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CSF proteomic fingerprints for HIV- associated cognitive impairment

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Abstract

Cognitive impairment remains a major complication of advanced human immunodeficiency virus (HIV) infection despite the wide spread use of anti-retroviral therapy. Diagnosis is made by exclusion making biomarkers of great potential use. Thus, we used an integrated proteomics platform to assess cerebrospinal fluid protein profiles from 50 HIV-1 seropositive Hispanic women. Nine of 38 proteins identified were unique in those patients with cognitive impairment. These proteins were linked to cell signaling, structural function, and antioxidant activities. This work highlights, in a preliminary manner, the utility of proteomic profiling for biomarker discovery for HIV-1 associated cognitive dysfunction.

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Keywords

HIV-associated cognitive impairment; biomarkers; proteomics; CSF

1. Introduction

Cognitive, behavioral, and motor dysfunction induced as a consequence of HIV-1 infection remain chronic and debilitating despite the advent of anti-retroviral therapy (McArthur, 2004). In its most severe form, HIV-associated cognitive impairment is termed HIV-associated dementia (HAD). Anti-retroviral therapy has altered the magnitude of HIV-associated cognitive impairments as patients present milder forms of disease and show different patterns of mental dysfunction. This represents significant challenges in making a firm diagnosis to the exclusion of opportunistic brain infections, tumors, or psychiatric and behavioral disorders (Everall et al., 2005; Wojna and Nath, 2006; Ances and Ellis, 2007). In era of active antiretroviral therapy, the term HIV-associated cognitive impairment (CI) is preferred over HAD. Indeed, cognitive dysfunction is manifest with milder forms of impairment and present with increased CD4+ T cell counts (Navia and Rostasy, 2005; Wojna et al., 2006; Woods et al., 2007).

Viral and immune biomarkers associated with the development of HIV-associated cognitive impairments are neither diagnostic nor predictive (Anthony et al., 2005; St. Hillaire et al., 2005). More commonly, biomarkers associated with cognitive impairments are linked to a cascade of neuroinflammatory events perpetrated by HIV-1-infected and immune competent brain mononuclear phagocytes (MP; microglia and blood-borne macrophages) (Kadiu et al., 2005).

Cerebrospinal fluid (CSF) contains a number of immunoregulatory proteins (Yuan and Desiderio, 2005), making it a likely source for analysis of the brain's microenvironment (Huhmer et al., 2006). Thus, we used CSF to test for biomarkers linked to CI during progressive HIV-1 disease. In this study, we used a proteomics platform consisting of surface enhanced laser desorption ionization time of-flight (SELDI-TOF), reverse phase high performance liquid chromatography (RP-HPLC) sample fractionation, 1D SDS PAGE electrophoresis, and liquid chromatography mass spectrometry (LC-MS/MS). This approach yielded signature spectra for HIV-associated CI on SELDI-TOF that coincided with specific protein identification. These results, while preliminary, support the use of proteomic protein profiling as an important tool in the biomarker discovery applied to NeuroAIDS.

2. Methods

2.1 Patient cohort

The study was conducted as part of the NeuroAIDS Specialized Neuroscience Research Program at the University of Puerto Rico Medical Sciences Campus (UPR-MSC). HIV-1 seropositive women were recruited from primary HIV clinics at the Puerto Rico Medical Center and the UPR-MSC. The study had the approval of the Institutional Review Board and was conducted with the informed consent of all participants. The inclusion criteria, recruitment, and evaluation have been described previously (Wojna et al., 2004a; Wojna et al., 2006). HIV-seropositive women with $CD4^+$ T lymphocyte counts ≤500 cells/cubic mm and/or viral load > 1,000 copies/mL while on ART were characterized for neurological and neuropsychological functions. All participants had at least a 9^{th} grade education and showed no evidence of systemic infection or concomitant neurological disorders (Wojna et al., 2004a). Plasma and CSF viral loads were determined with an Ultrasensitive RNA Roche Amplicor at an ACTG-certified laboratory with a detection range of 50 to 75000 copies of RNA/mL.

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2.2 Neurological and neuropsychological evaluations

Neurological examination consisted of a mental status evaluation, testing of sensory (including speed of thought and language), behavior and mood functions as well as standard evaluations of cranial nerves, cerebellar, motor, reflexes, and sensation. The neuropsychological evaluations included evaluation to determine pre-morbid vocabulary and reading scores. The neurocognitive evaluations included tests of verbal memory (trial 5, delay recall, and recognition of the Rey Auditory Verbal Learning Test), frontal executive function (Stroop word/color and Trail Making B), psychomotor speed (Symbol Digit Modalities Test and visual and auditory reaction time non dominant hand), motor speed (Trail Making A and Grooved Pegboard dominant and non dominant hand), and Beck Depression Index. The neuropsychological evaluations have been described previously (Wojna et al., 2006), and are detailed in Table 1. All tests were conducted on all patients in Spanish. We calculated z-scores of the neuropsychological tests in 34 HIV-1 seronegative Puerto Rican women. These women did not differ from the HIV-infected group with regards to age, education, and socio-demographics status. The neurologist and the neuropsychologist were blinded to each other's findings.

Cognitive impairment was determined using the American Academy of Neurology HIV-associated dementia criteria (AAN criteria) (American Academy of Neurology AIDS Task Force, 1991, 1996) modified to include an asymptomatic cognitively impaired group (m-AAN). An asymptomatic cognitively impaired group is defined as patients with abnormal neuropsychological tests (1 SD [on two or more tests] or 2 SD [on one or more tests] below the normal control group) but who presented neither functional/emotional nor neurological findings. According to the m-AAN, the patient groups were as follows: normal, asymptomatic cognitive impairment, minor cognitive motor disturbance (MCMD), and HAD (Wojna et al., 2006). For this study, patients with asymptomatic cognitive impairment, MCMD, or HAD were grouped together as CI and compared with patients having normal cognition (NC). Of the 50 patients included in this study, 13 had NC and 37 were CI. Three limited subsets of those 50 patients, e.g., n= 37, n=7, and n=18, were chosen for different assays dependent on availability of sample volume and protein concentration.

2.3. CSF collection and processing

*B*lood and CSF samples were collected from the patients on the day of Neurological and Neuropsychological evaluations. Blood was processed for monocyte isolation while CSF was immediately subjected to centrifugation in cold. Cell free CSF was mixed with 5% protease inhibitor cocktail (Sigma), and stored at -80°C for viral load and proteomics studies. CSF samples have been collected once a year and stored at −80°C within one hour of collection.

2.4. SELDI-TOF profiling

A pilot study was conducted to determine optimal experiment conditions, whole CSF was initially spotted in quadruplicates on three different protein chip array surfaces: CM10 (weak cation exchange), Q10 (strong anion exchange), and IMAC 30 (metal affinity). Each different surface property retains a specific subset of proteins from the sample. The binding buffers for the CM10 chip (0.1 M ammonium acetate, pH 4-10) and Q10 chip (50 mM Tris-HCl, pH 4-10) were optimized before analysis. Of the three surfaces tested (IMAC, CM10, and Q10) (Figure 1A), the ProteinChip® CM10 provided spectra with the best peak intensity and resolution. Therefore, CM10 was selected for analysis of whole and fractionated CSF. SELDI-TOF reproducibility of CSF samples spotted on CM10 chips is shown in Figure 1B.

Whole CSF from 37 patients (8 NC and 29 CI; Experiment 1) and fractionated CSF from 7 patients (2 NC and 5 CI; Experiment 2) were analyzed in quadruplicates by SELDI –TOF using CM10 chips. The spot surface of each CM10 chip was equilibrated with binding buffer (pH

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4). An aliquot (25 μg) of whole CSF was applied to each spot and incubated in a bioprocessor (Ciphergen, Inc.) at room temperature for 30 minutes with shaking. Unbound proteins were removed by washing spots twice with binding buffer and HPLC-graded water. After drying the spot, 50% sinapic acid (SPA) was applied once and air-dried. SPA was prepared as a saturated solution containing 30% acetonitrile (ACN), 15% isopropanol, 0.5% trifluoroacetic acid, and 0.05% Triton X-100. The ionized proteins and their molecular mass/charge (m/z) ratios were detected using a ProteinChip® Series 4000 reader at Bio-Rad Biomarker Research Center (Malvern, PA) and analyzed using ProteinChip® software 3.2 (Ciphergen Biosystems, Inc.). Data were acquired in the range of 3,000-20,000 m/z with laser intensity set at 3000 and detector sensitivity at 100. The ProteinChip® Reader was externally calibrated for each analysis using six standard proteins: human ACTH peptide 1-24 (2,933.5 Da), bovine ubiquitin (5,733.6 Da), bovine cytochrome C (12,230.9 Da), bovine SOD (15,591.4 Da), equine cardiac myoglobin (16,951.5 Da), and beta-lactoglobulin (18,363.3 Da). Peaks were automatically detected using the Biomarker Wizard® provided within the ProteinChip® software 3.2. The following parameters for peak detection were used: first pass signal/noise (S/N) ratio=5, second pass S/N ratio=2, mass tolerance=0.5%. Estimated peaks were included in completion of clustering.

