked immunosorbent assay or Western blot. In conclusion, we have identified potential candidates that could be involved in the pathogenesis of obesity. Further studies addressed to investigating the precise role of these proteins in the pathogenesis of obesity and their potential therapeutic implications are needed.

INTRODUCTION

With its rapid increase in prevalence during the last two decades, obesity has become one of the main threats to public health in the Western world (1). In healthy humans, body weight control is tightly regulated in the hypothalamus, a specialized region of the brain, where various neuropeptide systems receive the cues from peripheral tissues and produce an appropriate physiological response (2, 3).

The inaccessibility of the human brain tissue can be partially solved by using cerebrospinal fluid (CSF) as a tool for assessing the central nervous system (CNS) production of various orexigen and anorexigen factors (4, 5). Nonetheless, the collection of CSF is invasive and rather tricky in obese subjects. In addition, the volume of CSF obtained after lumbar puncture is limited to only few mL and, therefore, only a few peptides can be analysed simultaneously.

The recent development of proteome analysis has made it feasible to assess changes in the protein profiles with only a small sample in various cells, tissues and body fluids. In this way, proteome analysis of CSF from neurological diseases like Alzheimer, Parkinson, epilepsy and psychosis has been performed (6-8). Therefore, the study of changes in protein profile pattern of CSF from obese subjects would be useful in identifying new candidates involved in the pathogenesis of obesity or at least will permit us to gain new insights in the brain-derived molecules associated with its development. However, to the best of our knowledge, proteome analysis in human CSF has never been previously performed in the setting of obesity.
To investigate the differentially displayed proteins in human CSF from obese patients we have conducted a case-control study using CSF obtained from obese (cases) and non-obese (controls) women with idiopathic intracranial hypertension (IIH). To avoid the potential confounding effect of intracranial hypertension, CSF samples were always collected at least 12 months after the implantation of a CSF shunt and after assuring that intracranial pressure (ICP) was normal by lumbar puncture. IIH, also known as pseudotumour cerebri, is a condition that predominantly affects obese, premenopausal women and it is characterized by raised ICP without any identifiable pathology in the brain and with normal CSF chemical composition (9). To carry out the CSF comparative proteomic analysis isotope coded protein label (ICPL) associated to mass spectrometry (MS) and database searching was used for proteins larger than 10 kDa, and a label-free liquid chromatography-MS approach was used for lower molecular weight components. This technique provides an accurate high-throughput quantitative proteome profiling of the two groups of samples, thus permitting us to identify new candidates potentially involved in the pathogenesis of obesity (10).

MATERIALS AND METHODS

Design of the study and description of study population

Eight CSF samples from obese (cases) and eight from non-obese (controls) women with IIH attending the outpatient Neurosurgery Department were prospective recruited for the study over a 6-month period (table 1). All patients required previous placement of a ventriculo- or lumboperitoneal shunt to normalize ICP. Normal ICP (CSF pressure below 20 cm H₂O) was confirmed in all patients by CSF pressure measurement before samples collection and, therefore, there was no difference in ICP between groups. In addition both groups were well matched by age.
Obesity was defined in accordance with the World Health Organization’s definition as BMI of at least 30 kg/m². In addition, the percentage of fat mass was estimated by using a tetrapolar body electrical bioimpedance between 8:30 AM and 9:00 AM (Biodynamics, model 30, version 7.1).

IIH was diagnosed according to the following criteria: i) signs and symptoms of increased ICP, ii) no localizing signs except abducens nerve palsy, iii) intracranial hypertension diagnosed by continuous ICP monitoring using an epidural sensor (11), iv) CSF with normal chemical composition, and v) normal neuroimaging (9). Time elapsed between the placement of CSF shunt and the CSF sample collection was more than 12 months in all subjects. The presence of a CSF shunt ruled out a confounding factor like is the role of intracranial hypertension in brain protein production.

The exclusion criteria included persistence of increased CSF pressure, changes in weight higher than 5 kg or treatment with antiobesity therapies during the previous 12 months, CSF protein level higher than 0.45 g/L, CSF erythrocyte contamination or pleocytosis, neuropsychiatric diseases including eating disorders, and other chronic diseases apart from obesity.

