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Relative Quantification of Proteins in Human Cerebrospinal Fluids by MS/MS Using 6-Plex Isobaric Tags

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A new 6-plex isobaric mass tagging technology is presented, and proof of principle studies are carried out using standard protein mixtures and human cerebrospinal fluid (CSF) samples. The Tandem Mass Tags (TMT) comprise a set of structurally identical tags which label peptides on free amino-terminus and epsilon-amino functions of lysine residues. During MS/MS fragmentation, quantification information is obtained through the losses of the reporter ions. After evaluation of the relative quantification with the 6-plex version of the TMT on a model protein mixture at various concentrations, the quantification of proteins in CSF samples was performed using shotgun methods. Human postmortem (PM) CSF was taken as a model of massive brain injury and comparison was carried out with antemortem (AM) CSF. After immunoaffinity depletion, triplicates of AM and PM CSF pooled samples were reduced, alkylated, digested by trypsin, and labeled, respectively, with the six isobaric variants of the TMT (with reporter ions from $m/z = 126.1$ to 131.1 Th). The samples were pooled and fractionated by SCX chromatography. After RP-LC separation, peptides were identified and quantified by MS/MS analysis with MALDI TOF/TOF and ESI-Q-TOF. The concentration of 78 identified proteins was shown to be clearly increased in PM CSF samples compared to AM. Some of these proteins, like GFAP, protein S100B, and PARK7, have been previously described as brain damage biomarkers, supporting the PM CSF as a valid model of brain insult. ELISA for these proteins confirmed their elevated concentration in PM CSF. This work demonstrates the validity and robustness of the tandem mass tag (TMT) approach for quantitative MS-based proteomics.

The measurement of the relative protein abundance in cells, tissues, or body fluids is a major task in proteomics. In the field of biomarker discovery, in particular, proteins that are found to exhibit significant up- or down-regulation in disease-state samples compared to healthy-state samples are selected and investigated as potential biochemical markers of the considered pathologies. Several quantification methods have therefore been developed based either on protein differential gel electrophoresis and comparison of staining intensities[1] or proteolytic peptide mass spectrometry (MS) and comparison of ion counts.[2,3] MS-based quantification is achieved "label-free" or via differential biological or chemical incorporation of stable isotopes into the intact proteins or into the proteolytic peptides. Basically, a sample labeled with a "light" isotope is mixed with another sample labeled with a "heavy" isotope and introduced into a mass spectrometer, after prior separation by gel electrophoresis or liquid chromatography. The analyzed proteolytic peptides from both samples share the same physicochemical properties but are detected differentially in the mass spectrometer due to their mass difference, thus enabling the comparison of their ion relative abundance and, by extension, the relative abundance of the proteins from which they derive. Since the introduction of the isotope-coded affinity tag (ICAT),[4] chemical labeling has been widely used in quantitative MS-based proteomics. At variance with the ICAT strategy that focuses on the targeted quantification of cysteine-containing peptides, primary amine coding has been implemented for global proteomic analyses.[5] Nevertheless, using these techniques, the peptide identification and their relative quantification are achieved sequentially through tandem MS (MS/MS) and MS, respectively.

MS-based quantification using Tandem Mass Tags (TMTs) has first addressed this issue.[6] The TMTs permit simultaneous identification and relative quantification during the MS/MS mode.

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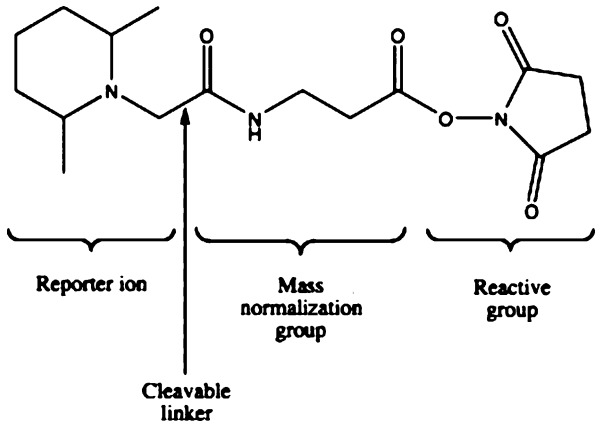
Scheme 1. Structure of the Tandem Mass Tag (TMT)[a]



[a] The reporter ion provides the abundance of a peptide upon MS/MS in individual samples being mixed. The cleavable linker enables the release of the reporter ion from the whole tag upon MS/MS. The mass normalization group balances mass differences from individual ion reporter to ensure the same overall mass of the label reagents. The reactive group labels amino groups.

Furthermore, the TMTs are reactive toward free amino-terminus peptides and epsilon-amino functions of lysine residues and are therefore not restricted to a particular class of peptides. An important advantage of such tags is that identical peptides in different samples remain isobaric after tagging and appear as single peaks in MS scans, thus enhancing the peptide detection and reducing the probability of peak overlaps. The tags are composed of a primary amine reactive group and an isotopic reporter group linked by an isotopic balancer group for the normalization of the total mass of the tags. The reporter group serves for quantification since it is cleaved during collision-induced dissociation (CID) to yield a characteristic isotope fragment. Since the commercial introduction of the iTRAQ reagents[7] several groups have used such an approach for the relative quantification of proteins between biological extracts. Recently, the iTRAQ strategy has been used for the comparison of cerebrospinal fluid (CSF) protein abundance in male vs female subjects[8] and in patients with neurodegenerative disorders[9] using 4-plex reagents. The quantification of changes in CSF protein expression in individuals undergoing intravenous immunoglobulin treatment for Alzheimer's disease using a prototype 8-plex reagent was also carried out.[10]

The 6-plex Tandem Mass Tags are newly designed isobaric tagging reagents (Scheme 1). Their chemical structure enables the introduction of five "heavy" isotopes (^{13}C or ^{15}N) in the reporter group and five "heavy" isotopes (^{13}C or ^{15}N) in the balancer group to provide six isobaric tags for the comparison of up to six different extracts. With respect to the first generation of 2-plex TMTs,[6] the 6-plex TMTs are structurally simpler, have smaller mass, and have been engineered in order to promote enhanced cleavage of the reporter groups, which are released in a pseudo-quiet region of the tandem mass spectra. The use of C and N isotopes instead of H isotopes guaranties the coelution of the differentially labeled peptides during liquid chromatography separation[11] in addition to other identical physicochemical properties such as ionization efficiency. In this work, the 6-plex TMTs were employed first on a model protein mixture for the relative quantification of six levels of protein abundance ranging from 1 to 10. The accuracy of the relative quantification, its relative standard deviation, and its dynamic range were assessed. Body fluids are widely studied to find biomarkers for the diagnosis and prognosis of diseases.[12] Notably, CSF is a remarkable sample to study brain-related disorders. In previous works,[13−15] it has been shown that post-mortem (PM) CSF can be used as a model of massive brain injury for the identification of potential brain damage biomarkers. The TMT technology was therefore used herein for the further assessment of protein differential abundance in antemortem (AM) and PM CSF. In order to increase the confidence in the quantification values, intrarun triplicates of both CSF samples were performed using each of the 6-plex TMTs. In addition, before any handling, CSF samples were spiked with an equivalent amount of a bovine protein to correct potential bias induced during the subsequent analytical workflow. The analysis protocol combined techniques such as immunoaffinity depletion of abundant proteins, trypsin digestion followed by differential TMT labeling, strong cation-exchange (SCX) chromatography, reversed-phase high performance liquid chromatography (RP-HPLC), and MS/MS. A total of 238 proteins were identified, 78 of which appeared clearly as increased in concentration in PM CSF samples. These results were then assessed with respect to the recognized or potential interest of these proteins as brain damage biomarkers. Some of these proteins, like GFAP, protein S100B, or PARK7, were also tested by enzyme-linked immunosorbent assays (ELISA) to confirm their elevated concentration in PM CSF. This work demonstrates the general applicability of the TMT approach for quantitative MS-based proteomics and biomarker discovery.

