Proteomic analysis of cerebrospinal fluid of amyotrophic lateral sclerosis patients in the presence of autologous bone marrow derived mesenchymal stem cells

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Abstract

Background

Amyotrophic lateral sclerosis (ALS) is a fatal and fast progressive motoneuron degenerative disorder. There are still no drugs capable to slower disease evolution or to improve life quality of ALS patients. In that scenario, the cell therapy has emerged as an alternative to be investigated in clinical ALS.

Method

Taking the advantage of Proteomics and Protein-Protein Interaction Network analyses combined to bioinformatics, possible cellular mechanisms and molecular targets related to mesenchymal stem cells (MSC, $1x10^6$ cells/kg, intrathecally in the lumbar region of the spine) were investigated in cerebrospinal fluid (CSF) of ALS patients who received intrathecal infusions of autologous bone marrow-derived MSC thirty days after cell therapy.

Results

Proteomics showed 220 deregulated proteins in CSF of ALS subjects. Bioinformatic enriched analyses evidenced APOA1, APOE, APP, C4A, C5, FGA, FGB, FGG and PLG, as highlighted targets as well as extracellular matrix and cell adhesion molecules as possible mechanisms related to the presence of MSC in CSF of ALS subjects.

Conclusions

We have demonstrated a possible role of extracellular matrix/cell adhesion molecules and their related highlighted targets to the presence of autologous MSC in CSF ALS patients.

Trial Registration:

Clinicaltrial.gov identifier NCT0291768. Registered 28 September 2016.

Background

Mesenchymal stem cells (MSC) have been tested clinically for amyotrophic lateral sclerosis (ALS), a fatal motor neurodegenerative disease (1, 2). Indeed, indications of MSC-induced motor neuron protection experimentally (3, 4) as well as clinically (5–10) in ALS have been obtained.

Recent reports have shown that the ability of MSC to induce neuroprotection and repair events (11, 12) in neurodegenerative disorders may involve their paracrine ability to interact with diseased milieu (13, 14),

thus further indicating the importance of extracellular matrix (ECM) in local MSC effects (15). Indeed, MSC are able to detect and to react to specific local molecular signs (16, 17) that in turn may produce and secrete soluble bioactive molecules and extracellular vesicles (13, 18) with potential impact to neurodegenerative processes.

Studies have explored the regulation of ECM proteins and cell adhesion molecules in the search for specific molecular targets of cellular events related to neurodegeneration (19), neurodegenerative disorders (20, 21) and neuroprotection (20), however it is still lacking information on specific molecular responses and related possible mechanisms of MSC in neurodegeneration experimentally or clinically (22, 23).

Therefore, this study used a large Proteomic analysis in combination to Protein interaction network and molecular modeling to obtain further indications on cellular mechanisms and related molecular targets in the CSF of ALS subjects thirty days after an intrathecal deliver of autologous bone marrow-derived MSC.

MSC benefits for ALS are tested due to its potential ability to trigger motor neuron protective events (24, 25), which might be mediated by MSC paracrine mechanisms involving specific molecules, a matter that deserve further investigation (25, 26).

Methods

ALS Subjects, MSC Infusion and CSF Withdrawn

This study is a subproject of a Phase I/II Clinical Trial (www.clinicaltrials.gov; NCT02917681) that tested safety and preliminary effects of intrathecal (subarachnoid space of lumbar vertebrae, L3-L5) autologous bone marrow-derived mesenchymal stem cell (MSC) infusion (10^6 cells/kg^{-1} body weight) that was conducted (2016–2019) at Neurology Division of Clinics Hospital of Medical School of University of Sao Paulo, Brazil. Study was approved by local Ethics Committee. Patients were clinically evaluated to inclusion/exclusion criteria and had their ALS diagnosis rechecked. Once included, ALS subjects signed informed consent. Subjects were accompanied monthly for three and seven months, respectively, before and after cell infusion. After bone marrow aspiration of ALS subjects, MSCs were individually isolated and expanded at Core for Cell Technology, Pontifical Catholic of University of Parana, Brazil, according previous description (27). CSF (10 ml) was collected from subarachnoid lumbar space of ALS subjects immediately before MSC infusion and also 30 days later. The first 5 ml were delivered for standard clinical laboratory tests, including bacteriological and biochemical analyses, and next 5 ml were used for molecular analysis of this study. CSF samples were centrifuged at 1,000 × g for 10 minutes at 4 ºC, aliquoted (1 ml) into polypropylene cryogenic tubes and stored at -80°C until further analyses. All samples were processed within 30 minutes of collection.

Proteomics

Mass spectrometry-based proteomic analysis of CSF from ALS subjects. CSF (1 ml) of ALS subjects were filtered using ultracentrifugation devices with a molecular cut-off of 10 kDa. Proteins in retentate were reduced by addition of dithiothreitol to a final concentration of 10 mM, alkylated with iodoacetamide (final concentration of 40 mM) and digested with trypsin (1:50 enzyme to protein ratio). Reaction was stopped (1% trifluoroacetic acid), resulting peptides were purified (primed Oligo R3 reversed phase SPE micro-column) and dried (28). Samples were then verified by a nLC-MS/MS analysis using an analytical platform, notably the nanoflow liquid chromatography with linear trap quadrupole (LTQ) Orbitrap mass spectrometers. Peptides were separated by nano ultra-high performance liquid chromatography tandem mass spectrometry (nUHPLC LC-MS/MS) according previous description (29).

Deregulated proteins using LTQ Orbitrap. Proteins that were identified to be deregulated in CSF of ALS subjects 30 days after MSC infusion in comparison to CSF of same subjects before cell delivery were selected and their proteotypic peptides mapped in PeptideAtlas database. Selected m/z values were monitored across all gradient and their MS/MS spectra were recorded in order to perform a database search using MaxQuant software (30). Specifically, http://www.mcponline.org/ downloaded from 17 engine Andromeda (31) was used to search for MS/MS spectra against a database composed by Uniprot Human Protein Database (32) with a 4.5ppm tolerance level for MS, and 20ppm for MS/MS. Furthermore, ceruloplasmin and reelin proteins, were selected as internal control. Proteins detected in seven out of eight samples with their peptides identified by at least 6 samples with MS/MS spectral search were considered for further analyses. At the end, label free quantification normalized values were used. Bioinformatics and statistical details are described below.

Statistical Analysis. All datasets were tested for normal distribution before applying parametric tests. Proteomic data were processed using Perseus computational platform v.1.6.14.0 (https://cox-labs.github.io/coxdocs/). Label Free Quantitation (LFQ) data were log2-transformed, protein reverse, contaminants and only by site were removed. Imputation was performed by replace missing values from normal distribution with a width of 0.3 and down shift of 1.8. Statistical analysis of LFQ data employing the paired t-test and Benjamini-Hochberg correction, FDR < 0.05, $p \leq 0.05$ (Graphpad Prism) identified deregulated proteins in CSF of ALS subjects 30 days after MSC infusion compared to CSF of subjects before cells.

