Clinical Proteomics Journal

ISSN 1542-6416/04/01:333–364/$25.00

Original Article

In-Depth Analysis of the Human CSF Proteome Using Protein Prefractionation

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Abstract

The identification of disease markers in human body fluids requires an extensive and thorough analysis of its protein constituents. In the present study, we have extended our analysis of the human cerebrospinal fluid (CSF) proteome using protein prefractionation followed by shotgun mass spectrometry. After the removal of abundant protein components from the mixture with the help of immunodepletion affinity chromatography, we used either anion exchange chromatography or sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to further subfractionate the proteins present in CSFs. Each protein subfraction was enzyme digested and analyzed by tandem mass spectrometry and the resulting data evaluated using the Spectrum Mill software. Different subfractionation methods resulted in the identification of a grant total of 259 proteins in CSF from a patient with normal pressure hydrocephalus. The greatest number of proteins, 240 in total, were identified after prefractionating the CSF proteins by immunodepletion and SDS-PAGE. Immuno-depletion combined with anion exchange fractionation resulted in 112 proteins and 74 proteins were found when only immunodepletion of the CSF sample was carried out. All methods used showed a significant increase in the number of identified proteins as compared with nondepleted and unfractionated CSF sample analysis, which yielded only 38 protein identifications. The present work establishes a platform for future studies aimed at a detailed comparative proteome analysis of CSFs from different groups of patients suffering from various psychiatric and neurological disorders.

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Key Words: Brain disease; cerebrospinal fluid; proteome; protein prefractionation.

Introduction

A study by the World Health Organization has found that diseases of the central nervous system are affecting a significant portion of the population and are among the leading causes of death in Western societies (*1*). Although great advances have been made in our understanding of the pathophysiology of psychiatric and neurological diseases, significant gaps remain in our knowledge about their ultimate causes.

Because it constantly perfuses brain tissue, cerebrospinal fluid (CSF) represents a lavage of sorts and hence contains mediators that reflect metabolic processes in the brain. In this regard, it is likely that CSF contains proteins that are secreted or shed from brain cells and can therefore be used for diagnostic purposes to analyze and quantify proteins and peptides derived from the brain. Furthermore, CSF can be obtained in a controlled fashion, minimizing the dangers of variability introduced at the sample collection step. For these reasons, CSF has been used for the analysis of specific proteins in screening several psychiatric and neurological disorders by various immunoassays *(2)*. It is hypothesized that the changes in CSF proteins reflect the pathological alterations in the function of the central nervous system. Therefore, the comparative analysis of the proteomes of human CSF from diseased and healthy subjects is becoming increasingly important for the identification of disease-specific proteins. It is hoped that these studies will ultimately result in the discovery of novel markers of disease and suggest mechanisms and treatments for psychiatric and neurological disorders. For this purpose, CSF samples from healthy subjects and patients with psychiatric and neurological diseases can be compared using various proteomic methods. In addition, CSF samples from patients before and after treatment with specific drugs may be able to reveal important differences in an individual's response to a certain drug treatment and lead to personalized medicine approaches. Hence, it is hoped that protein profiling of CSF will ultimately result in a better understanding of disease mechanisms and the molecular effects of drugs.

The analysis of entire proteomes from a clinical specimen is by no means straightforward *(3)*. The major hurdle when it comes to patient samples is the limiting amount of starting material that is available to carry out the analysis. Clinical samples, such as body fluids, frequently cannot be obtained in large enough amounts. The other reason that the analysis of the proteome in CSF is difficult is because of the large dynamic range of protein concentrations in such fluids, which can be up to 12 orders of magnitude between the highest and lowest expressed proteins *(3)*. This range of protein concentrations exceeds the current capabilities of proteomic analysis and is a major barrier in the identification of low abundant proteins in body fluids. Further complicating the analysis of CSF is the possible infiltration of serum proteins that is caused by a leaky blood–brain barrier that is especially pronounced in patients with brain disorders. As a consequence, it is often impossible to know if a protein that is found in CSF is derived from the brain or serum.

Proteome analysis in general involves two stages: protein separation followed by identification and analysis. Multidimensional separations are required in order to result in an adequate resolution of complex protein mixtures in body fluids. Classical proteomic approaches use fractionation on the protein level with the help of two-dimensional-polyacrylamide gel electrophoresis (2D-PAGE) *(4,5)*. This technique produces high-resolution protein separations resulting in the display of potentially thousands of protein spots. Alternatively, tryptic peptides derived from the

proteins in the mixture can be subjected to shotgun mass spectrometry analysis. In the shotgun mass spectrometry approach, proteins are digested by specific enzymes into small peptides and analyzed on-line by mass spectrometry *(6)*. A major advantage of the shotgun mass spectrometry approach is that low abundant proteins can be identified in the presence of high abundant proteins, a scenario that is often encountered when analyzing protein mixtures from body fluids like serum or CSF *(3)*.

In a previous study, we have described our attempts to identify a large number of the constituents of the human CSF proteome *(7)* using the shotgun mass spectrometry methodology *(8)*. This was achieved by depletion of the most abundant proteins from the mixture with the help of an immunoaffinity column and subsequent multidimensional fractionation of the peptides resulting from an Endoproteinase Lys-C, trypsin double digest of the remaining proteins and shotgun mass spectrometry analysis. In the present study, we have extended the human CSF proteome analysis by using prefractionation of the immunodepleted sample on the protein level. Using anion exchange chromatography (AEC) or sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for protein separation, we have been able greatly increase the number of CSF proteins identified. The described in-depth CSF proteome analysis will greatly assist our efforts to identify biomarkers for psychiatric and neurological disorders.

Materials and Methods

Collection of Human CSF

With the subject in the sitting position, lumbar puncture was performed at the fourth lumbar intervertebral space using an atraumatic needle without local anesthesia. For this study, CSF from a patient with normal pressure hydrocephalus was used. In order to remove cellular debris, we centrifuged the CSF sample and then aliquoted and transferred it to a −80°C freezer. The CSF sample used for the studies had the following protein concentrations: total protein 266 μg/mL, human serum albumin (HSA) 158 μg/mL, immunoglobulin (Ig)G 22.2 μg/mL.

Immunodepletion

A total of 6 mL CSF from a patient with normal pressure hydrocephalus was thawed on ice and concentrated to 100 μL on a Vivaspin 5-kDa cartridge (Vivascience AG, Hannover, Germany) by centrifugation at 2380*g* at 4°C. This cartridge retains proteins with a molecular mass >5 kDa; at the same time, it removes salts and other low-molecular-weight compounds.

The Vivaspin retentate was washed with 1 mL of buffer A (phosphate salt solution, pH 7.4, containing 0.02% NaN_3; Agilent Technologies, Wilmington, DE). This step was repeated three times and the sample concentrated to a final volume of 100 μL. Fifty-microliter aliquots of the concentrated CSF sample were then loaded onto the Multiple Affinity Removal System immuno affinity column, 0.46 × 50 mm (Agilent Technologies). The Multiple Affinity Removal System column removed the following proteins from the CSF mixture: HSA, transferrin, haptoglobin, IgG, IgA, and antitrypsin. During the immunoaffinity high-pressure liquid chromatography (HPLC) run, the column effluents were monitored at 280 nm with a flow rate of 250 μL/min for sample loading and collection of the flow-through fraction. For column elution, the flow was increased to 1 mL/min. Typically, the flow-through fraction containing the depleted CSF protein mixture eluted in a volume of 1.5–2 mL. Elution of the absorbed proteins was carried out with a urea buffer at pH 2.5 (Buffer B; Agilent Technologies) at a flow rate of 1 mL/min.

Sample Preparation for Direct Shotgun Mass Spectrometry

The immunoaffinity column flow-through fraction was concentrated on a Vivaspin 5-kDa cartridge (Vivascience AG) as described above, washed three times with 1 mL 200 m*M* NH_4HCO_3 at pH 8.5 and concentrated to a final volume of 50 µL. For digestion, the sample was adjusted to 8*M* urea and proteins reduced with 10 m*M* dithiothreitol (DTT) at 37°C in the dark for 1 h and alkylated with 50 m*M* iodoacetamide at 37°C in the dark for 30 min. The sample was then diluted to 2 *M* urea with 200 m*M* NH_4HCO_3 at pH 8.5 and digested with 10 µg Endoproteinase Lys-C (Roche Diagnostics, Indianapolis, IN) at 37°C overnight. The next day, 10 µg of trypsin (Promega, Madison, WI) was added and the incubation continued at 37°C overnight. The treatment with two enzymes leads to a more efficient digestion of the substrate proteins. In addition, peptide bonds after lysine residues that are preceded by a proline residue are not cleaved by trypsin but are amenable to cleavage with Endoproteinase Lys-C.

After digestion, salts were removed by using a SPEC Plus PT pipette tip solid phase extraction cartridge (Varian Inc., Palo Alto, CA) according to the manufacturer's instructions. Peptides were eluted from the SPEC cartridge with two aliquots of 20 µL 95% acetonitrile, 0.1% formic acid. Finally, the acetonitrile was removed by vacuum centrifugation and the remaining mixture dissolved in 40 µL 5 m*M* KH_2PO_4, 25% acetonitrile at pH 3.0.

Anion Exchange Chromatography

The depleted CSF sample was washed three times with 20 m*M* Tris-HCl at pH 8.0, using a Vivaspin 5-kDa cartridge (Vivascience AG) as described above and concentrated to 100 µL. The sample was then manually loaded onto a strong anion exchange macro trap cartridge, 3 × 8 mm (Michrom BioSources, Inc., Auburn, CA). Proteins were eluted with 100 µL 20 m*M* Tris-HCl, pH 8.0, buffer containing 20 m*M*, 50 m*M*, 100 m*M*, 150 m*M*, 200 m*M*, 300 m*M*, 400 m*M*, and 500 m*M* of NaCl. The eluted fractions were collected and concentrated and washed three times with 200 m*M* NH_4HCO_3 at pH 8.5 to a final volume of 50 µL with a Vivaspin 5-kDa cartridge (Vivascience AG) as described above. The protein mixtures from the eight salt fractions were adjusted to 8 *M* urea and reduced and alkylated as described above. The samples were then diluted to 2 *M* urea with 200 m*M* NH_4HCO_3 at pH 8.5 and digested and processed as described above.

SDS-Polyacrylamide Gel Electrophoresis

The depleted CSF was washed three times with 200 m*M* NH_4HCO_3 at pH 8.5, concentrated to 50 µL with a Vivaspin 5-kDa (Vivascience AG) cartridge, and reduced and alkylated as described above. The dried sample was dissolved in 30 µL of SDS sample buffer (125 m*M* Tris-HCl, pH 8.0, 9.2% SDS, 0.39 *M* DTT, 40% glycerol, 0.001% bromophenol blue) and loaded onto a 12% SDS polyacrylamide gel using a Protean Plus Cell apparatus (Bio-Rad Laboratories, Hercules, CA). After electrophoresis, the proteins were stained with colloidal Coomassie Blue and scanned with a densitometer (Bio-Rad Laboratories). The protein-containing gel lane was cut in 5 × 1 mm slices, which were destained two times with 70 µL acetonitrile/20 m*M* NH_4HCO_3 at pH 8.5 (1:1) at 37°C for 30 min and then dried under a fume hood. For digestion, 1 µg trypsin in 30 µL 20 m*M* NH_4HCO_3 at pH 8.5 was added to each gel slice. The enzymatic reaction was carried out at 37°C overnight. After the digestion, the peptides were extracted by adding 5% formic acid at 37°C for 30 min. The gel pieces were spun down and the liquid collected. The extraction was repeated twice. Finally, the extracted

digest peptide mixture was lyophilized to dryness and dissolved in 12 µL 0.1% formic acid.

Mass Spectrometry Analysis

Strong Cation Exchange Chromatography

After digestion and desalting, the CSF digest peptide mixture was loaded onto an SCX cation exchange column, 0.5 × 15 mm (Dionex, Sunnyvale, CA) that had been equilibrated in 5 m*M* KH_2PO_4, 25% acetonitrile at pH 3.0 at a flow rate of 30 µL/min. After washing the column with buffer A for 5 min, peptides were eluted by applying a linear gradient from 0 to 50% of 25% acetonitrile, 350 m*M* KCl at pH 3.0. Fractions were collected at 1-min intervals and lyophilized to dryness. The peptides were then dissolved in 36 µL 0.1% formic acid.

Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS) Analysis

One-third of each cation exchange fraction (12 µL) was loaded onto a PicoFrit RP-C18 column, 0.075 × 10 mm (New Objective Inc., Woburn, MA) in 0.1% formic acid (solvent A1), washed with solvent A1 for 15 min, and eluted with a linear gradient of 95% acetonitrile/0.1% formic acid (solvent B1) over 90 min at a flow rate of 200 nL/min using the ULTIMATE nano-HPLC system (Dionex). Column effluents were directly infused into an LCQ DECA XP PLUS iontrap mass spectrometer (Thermo Electron Corp., San Jose, CA). For mass spectrometry analysis, three separate runs were carried out for each cation exchange fraction. In each run, the four most abundant precursor ions were subjected to fragmentation and MS/MS analysis. Different mass/charge (m/z) limits were used for the full scan in each run (400–900, 900–1500, 1500–2000). The mass spectrometer was operated with an ion spray voltage of 1.1 kV, a normalized collision energy of 35%, a default charge state of +3, an isolation width of 4 amu, a reject mass width of 0.5, a dynamic exclusion of 3 min, an exclusion mass width of 3 amu, a repeat count of 2, and a repeat duration of 0.35 min.