EXPERIMENTAL SECTION

Materials. Myoglobin (MYO) from horse heart (≥90%), β-lactoglobulin (LACB) from bovine milk (~90%), and trypsin from porcine pancreas (16 000 units mg^{-1}) were purchased from Sigma (St. Louis, MO). Lysozyme (LYS) from hen egg white (10 500 units mg^{-1}) and albumin (ALBU) from bovine serum (≥98%) were from Fluka (Büchs, Switzerland). Triethylammonium hydrogen carbonate buffer (TEAB) 1 M pH = 8.5, sodium dodecyl sulfate (SDS, ≥98%), orthophosphoric acid (85%), trifluoroacetic acid

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(TFA, ≥99.5%), formic acid (FA, ≥98%), and hydrochloric acid fuming (37%) were from Fluka. Iodoacetamide (IAA, ≥99%), tris-(2-carboxyethyl) phosphine hydrochloride (TCEP) 0.5 M, potassium phosphate monobasic (99%), and α-cyano-4-hydroxycinnamic acid were from Sigma. Hydroxylamine solution 50 wt % in H_2O (99.999%) was from Aldrich (Milwaukee, WI). Potassium chloride (>99.5%) was from Merck (Darmstadt, Germany). Water for chromatography LiChrosolv and acetonitrile Chromasolv for HPLC (≥99.9%) were, respectively, from Merck and Sigma-Aldrich (Büchs, Switzerland). The 6-plex Tandem Mass Tags were provided by Proteome Sciences (Frankfurt am Main, Germany).

CSF Collection. Postmortem (PM) CSF samples from different patients ($n = 4$) were collected by ventricular puncture at autopsy, 6 h after death on average. Control antemortem (AM) CSF samples were collected by routine diagnostic lumbar puncture from living healthy patients ($n = 4$). Clinical data of deceased and living patients have been previously reported.[15] Each patient or patient's relatives gave informed consent prior to enrollment. The local institutional ethical committee board approved the clinical protocol.

Immunoaffinity Depletion, Protein Concentration, and Bradford Assay. An amount of 1 mL of pooled CSF ($n = 4$, spiked with 1 μg of LACB) was diluted 1:5 in MARS buffer A (Agilent, Palo Alto, CA) and passed through a 0.20 μm filter (Vivascience, Hannover, Germany). Aliquots of 1 mL were injected on a 4.6 mm × 100 mm MARS column (Agilent) that removes some of the more abundant CSF proteins (albumin, transferrin, IgG, IgA, antitrypsin, and haptoglobin). The flow-through fractions were collected, pooled, and concentrated, after prior buffer exchange with H_2O, to approximately 1 mL using 10 kDa MWCO centrifugal filter devices (Centricon Plus-20, Millipore, Billerica, MA). Three identical AM and PM samples were prepared this way. A Bradford assay (Protein Assay, Bio-Rad, Hercules, CA) gave, respectively, a mean amount of 34 and 63 μg of proteins in 1 mL of the depleted AM and PM CSF samples. The six final samples were evaporated under speed-vacuum.

Reduction, Alkylation, Digestion, and Labeling with TMT of a Model Protein Mixture. An amount of 250 μg of ALBU, MYO, LYS, and LACB was dissolved in 400 μL TEAB, 0.5 M, pH = 8.5. TCEP at 50 mM concentration in water (20 μL) was added. The reaction was performed for 60 min at 60 °C. IAA at a concentration of 400 mM was then added (10 μL), and the mixture reacted for 30 min in the dark at room temperature (RT). Freshly prepared trypsin (0.2 μg μL^{-1}) was added (100 μL to obtain a ratio 1:50 w/w), and the digestion was performed overnight at 37 °C. Aliquots of the digested sample were prepared to obtain a total amount of 10, 20, 30, 30, 50, and 100 μg of tryptic peptides. TMT labeling was performed on each aliquot with TMT with respective reporters at m/z = 126.1, 127.1, 128.1, 129.1, 130.1 and 131.1 Thomson (Th) in 40.2 μL CH_3CN. After 60 min of reaction at RT, 8 μL hydroxylamine 5% (w:V) was added in each tube, and mixed for 15 min. The aliquots were then combined and the pooled sample was evaporated under vacuum. The sample was then dissolved in 1894 μL H_2O/TFA 99.9%/0.1% before LC-MS analysis.

Reduction, Alkylation, Digestion, and Labeling with TMT of the CSF Samples. The six depleted CSF samples were dissolved in 40 μL of TEAB, 0.5 M, adjusted at pH = 8.2 with diluted HCl. SDS 1% in water (w:w) was added (5 μL). An amount of 2 μL of 50 mM TCEP was added. The reaction was performed for 60 min at 60 °C. An amount of 1 μL of IAA at 400 mM was then added, and the mixtures were reacted for 30 min in the dark at RT. An amount of 10 μL of freshly prepared trypsin at 0.2 μg μL^{-1} concentration in TEAB (0.5 M) were added. The digestion was performed overnight at 37 °C. The three AM samples were labeled by TMT with reporters at m/z = 126.1, 128.1, and 130.1 Th in 40.2 μL of CH_3CN and the three PM samples by TMT with reporters at m/z = 127.1, 129.1, and 131.1 Th in 40.2 μL of CH_3CN. After 60 min of reaction at RT, 8 μL of 5% hydroxylamine (w/V) was added in each tube and mixed for 15 min. The six samples were pooled in a new tube and dried for storage at −20 °C.

SCX Fractionation of the Pooled TMT-Labeled CSF Samples. The pooled TMT-labeled CSF sample was fractionated by strong cation-exchange (SCX) chromatography using a 30 min gradient at 0.2 mL min^{-1} on a 2.1 mm × 100 mm, (5 μm, 200 Å) Polysulfoethyl A column (PolyLC, Columbia, MD). The gradient was run as follows: 0−3 min 100% A (10 mM KH_2PO_4, 25% CH_3CN, adjusted to pH = 3 with H_3PO_4), then to 50% A and 50% B (10 mM KH_2PO_4, 500 mM KCl, 25% CH_3CN, adjusted to pH = 3 with H_3PO_4) at 23 min, 100% B at 30 min. Fractions were collected every minute. The fractions were dried under vacuum. They were dissolved in 20 μL of H_2O/TFA 99.9%/0.1% for further LC−MS/MS analysis.

LC−MS/MS. ESI-MS experiments were carried out on a Micromass Q-Tof1 from Waters (Mildford, MA). The mass spectrometer was hyphenated with an LC Packings Ultimate (Dionex, Olten, Switzerland) RP-HPLC system equipped with an autosampler, a valve switching system, and a UV detector. A 5 μm 200 Å Magic C18 AQ (Michrom Bioresources, Inc., Auburn, CA) 0.075 mm × 150 mm column was home-packed. After the sample was washed (5 μL) for 10 min with H_2O/TFA 99.9%/0.1% on a C18 Pepmap100 0.3 mm × 5 mm μ-precolumn (Dionex), the separation was run for 85 min using a gradient of H_2O/CH_3CN/FA 98%/2%/0.1% (solvent A) and H_2O/CH_3CN/FA 20%/80%/0.085% (solvent B). The gradient was run as follows: 0−15 min 100% A, then to 90% A and 10% B at 20 min, 60% A and 40% B at 75 min, 45% A and 55% B at 80 min, 35% A and 65% B at 83 min, and 96% B at 85 min at a flow rate of ∼200 nL min^{-1}. Silica tips (New Objective, Woburn, MA) with an internal diameter of 75 μm and tip size of 15 μm were used. The mass spectrometer voltage was set to 2600 V, the cone voltage to 40 V. The heated capillary was kept at 140 °C. All mass spectra were acquired in the positive ionization mode with an m/z scan range of 300−2000 Th. After selection of up to the three most intense precursor masses in MS mode (1.1 s scan duration), MS/MS scan functions were carried out with scan duration of 1.1 s until the signal fell under 10 counts or 4 s of recording. Tandem mass spectra were recorded with an m/z scan range of 50−2000 Th. The CID energy was set to 35 eV with a pressure of 1.4 bar of argon in the collision cell.