2.5 CSF fractionation

Subsequent to initial screening of whole CSF samples by SELDI-TOF (Experiment 1), CSF samples from 18 patients were fractionated by RP-HPLC. This procedure enhanced peak detection in the low mass range for SELDI-TOF analysis by making samples less complex, and facilitated protein isolation for sequencing. Pre-fractionation has been previously utilized to effectively separate complex biological samples, allowing isolation of specific low-abundance proteins and avoiding suppression by high-abundance proteins, such as albumin and immunoglobulin present in CSF. Indeed, previous studies demonstrated that CSF profiling was improved by pre-fractionation (Yuan and Desiderio, 2005; Ciborowski et al. 2007). This method does not remove high-abundance proteins, but separates them in different fractions, thereby effectively reducing their interference and allowing detection of low-abundant proteins. Briefly, CSF samples were fractionated using an Agilent 1100 Hewlett Packard HPLC system (Agilent Technologies, Palo Alto, CA). The mobile phases consisted of 100% HPLC-graded water with 0.1% formic acid (mobile phase A) and 100% HPLC-graded acetonitrile with 0.1% formic acid (mobile phase B). Chromatographic separations were achieved using a reverse phase 0.21 cm × 5 cm C8 column (Grace Vydac, Hesperia, CA). The flow rate was set at 0.1 mL/min. Chromatographic column was equilibrated with 100% HPLC-graded water before each run. A total of 100 μL of whole CSF was injected onto the column each time. This step was repeated five times for each patient sample. Since such a high volume of sample was necessary for fractionation and subsequent analysis, our sample size for subsequent assays was considerably reduced. After sample injection, an isocratic wash with mobile phase A to B ratio at 95:5 occurred for 20 minutes. A linear gradient of 90% mobile phase B was applied for 85 minutes to elute proteins. Four specific CSF fractions were obtained that contained sufficient amount of protein for further analysis. The fractions were eluted in 5-minute intervals: 50-55, 55-60, 60-65, and 65-70 minutes. The resultant fractions were dried using a speedvac and resuspended in 60 mL of HPLC-graded water. Each CSF fraction from NC and CI was pooled, one portion of which was tested by SELDI-TOF for detection of differentially expressed proteins in CI and NC patient groups, and the other portion was stored at -80°C prior to 1D SDS PAGE analysis.

2.6 1D SDS PAGE

Pooled CSF fractions from 18 patients were diluted with NuPAGE® LDS buffer (Invitrogen, Carlsbud, CA) and proteins were further separated by one dimensional electrophoresis. Duplicates of 20 μg of each fraction were loaded on a NuPAGE® Novex 10% Bis-Tris

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(Invitrogen) gel and after electrophoresis stained by Coomassie Brilliant Blue (BioRad, Hercules, CA). Protein bands corresponding to molecular weights of interest were cut out using a razor blade. Proteins in gel cubes were subjected to in-gel tryptic digestion as described previously (Ciborowski et al., 2007). Briefly, after distaining with 50% ACN, 50 mM NH_4HCO_3/50% ACN, and 10 mM NH_4HCO_3/50%, the gel slices were dried and then incubated with trypsin (Promega, Madison, WI) for 12-16 hours. All peptides extracted by 0.1% trifluoroacetic acid /60% ACN were pooled into a glass tube and dried prior to LC-MS/MS analysis. Resulting peptides were used for protein identification by LC-MS/MS peptide sequencing and database search.

2.7 Protein identification

The peptides were resuspended in 0.1% formic acid in HPLC-graded water and the ionized peptides were detected on a ProteomeX LC-MS/MS system (Thermo Electron Corporation, Waltham, MA) as previously described (Ciborowski et al., 2007). The amino acid sequence data from LC-MS/MS analysis were blasted against the protein database to search for the matching protein using BioWorks 3.1SR. Protein identifications were accepted as true positive on the basis of arbitrarily set up parameters as previously published (Omenn et al., 2005). Briefly, the criteria were: BioWorks® unified score was ≥3000, Xcorr for doubly charged precursor ion was ≥2.5, DeltaCn was ≥0.3, there were more than 60% of fragment ions per sequenced peptide, and at least two peptides per protein were identified.

2.8 Protein expression by Western blots

Western blot analysis was performed to examine the effect of CI on protein expression. Protein samples from ~20 to 80μg of CSF, from 6 patients with CI and 6 controls with normal cognition, were denatured and separated by SDS-PAGE on a 10% Tris-HCl Ready Gel in duplicates (Bio-Rad Laboratories) and transferred to nitrocellulose membranes (Bio-Rad Laboratories). Membranes were blotted overnight at 4°C either with or without a primary antibody. Immunoblot detection of candidate proteins was performed with the following antibodies: sheep polyclonal antibody against human Cu/Zn SOD-1 (Santa Cruz), polyclonal rabbit anti-human α-MIF (Santa Cruz); polyclonal rabbit anti-human α-VGF (Santa Cruz); and a monoclonal anti-human α-Galectin-7 (R&D Systems). All membranes were incubated for 1 hr at room temperature with horse radish peroxidase enzyme (HRP)-conjugated secondary antibody at a 1:2000 dilution. Secondary antibodies used were donkey anti-sheep (US Biologicals), goat anti rabbit, and donkey anti mouse (Jackson Immunoresearch). Expressed proteins were detected using an ultra sensitive HRP detection system (femtoLucent™ PLUS-HRP Reagent Kit, Geno Technology). The density of the bands was quantified using a scanning densitometer (VersaDoc Imaging System, Bio-Rad Laboratories). Uniformity of protein loading was confirmed by staining of the blots with Ponceau S staining (Sigma).

2.9. Statistical Analyses

Protein profiling spectra from whole CSF of 37 patients were normalized after baseline subtraction and calibration for mass accuracy. Peak detection and clustering was performed using Biomarker Wizard® software 3.2. Data were exported to Microsoft Excel for analysis using SAS® software (SAS Institute, Cary, NC). Protein CSF profiles obtained from patients with NC and CI were compared on the basis of peak intensity or normalized peak height. Repeated measures analysis of variance was used to identify peaks for which there was evidence of statistically significant differences in the distribution of intensity scores among the subjects. The raw intensity values were found to be asymmetrical and adjusted prior to analysis using the following transformation: Y = log2 (X + SQRT[X**2+ 1]), where ‘X’ is the observed intensity. This transformation has been previously used to stabilize intensity variance and make data more nearly normally distributed, and it has the advantage over a log-transformation of

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being able to handle negative intensities (Beyer et al., 2006). An “adjusted p-value” (a “q-value”) was computed to address the issue of multiple comparisons, by which the false discovery rate was controlled at 0.10 (i.e., no more than 10% expected false positives out of differentially expressed ones) (Storey et al., 2004). These comparisons were made using generalized estimating equation (GEE) statistics.

Data from fractionated CSF from 7 patients were analyzed similarly with the exception that the Bonferroni correction procedure was used instead of the false discovery rate due to the smaller number of comparisons. Following the recommendations of Yasui and coworkers (Yasui et al., 2003), only peaks identified in the m/z range of 3,000-20,000 were investigated in these analyses. The Random Forest technique was applied for determination of sensitivity and specificity of 20 differentially expressed peaks from whole CSF in discriminating between normal cognition and cognitive impairment as done previously (Luo et al., 2003). Briefly, this method can generate many classification trees and then estimate the importance of each variable (a peak in this case) by random permutation.

