Liquid Chromatography Mass Spectrometry to the Study of Plasma Biomarkers in Multiple Sclerosis

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Research Article

Keywords: multiple sclerosis, plasma, proteomics, biomarkers

Posted Date: March 2nd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1309240/v1

Abstract

Objective

Here, Our study aimed to find the plasma biomarkers related to the diagnosis of multiple sclerosis (MS) through Liquid chromatography Mass Spectrometry (LC/MS) technology.

Methods

Tandem mass tag (TMT) quantitative LC/MS proteomics method was used to determine the differentially expressed proteins (DEPs) in the plasma samples of 22 MS patients and 22 healthy controls. and the functional annotations of the DEPs were analyzed by GO and IPA. The candidate protein was validated by ELISA. The receiver operating characteristic (ROC) curves were used to determine the predictive potential of the biomarker.

Results

LC/MS analysis of plasma proteomic identified 88 DEPs among the quantified 375 proteins, of which 39 proteins were up-regulated and 49 proteins were down-regulated. These proteins are involved in immunity/inflammation and Neurological diseases related pathways. A protein panel consisting of Alpha-1-antitrypsin (SERPINA1) and Protein S100-A9 (S100A9) were with an area under the curve (AUC) of 0.991. A MS treatment related protein, DPP4, was validated by ELISA.

Conclusion

DEPs related to MS can be found in the plasma proteome, which may become biomarkers for MS diagnosis. Our study layed the foundation for the further application plasma proteomics in the diagnosis of MS.

Introduction

Multiple sclerosis (MS) is a chronic demyelinating inflammatory disease of the central nervous system (CNS)[1]. In most patients who later develop MS, the disease usually initiates with single acute or subacute episode of demyelination in the CNS when other diseases are excluded, which known as a clinically isolated syndrome (CIS)[2]. Two thirds of patients with CIS will have further episodes of neurological dysfunction and convert to MS [3].MS related to the chronic degeneration of the CNS and may lead to permanent neurological problems and considerable disability[1].One of the main characteristics of MS is its multiple time and space, the current pathogenesis of MS is dynamic and complex[1, 4].

The current diagnosis of MS is mainly based on the combination of magnetic resonance imaging (MRI) and the identification of oligoclonal IgG in cerebrospinal fluid[5]. However, since neither MRI nor oligoclonal bands are specific for MS[6], some patients fail to detect early in the disease, MS diagnosis and the start of treatment is often delayed from waiting for a second clinical relapse or after confirming neuroimage dissemination of lesions in space and time[7].Early diagnosis and treatment is essential to minimize the occurrence of further attacks and the accumulation of permanent disability[8].Therefore, there is an urgent need for biomarkers to achieve early diagnosis. However, there is still a severe lack of MS biomarkers based on body fluids [9, 10].

Proteomics has been developed with the support of mass spectrometry and bioinformatics technology which provides a high-resolution, high-accuracy strategy to identify novel MS biomarkers[11, 12].In the past two decades, several research groups have provided proteomic analysis of MS. For cerebrospinal fluid (CSF) proteomics, for the first time, Dumont, D et al and Hammack, B.N et al in 2004 used LC-ESI-MS/MS technology to analyze the proteomics of MS cerebrospinal fluid showing the changes of 65 and 61 proteins respectively[13, 14].Then, Violaine K. Harris et al in 2010 used SELDI-TOF-MS technology and identified CSF levels of Bri2-23 may serve as a biomarker of MS[15]. O. Timirci-Kahraman, et al in 2019used LC-MS/MS technology to identify in the proteome of the cerebrospinal fluid of MS that HOXB3 might be a new biomarker to predict the clinical conversion of CIS-MS[16].For blood proteomics, Jianghong Liu in 2012 used MALDI-TOF-MS technology and identified Serum nucleolin as a possible biomarker of relapsing-remitting multiple sclerosis [17].Arjan Malekzadeh et al in 2019 used multiple aptamer proteomics technology to evaluate a large number of plasma proteins in a large number of MS patients with different disease progression rates, and revealed 8 new biomarkers related to the clinical and radiological progression of MS[1].The best effect is Huang J et al in 2020 used proximity extension assay to analyze the proteomics of MS cerebrospinal fluid showing that the 10 cerebrospinal fluid proteins had a good combined diagnostic efficacy (area under the curve [AUC] = 0.95)[18].

Previous studies have provided a variety of MS proteome database analysis and disease difference analysis. And so far, most of the focus of the discovery of protein biomarkers in mass spectrometry has been on CSF proteomics, and the research on plasma proteomics needs to be further strengthened[1].

