

SUPPLEMENTARY MATERIALS OF:

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

Damiana Pieragostino^{a,b}, Paola Lanuti^{b,c}, Ilaria Cicalini^{b,c}, Maria Concetta Cufaro^{b,d}, Fausta Ciccocioppo^{b,c}, Maurizio Ronci^{a,b}, Pasquale Simeone^{b,c}, Marco Onofri^{e,f}, Edwin van der Pol^g, Antonella Fontana^d, Marco Marchisio^{b,c} and Piero Del Boccio^{b,d,*}

¹Department of Medical, Oral and Biotechnological Sciences, University “G. d’Annunzio” of Chieti-Pescara, Chieti, Italy

²Centre on Aging Sciences and Translational Medicine (Ce.SI-MeT), University “G. d’Annunzio” of Chieti-Pescara, Chieti, Italy.

³Department of Medicine and Aging Sciences, University “G. d’Annunzio” of Chieti-Pescara, Chieti, Italy

⁴Department of Pharmacy, University “G. d’Annunzio” of Chieti-Pescara, Chieti, Italy

⁵Unit of Neurology Ss Annunziata Hospital, Chieti, Italy

⁶Department of Neuroscience, Imaging and Clinical Sciences University “G. d’Annunzio” of Chieti-Pescara, Chieti, Italy.

⁷Biomedical Engineering and Physics, Laboratory Experimental Clinical, Vesicle Observation Center, Amsterdam University Medical Center, University of Amsterdam, Meibergdreef, Amsterdam, The Netherlands.

Keywords: Proteomics; Extracellular vesicles; FACS sorting, CSF; Tears; Multiple sclerosis.

*Corresponding Author:

Prof. Piero Del Boccio, PhD

Department of Pharmacy, University “G. d’Annunzio” of Chieti-Pescara, Chieti, Italy.

e-mail: piero.delboccio@unich.it

Phone: +39 0871 3554516

Fax: +39 0871 541598

Table S1. List of flow cytometry specificities and reagents					
Detection	Fluorochrome	Vendor	Ab Clone	Catalogue Number	Amount per test
Phalloidin	FITC	Sigma-Aldrich	-	P5282	0.2 μ l of stock solution (0.5 mg/ml)
CD171	BV421	BD Biosciences	5G3	565732	1.5 μ l
CD56	PerCP-Cy5.5	BD Biosciences	B159	560842	3 μ l
CD45	APC-H7	BD Biosciences	2D1	560178	2 μ l
Keys: Fluorescein Isothiocyanate (FITC); Brilliant Violet 421 (BV421); Peridinin-chlorophyll protein-Cyanine 5.5 (PerCP-Cy 5.5); Allophycocyanin (APC) -Hilite®7 (H7). Sigma-Aldrich Corp. (St. Louis, MO, USA); Becton Dickinson (BD) Biosciences (San Jose, CA, USA).					

Table S2 caption

Identified proteins in the whole biofluids, in sorted CSF and in ultra-centrifugated CSF. Table reports the Protein ID, Accession, the significance of the identification as $-10\lg P$, the Sequence Coverage as %; the numbers of matched Peptides; the Unique identified peptides; the Post Translational modification (PTM) occurred; the Average Mass and the Description name. Table S2 is reported as supplementary file named Table S2.

Table S3 caption

Identified proteins in the Sheat Fluid and in EVs from each biofluids and in each condition: HC CSF; MuS CSF; HC Tears; MuS Tears. Table reports the Protein ID, Accession, the significance of the identification as $-10\lg P$, the Sequence Coverage as %; the numbers of matched Peptides; the Unique identified peptides; the Post Translational modification (PTM) occurred; the Average Mass and the Description name. Table S3 is reported as supplementary file named Table S3.

Table S4

Significant Cellular Components obtained by FunRich enrichment analysis based on proteomics data of CSF HC, CSF MuS, Tears HC, and Tears MuS. We report the percentage of genes of each dataset involved in each Cellular Component, and the relative p-value at Hypergeometric test.

P-value<0.05 is considered significant.