MALDI-TOF/TOF MS was performed on a 4800 Proteomics Analyzer from Applied Biosystems (Foster City, CA). Samples (5 μL) were first separated by LC using an Alliance system from Waters equipped with a flow splitter. A home-packed 5 μm 200 Å Magic C18 AQ 0.1 mm × 100 mm column was used. The separation was run for 60 min using a gradient of H_2O/CH_3CN/

Table 1. Proteins Observed to Increase in Abundance in PM CSF by TOF/TOF MS/MS Analysis (As a Function of Their Ratio PM CSF/AM CSF Ranges)

protein name	UniProt-Swiss-Prot/TrEMBL ID	normalized mean abundance in AM CSF	normalized mean abundance in PM CSF	p^e
10 > PM/AM > 3				
alpha-1-acid glycoprotein 1 precursor[a,d]	A1AG1_HUMAN	0.19 ± 0.08	0.81 ± 0.05	0.0003
alpha-1-antichymotrypsin precursor[a,d]	AACT_HUMAN	0.25 ± 0.06	0.75 ± 0.07	0.0006
bis(5′-adenosyl)-triphosphatase[c,d]	FHIT_HUMAN	0.18 ± 0.19	0.82 ± 0.14	0.0099
elongation factor 1-beta[b] (or elongation factor 1-delta, EF1D_HUMAN)	EF1B_HUMAN	0.10 ± 0.11	0.90 ± 0.07	0.0004
ezrin[a,d]	EZRI_HUMAN	0.16 ± 0.06	0.84 ± 0.02	<0.0001
ferritin heavy chain[a,d]	FRIH_HUMAN	0.15 ± 0.06	0.85 ± 0.05	0.0001
fibrinogen alpha chain precursor[a,d]	FIBA_HUMAN	0.12 ± 0.07	0.88 ± 0.01	<0.0001
fibrinogen beta chain precursor[a,d]	FIBB_HUMAN	0.13 ± 0.13	0.87 ± 0.02	0.0007
filamin-A[a,d]	FLNA_HUMAN	0.11 ± 0.11	0.89 ± 0.05	0.0003
fructose-bisphosphate aldolase A[c]	ALDOA_HUMAN	0.11 ± 0.19	0.89 ± 0.17	0.006
fructose-bisphosphate aldolase C[b,d]	ALDOC_HUMAN	0.18 ± 0.08	0.82 ± 0.02	0.0002
glial fibrillary acidic protein[a,d]	GFAP_HUMAN	0.10 ± 0.12	0.88 ± 0.10	0.001
glyceraldehyde-3-phosphate dehydrogenase[b,d]	G3P_HUMAN	0.10 ± 0.03	0.90 ± 0.03	<0.0001
hepatoma-derived growth factor[b]	HDGF_HUMAN	0.22 ± 0.06	0.78 ± 0.04	0.0002
lambda-crystallin homologue[c,d]	CRYL1_HUMAN	0.16 ± 0.14	0.84 ± 0.04	0.0012
malate dehydrogenase, mitochondrial precursor[c,d]	MDHM_HUMAN	0.23 ± 0.05	0.77 ± 0.07	0.0005
moesin[a,d]	MOES_HUMAN	0.15 ± 0.12	0.85 ± 0.07	0.0009
myb-binding protein 1A[b]	MBB1A_HUMAN	0.16 ± 0.16	0.84 ± 0.39	0.052*f*
NG,NG-dimethylarginine dimethylaminohydrolase 1[a,d]	DDAH1_HUMAN	0.17 ± 0.11	0.83 ± 0.07	0.0009
nucleoprotein TPR[c]	TPR_HUMAN	0.13 ± 0.12	0.87 ± 0.09	0.0011
peroxiredoxin-2[a]	PRDX2_HUMAN	0.11 ± 0.10	0.89 ± 0.11	0.0008
pyruvate kinase isozymes M1/M2[b,d]	KPYM_HUMAN	0.19 ± 0.05	0.81 ± 0.05	0.0002
superoxide dismutase [Cu−Zn][a] (or uncharacterized protein SOD1, A6NHJ0_HUMAN[d])	Q6NR85_HUMAN	0.12 ± 0.08	0.86 ± 0.05	0.0002
transformation/transcription domain-associated protein[a]	TRRAP_HUMAN	0.24 ± 0.11	0.76 ± 0.10	0.0033
transforming growth factor-beta-induced protein ig-h3 precursor[c]	BGH3_HUMAN	0.24 ± 0.42	0.76 ± 0.27	0.15 *f*
tubulin polymerization-promoting protein family member 3[b,d]	TPPP3_HUMAN	0.23 ± 0.04	0.77 ± 0.08	0.0005
ubiquitin[c,d]	UBIQ_HUMAN	0.11 ± 0.20	0.89 ± 0.13	0.0047
ubiquitin carboxyl-terminal hydrolase 10[a]	UBP10_HUMAN	0.20 ± 0.11	0.80 ± 0.32	0.039
20 > PM/AM > 10				
14-3-3 protein theta[c] (or 14-3-3 protein beta/alpha, 1433B_HUMAN; 14-3-3 protein zeta/delta, 1433Z_HUMAN[d])	1433T_HUMAN	0.05 ± 0.08	0.95 ± 0.03	<0.0001
actin, α cardiac muscle 1[a,d] (or actin, alpha skeletal muscle, ACTS_HUMAN[d])	ACTC_HUMAN	0.09 ± 0.10	0.91 ± 0.04	0.0002
annexin A6[c]	ANXA6_HUMAN	0.09 ± 0.15	0.91 ± 0.33	0.0166
ATPase inhibitor, mitochondrial precursor[c,d]	ATIF1_HUMAN	0.07 ± 0.12	0.93 ± 0.13	0.0012
carbonic anhydrase 2[a,d]	CAH2_HUMAN	0.05 ± 0.05	0.95 ± 0.02	<0.0001
carbonyl reductase[a]	CBR1_HUMAN	0.06 ± 0.10	0.94 ± 0.15	0.0009
coactosin-like protein[c]	COTL1_HUMAN	0.05 ± 0.08	0.95 ± 0.13	0.0006
creatine kinase B-type[a,d]	KCRB_HUMAN	0.08 ± 0.07	0.92 ± 0.08	0.0002
heat shock protein HSP 90-alpha[a,d]	HS90A_HUMAN	0.07 ± 0.06	0.93 ± 0.02	<0.0001
hemoglobin subunit alpha[a,d]	HBA_HUMAN	0.07 ± 0.09	0.93 ± 0.09	0.0003
hemoglobin subunit delta[b,d]	HBD_HUMAN	0.09 ± 0.08	0.91 ± 0.14	0.001
L-lactate dehydrogenase B chain[b,d]	LDHB_HUMAN	0.05 ± 0.09	0.95 ± 0.08	0.0002
LOC63920 protein[c] (or CDNA: FLJ23101 fis, clone LNG07536, Q9H5S8_HUMAN; transposase-like protein Q9UH87_HUMAN)	Q8IZ13_HUMAN	0.05 ± 0.08	0.95 ± 0.13	0.0006
malate dehydrogenase, cytoplasmic[c,d]	MDHC_HUMAN	0.08 ± 0.15	0.92 ± 0.15	0.0025
peptidyl-prolyl *cis-trans* isomerase A[b] (or peptidyl-prolyl *cis-trans* isomerase B precursor, PPIB_HUMAN; peptidyl-prolyl *cis-trans* isomerase C, PPIC_HUMAN)	PPIA_HUMAN	0.06 ± 0.07	0.94 ± 0.16	0.0009
peroxiredoxin-1[a,d]	PRDX1_HUMAN	0.06 ± 0.02	0.94 ± 0.07	<0.0001
phosphoglycerate mutase 2[b,d] (or phosphoglycerate mutase 1, PGAM1_HUMAN[d])	PGAM2_HUMAN	0.07 ± 0.09	0.93 ± 0.02	<0.0001
phosphoserine aminotransferase 1[c,d]	Q5T7G5_HUMAN	0.05 ± 0.09	0.95 ± 0.06	0.0001
quinone oxidoreductase[c,d]	QOR_HUMAN	0.06 ± 0.10	0.94 ± 0.10	0.0004
spectrin beta chain, brain 1[b,d]	SPTB2_HUMAN	0.06 ± 0.11	0.94 ± 0.07	0.0003
PM/AM > 20				
acyl-CoA-binding protein[a,d]	ACBP_HUMAN	0.04 ± 0.07	0.96 ± 0.11	0.0003
CDNA FLJ35966 fis, clone TESTI2012628, moderately similar to Mus musculus STAP sperm tail associated protein[b] (or putative uncharacterized protein DKFZp434G131, Q9H0H4_HUMAN; polyamine modulated factor 1-binding protein 1, Q8TBY8_HUMAN)	Q8NA09_HUMAN	0.04 ± 0.08	0.96 ± 0.08	0.0002
CDNA FLJ43653 fis, clone SYNOV4002346[c] (or Cdc42-associated guanine nucleotide exchange factor ACG/DOCK11, Q66M66_HUMAN; uncharacterized protein DOCK11, A6NMF0_HUMAN; dedicator of cytokinesis protein 11, Q5JSL3_HUMAN)	Q6ZUJ5_HUMAN	0.05 ± 0.08	0.95 ± 0.03	<0.0001