Protein-Protein Interaction Network

Interactions among proteomic deregulated proteins in Network were evaluated using Cytoscape GeneMANIA plug-in (version 3.8.2), by highlighting “path” and “physical” interactions (33). Subsequently, Network nodes were obtained using the centrality parameters “degree” and “betweenness” (Cytoscape CentiScaPe plug-in). Node degree is a measure of local structure in networks that determines the number of edges at each node, and betweenness is a global structure measure in networks that identify the number of shortest paths that pass through a specific node when directly or indirectly connecting pairs of nodes (34). Furthermore, it was created a set of top 15 proteins with the highest betweenness and degree values. After elimination of repetitions, a set high representative molecules in Network was created based on 220 deregulated proteins.

Bioinformatics

In order to evaluate possible mechanisms, and their related molecular targets, to the presence of MSC delivered in CSF of ALS subjects, deregulated proteins described by Proteomics were submitted to cellular/molecular functional enrichments by employing specific bioinformatics tools described below.

Functional Enrichment Analysis. Deregulated proteins pointed by proteomic study were analyzed by means of Database for Integrated Annotation, Visualization and Discovery (DAVID, https://david.ncifcrf.gov) that identified pathways (KEGG - Kyoto Encyclopedia of Genes and Genomes) and Gene Ontology categories (Biological Process, Cellular component and Molecular Function) based on their specific set of deregulated proteins (35, 36), according to specific levels of significance for KEGG and Gene Ontology ($0.00001 \leq p \geq 0.01$, see legend of Table 2).

REVIGO. In order to further highlight cellular and molecular mechanisms among described enriched DAVID categories, REVIGO (http://revigo.irb.hr) was applied (37) to group such categories in Superclusters, based on distribution of the SimRel semantic similarity measure (default in REVIGO). REVIGO summarizes long Gene Ontology categories (Biological Process, Cellular Component and Molecular function) by reducing functional redundancies, and also visualizes the remaining Gene Ontology categories.

Highlighted Deregulated Proteins in CSF. Proteins of REVIGO Gene Ontology Superclusters were identified. Following, intersections of sets of proteins of Superclusters identified common molecules by means of Venn Diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/). Intersections among Superclusters were considered for Biological Process (up to 3), Cellular Component (up to 4), and Molecular Function (up to 2), to reach a maximal 27 molecules of each intersection. Thus, three Supercluster sets of overlapped proteins of Biological Process, Cellular Component, and Molecular Function were created. Subsequently, intersections of these 3 Supercluster sets and the Set of high representative molecules in the Network identified common molecules in the sets, considering their presence in at least 3 sets as well as a presence of a minimal of 1 molecule from Network set. Following these criteria, Highlighted molecules with 100% representation (presented in the 4 sets) and 75% representation (presented in 3 sets) were identified. Indeed, the nine final molecules with the greatest intersections were considered as possibly more prominent molecular targets and related molecular/cellular mechanisms related to the presence of MSC in CSF of ALS subjects.

Extracellular Matrix and Cell Adhesion Molecules MeSH. Based on the fact that biological/molecular aspects of “Extracellular Matrix” and “Cell Adhesion Molecules” have been well described in biological events related to MSC function in injured tissues, the Medical Subject Headings (MeSH) “Matrix extracellular” and “Cell Adhesion Molecules” were used to point out their related categories among all described KEGG, Biological Process, Cellular Component, and Molecular Function categories, whose terms indicated similarity to above MeSH terms. Proteins of those “Matrix extracellular” and “Cell Adhesion Molecules” MeSH-related categories were indicated (symbols) in the list of 220 proteomic

identified deregulated proteins (* for “Extracellular Matrix” and # for “Cell Adhesion Molecules”; see results). Subsequently, the number of molecules belonging to those categories were defined and corresponded percentages of total number of deregulated proteins were calculated.

Results

Demographic Information of ALS Subjects

Demographic Information of 24 ALS subjects included in the study are summarized in Table S1. Subjects were Caucasian (14 males and 10 females), who showed clinical history of spinal (n = 19) and bulbar (n = 5) disease onset. The averages of patient age at the time of disease onset and of disease evolution until the first CSF collection were 52.12 years and 53.21 months, respectively.

Deregulated Proteins in CSF of ALS Subjects

Mass spectrometry-based proteomics identified two hundred-twenty deregulated proteins [n = 86 (fold > 1.0) upregulated and n = 134 (fold < 1.0) downregulated] in the CSF of ALS subjects 30 days after MSC intrathecal infusion compared to CSF of subjects collected before cells (Table 1). Deregulated proteins were statistically significant with a q-value of less than 0.1 (Table 1).

Table 1
Deregulated proteins in CSF of ALS subjects 30 days after MSC infusion

Protein Name	Symbol	Fold	q-values
Alpha-2-macroglobulin	A2M *	1.07	0.06
Actin, alpha cardiac muscle 1	ACTC1 *	1.33	0.08
Actin, cytoplasmic 2	ACTG1 *	1.25	0.08
Agrin	AGRN *	0.91	0.03
Alpha-2-HS-glycoprotein	AHSG *	1.08	0.09
CD166 antigen	ALCAM *#	0.87	0.08
Fructose-bisphosphate aldolase C	ALDOC *	0.89	0.03
Protein AMBP	AMBP *#	1.12	0.03
Angiogenin	ANG *	0.89	0.03
Amyloid-like protein 1	APLP1 #	0.93	0.03
Amyloid-like protein 2	APLP2 *	0.86	0.04
Apolipoprotein A-I	APOA1 *	1.18	0.03
Apolipoprotein A-II	APOA2 *	1.16	0.03
Apolipoprotein A-IV	APOA4 *	1.12	0.08
Apolipoprotein B-100	APOB *	2.25	0.04
Apolipoprotein E	APOE *	0.92	0.03
Apolipoprotein L1	APOL1 *	1.18	0.05
Apolipoprotein M	APOM *	1.25	0.02
Amyloid beta A4 protein	APP *#	0.82	0.02
N-acetyllactosaminide beta-1,3-N-acetylglucosaminyltransferase 2	B3GNT2 *	0.55	0.09
Beta-1,4-glucuronyltransferase 1	B4GAT1 *	0.88	0.02
Brevican core protein	BCAN #	0.86	0.01

Two hundred-twenty deregulated proteins [n = 86 (fold > 1.0) upregulated and n = 134 (fold < 1.0) downregulated] were identified in cerebrospinal fluid (CSF) of amyotrophic lateral sclerosis (ALS) subjects 30 days after mesenchymal stem cell (MSC) intrathecal infusion compared to CSF of subjects collected before cells (n = 24). Fold refers to mean of LFQ intensities of CSF 30 days after MSC infusion by mean of LFQ intensities of CSF before infusion (n = 24). Molecules related to Medical Subject Headings (MesH) “Extracellular Matrix” and “Cell Adhesion Molecules”, are indicated with symbols * and #, respectively (see text for details.

