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Original Article

Proteomics characterization of extracellular vesicles sorted by flow cytometry reveals a disease-specific molecular cross-talk from cerebrospinal fluid and tears in multiple sclerosis

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ABSTRACT

Several proteomics studies have been conducted to identify new cerebrospinal fluid (CSF) biomarkers in Multiple Sclerosis (MuS). However, the complexity of CSF and its invasive collection, limits its use. Therefore, the goal of biomarker research in MuS is to identify novel distinctive targets in CSF or in easily accessible biofluids. Tears represent an interesting matrix for this purpose, because (1) tears are related to the central nervous system (CNS) and (2) the CNS contains Extracellular Vesicles (EVs) derived from brain cells. These EVs are emerging new biomarkers associated to several neurological disorders. Here we applied an optimized flow cytometer for the identification and subtyping of EVs from CSF and tears. We found, for the first time, microglia-derived and neural-derived EVs in tears. The flow cytometer was used to sort and purify 10^6 EVs from untouched CSF and tears of MuS patients and healthy subjects. Purified EVs were analyzed with shotgun proteomics analysis, revealing that EVs from both CSF and tears of MuS patients conveyed similar proteins. Our data demonstrated a specific EVs-mediated molecular cross talk between CSF and tears, which opens the door to new diagnostic perspectives for MuS.

Data are available via ProteomeXchange with identifier PXD013794.

Significance: Proteomics characterization of released Extracellular Vesicles (EVs) in CSF and tears of Multiple Sclerosis patients represents a pioneering application that helped in recognizing information about the biologically relevant molecules. We found, for the first time, microglia-derived and neural-derived EVs in tears. Moreover, purified EVs revealed that both CSF and tears of Multiple Sclerosis patients conveyed similar proteins involved in inflammation, angiogenesis and immune response signalling. We think that our data will contribute to enhance knowledge in Multiple Sclerosis mechanisms and help in biomarker discovery. Moreover tears represent one of the most convenient body fluid for biomarker discovery in Multiple Sclerosis, since it is an high informative and easy accessible. The opportunity to export such a platform to a territory monitoring plan opens the door to new diagnostic perspectives for Multiple Sclerosis.

1. Introduction

Multiple Sclerosis (MuS) is a multifactorial neurodegenerative disorder characterized by loss of axonal myelin sheets and resulting in increasing disability. MuS diagnosis is based on the assessment of both clinical features and results of the diagnostic work-up, which should include magnetic resonance imaging of the brain, spinal cord and cerebrospinal fluid (CSF) analysis for the search of oligoclonal bands

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(OCBs) [1]. Such a complex phenotypic scenery requires the identification of new robust biomarkers, that will eventually improve the sensitivity and specificity of diagnosis, helping in the clinical decision-making [2]. Several proteomics studies have been conducted to identify new CSF biomarkers in MuS [3,4]. However, the complexity of CSF makes this field of application affected by confounding factors. Furthermore, CSF collection is an invasive procedure that, for obvious ethical reasons, cannot be easily repeated. Therefore, the goal of biomarker research in MuS is to identify novel distinctive targets by studying the aliquots of the sampled CSF and, successively, try to transfer results obtained from CSF into a more easily accessible biological fluid. Tears may represent a candidate biofluid for this purpose, as the eye is an extension of the brain. For example, OCBs were found in tears of patients with MuS [5–7], which indicates that tears contain information about the state of the central nervous system (CNS). Moreover, in patients with clinically isolated syndrome, tear OCBs were detected in 42% of cases, compared with 63% for CSF OCBs. In these studies, all patients with tear OCBs had CSF OCBs, confirming an high specificity of this detection. More recently, “omics” technologies were used to identify molecular alterations in tears. Through proteomics study in tears, elevated levels of alpha-1- antichymotrypsin in MuS patients were found [8], suggesting that this can be a biomarker for MuS. Despite the lively research in the last years, a specific and sensitive MuS biomarker is still lacking. The high dynamic range of protein abundance of biofluids can limits the identification of lower abundant protein biomarkers. Therefore, to focus on proteins contained within Extracellular Vesicles (EVs) can reveal an underappreciated complexity of the circulating proteome [9].

There is evidence that distinct types of brain cells release EVs, functioning as shuttles for the delivery of cargo among different cells [10]. For example, CNS-derived EVs were indicated as emerging new biomarkers for several neurological disorders [11]. Recent evidence shows an increased concentration of EVs in several autoimmune diseases, including MuS [12]. In 2012, Verderio et al. already demonstrated elevated level of myeloid EVs in MuS patients, suggesting a pathogenic role for myeloid EVs in such a disease [13].However, most of the studies of CNS-derived EVs have mainly focused on the EV concentration in CSF [14], with limited attention to their molecular content. EVs are a heterogeneous group of cell-derived membranous structures. The sub-micrometer size and heterogeneity of EVs have complicated their detection and classification. Although these EVs were initially considered waste transporters, we now know that EVs are more than that, and research is now focused on their ability to deliver components among cells and to act as signalling vehicles. In fact, during cell-to-cell transit, EVs protect their luminal contents (lipids, proteins, enzymes, mRNA, miRNA, DNA of the origin cells) from degradation by extracellular proteases. An appealing feature for biomarker recovery is the substantial reduction in the complexity of isolated EVs compared to whole bodily fluids [15]. Therefore, studies for depicting the EVs molecular cargo are emerging, especially through proteomics, metabolomics and miRNomics approaches. However, to obtain reliable omics data and identify EVs-specific functions and biomarkers, implementation of high purification techniques is a prerequisite [16]. Protocols for typical EV omics assays require centrifugation, precipitation and ultracentrifugation steps, thus rely on the analysis of manipulated material. Therefore, such protocols and measurements might not reflect the initial characteristics of the EVs [17–19]. Instead, flow cytometer with sorting capability can be used to identify, enumerate and purify EVs. Here, we have applied a simple, manipulation-free, no-lyse and no-wash procedure in combination with a recently developed polychromatic flow cytometry (PFC) protocol (Patent pending Code: 102018000003981) for absolute volumetric counting, to identify, enumerate, subtype and separate EVs in untouched CSF and tears from MuS patients and healthy subjects. EVs were isolated directly from biofluids through flow cytometry sorting and characterized by proteomics. This method, avoiding sample manipulation, affords unbiased isolation of EVs from whole CSF and tears. We show, for the first time, similar modulated protein cargoes and similar disease-specific biological pathways in EVs purified from both biofluids of MuS subjects, demonstrating a molecular cross talk between CSF and tears that will open the door to new diagnostic perspectives and monitoring plans for MuS.