Informed written consent was obtained from all participants prior to collection of CSF and the study was approved by the hospital’s human ethics committee (Hospital Universitari Vall d’Hebrón). The study was conducted in accordance with the principles of the Declaration of Helsinki.

Collection of CSF and blood samples

Patients were admitted to the hospital and stay overnight before CSF samples were collected in a standardized fashion by the same team of experienced clinicians. Lumbar CSF samples (3 mL) were taken between 8:00 and 10:00 am, after at least 8 hours of fasting and bed rest. CSF was collected in plastic tubes containing Trasylol (1000 klU/ml) to prevent
proteolysis. The samples left after clinical analyses were subjected to 10,000 x g for 10 min to remove particulate matter, immediately frozen at –20°C, and stored at –80°C till further use.

Venous blood was collected from the antecubital vein just after the collection of CSF samples. Samples were separated by centrifugation (2,000 x g at 4°C for 20 min) and aliquots were frozen at -80°C for batched analysis.

**Sample preparation for proteomic screening**

Two different pools of four samples from obese and non-obese patients were prepared (obese1, obese2, non-obese1, non-obese2). The pooled samples were concentrated by ultrafiltration using 10 kDa-cutoff Amicon Ultra devices (Millipore, Billerica, MA, USA), to a final volume of ca. 60 µL. The concentrated samples (fraction retained >10 kDa) were further purified by a modified acetone-TCA precipitation (CleanUp Kit, GE Healthcare, Fairfield, CT, USA), redissolved in 6M guanidinium chloride and kept at -80°C until further analysis. The filtered fractions (<10 kDa) were concentrated using Sep-Pak Light C8 devices (Waters, Milford, MA, USA). The samples were first acidified to pH = 4 by the addition of trifluoroacetic acid (TFA). The columns were equilibrated in aqueous 0.1% TFA (2 x 1 mL) prior to the loading of the sample and then washed with 0.1% TFA (2 x 1 mL). The proteins were then eluted in 0.7 mL of aqueous 0.1% TFA: acetonitrile 1:1, the solvent was evaporated to dryness on a vacuum centrifuge, and finally were dissolved in 20 µL of 4M Urea, 50 mM ammonium bicarbonate.

**ICPL labelling**

The protein samples corresponding to the >10 kDa fractions (about 100 µg each) were subjected to ICPL labelling as previously described (12). $^{12}$C-Nicotinamide-reagent was used to label obese1 pool sample and $^{13}$C-Nicotinamide-reagent to label the non-obese1 pool sample. On an independent experiment, reverse-labelling was performed on obese2 pool protein and non-obese2 pool protein extract. Each pair of differentially labelled obese and non-obese pool
samples was combined and separated by 1D-electrophoresis in a 12.5% polyacrylamide 1D-gel. Each gel lane was cut into twenty horizontal slices and each slice was subjected to tryptic digestion with modified porcine trypsin (Promega, Madison, USA).

**ICPL liquid chromatography - mass spectrometry analysis**

ICPL-labeled tryptic digests were analyzed on an Esquire HCT ion trap mass spectrometer (Bruker, Bremen, Germany), coupled to a nanoHPLC system (Ultimate, LC-Packings, Netherlands), as described (12). For protein identification through database search of MS/MS spectra the International Protein Index (IPI)-Human 3.26 database (67665 sequences, 28462007 residues) was used (13). Data processing, search parameters, and quantification were performed as described (12). For protein quantification, (H/L) ratios were calculated averaging the measured (H/L) ratios for the observed peptides, after discarding outliers. For selected proteins of interest, quantification data obtained from the automated analysis was manually reviewed.