<i>Table CC</i>		<i>CSF_HC</i>		<i>CSF_MuS</i>		<i>Tears_HC</i>		<i>Tears_MuS</i>	
CC code	CC name	% genes	P-value	% genes	P-value	% genes	P-value	% genes	P-value
CC01	Exosomes	64.70	1.28E-07	65.38	5.27E-41	68.96	1.2E-13	68.81	9.07E-41
CC02	Cornified envelope	17.64	5.51E-07	-	-	6.89	0.0003	-	-
CC03	Extracellular	52.94	7.34E-06	25.00	3.19E-06	37.93	3.75E-05	19.35	0.002
CC04	Cytoplasm	82.35	9.96E-06	-	-	58.62	0.001	39.78	0.022
CC05	Lysosome	41.17	0.0002	29.80	2.39E-10	34.48	7.88E-05	29.03	6.45E-09
CC06	Extracellular region	23.52	0.0005	14.42	1.53E-08	-	-	-	-
CC07	Catenin TCF7L2 complex	5.88	0.0008	-	-	-	-	-	-
CC08	Gamma catenin TCF7L2 complex	5.88	0.0008	-	-	-	-	-	-
CC09	Plasma membrane	41.17	0.02	-	-	34.48	0.026	-	-
CC10	Centrosome	-	-	39.42	4.31E-33	31.03	3.22E-07	-	-
CC11	Nucleolus	-	-	42.30	3.72E-25	37.93	1.03E-06	38.70	2.82E-19
CC12	Ribosome	-	-	14.42	1.68E-15	13.79	6.19E-05	10.75	1.92E-09
CC13	Nucleosome	-	-	8.65	1.68E-12	-	-	9.67	6.01E-13
CC14	Cytosolic small ribosomal subunit	-	-	7.69	1.49E-11	-	-	5.37	7.95E-07
CC15	Nucleus	-	-	59.61	6.26E-10	51.72	0.013	70.96	8.37E-16
CC16	Cytosolic large ribosomal subunit	-	-	6.73	8.09E-10	-	-	3.22	0.0006
CC17	Cytosol	-	-	22.11	5.91E-08	27.58	0.0002	22.58	1.51E-07
CC18	Cytoskeleton	-	-	13.46	7.4E-08	13.79	0.003	-	-
CC19	Fibrinogen complex	-	-	2.88	5.29E-06	-	-	-	-
CC20	Collagen type I	-	-	1.92	2.9E-05	-	-	-	-
CC21	Platelet alpha granule lumen	-	-	3.84	3.72E-05	-	-	-	-
CC22	Small ribosomal subunit	-	-	2.88	4.23E-05	3.44	0.019	3.22	3.02E-05
CC23	Phagocytic vesicle membrane	-	-	-	-	3.44	0.004	1.07	0.014
CC24	Eukaryotic translation elongation factor 1 complex	-	-	-	-	3.44	0.004	1.07	0.014
CC25	Extrinsic to internal side of plasma membrane	-	-	-	-	3.44	0.006	1.07	0.019
CC26	DNA dependent protein kinase DNA ligase 4 complex	-	-	-	-	3.44	0.006	1.07	0.019
CC27	SUN KASH complex	-	-	-	-	3.44	0.007	1.07	0.023
CC28	Nonhomologous end joining complex	-	-	-	-	3.44	0.009	1.07	0.028
CC29	Gap junction	-	-	-	-	3.44	0.010	-	-
CC30	Postsynaptic membrane	-	-	-	-	3.44	0.010	1.07	0.033
CC31	Intermediate filament cytoskeleton	-	-	-	-	3.44	0.016	-	-
CC32	Sarcomere	-	-	-	-	3.44	0.022	3.22	4.78E-

