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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 rapidly progressive motoneuron degenerative disorder. There are still no drugs capable of slowing disease evolution or improving life quality of ALS patients. Thus, autologous stem cell therapy has emerged as an alternative treatment regime to be investigated in clinical ALS.

Method Using Proteomics and Protein-Protein Interaction Network analyses combined with bioinformatics, the possible cellular mechanisms and molecular targets related to mesenchymal stem cells (MSCs, 1×10^6 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 MSCs thirty days after cell therapy. Data are available via ProteomeXchange with identifier PXD053129.

Results Proteomics revealed 220 deregulated proteins in CSF of ALS subjects treated with MSCs compared to CSF collected from the same patients prior to MSCs infusion. Bioinformatics enriched analyses highlighted events of Extracellular matrix and Cell adhesion molecules as well as related key targets APOA1, APOE, APP, C4A, C5, FGA, FGB, FGG and PLG in the CSF of cell treated ALS subjects.

Conclusions Extracellular matrix and cell adhesion molecules as well as their related highlighted components have emerged as key targets of autologous MSCs in CSF of ALS patients.

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Keywords Amyotrophic lateral sclerosis, Mesenchymal stem cells, Proteomics, Protein-protein interaction network, Cerebrospinal fluid

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Background

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

Stem cell therapies for neurodegenerative disorders were proposed decades ago [11, 12]. Questions regarding their ability to promote clinically relevant outcomes have been raised since that time [13, 14]. These questions are based on the theory that functional neurons would be produced from these stem cells in vivo [15]. Indeed, despite the ability of adult stem cells to differentiate into neurons in vitro and in vivo under controlled experimental conditions [16], it was hard to accept that new neurons could regenerate long neuronal pathways or fully integrate in such morphologically and physiologically complex nervous tissue parenchyma leading towards functional restoration. That skepticism changed with the demonstration that MSCs could induce neuroprotection, reduce inflammation and contribute to functional neuronal repair in some neurodegenerative disorders [17, 18] via processes that may involve their paracrine ability to interact with the diseased milieu [19, 20]. Indeed, a wide range of complex surface receptors allow MSCs to detect and to react to specific local molecular signals [21, 22] and in response, may produce and secrete soluble bioactive molecules and extracellular vesicles [19, 23] with the potential to positively impact neurodegenerative processes. Remarkably, activated MCSs may modulate glial reaction, endothelial state, immune cell responses and endogenous stem cells, all able to interfere with neuroprotective events [16–23]. Additionally, these paracrine actions of MSCs on the pathophysiology of nervous tissue has highlighted the importance of the Extracellular matrix in local MSCs effects [24].

Studies have explored the regulation of Extracellular matrix proteins and Cell adhesion molecules in the search for specific molecular targets of cellular events related to neurodegeneration [25], neurodegenerative disorders [26, 27] and neuroprotection [26]. However, information on the specific molecular responses and related mechanisms of MSCs in combating neurodegeneration experimentally or clinically are still lacking [28, 29].

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

MSCs benefits for the treatment of ALS were tested due to their potential ability to trigger motor neuron protective events [30, 31]. This study investigated whether such benefits might be mediated by MSCs paracrine mechanisms and was designed to identify specific molecules associated with these paracrine interactions [31, 32].

