Possible Strategies to Optimize a Biomarker Discovery Approach to Correlate with Neurological Outcome in Patients with Spinal Cord Injury: A Pilot Study

Possible Strategies to Optimize a Biomarker Discovery Approach to Correlate with Neurological Outcome in Patients with Spinal Cord Injury: A Pilot Study

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Abstract

The lack of reliable diagnostic and prognostic markers for spinal cord injured (SCI) patients is a severe obstacle in development and testing of new therapies, and it also impairs appropriate rehabilitation care. The sparse available data on the biochemical composition of cerebrospinal fluid (CSF) during the acute and/or chronic phase of the lesion provide, up until now, inconsistent results. In this pilot study, we then explored the possibility of combining a multi-parametric and bioinformatic analysis of CSF for its biological properties tested on different cells types, suitable for investigating inflammation and re-myelination. The patient enrollment was based on stringent inclusion criteria; that is, cervical and thoracic SCI trauma, CSF collection within 24 h of trauma, type of surgical approach for spine stabilization, and absence of steroid therapy before CSF collection. Eleven SCI patients and four healthy controls were included, and in three patients, CSF was also collected at 3 months after lesion. We identified 19 proteins among the 60 investigated cytokines, chemokines, growth factors, and structural biomarkers, which are transiently regulated 24 h after SCI. A bioinformatic analysis indicated that interleukin (IL)-6 and IL-10 are in the core of the interconnected net of activated proteins. Cell-based experiments indicate that CSF from SCI patients stimulates astroglia derivation from neural precursor cells, and an inverse correlation between IL-8 CSF level and oligodendrocyte precursor cells generated from neural stem cells was also observed. Results from this pilot study suggest that using a combined bioanalytic and biological approach to analyze SCI CSF at different times after injury could be a useful approach for identifying reliable diagnostic and prognostic markers in SCI.

Keywords: biomarkers; CSF; IL-8; inflammatory cytokines; multi-parametric assay; oligodendrocyte precursor cells; SCI

Introduction

Spinal cord injury (SCI) represents a global medical and social problem that causes death and disability, the incidence of which varies from ~15–54 cases per 1,000,000.[1–3] In spite of the impressive advancement in medical and surgical treatments and the large body of pre-clinical evidence on the cellular and molecular mechanisms leading to the spinal cord damage following trauma, SCI is considered a incurable condition. In fact, currently, pharmacological interventions are still limited and are aimed at reducing edema, and the routine use of steroids has been largely abandoned and considered a ''harmful standard of care.''[4] Some drugs, approved for other neurological diseases, are currently being tested (e.g., riluzole, erythropoietin, Nogo-A targeting, Rho inhibitor cethrin, and minocycline). However, to date, no studies have shown evidence of recovery of neurological function. In addition, many of the current therapeutic attempts focus on complications.[5]

Pre-clinical and translational studies have highlighted the molecular pathology that follows trauma, divided into three phases: acute (a few seconds or minutes after the injury), secondary (from a few minutes to a few weeks after the injury), and chronic (some months to years after the injury).[6] In the acute phase, mechanic and

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vascular events are prevalent, such as edema and alterations of the chemical microenvironment, where excitotoxicity and infiltration by circulating macrophages prevails. Many of these events are also present in the secondary phase, in particular oxidative stress, inflammation, and immunological reaction also mediated by microglial cells, which lead to the initiation of astroglial scarring, extensive demyelination, and electrophysiological collapse. In the chronic phase, demyelination, astroglial reaction, and central cavitation continue and are prevalent. Regeneration attempts; for example, axonal sprouting by some neurons, actually exacerbates alterations in the anatomy and physiology of microcircuits, such as hematoma, ischemia, necrosis, and peri-hemorrhagic edema. Remyelination attempts are also present in this context, but many axons remain demyelinated,[7] probably because oligodendrocyte precursor cells (OPCs), the re-myelinating cells in the central nervous system (CNS), fail to mature into myelinating oligodendrocytes because of severe tissue inflammation.[8,9]

Therefore, biochemical analysis of cerebrospinal fluid (CSF) composition at specific times after the trauma has been pursued for lesion severity and prognostic biomarker discovery. However, results from these studies are poor and contrasting.[10,11] In order to try to improve biomarker discovery strategy in acute SCI, we correlated the biochemical analysis of CSF performed by a multiparametric approach with the *in vitro* study of the biological properties of CSF in suitable cell systems (for example: to explore impact on inflammatory cells and re-myelinating cells). In this pilot study, we investigated the CSF derived from 11 patients subjected to traumatic spinal cord injury, collected within 24 h of the injury, and 4 healthy controls, in order to (1) measure the level of 60 inflammatory cytokines, growth factors, and structural biomarkers using multiparametric assays; and (2) investigate biological properties of these CSF samples in cell cultures dealing with two of the main pathogenic events in early SCI; that is, inflammation (RAW 264.7 macrophage cell line) and demyelination (primary OPCs).

Methods

Patient enrolment and CSF sampling

Patients were enrolled at the Maggiore Hospital Trauma Centre, where they were admitted by the 118-Bologna emergency service in accordance with the regional protocol of Emilia Romagna Health Service for severe traumatic injuries. The enrolment criteria were the following: (1) presence of cervical and/or thoracic SCI, (2) surgical spine stabilization with posterior approach (this makes it possible to obtain the CSF sample by lumbar puncture at the beginning of the surgical procedure) and CSF collection performed within 12–24 h of the injury, and (3) age 18–74 years. Patients treated with steroid therapy in the early acute phase after SCI were excluded. Only patients able to submit their informed consent in person before the first CSF sample collection were recruited. In cases in which patients were not able to write, a witness, not related to the patient and not involved in the study, signed the written informed consent. After the early acute phase at the Maggiore Hospital Trauma Centre, patients were then transferred to the Spinal Unit of the Montecatone Rehabilitation Institute to undergo their rehabilitation process. A second CSF sample collection was planned 3 months after the first one, during this stay. Control CSF derived from healthy subjects was purchased from PrecisionMed (Human Biological Material, Solana Beach, CA).

The study was approved by the Ethics Committees of Azienda Unità Sanitaria Locale di Bologna (n. CE 12105, January 18, 2013) and Azienda Ospedaliera Universitaria S. Orsola Malpighi (notification acceptance n. 976/2013, March 12, 2013). It was registered on ClinicalTrials.gov (NCT01861808).

All animal protocols described herein were conducted according to the European Community Council Directives (86/609/EEC), and comply with the guidelines published in the *NIH Guide for the Care and Use of Laboratory Animals*.