									05
CC33	Apical membrane	-	-	-	-	3.44	0.023	-	-
CC34	Stored secretory granule	-	-	-	-	3.44	0.028	-	-
CC35	Dendrite	-	-	-	-	3.44	0.039	-	-
CC36	Golgi apparatus	-	-	-	-	13.79	0.044	-	-
CC37	Sarcoplasmic reticulum	-	-	-	-	3.44	0.047	-	-
CC38	Synapse	-	-	-	-	3.44	0.048	-	-
CC39	Mitochondrion	-	-	-	-	-	-	15.05	0.002
CC40	Ribonucleoprotein complex	-	-	-	-	-	-	3.22	0.003
CC41	Actomyosin, actin part	-	-	-	-	-	-	1.07	0.004
CC42	Extracellular vesicular exosome	-	-	-	-	-	-	1.07	0.004
CC43	Caspase complex	-	-	-	-	-	-	1.07	0.004
CC44	Actin filament	-	-	-	-	-	-	2.15	0.005
CC45	ER Golgi intermediate compartment	-	-	-	-	-	-	2.15	0.009
CC46	Intermediate filament	-	-	-	-	-	-	2.15	0.013
CC47	Barr body	-	-	-	-	-	-	1.07	0.014
CC48	Laminifilament	-	-	-	-	-	-	1.07	0.014
CC49	Nuclear lamina	-	-	-	-	-	-	1.07	0.019
CC50	I band	-	-	-	-	-	-	1.07	0.028
CC51	Polysome	-	-	-	-	-	-	1.07	0.033
CC52	Filopodium	-	-	-	-	-	-	1.07	0.0473

Table S5: List of significant Biological Pathways and the respective Codes, resulted by FunRich enrichment analysis based on proteomics data of CSF HC, CSF MuS, Tears HC, and Tears MuS. In table were reported the percentage of genes in our dataset, and the p-value at Hypergeometric test. P-value<0.05 is considered significant. Each group of similar Biological Pathways are encoded, as reported in the first column. The most important Biological Pathway (reported as BP code) groups are reported in the Chord Diagram in Figure 5, in which we considered the significant Biological Pathways characterized by more than the 30% of features presents in our datasets.

Table BP		CSF_HC		CSF_MuS		Tears_HC		Tears_MuS	
BP code	BP name	% genes	P-value	% genes	P-value	% genes	P-value	% genes	P-value
BP01	3' UTR mediated translational regulation	-	-	16.34	4.88E-21	10.34	0.0005	10.75	6.30E-11
BP02	Activation of the mRNA upon binding of the cap binding complex and eIFs, and subsequent binding to 43S	-	-	7.69	5.49E-10	-	-	5.37	6.82E-06
BP03	Adaptive Immune System	-	-	6.73	0.0003	-	-	-	-
BP04	Adherens junctions interactions	5.88	0.024	-	-	-	-	-	-
BP05	Alpha9 beta1 integrin signaling events	-	-	14.42	0.0043	-	-	11.83	0.04932
BP06	AP 1 transcription factor network	-	-	-	-	-	-	8.60	1.04E-02
BP07	Apoptosis	-	-	5.77	0.0002	6.90	0.0236	6.45	0.0001
BP08	Arf6 pathway	-	-	-	-	-	-	11.83	0.046
BP09	Beta1 integrin cell surface interactions	-	-	16.35	0.0009	-	-	-	-
BP10	Cap dependent Translation Initiation	-	-	16.35	0.0000	10.34	0.0006	10.75	1.34E-10
BP11	CDC42 signaling events	-	-	-	-	-	-	9.68	0.01
BP12	Cell cell junction organization	5.88	0.039	-	-	-	-	-	-
BP13	Cell Cycle, Mitotic	-	-	-	-	-	-	6.45	0.004
BP14	Class I PI3K signaling events	-	-	14.42	0.0038	-	-	11.83	0.046
BP15	Destabilization of mRNA by AUF1 (hnRNP D0)	-	-	-	-	-	-	5.38	2.4E-05
BP16	Developmental Biology	-	-	16.35	1.58E-10	10.34	0.0267	12.90	1.14E-06
BP17	Diabetes pathways	-	-	18.27	5.31E-18	10.34	0.0041	12.90	5.57E-10
BP18	DNA Repair	-	-	-	-	6.90	0.0111	6.45	1.26E-05
BP19	DNA Replication	-	-	-	-	-	-	6.45	0.002
BP20	E cadherin signaling events	11.76	0.025	-	-	-	-	-	-
	E cadherin signaling in keratinocytes	5.88	0.018	-	-	-	-	-	-
	E cadherin signaling in the nascent adherens junction	11.76	0.024	-	-	-	-	-	-
BP21	EGF receptor (ErbB1) signaling pathway	-	-	14.42	0.004	-	-	11.83	0.046
	EGFR dependent Endothelin signaling events	-	-	14.42	0.004	-	-	-	-
BP22	Endothelins	-	-	15.38	0.002	-	-	11.83	0.050
BP23	Epithelial to mesenchymal transition	-	-	-	-	6.90	0.0315	-	-
BP24	ErbB receptor signaling network	-	-	14.42	0.004	-	-	-	-
BP25	ErbB1 downstream signaling	-	-	14.42	0.004	-	-	11.83	0.046
BP26	Eukaryotic Translation Elongation	-	-	17.31	3.01E-24	13.79	0.0000	11.83	2.93E-13
	Eukaryotic Translation Initiation	-	-	16.35	1.9E-20	10.34	0.0006	10.75	1.34E-10
	Eukaryotic Translation Termination	-	-	16.35	8.97E-23	10.34	0.0003	10.75	6.85E-12
BP27	Factors involved in megakaryocyte development and platelet production	-	-	13.46	2.75E-15	-	-	15.05	5.42E-16
BP28	Formation of a pool of free 40S subunits	-	-	16.35	7.48E-22	10.34	0.0004	10.75	2.22E-11
BP29	Formation of the ternary complex, and subsequently, the 43S complex	-	-	7.69	1.47E-10	-	-	5.38	3.1E-06
	FOXA2 and FOXA3 transcription factor networks	5.88	0.037	-	-	-	-	-	-