**Label-free liquid chromatography - mass spectrometry analysis**

The samples corresponding to the <10 kDa fractions were first subjected to reduction and alkylation under the same conditions as above, and then digested with trypsin, after dilution of the urea concentration to 1 M. The protein digests were analyzed in triplicate by liquid chromatography-MS as described (12) (figure 1). Relative protein quantification was calculated on the basis of the number of MS/MS spectra matching to peptides corresponding to each of the proteins detected (spectral count). The ratio values obese/non-obese for each protein were calculated after averaging the spectral counts observed in the six runs corresponding to the triplicate analysis of the two different pools analyzed for each condition.
Western blot analysis

Neurosecretory protein VGF (non-acronymic) was determined on all 16 original samples by Western Blot in 15 μg of CSF total proteins resolved by 12.5 % SDS-PAGE. The protein concentration was determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Gels were transferred to a nitrocellulose membrane (GE Healthcare, Piscataway, NJ). The membranes were incubated with rabbit primary antibody against neurosecretory protein VGF (non-acronymic) (diluted 1:750; Abcam, Cambridge, UK) and further incubated with goat anti rabbit horseradish peroxidase-conjugated secondary antibody (diluted 1:5000; Dako, Glostrup, Denmark). Finally, proteins were visualized using the enhanced chemiluminescence detection system (Immobilon, Millipore, MA). Densitometric analysis was performed with a GS-800 calibrated densitometer (Bio-Rad Laboratories, Hercules, CA) and analyzed with the Quantity One 4.6.2 software (Bio-Rad Laboratories, Hercules, CA).

Enzyme-linked immunosorbent assays

Quantitative determination of osteopontin was performed on all 16 original samples in duplicated by a commercial enzyme-linked immunosorbent assay (ELISA) (R&D System, MN, USA) and following the manufacturer’s instructions. The assay lowest limit of detection was 0.011 mg/mL. Samples (CSF and serum) required dilution (1:500 and 1:10, respectively). Neurosecretory protein VGF quantification was performed similarly by a commercial ELISA (USCN, Wuhan, China). The assay lowest limit of detection was 30 pg/mL. The fibrinogen gamma chain was quantified by ELISA (Cusabio Biotech Ltd., Wuhan, China) with a lowest detectable dose of 25 ng/mL. The dilution factor was 1:30 and 1:4000 for CSF and serum, respectively. Chromogranin A was also determined by ELISA (Dako, Glostrup, DK). The detection limit of the assay was 2.0 U/L. CSF samples were diluted 1:10.
**Statistical analysis**

Normal distribution of the variables was evaluated using the Kolmogorov-Smirnov test. Data were expressed as the mean ± SD or median (range) for variables that were not normally distributed. For comparisons between groups Student-t or Mann–Witney tests were used. The relationship between the continuous variables was examined by the Pearson linear correlation test. A p value of less than 0.05 was considered statistically significant.

For ICPL experiment data (table 2), the ratios of protein abundances between obese and non-obese CSF were calculated averaging the ratios for the observed peptides. As a measurement of the technical reproducibility of the measured ratios, the number of peptide values averaged for the quantification of each protein, the resulting average ratio and its standard deviation are given. Only proteins with at least two different peptide ratio values were considered. Percent coefficient of variation of the measured ratios for the proteins in table 2 ranges from 5.8 to 22%. Only proteins presenting a consistent change in the two independent ICPL experiments were considered as potentially differential. Abundance ratio values lower than 1 have been converted to -1/ratio, to indicate fold decrease.

For label-free proteomic analysis data (table 3), significance of the protein ratios determined was estimated using an unpaired Student’s t-test to compare the two groups of values resulting from triplicate analysis of two different pooled samples per condition (n=6 biological + technical replicates).

**RESULTS**

**Proteomic screening**

A total of 348 proteins were identified in the >10 kDa fraction of the CSF samples, and 28 proteins in the <10 kDa fraction, following liquid-chromatography-MS analysis. Selecting an abundance ratio of obese:non-obese of 1.4-fold as the threshold for study, we were able to identify 18 differentially produced proteins (table 2 and table 3). Thirteen proteins were
increased in the CSF of obese patients in comparison with non-obese subjects (in order of increased abundance): osteopontin, metallothionein-1E, neurosecretory protein VGF, neuroendocrine protein 7B2, chromogranin-A, secretogranin-1 precursor (chromogranin-B), fibrinogen gamma chain, testican-1, fibrinogen beta chain, isoform 10 of fibronectin, metallothionein-3, alpha-1-acid glycoprotein 2, and haptoglobin. We also found five proteins that were underproduced in obese subjects (in order of decreased abundance): ProSAAS, autotaxin (ectonucleotide pyrophosphatase/phosphodiesterase 2), prostaglandin-H2 D-isomerase, metallopeptidase inhibitor type 2 (TIMP-2), and galectin-3 binding protein (figure 1).