Table 1. (Continued)

protein name	UniProt-Swiss-Prot/TrEMBL ID	normalized mean abundance in AM CSF	normalized mean abundance in PM CSF	p^e
cystatin-B[a,d]	CYTB_HUMAN	0.02 ± 0.04	0.98 ± 0.31	0.0064
cytochrome *c*[c,d]	CYC_HUMAN	0.00 ± 0.00	1.00 ± 0.15	-
death-associated protein 1[c]	DAP1_HUMAN	0.04 ± 0.08	0.96 ± 0.27	0.0051
enolase (fragment)[c]	A4UCS8_HUMAN	0.03 ± 0.06	0.97 ± 0.06	<0.0001
ezrin-radixin-moesin-binding phosphoprotein 50[a,d]	NHERF_HUMAN	0.04 ± 0.06	0.96 ± 0.02	<0.0001
fatty acid-binding protein, epidermal[c]	FABPE_HUMAN	0.01 ± 0.02	0.99 ± 0.28	0.004
FLJ00310 protein (fragment)[b]	Q8NF67_HUMAN	0.04 ± 0.07	0.96 ± 0.04	<0.0001
glucose-6-phosphate isomerase[a,d]	G6PI_HUMAN	0.03 ± 0.04	0.97 ± 0.18	0.0008
metallothionein-1E[c] (or metallothionein-2, MT2_HUMAN; metallothionein-1I, MT1I_HUMAN; metallothionein-1X, MT1X_HUMAN; metallothionein-1G, MT1G_HUMAN)	MT1E_HUMAN	0.04 ± 0.07	0.96 ± 0.12	0.0004
methylenetetrahydrofolate reductase[b]	MTHR_HUMAN	0.02 ± 0.03	0.98 ± 0.18	0.0009
microfibril-associated glycoprotein 4 precursor[c]	MFAP4_HUMAN	0.02 ± 0.04	0.98 ± 0.41	0.016
probable G-protein coupled receptor 110 precursor[b]	GP110_HUMAN	0.05 ± 0.08	0.95 ± 0.03	<0.0001
protein DJ-1[b,d]	PARK7_HUMAN	0.02 ± 0.03	0.98 ± 0.10	<0.0001
putative uncharacterized protein (fragment)[c]	A0JLN5_HUMAN	0.03 ± 0.05	0.97 ± 0.23	0.0025
spectrin alpha chain, brain[c,d]	SPTA2_HUMAN	0.04 ± 0.08	0.96 ± 0.15	0.0007
thymosin beta-4[a,d]	TYB4_HUMAN	0.05 ± 0.08	0.95 ± 0.28	0.0056

[a] Proteins identified and quantified with at least two different peptides. [b] Proteins identified and quantified several times with the same peptide. [c] Proteins identified and quantified with a single peptide. [d] Proteins also identified by the Mascot search engine. [e] Calculation based on the unpaired *t* test ($p < 0.05$ considered as significant). [f] Insignificant value.

TFA 97%/3%/0.1% (solvent C) and H_2O/CH_3CN/TFA 5%/95%/0.085% (solvent D). The gradient was run as follows: 0−10 min 100% C, then to 90% C and 10% D at 12 min, 50% C and 50% D at 55 min, and 98% D at 60 min at a flow rate estimated to be 400 nL min^{-1}. One minute fractions were deposited onto the MALDI plates using a homemade LC-robot. The matrix (α-cyano-4-hydroxycinnamic acid in H_2O/CH_3CN 50%/50% with 10 mM NH_4-PO_4) was then spotted onto the plates. All mass spectra were acquired in the positive ionization mode with an m/z scan range of 800−4000 Th (1000 shoots with a laser intensity of 3900 in arbitrary units). After selection of 20 precursors at the maximum, MS/MS experiments (1500 shoots with a laser intensity of 4000 in arbitrary units) were realized at medium collision energy.

Protein Identification. Peak lists were generated using the respective software of each mass spectrometer. The resulting pkl or mgf files, combined from the different analyzed fractions for the CSF experiments, were searched against UniProt-Swiss-Prot/TrEMBL database (Swiss-Prot Release 54.4 of October 23, 2007, 287 050 entries and TrEMBL release 37.4 of October 23, 2007, 4 988 379 entries) using Phenyx[16] (Gene Bio, Geneva, Switzerland) and Mascot[17] (version 2.2.03, Matrix Sciences, London, U.K.) operating on a local server. No taxonomy was used for the model protein mixture and *Homo sapiens* (and separately *Bos taurus* to retrieve the spiked LACB) was specified for the CSF experiments database searching. Variable amino acid modifications were oxidized methionine and TMT-labeled peptides on the amino terminus (+229.1629 Da). Glutamine and asparagine deamidation was specified as a variable modification when the TOF/TOF mass spectrometer was used. TMT-labeled lysine and carbamidomethylation of cysteines was set as a fixed modification. Trypsin was selected as the enzyme, with three potential missed cleavages. The peptide and fragment ion tolerance was, respectively, 0.5 and 0.4 Da for the Mascot search. For Phenyx, two search rounds were used. In the first round, one missed cleavage was allowed and the normal cleavage mode was used. This round was selected in "turbo" search mode. In the second round, two missed cleavages were allowed and the cleavage mode was set to half-cleaved. The minimum peptide length allowed was six and five amino acids, respectively, for the first and second run. The acceptance criteria were slightly lowered in the second round search (round 1, AC score 7.0, peptide *Z*-score 7.0, peptide *p* value 1×10^{-7}; round 2, AC score 6.0, peptide *Z*-score 6.0, peptide *p* value 1×10^{-6}). The parent ion tolerance was set to 1 Da for each round. In case of several matching entries, Swiss-Prot entries were preferred to TrEMBL entries.

Protein Quantification. Protein quantification was obtained from Phenyx data (see Supporting Information SI5 and SI6). The quantification values were extracted from the reporter peak intensities, assuming identical labeling efficiencies for each sample. Thanks to the spiking of LACB inside the CSF samples, a normalization of the data was achieved to reduce the manipulation bias of the CSF analysis. Attention was paid not to give any preponderant weight to any reporters. In this way, a normalization of the reporter intensities by the sum of all the reporter intensities was made in order to determine the relative abundance of each reporter. The quantification was assessed whatever the number of identified peptides, giving the same weight to the valid peptides even in case of redundancy. For the protein model mixture, if the minimum ion count of one of the reporter ions was less than 50 counts, the data were omitted from the quantification. For the CSF samples, the data were removed from the quantification when the sum of the 6 reporter ions was inferior to 300 counts. Peptides

(16) Heller, M.; Ye, M. L.; Michel, P. E.; Morier, P.; Stalder, D.; Junger, M. A.; Aebersold, R.; Reymond, F. R.; Rossier, J. S. *J. Proteome Res.* **2005**, *4* (6), 2273−2282.

(17) Perkins, D. N.; Pappin, D. J. C.; Creasy, D. M.; Cottrell, J. S. *Electrophoresis* **1999**, *20* (18), 3551−3567.