Protein Name	Symbol	Fold	q-values
Complement C1q subcomponent subunit A	C1QA *	1.16	0.05
Complement C1q subcomponent subunit B	C1QB *	1.25	0.01
Complement C1q subcomponent subunit C	C1QC *	1.22	0.03
Complement C1r subcomponent	C1R *	1.16	0.03
Complement C1r subcomponent-like protein	C1RL *	1.22	0.01
Complement C1s subcomponent	C1S *	1.14	0.01
Complement C2	C2 *	1.08	0.07
Complement C3	C3 *	1.10	0.03
Complement C4-A	C4A *	1.12	0.08
C4b-binding protein alpha chain	C4BPA *	1.69	0.03
Neuropeptide-like protein C4orf48	C4orf48 *	0.90	0.05
Complement C5	C5 *	1.14	0.02
Complement component C6	C6 *	1.18	0.01
Complement component C8 alpha chain	C8A *	1.10	0.03
Complement component C8 beta chain	C8B *	1.12	0.03
Complement component C9	C9 *	1.10	0.08
Carbonic anhydrase 1	CA1 *	29.56	0.01
Voltage-dependent calcium channel subunit alpha-2/delta-1	CACNA2D1 *	0.91	0.02
Cell adhesion molecule 1	CADM1 *#	0.89	0.02
Cell adhesion molecule 2	CADM2 #	0.87	0.02
Cell adhesion molecule 3	CADM3 #	0.88	0.01
Calreticulin	CALR *	0.92	0.03
Cerebellin-1	CBLN1 *	0.58	0.05

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Protein Name	Symbol	Fold	q-values
Cerebellin-3	CBLN3 *	0.86	0.09
Monocyte differentiation antigen CD14	CD14 *	1.13	0.07
Complement decay-accelerating factor	CD55 *	0.85	0.04
CD59 glycoprotein	CD59 *	0.87	0.03
CD5 antigen-like	CD5L *	1.55	0.02
Cadherin-10	CDH10	0.74	0.02
Cadherin-13	CDH13 *#	0.92	0.07
Complement factor B	CFB *	1.12	0.03
Complement factor D	CFD *	1.07	0.07
Complement factor H	CFH *	1.10	0.03
Complement factor I	CFI *	1.12	0.02
Cofilin-1	CFL1 *	0.42	0.05
Secretogranin-1	CHGB *	0.91	0.05
Chitinase-3-like protein 1	CHI3L1 *	0.84	0.03
Neural cell adhesion molecule L1-like protein	CHL1 *#	0.93	0.05
Calsyntenin-1	CLSTN1 *#	0.82	0.01
Calsyntenin-3	CLSTN3 *	0.80	0.02
Beta-Ala-His dipeptidase	CNDP1 *	0.93	0.08
Ciliary neurotrophic factor receptor subunit alpha	CNTFR	0.83	0.03
Contactin-2	CNTN2 #	0.87	0.01
Contactin-associated protein-like 4	CNTNAP4 #	0.77	0.04
Collagen alpha-1(I) chain	COL1A1 *#	1.48	0.00
Collagen alpha-2(I) chain	COL1A2 *	1.53	0.00

Two hundred-twenty deregulated proteins [n = 86 (fold > 1.0) upregulated and n = 134 (fold < 1.0) downregulated] were identified in cerebrospinal fluid (CSF) of amyotrophic lateral sclerosis (ALS) subjects 30 days after mesenchymal stem cell (MSC) intrathecal infusion compared to CSF of subjects collected before cells (n = 24). Fold refers to mean of LFQ intensities of CSF 30 days after MSC infusion by mean of LFQ intensities of CSF before infusion (n = 24). Molecules related to Medical Subject Headings (MesH) “Extracellular Matrix” and “Cell Adhesion Molecules”, are indicated with symbols * and #, respectively (see text for details.

Protein Name	Symbol	Fold	q-values
Collagen alpha-1(III) chain	COL3A1 *	1.48	0.00
Collagen alpha-1(VI) chain	COL6A1 *#	0.91	0.03
Carboxypeptidase B2	CPB2 *	1.16	0.03
Carboxypeptidase E	CPE *#	0.83	0.02
Carboxypeptidase Q	CPQ *	0.85	0.03
Cartilage acidic protein 1	CRTAC1 *	0.94	0.06
Cystatin-C	CST3 *	0.93	0.08
Cathepsin D	CTSD *	0.89	0.02
Protein CutA	CUTA *	0.84	0.09
Stromal cell-derived factor 1	CXCL12 * #	1.40	0.01
C-X-C motif chemokine 16	CXCL16 *	0.84	0.03
Decorin	DCN *	0.90	0.09
Delta and Notch-like epidermal growth factor-related receptor	DNER	0.82	0.02
Extracellular matrix protein 1	ECM1 *	0.95	0.09
Endothelin-3	EDN3 *	0.85	0.03
Gamma-enolase	ENO2 *	0.84	0.03
Ectonucleotide pyrophosphatase/ phosphodiesterase family member 2	ENPP2 *	0.90	0.03
Ephrin type-A receptor 4	EPHA4 #	0.89	0.03
Ephrin type-A receptor 5	EPHA5	0.62	0.03
Coagulation factor XII	F12 *	1.07	0.07
Prothrombin	F2 *	1.11	0.01
Coagulation factor V	F5 *	0.89	0.01
Protein lifeguard 2	FAIM2	0.87	0.07

Two hundred-twenty deregulated proteins [n = 86 (fold > 1.0) upregulated and n = 134 (fold < 1.0) downregulated] were identified in cerebrospinal fluid (CSF) of amyotrophic lateral sclerosis (ALS) subjects 30 days after mesenchymal stem cell (MSC) intrathecal infusion compared to CSF of subjects collected before cells (n = 24). Fold refers to mean of LFQ intensities of CSF 30 days after MSC infusion by mean of LFQ intensities of CSF before infusion (n = 24). Molecules related to Medical Subject Headings (MesH) “Extracellular Matrix” and “Cell Adhesion Molecules”, are indicated with symbols * and #, respectively (see text for details.

Protein Name	Symbol	Fold	q-values
Protein FAM3C	FAM3C *	0.91	0.03
Protocadherin Fat 2	FAT2 *	0.80	0.05
Fibulin-5	FBLN5 *	0.93	0.06
Fetuin-B	FETUB *	1.11	0.07
Fibrinogen alpha chain	FGA *#	1.35	0.01
Fibrinogen beta chain	FGB *#	1.22	0.04
Fibroblast growth factor receptor 2	FGFR2 *	0.85	0.02
Fibrinogen gamma chain	FGG *#	1.24	0.03
Fibroleukin	FGL2 *	1.41	0.02
Folate receptor beta	FOLR2 *	1.44	0.03
Follistatin-related protein 4	FSTL4 *	0.80	0.07
Plasma alpha-L-fucosidase	FUCA2	0.88	0.05
Polypeptide N-acetylgalactosaminyltransferase 2	GALNT2 *	1.20	0.08
Vitamin D-binding protein	GC *	1.07	0.07
Rab GDP dissociation inhibitor alpha	GDI1	2.46	0.05
Glypican-1	GPC1 *	0.81	0.02
Glutamate receptor 4	GRIA4	0.86	0.07
Hyaluronan-binding protein 2	HABP2 *#	1.20	0.03
Protein HEG homolog 1	HEG1 *	0.86	0.08
Beta-hexosaminidase subunit alpha	HEXA *	0.81	0.03
Haptoglobin-related protein	HPR *	1.31	0.03
Histidine-rich glycoprotein	HRG *	1.13	0.03
Serine protease HTRA1	HTRA1 *	0.85	0.01

Two hundred-twenty deregulated proteins [n = 86 (fold > 1.0) upregulated and n = 134 (fold < 1.0) downregulated] were identified in cerebrospinal fluid (CSF) of amyotrophic lateral sclerosis (ALS) subjects 30 days after mesenchymal stem cell (MSC) intrathecal infusion compared to CSF of subjects collected before cells (n = 24). Fold refers to mean of LFQ intensities of CSF 30 days after MSC infusion by mean of LFQ intensities of CSF before infusion (n = 24). Molecules related to Medical Subject Headings (MesH) “Extracellular Matrix” and “Cell Adhesion Molecules”, are indicated with symbols * and #, respectively (see text for details.