It should be noted that proteomic screening provides only a potential differential abundance of proteins and further validation in a larger group of individual samples is required. In addition, many of the proteins found in the analysis of the <10 kDa fraction were in fact fragments of higher molecular weight proteins, thus resulting from proteolytic processing or degradation. This was the case for most of the differential proteins reported in table 3 (with the exception of metallothioneins 1-E and 3). The identification by mass spectrometry of many peptides that presented non-tryptic derived ends provides further evidences of the endogenous proteolytic cleavage of those proteins (supplemental table S1). It can not be determined from the present data whether the higher abundance of these fragments results from the higher abundance of the protein or from the greater degree of its proteolysis.

**Validation by ELISA or Western blot analysis**

The different abundance of osteopontin, VGF, fibrinogen gamma-chain and chromogranin-A were validated by ELISA in all individual samples (8 from obese and 8 from non-obese subjects). VGF was also validated by Western blot.

Osteopontin levels in CSF were 10-fold higher than in serum (453.7 ± 400.5 vs. 44.4 ± 15.5 ng/mL, p = 0.001). Although no differences in serum levels were observed between obese and non-obese groups (43.8 ± 10.2 vs. 45.1 ± 20.2 ng/mL, p = 0.867), osteopontin CSF
concentrations were significantly higher in obese women (670.9 ± 479.9 vs. 236.5 ± 74.77 ng/mL, p = 0.038) (figure 2). In addition, a positive correlation between osteopontin CSF levels and BMI was observed (r = 0.646, p = 0.007).

No differences in serum levels of neurosecretory protein VGF was observed between obese and non-obese women (6.8 ± 2.5 vs. 7.6 ± 0.8 pg/mL, p = 0.401). In CSF, neurosecretory protein VGF was detectable by ELISA only in 7 patients. However, analysis of CSF neurosecretory protein VGF by Western Blot showed higher concentrations in obese in comparison with non-obese patients (0.5 ± 0.3 vs. 0.2 ± 0.1 arbitrary units, p = 0.044) (figure 2). In addition, a positive significantly correlation with BMI was also observed (r = 0.618, p = 0.001).

Serum levels of chromogranin-A were significantly higher in obese patients in comparison with non-obese women (15.7 U/L [9.5-94.8] vs. 8.6 U/L [6.5-21.7]; p=0.014). In CSF, chromogranin-A concentration was higher in obese than in non-obese subjects (1109 U/L [213-3348] vs. 187 U/L [4-779]; p= 0.046) (figure 2). Chromogranin-A levels in CSF were 70-fold higher than in serum in obese women. Chromogranin-A levels in CFS were not significantly correlated with BMI (r= 0.41; p= 0.11), but strongly correlated with the CSF concentration of osteopontin and fibrinogen gamma chain (r= 0.75, p= 0.001, and r= 0.79, p<0.001, respectively).

Regarding the fibrinogen gamma chain, no differences in serum concentration between obese and non-obese women were observed (10.6 ± 5.9 vs. 7.1 ± 6.0 μg/ml; p= 0.67). In CSF, fibrinogen gamma chain levels were significantly higher in obese women (10.7 μg/ml (6.4-21.8) vs. 2.9 μg/ml (2.6-11.2); p= 0.036) (figure 2). No correlation between CSF levels of fibrinogen gamma chain and BMI was detected (r= 0.36; p= 0.16).
DISCUSSION

To study the pathophysiology of obesity, CSF is of particular interest since its protein content is expected to reflect changes taking place in the brain. In the present study we applied quantitative liquid chromatography-MS proteomic analysis to identify candidate biomarkers and further to uncover hitherto unknown biological processes related to obesity. Although there have been previous studies characterizing CSF proteins in obesity, to the best of our knowledge, this is the first comprehensive proteomic study profiling differential protein expression in the CSF of obese patients. Using this new approach, 18 proteins were identified as being differentially produced in the CSF of obese in comparison with non-obese patients. However, only 4 out of 18 proteins have been further validated in all samples: osteopontin, VGF, fibrinogen gamma-chain and chromogranin-A.

