Szkoła Doktorska Nauk Ścisłych i Przyrodniczych
Uniwersytetu Łódzkiego

Karina Wasilewska

Charakterystyka kompleksów płytkowo-leukocytarnych i analiza ekspresji miRNA jako wskaźników stanu zapalnego i neurodegeneracji w rzutowo-remisyjnej i wtórnie postępującej postaci stwardnienia rozsianego

Characterization of Platelet-Leukocyte Complexes and Analysis of miRNA Expression as Indicators of Inflammation and Neurodegeneration in Relapsing-Remitting and Secondary Progressive Multiple Sclerosis

Praca doktorska wykonana
pod kierunkiem promotora
prof. dr hab. Joanny Saluk
oraz promotora pomocniczego
dr Angeli Dziedzic
w Katedrze Biochemii Ogólnej
Wydziału Biologii i Ochrony Środowiska

Łódź 2025

Dla Mamy Wioletty i Taty Grzegorza

Składam serdeczne podziękowania mojej Promotorce,
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mnie swoją pewnością w działaniu.

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SPIS TREŚCI

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Badania przeprowadzone w ramach niniejszej rozprawy doktorskiej zostały sfinansowane z następujących źródeł:

OPUS 16

Grant badawczy OPUS 16 nr UMO-2018/31/B/NZ4/02688 finansowany przez Narodowe Centrum Nauki, pt. „Badanie mechanizmów komunikacji międzykomórkowej, odpowiedzialnych za zależną od płytek krwi regulację odporności nabytej w stwardnieniu rozsianym".

Kierownik projektu: prof. dr hab. Joanna Saluk.

NARODOWE CENTRUM NAUKI

IDUB #UniLodz

Grant badawczy UŁ IDUB #UniLodz nr 65/2021, pt. „Analiza wybranych osoczowych cząsteczek miRNA w celu wytypowania potencjalnych markerów parametru NEDA w stwardnieniu rozsianym".

Kierownik projektu: prof. dr hab. Joanna Saluk.

UNIWERSYTET ŁÓDZKI

Szkoła Doktorska Nauk Ścisłych i Przyrodniczych UŁ

Dofinansowania działalności naukowej doktorantów Szkoły Doktorskiej Nauk Ścisłych i Przyrodniczych UŁ w latach 2021–2024.

SZKOŁA DOKTORSKA NAUK ŚCISŁYCH I PRZYRODNICZYCH
Uniwersytet Łódzki

WSPÓŁPRACE

Część badań przedstawionych w cyklu złożonym z opublikowanych artykułów i przesłanego do recenzji manuskryptu, stanowiącym podstawę niniejszej rozprawy doktorskiej, przeprowadzono we współpracy z poniższymi jednostkami naukowymi:

University of Bergen

- Neuro-SysMed – Centre For Clinical Treatment Research, Department of Neurology, Haukeland University Hospital, Bergen, Norwegia.

Współpraca w ramach programu wyjazdów zagranicznych ERASMUS+ PRAKTYKI 2023/2024.

UNIVERSITY OF BERGEN

Uniwersytet Medyczny w Łodzi

- Uniwersyteckie Laboratorium Naukowe CoreLab
- Klinika Rehabilitacji Neurologicznej, Wydział Nauk o Zdrowiu

UNIWERSYTET MEDYCZNY W ŁODZI

Instytut Immunologii i Terapii Doświadczalnej im. Ludwika Hirszfelda Polskiej Akademii Nauk

- Laboratorium Genomiki i Bioinformatyki

DOROBEK NAUKOWY

| Artykuły wchodzące w skład rozprawy doktorskiej

Na niniejszą rozprawę doktorską składa się cykl powiązanych tematycznie prac naukowych złożony z czterech opublikowanych artykułów oraz jednego manuskryptu, który został wysłany do recenzowanego czasopisma naukowego. Wartość współczynnika wpływu Impact Factor (IF) oraz liczbę punktów Ministerstwa Nauki i Szkolnictwa Wyższego (MNiSW) dla poszczególnych publikacji wskazano zgodnie z rokiem ich opublikowania.

Publikacje przeglądowe

1. **Maciak K**, Dziedzic A, Miller E, Saluk-Bijak J. "miR-155 as an Important Regulator of Multiple Sclerosis Pathogenesis. A Review". *Int. J. Mol. Sci.* 2021, 22(9), 4332

   Punkty MNiSW = 140; IF = 6,208

2. **Maciak K**, Dziedzic A, Saluk J. "Remyelination from the miRNA perspective". *Front Mol Neurosci.* 2023, 16:1199313

   Punkty MNiSW = 140; IF = 3,5

3. Anandan S, **Maciak K**, Breinbauer R, Mostafavi S, Kvistad CE, Torkildsen O, Myhr KM. "Brain-derived blood biomarkers in multiple sclerosis-current trends and beyond". *Front Immunol.* 2025, 16:1569503

   Punkty MNiSW = 140; IF = 5,9

Publikacje doświadczalne

1. **Maciak K**, Dziedzic A, Szymański J, Studzian M, Redlicka J, Miller E, Michlewska S, Jóźwiak P, Saluk J. "Human B-cells can form Hetero-aggregates with Blood Platelets: A Novel Insight into Adaptive Immunity Regulation in Multiple Sclerosis". *J Mol Biol.* 2025, 437(2):168885

   Punkty MNiSW = 140; IF = 4,5

2. Anandan S, **Maciak K**, Breinbauer R, Otero-Ortega L, Feliciello G, Stojanović Gužvić N, Torkildsen O, Myhr KM. "In-Depth Characterization of L1CAM^{+} Extracellular Vesicles as Potential Biomarkers for Anti-CD20 Therapy Response in Relapsing–Remitting Multiple Sclerosis". *Int. J. Mol. Sci.* 2025, 26(15), 7213

   Punkty MNiSW = 140; IF = 4,9

Nieopublikowany manuskrypt

1. **Wasilewska K**, Dziedzic A, Anandan S, Miller E, Łaczmański Ł, Zajdel R, Michlewska S, Kujawa D, Gancarek M, Raczkowska J, Włodarczyk L, Nowak P, Saluk J. "Extracellular vesicle-derived miR-760 as a novel candidate marker differentiating stable RRMS from SPMS".

Sumaryczna liczba punktów MNiSW cyklu publikacji: **700**

Sumaryczny IF cyklu publikacji: **25,008**

| Pozostałe artykuły stanowiące dorobek naukowy

1. **Maciak K**, Pietrasik S, Dziedzic A, Redlicka J, Saluk-Bijak J, Bijak M, Włodarczyk T, Miller E. "Th17-Related Cytokines as Potential Discriminatory Markers between Neuromyelitis Optica (Devic's Disease) and Multiple Sclerosis-A Review". *Int J Mol Sci.* 2021, 22(16):8946

   Punkty MNiSW = 140, IF = 6,208

2. Szelenberger R, Karbownik MS, Kacprzak M, **Maciak K**, Bijak M, Zielińska M, Czarny P, Śliwiński T, Saluk-Bijak J. "Screening Analysis of Platelet miRNA Profile Revealed miR-142-3p as a Potential Biomarker in Modeling the Risk of Acute Coronary Syndrome". Cells. 2021, 10(12):3526

   Punkty MNiSW = 140, IF = 7,666

3. **Maciak K**, Dziedzic A, Saluk J. "Possible role of the NLRP3 inflammasome and the gut-brain axis in multiple sclerosis-related depression". *FASEB J.* 2023, 37(1):e22687

   Punkty MNiSW = 140, IF = 4,4

4. Pogoda-Wesołowska A, Dziedzic A, **Maciak K**, Stępień A, Dziaduch M, Saluk J. "Neurodegeneration and its potential markers in the diagnosing of secondary progressive multiple sclerosis. A review". *Front Mol Neurosci.* 2023, 12;16:1210091

   Punkty MNiSW = 140, IF = 3,5

5. Dziedzic A, **Maciak K**, Miller ED, Starosta M, Saluk J. "Targeting Vascular Impairment, Neuroinflammation, and Oxidative Stress Dynamics with Whole-Body Cryotherapy in Multiple Sclerosis Treatment". *Int J Mol Sci.* 2024, 25(7):3858

   Punkty MNiSW = 140, IF = 4,9

6. Dziedzic A, **Maciak K**, Bliźniewska-Kowalska K, Gałecka M, Kobierecka W, Saluk J. "The Power of Psychobiotics in Depression: A Modern Approach through the Microbiota-Gut-Brain Axis: A Literature Review". *Nutrients.* 2024, 6(7):1054

   Punkty MNiSW = 140, IF = 5,0

Sumaryczna liczba punktów MNiSW publikacji stanowiących pozostały dorobek naukowy: **840**
Sumaryczny IF publikacji stanowiących pozostały dorobek naukowy: **31,674**

Sumaryczna liczba punktów MNiSW publikacji stanowiących całkowity dorobek naukowy: **1540**
Sumaryczny IF publikacji stanowiących całkowity dorobek naukowy: **56,682**
H-index: **7**
Liczba cytowań bez autocytowań wg bazy Web of Science: **156**

| Doniesienia konferencyjne

1. **Maciak K**, Sikorska A, Okła E, Sadzińska K, Słoma K, Rusek P, Sęczkowska K, Rudnicka K, Chmiela M. „β-glucan hamuje adhezję pałeczek *Helicobacter pylori* do komórek żołądka – wstępne wnioski z badań *in vitro*"

   POSTER | II Sesja Młodych Mikrobiologów Środowiska Łódzkiego Łódź, 08.06.2018 r.

2. **Maciak K**, Drzewiecka D. "Competition between *Proteus mirabilis* clinical strains belonging to O77 and O78 serogroups"

   POSTER | National Scientific Conference "Knowledge – Key to Success" Toruń, 19.01.2019 r.

3. **Maciak K**, Drzewiecka D. „Typ antygenu O nie warunkuje przewagi w konkurencji szczepów klinicznych *Proteus mirabilis*"

   POSTER | III Sesja Młodych Mikrobiologów Środowiska Łódzkiego Łódź, 07.06.2019 r.

4. Drzewiecka D, **Maciak K**, Szczerbiec D. "Antagonism and self-competition between clinical *Proteus mirabilis* strains"

   POSTER | 8th International Weigl Conference "Human Welfare and Infectious Diseases in a New Microbiome Research Era. Microorganisms in industrial and medical biotechnology" | Łódź, 26-28.06.2019 r.

5. **Maciak K**, Saluk-Bijak J. „miR-155 jako potencjalny biomarker patogenezy stwardnienia rozsianego"

   REFERAT | Ogólnopolska Konferencja Naukowa „Choroby neurodegeneracyjne – objawy, diagnostyka, leczenie" | Online, 18.06.2021 r.

6. **Maciak K**, Dziedzic A, Saluk-Bijak J. "Immunophenotyping and analysis of mutual interactions of platelet-leukocyte aggregates in secondary-progressive multiple sclerosis".

   POSTER | The FEBS-IUBMB-PABMB 2022 Congress: The Biochemistry Global Summit | Lizbona, 09-14.07.2022 r.

7. **Maciak K**, Dziedzic A, Saluk J. “Pro-thrombotic and pro-inflammatory phenotype of blood platelets in secondary progressive multiple sclerosis”

   REFERAT | International Medical Congress of Silesia (SIMC 2023) Katowice, 17-19.05.2023 r.

   2 nagroda w sesji “Experimental Medicine Session”

8. **Maciak K**, Dziedzic A, Saluk J. “Platelet-lymphocytic co-activation in secondary progressive multiple sclerosis”

   POSTER | MSMilan2023 – The 9th Joint ECTRIMS-ACTRIMS Meeting Mediolan, 11-13.10.2023 r.

9. **Maciak K**, Saluk J, Dziedzic A. “Are B-cells an unfairly missing player in the studies of platelet-leukocyte cross-talk in multiple sclerosis?”

   REFERAT | VI Zjazd Naukowy Polskiego Towarzystwa Biologii Medycznej Warszawa, 19-21.09.2024 r.

10. Anandan S, **Maciak K**, Breinbauer R, Otero-Ortega L, Torkildsen O, and Myhr KM. “Brain-derived blood extracellular vesicles as potential biomarkers in multiple sclerosis (MS): Pilot results from relapsing MS patients receiving anti-CD20 therapy”

    POSTER | MEDPOSTDOC program, MED faculty | Voss, 16-17.01.2025 r.

11. Anandan S, **Maciak K**, Breinbauer R, Otero-Ortega L, Torkildsen O, and Myhr KM. “Brain-derived blood extracellular vesicles as potential biomarkers in multiple sclerosis (MS): Pilot results from relapsing MS patients receiving anti-CD20 therapy”

    REFERAT | 19th Annual Research Presentations-Research School in Clinical Medicine | Bergen, 29-31.01.2025 r.

12. Anandan S, **Maciak K**, Breinbauer R, Otero-Ortega L, Torkildsen O, and Myhr KM. “Brain-derived blood extracellular vesicles as potential biomarkers in multiple sclerosis (MS): Pilot results from relapsing MS patients receiving anti-CD20 therapy”

    REFERAT | OddFellows Research Funds - Annual Steering Group Meeting Oslo, 02.03.2025 r.

13. Nowak P, **Maciak K**, Dziedzic A, Saluk J. “miR-98 and miR-760 as potential regulators of IL-17 mediated pathophysiology in multiple sclerosis”

    REFERAT | BioOpen – 10th International Doctoral Students' Conference in Life Sciences | Łódź, 15-16.05.2025 r.

14. Anandan S, **Maciak K**, Breinbauer R, Otero-Ortega L, Torkildsen O, and Myhr KM. “Brain-derived blood extracellular vesicles as potential biomarkers in multiple sclerosis (MS): Pilot results from relapsing MS patients receiving anti-CD20 therapy”

    REFERAT | BBB Seminar - Technicians Day at Institute of Biomedicine, UiB Bergen 22.05.2025 r.

15. Anandan S, **Maciak K**, Breinbauer R, Otero-Ortega L, Torkildsen O, and Myhr KM. “Brain-derived blood extracellular vesicles as potential biomarkers in multiple sclerosis (MS): Pilot results from relapsing MS patients receiving anti-CD20 therapy”

    REFERAT | Annual Meeting of NOR-EV Society | Oslo, 04-05.06.2025 r.

16. Anandan S, **Maciak K**, Breinbauer R, Otero-Ortega L, Torkildsen O, and Myhr KM. “Brain-derived blood extracellular vesicles as potential biomarkers in multiple sclerosis (MS): Pilot results from relapsing MS patients receiving anti-CD20 therapy”

    REFERAT | Hormone Research Laboratory, UiB | Bergen, 12.09.2025 r.

| Nagrody

1. Nagroda Rektora dla Doktorantów Uniwersytetu Łódzkiego za aktywne uczestnictwo w życiu Uczelni w roku akademickim 2020/2021 (semestr letni).
2. Nagroda Rektora dla Doktorantów Uniwersytetu Łódzkiego za aktywne uczestnictwo w życiu Uczelni w roku akademickim 2021/2022 (semestr zimowy).
3. Nagroda Rektora dla Doktorantów Uniwersytetu Łódzkiego za aktywne uczestnictwo w życiu Uczelni w roku akademickim 2021/2022 (semestr letni).
4. Nagroda Rektora dla Doktorantów Uniwersytetu Łódzkiego za aktywne uczestnictwo w życiu Uczelni w roku akademickim 2022/2023 (semestr zimowy).
5. Nagroda Specjalna Rektora dla członków Uczelnianej Rady Samorządu Doktorantów UŁ na zakończenie kadencji 2020/2022.

| Projekty

1. Kierownik projektu „Analiza współzawodnictwa pomiędzy szczepami klinicznymi *Proteus mirabilis* w odniesieniu do ich serotypu O” finansowanego ze środków konkursu Studenckie Granty Badawcze Uniwersytetu Łódzkiego.
2. Wykonawca w projekcie „Wzmocnienie bezpieczeństwa w zakresie CBRNE – koordynacja i standaryzacja” (nr 7/NMF/CBRNE/2020) finansowanym ze środków Norweskiego Mechanizmu Finansowego 2014-2021. Kierownik projektu: dr Marcin Niemcewicz.
3. Wykonawca w projekcie „Badanie mechanizmów komunikacji międzykomórkowej, odpowiedzialnych za zależną od płytek krwi regulację odporności nabytej w stwardnieniu rozsianym” (nr UMO-2018/31/B/NZ4/02688) finansowanym ze środków Narodowego Centrum Nauki w ramach grantu OPUS 16. Kierownik projektu: prof. dr hab. Joanna Saluk.
4. Wykonawca w projekcie „Analiza wybranych osoczowych cząsteczek miRNA w celu wytypowania potencjalnych markerów parametru NEDA w stwardnieniu rozsianym” (nr 65/2021) finansowanym ze środków konkursu IDUB #UniLodz. Kierownik projektu: prof. dr hab. Joanna Saluk.

| Szkolenia

1. „Jak uzyskać wiarygodne wyniki real-time PCR oraz digital PCR?" | ThermoFisher Scientific | 15.09.2022 r.
2. "Cytometry today and tomorrow. School of Cytometry" | BD Biosciences | 19.10.2022 r.
3. „Hodowla komórkowa – kurs praktyczny" | Centrum Medyczne Kształcenia Podyplomowego w Warszawie | 31.01-03.02.2023 r.
4. „Charakterystyka białek: elektroforeza SDS PAGE i Western blotting" | Centrum Medyczne Kształcenia Podyplomowego w Warszawie | 01-03.03.2023 r.
5. "SDS-PAGE, Multiplex Western Blot and Stain-Free Protein Gel Visualization using Mini-Protean Tetra Cell, Trans-Blot Turbo and ChemiDoc MP Systems" | Bio-Rad Laboratories | 09-10.03.2023 r.
6. „Projektowanie starterów i sond do PCR i qPCR" | MBS | 24.03.2023 r.
7. ICH Good Clinical Practice (GCP) E6 R3 | Stowarzyszenie na Rzecz Dobrej Praktyki Badań Klinicznych w Polsce (GCPpl) | 14.07.2025 r.

| Działalność organizacyjna

1. Członkini Uczelnianej Rady Samorządu Doktorantów UŁ | kadencja 2020/2022
2. Członkini Komitetu Naukowego VI Ogólnopolskiej Konferencji Doktorantów Nauk o Życiu BioOpen | 15-16.04.2021 r.
3. Organizacja i promocja IV Dnia Doktoranta UŁ | 11.06.2021 r.
4. Członkini Uczelnianej Komisji ds. przyznawania miejsc w Domach Studenckich dla Doktorantów UŁ | rok akademicki 2021/2022 oraz 2022/2023
5. Członkini Uczelnianej Komisji Stypendialno-Socjalnej Doktorantów UŁ | rok akademicki 2021/2022 oraz 2022/2023
6. Przewodnicząca Komitetu Organizacyjnego VII i VIII Ogólnopolskiej Konferencji Doktorantów Nauk o Życiu BioOpen | 07-08.04.2022 r. oraz 13-14.04.2023 r.
7. Organizacja i promocja V Dnia Doktoranta UŁ | 30.06.2022 r.
8. Wiceprzewodnicząca Komitetu Organizacyjnego IX Ogólnopolskiej Konferencji Doktorantów Nauk o Życiu BioOpen | 11-12.04.2024 r.
9. Członkini Komitetu Organizacyjnego konferencji 10th International Doctoral Students' Conference in Life Sciences | 15-16.05.2025 r.

| Stowarzyszenia

1. Polskie Towarzystwo Biochemiczne | 01.2023 r. – obecnie
2. Stowarzyszenie na Rzecz Dobrej Praktyki Badań Klinicznych w Polsce (GCPpl) | 07.2025 r. – obecnie

STRESZCZENIE

Stwardnienie rozsiane (ang. *multiple sclerosis*, MS) jest przewlekłą chorobą neurodegeneracyjną o podłożu autoimmunizacyjnym. Schorzenie obejmuje głównie ośrodkowy układ nerwowy (ang. *central nervous system*, CNS), prowadząc do ogniskowych i rozsianych uszkodzeń w mózgu i rdzeniu kręgowym. Rozwój MS napędzany jest poprzez utrzymującą się reakcję zapalną z udziałem autoreaktywnych limfocytów T i B skierowanych przeciwko antygenom mieliny. Proces zapalny w CNS jest nasilany przez zwiększoną przepuszczalność uszkodzonej bariery krew-mózg (ang. *blood-brain barrier*, BBB), z racji czego patofizjologia MS obejmuje nie tylko podłoże neurozapalne, ale także naczyniowe.

MS jest schorzeniem niejednorodnym, trudnym do sklasyfikowania, o zróżnicowanym przebiegu i zmiennym fenotypie. Najczęściej występującą formą choroby jest zapalna postać rzutowo-remisyjna, (ang. *relapsing-remitting multiple sclerosis*, RRMS), z kolei postać wtórnie postępująca (ang. *secondary progressive multiple sclerosis*, SPMS), rozwijająca się zwykle w następstwie RRMS, wiąże się z postępującymi procesami neurodegeneracyjnymi, bez okresów rzutów i remisji. Ostatnie doniesienia wskazują jednak na współistnienie zarówno cech zapalnych, jak i neurodegeneracyjnych w obu fenotypach choroby, co utrudnia wczesne różnicowanie, decyzje terapeutyczne i prognozowanie postępu schorzenia.

Nadrzędnym celem badań podjętych w niniejszej rozprawie doktorskiej była identyfikacja markerów umożliwiających różnicowanie fenotypów choroby u pacjentów z RRMS w fazie remisji oraz SPMS. W pierwszym etapie badań scharakteryzowano agregaty płytkowo-leukocytarne (ang. *platelet-leukocyte hetero-aggregates*, PLAs) jako element łączący patogenezę uszkodzeń naczyniowych i rozwój procesu zapalnego. Z wykorzystaniem testu migracji, obrazowania mikroskopowego i cytometrii przepływowej wykazano w MS zwiększoną chemotaksję leukocytów w kierunku płytek krwi i tworzenie PLAs, wśród których dominowały kompleksy z udziałem limfocytów B. Dodatkowo, stwierdzono prawdopodobną rolę osi CD40-CD40L w tworzeniu PLAs, odnotowując istotną korelację między ekspresją płytkowego CD40L i limfocytarnego CD40, najsilniej wyrażoną w badaniu koekspresji tych antygenów na płytkach krwi i komórkach B w SPMS.

W drugim etapie badań przeprowadzono przesiewową analizę różnicowej ekspresji cząsteczek mikroRNA (ang. *microRNA*, miRNA) pochodzących z pęcherzyków zewnątrzkomórkowych (ang. *extracellular vesicles*, EVs) metodą sekwencjonowania RNA, walidację wyników za pomocą RT-qPCR, a także oznaczenie stężenia osoczowych cytokin zapalnych i markerów uszkodzeń

neuronów/gleju przy użyciu odpowiednio systemu Bio-Plex oraz techniki ELISA. Następnie wykonano integracyjną analizę bioinformatyczną wyników. Zidentyfikowano cztery miRNA różnicujące RRMS i SPMS (miR-760, miR-98-5p, miR-301a-3p, miR-223-3p), przy czym miR-760 okazał się najsilniejszym pojedynczym predyktorem klasyfikacji do fenotypu RRMS. W SPMS odnotowano swoiste korelacje – między miR-760 a interleukiną (ang. *interleukin*, IL) 4 i IL-17 oraz między miR-98-5p a IL-17. Model łączący ekspresję miRNA z poziomem podstawowego czynnika wzrostu fibroblastów (ang. *fibroblast growth factor basic*, FGF basic) uzyskał AUC 0,97 (czułość 93,3%, swoistość 90%), potwierdzając wysoką zdolność dyskryminacji RRMS wobec SPMS.

W trzecim etapie kompleksowo scharakteryzowano EVs prezentujące cząsteczkę adhezyjną L1 (ang. *L1 cell adhesion molecule*, L1CAM) w surowicy i w płynie mózgowo-rdzeniowym (ang. *cerebrospinal fluid*, CSF). L1CAM$^+$ jest białkiem wykorzystywanym w celu wzbogacenia frakcji EVs pochodzenia neuronalnego podczas ich izolacji. Badania przeprowadzono pod kątem rozmiaru pęcherzyków, ich stężenia, morfologii, ładunku białkowego i fenotypu antygenów powierzchniowych. W analizach, oceniono przydatność L1CAM$^+$ EVs jako dynamicznych biomarkerów do monitorowania odpowiedzi na leczenie rytuksymabem, monoklonalnym przeciwciałem anty-CD20, u pacjentów z RRMS. Na tej podstawie uzyskano odmienne profile immunologiczne przed i po terapii, co podkreśla potencjał L1CAM$^+$ EVs w monitorowaniu leczenia immunosupresyjnego, skierowanego na deplecję limfocytów B.

Podsumowując, zrealizowane w ramach niniejszej pracy doktorskiej badania poszerzają wiedzę na temat mechanizmów patofizjologicznych MS, łącząc odpowiedź immunologiczną zależną od limfocytów z uszkodzeniami naczyniowymi. Ponadto, praca identyfikuje możliwości zastosowania potencjalnych nieinwazyjnych biomarkerów służących różnicowaniu RRMS i SPMS oraz monitorowaniu immunopatologii CNS i odpowiedzi na leczenie.

ABSTRACT

Multiple sclerosis (MS) is a chronic neurodegenerative disease characterized by an autoimmune background. The condition primarily affects the central nervous system (CNS), leading to focal and disseminated lesions in the brain and spinal cord. The development of MS is driven by a persistent inflammatory response involving autoreactive T and B-cells directed against myelin antigens. The inflammatory process in the CNS is exacerbated by increased permeability of a disrupted blood-brain barrier (BBB), which is why the pathophysiology of MS involves not only neuroinflammatory but also vascular factors.

MS is a heterogeneous disease, difficult to categorize, with a varied course and variable phenotype. The most common form of the disease is the inflammatory relapsing-remitting MS (RRMS), whereas the secondary progressive MS (SPMS), which typically develops after RRMS, is characterized by progressive neurodegenerative processes without periods of relapses and remissions. However, recent reports indicate the coexistence of inflammatory and neurodegenerative features in both phenotypes, which complicates early differentiation, therapeutic decision-making, and prognosis of disease progression.

The primary objective of the research undertaken in this doctoral dissertation was to identify markers enabling the differentiation of disease phenotypes in patients with RRMS in remission and SPMS. In the first stage of the study platelet-leukocyte hetero-aggregates (PLAs) were characterized as elements linking vascular injury pathogenesis with inflammatory process development. Using migration assays, microscopic imaging, and flow cytometry, increased leukocyte chemotaxis toward platelets and the formation of PLAs, predominantly involving B-cells, were demonstrated in MS. Moreover, a potential role of the CD40-CD40L axis in the formation of PLAs was identified, with a significant correlation between platelet CD40L and lymphocyte CD40, the strongest in the analysis of co-expression of these antigens on platelets and B-cells in SPMS.

In the second stage of the study a screening analysis of the differential expression of microRNA (miRNA) originating from extracellular vesicles (EVs) using RNA sequencing was conducted, followed by a validation of the results using RT-qPCR, determination of the concentration of plasma inflammatory cytokines and markers of neuronal/glial damage using the Bio-Plex system and ELISA technique, respectively. This was followed by an integrative bioinformatic analysis of the results. Four miRNAs differentiating RRMS and SPMS were identified (miR-760, miR-98-5p, miR-301a-3p, miR-223-3p), with miR-760 emerging as the strongest single predictor of classification into

the RRMS phenotype. In SPMS, specific correlations were observed between miR-760 and both interleukin (IL) 4 and IL-17, as well as between miR-98-5p and IL-17. A model combining miRNA expression with the level of basic fibroblast growth factor (FGF basic) achieved an AUC of 0.97 (sensitivity 93.3%, specificity 90%), confirming its high ability to discriminate RRMS vs SPMS.

In the third stage, EVs presenting the L1 cell adhesion molecule (L1CAM) in serum and cerebrospinal fluid (CSF) were comprehensively characterized. $L1CAM^+$ is a protein used to enrich the neuronal fraction of EVs during their isolation. The studies were conducted in terms of vesicle size, concentration, morphology, protein cargo, and surface antigens phenotype. Analyses evaluated the usefulness of $L1CAM^+$ EVs as dynamic biomarkers for monitoring response to rituximab treatment, an anti-CD20 monoclonal antibody, in RRMS patients. On this basis, different immune profiles were obtained before and after therapy, highlighting the potential of $L1CAM^+$ EVs in monitoring immunosuppressive treatment targeting B-cell depletion.

In summary, the research conducted in this doctoral thesis expands our understanding of the pathophysiological mechanisms of MS, linking the lymphocyte-dependent immune response with vascular injury. Furthermore, the study identifies opportunities for the use of potential non-invasive biomarkers to differentiate RRMS and SPMS, as well as and to monitor CNS immunopathology and treatment response.

WYKAZ SKRÓTÓW

AKT (ang. *protein kinase B*)

AMPK (ang. *AMP-activated protein kinase*)

ANXA2 – aneksyna 2 (ang. *annexin 2*)

AUC (ang. *area under the curve*)

BBB – bariera krew-mózg (ang. *blood-brain barrier*)

BDNF – neurotroficzny czynnik pochodzenia mózgowego (ang. *brain-derived neurotrophic factor*)

BTK – kinaza Brutona (ang. *Bruton's kinase*)

C/EBPβ (ang. *CCAAT/enhancer-binding protein beta*)

CCL2 (ang. *C-C motif chemokine ligand 2*)

CCL5 (ang. *C-C motif chemokine ligand 5*)

Ccnd2 – cykliny D2 (ang. *cyclin D2*)

CCR5 (ang. *C-C chemokine receptor 5*)

CCR7 (ang. *C-C chemokine receptor 7*)

cDNA – komplementarny DNA (ang. *complementary DNA*)

CHI3L1 (ang. *chitinase-3-like protein 1*)

CHIT1 – chitotriozydaza (ang. *chitotriosidase*)

CIS – zespół klinicznie izolowany (ang. *clinically isolated syndrome*)

CLDN-1 – klaudyna 1 (ang. *claudin-1*)

CNS – ośrodkowy układ nerwowy (ang. *central nervous system*)

CPDA-1 – cytrynian fosforanu deoksyrybozy adeniny 1 (ang. *citrate phosphate dextrose adeninę 1*)

CSF – płyn mózgowo-rdzeniowy (ang. *cerebrospinal fluid*)

CSF1 (ang. *colony-stimulating factor 1*)

CXCL13 (ang. *C-X-C motif chemokine ligand 13*)

DLS – dynamiczne rozpraszanie światła (ang. *dynamic light scattering*)

DMTs – terapie modyfikujące przebieg choroby (ang. *disease modyfying therapies*)

DOCK-1 (ang. *dedicator of cytokinesis 1*)

EAE – eksperymentalne autoimmunizacyjne zapalenie mózgu i rdzenia kręgowego (ang. *experimental autoimmune encephalomyelitis*)

EBNA1 (ang. *Epstein-Barr nuclear antigen 1*)

EBV – wirus Epsteina-Barr (ang. *Epstein-Barr virus*)

ErbB (ang. *erythroblastic leukemia viral oncogene homolog*)

ERK (ang. *extracellular signal-regulated kinase*)

EVs – pęcherzyki zewnątrzkomórkowe (ang. *extracellular vesicles*)

FC – krotność zmiany (ang. *fold change*)

FGF basic – podstawowy czynnik wzrostu fibroblastów (ang. *fibroblast growth factor basic*)

FoxO (ang. *forkhead box O*)

GDA – powiązanie „gen-choroba" (ang. *gene-disease association*)

GFAP – kwaśne białko włókienkowe gleju (ang. *glial fibrillary acid protein*)

GLAST – transporter glutaminianu i asparaginianu (ang. *glutamate aspartate transporter*)

GM-CSF – czynnik stymulujący tworzenie kolonii granulocytów i makrofagów (ang. *granulocyte-macrophage colony-stimulating factor*)

GO (ang. *gene ontology*)

GP – glikoproteina (ang. *glycoprotein*)

Hes5 (ang. *hairy and enhancer of split-5*)

ICAM-1 – międzykomórkowa cząsteczka adhezyjna (ang. *intercellular adhesion molecule-1*)

IFN-γ – interferon (ang. *interferon gamma*)

IgG OCB – prążki oligoklonalne immunoglobulin G (ang. *immunoglobulin G oligoclonal bands*)

IL – interleukina (ang. *interleukin*)

IL-1ra – antagonista receptora interleukiny 1 (ang. *interleukin-1 receptor antagonist*)

KEGG (ang. *Kyoto Encyclopedia of Genes and Genomes*)

L1CAM – cząsteczka adhezyjna L1 (ang. *L1 cell adhesion molecule*)

MAP2K7 (ang. *mitogen-activated protein kinase kinase 7*)

MAPK (ang. *mitogen-activated protein kinase*)

MDSCs – mieloidalne komórki supresorowe (ang. *myeloid-derived suppressor cells*)

MIP-1α – białko zapalne makrofagów 1α (ang. *macrophage inflammatory protein-1 alpha*)

MIP-1β – białko zapalne makrofagów 1β (ang. *macrophage inflammatory protein-1 beta*)

miRNA – mikroRNA (ang. *microRNA*)

MOG – glikoproteina mieliny oligodendrocytów (ang. *myelin oligodendrocyte glycoprotein*)

MRI – obrazowanie za pomocą rezonansu magnetycznego (ang. *magnetic resonance imaging*)

MS – stwardnienie rozsiane (ang. *multiple sclerosis*)

mTOR (ang. *mechanistic target of rapamycin*)

NF-κB (ang. *nuclear factor kappa-light-chain-enhancer of activated B cells*)

NfL – neurofilament lekki (ang. *neurofilament light chain*)

NGS – sekwencjonowanie nowej generacji (ang. *next generation sequencing*)

NTA – analiza śledzenia nanocząstek (ang. *nanoparticle tracking analysis*)

OPCs – prekursory oligodendrocytów (ang. *oligodendrocyte progenitor cells*)

PBMCs – jednojądrzaste komórki krwi obwodowej (ang. *peripheral blood mononuclear cells*)

PDGFRα – receptor czynnika wzrostu pochodzącego z płytek krwi α (ang. *platelet-derived growth factor receptor alpha*)

PEA (ang. *proximity extension assay*)

PI3K (ang. *phosphoinositide 3-kinase*)

PIK3CA (ang. *phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha*)

PIK3R1 (ang. *phosphoinositide-3-kinase regulatory subunit*)

PLAs – agregaty płytkowo-leukocytarne (ang. *platelet-leukocyte aggregates*)

PPMS – pierwotnie postępujące stwardnienie rozsiane (ang. *primary progressive multiple sclerosis*)

RANTES (ang. *regulated upon activation, normal T cell expressed and secreted*)

ROC (ang. *receiver operating characteristic*)

RORγt (ang. *RAR-related orphan receptor gamma t*)

RRMS – rzutowo-remisyjne stwardnienie rozsiane (ang. *relapsing-remitting multiple sclerosis*)

S1PR1 (ang. *sphingosine-1-phosphate receptor 1*)

S1PR5 (ang. *sphingosine-1-phosphate receptor 5*)

SDCBP (ang. *syndecan binding protein*)

SEM – skaningowa mikroskopia elektronowa (ang. *scanning electron microscopy*)

SHIP1 (ang. *SH2-containing inositol phosphatase 1*)

SOCS1 – supresor sygnalizacji cytokinowej 1 (ang. *suppressor of cytokine signaling 1*)

Sox (ang. *SRY-box transcription factor*)

SPMS – wtórnie postępujące stwardnienie rozsiane (ang. *secondary progressive multiple sclerosis*)

STAT3 (ang. *signal transducer and activator of transcription 3*)

sTREM2 (ang. *soluble triggering receptor expressed on myeloid cells 2*)

Tcyt – limfocyty T cytotoksyczne (ang. *T cytotoxic cells*)

TEM – transmisyjna mikroskopia elektronowa (ang. *transmission electron microscopy*)

Th – limfocyty T pomocnicze (ang. *T helper cells*)

TNF – czynnik martwicy nowotworu (ang. *tumor necrosis factor*)

TNFRSF13B (ang. *tumor necrosis factor receptor superfamily member 13B*)

Treg – limfocyty T regulatorowe (ang. *T regulatory cells*)

VCAM-1 – naczyniowa cząsteczka adhezyjna 1 (ang. *vascular cell adhesion molecule 1*)

VSNL1 (ang. *visinin-like protein 1*)

WPROWADZENIE

Stwardnienie rozsiane (ang. *multiple sclerosis*, MS) jest chorobą o złożonej patofizjologii, obejmującej przewlekły stan zapalny w obrębie ośrodkowego układu nerwowego (ang. *central nervous system*, CNS) oraz postępującą demielinizację, prowadzącą do uszkodzenia aksonów i neurodegeneracji. Charakterystyczną cechą MS jest wczesny wiek zachorowania – pierwsze objawy kliniczne pojawiają się zazwyczaj między 20. a 40. rokiem życia, co wiąże się z narastającą niepełnosprawnością i częstym wykluczeniem społeczno-ekonomicznym młodych dorosłych [1].

Pod względem klinicznym MS charakteryzuje się znacznym zróżnicowaniem fenotypowym. Większość pacjentów początkowo prezentuje tzw. zespół klinicznie izolowany (ang. *clinically isolated syndrome*, CIS), definiowany jako ogniskowy epizod neurologiczny, utrzymujący się co najmniej 24 godziny. CIS stanowi zwykle zapowiedź rozwijającego się MS i najczęściej przechodzi w postać rzutowo-remisyjną (ang. *relapsing-remitting multiple sclerosis*, RRMS). Zaostrzenia występują średnio ok. 0,4 – 1,2 raza w roku i we wczesnej fazie choroby zazwyczaj okresowo całkowicie ustępują, jednak ich częstość i intensywność rosną wraz z jej postępem. U większości chorych, po ok. 10-20 latach od pojawienia się pierwszych objawów, w wyniku kumulujących się uszkodzeń neurologicznych dochodzi do przejścia choroby w postać wtórnie postępującą (ang. *secondary progressive MS*, SPMS), charakteryzującą się stałym postępem neurodegeneracji skutkującej narastającą niepełnosprawnością chorego. Niewielki odsetek pacjentów rozwija postać pierwotnie postępującą (ang. *primary progressive multiple sclerosis*, PPMS), w której stopniowe pogarszanie funkcji neurologicznych występuje od początku choroby, bez okresów remisji [2].

