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Brain and immune system-derived extracellular vesicles mediate regulation of complement system, extracellular matrix remodeling, brain repair and antigen tolerance in Multiple sclerosis

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ABSTRACT

Background: Multiple sclerosis (MS) is an immune-mediated central nervous system disease whose course is unpredictable. Finding biomarkers that help to better comprehend the disease's pathogenesis is crucial for supporting clinical decision-making. Blood extracellular vesicles (EVs) are membrane-bound particles secreted by all cell types that contain information on the disease's pathological processes.

Purpose: To identify the immune and nervous system-derived EV profile from blood that could have a specific role as biomarker in MS and assess its possible correlation with disease state.

Results: Higher levels of T cell-derived EVs and smaller size of neuron-derived EVs were associated with clinical relapse. The smaller size of the oligodendrocyte-derived EVs was related with motor and cognitive impairment. The proteomic analysis identified mannose-binding lectin serine protease 1 and complement factor H from immune system cell-derived EVs as autoimmune disease-associated proteins. We observed hepatocyte growth factor-like protein in EVs from T cells and inter-alpha-trypsin inhibitor heavy chain 2 from neurons as white matter injury-related proteins. In patients with MS, a specific protein profile was found in the EVs, higher levels of alpha-1-microglobulin and fibrinogen β chain, lower levels of C1S and gelsolin in the immune system-released vesicles, and Talin-1 overexpression in oligodendrocyte EVs. These specific MS-associated proteins, as well as myelin basic protein in oligodendrocyte EVs, correlated with disease activity in the patients with MS.

Conclusion: Neural-derived and immune-derived EVs found in blood appear to be good specific biomarkers in MS for reflecting the disease state.

1. Introduction

Multiple sclerosis (MS) is a disabling immune-mediated disease affecting approximately 2.8 million people worldwide (Bierhansl et al., 2022), in which T cells escape tolerance to not well-characterized myelin proteins of the central nervous system (CNS). This response of

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self-reactive T cells against myelin proteins triggers B cells to produce antibodies, resulting in astrocyte activation, microglia reactions, extracellular matrix degradation and apoptosis of oligodendrocytes, leading to a demyelinating condition of the CNS and, consequently, axonal loss and neuronal death (Friese et al., 2014). Although this explanation is the most widely accepted for MS pathogenesis in the scientific community, it remains unknown why the MS course in each individual patient is unpredictable, which complicates decision-making in patient management. This heterogeneity in the disease course might be due to a single dominant mechanism in the breakdown of the immune-brain alliance. This scenario highlights the importance of finding new biomarkers to better understand the mechanisms underlying this complex interplay between the immune and nervous systems in MS, an understanding that is crucial for improving personalized MS management (Gutiérrez-Fernández et al., 2021).

Extracellular vesicles (EVs) are one of the most important mediators in the crosstalk between the immune and nervous systems. EVs are micro- and nano-sized cell-derived vesicles that regulate a wide range of biological processes in the brain-immune interaction by the intercellular transfer of molecules (lipids, proteins and genetic material) among immune and neural cells (Gutiérrez-Fernández et al., 2021). During MS, the immune cells release EVs into the bloodstream, revealing important information regarding the pathological reaction of self-reactive T and B cells against the nervous system (Dolcetti et al., 2020). Similarly, neural cells also secrete EVs that reach the blood, providing information on demyelination, axonal loss, oligodendrocyte damage and neuronal death (Gutiérrez-Fernández et al., 2021; Mustapic et al., 2017). Taken together, circulating immune and nervous system-derived EVs could act as potential markers of the autoimmune response and white matter damage taking place in MS. This research could help advance the

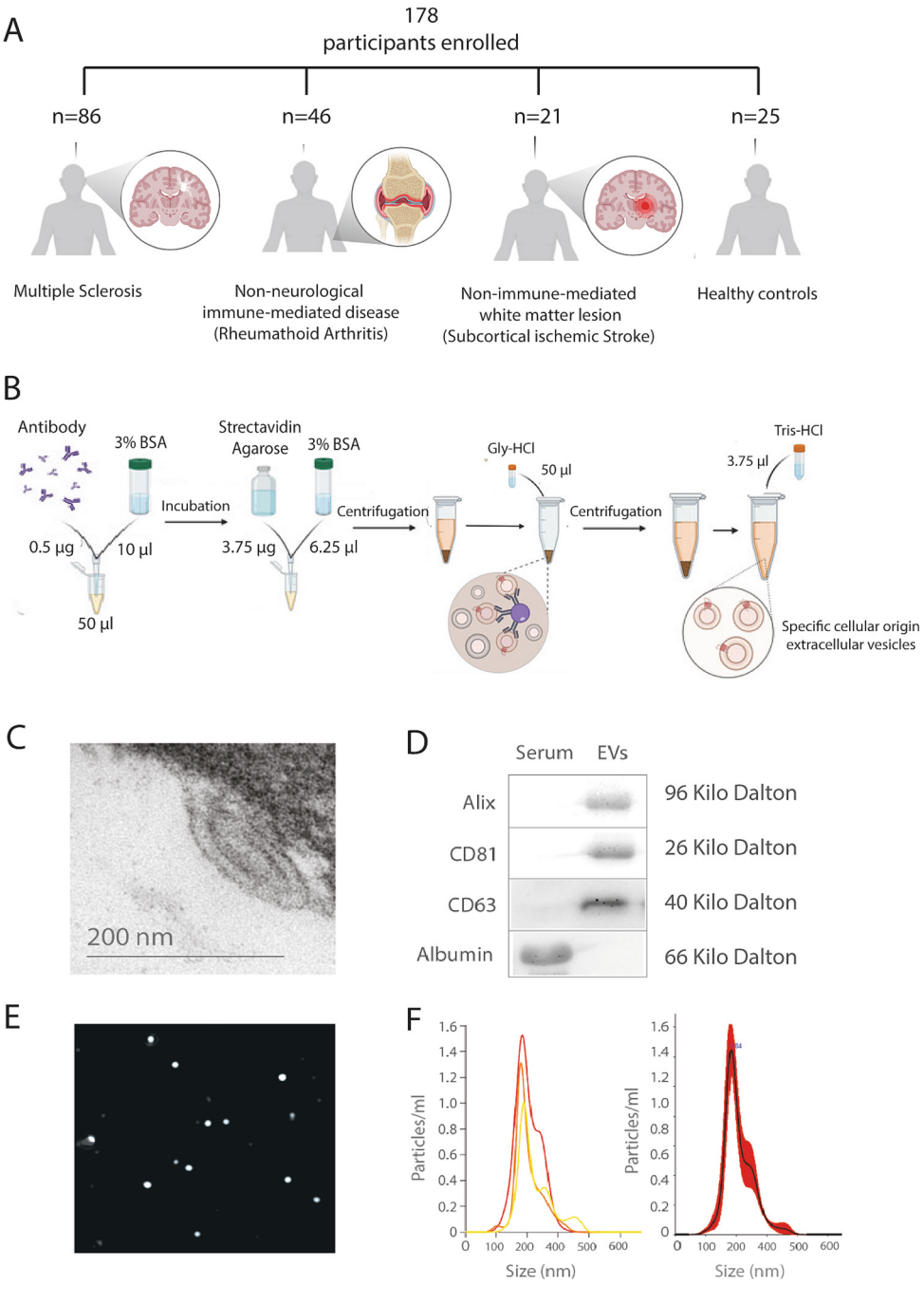

Fig. 1. Study participants, EV immunoprecipitation and characterization. A) Number of patients enrolled in the study; B) Immunoprecipitation protocol of neuronal, oligodendroglial, B and T cell-derived EV isolation; C) Electron microscope image of an EV smaller than 200 nm; D) Representative western blot demonstrating the detection of specific markers in the EV membrane (positive: Alix, CD81 and CD63; negative: Albumin). Negative control samples are serum. The gel image was cropped; E) Image of EV by NanoSight microscope. F) Size and concentration of the EV sample analyzed by NTA. Abbreviations: EVs, Extracellular vesicles.

understanding of the disease's pathogenesis (Dolcetti et al., 2020; Mustapic et al., 2017; Marostica et al., 2021).

Determining the role of EVs in the pathogenesis of MS could contribute to a better understanding of the processes involved in the heterogeneity of the course and progression of MS. This study aimed to identify the immune and nervous system-associated EV profile in blood that could have a specific role as biomarker in MS. We also assessed the possible correlation between this EV profile with disease activity and cognitive and motor dysfunction in patients with MS. For this propose, we analyzed the levels, diameter and protein content of circulating nervous and immune system-derived EVs in the blood of patients with MS compared to the EVs found in another immune-mediated disease (rheumatoid arthritis) and in another white matter lesion (subcortical stroke) to determine the contribution of these EVs to autoimmunity and brain damage.

2. Methods

2.1. Study design

A prospective, observational and single-center study was conducted at La Paz University Hospital (Madrid, Spain), recruiting patients aged 18 years or older diagnosed with relapsing-remitting MS according to the McDonald criteria (Thompson et al., 2018) and who attended the Neuroimmunology Unit of the Neurology Department. The study also included three control groups: 1) non-neurological immune-mediated disease control group: patients (≥18 years) diagnosed with rheumatoid arthritis according to the American College of Rheumatology criteria (Aletaha et al., 2010) and who attended the hospital's Rheumatology Department; 2) non-immune white matter lesion control group: patients (≥18 years) with strictly subcortical cerebral ischemic stroke with white matter lesion that clinically caused lacunar syndrome and with a previous Modified Rankin Score (mRS) ≤ 1 treated in the Stroke Unit of the Department of Neurology; and 3) healthy volunteer controls matched for age and sex who stated having no known disease (Fig. 1A). The shared exclusion criteria were pregnancy or breastfeeding, drug or alcohol dependence, severe concomitant disease or autoimmune disease, and follow-up and participation in a clinical trial.

The study was approved by the Research Ethics Committee of La Paz University Hospital (*PI-2416,PI-2562*), and all patients signed the informed consent. All data management was governed by the principles of Spanish Law 14/2007 on July 3 on biomedical research, ensuring the confidentiality of all personal data. The work described has been carried out in accordance with the code of ethics of the world medical association. The original data are available upon reasonable request.