Although the roles of these proteins remain obscure as pathogenic elements in the development of obesity, many of them lay under three main groups based on their biochemical characteristics and functions: inflammation, neuroendocrine mediators, and brain plasticity. In addition, also differences in antioxidant proteins and somnogenic factors in obese subjects have been detected.

**Inflammatory markers**

Osteopontin, which was first identified in 1986 in osteoblasts, is highly secreted by macrophages at inflammation sites where it mediates monocyte adhesion, migration, and differentiation as well as phagocytosis (14, 15). Elevated plasma levels of osteopontin have been associated with human and mice obesity, and weight loss after low-caloric diets was associated with a reduction of osteopontin plasma levels in obese patients (16, 17). However, osteopontin CSF levels have not been previously evaluated in the setting of obesity. In addition, four acute-phase reactants (fibrinogen gamma and beta chain, alpha-1-acid glycoprotein 2, and
haptoglobin) have been found simultaneously increased in obese patients relative to non-obese subjects.

Given that obesity is associated with a systemic low-grade of inflammation it is possible that the increase of these inflammatory markers in CSF reflects its blood levels and/or increased permeability across the blood-brain barrier (18). However, in the present study, osteopontin concentration in CSF was 10-fold higher than in serum, suggesting the contribution from CNS in the CSF osteopontin concentrations. In fact, inflammatory mediators could play a role in the development of obesity. In this regard, it should be noted that inflammatory activation within the hypothalamus of rodents produces molecular and functional resistance to the anorexigen hormones insulin and leptin, and is a key factor to promote elevated body weight (19, 20).

**Neuroendocrine mediators**

CSF levels of four neuroendocrine proteins [neurosecretory protein VGF, neuroendocrine protein 7B2, chromogranin-A, and secretogranin-1 precursor (chromogranin-B)] have been found simultaneously increased in obese patients relative to non-obese subjects. Although an imbalanced availability of chromogranins has been implicated in neurological diseases (21, 22), no study linking either chromogranins or neuroendocrine protein 7B2 to obesity has been reported yet.

Neurosecretory protein VGF is synthesized by neurons in the central and peripheral nervous systems, as well as in the adult pituitary, adrenal medulla, endocrine cells of the stomach and pancreatic beta cells (23). Thus, the distribution of VGF is similar to a number of peptides that have been implicated in the control of feeding behavior, facilitation of nutrient uptake and regulation of gastric contractility, including ghrelin and cholecystokinin. In fact, VGF plays a crucial role in the regulation of energy balance and the control of feeding behaviour in mice (24). Recent analysis of hypothalamic gene expression in VGF knockout mice, which are lean, hypermetabolic and hyperactive, found elevated mRNA levels of two powerful orexigenic neuropeptides (neuropeptide Y and Agouti-related protein) and decreased
levels mRNA of pro-opiomelanocortin which encodes the satiety-inducing peptide alpha-
melanocyte stimulating hormone (25). However, nothing was known about VGF levels in CSF from humans. In the present study we provide first evidence of higher levels of VGF in CSF from obese subjects, thus suggesting that in humans VGF may also participate in the development of obesity.

A fifth neuroendocrine protein mediator, proSAAS, was found decreased in obese patients. An ambiguous role of this peptide in regulating body weight has been proposed. Transgenic mice overexpressing proSAAS showed a normal body weight until approximately 10-12 weeks, and then increased 30-50% over wild-type littermates (26). By contrast, proSAAS derived peptides were found to be elevated by 48 hours food deprivation in mice hypothalamus (27). The changes in proSAAS detected in the present study suggest a potential role of this peptide in body weight control.