In our study, we used Tandem mass tag (TMT) absolute quantitative labeling combined with high-resolution LC/MS analysis to reveal the plasma differential proteins in MS patients and healthy controls, a total of 22 MS patients and 22 healthy controls were included. A detailed proteome functional annotations of differentially expressed proteins (DEPs) were provided through GO and IPA analysis, and finally used ELISA technology to verify candidate biomarkers(Figure 1). In this study we aimed to discover the potential biomarkers through a comparative analysis of plasma samples collected from MS patients and controls. This research helped to the understanding of MS and promoted the application of MS in clinical research.

Materials And Methods

Reagents and Instruments

Dithiothreitol (DTT), iodoacetamide (IAM), formic acid, Acetonitrile (ACN), were purchased from Sigma (St. Louis, MO, United States). Sequencing-grade trypsin was purchased from Promega.

The Q Exactive Plus Orbitrap MS (Thermo Scientific,Bremen) coupled with an EASY-nLC 1200 was used for the MS analysis in the DDA-MS modes.

Clinical Materials

The plasma samples for dataset establishment were obtained from 22 patients with MS and 22 healthy people. The average age of MS patients was 31.40±12.60 years old, and the median age was 31.5 years old. among them, 5 are males and 18 are females. The average age of healthy person was 30.52±20.48 years old. and the median age was 29.5 years old. among them, 8 are males and 14 are females (detailed data in Supplementary Table S1).

Protein Extraction

First, remove high-abundance protein, Mix the plasma with the matrix in the high-abundance cartridge, incubate for 10 minutes, open the closed end of the cartridge, put the cartridge into a 1.5ml centrifuge tube, centrifuge at 1,000 × g for 2 minutes, and place the collected sample at -20 Store at ℃, and then apply BCA spectrophotometry to quantify the protein after depletion of high abundance.

Protein Digestion

Take 50μg of the sample to be tested and denature it with (8mol guanidine hydrochloride, 50mmol PBS) solution. Add 1 mol of DTT solution and let stand at 37°C for 1 hour. Add 1 mol of iodoacetamide solution. 30℃, avoid light for 30min. The sample was transferred to a 10kd ultrafiltration membrane and washed twice with buffer (7mol/L urea and 50mmol/L Tris), and then washed twice with 100mm triethylammonium bicarbonate solution. The treated sample was digested with trypsin in a 100mm triethylammonium bicarbonate solution at 37°C overnight. Centrifuge the ultrafiltration tube at 12 000 × g/min at 4°C for 20 min to collect the enzymatic hydrolysis products. Store the mixed peptides in a refrigerator at -20℃.

TMT labeling

Peptides from the plasma samples were labeled using TMT 10-plex™Label Reagent set as per the manufacturer's instruction. Before use, the TMT labeling reagents were removed from the freezer and stabilized at room temperature (approximately 30 min). Then, 41 mL of ACN was added to each channel, dissolved through vortexing centrifuged, and set aside. The dissolved reagent was added to 100 mg of sample (mix 1:1). The solution was allowed to stand for at room temperature for 1 h. After adding 4 mL 5% of hydroxylamine, the solution was incubated at room temperature for 15 min to terminate the

reaction. The samples of each group from 10 channels were combined separately, and the vortexed to allow complete mixing. Salt and other impurities were removed from the sample before freezing at -80°C.

LC/MS Analysis

Q Exactive Plus Orbitrap MS (Thermo Scientific) coupled with the EASY-nLC1200 used for the MS analysis in the DDA-MS modes. The digested peptides were separated on an C18 self-packing capillary LC column (50 mm*15cm, 3μm).The eluted gradient was 2%-100% buffer B2 (0.1% formic acid, 80% ACN; flow rate,0.3μl/min) for 120 min.

For the generation of the spectral library, the DDA mode was used for analysis. The parameters were set as follows: The top 20 precursor ions are broken by high energy collision (HCD), and the normalization (NCE) is 30. The capillary temperature is 275°C, and the spray voltage is 1800v. The product ion resolution is 3500 (AGC 2.2e4). The maximum fill time for full scan and MS/MS is set to 50 ms and 45 ms, respectively, and the dynamic rejection time is set to 30s.

Data Analysis

The DDA data were processed using the Proteome Discoverer (Thermo Scientific, Germany) software, The analysis parameters are set to: parent ion mass tolerance: 10 ppm, secondary spectrum mass tolerance: 0.02 u, fixed modification Carbamidomethyl (C), variable modification to Deamidation (NQ), Oxidation (M), trypsin specific enzyme digestion in principle. Select FDR ≤ 1% as the identified peptide, and FDR ≤ 1% as the identified protein.

Bioinformatic Analysis

Protein classification was performed based on functional annotations using Gene Ontology (GO) for biological processes, molecular function, and cellular component categories.