Protein Name	Symbol	Fold	q-values
Iduronate 2-sulfatase	IDS	0.83	0.03
Insulin-like growth factor II	IGF2 *	0.90	0.06
Insulin-like growth factor-binding protein complex acid labile subunit	IGFALS *#	1.17	0.05
Insulin-like growth factor-binding protein 2	IGFBP2 *	1.06	0.08
Insulin-like growth factor-binding protein 7	IGFBP7 *#	0.88	0.02
Ig alpha-1 chain C region	IGHA1 *	1.09	0.03
Ig alpha-2 chain C region	IGHA2 *	1.16	0.04
Ig mu chain C region	IGHM *	1.57	0.01
Immunoglobulin J chain	IGJ	1.28	0.04
Immunoglobulin lambda-like polypeptide 5	IGLL5 *	1.38	0.03
Inositol monophosphatase 3	IMPAD1	0.88	0.08
Inter-alpha-trypsin inhibitor heavy chain H1	ITIH1 *	1.19	0.02
Inter-alpha-trypsin inhibitor heavy chain H2	ITIH2 *	1.21	0.01
Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4 *	1.14	0.01
Inter-alpha-trypsin inhibitor heavy chain H5	ITIH5 *	0.87	0.05
Kallikrein-6	KLK6 *	0.87	0.03
Kininogen-1	KNG1 *	1.07	0.09
Lysosome-associated membrane glycoprotein 2	LAMP2 *	0.83	0.09
Phosphatidylcholine-sterol acyltransferase	LCAT *	0.87	0.03
Plastin-2	LCP1 *	1.27	0.01
Galectin-1	LGALS1 *	1.15	0.09
Prolow-density lipoprotein receptor-related protein 1	LRP1	0.87	0.03
Leucine-rich repeat-containing protein 4B	LRRC4B #	0.91	0.07

Two hundred-twenty deregulated proteins [n = 86 (fold > 1.0) upregulated and n = 134 (fold < 1.0) downregulated] were identified in cerebrospinal fluid (CSF) of amyotrophic lateral sclerosis (ALS) subjects 30 days after mesenchymal stem cell (MSC) intrathecal infusion compared to CSF of subjects collected before cells (n = 24). Fold refers to mean of LFQ intensities of CSF 30 days after MSC infusion by mean of LFQ intensities of CSF before infusion (n = 24). Molecules related to Medical Subject Headings (MesH) “Extracellular Matrix” and “Cell Adhesion Molecules”, are indicated with symbols * and #, respectively (see text for details.

Protein Name	Symbol	Fold	q-values
Limbic system-associated membrane protein	LSAMP #	0.92	0.04
Latent-transforming growth factor beta-binding protein 2	LTBP2 *	1.13	0.04
Latent-transforming growth factor beta-binding protein 4	LTBP4 *	0.85	0.03
Lumican	LUM *	1.08	0.07
Lymphatic vessel endothelial hyaluronic acid receptor 1	LYVE1 *#	0.82	0.05
Lysozyme C	LYZ *	1.35	0.01
Cell surface glycoprotein MUC18	MCAM *#	0.89	0.02
Multiple epidermal growth factor-like domains protein 8	MEGF8 *	0.88	0.02
72 kDa type IV collagenase	MMP2 *	1.10	0.02
Moesin	MSN *#	6.99	0.02
Neural cell adhesion molecule 1	NCAM1 *#	0.94	0.05
Neurocan core protein	NCAN *#	0.92	0.04
Neuronal growth regulator 1	NEGR1 *#	0.84	0.00
Protein kinase C-binding protein NELL2	NELL2 *	0.90	0.01
Neogenin	NEO1 #	0.92	0.03
Neurofascin	NFASC *#	0.90	0.05
Nidogen-1	NID1 *	1.23	0.01
C-type natriuretic peptide	NPPC *	0.80	0.03
Neuronal pentraxin-1	NPTX1	0.84	0.03
Neuronal pentraxin receptor	NPTXR	0.88	0.03
Neuronal cell adhesion molecule	NRCAM *#	0.89	0.01
Neuritin	NRN1 *	0.91	0.09
Neurexin-1	NRXN1 #	0.85	0.01

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Protein Name	Symbol	Fold	q-values
Neurexin-2	NRXN2 #	0.88	0.03
Neurexin-3	NRXN3 #	0.89	0.03
Neurotrimin	NTM #	0.90	0.04
Oligodendrocyte-myelin glycoprotein	OMG #	0.87	0.01
Opioid-binding protein/cell adhesion molecule	OPCML *#	0.87	0.04
Alpha-1-acid glycoprotein 1	ORM1 *	1.12	0.05
Protocadherin-9	PCDH9	0.83	0.02
Procollagen C-endopeptidase enhancer 1	PCOLCE *	1.06	0.06
Phosphatidylethanolamine-binding protein 1	PEBP1 *	0.90	0.03
Phosphatidylethanolamine-binding protein 4	PEBP4 *	0.90	0.04
Profilin-1	PFN1 *	1.61	0.03
N-acetylmuramoyl-L-alanine amidase	PGLYRP2 *	1.10	0.03
Phospholipase D3	PLD3 *	0.78	0.04
Plasminogen	PLG *	1.09	0.03
Plexin-B2	PLXNB2 *	0.90	0.04
Protein O-linked-mannose beta-1,2-N-acetylglucosaminyltransferase 1	POMGNT1	0.77	0.00
Serum paraoxonase/arylesterase 1	PON1 *	1.17	0.02
Peptidyl-prolyl cis-trans isomerase A	PPIA *	1.30	0.04
Lysosomal Pro-X carboxypeptidase	PRCP *	0.86	0.03
Proline-rich transmembrane protein 3	PRRT3	0.90	0.07
Prosaposin	PSAP *	0.94	0.07
Prostaglandin-H2 D-isomerase	PTGDS *	0.92	0.05
Receptor-type tyrosine-protein phosphatase gamma	PTPRG *	0.83	0.02

Two hundred-twenty deregulated proteins [n = 86 (fold > 1.0) upregulated and n = 134 (fold < 1.0) downregulated] were identified in cerebrospinal fluid (CSF) of amyotrophic lateral sclerosis (ALS) subjects 30 days after mesenchymal stem cell (MSC) intrathecal infusion compared to CSF of subjects collected before cells (n = 24). Fold refers to mean of LFQ intensities of CSF 30 days after MSC infusion by mean of LFQ intensities of CSF before infusion (n = 24). Molecules related to Medical Subject Headings (MesH) “Extracellular Matrix” and “Cell Adhesion Molecules”, are indicated with symbols * and #, respectively (see text for details.