Chociaż MS tradycyjnie postrzegane jest jako schorzenie neurozapalne i neurodegeneracyjne, coraz więcej dowodów wskazuje na ważny udział mechanizmów naczyniowych w jego patogenezie [3]. Bodźce zapalne prowadzą do aktywacji i/lub uszkodzenia komórek odpowiedzialnych za hemostazę naczyniową. Silne pobudzenie płytek krwi w MS wzmaga procesy komunikacji międzykomórkowej z komórkami układu odpornościowego, co sprzyja dysfunkcji śródbłonka w wyniku adhezji powstających agregatów płytkowo-leukocytarnych (ang. *platelet-leukocyte aggregates*, PLAs), rekrutacji i infiltracji leukocytów oraz prowadzi do naruszenia integralności bariery krew-mózg (ang. *blood-brain barrier*, BBB) [4]. Płytki krwi stanowią zatem kluczowe ogniwo łączące hemostazę z zapaleniem, modulując lokalne środowisko naczyniowe, które warunkuje napływ komórek zapalnych do CNS (Figura 1).

W kontekście patogenezy MS, szczególnego znaczenia nabiera fakt, że aktywowane płytki krwi sa głównym źródłem krążących pęcherzyków zewnątrzkomórkowych (ang. *extracellular vesicles*, EVs) [5]. EVs, uwalniane przez niemal wszystkie typy komórek, stanowią nośnik lipidów, białek, kwasów nukleinowych oraz mitochondrialnego DNA, które mogą być transportowane lokalnie oraz obwodowo, co podkreśla istotną rolę EVs w komunikacji międzykomórkowej [6]. Co ważne, EVs mogą swobodnie przekraczać BBB, co czyni je szczególnie cennym obiektem badań w aspekcie łatwej i szybkiej diagnostyki zmian patologicznych w obrębie CNS z wykorzystaniem obwodowych płynów ustrojowych [7]. Szczególne zainteresowanie budzą cząsteczki mikroRNA (ang. *microRNA*, miRNA) transportowane w EVs, które m.in. regulują szlaki zapalne oraz procesy neurodegeneracyjne i potencjalnie mogą pełnić funkcję biomarkerów, których ekspresja odzwierciedla zmiany zależne od statusu choroby [8,9]. Równolegle, rozwój ultraczułych metod umożliwiających detekcję białek pochodzenia mózgowego we krwi wzmacnia koncepcję nieinwazyjnego monitorowania MS, stanowiąc alternatywę dla inwazyjnych badań płynu mózgowo-rdzeniowego (ang. *cerebrospinal fluid*, CSF) [10–12].

Identyfikacji neuronalnego pochodzenia EVs dokonuje się często w oparciu o obecność cząsteczki adhezyjnej L1 (ang. *L1 cell adhesion molecule*, L1CAM), będącej glikoproteiną transbłonową kluczową dla rozwoju neuronów [13]. Badania nad L1CAM$^+$ EVs w surowicy i CSF sugerują, że ich liczebność, morfologia, profil białkowy oraz fenotyp powierzchniowy mogą odzwierciedlać aktywność immunologiczną w obrębie CNS [14–16] i tym samym stanowić biomarker odpowiedzi na leczenie u pacjentów z MS. Taka strategia mogłaby uzupełniać diagnostykę obrazową MS, która, choć odgrywa zasadniczą rolę w rozpoznaniu i monitorowaniu choroby, nie oddaje w pełni dynamiki i złożoności procesów patofizjologicznych na poziomie molekularnym [17].

Nowatorski charakter niniejszej pracy doktorskiej wiąże się z istniejącą luką badawczą w obszarze identyfikacji nieinwazyjnych markerów w MS, które różnicują fenotypy choroby oraz z ciągłą potrzebą poszukiwania narzędzi pozwalających monitorować odpowiedź na leczenie. Podjęte badania prowadzone były głównie w oparciu o analizę EVs oraz w odniesieniu do naczyniowo-immunologicznych patomechanizmów MS. W cyklu publikacji naukowych składających się na niniejszą rozprawę doktorską przedstawiono wyniki:

- badań funkcjonalnych i obrazowych nad powinowactwem płytek krwi do leukocytów oraz ich zdolnością do formowania agregatów z subpopulacjami limfocytów,
- analiz frakcji L1CAM$^+$ EVs jako wskaźników odpowiedzi na leczenie anty-CD20,
- profilowania EV-miRNA i budowy modeli statystycznych integrujących markery białkowe i miRNA do stratyfikacji fenotypów MS.

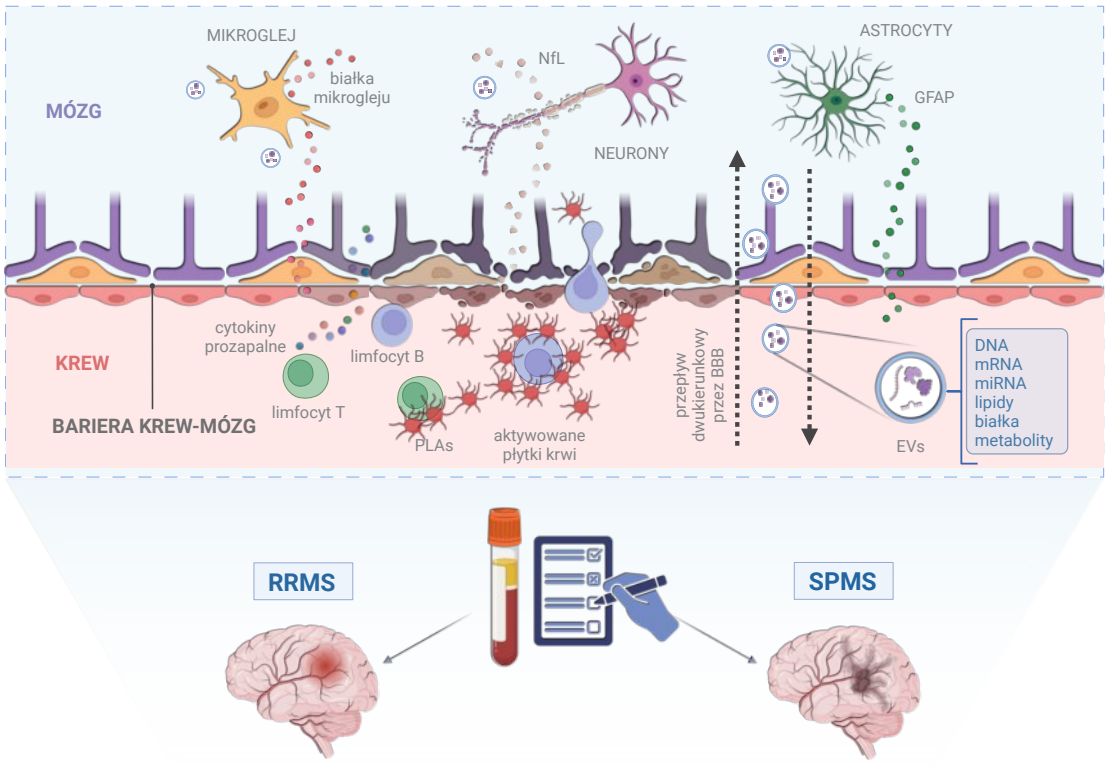

Figura 1. Schemat ilustruje kluczowe elementy patofizjologii stwardnienia rozsianego. Dysfunkcja bariery krew-mózg (BBB) prowadzi do napływu cytokin prozapalnych i tworzenia agregatów płytkowo-leukocytarnych (PLAs), które nasilają jej mechaniczne uszkadzanie, promują migrację limfocytów do ośrodkowego układu nerwowego i neurozapalenie. Aktywowane komórki mózgu (mikroglej, neurony, astrocyty) uwalniają biomarkery rozpuszczalne, które przenikają do krwioobiegu. Pęcherzyki zewnątrzkomórkowe (EVs) przenoszące m.in. DNA, RNA, białka, lipidy i metabolity przekraczają BBB krążąc w obu kierunkach, odzwierciedlając stan immunopatologii. Analiza PLAs, białkowych markerów zapalenia i EVs we krwi i płynie mózgowo-rdzeniowym umożliwia różnicowanie fenotypów rzutowo-remisyjnego (RRMS) i wtórnie postępującego stwardnienia rozsianego (SPMS) oraz monitorowanie odpowiedzi na leczenie. Opracowanie własne przy użyciu BioRender.

Skróty: BBB – bariera krew-mózg (ang. *blood-brain barrier*); EVs – pęcherzyki zewnątrzkomórkowe (ang. *extracellular vesicles*); GFAP – kwaśne białko włókienkowe gleju (ang. *glial fibrillary acid protein*); miRNA – mikroRNA (ang. *microRNA*); NfL – neurofilament lekki (ang. *neurofilament light chain*); PLAs – agregaty płytkowo-leukocytarne (ang. *platelet-leukocyte aggregates*); RRMS – rzutowo-remisyjne stwardnienie rozsiane (ang. *relapsing-remitting multiple sclerosis*); SPMS – wtórnie postępujące stwardnienie rozsiane (ang. *secondary progressive multiple sclerosis*)

CEL PRACY

Celem niniejszej pracy była ilościowa, jakościowa i funkcjonalna charakterystyka PLAs jako czynnika patofizjologii MS oraz analiza ekspresji cząsteczek miRNA pochodzących z krążących EVs, z oceną ich potencjału jako markerów fenotypu choroby u pacjentów z RRMS oraz SPMS. Uzupełnieniem badań była analiza profilu białek zaangażowanych w immunologiczną odpowiedź organizmu przenoszonych przez EVs pochodzenia neuronalnego ($L1CAM^+$) oraz ich roli jako potencjalnych biomarkerów służących do monitorowania odpowiedzi na leczenie rytuksymabem w RRMS.

Powyższe cele zostały zrealizowane poprzez następujące zadania badawcze:

1. Określenie stanu czynnościowego płytek krwi, ich potencjału chemotaktycznego wobec leukocytów oraz zdolności do tworzenia PLAs u pacjentów z MS;
2. Immunofenotypowanie subpopulacji limfocytów uczestniczących w tworzeniu PLAs;
3. Ocenę aktywności szlaku sygnałowego CD40-CD40L w formowaniu się PLAs z limfocytami T i B w RRMS i SPMS;
4. Analizę stężeń białkowych mediatorów zapalnych oraz markerów neurodegeneracji w osoczu krwi;
5. Oznaczenie ekspresji wyselekcjonowanych cząsteczek miRNA wyizolowanych z EVs;
6. Charakterystykę $L1CAM^+$ EVs w surowicy i CSF oraz określenie zmian w białkowym profilu immunologicznym ich zawartości przed i po leczeniu anty-CD20 w RRMS.

MATERIAŁY I METODY

Materiały i metody wykorzystane w niniejszych badaniach zostały szczegółowo opisane w artykułach doświadczalnych oraz w nieopublikowanym manuskrypcie, stanowiących podstawę rozprawy doktorskiej. Badania zostały przeprowadzone zgodnie z Deklaracją Helsińską i zatwierdzone przez Komisję ds. Bioetyki Badań Naukowych Uniwersytetu Łódzkiego, Uchwałą nr 3/KBBN-UŁ/IV/2018 oraz przez Regional Committee for Medical and Health Research Ethics, Western Norway – REC West ID: 66391.

| Materiał badany

Do badań wykorzystano następujące materiały:

- krew pełna pobrana na antykoagulant zawierający cytrynian fosforanu deoksyrybozy adeniny 1 (ang. *citrate phosphate dextrose adeninę 1*, CPDA-1) od pacjentów z RRMS w fazie remisji i SPMS hospitalizowanych na Oddziale Rehabilitacji Neurologicznej Miejskiego Centrum Medycznego im. Karola Jonschera w Łodzi; do grupy kontrolnej włączono zdrowych ochotników zrekrutowanych w Centrum Diagnostyki Laboratoryjnej w Łodzi, ul. Jaracza 85 (publikacja doświadczalna 1 i nieopublikowany manuskrypt);
- krew pełna pobrana bez użycia antykoagulantu od nowo zdiagnozowanych pacjentów z RRMS poddawanych leczeniu rytuksymabem (anty-CD20) w ramach randomizowanego badania klinicznego OVERLORD-MS (clinicaltrials.gov ID: NCT04578639) prowadzonego w szpitalu Haukeland University Hospital w Bergen, Norwegia (publikacja doświadczalna 2);
- CSF pobrany od nowo zdiagnozowanych pacjentów z RRMS poddawanych leczeniu rytuksymabem (anty-CD20) w ramach randomizowanego badania klinicznego OVERLORD-MS (clinicaltrials.gov ID: NCT04578639) prowadzonego w szpitalu Haukeland University Hospital w Bergen, Norwegia (publikacja doświadczalna 2).

| Metody badawcze

W pracach doświadczalnych zastosowano następujące metody preparatywne oraz analityczne:

- izolacja płytek krwi z osocza bogatopłytkowego przy użyciu kuleczek magnetycznych sprzężonych z monoklonalnymi przeciwciałami anty-CD45 i anty-CD235a (publikacja doświadczalna 1);
- izolacja jednojądrzastych komórek krwi obwodowej (ang. *peripheral blood mononuclear cells*, PBMCs) metodą wirowania w gradiencie gęstości z użyciem Gradisolu G (1,115 g/mL) i oczyszczanie leukocytów za pomocą kuleczek magnetycznych sprzężonych z przeciwciałami anty-CD45 (publikacja doświadczalna 1);

- izolacja EVs z osocza krwi (nieopublikowany manuskrypt), surowicy oraz CSF (publikacja doświadczalna 2) metodą precypitacji;
- oczyszczanie populacji L1CAM^{+} EVs przy użyciu kuleczek magnetycznych sprzężonych z przeciwciałem monoklonalnym anty-CD171 (L1CAM) (publikacja doświadczalna 2);
- charakterystyka i ocena jakości wyizolowanych EVs:
  - wizualizacja metodą transmisyjnej mikroskopii elektronowej (ang. *transmission electron microscopy*, TEM) (nieopublikowany manuskrypt oraz publikacja doświadczalna 2),
  - ocena rozkładu wielkości i jednorodności populacji EVs metodą dynamicznego rozpraszania światła (ang. *dynamic light scattering*, DLS) (nieopublikowany manuskrypt),
  - oznaczenie ekspresji charakterystycznych dla EVs antygenów powierzchniowych, CD63 i CD81, metodą cytometrii przepływowej (nieopublikowany manuskrypt),
  - pomiar ekspresji 37 markerów powierzchniowych EVs metodą cytometrii przepływowej – pełna lista użytych przeciwciał została zamieszczona w suplemencie do publikacji doświadczalnej 2, w tabeli S6 (publikacja doświadczalna 2),
  - charakterystyka EVs przy użyciu metody analizy śledzenia nanocząstek (ang. *nanoparticle tracking analysis*, NTA) (publikacja doświadczalna 2);
- oznaczenie stężeń panelu 21 białek w EVs, wyizolowanych z surowicy i CSF, związanych z odpowiedzią immunologiczną i stanem zapalnym metodą PEA (ang. *proximity extension assay*) w zewnętrznym laboratorium Olink Proteomics (Bevital AS, Bergen, Norwegia) – pełna lista białek została zamieszczona w suplemencie do publikacji doświadczalnej 2, w tabeli S2 (publikacja doświadczalna 2);
- multipleksowy pomiar stężenia panelu 27 cytokin w osoczu przeprowadzony metodą Luminex z zastosowaniem systemu Bio-Plex – pełna lista cytokin została zamieszczona w manuskrypcie artykułu (publikacja doświadczalna 1 i nieopublikowany manuskrypt);
- immunoenzymatyczny pomiar osoczowego stężenia wybranych białek: neurofilamentu lekkiego (ang. *neurofilament light chain*, NfL) oraz kwaśnego białka włókienkowego gleju (ang. *glial fibrillary acidic protein*, GFAP), wykorzystywanych jako markery neurodegeneracji, metodą ELISA (nieopublikowany manuskrypt);
- izolacja całkowitego RNA z EVs (nieopublikowany manuskrypt);
- synteza komplementarnego DNA (ang. *complementary DNA*, cDNA) na matrycy miRNA (nieopublikowany manuskrypt);
- sekwencjonowanie RNA w technologii sekwencjonowania nowej generacji (ang. *next generation sequencing*, NGS) na platformie Illumina NextSeq 500/550 (nieopublikowany manuskrypt);

- analiza różnicowej ekspresji miRNA metodą RT-qPCR z normalizacją do genu referencyjnego (nieopublikowany manuskrypt);
- ocena stopnia migracji leukocytów w kierunku płytek krwi *in vitro* przy użyciu komory Boydena (publikacja doświadczalna 1);
- wizualizacja morfologii PLAs przy użyciu skaningowej mikroskopii elektronowej (ang. *scanning electron microscopy*, SEM) (publikacja doświadczalna 1);
- ocena ekspresji markerów powierzchniowych na płytkach krwi i limfocytach tworzących PLAs metodą cytometrii przepływowej składająca się na (publikacja doświadczalna 1):
  - pomiar ekspresji glikoproteiny (ang. *glycoprotein*, GP) VI, receptora kolagenu, na płytkach krwi,
  - pomiar aktywnych konformacji GPIIb/IIIa, receptora fibrynogenu, przy użyciu przeciwciała PAC-1,
  - pomiar ekspresji powierzchniowej P-selektyny (CD62P) na płytkach krwi jako markera aktywacji,
  - pomiar ekspresji markerów limfocytarnych (CD3, CD4, CD8, CD25) na komórkach wchodzących w skład PLAs,
  - pomiar ekspresji antygenów CD40 i CD40L na płytkach krwi oraz limfocytach T i B;
- znakowanie immunofluorescencyjne i wizualizacja PLAs za pomocą mikroskopii konfokalnej wraz z odsetkową oceną udziału limfocytów T i B w tworzeniu kompleksów (publikacja doświadczalna 1);
- bioinformatyczna analiza danych surowych uzyskanych z sekwencjonowania RNA (nieopublikowany manuskrypt):
  - kontrola jakości odczytów przy użyciu FastQC, fastp oraz miRTrace,
  - mapowanie odczytów do ludzkiego genomu referencyjnego (GRCh38.p14/hg38) za pomocą programu Bowtie,
  - analiza różnicowej ekspresji miRNA przy użyciu pakietu DESeq2 oraz narzędzia miRTop;
- predykcja genów docelowych miRNA i analiza wzbogacenia funkcjonalnego (nieopublikowany manuskrypt):
  - predykcja genów docelowych przy użyciu baz danych miRecords, miRTarBase i TarBase za pomocą pakietu multiMiR [18] w programie R,
  - analizy wzbogacenia w oparciu o bazę GO (ang. *Gene Ontology*) [19,20], KEGG (ang. *Kyoto Encyclopedia of Genes and Genomes*) [21] oraz DisGeNET [22,23] za pomocą pakietu clusterProfiler [24] w oprogramowaniu R;
- analiza statystyczna wyników za pomocą oprogramowania GraphPad Prism oraz STATISTICA;
- wizualizacja wyników przy użyciu programów GraphPad Prism, R, Cytoscape oraz GIMP.

OMÓWIENIE PUBLIKACJI NAUKOWYCH I MANUSKRYPTU

Wyniki badań przeprowadzonych w niniejszej rozprawie doktorskiej zostały opublikowane w formie cyklu powiązanych tematycznie artykułów naukowych, obejmujących część teoretyczną oraz część eksperymentalną niniejszej pracy oraz zawarte w manuskrypcie przesłanym do recenzji.

| Publikacja przeglądowa 1

Zbiór publikacji otwiera praca przeglądowa pt. **"miR-155 as an Important Regulator of Multiple Sclerosis Pathogenesis. A Review"** [25], stanowiąca syntetyczne omówienie roli cząsteczki miRNA-155 (miR-155) w patogenezie MS, określanej jest "inflamma-miR", ze względu na jej zdolność do silnej aktywacji szlaków zapalnych. W artykule omówiono aktualny stan wiedzy w odniesieniu do wpływu miR-155 na procesy neurozapalne, demielinizację, uszkodzenie BBB, ból neuropatyczny oraz objawy neuropsychiatryczne.

We wstępie zarysowano mechanizmy działania cząsteczek miRNA oraz ich potencjał jako biomarkerów i celów terapeutycznych w różnych chorobach, w tym autoimmunizacyjnych. Następnie usystematyzowano dane dotyczące roli miR-155 w regulacji odpowiedzi immunologicznej, poprzez modulację polaryzacji komórek mieloidalnych, różnicowania limfocytów T oraz funkcji limfocytów B. Punktem wyjścia omawianej pracy jest fakt, że według doniesień literaturowych, nadekspresję miR-155 wykazano zarówno w aktywnych zmianach w mózgu, jak i we krwi obwodowej chorych na MS oraz w eksperymentalnym autoimmunizacyjnym zapaleniu mózgu i rdzenia kręgowego (ang. *exterimental autoimmune encephalomyelitis*, EAE) – zwierzęcym modelu choroby. Jako kluczowe efektory aktywności miR-155 wskazano SOCS1 (ang. *suppressor of cytokine signaling 1*), SHIP1 (ang. *SH2-containing inositol phosphatase 1*), C/EBPβ (ang. *CCAAT/enhancer-binding protein beta*) oraz elementy szlaku PI3K (ang. *phosphoinositide 3-kinase*): PIK3R1/PIK3CA (ang. *phosphoinositide-3-kinase regulatory subunit / phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha*).

Szczególną uwagę poświęcono w pracy wpływowi miR-155 na integralność BBB. W warunkach zapalnych, pod wpływem cytokin takich jak np. czynnik martwicy nowotworu (ang. *tumor necrosis factor*, TNF) i interferon γ (ang. *interferon gamma*, IFN-γ), nadekspresja miR-155 nasila przepuszczalność BBB poprzez obniżenie syntezy białek połączeń ścisłych (m.in. klaudyny 1 (ang. *claudin-1*, CLDN-1), aneksyny 2 (ang. *annexin 2*, ANXA2), SDCBP (ang. *syndecan binding protein*), DOCK-1 (ang. *dedicator of cytokinesis 1*)) oraz poprzez promowanie adhezji leukocytów w wyniku zwiększenia poziomu

glikoprotein śródbłonka (m.in. naczyniowej cząsteczki adhezyjnej 1 (ang. *vascular cell adhesion molecule 1*, VCAM-1) oraz międzykomórkowej cząsteczki adhezyjnej 1 (ang. *intercellular adhesion molecule-1*, ICAM-1)). Mechanizm ten łączy wczesny epizod naczyniowy z napływem komórek efektorowych do CNS.

W kontekście demielinizacji, zebrane dane wskazują, że nadekspresja miR-155 sprzyja aktywacji mikrogleju do prozapalnego fenotypu M1 i polaryzacji astrocytów w kierunku fenotypu A1, prowadząc do destrukcji osłonek mielinowych otaczających aksony. Ponadto, miR-155 reguluje poziom białka CD47, pełniącego funkcję sygnału „nie jedz mnie" (ang. "*don't eat me*"), którego obniżenie skutkuje nasileniem fagocytozy mieliny przez makrofagi i mikroglej.

W publikacji omówiono również udział miR-155 w patogenezie bólu neuropatycznego, który jest częstym objawem występującym w MS. miR-155 wpływa na ekspresję cytokin prozapalnych i aktywuje szlaki p38 MAPK (ang. *mitogen-activated protein kinase*) i NF-κB (ang. *nuclear factor kappa-light-chain-enhancer of activated B cells*) uczestniczące w aktywności mechanizmów odczuwania bólu. Natomiast inhibicja miR-155 zastosowana w modelach zwierzęcych prowadziła do redukcji bólu oraz zmiany polaryzacji mikrogleju z fenotypu M1 na przeciwzapalny M2.

W kontekście objawów neuropsychiatrycznych, takich jak depresja i lęk, współwystępujących u pacjentów z MS, wskazano rolę miR-155 w regulacji zapalenia w obrębie hipokampa oraz jego wpływ na czynniki neurotroficzne, np. neurotroficzny czynnik pochodzenia mózgowego (ang. *brain-derived neurotrophic factor*, BDNF). Eksperymenty na myszach *knockout* dla miR-155 wykazały ograniczenie zachowań depresyjnych i zmniejszenie ekspresji genów prozapalnych w mózgu.

W końcowej części pracy omówiono perspektywy kliniczne, biorąc pod uwagę, że cząsteczki miRNA spełniają kluczowe kryteria dobrych kandydatów na biomarkery (m.in. stabilność w płynach ustrojowych, możliwość izolacji w sposób nieinwazyjny, czy zmienność ekspresji skorelowaną z aktywnością choroby). Ze względu na szeroki zakres działania oraz potwierdzoną możliwość skutecznego wyciszania za pomocą cząsteczek anty-miR, miR-155 przedstawia się jako obiecujący cel terapeutyczny oraz potencjalny biomarker do monitorowania aktywności choroby i odpowiedzi na leczenie. Jednocześnie, w pracy podkreślono, że translacja miRNA do praktyki klinicznej wymaga większej powtarzalności wyników, zewnętrznej walidacji oraz standaryzacji metod badawczych.

Publikacja przedstawia miR-155 jako przykład węzła regulacyjnego, łączącego procesy immunologiczne i neurodegeneracyjne. W kontekście niniejszej rozprawy, artykuł uzasadnia także celowość badań nad krążącymi miRNA jako nieinwazyjnymi wskaźnikami aktywności procesów patofizjologicznych w MS.

| Publikacja przeglądowa 2

Drugą pracę przeglądową wchodzącą w skład rozprawy doktorskiej stanowi artykuł zatytułowany: **"Remyelination from the miRNA perspective"** [26], pogłębiający zagadnienie regulacji neurozapalnej przez cząsteczki miRNA, koncentrujący się na kluczowym mechanizmie naprawczym w MS jakim jest remielinizacja. W pracy zwrócono uwagę, że dostępne terapie modyfikujące przebieg choroby (ang. *disease modyfying therapies*, DMTs) skutecznie wyciszają aktywność zapalną, natomiast brak jest postępowania jednoznacznie przyspieszającego odbudowę mieliny, jako następstwa wdrażanego leczenia z udziałem DMTs. Zawarta w publikacji charakterystyka procesu remielinizacji opisuje mechanizmy angażujące mikroglej, prekursory oligodendrocytów (ang. *oligodendrocyte progenitor cells*, OPCs) oraz dojrzałe oligodendrocyty, przebiegające z udziałem szlaków sygnałowych Wnt/β-katenina (ang. *Wnt/β-catenin*), PI3K/AKT/mTOR (ang. *protein kinase B / mechanistic target of rapamycin*) oraz MAPK/ERK (ang. *extracellular signal-regulated kinase*).

W publikacji omówiono udział wybranych miRNA w poszczególnych etapach procesu remielinizacji. W fazie oczyszczania ognisk demielinizacyjnych miR-223 sprzyja aktywacji komórek mikrogleju i fagocytozie resztek mieliny, torując drogę remielinizacji. Natomiast miR-155-3p, indukowany po uszkodzeniu osłonki mielinowej, nasila środowisko prozapalne przez hamowanie SOCS1. W fazie rekrutacji i różnicowania OPCs, miR-204, indukowany przez czynnik transkrypcyjny Sox (ang. (ang. *SRY-box transcription factor*) 10, ogranicza nadmierną proliferację i promuje różnicowanie prekursorów poprzez hamowanie m.in. regulatorów cyklu komórkowego – cykliny D2 (ang. *cyclin D2*, Ccnd2) i Sox4. Główną rolę w epigenetycznej regulacji procesu mielinizacji przypisuje się miR-219, który umożliwia przejście OPCs w dojrzałe oligodendrocyty poprzez represję inhibitorów różnicowania, w tym receptora czynnika wzrostu pochodzącego z płytek krwi α (ang. *platelet-derived growth factor receptor alpha*, PDGFRα), Sox6 i Hes5 (ang. *hairy and enhancer of split-5*). W fazie dojrzewania oligodendrocytów i formowaniu osłonek mielinowych opisano udział miR-146a, miR-138, miR-145 i miR-338, promujących proces naprawy. Zidentyfikowano również cząsteczki miRNA o działaniu przeciwnym – miR-27a, który powoduje deregulację szlaku Wnt/β-katenina oraz miR-125a-3p, którego nadekspresja blokuje dojrzewanie oligodendrocytów.

Końcowa część pracy dotyczy badań translacyjnych, omawia strategie terapeutyczne ukierunkowane na dostarczanie miRNA do CNS za pośrednictwem EVs, liposomów, nanocząstek polimerowych oraz układów rusztowań hydrożelowo-włóknowych, działających jako nośniki zabezpieczające cząsteczki miRNA przed degradacją i ułatwiające ich wychwyt przez komórki docelowe.

Dane literaturowe podkreślają, że EVs zawierające miR-219a-5p przekraczają BBB, promują różnicowanie OPCs i łagodzą przebieg EAE.

Podsumowując, miR-219 zajmuje kluczową pozycję w sieci regulacyjnej wspierającej naprawę uszkodzeń mieliny, wspomagany przez miR-338, miR-138, miR-145, miR-146a i miR-23a, podczas gdy miR-155/miR-155-3p może utrwalać środowisko anty-remielinizacyjne.

Włączenie publikacji do cyklu prac stanowiących rozprawę doktorską stanowi uzasadnienie wyboru krążących miRNA jako celu badawczego, zarówno w kontekście ich potencjału jako biomarkerów przebiegu choroby i odpowiedzi na leczenie, jak i perspektywy przyszłych interwencji terapeutycznych ukierunkowanych na remielinizację.

| Publikacja przeglądowa 3

Trzecią pracą przeglądową włączoną do rozprawy jest artykuł pt. **"Brain-derived blood biomarkers in multiple sclerosis – current trends and beyond"** [27]. Publikacja stanowi domknięcie zagadnień obejmujących przejście od mechanizmów patogenezy do zastosowań w dziedzinie diagnostyki i monitorowania MS, stawiając tezę, że biomarkery pochodzenia mózgowego oznaczane we krwi mogą umożliwić nieinwazyjne śledzenie aktywności choroby i odpowiedzi na leczenie.

Na wstępie praca podsumowuje aktualne filary diagnostyki MS, tj. udokumentowanie rozsianych zmian patologicznych w czasie i przestrzeni poprzez obrazowanie za pomocą rezonansu magnetycznego (ang. *magnetic resonance imaging*, MRI) oraz analizę CSF. Jednocześnie, artykuł podkreśla istotę rozwoju czułych metod analitycznych, pozwalających obniżać granicę wykrywalności identyfikowanych związków, w tym białek pochodzenia mózgowego we krwi. W tym kontekście omówiono detekcję NfL jako markera uszkodzeń neuroaksonalnych oraz GFAP jako wskaźnika aktywacji astrogleju. Szczególną uwagę zwrócono na EVs pochodzenia mózgowego, które dzięki zdolności przekraczania BBB oraz obecności identyfikujących je białek błonowych, typowych dla tzw. komórki rodzicielskiej, takich jak L1CAM dla neuronów, transporter glutaminianu i asparaginianu (ang. *glutamate aspartate transporter*, GLAST) dla astrocytów, glikoproteina mieliny oligodendrocytów (ang. *myelin oligodendrocyte glycoprotein*, MOG) dla oligodendrocytów, mogą odzwierciedlać stan CNS w badaniu krwi.

W dalszej części pracy przedstawiono patofizjologię wczesnej fazy MS, w której nawracające nacieki limfocytów T i B inicjują ogniska zapalne, demielinizację i uszkodzenie aksonów widoczne w MRI. Następnie omówiono przejście z fazy nawracającej w kierunku przewlekłego,

tzw. „tlącego się" neurozapalenia (ang. *"smoldering" neuroinflammation*) w obrębie CNS, odpowiadającego za progresję MS niezależną od rzutów. W chronicznie aktywnych zmianach kluczową rolę odgrywają mikroglej i astrocyty, a w płynach ustrojowych obserwuje się wzrost stężeń białek takich jak sTREM2 (ang. *soluble triggering receptor expressed on myeloid cells 2*), chitotriozydaza (ang. *chitotriosidase*, CHIT1), CHI3L1 (ang. *chitinase-3-like protein 1*) i GFAP.

W dalszej części publikacji opisano udział EVs w zaburzeniach integralności BBB oraz regulacji odpowiedzi immunologicznej. Wykazano zależności pomiędzy podstawowymi mechanizmami patogenezy MS a kluczowymi cechami strukturalnymi i funkcjonalnymi EVs. Jak podkreślono w pracy, EVs limfocytarne nasilają ekspresję cząsteczek adhezyjnych śródbłonka i ułatwiają migrację komórek immunologicznych do CNS, EVs komórek dendrytycznych przenoszą cząsteczki kostymulujące aktywację limfocytów T, a EVs mikrogleju transportują mediatory prozapalne. Zwrócono również uwagę na potencjał diagnostyczny białek mieliny w EVs oligodendrocytarnych oraz wpływ EVs astrocytarnych na profil wydzielniczy limfocytów T.

Sekcję poświęconą biomarkerom krwi i CSF osadzono w perspektywie zastosowań klinicznych. W pracy zamieszczono obszerną tabelę "Tab. 1. Summary of fluid biomarkers in multiple sclerosis", która porządkuje i charakteryzuje najważniejsze biomarkery w MS możliwe do oznaczania w płynnej biopsji. Przykładowo, surowiczy NfL może być użyteczny w przewidywaniu ryzyka rzutów, natomiast CHI3L1, GFAP i prążki oligoklonalne immunoglobulin G (ang. *immunoglobulin G oligoclonal bands*, IgG OCB) częściej wiążą się z fenotypem progresywnym. Podkreślono, że markery aktywacji mikrogleju (CHIT1, sTREM2), astrogleju (CHI3L1, GFAP), patologii limfocytów B (CXCL13 (ang. *C-X-C motif chemokine ligand 13*)) i uszkodzenia neuroaksonalnego (NfL) uzupełniają się w ocenie aktywności choroby. Zwrócono uwagę na ograniczenia translacyjne, takie jak nieswoistość NfL, potrzebę standaryzacji warunków izolacji i wzbogacania frakcji EVs pochodzenia mózgowego, ujednolicenia analityki i lepszego poznania biodystrybucji. Wskazano, że praktyczne wdrożenie może wymagać łączenia markerów w zintegrowane profile obejmujące krew, CSF oraz neuroobrazowanie, korzystania z rozwiązań kliniczno-obrazowych oraz wsparcia algorytmami sztucznej inteligencji.

Praca akcentuje przesunięcie paradygmatu od tradycyjnej analizy CSF ku biomarkerom pochodzenia mózgowego oznaczanym we krwi, co otwiera drogę do mniej inwazyjnego monitorowania MS i ułatwionej, bardziej precyzyjnej stratyfikacji pacjentów.

W dalszej części omówienia niniejszej rozprawy doktorskiej podsumowane zostaną prace eksperymentalne, stanowiące kamienie milowe odzwierciedlające realizację poszczególnych celów badawczych.

| Publikacja doświadczalna 1

Publikacja doświadczalna pt. **"Human B-cells can form Hetero-aggregates with Blood Platelets: A Novel Insight into Adaptive Immunity Regulation in Multiple Sclerosis"** [28] prezentuje wyniki badań ukierunkowanych na charakterystykę zjawiska tworzenia PLAs jako niezbadanego dotychczas elementu patofizjologii MS w kontekście naczyniowo-immunologicznym.

Jedna z hipotez patogenezy MS zakłada naczyniowe podłoże choroby, związane ze zmianami śródbłonka wewnątrzczaszkowych naczyń krwionośnych w prozapalnym środowisku. Zaburzenie integralności ściany naczyniowej skutkuje aktywacją płytek krwi, ich adhezją do zapalnie zmienionego śródbłonka oraz rekrutacją leukocytów, co prowadzi do patologicznego formowania PLAs. Obecność tych struktur sprzyja rozszczelnieniu BBB i ułatwia migrację komórek immunologicznych do CNS.

Do badania zakwalifikowano 38 chorych na RRMS oraz 55 chorych na SPMS. Grupę kontrolną stanowiło 55 ochotników bez chorób układu nerwowego oraz zaburzeń sercowo-naczyniowych. W pierwszym etapie badań przeprowadzono test migracji w komorze Boydena [29], który miał na celu ocenę chemotaktycznych zdolność płytek krwi względem leukocytów krwi obwodowej, bez bezpośredniego kontaktu komórkowego. Następnie, wykorzystano obrazowanie z użyciem SEM oraz mikroskopię konfokalną do identyfikacji PLAs w MS oraz oceny ich morfologii. Równolegle, metodą cytometrii przepływowej analizowano: stopień aktywacji płytek krwi, skład PLAs w oparciu o immunofenotypowanie subpopulacji limfocytów zaangażowanych w tworzenie tych kompleksów, jak również ekspresję składowych szlaku kostymulującego CD40-CD40L, będącą wyznacznikiem jego zaangażowania w bezpośrednie oddziaływania komórkowe płytek krwi z limfocytami.