3. Results

3.1 Neuropsychological, immune, and viral load determinations

Fifty HIV-seropositive women characterized for cognitive function from the Hispanic Latino NeuroAIDS cohort were selected for this study. Of these, 13 patients had normal cognition and 37 were cognitively impaired. All of the patients selected were negative for hepatitis C and drug toxicology profile. CSF cell counts, protein and glucose determinations have been obtained for the first thirty patients entered into the cohort. All together as these measures were not altered by patient profiles, levels of immune dysfunction or viral load (data not shown).

The demographics of the patients included in this study by cognitive status category are described in Table 2. The overall group mean of age was as follows (mean ± SD): 38 ± 6 years old, with current CD4 count of 380 + 248 cells/mm^3, CD4 nadir of 217 ± 27 cells/mm^3, plasma viral load of 45,061 ± 144,760 copies/mL, and CSF viral load of 2,502 ± 8,334 copies/mL. Using analysis of variance, there were no significant differences between age (p=0.425), CD4 cell count (p=0.159), CD4 nadir cell count (p=0.753), plasma HIV RNA (p=0.189), or CSF HIV RNA (p=0.977) between patients with NC and CI. Among patients without cognitive impairment reporting therapy use (12/13 (92.3%) reported using HAART. This was not significantly different than the distribution of therapy among those with asymptomatic cognitive impairment (11/15 HAART, 2/15 mono, and 2/15 naive), those with MCMD (2/2 HAART), and those with HIV-D (11/17 HAART, 1/17 naive, and 1/17 ART) (p=0.616).

Figure 2 shows the distribution of scores on the cognitive domains. There were significant differences in neuropsychological performance (NP) on all the cognitive domains: verbal memory (p=0.009) frontal executive function (p=0.000), psychomotor speed (p=0.000), motor speed (p=0.00), and NPZ (p=0.000) between normal cognition and CI group (A, MCMD and HAD). These differences in NP have been reported previously in a larger group of patients (Wojna et al., 2006).

3.1 Protein profiling

Whole CSF from 37 patients was analyzed on a CM10 chip and tested in quadruplicate sets. One hundred forty-eight spectra, which included biological and technical replicates, were analyzed. Of the 357 detected peaks, five showed significantly lower intensities ($q \leq 0.10$) and 14 peaks showed significantly higher intensities in CI when compared to NC (Table 3). The differences in intensities between NC and CI is illustrated in Figure 3A.

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Random Forest statistical analyses were performed on whole CSF to determine the sensitivity and specificity of the protein peaks in distinguishing NC and CI. We found that the 20 peaks analyzed had either high sensitivity and low specificity, or low sensitivity and high specificity (Table 4A). Four most important peaks were selected by Random Forest analysis (3101, 3520, 5316 and 16636) that differentiated NC and CI groups. The sensitivity and specificity values of these peaks are included in Table 4B. The peaks 16636 and 3520 had relatively low sensitivity values. However, a combination of these two peaks was necessary to obtain high sensitivity (90%) and specificity (100%) (Table 4B). Unfortunately, these peaks showed very low intensities to detect them with naked eye most probably due to high abundant proteins present in whole CSF samples.

Protein profiles from whole CSF from patients with NC and those CI show multiple peaks with limited resolution in the lower mass range (Figure 4). Thus, 1D SDS PAGE was performed on CSF samples to assess the possibility that high abundant proteins present in all samples, could saturate the ProteinChip® detector and reduce peak intensities. These samples showed concordant distribution of up to 50% of the total protein content with immunoglobulin and albumin present among patient groups (data not shown). To improve resolution of proteins that could be differentially expressed amongst the CI and NC groups in SELDI-TOF analysis, we next performed CSF fractionation. Each of the four fractions obtained by RP-HPLC was analyzed on a CM10 chip and tested in quadruplicate sets as described in methods. Representative SELDI-TOF spectra are displayed for each fraction and for whole CSF (Figure 5). Twenty eight spectra, which included biological and technical replicates, were analyzed. These spectra represent RP-HPLC fractionated (fraction 2) CSF samples from 5 CI subjects and 2 subjects with NC. In fractionated CSF, of the 26 peaks detected, only the protein peaks with m/z of 4320, 5389, 5597 and 6982 were significantly lower ($q<0.10$) in CI, whereas the protein peaks with m/z of 6695 6799, 13376, 13579, 13811 were significantly higher ($q<0.10$) in CSF of patients with CI (Figure 6).

3.2 Protein identification

Subsequently, all four fractions derived from RP-HPLC fractionated CSF of 18 patients were separated in duplicates by 1D SDS PAGE gels with equivalent protein content. Bands corresponding to regions of interest in SELDI-TOF spectra and others corresponding to higher molecular weights were excised and proteins digested with trypsin. The derived peptides were sequenced by the LC-MS/MS, and eventually proteins were identified. Sixteen proteins were found in CSF from NC and CI meeting all five criteria for protein identification (Table 5). Nine proteins were identified as unique to CSF of CI. These were: soluble superoxide dismutase (SOD 1; related to amyotrophic lateral sclerosis (ALS)), migration inhibitory factor (MIF) - related protein 14, macrophage capping protein, neurosecretory protein VGF, galectin-7, L-plastin, acylphosphatase 1, and a tyrosine 3/tryptophan 5-monooxygenase activation protein. Eleven proteins were identified as unique to CSF of NC (Table 5). Although these proteins were matched only with one peptide, they satisfied four of the five criteria. We accepted these proteins as “true positive” identifications on the basis of results published by the Plasma Proteome Project performed by the Human Proteome Organization (Omenn et al., 2005), which showed that as many as 25% identifications based on the sequence of one peptide were eventually confirmed as “true positive.” It is important to note that we performed a careful inspection of MS/MS data for these peptides and increased a requirement of the number of fragment ions from >60% to >80%.

3.3 Protein validation by Western Blots

Western blots were performed initially for SOD-1 with 11 samples (5 NC and 6 with CI). SOD was recognized by specific-antibodies at the expected size of 16kDa thus demonstrating that it was expressed in CSF of HIV-infected individuals with and without CI. Relative abundance

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of SOD was higher in CI than NC patients (Figure 7). Subsequent Western blots assays were performed on 13 additional CSF samples from NC (3) and CI (10) for MIF, and galectin-7. Results for these proteins were generally increased in CI but not consistently so (data not shown).

4. Discussion

To our knowledge this report is one of two comprehensive proteomic platform analyses performed on CSF of HIV-1 seropositive patients with or at risk for CI (Berger et al. 2005). We identified unique spectral differences amongst CI and NC patient groups with putative proteins identified by tandem mass spectrometry. Nonetheless, identification of CSF protein biomarkers using a proteomics platform was completed for other neurodegenerative diseases including Alzheimer's disease (Carrette et al., 2003), ALS (Ranganathan et al., 2005) and frontotemporal dementia (Pasinetti et al., 2006). In those studies, SELDI-TOF provided important information as to differential expression of proteins between patients with neurodegenerative diseases and healthy individuals. Proteomics has also been applied successfully by our group to fingerprint macrophages from the Hispanic cohort and from *in vitro* HIV infected macrophages (Luo et al., 2003; Wojna et al., 2004a; Ciborowski et al. 2007). We have used the current platform to differentiate macrophage populations from different tissues including those of spleen, bone marrow, and brain (Enose et al., 2005) and response of mouse microglia to injured nerve (Glanzer et al. 2007). Differentially expressed proteins were found in the three distinct macrophage populations by combining SELDI-TOF, RP-HPLC, and peptide sequencing. We extended this proteomic platform to study CSF protein profiles and identify possible biomarkers for HIV-associated cognitive impairment. As part of this platform, we initially performed SELDI-TOF profiling of 37 whole CSF samples from women evaluated for cognitive function from the Hispanic women cohort (Experiment 1). This method yielded 20 differentially expressed protein peaks, of which 15 were over expressed and 5 under expressed in women with CI. A combination of protein peaks was necessary to obtain increased sensitivity and specificity of protein peaks for diagnostic studies. These included the 16.6 + 3.5 kDa protein peaks for 90% sensitivity and 100% specificity. Following RP-HPLC fractionation, 9 differentially expressed protein peaks were detected, 5 of which were over expressed and 4 under expressed in CI (Experiment 2). Of these, 2 protein peaks with m/z of ~5.3 and 6.9 kDa were detected in both whole CSF and fractionated CSF. In case of the 6.9 kDa peak we obtained inconsistent results. Of the protein peaks detected in fractionated CSF, we noticed that two similar peaks with m/z of 6.8 and 5.4 kDa have been observed as up-regulated and down-regulated in previous studies of peripheral blood monocyte-derived macrophages of one patient with CI, who was also included in this study (Wojna et al., 2004b).