For the Ingenuity Pathway Analysis (IPA), the Swiss Prot accession numbers were uploaded to the IPA software (Ingenuity Systems, Mountain View, CA, United States). The proteins were mapped to the disease and function categories, and canonical pathways available in ingenuity, and other databases that were ranked by the P-value.

Enzyme-Linked Immunosorbent Assay (ELISA)

A validation experiment was conducted using ELISA. To verify the candidate marker DPP4, the patient’s plasma sample was used and the ELISA reagent was used to determine it. The experiments were performed in accordance with the manufacturer’s instructions.

Results

Proteomic results of the MS proteome

For LC/MS analysis, a total of 375 proteins were quantified. After deleting the missing value greater than one-half of the total number of samples, there were 274 proteins remaining (detailed data in Supplementary Table S2).

The plasma DEPs analysis was performed on the MS group and the control group. According to the screening criteria of P<0.05, the volcano plot obtained 88 DEPs, of which 39 proteins (44%) were up-regulated differential proteins, and 49 proteins (56%) were down-regulated differential proteins (Figure 2A).

The cluster analysis of DEPs expression clearly showed that the expression patterns of MS patients (P) and healthy controls (C) are different, and the protein expression of each group is clustered together (Figure 2B).

Compare our work with previous MS proteomics research (Figure 2C, Supplementary Table S4). We have reviewed 29 articles in total. A total of 134 proteins were found in the blood and 516 proteins in the CSF.16 proteins (18%) in our study overlap with previous blood proteomics, and 36(41%) in our study overlap with previous CSF proteomics.

Functional Categorization of Differentially Expressed Proteins in MS Patients

In GO analysis, MS was mainly involved in biological processes such as immune/inflammatory response. In the cellular component category, MS proteins were enriched in the extracellular region. The main molecular functions of the MS proteins were binding and transport activity (Figure 3A).

The IPA pathway analysis revealed that MS DEPs are primarily involved in coagulation-related pathways (coagulation system, intrinsic prothrombin activation pathway, extrinsic prothrombin activation pathway), Immune/Inflammatory-related pathways (acute phase response signaling, Primary immunodeficiency signal, and dendritic cell maturation),and signal transduction-related pathways Synaptogenesis Signaling Pathway, Reelin Signaling in Neurons) (Figure 3B Supplementary Table S4).

The IPA function annotation revealed that the MS DEPs mainly regulate Immune/Inflammatory functions (Chemotaxis of T lymphocytes, Systemic autoimmune syndrome, Chronic inflammatory demyelinating polyradiculoneuropathy), Neurological disease Inflammation of central nervous system, Progressive neurological disorder) (Figure 3C, Supplementary Table S4 .

Evaluation of the Diagnostic Efficacy of the candidate protein in MS

The AUC was used to analyze the relationship between the MS group and the control group's DEPs. Overall, the AUC area of all DEPs was above 0.6, and that of 7 proteins had good diagnostic value with an AUC above 0.9, in which SERPINA1 is the best predictive DEP with an area under the receiver operating characteristic (ROC) curve of 0.97 with 100% sensitivity and 90.9% specificity, and the ROC curve of S100A9 (AUC-0.93) with 86.4% sensitivity and 95.5% specificity also has good predictability (Figure 4A). Combine the two protein ROC curves as a panel, and the AUC is 0.991 (Figure 4A).

Validation of DEP using ELISA

From the IPA function, among a group of proteins in the systemic autoimmune syndrome pathway closely related to MS, DPP4 protein may be used as an inhibitor in clinical treatment of MS[19], DPP4 inhibitor was successfully used to treat an animal model of MS[20]. So, we selected DPP4 was validated using the commercial ELISA kit. Compared with the control group, the DPP4 protein was significantly down-regulated in the MS group, which is consistent with the results of TMT analysis (Figure 4B).

Discussion

In this study We found 88 DEPs (Fig. 2C), mainly associated with immune/ inflammation function, and Neurological disease (Fig. 3A,3B,3C). A panel of Alpha-1-antitrypsin (SERPINA1) and S100-A9 (S100A9) could achieve AUC 0.991. Finally, we verified the DPP4 protein with ELISA.

In the function analysis DEPs were mostly involved in immune/inflammatory-related activity (Chemotaxis of T lymphocytes、Systemic autoimmune syndrome、Chronic inflammatory demyelinating polyradiculoneuropathy) (Fig. 3C) .Inflammation is present in all subgroups of MS[21], The diagnostic hallmark of MS pathology is the presence of a large number of confluent demyelinating lesions in the gray matter and white matter of the CNS[22, 23].It is generally believed that inflammation causes demyelination damage and disrupts neuronal signal transmission in the affected area[24]. Demyelination can occur in the brain, cerebellum, brainstem, basal ganglia, spinal cord and other parts[22]. After the nerve fiber loses myelin sheath, there will be a series of motor and sensory disorders[25], and in our function annotation enriched to Urination disorder、Mild cognitive impairment、Neuromuscular disease, etc. which are consistent with previous studies(Supplementary Table S4, S5).