W badaniach wykazano, że płytki krwi w MS tworzą środowisko sprzyjające rekrutacji leukocytów oraz bezpośrednim interakcjom płytka-limfocyt. W teście w komorze Boydena, w przypadku MS odnotowano istotny, ponad 2-krotny wzrost migracji leukocytów w kierunku płytek krwi w odniesieniu do kontroli. Uzyskany wynik ma swoje uzasadnienie w istotnie podwyższonym stężeniu płytkowej cytokiny CCL5 (ang. *C-C motif chemokine ligand 5*), dawniej RANTES (ang. *regulated upon activation, normal T cell expressed and secreted*), w osoczu (p<0,0001): 2,4-krotnie w RRMS i 3,1-krotnie w SPMS, będącej silnym

chemoatraktantem komórek układu odpornościowego. W kontekście uzyskanych wyników można wnioskować, że płytki krwi chorych na MS wykazują podwyższone zdolności aktywnego przyciągania leukocytów *in vivo*, co stanowi istotny element patogenezy choroby, poprzez ich rekrutację i tworzenie PLAs o wysokich zdolnościach adhezyjnych do śródbłonka naczyniowego, ułatwiających diapedezę przez ścianę naczynia.

Obrazowanie mikroskopowe dostarczyło bezpośrednich dowodów potwierdzających formowanie PLAs w MS. W próbkach od pacjentów z MS zaobserwowano liczne, nieregularne płytki krwi z rozbudowanymi filopodiami i lamellipodiami, tworzące zbite homo-agregaty oraz PLAs. W bardziej zaawansowanych stadiach aktywacji płytek widoczny był wyraźny płaszcz fibrynowy, działający jak rusztowanie zatrzymujące leukocyty i służący do tworzenia stabilnego czopu hemostatycznego. Masywne, usztywnione agregaty widoczne w próbkach MS mogą nie tylko zwiększać siłę przylegania do śródbłonka, lecz także mechanicznie obciążać ścianę naczyniową, sprzyjając rozwojowi lokalnego stanu zapalnego w obrębie dysfunkcyjnej BBB oraz dalszej infiltracji mediatorów zapalnych do CNS. PLAs o takim pokroju morfologicznym były obserwowane jedynie w próbkach pochodzących od pacjentów z MS, podczas gdy w próbkach kontrolnych płytki krwi zachowywały kształt kulisty, wykazywały minimalne cechy aktywacji i nie tworzyły złożonych strukturalnie PLAs.

Badania z zastosowaniem cytometrii przepływowej potwierdziły stan nadmiernej aktywacji płytek krwi, sprzyjającej tworzeniu PLAs i interakcji ze śródbłonkiem. Wykazano istotnie podwyższoną powierzchniową ekspresję aktywnej formy receptora GPIIb/IIIa (mierzoną za pomocą PAC-1) – 1,9-krotnie w RRMS (p=0,017) i 1,65-krotnie w SPMS (p=0,029) oraz znacznie podwyższoną ekspresję powierzchniową CD62P (P-selektyny, receptora odpowiedzialnego za interakcje komórkowe) – 3,06-krotnie w RRMS (p=0,0002) i 2,9-krotnie w SPMS (p=0,014). Natomiast ekspozycja GPVI (płytkowego receptora dla kolagenu) była istotnie wyższa jedynie u pacjentów z SPMS (2,13-krotnie, p=0,014), co może potwierdzać większe uszkodzenie ściany naczyniowej w progresywnym fenotypie choroby, w wyniku wzmożonej adhezji chronicznie pobudzonych płytek krwi do komórek warstwy śródbłonkowej.

Kolejny etap badań stanowiło określenie odsetka poszczególnych subpopulacji limfocytów tworzących PLAs u pacjentów z MS. Zarówno w RRMS jak i SPMS stwierdzono istotną przewagę PLAs z udziałem limfocytów B – odpowiednio 1,55-krotnie (p=0,009) i 1,94-krotnie (p<0,0001) względem kontroli. Obrazowanie konfokalne potwierdziło zwiększoną obecność PLAs z udziałem limfocytów B, co w świetle dotychczasowych doniesień stanowi nowy element charakterystyki patomechanizmów MS.

Największy odsetek PLAs z zaangażowanymi limfocytami T był widoczny w SPMS – 1,66-krotnie wyższy względem kontroli (p=0,005) i 1,45-krotnie wyższy względem RRMS (p=0,0002). Przy czym odsetek limfocytów T pomocniczych (ang. *T helper*, Th) w utworzonych PLAs był istotnie podwyższony w RRMS, podczas gdy limfocyty T cytotoksyczne (ang. *T cytotoxic*, Tcyt) oraz limfocyty T regulatorowe (ang. *T regulatory*, Treg) w agregatach wzrastały istotnie wyłącznie w SPMS.

Aby ocenić udział kostymulującego szlaku sygnalizacyjnego CD40-CD40L, kluczowego dla rozwoju reakcji immunologicznych, w tworzeniu PLAs z limfocytami, zbadano poziom powierzchniowej ekspresji receptorów osi CD40-CD40L na omawianych komórkach. Na płytkach krwi stwierdzono istotny wzrost ekspresji zarówno CD40 jak i CD40L w RRMS i SPMS względem kontroli. Korelacja między płytkowym CD40 a CD40L na limfocytach była wyraźnie silniejsza dla limfocytów B niż T, szczególnie w SPMS (odpowiednio r=0,64, p<0,001 i r=0,402, p=0,037), co jest spójne z wyraźną tendencją do tworzenia PLAs z zaangażowanymi limfocytami B. Powyższe wyniki wspierają zatem hipotezę, że aktywowane płytki krwi mogą wzmacniać odpowiedź B-komórkową poprzez sygnalizację CD40-CD40L. Może to tłumaczyć efektywność terapii skierowanych przeciwko limfocytom B, tj. terapii anty-CD20 czy leczenia z użyciem inhibitorów kinazy Brutona (ang. *Bruton's tyrosine kinase*, BTK) w MS, regulującej proliferację, przeżycie i sygnalizację tych komórek.

Zintegrowanie danych funkcjonalnych, morfologicznych i fenotypowych tworzy spójny obraz, w którym płytki krwi w MS są nadaktywne i proadhezyjne, przyciągają leukocyty i tworzą z nimi stabilne agregaty ze szczególną przewagą PLAs z limfocytami B. Model ten łączy komponent naczyniowy z odpowiedzią immunologiczną i wskazuje PLAs jako istotny, dotąd niedoszacowany element patofizjologii MS.

| Manuskrypt artykułu doświadczalnego

W związku z potrzebą identyfikacji biomarkerów odzwierciedlających przebieg procesów patofizjologicznych, w tym przejścia pomiędzy fenotypami MS, kolejny etap badań ukierunkowano na krążące EVs, a zwłaszcza obecne w nich miRNA, które wykazują szczególny potencjał jako nieinwazyjne markery biologiczne. Wyniki tych badań zostały przedstawione w manuskrypcie pracy doświadczalnej, przesłanym do recenzji, pt. **"Extracellular vesicle-derived miR-760 as a novel candidate marker differentiating stable RRMS from SPMS"**, której celem była analiza różnicowej ekspresji cząsteczek miRNA *ex vivo* oraz predykcja ich genów docelowych, potencjalnych szlaków sygnałowych i procesów biologicznych metodami *in silico*.

Pomimo znaczącego postępu w diagnostyce MS w ostatnich latach, rozpoznanie choroby wciąż w dużej mierze opiera się na ocenie obrazu klinicznego. Rutynowe badania krwi, takie jak oznaczenie stężenia NfL, który nie jest w pełni specyficznym markerem, cechują się ograniczoną swoistością i mogą prowadzić do błędnych rozpoznań. W tym kontekście profilowanie cząsteczek miRNA przenoszonych przez krążące EVs może stanowić obiecującą alternatywę, oferując wyższą wartość diagnostyczną niż analiza pełnej krwi, ponieważ przenoszone przez nie cząsteczki odzwierciedlają stan komórki, z której pochodzą, a dzięki zdolności do przekraczania BBB stanowią potencjalne nieinwazyjne źródło informacji o toczących się aktualnie procesach patologicznych w CNS.

Założeniem pracy było znalezienie wiarygodnych sygnatur EV-miRNA różnicujących RRMS i SPMS oraz ich interpretacja w kontekście mechanizmów molekularnych i procesów patologicznych mogących odzwierciedlać dynamikę choroby.

Do badań włączono 30 pacjentów z RRMS oraz 30 pacjentów z SPMS, a także 30 zdrowych ochotników w ramach grupy kontrolnej. W pierwszym etapie przeprowadzono wieloparametryczną analizę panelu 27 cytokin w systemie Bio-Plex, która pozwoliła określić profil zapalny osocza, obejmujący elementy zarówno pro-, jak i przeciwzapalnych mechanizmów regulacji. Równolegle, w osoczu oznaczono stężenia markerów uszkodzenia aksonów (NfL) i reaktywności astrogleju (GFAP) metodą ELISA. Analiza proteomiczna wykazała istotne zmiany stężenia 16 cytokin zarówno u pacjentów z RRMS, jak i SPMS w porównaniu z grupą kontrolną oraz jednego markera neurodegeneracji (NfL). W sposób najwyraźniejszy podwyższone stężenie u chorych względem kontroli wykazano w przypadku czynnika stymulującego tworzenie kolonii granulocytów i makrofagów (ang. *granulocyte-macrophage colony-stimulating factor*, GM-CSF), antagonisty receptora interleukiny 1 (ang. *interleukin-1 receptor antagonist*, IL-1ra), białka zapalnego makrofagów 1β (ang. *macrophage inflammatory protein-1 beta*, MIP-1β) oraz białka zapalnego makrofagów 1α (ang. *macrophage inflammatory protein-1 alpha*, MIP-1α), TNF oraz interleukiny (ang. *interleukin*, IL) 8. Co ciekawe, stężenia analizowanych markerów w większości były wyższe w SPMS niż RRMS, zwracając po raz kolejny uwagę na coraz szerzej opisywane zjawisko współwystępowania procesów zapalnych nie tylko w fenotypie RRMS, lecz także w SPMS.

W analizie porównawczej między grupami pacjentów, wykazano wyższe stężenia IL-4 (1,27-krotny wzrost, p=0,004) i IL-17 (1,26-krotny wzrost, p=0,033) w SPMS względem RRMS, co może odzwierciedlać współwystępowanie komponentów odpowiedzi regulacyjnej (Th2) i efektorowej (Th17), charakterystyczne

dla „tlącego się" stanu zapalnego w postępującym fenotypie choroby. Jednocześnie podwyższony poziom podstawowego czynnika wzrostu fibroblastów (ang. *fibroblast growth factor basic*, FGF basic) (1,33-krotny wzrost, p=0,01) może świadczyć o endogennej odpowiedzi proregeneracyjnej, potwierdzając doniesienia naukowe dotyczące udziału FGF basic w promowaniu remielinizacji.

Po przeprowadzeniu analizy porównawczej stężeń markerów zapalnych i neurodegeneracyjnych w osoczu w obu fenotypach choroby, kolejny etap badań stanowiło oznaczenie ekspresji cząsteczek miRNA w RRMS oraz SPMS, celem wykrycia potencjalnych oddziaływań regulacyjnych miRNA, kształtujących obraz profilu immunologicznego badanych pacjentów.

Izolację EVs z osocza przeprowadzono metodą precypitacji z uprzednim traktowaniem próbek RNazą w celu usunięcia wolnych, niezamkniętych w EVs cząsteczek RNA i zwiększenia specyficzności analizy miRNA na rzecz komponentu rzeczywiście przenoszonego przez EVs. Jakość frakcji EVs potwierdzono obrazowaniem TEM, DLS (dominujący rozmiar ~45 nm, umiarkowana monodyspersyjność) oraz ekspresją tetraspanin (CD63, CD81) w metodzie cytometrii przepływowej. Następnie, z pozyskanych próbek EVs wyizolowano całkowite RNA, zawierające frakcję miRNA.

Analiza ekspresji cząsteczek miRNA obejmowała etap eksploracyjny oparty na sekwencjonowaniu transkryptomu w 9 losowo wybranych próbkach oraz etap walidacji metodą RT-qPCR (w każdej próbce pojedynczo). Dodatkowo, do analiz metodą RT-qPCR wybrano dziewięć kolejnych cząsteczek (miR-155-5p, miR-326, miR-301a-3p, miR-191-5p, miR-223-3p, miR-181c-5p, miR-146a-5p, miR-23a-3p i miR-16-5p) w oparciu o ich udokumentowaną bądź przewidywaną rolę w regulacji markerów zapalenia i neurodegeneracji, na podstawie danych literaturowych oraz algorytmicznych predykcji interakcji miRNA-gen pozyskanych z bazy miRDB.

Analiza wyników uzyskanych w RT-qPCR wykazała istotne różnice w ekspresji miR-301a-3p, miR-181c-5p, miR-98-5p oraz miR-760 w grupach RRMS i SPMS w porównaniu z grupą kontrolną. Względne poziomy ekspresji miRNA obliczano z wykorzystaniem metody ΔΔCt. Dla każdej próbki wartość ΔCt obliczano jako różnicę pomiędzy wartością Ct badanego miRNA a wartością Ct endogennego genu referencyjnego (miR-451a). Średnia wartość ΔCt w grupie kontrolnej została przyjęta jako punkt odniesienia. Wartość ΔΔCt dla każdej próbki w grupach RRMS i SPMS wyliczano jako różnicę pomiędzy jej ΔCt a średnią ΔCt grupy kontrolnej. Wartość krotności zmiany (ang. *fold change*, FC) obliczano na podstawie wzoru $2^{-\Delta\Delta Ct}$, a następnie poddawano transformacji $\log_2$ w celu zwiększenia przejrzystości prezentacji wyników.

Ekspresja miR-301a-3p, powiązanego z osią Th17 i szlakami STAT3 (ang. *signal transducer and activator of transcription 3*), RORγt (ang. *RAR-related orphan receptor gamma t*), była istotnie obniżona w obu grupach pacjentów (szczególnie w RRMS) względem kontroli (p<0,0001). Natomiast miR-181c-5p, według danych literaturowych, uczestniczący w regulacji progu aktywacji limfocytów T oraz odpowiedzi mikrogleju i szlaków zapalnych (m.in. MAPK/NF-κB), był istotnie podwyższony zarówno w RRMS, jak i SPMS (p=0,037). miR-98-5p wykazywał istotną nadekspresję zarówno w grupie pacjentów z RRMS, jak i SPMS w porównaniu z grupą kontrolną (p<0,0001). Zgodnie z opisywaną w literaturze rolą miR-98-5p jako regulatora odpowiedzi Th17 i ochrony BBB, uzyskany wynik sugeruje jego zaangażowanie w wyciszanie stanu zapalnego poprzez hamowanie odpowiedzi limfocytów Th17 w postępującym fenotypie choroby. Z kolei miR-760, biorący udział w regulacji procesów remielinizacji i neuroprotekcji poprzez modulowanie szlaków stresu oksydacyjnego i mechanizmów śmierci komórkowej, wykazywał istotnie obniżoną ekspresję w RRMS względem kontroli.

Biorąc pod uwagę istotne różnice w ekspresji miRNA, jak i w stężeniach markerów białkowych, w kolejnym kroku oceniono ich powiązania za pomocą analizy korelacji. Przeprowadzona analiza wykazała istotną, negatywną korelację ekspresji miR-98-5p ze stężeniem IL-17 w SPMS (r=-0,447, p=0,013), natomiast w przypadku miR-760 – istotną, negatywną korelację ekspresji miR-760 ze stężeniem IL-4 (r=-0,472; p=0,008) i IL-17 (r=-0,52; p=0,003) w SPMS. Zależności tej nie zaobserwowano w RRMS, co wskazuje na potencjalnie fenotypowo swoistą dla SPMS rolę immunoregulacyjną względem Th2/Th17.

Ponadto, analiza wyników różnicowej ekspresji miRNA między RRMS i SPMS, wykonana metodą $2^{-\Delta\Delta Ct}$, wykazała istotnie podwyższoną ekspresję w grupie SPMS w przypadku miR-98-5p (p=0,001), miR-760 (p<0,0001), miR-301a-3p (p=0,01) i miR-223-3p (p=0,026). miR-760 pełni wielokierunkowe funkcje, m.in. promuje remielinizację oraz może regulować szlaki stresu oksydacyjnego i śmierci komórkowej, a obniżenie jego ekspresji prowadzi do aktywacji prozapalnego szlaku NF-κB oraz autoreaktywnych limfocytów B. miR-98-5p, opisywany jest jako regulator promujący działanie ochronne poprzez zapobieganie dysfunkcji BBB, hamowanie neurozapalenia oraz ograniczanie różnicowania limfocytów Th17 w modelu EAE. Natomiast wykazane różnice w ekspresji miR-301a-3p, związanego z subpopulacją komórek Th17, wspierają hipotezę o jego udziale w autoimmunizacji, demielinizacji i neurodegeneracji. Z kolei miR-223-3p pełni rolę immunoregulatora, ograniczając produkcję cytokin prozapalnych, a także wspiera funkcję mieloidalnych komórek supresorowych (ang. *myeloid derived supressor cells*, MDSCs), bierze udział w różnicowaniu

komórek Th1, polaryzacji makrofagów M2 oraz procesach usuwania resztek mieliny. Doniesienia naukowe podkreślają rolę miR-223-3p w różnicowaniu fenotypów MS, a także w monitorowaniu aktywności choroby i odpowiedzi na leczenie. Powyższe wyniki wskazują, że miR-760, miR-98-5p, miR-301a-3p i miR-223-3p mogą nie tylko różnicować RRMS i SPMS, ale także odzwierciedlać kluczowe mechanizmy związane z przejściem do progresywnego fenotypu choroby.

Kolejny etap badań stanowiła bioinformatyczna analiza wyników prac eksperymentalnych, obejmująca identyfikację genów docelowych dla analizowanych miRNA przy użyciu baz zwalidowanych interakcji miRNA-mRNA (miRecords, miRTarBase i TarBase). Cele miRNA wyselekcjonowano przy użyciu pakietu multiMiR (R), uzyskując łącznie 16 085 genów, przy czym najwięcej interakcji wykazywał miR-98-5p. W analizie wzbogacenia GO uzyskano 2899 wyników powiązanych z genami docelowymi różnicujących miRNA (miR-98-5p, miR-760, miR-301a-3p, miR-223-3p) (p<0,05), w tym 2214 związanych z procesami biologicznymi, 351 z komponentami komórkowymi i 334 z funkcjami molekularnymi. Analiza z wykorzystaniem bazy KEGG wykazała udział genów docelowych w 144 szlakach. We wszystkich analizach nadreprezentacji użyto pakietu clusterProfiler, stosując korekcję Benjaminiego-Hochberga i próg istotności p<0,05. Szlaki porządkowano według GeneRatio (proporcji genów z listy wejściowej przypadających na dany szlak) oraz skorygowanych wartości p. W celu porównania fenotypów RRMS i SPMS dokonano integracji publicznie dostępnych genów związanych z chorobą z eksperymentalnie potwierdzonymi transkryptami docelowymi dla cząsteczek miR-98-5p, miR-760, miR-301a-3p oraz miR-223-3p. Geny powiązane z jednostką chorobową pozyskano z bazy DisGeNET, wyszukując hasła “secondary progressive multiple sclerosis” (SPMS) oraz “multiple sclerosis relapse” i “multiple sclerosis exacerbation” (RRMS). Dla każdego z fenotypów wybrano po 30 genów o najwyższych wartościach tzw. wskaźnika powiązania „gen-choroba” (ang. *gene-disease association*, GDA), który odzwierciedla siłę i wiarygodność powiązania danego genu z jednostką chorobową, na podstawie zintegrowanych dowodów z wielu źródeł [22]. W dalszym etapie, w celu identyfikacji interakcji swoistych dla poszczególnych fenotypów zestawiono listy genów chorobowych ze zbiorem celów miRNA. W celu zwiększenia specyficzności analizy wykluczono geny wspólne dla RRMS i SPMS, koncentrując się na unikalnych sygnaturach molekularnych różnicujących oba fenotypy. Uzyskane pary miRNA-mRNA posłużyły następnie do konstrukcji sieci interakcji w programie Cytoscape. Analiza funkcjonalna celów miRNA wykazała nadreprezentację procesów komórkowych małych GTPaz, autofagii i proteostazy oraz szlaków sygnałowych obejmujących MAPK, AMPK (ang. *AMP-activated protein kinase*), FoxO (ang. *forkhead box O*), Hippo

oraz ErbB (ang. *erythroblastic leukemia viral oncogene homolog*), a także powiązań z neurodegeneracją (ścieżki choroby Alzheimera, stwardnienie zanikowe boczne, ataksje rdzeniowo-móżdżkowe). Sieci regulacyjne różnicowały fenotypy: w SPMS dominowały cele immunologiczne (*IL10*, *IL7*, *CSF1* (ang. *colony-stimulating factor 1*), *CD8A*, *CCR5* (ang. *C-C chemokine receptor 5*), *CCR7* (ang. *C-C chemokine receptor 7*)), typowe dla przewlekłego zapalenia CNS, natomiast w RRMS pojawiały się dodatkowo geny receptorów apoptozy (*FAS*), receptorów sygnalizacji sfingolipidowej (*S1PR1* (ang. *sphingosine-1-phosphate receptor 1*), *S1PR5* (ang. *sphingosine-1-phosphate receptor 5*)) i elementów odpowiedzi stresowej (*MAP2K7* (ang. *mitogen-activated protein kinase kinase 7*)). Na tym tle rola miR-98-5p – łączącego regulację odpowiedzi Th17 i ochrony BBB oraz miR-760 – związanego z regulacją odpowiedzi zapalnej i procesów dojrzewania/ochrony neuronów, wydaje się szczególnie istotna.

W ostatnim etapie przeprowadzono wnioskowanie statystyczne stosując analizę jednoczynnikową z użyciem regresji logistycznej w celu identyfikacji istotnych zmiennych ($p<0,05$), które następnie włączano do wieloczynnikowego modelu regresji logistycznej oceniającego prawdopodobieństwo określonego wyniku klinicznego (RRMS/SPMS). Wyselekcjonowano potencjalne markery miRNA oraz białkowe, dla których wyznaczono krzywą ROC (ang. *receiver operating characteristic*) w celu oceny przydatności zmiennych mierzalnych do różnicowania fenotypów choroby.

Na podstawie zastosowanego modelu analizy jednoczynnikowej wykazano istotny związek z klasyfikacją do fenotypu RRMS dla trzech miRNA. Mianowicie, każdy jednostkowy wzrost wartości ΔCt dla miR-301a-3p oraz miR-146a-5p wiązał się odpowiednio z 17,8% i 26,8% wzrostem szans klasyfikacji do RRMS, natomiast najsilniejszy efekt odnotowano dla miR-760 (107,5%). Opracowany następnie model regresji wieloczynnikowej, obejmujący FGF basic, miR-760, miR-301a-3p, miR-191-5p i miR-146a-5p, wykazał miR-760 jako najsilniejszy predyktor klasyfikacji RRMS, następnie miR-301a-3p oraz miR-146a-5p. Z kolei wyższe poziomy FGF basic i miR-191-5p wiązały się ze zmniejszonym prawdopodobieństwem klasyfikacji RRMS. Analiza krzywej ROC wykazała wysoką moc dyskryminacyjną modelu końcowego, uzyskując wartość pola pod krzywą (ang. *area under the curve*, AUC) równą 0,973. Model charakteryzował się czułością na poziomie 93,33% i swoistością 90%.

Podsumowując, zintegrowanie profilu cytokin, miRNA pochodzącego z EVs oraz analiz sieciowych ujawnia fenotypowo swoiste osie regulacji. Z punktu widzenia translacyjnego istotne jest, że komponent miRNA może dostarczać informacji uzupełniających wobec profilu markerów białkowych we krwi, tworząc przesłanki do budowy prostych algorytmów stratyfikacyjnych dla RRMS i SPMS.

| Publikacja doświadczalna 2

Publikacja doświadczalna pt. **"In-Depth Characterization of L1CAM⁺ Extracellular Vesicles as Potential Biomarkers for Anti-CD20 Therapy Response in Relapsing–Remitting Multiple Sclerosis"** prezentuje kolejny etap badań, którego celem było określenie czy krążące we krwi L1CAM⁺ EVs odzwierciedlają stan immunopatologiczny CNS oraz czy ich stężenie i profil fenotypowy reagują na deplecję limfocytów B u pacjentów z RRMS.

Terapia anty-CD20, ukierunkowana na białko znajdujące się na powierzchni limfocytów B, skutecznie hamuje aktywność zapalną w MS poprzez szybką i efektywną eliminację krążących we krwi limfocytów B cechujących się wysoką ekspresją tego antygenu. Jednak z uwagi na możliwość wystąpienia poważnych skutków ubocznych i brak biomarkerów umożliwiających precyzyjne dostosowanie dawki jest obecnie najczęściej prowadzona w schemacie podtrzymującym.

Założeniem pracy było, że EVs wykazujące ekspresję L1CAM, markera szeroko stosowanego do selekcji frakcji EVs pochodzenia mózgowego, mogą odzwierciedlać stan immunologiczny CNS i dynamicznie reagować na leczenie rytuksymabem, dostarczając nieinwazyjnych wskaźników odpowiedzi terapeutycznej w MS.

Do analizy włączono sparowane próbki surowicy, przed i po 6 miesiącach leczenia, od nowo rozpoznanych pacjentów z RRMS włączonych do badania klinicznego OVERLORD-MS (NCT05296161) oraz próbki CSF od tych samych pacjentów pobrane przy rozpoznaniu (przed wdrożeniem leczenia). Grupę kontrolną stanowili ochotnicy, od których pozyskiwano surowicę.

EVs izolowano z surowicy i CSF, a następnie immunoselektywnie wzbogacano frakcję L1CAM⁺ przy użyciu przeciwciała anty-CD171 sprzężonego ze streptawidyną, co umożliwiło uzyskanie EVs pochodzenia mózgowego. Morfologię EVs zweryfikowano za pomocą obrazowania TEM, a stężenie i rozmiar przy użyciu NTA. Immunofenotypowanie 37 markerów na powierzchni EVs przeprowadzono za pomocą metody cytometrii przepływowej, a profil białkowy przeanalizowano metodą PEA dla panelu 21 analitów. Taki układ metod umożliwił jednoczesną kwantyfikację ilościową L1CAM⁺ EVs i analizę jakościową poprzez identyfikację fenotypu ich receptorów powierzchniowych oraz ocenę zmian przenoszonego przez nie ładunku białkowego.

Wykazano, że w surowicy pacjentów przed rozpoczęciem terapii stężenie L1CAM⁺ EVs było istotnie wyższe w porównaniu z grupą kontrolną (p<0,0001), sugerując nasiloną aktywność procesów neuroimmunologicznych. Równolegle, w proteomie surowiczych EVs dla ekspresji CD79B, podjednostki białkowej receptora komórek B, odnotowano FC=0,24; p=0,03, a dla CCL2 (ang. *C-C motif*

chemokine ligand 2) FC=0,5; *p*=0,02 względem kontroli; po korekcie wielokrotnych porównań różnice te nie osiągnęły istotności statystycznej (ang. *non-significant*, NS), jednak kierunek zmian pozostaje biologicznie spójny z aktywacją szlaków limfocytów B oraz chemotaksji leukocytów. Z kolei w próbkach EVs pochodzących z CSF pacjentów przed leczeniem stwierdzono podwyższone stężenia CCL2 i TREM2 – odpowiednio FC=9,35 (NS) i FC=10,71 (NS) względem wartości referencyjnych. Natomiast dla L1CAM$^+$ EVs pochodzących z CSF odnotowano podwyższone stężenia IL-4 (FC=9,35, NS) oraz TREM2 (FC=3,2, NS). Chociaż wyniki nie wykazały istotności statystycznej, zaobserwowany trend można interpretować jako współwystępowanie sygnałów przeciwzapalnych (IL-4) i aktywacji mikrogleju (TREM2, CCL2) w CNS.

Immunofenotypowanie markerów powierzchniowych wykazało podwyższoną ekspresję CD41b, CD42a oraz CD29 na surowiczych L1CAM$^+$ EVs przed leczeniem w porównaniu do kontroli. CD41b/CD42a są markerami płytkowo-pochodnych mikropęcherzyków, natomiast CD29 (integryna β1) uczestniczy w adhezji i migracji komórkowej. Wskazuje to na wzrost odsetka mikropęcherzyków pochodzenia płytkowego w krążących EVs przy nasileniu stanu zapalnego, ale może też świadczyć o częściowym wychwycie nie-neuronalnych EVs podczas selekcji L1CAM. Jednocześnie w całkowitej puli EVs z CSF odnotowano wyższe poziomy CD1c i CD24, co wskazuje na udział komórek dendrytycznych (CD1c) oraz komponenty neuronalno-glejowej (CD24) w kształtowaniu środowiska EVs w CNS. W przypadku L1CAM$^+$ EVs z CSF wykazano podwyższoną ekspresję T-komórkowych antygenów CD3 i CD56 (NS).

Warto podkreślić, że zgodnie z danymi literaturowymi, EVs są uznawane za nośnik informacji o toczących się procesach patofizjologicznych w MS, jednak większość dotychczasowych badań koncentrowała się na puli całkowitych EVs, bez wyróżnienia subpopulacji ze względu na ich pochodzenie komórkowe. Wyniki niniejszych badań dodają do dotychczasowego stanu wiedzy nowatorski aspekt związany z kluczowym komponentem EVs jakim jest subpopulacja pochodzenia neuronalnego, w tym wskazują na udział szlaku sygnałowego CCL2/CCR2, kluczowego dla migracji leukocytów przez BBB.

Po 6 miesiącach terapii rytuksymabem istotnie obniżyła się liczba L1CAM$^+$ EVs w surowicy (p<0,0001) w porównaniu z wartością wyjściową. Efekt ten współwystępował z istotnym obniżeniem stężenia TNFRSF13B (ang. *tumor necrosis factor receptor superfamily member 13B*) w proteomie surowiczych EVs (FC=-0,49, p=0,0004), kluczowego elementu sygnalizacji limfocytów B, a także obniżeniem stężenia CD79B (FC=-0,02, NS). Odnotowano także obniżenie stężenia CCL2 w L1CAM$^+$ EVs (FC=-0,14, NS) oraz wzrost TNF, IL-4 i VSNL1 (ang. *visinin-like protein 1*) (odpowiednio FC=0,11; FC=1,7; FC=0,09, NS).

Ponadto, profilowanie receptorów powierzchniowych po 6 miesiącach leczenia wykazało podwyższoną ekspresję CD29, CD42a, CD41b i CD62p (NS) w surowiczych EVs oraz wzrost CD42a, CD24, CD69 i CD25 w surowiczych L1CAM$^+$ EVs (NS).

Zestawienie tych obserwacji sugeruje, że deplecji limfocytów B, za którą idzie obniżenie poziomów komponentów odpowiedzi B-komórkowej, TNFRSF13B oraz CD79B, towarzyszy przebudowa środowiska chemokin (spadek CCL2) oraz potencjalne elementy równoważące (wzrost IL-4). Wzrost neuronalnego białka VSNL1, choć nieistotny statystycznie, może odzwierciedlać subtelne zmiany w homeostazie neuronalnej.

Zmiany zaobserwowane w immunofenotypowaniu mogą odzwierciedlać modyfikacje ekspresji markerów wczesnej aktywacji odpowiedzi immunologicznej po deplecji limfocytów B. Uzyskane wyniki wskazują, że w proces ten mogą być zaangażowane także populacje T-komórkowe (CD69, CD25) oraz komponenta płytkowa (CD42a).

W związku z tym, że zakażenie wirusem Epsteina-Barr (ang. *Epstein-Barr virus*, EBV) jest uważane za silny warunek wstępny rozwoju MS, w badaniu przeanalizowano również subpopulację L1CAM$^+$ EVs wykazujących ekspresję antygenu EBNA1 (ang. *Epstein-Barr nuclear antigen 1*), będącego markerem utajonych zakażeń EBV. Badanie NTA wykazało istotnie zwiększoną liczbę L1CAM$^+$ EBNA1$^+$ EVs (p<0,01) w surowicy przed leczeniem w porównaniu z grupą kontrolną, co sugeruje ich potencjał jako markera odpowiedzi na terapię rytuksymabem.

W niniejszej pracy oceniono, czy krążące we krwi L1CAM$^+$ EVs odzwierciedlają stan immunopatologiczny CNS oraz czy ich liczba i profil przenoszonych przez nie białek reagują na deplecję limfocytów B u pacjentów z RRMS w trakcie terapii anty-CD20. Przeprowadzone badania wskazują, że terapia anty-CD20 powoduje spadek liczby L1CAM$^+$ EVs po leczeniu oraz jednoczesne zmiany w profilu białkowym związanym z aktywnością limfocytów B (TNFRSF13B, CD79B). Na tej podstawie można wysnuć wniosek, że pęcherzyki L1CAM$^+$ EVs w surowicy mogą stanowić obiecujący biomarker odpowiedzi na leczenie rytuksymabem w RRMS.

PODSUMOWANIE I WNIOSKI

Biorąc pod uwagę złożoność mechanizmów patogenezy MS oraz heterogenny przebieg tego schorzenia, znalezienie markerów precyzyjnie odwzorowujących zmiany patofizjologiczne oraz skutecznych w monitorowaniu odpowiedzi na leczenie wciąż pozostaje wyzwaniem dla naukowców i klinicystów.

Zróżnicowane metody badawcze wykorzystane w niniejszej rozprawie doktorskiej dostarczyły wyników rzucających nowe światło na udział PLAs w naczyniopochodnym aspekcie patogenezy MS. Dodatkowo, przeprowadzone badania pozwoliły zgłębić potencjał EVs pochodzenia mózgowego jako źródła biomarkerów odpowiedzi na leczenie. Możliwość ich identyfikacji i łatwego pozyskania z krwi umożliwia rozwój diagnostyki procesów patofizjologicznych w obrębie CNS metodami nieinwazyjnymi. Ponadto, uzyskane wyniki wydają się obiecujące w kontekście wykorzystania wybranych cząsteczek EV-miRNA do różnicowania fenotypów MS.

Na podstawie uzyskanych wyników sformułowano następujące wnioski:

1. W MS płytki krwi wykazują wzmożoną aktywność, w tym podwyższoną zdolność tworzenia stabilnych PLAs z różnymi subpopulacjami limfocytów, przy udziale szlaku CD40-CD40L, co może sprzyjać rekrutacji oraz transmigracji leukocytów do CNS i promować przewlekłe neurozapalenie.
2. Uzyskane wyniki badań obejmujących charakterystykę PLAs wskazują na istotne znaczenie interakcji płytka-limfocyt B w przebiegu patofizjologii MS, szczególnie postaci progresywnej, łącząc mechanizmy prozakrzepowe z przewlekłym zapaleniem.
3. Fenotypy RRMS i SPMS wykazują odmienną ekspresję cząsteczek miRNA wyizolowanych z EVs, swoiste sygnatury wzbogacenia funkcjonalnego miRNA oraz odrębne profile sieci powiązań miRNA-mRNA, odzwierciedlając różne mechanizmy patobiologiczne obu fenotypów choroby.
4. Panel obejmujący miR-760, miR-98-5p, miR-301a-3p, miR-223-3p i FGF basic wykazuje wysoką zdolność dyskryminacji fenotypów RRMS i SPMS, dzięki czemu, po walidacji zewnętrznej, może być brany pod uwagę w kontekście markerów wspierających nieinwazyjne monitorowanie choroby.
5. W przebiegu leczenia anty-CD20 u pacjentów z RRMS obserwuje się obniżenie liczby $L1CAM^+$ EVs w surowicy oraz modulację profilu przenoszonych przez nie białek odpowiedzi immunologicznej, wskazując na ich wrażliwość na deplecję limfocytów B.
6. $L1CAM^+$ EVs mogą stanowić dynamiczny, nieinwazyjny biomarker służący do monitorowania odpowiedzi na leczenie anty-CD20 u pacjentów z RRMS, który, po odpowiedniej walidacji, może wspierać zindywidualizowane schematy dawkowania rytuksymabu i tym samym ograniczać ryzyko nadmiernej immunosupresji.

BIBLIOGRAFIA

1. Khan, G. & Hashim, M. J. Epidemiology of Multiple Sclerosis: Global, Regional, National and Sub-National-Level Estimates and Future Projections. *J Epidemiol Glob Health* 15, 21 (2025).
2. Brownlee, W. J. *et al.* Towards a Unified Set of Diagnostic Criteria for Multiple Sclerosis. *Annals of Neurology* 97, 571–582 (2025).
3. Parsons, M. *et al.* Characterisation of Platelet Releasate Proteome in Relapsing-Remitting Multiple Sclerosis Reveals Dysregulation of Inflammatory Signalling and Extracellular Vesicle Dynamics. *PROTEOMICS – Clinical Applications* 19, e202400019 (2025).
4. Lan, Y., Ding, J., Yu, T. & Cheng, C. Research progress of platelets in neurodegenerative diseases. *Front. Aging Neurosci.* 17, (2025).
5. Muttiah, B., Ng, S. L., Lokanathan, Y., Ng, M. H. & Law, J. X. Beyond Blood Clotting: The Many Roles of Platelet-Derived Extracellular Vesicles. *Biomedicines* 12, 1850 (2024).
6. Xiang, H. *et al.* Extracellular vesicles (EVs)' journey in recipient cells: from recognition to cargo release. *J. Zhejiang Univ. Sci. B* 25, 633–655 (2024).
7. Bhom, N., Somandi, K., Ramburrun, P. & Choonara, Y. E. Extracellular nanovesicles as neurotherapeutics for central nervous system disorders. *Expert Opinion on Drug Delivery* 22, 69–84 (2025).
8. Kalluri, R. The biology and function of extracellular vesicles in immune response and immunity. *Immunity* 57, 1752–1768 (2024).
9. Liu, X., Shen, L., Wan, M., Xie, H. & Wang, Z. Peripheral extracellular vesicles in neurodegeneration: pathogenic influencers and therapeutic vehicles. *J Nanobiotechnol* 22, 170 (2024).
10. Kobeissy, F. *et al.* The game changer: UCH-L1 and GFAP-based blood test as the first marketed in vitro diagnostic test for mild traumatic brain injury. *Expert Review of Molecular Diagnostics* 24, 67–77 (2024).
11. Xu, X. *et al.* Brain-derived extracellular vesicles: Potential diagnostic biomarkers for central nervous system diseases. *Psychiatry and Clinical Neurosciences* 78, 83–96 (2024).
12. Bjursten, S. *et al.* Concentrations of S100B and neurofilament light chain in blood as biomarkers for checkpoint inhibitor–induced CNS inflammation. *eBioMedicine* 100, (2024).
13. Nogueras-Ortiz, C. J. *et al.* Single-extracellular vesicle (EV) analyses validate the use of L1 Cell Adhesion Molecule (L1CAM) as a reliable biomarker of neuron-derived EVs. *Journal of Extracellular Vesicles* 13, e12459 (2024).
14. Li, D., Zou, S., Huang, Z., Sun, C. & Liu, G. Isolation and quantification of L1CAM-positive extracellular vesicles on a chip as a potential biomarker for Parkinson's Disease. *J Extracell Vesicles* 13, e12467 (2024).