Methods

ALS subjects, MSCs infusion and CSF withdrawal

This study is a subproject of a Phase I/II Clinical Trial (www.clinicaltrials.gov; NCT02917681) that tested the safety and preliminary effects of intrathecal (subarachnoid space of lumbar vertebrae, L3-L5) autologous bone marrow-derived mesenchymal stem cells (MSCs) infusion (10^6 cells/kg^{-1} body weight). The study was conducted (2016–2019) at the Neurology Division of the Clinics Hospital of the Medical School of the University of Sao Paulo, Brazil. The study was approved by the Ethics Committee of the Clinics Hospital of the University of Sao Paulo. Patients were clinically evaluated to inclusion/exclusion criteria and had their ALS diagnosis reconfirmed. Once included, ALS all subjects signed informed consent forms. 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 the Core for Cell Technology, Pontifical Catholic of University of Parana, Brazil, according to the previously described protocols [33]. A rigorous analysis of cell quality was performed in the amplified MSCs of ALS patients before injection, in order to obtain information of specific cell identity, cell viability and karyotype, based on the fact that laboratorial handling may impact the final cell set to be injected as it has been demonstrated in experimental animal research [34]. Only high quality MSCs were injected in the ALS subjects, according to well-stablished international criteria [33]. CSF (10 ml) was collected from the subarachnoid lumbar space of ALS subjects immediately before MSCs infusion and also 30 days later. The first 5 ml were delivered for standard clinical laboratory tests, including bacteriological and biochemical analyses, and the remaining 5 ml were used for molecular analysis in this study. CSF samples were centrifuged at 1,000 × g for 10 min at 4 °C, aliquoted (1 ml) into polypropylene cryogenic tubes and stored at -80 °C until further analyses. All samples were processed within 30 min 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 the retentate were denatured in 8 M urea, reduced by addition of dithiothreitol (final concentration

of 10 mM DTT), alkylated with iodoacetamide (final concentration of 40 mM) and digested with trypsin (1:50 enzyme to protein ratio). The reaction was stopped (1% trifluoroacetic acid), resulting peptides were purified (primed Oligo R3 reversed phase SPE micro-column) and dried [35].

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 (see below). Peptides were separated by nano ultra-high performance liquid chromatography tandem mass spectrometry (nUHPLC LC-MS/MS) according to the previous description [36–38].

The nanoLC was connected online to a QExactive HF Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) operating in positive ion mode and using data-dependent acquisition [37, 38].

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [39–41] partner repository with the dataset identifier PXD053129.

Deregulated proteins using LTQ Orbitrap

Proteins that were identified to be deregulated in the CSF of ALS subjects 30 days after MSCs infusion in comparison to CSF of same subjects before cell delivery were selected and their proteotypic peptides mapped in the PeptideAtlas database. Selected m/z values were monitored across all gradients and their MS/MS spectra were recorded in order to perform a database search using MaxQuant software [42]. Specifically, http://www.mcponline.org/ downloaded from 17 engine Andromeda [43] was used to search for MS/MS spectra against a database composed from the Uniprot Human Protein Database [44] with a 4.5ppm tolerance level for MS, and 20ppm for MS/MS. Furthermore, ceruloplasmin and reelin proteins, were selected as internal controls. Proteins detected in seven out of eight samples with their peptides identified by at least 6 samples with the MS/MS spectra search were considered for further analyses [37, 38]. Finally, 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 replacing missing values from the 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 MSCs infusion, compared to CSF of subjects before cell infusions [37, 38].

Protein-protein interaction network

Interactions among identified deregulated proteins in Network were evaluated using Cytoscape GeneMANIA plug-in (version 3.8.2), by highlighting “path” and “physical” interactions [45]. 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 [46]. Furthermore, a set of top 15 proteins was created with the highest betweenness and degree values. After elimination of repetitions, a set of final high representative molecules in Network was created based on 220 deregulated proteins.

Bioinformatics

We next evaluated possible mechanisms of action, and their related molecular targets, of the deregulated proteins identified by our proteomic analysis above. The data were further analyzed for cellular/molecular functional enrichments by employing specific bioinformatics tools described below.

Functional enrichment analysis

Deregulated proteins identified by the proteomic study were analyzed by means of Database for Integrated Annotation, Visualization and Discovery (DAVID, https://david.ncifcrf.gov). This analysis 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 [47, 48], 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 [49] 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. Subsequently, 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 each Network set. Following these criteria, highlighted molecules with 100% representation (present in the 4 sets) and 75% representation (present in 3 sets) were identified. Finally, the nine final molecules with the greatest intersections were considered as potentially prominent molecular targets and related molecular/cellular mechanisms in the CSF associated with the presence of MSCs in 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 MSCs function in injured tissues, the Medical Subject Headings (MeSH) “Extracellular Matrix” 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 52.13 years and 16.88 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 MSCs 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).