Table 2. Proteins Observed to Increase in Abundance in PM CSF by Q-TOF MS/MS Analysis (As a Function of Their Ratio PM CSF/AM CSF Ranges)

protein name	UniProt-Swiss-Prot/TrEMBL ID	normalized mean abundance in AM CSF	normalized mean abundance in PM CSF	p[e]
10 > PM/AM > 3				
actin, cytoplasmic 1[a,d] (or actin, cytoplasmic 2, ACTG_HUMAN[d]; actin, gamma-enteric smooth muscle, ACTH_HUMAN[d]; actin, aortic smooth muscle, ACTA_HUMAN[d]; actin, alpha cardiac muscle 1, ACTC_HUMAN[d]; actin, alpha skeletal muscle, ACTS_HUMAN[d])	ACTB_HUMAN	0.14 ± 0.03	0.86 ± 0.14	0.001
calcyphosin[c]	CAYP1_HUMAN	0.14 ± 0.04	0.86 ± 0.14	0.001
carbonic anhydrase 2[a,d]	CAH2_HUMAN	0.12 ± 0.02	0.88 ± 0.10	0.0003
ferritin light chain[b,d]	FRIL_HUMAN	0.15 ± 0.07	0.85 ± 0.10	0.0005
heat shock cognate 71 kDa protein[c]	HSP7C_HUMAN	0.22 ± 0.05	0.78 ± 0.16	0.0041
hemoglobin beta chain (fragment)[a,d]	Q6VFQ5_HUMAN	0.10 ± 0.04	0.90 ± 0.12	0.0004
NG,NG-dimethylarginine dimethylaminohydrolase 1[c]	DDAH1_HUMAN	0.16 ± 0.01	0.84 ± 0.12	0.0006
phosphoglycerate mutase 2[c] (or phosphoglycerate mutase 1, PGAM1_HUMAN)	PGAM2_HUMAN	0.24 ± 0.05	0.76 ± 0.03	<0.0001
protein DJ-1[b,d]	PARK7_HUMAN	0.12 ± 0.03	0.88 ± 0.25	0.0066
superoxide dismutase [Cu−Zn][a,d] (or uncharacterized protein SOD1, A6NHJ0_HUMAN[d])	Q6NR85_HUMAN	0.19 ± 0.04	0.81 ± 0.15	0.0026
tubulin beta-4 chain[b,d] (or tubulin beta chain, TBB5_HUMAN[d]; tubulin beta-2C chain, TBB2C_HUMAN[d])	TBB4_HUMAN	0.24 ± 0.10	0.76 ± 0.07	0.0021
20 > PM/AM > 10				
fibrinogen alpha chain precursor[c]	FIBA_HUMAN	0.07 ± 0.05	0.93 ± 0.19	0.0017
peptidyl-prolyl *cis-trans* isomerase B precursor[b] (or peptidyl-prolyl *cis-trans* isomerase C, PPIC_HUMAN)	PPIB_HUMAN	0.09 ± 0.04	0.91 ± 0.14	0.0006
peroxiredoxin−5, mitochondrial precursor[c]	PRDX5_HUMAN	0.06 ± 0.05	0.94 ± 0.09	0.0001
protein S100-B[d]	S100B_HUMAN	0.06 ± 0.04	0.94 ± 0.05	<0.0001
PM/AM > 20				
glyceraldehyde-3-phosphate dehydrogenase[b,d]	G3P_HUMAN	0.03 ± 0.04	0.97 ± 0.39	0.014
heat shock protein HSP 90-beta[a] (or heat shock protein HSP 90-alpha, HS90A_HUMAN)	HS90B_HUMAN	0.00 ± 0.00	1.00 ± 0.21	0.0012
plasma membrane calcium-transporting ATPase 1[a]	AT2B1_HUMAN	0.01 ± 0.02	0.99 ± 0.14	0.0003

[a] Proteins identified and quantified with at least two different peptides. [b] Proteins identified and quantified several times with the same peptide. [c] Proteins identified and quantified with a single peptide. [d] Proteins also identified by the Mascot search engine. [e] Calculation based on the unpaired t test ($p < 0.05$ considered as significant). [f] Insignificant value.

identified as TMT-unlabeled but exhibiting the reporter peaks were considered as false positive matches and were removed from the quantification. The protein ratios PM CSF/AM CSF were obtained from the average values of the intrarun triplicates. Therefore, the quantification data obtained with a single identified peptide are based on three abundance measurements both for AM and PM CSF. The mean abundance and standard deviation in Tables 1 and 2 were obtained from these three averaged or single measurements. An unpaired t test was assessed to test the significance of the abundance difference obtained in AM ($n = 3$) vs PM ($n = 3$) CSF. A p value inferior to 0.05 was considered as significant.

Protein Validation. GFAP and S100B were validated using commercial ELISA kits from Abnova Corp. (Taipei City, Taiwan) according to the manufacturer's recommendations. Because no commercial assay is currently available for the detection of protein DJ-1 (PARK 7), a sandwich homemade ELISA was used as previously described by L. Allard et al.[18] Reproducibility tests showed less than 20% relative standard deviation.

A 100 μL portion of CSF samples diluted 2-fold for PARK7, 3-fold for GFAP, and 4-fold for S100B were tested. Each sample was assayed in duplicate and distributed randomly on the microtiter plates. Calibrator samples corresponding to the recombinant proteins were run on the same plate to calculate protein concentrations in CSF samples thanks to the linear regression. Statistical analyses were performed using Graph Pad Prism software version 4.3 (Graphpad Software Inc, San Diego, CA). Nonparametric Mann−Whitney tests were performed to assess the capability of GFAP, S100B, and PARK7 to discriminate AM vs PM CSF.

RESULTS AND DISCUSSION

Model Protein Mixture. The 6-plex TMT quantification is achieved through the inspection of the reporter ion region in tandem mass spectra. Each of the six tags has a specific reporter ion that appears at m/z = 126.1, 127.1, 128.1, 129.1, 130.1, and 131.1 Th, respectively (Figure 1). A mixture of albumin (ALBU) from bovine serum, myoglobin (MYO) from horse heart, β-lactoglobulin (LACB) from bovine milk, and lysozyme (LYS) from hen egg in equal weight was tested. In Figure 2 are summarized the results for the reporter relative abundance intensity for six combined samples with theoretical protein ratios 1:2:3:3:5:10. The mean absolute error with respect to the expected theoretical value was about 10% with obtained protein ratios 1.2:2.4:2.8:3.2:5.0:9.4. When processing the data to extract relative quantification ratios (see Experimental Section), giving any preponderant weight to any reporter was avoided. A normalization of the reporter

(18) Allard, L.; Burkhard, P. R.; Lescuyer, P.; Burgess, J. A.; Walter, N.; Hochstrasser, D. F.; Sanchez, J. C. *Clin. Chem.* **2005**, *51* (11), 2043−2051.