15. Zhang, H. *et al.* Proteomic study of plasma and L1CAM-captured exosomal proteins in children with autism spectrum disorders. *Journal of Pharmaceutical and Biomedical Analysis* 264, 116965 (2025).
16. Gomes, D. E. & Witwer, K. W. L1CAM-associated extracellular vesicles: A systematic review of nomenclature, sources, separation, and characterization. *Journal of Extracellular Biology* 1, e35 (2022).
17. Di Filippo, M. *et al.* Fluid biomarkers in multiple sclerosis: from current to future applications. *Lancet Reg Health Eur* 44, 101009 (2024).
18. Ru, Y. *et al.* The multiMiR R package and database: integration of microRNA–target interactions along with their disease and drug associations. *Nucleic Acids Research* 42, e133 (2014).
19. Ashburner, M. *et al.* Gene Ontology: tool for the unification of biology. *Nat Genet* 25, 25–29 (2000).
20. The Gene Ontology Consortium *et al.* The Gene Ontology knowledgebase in 2023. *Genetics* 224, iyad031 (2023).
21. Kanehisa, M. & Goto, S. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* 28, 27–30 (2000).
22. DISGENET: Database Information. https://www.disgenet.com/About.
23. Piñero, J. *et al.* The DisGeNET knowledge platform for disease genomics: 2019 update. *Nucleic Acids Research* 48, D845–D855 (2020).
24. Yu, G., Wang, L.-G., Han, Y. & He, Q.-Y. clusterProfiler: an R Package for Comparing Biological Themes Among Gene Clusters. *OMICS* 16, 284–287 (2012).
25. Maciak, K., Dziedzic, A., Miller, E. & Saluk-Bijak, J. miR-155 as an Important Regulator of Multiple Sclerosis Pathogenesis. A Review. *Int J Mol Sci* 22, 4332 (2021).
26. Maciak, K., Dziedzic, A. & Saluk, J. Remyelination in multiple sclerosis from the miRNA perspective. *Front. Mol. Neurosci.* 16, (2023).
27. Anandan, S. *et al.* Brain-derived blood biomarkers in multiple sclerosis—current trends and beyond. *Frontiers in Immunology* 16, 1569503 (2025).
28. Maciak, K. *et al.* Human B-cells can form Hetero-aggregates with Blood Platelets: A Novel Insight into Adaptive Immunity Regulation in Multiple Sclerosis. *Journal of Molecular Biology* 437, 168885 (2025).
29. Boyden, S. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. *J Exp Med* 115, 453–466 (1962).

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Review

miR-155 as an Important Regulator of Multiple Sclerosis Pathogenesis. A Review

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Abstract: Multiple sclerosis (MS) is a chronic, immune-mediated disease and the leading cause of disability among young adults. MicroRNAs (miRNAs) are involved in the post-transcriptional regulation of gene expression. Of them, miR-155 is a crucial regulator of inflammation and plays a role in modulating the autoimmune response in MS. miR-155 is involved in blood–brain barrier (BBB) disruption via down-regulation of key junctional proteins under inflammatory conditions. It drives demyelination processes by contributing to, e.g., microglial activation, polarization of astrocytes, and down-regulation of CD47 protein and affecting crucial transcription factors. miR-155 has a huge impact on the development of neuropathic pain and indirectly influences a regulatory T (Treg) cell differentiation involved in the alleviation of pain hypersensitivity. This review also focused on neuropsychiatric symptoms appearing as a result of disease-associated stressors, brain atrophy, and pro-inflammatory factors. Recent studies revealed the role of miR-155 in regulating anxiety, stress, inflammation in the hippocampus, and treatment-resistant depression. Inhibition of miR-155 expression was demonstrated to be effective in preventing processes involved in the pathophysiology of MS. This review aimed to support the better understanding the great role of miR-155 dysregulation in various aspects of MS pathophysiology and highlight future perspectives for this molecule.

Keywords: miR-155; microRNA (miRNA); neuroinflammation; multiple sclerosis (MS); autoimmunity; biomarkers

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1. Introduction

Multiple sclerosis (MS) is a chronic, untreatable disease of the central nervous system (CNS) and the most common cause of neurological disability diagnosed in young adults. It is estimated that the average age of sufferers globally is 32 years. This distinguishes the condition from other neurodegenerative diseases and makes it an increasing socioeconomic and medical problem. As of 2020, there were an estimated 2.8 million people diagnosed with MS worldwide; in 2013, the number was 2.3 million [1].

In MS, complex processes involving both genetic (e.g., polymorphism of HLA-DPB1 allele), epigenetic (e.g., miRNAs) and environmental (e.g., smoking, infectious diseases, vitamin D deficiency) determinants lead to autoimmunity and, consequently, demyelination, axons damage, and neurodegeneration [2–5]. However, the picture of causative factors underlying the disease remains unclear. Additionally, MS is a heterogeneous disease with a highly individualized clinical course. This makes differential diagnosis and monitoring of treatment efficacy difficult, as well as applying effective therapy without the risk of complications. There is a necessity to search for specific biochemical, molecular, and genetic factors that can reliably correlate with the disease development degree.

Numerous studies confirm a pivotal role of small (21–23 nucleotides) non-coding RNAs—microRNAs (miRNAs) as crucial regulators of biological processes associated with

the pathophysiology of various conditions including cancer, autoimmune, neurodegenerative, and infectious diseases [6–10]. Moreover, miRNAs are likely to meet the criteria for a potentially good prognostic marker. Namely, one of the crucial characteristics of the biomarker is the ability to be detected in a non-invasive manner. Moreover, the biomarker should be specific, sensitive, and susceptible to change with disease progression or remission. The final important criterion is its applicability in the bench-to-bedside approach [11]. miRNAs are easy to obtain as they occurred in body fluids so there is no necessity to isolate them in an invasive way from cells or tissues. These molecules could be stored for a long period, subjected to freezing and thawing, and are resistant to pH extremes. Moreover, blood miRNAs show resistance to ribonucleases and have an average half-life of 5 days, as shown in one study [10,11].

Available data identify miR-155 as so-called inflamma-miR, a powerful activator of the inflammation, the essential miR in the autoimmune diseases' pathogenesis because of its influence on myeloid cell polarization to a phenotypic and functional pro-inflammatory form [12,13]. Additionally, miR-155 dysregulation has a role in many human cancers, hematological malignancies, and reactions to viral infections [14]. miR-155 can serve as a significant player in the pathogenesis of MS. Successfully investigating how miR-155 contributes to MS may provide innovative determinants of neurodegeneration with the great potential for future use in diagnostics.

miR-155 activity in peripheral immune cells and brain resident cells in the context of MS was analyzed in detail in a review by McCoy [15]. This paper aims to consolidate the results of studies reflecting the consequences of miR-155 activity in certain elements of the pathophysiology of MS. Collecting the emerging data on this topic may contribute to increase understanding of the miR-155 operation and emphasize the potential of this molecule to be used in future research toward advancing the diagnosis and treatment of MS. This review will initially focus on summarizing the role of miR-155 in the context of two phenomena underlying the initiation of the MS disease process—blood–brain barrier (BBB) damage and demyelination. Second, an attempt will be made to present the available studies shedding light on miR-155 as a relevant element of the pathophysiology of neuropathic pain and cognitive impairment, which are serious dysfunctions of the complex clinical symptomatology of MS.

2. Multiple Sclerosis

Clinically, several subtypes of MS can be distinguished. The most prevalent form is relapsing-remitting multiple sclerosis (RRMS), characterized by periods of flares and silencing (remission). In most cases, as a result of accumulating neurological injuries, secondary progressive multiple sclerosis (SPMS) develops, and recovery phases are no longer observed. A constant progression of nervous system symptoms and occasional phases of stabilization or remission are characteristic of primary progressive multiple sclerosis (PPMS). Progressive-relapsing multiple sclerosis (PRMS) is observed when the disease progression is continuous and acute throwing episodes occur regularly [16,17].

The pathophysiology of MS is based on a persistent inflammatory state involving immune cells, such as CD4+ T-cells, CD8+ T-cells, and B-cells response, reactive against myelin antigens. Under physiological conditions, the BBB has a protective function, while in the course of MS, it becomes dysfunctional and allows CD4+ T-cells, CD8+ T-cells, and B-cells to infiltrate into the brain [18]. This results in damage to the myelin sheaths surrounding the axons, followed by axonal injury leading to the progression of the patient's disability [19]. Disruption of the BBB is one of the critical, specifically targeted incidents that initiate MS. The activity of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interferon-γ (INF-γ) leads to the malfunction of this protective barrier [20]. The main hallmark of MS is demyelination of the cerebral white matter. Spinal cord and cerebral cortex demyelination also contribute to permanent neurological disability in this disorder [21]. Destruction of the myelin sheaths is a consequence of an autoimmune process

directed against a putative myelin autoantigen mediated by CD4+ T-cells, especially T helper (Th) 1 cells producing IFN-γ and Th17 producing interleukin (IL)-17 [15,22].

The specific signs of MS are gadolinium-enhancing inflammatory lesions (plaques) mainly in the white matter of the CNS, visible on magnetic resonance imaging (MRI), which must be characterized by dissemination in time (DIT) and dissemination in space (DIS), according to the McDonald's criteria. Radiological and laboratory tests are performed to confirm the diagnosis [23]. The clinical manifestations of MS are individually differentiated neurological symptoms, including, e.g., impaired vision and sensation deficits [16]. A common MS symptom that results from the central nerve injury is neuropathic pain. It leads to sleep disorders, anxiety, and depression and contributes to a decrease in the quality of life and socioeconomic issues. It manifests, e.g., by hyperalgesia, spontaneous pain, and allodynia [24]. It occurs in up to 75% of patients and its treatment is often unsatisfactory for them, even though pain treatment accounts for as much as 30% of drugs used to alleviate the symptoms of MS [25]. Damage to the white and gray matter of the CNS resulting from inflammation and demyelination leads not only to physical disability but also to neuropsychiatric symptoms in MS patients. These signs are observed even in the early phase of the disease and constitute an important component of the diagnostic process and the therapy. The category of neuropsychiatric symptoms in MS covers a broad range of features that distinguishes between cognitive disorders and disturbances impacting mood, behavior, and affect [26,27].

Other symptoms include urinary tract dysfunction, intestinal disorders, spasticity, sexual dysfunctions, dizziness, and fatigue [28]. Non-specific signs such as migraine (alone or in combination with other disorders), fibromyalgia, or non-specific/non-localizing neurologic symptoms with abnormal MRI may lead to misdiagnosis. The 2017 McDonald's criteria emphasize the role of paraclinical testing on the way to distinguish MS from other diseases with overlapping symptoms, e.g., neuromyelitis optica spectrum disorders (NMOSDs) [29,30]. The main tests used for diagnosis and to support the treatment are cerebrospinal fluid (CSF) analysis in the context of the presence of oligoclonal bands, MRI for active lesions in the brain white matter, and John Cunningham (JC) virus antibody titers [31]. The concept of no evidence of disease activity (NEDA) was introduced to evaluate the efficacy of the treatment used. NEDA generally refers to disease stabilization as evidenced by the lack of clinical relapses, no disease progression as measured by the Expanded Disability Status Scale (EDSS), and the absence of new disease activity on MRI. Recently, an extension of the concept of the NEDA to include brain atrophy and analysis of cognitive function measurement has been proposed [32].

There is no available treatment for MS. Currently approved disease-modifying therapies (DMTs) that act via immunomodulatory/immunosuppressive mechanisms apply to RRMS to reduce relapses frequency and delay disease progression. The DMTs used include interferon-β (INF-β), glatiramer acetate, or natalizumab, which interfere with inflammation. However, recent recommendations point to siponimod and ocrelizumab as drugs that may reduce the progression of SPMS and PPMS, respectively [33,34]. The process of finding the most optimal therapy is highly individualized due to the dynamic and diverse course of the disease. Deciding between choosing more effective but also riskier DMTs in the early stage of MS remains a challenge for clinicians. The methods currently used for the diagnostic and prediction of the DMTs effectiveness have limitations of specificity and sensitivity [35].

3. miRNA, Facts, and Expectations

Features such as stability, convenience in extraction from body fluids, and the ability to change the expression profile in the early stages of a specific disease allow miRNAs to be considered as potentially effective diagnostic and prognostic factors [36]. In 2009, Otaegui et al. suggested miRNAs as promising biomarkers in MS and selected those that may be indicators of relapse phase [37]. Regev et al. have already demonstrated that it is a useful tool to discriminate MS patients from healthy controls. Furthermore, 10 particular

miRNAs were correlated with EDSS score [38]. The review by Baulina et al. presented the list of miRNAs with altered expression in the course of MS obtained from different sources (CSF, demyelinating plaques, whole blood, plasma, MNCs, T-cells, B-cells), which includes, e.g., miR-155, miR-146a, miR-181c, miR-326, miR-346, miR-17, miR-320a, miR-34a, miR-340, miR-132, and their predicted target genes [39]. However, the functions of miRNAs in the context of MS pathophysiology remain largely unknown.

The transcription of genes encoding miRNAs takes place with the participation of RNA II polymerase, leading to the formation of long primary miRNAs (pri-miRNAs) [40]. Subsequently, the microprocessor complex of RNAse III type Drosha enzyme and DiGeorge syndrome critical region 8 (DCGR8) protein cleaves pri-miRNA in the nucleus into hairpin sequences of precursor miRNAs (pre-miRNA) [41]. Here, the nuclear phase of the processes ends, and the pre-miRNA is transported by the Exportin-5 protein to the cytoplasm. There, pre-miRNA is cleavage by RNase III type Dicer forming an RNA duplex with a passenger strand and a guide strand [42]. This molecule contains mature miRNA sequences that can form an integral compound within the core of a multiprotein RNA-induced Silencing Complex (RISC). The core of a mature RISC consists of Argonaute family proteins (Ago) that remove the passenger strand—typically the guide strand is the one with lower thermodynamical stability of 5′ [43].

Single-stranded miRNA is a template for the recognition of complementary sequences known as miRNA response elements (MREs), located mostly within 3′-untranslated regions (3′UTR) of the mRNA transcript. Within the miRNA, there is a so-called "seed" region located at nucleotides 2–8 from the 5′ end that designates a target. miRNAs play a role in a natural mechanism of specific gene silencing after transcription known as RNA interference (RNAi). The mechanism of gene silencing depends on the degree of match with the target with two types of effects occurring in most cases. Perfect complementarity leads to the cleavage of mRNA by the catalytic Ago2 protein and destabilization and degradation of the transcript [11,14,44–46]. If the complementarity is non-canonical, which means, not full, it results in translational repression—this type concerns 60% of interactions [47]. The consequences of the miRNA regulatory mechanism are changes in biological processes such as inflammation, proliferation, differentiation, and cells apoptosis [36,37]. Moreover, miRs modify the transcripts of proteins involved in neurogenesis, gliogenesis, and myelin repair. Dysregulation of specific miRNAs expression may underlie neurodegenerative processes and autoimmunity [48].

Many miRNAs circulate in biological fluids, derived from active transport or revealed through damaging processes such as apoptosis. Circulating miRNAs are packed into vesicles called exosomes (10% of circulating miRNAs) or form complexes with proteins (90% of circulating miRNAs) that prevent digestion by RNases. The miRNAs circulating in the blood are much better detectable than intracellular ones [11,49].

A given mRNA can be targeted by multiple miRNAs, as well as a particular miRNA molecule can influence hundreds of mRNA transcripts, since various genes share the same miRNA recognition site. Nevertheless, the biological significance of various targets can be different [45].

4. miR-155 and Immune Response

miR-155 is among those miRNAs that are most strongly implicated in autoimmune diseases [13]. It is encoded by the miR-155 host gene (*mir155hg*) that produces miR-155-3p and miR-155-5p forms. It was formerly called the B-cell integration cluster (BIC) and was found to be strongly overexpressed in B-cell-activated lymphomas [50]. Further research demonstrated that miR-155 occurs in human tissues such as the spleen, thymus, liver, lung, and kidney and is specific for hematopoietic cells [51,52]. The available data suggest the high significance of miR-155 for the homeostasis and the immune response. Namely, the alignment of B-cells functions such as antibody differentiation and production, modulation of T-cells, including CD8+, differentiation of CD4+ toward Th1, Th2, Th17, and regulatory T (Treg) cells, regulation of dendritic cells, cytokines, chemokines, and transcription factors

stimulation [14,53]. Furthermore, miR-155 increases activation of astrocytes and affects microglia and macrophages, causing pro-inflammatory polarization to M1-like phenotype, and neurotoxicity [54].

miR-155 is a pleiotropic miRNA characterized by a significant association with genes related to the immune response among which are TNF-α and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcripts. It is the main promoter of the development of neuroinflammation manifested by glial activation and pro-inflammatory cytokines production by the CNS-resident cells, a process occurring in neurodegenerative disorders [55–57]. After activation of toll-like receptors (TLRs), miR-155 modulates inhibitors of inflammation such as suppressor of cytokine signaling 1 (SOCS1), Src homology-2 domain-containing inositol 5-phosphatase 1 (SHIP1), transcription factor enhancer-binding protein beta (C/EBP-beta), interleukin 13 receptor, and alpha 1 (IL13Rα1) within macrophages/microglia [58–61]. The pro-inflammatory action of miR-155 in microglia may also be activated by the transcriptional factor p53. In this case, the target of miR-155 becomes the transcription factor c-Maf that normally induces anti-inflammatory processes in the immune cells. The effect of this action is the promotion of inflammation [62].

Overexpression of miR-155 has been observed in a range of samples, including resident brain cells, active brain lesions, as well as blood cells from MS patients [15]. Junker et al. demonstrated that miR-155 was up-regulated (fold change = 11.9; $p < 0.01$) in active white matter lesions compared with healthy controls [63]. Paraboschi et al. carried out a study that investigated the expression levels of 22 miRNAs associated with immunity and located in the peripheral blood mononuclear cells (PBMCs) of RRMS patients in the remission phase compared with healthy controls. As a result, miR-155 was found to be the most up-regulated (fold change = 3.30; $p = 0.013$) indicating its involvement in the regulation of MS pathophysiology [64]. The study carried out on an experimental autoimmune encephalomyelitis (EAE), an animal model for MS, revealed overexpression in CD4+ T-cells from the spleen, lymph nodes, and CNS compared with controls and thus showed that miR-155 confers susceptibility to EAE [65]. It has been found that miR-155 is one of the most crucial miRNAs in MS as it regulates MS risk genes *PIK3R1* and *PIK3CA* and correlates with severity of the disease [49]. These genes encode p85-α and p110-α proteins, members of phosphoinositide 3-kinase (PI3K) family [66,67]. Abnormalities in PI3K contribute to, e.g., cancer development, neurological and immunological dysfunctions, dendritic cells functioning, EAE pathogenesis and demyelination in MS [49]. The results of further research in the context of MS and EAE confirmed the association of miR-155 with these disease states. Up-regulation of miR-155 might indicate severe condition course and poor prognosis in MS patients [68–70]. The pro-inflammatory effect of miR-155 affects microglia activation, phagocytosis of myelin by macrophages [63], differentiation of T-cells towards Th1 or Th2 [71], contribution to increased permeability of the BBB, and infiltration of peripheral immune cells [72]. In recent studies, attempts have been made to check potential links between the miR-155 expression level and depression, cognitive functions, and neuropathic pain [55,73–76]. As miR-155 is generally associated with inflammation, it is likely to be involved in various pathophysiological processes and symptoms of MS.

5. miR-155 and Blood–Brain Barrier

The BBB vascularizes the CNS; selects the movement of leukocytes, pathogens, nutrients, and proteins from blood to the brain; and takes part in cell-to-cell interactions. It is a unique semi-permeable structure of brain capillaries, formed by cerebral endothelial cells (ECs), pericytes, and astrocytes. ECs are essential for the integrity of BBB and cerebral homeostasis. Cellular junctions consist of selective complexes, mainly tight junctions (TJs) and adherens junctions (AJs), composed of smaller subunits of transmembrane proteins, such as occludin, claudins, junctional adhesion molecules, zonula occludens, cadherins, and catenins [16,77,78].

Although the mechanism of BBB disassembly is not fully elucidated, it is believed that the pro-inflammatory cytokines contribute to this phenomenon, among others, at the transcriptional stage and/or during post-transcriptional regulation of gene expression. Moreover, it is known that vascular endothelial cells are abundant in miRNAs [79].

Lopez-Ramirez et al. confirmed that the activity of miR-155 affects the function of BBB at the neurovascular unit (NVU) components in both brain of MS patients and spinal cord of EAE mouse [72,80]. The NVU is a comparatively new concept in neuroscience, defined as a multicellular structure composed of neurons, perivascular astrocytes, microglia, pericytes, BBB ECs, and the basement membrane. The NVU is the fundamental driver of neurovascular coupling that plays an essential role in all stages of BBB development and maintenance. The NVU can act in a synchronized, functional way and regulate cerebral blood flow maintaining brain homeostasis. Damage to NVU components is connected with reduction in permeability as well as selectivity of BBB [80]. miR-155 was demonstrated to be up-regulated in mice, and loss of this regulator caused higher tightness of BBB within the neuroinflammatory loci. Furthermore, the expression level of miR-155 is increased in brain endothelium in active MS lesions, and the analysis performed on cell culture of brain endothelial cells (BECs) confirmed that miR-155 overexpression is stimulated by pro-inflammatory cytokines, TNF-α, and IFN-γ. Therefore, it is suggested that brain endothelial miR-155 is induced by inflammation and subsequently stimulates an early inflammatory effect at the NVU. The up-regulation of miR-155 influenced BBB permeability mimicking the alternations that were induced by cytokines. The mechanism by which miR-155 exacerbated BBB disruption relies on down-regulation of junctional proteins, significant for BBB integrity: claudin-1 (CLDN-1), annexin-2 (ANXA-2), syntenin-1 (SDCBP), and dedicator of cytokinesis-1 (DOCK-1) (Figure 1). CLDN-1 and ANXA-3 belong to interendothelial junctional complex molecules, while SDCBP and DOCK-1 constitute focal adhesion components. Thus, overexpression of miR-155 indirectly contributes to BBB malfunction by negatively regulating elements of the cell-to-cell and cell-to-matrix adhesion pathways [72,81].

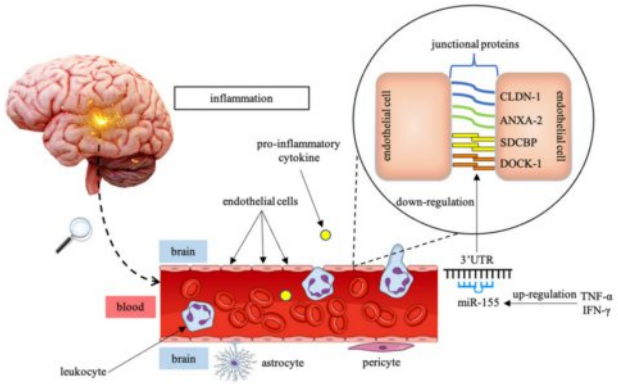

Figure 1. Operation of miR-155 in disruption of the BBB under inflammatory conditions. miR-155 is up-regulated due to pro-inflammatory cytokines activity, such as TNF-α and IFN-γ. Overexpression of miR-155 enhances an inflammatory effect contributing indirectly to BBB permeability via down-regulation of junctional proteins between endothelial cells: CLDN-1, ANXA-2, SDCBP, and DOCK-1. Abbreviations: BBB—blood–brain barrier; TNF-α—tumor necrosis factor-α; IFN-γ—interferon-γ; CLDN-1—claudin-1; ANXA-2—annexin-2; SDCBP—syntenin-1, DOCK-1—dedicator of cytokinesis-1.

The following study conducted on the cytokine-stimulated cultured human brain endothelium revealed the contribution of miR-155 up-regulation to increased adhesion of monocytes and T-cells to the BBB during neuroinflammation. It has been suggested that

miR-155 acts via modulation of endothelial adhesion molecules, vascular cell adhesion molecule 1 (VCAM1), and intercellular adhesion molecule 1 (ICAM1) [79]. Noteworthy is the study carried out on an animal model of stroke, which supports brain endothelial miR-155 as an important player in the process of neuroinflammation and disruption of the BBB integrity. Additionally, it presents a protective effect of anti-microRNA-155 in vivo [82]. In contrast, increased expression of other miRNAs, miR-98 and let-7 in vitro and in vivo decreased leukocyte adhesion to and infiltration across ECs, inhibited pro-inflammatory cytokines, and supported BBB during neuroinflammation [83]. It is also worth mentioning that the importance of miR-155 modulation in the context of BBB disruption has been confirmed in several studies associated with post-ischemic endothelial injury and recovery [82,84,85].

Interestingly, the lipopolysaccharide (LPS)-activated layer of epithelial cells composing the blood-CSF barrier (BCSFB) called choroid plexus epithelium (CPE) has been shown to release exosomes into the CSF during inflammation. These extracellular vesicles (EVs) containing miR-155 reach the brain parenchyma and stimulate cytokines such as TNF, IL-1, and IL-6 giving rise to inflammation. Although the BBB function is undeniably important in the blood-to-brain signaling and the production of EVs by ECs has not been excluded, it has been emphasized that ECs manifest low vesicle transport activity. Moreover, the BBB integrity was not disrupted by LPS activity; this fact is argued against the role of BBB permeability in the inflammation-induced EVs increase in the CSF [86].

6. miR-155 and Demyelination

The main cells that are responsible for myelin production in the CNS are oligodendrocytes differentiated from the oligodendrocyte precursor cells (OPCs). The phenomenon of myelin loss occurs when the differentiation process is compromised, e.g., by the resident innate immune cells of the CNS, involved in antigen presentation and secretion of pro-inflammatory factors—microglia, leading to the destruction of the myelin sheath or/and oligodendrocytes [87].

miR-155 contributes to the activation of the microglia, which produces pro-inflammatory factors including TNF-α, IL-1b, IL-6, interferon-inducible protein 10 (IP-10), macrophage inflammatory protein-1α (MIP-1α), monocyte chemoattractant protein-1 (MCP-1), and nitric oxide (NO) [16]. Interestingly, miR-155 has been shown to promote the polarization of astrocytes towards their activated neurotoxic form A1 [15]. Astrocytes can adopt at least two different reactive phenotypes (A1 and A2) in response to CNS insult. The former type is induced as a result of CNS disease, acute injury, and LPS-induced neuroinflammation and can lead to the death of neurons and oligodendrocytes. In contrast, astrocytes A2 are induced by ischemia and may act protective by upregulating neurotrophic factors. Under normal conditions, astrocytes contribute to neuronal survival, however, subtype A1 has lost this function. It has been revealed that activated microglia might induce the astrocytes' transformation into the A1 form by releasing interleukin 1-α (IL-1α), TNF, and complement 1, and subcomponent q (C1q). The enhanced level of A1 astrocytes was reported in different human neurodegenerative diseases, including MS [15,16]. The level of the transmembrane CD47 protein, which reduces microglial activation, is decreased by overexpression of miR-155 in brain active lesions. CD47 acts by inducing a "do not eat me" signal on cells adjacent to the microglia. Overexpression of miR-155 is involved in macrophages activation by decreasing CD47 expression in astrocytes and oligodendrocytes, and also causes myelin phagocytosis by macrophages, affecting them directly [63].

In one study, Mycko et al. analyzed the alternations of miR-155, miR-301a, and miR-21 expression profiles in the context of CD4+ T-cells. The research was conducted on the animal model of the disease, which was induced by immunization with the CNS antigen, myelin-oligodendrocyte glycoprotein (MOG) 35–55. The results demonstrated that all miRs studied, including miR-155, were significantly elevated in vitro and in vivo. Interestingly, in this study, only miR-301a was found to be involved in Th17 differentiation and the pathogenesis of demyelination via Protein Inhibitor of Activated STAT 3 (PIAS3) molecule

(affecting IL-6/23–STAT3 pathway) as a direct target in CD4+ T-cells. Inhibition of miR-155 and miR-21 by specific inhibitors, called antagomirs (anti-miRs) caused down-regulation of IFN-γ and up-regulation of IL-4 secretion [88]. However, the crucial role of miR-155 in Th17 cells has been demonstrated in the context of autoimmune inflammation during EAE via the action involving transcription factor Ets1 and the clinically relevant IL-23-IL-23R pathway [89]. Another study demonstrated that miR-155-3p targets two genes in CD4+ T-cells: *Dnaja2* and *Dnajb1*, which are related to the down-regulation of Th17 lymphocytes and their infiltration into the brain. It has been suggested that miR-155-3p operates in the process of autoimmune demyelination with selective expression during EAE [22]. The results of recent studies based on the EAE model induced by cuprizone (a copper chelator molecule that affects oligodendrocytes) confirmed that decreasing expression of miR-155-3p can prevent microglia activation, myelin damage, and MS progression [90,91]. Analysis of miR-155 expression carried out on a mouse model allowed revealing the effect of the probiotic bacteria, *Lactobacillus casei,* on the recovery of demyelinated animals by regulating the immune response [92]. Another research conducted on the cuprizone-induced demyelination model evaluated the miRNAs expression and predicted their target genes according to the micro-RNA database (miRDB). miR-155-5p was among the upregulated miRNAs with fold change ≥ 1.5, compared with the control group. Prediction of target genes could reveal connections between miRs modulation and the cascade of processes during demyelination induced by cuprizone administration. Among the targets was a transcription factor mothers against decapentaplegic homolog 2 (SMAD2), mediating the anti-inflammatory effects of transforming growth factor-β (TGF-β), the pathway of which was previously considered one of the regulatory demyelination pathways. Up-regulated miR-155-5p may play a role in the mechanism of demyelination through a suppressive effect on Smad molecular cascades, leading to upregulation of the Nogo receptor (NgR) present on axons to inhibit neurite outgrowth [93].

7. miR-155 and Neuropathic Pain

Emerging data suggest neuropathic pain as a neuro-immune dysfunction with increased activation of the immune system. miR-155 has a well-studied influence on inflammatory factors such as IL-1β, IL-6, TNF-α, NF-κB, and p38 mitogen-activated protein kinase (MAPK), associated with neuropathic pain, [94–96]. miR-155 was demonstrated to be up-regulated in the prefrontal cortex of mice with inflammatory pain [97]. A rat model of neuropathic pain, chronic constrictive injury (CCI), allowed noting increased expression level of miR-155 in spinal microglia, while its inhibition alleviated neuropathic pain and neuroinflammation. It has been investigated that suppression of miR-155 caused inhibition of NF-κB and p38 MAPK activation by mediating a negative regulator of inflammation, SOCS1 [98]. Moreover, miR-155 has been demonstrated to modulate an inflammation-related serum and glucocorticoid regulated protein kinase 3 (SGK3) in neuropathic pain rats. Down-regulation of miR-155 and stimulation of SGK3 expression resulted in alleviation of the nerve pain and lower pain threshold [99]. Both miR-155 and miR-124a are predicted to target the histone deacetylase sirtuin 1 (SIRT1). This interaction enhances the development of anti-inflammatory Tregs by increasing the expression of transcription factor forkhead box-p3 (Foxp3), which is a key regulator of Tregs differentiation. Promotion of Tregs leads to alleviation of pain hypersensitivity suggesting a possible role of these cells in the mitigation of the pain-promoting inflammatory response [100].

Another gene regulated by miR-155 is NADPH oxidase (NOX2), an inducer of reactive oxygen species (ROS) in macrophages/microglia [101]. NOX2 regulation allows manipulating the proportion of pro-inflammatory (M1) and anti-inflammatory (M2) phenotypes of macrophages/microglia [102,103]. Increased expression of this enzyme has been reported after spinal cord injury. It contributes to neuropathic pain development via M1 microglia/macrophages activation and inflammatory-related cascades [104]. Inhibition of NOX2 reduces ROS production, stimulates microglia differentiation towards M2, increases expression of anti-inflammatory cytokine IL-10, and decreases expression

of pro-inflammatory factors, including miR-155 [100,105]. It has been demonstrated that miR-155 expression, which is elevated in the spinal cord injury (SCI) model, is lower in NOX2−/− mice and it may be controlled by IL-10 [101,106]. Microglial miR-155 has recently been investigated as an important player in neuropathic pain development by engaging in M1/M2 polarization switch. The research was conducted on the BV-2 type microglial cells model, and the M1/M2 polarization was induced by LPS/IL-4. The neuropathic pain model was created by generating spinal nerve ligation in rats. The elevated miR-155 expression has been noted in the M1 microglia phenotype and lowered in M2. Suppression of miR-155 reduces the secretion of IL-1β and TNF-α and alleviates neuropathic pain by stimulating the switch from pro-inflammatory M1 to anti-inflammatory M2 polarization [76]. Another study investigated the role of miR-155 in a disorder manifested by a chronic neuropathic pain associated with demyelination—trigeminal neuralgia (TN). It was demonstrated that miR-155-5p is overexpressed in the serum of TN patients and inhibits nuclear factor erythroid 2-related factor 2 (Nrf2) expression, which was previously shown as a neuroprotective factor in diabetic peripheral neuropathy [107,108].

8. miR-155 and Neuropsychiatric Symptoms

Damage to the BBB, loss of myelin and axons, synaptic dysfunction, and dysregulated neurotransmitter production due to acute inflammation result in brain atrophy and progressive cognitive impairment [48]. Depression and anxiety appear to worsen memory, information processing speed, and daily functioning in people with MS [26].

It is estimated that the prevalence of depressive disorders among people with MS is up to 50%, which means 2–3 times higher than in the general population. Besides anxiety and stressors related to the presence of the disease, pathophysiological mechanisms such as brain atrophy, activation of pro-inflammatory cytokines, astrocytes and microglia, and lesion burden contribute to depressive disorders [109]. It is believed that mood disorders development results from complex mechanisms related to genetic predisposition and environmental factors. It is suggested that epigenetic mechanisms have a great role in pathogenesis. Research results revealed alterations in miRNAs expression levels, including miR-155, targeting neurotrophic factors (brain-derived neurotrophic factor, BDNF, TGF-β) and inflammatory mechanisms in patients with depressive disorders [73,110,111]. In the CSF and blood samples of people suffering from a major depressive disorder (MDD), the expression of miR-155 was elevated [112,113].

Fonken et al. probably were the first to demonstrate the effect of experimental modulation of miR-155 expression on anxiety- and depressive-like behaviors in an animal model. Mentioned behaviors, as well as sensorimotor function and social behavior, have been assessed in miR-155 knockout and wild-type mice. Additionally, the expression of genes related to inflammatory and neurotrophic factors was evaluated in the hippocampus, since changes in this structure are associated with mood disorders. In mice with the miR-155 deletion, a reduction in anxiety and depressive behaviors was observed, however, no effects on memory, learning, social behavior, and sensorimotor skills were found. This demonstrates the specificity of miR-155 activity. Analysis of changes in miR-155 expression levels revealed that inactivation of this factor reduces inflammation in the hippocampus by decreasing the expression of genes encoding the pro-inflammatory cytokines, IL-6, and TNF-α. Furthermore, mRNA encoding a neurotrophic cytokine that promotes neurogenesis in several regions in the brain, ciliary neurotrophic factor (CNTF), was increased in the hippocampi of female mice lacking miR-155 [73]. The study conducted on mice cell lines revealed a possible role of miR-155 in the context of treatment-resistant depression. miR-155 down-regulated SOCS1, leading to the activation of microglia that contributes to inflammatory injury of hippocampal neurons [114]. In contrast, the correlation analysis of miR-155 expression in RR-MS patients (in the remission phase) with the Beck Depression Index (BDI) scale and with Montreal Cognitive Assessment (MoCA) values did not reveal to be statistically significant [74].

Chronic stress occurring simultaneously with EAE has been shown to exacerbate the clinical manifestations of the disease. Furthermore, deep sequencing revealed changes in the expression of miRNAs that are important in human MS. Both miR-155 and another epigenetic hallmark of MS, miR-146a, were up-regulated in the lumbar spinal cord during EAE, as well as a result of stress exposure. It has been suggested that stress may synergistically exacerbate the severity of EAE through changes in the expression of epigenetic factors. This supports the role of MS-associated stress in disease worsening and the significance of miR-155 as a biomarker for monitoring disease progression [115].