2. Material and methods

2.1. Ethics statement

The study design was made following the guidelines for the local Ethics Committee that approved the study (n. 18 of 31 October 2013, protocol n. 176, Ethic committee of “G. d'Annunzio” University and ASL N.2 Lanciano-Vasto-Chieti, Italy), and conducted according to Declaration of Helsinki (World Medical Association, 1997). All patients were informed about the procedures and provided written informed consent to participate in the study. In order to protect human subject identity, a number code was employed for specimen identification.

2.2. Patients

MuS patients, in agreement with the 2010 Polman's criteria [1] were included in this study. Clinical diagnosis was confirmed by MRI studies and by the presence of OCBs in CSF. To be enrolled in the study, patients should not have been treated with steroids in the month before study entry and should never have been treated with immunomodulatory or immunosuppressive drugs. Matched Healthy Controls (HCs) are a selection of people who underwent lumbar puncture for suspected CNS infection (resulted negative after the diagnosis). Exclusion criteria for tears collection were ocular surface inflammatory diseases, glaucoma, and use of contact lenses. Exclusion criteria for normal controls were history of systemic or topical therapy, ocular or systemic diseases in the previous 12 months, pregnancy, and use of contact lenses. MuS patients and HCs for CSF and tears were selected to mach age, sex and ethnicity (Caucasian people).

2.3. CSF and tears samples collection

CSF samples, taken by a routine lumbar puncture at L3/L4 or L4/L5 interspace, were always collected in the morning on the first day of patient observation. Each sample (around 3 mL) was centrifuged 200 rcf at 4 °C, for 10 min. 2.5 mL of supernatant was divided into aliquots and snap-frozen at −80 °C. About 200 μL of CSF was used for phenotyping study of EVs through flow cytometry. Pooled CSF was used for methodological assessment of EV purification and for proteomics analysis of isolated CSF EVs.

Tear samples from HCs and MuS patients were collected on graduated Schirmer's strip (EasyOpht, Busto Arsizio, VA, Italy) as previously described [20]. Briefly, tears were collected by gently putting the strip in the lower lid and waiting for 5 min the imbibition of the strip. Then the imbibed strip was placed in a 2.0 mL Eppendorf tube and stored at −80 °C.

2.4. Tears sample extraction from imbibed Shirmer's strip

Imbibed Schirmer's strips were treated as previously reported [21]. Briefly, the Schirmer's strips were cut into 2–3 mm paper pieces and transferred into 0.5 mL microcentrifuge tube (Eppendorf®, Hamburg, Germany), paying attention to wash the required equipment with EtOH before each sample preparation. After adding 200 μL of 0.01 M PBS, each sample was gently mixed. After, the 0.5 mL tubes containing the piece of the Strip in PBS were cut off on the bottom and then put into another 2.0 mL tube and centrifuged. The extracted liquid solutions were transferred into 1.5 mL microcentrifuge tube and used for EV sorting and analysis.

2.5. Flow cytometry analysis of EVs from CSF and tears

In order to identify, phenotype and enumerate EVs, flow cytometry was applied to each enrolled subject. For each sample, 100 μL of CSF or tears were processed by a common flow cytometry no-lyse and no-wash method [22]. Briefly, samples were stained using 1 μL of 0.2 mM lipophilic cationic dye (LCS; stock solution), a Pan-EV dye recently optimized in our laboratories (patent pending, code: 102018000003981), together with a mix of reagents, as detailed in Supplementary Table S1. Samples were incubated at RT for 45 min in the dark; 300 μL of 1 × binding buffer (BD Biosciences) were finally added to each tube and 1×10^6 events/sample were acquired by flow cytometry (FACSVerse, BD - three laser, eight colour configuration), by setting a threshold of 200 a.u. on the channel in which the LCD emits (APC-H channel). Amplifier settings for forward scatter (FSC) and side scatter (SSC) as well as for any fluorescence channel was set in logarithmic mode, and all parameters were visualized with respect to the relative height (H) signal.

Scatter channel calibration was obtained by Rosetta calibration beads (Exometry, Amsterdam, The Nederland) and EV size characteristic have been reported in Result section. Each antibody/reagent was titrated (8 points titration) under assay conditions; dilutions were established based on achieving the highest signal (mean fluorescence intensity, MFI) for the positive population and the lowest signal for the negative population, representing the optimal signal to noise ratio, and stain indexes were calculated [22]. Immune complex formation and unspecific background linked to antibody aggregation was prevented by spinning the antibody stock solution before use, at 21,000 g for 12 min. Instrument performances and data reproducibility were implemented and checked by the Cytometer Setup & Tracking Module (BD Biosciences). In order to evaluate non-specific fluorescence, Fluorescence Minus One (FMO) controls were used [23]. Compensation were assessed using CompBeads (BD) and single stained fluorescent samples. Data were analyzed using FACSDiva v 6.1.3 (BD), FACSuite v 1.0.5 (BD) and FlowJo v 8.8.6 (TreeStar, Ashland, OR, USA) software. EV concentration were obtained by volumetric count.

2.6. Flow cytometry purification of EVs

EVs were separated by instrumental cell sorting (100 μm nozzle, FACSAria III, BD Biosciences), on the basis of their positivity to LCD and negativity to Phalloidin FITC-conjugated (Sigma-Aldrich, Cat. P5282), as shown in Fig. 1. Pure EVs (> 92% of purity) were analyzed for their size using Rosetta Calibration beads and confirmed by Dynamic Light Scattering (DLS).

2.7. Dynamic laser light scattering

Purified EV dimensions have been determined through DLS (90Plus/BI-MAS Zeta Plus, Brookhaven Instruments Corp.). The size of the EVs has been calculated from the translational diffusion coefficient by using the Stokes-Einstein equation [$d = k_BT/3\pi\eta D$, where d is the particle diameter, k_B is the Boltzmann's constant, T is the absolute temperature, η is the fluid viscosity, D is the translational diffusion coefficient]. Data have been analyzed in terms of both scattering intensity and particles number by using either a monomodal or a multimodal size distribution software depending on the specific sample. The polydispersity index has been used as a measure of the homogeneity of the EV population.