Protein Name	Symbol	Fold	q-values
Receptor-type tyrosine-protein phosphatase zeta	PTPRZ1	0.90	0.03
Nectin-1	PVRL1	0.89	0.09
Dihydropteridine reductase	QDPR *	0.85	0.02
Retinoic acid receptor responder protein 2	RARRES2 *	0.87	0.01
Reelin	RELN *#	0.82	0.03
RGM domain family member B	RGMB #	0.86	0.03
Ribonuclease pancreatic	RNASE1 *	0.88	0.03
Reticulon-4 receptor	RTN4R *	0.81	0.03
Reticulon-4 receptor-like 2	RTN4RL2 *	0.85	0.05
Serum amyloid A-4 protein	SAA4 *	1.16	0.04
Secretogranin-2	SCG2 *	0.93	0.08
Secretogranin-3	SCG3 *	0.91	0.03
Semaphorin-7A	SEMA7A *	0.83	0.03
Kallistatin	SERPINA4 *	0.71	0.09
Corticosteroid-binding globulin	SERPINA6 *	1.15	0.08
Antithrombin-III	SERPINC1 *	1.10	0.02
Alpha-2-antiplasmin	SERPINF2 *	1.12	0.02
Plasma protease C1 inhibitor	SERPING1 *	1.08	0.03
Neuroserpin	SERPINI1 *	0.76	0.05
Seizure protein 6 homolog	SEZ6 *	0.90	0.05
Seizure 6-like protein	SEZ6L	0.90	0.08
Seizure 6-like protein 2	SEZ6L2	0.89	0.03
Tyrosine-protein phosphatase non-receptor type substrate 1	SIRPA *#	0.85	0.09

Two hundred-twenty deregulated proteins [n = 86 (fold > 1.0) upregulated and n = 134 (fold < 1.0) downregulated] were identified in cerebrospinal fluid (CSF) of amyotrophic lateral sclerosis (ALS) subjects 30 days after mesenchymal stem cell (MSC) intrathecal infusion compared to CSF of subjects collected before cells (n = 24). Fold refers to mean of LFQ intensities of CSF 30 days after MSC infusion by mean of LFQ intensities of CSF before infusion (n = 24). Molecules related to Medical Subject Headings (MesH) “Extracellular Matrix” and “Cell Adhesion Molecules”, are indicated with symbols * and #, respectively (see text for details.

Protein Name	Symbol	Fold	q-values
Superoxide dismutase [Cu-Zn]	SOD1 *	0.89	0.04
SPARC	SPARC *	0.84	0.02
SPARC-like protein 1	SPARCL1 *	0.91	0.03
Testican-1	SPOCK1 *#	0.85	0.03
Spondin-1	SPON1 *#	0.88	0.03
Transforming growth factor-beta-induced protein ig-h3	TGFBI *#	1.20	0.01
Thy-1 membrane glycoprotein	THY1 *#	0.90	0.08
Metalloproteinase inhibitor 1	TIMP1 *	1.32	0.01
Transmembrane protein 132A	TMEM132A*	0.87	0.03
Tripeptidyl-peptidase 1	TPP1 *	0.76	0.01
Transthyretin	TTR *	0.90	0.06
Vitronectin	VTN *#	1.11	0.03
WAP four-disulfide core domain protein 1	WFDC1 *	0.84	0.09
Kunitz and NTR domain-containing protein 2	WFIKKN2 *	0.86	0.02

Two hundred-twenty deregulated proteins [n = 86 (fold > 1.0) upregulated and n = 134 (fold < 1.0) downregulated] were identified in cerebrospinal fluid (CSF) of amyotrophic lateral sclerosis (ALS) subjects 30 days after mesenchymal stem cell (MSC) intrathecal infusion compared to CSF of subjects collected before cells (n = 24). Fold refers to mean of LFQ intensities of CSF 30 days after MSC infusion by mean of LFQ intensities of CSF before infusion (n = 24). Molecules related to Medical Subject Headings (MesH) “Extracellular Matrix” and “Cell Adhesion Molecules”, are indicated with symbols * and #, respectively (see text for details.

Table 2
KEGG pathways and Gene Ontology categories

K	ID	KEGG pathways	n
K1	hsa04610	Complement and coagulation cascades	33
K2	hsa05150	Staphylococcus aureus infection	15
K3	hsa05133	Pertussis	13
K4	hsa05322	Systemic lupus erythematosus	13
K5	hsa04514	Cell adhesion molecules (CAMs)	13
K6	hsa05020	Prion diseases	10
K7	hsa04512	ECM-receptor interaction	7
BP	**ID**	**Biological Process**	**n**
BP1	GO:0007155	cell adhesion	34
BP2	GO:0002576	platelet degranulation	28
BP3	GO:0010951	negative regulation of endopeptidase activity	27
BP4	GO:0006508	Proteolysis	27
BP5	GO:0045087	innate immune response	25
BP6	GO:0006958	complement activation, classical pathway	22
BP7	GO:0030198	extracellular matrix organization	19
BP8	GO:0006898	receptor-mediated endocytosis	18
BP9	GO:0030449	regulation of complement activation	15
BP10	GO:0006956	complement activation	15
BP11	GO:0044267	cellular protein metabolic process	14
BP12	GO:0007411	axon guidance	13
BP13	GO:0001523	retinoid metabolic process	11
BP14	GO:0007417	central nervous system development	11
BP15	GO:0042730	Fibrinolysis	10

KEGG pathways (K) and Gene Ontology categories by means of DAVID (Database for Annotation, Visualisation, and Integrated Discovery) analysis based on 220 deregulated proteins identified in cerebrospinal fluid (CSF) of amyotrophic lateral sclerosis subjects 30 days after mesenchymal stem cell intrathecal infusion compared to CSF collected before cells. Corrected p-values for multiple tests using Benjamin-Hochberg (FDR) method are: $p < 0.01$, categories of K; $p < 0.00001$, categories of Biological Process (BP) and Cellular Component (CC); $p < 0.0001$, categories of Molecular Function (MF). Categories of KEGG and GO strands were ranked according to their number (n) of proteins.

K	ID	KEGG pathways	n
BP16	GO:0042157	lipoprotein metabolic process	9
BP17	GO:0008203	cholesterol metabolic process	9
BP18	GO:0022617	extracellular matrix disassembly	9
BP19	GO:0001558	regulation of cell growth	9
BP20	GO:0006957	complement activation, alternative pathway	8
BP21	GO:0042158	lipoprotein biosynthetic process	6
BP22	GO:0034375	high-density lipoprotein particle remodeling	6
BP23	GO:0007597	blood coagulation, intrinsic pathway	6
BP24	GO:0043691	reverse cholesterol transport	6
BP25	GO:0019835	Cytolysis	6
BP26	GO:0034380	high-density lipoprotein particle assembly	5
BP27	GO:0051918	negative regulation of fibrinolysis	5
CC	**ID**	**Cellular Component**	**n**
CC1	GO:0070062	extracellular exosome	146
CC2	GO:0005615	extracellular space	111
CC3	GO:0005576	extracellular region	111
CC4	GO:0005886	plasma membrane	83
CC5	GO:0072562	blood microparticle	46
CC6	GO:0031012	extracellular matrix	29
CC7	GO:0009986	cell surface	26
CC8	GO:0005578	proteinaceous extracellular matrix	23
CC9	GO:0009897	external side of plasma membrane	19
CC10	GO:0031093	platelet alpha granule lumen	17
CC11	GO:0043025	neuronal cell body	17

KEGG pathways (K) and Gene Ontology categories by means of DAVID (Database for Annotation, Visualisation, and Integrated Discovery) analysis based on 220 deregulated proteins identified in cerebrospinal fluid (CSF) of amyotrophic lateral sclerosis subjects 30 days after mesenchymal stem cell intrathecal infusion compared to CSF collected before cells. Corrected p-values for multiple tests using Benjamin-Hochberg (FDR) method are: $p < 0.01$, categories of K; $p < 0.00001$, categories of Biological Process (BP) and Cellular Component (CC); $p < 0.0001$, categories of Molecular Function (MF). Categories of KEGG and GO strands were ranked according to their number (n) of proteins.