The study conducted on pediatric multiple sclerosis (PedMS) patients demonstrated that the expression panel of 11 miRNAs (miR-181a-5p, miR-99b-5p, miR-25-3p, miR-148b-3p, miR-125a-5p, miR-185-5p, miR-182-5p, miR-320a, miR-652-3p, miR-942-5 and miR-221-3p; out of 13 primarily selected), derived from the peripheral blood samples, were associated with genes such as *NTNG2*, *BST1*, *STAB1*, and *SPTB*, possibly related to cognitive abilities. The selection of miRs for the study was based on significant differences in their expression between PedMS patients and healthy controls. These results may be a step towards finding reliable molecular indicators of cognitive impairment in MS and effective monitoring disease progression, however, the authors emphasized the limitations of this research due to small study group (n = 19) or inability to compare results with adult MS patients [116]. To date, no studies referred to cognitive impairment in adult MS patients have been published. As such, Varma-Doyle et al. provided a scoping review of miRs that are dysregulated in both MS and in cognitive disorders, including dementia, to find the overlapping ones and explore their possible role in MS-associated cognitive impairment. The miRs analyzed were found to be capable of modulating proteins with neuroprotective and/or neurodegenerative properties. Of them, miR-155, miR-15, miR-132, miR-138, and let-7 contributed to the elevation of neurodegenerative proteins, e.g., SRY-Box transcription factor 4 (SOX4). miR-155 was one of the dysregulated miRNAs overlapping MS and dementia via association with neuroinflammation and activation of microglia. This study further identified overlapping dysregulated miRs affecting various processes, including neuronal repair and remyelination, apoptosis, glutamate toxicity, and amyloid deposition that might play a role in dementia in MS patients. The need for further research to investigate the role of these miRs specifically in the context of cognitive changes in MS has been emphasized [48].

Up-regulated miR-155 located on the triplicated chromosome 21 has been shown to play a key role in the development of dementia in individuals with Down syndrome [117]. In Alzheimer's Disease (AD), miR-155 contributed to the regulation of cognitive functions by participating in neuroinflammatory processes, which was demonstrated in AD rats with memory impairment [118]. In contrast, the analysis of serum exosomal miRNA in patients with dementia did not reveal a significant change in expression of miR-155 compared with controls, pointing to another inflammation-related miR—miR-223 as a valuable diagnostic parameter. The levels of miR-223 were significantly decreased and negatively correlated with inflammatory markers. This result was supported by dementia severity assessment tools and magnetic resonance spectroscopy [119].

9. Future Perspectives

Following the databases, there is strong evidence that miR-155 is of interest to researchers and clinicians as an effective biomarker candidate for monitoring treatment efficacy as well as a therapeutic target, considering the results of studies in which anti-miRs are tested.

Presently, there is an ongoing clinical study that aims to investigate the correlation between miR-155 and miR-150 with different MS phenotypes, disability status, and the patient demographic data [120,121]. Worth attention are the actions of the American company miRagen Therapeutics that developed a drug cobomarsen (MRG-106) designed to inhibit the activity of miR-155 in patients with cutaneous T-cell lymphoma (CTCL) and mycosis fungoides (MF) subtype [122,123]. Clinically, there were no serious adverse reactions

attributed to these anti-miR-155 molecules, as well as no evidence of immunosuppression over nearly 2 years [124]. miRagen Therapeutics has also a completed preclinical trial for drug MRG-107, targeting miR-155 designed for the treatment of amyotrophic lateral sclerosis (ALS). What is more, the biotech company DiamiR provided miRNA diagnostic blood tests used in studies on biomarkers for detection and differentiation of neurodegenerative diseases [7,125]. The drug discovery development of miRNA and their status in clinical trials have been recently reviewed by Chakraborty et al., who placed great emphasis on the essence of conducting more clinical trials and supporting future perspectives in this field [126].

Increasing understanding and exploration of miR-155 mechanisms may contribute to the initiation of further studies on the application of these molecules in clinical practice. Currently, numerous pharmaceutical companies are working on the development of miRNAs as novel biomarkers and therapeutic targets of various diseases, including MS.

10. Conclusions

Abnormalities in the pathogenesis of MS may originate from genetic, exogenous, and environmental factors. The search for relatively simple, specific, and sensitive methods of diagnosing and monitoring the course of MS remains a challenge. The results of the analyses performed so far indicate that miRNAs may represent a sensitive and specific determinant for this condition stage. Of them, miR-155 has a great potential to be implemented into clinical trials toward qualifying it as a biomarker due to its extensive impact on inflammatory molecular pathways, detectable alternations in expression levels, and ability to be inhibited by specific anti-miRs. As suggested by the research findings, miR-155 affects key components of the BBB under inflammatory conditions contributing to its disruption and has a huge impact on demyelination by stimulating various pro-inflammatory factors. Emerging studies indicate a significant role of miR-155 in regulating the development of neuropathic pain in general, which may be a great hint for further research specifically on MS. This epigenetic factor is also suggested to be an important player in complex processes involved in neuropsychiatric symptoms development appearing as a serious problem affecting the quality of life in MS patients. It may be considered as a possibly good candidate for further research toward innovative therapeutic targets because of the ability to obtain an effect on a wide range of pathophysiological factors simultaneously. However, targeting multiple transcripts may be also restrictive due to the limited specificity of the action. miR-155 has been already evaluated as a general regulator of inflammation. Successive exploration of its specific role, in particular, pathophysiological processes in MS, is a promising path toward a better understanding of the mechanisms of this complex disease and more effectively diagnosing, treating, and monitoring it.

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References

1. *Mapping Multiple Sclerosis around the World Key Epidemiology Findings, Atlas of MS*, 3rd ed.; The Multiple Sclerosis International Federation (MSIF): London, UK, 2020; Available online: www.atlasofms.org (accessed on 1 December 2020).
2. Field, J.; Browning, S.R.; Johnson, L.J.; Danoy, P.; Varney, M.D.; Tait, B.D.; Gandhi, K.S.; Charlesworth, J.C.; Heard, R.N.; Stewart, G.J.; et al. A Polymorphism in the HLA-DPB1 Gene Is Associated with Susceptibility to Multiple Sclerosis. *PLoS ONE* **2010**, *5*, e13454. [CrossRef] [PubMed]

3. Handel, A.E.; Williamson, A.J.; Disanto, G.; Handunnetthi, L.; Giovannoni, G.; Ramagopalan, S.V. An Updated Meta-Analysis of Risk of Multiple Sclerosis following Infectious Mononucleosis. *PLoS ONE* **2010**, *5*, e12496. [CrossRef] [PubMed]
4. Handel, A.E.; Williamson, A.J.; Disanto, G.; Dobson, R.; Giovannoni, G.; Ramagopalan, S.V. Smoking and Multiple Sclerosis: An Updated Meta-Analysis. *PLoS ONE* **2011**, *6*, e16149. [CrossRef] [PubMed]
5. O'Connell, R.M.; Rao, D.S.; Chaudhuri, A.A.; Baltimore, D. Physiological and pathological roles for microRNAs in the immune system. *Nat. Rev. Immunol.* **2010**, *10*, 111–122. [CrossRef]
6. Zhang, K.; Wang, Y.-W.; Wang, Y.-Y.; Song, Y.; Zhu, J.; Si, P.-C.; Ma, R. Identification of microRNA biomarkers in the blood of breast cancer patients based on microRNA profiling. *Gene* **2017**, *619*, 10–20. [CrossRef]
7. Sheinerman, K.S.; Toledo, J.B.; Tsivinsky, V.G.; Irwin, D.; Grossman, M.; Weintraub, D.; Hurtig, H.I.; Chen-Plotkin, A.; Wolk, D.A.; McCluskey, L.F.; et al. Circulating brain-enriched microRNAs as novel biomarkers for detection and differentiation of neurodegenerative diseases. *Alzheimer's Res. Ther.* **2017**, *9*, 1–13. [CrossRef]
8. Barwari, T.; Joshi, A.; Mayr, M. MicroRNAs in Cardiovascular Disease. *J. Am. Coll. Cardiol.* **2016**, *68*, 2577–2584. [CrossRef]
9. Tribolet, L.; Kerr, E.; Cowled, C.; Bean, A.G.D.; Stewart, C.R.; Dearnley, M.; Farr, R.J. MicroRNA Biomarkers for Infectious Diseases: From Basic Research to Biosensing. *Front. Microbiol.* **2020**, *11*, 1197. [CrossRef]
10. Long, H.; Wang, X.; Chen, Y.; Wang, L.; Zhao, M.; Lu, Q. Dysregulation of microRNAs in autoimmune diseases: Pathogenesis, biomarkers and potential therapeutic targets. *Cancer Lett.* **2018**, *428*, 90–103. [CrossRef]
11. Condrat, C.E.; Thompson, D.C.; Barbu, M.G.; Bugnar, O.L.; Boboc, A.; Cretoiu, D.; Suciu, N.; Cretoiu, S.M.; Voinea, S.C. miRNAs as Biomarkers in Disease: Latest Findings Regarding Their Role in Diagnosis and Prognosis. *Cells* **2020**, *9*, 276. [CrossRef]
12. Moore, C.S.; Rao, V.T.; Durafourt, B.A.; Bedell, B.J.; Ludwin, S.K.; Bar-Or, A.; Antel, J.P. miR-155 as a multiple sclerosis-relevant regulator of myeloid cell polarization. *Ann. Neurol.* **2013**, *74*, 709–720. [CrossRef]
13. Leng, R.-X.; Pan, H.-F.; Qin, W.-Z.; Chen, G.-M.; Ye, D.-Q. Role of microRNA-155 in autoimmunity. *Cytokine Growth Factor Rev.* **2011**, *22*, 141–147. [CrossRef]
14. Seddiki, N.; Brezar, V.; Ruffin, N.; Lévy, Y.; Swaminathan, S. Role of miR-155 in the regulation of lymphocyte immune function and disease. *Immunology* **2014**, *142*, 32–38. [CrossRef]
15. McCoy, C.E. miR-155 Dysregulation and Therapeutic Intervention in Multiple Sclerosis. In *Advances in Experimental Medicine and Biology*; Springer Science and Business Media LLC: Berlin, Germany, 2017; Volume 1024, pp. 111–131.
16. Miller, E. Multiple Sclerosis. *Chem. Biol. Pteridines Folates* **2012**, *724*, 222–238. [CrossRef]
17. Lublin, F.D.; Reingold, S.C. Defining the clinical course of multiple sclerosis: Results of an international survey. *Neurology* **1996**, *46*, 907–911. [CrossRef]
18. Kasper, L.H.; Shoemaker, J. Multiple sclerosis immunology: The healthy immune system vs. the MS immune system. *Neurology* **2009**, *74*, S2–S8. [CrossRef]
19. Alvarez, J.I.; Cayrol, R.; Prat, A. Disruption of central nervous system barriers in multiple sclerosis. *Biochim. Biophys. Acta Mol. Basis Dis.* **2011**, *1812*, 252–264. [CrossRef]
20. Kamphuis, W.W.; Troletti, C.D.; Reijerkerk, A.A.; Romero, I.; De Vries, E.H. The Blood-Brain Barrier in Multiple Sclerosis: microRNAs as Key Regulators. *CNS Neurol. Disord. Drug Targets* **2015**, *14*, 157–167. [CrossRef]
21. Trapp, B.D.; Vignos, M.; Dudman, J.; Chang, A.; Fisher, E.; Staugaitis, S.M.; Battapady, H.; Mork, S.; Ontaneda, D.; Jones, E.S.; et al. Cortical neuronal densities and cerebral white matter demyelination in multiple sclerosis: A retrospective study. *Lancet Neurol.* **2018**, *17*, 870–884. [CrossRef]
22. Mycko, M.P.; Cichalewska, M.; Cwiklinska, H.; Selmaj, K.W. miR-155-3p Drives the Development of Autoimmune Demyelination by Regulation of Heat Shock Protein 40. *J. Neurosci.* **2015**, *35*, 16504–16515. [CrossRef] [PubMed]
23. Losy, J.; Bartosik-Psujek, H.; Członkowska, A.; Kurowska, K.; Maciejek, Z.; Mirowska-Guzel, D.; Potemkowski, A.; Ryglewicz, D.; Stępień, A. Rekomendacje i Zalecenia Ekspertów 80 Leczenie stwardnienia rozsianego Zalecenia Polskiego Towarzystwa Neurologicznego. *Polski Przegląd Neurologiczny* **2016**, *12*, 80–95. Available online: www.ppn.viamedica.pl (accessed on 4 December 2020).
24. De Courcy, J.; Liedgens, H.; Obradovic, M.; Holbrook, T.; Jakubanis, R. A burden of illness study for neuropathic pain in Europe. *Clin. Outcomes Res.* **2016**, *8*, 113–126. [CrossRef] [PubMed]
25. Solaro, C.; Trabucco, E.; Uccelli, M.M. Pain and Multiple Sclerosis: Pathophysiology and Treatment. *Curr. Neurol. Neurosci. Rep.* **2012**, *13*, 1–9. [CrossRef] [PubMed]
26. Kalb, R.; Beier, M.; Benedict, R.H.; Charvet, L.; Costello, K.; Feinstein, A.; Gingold, J.; Goverover, Y.; Halper, J.; Harris, C.; et al. Recommendations for cognitive screening and management in multiple sclerosis care. *Mult. Scler. J.* **2018**, *24*, 1665–1680. [CrossRef]
27. Paparrigopoulos, T.; Ferentinos, P.; Kouzoupis, A.; Koutsis, G.; Papadimitriou, G.N. The neuropsychiatry of multiple sclerosis: Focus on disorders of mood, affect and behaviour. *Int. Rev. Psychiatry* **2010**, *22*, 14–21. [CrossRef]
28. Samkoff, L.M.; Goodman, A.D. Symptomatic Management in Multiple Sclerosis. *Neurol. Clin.* **2011**, *29*, 449–463. [CrossRef]
29. Solomon, A.J.; Bourdette, D.N.; Cross, A.H.; Applebee, A.; Skidd, P.M.; Howard, D.B.; Spain, R.I.; Cameron, M.H.; Kim, E.; Mass, M.K.; et al. The contemporary spectrum of multiple sclerosis misdiagnosis: A multicenter study. *Neurology* **2016**, *87*, 1393–1399. [CrossRef]
30. Thompson, A.J.; Banwell, B.L.; Barkhof, F.; Carroll, W.M.; Coetzee, T.; Comi, G.; Correale, J.; Fazekas, F.; Filippi, M.; Freedman, M.S.; et al. Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. *Lancet Neurol.* **2018**, *17*, 162–173. [CrossRef]

31. Housley, W.J.; Pitt, D.; Hafler, D.A. Biomarkers in multiple sclerosis. *Clin. Immunol.* **2015**, *161*, 51–58. [CrossRef]
32. Giovannoni, G.; Tomic, D.; Bright, J.R.; Havrdová, E. "No evident disease activity": The use of combined assessments in the management of patients with multiple sclerosis. *Mult. Scler. J.* **2017**, *23*, 1179–1187. [CrossRef]
33. Montalban, X.; Hauser, S.L.; Kappos, L.; Arnold, D.L.; Bar-Or, A.; Comi, G.; De Seze, J.; Giovannoni, G.; Hartung, H.-P.; Hemmer, B.; et al. Ocrelizumab versus Placebo in Primary Progressive Multiple Sclerosis. *New Engl. J. Med.* **2017**, *376*, 209–220. [CrossRef]
34. Gajofatto, A.; Turatti, M. Siponimod to treat secondary progressive multiple sclerosis. *Drugs Today* **2020**, *56*, 37–46. [CrossRef]
35. Wingerchuk, D.M.; Weinshenker, B.G. Disease modifying therapies for relapsing multiple sclerosis. *BMJ* **2016**, *354*, i3518. [CrossRef]
36. Szelenberger, R.; Kacprzak, M.; Saluk-Bijak, J.; Zielinska, M.; Bijak, M. Plasma MicroRNA as a novel diagnostic. *Clin. Chim. Acta* **2019**, *499*, 98–107. [CrossRef]
37. Otaegui, D.; Baranzini, S.E.; Armañanzas, R.; Calvo, B.; Muñoz-Culla, M.; Khankhanian, P.; Inza, I.; Lozano, J.A.; Castillo-Triviño, T.; Asensio, A.; et al. Differential Micro RNA Expression in PBMC from Multiple Sclerosis Patients. *PLoS ONE* **2009**, *4*, e6309. [CrossRef]
38. Regev, K.; Paul, A.; Healy, B.; Von Glenn, F.; Diaz-Cruz, C.; Gholipour, T.; Mazzola, M.A.; Raheja, R.; Nejad, P.; Glanz, B.I.; et al. Comprehensive evaluation of serum microRNAs as biomarkers in multiple sclerosis. *Neurol. Neuroimmunol. Neuroinflamm.* **2016**, *3*, e267. [CrossRef]
39. Baulina, N.M.; Kulakova, O.G.; Favorova, O.O. MicroRNAs: The Role in Autoimmune Inflammation. *Acta Nat.* **2016**, *8*, 21–33. [CrossRef]
40. Ha, M.; Kim, V.N. Regulation of microRNA biogenesis. *Nat. Rev. Mol. Cell Biol.* **2014**, *15*, 509–524. [CrossRef]
41. Macias, S.; Cordiner, R.A.; Caceres, J.F. Cellular functions of the microprocessor. *Biochem. Soc. Trans.* **2013**, *41*, 838–843. [CrossRef]
42. Song, M.-S.; Rossi, J.J. Molecular mechanisms of Dicer: Endonuclease and enzymatic activity. *Biochem. J.* **2017**, *474*, 1603–1618. [CrossRef]
43. Meijer, H.A.; Smith, E.M.; Bushell, M. Regulation of miRNA strand selection: Follow the leader? *Biochem. Soc. Trans.* **2014**, *42*, 1135–1140. [CrossRef]
44. Piket, E.; Zheleznyakova, G.Y.; Kular, L.; Jagodic, M. Small non-coding RNAs as important players, biomarkers and therapeutic targets in multiple sclerosis: A comprehensive overview. *J. Autoimmun.* **2019**, *101*, 17–25. [CrossRef]
45. O'carroll, D.; Schaefer, A. General Principals of miRNA Biogenesis and Regulation in the Brain. *Neuropsychopharmacology* **2012**, *38*, 39–54. [CrossRef]
46. Mahesh, G.; Biswas, R. MicroRNA-155: A Master Regulator of Inflammation. *J. Interf. Cytokine Res.* **2019**, *39*, 321–330. [CrossRef]
47. Helwak, A.; Kudla, G.; Dudnakova, T.; Tollervey, D. Mapping the Human miRNA Interactome by CLASH Reveals Frequent Noncanonical Binding. *Cell* **2013**, *153*, 654–665. [CrossRef]
48. Varma-Doyle, A.V.; Lukiw, W.J.; Zhao, Y.; Lovera, J.; Devier, D. A hypothesis-generating scoping review of miRs identified in both multiple sclerosis and dementia, their protein targets, and miR signaling pathways. *J. Neurol. Sci.* **2021**, *420*, 117202. [CrossRef]
49. Luo, D.; Wang, J.; Zhang, X.; Rang, X.; Xu, C.; Fu, J. Identification and functional analysis of specific MS risk miRNAs and their target genes. *Mult. Scler. Relat. Disord.* **2020**, *41*, 102044. [CrossRef] [PubMed]
50. Eis, P.S.; Tam, W.; Sun, L.; Chadburn, A.; Li, Z.; Gomez, M.F.; Lund, E.; Dahlberg, J.E. Accumulation of miR-155 and BIC RNA in human B cell lymphomas. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 3627–3632. [CrossRef] [PubMed]
51. Tam, W. Identification and characterization of human BIC, a gene on chromosome 21 that encodes a noncoding RNA. *Gene* **2001**, *274*, 157–167. [CrossRef]
52. Landgraf, P.; Rusu, M.; Sheridan, R.; Sewer, A.; Iovino, N.; Aravin, A.; Pfeffer, S.; Rice, A.; Kamphorst, A.O.; Landthaler, M.; et al. A Mammalian microRNA Expression Atlas Based on Small RNA Library Sequencing. *Cell* **2007**, *129*, 1401–1414. [CrossRef] [PubMed]
53. Rodriguez, A.; Vigorito, E.; Clare, S.; Warren, M.V.; Couttet, P.; Soond, D.R.; Van Dongen, S.; Grocock, R.J.; Das, P.P.; Miska, E.A.; et al. Requirement of bic/microRNA-155 for Normal Immune Function. *Science* **2007**, *316*, 608–611. [CrossRef]
54. Gaudet, A.D.; Fonken, L.K.; Watkins, L.R.; Nelson, R.J.; Popovich, P.G. MicroRNAs: Roles in Regulating Neuroinflammation. *Neuroscientist* **2018**, *24*, 221–245. [CrossRef]
55. Bradburn, S. Mechanisms Linking Depression, Immune System and Epigenetics During Aging. *Inflamm. Immun. Depress.* **2018**, 339–356. [CrossRef]
56. Slota, J.A.; Booth, S.A. MicroRNAs in Neuroinflammation: Implications in Disease Pathogenesis, Biomarker Discovery and Therapeutic Applications. *Non Coding RNA* **2019**, *5*, 35. [CrossRef]
57. Ransohoff, R.M. How neuroinflammation contributes to neurodegeneration. *Science* **2016**, *353*, 777–783. [CrossRef]
58. Cardoso, A.L.; Guedes, J.R.; De Almeida, L.P.; De Lima, M.C.P. miR-155 modulates microglia-mediated immune response by down-regulating SOCS-1 and promoting cytokine and nitric oxide production. *Immunology* **2011**, *135*, 73–88. [CrossRef]
59. O'Connell, R.M.; Chaudhuri, A.A.; Rao, D.S.; Baltimore, D. Inositol phosphatase SHIP1 is a primary target of miR-155. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 7113–7118. [CrossRef]
60. Worm, J.; Stenvang, J.; Petri, A.; Frederiksen, K.S.; Obad, S.; Elmén, J.; Hedtjärn, M.; Straarup, E.M.; Hansen, J.B.; Kauppinen, S. Silencing of microRNA-155 in mice during acute inflammatory response leads to derepression of c/ebp Beta and down-regulation of G-CSF. *Nucleic Acids Res.* **2009**, *37*, 5784–5792. [CrossRef]

61. Martinez-Nunez, R.T.; Louafi, F.; Sanchez-Elsner, T. The Interleukin 13 (IL-13) Pathway in Human Macrophages Is Modulated by MicroRNA-155 via Direct Targeting of Interleukin 13 Receptor α1 (IL13Rα1). *J. Biol. Chem.* **2011**, *286*, 1786–1794. [CrossRef]
62. Su, W.; Hopkins, S.; Nesser, N.K.; Sopher, B.; Silvestroni, A.; Ammanuel, S.; Jayadev, S.; Möller, T.; Weinstein, J.; Garden, G.A. The p53 Transcription Factor Modulates Microglia Behavior through MicroRNA-Dependent Regulation of c-Maf. *J. Immunol.* **2014**, *192*, 358–366. [CrossRef]
63. Junker, A.; Krumbholz, M.; Eisele, S.; Mohan, H.; Augstein, F.; Bittner, R.A.; Lassmann, H.; Wekerle, H.; Hohlfeld, R.; Meinl, E. MicroRNA profiling of multiple sclerosis lesions identifies modulators of the regulatory protein CD47. *Brain* **2009**, *132*, 3342–3352. [CrossRef] [PubMed]
64. Paraboschi, E.M.; Soldà, G.; Gemmati, D.; Orioli, E.; Zeri, G.; Benedetti, M.D.; Salviati, A.; Barizzone, N.; Leone, M.; Duga, S.; et al. Genetic Association and Altered Gene Expression of Mir-155 in Multiple Sclerosis Patients. *Int. J. Mol. Sci.* **2011**, *12*, 8695–8712. [CrossRef] [PubMed]
65. Murugaiyan, G.; Beynon, V.; Mittal, A.; Joller, N.; Weiner, H.L. Silencing MicroRNA-155 Ameliorates Experimental Autoimmune Encephalomyelitis. *J. Immunol.* **2011**, *187*, 2213–2221. [CrossRef] [PubMed]
66. Gavgani, F.M.; Arnesen, V.S.; Jacobsen, R.G.; Krakstad, C.; Hoivik, E.A.; Lewis, A.E. Class I Phosphoinositide 3-Kinase PIK3CA/p110α and PIK3CB/p110β Isoforms in Endometrial Cancer. *Int. J. Mol. Sci.* **2018**, *19*, 3931. [CrossRef]
67. Urick, M.E.; Rudd, M.L.; Godwin, A.K.; Sgroi, D.C.; Merino, M.J.; Bell, D.W. PIK3R1 (p85α) Is Somatically Mutated at High Frequency in Primary Endometrial Cancer. *Cancer Res.* **2011**, *71*, 4061–4067. [CrossRef]
68. Ma, X.; Zhou, J.; Zhong, Y.; Jiang, L.; Mu, P.; Li, Y.; Singh, N.; Nagarkatti, M.; Nagarkatti, P. Expression, Regulation and Function of MicroRNAs in Multiple Sclerosis. *Int. J. Med. Sci.* **2014**, *11*, 810–818. [CrossRef]
69. Zhang, J.; Cheng, Y.; Cui, W.; Li, M.; Li, B.; Guo, L. MicroRNA-155 modulates Th1 and Th17 cell differentiation and is associated with multiple sclerosis and experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* **2014**, *266*, 56–63. [CrossRef]
70. Ksiazek-Winiarek, D.; Szpakowski, P.; Turniak, M.; Szemraj, J.; Glabinski, A. IL-17 Exerts Anti-Apoptotic Effect via miR-155-5p Downregulation in Experimental Autoimmune Encephalomyelitis. *J. Mol. Neurosci.* **2017**, *63*, 320–332. [CrossRef]
71. Ksiazek-Winiarek, D.J.; Kacperska, M.J.; Glabinski, A. MicroRNAs as Novel Regulators of Neuroinflammation. *Mediat. Inflamm.* **2013**, *2013*, 1–11. [CrossRef]
72. Lopez-Ramirez, M.A.; Wu, D.; Pryce, G.; Simpson, J.E.; Reijerkerk, A.; King-Robson, J.; Kay, O.; De Vries, H.E.; Hirst, M.C.; Sharrack, B.; et al. MicroRNA-155 negatively affects blood–brain barrier function during neuroinflammation. *FASEB J.* **2014**, *28*, 2551–2565. [CrossRef]
73. Fonken, L.K.; Gaudet, A.D.; Gaier, K.R.; Nelson, R.J.; Popovich, P.G. MicroRNA-155 deletion reduces anxiety- and depressive-like behaviors in mice. *Psychoneuroendocrinology* **2016**, *63*, 362–369. [CrossRef] [PubMed]
74. Niwald, M.; Migdalska-Sęk, M.; Brzeziańska-Lasota, E.; Miller, E. Evaluation of Selected MicroRNAs Expression in Remission Phase of Multiple Sclerosis and Their Potential Link to Cognition, Depression, and Disability. *J. Mol. Neurosci.* **2017**, *63*, 275–282. [CrossRef]
75. Shademan, B.; Nourazarian, A.; Nikanfar, M.; Avci, C.B.; Hasanpour, M.; Isazadeh, A. Investigation of the miRNA146a and miRNA155 gene expression levels in patients with multiple sclerosis. *J. Clin. Neurosci.* **2020**, *78*, 189–193. [CrossRef]
76. Zhang, Y.; Chen, Q.; Nai, Y.; Cao, C. Suppression of miR-155 attenuates neuropathic pain by inducing an M1 to M2 switch in microglia. *Folia Neuropathol.* **2020**, *58*, 70–82. [CrossRef]
77. Daneman, R.; Prat, A. The Blood–Brain Barrier. *Cold Spring Harb. Perspect. Biol.* **2015**, *7*, a020412. [CrossRef]
78. Ma, F.; Zhang, X.; Yin, K.-J. MicroRNAs in central nervous system diseases: A prospective role in regulating blood-brain barrier integrity. *Exp. Neurol.* **2020**, *323*, 113094. [CrossRef]
79. Cerutti, C.; Soblechero-Martin, P.; Wu, D.; Lopez-Ramirez, M.A.; De Vries, H.; Sharrack, B.; Male, D.K.; Romero, I.A. MicroRNA-155 contributes to shear-resistant leukocyte adhesion to human brain endothelium in vitro. *Fluids Barriers CNS* **2016**, *13*, 8. [CrossRef] [PubMed]
80. Muoio, V.; Persson, P.B.; Sendeski, M.M. The neurovascular unit—Concept review. *Acta Physiol.* **2014**, *210*, 790–798. [CrossRef]
81. Lopez-Ramirez, M.A.; Reijerkerk, A.; De Vries, H.E.; Romero, I.A. Regulation of brain endothelial barrier function by microRNAs in health and neuroinflammation. *FASEB J.* **2016**, *30*, 2662–2672. [CrossRef]
82. Caballero-Garrido, E.; Pena-Philippides, J.C.; Lordkipanidze, T.; Bragin, D.; Yang, Y.; Erhardt, E.B.; Roitbak, T. In Vivo Inhibition of miR-155 Promotes Recovery after Experimental Mouse Stroke. *J. Neurosci.* **2015**, *35*, 12446–12464. [CrossRef]
83. Rom, S.; Dykstra, H.; Zuluaga-Ramirez, V.; Reichenbach, N.L.; Persidsky, Y. miR-98 and let-7g* Protect the Blood-Brain Barrier Under Neuroinflammatory Conditions. *Br. J. Pharmacol.* **2015**, *35*, 1957–1965. [CrossRef] [PubMed]
84. Pena-Philippides, J.C.; Gardiner, A.S.; Caballero-Garrido, E.; Pan, R.; Zhu, Y.; Roitbak, T. Inhibition of MicroRNA-155 Supports Endothelial Tight Junction Integrity Following Oxygen-Glucose Deprivation. *J. Am. Heart Assoc.* **2018**, *7*, e009244. [CrossRef] [PubMed]
85. Jiang, T.; Zhou, S.; Li, X.; Song, J.; An, T.; Huang, X.; Ping, X.; Wang, L. microRNA 155 induces protection against cerebral ischemia/reperfusion injury through regulation of the Notch pathway in vivo. *Exp. Ther. Med.* **2019**, *18*, 605–613. [CrossRef] [PubMed]
86. Almutairi, M.M.A.; Gong, C.; Xu, Y.G.; Chang, Y.; Shi, H. Factors controlling permeability of the blood–brain barrier. *Cell. Mol. Life Sci.* **2016**, *73*, 57–77. [CrossRef]

87. Luo, C.; Jian, C.; Liao, Y.; Huang, Q.; Wu, Y.; Liu, X.; Zou, D.; Wu, Y. The role of microglia in multiple sclerosis. *Neuropsychiatr. Dis. Treat.* **2017**, *13*, 1661–1667. [CrossRef]
88. Mycko, M.P.; Cichalewska, M.; Machlanska, A.; Cwiklinska, H.; Mariasiewicz, M.; Selmaj, K.W. microRNA-301a regulation of a T-helper 17 immune response controls autoimmune demyelination. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, E1248–E1257. [CrossRef]
89. Hu, R.; Huffaker, T.B.; Kagele, D.A.; Runtsch, M.C.; Bake, E.; Chaudhuri, A.A.; Round, J.L.; O'Connell, R.M. MicroRNA-155 Confers Encephalogenic Potential to Th17 Cells by Promoting Effector Gene Expression. *J. Immunol.* **2013**, *190*, 5972–5980. [CrossRef]
90. Mazloumfard, F.; Mirian, M.; Eftekhari, S.-M.; Aliomrani, M. Hydroxychloroquine effects on miR-155-3p and miR-219 expression changes in animal model of multiple sclerosis. *Metab. Brain Dis.* **2020**, *35*, 1–9. [CrossRef]
91. Gholami, S.; Mirian, M.; Eftekhari, S.M.; Aliomrani, M. Apamin administration impact on miR-219 and miR-155-3p expression in cuprizone induced multiple sclerosis model. *Mol. Biol. Rep.* **2020**, *47*, 9013–9019. [CrossRef]
92. Digehsara, S.G.; Name, N.; Esfandiari, B.; Karim, E.; Taheri, S.; Tajabadi-Ebrahimi, M.; Arasteh, J. Effects of Lactobacillus casei Strain T2 (IBRC-M10783) on the Modulation of Th17/Treg and Evaluation of miR-155, miR-25, and IDO-1 Expression in a Cuprizone-Induced C57BL/6 Mouse Model of Demyelination. *Inflammation* **2021**, *44*, 334–343. [CrossRef]
93. Han, S.R.; Kang, Y.H.; Jeon, H.; Lee, S.; Park, S.J.; Song, D.-Y.; Min, S.S.; Yoo, S.-M.; Lee, S.; Lee, M.-S.; et al. Differential Expression of miRNAs and Behavioral Change in the Cuprizone-Induced Demyelination Mouse Model. *Int. J. Mol. Sci.* **2020**, *21*, 646. [CrossRef]
94. Vallejo, R.; Tilley, D.M.; Vogel, L.; Benyamin, R. The Role of Glia and the Immune System in the Development and Maintenance of Neuropathic Pain. *Pain Pract.* **2010**, *10*, 167–184. [CrossRef]
95. Ledeboer, A.; Gamanos, M.; Lai, W.; Martín, D.; Maier, S.F.; Watkins, L.R.; Quan, N. Involvement of spinal cord nuclear factor κB activation in rat models of proinflammatory cytokine-mediated pain facilitation. *Eur. J. Neurosci.* **2005**, *22*, 1977–1986. [CrossRef]
96. Xu, L.; Huang, Y.; Yu, X.; Yue, J.; Yang, N.; Zuo, P. The Influence of p38 Mitogen-Activated Protein Kinase Inhibitor on Synthesis of Inflammatory Cytokine Tumor Necrosis Factor Alpha in Spinal Cord of Rats with Chronic Constriction Injury. *Anesthesia Analg.* **2007**, *105*, 1838–1844. [CrossRef]
97. Pohl, K.-W.; Yeol, J.-F.; Ongl, W.-Y. MicroRNA changes in the mouse prefrontal cortex after inflammatory pain. *Eur. J. Pain* **2011**, *15*, 801.e1–801.e12. [CrossRef]
98. Tan, Y.; Yang, J.; Xiang, K.; Tan, Q.; Guo, Q. Suppression of MicroRNA-155 Attenuates Neuropathic Pain by Regulating SOCS1 Signalling Pathway. *Neurochem. Res.* **2014**, *40*, 550–560. [CrossRef]
99. Liu, S.; Zhu, B.; Sun, Y.; Xie, X. miR-155 modulates the progression of neuropathic pain through targeting SGK3. *Int. J. Clin. Exp. Pathol.* **2015**, *8*, 14374–14382.
100. Heyn, J.; Luchting, B.; Hinske, L.C.; Hübner, M.; Azad, S.C.; Kreth, S. miR-124a and miR-155 enhance differentiation of regulatory T cells in patients with neuropathic pain. *J. Neuroinflamm.* **2016**, *13*, 1–11. [CrossRef]
101. Sabirzhanov, B.; Li, Y.; Coll-Miro, M.; Matyas, J.J.; He, J.; Kumar, A.; Ward, N.; Yu, J.; Faden, A.I.; Wu, J. Inhibition of NOX2 signaling limits pain-related behavior and improves motor function in male mice after spinal cord injury: Participation of IL-10/miR-155 pathways. *Brain Behav. Immun.* **2019**, *80*, 73–87. [CrossRef]
102. Kumar, A.; Barrett, J.P.; Alvarez-Croda, D.-M.; Stoica, B.A.; Faden, A.I.; Loane, D.J. NOX2 drives M1-like microglial/macrophage activation and neurodegeneration following experimental traumatic brain injury. *Brain Behav. Immun.* **2016**, *58*, 291–309. [CrossRef]
103. Choi, S.-H.; Aid, S.; Kim, H.-W.; Jackson, S.H.; Bosetti, F. Inhibition of NADPH oxidase promotes alternative and anti-inflammatory microglial activation during neuroinflammation. *J. Neurochem.* **2012**, *120*, 292–301. [CrossRef]
104. Kim, D.; You, B.; Jo, E.-K.; Han, S.-K.; Simon, M.I.; Lee, S.J. NADPH oxidase 2-derived reactive oxygen species in spinal cord microglia contribute to peripheral nerve injury-induced neuropathic pain. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 14851–14856. [CrossRef] [PubMed]
105. Hassler, S.N.; Johnson, K.M.; Hulsebosch, C.E. Reactive oxygen species and lipid peroxidation inhibitors reduce mechanical sensitivity in a chronic neuropathic pain model of spinal cord injury in rats. *J. Neurochem.* **2014**, *131*, 413–417. [CrossRef]
106. McCoy, C.E.; Sheedy, F.J.; Qualls, J.E.; Doyle, S.L.; Quinn, S.R.; Murray, P.J.; O'Neill, L.A. IL-10 Inhibits miR-155 Induction by Toll-like Receptors. *J. Biol. Chem.* **2010**, *285*, 20492–20498. [CrossRef] [PubMed]
107. Li, X.; Wang, D.; Zhou, J.; Yan, Y.; Chen, L. Evaluation of circulating microRNA expression in patients with trigeminal neuralgia. *Medicine* **2020**, *99*, e22972. [CrossRef] [PubMed]
108. Chen, J.; Li, C.; Liu, W.; Yan, B.; Hu, X.; Yang, F. miRNA-155 silencing reduces sciatic nerve injury in diabetic peripheral neuropathy. *J. Mol. Endocrinol.* **2019**, *63*, 227–238. [CrossRef] [PubMed]
109. Patten, S.B.; Marrie, R.A.; Carta, M.G. Depression in multiple sclerosis. *Int. Rev. Psychiatry* **2017**, *29*, 463–472. [CrossRef]
110. Brites, D.; Fernandes, A. Neuroinflammation and Depression: Microglia Activation, Extracellular Microvesicles and microRNA Dysregulation. *Front. Cell. Neurosci.* **2015**, *9*, 476. [CrossRef]
111. Pittenger, C.; Duman, R.S. Stress, Depression, and Neuroplasticity: A Convergence of Mechanisms. *Neuropsychopharmacology* **2008**, *33*, 88–109. [CrossRef]
112. Wan, Y.; Liu, Y.; Wang, X.; Wu, J.; Liu, K.; Zhou, J.; Liu, L.; Zhang, C. Identification of Differential MicroRNAs in Cerebrospinal Fluid and Serum of Patients with Major Depressive Disorder. *PLoS ONE* **2015**, *10*, e0121975. [CrossRef]

113. Wang, X.; Wang, B.; Zhao, J.; Liu, C.; Qu, X.; Li, Y. MiR-155 is involved in major depression disorder and antidepressant treatment via targeting SIRT1. *Biosci. Rep.* **2018**, *38*. [CrossRef]
114. Sun, X.; Song, M.; Song, H.; Wang, Y.; Luo, M.; Yin, L. miR 155 mediates inflammatory injury of hippocampal neuronal cells via the activation of microglia. *Mol. Med. Rep.* **2019**, *19*, 2627–2635. [CrossRef]
115. Gerrard, B.; Singh, V.; Babenko, O.; Gauthier, I.; Yong, V.W.; Kovalchuk, I.; Luczak, A.; Metz, G.A. Chronic mild stress exacerbates severity of experimental autoimmune encephalomyelitis in association with altered non-coding RNA and metabolic biomarkers. *Neuroscience* **2017**, *359*, 299–307. [CrossRef]
116. Liguori, M.; Nuzziello, N.; Simone, M.; Amoroso, N.; Viterbo, R.G.; Tangaro, S.; Consiglio, A.; Giordano, P.; Bellotti, R.; Trojano, M. Association between miRNAs expression and cognitive performances of Pediatric Multiple Sclerosis patients: A pilot study. *Brain Behav.* **2019**, *9*, e01199. [CrossRef]
117. Tili, E.; Mezache, L.; Michaille, J.-J.; Amann, V.; Williams, J.; Vandiver, P.; Quinonez, M.; Fadda, P.; Mikhail, A.; Nuovo, G. microRNA 155 up regulation in the CNS is strongly correlated to Down's syndrome dementia. *Ann. Diagn. Pathol.* **2018**, *34*, 103–109. [CrossRef]
118. Liu, D.; Zhao, D.; Zhao, Y.; Wang, Y.; Zhao, Y.; Wen, C. Inhibition of microRNA-155 Alleviates Cognitive Impairment in Alzheimer's Disease and Involvement of Neuroinflammation. *Curr. Alzheimer Res.* **2019**, *16*, 473–482. [CrossRef]
119. Wei, H.; Xu, Y.; Xu, W.; Zhou, Q.; Chen, Q.; Yang, M.; Feng, F.; Liu, Y.; Zhu, X.; Yu, M.; et al. Serum Exosomal miR-223 Serves as a Potential Diagnostic and Prognostic Biomarker for Dementia. *Neuroscience* **2018**, *379*, 167–176. [CrossRef]
120. Liddelow, S.A.; Guttenplan, K.A.; Clarke, L.E.; Bennett, F.C.; Bohlen, C.J.; Schirmer, L.; Bennett, M.L.; Münch, A.E.; Chung, W.-S.; Peterson, T.C.; et al. Neurotoxic reactive astrocytes are induced by activated microglia. *Nature* **2017**, *541*, 481–487. [CrossRef]
121. ClinicalTrials.gov. MicroRNA-150 and microRNA-155 in Multiple Sclerosis—Full Text View. Available online: https://clinicaltrials.gov/ct2/show/NCT04300543?term=miR-155&cond=Multiple+Sclerosis&draw=2&rank=1 (accessed on 2 April 2021).
122. ClinicalTrials.gov. SOLAR: Efficacy and Safety of Cobomarsen (MRG-106) vs. Active Comparator in Subjects with Mycosis Fungoides—Tabular View. Available online: https://clinicaltrials.gov/ct2/show/record/NCT03713320 (accessed on 2 April 2021).
123. ClinicalTrials.gov. PRISM: Efficacy and Safety of Cobomarsen (MRG-106) in Subjects with Mycosis Fungoides Who Have Completed the SOLAR Study—Full Text View. Available online: https://clinicaltrials.gov/ct2/show/NCT03837457 (accessed on 2 April 2021).
124. Witten, L.; Slack, F.J. miR-155 as a novel clinical target for hematological malignancies. *Carcinogenesis* **2019**, *41*, 2–7. [CrossRef]
125. Sheinerman, K.S.; Umansky, S.R. Circulating cell-free microRNA as biomarkers for screening, diagnosis and monitoring of neurodegenerative diseases and other neurologic pathologies. *Front. Cell. Neurosci.* **2013**, *7*, 150. [CrossRef]
126. Chakraborty, C.; Sharma, A.R.; Sharma, G.; Lee, S.-S. Therapeutic advances of miRNAs: A preclinical and clinical update. *J. Adv. Res.* **2021**, *28*, 127–138. [CrossRef] [PubMed]

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Remyelination in multiple sclerosis from the miRNA perspective

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Remyelination relies on the repair of damaged myelin sheaths, involving microglia cells, oligodendrocyte precursor cells (OPCs), and mature oligodendrocytes. This process drives the pathophysiology of autoimmune chronic disease of the central nervous system (CNS), multiple sclerosis (MS), leading to nerve cell damage and progressive neurodegeneration. Stimulating the reconstruction of damaged myelin sheaths is one of the goals in terms of delaying the progression of MS symptoms and preventing neuronal damage. Short, noncoding RNA molecules, microRNAs (miRNAs), responsible for regulating gene expression, are believed to play a crucial role in the remyelination process. For example, studies showed that miR-223 promotes efficient activation and phagocytosis of myelin debris by microglia, which is necessary for the initiation of remyelination. Meanwhile, miR-124 promotes the return of activated microglia to the quiescent state, while miR-204 and miR-219 promote the differentiation of mature oligodendrocytes. Furthermore, miR-138, miR-145, and miR-338 have been shown to be involved in the synthesis and assembly of myelin proteins. Various delivery systems, including extracellular vesicles, hold promise as an efficient and non-invasive way for providing miRNAs to stimulate remyelination. This article summarizes the biology of remyelination as well as current challenges and strategies for miRNA molecules in potential diagnostic and therapeutic applications.