Studies on the immune system of MS patients indicate that MS is an autoimmune disease, T cells、 B cells and autoantibodies may be the most important factors in the pathogenesis of its immune system[26, 27] .And it is generally believed that the inflammatory process of MS is caused by the autoimmune cascade, including the main target of T cells (Th17 phenotype) is the self-myelin antigen[24] The autoimmune pathogenesis of MS has been confirmed in its animal model (EAE)[28].EAE is caused by the subject's subcutaneous injection of CNS myelin antigen in complete Freund's adjuvant or adoptive transfer of newly activated neural antigen/myelin specific CD4 + T helper cell 1 (Th1) or Th17 cells[28] .Therefore, self-reactive CD4 + T cells are sufficient to induce MS-like inflammatory demyelinating diseases. Of which Macrophages are the most prominent contributor to the pathological changes of MS, in the early stage of MS, the M1 phenotype of macrophages releases inflammatory factors to infiltrate the CNS, thereby promoting neuroinflammation and neurodegenerative development, in the later stage of MS, its M2 phenotype is mainly responsible for removing fragments of myelin and assisting in the repair of the CNS, which is a sign of MS[29, 30]. Complement deposition is found in the cortical gray matter of MS. Studies have confirmed that the genetic variation of early complement components is related to the retinal neurodegeneration of MS[31].

DEPs are mostly enriched in blood coagulation system-related pathways (Fig. 3B).Previous studies have shown that a variety of coagulation factors act as inflammatory driving factors in EAE[32], and a variety of coagulation factors are dysregulated in MS[33], suggesting that the coagulation system plays an important role in MS. DEPs are also enriched in Synaptogenesis Signaling Pathway. Synaptogenesis Signaling Pathway is becoming a key determinant of early neurodegeneration of MS[34]. Previous studies used immunohistochemical methods to study the degree of synapse loss in the spinal cord (18 patients with chronic MS, 8 healthy controls) after death. A large amount (58–96%) of synapse loss was detected in the entire spinal cord, and moderate (47%) loss of neurons in the anterior horn especially in demyelinating MS lesions. It indicates that synaptic damage in chronic MS may lead to the accumulation of disability[35]. Moreover, in animal models of MS, the loss of synaptic signals has been shown to be related to changes in the balance of inhibitory and excitatory neurotransmission caused by inflammation[36]. Moreover, many DEPS were correlated with energy metabolism, Glycolysis is another source of energy in the synapse[37],and MS patients can result into energy failure in mitochondria and cytosol[37].

In our study, from the ROC curve, We found a good predictive panel biomarkers with SERPINA1 and S100A9(Figure 4A). Previous studies have revealed the presence of SERPINA1 is a serine protease inhibitor, which not only has the ability to inhibit serine proteases, but also exerts anti-inflammatory and tissue protective effects independent of inhibiting proteases[38].SERPINA1 can modify the maturation of dendritic cells and can promote T cells to regulate and differentiate[38]. Past studies have shown that sustained levels of circulating SERPINA1 profoundly inhibit induction of clinical and histological signs of EAE[39]. It suggests that SERPINA1 may have the ability to interfere with the progression of MS[39]. SERPINA1 also enriched in our Inflammatory response, Progressive neurological disorder process.

S100A9 is a calcium-zinc-binding protein that plays a prominent role in the regulation of inflammation and immune response[40], past studies have shown that S100A9 induces the activation of microglia by activating the NF-kB signaling pathway, and promotes the production of pro-inflammatory factors, thereby aggravating the damage of oligodendrocyte precursor cells (OPCs), thereby affecting the damage of MS disease[41]. And in two independent EAE models, it was found that the protein expression was significantly different during the disease[42], and enriched in our Chronic inflammatory disorder Progressive neurological disorder process etc. It further confirms the important value of S100A9 as a potential plasma marker of MS.