Neurological evaluations

The severity of neurological impairment was graded by experienced physicians according to the International Standards for Neurological Classification of SCI (ISNCSCI); that is with the American Spinal Injury Association (ASIA) Impairment Scale for completeness/incompleteness of the lesion and the ASIA neurological level (http://www.asia-spinalinjury.org).

CSF collection and storage

CSF collection was performed at the Maggiore Hospital within 24 h of the injury. CSF samples were centrifuged (2000*g*, 10 min, 4°C) and supernatant was aliquoted, then immediately deep frozen and stored at -80°C at the hospital laboratory. The CSF samples collected within 24 h of the injury have been named T0. Three months after the injury, CSF was collected at the Montecatone Rehabilitation Institute and immediately transported to the analysis laboratory (22.5 km distance, approved as ''health laboratory'' for human samples analysis by the competent authorities), keeping samples at +4°C. Upon arrival, CSF samples were centrifuged, aliquoted, snap-frozen, and conserved at -80°C until the day of the assay. T3 indicates the CSF collected 3 months after trauma. In order to optimize the reproducibility, all samples were analyzed in two runs, the first including patients 1–12, the second including patients 13–17.

Biochemical analysis

The multi-parametric protein/biomarker quantification was performed through Luminex xMAP technology and the MAGPIX platform. This technique allows the contemporaneous assay of up to 50 analytes in maximum 50μl CSF. Kits used including cytokine, chemokines, growth factors, neurological disease, and neurological disorders biomarkers (see Supplementary Methods for the complete list of analytes).

Cell cultures and treatments

The RAW 264.7 murine macrophages (ATCC-TIB-71 1 RAW 264.7) (ATCC) were obtained from the Cell Bank of Type Culture Collection (Manassas, VA, in partnership with LGC Standards S.r.L., Milan, Italy) and cultured as specified by the ATCC. Cells were exposed to 10% CSF from healthy control subjects (CNT-CSF) or SCI subjects for 4 h, and then cells were processed for RNA isolation (see Supplementary Methods). The experimental design is included in Figure 1A.

OPCs were derived from fetal neural stem/precursor cells (NSCs) as already described,[12] with some modifications. OPCs were exposed to 10% CSF from healthy control subjects or from SCI subjects. The experimental design is included in Figure 1B.

RNA isolation, retrotranscription and quantitative polymerase chain reaction (qPCR)

All details concerning procedures for RNA isolation, retrotranscription, and qPCR are described in the Supplementary Methods.

MitoTracker® staining

OPC were stained with MitoTracker Orange (Thermo Scientific, Waltham) following the manufacturer's instructions (see Supplementary Methods).

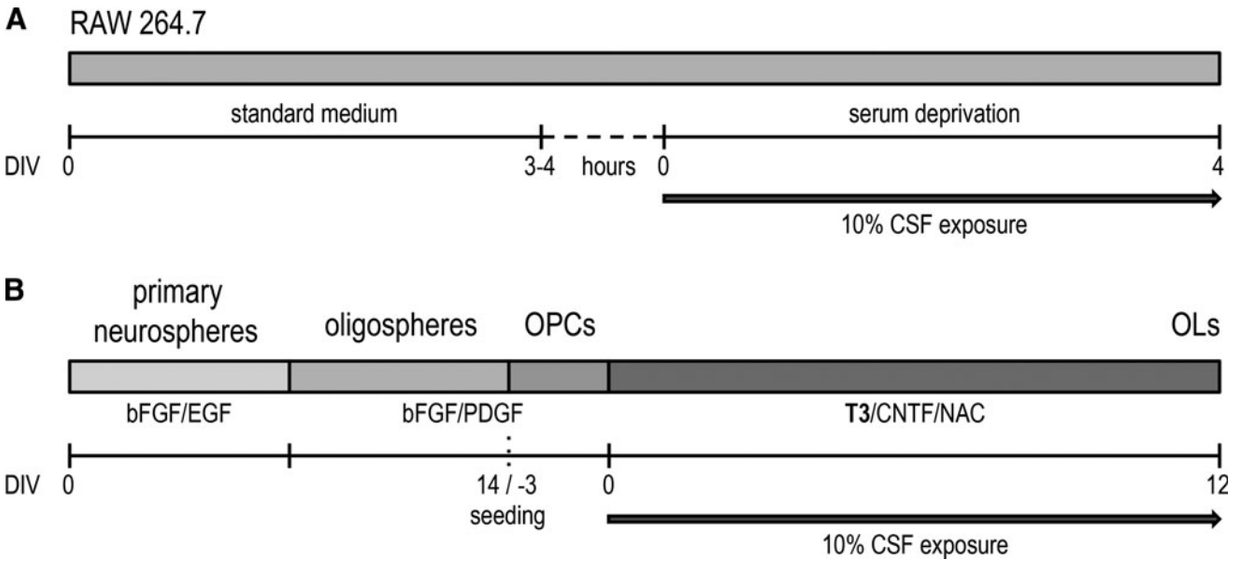

FIG. 1. **(A)** RAW 264.7 cell cultures experimental design. Cells were seeded and after 3–4 days reached 90% of confluency. At this step, cells were serum deprived and exposed at 10% of cerebrospinal fluid (CSF), from spinal cord injury (SCI) patients or controls, for 4 h (thick black arrow). **(B)** Oligodendrocyte precursor cell (OPC) culture experimental design. Neural stem cells were cultured in suspension as primary neurospheres in the presence of specific growth factors (basic fibroblast growth factor [bFGF] and epidermal growth factor [EGF]). When spheres reached 100 μm diameter, they were split and cultured as oligospheres, in the presence of platelet-derived growth factor (PDGF) and bFGF. When oligospheres reached 100 μm diameter, they were dissociated and seeded (-3 days in vitro, DIVs) in the same culture medium. After 3 DIVs (0 DIV), the culture medium was substituted with the differentiation medium, containing the differentiation factor T3. At this step, cells were exposed to 10% of CSF from SCI patients or controls (thick black arrow), for 12 DIVs.

Immunocytochemistry

OPCs were processed for immunocytochemistry after 12 days of differentiation (see Supplementary Methods).

Cell-based high content screening

Analysis of mitochondrial depolarization, condensed nuclei, cell number, and specific marker identification in OPCs was performed with Cell Insight™ CX5 High Content Screening (HCS) (Thermo Scientific), using the Compartmental analysis BioApplication (see Supplementary Methods).