2.8. Label free proteomics by liquid chromatography tandem mass spectrometry

One milion of purified EVs from pooled CSF and tears were employed for proteomics investigation. Filter-aided proteome preparation (FASP) tryptic digestion was carried out overnight at 37 °C using trypsin (Promega, Madison, WI). EVs digested proteins from each sample were analyzed in triplicate by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) using a Proxeon EASY-nLCII (Thermo Fisher Scientific, Milan, Italy) chromatographic system coupled to a Maxis HD UHR-TOF (Bruker Daltonics GmbH, Bremen, Germany) mass spectrometer. Peptides were loaded on the EASY-Column C18 trapping column (2 cm L., 100 μm I.D, 5 μm ps, Thermo Fisher Scientific), and subsequently separated on an Acclaim PepMap100 C18 (75 μm I.D., 25 cm L, 5 μm ps, Thermo Fisher Scientific) nano scale chromatographic column. The flow rate was set to 300 nL/min with a total run time of 90 min and the following chromatographic gradient: from 2 to 10% of B in 30′ followed by 10 to 18% in 20′, from 18 to 26% in 10′, from 26 to 50% in 25′ and finally from 50 to 90% in 15′. Mobile phase A was 0.1% formic acid in H2O and mobile phase B was 0.1% formic acid in acetonitrile. The mass spectrometer, typically providing a 60,000 full width at half maximum (FMHW) resolution throughout the mass range, was equipped with a nanoESI spray source. The mass spectrometer was operated in positive ion polarity and Auto MS/MS mode (Data Dependent Acquisition - DDA), using N2 as collision gas for CID fragmentation. Precursors in the range 350 to 2200 *m/z* (excluding 1220.0–1224.5 m/z) with a preferred charge state +2 to +5 (excluding singly charged ions) and absolute intensity above 4706 counts were selected for fragmentation in a maximum cycle time of 3 s. After acquiring one MS/MS spectrum, the precursors were actively excluded from selection for 30 s but reconsidered if the intensity increased > 10 times compared to the previous measured value. Isolation width and collision energy for MS/MS fragmentation were set according to the mass and charge state of the precursor ions (from 3 to 9 Da and from 21 eV to 55 eV). In-source reference lock mass (1221.9906 *m/z*) was acquired online throughout the runs.

2.9. Bioinformatics processing

Proteomics raw data were processed using PEAKS Studio v7.5 software (Bioinformatic Solutions Inc., Waterloo, Canada) using the function 'correct precursor only'. The mass lists were searched against nextprot database (including isoforms as of June 2017; 42,151 entries). Carbamidomethylation of cysteines was selected as fixed modification and oxidation of methionines and deamidation of asparagine and glutamine were set as variable modifications. Nonspecific cleavage to both ends of the peptides were allowed with maximum of 2 missed cleavages. 10 ppm and 0.05 Da were set as the highest error mass tolerances for precursors and fragments, respectively. Quantitative information on the proteins detected was obtained by label free quantification analysis performed using the integrated tool PEAKS-Q, part of the PEAKS Studio suite, by applying 1.5 as fold change ratio.

Protein abundance used for the proteomics assessment of sorted EVs from CSF, was measured as -10LogP/MW, after Peaks data processing [24]. D'Agostino and Pearson omnibus normality test, Student's *t*-test, and Mann Whitney *U* test were performed for data comparisons, using GraphPad Prism v.5 (GraphPad software, Inc. USA).

Functional enrichment and interaction network analysis of proteins was obtained through Functional Enrichment analysis tool (FunRich software) and depicted graphically in the form of Chord diagrams (Circos software).

STRING database was used to highlight Protein Protein Interaction (PPI), including direct (physical) and indirect (functional) associations of identified proteins in each condition.

Protein ratio obtained in each comparison were undergone to "Core Analysis" through Ingenuity Pathway Analysis (IPA) (Qiagen, Hilden, Germany) bioinformatics tool, for functional annotation of proteins, as well as canonical pathway analysis, network discovery, functional analysis and upstream analysis. IPA were also used to identify functionally related genes that correspond to specific canonical pathways that were most significant to the data set. We considered molecules and/or relationships in all species and a confidence setting as high

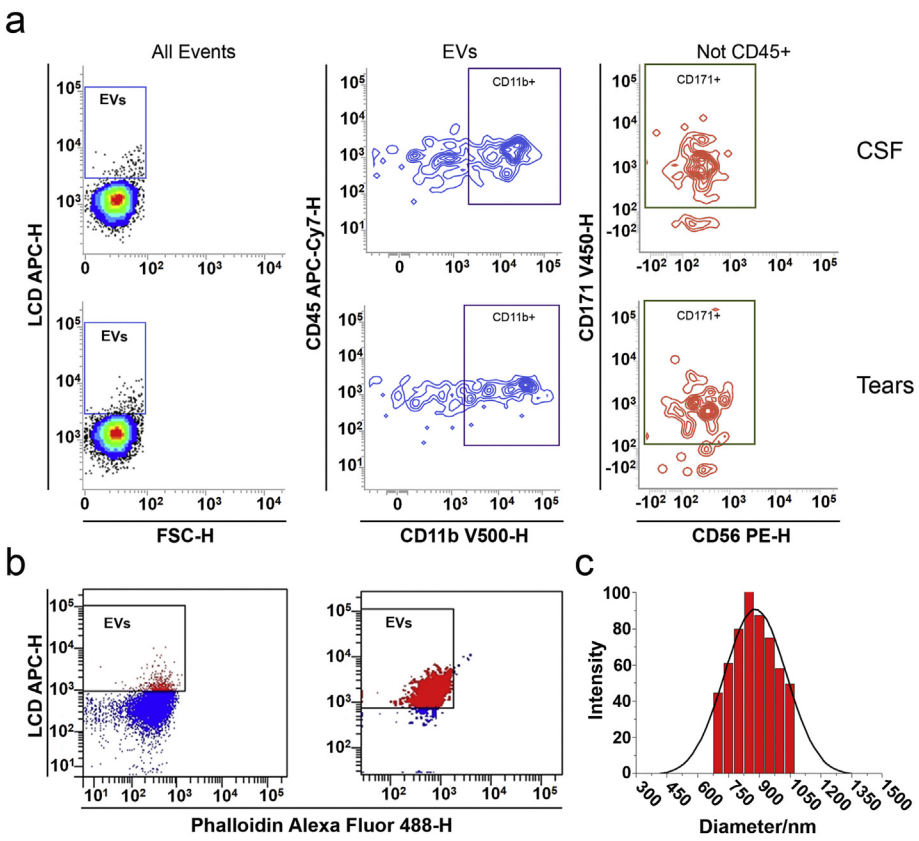

Fig. 1. Gating strategy for EV identification and subtyping. The whole population of events was analyzed for its positivity to LCD-H, and LCD + vesicles were gated and identified as EVs. EVs were then subtyped: microglia-derived EVs (CD11 + events) were gated on the basis of their positivity to CD11b (all CD11b positive events resulted negative for CD45). The gate "NOT-CD45" was obtained and, in such a population, neuronal-derived EVs (CD171 + events) were identified. Data are representative of all analyzed CSF (panel a) and Tears (Panel b) samples. Panel c shows purified EVs re-analyzed by flow cytometry, on the left, and the Gaussian distribution to Dynamic Laser Light Scattering (DLS) on the right.

predicted or experimental observed (excluding medium predicted).