We used ELISA to perform follow-up verification of DPP4-dipeptidyl peptidase 4 (DPP4) protein, DPP4 is a serine protease, also known as the lymphocyte surface protein CD26, which plays a central role in T cell immunity [43]. In a variety of autoimmune diseases, the expression and activity of CD26 in serum have changed, suggesting that it may be involved in the pathogenesis of autoimmune diseases[43]. The study have found that in the magnetic resonance imaging of patients with MS subtypes, there was a correlation between changes in the frequency of CD26-expressing T cells and disease activity, which was consistent with the function of CD26 to regulate the activation state of T cells[44]. These data indicated that CD26 is

associated with the expression of human CD4 + T cells in MS disease activity. So, CD26 has attracted great interest as a potential target for MS immunity therapy. A single synthetic CD26 inhibitor was successfully used to treat an animal model of MS[20].Importantly, studies have shown that CD26 inhibitor combination therapy can reduce the risk of complex autoimmune diseases (RA, IBD, MS, and SLE)[45]. This study used ELISA to verify that CD26 was consistent with our mass spectrometry results[19], confirming that DPP4 may be used as an inhibitor in clinical treatment of MS diseases. However, before advocating any clinical application, it is very important to define and optimize the molecular mechanism of such drugs, and we need further research.

Comparison Of Our Study, Ms-blood, And Ms-csf Proteome

In our study, we summarized our study with previous blood proteomics research, and compared it with past years of CSF proteomics. (Fig. 5A 5BSupplementary Table S5).

The blood-brain barrier is (or is) interrupted in most MS patients, the CNS protein substances released into the circulation can be assessed by proteomics analysis [46].However, due to the limited clinical utility of cerebrospinal fluid biomarkers, the lumbar puncture process for obtaining cerebrospinal fluid is invasive and associated with risks(19), And, it was not always feasible to collect CSF, especially for individuals and children who had never developed symptoms[47].Instead, blood can be routinely collected for the discovery of early protein biomarkers, and plasma contains proteins from almost all cell types in the organism(20), indicating that blood can be a viable alternative biological sample for patients with MS.

For MS-CSF proteomics: as shown in (Figure 5A, Supplementary Table S5)CSF proteins were more remarkably involved in the CNS process and MS central nervous system cancer, Astrocytoma, Multiple sclerosis, Morphology of nervous system, Degeneration of neurons).In the pathway analysis in Figure 5B CSF proteins were more remarkably involved in the Axonal Guidance SignalingSynaptogenesis Signaling Pathway et al. Axonal degeneration as a cause of irreversible neurological impairment during MS[48].This result reflects that the CSF protein may better reflect the pathological characteristics of MS.

Overall ,for MS-Blood and MS-CSF proteomics:(Figure 5A, Supplementary Table S5), the protein pathways/functions of the blood enrichment were aggressively concentrated on the immune/inflammation-related activity (Systemic autoimmune syndromeDendritic Cell Maturation Chronic inflammatory demyelinating polyradiculoneuropathy),which consisting with our study (Fig. 5A 5B).

For the MS-Blood enrichment: Compared to MS-CSF proteomics, the central nervous system functions associated with MS disease are also altered (Glioblastoma). Also, the blood protein enrichment is significantly involved in immune/inflammatory pathways (macrophage activation process, systemic inflammatory response syndrome, etc.). These immune/inflammatory and neurological disease

pathways may also reflect pathological changes in MS(Fig. 5A□5B). This result reflects that the blood protein also could reflect the pathological characteristics of MS.

Conclusionss

In our plasma proteome research, we have found markers that can reflect the disease, laying a foundation and reference for the diagnosis of MS and the future development of MS plasma proteome. And help in the application of MS. This is a single-center study of plasma proteomics. In the future, we will continue to conduct in-depth multi-center research on the mechanism of MS.

Declarations

Ethics approval and consent to participate:

Prior to study enrollment, all volunteers were given a verbal explanation of the study, and each participant provided a signed informed consent. The consent procedure and research protocol for this study have been approved by the Ethics Committee of the Second People's Hospital of Lishui. The study methodologies conformed to the standards set by the Declaration of Helsinki.

Consent for publication:

The authors affirm that human research participants provided informed consent for publication.

Data availability statement□

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: iProx (https://www.iprox.cn/page/PSV023.html;?url=1641896103480G9CY password: Dcew□

IPX□0003929000

Availability of data and materials:

1. All data generated or analyzed in this study are partially included in this published article [and its supplementary information document].

2.The datasets generated and/or analysed during the current study are available in the Integrated Proteome Resources repository, The names of the repository/repositories and accession number(s) can be found below: iProx (https://www.iprox.cn/page/PSV023.html;?url=1641896103480G9CY password: Dcew□

IPX□0003929000

Competing interests:

The authors have no relevant financial or non-financial interests to disclose.

Funding:

This work was supported in part by grants from the Preliminary study on the diagnosis and outcome of early multiple sclerosis based on proteomics, Lishui city-level key research and development plan , China (2017ZDYF15).