2.2. Disease activity and motor and cognitive dysfunction evaluation

The study collected demographic, disease duration, activity and disability data (motor and cognitive impairment). MS disease activity was evaluated based on the occurrence of clinical relapses or increase in brain or spinal cord lesions observed on magnetic resonance imaging (MRI) in the past year (Jones, 2016). Motor dysfunction was assessed with the Expanded Disability Status Scale (EDSS) and the 9- hole peg test (9HPT), and cognitive impairment was measured by the Symbol Digit Modalities Test (SDMT) (Table 1). For 9HPT, a good score was considered if male patients complete the test in 19.0 s or less with the right hand, and in 20.6 s with the left hand. For female patients, the 9HPT was considered a good score if it was completed in 17.9 s or less and 19.6 s with the right and left hand, respectively (Figueiredo, 2011). For SDMT, a good score was considered if patients performed a total score of 55 or higher (Parmenter et al., 2007).

2.3. Blood extraction, extracellular vesicle isolation, and characterization

A total of 7 mL of peripheral blood was collected from each participant during the medical consultation. The tubes were centrifuged at 3000 × g for 15 min at 4 °C, and the samples were stored at − 80 °C until analysis. For EV isolation, we specifically addressed their cell origin in an in-depth study of those originating from the nervous and immune systems. Nervous system-associated EVs consisted of neuron-derived or oligodendrocyte-derived populations, and immune system-derived EVs came from B and T cells. To this end, we used a two-step isolation for EV subpopulations by a combination of precipitation and immunoisolation. For precipitation, we employed a commercially available high-throughput particle precipitation method (ExoQuick EV isolation kit; System Biosciences, USA), as previously described (Coughlan et al., 2020). This method efficiently isolates total EVs, followed by immunoprecipitation with biotinylated antibodies against EV surface markers to isolate subpopulations. Primary biotinylated anti-L1CAM antibody (Thermofisher Scientific, USA) was employed to obtain enriched blood EVs from neuronal origin, biotinylated anti-MOG antibody (Merck Millipore, Germany) for those of oligodendrocyte origin, biotinylated anti-CD20 antibody (Merck Millipore, Germany) for B cells, and biotinylated anti-CD3 antibody (Merck Millipore, Germany) for T cells. Streptavidin agarose beads (Thermo Fisher Scientific, USA) were employed to precipitate EVs (Mustapic et al., 2017) (Fig. 1B).

Purified EVs were characterized by transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA) and western blot. TEM (JEOL JEM1010) was employed to visualize EV morphology and measure the size (Fig. 1C), as previously described (Otero-Ortega et al., 2021). We performed western blotting to analyze specific surface tetraspanin proteins using antibodies anti-Alix (1:250, Cell Signal, USA), anti-CD81 (1:250, Abcam, UK) and anti-CD63 (1:250, Abcam, UK), followed by goat anti-mouse or anti-rabbit Alexa Fluor 488 antibodies (1:750, Invitrogen, USA) (Fig. 1D). We used albumin (1:1000, Abcam, UK) as a purity control. The blots were visualized using ECL Pierce chemiluminescence (Thermo Fisher Scientific, USA) and an Uvitec–Cambridge imaging system. NTA was performed by using the

Table 1
Demographic and clinical data of the study participants.

	MS	NNID	NIWML	HC	*P*
Demographics					
Age, years	42.75 (10.01)	52.64 (10.69)*	62.3 (12)*	51.79 (17.72)	0.001
Men, n (%)	25 (35.7)*	26 (56.5)	16 (76.1)*	10 (41.7)	0.028
Clinical data					
Disease duration, months	121.30 (122.19)	–	–	–	–
Active disease, n (%)	54 (62.9%)	–	–	–	–
Expanded disability status score	2.36 (4.75)	–	–	–	–
Symbol digit score	46.44 (14.24)	–	–	–	–
9-hole Peg Test score, dominant hand	24.57 (6.5)	–	–	–	–
9-hole Peg Test score, non-dominant hand	24.47 (6.21)	–	–	–	–

* All values are mean (SD) unless otherwise noted.
*Mann–Whitney *U* test for continuous variables and Fisher's exact test for categorical variables were employed to determine statistically significant differences between groups. *P*-values < 0.05 are in bold. *Age showed significant differences for NNID and NIWML when compared with MS (p = 0.001). *Sex showed significant differences in the NNID group compared with the NIWML group (p = 0.011) and MS group (p = 0.014).
Abbreviations: MS, multiple sclerosis; N, number; SD, standard deviation; NNID, non-neurological immune-mediated disease (rheumatoid arthritis); NIWML, non-immune-mediated white matter lesion (subcortical stroke); HC, healthy controls.

NanoSight NS500 nanoparticle analyzer (Malvern Instruments, UK) to measure the size distribution and concentration of the purified EVs. The EV samples were diluted in PBS, and the movement of the particles was recorded within three 60 s videos recorded at a detection threshold of 3, which were subsequently analyzed using NTA Software 2.3 (Malvern Instruments, UK) *(*Fig. 1E and F*)*.

2.4. EV quantification and size assessment

We analyzed the levels and size of circulating EVs from neurons, oligodendrocytes, and B and T cells and compared them among the MS group, the non-neurological immune-mediated disease group, the non-immune white matter lesion group and the healthy control group, using the NanoSight NS500 nanoparticle analyzer (Malvern Instruments, UK). Three 60 s videos were recorded at a detection threshold of 3. This process was run in triplicate.

2.5. Proteomic analysis

The protein content of each cellular origin-derived vesicle was analyzed and compared among the groups using a new "label-free" method known as sequential window acquisition of all theoretical mass spectra (SWATH) and data validation was done by data dependent acquisition (DDA). We performed three sample pools from each condition to obtain biological replicates to obtain a relevant biological average. Protein identification of each sample was done in a two-step analysis using a combination of liquid chromatography and mass spectrometry and followed by a data-dependent acquisition method, as previously described (Peñas-Martínez et al., 2021). Data was acquired in a TripleTOF 6600 System (Sciex, CA) and processed using ProteinPilot™ 5.0.1 software from Sciex, using the Paragon™ algorithm for the database search. Protein quantification was calculated by SWATH, as previously described (Peñas-Martínez et al., 2021). We used normalized spectral abundance factor (NSAF) values as a measure of relative abundance. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD038003.

2.6. Bioinformatic analysis

The functions of the set of identified proteins in EVs were analyzed by a computational analysis of Gene Ontology (GO) terms. The GO analysis consisted of three ontologies to describe the biological processes, molecular functions, and cellular component attributes of the identified proteins. Molecular pathway, molecular function and protein class were powered by the Gene Ontology enRIchment anaLysis and visuaLizAtion (GORILLA) tool (Eden et al., 2009). The network of biological processes was analyzed and represented by using the Protein ANalysis THrough Evolutionary Relations (PANTHER) system (Paul et al., 2022).

2.7. Statistics

The statistical analysis was performed using SPSS 23.0 for Windows (SPSS Inc, IBM, USA). Categorical variables are described as percentages, proportions between groups were compared using the chi-squared test, and Fisher's exact test was employed for dichotomous variables. Continuous variables are expressed as mean (standard deviation) or median (interquartile range). For normally distributed data, Student's *t*-test and ANOVA with Bonferroni's post hoc correction were employed for multiple comparisons. The comparison of data sets with non-normal distribution was performed using the Kruskal-Wallis test or Mann-Whitney *U* test. Any data non outlier was removed before analysis. The data were represented using GraphPad Prism 8 software (GraphPad software, USA) and Adobe Illustrator (Adobe Inc., USA).

3. Results

The study enrolled 86 patients with MS, 46 patients with non-neurological immune-mediated disease (rheumatoid arthritis), 21 patients with non-immune-mediated white matter lesions (subcortical ischemic stroke) and 25 healthy controls (Fig. 1A). Table 1 lists their demographic and clinical characteristics.

3.1. EV characterization: size, morphology, and EV surface-specific tetraspanin proteins

Purified EVs were characterized by TEM, western blot, and NTA. TEM showed the typical morphology of double membrane and circular shape of EVs. The western blot analysis showed the presence of the EV-specific markers Alix, CD81, and CD63 in the EV membrane. Albumin was used as a purity control marker and was not found in the EV sample. NTA showed that the purified EVs display heterogeneous population, with typical EV-like sizes of 30–300 nm. These approaches allowed for robust characterization of the EV sample based on size, morphology and tetraspanin profiles.

3.2. Levels of T cell-derived EVs were similar in both immune-mediated diseases

Non-significant differences were found in the levels of T cell-derived EVs between the MS group ($1.4 \times 10^9 \pm 1.3 \times 10^9$ particles/mL) and the non-neurological immune-mediated disease group ($2.4 \times 10^9 \pm 6.5 \times 10^9$ particles/mL). However, these levels were significantly lower in the MS group ($1.4 \times 10^9 \pm 1.3 \times 10^9$ particles/mL) than in the non-immune white matter lesion control group ($3.6 \times 10^9 \pm 2.3 \times 10^9$ particles/mL; $p = 0.001$) (Fig. 2A). Nonetheless, the diameter of these vesicles differed between the MS group (182.16 ± 43.49 nm) and the other control groups [immune-mediated disease control group (176.67 ± 44.37 nm; $p = 0.023$) and non-immune white matter lesion group (203.91 ± 33.09 nm; $p = 0.017$)] (Fig. 2A).

3.3. The MS group released different EVs, in terms of level and size, from B cells than those of the patients with other immune-mediated disease and non-immune-mediated white matter lesions

The levels and size of the B cell-derived EVs differed significantly between the MS group ($3.86 \times 10^8 \pm 4.02 \times 10^8$ particles/mL and 123.75 ± 47.96 nm, respectively) and the control group with other immune-mediated disease ($6.64 \times 10^8 \pm 5.82 \times 10^8$ particles/mL and 110.62 ± 20.14 nm, respectively; $p = 0.006$ and $p = 0.026$) and the control group with non-immune white matter lesions ($5.64 \times 10^8 \pm 2.37 \times 10^8$ particles/mL and 148.63 ± 19.37 nm, respectively; $p = 0.001$ and $p = 0.001$) (Fig. 2A).