Data Analysis

All MS/MS spectra were processed and analyzed using the Spectrum Mill software and default parameters (Agilent Technologies, Santa Clara, CA) *(9)*. After the extraction step during which MS/MS spectra of low quality were removed, the spectra were searched against the human international protein index (IPI) database, version 2.29, 03/05/2004 (EBI, Cambridgeshire, UK) with the static chemical modification of +57 Dalton (Cys-carboxyamidomethyl) and a precursor ion accuracy of 3 Dalton. For a protein identification to be autovalidated in Spectrum Mill, the protein needs to have a score (sum of peptide scores that map to that protein) of >20. For a "one-hit wonder" to pass Spectrum Mill's autovalidation routine, in peptide validation mode, the peptide must have a score of 13 or greater. In addition, using the same parameters, all MS/MS spectra were also searched against a reversed human IPI database in order to assess the frequency of false-positive hits.

Results and Discussion

A major hurdle of the proteome analysis of body fluids such as CSF is the rather large dynamic range of the proteins present in the mixture. It is estimated that in CSF and serum the range of protein concentrations spans 12 orders of magnitude *(3)*. As a consequence, the identification of proteins lower in concentration than the very abundant HSA and Ig is extremely difficult. To circumvent the problem caused by the large dynamic range of protein expression, several approaches have been developed that will deplete the most abundant proteins from body fluids. In our previous studies, we have shown that an immunoaffinity

matrix that is made up of affinity-purified polyclonal antibodies against the six major proteins in serum (HSA, transferrin, haptoglobin, IgG, IgA, and antitrypsin) also removes the six most abundant proteins from CSF and thereby allows the identification by mass spectrometry of a great number of low abundant proteins in CSF *(7)*. Because of its great specificity, we have been using the Multiple Affinity Removal System for all our subsequent studies that are geared toward an in-depth proteome analysis of human CSF.

CSF from a patient with normal pressure hydrocephalus was used for all our studies because of the relatively large volume of fluid that can be obtained. This is critical because it allows one to perform several experiments with the same CSF sample whose results can be compared for proteomic method development.

In our previous CSF proteomic studies, we used immunodepletion followed by shotgun mass spectrometry analysis of the peptide *(7)*. In order to extend our proteome mining efforts and increase the number of identified proteins, we reasoned that further fractionation on the intact protein level of the CSF sample mixture is necessary. For this purpose, we used either AEC or SDS-PAGE (Fig. 1). In the case of AEC, absorbed proteins from the immunodepleted CSF sample were eluted in a stepwise fashion with increasing concentrations of salt. An aliquot of each fraction was analyzed by SDS-PAGE in order to gauge the success of the separation (Fig. 2A). The proteins from each salt fraction were digested and the resultant peptide mixtures subjected to two rounds of chromatography (strong cation exchange and reversed phase) and on-line tandem mass spectrometry *(7)*.

In the other prefractionation experiment, we loaded the immunodepleted and concentrated CSF protein mixture onto an SDS polyacrylamide gel. After electrophoresis and gel staining (Fig. 2B), the lane containing the CSF sample was cut into 1-mm slices. Each of a total of 146 slices was then subjected to an in-gel digest followed by shotgun mass spectrometry.

For all analyses, the nano reversed phase column-eluting peptides were processed on-line by tandem mass spectrometry of the four strongest signals from each full scan. The MS/MS spectra acquired by the mass spectrometer were analyzed for matching peptide sequences against the human IPI protein database and a reversed sequence version of the same database using the Spectrum Mill software *(9)*. In the case of single peptide hits, the MS/MS spectra were further inspected manually to confirm the obtained sequence.

Processing the CSF sample by only immunodepletion and no further protein prefractionation followed by shotgun mass spectrometry resulted in 74 proteins identified. Protein separation of the immunodepleted CSF sample with the help of AEC with eight salt fractions collected yielded 112 protein hits. Prefractionation of the same sample by SDS-PAGE resulted in the most protein hits of all the analyses that were performed. A total of 240 proteins were identified from the human IPI database using default Spectrum Mill parameters (Fig. 3). A grand total of 259 proteins were identified when data form all three methods were considered.

In order to assess the probability of false-positive hits, all MS/MS spectra were searched against a reversed sequence IPI database using the same default Spectrum Mill parameters. This allows one to obtain a fairly accurate measure of the false-positive rate of the search data. The results of both searches shown in Fig. 3 reveal that the estimated number of false-positive protein hits depends on the number of MS/MS spectra that are included in the search. Whereas a rather low percentage of false-positive protein hits was obtained for the imunodepleted analysis with no further subfractionation of the

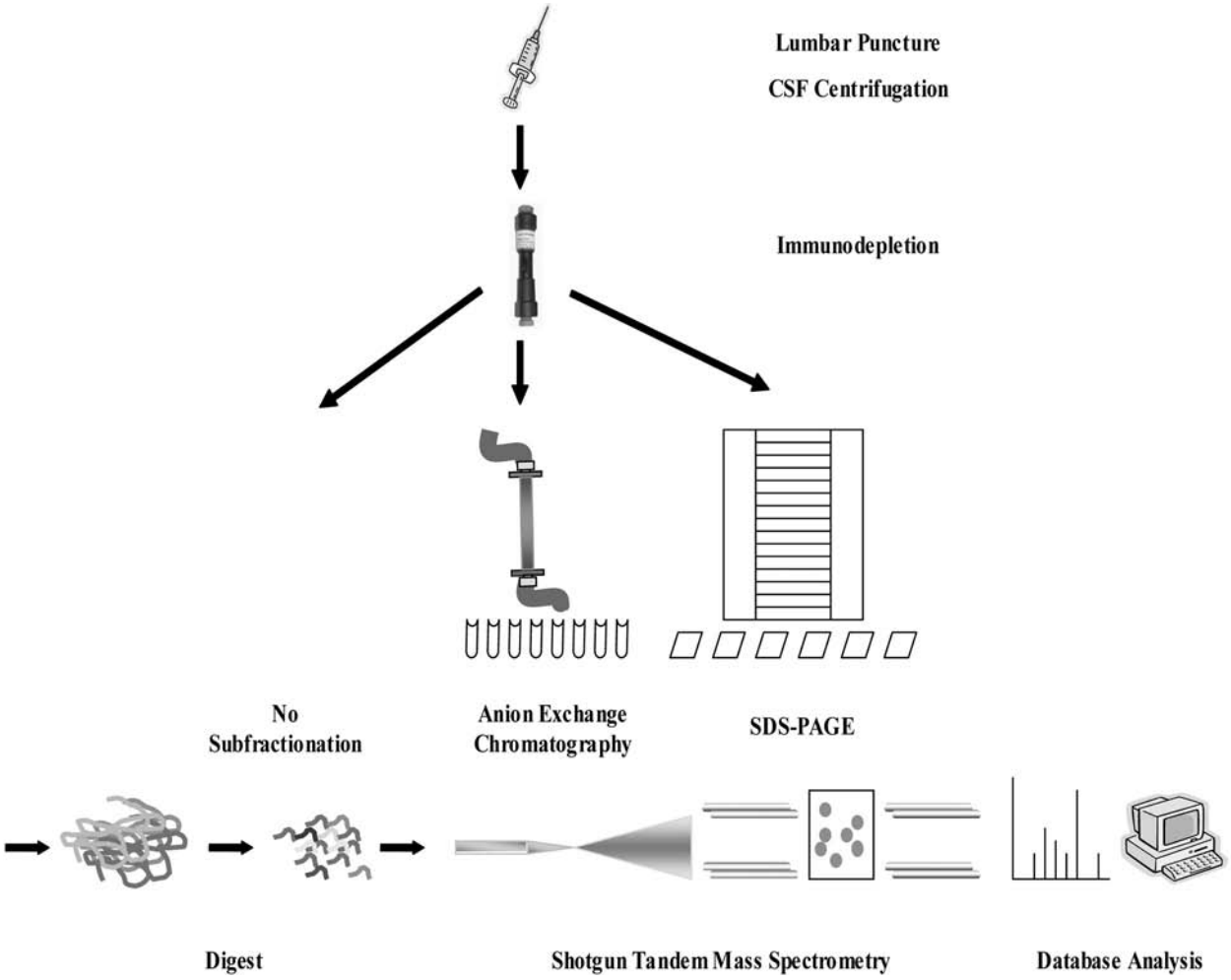

Fig. 1. Cerebrospinal fluid (CSF) proteomic workflow. In all cases, CSF was immunodepleted and either underwent no further protein subfractionation, or anion exchange chromatography resulting in eight salt fractions, or sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 146 gel slice fractions. All protein fractions were further processed by enzymatic digestion followed by shotgun mass spectrometry of the peptide digest mixture. Data-dependent mass spectrometry involved a full scan followed by data-dependent tandem meass spectrometry (MS/MS) scans of the four most intense peptide ions under dynamic exclusion conditions.

proteins (two estimated false-positive protein hits corresponding to a false-positive rate of 2.7%), the number of the estimated false-positive hits goes up slightly for data files that contain significantly more MS/MS spectra. Three and 12 protein hits were obtained when the AEC and SDS-PAGE subfractionated MS/MS data were searched against the reversed sequence IPI database, respectively. This corresponds to a false-positive rate of 4% and 5%, respectively. All false-positive hits fall in the category of "one-hit wonders"; in other words, only one peptide sequence produced the respective protein hit. A manual validation in the case of single peptide hits is therefore warranted, as there is less confidence in these assignments. No hits corresponding to "real" peptide sequences that are present in the IPI database were found during the reversed sequence IPI database search.

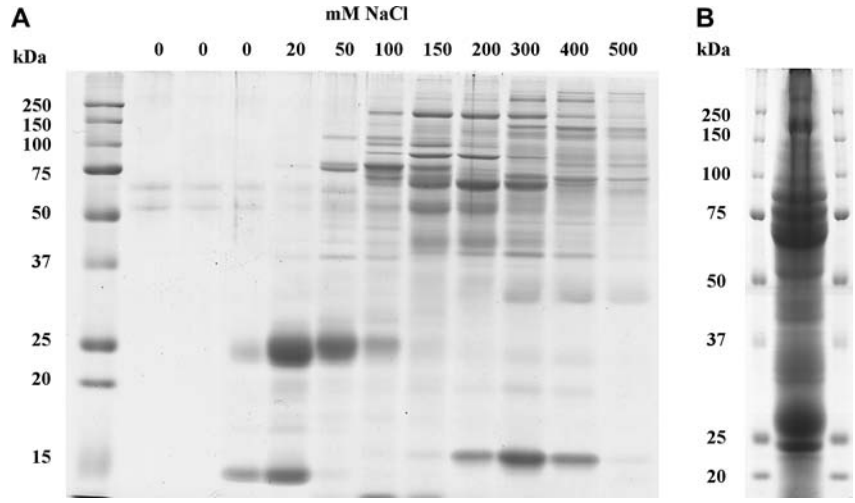

Fig. 2. **(A)** SDS polyacrylamide gel image of the eight salt fractions resulting from anion exchange chromatography of immunodepleted cerebrospinal fluid (CSF). The first three lanes after the standards are breakthrough and wash fractions. **(B)** SDS polyacrylamide gel image of immunodepleted CSF that was subsequently cut into 146 slices for further processing. The positions of the molecular weight markers are indicated.

The number of proteins resulting from "one-hit wonders" that were deleted from the list after manual inspection of the MS/MS spectra were 12, 3, and 0 for the SDS-PAGE-subfractionated, AEC-subfractionated, and unfractionated protein runs, respectively. These numbers are close to the predicted false-positive rate based on the search against the reversed sequence human IPI database (Fig. 3). A total of 46 common proteins were identified from all three runs and 95 common proteins were identified in the AEC- and SDS-PAGE-subfractionated sample runs (Table 1). This rather low overlap of protein identifications from different runs is typical for the shotgun mass spectrometry method. In Table 1, proteins are ranked according to the number of unique peptides that were identified for all three runs using the Spectrum Mill software. Hence, the list is a reflection of protein abundance in human CSF with high abundant proteins listed in the upper part of Table 1.

The proteins found in normal pressure hydrocephalus CSF can be classified into several groups based on molecular function, biological process, and cellular compartment distribution (Fig. 4 and Table 1). A great number of these proteins are of low abundance and in many cases represent intracellular components such as signaling proteins and transcription factors. These findings indicate that intracellular contents of cells and tissues are released into CSF, presumably through apoptotic and necrotic mechanisms.