Authors' contributions:

(I) † These authors contributed equally to this work and should be considered co-first authors (II)LYZ 4 JS1 and WY1 contributed to conception and design of the study. (III) LYZ 4 and SW3 organized the database. (IV) BS2 and SW3 performed the statistical analysis. (V) WY wrote the first draft of the manuscript. (VI) SW3LJZ4,XY4 and HZ5 wrote sections of the manuscript. (VII) JS1 and LYZ4, the two authors who contributed the same to the article, are both the corresponding authors of the article. ( ) All authors contributed to manuscript revision, read, and approved the submitted version.

Acknowledgements:

We thank Mr. Sun Wei from Peking Union Medical College for his help and support

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Figures

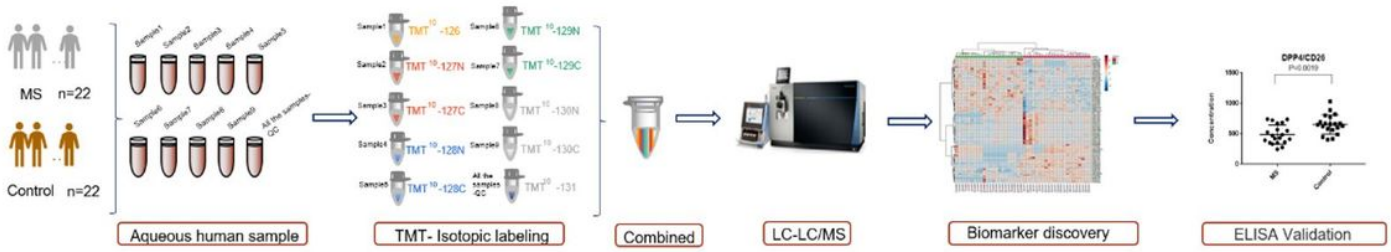

Figure 1

Workflow for the discovery and verification of DEPs in MS.

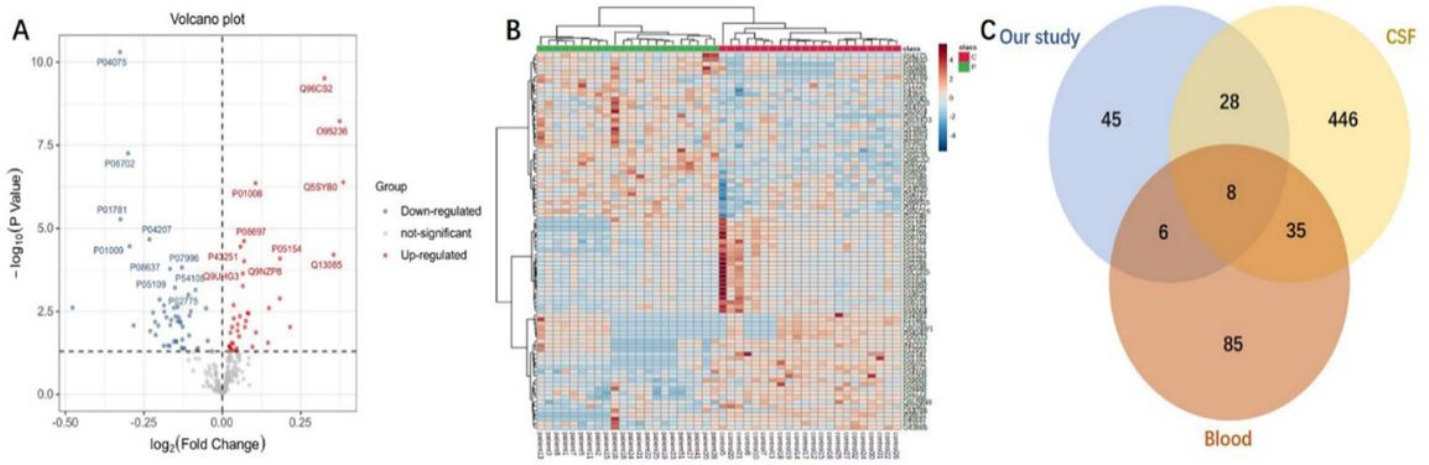

Figure 2

The MS proteome analysis of the present study. (A)The volcano plot was drawn using two factors, the fold change (Log2) between the two groups of samples and the p-value (−Log10), to show the significance of differences in the data between the two groups of samples. The orange dots and blue dots in the figure are proteins that were significantly upregulated and downregulated, respectively; the gray dots are proteins with no significant difference. (B)Hierarchical clustering analysis of DEPs in plasme between patients with MS (green) and healthy controls (red). Each row in the figure represents a protein, and each column represents a sample. Red, high expression; dark blue, low expression. Two main clusters of proteins were observed, one of which was downregulated and one of which upregulated in patients with MS.(C)The Venn diagram of the identified proteins and comparison of the present results with the previous MS-CSF and MS-Blood proteome data.