3.4. Neuron-derived EV subpopulations differed between the MS group and all control groups

The levels and size of the neuron-derived EVs were significantly smaller in the MS group ($9.34 \times 10^8 \pm 1.08 \times 10^8$ particles/mL and 119.43 ± 22.19 nm, respectively) than in the control group with other immune-mediated disease ($9.88 \times 10^8 \pm 1.14 \times 10^9$ particles/mL and 126.64 ± 27.79 nm, respectively; $p = 0.017$ and $p = 0.039$) or other white matter lesions ($2.04 \times 10^9 \pm 1.10 \times 10^9$ particles/mL and 193.48 ± 23.74 nm, respectively; $p = 0.001$ and $p = 0.036$) (Fig. 2B).

3.5. Oligodendrocytes release different EVs according to white matter lesion origin

The levels and size of the oligodendrocyte-derived EVs in the MS group ($1.70 \times 10^9 \pm 2.20 \times 10^9$ particles/mL and 117.08 ± 25.44 nm, respectively) were significantly smaller than those in the non-immune

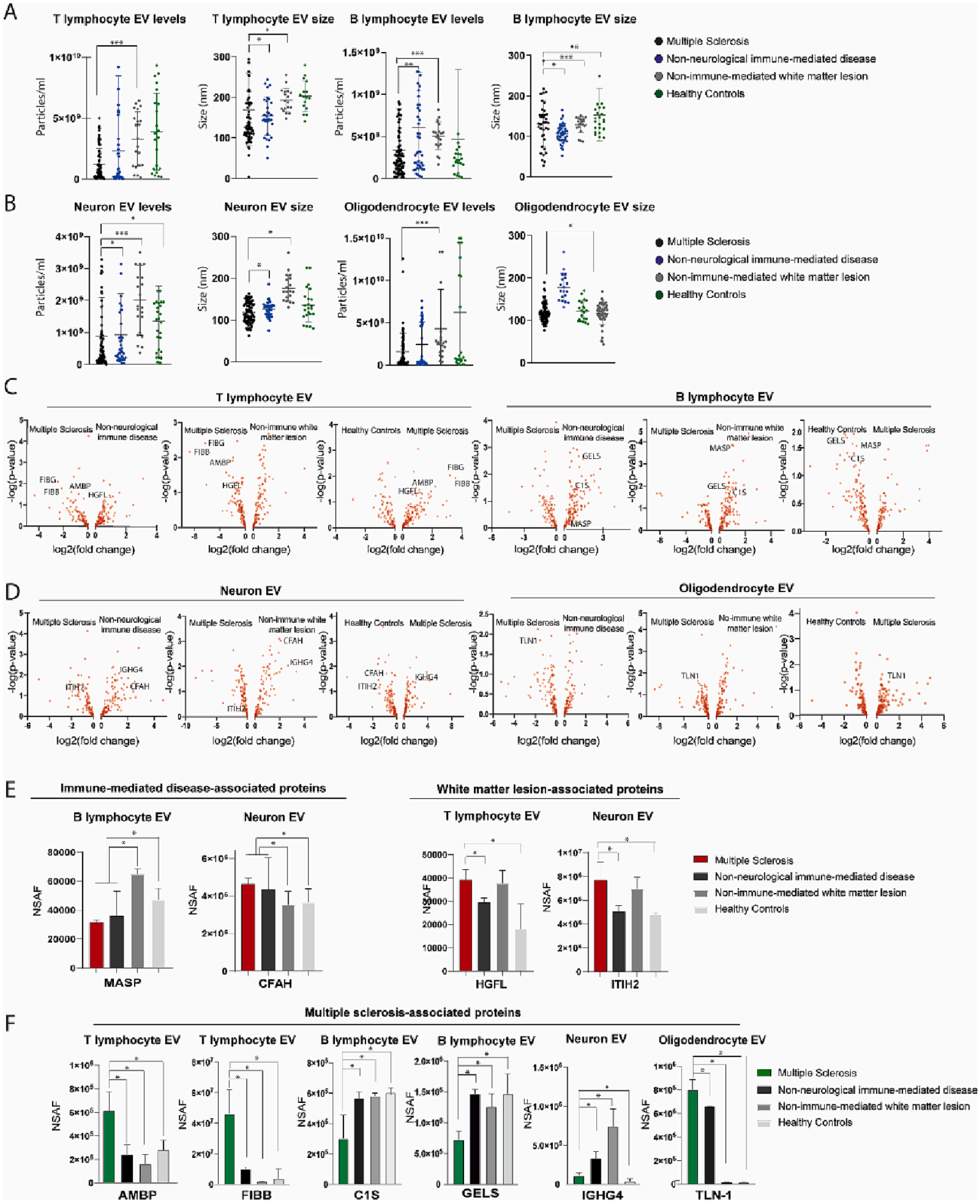

Fig. 2. Levels, size and protein content comparison of immune and nervous system-derived EVs in MS compared with the control groups. A) Immune system (T and B cell)-derived EV levels and size found in MS compared with the other immune-mediated disease, other white matter lesion-associated disease and healthy controls. B) Nervous system (neurons and oligodendrocyte)-derived EV levels and size in the MS group compared with the aforementioned control groups. C and D) Volcano plots comparing the MS group with the control groups from immune and nervous system-derived EVs, respectively. E) Proteins found in common in both immune-mediated disease (MS and rheumatoid arthritis) and in both white matter disease (MS and subcortical stroke). F) Proteins that showed statistically significant differences in their levels between the MS group and all control groups. Data are expressed as mean ± SD. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Abbreviations: EVs, Extracellular vesicles; NSAF, normalized spectral abundance factor.

white matter lesion control group ($3.85 \times 10^9 \pm 4.68 \times 10^9$ particles/mL and 131.40 ± 13.66 nm, respectively; p = 0.001 and p = 0.022; Fig. 2B).

3.6. Mannose-binding lectin serine protease 1 from immune system-derived EVs and complement factor H from neurons were associated with autoimmunity

The vesicles' protein content was analyzed by mass spectrometry and compared among the groups. Similar levels of mannose-binding lectin serine protease 1 (MASP-1) from B cell-derived EVs (31525.66 ± 1654.58 and 35966 ± 17173.47 NSAF; p = 0.79) and complement factor H (CFAH) from neuron-derived EVs (4531273.02 ± 1975964.43 and 4353936.23 ± 1975964.43 NSAF; p = 0.11) were found in both autoimmune diseases (MS and non-neurological immune-mediated disease). However, we observed significantly different levels of MASP in the B cell-derived EVs [non immune-mediated white matter lesion (65004.33 ± 3806.92 NSAF; p = 0.001) and healthy controls (47121.66 ± 7942.03 NSAF; p = 0.02)] and CFAH from neurons [non immune-mediated white matter lesion (4035014.54 ± 1975964.43 NSAF; p = 0.01) and healthy controls (3656196.37 ± 1975964.43 NSAF; p = 0.02)] (Fig. 2C, D and E).

3.7. Hepatocyte growth factor-like protein and inter-alpha-trypsin inhibitor heavy chain 2 are enriched proteins in both diseases with white matter involvement

We observed similar levels of hepatocyte growth factor-like protein (HGFL) from T cell-derived EVs (39114.66 ± 4500.10 and 37743.66 ± 5494.22 NSAF; p = 0.058) and inter-alpha-trypsin inhibitor heavy chain 2 (ITIH2) in EVs from neurons (7696566.66 ± 1510317.91 and 6973066.67 ± 952871.037 NSAF; p = 0.052) in the MS group and the white matter lesion control group. Nevertheless, there were differing levels of HGFL [in the other autoimmune disease group (29379.66 ± 2039.48 NSAF; p = 0.04) and healthy controls (18063.46 ± 10765.24 NSAF; p = 0.04)] and of ITIH2 [other autoimmune disease (5024933.33 ± 537280.19 NSAF; p = 0.04) and healthy controls (4840000 ± 129950.91 NSAF; p = 0.008)] (Fig. 2C, D and E).

3.8. Alpha-1-microglobulin, fibrinogen β chain, C1S and gelsolin from immune system-derived EVs are MS-specific proteins

Higher levels of alpha-1-microglobulin (AMBP) and fibrinogen β chain (FIBB) were found in the EVs released from the T cells of the MS group ($6.1 \times 10^5 \pm 1.6 \times 10^5$ and $4.63 \times 10^7 \pm 1.56 \times 10^7$ NSAF) than in all control groups [non-neurological immune-mediated disease ($2.3 \times 10^4 \pm 8.7 \times 10^4$ and $1.03 \times 10^7 \pm 1.1 \times 10^6$ NSAF; p = 0.02 and p = 0.01), non-immune-mediated white matter lesion ($1.5 \times 10^5 \pm 8.4 \times 10^4$ and $0.2 \times 10^7 \pm 4 \times 10^4$ NSAF; p = 0.01 and p = 0.006) and healthy controls ($2.7 \times 10^4 \pm 8.4 \times 10^4$ and $0.39 \times 10^7 \pm 6.4 \times 10^6$ NSAF; p = 0.03 and p = 0.01)(Fig. 2C, D and F)], while the levels of these proteins were similar throughout the control groups.

Similarly, the MS group showed lower levels of C1S and Gelsolin (GELS) in B cell-derived EVs ($3.03 \times 10^5 \pm 1.53 \times 10^5$ and $7.28 \times 10^5 \pm 1.38 \times 10^5$ NSAF) than in all control groups [non-neurological immune-mediated disease ($5.8 \times 10^5 \pm 1.8 \times 10^4$ and $1.47 \times 10^6 \pm 7.03 \times 10^4$ NSAF; p = 0.03 and p = 0.001), non-immune-mediated white matter lesion ($6.04 \times 10^5 \pm 4.8 \times 10^4$ and $1.26 \times 10^6 \pm 2.12 \times 10^5$ NSAF; p = 0.03 and p = 0.02) and healthy controls ($5.67 \times 10^5 \pm 4.2 \times 10^4$ and $1.47 \times 10^6 \pm 3.17 \times 10^6$ NSAF; p = 0.04 and p = 0.02)], while the levels of these proteins were similar in all the controls (Fig. 2C, D and F).