Functional enrichment analysis

Cellular and molecular events possibly related to MSCs 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 indicated 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 the proposed method, are seen in Table 3, thus highlighting important proteins of the DAVID enriched analysis among those proteomics-indicated 220 deregulated molecules.

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. Including elimination of repetitions, the resulting set of twenty-four Network relevant proteins is shown in the legend of Table 4. The Network is illustrated in Figure S2 of supplementary material.

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. The majority of proteomics-indicated deregulated proteins were encountered in the 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 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
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
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

Table 1 (continued)

Protein Name	Symbol	Fold	q-values
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
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
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

Table 1 (continued)

Protein Name	Symbol	Fold	q-values
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
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
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

Table 1 (continued)

Protein Name	Symbol	Fold	q-values
Neurexin-1	NRXN1 #	0.85	0.01
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
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-7 A	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
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

Table 1 (continued)

Protein Name	Symbol	Fold	q-values
Thy-1 membrane glycoprotein	THY1 *#	0.90	0.08
Metalloproteinase inhibitor 1	TIMP1 *	1.32	0.01
Transmembrane protein 132 A	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)

Highlighted molecules related to MSCs infusion

Highlighted Molecules with a high presence (100% or 75%, according to defined criteria described in methods) were seen in Table 6. See details also in Table 6 legend. APOA1, APOE, APP, and PLG reached 100% representation. C4A, C5, FGA, FGB, FGG) reached 75% representation (Table 6). Specifically, APOA1, C4A, C5, FGA, FGB, FGG and PLG are upregulated and APOE and APP are downregulated, as indicated by our proteomic analysis. (Table 2). All Highlighted Molecules were verified to belong to pathways/categories related to MESH "Extracellular Matrix" and "Cell Adhesion Molecules" (Table 6).

Discussion

MSCs as a promising approach for effective drug discovery in clinical ALS

MSCs have emerged as a promising therapy for the treatment of human ALS [10]. Indeed, recent clinical trials have revealed positive effects of MSCs for the treatment of some neurodegenerative diseases [50, 51], including ALS [7, 8, 52, 53]. However, information on the putative cellular/molecular mechanisms underlying MSCs-induced neuroprotection [18, 20, 54], and thus counteracting motor neuron death in ALS [3, 55] is lacking. Interestingly, the identification of molecules involved in mediating the effects of MSCs on neurons has increased experimentally [17, 20] but our clinical understanding of such phenomena has not kept pace [54, 56]. Given the failure to translate therapeutic targets for ALS from bench to bed side [57, 58], there is a need to explore, in detail, the cellular mechanisms and corresponding molecules related to the use of MSCs in the treatment of ALS. Based on the absence of a reliable, long-lasting cell therapy for chronic neurodegenerative disorders, MSCs infusion in ALS patients may lead to the discovery of critical molecular targets. These could, in turn, contribute to the development of an effective pharmacological therapy to replace cell therapy in ALS and potentially, other neurodegenerative diseases.

Advantage of large proteomic analysis of CSF from MSCs treated ALS patients over sampled immune assays

The present study was the first to combine large omics analyses, specifically Proteomics and Protein Interaction Network, with well-defined criteria for molecular modeling in order to identify novel cellular mechanisms and their related molecules in the CSF of ALS subjects 30 days after intrathecal infusion of autologous bone marrow-derived MSCs. Detailed methods and the set of proteomically identified deregulated proteins described herein have also been shown in preprint form [59] according to BMC's publication policy. Importantly, our results are in agreement with those of previous reports that investigated the molecular responses in CSF after local delivery of MSCs in ALS patients by applying different methodologies [2, 60–62]. Moreover, while previous investigations have identified the molecular responses to MSCs in blood serum in clinical ALS [63, 64] and also in striatal muscles in experimental ALS [65, 66], the CSF is considered a more physiologically and clinically relevant body compartment for molecular investigation. This is due to the CSF's anatomical proximity to diseased neurons as well as for its containment of bio molecular signatures of altered biochemical processes related to central nervous system pathophysiology [67, 68].