KEYWORDS

remyelination, miRNA, microRNA, multiple sclerosis, oligodendrocyte, demyelination, myelin, oligodendrocyte precursor cells

1. Introduction

Multiple sclerosis (MS) is a chronic autoimmune disease that leads to the deterioration of the myelin sheath in the brain, spinal cord, and optic nerves. It is a relatively common condition, with an estimated 2.8 million people worldwide living with the disease ("Atlas of MS 2020 – Epidemiology Report, 2020"). It represents a significant burden on healthcare systems, both in terms of the cost of treatment and the need for ongoing monitoring and care (Filippi et al., 2018).

There are four main subtypes of MS, each with unique features and patterns of disease progression. Relapsing–remitting (RR) MS is the most common form of the disease, characterized by relapses of neurological symptoms, followed by periods of remission, during which signs of the disease improve or disappear. Secondary-progressive (SP) MS follows the RRMS and is characterized by a steady progression of disability, with or without relapses and remissions. Primary-progressive (PP) MS is characterized by a gradual onset of symptoms that worsen over time, with little to no remission. Progressive-relapsing (PR) MS is a rare subtype, characterized by a steady progression of disability, with occasional relapses and remissions (Filippi et al., 2018).

Demyelination is a key feature of MS and is believed to play a central role in the pathogenesis of the disease (Lassmann, 2018). Activation of immune cells, such as T and B lymphocytes, defective

functioning of regulatory cells, release of pro-inflammatory cytokines, including interferon (IFN)-γ and interleukin (IL)-17A, and neuroinflammation are key steps in the development of autoimmune demyelination in MS (Dendrou et al., 2015). Autoreactive T cells infiltrate the CNS and secrete cytokines that trigger macrophages, leading to the creation of demyelinating lesions in the white matter. As a result of the activation of T cells and the release of lymphokines, B cells are induced to transform into plasma cells, which generate autoantibodies that cause neurodegeneration by degrading the myelin sheath surrounding nerve fibers (Engelhardt and Ransohoff, 2005). The effect of immune cells-derived cytokines on glia and neurons causes damage to myelin membranes and a reduction in their integrity (Schmitz and Chew, 2008). Another important molecular aspect of demyelination in MS is the role of antigen-presenting cells (APCs) in the autoimmune process. APCs present myelin antigens on their surface, leading to the activation and proliferation of myelin-targeted autoreactive T cells and subsequent destruction of myelin membranes (van Langelaar et al., 2020). At first glance, demyelination in various CNS pathologies is quite similar; however, the stress to which brain cells are subjected during these pathologies is somewhat different. In contrast to MS, ischemic stroke (IS) is characterized by reduced cerebral blood flow, which limits the availability of glucose and oxygen (hypoxia), especially in neurons leading to immediate cell death (Qin et al., 2022), while trauma injury (TI) refers to severe sudden CNS damage, which requires instant medical attention (Ray et al., 2002). Contrary to chronic, multifocal demyelination of the CNS (brain and spinal cord) with clinical and/or radiological evidence of 'dissemination in space' (DIS, demyelination lesions at more than one place in the nervous system) and 'dissemination in time' (DIT, demyelination lesions have occurred more than once) in MS (Lopaisankrit and Thammaroj, 2023), major changes after IS and TI occur mainly in the brain microcirculation. With ongoing hypoxia/ischemia and circulating cell retention, there is a potential for vascular edema, preventing rapid recovery of normal blood flow after fluid resuscitation, which may result in immediate cell death. In both, disruption of energy leads to mitochondrial dysfunction and oxidative stress-induced injury, triggered by excessive production of reactive oxygen species (ROS; Hiebert et al., 2015; Jia et al., 2021). Simultaneously, energy deficiency contributes to an ionic imbalance that affects Na^+, K^+, and Ca^{2+} levels, causing the brain cell depolarization (mainly disturbances in macrophages pro-inflammatory and anti-inflammatory phenotype ratio) and inducing massive glutamate release, which activates N-methyl-D-aspartate receptors, inducing toxicity, severe injury, and finally CNS cell death (Ladak et al., 2019; Qin et al., 2022). According to our assumption, the key element that differentiates MS from other CNS pathologies is the possibility of remyelination. The process of demyelination in MS is mostly gradual, while in the case of IS or TI there is an immediate death of brain cells, with no chance of starting their repair mechanisms. Demyelination is a progressive process that causes permanent nerve damage and neurological loss. However, MS is characterized by the ability to remyelinate, i.e., repairing the myelin sheath surrounding the axons, which can be a natural process or the result of therapy (Chari, 2007).

Remyelination is the process by which CNS glial cells rebuild damaged myelin sheaths. Various cell types are involved in the remyelination process, including oligodendrocytes, oligodendrocyte precursor cells (OPCs), microglia, and astrocytes. Oligodendrocytes are the main myelin-producing cells in the CNS and are responsible for rebuilding myelin sheaths after damage. OPCs are stem cells that can differentiate into oligodendrocytes and play a role in remyelination. Microglia and astrocytes are crucial in the repair process, as they are involved in removing damaged cells and substances from the site of damage, as well as in the production of growth factors and cytokines (Uyeda and Muramatsu, 2020). A new myelin sheath can be formed by two different processes: remyelination or myelinogenesis. Remyelination is an intrinsic process of the repair of myelin within a partially damaged oligodendrocyte through cellular repair aided by cellular and non-cellular pro-repair extrinsic factors. Myelinogenesis relies on a replacement of a completely damaged oligodendrocyte by a new cell through recruitment and differentiation of OPCs (Franklin and Ffrench-Constant, 2008; Wlodarczyk et al., 2017). Here, we focus on the restoration process of the damaged myelin sheath, which is a typical sign of the pathophysiology of MS.

In the case of MS, remyelination is impaired by the presence of inflammation around the nerves and is insufficient to compensate for myelin loss and an unsuccessful differentiation of OPCs. Mechanisms included in this pathology may be associated with a lack of myelination stimulators or molecular inhibitory factors, suppressing OPCs differentiation and myelination (Chari, 2007).

Various molecular factors affect the ability of oligodendrocytes to remyelinate, among which microRNAs (miRNAs) have been shown to play an important regulatory role. These short non-coding RNA molecules act by binding to target mRNAs and inhibiting the process of translation or promoting their degradation. In the biogenesis process, miRNA is transcribed from DNA as a longer precursor, which is then processed by an enzyme complex called Dicer-dependent RNA into a short, double-stranded miRNA molecule. MiRNA molecules interact with proteins in the RNA-induced silence complex (RISC) to form the RISC-miRNA complex, which binds to complementary RNA sequences in the cell. This process results in inhibition of mRNA translation or destabilization, leading to a decrease in the level of protein encoded by mRNA (Gebert and MacRae, 2019). Recent studies have demonstrated that miRNAs are involved in various biological processes, including remyelination in the CNS (Duffy and McCoy, 2020). They operate through the regulation of gene expression related to oligodendrocyte proliferation, myelin production, the process of removing dead or damaged cells, and the expression of genes related to the production of cytokines and chemokines. It appears that inhibition of miRNAs involved in the processing of mature OPCs interrupts normal CNS myelination and that OPCs lacking mature miRNAs are not capable of differentiating properly, as has been shown *in vitro* and *in vivo* (Dugas et al., 2010). The research results also suggest that the specific miRNA restricts neuroinflammation while also promoting remyelination and repair in the CNS after demyelination (Galloway et al., 2019).

In this article, we briefly summarize the biology of remyelination and the current challenges and strategies to induce this process after damage. We focus particular attention on miRNA molecules that are involved in remyelination by regulating gene expression and may be applied in novel diagnostic and therapeutic strategies.

2. Remyelination

The degree of remyelination observed between MS lesions is highly variable and depends on the stage of disease progression, the activity of the lesions, and the variation in the underlying pathogenic mechanisms (Patrikios et al., 2006; Wiggermann et al., 2023).

Furthermore, the level of remyelination is directly correlated with both the number of oligodendrocytes and macrophages in the lesions (Lucchinetti et al., 1999; Kamma et al., 2022). The process of remyelination involves the migration, proliferation, and differentiation of OPCs that come into contact with the axons, ultimately forming myelin sheaths (Chari, 2007). Moreover, T- and B-cells have also been found to affect the remyelination process. Tregs were shown to promote oligodendrocyte differentiation and remyelination, while Treg-deficient mice have reduced remyelination and differentiation that can be restored by adoptive transfer of Tregs. These cells directly promote the differentiation of OPCs and myelination *in vitro*, and CCN3 was newly identified as a Treg-derived factor that helps with oligodendrocyte differentiation and myelination. These results indicated that Treg cells exhibit a new regenerative role in the CNS that is different from their immunomodulatory function (Dombrowski et al., 2017). Furthermore, the recent study examined how the adoptive transfer of IL-10+ regulatory B cells (Bregs) in female mice with experimental autoimmune encephalomyelitis (EAE) can reverse the disease and promote the expansion of peripheral and CNS-infiltrating IL-10+ T cells. Bregs transfusion resulted in clinical improvement and spinal cord remyelination in EAE Bregs-treated mice, along with the normalization of the immune environment of the CNS and activation of OPCs with subsequent remyelination (Pennati et al., 2020).

In response to demyelination, microglia are activated and migrate to the injury site, where they phagocytose myelin debris and release cytokines and chemokines to attract other cells to the site (Fu et al., 2014). Once the microglia have cleared the debris, the next step is the recruitment and proliferation of OPCs. In the next stage, called the differentiation phase, the OPCs differentiate into pre-myelinating oligodendrocytes, which then contact the demyelinated axons and differentiate into mature, myelinating oligodendrocytes that form functional myelin sheaths (Tepavčević and Lubetzki, 2022). This step is regulated by several molecular signaling pathways, including the Wnt/β-catenin, PI3K/AKT/mTOR, and ERK/MAPK (Gaesser and Fyffe-Maricich, 2016). During remyelination, axons secrete signaling factors such as neuronal growth factor (NGF) and glial cell growth factor (GFAP), which stimulate the migration and differentiation of OPCs. The final step in the remyelination process is the synthesis and assembly of myelin proteins, which are essential for the formation of new myelin sheaths. Myelin proteins are synthesized by mature oligodendrocytes and are assembled into myelin sheaths around axons (Franklin and Ffrench-Constant, 2008). Various proteins and regulatory factors are involved in myelin synthesis, including myelin basic protein (MBP), myelin proteolipid protein (PLP) and myelin-associated glycoprotein (MAG). These proteins bind to myelin, forming insulating layers around axons that prevent the loss of nerve signals (Meschkat et al., 2022).

OPCs are the primary cells responsible for remyelination in the CNS (Bottes and Jessberger, 2021). Molecular markers that identify OPCs or their progeny are useful to detect and quantify remyelination (Valério-Gomes et al., 2018). The transcription factor Olig2 is critical for oligodendrocyte development, but a subset of OPCs in the brain do not express Olig2 throughout life, and this population appears to coincide with changes in brain activity (Fang et al., 2023). Another molecular marker of remyelination is MBP, a component of the myelin sheath. MBP is expressed by mature oligodendrocytes and is down-regulated during demyelination. However, in remyelinating lesions, MBP expression is restored as new myelin sheaths are formed. Detection of MBP expression in tissue sections can be used as an indicator of remyelination (Lindner et al., 2008). In addition to OPCs and myelin components, other molecular markers of remyelination have been identified. One such marker is the transcription factor Sox10, which is expressed in OPCs and their progeny, as well as in Schwann cells (Stolt et al., 2002). Schwann cells are a type of glial cells that normally myelinates peripheral nerves but can also contribute to remyelination in the CNS (Chen et al., 2021). Other molecular markers of remyelination include growth factors and cytokines that regulate the proliferation and differentiation of OPCs. For example, insulin-like growth factor-1 (IGF-1; Mason et al., 2000) and platelet-derived growth factor (PDGF; Woodruff et al., 2004) are important regulators of OPC proliferation and differentiation. These growth factors are up-regulated in remyelinating lesions and can be used as markers of remyelination.

There are several molecular factors that challenge the proper remyelination process during MS (Gruchot et al., 2019). Along with aging and disease duration, the remyelination decreases, which may be due to a decrease in the number of OPCs (Goldschmidt et al., 2009; Hart et al., 2012; Frischer et al., 2015; Neumann et al., 2017). Overactive inflammation, unfavorable microenvironment, the presence of inhibitors and the lack of stimulators can also hinder the process of remyelination (Wang et al., 1998; Charles et al., 2000; Mi et al., 2005; Kuhlmann et al., 2008; Kremer et al., 2019). Researchers are currently investigating various approaches to enhance remyelination in MS (Jolanda Münzel and Williams, 2013; Kremer et al., 2019). These include replacement therapies (Huang and Franklin, 2012), the modifications of stem cells (Uchida et al., 2012), drugs stimulating growth factor production, and immunomodulatory therapies (Kremer et al., 2015) that can reduce inflammation and create a more favorable environment for remyelination. Enhancing endogenous remyelination can be achieved by targeting specific signaling pathways, such as Wnt (Fancy et al., 2009), Neurogenic locus notch homolog (Notch; Aparicio et al., 2013), and Sonic hedgehog (Shh; Loulier et al., 2006), which are involved in the regulation of OPCs differentiation and remyelination. Researchers are also investigating small-molecule therapies that can promote remyelination by stimulating the differentiation of OPCs, improving myelin protein synthesis, or hampering inhibitors of myelination (Medina-Rodríguez et al., 2017). Furthermore, gene therapy is a promising approach that involves the delivery of genetic material to cells to promote remyelination (Billinghurst et al., 1998).

It is likely that a combination of the above approaches will be required to achieve effective remyelination in MS patients.

3. MiRNA in remyelination – mechanisms and capabilities

MiRNAs play an essential role in the regulation of the remyelination process at various stages. They act as fine-tuners of gene expression, and their dysregulation has been implicated in various demyelinating diseases such as MS (Dolati et al., 2018).

The use of the EAE mouse model allowed to show that miR-223 is required to efficiently remove myelin debris and promote remyelination (Galloway et al., 2019). miR-223 was needed to efficiently perform the activation and phagocytosis of debris M2 myeloid cells. MiR-233-deficient mice showed impaired CNS remyelination (Galloway et al., 2019). Furthermore, miR-223 has been reported to be dysregulated in

myeloid cells from MS patients and to contribute to reparative activation of myeloid cells and remyelination of the CNS (Galloway et al., 2019). In turn, in the murine BV_2 microglia cells, miR-155-3p has been shown to be up-regulated in response to demyelination and to promote microglia activation and increase the production of pro-inflammatory mediators, such as tumor necrosis factor (TNF)-α, interleukins (IL-1, IL-6) and nitric oxide (NO), through the negative regulation of the SOCS1 signaling pathway (Zheng et al., 2018). Although microglial activation is a key step in initiating the remyelination process, its chronic and uncontrolled activation can lead to neurotoxic effects (Liu et al., 2013). Therefore, miR-155-3p has appeared to be a good target for restoring dysfunctional microglia and promoting myelination (Butovsky et al., 2015).

Transformation of OPCs to myelinating oligodendrocytes is a complex process, greatly influenced by transcription factors, particularly the Sox protein family (Stolt et al., 2002, 2003, 2006). Recent research has identified miR-204 as a new target gene of Sox10, a critical regulator in the development of oligodendroglia (Stolt et al., 2006). MiR-204 has been found to suppress OPC proliferation and promote oligodendrocyte differentiation by inhibiting pro-proliferative Ccnd2 and differentiation-inhibiting Sox4 (Wittstatt et al., 2020). The study by Wittstatt et al. (2020) shows that Sox10 plays a key role in driving oligodendroglial cells to exit the cell cycle and initiate differentiation through miR-204 induction (Wittstatt et al., 2020).

The improper balance of the components of the RISC complex in oligodendrocytes has been shown to result in decreased levels of miRNAs that are crucial for oligodendrocyte differentiation, survival, and myelin synthesis: miR-106b-5p, −15a-5p, −15b-5p, −181a-5p, −181c-5p, −181d-5p, −20b-5p, −320-3p, −328-3p, −338-3p, −20a, and −92a-1. Meanwhile, the formation of abnormal RISC in T-cells that infiltrate the brain contributed to the polarization of miRNA-dependent proinflammatory helper T (Th)-cells. Based on the research findings, it is suggested that the dysregulation of miRNA in EAE/MS might be caused by the defective assembly of RISC, which allows autoreactive effector T-cells to maintain a highly specific proinflammatory program (Lewkowicz et al., 2015). MiR-92a has also been reported to drive autoimmunity in the CNS by maintaining the imbalance of the regulatory T (Treg)/Th17 ratio in MS patients (Fujiwara et al., 2022). Moreover, a new subpopulation of myelin-specific CD49d + CD154+ lymphocytes present in the peripheral blood of MS patients during remission were found to have the unique ability to migrate to maturing OPCs and synthesize proinflammatory chemokines/cytokines, which interferes with the remyelination process (Piatek et al., 2020). The presence of myelin-specific CD49d + CD154+ lymphocytes close to maturing OPCs and remyelinating plaques has been confirmed in mice during disease remission. CD49d + CD154+ cells affected the maturation of OPCs toward immune reactive oligodendrocytes, which were characterized by uneven production of MBP and PLP and pro-inflammatory mediators. The examination of cellular pathways responsible for reprogramming of the oligodendrocytes revealed that CD49d + CD154+ lymphocytes had an impact on miRNA production by disrupting polymerase II activity. CD49d + CD154+ targeted miR-665 and ELL3 and when the high level of miR-665 was neutralized, miRNA and MBP/PLP synthesis were restored (Piatek et al., 2019).

Furthermore, miR-219 has been shown to rapidly promote OPCs differentiation in mature oligodendrocytes by targeting the expression of platelet-derived growth factor receptor (PDGFR)α, Sox6, FoxJ3, and zinc finger protein (ZFP) 238, which result in inhibition of OPCs proliferation and pass to the oligodendrocyte differentiation phase (Dugas et al., 2010). Inhibition of miR-219 strongly affects the differentiation of oligodendrocytes, while miR-219 alone can instigate oligodendrocytes differentiation in OPCs immersed in mitogens and can also partially rescue the differentiation deficit caused by the loss of mature miRNA production in oligodendrocytes (Dugas et al., 2010). Overexpression of miR-219 has been found to promote precocious oligodendrocyte maturation and regeneration processes in mice. The study also identified a network for miR-219 targeting of differentiation inhibitors, including Lingo1 and Etv5, and inhibition of these factors partially rescued differentiation defects of miR-219-deficient oligodendrocyte precursors (Wang H. et al., 2017).

By selectively deleting the miRNA processing enzyme Dicer1 in oligodendrocyte lineage cells, it was found that mice lacking Dicer1 had severe myelinating deficits despite the expansion of the oligodendrocyte progenitor pool. Further experiments identified miR-219 and miR-338 as oligodendrocyte-specific miRNAs that promote oligodendrocyte differentiation by repressing negative regulators of oligodendrocyte differentiation, including transcription factors Sox6 and Hes5 (Zhao et al., 2010). Based on these findings, a study including cerebrospinal fluid (CSF) miR-219 obtained from MS patients has been carried out. The lack of detection of CSF miR-219 has been associated with MS in all three cohorts of patients (RRMS, SPMS, PPMS) compared to controls (Bruinsma et al., 2017).

The effect of miR-125a-3p on oligodendroglial maturation in cultured OPCs has also been studied. It has been suggested that over-expression of miR-125a-3p impairs maturation, while inhibiting it stimulates maturation. The abnormally high levels of miR-125a-3p in the CSF of MS patients with active demyelinating lesions have been reported. This miRNA molecule was also upregulated in active lesions of MS patients and in OPCs isolated from the spinal cord of EAE. The study identified Slc8a3 and Gas7 as targets of miR-125a-3p, with Gas7 necessary for correct oligodendrocyte terminal maturation (Marangon et al., 2020). This suggests that the overexpression of miR-125a-3p may contribute to the development of MS by blocking OPC differentiation, which impairs the repair of demyelinated lesions (Lecca et al., 2016).

Another study conducted in rats showed that miR-212 level was reduced at the spinal cord injury site after injury and that it is expressed in oligodendrocytes and glial progenitor cells in the adult CNS. The researchers found that reducing the expression of miR-212 improved the cell process outgrowth of oligodendrocytes and up-regulated genes associated with their differentiation and maturation, including OLIG1, SOX10, MBP, and PLP1. On the other hand, increased expression of miR-212 in glial progenitor cells or OPCs decreased the expression of these genes. The study also showed that PLP1 is a direct target molecule of miR-212 and that its overexpression inhibited oligodendrocyte maturation-associated proteins, including 2′, 3′-cyclic nucleotide 3′-phosphodiesterase (CNPase), MBP, and PLP, and the oligodendrocyte extension process (Wang C.-Y. et al., 2017).

A study by Morris et al. (2015) suggested the role of neuronal miRNA, miR-124, in controlling gene expression in oligodendrocytes. Loss of miR-124 resulted in a decrease in the number of oligodendrocyte cells and myelination of axonal projections in the ventral hindbrain model of zebrafish embryos (*Danio rerio*) model. When miR-124 levels were reduced, there was a decrease in the

number of MBP-positive oligodendrocytes and MBP RNA did not pass through the oligodendrocyte processes (Morris et al., 2015).

The study by Tripathi et al. (2019) demonstrated that increased levels of miR-27a were found in OPCs associated with MS lesions and in animal models of demyelination. Increased levels of miR-27a led to inhibition of OPCs proliferation and impaired differentiation of human OPCs by dysregulating the Wnt-β-catenin signaling pathway. Administration of miR-27a led to suppression of myelinogenic signals, resulting in loss of endogenous myelination and remyelination. These findings suggest that a steady-state level of oligodendrocyte-specific miR-27a is critical in supporting multiple steps in the complex process of OPCs maturation and remyelination (Tripathi et al., 2019).

Interestingly, miR-297c-5p has been found to play a dual role in remyelination. It acts as a negative regulator of OPCs proliferation and a positive regulator of oligodendrocyte maturation by targeting cyclin T2 (CCNT2), the regulatory subunit of positive transcription elongation factor b, which inhibits oligodendrocyte maturation. When miR-297c-5p was overexpressed in mouse embryonic fibroblasts and rat OPCs, it promoted G1/G0 arrest and increased the number of O1+ rat OPCs during differentiation. These findings suggest that miR-297c-5p may be a potential target for promoting oligodendrocytes maturation and enhancing remyelination in diseases with a demyelinating component (Kuypers et al., 2016).

The role of miR-23a in enhancing oligodendrogenesis and myelin synthesis *in vivo* has been analyzed. Previous research showed that miR-23 can enhance oligodendrocytes differentiation and that lamin B1 negatively regulates oligodendrocytes (Lin and Fu, 2009). The study used mice in which miR-23a is overexpressed by an oligodendrocyte-specific promoter to investigate the effects of miR-23a. It has been found that miR-23a modulates the expression of phosphatase and tensin homolog on chromosome 10 (PTEN) and a long noncoding RNA (lncRNA) – 2700046G09Rik, which fine-tunes activities of the Akt/mTOR and MAPK pathways, promoting myelin gene expression. The results suggest that myelination requires tightly regulated multilayer signaling pathways (Lin et al., 2013).

In the attachment and extension of oligodendrocyte processes, miR-219 has been shown to be involved by promoting the expression of integrins and adhesion molecules. It has been reported that the myelination defects observed *in vitro* and *in vivo* are directly caused by the loss of Dicer1 function in OPCs and mature oligodendrocytes, resulting in ineffective action of miRNA (Dugas et al., 2010). Furthermore, studies on Dicer-floxed mice specific for oligodendrocytes showed that miR-219 targeting the elongation of the very long chain fatty acid protein (ELOVL7) plays a role in the maintenance of lipids and redox homeostasis in mature oligodendrocytes, required for the formation and integrity of myelin (Shin et al., 2009).

In the synthesis and assembly of myelin proteins, several miRNAs have been shown to be involved, including miR-138, miR-145, and miR-338. These miRNAs promote the expression of myelin genes by targeting negative regulators of the cAMP signaling pathway and the epigenetic regulator HDAC2. The overexpression of miR-146a in primary OPCs increased their expression of myelin proteins, while the reduction of endogenous miR-146a levels inhibited the generation of these proteins (Liu et al., 2017). The study also found that miR-146a inversely regulated the expression of its target gene-IRAK1 in OPCs, and suppressing the expression of IRAK1 in OPCs significantly increased myelin proteins and decreased OPC apoptosis (Liu et al., 2017).

The contribution of selected miRNAs to the particular stages of remyelination is summarized in the Figure 1.

4. The concept of miRNA as a biomarker for MS

In addition to the usefulness of miRNAs as candidates for the development of novel therapeutic strategies for remyelination, they have also been proposed as biomarkers for the diagnosis and prognosis of MS (Regev et al., 2016). While miRNA profiling from biological fluid poses great promise as a biomarker for MS, it must be noted that often conflicting results, high heterogeneity, and lack of repeatability are becoming challenging in the field (Piket et al., 2019). However, the lack of compliance is not unfamiliar to all biomarker projects and underscores only the logistical difficulties in such kind of research (Zurawska et al., 2019).

Currently used biomarkers for MS diagnosing are insufficient in terms of sensitivity and specificity, and prevent recognizing patients who are in the asymptomatic phase of MS, prior to the onset of clinical manifestations. In MS, the disease progression and the presence of active inflammation within the brain are usually monitored by gadolinium (Gd) enhanced magnetic resonance imaging (MRI) technique. Muñoz-San Martín et al. (2019) showed a positive correlation between miR-21, miR-146a, and miR-146b upregulation in CSF from MS patients with Gd+lesions suggesting that analyzed miRNAs are useful biomarkers in identifying the active lesions (Muñoz-San Martín et al., 2019). Selmaj et al. (2017) isolated exosomes from serum from RRMS patients (33 with relapse and 30 with remission) and a control group. Specimens from all patients were sampled before methylprednisolone administration, and patients in remission did not receive DMT for at least 6 months. Using the qPCR technique demonstrated that miR-122-5p, miR-196b-5p, and miR-532-5p were significantly downregulated in relapse patients compared to remission patients. Furthermore, by ROC analysis, a combination of miR-122-5p and miR-196b-5p gave AUC of 0.866 for distinguishing RRMS relapse from RRMS remission. While lower levels of miR-122-5p, miR-196b-5p, miR-301a-3p, and miR-532-5p were related to disease activity (Selmaj et al., 2017). Regev et al. (2018) reported 5 miRNAs (miR-484, miR-140-5p, miR-320a, miR-486-5p, and miR-320c), which demonstrated an essential difference between MS patients and the control group, according to data from 4 cohort studies (Regev et al., 2018). Other study performed on CSF from 53 MS patients and 39 healthy volunteers demonstrated that miR-181c, miR-633, and miR-922 were specifically expressed only in MS patients (Haghikia et al., 2012). A large-scale study by Vistbakka et al. (2018) showed that miR-191-5p and miR-24-3p were significantly up-regulated in both RRMS and PPMS compared to the control group (Vistbakka et al., 2018). Another large-scale study by Nuzziello et al. (2018) that included whole blood samples from 58 MS patients (54 RRMS and 4 SPMS), and 20 healthy controls reported that miR-320a, miR-125a-5p, miR-652-3p, miR-185-5p, miR-942-5p, and miR-25-3p were significantly upregulated in MS compared to the control group. The area under the curve (AUC) values for validated miRNAs ranged from 0.701 to 0.735 and are fair tests to discriminate MS patients from controls, with miR-320a having the highest AUC value (0.735) (Nuzziello et al., 2018).

In addition, there are few studies that miRNA profiling is a useful tool to identify MS subtypes. A cohort study on a large group of MS

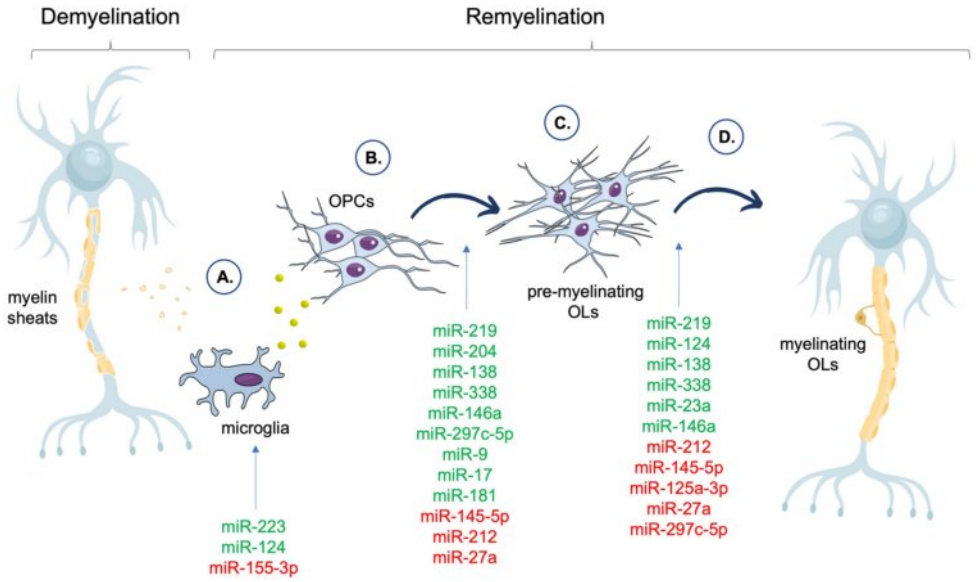

FIGURE 1
Summary of the miRNAs associated with different phases of the remyelination process. In the figure, miRNAs that have a positive impact on a particular stage of remyelination are shown in green, whereas miRNAs that have a negative effect on a particular stage of remyelination are shown in red. **(A)** Clearance of myelin debris and secreted of factors by microglia. **(B)** OPCs recruitment and proliferation. **(C)** OPCs differentiation and maturation. **(D)** Myelin proteins assembly. OPCs, Oligodendrocyte Precursor Cells (OPCs); OLs, oligodendrocytes.

patients ($n = 1,088$) was based on an examination of the relationship between MRI brain imaging and miRNA profiling. Surprisingly, each MRI-phenotypes (different in terms of brain atrophy and cerebral T2-hyperintense lesion volume) was linked with a characteristic miRNA signature, especially miR-22-3p, miR-361-5p, and miR-345-5p, which were the most valid differentiators of the MRI-phenotypes in MS patients (Hemond et al., 2019). Ebrahimkhani et al. (2017) using NGS identified 9 miRNAs dysregulated in RRMS compared to progressive MS patients (SPMS/PPMS) (6 upregulated: miR-15b-5p, miR-23a-3p, miR-223-3p, miR-30b-5p, miR-342-3p, miR-374a-5p, and 3 downregulated: miR-432-5p, miR-433-3p, miR-485-5p). Furthermore, ROC analysis allowed distinguishing RRMS from SPMS/PPMS patients with AUC values for miR-433-3p, miR-432-5p, and miR-485-5p were 0.93, 0.86, and 0.87, respectively (Ebrahimkhani et al., 2017). The recent study by Edgünlü et al. (2022) showed that the expression of miR-181a-5p was downregulated and associated with an increased risk of MS ($p = 0.012$). Furthermore, *in silico* analyses showed that circulating miR-181a-5p can participate in MS pathology by targeting genes involved in inflammation and neurodegeneration molecular pathways, such as MAP2K1, CREB1, ATXN1, and ATXN3 (Edgünlü et al., 2022).

5. MiRNA in novel therapeutic and diagnostic strategies of remyelination – facts and expectations

Although there are various treatments available that aim to decrease the immune response in MS, currently there is no treatment that encourages the regeneration of myelin. The disease-modifying therapies (DMTs) are a key component of MS symptoms management; however, the effects of these treatments vary depending on the individual and the type of MS. Moreover, they may not provide complete symptom management and can lead to significant side effects. They are also insufficient to prevent the accumulation of permanent disability caused by neurodegeneration, especially in the progressive phase of the disease (Goldenberg, 2012). As a result, alternative approaches are emerging. The identification of miRNAs as regulators of the remyelination process has led to the development of novel therapeutic strategies targeting these molecules.