3.9. Neural immunoglobulin heavy constant gamma 4 and oligodendroglial talin-1 were specifically associated with MS

The MS group showed significant different levels of immunoglobulin heavy constant gamma 4 (IGHG4) in the cargo of neuron-derived EVs ($1.1 \times 10^5 \pm 3.13 \times 10^4$) and talin-1 (TLN-1) in oligodendrocyte-derived EVs ($8.01 \times 10^5 \pm 8.4 \times 10^4$ NSAF) compared with those of all control groups [non-neurological immune-mediated disease ($3.3 \times 10^5 \pm 8.5 \times 10^4$ and $2.0 \times 10^4 \pm 251.9$ NSAF; p = 0.01 and p = 0.014), non-immune-mediated white matter lesion ($7.4 \times 10^4 \pm 2.2 \times 10^5$ and $1.8 \times 10^4 \pm 1.1 \times 10^3$ NSAF; p = 0.009 and p = 0.02) and healthy controls ($4.07 \times 10^4 \pm 3.36 \times 10^4$ and $6.61 \times 10^5 \pm 1.2 \times 10^4$ NSAF; p = 0.04 and p = 0.05)], while all the control groups showed similar levels of these proteins (Fig. 2C, D and F).

3.10. Levels of T cell-derived EVs and the size of those from neurons correlate with disease activity while oligodendrocyte-derived EVs are associated with cognitive and motor dysfunction in the MS group

The EV samples from the MS group underwent further analysis, including examining the possible correlation between levels and size of EVs with disease activity, cognitive and motor dysfunction. The patients with clinical relapse showed higher levels of T cell-derived EVs ($1.78 \times 10^9 \pm 1.65 \times 10^9$ particles/mL) compared with the patients with stable disease ($8.69 \times 10^8 \pm 9.19 \times 10^8$ particles/ mL; p = 0.002) (Fig. 3A). The patients with clinical relapse (111.9 ± 17.09 nm) and those with new MRI lesions (199.02 ± 28.7 nm) showed significant differences in the size of the neuron-derived EVs when compared with the patients with stable disease or with no increase in new MRI lesions (121.28 ± 23.02 and 175.44 ± 33.03 nm; respectively; p = 0.042 and p = 0.04) (Fig. 3B and D). While there was no correlation when analyzing any of the cellular origin-derived EVs with the EDSS, the smaller size of oligodendrocyte-derived EVs correlated with poorer scores in both the cognitive impairment test by SDMT (P = 0.01; R = -989) and the motor function test 9HPT (using the dominant hand [p = 0.009; R = -997] and non-dominant hand [p = 0.022; R = -991]) (Fig. 3E).

3.11. Gene ontology enrichment analysis of EV release revealed functions related to B-cell activation, inflammation and p53 pathway in the MS group

The specific functions of proteins carried in the EVs of the MS group were also explored on a global level using functional gene ontology analysis, which helped identify statistically relevant associations with the functional processes of proteins. The results of these analyses are shown in Fig. 4, which presents the gene ontology analysis of biological processes, molecular pathway, molecular function, and protein class of the proteins identified by mass spectrometry in the MS group. The network shows that most of the biological processes are associated with cellular response to stimulus, regulation of localization, regulation of system processes, cellular protein metabolic processes, and cellular response to hormone stimulus, among others. There are also numerous smaller but significant molecular pathways and molecular functions present in the analysis including those associated with B-cell activation, inflammation mediated by chemokines and cytokines, p53 pathway, growth, different immune system processes, cell adhesion molecules, and carrier proteins (Fig. 4).

3.12. The MS-associated proteins AMBP, FIBB, GELS and IGHG4 are related to disease activity in the MS group

The proteins carried in the EV samples from the MS group underwent further analysis, identifying those proteins related to disease activity. While no protein associated with immune-mediated disease (CFAH, MASCP) or white matter damage (HGFL and ITIH2) was identified as related to disease activity, different levels of MS-differentially expressed proteins have been found in patients with active disease compared with those with stable disease. Thus, there was an increase in AMPB and FIBB from T cell-derived EVs in the patients with active disease ($3.42 \times 10^5 \pm 2.77 \times 10^5$ and $5.15 \times 10^7 \pm 1.14 \times 10^7$ NSAF) compared with the

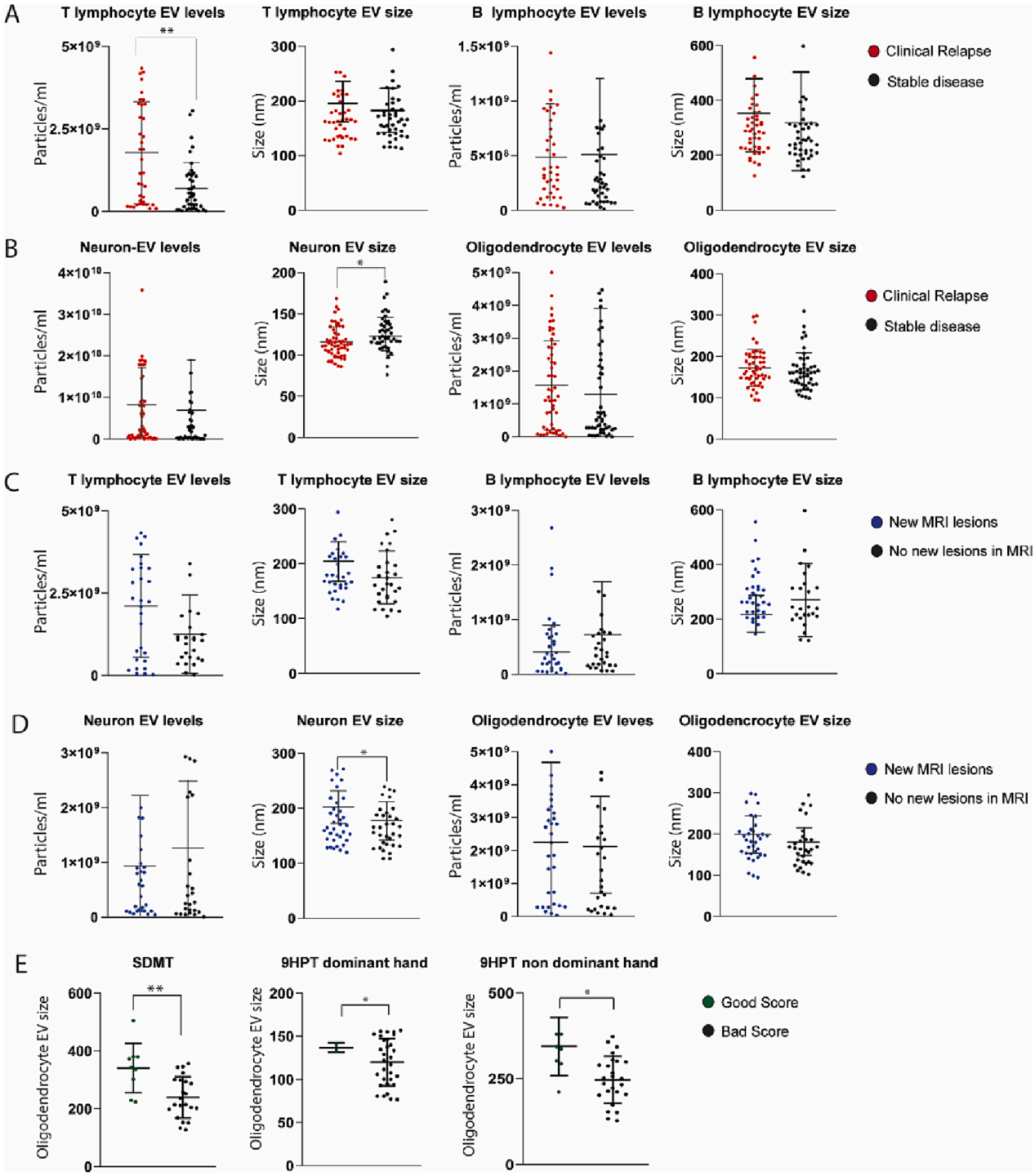

Fig. 3. Quantitative analysis of the levels and size of circulating EVs released from the immune and nervous system of the MS group related to the clinical settings. A) T and B cell-derived EVs and B) neuron and oligodendrocyte-derived EVs in the MS group and clinical relapse compared with those with disease remission. C) Comparison of T and B cell-derived EVs and D) neuron and oligodendrocyte-derived EVs in patients with and without new MRI lesions. E) Relation between oligodendrocyte-derived EVs and cognitive and motor dysfunction in the MS group. Abbreviations: EVs, Extracellular vesicles; MRI, magnetic resonance imaging; SDMT, Symbol Digit Modalities Test; 9HPT, 9 hole peg test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

patients with stable disease ($1.68 \times 10^5 \pm 4.29 \times 10^4$ and $1.93 \times 10^5 \pm 8.72 \times 10^4$ NSAF; $p = 0.004$ and $p = 0.001$). Furthermore, the patients with active disease showed a decrease of GELS from B cell-derived EVs ($6.78 \times 10^5 \pm 8.97 \times 10^4$ NSAF) and IGHG4 from EV released from neurons ($2.90 \times 10^5 \pm 6.0 \times 10^4$ NSAF) when compared with the patients with stable disease ($1.3 \times 10^6 \pm 1.07 \times 10^5$ and $6.60 \times 10^6 \pm 2.59 \times 10^6$ NSAF; respectively; $p = 0.001$ and $p = 0.005$).

3.13. Myelin protein content of extracellular vesicles from oligodendrocytes differ in patients with active disease compared to non-active patients.