**Brain plasticity markers**

Quantitative differences in proteins related with synaptic plasticity and components of the extracellular matrix (ECM) have been found in the present study. Recent evidence points to synaptic plasticity as a major way through which peripheral metabolic hormones influence hypothalamic regulation of feeding behaviour and body weight (28). However, the central molecules regulating these synaptic changes are not known, and no relation with obesity has been previously described for testican-1 precursor, isoform 10 of fibronectin, galectin-3 binding protein, nor metalloproteinase inhibitor type 2. Only autotaxin, which is involved in motility-related processes such as angiogenesis and neurite outgrowth, has been found up-regulated in serum from massively obese subjects (29).
Antioxidant and somnogenic factors

The metallothionein (MT) are a family of proteins with low molecular mass, high affinity to certain metals, and a potent antioxidant action against various oxidative damages that exist in multiple organs with several molecular forms, including isoforms of MT1, MT2, MT3, and MT4 (30, 31, 32). Although their role in obesity development remains to be elucidated, experimental studies have shown that disruption of MT1 and MT2 genes induce moderate obese mice (33). In addition, the level of MT2A mRNA in human subcutaneous adipose tissue from obese subjects was higher than in lean subjects (34). Taking together, our data suggest that the increased production of both MT1 and MT3 in the CNS, and its secretion into the CSF, might be contemplated as compensatory mechanism, thus protecting against the deleterious effects of inflammatory mediators and oxidative stress associated with obesity. In a similar way, a rapid and dramatically increase of the MT3 gene expression has been recently shown in the human adipocytes induced by hypoxia (35).

Prostaglandin D2 synthase (PGDS) is produced mainly in the leptomeninges and choroids plexus, being the second most abundant protein in CSF after albumin (36). Interestingly, PGDS is a brain enzyme which produces prostaglandin D2 (PGD2), a substance suggested to be a sleep hormone by its somnogenic effect (37). In this way, in patients with obstructive sleep apnea syndrome, those with excessive daytime sleepiness (EDS) had increased levels of circulating PGDS in comparison with those without EDS (38).

There are three main limitations to the present study. The first factor that could lead to misinterpretation of the results is the selection of women with IIH, a disorder with a poorly understood pathogenesis that leads to high ICP, which might influence the brain-derived proteins. However, we tried to solve this limitation including only patients with normalized ICP through the placement of a CSF shunt. In addition, all patients included in the study presented a very similar degree of IIH severity and, in fact, only BMI was the differential feature between groups (cases and controls). It could be argued that subjects without neurological pathology
would be better candidates for the purpose of the study but lumbar puncture is too much aggressive to be accepted for both patients and the Ethical’s Committee. The second limitation involves the lack of a more lean control group that may consequently impede to find a wider range of proteins differentially expressed in the CSF of obese patients. Nevertheless, although additional differentially expressed proteins could probably be identified by the analysis of more differentiated set of samples, those reported here would probably also be found. The third limiting factor is the small sample size. This has been mainly due to the rigorous exclusion criteria and because cases and controls were close-matched. Nevertheless, this selection process enabled us to minimize the dispersion of measurements in each group. Another controversial aspect of studies on concentrations of neuropeptides measured at the lumbar level is the presence of gradients between ventricular and lumbar CSF in several neurotransmitters and neuropeptides (39). However, measurements of the lumbar CSF concentrations of different substances have been an important research tool for several years, and the accuracy of this approach has been repeatedly corroborated both in experimental and clinical studies by parallel determinations of brain tissue and CSF peptide concentrations (40). In the present study, CSF was obtained at the lumbar level in all patients and also at the same time of day to eliminate the effects of different locations and possible circadian rhythms on the neuropeptides studied.