Although it is difficult to know how many proteins are exclusively derived from the brain because many proteins are likely expressed in many tissues and therefore could potentially be introduced into CSF through its exchange with serum, we now have evidence that many of the proteins that were identified in CSF are in fact derived from brain tissue. This is based on a comparison of the identified CSF proteins with two recently published human serum protein databases *(10,11)*. The comparison revealed that 122 proteins identified in our CSF analysis were also found during an in-depth analysis of the human serum proteome (indicated with a bold font in the "Protein

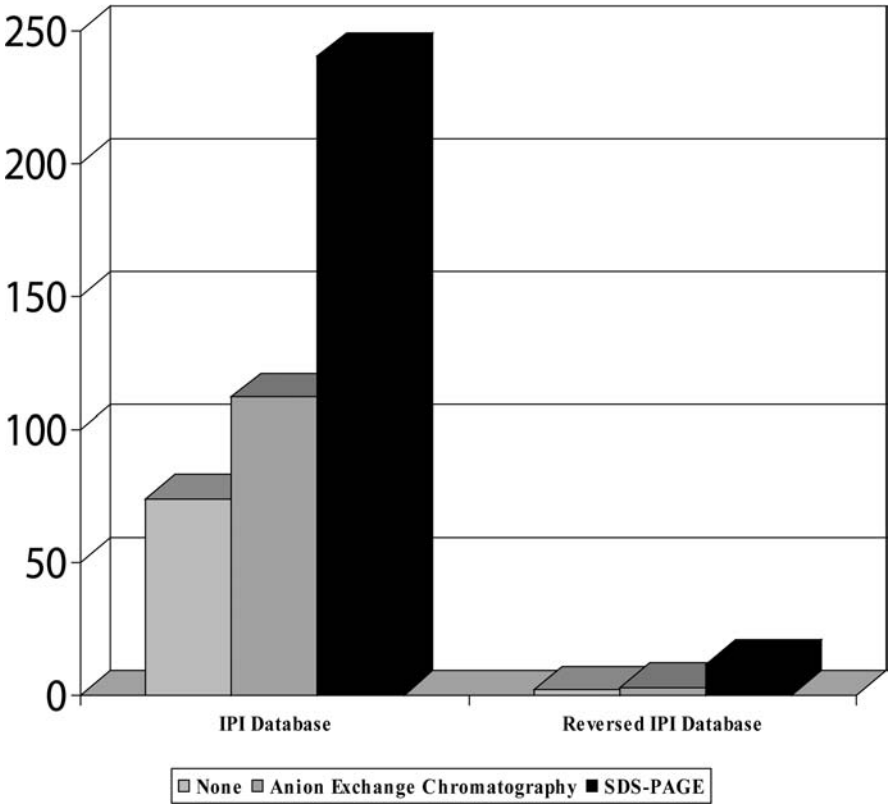

Fig. 3. Results of the mass spectra data analysis using the Spectrum Mill software. The number of proteins that were identified with default search parameters for all three analyses are plotted for the human international protein index (IPI) and reversed sequence human IPI database searches.

Name" column of Table 1). Of the 122 overlaps between CSF and serum, 15 proteins (HSA, transferrin, IgG, fibrinogen, macroglobulin, antitrypsin, C3 complement, apolipoprotein A-I, apolipoprotein, α-1-acid glycoprotein, complement factor H, ceruloplasmin, C9 complement, C1q complement, C8 complement) are considered to be of high abundance in serum *(11)*. Based on the ranking by Spectrum Mill, 5 of these proteins are also of high abundance in human CSF (macroglobulin, C3 complement, apolipoprotein A–I, complement factor H, ceruloplasmin; Table 1). Furthermore, 4 proteins (HSA, transferrin, IgG, antitrypsin) known to be abundant in CSF *(7)* had been depleted by previous immunoaffinity absorption.

As was the case in our previous analysis *(7)*, we found proteins with a wide range of isoelectric points using the shotgun mass spectrometry approach (Fig. 5). In order to achieve a comparable coverage of proteins with 2D-PAGE analysis, one needs to run several gels with overlapping pH gradients. This requires more sample and is often prohibitive when dealing with human body fluids and tissues. Some peptides derived from the six proteins that the Multiple Affinity Removal System was designed to remove were found in our analyses. They are most likely derived from degradation products of these proteins. One indication that this is indeed the case stems from the mobility of the protein during SDS-PAGE, which indicated a smaller molecular

Table 1
Summary of Proteins That Were Identified in Human CSF From a Patient With Normal Pressure Hydrocephalus Using Three Different Sample Preparation Methods

Unique peptides	Score	Protein name	Molecular function	Biological process	Cellular compartment
63	1073.26	**Complement C3 precursor**	Complement activity	Immune response	Extracellular
55	980.42	**Complement C4 precursor**	Complement activity	Immune response	Extracellular
47	792.96	**Alpha-2-macroglobulin precursor**	Protease Inhibitor activity	Protein metabolism	Extracellular
46	846.99	**Serum albumin precursor**	Transporter activity	Transport	Extracellular
41	731.68	**Serotransferrin precursor**	Transporter activity	Transport	Extracellular
37	633.10	**Fibronectin precursor**	Extracellular matrix protein	Cellular organization and biogenesis	Extracellular
32	533.75	**Complement factor H**	Complement activity	Immune response	Extracellular
28	484.25	**Ceruloplasmin precursor**	Transporter activity	Transport	Extracellular
27	455.14	**Plasminogen precursor**	Enzymatic activity	Protein metabolism	Extracellular
26	453.83	**Gelsolin precursor**	Structural constituent of cytoskeleton	Cellular organization and biogenesis	Extracellular
25	414.78	Cell adhesion molecule L1-like	Cell adhesion molecule activity	Signal transduction; Cell communication	Plasma membrane
24	400.48	**Complement component C7**	Complement activity	Immune response	Extracellular
20	342.24	**Complement C1r component**	Complement activity	Immune response	Extracellular
20	326.75	**Complement factor B**	Complement activity	Immune response	Extracellular
19	345.99	**Vitamin D-binding protein**	Transporter activity	Transport	Extracellular
18	305.84	**Apolipoprotein E precursor**	Transporter activity	Transport	Extracellular
18	288.27	**Contactin**	Cell adhesion molecule activity	Cellular organization and biogenesis	Plasma membrane
18	243.90	Ubiquitin	Structural constituent of ribosome	Metabolism	Intracellular
17	310.40	**Pigment epithelium-derived factor**	Serine-type peptidase activity	Signal transduction; Cell communication	Extracellular
17	297.15	Neuronal cell adhesion molecular precursor	Cell adhesion molecule activity	Signal transduction; Cell communication	Plasma membrane
16	289.45	**Alpha-1-antitrypsin**	Transport	Transporter activity	Extracellular
16	283.16	**Apolipoprotein A-I**	Transporter activity	Transport	Extracellular
16	272.18	**Alpha-1-antichymotrypsin**	Protease inhibitor activity	Protein metabolism	Extracellular
15	273.66	Glutamate carboxypeptidase-like protein 2	Transport/Enzymatic activity	Metabolism	Extracellular
15	245.36	Fc fragment of IgG binding protein	Receptor activity	Signal transduction; Cell communication	Plasma membrane
14	284.38	**Transthyretin precursor**	Transporter activity	Transport	Extracellular
14	239.01	Alpha 1B-glycoprotein	Unknown	Unknown	Extracellular
14	235.87	**Antithrombin-III**	Protease Inhibitor activity	Protein metabolism	Extracellular
14	220.37	Apolipoprotein A-IV	Transporter activity	Transport	Extracellular
14	213.52	Chitinase-3 like protein 1	Extracellular matrix protein	Cellular organization and biogenesis	Extracellular
13	255.45	**Angiotensinogen**	Peptide hormone	Signal transduction; Cell communication	Extracellular
13	242.25	SPARC-like protein 1	Unknown	Signal transduction; Cell communication	Extracellular
13	230.07	Neural cell adhesion molecule 1	Cell adhesion molecule activity	Signal transduction; Cell communication	Plasma membrane
13	216.07	**Clusterin**	Complement activity	Immune response	Extracellular

Immunodepletion		Immunodepletion and AEC		Immunodepletion and SDS-PAGE				
Number of spectra	Mean intensity	Number of spectra	Mean intensity	Number of spectra	Mean intensity	Mw	pl	Accession number
57	5.68e + 007	88	4.76e + 006	326	1.35e + 008	187236.1	6	IPI:IPI00164623.2
45	5.07e + 007	22	4.31e + 006	332	1.27e + 008	192772.5	6.66	IPI:IPI00032258.4
14	2.79e + 007	51	6.10e + 006	217	2.27e + 008	163278.8	5.95	IPI:IPI00032256.1
87	6.05e + 007	262	1.87e + 007	384	1.82e + 008	69367.1	5.47	IPI:IPI00022434.1
10	2.85e + 007	47	8.73e + 006	218	1.92e + 008	77050.4	6.7	IPI:IPI00022463.1
4	2.31e + 007	9	3.40e + 006	76	2.38e + 008	262607.9	5.38	IPI:IPI00022418.1
4	6.95e + 007	30	8.04e + 006	109	3.11e + 008	139126.3	6.19	IPI:IPI00029739.3
31	6.60e + 007	3	6.43e + 006	83	3.12e + 008	122205.8	5.41	IPI:IPI00017601.1
14	3.11e + 007	15	5.39e + 006	59	1.33e + 008	90569.6	7.08	IPI:IPI00019580.1
8	5.63e + 007	27	4.32e + 006	227	2.52e + 008	85697.9	5.72	IPI:IPI00026314.1
9	6.42e + 006	14	3.95e + 006	50	3.46e + 008	136654.8	5.46	IPI:IPI00299059.3
1	2.20e + 006	4	3.69e + 006	65	1.68e + 008	97861.0	6.09	IPI:IPI00296608.4
0	0.00e + 000	2	7.44e + 006	66	1.65e + 008	80174.1	5.82	IPI:IPI00296165.3
0	0.00e + 000	0	0.00e + 000	81	1.09e + 008	85533.4	6.66	IPI:IPI00019591.1
8	2.65e + 007	31	7.53e + 006	142	2.32e + 008	57109.8	5.22	IPI:IPI00298853.4
0	0.00e + 000	38	2.70e + 006	240	2.14e + 008	36154.3	5.52	IPI:IPI00021842.1
1	4.64e + 007	8	3.52e + 006	37	1.43e + 008	113321.0	5.58	IPI:IPI00029751.1
0	0.00e + 000	9	6.08e + 006	18	1.65e + 008	77039.0	7.58	IPI:IPI00220286.1
0	0.00e + 000	11	3.21e + 006	126	2.14e + 008	46342.5	5.90	IPI:IPI00006114.2
4	1.46e + 007	11	4.07e + 006	77	3.19e + 008	143894.4	5.28	IPI:IPI00333776.1
27	4.81e + 007	18	4.68e + 006	61	1.81e + 008	46736.8	5.37	IPI:IPI00305457.3
12	1.59e + 007	83	7.94e + 006	82	3.55e + 008	30778.0	5.27	IPI:IPI00021841.1
0	0.00e + 000	34	6.72e + 006	59	1.48e + 008	48637.3	5.32	IPI:IPI00032215.2
5	3.83e + 006	72	5.83e + 006	51	1.90e + 008	56779.6	5.12	IPI:IPI00064667.1
0	0.00e + 000	3	5.73e + 006	25	4.82e + 007	572099.9	5.03	IPI:IPI00242956.2
27	1.66e + 007	28	4.10e + 006	927	7.70e + 008	15887.1	5.27	IPI:IPI00022432.1
42	2.76e + 007	12	7.95e + 006	48	2.13e + 008	54253.8	5.50	IPI:IPI00216722.1
0	0.00e + 000	17	6.31e + 006	35	1.25e + 008	52602.7	5.95	IPI:IPI00032179.1
6	3.05e + 007	15	2.54e + 006	31	2.97e + 007	45371.3	5.18	IPI:IPI00304273.1
1	2.04e + 007	8	5.43e + 006	40	9.90e + 007	42613.6	8.65	IPI:IPI00002147.3
2	3.30e + 006	1	7.69e + 007	148	1.75e + 008	53154.5	5.60	IPI:IPI00032220.1
8	5.42e + 006	66	3.88e + 006	19	1.86e + 008	75216.1	4.66	IPI:IPI00296777.3
0	0.00e + 000	3	6.02e + 006	53	1.22e + 008	83770.5	4.77	IPI:IPI00220737.1
0	0.00e + 000	10	4.30e + 006	208	1.21e + 008	52494.9	5.89	IPI:IPI00291262.3

(continued)

Table 1 (continued)

Unique peptides	Score	Protein name	Molecular function	Biological process	Cellular compartment
13	213.68	**Splice isoform 2 of Inter-alpha-trypsin inhibitor heavy chain H4 precursor**	Protease Inhibitor activity	Unknown	Extracellular
12	213.21	Fibulin-1	Extracellular matrix protein	Cellular organization and biogenesis	Extracellular
12	211.17	**Hemopexin**	Transporter activity	Transport	Extracellular
12	177.50	Procollagen C-proteinase enhancer protein	Extracellular matrix protein	Cellular organization and biogenesis	Extracellular
11	194.54	Plasma protease C1 inhibitor	Protease Inhibitor activity	Protein metabolism	Extracellular
11	193.63	**Alpha-2-HS-glycoprotein**	Unknown	Signal transduction; Cell communication	Extracellular
11	181.66	Ectonucleotide pyrophosphatase/ phosphodiesterase 2	Phosphoric diester hydrolase activity	Metabolism; Energy pathways	Extracellular
11	180.82	**Afamin**	Structural molecule activity	Cellular organization and biogenesis	Extracellular
11	169.91	**Complement C1s component**	Complement activity	Immune response	Extracellular
10	175.87	**Histidine-rich glycoprotein**	Transporter activity	Transport	Extracellular
10	175.59	**Prothrombin**	Blood coagulation factor activity	Protein metabolism	Extracellular
10	175.02	Cystatin C	Protease Inhibitor activity	Protein metabolism	Extracellular
10	171.60	**Tetranectin**	Unknown	Unknown	Extracellular
10	170.58	Amyloid-like protein 1 precursor	Transcription regulator activity	Signal transduction; Cell communication	Plasma membrane
10	166.36	Calsyntenin-1	calcium ion binding	homophilic cell adhesion	Plasma membrane
10	164.57	**Zinc-alpha-2-glycoprotein**	Unknown	Immune response	Extracellular
10	160.56	Neurocan core protein	Extracellular matrix protein	Signal transduction; Cell communication	Extracellular
10	147.15	Protein kinase C-binding protein NELL2	calcium ion binding	cell adhesion	Extracellular
9	156.14	Kallikrein 6	Serine-type peptidase activity	Protein metabolism	Cytoplasm
9	155.23	**Splice Isoform: LMW of Kininogen**	Blood coagulation factor activity	Protein metabolism	Extracellular
9	154.44	AMBP protein	Unknown	Immune response	Extracellular
9	151.51	Dihydropyridine-sensitive L-type	Voltage-gated ion channel activity	Transport	Sarcoplasmic Reticulum
9	150.84	Contactin 2	Cell adhesion molecule activity	Signal transduction; Cell communication	Plasma membrane
9	148.54	**Complement factor I**	Unknown	Immune response	Extracellular
9	144.92	**Keratin**	Structural constituent of cytoskeleton	Cellular organization and biogenesis	Cytoplasm
9	143.00	**Amyloid beta A4 protein**	Receptor activity	Signal transduction; Cell communication	Plasma membrane
9	136.24	**Fibrinogen alpha/alpha-E chain precursor**	Blood coagulation factor activity	Protein metabolism	Extracellular