One prior study used SELDI-TOF to profile CSF from HAD patients. However, the study was limited in number of patients evaluated in that six HIV-seropositive men, two with NC, two with mild dementia, and two with HAD (Berger et al., 2005). Using metal binding chips (IMAC), those investigators found a protein peak with m/z of 6.7 kDa that was under expressed in CI, and a peak with m/z of 8.9 kDa that was over expressed in CI. Using anionic chips WCX2 (equivalent to the CM10), they found a peak with an m/z of 2.0 kDa that was over expressed in CI and a peak with m/z of 4.9 kDa that was under expressed in CI. In this our study, we also detected a peak with m/z of 6.7 kDa as differentially expressed in fractionated CSF of NC compared to and CI. However, this peak was found using anionic chips CM10, as over expressed in the CSF of women with CI. The protein found by Berger and coworkers (2005) could be distinct from what we observed in this study since it was metal binding (negatively charged) and they did not detect it in the WCX2 chips. The differences between the two studies could be due to variations in the patient classification criteria for cognitive dysfunction, the

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cohort composition (proportion of men and women vs. women only), or ethnicity (Meléndez et al., 2007).

In an attempt to identify these differentially expressed proteins, we performed LC MS/MS of pooled CSF fractions. Samples were pooled by fractions within each patient group. Interestingly, five of the nine proteins identified in the CSF of CI patients (migration inhibitory factor (MIF) -related protein 14, neurosecretory protein VGF, galectin-7, acylphosphatase 1, and a tyrosine 3/tryptophan 5-monooxygenase activation protein) are important in cell signaling. One of these, MIF is secreted by macrophages during inflammation and is an inhibitor of protein kinases. MIF was originally discovered as a lymphokine involved in delayed hypersensitivity and various macrophage functions, including phagocytosis, spreading, and tumoricidal activity (Nishihira, 2000). To determine if MIF could increase with the development of CI, we performed Western blots. Indeed, after densitometry analyses MIF showed a biphasic mode where it was increased in HAD. Additional studies will be pursued with increased number of patients to confirm the validity of MIF as a biomarker for stages of CI associated with HIV. However, the ideal biomarker will be one that increases with the degree of CI.

VGF has been reported as decreased in ALS (Pasinetti et al., 2006), Alzheimer's disease (Carrete et al., 2003), and frontotemporal dementia (Rüetschi et al., 2005). VGF is a nerve growth factor that could be produced in response to neuronal damage during the development of CI. We performed Western blots on additional CSF samples from the cohort using a polyclonal antibody against VGF. However, this antibody reacted with a higher mw band (~98 kDa) and lower molecular masses (~49 and 62 kDa) than the theoretical band expected from mass spectrometry analyses (~67kDa). Despite this cross reactivity, we could not detect differences between the groups.

Galectin-7 is involved in cell-cell and/or cell-matrix interactions necessary for normal growth control and is pro-apoptotic; an event that is known to occur during HIV associated CI (Kaul and Lipton, 2006). We pursued Western blot analyses on Galectin-7 using a monoclonal antibody that recognized a ~72kDa and 42kDa bands, but nothing in the expected size (15-17kDa). This may represent a truncated fragment of Galectin-7 not detectable by the monoclonal antibody.

Two of the nine proteins identified in CSF of CI patients have a structural role in actin filaments (macrophage capping protein and L-plastin). Macrophage capping protein is calcium sensitive, and has an important role in macrophage function by regulating cytoplasmic and nuclear structures. Both L-plastin and human macrophage capping protein are associated with phagocytosis, an important macrophage function that leads to the release of inflammatory mediators. Disruption of these structural filaments is well known to alter macrophage function (Hartwig and Yin, 1988). Indeed, we identified L-plastin in macrophages infected in vitro with HIV isolates from CI, and it was not present in uninfected controls or in macrophages infected with other isolates (Toro et al., manuscript in preparation). We could not confirm L-plastin in the CSF samples from the cohort by Western blot analyses at the moment as there is no antibody commercially available to test L-plastin.

One protein was found to be related to antioxidant activity, SOD 1, a variant of Cu/Zn superoxide dismutase related to ALS, is the primary enzyme in the antioxidant pathways and was identified only in CSF of CI patients. This finding was intriguing since this mutation is mostly associated with familial ALS, a genetic neurodegenerative disease. However, several reports show an ALS-like syndrome occurring in association with HIV infection (von Giesen et al., 2002; Cone et al., 2002; Ahmad, 2001; MacGowan et al., 2001; Moulingnier et. al., 2001; Verma and Berger, 2006). Cu/Zn SOD 1 is a cytosolic enzyme produced during an

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inflammatory response to neutralize reactive oxygen species such as superoxide anions that induce oxidative damage. Elevated SOD 1 was found in a subset of 6 CSF samples from patients with HAD by Western blot. When attempting to correlate this result with the SELDI-TOF experiments, we found that in whole CSF there was a peak with an m/z of 16636 Da that decreased with CI (Table 3). We believe that this apparent discrepancy could be due to distinct proteins, that SOD was modified, or that the protein did not bind well to the chip's surface under conditions used in this study. Such differences are not uncommon in proteomics analyses and the most likely conclusions are that Table 3 and Fig. 7 are both correct but represent different proteins. Ongoing studies at our laboratories suggest that there may be changes in SOD related to the degree of CI. High SOD1 was previously reported in HIV-1-infected macrophages present in brain tissues of patients who died with HAD (Treitinger et al., 2000; Boven et al., 1999; Mollace et al., 2001). As the primary infected cells in the brain, HIV-infected macrophages can respond to oxidative stress by producing a number of viral and cellular factors that affect disease pathogenesis. Ongoing studies on SOD-1 expression and activity are active in our laboratory from body fluids derived from our patient cohort and from *in vitro* infected macrophages. Preliminary studies suggest that SOD expression is decreased during monocyte-macrophage differentiation.

Additional proteins found common to NC and CI, such as Apo E, APP, and cystatin C have been associated with HAD and other neurodegenerative diseases such as Alzheimer's, multiple sclerosis (MS), and ALS (Vehmas et al., 2004; Irani et al., 2006; Pasinetti et al., 2006, Pocernish et al., 2004). The findings of increased enzymes in the CSF is consistent with the reported high lysosomal expansion that occurs during HAD (Gelman et al., 2005). Although found common to NC and CI, it is still possible that they may differ in abundance between both groups. A peak of 13.4 kDa detected by SELDI-TOF and corresponding to cystatin C was decreased in ALS and MS (Pasinetti et al., 2006; Irani et al., 2006). Interestingly, we found that this peak was significantly higher in CSF of patients with CI analyzed in this study. In a parallel study, we recently found increased expression of cystatin B, the non-secreted form of cystatin C, to be related to the levels of viral replication in macrophages (Luciano-Montalvo et al., manuscript in preparation). Cystatin family members are cysteine proteinase inhibitors of cathepsins, which are released during pain and inflammation and can interfere with antigen processing by monocytes (Mannes et al., 2003; Greiner et al., 2003). Therefore, increased cystatin C may play a protective role against neuronal apoptosis during HIV-cognitive impairment.

PTP was found among the 11 proteins unique to the NC group. The finding of this protein, CD45, in macrophages suggests another linkage of these cells to CSF of women with normal cognition. Current efforts in our laboratories are directed at detecting the proteins common to CSF and MDM from selected members of the Hispanic women cohort.

Overall, our study objective was to apply a proteomics platform to the study of CSF to identify proteins that would differentiate CI from NC. We were able to successfully identify several candidate biomarkers related to signaling, macrophage function, and redox, namely, MIF, SOD 1, cystatin C, and VGF among others. Validation of these proteins will require additional efforts to find the correct experimental conditions and disease state that is linked to CI. Our study has several limitations that need to be addressed. These include the small number of patients with NC compared to CI and the low protein concentration in CSF. These are inherent obstacles towards the precise identification, and validation of all protein candidates. Nonetheless, these may be overcome, in part, by increasing the CSF sample volume collection. Additional experiments are being conducted to improve this platform to minimize the amount of protein required for identification. Combining measures of enzymes and their activity with other CSF biomarkers uncovered through such screening may offer complementary information on the underlying pathology and prognosis in an individual patient who is susceptible to virus-associated brain disease. It may ultimately provide unique insights into the timing and

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mechanism of brain injury. The search for biomarkers in patients under HAART must continue. Ultimately a better understanding of immune responses to viral infection will lead not only to a better understanding of disease and disease mechanisms but also improved measures to diagnose, follow, and treat a most devastating complication of progressive infection in the human host.