MSCs performed in this trial may facilitate cell signals reaching neurodegeneration zones in ALS subjects, as discussed elsewhere [3, 17, 61]. This is in contrast to previous clinical designs that analyzed molecular responses to MSCs after intra-muscular delivery [30, 69]. It should be mentioned that this study has employed an endogenous control (CSF before MSC infusion) to evaluate molecular changes in the CSF after cell therapy, instead of CSF from healthy subjects, based on the well-known difficulty to eliminate the influence of the specific clinical situation that led a CSF withdrawn on the molecular signaling in central nervous system [70].

Furthermore, this study is the first to employ Proteomics by means of mass spectrometry to determine the molecular profile of CSF after intrathecal autologous

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
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
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

Table 2 (continued)

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

MSCs treatment of ALS subjects. Recently, a similar methodology has been employed in a biomarker discovery program in CSF of ALS patients [71–73]. Previously, the molecular regulation within CSF of MSCs-treated ALS patients has been performed using classical, non-proteomic methodology [2, 62]. As with our study, the majority of ALS clinical trials on MSC-delivered to CSF have employed autologous bone marrow-derived MSCs [2, 6, 60, 74, 75], rather than stem cells derived from adipose tissue, umbilical cord or other sources that are mainly employed in experimental investigations [61]. The advantages of bone marrow-derived MSCs for clinical application, especially in neurodegenerative disorders, have been well described, particularly their ability to interact in an autocrine/paracrine manner with injured tissue [17–20, 76]. Indeed, it has been proposed that the paracrine molecular crosstalk between MSCs and nervous tissue cells might interfere with inflammatory events at the wound site, with the potential to modify the progression of neurodegeneration [77, 78]. Indeed, MSCs paracrine signaling might be important in counteracting neuronal cell death in progressive neurodegenerative disorders like ALS [77, 78]. Thus, it is clear that there are distinct advantages of employing large omics analyses (like proteomics), over sampled immune assays of predefined proteins, in the search for effective and reliably translatable therapeutic targets in the CSF of ALS patients. The power of large proteomics may be amplified with the combination of specific criteria for molecular modeling in the search for key molecules among all deregulated proteins.

Our proteomic analysis has identified 220 deregulated proteins in the CSF of ALS subjects 30 days after autologous bone marrow-derived MSCs intrathecal delivery. These data provide an extensive set of molecular responses to the presence of MSCs in ALS subjects. In addition, our results provide a much more extensive database than the set of deregulated molecules described by similar clinical trials on ALS that have not applied omics technology in the screening of molecular biomarkers [2, 60]. Among those deregulated proteins identified in our study, upregulated and downregulated molecules might contribute to our understanding of the mechanisms related to the efficacy of MSCs therapy for ALS. Moreover, our results may identify important biomarkers of MSCs effects on the progression of ALS in future investigations.

Enrichment analysis described key mechanisms and targets related to cell therapy

The present study has contributed useful data to the original descriptions of the cellular mechanisms and related molecular targets associated with intrathecal infusion of MSCs in ALS patients by employing enrichment analysis of deregulated molecules [48, 49]. REVIGO analysis of deregulated proteins has identified a set of clusters and superclusters of cellular/molecular mechanisms possibly related to MSCs actions 30 days after intrathecal MSCs delivery in ALS patients. Interestingly, Extracellular matrix and Cell adhesion terms were highlighted among these superclusters, thus highlighting the usefulness of this powerful methodology. In fact, despite the development of REVIGO clusterization analyses 13 years ago [49], our study is the first to employ this approach to identify mechanisms related to MSCs effects in ALS patients. Moreover, the literature analysis of "Extracellular matrix" and "Cell adhesion molecules" MeSHs indicated a significant involvement of such factors in the context of ALS as

Table 3 Highly representative molecules in superclusters

	Superclusters	N	Overlapping	Proteins
Biological process				
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				
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				
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
			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

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)

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)

well as MSCs. Our study determined that 92% of the 220 deregulated molecules identified by proteomic analysis belonged to Pathways/Categories related to "Extracellular matrix" and/or "Cell adhesion molecules" MeSHs. Our data thus clearly implicate Extracellular matrix and Cell adhesion molecules as factors associated with the putative paracrine signaling of MSCs delivered to the CSF of ALS subjects.