BP31	Gene Expression	-	-	19.23	1.27E-14	13.79	0.0024	11.83	2.11E-06
BP32	Glypican 1 network	23.53	0.024	14.42	0.004	-	-	11.83	0.048
BP33	Glypican pathway	23.53	0.027	14.42	0.005	-	-	-	-
BP34	GMCSF mediated signaling events	-	-	14.42	0.003893	-	-	11.83	0.046
BP35	GTP hydrolysis and joining of the 60S ribosomal subunit	-	-	16.35	5.81E-21	10.34	0.0005	10.75	6.95E-11
BP36	Hemostasis	-	-	21.15	5.84E-17	-	-	17.20	3.09E-11
BP37	IFN gamma pathway	-	-	14.42	0.004007	-	-	11.83	0.047
BP38	IGF1 pathway	-	-	14.42	0.004	-	-	11.83	0.046
BP39	IL3 mediated signaling events	-	-	14.42	0.003978	-	-	11.83	0.046
BP40	Immune System	-	-	6.73	0.023	-	-	-	-
BP41	Influenza Infection	-	-	16.35	1.34E-18	10.34	0.0012	11.83	7.5E-11
	Influenza Life Cycle	-	-	16.35	7.12E-19	10.34	0.0011	11.83	5.05E-11
	Influenza Viral RNA Transcription and Replication	-	-	16.35	2.36E-21	10.34	0.0004	11.83	1.46E-12
BP42	Innate Immune System	-	-	-	-	-	-	5.38	0.0020
BP43	Insulin Pathway	-	-	14.42	0.004	-	-	11.83	0.046
BP44	Insulin Synthesis and Processing	-	-	16.35	4.8E-19	10.34	0.0010	10.75	8.2E-10
<i>Table BP</i>		<i>CSF_HC</i>		<i>CSF_MuS</i>		<i>Tears_HC</i>		<i>Tears_MuS</i>	
BP code	BP name	% genes	P-value	% genes	BP code	BP name	% genes	P-value	% genes
BP45	Integrin cell surface interactions	-	-	5.77	2.53E-06	-	-	-	-
	Integrin family cell surface interactions	-	-	17.31	0.0004	-	-	-	-
BP46	Integrin linked kinase signaling	-	-	-	-	-	-	8.60	0.014
BP47	Internalization of ErbB1	-	-	14.42	0.004	-	-	11.83	0.046
BP48	L13a mediated translational silencing of Ceruloplasmin expression	-	-	16.35	0.000	10.34	0.0005	10.75	6.30E-11
BP49	LKB1 signaling events	-	-	14.42	0.004	-	-	11.83	0.050
BP50	Membrane Trafficking	-	-	-	-	-	-	5.38	5.42E-05
BP51	Metabolism	-	-	20.19	2.5E-09	17.24	0.0072	16.13	9.02E-06
BP52	Metabolism of mRNA	-	-	20.19	5.22E-20	13.79	0.0004	13.98	1.01E-10
	Metabolism of proteins	-	-	18.27	1.6E-16	13.79	0.0006	11.83	4.92E-08
	Metabolism of RNA	-	-	20.19	2.35E-18	13.79	0.0008	13.98	9.61E-10
BP53	Mitotic M M/G1 phases	-	-	-	-	-	-	6.45	0.001
BP54	mTORsignaling pathway	-	-	14.42	0.004	-	-	11.83	0.046
BP55	N cadherin signaling events	11.76	0.020	-	-	-	-	-	-
BP56	NCAM signaling for neurite out growth	5.88	0.049	-	-	-	-	-	-
BP57	NCAM1 interactions	5.88	0.020	-	-	-	-	-	-
BP58	Nectin adhesion pathway	-	-	14.42	0.004	-	-	11.83	0.047
BP59	Nonsense Mediated Decay	-	-	16.35	6.92E-21	10.34	0.0005	10.75	7.66E-11
BP60	Nonsense Mediated Decay Independent of the Exon Junction Complex	-	-	16.35	2.68E-22	10.34	0.0003	10.75	1.26E-11
BP61	Nucleotide binding domain, leucine rich repeat containing receptor (NLR) signaling pathways	-	-	-	-	-	-	5.38	1.98E-06
BP62	PAR1 mediated thrombin signaling events	-	-	14.42	0.004094	-	-	11.83	0.048
BP63	PDGF receptor signaling network	-	-	14.42	0.004	-	-	11.83	0.047
	PDGFR beta signaling pathway	-	-	14.42	0.003782	-	-	-	-
BP64	Peptide chain elongation	-	-	17.31	1.48E-24	13.79	0.0000	11.83	1.95E-13
BP65	Plasma membrane estrogen receptor signaling	-	-	14.42	0.004153	-	-	12.90	0.022
BP66	Posttranslational regulation of adherens junction stability and disassembly	11.76	0.017	-	-	-	-	-	-
BP67	Proteoglycan syndecan mediated signaling events	-	-	14.42	0.005637	-	-	-	-
BP68	Regulation of beta cell development	-	-	16.35	1.36E-20	10.34	0.0006	10.75	1.12E-10
BP69	Regulation of CDC42 activity	-	-	-	-	-	-	9.68	0.012
BP70	Regulation of gene expression in beta cells	-	-	16.35	2.36E-21	10.34	0.0004	10.75	4.21E-11
BP71	Regulation of mRNA Stability by Proteins that Bind AU rich Elements	-	-	5.77	2.14E-05	-	-	5.38	1.50E-04