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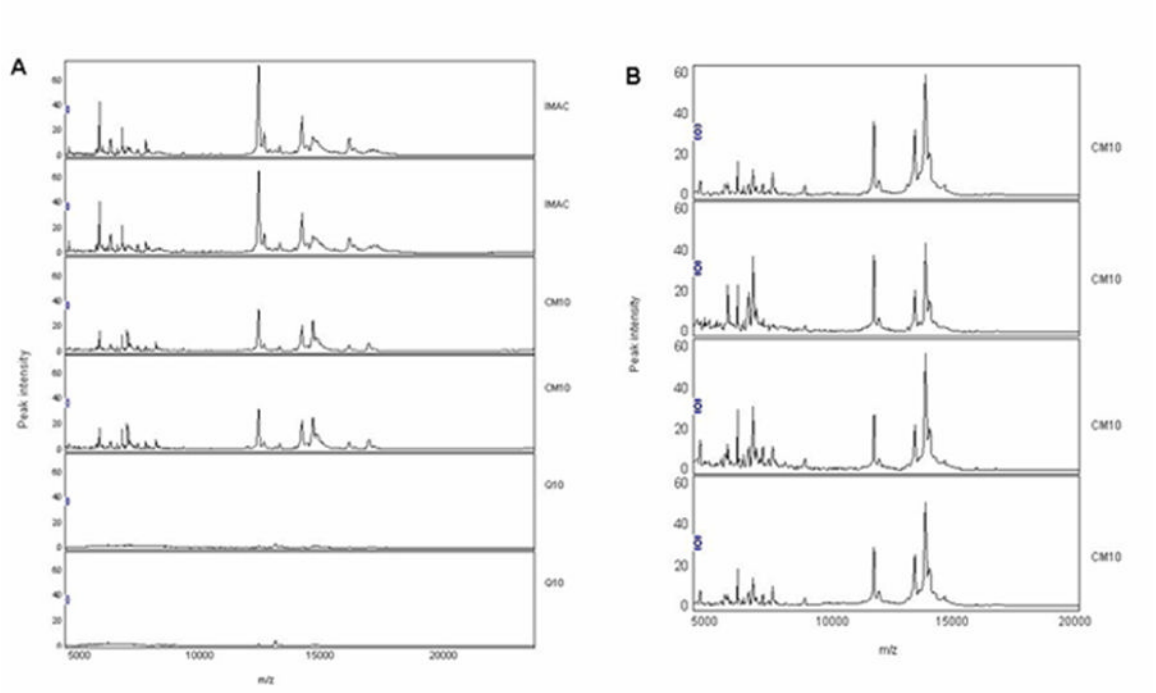

Figure 1.

SELDI-TOF reproducibility of whole CSF on different chip arrays. Whole CSF was analyzed on three different types of Protein Chip array surfaces: IMAC chips (top two spectra), CM10 chips (middle two spectra), and Q10 chips (bottom two spectra) (panel A). Samples were run in quadruplicates and displayed in duplicate. A representative sample showing good reproducibility on CM10 is illustrated (panel B).

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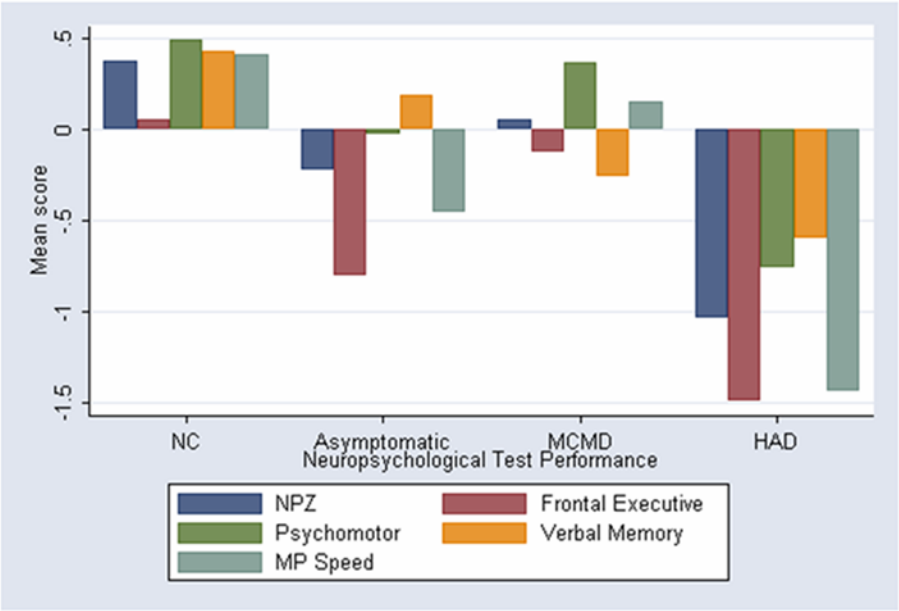

Figure 2.
Neuropsychological performance by cognitive domain among women with HIV infection by modified American Academy of Neurology diagnostic criteria for HIV-associated dementia (mAAN). Neuropsychological performance was significantly different in CI group (A, MCMD, and HAD) compared to normal cognition by ANOVA ($p<0.001$). A, asymptomatics; MCMD, HIV-associated minor cognitive-motor disorder; HAD, HIV-associated dementia.

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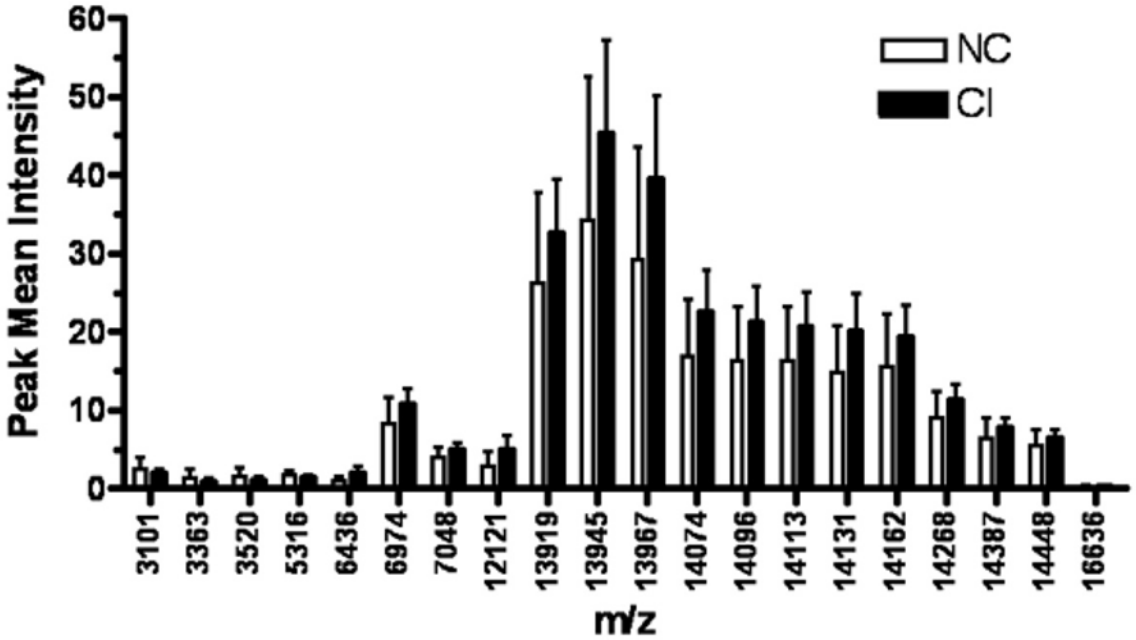

Figure 3.
Differentially expressed protein peaks of whole CSF from NC and CI groups. One hundred and forty-eight spectra from CSF of 29 CI patients and 8 patients with NC were compared using generalized estimating equations. This analysis revealed significant differences ($p<0.10$) between the peak intensities of both patient groups.