Immunodepletion		Immunodepletion and AEC		Immunodepletion and SDS-PAGE				
Number of spectra	Mean intensity	Number of spectra	Mean intensity	Number of spectra	Mean intensity	Mw	pl	Accession number
0	0.00e + 000	0	0.00e + 000	25	1.25e + 008	101242.5	6.21	IPI:IPI00218192.1
0	0.00e + 000	0	0.00e + 000	34	8.11e + 007	77261.8	5.07	IPI:IPI00296534.1
14	3.83e + 007	53	1.12e + 007	147	1.40e + 008	51676.7	6.43	IPI:IPI00022488.1
1	1.56e + 007	0	0.00e + 000	38	8.46e + 007	47972.8	7.55	IPI:IPI00299738.1
6	1.87e + 007	38	4.74e + 006	28	1.51e + 008	55154.5	5.97	IPI:IPI00291866.1
13	1.64e + 007	61	1.32e + 007	25	1.40e + 008	39324.9	5.36	IPI:IPI00022431.1
6	4.45e + 007	0	0.00e + 000	18	8.04e + 007	99004.2	7.43	IPI:IPI00156171.2
0	0.00e + 000	2	3.30e + 006	18	9.99e + 007	69069.6	5.58	IPI:IPI00019943.1
0	0.00e + 000	13	4.26e + 006	23	1.45e + 008	76684.9	4.86	IPI:IPI00017696.1
8	1.33e + 007	5	4.03e + 006	18	9.29e + 007	59578.6	7.03	IPI:IPI00022371.1
8	3.59e + 007	3	2.48e + 006	25	1.81e + 008	70037.3	5.24	IPI:IPI00019568.1
3	3.06e + 008	0	0.00e + 000	299	1.26e + 009	15799.3	8.75	IPI:IPI00032293.1
0	0.00e + 000	2	4.28e + 006	51	6.81e + 007	22567.0	5.80	IPI:IPI00009028.1
22	7.93e + 006	0	0.00e + 000	48	1.41e + 008	72176.8	5.37	IPI:IPI00020012.2
6	1.87e + 007	38	4.74e + 006	28	1.51e + 008	55154.5	5.97	IPI:IPI00291866.1
13	1.64e + 007	61	1.32e + 007	25	1.40e + 008	39324.9	5.36	IPI:IPI00022431.1
6	4.45e + 007	0	0.00e + 000	18	8.04e + 007	99004.2	7.43	IPI:IPI00156171.2
0	0.00e + 000	2	3.30e + 006	18	9.99e + 007	69069.6	5.58	IPI:IPI00019943.1
0	0.00e + 000	13	4.26e + 006	23	1.45e + 008	76684.9	4.86	IPI:IPI00017696.1
8	1.33e + 007	5	4.03e + 006	18	9.29e + 007	59578.6	7.03	IPI:IPI00022371.1
8	3.59e + 007	3	2.48e + 006	25	1.81e + 008	70037.3	5.24	IPI:IPI00019568.1
3	3.06e + 008	0	0.00e + 000	299	1.26e + 009	15799.3	8.75	IPI:IPI00032293.1
0	0.00e + 000	2	4.28e + 006	51	6.81e + 007	22567.0	5.80	IPI:IPI00009028.1
22	7.93e + 006	0	0.00e + 000	48	1.41e + 008	72176.8	5.37	IPI:IPI00020012.2
0	0.00e + 000	7	4.30e + 006	32	1.41e + 008	109793.3	4.77	IPI:IPI00007257.1
0	0.00e + 000	6	3.78e + 006	46	4.13e + 007	34258.9	5.58	IPI:IPI00166729.1
0	0.00e + 000	0	0.00e + 000	17	8.76e + 007	142973.9	5.20	IPI:IPI00159927.1
0	0.00e + 000	1	4.32e + 006	25	1.90e + 008	91347.2	5.40	IPI:IPI00015260.1
0	0.00e + 000	6	4.18e + 006	71	5.60e + 007	26855.9	6.91	IPI:IPI00023845.1
0	0.00e + 000	2	3.82e + 006	32	1.23e + 008	47883.5	6.29	IPI:IPI00215894.1

(continued)

Table 1 (continued)

Unique peptides	Score	Protein name	Molecular function	Biological process	Cellular compartment
8	155.17	Chromogranin A	Unknown	Signal transduction; Cell communication	Extracellular
8	150.27	Prostaglandin-H2 D-isomerase	Catalytic activity	Metabolism; Energy pathways	Cytoplasm
8	141.03	**Apolipoprotein D**	Transporter activity	Transport	Extracellular
8	139.15	Neural cell adhesion molecule 2	Cell adhesion molecule activity	Cellular organization and biogenesis	Plasma membrane
8	133.09	Keratin, type II cuticular HB3	Structural constituent of cytoskeleton	Cellular organization and biogenesis	Cytoplasm
8	130.72	N-acetyllactosaminide beta-1,3-N-acetylglucosaminyltransferase	Enzymatic activity	Biosynthesis	Plasma membrane
8	130.61	**Inter-alpha-trypsin inhibitor heavy chain H2**	Protease Inhibitor activity	Unknown	Extracellular
8	119.64	**Apolipoprotein B-100**	Transporter activity	Transport	Endoplasmic reticulum
7	137.33	**Beta-2-glycoprotein I**	Transporter activity	Transport	Extracellular
7	136.99	**Fibrinogen gamma chain**	Blood coagulation factor activity	Protein metabolism	Extracellular
7	119.14	**Basement membrane-specific heparan sulfate proteoglycan**	Extracellular matrix protein	Cellular organization and biogenesis	Extracellular
7	113.05	Multiple EGF-like-domain protein 4	Unknown	Unknown	Membrane protein
7	112.89	**Complement component C8 gamma chain**	Complement activity	Immune response	Extracellular
7	111.94	Immunoglobulin superfamily, member 8	Protein binding	Cell proliferation	Integral to membrane
7	108.53	Type I hair keratin 1	Structural constituent of cytoskeleton	Cellular organization and biogenesis	Cytoplasm
6	117.44	Phosphatidylethanolamine-binding protein	Protease Inhibitor activity	Signal transduction; Cell communication	Cytoplasm
6	112.06	Secretogranin III	Peptide hormone	Signal transduction; Cell communication	Extracellular
6	111.66	Osteopontin	Cell adhesion molecule activity	Cellular organization and biogenesis	Extracellular
6	108.39	**Inter-alpha-trypsin inhibitor heavy chain H1**	Protease Inhibitor activity	Protein metabolism	Extracellular
6	107.58	Monocyte differentiation antigen CD14	Receptor activity	Immune response	Plasma membrane
6	104.08	Immunoglobulin superfamily	Cell adhesion molecule activity	Signal transduction; Cell communication	Plasma membrane
6	102.38	Plasma retinol-binding protein	Transporter activity	Transport	Extracellular
6	98.27	Splice isoform 2 of Protein-tyrosine phosphatase non-receptor type substrate 1 precursor	Receptor activity	Signal transduction; Cell communication	Plasma membrane
6	92.71	Protein FAM3C	Receptor activity	Unknown	Extracellular
6	92.62	Neogenin	Receptor activity	Signal transduction; Cell communication	Plasma membrane
6	92.56	Chondroitin sulfate proteoglycan BEHAB	Extracellular matrix protein	Signal transduction; Cell communication	Extracellular

Immunodepletion		Immunodepletion and AEC		Immunodepletion and SDS-PAGE				
Number of spectra	Mean intensity	Number of spectra	Mean intensity	Number of spectra	Mean intensity	Mw	pl	Accession number
0	0.00e + 000	1	2.33e + 006	27	9.67e + 007	38999.7	5.91	IPI:IPI00022426.1
0	0.00e + 000	0	0.00e + 000	14	1.16e + 008	123184.0	4.96	IPI:IPI00306196.1
0	0.00e + 000	0	0.00e + 000	15	2.09e + 008	113393.9	7.35	IPI:IPI00024966.1
0	0.00e + 000	0	0.00e + 000	22	4.09e + 007	65720.7	7.38	IPI:IPI00291867.3
0	0.00e + 000	0	0.00e + 000	13	2.70e + 007	66067.0	8.82	IPI:IPI00220327.1
11	2.01e + 007	10	2.93e + 006	17	8.84e + 007	86943.8	4.72	IPI:IPI00006608.1
5	1.19e + 007	2	2.89e + 006	11	4.21e + 007	94973.5	5.65	IPI:IPI00021885.1
5	2.41e + 007	65	6.71e + 006	1	7.66e + 006	50730.7	4.56	IPI:IPI00290315.3
92	9.50e + 006	24	1.31e + 007	280	1.39e + 008	21028.9	8.37	IPI:IPI00013179.1
0	0.00e + 000	2	5.19e + 006	81	7.62e + 007	21275.7	5.20	IPI:IPI00006662.1
0	0.00e + 000	0	0.00e + 000	16	7.61e + 007	93046.7	5.33	IPI:IPI00376427.1
0	0.00e + 000	0	0.00e + 000	11	4.61e + 007	54214.8	5.20	IPI:IPI00297795.3
3	1.92e + 007	0	0.00e + 000	50	1.16e + 008	47119.3	6.56	IPI:IPI00009997.1
3	3.27e + 007	0	0.00e + 000	11	1.51e + 008	106596.2	5.75	IPI:IPI00305461.1
0	0.00e + 000	1	4.29e + 006	7	8.19e + 007	515565.2	6.59	IPI:IPI00022229.1
0	0.00e + 000	0	0.00e + 000	35	1.25e + 008	38298.4	8.37	IPI:IPI00298828.1
9	8.44e + 006	3	2.06e + 006	26	1.08e + 008	51511.9	5.24	IPI:IPI00021891.5
0	0.00e + 000	1	2.80e + 006	10	4.96e + 007	468827.8	6.03	IPI:IPI00024284.2
0	0.00e + 000	0	0.00e + 000	8	1.22e + 008	305565.3	6.57	IPI:IPI00027310.3
3	1.57e + 007	0	0.00e + 000	14	3.57e + 007	22219.5	8.87	IPI:IPI00011261.1
0	0.00e + 000	0	0.00e + 000	13	1.69e + 007	65034.6	9.15	IPI:IPI00056478.1
0	0.00e + 000	0	0.00e + 000	13	8.28e + 007	47237.3	4.65	IPI:IPI00383759.1
1	5.09e + 007	0	0.00e + 000	16	3.55e + 007	21056.9	7.84	IPI:IPI00219446.1
8	1.40e + 007	47	7.06e + 006	3	7.21e + 007	52977.5	4.94	IPI:IPI00292071.3
19	7.81e + 006	36	1.31e + 007	61	5.49e + 007	35422.9	4.35	IPI:IPI00021000.1
0	0.00e + 000	0	0.00e + 000	9	1.29e + 008	101389.7	6.32	IPI:IPI00292530.1

(continued)

Table 1 (continued)

Unique peptides	Score	Protein name	Molecular function	Biological process	Cellular compartment
6	92.46	Metalloproteinase inhibitor 1	Extracellular matrix protein	Cellular organization and biogenesis	Extracellular
6	89.21	Mimecan	Growth factor activity	Signal transduction; Cell communication	Extracellular
5	95.80	Fructose-bisphosphate aldolase C	Aldolase activity	Metabolism; Energy pathways	Cytoplasm
5	95.16	Insulin-like growth factor binding protein 2	Cell adhesion molecule activity	Signal transduction; Cell communication	Extracellular
5	94.78	Neural-cadherin	Cell adhesion molecule activity	Signal transduction; Cell communication	Plasma membrane
5	93.22	Aspartate aminotransferase	Unknown	Metabolism; Energy pathways	Cytoplasm
5	92.45	Superoxide dismutase	Superoxide dismutase activity	Metabolism; Energy pathways	Cytoplasm
5	92.12	**Fibrinogen beta chain**	Blood coagulation factor activity	Protein metabolism	Extracellular
5	90.84	Hypothetical protein KIAA0830	Enzyme activity	Unknown	Extracellular
5	89.55	Dickkopf related protein-3	Receptor binding	Signal transduction; Cell communication	Extracellular
5	89.30	Coagulation factor XII	Blood coagulation factor activity	Protein metabolism	Extracellular
5	87.99	Neuronal pentraxin I	Transporter activity	Signal transduction; Cell communication	Extracellular
5	86.32	Ribonuclease 4	Enzymatic activity: Ribonuclease activity	Of nucleobase, nucleoside, nucleotide and nucleic acid	Extracellular
5	86.31	Galectin 3 binding protein	Extracellular matrix protein	Immune response	Extracellular
5	85.46	**Alpha-1-acid glycoprotein 1**	Unknown	Immune response	Extracellular
5	83.24	**Serum paraoxonase**	Hydrolase activity, acting on ester bonds	Metabolism; Energy pathways	Extracellular
5	81.19	Protein tyrosine phosphatase, receptor type, sigma isoform 2	tor signaling protein tyrosine phosphatase	Signal transduction; Cell communication	Plasma membrane
5	78.79	EGF-containing fibulin-like extracellular matrix protein 1 isoforr	Extracellular matrix protein	Cellular organization and biogenesis	Extracellular
5	75.50	**Hypothetical protein**	Unknown	Unknown	Unknown
5	71.25	Cathepsin D	Enzymatic activity: ysteine-type	Protein metabolism	Lysosome
4	76.81	Ganglioside GM2 activator	Transporter activity	Metabolism; Energy pathways	Lysosome
4	76.54	Complement factor D precursor	Serine-type peptidase activity	Immune response	Extracellular
4	73.76	Epididymal secretory protein E1	Transporter activity	Metabolism; Energy pathways	Lysosome
4	73.43	**Complement component C6**	Complement activity	Immune response	Extracellular
4	71.81	Proprotein convertase subtilisin	Peptide hormone	Signal transduction; Cell communication	Extracellular