Statistical analysis

Data are reported as mean ± standard error of the mean (SEM). Prism software (GraphPad) was used for statistical analyses and graph generation. Multi-parametric assay data were analyzed using the nonparametric Mann–Whitney *U* test in order to compare SCI versus control CSF samples. The value of the ''limit of detection'' (LOD) (provided by kit's manufacture) was assigned to biomarkers that were out of range below (< OOR) in CNTs-, T0- or T3-CSF experimental groups. Biomarkers giving < OOR value in all the experimental groups were declared as ''undetectable.'' Values above the higher standard of the standard curve (out of range above [> OOR]) were assigned the higher standard value. In order to analyze the effect of single SCI- versus control-CSF exposure in cell culture experiments, the Student's *t* test was used. Finally, correlation analysis between cytokines and cell culture readouts was performed by Pearson's test. A p value <0.05 was considered statistically significant.

Results

Fifteen subjects (4 controls and 11 SCI patients) were enrolled in the study. Three patients agreed to undergo the second CSF sampling 3 months after the first one (T3). One patient (ID 9) was later excluded, because his CSF sample was grossly contaminated with blood.[13] One patient (ID 8) agreed to CSF sample collection only at the second time point studied (at 3 months). The main demographic and clinical data of the enrolled patients are reported in Table 1.

Biochemical analysis of CSF

Results from the multi-parametric assay performed on CSF collected within 24 h after trauma (T0) and after 3 months (T3) are reported in Table 2, where T0 refers to the CSF collected within 24 h of trauma, T3 refers to 3 months after trauma, and ~~CTN~~, refers to control subjects. Individual data plots for all the detectable proteins have been included in Supplementary Figures S1 and S2. Because of the different T0 and T3 group composition, the statistical analysis was performed separately for these two groups, and results are expressed as ''p'' value in the respective columns.

Many of the investigated proteins show a transient, significant increase at T0 compared with control subjects (interleukin [IL]-10, IL-13, IL-16, IL-18, IL-1rα, IL-6, IL-8, IL-9, monocyte chemoattractant protein [MCP]-1, macrophage inflammatory protein [MIP]-1α, MIP-1β, RANTES, tumor necrosis factor (TNF)-α, TNF-related apoptosis-inducing ligand [TRAIL], granulocyte-colony stimulating factor [G-CSF], GRO-α, nerve growth factor [NGF]-β, myeloperoxidase [MPO], plasminogen activator inhibitor [PAI]-1). Within these, some cytokines and chemokines that are undetectable in control subjects are instead measurable at T0 (Eotaxin, IL-10, IL-1rα, IL-9, MCP-1, MIP-1α, RANTES, G-CSF, and GRO-α). Some upregulated proteins at T0 are significantly downregulated at T3 (Eotaxin, interferon [IFN]-γ, IL-10, IL-16, IL-1rα, IL-6, IL-8, IL-9, MCP-1, MIP-1α, MIP-1β, TNF-α, G-CSF, GRO-α, SCGF-β, macrophage

Table 1. Demographic and Main Clinical Data of Patients and Controls at Admission, Together with the Time Points of CSF Collection

ID	*Sex*	*Age*	*AIS grade*[a]	*Neurological level*[b]	*CSF at T0*	*CSF at T3*
3	Male	67	A	T6	✓	✓
4	Male	34	B	T4	✓	✓
5	Male	73	D	C5	✓	✓
6	Male	41	A	T7	✓	
7	Male	72	D	C5	✓	
8	Male	46	B	C4		✓
9	Female	41	A	T8	✓[c]	
10	Male	63	D	C5	✓	
13	Male	71	A	C4	✓	
14	Female	53	B	T11	✓	
16	Male	52	B	C4	✓	
17	Male	55	A	T3	✓	
C1	Male	41				
C2	Male	70				
C3	Female	45				
C4	Female	71				

[a]AIS, American Spinal Injury Association (ASIA) Impairment Score. A, motor and sensory complete spinal cord injury (SCI); B, motor complete and sensory incomplete SCI; C, motor and sensory incomplete SCI (key muscle functions below the single neurological level of injury [NLI] have a muscle grade <3); D, motor and sensory incomplete SCI (at least half of key muscle functions below the NLI have a muscle grade of ≥3).
[b]ASIA neurological level. C, cervical; T, thoracic.
[c]Later excluded because of gross blood contamination.
CSF, cerebrospinal fluid.

migration inhibitory factor [MIF], PAI-1). The anti-inflammatory cytokine IL-13 is instead significantly downregulated at T0 compared with control, and also remains low at T3. The neural cell adhesion molecule (NCAM) level is significantly lower at T3 compared with control. Notably, neuronal damage biomarkers, such as neuro-specific enolase (NSE), total Tau and p-Tau are unchanged at the investigated times in our experimental conditions. Finally, biomarkers IL-1α, IL-2, IL-3, IL-5, ~~IL-12p40~~, IL-12 heterodimeric p70 (IL-12p70), IL-17A, basic fibroblast growth factor (bFGF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) were not detectable at any of the investigated time points in SCI-CSFs or in control CSFs.

We then perfomed a bioinformatic cluster analysis using STRING software (version 10.0; European Molecular Biology Laboratory, Heidelberg, Germany; https://string-db.org/), to explore prevalent molecular pathways activated at T0 (Fig. 2). The software is able to connect query proteins by analyzing protein–protein interactions (PPI) elaborated from different databases, including PubMed queries, generating an interaction net. In order to fit the strength of the protein interactions, the most stringent (minimum) confidence (0.900) was used. All the proteins significantly modified at T0 were used as query proteins (Table 2). Moreover, 10 proteins from the first shell (direct interactions with the query proteins) were added by the software. This analysis revealed that the interactions among the query proteins are statistially significant, and the cluster analysis revealed that the largest cluster has IL-6 and IL-10 as the core proteins. Moreover, these two factors are also interacting with the proteins of the other smaller clusters.

Biological properties of CSF

In order to test if the observed biochemical changes in CSF composition may have an impact on spinal cord tissue and underlying ongoing pathology, we ran cell culture experiments by replacing 10% of the culture media with the CSF from SCI or control subjects. We focused on two of the main pathogenic events occurring in the spinal cord trauma: inflammation and demyelination. Thus, we used the well-established macrophage RAW 264.7 mouse cell line and mouse primary OPC.