Direct administration of miRNAs to the CNS is currently difficult and invasive, thus, to overcome this issue, various delivery systems, including poly (lactic-co-glycolic acid) (PLGA) nanoparticles, liposomes, and extracellular vesicles (EVs) have been developed. These delivery systems can protect miRNA from degradation and facilitate its uptake by oligodendrocytes and other cells in the CNS. The research results suggest that EVs hold the most promise as a non-invasive and efficient delivery system for miRNAs, as they were able to induce remyelination in an animal model. Moreover, it has been reported that miR-219a-5p encapsulated in EVs stimulate OPCs differentiation, can cross the blood–brain barrier (BBB) and improve the clinical transformation of EAE (Osorio-Querejeta et al., 2020). These findings seem promising in the context of a novel therapeutic approach for MS patients.

Interestingly, a recent study investigated the effect of exposure of aging rats to a young systemic environment on the production of serum exosomes involved in the promotion of remyelination by increasing the number and differentiation of OPCs. It has been shown

that the so-called environmental enrichment (social, cognitive, and physical exposition to the youth environment) of aging animals stimulates the production of exosomes that mimic the promyelinating effect (Pusic and Kraig, 2014). Environmental enrichment has previously been shown to improve memory and myelin production, alleviate the consequences of neurodegeneration (Fields, 2008), enhance immune system functioning (Pedersen and Hoffman-Goetz, 2000), and reduce oxidative stress (Arranz et al., 2010). Exosomes derived from both young and environmental enrichment animals were enriched in miR-219 molecules. The nasal administration of exosomes to aging rats improved myelination, which may be useful in novel remyelination strategies (Pusic and Kraig, 2014). Further studies support findings regarding the role of miR-219 in the pro-myelinating effect. Moreover, in this study, exosomal miR-9, miR-17, and miR-181 have been revealed to contribute to oligodendrocyte proliferation and anti-inflammatory process (Pusic et al., 2016).

The study by Li et al. aimed to investigate the therapeutic effects of M2 microglia-derived EVs in promoting white matter repair and functional recovery after cerebral ischemia in mice. The researchers found that M2-EVs treatment led to increased oligodendrogenesis and white matter repair, resulting in improved functional recovery. The therapeutic effect was attributed to the presence of miRNAs, including miR-23a-5p, miR-221-3p, miR-129-5p, and miR-155-5p, in M2-EVs, which promoted the survival and differentiation of OPCs. In particular, miR-23a-5p was identified as a key miRNA that promoted OPC differentiation by targeting Olig3 directly (Li et al., 2022).

Another study on potential miRNA delivery tools investigated the utility of using a biodegradable and biocompatible cationic polymer called PDAPEI to deliver miRNAs for therapeutic purposes. The study found that PDAPEI was less toxic and more efficient in delivering miR-221/222 to rat Schwann cells than another polymer called PEI25kDa. Upregulation of miR-221/222 in Schwann cells promoted the expression of NGF and MBP. The study also tested the effectiveness of PDAPEI/miR-221/222 complexes in promoting nerve regeneration in a mouse sciatic nerve crush injury model. The results showed that the complexes significantly enhanced remyelination and promoted nerve regeneration. In general, the study suggests that PDAPEI/miR-221/222 complexes may provide a safe and effective means of treating nerve crush injury (Song et al., 2017).

The next proposed platform to promote remyelination in the CNS was a scaffolding system for sustained nonviral delivery of miRNAs to promote the differentiation, maturation, and myelination of oligodendrocytes. The miRNAs were incorporated into a fiber-hydrogel scaffold. It has been found to promote the differentiation and myelination of oligodendrocytes *in vitro* and *in vivo* after spinal cord injury in rats. The miR-219/miR-338 treatment increased the number of oligodendrocytes and the rate and extent of their differentiation, resulting in more compact myelin sheaths and higher myelination (Milbreta et al., 2019).

Another study aimed to understand the molecular pathway by which NGF negatively regulates oligodendrogenesis by investigating downstream targets, focusing on miRNAs. The study used a mouse model deprivation of NGF and found that NGF inhibits oligodendrogenesis by negatively regulating the expression of miR-219a-5p, which is a positive regulator of oligodendrocyte differentiation and myelin repair. These findings suggest that NGF can be targeted to enhance myelination and promote remyelination in demyelinating diseases such as MS (Brandi et al., 2021).

As mentioned before, miR-223 expression is essential for the efficient clearing of myelin debris after demyelination and is upregulated in active MS lesions, likely due to macrophage infiltration and proliferation (Galloway et al., 2019). The NLR family pyrin domain containing 3 (NLRP3) inflammasome and miR-223-3p are up-regulated immediately after demyelination and returned to near baseline after remyelination (Galloway et al., 2022). The NLRP3 inflammasome is a protein complex that is part of the innate immune system, which is responsible for initiating the body's inflammatory response to potential threats, such as pathogens and tissue damage (Swanson et al., 2019). It has been revealed that the NLRP3 inflammasome was primarily expressed within activated macrophages/microglia, both in experimentally induced demyelination and mixed active/inactive MS lesions. *In vitro*, the small-molecule NLRP3 inhibitor, MCC950, and miR-223-3p mimics suppressed the activation of the NLRP3 inflammasome in macrophages and microglia. When delivering MCC950 to animals after lysolecithin-induced demyelination, the axonal injury within the demyelinated lesions was significantly reduced. The results suggest that the NLRP3 inflammasome plays a role in demyelinating injury and that NLRP3 inhibitors may serve as an effective new therapeutic strategy for treating MS (Galloway et al., 2022).

Otero-Ortega et al. (2017) made initial observations from deep RNA sequencing data of exosomes derived from mesenchymal stem cells (MSCs) and hypothesized that certain miRNAs, such as miR-199a-5p and miR-145, could be involved in oligodendrocyte maturation. In a model of subcortical ischemic stroke, MSC-derived exosomes were found to facilitate the differentiation of oligodendrocytes and the remyelination process. Upon intravenous administration, the authors observed increased levels of myelin protein and a greater number of myelinated axons (Otero-Ortega et al., 2017). These findings were consistent with the results of an *in vitro* model of ischemic stroke, which demonstrated that miR-134, obtained from bone marrow MSC exosomes, exerted a positive impact on rat oligodendrocytes by suppressing apoptosis by targeting caspase-8 (Xiao et al., 2019).

One study investigated the impact of hippocampal demyelination on neuronal gene expression and memory impairment in MS patients. Comparative analysis of miRNA profiles from myelinated and demyelinated hippocampi from the postmortem brain of MS revealed that demyelination led to increased expression of miR-124, which targets mRNAs encoding several neuronal proteins, including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA2) and AMPA3 receptors. It has been also observed that hippocampal demyelination in mice increased miR-124, reduced expression of AMPA receptors, and decreased memory performance in water maze tests. However, remyelination of the mouse hippocampus reversed these changes (Dutta et al., 2013).

A recent study investigated the effect of Hydroxychloroquine, an antimalarial immunomodulatory medication, on microglia and oligodendrocytes by regulating the expression of miR-219 and miR-155-3p in the cuprizone-induced demyelination mice model (Mazloumfard et al., 2020). The influence of Hydroxychloroquine on the activity of microglia and/or oligodendrocytes has previously been established (Koch et al., 2015). The study revealed that pharmacological strategies leading to miR-155-3p down-regulation may enhance remyelination in MS (Mazloumfard et al., 2020).

Another study on miR-219 and miR-155-3p expression levels has been carried out in the context of myelination with the use of Apamin in a cuprizone-induced demyelination mice model. Apamin exhibited

more impact on the reduction in miR-155-3p expression in the demyelination phase of the disease than the elevation of miR-219 in the remyelination phase and has been suggested as a therapeutic option to reduce plaque formation during the exacerbation phase of MS by reducing the expression of miR-155-3p (Gholami et al., 2020).

The common effect of the action of miR-219 and miR-338 to promote OPCs differentiation, maturation, and myelination may serve as a promising strategy for nerve repair, as has been reported using the scratch test, which recreated a nerve injury *in vitro* (Nguyen et al., 2019). A study by Diao et al. aimed to improve oligodendrocyte differentiation and maturation by developing a nanofiber-mediated miRNA delivery method to control the differentiation of OPCs through a combination of fiber topography and gene silencing. The study showed that nanofiber topography enhanced OPCs differentiation, while miRNA delivery further improved the results. Furthermore, nanofiber-mediated delivery of miR-219 and miR-338 promoted the maturation of oligodendrocytes. The study's results demonstrate the potential of nanofibers in providing topographical cues and miRNA delivery to direct OPCs differentiation and may find useful applications in treating CNS pathological conditions that require remyelination (Diao et al., 2015).

The use of miRNAs as biomarkers of remyelination in MS provided several advantages. Firstly, these molecules can be detected in easily accessible biofluids, such as CSF and blood, making them attractive as non-invasive biomarkers (McCoy, 2017). Furthermore, miRNAs are stable and can be reliably detected using common quantitative real-time PCR (qPCR), microarrays or next-generation sequencing (NGS) techniques (Moody et al., 2017). Then, differences in the expression of certain miRNAs seem to be specific to some biological and pathological processes and therefore can provide more accurate information about the disease process than other biomarkers (Sheinerman et al., 2017). Despite this, several challenges hamper their application into clinical diagnostic practice, such as the variability in miRNA expression across individuals, the lack of standardization in miRNA detection methods, and data normalization, which affect the accuracy and reproducibility of results (Piket et al., 2019).

One study aimed to identify miRNA expression patterns during the maturation of oligodendrocytes from human embryonic stem (hES) cells. The miRNA analysis in cells from eight stages of oligodendrocytes differentiation has been performed. MiRNA expression patterns have been found to be similar to those in rat and mouse CNS cells, with four distinct clusters of miRNA expression corresponding to different stages of oligodendrocyte maturation. The study also identified potential mRNA targets for these miRNAs, including factors involved in oligodendrocyte development and myelination, such as C11Orf9, CLDN11, MYTL1, MBOP, MPZL2, and DDR1. These findings provide insights into the molecular mechanisms of oligodendrocyte differentiation and may serve as markers for oligodendrocytes maturation (Letzen et al., 2010).

6. Conclusion

Over the last few years, significant advances have been made in understanding how miRNAs control gene expression post-transcriptionally to regulate CNS myelination. Studies indicate that miRNAs do not act alone, but rather influence multiple signaling and regulatory pathways, which may affect their effectiveness as therapeutic targets. Therefore, it is necessary to understand more complex regulatory mechanisms before miRNAs can be used to treat demyelinating diseases. The miRNA molecule miR-219 is highly expressed in mature myelinating oligodendrocytes and plays a crucial role in promoting the differentiation of precursor oligodendrocyte cells into mature oligodendrocytes. Reduced levels of miR-219 have been observed in MS patients, which can contribute to failed remyelination. Increasing miR-219 has been shown to enhance the maturation of oligodendrocyte precursor cells and is a promising target for remyelination. The use of drugs to stimulate myelin restoration in the CNS could greatly benefit patients by slowing or even protecting against neurodegeneration. However, most of the studies are developed in animal models, which retains several barriers before introducing remyelination strategies into clinical practice. Various models have been created to study inflammatory demyelinating diseases in animals, including immunization, virus-induced, genetic, and toxic models (Ransohoff, 2012). Nevertheless, none of them perfectly replicates the specific characteristics of MS lesions, complexity of the disease pathophysiology, integrating immune and nervous system, contribution of distinct environmental factors, role of T CD8+ cells, mechanisms of the disease progression and age-dependency (Lassmann and Bradl, 2017). Observing remyelination in human samples is difficult due to the limited access to histopathological material and insufficient reflection of remyelination processes in biomarkers derived from body fluids. EAE is, so far, the most commonly employed animal model, as it is able to reflect immune response, inflammation, demyelination, axonal loss, gliosis, and remyelination. Despite critical comments, studies often show that the results obtained from EAE and MS are comparable and are an integral tool in conducting MS research on aspects of autoimmunity, neuroinflammation, and neuronal loss (Birmpili et al., 2022). It should be borne in mind, first of all, that any hypothesis verified on an animal model must be tested on patient material and in clinical trials, which will be its only final confirmation.

Remyelination is believed to prevent progressive axonal injury and reduce long-term disability in MS patients. Therefore, there is a clear need for therapeutic approaches that can enhance the organism's own repair and remyelination mechanisms.

Author contributions

All authors contributed to the conception of the work, drafted and revised the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Aparicio, E., Mathieu, P., Pereira Luppi, M., Almeira Gubiani, M. F., and Adamo, A. M. (2013). The notch signaling pathway: its role in focal CNS demyelination and apotransferrin-induced remyelination. *J. Neurochem.* 127, 819–836. doi: 10.1111/jnc.12440

Arranz, L., De Castro, N. M., Baeza, I., Maté, I., Viveros, M. P., and De la Fuente, M. (2010). Environmental enrichment improves age-related immune system impairment: long-term exposure since adulthood increases life span in mice. *Rejuvenation Res.* 13, 415–428. doi: 10.1089/rej.2009.0989

Atlas of MS 2020 – Epidemiology Report (2020). MS International Federation. Available at: https://www.msif.org/resource/atlas-of-ms-2020/ (accessed March 28, 2023).

Billinghurst, L. L., Taylor, R. M., and Snyder, E. Y. (1998). Remyelination: cellular and gene therapy. *Semin. Pediatr. Neurol.* 5, 211–228. doi: 10.1016/s1071-9091(98)80036-3

Birmpili, D., Charmarke Askar, I., Bigaut, K., and Bagnard, D. (2022). The translatability of multiple sclerosis animal models for biomarkers discovery and their clinical use. *Int. J. Mol. Sci.* 23:11532. doi: 10.3390/ijms231911532

Bottes, S., and Jessberger, S. (2021). Live imaging of remyelination in the adult mouse corpus callosum. *Proc. Natl. Acad. Sci.* 118:e2025795118. doi: 10.1073/pnas.2025795118

Brandi, R., Fabiano, M., Giorgi, C., Arisi, I., La Regina, F., Malerba, F., et al. (2021). Nerve growth factor neutralization promotes oligodendrogenesis by increasing miR-219a-5p levels. *Cells* 10:405. doi: 10.3390/cells10020405

Bruinsma, I. B., van Dijk, M., Bridel, C., van de Lisdonk, T., Haverkort, S. Q., Runia, T. F., et al. (2017). Regulator of oligodendrocyte maturation, miR-219, a potential biomarker for MS. *J. Neuroinflammation* 14:235. doi: 10.1186/s12974-017-1006-3

Butovsky, O., Jedrychowski, M. P., Cialic, R., Krasemann, S., Murugaiyan, G., Fanek, Z., et al. (2015). Targeting miR-155 restores abnormal microglia and attenuates disease in SOD1 mice. *Ann. Neurol.* 77, 75–99. doi: 10.1002/ana.24304

Chari, D. M. (2007). Remyelination in multiple sclerosis. *Int. Rev. Neurobiol.* 79, 589–620. doi: 10.1016/S0074-7742(07)79026-8

Charles, P., Hernandez, M. P., Stankoff, B., Aigrot, M. S., Colin, C., Rougon, G., et al. (2000). Negative regulation of central nervous system myelination by polysialylated-neural cell adhesion molecule. *Proc. Natl. Acad. Sci. U. S. A.* 97, 7585–7590. doi: 10.1073/pnas.100076197

Chen, C. Z., Neumann, B., Förster, S., and Franklin, R. J. M. (2021). Schwann cell remyelination of the central nervous system: why does it happen and what are the benefits? *Open Biol.* 11:200352. doi: 10.1098/rsob.200352

Dendrou, C. A., Fugger, L., and Friese, M. A. (2015). Immunopathology of multiple sclerosis. *Nat. Rev. Immunol.* 15, 545–558. doi: 10.1038/nri3871

Diao, H. J., Low, W. C., Milbreta, U., Lu, Q. R., and Chew, S. Y. (2015). Nanofiber-mediated microRNA delivery to enhance differentiation and maturation of oligodendroglial precursor cells. *J. Control. Release* 208, 85–92. doi: 10.1016/j.jconrel.2015.03.005

Dolati, S., Marofi, F., Babaloo, Z., Aghebati-Maleki, L., Roshangar, L., Ahmadi, M., et al. (2018). Dysregulated network of miRNAs involved in the pathogenesis of multiple sclerosis. *Biomed. Pharmacother.* 104, 280–290. doi: 10.1016/j.biopha.2018.05.050

Dombrowski, Y., O'Hagan, T., Dittmer, M., Penalva, R., Mayoral, S. R., Bankhead, P., et al. (2017). Regulatory T cells promote myelin regeneration in the central nervous system. *Nat. Neurosci.* 20, 674–680. doi: 10.1038/nn.4528

Duffy, C. P., and McCoy, C. E. (2020). The role of MicroRNAs in repair processes in multiple sclerosis. *Cells* 9:1711. doi: 10.3390/cells9071711

Dugas, J. C., Cuellar, T. L., Scholze, A., Ason, B., Ibrahim, A., Emery, B., et al. (2010). Dicer1 and miR-219 are required for normal oligodendrocyte differentiation and myelination. *Neuron* 65, 597–611. doi: 10.1016/j.neuron.2010.01.027

Dutta, R., Chomyk, A. M., Chang, A., Ribaudo, M. V., Deckard, S. A., Doud, M. K., et al. (2013). Hippocampal demyelination and memory dysfunction are associated with increased levels of the neuronal microRNA miR-124 and reduced AMPA receptors. *Ann. Neurol.* 73, 637–645. doi: 10.1002/ana.23860

Ebrahimkhani, S., Vafaee, F., Young, P. E., Hur, S. S. J., Hawke, S., Devenney, E., et al. (2017). Exosomal microRNA signatures in multiple sclerosis reflect disease status. *Sci. Rep.* 7:14293. doi: 10.1038/s41598-017-14301-3

Edgünlü, T. G., Yılmaz, Ş. G., Emre, U., Taşdelen, B., Kuru, O., Kutlu, G., et al. (2022). miR-181a-5p is a potential candidate epigenetic biomarker in multiple sclerosis. *Genome* 65, 547–561. doi: 10.1139/gen-2022-0040

Engelhardt, B., and Ransohoff, R. M. (2005). The ins and outs of T-lymphocyte trafficking to the CNS: anatomical sites and molecular mechanisms. *Trends Immunol.* 26, 485–495. doi: 10.1016/j.it.2005.07.004

Fancy, S. P. J., Baranzini, S. E., Zhao, C., Yuk, D.-I., Irvine, K.-A., Kaing, S., et al. (2009). Dysregulation of the Wnt pathway inhibits timely myelination and remyelination in the mammalian CNS. *Genes Dev.* 23, 1571–1585. doi: 10.1101/gad.1806309

Fang, L.-P., Liu, Q., Meyer, E., Welle, A., Huang, W., Scheller, A., et al. (2023). A subset of OPCs do not express Olig2 during development which can be increased in the adult by brain injuries and complex motor learning. *Glia* 71, 415–430. doi: 10.1002/glia.24284

Fields, R. D. (2008). White matter in learning, cognition and psychiatric disorders. *Trends Neurosci.* 31, 361–370. doi: 10.1016/j.tins.2008.04.001

Filippi, M., Bar-Or, A., Piehl, F., Preziosa, P., Solari, A., Vukusic, S., et al. (2018). Multiple sclerosis. *Nat. Rev. Dis. Primers.* 4, 1–27. doi: 10.1038/s41572-018-0041-4

Franklin, R. J. M., and Ffrench-Constant, C. (2008). Remyelination in the CNS: from biology to therapy. *Nat. Rev. Neurosci.* 9, 839–855. doi: 10.1038/nrn2480

Frischer, J. M., Weigand, S. D., Guo, Y., Kale, N., Parisi, J. E., Pirko, I., et al. (2015). Clinical and pathological insights into the dynamic nature of the white matter multiple sclerosis plaque. *Ann. Neurol.* 78, 710–721. doi: 10.1002/ana.24497

Fu, R., Shen, Q., Xu, P., Luo, J. J., and Tang, Y. (2014). Phagocytosis of microglia in the central nervous system diseases. *Mol. Neurobiol.* 49, 1422–1434. doi: 10.1007/s12035-013-8620-6

Fujiwara, M., Raheja, R., Garo, L. P., Ajay, A. K., Kadowaki-Saga, R., Karandikar, S. H., et al. (2022). microRNA-92a promotes CNS autoimmunity by modulating the regulatory and inflammatory T cell balance. *J. Clin. Invest.* 132:e155693. doi: 10.1172/JCI155693

Gaesser, J. M., and Fyffe-Maricich, S. L. (2016). Intracellular signaling pathway regulation of myelination and remyelination in the CNS. *Exp. Neurol.* 283, 501–511. doi: 10.1016/j.expneurol.2016.03.008

Galloway, D. A., Blandford, S. N., Berry, T., Williams, J. B., Stefanelli, M., Ploughman, M., et al. (2019). miR-223 promotes regenerative myeloid cell phenotype and function in the demyelinated central nervous system. *Glia* 67, 857–869. doi: 10.1002/glia.23576

Galloway, D. A., Carew, S. J., Blandford, S. N., Benoit, R. Y., Fudge, N. J., Berry, T., et al. (2022). Investigating the NLRP3 inflammasome and its regulator miR-223-3p in multiple sclerosis and experimental demyelination. *J. Neurochem.* 163, 94–112. doi: 10.1111/jnc.15650

Gebert, L. F. R., and MacRae, I. J. (2019). Regulation of microRNA function in animals. *Nat. Rev. Mol. Cell Biol.* 20, 21–37. doi: 10.1038/s41580-018-0045-7

Gholami, S., Mirian, M., Eftekhari, S. M., and Aliomrani, M. (2020). Apamin administration impact on miR-219 and miR-155-3p expression in cuprizone induced multiple sclerosis model. *Mol. Biol. Rep.* 47, 9013–9019. doi: 10.1007/s11033-020-05959-6

Goldenberg, M. M. (2012). Multiple sclerosis review. *P T* 37, 175–184.

Goldschmidt, T., Antel, J., König, F. B., Brück, W., and Kuhlmann, T. (2009). Remyelination capacity of the MS brain decreases with disease chronicity. *Neurology* 72, 1914–1921. doi: 10.1212/WNL.0b013e3181a8260a

Gruchot, J., Weyers, V., Göttle, P., Förster, M., Hartung, H.-P., Küry, P., et al. (2019). The molecular basis for remyelination failure in multiple sclerosis. *Cells* 8:825. doi: 10.3390/cells8080825

Haghikia, A., Haghikia, A., Hellwig, K., Baraniskin, A., Holzmann, A., Décard, B. F., et al. (2012). Regulated microRNAs in the CSF of patients with multiple sclerosis: a case-control study. *Neurology* 79, 2166–2170. doi: 10.1212/WNL.0b013e3182759621

Hart, A. D., Wyttenbach, A., Perry, V. H., and Teeling, J. L. (2012). Age related changes in microglial phenotype vary between CNS regions: grey versus white matter differences. *Brain Behav. Immun.* 26, 754–765. doi: 10.1016/j.bbi.2011.11.006

Hemond, C. C., Healy, B. C., Tauhid, S., Mazzola, M. A., Quintana, F. J., Gandhi, R., et al. (2019). MRI phenotypes in MS: longitudinal changes and miRNA signatures. *Neurol Neuroimmunol Neuroinflamm* 6:e530. doi: 10.1212/NXI.0000000000000530

Hiebert, J. B., Shen, Q., Thimmesch, A. R., and Pierce, J. D. (2015). Traumatic brain injury and mitochondrial dysfunction. *Am J Med Sci* 350, 132–138. doi: 10.1097/MAJ.0000000000000506

Huang, J. K., and Franklin, R. J. M. (2012). "Chapter 12 - Current status of myelin replacement therapies in multiple sclerosis," in *Progress in brain research, functional neural transplantation III*. eds. S. B. Dunnett and A. Björklund (Elsevier), 219–231.

Jia, J., Jin, H., Nan, D., Yu, W., and Huang, Y. (2021). New insights into targeting mitochondria in ischemic injury. *Apoptosis* 26, 163–183. doi: 10.1007/s10495-021-01661-5

Jolanda Münzel, E., and Williams, A. (2013). Promoting remyelination in multiple sclerosis—recent advances. *Drugs* 73, 2017–2029. doi: 10.1007/s40265-013-0146-8

Kamma, E., Lasisi, W., Libner, C., Ng, H. S., and Plemel, J. R. (2022). Central nervous system macrophages in progressive multiple sclerosis: relationship to neurodegeneration and therapeutics. *J. Neuroinflammation* 19:45. doi: 10.1186/s12974-022-02408-y

Koch, M. W., Zabad, R., Giuliani, F., Hader, W., Lewkonia, R., Metz, L., et al. (2015). Hydroxychloroquine reduces microglial activity and attenuates experimental autoimmune encephalomyelitis. *J. Neurol. Sci.* 358, 131–137. doi: 10.1016/j.jns.2015.08.1525

Kremer, D., Akkermann, R., Küry, P., and Dutta, R. (2019). Current advancements in promoting remyelination in multiple sclerosis. *Mult. Scler.* 25, 7–14. doi: 10.1177/1352458518800827

Kremer, D., Küry, P., and Dutta, R. (2015). Promoting remyelination in multiple sclerosis: current drugs and future prospects. *Mult. Scler.* 21, 541–549. doi: 10.1177/1352458514566419

Kuhlmann, T., Miron, V., Cui, Q., Wegner, C., Antel, J., and Brück, W. (2008). Differentiation block of oligodendroglial progenitor cells as a cause for remyelination failure in chronic multiple sclerosis. *Brain* 131, 1749–1758. doi: 10.1093/brain/awn096

Kuypers, N. J., Bankston, A. N., Howard, R. M., Beare, J. E., and Whittemore, S. R. (2016). Remyelinating oligodendrocyte precursor cell miRNAs from the Sfmbt2 cluster promote cell cycle arrest and differentiation. *J. Neurosci.* 36, 1698–1710. doi: 10.1523/JNEUROSCI.1240-15.2016

Ladak, A. A., Enam, S. A., and Ibrahim, M. T. (2019). A review of the molecular mechanisms of traumatic brain injury. *World Neurosurg.* 131, 126–132. doi: 10.1016/j.wneu.2019.07.039

Lassmann, H. (2018). Multiple sclerosis pathology. *Cold Spring Harb. Perspect. Med.* 8:a028936. doi: 10.1101/cshperspect.a028936

Lassmann, H., and Bradl, M. (2017). Multiple sclerosis: experimental models and reality. *Acta Neuropathol.* 133, 223–244. doi: 10.1007/s00401-016-1631-4

Lecca, D., Marangon, D., Coppolino, G. T., Méndez, A. M., Finardi, A., Costa, G. D., et al. (2016). MiR-125a-3p timely inhibits oligodendroglial maturation and is pathologically up-regulated in human multiple sclerosis. *Sci. Rep.* 6:34503. doi: 10.1038/srep34503

Letzen, B. S., Liu, C., Thakor, N. V., Gearhart, J. D., All, A. H., and Kerr, C. L. (2010). MicroRNA expression profiling of oligodendrocyte differentiation from human embryonic stem cells. *PLoS One* 5:e10480. doi: 10.1371/journal.pone.0010480

Lewkowicz, P., Cwiklińska, H., Mycko, M. P., Cichalewska, M., Domowicz, M., Lewkowicz, N., et al. (2015). Dysregulated RNA-induced silencing complex (RISC) assembly within CNS corresponds with abnormal miRNA expression during autoimmune demyelination. *J. Neurosci.* 35, 7521–7537. doi: 10.1523/JNEUROSCI.4794-14.2015

Li, Y., Liu, Z., Song, Y., Pan, J., Jiang, Y., Shi, X., et al. (2022). M2 microglia-derived extracellular vesicles promote white matter repair and functional recovery via miR-23a-5p after cerebral ischemia in mice. *Theranostics* 12, 3553–3573. doi: 10.7150/thno.68895

Lin, S.-T., and Fu, Y.-H. (2009). miR-23 regulation of lamin B1 is crucial for oligodendrocyte development and myelination. *Dis. Model. Mech.* 2, 178–188. doi: 10.1242/dmm.001065

Lin, S.-T., Huang, Y., Zhang, L., Heng, M. Y., Ptácek, L. J., and Fu, Y.-H. (2013). MicroRNA-23a promotes myelination in the central nervous system. *Proc. Natl. Acad. Sci. U. S. A.* 110, 17468–17473. doi: 10.1073/pnas.1317182110

Lindner, M., Heine, S., Haastert, K., Garde, N., Fokuhl, J., Linsmeier, F., et al. (2008). Sequential myelin protein expression during remyelination reveals fast and efficient repair after central nervous system demyelination. *Neuropathol. Appl. Neurobiol.* 34, 105–114. doi: 10.1111/j.1365-2990.2007.00879.x

Liu, X. S., Chopp, M., Pan, W. L., Wang, X. L., Fan, B. Y., Zhang, Y., et al. (2017). MicroRNA-146a promotes oligodendrogenesis in stroke. *Mol. Neurobiol.* 54, 227–237. doi: 10.1007/s12035-015-9655-7

Liu, C., Li, Y., Yu, J., Feng, L., Hou, S., Liu, Y., et al. (2013). Targeting the shift from M1 to M2 macrophages in experimental autoimmune encephalomyelitis mice treated with fasudil. *PLoS One* 8:e54841. doi: 10.1371/journal.pone.0054841

Lopaisankrit, T., and Thammaroj, J. (2023). Brain and spinal cord MRI findings in thai multiple sclerosis patients. *J. Imaging* 9:27. doi: 10.3390/jimaging9020027

Loulier, K., Ruat, M., and Traiffort, E. (2006). Increase of proliferating oligodendroglial progenitors in the adult mouse brain upon Sonic hedgehog delivery in the lateral ventricle. *J. Neurochem.* 98, 530–542. doi: 10.1111/j.1471-4159.2006.03896.x

Lucchinetti, C., Brück, W., Parisi, J., Scheithauer, B., Rodriguez, M., and Lassmann, H. (1999). A quantitative analysis of oligodendrocytes in multiple sclerosis lesions. A study of 113 cases. *Brain* 122, 2279–2295. doi: 10.1093/brain/122.12.2279

Marangon, D., Boda, E., Parolisi, R., Negri, C., Giorgi, C., Montarolo, F., et al. (2020). In vivo silencing of miR-125a-3p promotes myelin repair in models of white matter demyelination. *Glia* 68, 2001–2014. doi: 10.1002/glia.23819

Mason, J. L., Ye, P., Suzuki, K., D'Ercole, A. J., and Matsushima, G. K. (2000). Insulin-like growth Factor-1 inhibits mature oligodendrocyte apoptosis during primary demyelination. *J. Neurosci.* 20, 5703–5708. doi: 10.1523/JNEUROSCI.20-15-05703.2000

Mazloumfard, F., Mirian, M., Eftekhari, S.-M., and Aliomrani, M. (2020). Hydroxychloroquine effects on miR-155-3p and miR-219 expression changes in animal model of multiple sclerosis. *Metab. Brain Dis.* 35, 1299–1307. doi: 10.1007/s11011-020-00609-z

McCoy, C. E. (2017). miR-155 dysregulation and therapeutic intervention in multiple sclerosis. *Adv. Exp. Med. Biol.* 1024, 111–131. doi: 10.1007/978-981-10-5987-2_5

Medina-Rodríguez, E. M., Bribián, A., Boyd, A., Palomo, V., Pastor, J., Lagares, A., et al. (2017). Promoting in vivo remyelination with small molecules: a neuroreparative pharmacological treatment for multiple sclerosis. *Sci. Rep.* 7:43545. doi: 10.1038/srep43545

Meschkat, M., Steyer, A. M., Weil, M.-T., Kusch, K., Jahn, O., Piepkorn, L., et al. (2022). White matter integrity in mice requires continuous myelin synthesis at the inner tongue. *Nat. Commun.* 13:1163. doi: 10.1038/s41467-022-28720-y

Mi, S., Miller, R. H., Lee, X., Scott, M. L., Shulag-Morskaya, S., Shao, Z., et al. (2005). LINGO-1 negatively regulates myelination by oligodendrocytes. *Nat. Neurosci.* 8, 745–751. doi: 10.1038/nn1460

Milbreta, U., Lin, J., Pinese, C., Ong, W., Chin, J. S., Shirahama, H., et al. (2019). Scaffold-mediated sustained, non-viral delivery of miR-219/miR-338 promotes CNS remyelination. *Mol. Ther.* 27, 411–423. doi: 10.1016/j.ymthe.2018.11.016

Moody, L., He, H., Pan, Y.-X., and Chen, H. (2017). Methods and novel technology for microRNA quantification in colorectal cancer screening. *Clin. Epigenetics* 9:119. doi: 10.1186/s13148-017-0420-9

Morris, J. K., Chomyk, A., Song, P., Parker, N., Deckard, S., Trapp, B. D., et al. (2015). Decrease in levels of the evolutionarily conserved microRNA miR-124 affects oligodendrocyte numbers in zebrafish, *Danio rerio*. *Invert. Neurosci.* 15:4. doi: 10.1007/s10158-015-0180-1

Muñoz-San Martín, M., Reverter, G., Robles-Cedeño, R., Buxò, M., Ortega, F. J., Gómez, I., et al. (2019). Analysis of miRNA signatures in CSF identifies upregulation of miR-21 and miR-146a/b in patients with multiple sclerosis and active lesions. *J. Neuroinflammation* 16:220. doi: 10.1186/s12974-019-1590-5

Neumann, B., Baror, R., van Wijngaarden, P., and Franklin, R. J. (2017). Remyelination of regenerating axons. *Acta Ophthalmol.* 95. doi: 10.1111/j.1755-3768.2017.03525

Nguyen, L. H., Ong, W., Wang, K., Wang, M., Nizetic, D., and Chew, S. Y. (2019). Effects of miR-219/miR-338 on microglia and astrocyte behaviors and astrocyte-oligodendrocyte precursor cell interactions. *Neural Regen. Res.* 15, 739–747. doi: 10.4103/1673-5374.266922

Nuzziello, N., Vilardo, L., Pelucchi, P., Consiglio, A., Liuni, S., Trojano, M., et al. (2018). Investigating the role of MicroRNA and transcription factor co-regulatory networks in multiple sclerosis pathogenesis. *Int. J. Mol. Sci.* 19:3652. doi: 10.3390/ijms19113652

Osorio-Querejeta, I., Carregal-Romero, S., Ayerdi-Izquierdo, A., Mäger, I., Nash, L. A., Wood, M., et al. (2020). MiR-219a-5p enriched extracellular vesicles induce OPC differentiation and EAE improvement more efficiently than liposomes and polymeric nanoparticles. *Pharmaceutics* 12:186. doi: 10.3390/pharmaceutics12020186

Otero-Ortega, L., Laso-García, F., Gómez-de Frutos, M. D. C., Rodríguez-Frutos, B., Pascual-Guerra, J., Fuentes, B., et al. (2017). White matter repair after extracellular vesicles administration in an experimental animal model of subcortical stroke. *Sci. Rep.* 7:44433. doi: 10.1038/srep44433

Patrikios, P., Stadelmann, C., Kutzelnigg, A., Rauschka, H., Schmidbauer, M., Laursen, H., et al. (2006). Remyelination is extensive in a subset of multiple sclerosis patients. *Brain* 129, 3165–3172. doi: 10.1093/brain/awl217

Pedersen, B. K., and Hoffman-Goetz, L. (2000). Exercise and the immune system: regulation, integration, and adaptation. *Physiol. Rev.* 80, 1055–1081. doi: 10.1152/physrev.2000.80.3.1055

Pennati, A., Nylen, E. A., Duncan, I. D., and Galipeau, J. (2020). Regulatory B cells normalize CNS myeloid cell content in a mouse model of multiple sclerosis and promote oligodendrogenesis and remyelination. *J. Neurosci.* 40, 5105–5115. doi: 10.1523/JNEUROSCI.2840-19.2020

Piatek, P., Namiecinska, M., Domowicz, M., Przygodzka, P., Wieczorek, M., Michlewska, S., et al. (2019). MS CD49d+CD154+ lymphocytes reprogram oligodendrocytes into immune reactive cells affecting CNS regeneration. *Cells* 8:1508. doi: 10.3390/cells8121508

Piatek, P., Namiecinska, M., Domowicz, M., Wieczorek, M., Michlewska, S., Matysiak, M., et al. (2020). Multiple sclerosis CD49d+CD154+ as myelin-specific lymphocytes induced during remyelination. *Cells* 9:15. doi: 10.3390/cells9010015

Piket, E., Zheleznyakova, G. Y., Kular, L., and Jagodic, M. (2019). Small non-coding RNAs as important players, biomarkers and therapeutic targets in multiple sclerosis: a comprehensive overview. *J. Autoimmun.* 101, 17–25. doi: 10.1016/j.jaut.2019.04.002

Pusic, A. D., and Kraig, R. P. (2014). Youth and environmental enrichment generate serum exosomes containing miR-219 that promote CNS myelination. *Glia* 62, 284–299. doi: 10.1002/glia.22606

Pusic, K. M., Pusic, A. D., and Kraig, R. P. (2016). Environmental enrichment stimulates immune cell secretion of exosomes that promote CNS myelination and may regulate inflammation. *Cell. Mol. Neurobiol.* 36, 313–325. doi: 10.1007/s10571-015-0269-4

Qin, C., Yang, S., Chu, Y.-H., Zhang, H., Pang, X.-W., Chen, L., et al. (2022). Signaling pathways involved in ischemic stroke: molecular mechanisms and therapeutic interventions. *Sig. Transduct. Target Ther.* 7, 215–229. doi: 10.1038/s41392-022-01064-1

Ransohoff, R. M. (2012). Animal models of multiple sclerosis: the good, the bad and the bottom line. *Nat. Neurosci.* 15, 1074–1077. doi: 10.1038/nn.3168

Ray, S. K., Dixon, C. E., and Banik, N. L. (2002). Molecular mechanisms in the pathogenesis of traumatic brain injury. *Histol. Histopathol.* 17, 1137–1152. doi: 10.14670/HH-17.1137