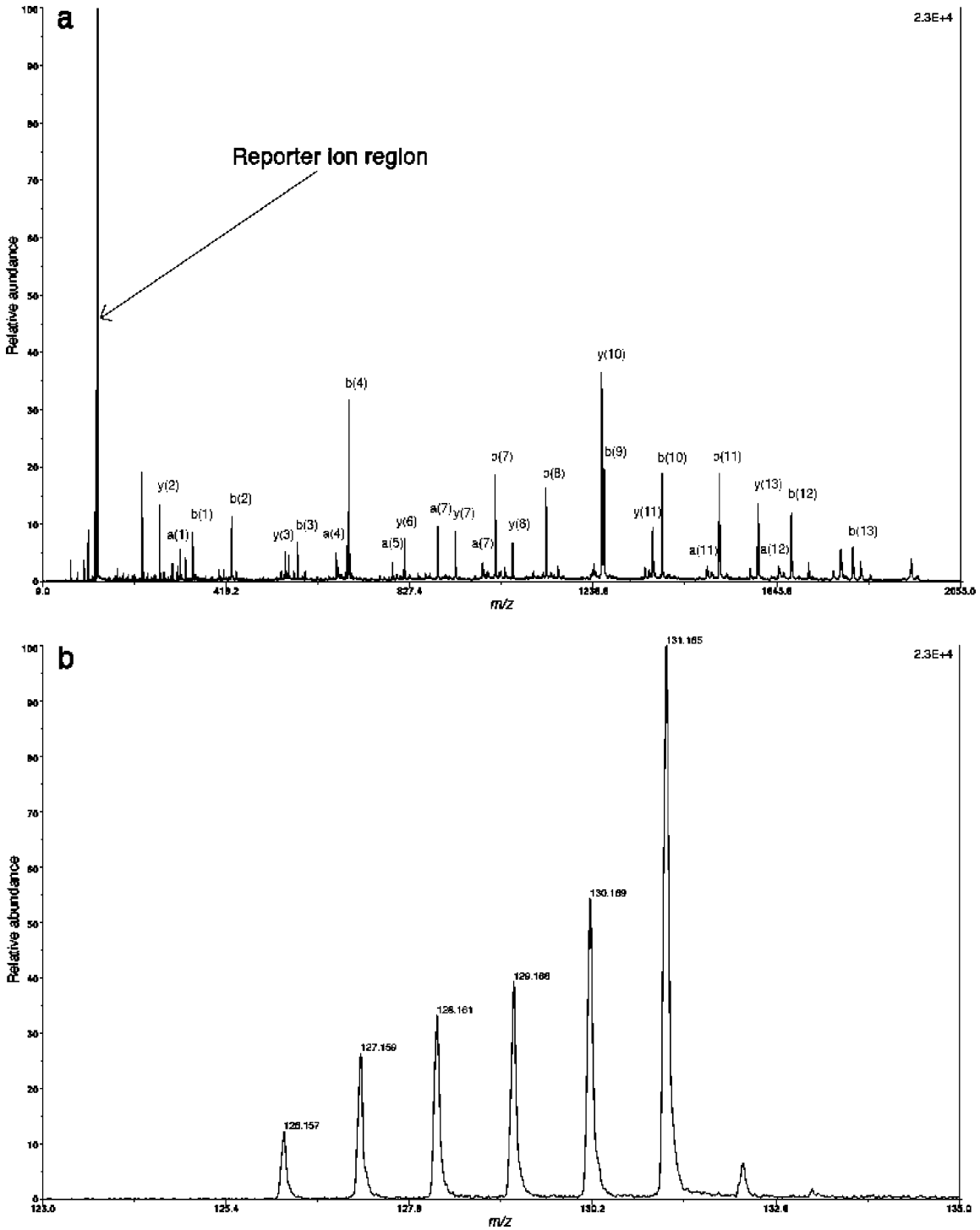

Figure 1. (a) Tandem mass spectrum of peptide at $m/z = 1944.902$ Th, identified as LSFNPTQLEEQCHI of β-lactoglobulin, labeled with TMT and iodoacetamide. (b) Zoom of the reporter ion region that provides the peptide relative quantification in a model four-protein mixture at six different concentrations (1:2:3:3:5:10) through the abundance of the reporter ions at $m/z = 126.1$, 127.1, 128.1, 129.1, 130.1, and 131.1 Th. Obtained by TOF/TOF MS/MS.

intensities by the sum of all the reporter intensities was made in order to determine the relative abundance of each reporter.

The differentially-TMT-labeled samples were analyzed independently at the same concentration to assess the apparent purity of each of the TMT labels. From the TOF/TOF MS/MS measurement, 89.4% of the mean apparent purity was obtained (results are given in Supporting Information SI1). Although this apparent purity does not represent the absolute chemical purity, it indicates to which extent the MS/MS quantification could be disturbed (e.g., isotopic purity, MS background, overlapping ions). For example, the label with reporter at $m/z = 128.1$ Th exhibits 2.1% of reporter at $m/z = 127.1$ Th and 7.3% of reporter at $m/z = 129.1$ Th. The label with reporter at $m/z = 131.1$ Th exhibits 2.9% of reporter at $m/z = 130.1$ Th and 4.7% of reporter at $m/z = 132.1$ Th, corresponding mainly to ^{12}C impurities in the incorporated ^{13}C and natural ^{13}C occurrence in the four remaining ^{12}C of the

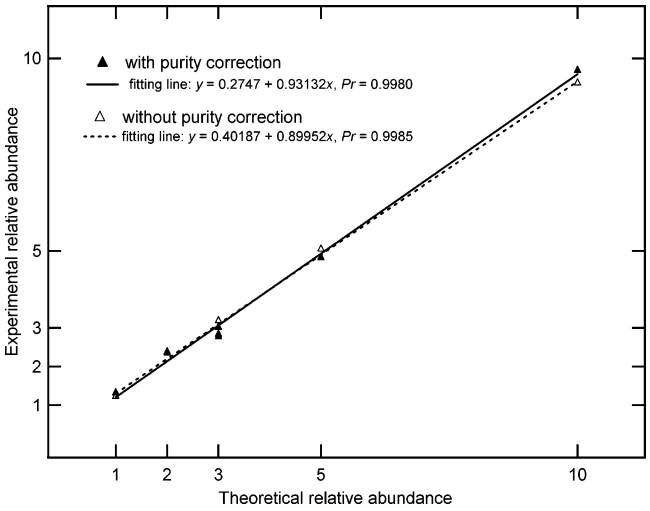

Figure 2. Calibration curve of the model protein mixture with six concentrations obtained by TOF/TOF MS/MS. The theoretical ratios are 1:2:3:3:5:10.

reporter group. A purity correction was thus applied to the previous quantification values.[19] Reporter intensities were corrected according to the following system of six equations with six unknowns:

$$I_i = i_i p_{i,i} + i_j p_{i,j} + i_k p_{i,k} + i_l p_{i,l} + i_m p_{i,m} + i_n p_{i,n}$$

where I_i and i_i are, respectively, the measured and the true intensity of the reporter at $m/z = i$, $p_{i,i}$ is the apparent purity of the TMT with reporter at $m/z = i$, and $p_{i,j}$, $p_{i,k}$, $p_{i,l}$, $p_{i,m}$, and $p_{i,n}$ are the five apparent impurity values at $m/z = i$ of each other reporter with true intensity i_j, i_k, i_l, i_m, and i_n. The use of the Maple software (Maple 11, Maple Inc., Waterloo, Canada) was convenient to solve the equation systems and obtained the six true reporter intensities i_{i-n} (see Supporting Information SI2).

Once corrected, the protein ratios were 1.3:2.4:2.8:3.0:4.8:9.7. The calibration curve plotted in Figure 2 clearly shows that the overall accuracy of the quantification with a regression coefficient of 0.931 for the fitting line was slightly better when the correction was applied. On two independent experiments analyzed twice by TOF/TOF MS/MS, the relative standard deviation for the quantification on each reporter was found to vary from 6.7 to 20.3% with a mean value of 13.6%. It clearly appeared that the low concentrated samples, corresponding to TMT reporters at m/z = 126.1 and 127.1 Th, give the least accurate results with absolute error values of 31.9 and 19.1% with respect to the expected theoretical values. The remaining quantification values provided absolute errors of 8.3, 3.4, 3.9 and 3.4% for the ratios obtained, respectively, from reporters at m/z = 128.1, 129.1, 130.1, and 131.1 Th. This is a relevant point to consider for the comparison of AM and PM CSF samples because a considerable difference in the abundance of potential protein biomarkers is expected. Notably, very high or low ratios of PM CSF/AM CSF cannot be considered as accurate as those close to unity because of the quantification dynamics issue.

(19) Shadforth, I. P.; Dunkley, T. P.; Lilley, K. S.; Bessant, C. *BMC Genomics* **2005**, *6*, 145.

The apparent purity values were found to be slightly dependent on the mass spectrometer used. When performing identical analysis on the Q-TOF apparatus, the mean purity was 88.8% (see Supporting Information SI3 for details) providing final protein ratios 1.3:2.4:2.9:3.5:4.7:9.3 on this mass spectrometer. The calibration curve is given in Supporting Information SI4.