IPA provides, also, the principal disease and function categories resulting from some of the modulated proteins of uploaded dataset.

Upstream regulator analysis by IPA, based on prior knowledge of expected effects between transcriptional regulators and their target genes, was performed [25]. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [26] partner repository with the dataset identifier PXD013794.

3. Results

3.1. Flow cytometry identification and sub-typing of EVs in untouched CSF and tears

To determine the concentration of EV populations, specific markers were added to the biofluids (see methods, patent pending, code: 102018000003981) any without further processing such as centrifugation. All events positive for the Lipophilic Cationic Dye (LCD-H) were gated and identified as EVs (Fig. 1). EVs were then sub-typed following a specific gating strategy. In details, given the fact that peripheral macrophages are CD45 + /CD11b +, events here identified as CD45-/CD11b + represent the microglia-derived compartment. The gate "NOT-CD45" (CD45 negative EVs) was obtained and, in such a population, neuronal-derived EVs (CD171 + events) were identified. Fig. 1a shows representative data of all analyzed CSF samples and tears. The contour plots highlight microglial and neuronal EVs in tear samples, suggesting a connection between CSF and lacrima. Distant fluids, such as urine or blood, show very low number of microglial EVs with respect to tears, as shown in Supplementary Fig. S1. The number of Neuronal EVs is, instead, high also in blood, probably due to the cross contamination of peripheral synthesis.

3.2. EVs purification

An innovative method based on FACS sorting purification of EVs from whole samples of CSF and tears was optimized and used to purify EVs for proteomics studies. Therefore, total and intact EVs in CSF and tears were identified as LCD + /Phalloidin- (Fig. 1 panel b on the left). Sorted LCD + /phalloidin- EVs were re-analyzed by flow cytometry (Fig. 1 panel b on the right) to confirm a high level of purity (> 92%). Size of EVs, was analyzed by Rosetta calibration beads and data show that EVs from tears have a size in the range 200–1490 nm (mean 1059; median 1078; SD 237) while EVs from CSF have a size in the range 333–1326 nm (mean 915; median 929; SD 212). Results were confirmed thought DLS experiment conducted on pure EVs; DLS data showed only one population (Fig. 1 panel c), having a homogenous polydispersity index and a mean diameter of 837 ± 62 nm, referable to the EV compartment. In total, 1.0×10^6 sorted EVs from each CSF and tear sample have been analyzed by proteomics.

3.3. Proteomics assessment of sorted EVs from CSF

Identified proteins from whole CSF were compared to proteins from purified EVs and ultra-centrifuged ones. A CSF pool was divided into three aliquots: whole biofluid, 1.0 x 10^6sorted EVs and an ultra-centrifuged aliquot. Supplementary Table S2 shows the proteins that were identified in all analyzed conditions (n = 3). Identified proteins in each condition were processed by Panther Cellular Component (CC) classification tool and reported as pie chart. Fig. 2a shows the CC classification of identified proteins in the whole biological fluid, highlighting > 40% of proteins reclassified as extracellular region. Fig. 2b shows the CC classification in enriched EVs from ultra-centrifuged CSF, indicating that the extracellular region proteins decrease to 31.9%. Finally, Fig. 2c shows CC classification of proteins obtained by sorting, highlighting the lowest (26.1%) percentage of extracellular region proteins. These data are verified by the results reported in Fig. 2d,

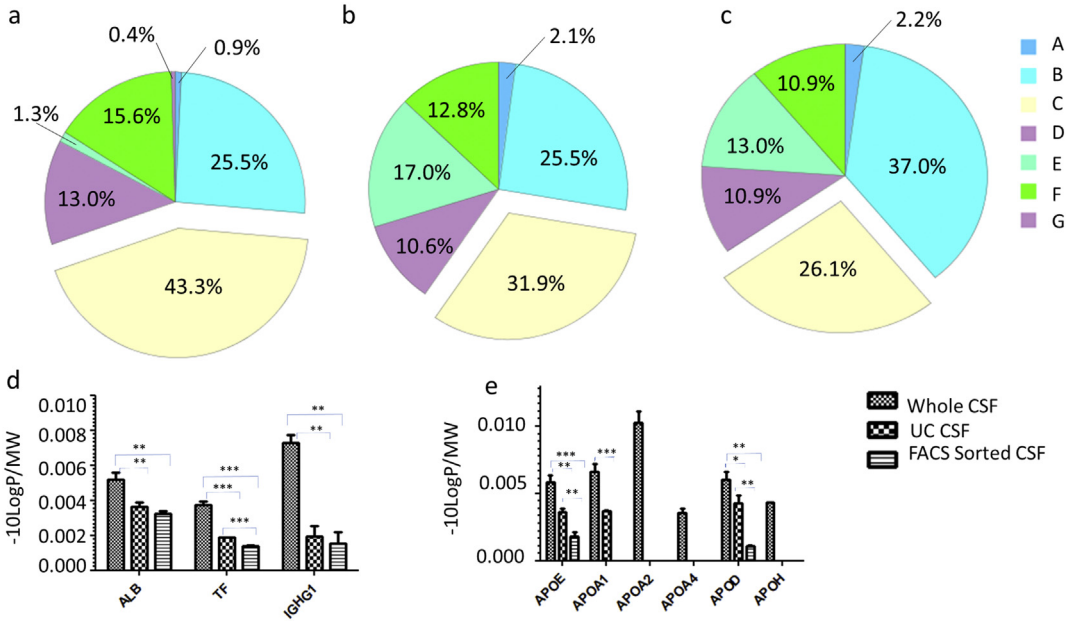

Fig. 2. Identified proteins analysis from each purification strategy used. Pie charts containing the Cellular Component classification of the identified proteins in: a) the whole CSF. b) enriched EVs from ultra-centrifugated CSF. c) purified EVs through FACS cell sorting protocol. d-e The -10logP/MW values for the most abundant CSF proteins identified in each purification strategy used (d: *ALB*, *TF* and *IGHG1*; e: Apolipoproteins).
* pValue < 0.05, ** pValue < 0.01, *** pValue < 0.001.
Cellular Component colour are the following: A: Cell junction; B: Cell; C: Extracellular region; D: Membrane; E: Organelle; F: Protein-containing complex; G: Supramolecular complex.

where the -10logP/MW values for the most abundant proteins (Albumin (*ALB)*, Serotransferrin (*TF*) and Ig gamma-1 immunoglobulin (*IGHG1)*) are shown. Results reported in Fig. 2e, suggest that the sorting method avoid lipoproteins contamination better than ultracentrifugation protocol. Our data confirm the efficiency of FACSorter in isolating EVs directly from the untouched biological fluid and in reducing contamination from circulating abundant proteins and apolipoproteins.