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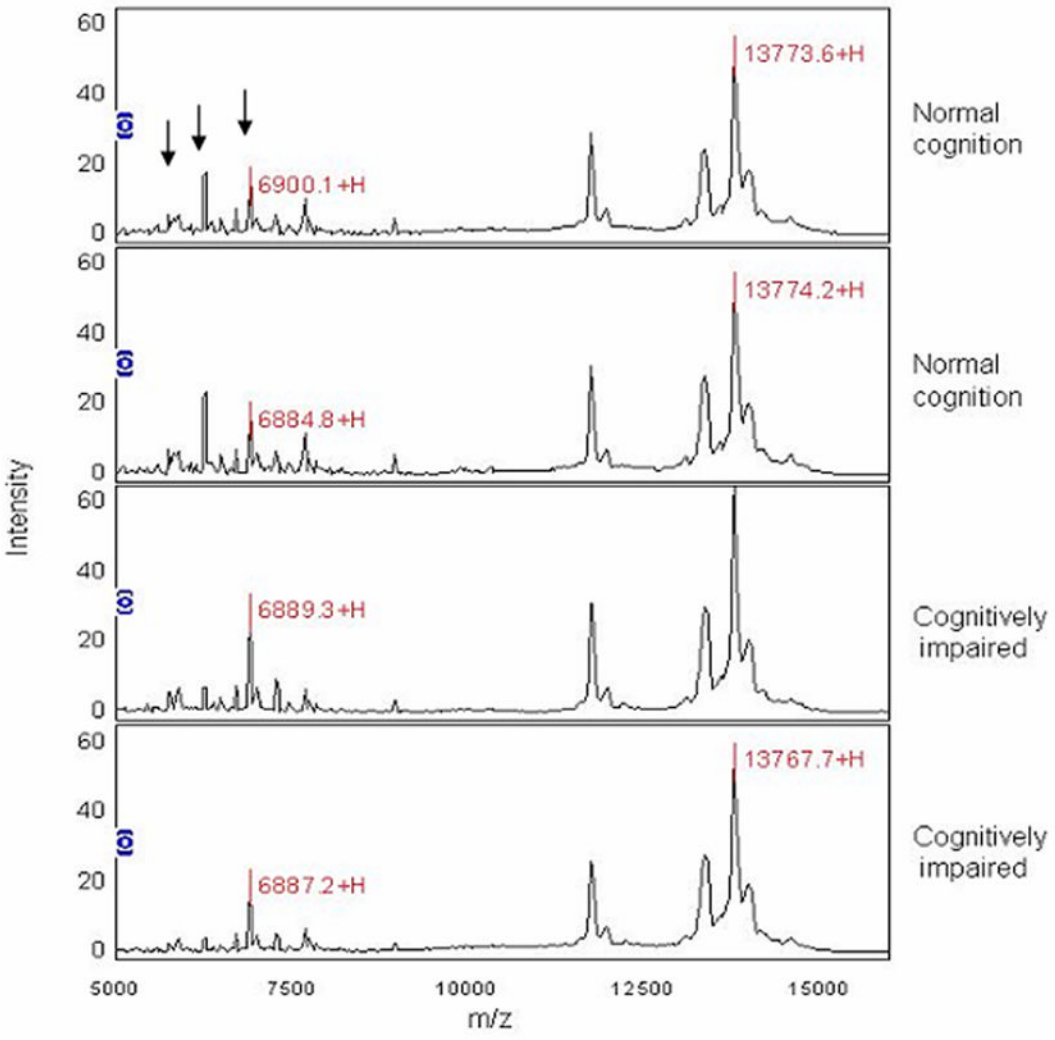

Figure 4.

Representative SELDI-TOF profile of whole CSF from NC and CI. SELDI-TOF spectra on CM10 chips show peaks at the higher end of the spectra and peaks with low intensities at lower m/z values. Samples were run in quadruplicates and displayed in duplicates to illustrate reproducibility. The first spectrum has three arrows that point out several differences in peak intensities between NC and CI.

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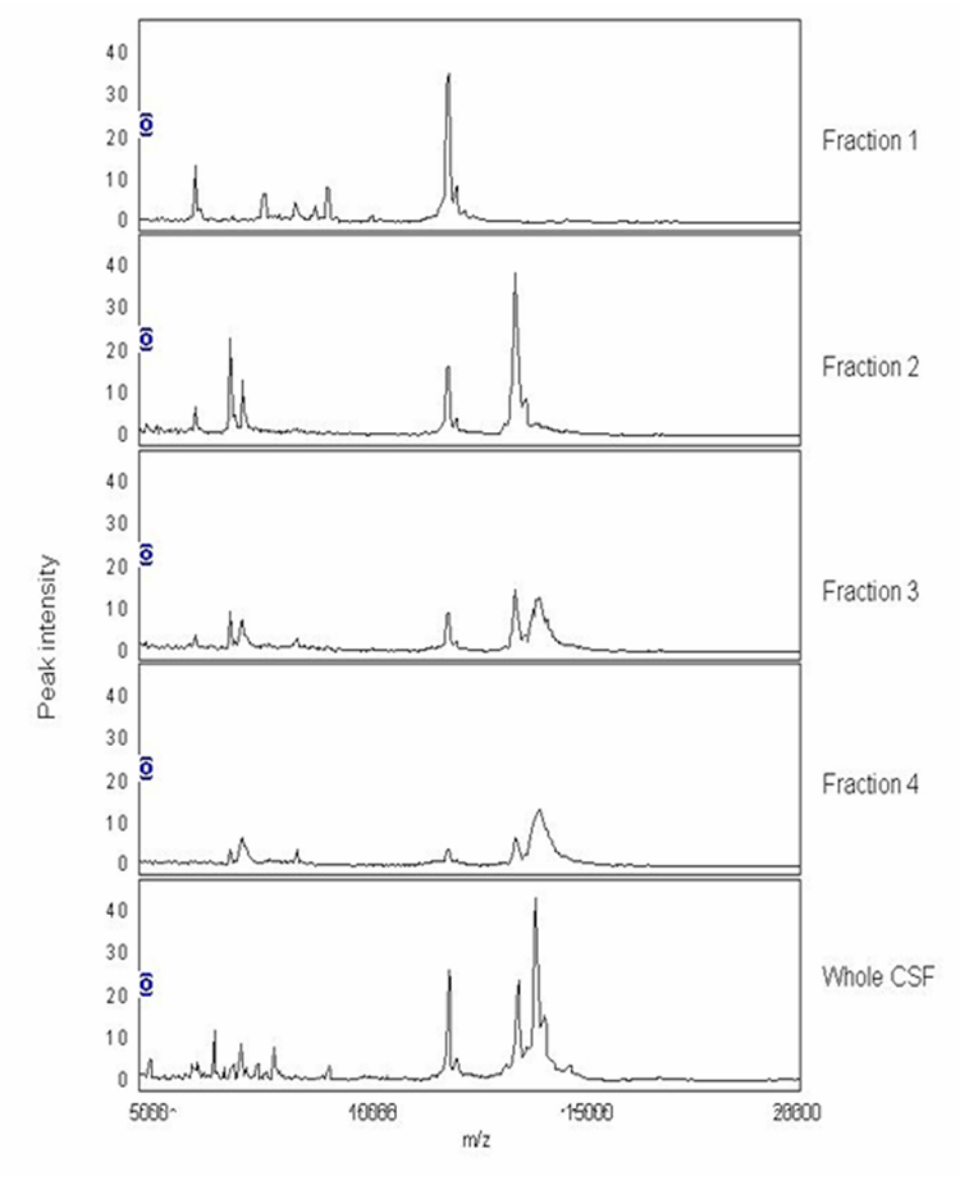

Figure 5.

Representative SELDI-TOF spectra for CSF fractions and whole CSF. Four fractions were obtained from RP-HPLC with elution times between 50-55, 55-60, 60-65, and 65-70 min. Each of the fractions was prepared on a CM 10 chip and run in quadruplicate sets. Only fraction 2 contained sufficient amount of protein for further analysis.