K	ID	KEGG pathways	n
CC12	GO:0031225	anchored component of membrane	15
CC13	GO:0043202	lysosomal lumen	13
CC14	GO:0034364	high-density lipoprotein particle	9
CC15	GO:0034361	very-low-density lipoprotein particle	7
CC16	GO:0005579	membrane attack complex	5
CC17	GO:0034366	spherical high-density lipoprotein particle	5
CC18	GO:0005577	fibrinogen complex	5
MF	**ID**	**Molecular Function**	**n**
MF1	GO:0005509	calcium ion binding	33
MF2	GO:0005102	receptor binding	26
MF3	GO:0004252	serine-type endopeptidase activity	23
MF4	GO:0004867	serine-type endopeptidase inhibitor activity	19
MF5	GO:0008201	heparin binding	14
MF6	GO:0050839	cell adhesion molecule binding	11
MF7	GO:0004866	endopeptidase inhibitor activity	9
MF8	GO:0005518	collagen binding	8
MF9	GO:0004869	cysteine-type endopeptidase inhibitor activity	6
MF10	GO:0017127	cholesterol transporter activity	5
MF11	GO:0005319	lipid transporter activity	5
MF12	GO:0060228	phosphatidylcholine-sterol O-acyltransferase activator activity	4

KEGG pathways (K) and Gene Ontology categories by means of DAVID (Database for Annotation, Visualisation, and Integrated Discovery) analysis based on 220 deregulated proteins identified in cerebrospinal fluid (CSF) of amyotrophic lateral sclerosis subjects 30 days after mesenchymal stem cell intrathecal infusion compared to CSF collected before cells. Corrected p-values for multiple tests using Benjamin-Hochberg (FDR) method are: $p < 0.01$, categories of K; $p < 0.00001$, categories of Biological Process (BP) and Cellular Component (CC); $p < 0.0001$, categories of Molecular Function (MF). Categories of KEGG and GO strands were ranked according to their number (n) of proteins.

Functional Enrichment Analysis

Cellular and molecular events possibly related to MSC therapy demonstrated by KEGG pathways and Gene Ontology categories are shown in Table 2. Respective number of molecules and p-value are also seen in Table 2. Furthermore, proteins related KEGG and Gene Ontology events are pointed in Table S2.

Additionally, Superclusters, as well as their respective protein number, that were formed by REVIGO from Gene Ontology categories of Biological Process, Cell Component and Molecular Function are shown in Table 3. Figure S1 illustrates an image of a REVIGO Biological Processes cluster. Importantly, the overlapped proteins among specific Gene Ontology Superclusters, according to proposed method, are seen in Table 3, thus highlighting important proteins of DAVID enriched analysis among those proteomics-indicated 220 deregulated molecules in CSF of ALS subjects 30 days after autologous MSC intrathecal infusion.

Table 3
Highly representative molecules in superclusters

Biological Process				
	Superclusters	**N**	**Overlapping**	**Proteins**
A	Lipoprotein biosynthesis	67	A B C D E G	APP
B	Receptor-mediated endocytosis	48	A B D E F	FGA
C	Cell adhesion	34	A B F G H	APOA1, APOA2, APOA4, APOE
D	Innate immune response	33	B D G H I	APOL1
E	Extracellular matrix organization	27	A B C E	VTN
F	Central nervous system development	26	A B D F	SERPING1
G	Cholesterol metabolismo	15	A B G H	APOB, LCAT
H	Lipoprotein metabolismo	9	B D E F	FGB
I	Cytolysis	6	B F G H	APOM
			A B C	AMBP
			A B D	CFD, CFI
			A B F	HRG, PLG, SERPINF2
			A C E	SPOCK1, TGFBI
			A C F	RELN
			A D I	C5
			A E G	TTR
			B E F	FGG
			B G H	LRP1
			C E F	NCAN
Cell Component				
	Superclusters	**N**	**Overlapping**	**Proteins**

REVIGO of Gene Ontology categories pointed in DAVID (Database for Annotation, Visualization, and Integrated Discovery) analysis using the 220 deregulated proteins identified in cerebrospinal fluid (CSF) of amyotrophic lateral sclerosis (ALS) subjects 30 days after intrathecal infusion of mesenchymal stem cells (MSC) compared to CSF collected before cells. Significantly enriched (FDR < 0.05) for Gene Ontology processes in each supercluster. Representatives are joined into superclusters of loosely related terms, visualized with different colors (illustrated in Figure S1). Molecules overlapping in supercluster of Biological Processes, Cell Components and Molecular Functions categories grouped by REVIGO. N: number of molecules present in each supercluster.

Biological Process				
	Superclusters	**N**	**Overlapping**	**Proteins**
A	Extracellular region	111	A B C D E	FGA, FGB, FGG, PLG, SPARC
B	External side of plasma membrane	95	A B C D	AGRN, HRG, KNG1
C	Fibrinogen complex	85	A B C E	AMBP, APOA1, CALR, FGFR2
D	Lysosomal lumen	30	A B C F	APOE, C4A, SOD1
E	Cell surface	26	A B D E	APP
F	Neuronal cell body	17	A B E G	CD55
G	Anchored component of membrane	15	A C D E	SERPINF2
			A C D G	BCAN
			B C D G	GPC1
			B E F G	CNTN2, RTN4R
Molecular Function				
	Superclusters	**N**	**Overlapping**	**Proteins**
A	Heparin binding	46	A B C	F2
B	Collagen Binding	38	A D E	APOE
C	Serine-type endopeptidase activity	23	B C D	C3
D	Phosphatidylcholine - sterol O - acyltransferase activator activity	13	A B	APP, ANG, HRG, KNG1, NID1, NRXN1, PCOLCE, SPARC, SPARCL, VTN
E	Lipid transporter activity	6	AC	C1S, C1R, F12, HABP2
			A E	APOB
			B C	PLG
			B D	A2M, C5
			C D	C4A

REVIGO of Gene Ontology categories pointed in DAVID (Database for Annotation, Visualization, and Integrated Discovery) analysis using the 220 deregulated proteins identified in cerebrospinal fluid (CSF) of amyotrophic lateral sclerosis (ALS) subjects 30 days after intrathecal infusion of mesenchymal stem cells (MSC) compared to CSF collected before cells. Significantly enriched (FDR < 0.05) for Gene Ontology processes in each supercluster. Representatives are joined into superclusters of loosely related terms, visualized with different colors (illustrated in Figure S1). Molecules overlapping in supercluster of Biological Processes, Cell Components and Molecular Functions categories grouped by REVIGO. N: number of molecules present in each supercluster.