Immunodepletion		Immunodepletion and AEC		Immunodepletion and SDS-PAGE				
Number of spectra	Mean intensity	Number of spectra	Mean intensity	Number of spectra	Mean intensity	Mw	pl	Accession number
0	0.00e + 000	2	3.65e + 006	45	1.60e + 008	40076.4	5.20	IPI:IPI00029260.2
0	0.00e + 000	0	0.00e + 000	15	4.05e + 007	42785.5	5.89	IPI:IPI00176427.1
0	0.00e + 000	0	0.00e + 000	13	4.16e + 007	23044.2	5.27	IPI:IPI00022420.2
2	3.318 + 007	7	3.45e + 006	9	3.38e + 007	55240.4	6.29	IPI:IPI00216031.1
3	1.51e + 007	0	0.00e + 000	21	2.37e + 007	24680.6	7.75	IPI:IPI00021923.1
0	0.00e + 000	0	0.00e + 000	6	8.20e + 007	159960.3	5.95	IPI:IPI00023814.1
0	0.00e + 000	2	2.29e + 006	10	4.14e + 007	99148.7	4.57	IPI:IPI00107634.2
0	0.00e + 000	0	0.00e + 000	23	7.95e + 007	23171.0	8.47	IPI:IPI00032292.1
0	0.00e + 000	0	0.00e + 000	20	2.27e + 007	33922.4	5.22	IPI:IPI00025465.1
0	0.00e + 000	0	0.00e + 000	15	4.46e + 007	39456.1	6.47	IPI:IPI00216976.1
3	1.95e + 007	0	0.00e + 000	19	5.85e + 007	35137.8	6.90	IPI:IPI00297284.1
0	0.00e + 000	6	3.44e + 006	21	1.23e + 008	99851.9	4.49	IPI:IPI00290085.1
0	0.00e + 000	0	0.00e + 000	7	5.23e + 007	46247.7	6.59	IPI:IPI00219029.1
0	0.00e + 000	37	1.37e + 007	9	2.09e + 007	15935.8	5.68	IPI:IPI00218733.1
0	0.00e + 000	0	0.00e + 000	23	4.95e + 007	55928.5	7.95	IPI:IPI00298497.3
0	0.00e + 000	5	4.89e + 006	16	2.79e + 007	59385.1	5.81	IPI:IPI00001952.2
0	0.00e + 000	12	6.85e + 006	25	1.02e + 008	38291.4	4.52	IPI:IPI00002714.1
1	2.06e + 007	0	0.00e + 000	8	1.25e + 008	67818.6	9.39	IPI:IPI00019581.1
0	0.00e + 000	0	0.00e + 000	13	4.27e + 007	47122.6	5.84	IPI:IPI00220562.1
0	0.00e + 000	0	0.00e + 000	13	4.08e + 007	16840.6	9.18	IPI:IPI00029699.1
0	0.00e + 000	0	0.00e + 000	12	9.18e + 007	65331.4	4.99	IPI:IPI00023673.1
27	9.08e + 007	12	6.43e + 006	26	5.46e + 007	23511.7	5.00	IPI:IPI00022429.1
0	0.00e + 000	0	0.00e + 000	12	3.98e + 007	39731.5	4.96	IPI:IPI00218732.1
0	0.00e + 000	3	1.90e + 006	9	6.23e + 007	212539.7	6.35	IPI:IPI00107764.1
0	0.00e + 000	0	0.00e + 000	26	5.86e + 007	52066.8	4.90	IPI:IPI00002365.1
0	0.00e + 000	0	0.00e + 000	8	5.99e + 007	54154.4	6.21	IPI:IPI00061977.1
0	0.00e + 000	0	0.00e + 000	13	5.99e + 007	44552.5	6.11	IPI:IPI00011229.1
4	5.56e + 006	2	4.67e + 006	8	3.62e + 007	20822.4	4.76	IPI:IPI00018236.1

(continued)

Table 1 (continued)

Unique peptides	Score	Protein name	Molecular function	Biological process	Cellular compartment
4	71.24	Complement C1q subcomponent	Complement activity	Immune response	Extracellular
4	69.91	Insulin-like growth factor binding protein 7	Cell adhesion molecule activity	Signal transduction; Cell communication	Extracellular
4	69.58	**Ig kappa chain C region**	antigen binding	immune response	Unknown
4	69.07	hypothetical protein MGC45438	Unknown	Unknown	Unknown
4	68.87	Actin, cytoplasmic 1	Structural constituent of cytoskeleton	Cellular organization and biogenesis	Cytoplasm
4	68.86	Peptidyl-prolyl cis-trans isomerase A	Chaperone activity	Unknown	Cytoplasm
4	68.72	Immunoglobulin superfamily, member 4B	Cell adhesion molecule activity	Signal transduction; Cell communication	Plasma membrane
4	68.54	Beta-2-microglobulin	ass II receptor activity MHC class I receptor	Immune response	Extracellular
4	65.99	CD163 antigen isoform b	Unknown	Immune response	Plasma membrane
4	63.80	**Vitronectin**	Extracellular matrix protein	Cellular organization and biogenesis	Extracellular
4	61.16	**Complement C5 beta chain**	Complement protein activity	Cellular organization and biogenesis	Extracellular
4	60.61	Extracellular matrix protein 1	Extracellular matrix protein	Cellular organization and biogenesis	Extracellular
4	59.10	SPARC precursor	Transport	Signal transduction; Cell communication	Extracellular
4	58.60	**Complement C1q subcomponent**	Immune response	Immune response	Extracellular
4	57.60	Glyceraldehyde-3-phosphate dehydrogenase	Catalytic activity	Metabolism; Energy pathways	Cytoplasm
4	57.13	Neurotrimin	Cell adhesion molecule activity	Signal transduction; Cell communication	Plasma membrane
4	56.93	Peptidyl-glycine alpha-amidating monooxygenase precursor	Transport	Metabolism	Plasma membrane
3	55.98	Neurexin 2-alpha precursor	Cell adhesion molecule activity	Signal transduction; Cell communication	Plasma membrane
3	55.12	Insulin-like growth factor binding protein 6	Cell adhesion molecule activity	Signal transduction; Cell communication	Extracellular
3	54.59	**Extracellular superoxide dismutase**	Superoxide dismutase activity	Metabolism; Energy pathways	Extracellular
3	53.90	Collagen alpha 1(XVIII)	Extracellular matrix protein	Cellular organization and biogenesis	Extracellular
3	53.20	Hypothetical protein	Unknown	Unknown	Unknown
3	52.64	Insulin-like growth factor II	Growth factor activity	Signal transduction; Cell communication	Extracellular
3	52.51	**Complement C1q subcomponent, B chain precursor**	Complement activity	Complement activity	Extracellular
3	50.84	Retinoic acid receptor responder protein	Unknown	Signal transduction; Cell communication	Plasma membrane

Immunodepletion		Immunodepletion and AEC		Immunodepletion and SDS-PAGE				
Number of spectra	Mean intensity	Number of spectra	Mean intensity	Number of spectra	Mean intensity	Mw	pl	Accession number
3	3.95e + 007	4	4.63e + 006	2	2.31e + 007	27004.1	6.85	IPI:IPI00019579.1
2	1.30e + 007	0	0.00e + 000	32	2.49e + 007	19657.9	6.95	IPI:IPI00301579.2
0	0.00e + 000	0	0.00e + 000	8	1.34e + 008	104844.8	6.11	IPI:IPI00009920.1
0	0.00e + 000	5	7.25e + 006	8	3.63e + 007	27372.5	6.24	IPI:IPI00002280.1
0	0.00e + 000	0	0.00e + 000	7	2.77e + 007	26016.7	9.34	IPI:IPI00022392.1
0	0.00e + 000	9	4.13e + 006	10	2.06e + 007	29130.5	8.06	IPI:IPI00016915.1
0	0.00e + 000	1	5.95e + 006	18	4.13e + 007	11608.9	5.53	IPI:IPI00004574.1
0	0.00e + 000	0	0.00e + 000	8	4.69e + 007	48766.4	5.70	IPI:IPI00296168.1
0	0.00e + 000	9	4.04e + 006	8	5.02e + 007	41737.0	5.29	IPI:IPI00021439.1
0	0.00e + 000	0	0.00e + 000	6	1.30e + 007	17881.4	9.14	IPI:IPI00006664.4
0	0.00e + 000	2	7.32e + 006	14	4.43e + 007	47021.2	5.07	IPI:IPI00009619.1
23	2.57e + 007	49	8.26e + 006	9	1.60e + 009	13714.6	6.07	IPI:IPI00004656.1
0	0.00e + 000	0	0.00e + 000	4	1.31e + 008	125353.4	5.69	IPI:IPI00104074.3
0	0.00e + 000	0	0.00e + 000	15	2.30e + 008	54305.9	5.47	IPI:IPI00298971.1
0	0.00e + 000	0	0.00e + 000	5	6.16e + 007	188332.4	5.01	IPI:IPI00032291.1
0	0.00e + 000	7	2.76e + 006	3	6.39e + 007	60704.5	6.19	IPI:IPI00003351.1
1	1.80e + 007	2	4.35e + 006	1	2.75e + 007	34632.4	4.66	IPI:IPI00014572.1
0	0.00e + 000	0	0.00e + 000	10	2.36e + 008	25773.8	8.33	IPI:IPI00022394.2
0	0.00e + 000	1	3.51e + 006	7	4.49e + 007	36053.4	9.30	IPI:IPI00219018.1
1	1.34e + 007	1	3.26e + 006	4	6.99e + 007	37971.7	6.01	IPI:IPI00009764.1
0	0.00e + 000	0	0.00e + 000	4	2.03e + 007	108403.7	5.83	IPI:IPI00177543.4
0	0.00e + 000	0	0.00e + 000	6	2.14e + 007	184983.0	5.55	IPI:IPI00007921.1
17	7.39e + 007	13	5.70e + 006	20	4.54e + 007	25322.6	7.94	IPI:IPI00029235.1
0	0.00e + 000	0	0.00e + 000	8	2.37e + 007	25881.1	6.32	IPI:IPI00027827.1
0	0.00e + 000	0	0.00e + 000	10	1.69e + 007	153827.4	5.40	IPI:IPI00022822.2
0	0.00e + 000	0	0.00e + 000	5	2.42e + 007	214847.3	6.77	IPI:IPI00374563.1
0	0.00e + 000	0	0.00e + 000	4	6.48e + 007	20140.5	9.50	IPI:IPI00001611.1

(continued)

Table 1 (continued)

Unique peptides	Score	Protein name	Molecular function	Biological process	Cellular compartment
3	49.69	Insulin-like growth factor binding protein 4	Cell adhesion molecule activity	Signal transduction; Cell communication	Extracellular
3	49.25	L-lactate dehydrogenase B chain	Catalytic activity	Metabolism; Energy pathways	Cytoplasm
3	48.49	**Peptidoglycan recognition protein 2**	Receptor activity	Immuno response	Intracellular/ Membrane protein
3	48.37	Sex hormone-binding globulin	Transporter activity	Transport	Extracellular
3	47.86	hypothetical protein MGC33530	Protein binding	Cell adhesion/ neurogenesis	Plasma membrane
3	47.26	hypothetical protein MGC33530	Unknown	Unknown	Unknown
3	47.14	Metalloproteinase inhibitor 2	Extracellular matrix protein	Cellular organization and biogenesis	Extracellular
3	46.83	Plasma glutathione peroxidase	Peroxidase activity	Metabolism; Energy pathways	Extracellular
3	46.04	Hypothetical protein FLJ10320	Cell adhesion molecule activity	Signal transduction; Cell communication	Extracellular
3	43.34	Protein-tyrosine phosphatase delta precursor	tor signaling protein tyrosine phosphatase	Signal transduction; Cell communication	Plasma membrane
3	39.26	**Leucine-rich alpha-3-glycoprotein**	Unknown	Unknown	Plasma membrane
3	38.51	**Complement C2**	Complement protein activity	Immune response	Extracellular
3	32.49	similar to Serine/threonine protein kinase PRKK\	Protein serine/threonine kinase activity	Signal transduction; Cell communication	Cytoplasm
3	32.46	Transforming growth factor-beta induced	Receptor binding	Signal transduction; Cell communication	Extracellular
2	43.72	Secretogranin I	Peptide hormone	Signal transduction; Cell communication	Extracellular
2	41.91	Neurexin 1-alpha	Cell adhesion molecule activity	Signal transduction; Cell communication	Plasma membrane
2	41.71	Proteoglycan 4	Unclassified	cell proliferation	Extracellular
2	41.36	**Splice isoform 1 of O94856 Neurofascin precursor**	Cell adhesion molecule activity	Signal transduction; Cell communication	Plasma membrane
2	40.41	Ribonuclease pancreatic	RNA binding	Of nucleobase, nucleoside, nucleotide and nucleic acid	Extracellular
2	38.21	Lysozyme C	Hydrolase activity	Metabolism; Energy pathways	Extracellular
2	38.12	Thy-1 membrane glycoprotein	Unknown	Immune response	Plasma membrane
2	38.10	Cartilage oligomeric matrix protein	Extracellular matrix protein	Cellular organization and biogenesis	Extracellular
2	37.91	Carboxypeptidase H	Enzymatic activity	metabolism	Plasma membrane
2	35.75	Chitinase-3 like protein 1		Signal transduction; Cell communication	Plasma membrane
				Transport	Extracellular
2	35.47	**Dystroglycan**	Cell adhesion molecule activity	Metabolism	Extracellular