The schema of the experiment using RAW 264.7 mouse cell line is presented in Figure 1A. Briefly, at 90% confluence, cells were serum deprived and treated with 10% CSF from healthy control subjects or SCI subjects (time T0) for 4 h. The mRNA expression level of genes encoding for cytokines and other gene markers of inflammation (TNF-α, IFγ, IL-10, IL-1β, inducible nitric oxide synthase [iNOS], cluster of differentiation [CD]40, triggering receptor expressed on myeloid cells [TREM]2, cyclooxigenase-2 [COX]-2), as well as proteins associated with cellular death (c-Fos, c-Jun, B-cell leukemia/lymphoma 2 [Bcl-2], Bcl-2 associated protein [Bax]), were analyzed. For all the investigated genes, values obtained from cells exposed to the CSF from each SCI patient were analyzed considering the group of cells exposed to control CSFs as the ''reference group'' (CNT-CSF = reference). Relative gene expression has been calculated by the $2^{(-\Delta\Delta Ct)}$ method. Significant results are presented in Figure 3. The white bar represents the control reference value, the gray bars represent the individual relative expression of cells exposed to the individual SCI CSFs, and the horizontal red line indicates the mean value among SCI patients. Panels E and F show representative micrographs of the cell system before (E) and after (F) CSF exposure. We observed that the mean value of CD40 and COX-2 mRNA expression level is higher after SCI-CSF exposure compared with control. Moreover, individual samples from SCI patients induce an upregulation of c-Fos, and downregulation of iNOS mRNA expression level. No changes were observed in the other investigated genes (es. IFN-γ, TNF-α, IL-β, IL-6, IL-10, Bax, BCl-2), either as mean or as individual values (data not shown).

We then studied the CSF effect on OPCs, the re-myelinating cell of the mature CNS. Cells cultures obtained from NSC-derived oligospheres prepared according to the proliferation and differentiation protocol illustrated in Figure 1B contain both OPC and astrocytes (in control cultures at days in vitro, DIVs 0: 80% OPCs, 15% of astrocytes, and 5% unidentified cells, data not shown). At 0 DIVs, cells were exposed to 10% CSFs (SCI and controls), and maintained in these differentiation conditions until 12 DIV, when they were processed for MitoTracker staining, immunocytochemistry, and analysis through HCS approach. Results are presented in Figure 4. The white bar indicates the control values, the gray bars represent the individual analysis of SCI patients, and the horizontal red line indicates the mean value among SCI patients. Micrographs of OPCs (platelet-derived growth factor-α receptor [PDGFαR]-positive cells, Fig. 4G) and mature/myelinating OLs (2′,3′-Cyclic-nucleotide 3′-phosphodiesterase [CNPase]/myelin basic protein [MBP]-positive cells, Fig. 4H) are also shown. The number of cells per well was significantly different only for CSF from subject 3 at T0 ($p = 0.0254$; Fig. 4A), whereas CSFs from two different subjects induced a significant increase in the percentage of cells showing mitochondrial depolarization as an early stage of cell distress (subject 5 at T0, $p = 0.0125$; subject 6 at T0, $p = 0.005$; Fig. 4B). No differences were detected for the percentage of cells showing condensed nuclei, corresponding to the last phases of apoptosis (Fig. 4C). We then analyzed the effect of SCI-CSF exposure on the differentiation of NSC-derived OPCs, using specific markers to identify astrocytes (glial fibrillary acidic protein [GFAP]), OPCs (PDGFαR), and myelinating OLs (MBP) at the end of the differentiation phase (12 DIV). The mean number of GFAP-positive cells in all SCI-CSFs exposed cultures is significantly higher than in

Table 2. Concentrations of the Detectable Investigated Cytokines and Chemokines, Growth Factors, Soluble Cell Adhesion Molecules, and Other Biomarkers in Control (CNT) and Spinal Cord Injured Subjects at Time 0 (T0) and 3 Months after Injury (T3), Reported as Mean ± SEM