In summary, we have shown that proteomic analysis could facilitate the identification of biomarkers differentially expressed in the CSF of obese patients. Although their involvement in the etiology of obesity is still speculative, it seems that changes in proteins involved in inflammation, neuroendocrine mediators, and brain plasticity are associated with obesity and it is possible that play a role in its development. Further studies addressed to confirm these results can help to elucidate the mechanisms of weight gain and may be useful for the design of new therapeutic strategies.
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REFERENCES


28.- Horvath TL. Synaptic plasticity in energy balance regulation. Obesity (Silver Spring) 2006; 14: 228S-233S.


FIGURE LEGENDS

Figure 1. Schematics of the proteomic analysis of the CSF samples and example of ICPL analysis results.

A) Two different samples of each group, consisting each of them of pooled CSF samples from four patients, were first separated by ultrafiltration through a 10kDa membrane. The retained protein fractions were differentially labeled with either of the two isotopic variants of ICPL reagents, mixed 1:1, and fractionated on a SDS-PAGE electrophoresis. The gel lane was cut in 20 slices that were then subjected to trypsin digestion and analyzed by liquid chromatography-MS/MS. The low molecular weight protein fractions were concentrated using reverse phase cartridges, digested with trypsin, and analyzed by liquid chromatography-MS/MS. Quantitative comparison was performed on the basis of spectral counts. B) Integrated MS spectra along the chromatographic peak corresponding to a peptide of fibrinogen gamma, showing the different abundances of the ICPL light and heavy forms, corresponding to samples from non-obese and obese individuals, respectively. C) Signals as in A, for a peptide from prostaglandin-H2 D-isomerase. In this case, ICPL light and heavy forms correspond to samples from obese and non-obese individuals, respectively.
Figure 2. Serum and CSF concentrations of osteopontin (A), fibrinogen gamma chain (B), and chromogranin A (C), and CSF levels of neurosecretory protein VGF (D) in the 16 patients included in the study (8 non-obese and 8 obese).

Black bars for serum; white bars for CSF. CSF: cerebrospinal fluid. NO: non-obese. O: obese. The Western Blot image of VGF in CSF (upper panel of D) corresponds to four representative samples.
Table 1. The main clinical and biochemical features, as well as CSF parameters, of the study population.

<table>
<thead>
<tr>
<th></th>
<th>Obese group (n = 8)</th>
<th>Non-obese group (n = 8)</th>
<th>p</th>
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<tr>
<td>Age (years)</td>
<td>48.1 ± 10.1</td>
<td>42.5 ± 15.9</td>
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<tr>
<td>BMI (Kg/m²)</td>
<td>36.7 ± 1.7</td>
<td>28.0 ± 1.9</td>
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<td>Fat mass (%)</td>
<td>41.3 ± 2.6</td>
<td>36.1 ± 5.1</td>
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<tr>
<td>Glucose (mmol/L)</td>
<td>5.2 ± 0.2</td>
<td>5.1 ± 0.5</td>
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<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.5 ± 0.6</td>
<td>1.5 ± 0.8</td>
<td>0.932</td>
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<tr>
<td>CSF protein (g/L)</td>
<td>0.29 ± 0.04</td>
<td>0.29 ± 0.10</td>
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<td>CSF glucose (g/L)</td>
<td>0.6 ± 0.1</td>
<td>0.5 ± 0.0</td>
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<td>CSF leucocytes (cel/μL)</td>
<td>6.5 ± 7.2</td>
<td>3.8 ± 7.3</td>
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<td>CSF erythrocytes (cel/μL)</td>
<td>4.0 ± 3.5</td>
<td>7.4 ± 14.3</td>
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</table>

Data are mean ± SD. BMI: body mass index; CSF: cerebrospinal fluid.
Table 2. Proteins with a molecular weight >10 kDa presenting significant differences in abundance in obese vs. non-obese CSF.