Immunodepletion		Immunodepletion and AEC		Immunodepletion and SDS-PAGE				
Number of spectra	Mean intensity	Number of spectra	Mean intensity	Number of spectra	Mean intensity	Mw	pI	Accession number
0	0.00e + 000	0	0.00e + 000	15	2.86e + 007	26703.9	8.85	IPI:IPI00218746.1
2	1.69e + 007	0	0.00e + 000	12	2.19e + 007	18617.7	8.99	IPI:IPI00019176.1
2	8.12e + 006	0	0.00e + 000	5	2.38e + 008	27934.2	6.87	IPI:IPI00305380.3
0	0.00e + 000	0	0.00e + 000	8	3.22e + 007	36638.7	5.72	IPI:IPI00219217.1
0	0.00e + 000	1	3.61e + 006	2	7.15e + 007	62217.3	7.64	IPI:IPI00163207.1
0	0.00e + 000	0	0.00e + 000	5	2.58e + 007	43779.4	5.83	IPI:IPI00023019.1
0	0.00e + 000	3	2.96e + 006	3	3.99e + 007	37308.6	8.87	IPI:IPI00013303.1
0	0.00e + 000	0	0.00e + 000	6	4.90e + 006	27049.6	8.87	IPI:IPI00290411.1
0	0.00e + 000	0	0.00e + 000	4	6.78e + 007	24399.4	6.48	IPI:IPI00027166.1
0	0.00e + 000	0	0.00e + 000	5	5.38e + 007	25505.6	7.80	IPI:IPI00026199.1
0	0.00e + 000	1	2.59e + 006	2	5.57e + 007	44861.6	5.31	IPI:IPI00018073.1
0	0.00e + 000	1	4.72e + 006	3	3.61e + 007	214761.0	6.10	IPI:IPI00011642.1
0	0.00e + 000	2	6.84e + 006	6	9.36e + 007	38178.1	5.66	IPI:IPI00022417.2
1	1.09e + 007	1	7.46e + 006	1	3.95e + 007	83268.3	7.57	IPI:IPI00303963.1
3	4.84e + 007	0	0.00e + 000	0	0.00e + 000	141149.6	11.51	IPI:IPI00399428.1
0	0.00e + 000	0	0.00e + 000	3	5.01e + 007	74681.3	7.37	IPI:IPI00018219.1
5	7.10e + 006	33	5.89e + 006	0	0.00e + 000	78246.6	5.02	IPI:IPI00006601.3
0	0.00e + 000	0	0.00e + 000	2	6.93e + 007	161883.8	5.61	IPI:IPI00006314.1
0	0.00e + 000	0	0.00e + 000	2	6.89e + 007	151091.9	10.15	IPI:IPI00024825.1
0	0.00e + 000	1	5.36e + 006	2	1.00e + 008	137619.5	5.73	IPI:IPI00394652.1
0	0.00e + 000	0	0.00e + 000	17	2.36e + 007	17644.4	8.98	IPI:IPI00014048.1
0	0.00e + 000	0	0.00e + 000	6	4.27e + 008	16537.1	9.28	IPI:IPI00019038.1
11	5.59e + 007	0	0.00e + 000	8	1.84e + 007	17934.8	9.16	IPI:IPI00022892.2
0	0.00e + 000	0	0.00e + 000	2	1.06e + 008	82832.9	4.35	IPI:IPI00028030.1
0	0.00e + 000	0	0.00e + 000	6	8.26e + 007	53150.9	4.98	IPI:IPI00031121.1
0	0.00e + 000	0	0.00e + 000	4	5.60e + 007	43501.0	7.77	IPI:IPI00019533.1
0	0.00e + 000	0	0.00e + 000	10	3.18e + 007	97581.2	9.36	IPI:IPI00028911.1
0	0.00e + 000	0	0.00e + 000	2	4.85e + 007	479394.8	5.00	IPI:IPI00302641.1

(continued)

Table 1 (continued)

Unique peptides	Score	Protein name	Molecular function	Biological process	Cellular compartment
2	35.44	Protocadherin Fat 2	Transport activity	Immune response	Extracellular
2	35.43	Cystatin M	Protease Inhibitor activity	Immune response	Plasma membrane
2	34.62	**Complement component C9**	Complement protein activity	Cellular organization and biogenesis	Extracellular
2	34.44	Semaphorin 7A	Receptor activity	Signal transduction; Cell communication	Plasma membrane
2	34.44	Nidogen-2	Extracellular matrix protein	Cellular organization and biogenesis	Extracellular
2	34.42	**Oligodendrocyte-myelin glycoprotein**	Cell adhesion molecule activity	Signal transduction; Cell communication	Plasma membrane
2	34.34	Alpha 2 type I collagen	Extracellular matrix protein	Cellular organization and biogenesis	Extracellular
2	34.14	CD59 glycoprotein	Receptor activity	Immune response	Plasma membrane
2	33.81	Neuroendocrine protein 7B2	Chaperone activity	Signal transduction	Extracellular
2	33.34	Microfibril-associated glycoprotein 4	Protein binding	Cell adhesion	Extracellular
2	33.17	**Neuronal pentraxin receptor**	Receptor molecule activity	Immune response	Plasma membrane
2	32.10	DJ68D18.2.5	Transport activity	Immune response	Plasma membrane
2	32.06	**Corticosteroid-binding globulin**	Transport	Transporter activity	Extracellular
2	31.75	Neuronal growth regulator 1	Adhesion molecule activity: Neural cell	Signal transduction	Extracellular
2	31.68	Seizure related 6 homolog	Unknown	Signal transduction; Cell communication	Membrane protein
2	31.61	Pyruvate kinase 3 isoform 2	Catalytic activity	Metabolism; Energy pathways	Cytoplasm
2	30.54	**Serum amyloid A-4 protein**	Transporter activity	Transport	Extracellular
2	30.12	Calreticulin	Chaperone activity	Protein metabolism	Endoplasmic Reticulum
2	30.11	Ecto-ADP-ribosyltransferase 3	Catalytic activity	Protein metabolism	Plasma membrane
2	29.52	Hypothetical protein	protein binding	Immuno response	Extracellular
2	29.40	Vitamin K-dependent protein C	Blood coagulation factor activity	Protein metabolism	extracellular
2	29.27	Cadherin-13 precursor	Cell adhesion molecule activity	Cellular organization and biogenesis	Plasma membrane
2	27.95	**Complement component C8**	complement activity	immuno response	Plasma membrane
2	26.41	Collagen alpha 1(VI) chain	Extracellular matrix protein	Cellular organization and biogenesis	Extracellular
2	26.32	Keratin, type I cuticular HA6	Structural molecule activity	Cellular organization and biogenesis	Extracellular
2	25.64	similar to contains transmembrane (TM) region	Binding protein	Immune response	Extracellular
2	25.25	Ribonuclease K6	Enzymatic activity: Ribonuclease activity	Of nucleobase, nucleoside, nucleotide and nucleic acid	Extracellular

Immunodepletion		Immunodepletion and AEC		Immunodepletion and SDS-PAGE				
Number of spectra	Mean intensity	Number of spectra	Mean intensity	Number of spectra	Mean intensity	Mw	pl	Accession number
0	0.00e + 000	0	0.00e + 000	4	1.12e + 008	16511.2	7.00	IPI:IPI00019954.1
0	0.00e + 000	0	0.00e + 000	5	6.65e + 007	63173.8	5.42	IPI:IPI00022395.1
0	0.00e + 000	2	5.98e + 006	2	6.92e + 007	74824.2	6.58	IPI:IPI00025257.1
0	0.00e + 000	0	0.00e + 000	2	1.11e + 007	151395.8	5.06	IPI:IPI00028908.3
0	0.00e + 000	9	4.85e + 006	0	0.00e + 000	49608.2	7.80	IPI:IPI00295832.1
0	0.00e + 000	0	0.00e + 000	2	1.14e + 008	129288.4	9.74	IPI:IPI00164755.2
0	0.00e + 000	0	0.00e + 000	4	7.08e + 006	14177.4	5.18	IPI:IPI00011302.1
3	5.80e + 006	2	2.05e + 006	0	0.00e + 000	23729.8	5.49	IPI:IPI00008944.2
0	0.00e + 000	0	0.00e + 000	2	1.20e + 007	28648.4	5.21	IPI:IPI00022792.3
0	0.00e + 000	14	6.15e + 006	8	2.60e + 007	52718.4	5.72	IPI:IPI00031289.1
1	8.49e + 006	5	3.64e + 006	0	0.00e + 000	50727.8	5.18	IPI:IPI00021230.2
3	1.24e + 007	1	3.51e + 006	2	5.75e + 007	45141.1	5.64	IPI:IPI00027482.1
0	0.00e + 000	0	0.00e + 000	6	6.18e + 007	38779.5	5.81	IPI:IPI00176221.1
1	3.20e + 007	0	0.00e + 000	3	2.63e + 008	107585.8	5.05	IPI:IPI00154734.1
0	0.00e + 000	0	0.00e + 000	5	3.39e + 007	58293.6	8.66	IPI:IPI00220644.5
0	0.00e + 000	2	2.23e + 006	6	2.06e + 007	14806.8	9.19	IPI:IPI00019399.1
1	2.21e + 007	0	0.00e + 000	2	1.26e + 008	48141.8	4.29	IPI:IPI00020599.1
0	0.00e + 000	2	5.22e + 006	3	2.78e + 007	43923.5	5.64	IPI:IPI00013682.2
0	0.00e + 000	1	4.31e + 006	1	8.78e + 007	67921.9	6.00	IPI:IPI00101462.1
0	0.00e + 000	0	0.00e + 000	2	9.58e + 006	52071.6	5.60	IPI:IPI00021817.1
0	0.00e + 000	1	3.90e + 006	4	1.31e + 007	78287.2	4.77	IPI:IPI00024046.1
2	1.45e + 007	0	0.00e + 000	3	3.13e + 007	65163.6	5.74	IPI:IPI00011252.1
0	0.00e + 000	0	0.00e + 000	2	6.45e + 007	108548.1	5.23	IPI:IPI00291136.3
0	0.00e + 000	0	0.00e + 000	3	5.50e + 007	52247.4	4.90	IPI:IPI00008692.1
0	0.00e + 000	0	0.00e + 000	2	9.76e + 006	30297.3	8.99	IPI:IPI00247243.2

(continued)

Table 1 (continued)

Unique peptides	Score	Protein name	Molecular function	Biological process	Cellular compartment
2	24.57	**Keratin, type I cytoskeletal 10**	Structural molecule activity	Cellular organization and biogenesis	Cytoplasm
2	23.94	RNA-binding protein regulatory subunit	RNA binding	Of nucleobase, nucleoside, nucleotide and nucleic acid	Cytoplasm
2	23.17	Splice Isoform: 2 of Reelin	Serine-type peptidase activity	Protein metabolism	Extracellular
2	22.19	**Breast cancer type 2 susceptibility protein**	Transcription regulator activity	Of nucleobase, nucleoside, nucleotide and nucleic acid	Nucleus
2	20.75	hypothetical protein FLJ10839	DNA binding	Signal transduction	Nucleus
1	22.50	Vesicular integral-membrane protein	Transporter activity	Immune response	Extracellular
1	22.37	Protein tyrosine phosphatase, receptor type, N polypeptide 2	Enzymatic activity:tyrosine phosphatase	Signal transduction; Cell communication	Endoplasmic reticulum
1	21 .78	Cell growth regulator with EF hand domain 1	Calcium ion binding	Cell cycle arrest	Unknown
1	21.35	**Hypothetical protein FLJ20539**	Unclassified	Unclassified	Unclassified
1	21.33	Ephrin type-A receptor 4	Receptor activity	Signal transduction	Plasma membrane
1	20.42	Phosphatidylcholine-sterol	Acyltransferase activity	Metabolism; Energy pathways	Extracellular
1	20.05	Ribonuclease T2	Enzymatic activity: Ribonuclease activity	Of nucleobase, nucleoside, nucleotide and nucleic acid	Extracellular
1	19.90	Cochlin	Extracellular matrix protein		Extracellular
1	19.88	Ciliary neurotrophic factor receptor alpha	Receptor activity	Signal transduction; Cell communication	Plasma membrane
1	19.83	Frizzled-related protein	Transmembrane receptor activity	Signal transduction; Cell communication	Plasma membrane, extracellular
1	19.81	Vacuolar ATP synthase subunit S1	Transport activity	Transport	Plasma membrane
1	19.81	Guanine deaminase	Catalytic activity	neurogenesis, metabolism	Intracellular
1	19.74	Ig superfamily protein	Neural cell adhesion molecule	Signal transduction	Extracellular
1	19.73	Alpha enolase, lung specific	Hydrolase activity	Metabolism; Energy pathways	Unknown
1	19.42	**Ferritin light chain (Ferritin L subunit)**	Transport activity	Transport	Cytoplasm
1	19.41	72 kDa type IV collagenase precursor	Enzymatic activity: Metallopeptidase	Protein metabolism	Extracellular
1	19.40	Triosephosphate isomerase	Isomerase activity	Metabolism; Energy pathways	Cytoplasm