BP72	Ribosomal scanning and start codon recognition	-	-	7.69	4.72E-10	-	-	5.38	6.22E-06
BP73	RNA Polymerase I Chain Elongation	-	-	13.46	3.06E-21	-	-	15.05	5.81E-22
	RNA Polymerase I Promoter Clearance	-	-	13.46	2.01E-20	-	-	15.05	3.82E-21
	RNA Polymerase I Promoter Opening	-	-	13.46	1.81E-24	-	-	15.05	3.41E-25
	RNA Polymerase I Transcription	-	-	13.46	3.56E-20	-	-	15.05	6.79E-21
	RNA Polymerase I, RNA Polymerase III, and Mitochondrial Transcription	-	-	13.46	6.59E-17	-	-	15.05	1.28E-17
BP74	SIP1 pathway	-	-	14.42	0.0038	-	-	11.83	0.046
BP75	Signal Transduction	-	-	11.54	0.0299	-	-	-	-
BP76	Signaling events mediated by focal adhesion kinase	-	-	14.42	0.0038	-	-	11.83	0.046
BP77	Signaling events mediated by Hepatocyte Growth Factor Receptor (c Met)	-	-	14.42	0.0039	-	-	11.83	0.047
BP78	Signaling events mediated by VEGFR1 and VEGFR2	-	-	14.42	0.0040	-	-	12.90	0.022
BP79	Sphingosine 1 phosphate (S1P) pathway	-	-	14.42	0.0045	-	-	-	-
BP80	Stabilization and expansion of the E cadherin adherens junction	11.764	0.024	-	-	-	-	-	-
BP81	Syndecan 1 mediated signaling events	-	-	14.42	0.0041	-	-	11.83	0.048
BP82	Thrombin/protease activated receptor (PAR) pathway	-	-	14.42	0.0041	-	-	11.83	0.048
BP83	TNF alpha/NF kB	-	-	7.69	0.0000	-	-	5.38	0.001
BP84	TRAIL signaling pathway	-	-	16.35	0.0007	-	-	15.05	0.004
BP85	Transcription	-	-	14.42	4.03E-14	-	-	15.05	1.59E-13
BP86	Translation	-	-	17.31	1.44E-21	13.79	0.00003	11.83	1.05E-11
BP87	Translation initiation complex formation	-	-	7.69	4.72E-10	-	-	5.38	6.22E-06
BP88	Urokinase type plasminogen activator (uPA) and uPAR mediated signaling	-	-	14.42	0.003782	-	-	11.83	0.046
BP89	VEGF and VEGFR signaling network	-	-	14.42	0.004	-	-	12.90	0.023
BP90	Viral mRNA Translation	-	-	16.35	8.97E-23	10.34	0.0003	10.75	6.85E-12