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" (*)

We are still unable to address whether the Extracellular matrix/Cell adhesion molecules (or some interaction of both) identified in this study are associated with the putative MSCs effects (after intrathecal delivery in ALS patients) on ongoing motor neuron degeneration. This question remains the subject of further investigation. Extracellular matrix and Cell adhesion molecules have been previously described in the context of ALS motor neuron degeneration [79–82] as well as with MSCs mechanisms of action [10, 55, 83]. Indeed, Extracellular Matrix and Cell adhesion molecules have been frequently associated with the mechanisms underlying both autocrine [84] and paracrine [85] cellular effects correlated with MSCs actions [54] and motor neuron degeneration/protection [51, 86]. These observations suggest the possibility that an integrated mechanism involving Extracellular matrix and Cell adhesion molecules might underlie the effects of MSCs in the injury sites of ALS. This interaction, in turn, could possibly interfere with motor neuron degeneration in this disorder. In sum, this study has highlighted, for the first time, the importance of the Extracellular matrix and Cell adhesion molecules as contributors to the neuroprotective effects of MSCs in ALS.

The identification of factors such as APOA1, APOE, APP, PLG, C4A, C5, FGA, FGB and FGG as possible key proteins in the CSF from ALS subjects treated with MSCs is an additional important contribution of this study. Moreover, we must highlight the critical contribution of the Protein Interaction Network analysis [87, 88] in establishing criteria for the identification and establishment of possible interactions between these identified 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 in CSF of ALS subjects 30 days after intrathecal MSCs cell delivery.

Indeed, all nine highlighted molecules are associated with elements of the Extracellular matrix and Cell adhesion [89–93] and have also been demonstrated to be associated with mechanisms of action of MSCs [94–97]. Thus, the Extracellular matrix and Cell adhesion

molecules represent important factors associated with the paracrine actions of these stem cells. These findings are a major and original contribution of this study. Additionally, they will lead to further studies addressing the precise roles of Extracellular Matrix and Cell Adhesion molecules in paracrine signaling associated with the neurotrophic effects of MSCs, with the ultimate goal of providing clinical benefits for ALS patients [53].

Furthermore, some of our highlighted molecules have been described in the context of neuronal degeneration/survival or with neurodegenerative disorders, as is the case for C4A, FGB, FGG and PLG [98–102]. Unfortunately little information is presently available on their involvement in these processes in ALS. Despite the fact that the majority of these key proteins (APOA1, APOE, APP, C4A, C5, FGA, FGG and PLG) have been investigated in the context of ALS [103–109], there is a lack of information on their roles associated with specific cellular/molecular mechanisms of motoneuron death in this disease. More specifically, despite the identification of APOE as a biomarker for the rare, genetic forms of ALS associated with dementia [53, 110], the role of APOE in neuronal death in the more prevalent sporadic form of ALS is still lacking. Nevertheless, APOE polymorphisms have been correlated with lysosomal dysfunction-induced neuronal death in ALS [110]. Also, activation of microglial APOE signaling has been associated with motoneuron cell death in ALS [111] and APOE signaling over nuclear TDP-43 seems to trigger motor neuron death in this disorder [112]. Importantly these detrimental effects of APOE on motoneuron survival in ALS correlate with a possible protective action of MSCs delivered to ALS subjects in our clinical trial, as a downregulation of APOE was seen in the CSF of ALS patients treated with MSCs.