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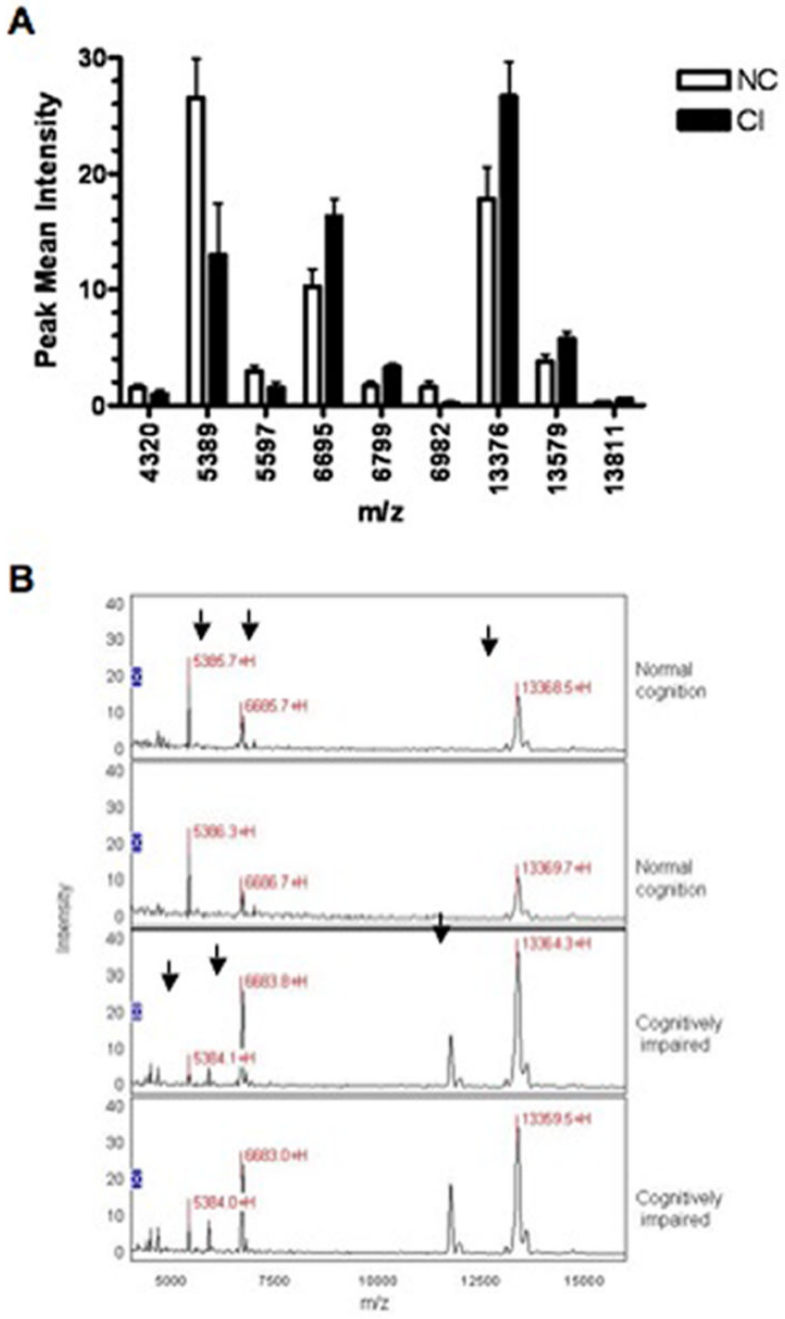

Figure 6.

Differentially expressed peaks in CSF of NC and CI. Twenty-eight spectra from CSF fraction 2 of 2 CI patients and 5 patients with NC were analyzed. Nine protein species were differentially expressed between the two groups ($p<0.10$; panel A). Representative spectra of showing several differentially expressed peaks (black arrows) from NC (upper spectra) and CI (bottom spectra) in duplicates to illustrate reproducibility (panel B).

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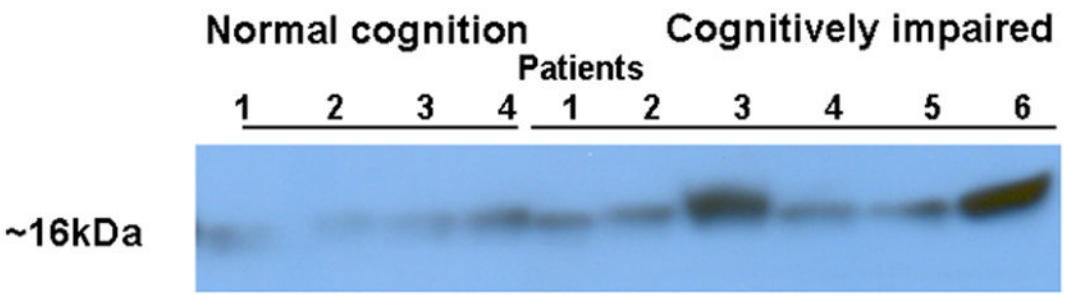

Figure 7.
Western blot analysis of Cu/Zn SOD-1 in CSF. A representative Western blot demonstrating SOD-1 (16KDa) in CSF of 11 of HIV-1 infected individuals with and without CI (6 CI and 5 NC).

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Table 1

Neurocognitive examination of Hispanic women

Neuropsychological domain	Test	Subtests
Verbal memory	Rey Auditory Verbal Learning Test[1]	Trial 5 Memory recall Delayed recognition
Frontal executive	Stroop	Word/color
	Trail Making B	Total score (seconds)
Psychomotor speed	Digit Symbol Modality Test	Total score
	Reaction Time	Visual & auditory non dominant hand
Motor speed	Trail Making A	Total score (seconds)
	Grooved Pegboard	Dominant & non dominant hand (seconds)
Visuoconstruction	Cube	Copy

[1]) Spanish translation, standardized with a similar reference control group (see text description)

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Table 2

Demographics, viral and immune parameters in HIV-seropositive women

	m-AAN criteria (n=50)[1]				
	Normal Cognition NC	**Cognitive Impairment CI**			**p-value[2]**
	Normal n=13	**Asymptomatic n=18**	**MCMD n=2**	**HAD n=17**	
Age Range	37 ± 5 29-44	36 ± 6 22-44	36 ± 1 35-37	39 ± 7 29-53	p= 0.425
CD4[3] Range	307.5 ± 147.9 60-596	327.6 ± 243.3 4-949	271.5 ± 92.6 206-337	482.9 ± 288.5 35-477	p= 0.159
CD4 nadir[3] Range	209.1 ± 139.5 28-309	256.1 ± 274.5 4-1058	181.5 ± 201.5 39-324	209.1 ± 139.5 35-477	p=0.753
Plasma HIV RNA[4] Range	18,871 ± 38,225 50-128,444	122,052 ± 257,624 50-892,916	13,857 ± 8,376 7,934-19,780	14,029 ± 23,401 50-72,329	p=0.189
CSF HIV RNA[4] Range	2,242-4,740 50-13,928	2,334 ± 6,028 50-23,616	1,170 ± 1,584 50-2,290	3,343 ± 12,795 50-51,319	p=0.977
Treatment					p=0.616
•No treatment	7.6% (1/13)	13.3% (2/15)	0	5.9% (1/17)	
•ART	0	13.3% (2/15)	0	5.9% (1/17)	
•HAART	92.3% (12/13)	73.3% (11/15)	100.00% (2/2)	88.2% (15/17)	

[1]) m-AAN, modified American Academy of Neurology HIV-associated dementia; MCMD, HIV-associated minor cognitive-motor disturbance; HAD, HIV-associated dementia; ART, antiretroviral treatment; HAART, highly active antiretroviral treatment.

[2]) ANOVA statistics.

[3]) CD4 cells/mm3.

[4]) HIV RNA or viral load in copies/mL.

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Table 3

Differentially expressed peaks in whole CSF of NC (n=8) and CI (n=29) patients

Peak Number	Median m/z	q-value[1]	NC	CI
1	3101	0.0571	HIGH	LOW
2	3363	0.0860	HIGH	LOW
3	3520	0.0571	HIGH	LOW
4	5316	0.0213	HIGH	LOW
5	6436	0.0545	LOW	HIGH
6	6974	0.0571	LOW	HIGH
7	7048	0.0832	LOW	HIGH
8	12121	0.0928	LOW	HIGH
9	13919	0.0213	LOW	HIGH
10	13945	0.0928	LOW	HIGH
11	13967	0.0911	LOW	HIGH
12	14074	0.0389	LOW	HIGH
13	14096	0.0213	LOW	HIGH
14	14113	0.0213	LOW	HIGH
15	14131	0.0213	LOW	HIGH
16	14162	0.0489	LOW	HIGH
17	14268	0.0213	LOW	HIGH
18	14387	0.0213	LOW	HIGH
19	14448	0.0213	LOW	HIGH
20	16636	0.0661	HIGH	LOW

[1]) The adjusted p-value ≤ 0.10, after correcting for multiple comparisons (Storey and Sigmund, 2004).