	CNT (n = *4*)	*T0* (n = *10*)	*T3* (n = *4*)	*CNT vs. T0* p *values*		*T0 vs. T3* p *values*
Cytokines and chemokines						
CTACK	14.12 ± 1.08	23.87 ± 4.02	21.28 ± 4.22	*0.081*		*0.767*
Eotaxin	2.5 ± 0.00	9.42 ± 2.91	2.5 ± 0.00	*0.087*	↓	***0.049***
IFN-a2	4.3 ± 0.00	4.28 ± 0.99	4.99 ± 0.64	*0.733*		*0.453*
IFN-γ	7.28 ± 0.88	24.66 ± 7.76	5.73 ± 0.67	*0.112*	↓	***0.017***
IL-10	0.3 ± 0.00	6.82 ± 3.4	0.3 ± 0.00	↑ ***0.047***	↓	***0.001***
IL-13	28.66 ± 9.21	3.11 ± 0.51	3.48 ± 0.98	↓ ***0.019***		*0.837*
IL-15	1.66 ± 0.74	1.64 ± 0.37	1.87 ± 0.53	*0.668*		*0.449*
IL-16	24.37 ± 1.55	192.1 ± 59.5	33.54 ± 8.17	↑ ***0.002***	↓	***0.008***
IL-18	0.98 ± 0.14	2.98 ± 0.61	1.97 ± 0.5	↑ ***0.004***		*0.289*
IL-1β	0.6 ± 0.00	1.37 ± 0.93	0.6 ± 0.00	*0.695*		*0.604*
IL-1rα	5.5 ± 0.00	60.18 ± 34.03	5.5 ± 0.00	↑ ***0.049***	↓	***0.002***
IL-2rα	12.58 ± 1.46	11.51 ± 1.46	14.59 ± 2.47	*0.733*		*0.453*
IL-4	0.7 ± 0.00	2.23 ± 0.64	0.7 ± 0.00	*0.178*		*0.101*
IL-6	6.14 ± 1.13	5587 ± 3442	8.08 ± 2.66	↑ ***0.014***	↓	***0.0059***
IL-7	9.56 ± 1.29	18.68 ± 4.36	20.73 ± 8.79	*0.364*		*0.758*
IL-8	30.66 ± 1.77	6569 ± 2313	45.83 ± 9.79	↑ ***0.007***	↓	***0.008***
IL-9	2.5 ± 0.00	31.73 ± 10.92	2.5 ± 0.00	↑ ***0.007***	↓	***0.002***
IP-10	3210 ± 1188	5819 ± 1557	2379 ± 811	***0.32***		*0.132*
MCP-1	145.2 ± 43.36	667.5 ± 91.09	257.6 ± 30.8	↑ ***0.014***	↓	***0.069***
MCP-3	1 ± 0.00	3.626 ± 2.27	0.71 ± 0.62	*0.505*		*0.555*
MIP-1α	1.6 ± 0.00	10.97 ± 6.63	0.86 ± 0.98	↑ ***0.049***	↓	***0.004***
MIP-1β	20.99 ± 1.76	352.8 ± 200.4	8.04 ± 0.98	***0.028***	↓	***0.002***
RANTES	1.8 ± 0.00	67.93 ± 38.8	4.34 ± 2.5	↑ ***0.04***		***0.068***
TNF-α	1.64 ± 0.09	7.92 ± 2.48	2.26 ± 0.31	↑ ***0.007***	↓	***0.014***
TRAIL	4.84 ± 1.82	13.15 ± 1.59	9.06 ± 3.07	↑ ***0.008***		*0.412*
Growth and other factors						
BDNF	0.28 ± 0.04	2.71 ± 1.28	0.46 ± 0.16	*0.340*		*0.535*
G-CSF	1.7 ± 0.00	324 ± 230	1.7 ± 0.00	↑ ***0.047***	↓	***0.001***
GRO-α	6.3 ± 0.00	8064 ± 3610	6.3 ± 0.00	↑ ***0.002***	↓	***0.002***
HGF	885.9 ± 147.5	906.8 ± 96.83	828.8 ± 193.2	*0.782*		*0.687*
M-CSF	0.9 ± 0.00	9.63 ± 7.26	0.9 ± 0.00	*0.177*		*0.177*
NGF-β	3.65 ± 0.33	5.42 ± 0.69	6.26 ± 1.22	↑ ***0.02***		*0.608*
PDGF-AA	10.25 ± 0.66	14.98 ± 1.83	10.33 ± 0.23	*0.228*		*0.290*
PDGF-AA/BB	17.01 ± 1.99	25.45 ± 6.3	16.3 ± 1.57	*0.864*		*0.657*
PDGF-BB	2.9 ± 0.00	8.78 ± 5.88	2.9 ± 0.00	*1*		*1*
SCF	29.1 ± 1.071	29.69 ± 3.11	27.01 ± 6.08	*0.132*		*0.539*
SCGF-β	9598 ± 1326	15225 ± 2106	5954 ± 1007	*0.052*	↓	***0.008***
SDF-1α	370.8 ± 17.26	468 ± 40.82	885.9 ± 145.7	*0.132*		*0.539*
VEGF	3.1 ± 0.00	2.85 ± 0.23	3.1 ± 0.00	*1*		*1*
Soluble cell adhesion molecules						
sVCAM	35138 ± 4557	35242 ± 5739	45765 ± 16399	*0.613*		*1*
sICAM	423.5 ± 37.02	436.3 ± 29.57	479.2 ± 66.39	*0.767*		*0.733*
NCAM[a]	57347 ± 1928	42169 ± 4579	45685 ± 3558	↓ *0.076*		*0.733*
Neurological biomarkers						
NSE	93.71 ± 9.52	89.83 ± 12.69	67.99 ± 14.27	*0.733*		*0.412*
p-TAU (nM)	1.37 ± 0.11	1.6 ± 0.54	1.05 ± 0.19	*0.976*		*0.577*
T-TAU	0.72 ± 0.08	0.89 ± 0.4	0.37 ± 0.09	*0.325*		*0.326*
Other biomarkers						
MIG	405.3 ± 193.7	512.3 ± 185.6	620.2 ± 246.5	*0.487*		*0.374*
MIF	1072 ± 408.9	4511 ± 2499	684.1 ± 166.2	*0.24*	↓	***0.036***
LIF	5.5 ± 0.00	25.52 ± 14.01	5.02 ± 0.479	↑ *0.288*	↓	*0.184*
Cathepsin-D	65696 ± 4424	62639 ± 9551	72757 ± 12825	*0.837*		*0.635*
MPO	401.6 ± 43.03	18693 ± 1331	462 ± 61.38	↑ ***0.041***		*0.819*
PAI-1	594.1 ± 106.9	7104 ± 1458	1026 ± 181.0	↑ ***0.002***	↓	***0.008***

The statistical analysis is performed through the non-parametric Mann–Whitney *U* test, by comparing the control versus the lesioned subjects at time 0 (CNT vs. T0), and the lesioned subjects at times 0 and 3 (T0 vs. T3). P values are shown in italic in the two right hand columns. Bold indicates significant differences.

[a]Biomarker showing significant differences when comparing control versus injured subjects at T3 (CNT vs. T3, NCAM, $p = 0.029$).

All abbreviations are described in Supplementary Table ~~ST1~~.

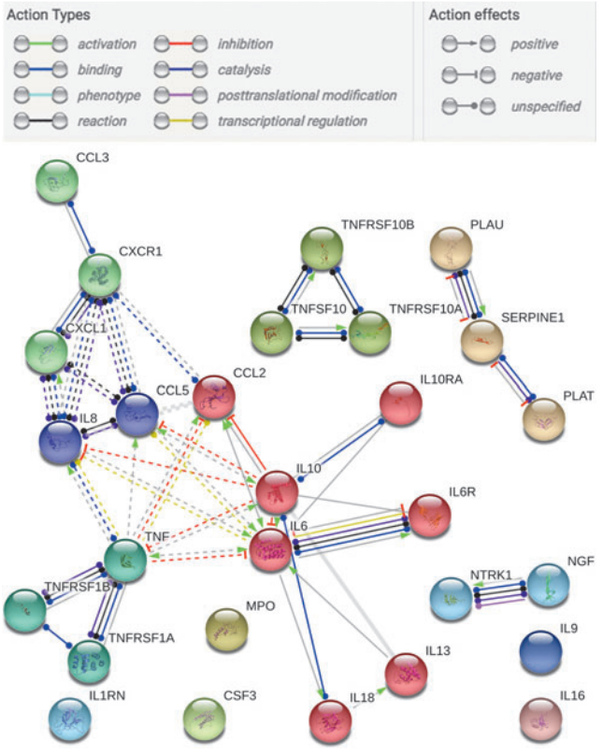

FIG. 2. Results from the STRING analysis of query proteins *plus* 10 proteins in the first shell. Query proteins were chosen as factors modified at T0 in the cerebrospinal fluid (CSF) of spinal cord injury (SCI) patients: interleukin (IL)-10, IL-13, IL-16, IL-18, IL-1rα, IL-6, IL-8, IL-9, monocyte chemoattractant protein (MCP)-1(C-C Motif Chemokine Ligand [CCL]2), macrophage inflammatory protein [MIP]-1α (CCL3), RANTES (CCL5), tumor necrosis factor (TNF)-α, TNF-related apoptosis-inducing ligand (TRAIL), granulocyte-colony stimulating factor (G-CSF), Chemokine (C-X-C motif) ligand 1, Melanoma Growth Stimulating Activity, alpha (GRO-α), nerve growth factor (NGF)*β*, myeloperoxidase enzyme (MPO), and plasminogen activator inhibitor (PAI)-1. Then, 10 proteins directly interacting with the query proteins were added in the interactions map, reaching a total of 28 nodes. Interactions were defined as molecular actions, based on text mining, experiments, and databases, using the highest stringency as minimum confidence (0.900). Different colors represent different clusters, generated by the Markov Cluster Algorithm (MCL) clusterization method (inflation parameter: 3). Statistical analysis: protein–protein interactions (PPI) enrichment *p* value: <1.0e-16.