3.4. Comparative proteomics of sorted EVs from CSF and tears of Multiple Sclerosis patient's vs healthy subjects

The workflow in Fig. 3 was used to collect CSF and tears (7 MuS patients, 7 HCs), sort 1.0×10^6 EVs per pooled sample, and apply LC-MS/MS in triplicate.

The list of identified proteins in at least two replicates for each condition were subtracted by the proteins found in the sheath fluid after it passed through the fluidic system of the cell sorter, called “sheath fluid” and reported in Table S3 in supplementary files. After this correction, we identified 101 EV proteins in CSF and 86 EV proteins in tears of MuS patients. Surprisingly, we identified only 17 EV proteins in CSF and 32 EV proteins in tears from HCs.

Fig. 4 shows the proteomics results obtained by functional enrichment protein analysis through FunRich Database tool and depicted as a chord diagram (details are reported in Supplementary Table S4). Fig. 4a shows the classification of EV proteins into Cellular Components (CCs), showing 53 significantly involved terms (Table S4). In particular “Exosomes” is the most covered CC in all physio-pathological conditions and in both biofluids. Other significant CCs for all experimental conditions are “Extracellular” and “Lisosome”; these data are consistent with EV origin and biogenesis. Intriguingly, the “Nucleosome” and “Cytosolic small and large ribosomal subunit” terms, are covered only by tears and CSF from MuS patients (orange and red cords). Moreover, 8 CC terms (orange and blue cords) are specific for tears from both HC and MuS patients, for example “Phagocytic vesicle membrane” and “Eukaryotic translation elongation factor 1 complex”.

Fig. 4b shows an overview of the identified proteins mapping into Biological Pathway (BP) categories, obtained by the same aforementioned approach and depicted as chord diagram. Details and p-values of this classification for each experimental condition are reported in Supplementary Table S5. Data demonstrate that BP are significant quite exclusively in disease condition, for both biofluids: tears and CSF. The identified proteins in HC, in both tears and CSF, are not significantly associated to any specific function, showing thinner portion of the circumference compared to the MuS one (black and blue, respectively). On the contrary, CSF and tears EV proteins from MuS patients show several activated functions, resulting in high number of chords and bigger portion of the circumference compared to HCs (red and orange, respectively). Between the significant BPs in MuS, there are: Integrin signalling events; *PI3K* signalling; *EGF* receptor (*ErbB1*) signalling pathway; *ErbB* receptor signalling network; *IFN* gamma pathway; *LKB1* signalling events; *PDGF* receptor signalling network; RNA Polymerase I Chain Elongation; *S1P1* pathway; Signalling events mediated by *VEGFR1* and *VEGFR2*; *TNF alpha/NF kB*; *TRAIL* signalling pathway. Surprisingly, as reported in the Venn diagram of Supplementary Fig. S2, the activated BPs found in EVs of MuS CSF present an overlapping of 73.1% with the BPs found in EVs of MuS tears. These results suggest that MuS EVs are more functional than HC EVs and that tears reflect the pathophysiological condition of the CSF, through EV protein cargo.

3.5. Functional interaction networks of EV proteins in multiple sclerosis

Identified proteins in each condition were uploaded into STRING software to be processed through Protein-Protein Interaction (PPI) analysis [27]. Proteins identified in CSF EVs of MuS patients shows a PPI enrichment p-value $< 1.0e^{-16}$, indicating that these proteins have more interactions among themselves than what would be expected for a random set of proteins as reported in Fig. 5a. Red dots in the Fig. 5a show as most of the proteins were referable to “extracellular exosome” and “membrane-bounded vesicle”, with FDR of $1.04e^{-29}$ and $5.50e^{-26}$, respectively. Based on the current knowledge on PPI, we identified two clusters of proteins in CSF EVs from MuS patients. The first cluster (green dots) play a role related to “Establishment of localization in cell”

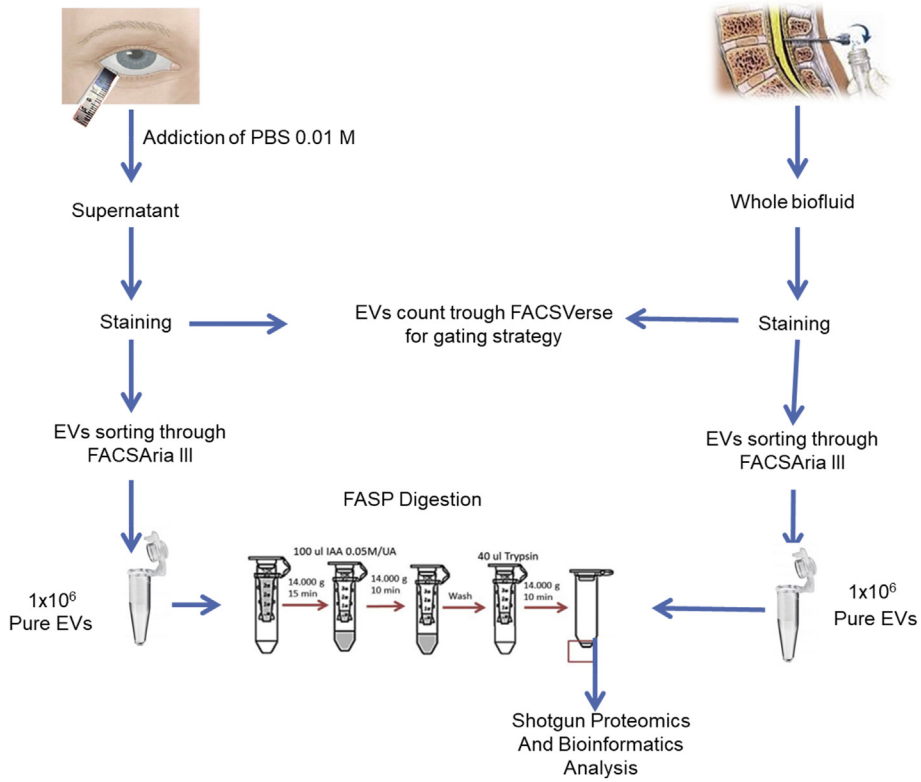

Fig. 3. Workflow scheme of the experimental strategy used for to analyze and purify CSF and tears derived EVs that underwent to shotgun proteomics analysis.