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Table 4A

Diagnostic value of the 20 differentially expressed peaks between NC and CI

Peak in m/z (mean intensity)	HIV-CI vs. HIV-NC	
	Sensitivity (%)	Specificity (%)
3101 (<2.453)[1]	93	63
3363 (<1.333)	83	63
3520 (<1.119)	55	100
5316 (<1.776)	97	63
6436 (>=0.906)	90	50
6974 (>=8.233)	83	63
7048 (>= 4.222)	62	88
12121 (>=4.196)	45	100
13919 (>=27.15)	62	88
13945 (>=37.1)	66	75
13967 (>=37.03)	41	100
14074 (>=20.09)	41	100
14096 (>=18.86)	45	100
14113 (>=18.77)	45	100
14131 (>=16.5)	48	100
14162 (>=18.3)	45	100
14268 (>=10.15)	48	100
14387 (>=6.996)	62	88
14448 (>=5.791)	66	88
16636 (<0.219)	69	100

[1]) For this entry, the m/z value is 3101 and the patient with mean intensity less than 2.453 is classified as **CI**

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Table 4B

Comparisons of most important protein peaks for diagnostic value[1]

Peak (m/z)	HIV-CI vs. HIV-NC	
	Sensitivity (%)	Specificity (%)
Single peak		
5316	97	63
16636	69	100
3101	93	63
3520	55	100
Combined peak		
16636 + 3520	90	100

[1]) Four most important peaks were selected based on Random Forest statistical analysis

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Table 5

Identified proteins in fractionated CSF of CI and NC patients.

Protein	NC	CI	NCBI[a]	SwissProt[b]	MW	Proposed function	Peptides
Acylphosphatase 1	-	+	1082173	P07311	11.3	Catalyzes the hydrolysis of the carboxyl-phosphate bond of acylphosphates	1
Albumin	+	+	23307793	Q56G89	69.1	Regulation of the colloidal osmotic pressure of blood	7
Alpha-1-antitrypsin	+	+	24438	P01009	46.7	Inhibitor of serine proteases.	5
Amyloid beta A4 protein precursor	+	+	28558768	P05067	86.9	Cell surface receptor relevant to neurite growth, neuronal adhesion and axonogenesis.	2
Antithrombin	+	+	4502261	Q5M7T5	52.2	Serpin; serine protease inhibitor.	2
Apolipoprotein A-I (and precursor)	+	+	37499465	P02647	30.8	Promotes cholesterol efflux from tissues and acts as a cofactor for the lecithin cholesterol acyltransferase. .	5
Apolipoprotein A-IV (and precursor)	+	+	37499461	P06727	45.4	Major component of HDL and chylomicrons and a potent activator of lecithin-cholesterol acyltransferase in vitro.	8
Apolipoprotein D	+	+	1246096	P05090	21.3	Occurs in the macromolecular complex with lecithin-cholesterol acyltransferase.	1
Apolipoprotein E (and precursor)	+	+	13097699	P02649	36.2	Mediates the binding, internalization, and catabolism of lipoprotein particles.	5
Apolipoprotein H (Beta-2-Glycoprotein-I)	+	-	543826	P02749	38.3	May prevent activation of the intrinsic blood coagulation cascade by binding to phospholipids on the surface of damaged cells.	1
Apolipoprotein J (Clusterin)	+	+	32891795	P10909	52.5	Associated with the clearance of cellular debris and apoptosis.	5
Brain-specific angiogenesis inhibitor 2	+	-	21928571	O60241	171.1	Might be involved in angiogenesis inhibition.	1
Cell surface glycoprotein MUC18	+	-	33989399	P43121	71.6	CD146. Plays a role in cell adhesion, and in cohesion of the endothelial monolayer at intercellular junctions in vascular tissue.	2
Chromogranin B precursor (secretogranin 1)	+	-	4502807	P05060	78.2	Neuroendocrine secretory granule protein.	1
Cystatin C (and precursor)	+	+	296643	P01034	15.8	Inhibitor of cysteine proteinases.	5
Dermcidin precursor	+	+	16751921	P81605	11.3	Antimicrobial activity.	1
Extracellular superoxide dismutase (Cu, Zn)	+	-	4507151	P08294	25.9	SOD 3, destroys radicals which are normally produced within the cells and which are toxic to biological systems.	1
Fibrinogen beta chain	+	-	399492	P02675	55.9	Yields monomers that polymerize into fibrin and acts as a cofactor in platelet aggregation.	1
Galectin-7	-	+	3891470	P47929	15.1	Pro-apoptotic protein upstream of JNK activation and cytochrome c release.	2
Gelsolin	+	+	4504165	P06396	85.7	Calcium-regulated, actin-modulating protein that binds to actin monomers or filaments, preventing monomer exchange	1
Hypothetical protein U55B	-	+	51874279	P52518	49.6	Herpes virus 7.	1
Kallikrein-6	+	-	15930186	Q92876	26.9	Serine endopeptidase.	1
L-plastin	-	+	14043359	P13796	70.3	Actin-binding protein.	1
Macrophage Capping Protein	-	+	21730367	P40121	38.5	Calcium-sensitive protein which reversibly blocks the barbed ends of actin filaments but does not sever preformed actin filaments.	1
MAP/ERK kinase kinase 4	+	-	50741728	Q9Y6R4	181.6	Activates the CSBP2, P38 and JNK MAPK pathways, but not the ERK pathway.	1
Migration inhibitory factor-related protein 14	-	+	4506773	P06702	13.2	Expressed by macrophages in acutely inflammated tissues and in chronic inflammations. Inhibitor of protein kinases.	1
N-acetyllactosaminide beta-1,3-N-acetylglucosaminyltransferase	+	-	18314366	O43505	47.1	Can initiate the synthesis or the elongation of the linear poly-N-acetyllactosaminoglycans.	1
Neurosecretory protein VGF (precursor)	-	+	20140360	O15240	67.3	May be involved in the regulation of cell-cell interactions or in synatogenesis during the maturation of the nervous system (By similarity).	1

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Protein	NC	CI	NCBI[a]	SwissProt[b]	MW	Proposed function	Peptides
Olfactory receptor 1B1	+	-	52219202	Q8NGR6	35.2	G-protein-coupled receptor responsible for the recognition and G protein-mediated transduction of odorant signals.	1
Orosmucoid -1 and -2	+	+	55958974, 4505529	P02763, P19652	23.5	Appears to function in modulating the activity of the immune system during the acute-phase reaction.	1
Osteopontin precursor	+	+	22761565	P10451	35.4	Acts as a cytokine involved in enhancing production of interferon-gamma and interleukin-12 and reducing production of interleukin-10 and is essential in the pathway that leads to type I immunity..	1
Prostaglandin D2 synthase	+	+	54696706	P41222	21.2	Involved in a variety of CNS functions, such as sedation, NREM sleep and PGE2-induced allodynia, and may have an anti-apoptotic role in oligodendrocytes.	3
Protein FAM3C	+	-	7661714	Q92520	24.7	Cytokine activity. Secreted protein.	1
Soluble superoxide dismutase (Cu, Zn; amyotrophic lateral sclerosis 1)	-	+	31615967	P00441	15.9	SOD 1, destroys radicals which are normally produced within the cells and which are toxic to biological systems.	1
Transferrin (and precursor)	+	+	4557871	Q9XT72	76.5	Glycoprotein that transports iron from the intestine, reticuloendothelial system, and liver parenchymal cells to all proliferating cells in the body.	9
Transthyretin (prealbumin)	+	+	48145933	P02766	15.9	Thyroid hormone-binding protein. Probably transports thyroxine from the bloodstream to the brain.	3
Tyrosine 3/tryptophan 5 - monooxygenase activation protein	-	+	4507953	P63104	27.7	Protein Kinase C inhibitor.	2
Tyrosine-protein phosphatase non-receptor type substrate 1 precursor	+	-	21619841	P78324	54.8	Immunoglobulin-like cell surface receptor for CD47. May play a key role in intracellular signaling during synaptogenesis and in synaptic function. Mediates negative regulation of phagocytosis, mast cell activation and dendritic cell activation.	2
Vitamin D Binding Protein	+	+	34785355	P02774	52.9	Carries vitamin D sterols and prevents polymerization of actin by binding its monomers.	2

[a] NCBI Accession Numbers.

[b] SwissProt Accession Numbers.