Immunodepletion		Immunodepletion and AEC		Immunodepletion and SDS-PAGE				
Number of spectra	Mean intensity	Number of spectra	Mean intensity	Number of spectra	Mean intensity	Mw	pl	Accession number
0	0.00e + 000	0	0.00e + 000	2	2.72e + 007	17196.3	9.22	IPI:IPI00004114.1
0	0.00e + 000	0	0.00e + 000	2	3.94e + 007	59518.9	5.13	IPI:IPI00009865.1
1	2.10e + 007	0	0.00e + 000	2	2.25e + 007	19891.2	6.40	IPI:IPI00298547.1
0	0.00e + 000	0	0.00e + 000	2	7.60e + 007	388217.7	5.54	IPI:IPI00241562.1
1	1.19e + 008	0	0.00e + 000	1	7.11e + 007	384360.4	6.03	IPI:IPI00293471.2
0	0.00e + 000	1	4.36e + 006	1	4.85e + 007	132706.6	8.99	IPI:IPI00217357.1
0	0.00e + 000	0	0.00e + 000	1	2.33e + 007	40228.9	6.06	IPI:IPI00009950.1
0	0.00e + 000	1	4.75e + 006	0	0.00e + 000	114795.6	5.59	IPI:IPI00024289.4
0	0.00e + 000	2	4.61e + 006	0	0.00e + 000	31976.7	4.39	IPI:IPI00008584.1
0	0.00e + 000	0	0.00e + 000	2	8.17e + 007	83181.7	5.28	IPI:IPI00301865.1
0	0.00e + 000	0	0.00e + 000	6	9.45e + 007	109860.6	6.32	IPI:IPI00008318.1
0	0.00e + 000	0	0.00e + 000	1	6.48e + 007	49578.2	5.72	IPI:IPI00022331.1
0	0.00e + 000	1	1.74e + 006	1	5.50e + 006	29708.2	6.2	IPI:IPI00299103.2
0	0.00e + 000	0	0.00e + 000	2	2.41e + 007	59483.3	8.24	IPI:IPI00012386.1
1	3.28e + 006	0	0.00e + 000	0	0.00e + 000	40633.4	6.27	IPI:IPI00003102.1
0	0.00e + 000	0	0.00e + 000	1	3.43e + 007	36254.2	8.63	IPI:IPI00294650.3
0	0.00e + 000	0	0.00e + 000	1	1.63e + 007	52154.2	5.28	IPI:IPI00020430.2
0	0.00e + 000	0	0.00e + 000	2	2.19e + 007	51003.3	5.44	IPI:IPI00032461.1
0	0.00e + 000	0	0.00e + 000	3	2.37e + 007	43987.3	5.92	IPI:IPI00027038.1
0	0.00e + 000	0	0.00e + 000	1	2.71e + 007	49477.6	5.78	IPI:IPI00013769.1
0	0.00e + 000	0	0.00e + 000	2	9.80e + 006	25826.4	5.29	IPI:IPI00074333.1
0	0.00e + 000	0	0.00e + 000	2	1.22e + 008	73882.7	5.26	IPI:IPI00027780.1
0	0.00e + 000	0	0.00e + 000	1	2.23e + 007	26538.4	6.51	IPI:IPI00328807.3
0	0.00e + 000	0	0.00e + 000	3	7.97e + 006	27745.3	4.73	IPI:IPI00021263.1
0	0.00e + 000	0	0.00e + 000	1	2.52e + 007	138884.1	7.98	IPI:IPI00297646.1

(continued)

Table 1 (continued)

Unique peptides	Score	Protein name	Molecular function	Biological process	Cellular compartment
1	19.39	14-3-3 protein zeta/delta (Protein kinase C inhibitor protein	Receptor signaling complex scaffold activity	Signal transduction; Cell communication	Cytoplasm
1	19.17	**Collagen alpha 1(I) chain**	Extracellular matrix protein	Cellular organization and biogenesis	Extracellular
1	19.14	Parvalbumin alpha	Calcium binding protein	Signal transduction; Cell communication	Cytoplasm
1	19.05	HGFL protein	Growth factor activity	Signal transduction; Cell communication	Extracellular
1	18.92	Keratin, type II	Structural constituent of cytoskeleton	Cellular organization and biogenesis	Cytoplasm
1	18.64	Hypothetical protein FLJ13813	Unknown	Unknown	Unknown
1	18.34	Malate dehydrogenase	Catalytic activity	Metabolism; Energy pathways	Cytoplasm
1	18.33	Hypothetical protein	Cell adhesion	Signal transduction	Plasma membrane
1	18.05	14-3-3 protein beta/alpha	Receptor signaling complex scaffold activity	Signal transduction; Cell communication	Cytoplasm
1	18.03	Nidogen	Extracellular matrix protein	Cellular organization and biogenesis	Extracellular
1	17.97	**Nucleobindin 1**	Calcium ion binding	Signal transduction; Cell communication	Golgi apparatus
1	17.61	Plexin domain containing 2 precursor	Receptor activity	Signal transduction; Cell communication	Extracellular
1	17.34	Neuroserpin	Protease Inhibitor activity	Protein metabolism	Extracellular
1	17.03	Lymphocyte antigen Ly-6H	Unknown	Immune response / neurogenesis	Plasma membrane
1	16.89	Major prion protein precursor	Unknown	Metabolism	Plasma membrane
1	16.61	Contactin associated protein-like 4	Cell adhesion	Signal transduction	Plasma membrane
1	16.52	**Apolipoprotein L1**	Transporter activity	Transport	Extracellular
1	16.31	MIC2L1 isoform E3′-E4′-E3-E4	Unknown	Unknown	Unknown
1	16.31	similar to cerebellin	Unknown	Unknown	Unknown
1	16.26	Thrombospondin 4 precursor	Extracellular matrix protein	Cellular organization and biogenesis	Extracellular
1	16.05	Collagen alpha 1(XV)	Extracellular matrix protein	Cellular organization and biogenesis	Extracellular
1	15.94	Extracellular matrix protein 2	Extracellular matrix protein	Cellular organization and biogenesis	Extracellular
1	15.72	Hypothetical protein FLJ90018	Calcium binding protein	Unknown	
1	15.68	Cystatin B	Protease Inhibitor activity	Protein metabolism	Nucleus
1	15.61	Neurexin 3-alpha precursor	Receptor activity	Cell adhesion	Plasma membrane
1	15.58	Ly-6/neurotoxin-like protein 1	Unknown	Unknown	Unknown
1	15.54	Plasma serine protease inhibitor	Protease Inhibitor activity	Unknown	Extracellular
1	15.51	Hypothetical protein KIAA0315	Receptor activity	development	Plasma membrane
1	15.44	**Cell surface glycoprotein MUC18**	Cell adhesion molecule activity	Signal transduction; Cell communication	Plasma membrane

Immunodepletion		Immunodepletion and AEC		Immunodepletion and SDS-PAGE				
Number of spectra	Mean intensity	Number of spectra	Mean intensity	Number of spectra	Mean intensity	Mw	pI	Accession number
0	0.00e + 000	2	2.43e + 006	0	0.00e + 000	12058.8	4.22	IPI:IPI00219703.1
0	0.00e + 000	2	3.15e + 006	0	0.00e + 000	28248.2	4.77	IPI:IPI00298388.1
0	0.00e + 000	0	0.00e + 000	1	1.06e + 008	56683.2	6.40	IPI:IPI00300053.3
0	0.00e + 000	0	0.00e + 000	1	2.89e + 007	25953.6	6.19	IPI:IPI00030385.1
0	0.00e + 000	0	0.00e + 000	1	1.73e + 007	36426.3	6.89	IPI:IPI00291005.2
0	0.00e + 000	0	0.00e + 000	9	8.01e + 006	24712.7	6.88	IPI:IPI00061246.1
0	0.00e + 000	0	0.00e + 000	1	2.11e + 006	28082.5	4.76	IPI:IPI00216318.1
0	0.00e + 000	0	0.00e + 000	1	5.36e + 006	136489.9	5.09	IPI:IPI00026944.1
0	0.00e + 000	13	3.72e + 006	0	0.00e + 000	53821.6	5.06	IPI:IPI00295542.1
0	0.00e + 000	5	3.91e + 006	0	0.00e + 000	59583.5	5.99	IPI:IPI00044369.1
0	0.00e + 000	0	0.00e + 000	2	4.24e + 007	46427.2	4.84	IPI:IPI00016150.1
0	0.00e + 000	0	0.00e + 000	6	1.57e + 007	14669.1	6.43	IPI:IPI00014964.1
0	0.00e + 000	0	0.00e + 000	2	1.03e + 007	27661.3	9.39	IPI:IPI00022284.1
0	0.00e + 000	0	0.00e + 000	1	2.87e + 007	145660.9	6.22	IPI:IPI00216250.3
0	0.00e + 000	0	0.00e + 000	1	4.17e + 007	44026.6	5.71	IPI:IPI00177869.4
0	0.00e + 000	2	4.06e + 006	0	0.00e + 000	27986.0	4.92	IPI:IPI00152491.1
0	0.00e + 000	0	0.00e + 000	1	1.46e + 007	21520.7	6.72	IPI:IPI00402157.1
0	0.00e + 000	0	0.00e + 000	2	3.91e + 006	105802.9	4.44	IPI:IPI00328550.2
0	0.00e + 000	0	0.00e + 000	3	1.06e + 007	142569.9	5.3	IPI:IPI00295414.3
0	0.00e + 000	0	0.00e + 000	1	6.71e + 007	79789.8	5.30	IPI:IPI00015315.1
0	0.00e + 000	0	0.00e + 000	1	1.43e + 008	68667.7	4.49	IPI:IPI00384073.1
0	0.00e + 000	0	0.00e + 000	1	7.41e + 007	11139.6	6.98	IPI:IPI00021828.1
0	0.00e + 000	0	0.00e + 000	1	1.32e + 008	169924.4	5.76	IPI:IPI00006300.2
0	0.00e + 000	0	0.00e + 000	13	5.39e + 007	12641.1	8.09	IPI:IPI00289058.3
0	0.00e + 000	0	0.00e + 000	3	1.22e + 007	45702.0	9.35	IPI:IPI00007221.1
0	0.00e + 000	0	0.00e + 000	1	1.07e + 008	205412.7	5.88	IPI:IPI00004462.1
0	0.00e + 000	2	4.79e + 006	0	0.00e + 000	71794.0	5.52	IPI:IPI00016334.1
0	0.00e + 000	4	4.57e + 006	0	0.00e + 000	61133.3	5.39	IPI:IPI00218413.1
0	0.00e + 000	0	0.00e + 000	6	2.32e + 007	40526.9	9.67	IPI:IPI00013909.1
0	0.00e + 000	0	0.00e + 000	1	7.91e + 006	20927.3	6.59	IPI:IPI00164876.1

(continued)

Table 1 (continued)

Unique peptides	Score	Protein name	Molecular function	Biological process	Cellular compartment
1	15.35	Biotinidase	Hydrolase activity	Metabolism; Energy pathways	Extracellular
1	14.92	Apoptosis-inducing factor (AIF)-like mitochondrion-associated inducer of death	Receptor activity	Signal transduction; Cell communication	Plasma membrane
1	14.91	BA145L22.1.2 (Myelin/oligodendrocyte glycoprotein (MOG)	Cell adhesion molecule activity	Signal transduction; Cell communication	Plasma membrane
1	14.72	Opioid binding protein	opioid receptor activity	Signal transduction; Cell communication	Plasma membrane
1	14.70	Phosphoglycerate mutase 2	Catalytic activity	Metabolism; Energy pathways	Cytoplasm
1	14.52	Phosphoglycerate kinase 1	Catalytic activity	Metabolism; Energy pathways	Cytoplasm
1	14.43	hypothetical protein FLJ10006	Unknown	Unknown	Unknown
1	14.08	**Keratin, type I**	Structural constituent of cytoskeleton	Cellular organization and biogenesis	Cytoplasm
1	13.84	hypothetical protein MGC22776	Unknown	Unknown	Unknown
1	13.82	Splice form of interleukin-6 receptor beta chain	Receptor activity: transmembrane	Immune response	Plasma membrane
1	13.68	Proenkephalin A	neuropeptide hormone activity	Signal transduction	Cytoplasm
1	13.66	GAS2-related protein	Unknown	Unknown	Unknown
1	13.65	similar to Ba1-651	Unknown	Unknown	Unknown
1	13.54	endoglycan	Binding:glycosaminoglycan binding		Plasma membrane
1	13.46	hypothetical protein XP_376158	Unknown	Unknown	Unknown
1	13.44	BA416N2.2	signal transduction	Signal transduction	Plasma membrane
1	13.21	Transgelin	Structural constituent of cytoskeleton	Cellular organization and biogenesis	Cytoplasm
1	13.04	Alpha-2-antiplasmin	Protease Inhibitor activity	Unknown	Extracellular
1	13.03	HGF activator like protein (Hyaluronan binding protein 2)	Binding protein	Cell adhesion	Extracellular

weight than the one predicted for the respective full-length protein.