Table S6 Common upstream regulators in MuS EVs in CSF and Tears. Data are obtained through Ingenuity Pathways analysis (IPA) after Comparison of the Core Analyses. Z-scores of the activated upstream are reported for both biofluids.

Upstream regulators	EVs CSF	EVs Tears
TGFB1	3.18	3.03
ANGPT2	3.12	2.20
NFE2L2	3.00	2.37
IL4	2.99	1.72
cisplatin	2.97	2.75
hydrogen peroxide	2.74	0.89
EGFR	2.72	2.34
OSM	2.66	0.85
indomethacin	2.65	1.96
PRL	2.63	1.69
IGF1	2.60	1.04

beta-estradiol	2.44	2.52
SMARCA4	2.43	1.98
HIF1A	2.40	2.36
progesterone	2.38	1.97
IL6	2.25	1.48
Jnk	2.21	1.98
IL5	2.20	2.38
MYCN	2.11	2.54
1,2-dithiol-3-thione	2.00	2.23
ESR1	2.00	0.82
inosine	1.98	1.96
ouabain	1.95	2.19
CD38	1.84	2.21
TCR	1.80	1.91
D-glucose	1.75	2.61
cyclosporin A	1.62	0.18
doxorubicin	1.62	0.88
STAT3	1.56	0.37
methapyrilene	1.41	1.00
Insulin	1.38	1.97
gentamicin	1.34	1.34
phorbol myristate acetate	1.21	0.93
CTNNB1	1.10	0.25
lipopolysaccharide	1.07	0.81
acetaminophen	1.00	1.00
FOS	0.96	1.98
SRF	0.95	2.37
AR	0.69	0.37
MKL1	0.62	1.95
KRAS	0.58	1.98
IL1B	0.57	0.86
tanespimycin	0.55	1.98
nitrofurantoin	0.54	1.34
GATA4	0.42	1.61
MYC	0.33	2.11
camptothecin	0.23	0.69
tretinoin	0.22	0.43
KLF4	0.16	1.41

Figure S1

EVs derived from Microglial (Panel a) and neuronal (Panel b) cells in CSF, Tears, blood and urine from HCs.