Interestingly, ablation of the APP gene in the murine model of ALS was able to counteract disease severity [113]. Furthermore, APP accumulation in the endoplasmic reticulum of motoneurons was correlated with cytoskeleton disruption, neurite retraction, accumulation of toxic molecules, i.e. TDP-43, SOD1 FUS, and motoneuron death in ALS [114]. In fact, misfolded APP toxicity might spread among motoneurons thus amplifying cell death in ALS [115]. These descriptions of APP toxicity in ALS are interesting, given our finding that APP is downregulated in the CSF of MSCs treated ALS subjects. This downregulation may thus be related to a protective effect of MSCs on motoneurons, thus representing another significant contribution of the present study.

Furthermore, the upregulation of complement 5 (C5) in the CSF of ALS subjects 30 days after MSCs infusion may be an indication of ongoing neurodegenerative events rather than a response to cell infusion. This conclusion is in view of the recent findings on the promising effects of the monoclonal antibody C5 complement inhibitor ravulizumab in a clinical ALS trial [116]. Interestingly, Mantovani et al. 2014 [106] have described increases of C4 and C5 in the blood of ALS patients while our proteomic analysis has shown upregulation of C4, C5 and several other molecules of complement system in the CSF of MSC-treated ALS subjects. Based on these observations, we speculate that a combination of monoclonal antibodies against several complement system molecules might amplify neurological benefits achieved with the C5 complement inhibitor ravulizumab [116].

The identification of the molecules APOA1, FGA, FGB, FGG and PLG represent an original contribution to our understanding of the molecular signaling associated with the Extracellular matrix and Cell adhesion underlying the putative protective effects of MSCs treatment of ALS. This represents another direction for further analysis.

Finally, additional criteria can be applied in future studies for molecular modeling among our 220 deregulated proteins. These studies could identify new key mechanisms and associated molecules related to MSCs treatment in clinical ALS. Thus, our present proteomic analysis provides an initial framework for further study and establishes the importance of a set of 220 deregulated proteins in the CSF of ALS patients 30 days after MSCs delivery.

Conclusion

A complex proteomics and network analysis revealed 220 deregulated proteins in the CSF of ALS subjects 30 days after intrathecal autologous MSCs infusion. Extracellular matrix and Cell adhesion molecules were identified as potential therapeutic targets related to stem cell therapy for the ongoing neurodegenerative events in ALS chronic progressive disorder. APOA1, APOE, APP, PLG, C4A, C5, FGA, FGB and FGG were identified as key molecules associated with those events.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13287-024-03820-2.

Supplementary Material 1

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and Associate Professor of Parasitology Department, Institute of Biomedical Sciences, USP.

Author contributions

GC, PRSB contributed to conception and design, manuscript writing, final approval of manuscript, general interpretation and responsibilities. ALGR was responsible for proteomics and corresponded data interpretation, review of literature, and manuscript writing. LAPCL, JP were responsible for bone marrow aspiration, HRG for CSF aspiration and WSP for MSCs infusion. ACS, CLKR, DRG were responsible for MSCs production and quality control. JRM and GC were responsible for bioinformatics, general statistical analysis, interpretation of data and manuscript writing. All authors read and approved the final manuscript.

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Data availability

Raw data are available via ProteomeXchange with identifier PXD053129. Additional data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Clinics Hospital of the University of Sao Paulo (Approval Number: 1.616.004; Date of Approval: June, 30th 2016). Project Registration Number: 37772414.0.0000.0068. Original Title of Approved Project: Estudo fase 1/2 da segurança e eficácia de duas doses intratecais de células-tronco mesenquimais autólogas (CTM), obtidas de células estromais da medula óssea, em pacientes com Esclerose Lateral Amiotrófica. English Title of Approved Project (Translated): Phase 1/2 clinical trial on security and efficiency of 2 doses of intrathecal infusion of bone marrow derived autologous Mesenchymal Stem Cell (MSC) in Amyotrophic Lateral Sclerosis ALS patients provided informed consent in accordance with the Helsinki declaration.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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