There was an increase in the levels of myelin basic protein (MBP) ($2.96x10^5 \pm 1.28 x10^5$ and $0.01x10^5 \pm 0.01x10^5$; $p = 0.016$) and a trend to increase in myelin proteolipid protein (PLP) ($3.00x10^5 \pm 1.70x10^5$ and $1.43x10^5 \pm 5.9x10^4$) in patients with active disease compared to

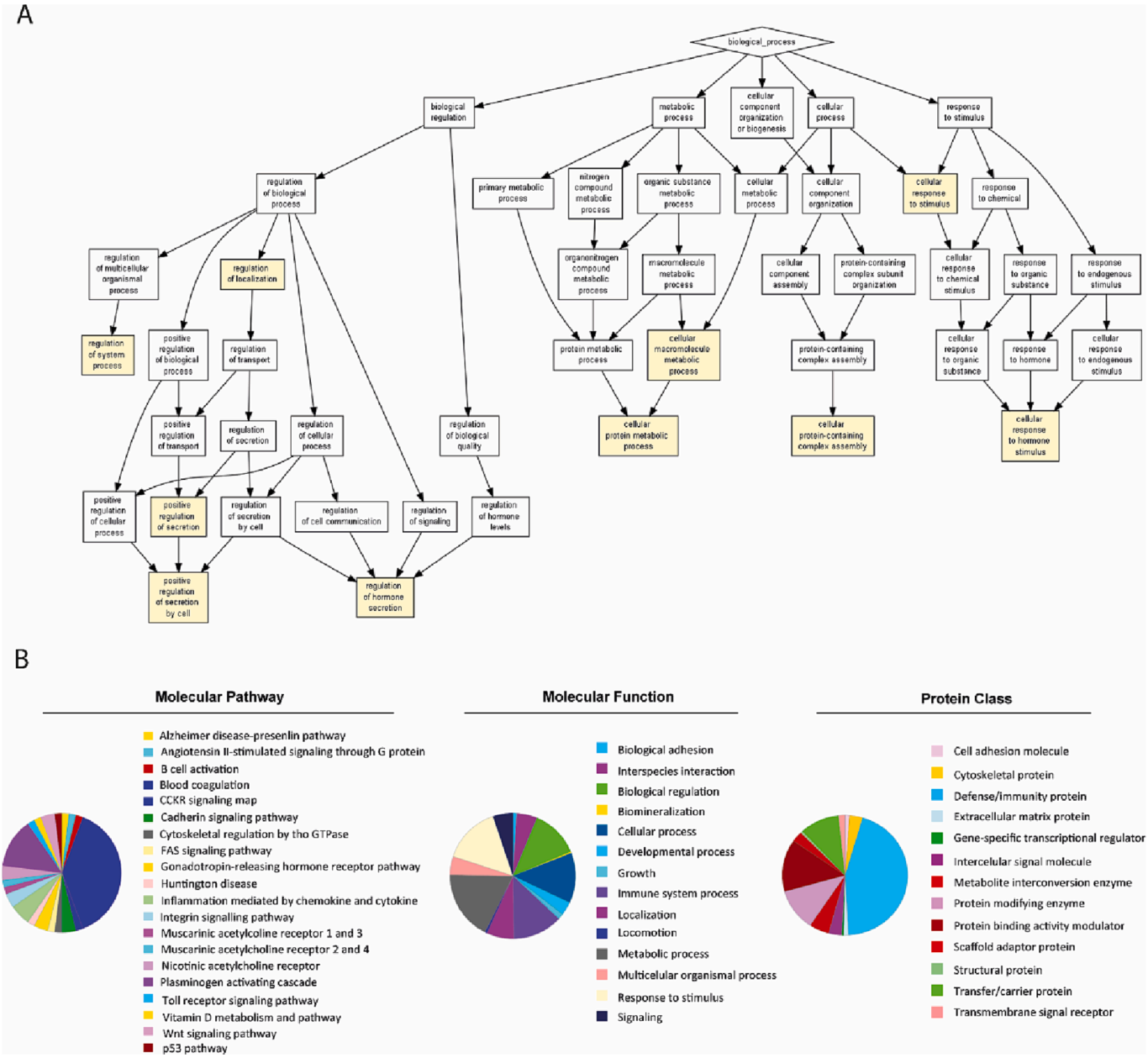

Fig. 4. Proteomic analysis of common proteins in all study groups. A) Gene ontology analysis of biological processes of the proteins identified by mass spectrometry. The deeper the color, the more statistically significant the function associations. B) Gene ontology analysis of molecular pathway, molecular function, and protein class.

stable disease (Fig. 5A and B).

3.14. The most abundant differentially expressed protein from the patients with disease activity and those with stable disease

Fig. 5C and supplementary Table 2 show the most abundant proteins found in the vesicles of the MS group. The most abundant proteins found in the patients with active disease but not in the patients with stable disease were ApoA, Clusterin and FGC. The most abundant proteins found in the patients with stable disease compared with those with active disease were ApoL, C1qA, C1qB, HC2, IαTIHC, Titin, keratin type I, SAPC and vitronectin.

4. Discussion

EVs are nano-sized and micro-sized lipid bilayer vesicles secreted by all eukaryote cells. EVs have emerged as potential carriers of disease-associated markers, playing an important role as a window into the pathogenic processes that occur during disease (Mustapic et al., 2017; Marostica et al., 2021; Vandendriessche et al., 2020). EVs have been proposed to be involved in diverse biological functions in MS; including mediating the inflammatory response (Manu et al., 2021; Mycko and Baranzini, 2020), T-cell stimulation (Mycko and Baranzini, 2020) and synaptic plasticity (Dolcetti et al., 2020). Although informative, these approaches do not capture the cell type-specific functions. Recent advances in isolation techniques provide new tools for studying blood EVs at the single cell origin-derived vesicle level, opening new horizons to examine previously intractable issues such as assessing the process in the brain and the involvement of the immune system in the disease. The present study aimed to identify the contribution of nervous system-associated EVs (consisting of neurons or oligodendrocyte subpopulations) and immune system-derived EVs from B and T cells in

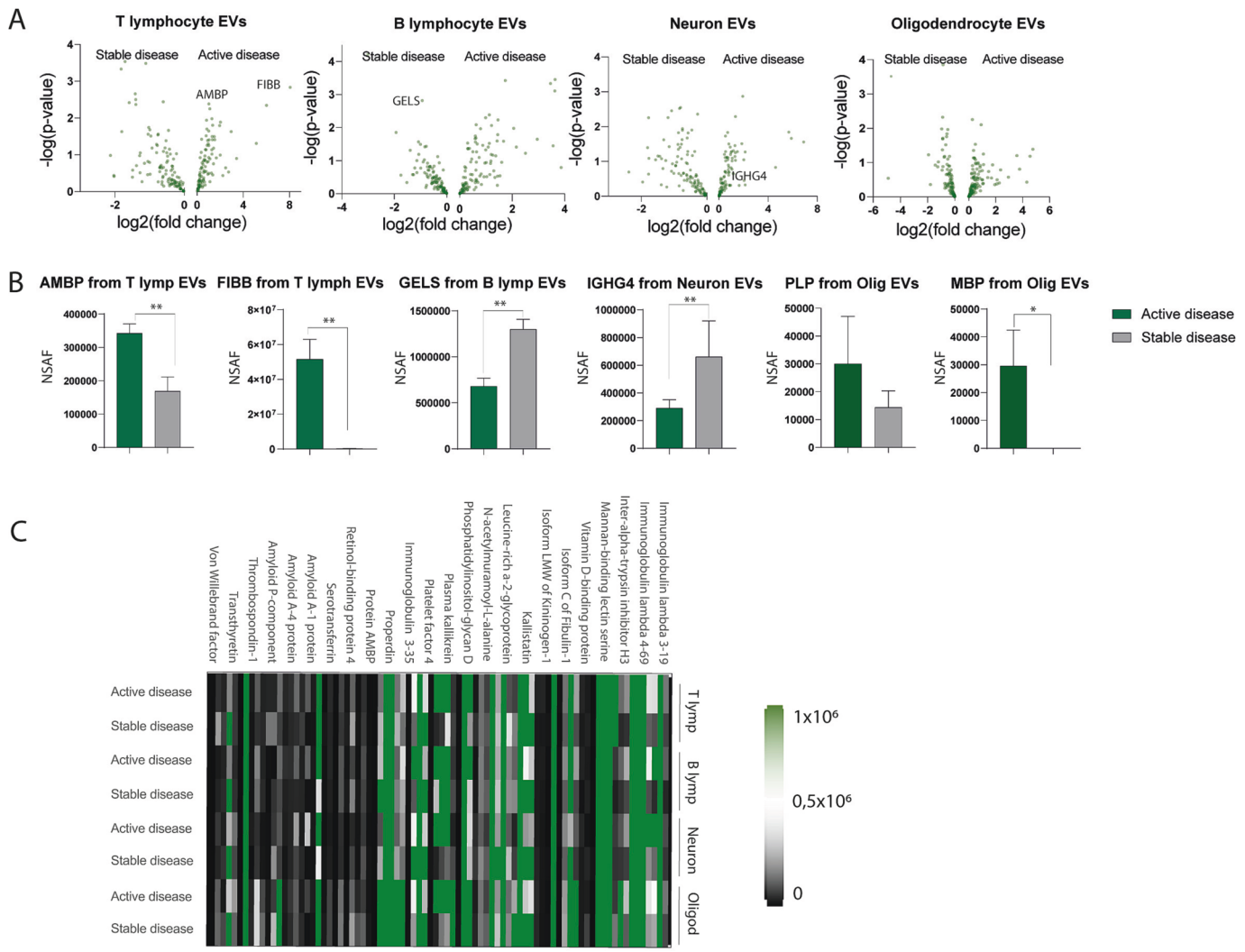

Fig. 5. Protein content comparison of immune and nervous system-derived EVs in the patients with active MS compared to those with stable disease. A) Volcano plots comparing proteins in immune and nervous system-derived EVs found in the patients with MS with active disease and stable disease. B) Comparison of MS differentially expressed proteins in the patients with active disease and stable disease. C) Heatmap showing the most abundant differentially expressed proteins in immune and nervous-system derived EVs in the patients with active and stable disease.

autoimmunity processes and brain damage. To this end, MS-specific EVs characteristics were compared with those observed in other non-neurological autoimmune diseases, patients with other white matter lesions and healthy controls. This "hot spot" mapping of the cells involved in the disease could optimize strategies to harvest the most relevant EVs to obtain detailed information on disease pathogenesis in MS.