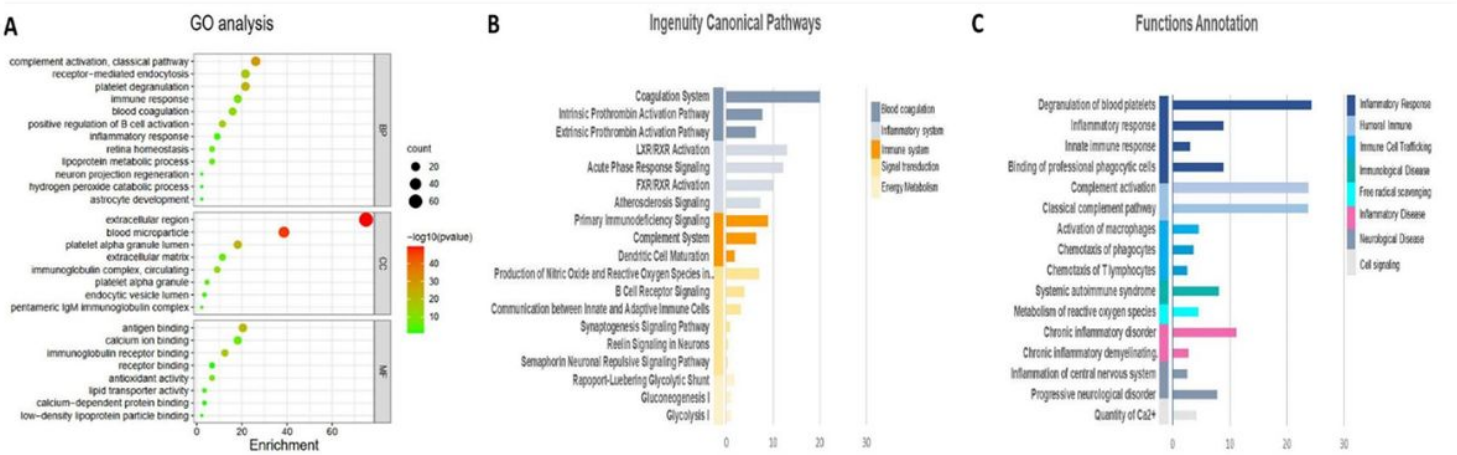

Figure 3

The DEP analysis of the MS proteome. (A) The GO pathway analyses of MS-related proteins. Classification of 88 DEPs based on biological processes (BP), molecular functions (MF), cellular components (CC). The abscissa represents the number of DEPs in each functional classification. (B)The

Ingenuity Pathway Analysis (IPA) canonical pathway analysis of MS-related proteins.(C) The function annotation of validated DEPs.

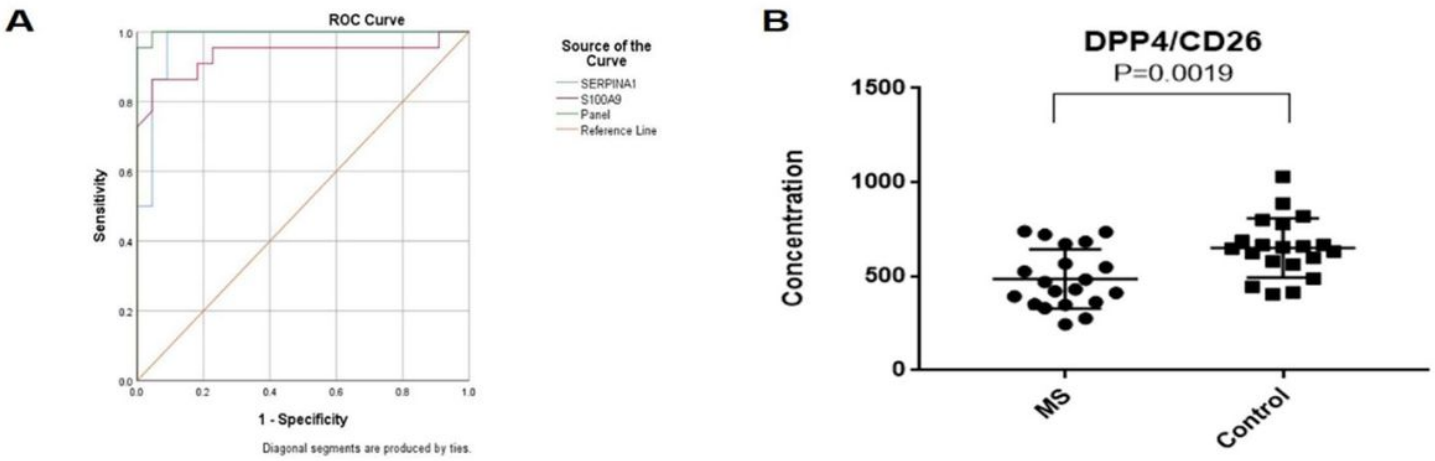

Figure 4

Efficacy of multiple sclerosis biomarkers and ELISA validation. (A) ROC curvers for the diagnostic value of SERPINA1 and S100A9 in MS.(B)Dot Plots showing levels of DPP4 in plasme samples of MS and controls.