(FDR = $7.11e^{-11}$), a Biological Process which includes any process that localizes a substance or cellular component. The second cluster (blue dots) includes proteins reclassified into "Nucleosome" (FDR = $9.62e^{-17}$), indicating as EVs bring primary packing unit of DNA, probably from the origin cell, but also the outfit of proteins for insert this information into target cells. These clusters are indicative of a pathological condition. Identified proteins in CSF EVs from HC (Fig. 5b) were also referable to "extracellular exosome" (FDR = $4.28e^{-7}$), however, no significant clusters of interaction between them were found. The link between CSF and tears is verified through PPI analysis of EV proteins identified in tears of MuS, showing the same significant network of interaction highlighted in CSF and confirming the absence of such network in HC (Fig. S3 in supplementary materials).

3.6. Upstream analysis

Quantitative proteomics data were obtained through Peaks software and compared by Core Analysis (IPA software) for each EV biofluid. Common activated upstream regulators in MuS CSF and tears were reported in Supplementary Table S6. Between these activated upstream regulators, the most significant are depicted in Fig. 6.

Upstream regulator analysis by IPA is based on prior knowledge of expected effects between transcriptional regulators and their target genes. This analysis, principally based on the input dataset, identifies known regulators and generates direct or indirect relationship between genes. If the observed direction of change is mostly consistent with either activation or inhibition of the transcriptional regulator, IPA system performs a prediction and generates a z score (z scores > 2.0 indicate that a molecule is activated, whereas z scores < −2.0 indicate the inhibition of target molecules) [28].

In particular, Fig. 6a shows the activation of *Transforming Growth Factor-Beta1*(*TGFB1*) gene, (*Z*-score = 3,17 and 3,03 in CSF and tears, respectively); Fig. 6b shows the activation of *Interleukin 4* (*IL4*) gene, *Interleukin 5* (*IL5*), *6* (*IL6*) and *Nuclear Factor* (*eriythroid-derived 2*)-*like2* (*NFE2L2*) genes, demonstrating a complex inflammatory process involving EVs. In Fig. 6c was shown the mechanistic network of activated Hypoxia Inducible Factor 1-alpha subunit (*HIF1A*), with z-score = 2. 40 and 2. 36 for CSF and tears, respectively. Moreover, Fig. 6d showed activated *Angiopoietin 2* (*ANGPT2*) gene with z-score of 3.12 and 2.20 for CSF and tears, respectively. Together with *ANGPT2*, we found activated *Vascular Endothelial Growth Factor A* (*VEGFA*) with high significance in CSF (z-score = 2.22). Between regulated upstream, *Betaestradiol* and progesterone were found significantly activated (Supplementary Fig. S4) in MuS of both CSF and tears fluids. These data demonstrate that EVs are active vehicles in inflammatory, angiogenetic and immunological processes and that this information can be found also in the tears.

4. Discussion

In this study we have applied an optimized PFC method [22] to detect and purify EVs from untouched body fluids, avoiding pre-analytical steps that can activate or damage cells, generate or disrupt EVs, or induce aggregation. In particular we applied such strategy for purifying EVs from CSF and tears of MuS and HC subjects, to further analyze their proteomics fingerprint. Our method resulted in samples with < type > EVs having a purity exceeding 92%. The obtained -10logP/MW values for the most unspecific and abundant proteins significantly decreased in EVs PFC-sorted samples, even with respect to EVs samples enriched by traditional methods (i.e. ultra-centrifugation) [29,30]. By the analysis of untouched CSF and tears we proved, for the first time, the presence of EVs from neuronal and microglial origin in tears. These data confirmed a strong connection between tears and CNS, as already supposed by different authors that found OCBs in tears of MuS patients [7]. Microglial EVs were already identified as important vehicle in MuS, since the increase of their production leads to loss of excitatory synapses, thanks to the role for microglia-enriched miRNAs released in association to EVs and able to silence key synaptic genes [31]. Based on these encouraging data, we studied EVs isolated from