Due to their small size and membrane composition, neural-derived EVs have the essential ability to cross major biological membranes, including the blood brain barrier, allowing vesicle dispersion to the bloodstream (Ramos-Zaldívar et al., 2022). This essential function provides data that was previously inaccessible due to the difficulty of brain sampling. The only currently available source for analyzing brain processes in MS in clinical practice is cerebrospinal fluid, which still involves an invasive procedure for its isolation. We therefore relied on EV analysis, isolating them from the blood. EVs have the promising properties of being readily available, easy to obtain and less invasive than lumbar puncture, allowing an assessment of the processes occurring in the CNS.

In the past few years, several studies have analyzed the levels and size of total circulating EVs in patients with MS to monitor disease progression, disease activity and differentiate patient disease phenotype (Dolcetti et al., 2020; Manu et al., 2021; Geraci et al., 2018). However, these studies analyze total circulating EVs; therefore, small changes in the EVs from single cells involved in the pathogenesis of MS could be masked by dilution or be overlooked. In our study, which focused on immune system-derived EVs, the levels of T cell-derived EVs were similar for both immune-mediated disease groups and differed from the other non-immunological white matter lesion group. Moreover, the patients with relapses had higher levels of T cell-derived EVs than those in remission. These results are consistent with the findings of a previous study, where a significant increase in T cell-derived EVs was observed in patients with MS who presented proinflammatory activity (Geraci et al., 2018). The authors analyzed EVs in cerebrospinal fluid, while we took a step towards translational research by isolating EVs from the blood, which involves a less invasive procedure. According to the immune system findings, the EVs released by the B cells had differing levels and sizes in the MS group than in the rest of the groups, a finding that agrees with a previously published study (Zinger et al., 2016) where B-cell EV levels were higher in patients with MS than in healthy controls. Our study also observed that B cells release MS-specific EV levels and sizes that differed from those in other non-neurological immune-mediated disease and other types of white matter lesion, which might indicate that the EV levels from B cells might be specific for patients with MS. Our

study also analyzed the neural source of EVs. The neuron-derived EV levels and size were smaller in the MS group than in the rest of the groups. Our finding differs from recent observations where the levels of blood EVs from neurons were similar between the MS group and controls, but the size was slightly greater in the MS group (Bhargava et al., 2021). These differences can be due to the fact that the previous published article only included patients in remission, in contrast to our work, which also enrolled patients with relapses. In our study, we demonstrated that patients with relapse showed smaller size of neuronal EVs than those in remission. This observation could explain why in our study the neuronal EVs of MS patients are smaller than in healthy controls, and in the previously published study these are greater. On the other hand, we observed that smaller oligodendrocyte-derived EVs correlated with motor and cognitive dysfunction. To the best of our knowledge, this study is the first to isolate EVs from oligodendrocytes in patients with MS, but our findings could identify a potential new role for these EVs as markers of neurological impairment.

Although the etiology of MS remains elusive, large scientific evidence suggests that it is immune-mediated. The etiology has been classically associated with the activation of T and B cells that act against CNS antigens, although recent studies have suggested that adaptive immunity loses importance with disease progression, while innate immunity, orchestrated by macrophages, microglia and astroglia, takes precedence (Bierhansl et al., 2022). The complement system is an important player in the interplay between innate and adaptive immunity, and its dysregulation has been involved in the pathogenesis of MS (Kwok et al., 2011). Various pathways have been identified that initiate complement system activation: the classical (initiated by the C1s protein (Degn et al., 2011) and the lectin pathway (initiated by the MASP-1 protein (Sekine et al., 2013). Our study showed lower MASP-1 levels in B cell-derived EVs in both autoimmune disease groups, compared with the non-autoimmune controls. We identified MASP-1 as one of the most abundant proteins in the EVs of the MS group. C1S is also underexpressed in B cell-derived EVs in a specific manner in the MS group. Other components related to C1S in the classical pathway of complement activation, such as C1q, C3 and C4, are some of the most abundant proteins in patients with MS. Our finding is consistent with recent observations that complement subunits such as C1q, C3 and C4 are carried in the EVs of patients with MS, although not from B cells but from astrocytes (Bhargava et al., 2021). Moreover, higher levels of CFAH were found in the EVs released from neurons in both autoimmune diseases when compared with the non-autoimmune controls. CFAH is one of the most abundant proteins in patients with MS, is an important regulator of the complement system and is largely responsible for inhibiting the inflammation propagation (Ingram et al., 2010). This result might demonstrate that EVs are not simply mediating the activation of the inflammatory response, as has been previously reported (Manu et al., 2021; Mycko and Baranzini, 2020). Overall, all of these identified proteins provide crucial insight into the significant role of EVs in the classical complement pathway, specifically in MS, and the nonspecific role of EVs in the lectin pathway, regulating complement system dysfunction.

Previous studies have reported the involvement of EVs in CNS inflammation and consequent degeneration in MS. Although these functional roles for EVs are being increasingly reported, our current knowledge is still limited. Conceptually, MS white matter lesions are characterized by abundant mononuclear cell infiltrates that consist mainly of lymphocytes and monocyte-derived macrophages. Infiltrated mononuclear cells produce an array of inflammatory mediators (Bierhansl et al., 2022) and reactive oxygen species, thereby influencing the degradation of the perivascular and perineuronal extracellular matrix (ECM) (Malemud, 2006). This degradation of the ECM is followed by a phase of attempted ECM remodeling, leading to the synthesis and aggregation of ECM molecules, which when deposited in MS lesions provide an altered microenvironment that exacerbates inflammatory responses within the lesions. Our study identified important ECM-associated proteins to be upregulated in the circulating EVs from patients with MS that participate in this biphasic dysregulation of ECM.

AMBP is a potent tissue-protective protein that participates as a scavenger, protecting ECM structures from reactive oxygen species during ECM degradation (Olsson et al., 2011). Our study observed AMBP overexpression specifically in the EVs released from T cells in the MS group compared with the control groups, and this overexpression was present in the patients with active rather than stable disease. To our knowledge, this is the first study to identify a role for this protein in MS. This result might indicate a specific role for EV-derived AMBP in protection from ECM degradation after the immune mediated-ECM injury that occurs in MS.

AMBP also cleaved into 3 polypeptides, one of which is the inter-alpha-trypsin inhibitor light chain, which binds to ITIH2 to greatly promote ECM stability and integrity (Bost et al., 1998). Our study observed higher ITIH2 levels in both white matter lesion disease groups compared with the non-neurological controls. These results might indicate that the AMBP polypeptide and ITIH2 complex could critically mediate ECM repair after white matter injury in MS but in a non-disease-specific manner, as this phenomenon also occurs in other white matter lesions. These results agree with those of previous studies that reported a role for AMBP in extracellular space remodeling by stabilizing new molecular aggregates in the ECM after brain injury (Romantsik et al., 2019; Pendlebury et al., 2014). Following this approach, this result might indicate a specific role for EV-derived AMBP and ITIH2 in ECM healing and repair in MS brain lesions.

Fibrinogen is a protein typically considered a player in hemostasis and coagulation. However, fibrinogen is also synthetized and assembled into the ECM as a substratum during an inflammation process (Pereira et al., 2002). Previous studies have identified fibrinogen deposits around endothelial cells in chronic active lesions in patients with MS (Sobel, 1998). This protein has also been shown to be abundant in the cortex of progressive MS lesions (Yates et al., 2017). These results agree with our study's findings, where specific overexpression of fibrinogen occurred in the T-cell derived EVs from the MS group when compared to the other groups. We also observed higher levels of fibrinogen in the patients with active disease than in those with stable disease and this protein has been identified as one of the most abundant proteins in the EVs of patients with active lesions. Fibrinogen is deposited in these MS lesions as fibrin, which is a potent activator of microglia and macrophages (Davalos et al., 2012). Until now, astrocytes, macrophages and microglia have been known to be the responsible cells for fibrinogen accumulation in the ECM within CNS (Asher et al., 2002). Our study, however, observed fibrinogen overexpression in the T cell-derived EVs, which might indicate that the T cell-derived EVs might have a role in the dysregulation of ECM molecules participating in the interaction between ECM and immune cell activation in MS lesions.

This remodeling of ECM components after injury appears to affect brain repair. In MS, inflammation regularly damages oligodendrocytes, resulting in demyelination and, consequently, axonal loss. To enable remyelination, oligodendrocyte progenitor cells (OPCs) need to be recruited to the zone of myelin loss and undergo further differentiation and maturation to become fully competent myelin-producing oligodendrocytes (Bierhansl et al., 2022). However, the ECM molecules deposited into MS lesions likely lead to a matrix that becomes unfavorable for repair, inhibiting OPC differentiation, newborn cell migration and guidance of new axonal and dendritic sprouting, which might lead to inefficient brain repair (Ulbrich et al., 2021; Ghorbani and Yong, 2021). Previous studies have shown that excess fibrinogen during ECM remodeling contributes to the non-permissive extracellular environment in demyelinating lesions and inhibits remyelination (Petersen et al., 2017). Fibrinogen also skews the OPC differentiation towards astrocyte-like cells instead of mature oligodendrocytes (Petersen et al., 2017). The fibrinogen overexpression in T cell-derived EVs leads us to consider the possibility that autoreactive T cells might contribute to restricting certain forms of brain plasticity and remyelination in the active lesions

of patients with MS that were thought to release EVs containing fibrinogen protein, which might contribute to ECM remodeling.

During brain repair processes, the links between ECM and newborn cells are critically important for cell growth, survival and differentiation. TLN-1 is a cytoplasmatic protein that has a role in the cell-ECM interaction, controlling the ECM adhesion of newborn cells and cell migration (Haage et al., 2018) TLN-1 also contributes to new blood vessel formation (Petersen et al., 2017) and markedly enhances axon growth after white matter injury (Pulous et al., 2021; Tan et al., 2015). Our study detected an MS-specific overexpression of TLN-1 in oligodendrocyte-derived EVs. This is the first study that has isolated EVs from oligodendrocytes in patients with MS. Previous studies have identified higher levels of soluble TLN-1 in the serum of patients with MS, although directly in blood. The difference with our study is that we observed TLN-1 in the oligodendrocyte-derived EVs instead of serum-soluble TLN-1. Our findings highlight a potential alternative role of oligodendrocyte-derived EVs releasing TLN-1, which might promote newborn cell migration, angiogenesis and axonal sprouting after white matter injury in patients with MS.