control-CSF exposed cultures (CNT-CSFs = 48.09 ± 2.171; SCI-CSFs = 55.28 ± 2.033; $p = 0.0298$; Fig. 4D). The effect of SCI-CSF exposure on OPC percentage yielded variable results (SCI-CSF mean = 57.16 ± 7.96), with two CSF samples determining an increase in PDGFαR-positive cell percentage (subject 6 at T0, $p = 0.0090$; subject 7 at T0, $p = 0.0064$), and one subject showing, in an opposite way, a significant decrease (subject 14 at T0, $p = 0.0046$; Fig. 4E). The SCI-CSF exposure had no significant effects on the percentage of myelinating OLs (Fig. 4F). However, the percentage of MBP-positive cells was lower in all the analyzed CSF-exposed groups (CNT- or SCI-CSF), compared with standard control cultures (CNT-CSF, mean = 30.24 ± 5.104; culture, mean = 44.24 ± 7.775).

Finally, in order to correlate bioanalytical and biological data obtained from individual CSF, we performed a correlation analysis between the upregulated analytes IL-6 IL-8, a MCP nd 1, and the biological parameters measured in the cell culture experiments. We observed a negative correlation between IL-8 CSF levels and the percentage of OPC (PDGFαR-positive cells) generated from oligospheres (Fig. 4I).

Discussion

The identification of robust and reliable diagnostic and prognostic biomarkers is recognized as a necessary prerequisite and beneficial strategy for the development of new treatments and to improve patient care in SCI. [11,14–17] In the recent past, several studies have attempted to define potential blood and/or CSF biomarkers.[10,11] Still, obtained results have been poor and inconsistent. One of the reasons for this might be the huge variability among SCI patients, in spite of the stringent inclusion criteria generally used in these studies. In an attempt to improve the predictive value of CSF biomarkers analysis, in this pilot study we combined a multi-parametric approach to study the CSF composition and the biological testing of CSF properties on *in vitro* cell cultures, using different cells types selected according to the pre-clinical studies describing SCI cellular pathology.[16]

The main features in the design of this pilot study are the following: (1) we enrolled patients using very stringent inclusion criteria, in terms of post-trauma time (≤ 24 h), trauma characteristics (cervical and thoracic SCI), and pharmacological treatments (no steroids); (2) we included control CSF obtained from non-pathological subjects; (3) we analyzed a large group of possible biomarkers ($n = 60$), related to inflammation, cell recruitment, myelination, and neurodegeneration, using a high-sensitivity methodology allowing the simultaneous analysis of dozens analytes in a small amount of CSF, with a wide dynamic range; and (4) we performed *in vitro* tests exposing different cell systems to CSF, focusing on the pro-inflammatory potential and toxicity for re-myelinating cells. We selected two ''pivot'' cells: macrophages and OPCs. Peripheral macrophages are in fact the first activated cells after SCI.[18] OPCs are the cells responsible for developmental myelination and myelin repair in cases of lesion during adulthood.

During the acute phase of brain injury and SCI, a chemical burst occurs, including cytokines, chemokines, growth factor, and extracellular matrix proteins, which are regarded as possible biomarkers to predict the functional outcome. Among the 60 investigated cytokines, chemokines, growth factors, and structural biomarkers, we found 19 transiently regulated proteins 24 h after SCI. Within the upregulated proteins, IL-6, IL-8, and MCP-1 have been already proposed as markers for injury severity and as outcome predictors.[19,20] These alterations were also coherent with available pre-clinical and clinical data, indicating that IL-6 is involved in generation and propagation of chronic inflammation, having a negative impact on OPC differentiation and myelination.[9,21] IL-8 (or CXCL8) is produced by multiple cell types including macrophages, monocytes, and endothelial cells. Moreover, OPCs derived from NSCs also produce IL-8 and express the related receptors CXCR1 and CXCR2.[22] IL-8 increases in the CSF of patients with multiple sclerosis (MS), a disease characterized by extensive inflammation and demyelination.[23] MCP-1 is a chemoattractant that regulates migration and infiltration of monocytes/macrophages. It is released by astrocytes, and its expression is upregulated by different CNS insults, including SCI.[19,20,24]

GRO-α (Chemokine C-X-C motif ligand-1, melanoma growth stimulating activity alpha) levels in SCI CSF are actually 3400fold higher than in controls, which is the highest regulation observed in