Conclusions

As was anticipated at the outset of these studies, we have confirmed that extensive prefractionation of complex protein mixtures leads to an increased coverage of the sample's protein constituents. This is exemplified by using AEC or SDS-PAGE to separate CSF protein mixtures that had been previously immunodepleted of the six most abundant

Immunodepletion		Immunodepletion and AEC		Immunodepletion and SDS-PAGE				
Number of spectra	Mean intensity	Number of spectra	Mean intensity	Number of spectra	Mean intensity	Mw	pl	Accession number
0	0.00e + 000	0	0.00e + 000	3	1.43e + 008	38007.8	5.89	IPI:IPI00001662.1
0	0.00e + 000	0	0.00e + 000	1	1.11e + 007	28850.3	9	IPI:IPI00218570.1
0	0.00e + 000	0	0.00e + 000	1	2.98e + 007	44615.0	9.22	IPI:IPI00169383.1
0	0.00e + 000	0	0.00e + 000	22	1.20e + 008	91955.0	4.43	IPI:IPI00296432.1
0	0.00e + 000	0	0.00e + 000	2	5.30e + 006	62064.6	5.14	IPI:IPI00019359.2
1	4.97e + 007	3	6.83e + 006	0	0.00e + 000	25431.2	7.82	IPI:IPI00163563.1
0	0.00e + 000	0	0.00e + 000	2	3.94e + 007	37484.7	5.46	IPI:IPI00218963.1
0	0.00e + 000	1	3.39e + 006	0	0.00e + 000	30787.2	5.44	IPI:IPI00000828.1
10	1.34e + 008	16	6.74e + 006	0	0.00e + 000	96520.4	10.21	IPI:IPI00169377.1
0	0.00e + 000	0	0.00e + 000	2	1.99e + 008	65929.3	5.27	IPI:IPI00402024.1
0	0.00e + 000	0	0.00e + 000	1	1.03e + 008	65076.1	4.12	IPI:IPI00024585.1
2	1.66e + 008	2	2.42e + 007	0	0.00e + 000	722928.1	6.04	IPI:IPI00400963.1
0	0.00e + 000	0	0.00e + 000	7	2.17e + 007	115717.9	8.75	IPI:IPI00400923.1
0	0.00e + 000	0	0.00e + 000	2	2.28e + 007	22475.9	8.88	IPI:IPI00216138.1
0	0.00e + 000	0	0.00e + 000	1	3.02e + 007	54566.1	5.87	IPI:IPI00029863.1
0	0.00e + 000	0	0.00e + 000	1	7.55e + 007	63832.5	6.1	IPI:IPI00041065.2

The proteins were identified by subjecting MS/MS data of the tryptic peptides to Spectrum Mill *(9)* analysis using default extraction and search parameters. The Spectrum Mill software ranks the proteins according to the number of unique peptides that were identified for all three runs. Proteins that are present in two comprehensive human serum databases *(10,11)* are indicated with a bold font in the "Protein name" column.

proteins. The subsequent shotgun mass spectrometry and protein identification analyses benefit from both the removal of abundant proteins as well as prefractionation of the CSF sample on the protein level. This is a result of the generation of a smaller dynamic range of the remaining protein mixture. In addition, because of the smaller amount of total protein that results from the combination of immunodepletion and prefractionation, a greater equivalent of the CSF sample can be used during the shotgun mass spectrometry analysis

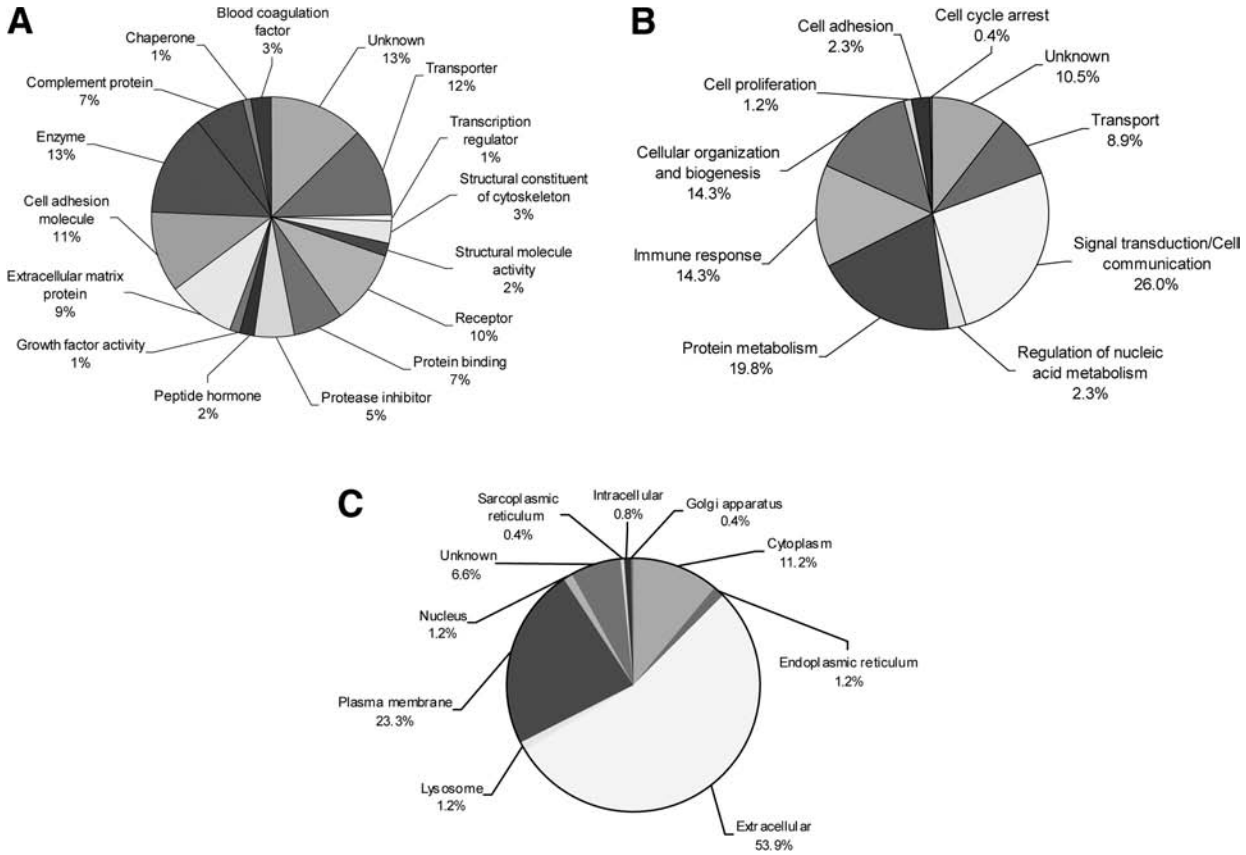

Fig. 4. Protein categories that were identified in cerebrospinal fluid from a patient with normal pressure hydrocephalus after immunodepletion and protein prefractionation followed by shotgun mass spectrometry analysis. Proteins are grouped according to the Gene Ontology Consortium (http://www.geneontology.org) classification by molecular function **(A)**, involvement in biological processes **(B)**, and cellular compartment location **(C)**.

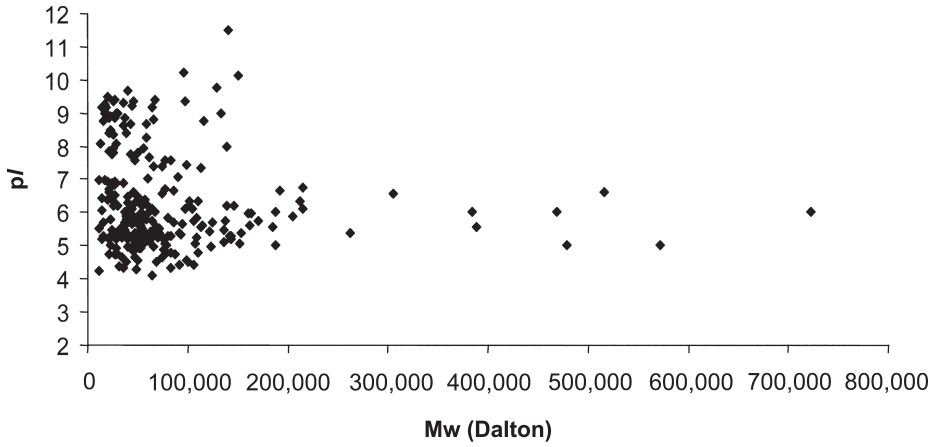

Fig. 5. Predicted isoelectric point (p*I*) and molecular weight (Mw) of proteins that were identified in the cerebrospinal fluid proteome. The information is based on the full-length sequences of the proteins as they are listed in the human IPI protein database.

without the risk of overloading the reversed-phase nano column that is used on-line with the mass spectrometer. Our previous attempts to use crude CSF (neither immunodepleted nor prefractionated) in a shotgun mass spectrometry analysis resulted in only 38 proteins that were identified as a result of the overwhelming presence of HSA, Ig, and other abundant protein-derived peptides (data not shown).

Approximately 47% of the CSF proteins that we identified were also found during in-depth proteomic analyses of human serum *(10,11)*. This partial overlap can be attributed to the following observations. On one hand, as discussed above, there is an inherently low overlap between shotgun analyses, especially when performed in different laboratories using different fractionation approaches and different equipment. Second, there is variability between the individuals whose body fluids were analyzed. Third, certain proteins are removed in the process of generating serum from blood and hence will not show up in the serum protein list. Finally, brain-derived proteins are present in larger amounts in CSF than in serum because of the proximity of CSF and the brain and the rather low exchange between the two body fluids. The latter reason makes CSF the most relevant source for a proteomics study that is aimed at the identification of markers for brain disorders. The CSF flow rate has been recognized as the key modulator of blood-derived and brain-derived proteins in this body fluid. A close approximation of the protein portion that is derived from blood can be gained by measuring the CSF/blood albumin ratio *(12)*. However, at the same time, it has been shown that blood-derived proteins in CSF follow a hyperbolic function, which depends primarily on the molecular weight of the proteins *(12)*.

Other reports that describe attempts to analyze the CSF proteome have been published. Most of these studies have used 2D-PAGE for sample preparation followed by mass spectrometric identification of the stained protein spots. The most detailed analysis of CSF protein constituents up to now has been performed by Sickmann et al. *(13)*. The analysis of over 480 protein spots from a 2D-PAGE fractionation of undepleted CSF resulted in the identification of 24 proteins from 65 spots, of which 15 were also found in our analyses. As expected, many proteins were identified multiple times because of the great number of protein isoforms found in CSF. In another report, Davidsson et al. used preparative liquid isoelectric focusing in combination with one-dimensional SDS gel electrophoresis for the identification of 32 CSF proteins, of which we identified 24 proteins in our analyses *(14)*. This procedure was able to identify a number of low abundant proteins in undepleted CSF samples because of the concentration in a few fractions of the abundant HSA and Ig proteins during the preparative isoelectric focusing step.

In our future studies, we will continue our CSF proteome mining efforts by trying to identify protein constituents of ever lower abundance. This will require not only the use of further fractionation steps, but, at the same time, the use of increased amounts of CSF material. Furthermore, we have begun to extend our strategies by establishing a proteomic platform that will allow us to compare the quantities of many proteins between different CSF samples. The quantitative approach will enable us to compare CSFs from different patient groups and result in the identification of patterns of proteins that are up- or down-regulated, leading to important markers of brain disease.

Acknowledgments

This work was supported by the Max Planck Society and a grant from the Bavaria California Technology Center (BaCaTeC).

References

1. Holsboer, F. (2001). Antidepressant drug discovery in the postgenomic era. World J. Biol. Psychiatry 2:165–177.
2. Reiber, H. (2001). Dynamics of brain-derived proteins in cerebrospinal fluid. Clin. Chim. Acta 310:173–186.
3. Anderson, N.L. and Anderson, N.G. (1998). Proteome and proteomics: new technologies, new concepts, and new words. Electrophoresis 19:1853–1861.
4. Klose, J. (1975). Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutations in mammals. Humangenetik 26:231–243.
5. O'Farrel, P.H. (1975). High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250:4007–4021.
6. Link, A.J. (2002). Multidimensional peptide separations in proteomics. Trends Biotechnol. 20(Suppl. 12):S8–S13.
7. Maccarrone, G., Milfay, D., Birg, I., Rosenhagen, M., Grimm, R., Bailey, J., et al. (2004). Mining the human CSF proteome by immunodepletion and shotgun mass spectrometry. Electrophoresis 25:2402–2412.
8. Eng, J., McCormack, J.R., and Yates, J. (1994). An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. J. Am. Soc. Mass Spectrom. 5:976–984.
9. Spectrum Mill MS Proteomics Workbench-Comprehensive tools for MS proteomics. 2003. Available at: http://www.agilent.com.
10. Anderson, N.L., Polanski, M., Pieper, R., Gatlin, T., Tirumalai, R.S., Conrads, T.P., et al. (2004). The human plasma proteome. Mol. Cell. Proteomics 3:311–326.
11. Chan, K.C., Lucas, D.A., Hise, D., Schaefer, C.F., Xiao, Z., Janini, G.M., et al. (2004). Analysis of the human serum proteome. Clin. Proteomics 1:101–225.
12. Reiber, H., Otto, M., Trendelenburg, C., and Wormek, A. (2001). Reporting cerebrospinal fluid data: knowledge base and interpretation software. Clin. Chem. Lab. Med. 39: 324–332.
13. Sickmann, A., Dormeyer, W., Wortelkamp, S., Woitalla, D., Kuhn, W., and Meyer, H.E. (2002). Towards a high resolution separation of human cerebrospinal fluid. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 771: 167–196.
14. Davidsson, P., Paulson, L., Hesse, C., Blennow, K., and Nilsson, C.L. (2001). Proteome studies of human cerebrospinal fluid and brain tissue using a preparative two-dimensional electrophoresis prior to mass spectrometry. Proteomics 1:444–452.