Last but not least, our study has showed lower levels and smaller oligodendrocyte-derived EVs in MS patients that in controls. The EVs analyzed in this study are a broad spectrum of vesicles with a distribution of range size from 50 to 500 nm in diameter, which consists of a population formed by small EVs called exosomes (50 to 120 nm in diameter) and medium EVs termed microvesicles (100 to 1000 nm in diameter). Considering that cells under stress are known to increase exosome secretion (Debbi et al., 2022), the fact that EVs were found to be smaller in MS patients could be due to oligodendrocytes, under the cell damage occurring in MS, releasing EVs mainly enriched in exosomes instead of microvesicles. In addition to the levels and size, we further analyze the cargo of the EVs released by oligodendrocytes in active and stable patients and we found higher expression of myelin proteins antigens in patients with active disease. Notably, the immune system in MS is derailed and generates immunity against itself through a process of loss of tolerance of oligodendrocyte-produced myelin proteins. Although the specific myelin antigen has not been identified, proteins such as MBP and PLP have been proposed as key antigens promoting immune reaction as they are the most abundant proteins in the outermost layer of oligodendrocytes' myelin sheath surfaces (Gutiérrez-Fernández et al., 2021). Our study has identified higher expression of MBP and a trend of PLP in oligodendrocytes-derived EVs during disease activity. These results agree with a previous study that have shown higher expression of other myelin protein called myelin oligodendrocyte glycoprotein in EVs from MS patients during disease activity (Galazka et al., 2018). These findings might indicate that during stable conditions, myelin proteins are "exported" via exosomes from the CNS parenchyma to the peripheral compartment of the immune system and, during periods of active demyelination, this process becomes enhanced. Interestingly, in this regard, recent studies have proposed these EVs as cell-free antigen-presenting platforms to induce tolerance of self-proteins. EVs facilitate the antigen delivery to monocytes, dendritic cells, macrophages, and microglia, which internalize antigen-presenting EVs, trigger antigen captures and induce immune tolerance (Getts et al., 2012). A previous study showed that oligodendrocyte-derived EVs loaded with MBP and PLP induced monocyte-mediated tolerance of these myelin proteins, which mediated disease suppression, in an animal model of experimental autoimmune encephalomyelitis. In the light of the recent reports, the results of the present study could indicate that oligodendrocytes secrete EVs containing myelin proteins as a strategy to induce restoration of myelin proteins-specific peripheral immune tolerance during disease exacerbation as an attempt to suppress CNS autoimmunity in a myelin antigen-specific manner.

In conclusion, our study of oligodendrocyte, neural and immune cell origin, levels, size, and the protein content of blood-derived EVs in MS patients, point to these vesicles as promising specific biomarkers in MS. The size of T cell- and neuron-derived EVs are able to reflect disease activity, and the size of oligodendrocyte-derived EVs correlates with motor and cognitive impairment in MS patients. We also report the role of AMBP, FIBB, C1S, GELS, IGHG4, and TLN-1 proteins contained in EV subclasses of neural and immune origin as disease-specific biomarkers in MS. More specifically, AMBP, FIBB, GELS, IGHG4, MBP reflect the patient's disease activity. These proteins are involved in non-canonical functions of EVs in MS with a focus on three highly promising areas: regulation of complement system, ECM remodeling, brain repair and antigen tolerance; which may represent new avenues of research yet to be explored.

5. Study limitations

The limitations of this study include the differing sample sizes between the study groups. Using the G power 3.1 software, we estimated that for a power of 80% and a significance value of 0.05, the sample size for the healthy control and subcortical ischemic stroke groups would be smaller than for the rest of the groups, given that the former has a homogeneous disease population. However, the G power 3.1 software estimated a larger sample size for rheumatoid arthritis due to the disease's heterogeneity. In the MS group, the sample size had to be further increased to achieve relevant statistical power to perform the subanalysis of the clinical settings correlation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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Informed Consent Statement

All participants or their proxies provided their informed consent after a detailed explanation of the nature and purpose of this study

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2023.06.025.

References

Aletaha, D., Neogi, T., Silman, A.J., et al., 2010. 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum. 62 (9), 2569–2581. https://doi.org/10.1002/art.27584.

Asher, R.A., Morgenstern, D.A., Shearer, M.C., Adcock, K.H., Pesheva, P., Fawcett, J.W., 2002. Versican is upregulated in CNS injury and is a product of oligodendrocyte