CSF Samples. A pooled sample of AM CSF and a pooled sample of PM CSF were spiked with the same amount of LACB and each divided into three samples. The six resulting samples were depleted of albumin, transferrin, IgG, IgA, antitrypsin, and haptoglobin. They were reduced, alkylated, digested by trypsin, and labeled with TMT. The three AM CSF samples were, respectively, labeled with TMT with reporter at m/z = 126.1, 128.1, and 130.1 Th whereas the three PM CSF samples were, respectively, labeled with TMT with reporter at m/z = 127.1, 129.1, and 131.1 Th. The six samples were pooled and fractionated by SCX and the fractions were analyzed by TOF/TOF MS/MS after the RP-HPLC fractions were deposited on a MALDI plate.

When the UniProt-Swiss-Prot/TrEMBL database was searched on a total of 9920 tandem mass spectra using Phenyx software (from 1500 precursor mass spectra), 1419 peptides were matched with the criteria indicated in the Experimental Section. A total of 220 protein hits were found. From these proteins, 170 were quantified, even with only one valid peptide (see Supporting Information SI5) and 80 proteins were quantified with at least two valid peptides.

The reporter peak intensities were first corrected from the apparent TMT impurities, and then the experimental bias were taken into account according to the LACB reporter intensities that should be equal. This last step seems to be relevant regarding the series of triplicates that should normally give identical quantitative values. It was noticed that some of the reporter peak intensities became slightly negative because of the impurity correction (see Supporting Information SI5). This artifact results from the absence or very low amount of detected reporters. In these cases, their intensity was corrected to zero. The average ratio PM CSF/AM CSF obtained from the average values of both triplicates was found to be equal to infinity, which theoretically indicates protein presence/absence, in one single case.

The proteins that were found to be present in the PM CSF much more were classified in three categories that are displayed in Table 1. Nineteen proteins were shown to be massively increased in PM CSF (ratio PM CSF/AM CSF > 20). Fourteen proteins had a ratio between 10 and 20. Twenty eight other proteins had a ratio PM CSF/AM CSF between 3 and 10. Therefore, 67 proteins (26 when considering those identified with at least 2 different peptides) can be estimated to be highly concentrated in PM CSF. A comparison with Mascot matches was made to further validate some of these proteins, notably those with only one identified peptide (Table 1). The Mascot search provided some other interesting identified proteins like glutathione S-transferase P (GSTP1), which was not found using the Phenyx software and was more concentrated in PM CSF.[14] The sample was also analyzed by ESI Q-TOF MS/MS (see Supporting Information SI6). Whereas less protein matches were found (61 matched), this technique allowed additional validation of some of the protein matches. Furthermore, some peptides, not seen by MALDI-TOF/TOF, revealed the up-concentration of an interesting brain biomarker, protein S100B, which was extensively assessed

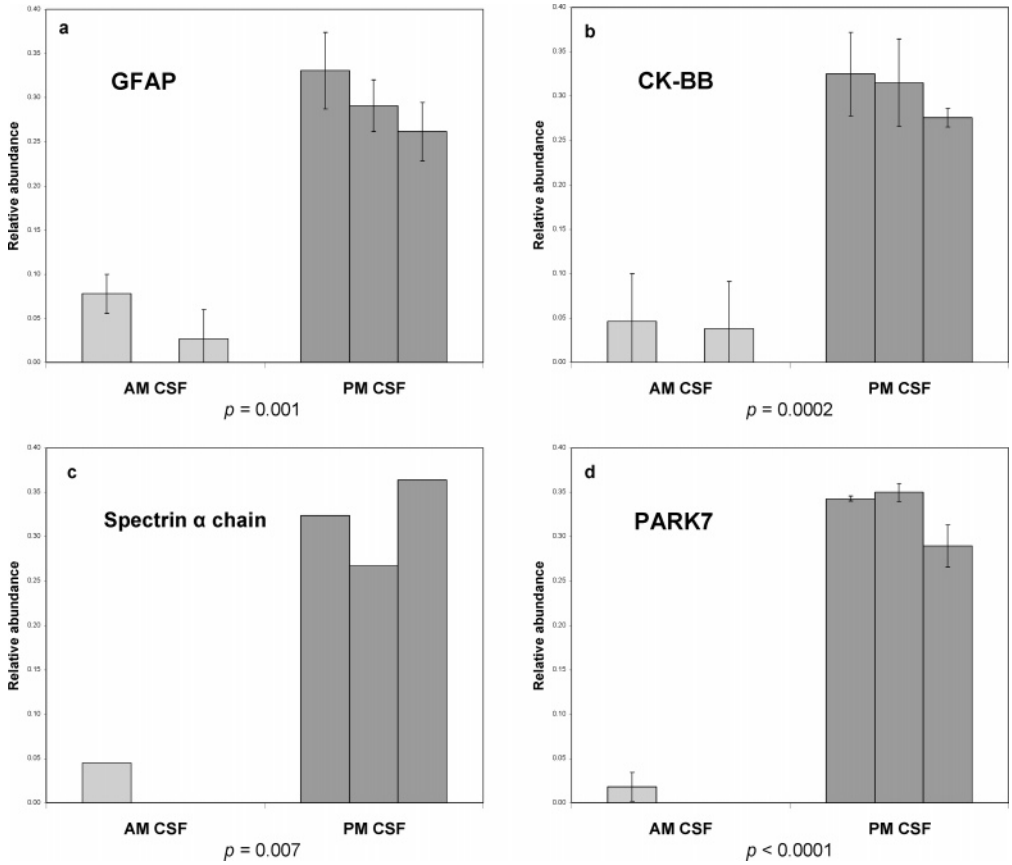

Figure 3. Relative quantification from intrarun triplicate AM and PM CSF samples for (a) glial fibrillary acidic protein (GFAP), (b) creatine kinase B-type (CK-BB), (c) spectrin α chain, and (d) protein DJ-1 (PARK7) using isobaric tagging and TOF/TOF MS/MS.

as a biomarker of stroke (Table 2).[20] In summary from both MS/MS experiments, 238 proteins were identified by Phenyx, 189 were quantified, and a total of 78 proteins showed a definite and marked increase in PM CSF vs AM CSF, i.e., ratio PM CSF/AM CSF > 3 (or, respectively, 91, 87, and 30 when considering those identified with at least two different peptides). Interestingly, nearly all of these 78 proteins have already been identified in previous studies on postmortem CSF,[14,15] strongly supporting the validity of the findings reported here.

The concentration comparison of glial fibrillary acidic protein (GFAP), creatine kinase B-type (CK-BB), spectrin α chain, and protein DJ-1 (PARK7) in AM and PM CSF is shown in Figure 3 and in Figure 4 for S100B. The level of GFAP was previously assessed in PM CSF by 2D gel electrophoresis immunoblot assays with a specific antibody. A strong increase was detected in deceased patients compared to healthy controls.[15] In the same study, PARK7 was determined to increase in deceased patients by comparison of spot intensities on 2D electrophoretic gels of AM and PM CSF. In order to confirm the MS-based quantitative measurements, ELISA validations of GFAP, S100B, and PARK7 on four different AM and PM CSF samples were carried out. The ELISA revealed a dramatic and significant ($p = 0.0286$, where p is the probability, from the Mann−Whitney nonparametric test, that the observed result is a random event) increase of their concentration in PM CSF (Figure 5). The ratios PM CSF/AM CSF were found to be 9.52, 19.45, and 58.00, respectively, for GFAP, S100B, and PARK7. These values correlated strikingly with those obtained by MS/MS which were, respectively, 8.44, 15.35 (obtained by ESI Q-TOF MS/MS), and 54.90. The ratio for PARK7 obtained by ESI Q-TOF MS/MS was 7.60. In Supporting Information SI7, a comparison of the quantitative values for the proteins identified on both apparatuses is presented. The highest differences were observed for the highest ratio PM CSF/AM CSF. For ratios PM CSF/AM CSF relatively close to 1, the differences of both measurements were almost in the range of the quantitative measurement errors obtained previously. The error and standard deviation for abundance measurements in AM and PM CSF are generally smaller when the abundances of each reporter ion are of the same magnitude (i.e., 0.5 < ratio PM CSF/AM CSF < 2) as calculated in Supporting Information SI5 and SI6. When the abundance differences between AM and PM CSF become larger (e.g., PM CSF/AM CSF > 3), the relative standard deviation increases for MS/MS measurements relative to AM CSF as a result of the overall decrease of the signal-to-noise ratio for the reporter ion peaks. Therefore, classifying proteins significantly increased in concentration in PM CSF by ratio PM CSF/AM CSF ranges (specifying p values based on the unpaired t test) appears more relevant than considering strict numerical values (Tables 1 and 2).