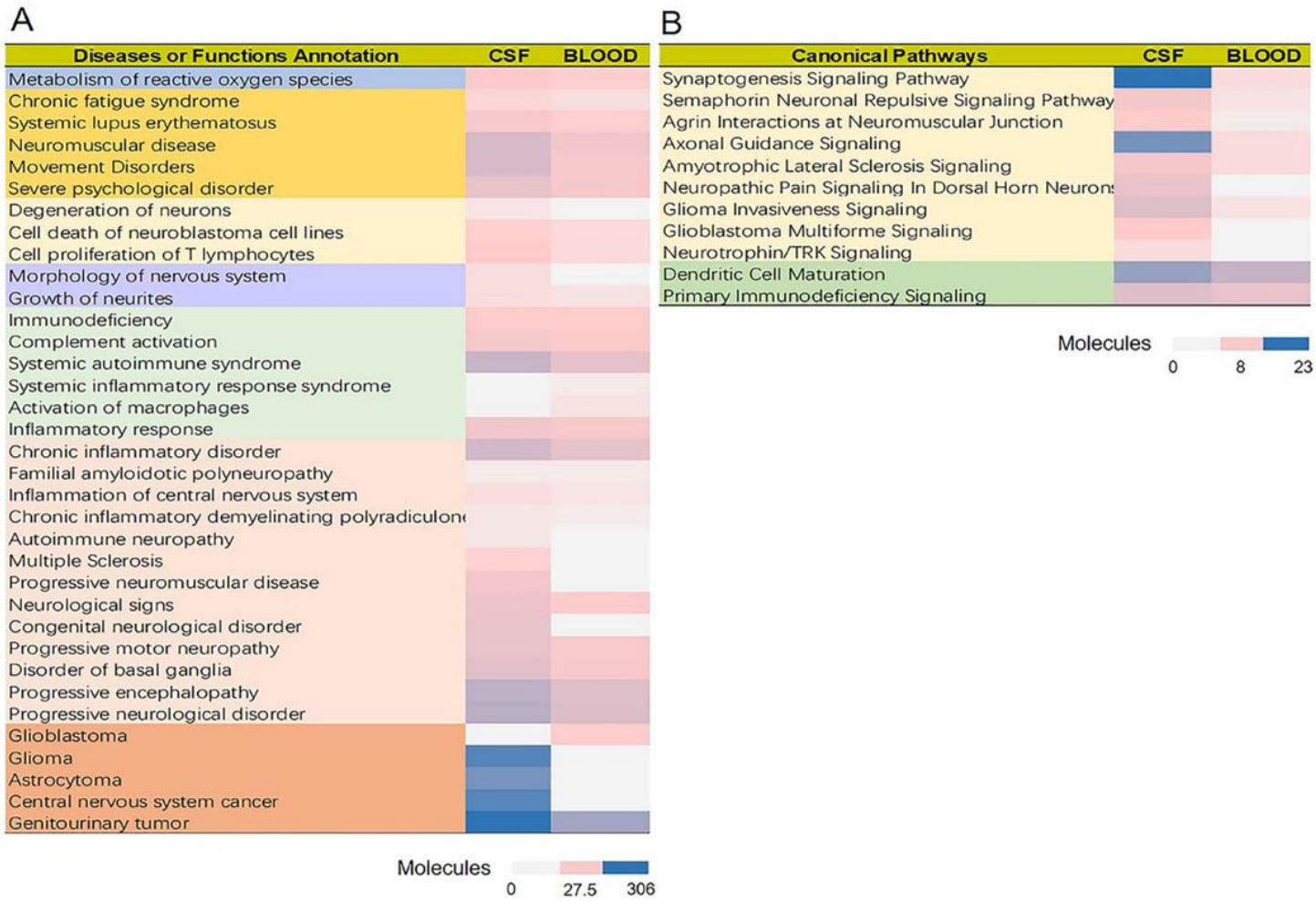

Figure 5

Comparison of the MS CSF and MS blood fluid proteome. (A) The functional comparison between MS-CSF proteins and MS-Blood proteins.(B) The pathway comparison between MS-CSF proteins and MS-Blood proteins.

Supplementary Files

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- SupplementarytableS1.xlsx
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- SupplementaryMaterial.pdf