lineage cells. J. Neurosci. 22 (6), 2225–2236. https://doi.org/10.1523/JNEUROSCI.22-06-02225.2002.
Bhargava, P., Nogueras-Ortiz, C., Kim, S., Delgado-Peraza, F., Calabresi, P.A., Kapogiannis, D., 2021. Synaptic and complement markers in extracellular vesicles in multiple sclerosis. Mult. Scler. 27 (4), 509–518. https://doi.org/10.1177/1352458520924590.
Bierhansl, L., Hartung, H.-P., Aktas, O., Ruck, T., Roden, M., Meuth, S.G., 2022. Thinking outside the box: non-canonical targets in multiple sclerosis. Nat. Rev. Drug Discov. 21 (8), 578–600. https://doi.org/10.1038/s41573-022-00477-5.
Bost, F., Diarra-Mehrpour, M., Martin, J.P., 1998. Inter-alpha-trypsin inhibitor proteoglycan family–a group of proteins binding and stabilizing the extracellular matrix. Eur. J. Biochem. 252 (3), 339–346. https://doi.org/10.1046/j.1432-1327.1998.2520339.x.
Coughlan, C., Bruce, K.D., Burgy, O., Boyd, T.D., Michel, C.R., Garcia-Perez, J.E., Adame, V., Anton, P., Bettcher, B.M., Chial, H.J., Königshoff, M., Hsieh, E.W.Y., Graner, M., Potter, H., 2020. Exosome isolation by ultracentrifugation and precipitation and techniques for downstream analyses. Curr. Protoc. Cell Biol. 88 (1), e110. https://doi.org/10.1002/cpcb.110.
Davalos, D., Kyu Ryu, J., Merlini, M., Baeten, K.M., Le Moan, N., Petersen, M.A., Deerinck, T.J., Smirnoff, D.S., Bedard, C., Hakozaki, H., Gonias Murray, S., Ling, J. B., Lassmann, H., Degen, J.L., Ellisman, M.H., Akassoglou, K., 2012. Fibrinogen-induced perivascular microglial clustering is required for the development of axonal damage in neuroinflammation. Nat. Commun. 3 (1) https://doi.org/10.1038/ncomms2230.
Debbi, L., Guo, S., Safina, D., Levenberg, S., 2022. Boosting extracellular vesicle secretion. Biotechnol. Adv. 59, 107983 https://doi.org/10.1016/j.biotechadv.2022.107983.
Degn, S.E., Jensenius, J.C., Bjerre, M., 2011. The lectin pathway and its implications in coagulation, infections and auto-immunity. *Curr Opin.* Organ Transplant. 16, 21–27. https://doi.org/10.1097/MOT.0b013e32834253df.
Dolcetti, E., Bruno, A., Guadalupi, L., Rizzo, F.R., Musella, A., Gentile, A., De Vito, F., Caioli, S., Bullitta, S., Fresegna, D., Vanni, V., Balletta, S., Sanna, K., Buttari, F., Stampanoni Bassi, M., Centonze, D., Mandolesi, G., 2020. Emerging Role of Extracellular Vesicles in the Pathophysiology of Multiple Sclerosis. Int. J. Mol. Sci. 21 (19), 7336. https://doi.org/10.3390/ijms21197336.
Eden, E., Navon, R., Steinfeld, I., Lipson, D., Yakhini, Z., 2009. GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. BMC Bioinf. 10 (1) https://doi.org/10.1186/1471-2105-10-48.
Figueiredo, S., 2011. Nine hole peg test (NHPT). Stroke Eng.
Friese, M.A., Schattling, B., Fugger, L., 2014. Mechanisms of neurodegeneration and axonal dysfunction in multiple sclerosis. Nat. Rev. Neurol. 10, 225–238. https://doi.org/10.1038/nrneurol.2014.37.
Galazka, G., Mycko, M.P., Selmaj, I., Raine, C.S., Selmaj, K.W., 2018. Multiple sclerosis: Serum-derived exosomes express myelin proteins. Mult. Scler. 24, 449–458. https://doi.org/10.1177/1352458517696597.
Geraci, F., Ragonese, P., Barreca, M.M., et al., 2018. Differences in intercellular communication during clinical relapse and gadolinium-enhanced MRI in patients with relapsing remitting multiple sclerosis: a study of the composition of extracellular vesicles in cerebrospinal fluid. Front. Cell. Neurosci. 12, 1–13. https://doi.org/10.3389/fncel.2018.00418.
Getts, D.R., Martin, A.J., McCarthy, D.P., Terry, R.L., Hunter, Z.N., Yap, W.T., Getts, M. T., Pleiss, M., Luo, X., King, N.JC., Shea, L.D., Miller, S.D., 2012. Microparticles bearing encephalitogenic peptides induce T-cell tolerance and ameliorate experimental autoimmune encephalomyelitis. Nat. Biotechnol. 30 (12), 1217–1224. https://doi.org/10.1038/nbt.2434.
Ghorbani, S., Yong, V.W., 2021. The extracellular matrix as modifier of neuroinflammation and remyelination in multiple sclerosis. Brain 144 (7), 1958–1973. https://doi.org/10.1093/brain/awab059.
Gutiérrez-Fernández, M., de la Cuesta, F., Tallón, A., Cuesta, I., Fernández-Fournier, M., Laso-García, F., Gómez-de Frutos, M.C., Díez-Tejedor, E., Otero-Ortega, L., 2021. Potential Roles of Extracellular Vesicles as Biomarkers and a Novel Treatment Approach in Multiple Sclerosis. Int. J. Mol. Sci. 22 (16), 9011. https://doi.org/10.3390/ijms22169011.
Haage, A., Goodwin, K., Whitewood, A., et al., 2018. Talin autoinhibition regulates Cell-ECM adhesion dynamics and wound healing in vivo. Cell Rep. 25 (9), 2401–2416.e5. https://doi.org/10.1016/j.celrep.2018.10.098.
Ingram, G., Hakobyan, S., Hirst, C.L., Harris, C.L., Pickersgill, T.P., Cossburn, M.D., Loveless, S., Robertson, N.P., Morgan, B.P., 2010. Complement regulator factor H as a serum biomarker of multiple sclerosis disease state. Brain 133 (6), 1602–1611. https://doi.org/10.1093/brain/awq085.
Jones, D.E., 2016. Early relapsing multiple sclerosis. Contin Lifelong Learn Neurol. 22 (3), 744–760. https://doi.org/10.1212/CON.0000000000000329.
Kwok, J.Y., Vaida, F., Augst, R.M., Yu, D.Y., Singh, K.K., 2011. Mannose binding lectin mediated complement pathway in multiple sclerosis. J. Neuroimmunol. 239 (1–2), 98–100. https://doi.org/10.1016/j.jneuroim.2011.08.018.
Malemud, C.J., 2006. Matrix metalloproteinases (MMPs) in health and disease: an overview. Front. Biosci. 11, 1696–1701. https://doi.org/10.2741/1915.
Manu, M.S., Hohjoh, H., Yamamura, T., 2021. Extracellular vesicles as pro- and anti-inflammatory mediators, biomarkers and potential therapeutic agents in multiple sclerosis. Aging Dis 12 (6), 1451. https://doi.org/10.14336/AD.2021.0513.
Marostica, G., Gelibter, S., Gironi, M., Nigro, A., Furlan, R., 2021. Extracellular vesicles in neuroinflammation. Front. Cell Dev. Biol. 8, 623039 https://doi.org/10.3389/fcell.2020.623039.
Mustapic, M., Eitan, E., Werner, J.K., Berkowitz, S.T., Lazaropoulos, M.P., Tran, J., Goetzl, E.J., Kapogiannis, D., 2017. Plasma extracellular vesicles enriched for neuronal origin: a potential window into brain pathologic processes. Front. Neurosci. 11 https://doi.org/10.3389/fnins.2017.00278.
Mycko, M.P., Baranzini, S.E., 2020. microRNA and exosome profiling in multiple sclerosis. Mult. Scler. 26 (5), 599–604. https://doi.org/10.1177/1352458519879303.
Olsson, M.G., Allhorn, M., Larsson, J., Cederlund, M., Lundqvist, K., Schmidtchen, A., Sørensen, O.E., Mörgelin, M., Åkerström, B.o., Chandra, D., 2011. Up-regulation of A1M/α1-microglobulin in skin by heme and reactive oxygen species gives protection from oxidative damage. PLoS One 6 (11), e27505. https://doi.org/10.1371/journal.pone.0027505.
Otero-Ortega, L., Alonso-López, E., Pérez-Mato, M., Laso-García, F., Gómez-de Frutos, M. C., Diekhorst, L., García-Bermejo, M.L., Conde-Moreno, E., Fuentes, B., de Leciñana, M.A., Bravo, S.B., Díez-Tejedor, E., Gutiérrez-Fernández, M., 2021. Circulating extracellular vesicle proteins and MicroRNA profiles in subcortical and cortical-subcortical ischaemic stroke. Biomedicines 9 (7), 786. https://doi.org/10.3390/biomedicines9070786.
Parmenter, B.A., Weinstock-Guttman, B., Garg, N., Munschauer, F., Benedict, R.HB., 2007. Screening for cognitive impairment in multiple sclerosis using the Symbol digit Modalities Test. Mult. Scler. 13 (1), 52–57. https://doi.org/10.1177/1352458506070750.
Paul, D.T., Dustin, E., Anushya, M., et al., 2022. PANTHER: making genome-scale phylogenetics accessible to all Protein. Society 31, 8–22. https://doi.org/10.1002/pro.4218.
Peñas-Martínez, J., Barrachina, M.N., Cuenca-Zamora, E.J., et al., 2021. Qualitative and quantitative comparison of plasma exosomes from neonates and adults. Int. J. Mol. Sci. 22 (4), 1–21. https://doi.org/10.3390/ijms22041926.
Pendlebury, D., Wang, R., Henin, R.D., Hockla, A., Soares, A.S., Madden, B.J., Kazanov, M.D., Radisky, E.S., 2014. Sequence and conformational specificity in substrate recognition: several human Kunitz protease inhibitor domains are specific substrates of mesotrypsin. J. Biol. Chem. 289 (47), 32783–32797. https://doi.org/10.1074/jbc.M114.609560.
Pereira, M., Rybarczyk, B.J., Odrljin, T.M., Hocking, D.C., Sottile, J., Simpson-Haidaris, P.J., 2002. The incorporation of fibrinogen into extracellular matrix is dependent on active assembly of a fibronectin matrix. J. Cell Sci. 115 (3), 609–617. https://doi.org/10.1242/jcs.115.3.609.
Petersen, M.A., Ryu, J.K., Chang, K.-J., Etxeberria, A., Bardehle, S., Mendiola, A.S., Kamau-Devers, W., Fancy, S.P.J., Thor, A., Bushong, E.A., Baeza-Raja, B., Syme, C. A., Wu, M.D., Rios Coronado, P.E., Meyer-Franke, A., Yahn, S., Pous, L., Lee, J.K., Schachtrup, C., Lassmann, H., Huang, E.J., Han, M.H., Absinta, M., Reich, D.S., Ellisman, M.H., Rowitch, D.H., Chan, J.R., Akassoglou, K., 2017. Fibrinogen activates BMP signaling in oligodendrocyte progenitor cells and inhibits remyelination after vascular damage. Neuron 96 (5), 1003–1012.e7. https://doi.org/10.1016/j.neuron.2017.10.008.
Pulous, F.E., Carnevale, J.C., Al-Yafeai, Z., Pearson, B.H., Hamilton, J.A.G., Henry, C.J., Orr, A.W., Petrich, B.G., 2021. Talin-dependent integrin activation is required for endothelial proliferation and postnatal angiogenesis. Angiogenesis 24 (1), 177–190. https://doi.org/10.1007/s10456-020-09756-4.
Ramos-Zaldívar, H.M., Polakovicova, I., Salas-Huenuleo, E., Corvalán, A.H., Kogan, M.J., Yefi, C.P., Andia, M.E., 2022. Extracellular vesicles through the blood-brain barrier: a review. Fluids Barriers CNS. 19 (1) https://doi.org/10.1186/s12987-022-00359-3.
Romantsik, O., Agyemang, A.A., Sveinsdóttir, S., Rutardóttir, S., Holmqvist, B.o., Cinthio, M., Mörgelin, M., Gumus, G., Karlsson, H., Hansson, S.R., Åkerström, B.o., Ley, D., Gram, M., 2019. The heme and radical scavenger α1-microglobulin (A1M) confers early protection of the immature brain following preterm intraventricular hemorrhage. J. Neuroinflamm. 16 (1) https://doi.org/10.1186/s12974-019-1486-4.
Sekine, H., Takahashi, M., Iwaki, D., et al., 2013. The role of MASP-1/3 in complement activation. Adv. Exp. Med. Biol. 735, 41–53. https://doi.org/10.1007/978-1-4614-4118-2_3.
Sobel, R.A., 1998. The extracellular matrix in multiple sclerosis lesions. J. Neuropathol. Exp. Neurol. 57, 205–217. https://doi.org/10.1097/00005072-199803000-00001.
Tan, C.L., Kwok, J.C., Heller, J.P., Zhao, R., Eva, R., Fawcett, J.W., 2015. Full length talin stimulates integrin activation and axon regeneration. Mol. Cell. Neurosci. 68, 1–8. https://doi.org/10.1016/j.mcn.2015.03.011.
Thompson, A.J., Banwell, B.L., Barkhof, F., Carroll, W.M., Coetzee, T., Comi, G., Correale, J., Fazekas, F., Filippi, M., Freedman, M.S., Fujihara, K., Galetta, S.L., Hartung, H.P., Kappos, L., Lublin, F.D., Marrie, R.A., Miller, A.E., Miller, D.H., Montalban, X., Mowry, E.M., Sorensen, P.S., Tintoré, M., Traboulsee, A.L., Trojano, M., Uitdehaag, B.M.J., Vukusic, S., Waubant, E., Weinshenker, B.G., Reingold, S.C., Cohen, J.A., 2018. Diagnosis of multiple sclerosis: 2017 revisions of the mcdonald criteria. Lancet Neurol 17 (2), 162–173. https://doi.org/10.1016/S1474-4422(17)30470-2.
Ulbrich, P., Khoshneviszadeh, M., Jandke, S., Schreiber, S., Dityatev, A., 2021. Interplay between perivascular and perineuronal extracellular matrix remodelling in neurological and psychiatric diseases. Eur. J. Neurosci. 53 (12), 3811–3830. https://doi.org/10.1111/ejn.14887.
Vandendriessche, C., Bruggeman, A., Van Cauwenberghe, C., Vandenbroucke, R.E., 2020. Extracellular vesicles in Alzheimer's and Parkinson's disease: small entities with large consequences. Cells 15, 2485. https://doi.org/10.3390/cells9112485.
Yates, R.L., Esiri, M.M., Palace, J., Jacobs, B., Perera, R., DeLuca, G.C., 2017. Fibrin (ogen) and neurodegeneration in the progressive multiple sclerosis cortex. Ann. Neurol. 82 (2), 259–270. https://doi.org/10.1002/ana.24997.
Zinger, A., Latham, S.L., Combes, V., Byrne, S., Barnett, M.H., Hawke, S., Grau, G.E., 2016. Plasma levels of endothelial and B-cell-derived microparticles are restored by fingolimod treatment in multiple sclerosis patients. Mult. Scler. 22 (14), 1883–1887. https://doi.org/10.1177/1352458516636959.