GFAP, S100B, CK-BB, or spectrin α chain are well documented biomarkers of acute brain damage and many studies have described their level modifications according to pathological situations. S100B is a calcium-binding, growth-regulating secretory protein that is expressed particularly in brain tissues, mainly in

(20) Persson, L.; Hardemark, H. G.; Gustafsson, J.; Rundstrom, G.; Mendel-Hartvig, I.; Esscher, T.; Pahlman, S. *Stroke* **1987**, *18* (5), 911−918.

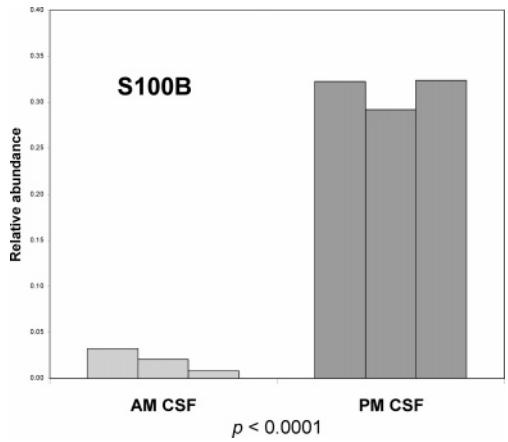

Figure 4. Relative quantification from intrarun triplicate AM and PM CSF samples for protein S100B using isobaric tagging and Q-TOF MS/MS.

glial and Schwann cells. Thus, S100B levels in CSF or blood are often used as a marker of brain insult or dysfunction. Increased levels of S100B have been reported in various neurological conditions including stroke,[21,22] traumatic brain injury,[23] and schizophrenia[24] and also in systemic metabolic disorders,[25,26] trauma of muscle and bone marrow,[27,28] and cardiac arrest.[29,30] GFAP is a monomeric intermediate filament protein of astrocytes. GFAP concentrations in the CSF have been found elevated in stroke and dementia.[21,31] Moreover, GFAP serum levels in stroke patients were found to correlate with both infarction volume and clinical outcome.[32] CK-BB is abundant in brain but normally not present in the CSF.[33] Various brain injuries such as aneurismal subarachnoid hemeorrhage,[34] traumatic brain injury,[35,36] and

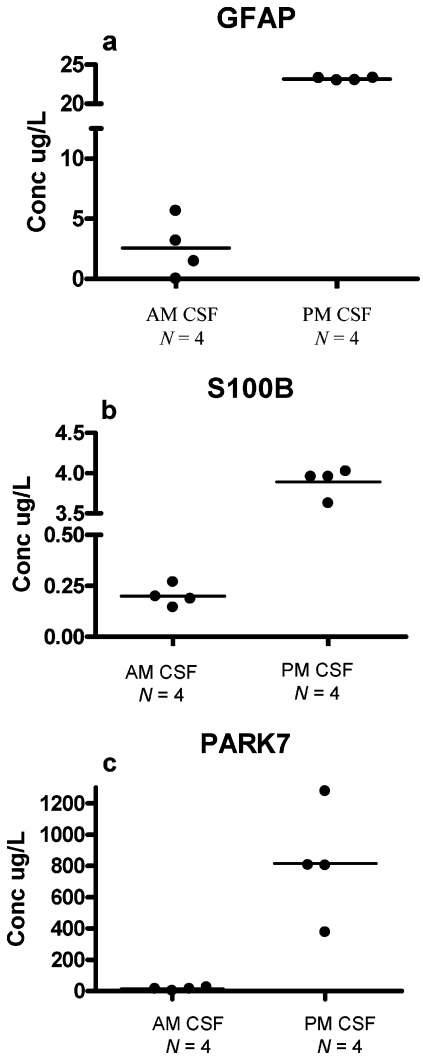

Figure 5. Immunovalidation of glial fibrillary acidic protein (GFAP), protein S100B, and DJ-1 (PARK7) in AM and PM CSF. (a) AM GFAP concentration (mean 2.57 μg L^{-1}; range 0−5.66 μg L^{-1}) vs PM concentration (mean 23.15 μg L^{-1}; range 22.99−23.33 μg L^{-1}), p = 0.0286. The ratio PM CSF/AM CSF = 9.52. (b) AM S100B concentration (mean 0.2 μg L^{-1}; range 0.145−0.268 μg L^{-1}) vs PM concentration (mean 3.89 μg L^{-1}; range 3.63−4.02 μg L^{-1}), p = 0.0286. The ratio PM CSF/AM CSF = 19.45. (c) AM PARK7 concentration (mean 14.04 μg L^{-1}; range 2.22−24.66 μg L^{-1}) vs PM concentration (mean 816.22 μg L^{-1}; range 377−1278 μg L^{-1}), p = 0.0286. The ratio PM CSF/AM CSF = 58.00.

cerebral ischemia following myocardial infarcts[37,38] cause the release of CK-BB into the CSF. The literature contains extensive information on the proteolytic degradation of spectrin α chain after traumatic brain injury. Spectrin is an actin-binding protein that forms with short actin filaments a cytoskeletal network on the cytoplasmic surface of the cells. The loss of cytoskeleton is

(21) Missler, U.; Wiesmann, M.; Friedrich, C.; Kaps, M. *Stroke* **1997**, *28* (10), 1956−1960.
(22) Buttner, T.; Weyers, S.; Postert, T.; Sprengelmeyer, R.; Kuhn, W. *Stroke* **1997**, *28* (10), 1961−1965.
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characteristic of a variety of central nervous system insults such as ischemic and traumatic brain injury.[39,40] Finally, PARK7 is a conserved protein widely expressed in many tissues including brain and heart.[41] PARK7 is expressed mostly in astrocytes and to a lesser extent in neurons.[42,43] Only few data are available about its function as potential biomarker. Point mutations in the PARK7 are responsible for a rare form of autosomal-recessive, early onset Parkinson's disease,[44] and the level of PARK7 was shown to increase after exposure to oxidative stress.[45] Furthermore, a former study conducted in our laboratory has demonstrated that PARK7 could be used as an early stroke biomarker because its level showed a statistically significant increase in the blood of stroke patients compared to control patients.[18] In addition, PARK7 has recently been described to present a neuroprotective role against neurodegeneration induced by cerebral ischemia.[46] In conclusion, the finding of these proteins as increased in the PM CSF is not unexpected and validates the use of the TMT strategy in biomarker discovery.

CONCLUSIONS

The TMT label procedure provides the simultaneous relative quantification of proteins in up to six different extracts from MS/MS experiments. The relative quantification for protein ratios from 1 to 10 has been shown to present accuracy and relative standard deviation in similar or better ranges than several other quantitative methods used in proteomics.

When comparing ante- and postmortem cerebrospinal fluid samples with TMT, it clearly appeared that some of the proteins that are present in postmortem CSF are almost absent in antemortem CSF. Some of these proteins have already been shown to have a great potential in the diagnosis or prognosis of brain-related diseases. The MS-based quantitative values for GFAP, S100B, and PARK7 were further validated by ELISA, which confirmed the excellent accuracy of the TMT relative quantification, even when high concentration differences exist between the samples. This work therefore demonstrated the usefulness of the TMT reagents for the discovery of biomarkers in a shotgun proteomics approach.

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SUPPORTING INFORMATION AVAILABLE

Table of the apparent purity of the TMT labels observed by TOF/TOF MS/MS, Maple program for the reporter intensity correction, table of the apparent purity of the TMT labels observed by Q-TOF MS/MS, calibration curve of the model protein mixture with six concentrations obtained by Q-TOF MS/MS, table of protein identification and quantification in AM and PM CSF obtained by TOF/TOF MS/MS, table of protein identification and quantification in AM and PM CSF obtained by Q-TOF MS/MS, and comparison of protein ratios PM CSF vs AM CSF according to the mass spectrometer used. This material is available free of charge via the Internet at http://pubs.acs.org.

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