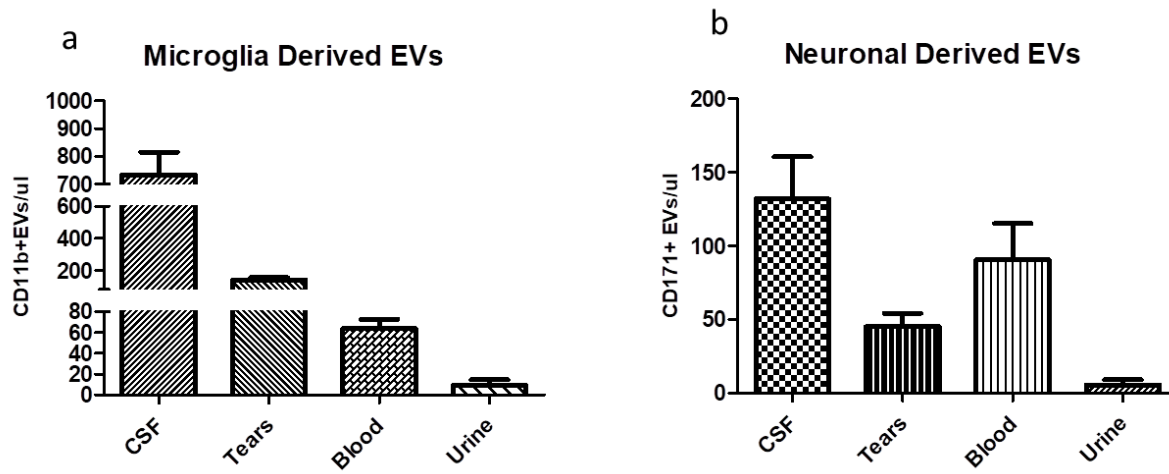


Figure S2

Venn diagram of the activated BPs found in EVs from MuS CSF and MuS Tears, showing 73.1% overlapping between biofluids.

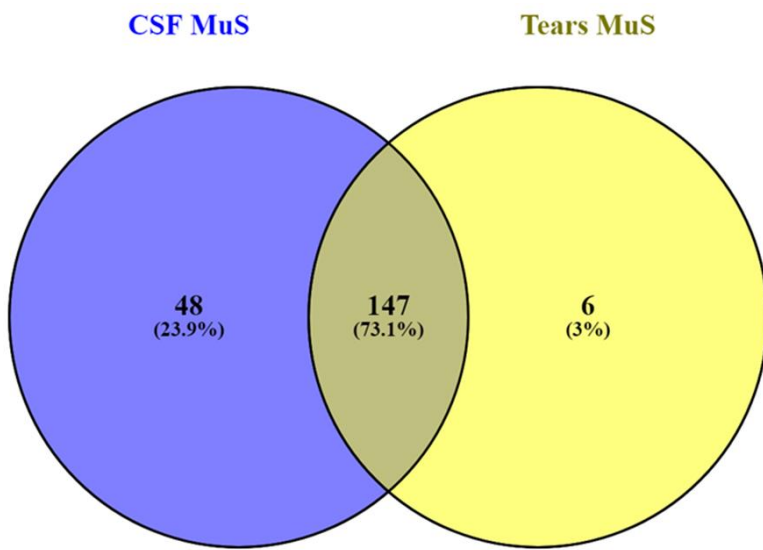


Figure S3: network generated by the proteins expressed in Tear EVs of MuS patients (panel a). Red dots show proteins referable to “extracellular exosome” and “membrane-bounded vesicle”. The first node (with green edges) includes several proteins related to “Establishment of localization in cell”. The second node (with blue edges) includes proteins reclassified into “Nucleosome”. Identified proteins in Tear EVs from HC are reported in panel b, with the same color code of panel a.