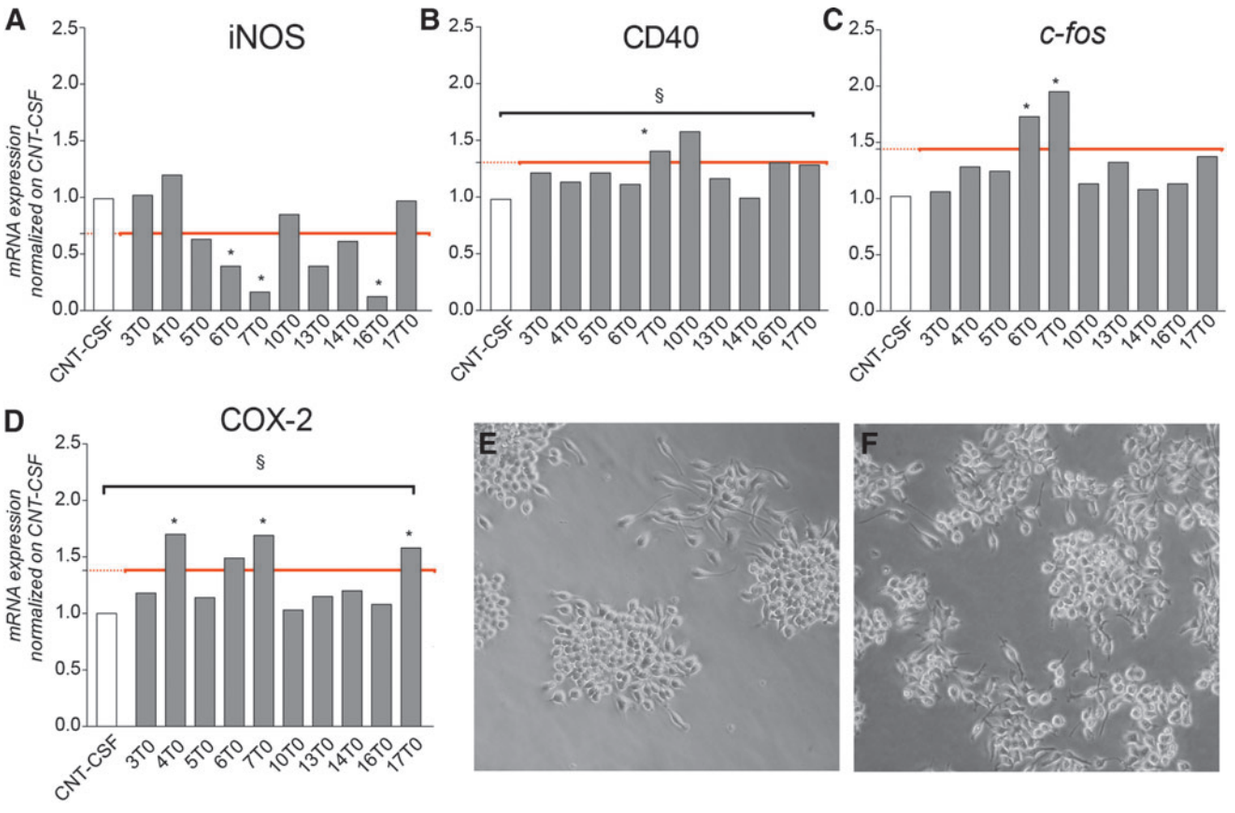

FIG. 3. Effect of cerebrospinal fluid (CSF) exposure on, RAW 264.7 cells. **(A–D)** mRNA expression level of inducible nitric oxide synthase (iNOS) **(A)**, cluster of differentiation (CD) 40 **(B)**, c-*fos* **(C)**, and cyclooxigenase-2 (COX)-2 **(D)**, normalized on the expression level in the control CSF-exposed group (CNT-CSF, white columns). White bar represents the mean of three different replicates of control CSF, gray bars represent mean of single-subject CSF, and the red line shows the mean of all the SCI-CSFs. **(E–F)** Representative micrographs of RAW 264.7 cells before **(E)** and after **(F)** CSF exposure. Statistical analysis: Student's *t* test. Asterisks represent differences between single SCI-CSFs and CNT-CSF ($*p < 0.05$); § represent the difference between the mean value of all SCI-CSFs and CNT-CSF ($\S p < 0.05$).

this study. GRO-α, which is expressed by macrophages, neutrophils and epithelial cells,[24] inhibits OPC migration during spinal cord development; therefore, its increase might negatively impact on OPC-dependent myelin repair. A transient increase in GRO-α has also been described in experimental SCI in rats.[24]

We also identified other potential biomarkers for the acute phase of SCI among cytokines and chemokines, including Eotaxin, INFγ, IL-10, IL-16, IL-18, IL-1rα, IL-9, MCP1, MIP-1α, RANTES, TNFα, and TRAIL, the upregulation of which could be mainly related to ongoing inflammation and cross-talk among the involved cell types.[25] Interestingly, we also observed that the anti-inflammatory cytokine IL-13 decreases at T0, and remains low at T3. This cytokine, when administered via cell therapy, improved functional and histopathological recovery in a SCI rat model.[26]

According to our results, the marker proteins for neuronal damage NSE, total Tau, and p-Tau are not modified at the investigated time points, compared with control CSF. Interestingly, other authors have described that these markers correlate with lesion severity (ASIA Impairment Scale [AIS] grade scale),[13–15] but no control CSF was included in these studies.

Finally, nine analytes were undetectable in all samples. At the moment, we can not state if this is a sensitivity issue or if the indicated analytes are not present in this biological matrix.

The interaction analysis among all modified proteins at T0 and performed by the STRING software, revealed an overall dysregulation of the cytokine interactions, generating small clusters of interacting proteins. In particular, IL-10 and IL-6 are in the center of the full net. These two interleukins are known to be tightly involved in different types of demyelinating diseases, and both participate in the microglia-oligodendrocyte cross-talk and re-myelination process.[27,28]

In this study, we also used a biological approach to analyze CSF properties in *in vitro* systems that mimic two main pathogenic events in early SCI. RAW 264.7 macrophage cell line was used to model inflammation, being the peripheral macrophages' first inflammatory cell recruited at the injury side, also responsible for driving microglia activation.[18,29] To the best of our knowledge, the murine RAW 264.7 cell line is the most used macrophage cell line in the context of inflammation, for anti-inflammatory drug screening, widely used in human-related studies. Moreover, it behaves like a human macrophage cell line (THP-1 human macrophage cell line) when compatively tested in anti-inflammatory drug screening protocols.[30–33] Neural stem cells (NSC) have been used to study the CSF impact on astroglial lineage and on OPC lineage and differentiation. This cells system is particularly suitable for investigating cell plasticity and activation of different cell types,