Biological Process			
Superclusters	**N**	**Overlapping**	**Proteins**
		DE	APOA1, APOA2, APOA4

REVIGO of Gene Ontology categories pointed in DAVID (Database for Annotation, Visualization, and Integrated Discovery) analysis using the 220 deregulated proteins identified in cerebrospinal fluid (CSF) of amyotrophic lateral sclerosis (ALS) subjects 30 days after intrathecal infusion of mesenchymal stem cells (MSC) compared to CSF collected before cells. Significantly enriched (FDR < 0.05) for Gene Ontology processes in each supercluster. Representatives are joined into superclusters of loosely related terms, visualized with different colors (illustrated in Figure S1). Molecules overlapping in supercluster of Biological Processes, Cell Components and Molecular Functions categories grouped by REVIGO. N: number of molecules present in each supercluster.

Protein-Protein Interaction Network

Two sets of Top 15 protein hubs in the Protein-protein Interaction Network, that were ranked according to values of their betweenness and degree nodes in the network, are seen in Table 4. Provided elimination of repetitions, the resulting set of twenty-four Network relevant proteins is shown in the legend of Table 4.

Table 4
Hubs of Protein interaction network

Ranking	Betweenness		Degree	
	Molecule	value	Molecule	value
1	APP	6.434	APP	28
2	NCAM1	2.147	PLG	21
3	C3	2.078	C3	20
4	APOA1	1.936	APOA1	18
5	FGFR2	1.641	FGA	18
6	CNTN2	1632	C1QA	17
7	PLG	1.586	C5	17
8	A2M	1.359	F2	16
9	MMP2	1.161	A2M	15
10	AGRN	1.049	ACTG1	15
11	COL1A2	980	FGB	15
12	NTM	838	FGG	15
13	ACTC1	775	C4A	14
14	LRP1	760	COL1A2	14
15	MSN	721	APOE	13

Protein Interaction Network analysis based upon 220 deregulated proteins identified in cerebrospinal fluid (CSF) of amyotrophic lateral sclerosis subjects 30 days after mesenchymal stem cell intrathecal infusion compared to CSF before cells indicated two sets of Top 15 hubs, which were ranked according to values of their betweenness (physical interactions) and degree (signalling pathways) in the Network. Provided elimination of repetitions, the resulting set of twenty-four Network relevant proteins were: A2M, ACTC1, ACTG1, AGRN, APOA1, APOE, APP, C1QA, C3, C4A, C5, CNTN2, COL1A2, F2, FGA, FGB, FGFR2, FGG, LRP1, MMP2, MSN, NCAM1, NTM, PLG. These proteins, which were called as “High Representative Molecules in Network” were employed in further analyses in order to obtain the Highlighted molecules among proteomics deregulated proteins (see below).

Molecular Representation of “Extracellular Matrix” and “Cell Adhesion Molecules” MESHs

Two KEGG pathways (K5, K7) and eight Gene Ontology categories (PB1, PB7, PB18, CC1, CC2, CC3, CC6, MF6) that are related to “Extracellular Matrix” or “Cell Adhesion Molecules” MeSHs were described in Table 5. Vast majority of proteomics-indicated deregulated proteins were encountered in above described “Extracellular Cellular Matrix” and “Cell Adhesion Molecules” -related pathways/categories (201 molecules, representing 92% of total). Specifically, 186 (84% of total) and 49 (22% of total) molecules

corresponded to pathways/categories that are related to “Extracellular Cellular Matrix” and “Cell Adhesion Molecules” MESHs, respectively (Table 5).

Table 5
DAVID representation of “Extracellular Matrix” and “Cell Adhesion Molecules” MeSH

MeSH	Pathways/Categories	Molecules: n (%)
Extracellular Matrix	K7, PB7, PB18, CC1, CC2, CC3, CC6	186 (84)
Cell Adhesion Molecules	K5, PB1, MF6	49 (22)
Extracellular Matrix + Cell Adhesion Molecules		201 (92)

DAVID Pathways/Categories, as well as the number (n) of their corresponded molecules, related to specific Medical Subject Headings (MesH) “Extracellular Matrix” and “Cell Adhesion Molecules” are shown. The number and ID of specific DAVID Pathways/Categories were described in Table 2. Furthermore, percentages (%) of molecules grouped in the MeSH-related Pathways/Categories in relation to 220 proteomics deregulated proteins are also shown. Additionally, Molecules of MeSH-related Pathways/Categories were pointed out (# and *, “Extracellular Matrix” and “Cell Adhesion Molecules”, respectively) in set of 220 deregulated proteins (Table 1) in cerebrospinal fluid of amyotrophic lateral sclerosis subjects 30 days after mesenchymal stem cell intrathecal infusion. Abbreviation: KEGG pathways (K), Biological Process (BP), Cellular Component (CC), and Molecular Function (MF).

Highlighted Molecules Related to MSC Infusion

Highlighted Molecules with a high presence (100% or 75%, according to defined criteria described in method) were seen in Table 6. See details also in Table 6 legend. APOA1, APOE, APP, and PLG reached 100% of representation. C4A, C5, FGA, FGB, FGG) reached 75% of representation (Table 6). Specifically, APOA1, C4A, C5, FGA, FGB, FGG and PLG are upregulated and APOE and APP are downregulated, as indicated by proteomics in CSF of ALS subjects 30 days after MSC intrathecal infusion compared to CSF collected before cells (Table 1). All Highlighted Molecules were verified to belong to pathways/categories related to MESH “Extracellular Matrix” and “Cell Adhesion Molecules” (Table 6).

Table 6
Highlighted Molecules

%	MOLECULES				
100%	APOA1*	APOE*	APP*#	PLG*	
75%	C4A*	C5*	FGA*#	FGB*#	FGG*#

Highlighted Molecules were evidenced according to their high presence in REVIGO Superclusters (see Table 3) based upon KEGG pathways and DAVID categories, provided their obligatory presence in the set of Network Relevant Proteins (see Table 4). Highlighted Molecules of 100% (belonging to Network plus 4 pathways/categories) or 75% (belonging to Network plus 3 pathways/categories) representation are seen. Specifically, APOA1, C4A, C5, FGA, FGB, FGG and PLG are upregulated and APOE and APP are downregulated, as indicated by proteomics in the cerebrospinal fluid (CSF) of amyotrophic lateral sclerosis subjects 30 days after mesenchymal stem cell intrathecal infusion compared to CSF collected before cells (Table 1). Remarkably, all Highlighted Molecules were verified to belong to pathways/categories related to Medical Subject Headings (MESH) “Extracellular Matrix” (#) and “Cell Adhesion Molecules” (*).

Discussion

MSC have emerged as a promise in the treatment of human ALS (10). Indeed, rather recent clinical trials have pointed out potential positive effects of MSC for neurodegenerative diseases (38, 39), including ALS (7, 8, 40, 41), it is still lacking information on putative cellular/molecular mechanisms underlying MSC-induced neuroprotection (12, 14, 42) as well as to counteract motor neuron death in ALS (3, 43). Remarkably, the descriptions of molecules possible involved in MSC effects on neurons have increased experimentally (11, 14) but not as far clinically (42, 44). Considering a well described failure to translate therapeutical targets for ALS from bench to bed side (45, 46), researches that explore in details cellular mechanisms and corresponded molecules related to MSC treatment in ALS disorder are desirable.