<table>
<thead>
<tr>
<th>Function</th>
<th>Protein name</th>
<th>Average ratio(^2)</th>
<th>N(^1)</th>
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<tr>
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<tr>
<td><strong>Inflammation</strong></td>
<td>Fibrinogen gamma chain</td>
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<tr>
<td></td>
<td>Fibrinogen beta chain</td>
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<td></td>
<td>Alpha-1-acid glycoprotein 2</td>
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<td>Haptoglobin</td>
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<tr>
<td></td>
<td>Galectin-3 binding protein</td>
<td>-1.45</td>
<td>5</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>Metallopeptidase inhibitor type 2</td>
<td>-1.52</td>
<td>2</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>Autotaxin (Ectonucleotide pyrophosphatase/phosphodiesterase 2)</td>
<td>-1.80</td>
<td>5</td>
<td>0.21</td>
</tr>
<tr>
<td><strong>Somnogenic</strong></td>
<td>Prostaglandin-H2 D-Isomerase</td>
<td>-1.72</td>
<td>10</td>
<td>0.13</td>
</tr>
<tr>
<td><strong>Neuroendocrine</strong></td>
<td>Secretogranin-1 precursor (chromogranin-B)</td>
<td>1.69</td>
<td>6</td>
<td>0.25</td>
</tr>
</tbody>
</table>

\(^1\) Number of ICPL-labeled peptide pairs corresponding to the protein for which an abundance ratio measure has been obtained.  
\(^2\) Protein abundance ratio calculated averaging the individual peptide ratios corresponding to the protein, and standard deviation of the peptide ratios.
Table 3. Low molecular weight proteins (<10 kDa) presenting significant differences in abundance in obese vs non-obese CSF.

<table>
<thead>
<tr>
<th>Function</th>
<th>Protein name</th>
<th>Average ratio</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammation</td>
<td>Osteopontin</td>
<td>&gt;10</td>
<td>0.0043</td>
</tr>
<tr>
<td>Brain plasticity</td>
<td>Testican-1 precursor</td>
<td>3.14</td>
<td>0.0001</td>
</tr>
<tr>
<td>Antioxidant</td>
<td>Metallothionein-1E</td>
<td>&gt;10</td>
<td>0.0219</td>
</tr>
<tr>
<td></td>
<td>Metallothionein-3</td>
<td>2.00</td>
<td>0.0029</td>
</tr>
<tr>
<td>Neuroendocrine</td>
<td>Neurosecretory protein VGF (non-acronymic)</td>
<td>5.50</td>
<td>0.0103</td>
</tr>
<tr>
<td></td>
<td>Neuroendocrin protein 7B2</td>
<td>4.60</td>
<td>0.0020</td>
</tr>
<tr>
<td></td>
<td>Chromogranin-A</td>
<td>4.33</td>
<td>0.0475</td>
</tr>
<tr>
<td></td>
<td>ProSAAS (proprotein convertase 1 inhibitor)</td>
<td>&lt; -10</td>
<td>0.0367</td>
</tr>
</tbody>
</table>

1 Average abundance ratio calculated on the basis of spectral count on three replicate LC-MS analysis of two different samples of each group. 2 p value of an unpaired Student’s t-test comparing the two groups of measurements.
A) Two different samples of each group, consisting of four of pooled CSF samples from four patients, were first separated by ultrafiltration through a 10kDa membrane. The retained protein fractions were differentially labeled with either of the two isotope variants of ICPL reagents, mixed 1:1, and then analyzed by LC-MS/MS electrophoresis. The gel lane was cut into 20 slices, that were then subjected to trypsin digestion and analyzed by LC-MS/MS. The low molecular weight protein fractions were concentrated using reverse phase cartridges, digested with trypsin, and analyzed by LC-MS/MS. Quantitative comparison was performed on the basis of spectral counts. B) Integrated MS spectra along the chromatographic peak corresponding to a peptide of fibrinogen gamma, showing the different abundances of the ICPL light and heavy forms, corresponding to samples from non-obese and obese individuals, respectively. C) Signals as in A, for a peptide from prostaglandin H2 D-isomerase. In this case, ICPL light and heavy forms correspond to samples from obese and non-obese individuals, respectively.
Black bars for serum; white bars for CSF. CSF: cerebrospinal fluid. NO: non-obese. O: obese. The Western Blot image of VGF in CSF (upper panel of D) corresponds to four representative samples.