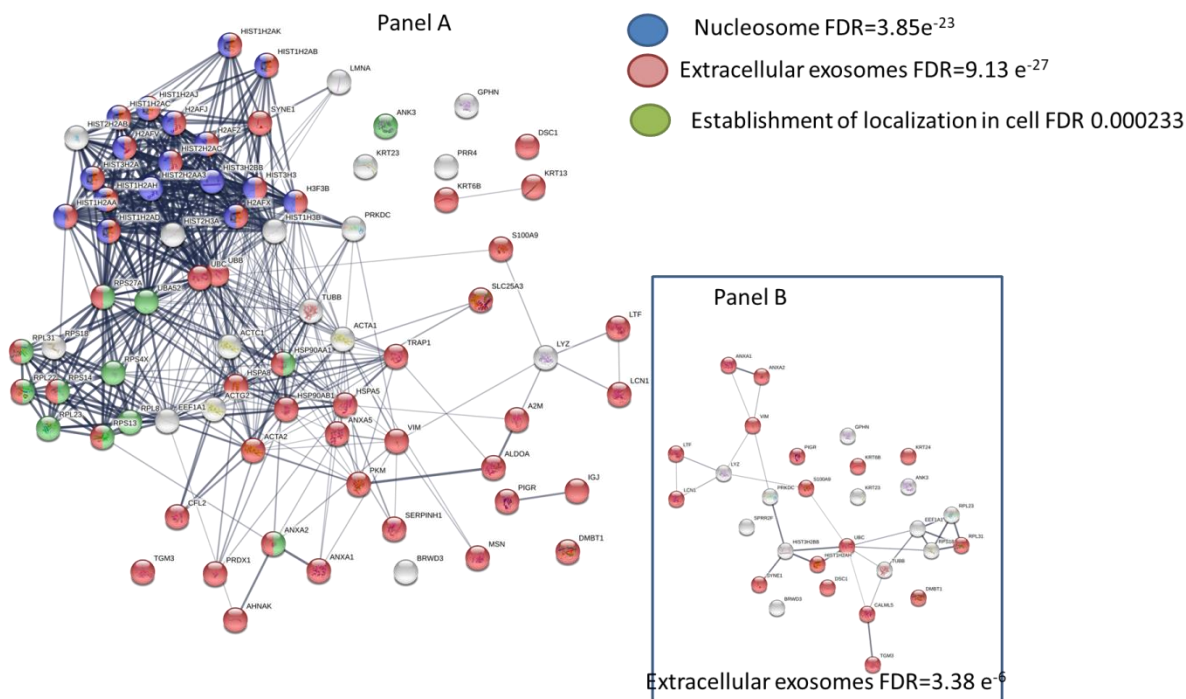
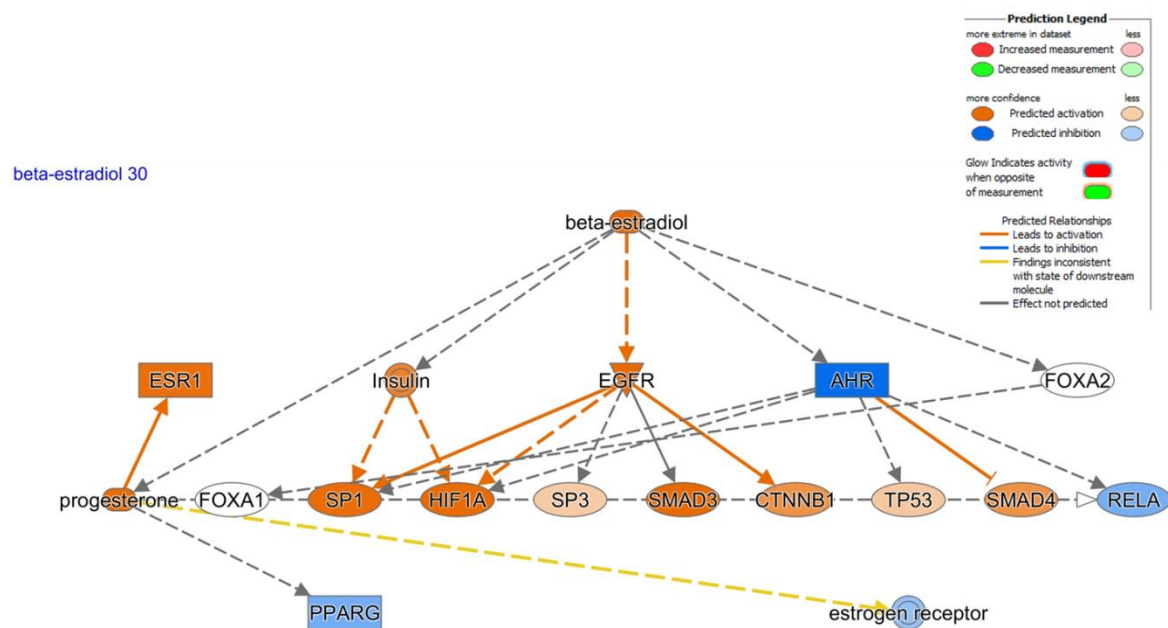


Figure S4: Upstream regulator analysis, using Ingenuity Pathway software, in MuS EVs from tears and CSF. Activated Beta estradiol and progesterone pathways is reported. Details of color code are reported in the legend.



© 2000-2018 QIAGEN. All rights reserved.

Figure S5: IPA downstream analysis highlights activated immune status related to an increase of cell proliferation of T-Lymphocytes and chemotaxis of phagocytes. Details of color code are reported in the legend.