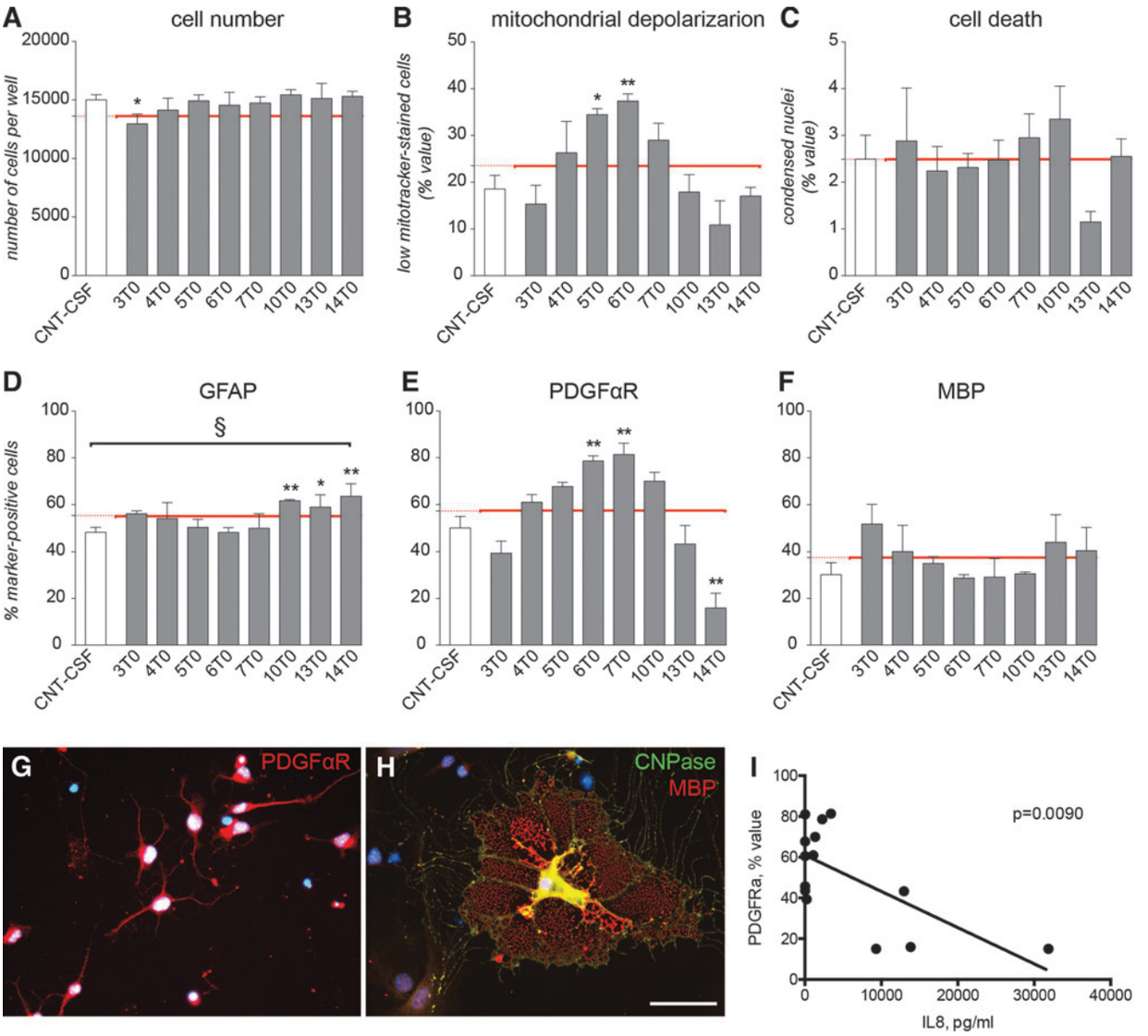

FIG. 4. Effect of cerebrospinal fluid (CSF) exposure on neural precursor-derived cells. **(A–F)** Number of cells per well **(A)**; percentage of cells showing low MitoTracker staining **(B)**; percentage of cells showing condensed nuclei **(C)**; percentage of glial fibrillary acidic protein (GFAP) **(D)**, platelet-derived growth factor-α receptor (PDGFαR)- **(E)**, and myelin basic protein (MBP)- **(F)** positive cells. The white bar represents the mean of three different replicates of control CSFs, gray bars represent the mean of single-subject CSFs, and the red line shows the mean of all the SCI-CSFs. **(G–H)** Representative micrographs of neural stem cell (NSC)-derived oligodendrocyte precursor cell (OPC) cultures, showing PDGFαR- (OPCs; **H**) and CNPase/myelin basic protein (MBP)- (mature/myelinating oligodendrocytes [OLs]; **I**) positive cells. Bar: 20 μm. Statistical analysis: Student's t test. Asterisks represent differences between single SCI-CSFs and CNT-CSF (*$p<0.05$; **$p<0.01$); § represent the difference between the mean value of all SCI-CSFs and CNT-CSF (§$p<0.05$). **(I)** Correlation analysis between interleukin (IL)-8 CSF levels and percentage of PDGFαR-positive OL precursor cells (OPC in control and SCI subjects. Statistical analysis: Pearson's test ($p=0.0090$; $R^2=0.45$).

including astrocytes and OPC, and has been widely used in our laboratory to study the impact of inflammation on OPC differentiation and remyelination, and for drug screening. [9,34–36] All CSF included in the biochemical assay was tested in triplicate for biological properties, and results were presented individually according to the donor and in comparison with control CSF. Although, as expected, a degree of variability among CSF patients was observed, some general trends appear from these experiments. In macrophages, iNOS mRNA expression generally decreases after exposure to pathological CSF, and this decrease was significant in three CSF patients. On the contrary, we observed a trend toward increasing in the mRNA expression level of CD40, a member of the TNF-receptor superfamily, which has been associated with severity and mortality in brain trauma injured patients,[37] and COX-2, an inducible factor in the core of many anti-inflammatory treatment attempts,[38] being significant in one and three cases, respectively. The concurrent increase of COX-2 and CD40 levels has been described in pre-clinical models of gliosis and neurodegeneration,

including experimental SCI, in which an increase in the CD40 expression in reactive glia leading to COX-2-induced cell death has been described.[39]

A remarkable effect of pathological CSF is observed on NSC lineage induction. In particular, a trend to favor astroglial lineage (GFAP-positive cells) by pathological CSF is observed, being significant in three patients. On the contrary, an increased percentage of undifferentiated precursor (PDGFαR-positive) cells, which is not accompanied by full OPC differentiation in mature oligodendrocytes (MBP-positive cells), is observed after exposure to SCI, compared with control CSF. These results support the hypothesis that an inflammatory microenvironment inhibits OPCs' differentiation into myelinating OL, as already suggested by us and others in several studies in animal models of inflammatory/demyelinating diseases.[9,40,41]

In order to correlate CSF composition with possible biological effects, we performed a correlation analysis among bioanalytical (protein analysis) and biological (cellular effects) parameters. Because inflammation impairs re-myelination, and this impacts on the clinical outcome of SCI,[42,43] we tested the correlation between OPC generation from NSC and IL-8 CSF level. In spite of the relatively exiguous number of subjects included in the study and in this analysis, we observed a significant negative correlation between IL-8 CSF level and the percentage of OPCs generated from neurospheres (Pearson correlation analysis). This suggests that the chemical composition of the CSF, and possibly the increased levels of IL-8, reduce the generation of new myelinating oligodendrocytes, thus impairing the re-myelination potential of SCI patients. IL-8 induces NSC death and has been indicated as a potential target to improve myelin repair in MS,[18] the most diffuse inflammatory/demyelinating disease. Notably, CXCR1, one of the IL-8 receptors, has been identified in the core of the protein net by the STRING analysis of the CSF biomarkers, and pre-clinical data in a rat model of SCI indicate that pharmacological inhibition of CXCR1 and CXCR2 chemokine receptors attenuates acute inflammation, preserves gray matter, and diminishes autonomic dysreflexia.[44]

This study has some obvious limitations, starting with the low number of enrolled subjects. Moreover, the limited CSF availablity affects the number of *in vitro* tests, and, therefore, of the cell types included in the study. However, results from this pilot study suggest that diagnostic and prognostic biomarker discovery for SCI may benefit from a translational approach that combines the proteomic analysis of the CSF and related bioinformatic analysis, with cell culture experiments, selected to mirror the sequence of pathogenic events occurring after the SCI.

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Author Disclosure Statement

No competing financial interests exist.

Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Table S1
Supplementary Table S2
Supplementary Table S3
Supplementary Methods

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