This manuscript innovated by combining large omics, specifically Proteomics and Protein Interaction Network, as well as defined criteria for molecular modeling in order to highlight cellular mechanisms and their related molecules in the CSF of ALS subjects 30 days after intrathecal infusion of autologous bone marrow-derived MSC. Nevertheless, our study is in agreement to previous reports that investigated molecular responses in CSF after local deliver of MSC in ALS patients by applying different methodologies (2, 47–49). Moreover, despites investigations have searched molecular responses to MSC in blood serum in clinical ALS (50, 51) as well as striatal muscles in experimental ALS (52, 53), CSF has been considered an important body compartment for molecular investigation due to CSF anatomical proximity to suffering neurons as well as for carrying bio molecular signatures of aberrant biochemical processes related to central nervous system pathophysiology (54, 55). In this context, it seems likely that CSF administration of autologous MSC performed in this trial may be relevant to facilitate cell signals to reach neurodegeneration zones in ALS, as discussed elsewhere (3, 11, 48), contrasting previous clinical design that analyzed molecular responses to MSC after muscular deliver (24, 56).

Furthermore, to our concern, this study is the first one to employ Proteomics by means of mass spectrometry for molecular investigation in CSF of intrathecal autologous MSC-treated ALS subjects, regardless the methodology has been recently employed on biomarker discovery program in CSF of ALS patients (57–59). Moreover, despite a lack of omics investigation on that matter, molecular regulation in CSF of MSC-treated ALS patients has been performed using classical non omics methodology (2, 49). In line to present study, majority of ALS clinical trials on MSC-delivered CSF have employed autologous bone marrow-derived MSC (2, 6, 47, 60, 61), rather than stem cells derived from adipose tissue (48), umbilical cord or other sources that are mainly employed in experimental investigations. The advantage of bone marrow-derived MSC to clinical application, specially in neurodegenerative disorders, has been well described, that is specially related to their ability to interact in an autocrine/paracrine matter to injured tissue (11–14, 62). Indeed, the molecular crosstalk among MSC and nervous tissue might interfere with inflammatory events at wound with the potential to modify the progression of neurodegeneration which is substantially important for progressive neurodegenerative disorders like ALS (63, 64).

Remarkably, proteomic analysis has pointed out 220 deregulated proteins in CSF of ALS subjects thirty days after autologous bone marrow-derived MSC intrathecal delivery. This result represents an important set of molecular responses to MSC presence in ALS indeed a number far way larger than the set of deregulated molecules described by similar clinical trials on ALS that have not applied omics technology (2, 47) in the screening of molecular biomarkers. Among those deregulated proteins, upregulated and downregulated molecules might be able to address mechanisms related to MSC in ALS or even may contribute as biomarkers of MSC effects in ALS in future investigations.

In fact, the present study has worthy contributed to original description of cellular mechanisms and related molecular targets facing intrathecal MSC in ALS by employing enrichment analysis of deregulated molecules (36, 37). The clusterization of deregulated proteins by means of REVIGO has pointed out a set of clusters and superclusters of cellular/molecular mechanisms possibly related to MSC actions thirty days after intrathecal delivery in ALS patients. Remarkably, extracellular matrix and cell adhesion terms were highlighted among superclusters thus representing an important contribution of herein employed methodology. In fact, despites REVIGO clusterization has been largely applied (37), it is an original contribution in the search for mechanism related to MSC in ALS. In fact, the literature analysis of “Extracellular matrix” and “Cell adhesion molecules” MeSHs indicated a huge involvement of such matters in the context of ALS as well as MSC. Our study remarkably highlighted specific Pathways/Categories related to “Extracellular matrix” and “Cell adhesion molecules” MeSHs. The observation that 92% of proteomics deregulated molecules belonged to Pathways/Categories related to “Extracellular matrix” and “Cell adhesion molecules” MeSHs strongly emphasized the possible involvement of Extracellular matrix and Cell adhesion molecules in the putative mechanisms of MSC delivered in CSF of ALS subjects thus representing an important contribution of this study.

It has to be mentioned that we are still not able to address whether Extracellular Matrix/Cell Adhesion Molecules highlighted in this study are related to putative MSC actions after intrathecal delivery in ALS patients, to ongoing ALS motor neuron degeneration or a possible interaction of both, a matter that must be the subject of further investigations. Anyhow, extracellular matrix and cell adhesion have been largely described in the context of ALS motor neuron degeneration (65–68) as well as MSC mechanisms of action (10, 43, 69). Actually, Extracellular Matrix and Cell Adhesion Molecules largely interact in the mechanisms of cell signaling driving autocrine (70) and paracrine (71) cellular mechanisms that indeed have been largely correlated to MSC actions (42) and motor neuron degeneration/protection (39, 72). Those observations remarkably open up the possibility for an integrated mechanism related to Extracellular Matrix and Cell Adhesion Molecules that might be involved in the effects of MSC in the injury sites of ALS thus possibly interfering with motor neuron degeneration in the disorder. All in all, this paper has highlighted for the first time the importance of Extracellular Matrix and Cell Adhesion Molecules in the interactive mechanisms of MSC and motor neuron death in ALS.

The demonstration of highlighted molecules APOA1, APOE, APP, PLG, C4A, C5, FGA, FGB and FGG as possible important proteins related to the presence of MSC in CSF of ALS subjects is an additional contribution of this study. Moreover, it should be emphasized the contribution of Protein Interaction

Network evaluation (73, 74) in the criteria herein applied to point out those highlighted molecules. Importantly, it is the first time APOA1, APOE, APP, C4A, C5, FGA, FGB, FGG and PLG have been mentioned in the context of biomarkers of MSC presence in \ CSF of ALS subjects 30 days after intrathecal cell delivery.

Indeed, all nine highlight molecules belong or signalize to elements of Extracellular Matrix and Cell Adhesion Molecules as well as they have been described to interact to stem cells in general or to MSC in particular (75–78). C4A, FGB, FGG and PLG have been described in the context of neuronal degeneration/survival or neurodegenerative disorders (79–83) and APOA1, APOE, APP, C5 and FGA have been investigated in the context of ALS (84–90).

All in all, the highlighted molecules described above have a potential possibility to be involved in the mechanisms of MSC in motor neuron degeneration in human ALS, a matter to be explored in future investigation.

Conclusion

Deregulated proteins that were indicated by means of proteomic analysis in CSF of ALS subjects 30 days after intrathecal autologous MSC infusion pointed out the importance of Extracellular matrix and Cell adhesion in the possible mechanisms of cell therapy. APOA1, APOE, APP, PLG, C4A, C5, FGA, FGB and FGG were highlighted as important molecules possibly participating in this process.

Declarations

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Availability of supporting data

Some of the data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GC, PRSB contributed to conception and design, manuscript writing, final approval of manuscript, general interpretation and responsibilities. GP responsible for proteomics and corresponded data interpretation, LAPCL, JP responsible for bone marrow aspiration, HRG responsible for SCF aspiration, WSP responsible for MSC infusion, ACS, CLKR, DRG responsible for MSC production and quality control, JRM responsible for bioinformatics, general statistical analysis, interpretation of data and manuscript writing. ALN responsible for data analysis, review of literature, interpretation of data and manuscript writing.

All authors read and approved the final manuscript.

Authors' information

All authors are Ph.D. and linked to research activities of corresponded public universities.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

Ethics approval and consent to participate

ALS patients were consented in accordance with the Helsinki declaration

Consent for publication

Not applicable.

Competing interests

Authors declare no conflate of interest

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