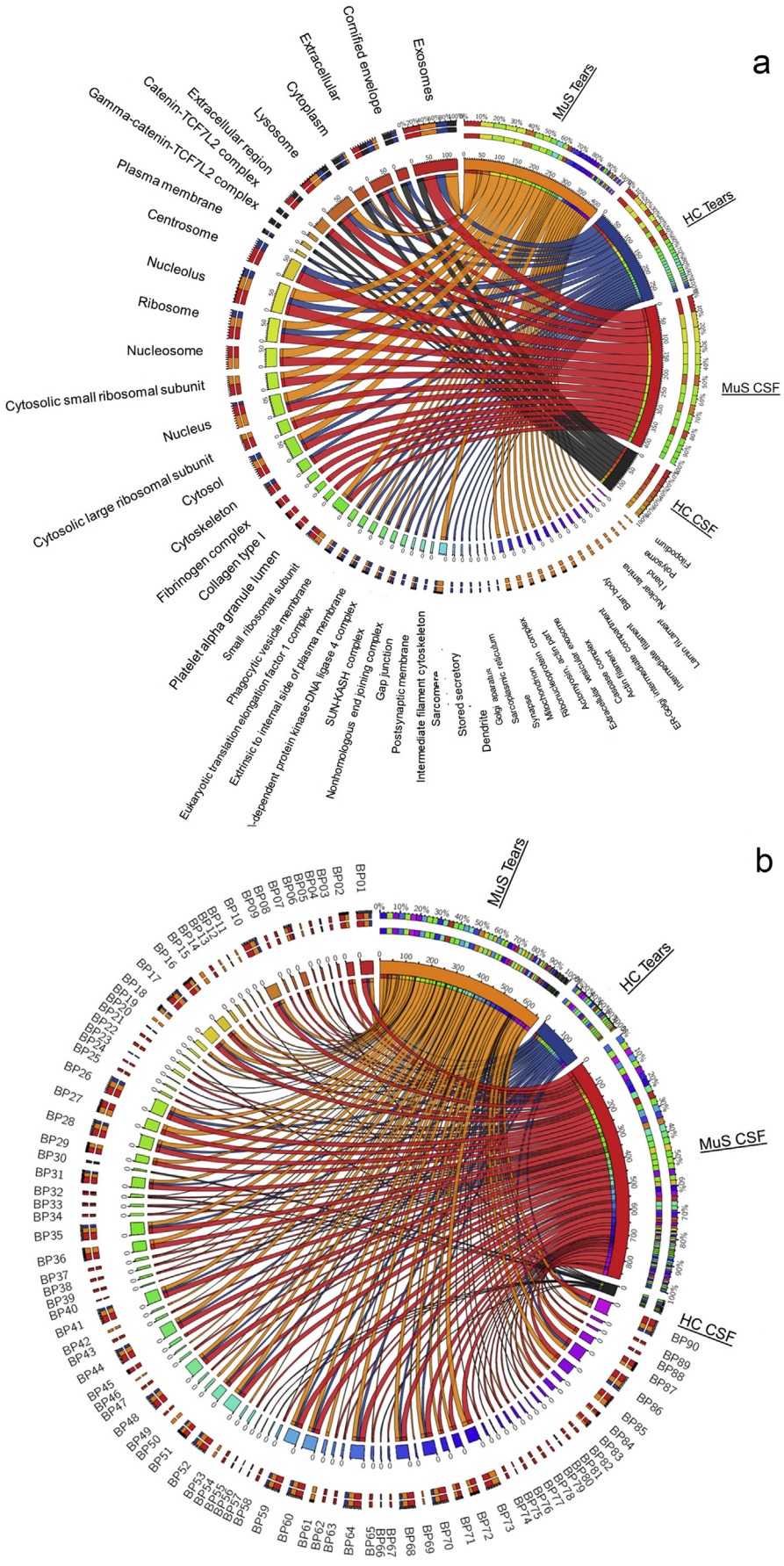

Fig. 4. Chord diagrams (Circos software) reporting in circular layout the proteomics results obtained by functional enrichment protein analysis (FunRich software). MuS CSF and tears data are reported as Red and Orange chords, respectively. HC CSF and tears data are reported as Black and Blue chords, respectively. a Mapping of Cellular Component (CC) categories obtained by the identified proteins in CSF and tears of isolated EVs. b Mapping of Biological Function categories obtained by the identified proteins in CSF and tears of isolated EVs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

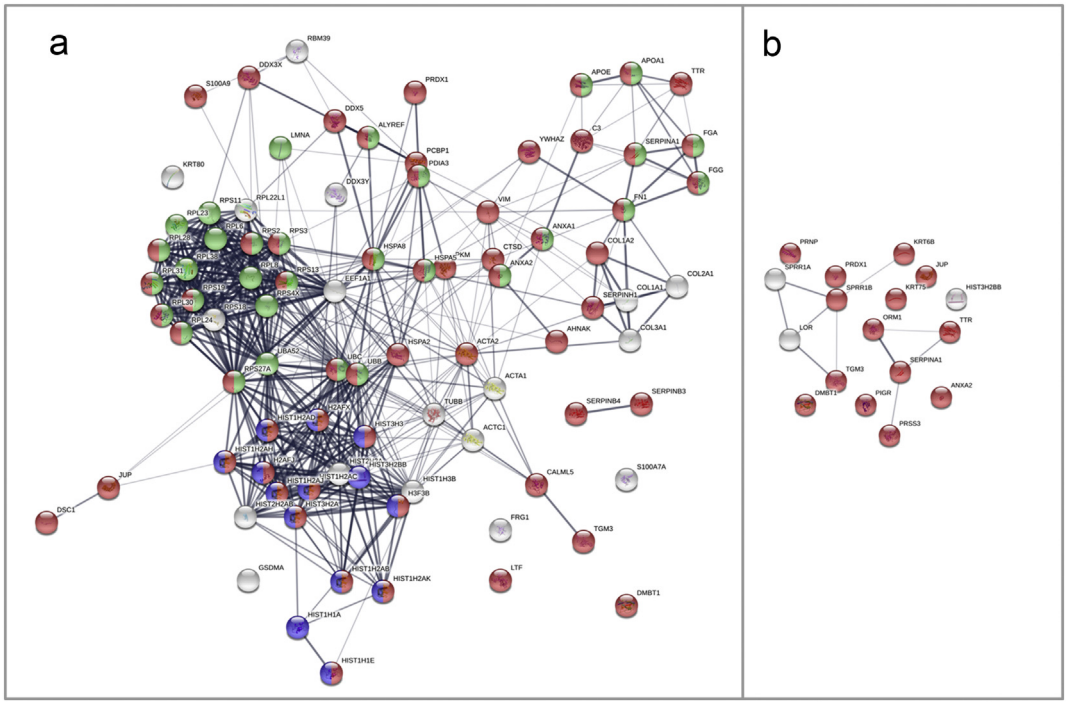

Fig. 5. Network of interaction obtained through STRING analysis of proteins identified in the isolated EVs from CSF. Gene Ontology classification of proteins are reported. Blue dots: Nucleosome; red dots: extracellular exosomes; green dots: Establishment of localization in cell. The semi-colored dots symbolized the proteins which are identified in more than one Gene Ontology classification, and white dots represent proteins not identified with the above mentioned Gene Ontology classification. a Network of interaction obtained by the identified proteins from EVs of MuS patients. b Network of interaction obtained by the identified proteins from EVs of HC. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tears and CSF obtained by MuS patients and HC subjects. One million of EVs per sample were collected through FACS sorter and used for shotgun proteomics analysis. Results highlighted that MuS EVs contain more proteins able to carry specific information toward target cells than HC EVs. Moreover, the isolated EVs from CSF and tears of MuS subjects contain about 70% of the same identified protein cargo, verifying data obtained by PFC analysis of EV phenotypes and confirming a EV-mediated molecular link between CSF and tears. Bioinformatics elaboration of these data showed that the protein cargo identified in pathological EVs are specifically organized in two main nodes of interaction which are “nucleosome” and “establishment of localization in cell”. On the other hand, proteins of EVs from HCs are not organized in any significant network.

Based to our data, we assert that the protein cargo of pathological EVs deliver nuclear information and the outfit of proteins programmed to insert these information into the target cells. Quantitative proteomics data were employed to perform “Core Analysis” through IPA, revealing many upstream genes activated in MuS biofluids with respect to HC ones. Between them, *TGFB1* was the most significant. This gene encodes a secreted ligand of the *TGFB1* superfamily of proteins which regulate cell proliferation, differentiation and growth, and can modulate expression and activation of other growth factors including interferon gamma and tumor necrosis factor alpha. The main molecular function of *TGFB1* is the controlling of the immune system through autocrine and paracrine activity [32]. This evidence may explain the immunosuppressive effects in EAE model of the secretome derived by MuS stem cells [33]. Another important regulated upstream gene resulted by a set of proteins found in MuS EVs is *ANGPT2*, activated in both tears and CSF. This data is consistent with the emerging evidences of the strong angiogenic response in plaques and surrounding white matter described in MuS [34]. In agreement with these results, we found a set of proteins in CSF EVs related to the activation of the *VEGFA* gene, probably due to the constant energy demand for responding to the inflammatory bearings into the CNS. The vascularization and angiogenesis may be turned on through hypoxia-inducible factor 1 (*HIF-1*), another activated upstream in both MuS biofluids and involved in the encoding of *VEGFA*. *Beta-estradiol* and *progesterone* (Supplementary Fig. S4) resulted activated in both biofluids. This result is corroborated considering that MuS has an higher female incidence [35] and that a protective effect during pregnancy is observed [35]. Moreover, both *beta-estradiol* and *ANGPT2* are indirectly activators of EGFR-HIF1A axis (Fig. 6 d and Fig. S4), which are able to modulate angiogenesis and MuS prognosis, since many data correlate neoangiogenesis to disease severity [36]. Furthermore, IPA downstream analysis highlighted that protein EV cargoes deliver an immune message, related to an increase of cell proliferation of T-Lymphocytes and chemotaxis of phagocytes (Supplementary Fig. S5), two crucial mechanisms in MuS adaptive immunity [37].

Despite the need of a more exhaustive investigation not only in MuS patients, our data show, for the first time, the ability of EVs to deliver information from the CNS into a peripheral biofluid, bringing the photo print of CNS immune status [8,38]. The major limitation of this study is pooling of biological samples that may not answer the actual expression of proteins, since does not represent closely the biological average of the individual samples. However, it remains a valid approach in the early stage of biomarkers discovery studies. Moreover, we provided a proteomics EVs study which is suitable for future in-depth functional studies of EVs from different body fluids and for different diseases.

Ethical standards

The authors declare that the experiments comply with the current lows of the country in which they were performed.

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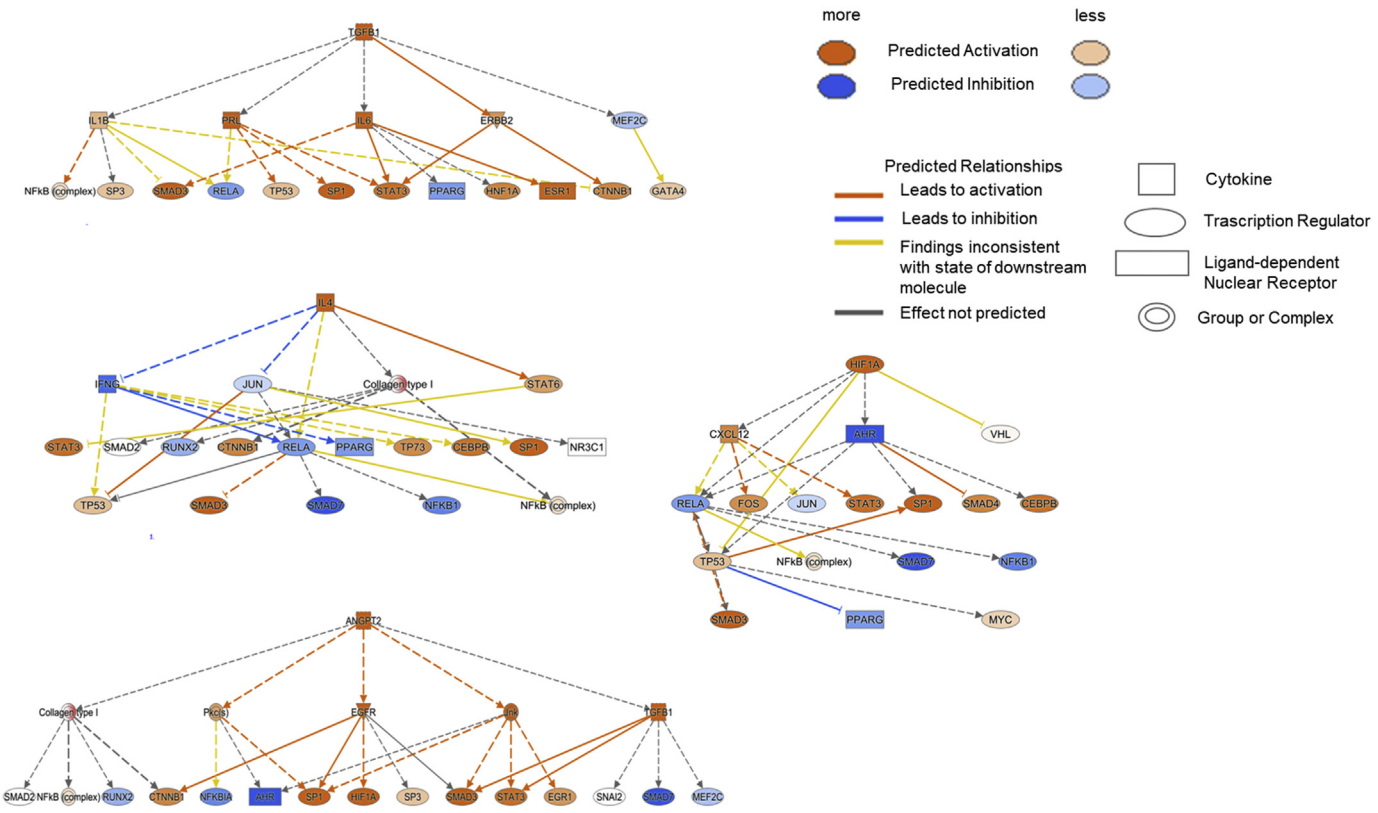

Fig. 6. Upstream regulator analysis, using Ingenuity Pathway software, in MuS EVs from tears and CSF. Orange and blue shapes represent predicted activation or inhibition, respectively. Colour intensity resulted to be directly proportional to the significance of the predicted activation or inhibition. The predicted relationship between genes may leads to direct activation (orange solid lines) or direct inhibition (blue solid lines); indirect relationship are represented by dotted lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Finally, yellow and grey lines means "inconsistent" or "not predicted" relationship effetcs, respectively.
Cytokine are enclosed in square forms, Trascription regulator are coded with the ellipse forms, ligand-dependent Nuclear Receptor are enclosed in the rectangular forms, while Group or Complex are indicated by the double circles.
Activated pathways in both biofluids are reported.
a TGFB1 gene; b IL4 gene; c HIF1A gene; d ANGPT2 gene. Details of colour code are reported in the legend.

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Appendix A. Supplementary data

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