Regev, K., Healy, B. C., Paul, A., Diaz-Cruz, C., Mazzola, M. A., Raheja, R., et al. (2018). Identification of MS-specific serum miRNAs in an international multicenter study. *Neurol Neuroimmunol. Neuroinflamm.* 5:e491. doi: 10.1212/NXI.0000000000000491

Regev, K., Paul, A., Healy, B., von Glenn, F., Diaz-Cruz, C., Gholipour, T., et al. (2016). Comprehensive evaluation of serum microRNAs as biomarkers in multiple sclerosis. *Neurol Neuroimmunol. Neuroinflamm.* 3:e267. doi: 10.1212/NXI.0000000000000267

Schmitz, T., and Chew, L.-J. (2008). Cytokines and myelination in the central nervous system. *ScientificWorldJournal* 8, 1119–1147. doi: 10.1100/tsw.2008.140

Selmaj, I., Cichalewska, M., Namiecinska, M., Galazka, G., Horzelski, W., Selmaj, K. W., et al. (2017). Global exosome transcriptome profiling reveals biomarkers for multiple sclerosis. *Ann. Neurol.* 81, 703–717. doi: 10.1002/ana.24931

Sheinerman, K. S., Toledo, J. B., Tsivinsky, V. G., Irwin, D., Grossman, M., Weintraub, D., et al. (2017). Circulating brain-enriched microRNAs as novel biomarkers for detection and differentiation of neurodegenerative diseases. *Alzheimers Res. Ther.* 9:89. doi: 10.1186/s13195-017-0316-0

Shin, D., Shin, J.-Y., McManus, M. T., Ptácek, L. J., and Fu, Y.-H. (2009). Dicer ablation in oligodendrocytes provokes neuronal impairment in mice. *Ann. Neurol.* 66, 843–857. doi: 10.1002/ana.21927

Song, J., Li, X., Li, Y., Che, J., Li, X., Zhao, X., et al. (2017). Biodegradable and biocompatible cationic polymer delivering microRNA-221/222 promotes nerve regeneration after sciatic nerve crush. *Int. J. Nanomedicine* 12, 4195–4208. doi: 10.2147/IJN.S132190

Stolt, C. C., Lommes, P., Sock, E., Chaboissier, M.-C., Schedl, A., and Wegner, M. (2003). The Sox9 transcription factor determines glial fate choice in the developing spinal cord. *Genes Dev.* 17, 1677–1689. doi: 10.1101/gad.259003

Stolt, C. C., Rehberg, S., Ader, M., Lommes, P., Riethmacher, D., Schachner, M., et al. (2002). Terminal differentiation of myelin-forming oligodendrocytes depends on the transcription factor Sox10. *Genes Dev.* 16, 165–170. doi: 10.1101/gad.215802

Stolt, C. C., Schlierf, A., Lommes, P., Hillgärtner, S., Werner, T., Kosian, T., et al. (2006). SoxD proteins influence multiple stages of oligodendrocyte development and modulate SoxE protein function. *Dev. Cell* 11, 697–709. doi: 10.1016/j.devcel.2006.08.011

Swanson, K. V., Deng, M., and Ting, J. P.-Y. (2019). The NLRP3 inflammasome: molecular activation and regulation to therapeutics. *Nat. Rev. Immunol.* 19, 477–489. doi: 10.1038/s41577-019-0165-0

Tepavčević, V., and Lubetzki, C. (2022). Oligodendrocyte progenitor cell recruitment and remyelination in multiple sclerosis: the more, the merrier? *Brain* 145, 4178–4192. doi: 10.1093/brain/awac307

Tripathi, A., Volsko, C., Garcia, J. P., Agirre, E., Allan, K. C., Tesar, P. J., et al. (2019). Oligodendrocyte intrinsic miR-27a controls myelination and remyelination. *Cell Rep.* 29, 904–919.e9. doi: 10.1016/j.celrep.2019.09.020

Uchida, N., Chen, K., Dohse, M., Hansen, K. D., Dean, J., Buser, J. R., et al. (2012). Human neural stem cells induce functional myelination in mice with severe dysmyelination. *Sci. Transl. Med.* 4:155ra136. doi: 10.1126/scitranslmed.3004371

Uyeda, A., and Muramatsu, R. (2020). Molecular mechanisms of central nervous system axonal regeneration and remyelination: a review. *Int. J. Mol. Sci.* 21:8116. doi: 10.3390/ijms21218116

Valério-Gomes, B., Guimarães, D. M., Szczupak, D., and Lent, R. (2018). The absolute number of oligodendrocytes in the adult mouse brain. *Front. Neuroanat.* 12:90. doi: 10.3389/fnana.2018.00090

van Langelaar, J., Rijvers, L., Smolders, J., and van Luijn, M. M. (2020). B and T cells driving multiple sclerosis: identity, mechanisms and potential triggers. *Front. Immunol.* 11:760. doi: 10.3389/fimmu.2020.00760

Vistbakka, J., Sumelahti, M.-L., Lehtimäki, T., Elovaara, I., and Hagman, S. (2018). Evaluation of serum miR-191-5p, miR-24-3p, miR-128-3p, and miR-376c-3 in multiple sclerosis patients. *Acta Neurol. Scand.* 138, 130–136. doi: 10.1111/ane.12921

Wang, C.-Y., Deneen, B., and Tzeng, S.-F. (2017). MicroRNA-212 inhibits oligodendrocytes during maturation by down-regulation of differentiation-associated gene expression. *J. Neurochem.* 143, 112–125. doi: 10.1111/jnc.14138

Wang, H., Moyano, A. L., Ma, Z., Deng, Y., Lin, Y., Zhao, C., et al. (2017). miR-219 cooperates with miR-338 in myelination and promotes myelin repair in the CNS. *Dev. Cell* 40, 566–582.e5. doi: 10.1016/j.devcel.2017.03.001

Wang, S., Sdrulla, A. D., diSibio, G., Bush, G., Nofziger, D., Hicks, C., et al. (1998). Notch receptor activation inhibits oligodendrocyte differentiation. *Neuron* 21, 63–75. doi: 10.1016/s0896-6273(00)80515-2

Wiggermann, V., Endmayr, V., Hernández-Torres, E., Höftberger, R., Kasprian, G., Hametner, S., et al. (2023). Quantitative magnetic resonance imaging reflects different levels of histologically determined myelin densities in multiple sclerosis, including remyelination in inactive multiple sclerosis lesions. *Brain Pathol.*:e13150. doi: 10.1111/bpa.13150

Wittstatt, J., Weider, M., Wegner, M., and Reiprich, S. (2020). MicroRNA miR-204 regulates proliferation and differentiation of oligodendroglia in culture. *Glia* 68, 2015–2027. doi: 10.1002/glia.23821

Wlodarczyk, A., Holtman, I. R., Krueger, M., Yogev, N., Bruttger, J., Khorooshi, R., et al. (2017). A novel microglial subset plays a key role in myelinogenesis in developing brain. *EMBO J.* 36, 3292–3308. doi: 10.15252/embj.201696056

Woodruff, R. H., Fruttiger, M., Richardson, W. D., and Franklin, R. J. M. (2004). Platelet-derived growth factor regulates oligodendrocyte progenitor numbers in adult CNS and their response following CNS demyelination. *Mol. Cell. Neurosci.* 25, 252–262. doi: 10.1016/j.mcn.2003.10.014

Xiao, Y., Geng, F., Wang, G., Li, X., Zhu, J., and Zhu, W. (2019). Bone marrow-derived mesenchymal stem cells-derived exosomes prevent oligodendrocyte apoptosis through exosomal miR-134 by targeting caspase-8. *J. Cell. Biochem.* 120, 2109–2118. doi: 10.1002/jcb.27519

Zhao, X., He, X., Han, X., Yu, Y., Ye, F., Chen, Y., et al. (2010). MicroRNA-mediated control of oligodendrocyte differentiation. *Neuron* 65, 612–626. doi: 10.1016/j.neuron.2010.02.018

Zheng, X., Huang, H., Liu, J., Li, M., Liu, M., and Luo, T. (2018). Propofol attenuates inflammatory response in LPS-activated microglia by regulating the miR-155/SOCS1 pathway. *Inflammation* 41, 11–19. doi: 10.1007/s10753-017-0658-6

Zurawska, A., Mycko, M. P., and Selmaj, K. W. (2019). Circular RNAs as a novel layer of regulatory mechanism in multiple sclerosis. *J. Neuroimmunol.* 334:576971. doi: 10.1016/j.jneuroim.2019.576971

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Brain-derived blood biomarkers in multiple sclerosis—current trends and beyond

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Multiple sclerosis (MS) is a chronic inflammatory and neurodegenerative disease of the nervous system and a main cause of neurological disability in young adults. Most disease-modifying therapies are administrated as long-term maintenance therapies and may, thereby, increase the risk of infections and other immune-mediated side effects. In the last years, several cerebrospinal fluid and soluble blood biomarkers have been suggested as potential key tools for diagnosis, prognosis, and treatment monitoring of MS. Recently, the specific ability of brain-derived blood extracellular vesicles (EVs) that cross the blood-brain barrier into the bloodstream, reflecting the current immune status of the central nervous system, has kindled interest as potential biomarkers. In this review, we discuss the current trends of clinical brain-derived blood biomarkers, with a special focus on the emerging role of brain-derived blood EVs in MS.

KEYWORDS

multiple sclerosis (MS), cerebrospinal fluid (CSF), brain-derived blood biomarkers, extracellular vesicles (EVs), magnetic resonance imaging (MRI)

1 Introduction

Almost 3 million people worldwide are affected by multiple sclerosis (MS), an immune-mediated inflammatory and degenerative disease of the central nervous system (CNS) (1). From a clinical perspective, MS is highly heterogeneous with most patients (85%–90%) experiencing an initial relapsing-remitting course (RRMS) marked by episodic inflammation and, if not treated effectively, followed by a secondary progressive (SPMS) phase, associated with gradual increasing disability (2). Epidemiological data suggest that Epstein–Barr virus is a prerequisite for developing MS, but the underlying pathogenic mechanisms are still unclear (3, 4).

The MS diagnosis relies on the combination of clinical and paraclinical findings, with no single definitive diagnostic test available (5). Currently, it is essential to determine

inflammatory immune-mediated damage affecting at least two distinct regions (dissemination in space) of the CNS at varied time points (dissemination in time) to establish an MS diagnosis. Since the incorporation in the diagnostic criteria (1983), magnetic resonance imaging (MRI) of the brain and spinal cord holds a pivotal role in the diagnostic process. In addition, cerebrospinal fluid (CSF) analysis detecting intrathecal immunoglobulin G (IgG) synthesis was highlighted in the update of the diagnostic criteria of MS in 2017 (5).

Recent advancements have shed light on detecting brain-derived proteins at remarkably low concentrations in blood, paving the way for the exploration of early blood-based biomarkers in MS (6). Specific markers of immunopathological processes including neuroaxonal damage [neurofilament light chain (NfL)] and astrocyte activation [glial fibrillary acidic protein (GFAP)] are already rapidly emerging (7, 8). Extracellular vesicles (EVs) are defined as membrane-bound particles, released from virtually all cell types, with a sophisticated sorting mechanism of their cargo inclusive of lipids, proteins, and nucleic acids, in addition to carrying specific membrane proteins, mainly reflecting their donor cell. This peculiarity, plus their ability to cross the blood-brain barrier (BBB) into the blood stream, increased stability, and involvement in the regulation of both the immune system and CNS homeostasis, features brain-derived blood EVs, as improved biomarkers in CNS diseases, including MS (9–12). This review aims to summarize the current CSF and blood biomarkers in MS, discussing the unmet needs and future perspectives.

2 MS pathogenesis and fluid biomarkers

In the early stages of MS, the recurrent invasion of T and B cells in the brain and spinal cord drives a cascade of pathophysiological processes within the CNS (13). Several fluid biomarkers have emerged as effective indicators of this complex interaction, which contributes to the diverse clinical manifestations observed in the disease (14). Early episodes of acute focal inflammation, demyelination, and axonal damage, driven by infiltrating immune cells (macrophages, $CD8^+$ T cells, $CD4^+$ T cells, B cells, and plasma cells), could be typically detected through conventional MRI, showing new lesions in T2-weighted and/or T1-weighted gadolinium enhancing lesions (15, 16). Infiltrating immune cells are attracted to the CNS by several chemotactic factors such as chemokine (C-X-C motif) ligand 13 (CXCL13) for B cells (Figure 1) (17).

Abbreviations: MS, multiple sclerosis; CNS, central nervous system; BBB, blood-brain barrier; CXCL13, chemokine (C-X-C motif) ligand 13; APCs, antigen-presenting cells; κ-FLCs, kappa-free light chains; NfL, neurofilament light chain; CSF, cerebrospinal fluid; sTREM2, soluble triggering receptor expressed on myeloid cells 2; CHIT1, chitotriosidase 1; CHI3L1, chitinase-3–like protein 1; GFAP, glial fibrillary acidic protein; EVs, extracellular vesicles; miRNA, microRNA; lncRNA, long non-coding RNA; MOG, myelin oligodendrocyte glycoprotein; MBP, myelin basic protein.

Invading T and B cells closely interact within the CNS (16, 17). In contrast to T cells, the immune pathways involving B cell activation have, so far, served as the most robust fluid biomarkers for MS. Mature plasma cells secrete IgG and IgM antibodies intrathecally, also leading to release of free light chains (due to a mismatch between immunoglobulin light- and heavy-chain synthesis) (18, 19). This inflammatory process results in axonal damage and release of neuronal markers like NfL (20). Over time, there is worsening of disability and accumulation of neurological deficits in the absence of concurrent relapses defined as "progression independent of relapse activity" (PIRA) (21). Underlying mechanism driving PIRA is increasingly understood as a pathophysiological continuum of the early "relapsing" phase driven by a chronic "smouldering" inflammatory process compartmentalized within the CNS, characterized by innate immune cells and astrocytes (22). Recent studies on positron emission tomography (PET) employing radioligands for innate immunity activation assessment have revealed an interestingly high prevalence of smouldering component in MS lesions (23). Chronically active MS lesions are slowly expanding over time or as paramagnetic rim lesions, expressing a dense network of activated iron-laden microglia/macrophages (24). Activated microglia and astrocytes release various mediators into the CSF, such as soluble triggering receptor expressed on myeloid cells 2 (sTREM2), chitinase 1 (CHIT1), chitinase-3–like protein 1 (CHI3L1), and GFAP, impacting axon, synaptic integrity, and function (25–30).

The critical role of the complement system in MS is underlined with the complement and Ig deposition across all areas of demyelination regardless of the plaque subtype, including complement-mediated myelin phagocytosis implying its importance once the disease is established. In progressive MS and long-standing disease patients, white matter plaques were consistently positive for complement proteins (C3, factor B, and C1q), regulators (factor H, C1inh, and clusterin) and activation products [C3b, iC3b, C4d, and terminal complement complex (TCC)] providing evidence that, once established, progression of inflammation in MS may not rely on infiltrating cells but rather on innate immune mechanisms including complement activation (31, 32).

EVs are pivotal in the intricate communication of neurons and glial cells of the CNS system holding neuroprotective and homeostatic effects but may have detrimental effects under pathological conditions (33, 34). EVs derived from T cells containing chemokine CCL5 and arachidonic acid can increase the expression of intercellular adhesion molecule 1 (ICAM-1) on endothelial cells and of Mac-1 on monocytes, contributing to the dysfunction of the BBB, leading to immune infiltration, a characteristic of MS pathogenesis (35–37). Dendritic cell (DCs) derived EVs carry cell surface molecules like major histocompatibility complex (MHC), ICAM-1, and other costimulatory molecules, which could aid in T-cell activation (38). EVs from activated microglia express pro-inflammatory mediators (Tumor Necrosis Factor-alpha (TNF-α) and Interleukin-1 (IL-1)) exhibiting a distinct proteomic profile enforcing inflammatory stimuli throughout the CNS (39). Recent studies show the role of astrocyte-derived EVs in the regulation of T-cell secretion and biomarker utility of myelin basic protein (MBP) and myelin

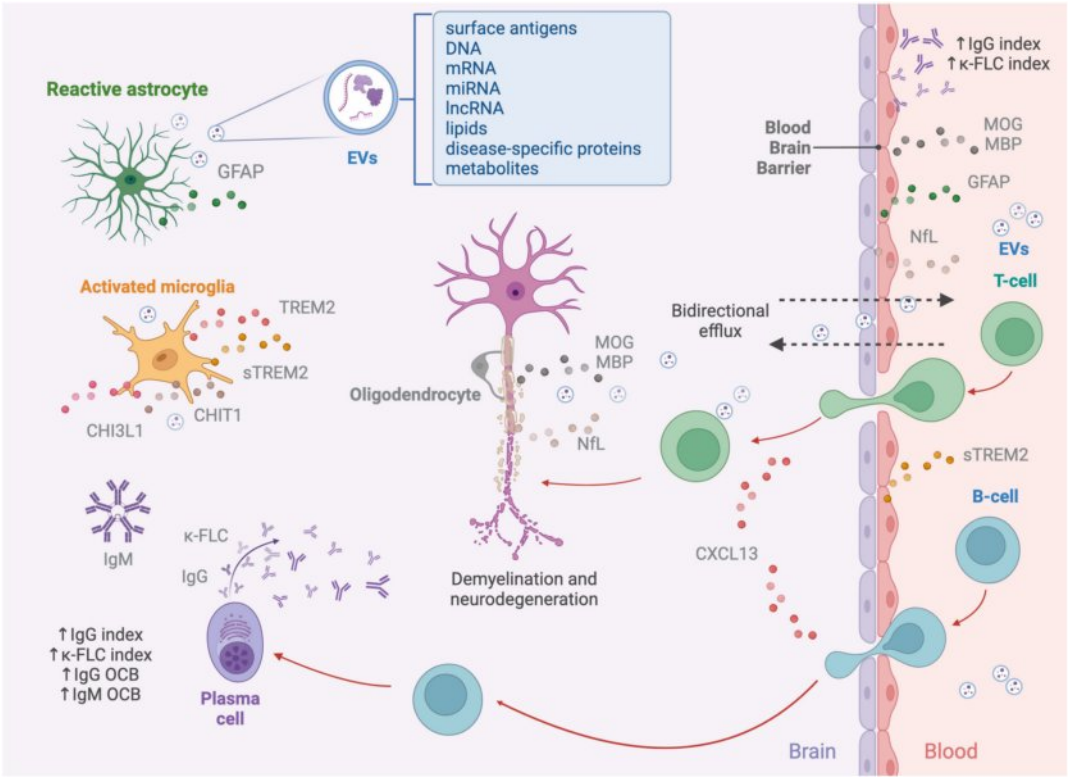

FIGURE 1
Pathophysiology of multiple sclerosis and associated biomarkers. The pathogenesis of MS begins with immune cells, including macrophages, autoreactive T cells targeting myelin, B cells, and plasma cells, infiltrating the CNS through a dysfunctional BBB. Lymphocyte recruitment is mediated by chemokines like CXCL13, which specifically attracts B cells. Within the CNS, T and B cells interact, to amplify the immune response, with T cells secreting cytokines and B cells acting as APCs. Activated B cells differentiate into plasma cells, producing immunoglobulins, including IgG and IgM and releasing κ-FLCs. The ongoing neuroinflammation leads to demyelination, axonal damage, and neurodegeneration, marked by NfL, which is released into the interstitial space, CSF, and bloodstream as a result of axonal injury, along with MOG and MBP, major proteins of the myelin sheet of oligodendrocytes. Resident immune cells within the CNS, such as microglia and astrocytes, contribute to the disruption of axonal integrity and synaptic function. While activated, microglia and astrocytes release various mediators into the CSF, including sTREM2, CHIT1, and CHI3L1. Additionally, astrocyte damage results in the release of structural proteins, such as GFAP, into the CSF and bloodstream. Another source of biomarkers reflecting pathological processes occurring in the CNS are EVs. These nanovesicles, secreted by various cell types, including neurons, astrocytes, microglia, and oligodendrocytes, carry a diverse molecular cargo, such as surface antigens, DNA, mRNA, miRNA, lncRNA, lipids, disease-specific proteins, and metabolites, acting as mediators of intercellular communication. The bidirectional efflux of EVs and soluble biomarkers across the compromised BBB enables their detection in peripheral fluids.

oligodendrocyte glycoprotein (MOG) content in oligodendrocytes-derived EVs (40). Most immune cell–derived EVs seem to be significantly higher in treatment naïve relapsing MS patients with low disability, and their functions might depend on the physiological environment, despite limited changes in circulating immune cells (33).

3 MS fluid biomarkers—current trends and beyond

The diagnostic criterion for MS underscores the importance of both MRI and biofluid biomarkers emphasizing the pivotal role of accurate diagnosis, prognosis, and treatment response in the management of the disease (5). In addition to advancements in MRI techniques (7-T MRI, PET, magnetization transfer imaging, diffusion tensor imaging, and myelin water imaging), integrating biofluid biomarkers would be beneficial because of their ability to directly reflect the pathophysiological processes involved in the MS disease course (41). Cumulative evidence shows that the blood-based biomarker sNfL can predict relapses in relapsing MS patients, whereas CSF IgM oligoclonal bands, CHI3L1, and GFAP seem to be associated with a more progressive phenotype. Different aspects of microglial involvement (CHIT1 and sTREM2), astroglia pathology (CHI3L1 and GFAP), B-cell–related pathology (CXCL13), and neuroaxonal damage (sNfL) have been evaluated in several studies aiding in classifying MS disease activity (Table 1) (25–30).

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TABLE 1 Summary of fluid biomarkers in multiple sclerosis.

Marker	Source	Measurement methods	Clinical significance and utility	Prognostic potential	Specificity to MS	Limitations	References
Validated and completely introduced into clinical practice							
IgG OCB	CSF	Isoelectric focusing with specific IgG staining	Indicates intrathecal IgG synthesis; evidence of CNS immune activity; high sensitivity for MS diagnosis and validated biomarker in clinical utility	Predicts CIS to MS conversion; linked to disability progression	Limitation: present in other inflammatory/infectious neurological conditions	Time-consuming, qualitative method	(43–47)
IgG index	CSF and serum	Calculated as (IgG in CSF/IgG in serum)/(albumin in CSF/albumin in serum)	Measures intrathecal IgG synthesis; assesses blood-CSF barrier function	Limited; weak correlation with MS severity but linked to future disability worsening	Limitation: affected by other CNS conditions	Low sensitivity for MS diagnosis	(43–46)
κ-FLC	CSF and serum	Nephelometry, turbidimetry, κ-FLC index	Less expensive, faster quantitative alternative to OCB; detects intrathecal inflammation	Limited; predicts early relapses and disease activity in MS and enables risk stratification of disease activity in OCB-positive MS patients but still not widely validated in clinical practice	Moderate; approximately 90% diagnostic sensitivity and specificity for distinguishing MS from other neurological disorders; not exclusive to MS	Elevated in other conditions with intrathecal Ig synthesis; includes IgA and IgM (not limited to IgG)	(48–50)
Validated and not completely introduced into clinical practice							
NfL	CSF and blood (serum, plasma)	Immunoassays (e.g., ELISA); ultrasensitive immunoassays (e.g., Simoa); automated assays (e.g., Lumipulse®)	Reflect severity of axonal damage; elevated in RRMS and progressive MS; normalizes post-treatment	Predicts CIS to MS conversion, relapses, EDSS worsening, and brain atrophy; elevated in serum before the onset of clinical symptoms; strong marker for tissue destruction and treatment efficacy	Moderate; specific for neuronal damage but not for a disease; elevated in other neurodegenerative disorders (e.g., Alzheimer's, traumatic brain injury)	Serum levels influenced by age and weight (can be corrected by z-score normalization); threshold values for treatment success and disease reactivation need standardization	(51–56)
Partially validated and not introduced into clinical practice							
IgM OCB	CSF	IgM index or non-linear formulas; immunoblotting; isoelectric focusing	Detects intrathecal IgM synthesis; linked to highly inflammatory RRMS and a subset of PPMS patients	Predicts shorter time to relapse and higher relapse rates; associated with disability progression and more aggressive PPMS	Moderate; found in approximately 40% of MS cases and also in other CNS conditions	Technical challenges in detection due to the high molecular weight of IgM; limited data compared to IgG OCB	(57–61)
CXCL13	CSF, serum	Immunoassays (e.g., ELISA), CXCL13 index	Elevated in early active and progressive MS; correlates with gadolinium-enhancing lesions, B-cell counts, IgG levels, κ-FLC index, relapse rate, and disease activity	Predicts CIS to MS conversion; monitors response to corticosteroids and long-term DMTs	High in CSF; independent of BBB dysfunction; undetectable in non-inflammatory controls; limitation in serum; elevated in other conditions like systemic autoimmune, inflammatory, infectious, and neoplastic diseases	Limited utility in serum—diagnostically irrelevant due to lack of CSF correlation and low specificity	(17, 62–65)
CHI3L1	CSF, serum	Immunoassays (e.g., ELISA)	Elevated in progressive MS; decreased during acute relapses compared to remission; unrelated to gadolinium lesions	Predicts CIS to MS conversion; correlates with disease progression in PPMS	Limitation: serum levels not significantly different between MS and healthy controls	Poor CSF-serum correlation; lacks specificity due to broad expression in other tissues beyond the CNS	(62, 66–69)

(Continued)

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TABLE 1 Continued

Marker	Source	Measurement methods	Clinical significance and utility	Prognostic potential	Specificity to MS	Limitations	References
Partially validated and not introduced into clinical practice							
CHIT1	CSF, brain tissue (post-mortem)	Immunoassays (e.g., ELISA); RNA analysis in white matter tissue	Specific to microglial activation; correlates with neuronal injury (NfL) and disease activity at follow-up (up to 6 years post-diagnosis); upregulated in chronic active lesions of MS	Predicts long-term disease activity and progression; CHIT1 RNA expression differentiates chronic active lesions from chronic inactive lesions	High specificity for chronic active lesions in MS	Limited longitudinal data; needs further validation	(70–73)
sTREM2	CSF and blood (serum, plasma)	Immunoassays (e.g., ELISA)	Elevated in MS; linked to microglial activity; normalizes with natalizumab; partially reduced by mitoxantrone	Moderate correlation with EDSS and MS severity score; lack of strong correlation with other clinical measures	Limitation: elevated in other inflammatory neurological conditions	Insufficient data; weak serum-CSF correlation; not reliable as a blood biomarker	(74–78)
GFAP	CSF and blood (serum)	Immunoassays (e.g., ELISA)	Indicates astrocyte activity; reflects neuroinflammation; elevated in RRMS relapses, progressive MS; correlates with brain atrophy	Predicts disability progression in both active and non-active MS; elevated levels post-treatment indicate progression	Moderate; elevated in MS and NMOSD (predicts activity in NMOSD remission)	Labile in CSF; highly sensitive to freeze-thaw cycles; serum levels influenced by age; requires standardization for comparisons across MS subtypes	(79–83)
Not validated and not introduced into clinical practice							
$CD62p^+$ EVs	Plasma	Flow cytometry	Elevated in MS vs. HC	Reflects platelet activation and monocyte interaction with damaged endothelium	Low; common in other thrombosis-related or inflammatory conditions	Overlap with other conditions	(84)
$CD61^+$ EVs, $CD14^+$ EVs, $CD45^+$ EVs	Plasma	Flow cytometry	Elevated CD61+ EVs in untreated MS vs. HC Elevated CD61+, CD14+, and CD45+ EVs in RRMS vs. HC and SPMS	Indicates platelet activation, monocyte, and leukocyte interaction with damaged endothelium	Low; signify broader immune activation rather than MS-specific inflammation	Limited specificity for MS pathology	(85)
MOG	Serum EVs	Western blotting, ELISA	Elevated MOG EV content in RRMS patients in relapse and SPMS vs. HC	Monitors disease activity	Moderate; marker implicated in other CNS autoimmune disorders	Cross-reactivity in assays	(86, 87)
TLR3 and TLR4	Serum EVs	ELISA	Decreased TLR3 and elevated TLR4 in RRMS EVs vs. HC	Suggests altered innate immune signaling	Low; TLR expression changes occur in other autoimmune and inflammatory conditions	Requires further validation in larger cohorts	(88, 89)
Synaptopodin and synaptophysin (NEVs), complement components (AEVs)	Plasma $L1CAM^+$ NEVs, plasma $GLAST^+$ AEVs	ELISA, Luminex®	Decreased synaptopodin and synaptophysin in NEVs in MS vs. HC Elevated C1q, C3, C3b/iC3b, C5, C5a, factor H in AEVs in MS vs. HC Strong inverse correlations between both types of biomarkers in MS patients	Indicates synaptic loss and complement activation	Moderate; synaptic and complement markers are also observed in neurodegenerative diseases	Complexity in distinguishing source biomarkers	(90–92)

(Continued)

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TABLE 1 Continued

Marker	Source	Measurement methods	Clinical significance and utility	Prognostic potential	Specificity to MS	Limitations	References
Not validated and not introduced into clinical practice							
Absence of CD3 and CD41; presence of CD31, CD105, and CD144	Plasma EVs	Flow cytometry	Elevated concentration of CNS endothelial-derived EV in active vs. stable MS and HC	Reflects BBB permeability and active disease	Moderate; endothelial-derived markers are seen in broader CNS pathologies, reducing specificity	Limited application outside severe cases	(93)
MBP	Serum EVs	ELISA	Elevated in CIS, RRMS, and PPMS vs. HC Elevated in PPMS vs. RRMS and CIS	Correlates with EDSS and MSSS Predicts disease subtype	High; marker strongly linked to demyelination, which is a hallmark of MS	Might be not cost-effective	(40, 86, 94)
EVs concentration	Plasma EVs	NTA	Increased after 5 h of treatment with fingolimod vs. pre-treatment	Monitors treatment response	Low; observed in other conditions involving immune activation	Requires specific equipment	(95)
$IB4^+$ EVs concentration	CSF	Flow cytometry	Increased in RRMS and CIS vs. HC	Reflects microglia/macrophage activation	Moderate; microglial activation is a common feature in other neuroinflammatory conditions	Limited EVs concentration in CSF	(96)
EVs concentration, $CCR3^+/CCR5^+$ EVs, $CD4^+/CCR3^+$ EVs, $CD4^+/CCR5^+$	CSF	Flow cytometry	Increased EVs in clinical relapse vs. remission Increased CCR3+/CCR5+ EVs, CD4+/CCR3+ EVs, and CD4+/CCR5+ EVs in patients with gadolinium-enhanced MR lesions	Identifies different MS phases	High; associated with active MS lesion pathology	Requires specialized equipment	(97)

MS, multiple sclerosis; HC, healthy controls; EV, extracellular vesicles; RRMS, relapsing-remitting MS; SPMS, secondary progressive MS; PPMS, primary progressive MS; CIS, clinically isolated syndrome; BBB, blood-brain barrier; EDSS, expanded disability status scale; MSSS, MS severity score; CSF, cerebrospinal fluid; CNS, central nervous system; OCB, oligoclonal bands; κ-FLC, kappa-free light chains; NGS, next-generation sequencing; NMOSD, neuromyelitis optica spectrum disorder; TLR, Toll-like receptor; NTA, nanoparticle tracking analysis; NEVs, neuron-derived extracellular vesicles; AEVs, astrocyte-derived extracellular vesicles; MOG, myelin oligodendrocyte glycoprotein; MBP, myelin basic protein; IFN-β, interferon-beta.

Brain-derived blood EVs (L1CAM, MOG, and GLAST) serve as potential windows into the CNS reflecting the underlying MS-related pathophysiology (Table 1) (33).

Certain limitations of the emerging fluid biomarkers intrude their clinical transition. For example, NfL is a promising biomarker but with limited diagnostic use due to its unspecific increase in the blood connected to several neurological conditions (42). EVs hold potential as biomarkers; however, existing knowledge gaps in terms of EVs biology, biodistribution, and assay standardization are yet to be fully elucidated (33). Although MS fluid biomarkers hold a promising frontier, addressing standardization, data validation, and accessibility are key in resolving ongoing challenges. Composite scoring with integrated clinical and MRI metrics [e.g., the MAGNIMS score or no evidence of disease activity 3 (NEDA-3) and NEDA-4] and multimodal biomarker profiling (CSF and blood-based biomarkers with neuroimaging) may be a way forward in MS management (41). Furthermore, artificial intelligence (automated lesion detection and improved diagnostic accuracy) holds transformative potential in enhancing clinical decision-making.

In conclusion, despite the limitations, the recent advances within the field hold a promising frontier, giving a paradigm shift from the conventional CSF (oligoclonal banding) analysis to a new era of brain-derived blood biomarkers (NfL, GFAP, and EVs), enabling improved longitudinal disease monitoring and personalized treatment.

Author contributions

SA: Conceptualization, Formal Analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing. KM: Formal Analysis, Investigation, Writing – original draft, Writing – review & editing. RB: Investigation, Writing – review & editing. SM: Investigation, Writing – review & editing. CK: Investigation, Writing – review & editing. OT: Investigation, Project administration, Writing – review & editing. KMM: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – review & editing.

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Conflict of interest

K-MM has received speaker honoraria from Biogen, Novartis, or Sanofi and has participated in clinical trials organized by Biogen, Merck, Novartis, Roche, and Sanofi. OT has participated in advisory boards and received speaker honoraria from Biogen, Merck, Novartis, Teva, Roche, Sanofi, and Bristol Myers Squibb and has participated in clinical trials organized by Merck, Novartis, Roche, and Sanofi.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

1. Walton C, King R, Rechtman L, Kaye W, Leray E, Marrie RA, et al. Rising prevalence of multiple sclerosis worldwide: Insights from the Atlas of MS, third edition. *Mult Scler*. (2020) 26:1816–21.

2. Thompson AJ, Baranzini SE, Geurts J, Hemmer B, Ciccarelli O. Multiple sclerosis. *Lancet*. (2018) 391:1622–36.

3. Fernández-Fournier M, López-Molina M, Torres Iglesias G, Botella L, Chamorro B, Laso-Garcia F, et al. Antibody content against epstein–barr virus in blood extracellular vesicles correlates with disease activity and brain volume in patients with relapsing–remitting multiple sclerosis. *Int J Mol Sci*. (2023) 24:14192.

4. Mrad MF, Saba ES, Nakib L, Khoury SJ. Exosomes from subjects with multiple sclerosis express EBV-derived proteins and activate monocyte-derived macrophages. *Neurol Neuroimmunology Neuroinflammation*. (2021) 8:e1004.

5. Thompson AJ, Banwell BL, Barkhof F, Carroll WM, Coetzee T, Comi G, et al. Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. *Lancet Neurology*. (2018) 17:162–73.

6. Teunissen CE, Kimble L, Bayoumy S, Bolsewig K, Burtscher F, Coppens S, et al. Methods to discover and validate biofluid-based biomarkers in neurodegenerative dementias. *Mol Cell Proteomics*. (2023) 22:100629.

7. Meier S, Willemse EAJ, Schaedelin S, Oechtering J, Lorscheider J, Melie-Garcia L, et al. Serum glial fibrillary acidic protein compared with neurofilament light chain as a biomarker for disease progression in multiple sclerosis. *JAMA Neurology*. (2023) 80:287–97.

8. Di Filippo M, Gaetani L, Centonze D, Hegen H, Kuhle J, Teunissen CE, et al. Fluid biomarkers in multiple sclerosis: from current to future applications. *Lancet Regional Health – Europe*. (2024) 44.

9. Gurunathan S, Kang MH, Jeyaraj M, Qasim M, Kim JH. Review of the isolation, characterization, biological function, and multifarious therapeutic approaches of exosomes. *Cells*. (2019) 8.

10. Huo L, Du X, Li X, Liu S, Xu Y. The emerging role of neural cell-derived exosomes in intercellular communication in health and neurodegenerative diseases. *Front Neurosci*. (2021) 15.

11. Mycko MP, Baranzini SE. microRNA and exosome profiling in multiple sclerosis. *Mult Scler*. (2020) 26:599–604.

12. Hornung S, Dutta S, Bitan G. CNS-derived blood exosomes as a promising source of biomarkers: opportunities and challenges. *Front Mol Neurosci*. (2020) 13.

13. Filippi M, Bar-Or A, Piehl F, Preziosa P, Solari A, Vukusic S, et al. Multiple sclerosis. *Nat Rev Dis Primers*. (2018) 4:43.

14. Engelhardt B, Comabella M, Chan A. Multiple sclerosis: Immunopathological heterogeneity and its implications. *Eur J Immunol*. (2022) 52:869–81.

15. Rocca MA, Preziosa P, Barkhof F, Brownlee W, Calabrese M, De Stefano N, et al. Current and future role of MRI in the diagnosis and prognosis of multiple sclerosis. *Lancet Regional Health – Europe*. (2024) 44.

16. Dendrou CA, Fugger L, Friese MA. Immunopathology of multiple sclerosis. *Nat Rev Immunol*. (2015) 15:545–58.

17. Novakova L, Axelsson M, Malmeström C, Zetterberg H, Blennow K, Svenningsson A, et al. NFL and CXCL13 may reveal disease activity in clinically and radiologically stable MS. *Multiple Sclerosis Related Disord*. (2020) 46:102463.

18. Hegen H, Walde J, Berek K, Arrambide G, Gnanapavan S, Kaplan B, et al. Cerebrospinal fluid kappa free light chains for the diagnosis of multiple sclerosis: A systematic review and meta-analysis. *Multiple Sclerosis J*. (2022) 29:169–81.

19. Hegen H, Arrambide G, Gnanapavan S, Kaplan B, Khalil M, Saadeh R, et al. Cerebrospinal fluid kappa free light chains for the diagnosis of multiple sclerosis: A consensus statement. *Multiple Sclerosis J*. (2022) 29:182–95.

20. Gaetani L, Blennow K, Calabresi P, Di Filippo M, Parnetti L, Zetterberg H. Neurofilament light chain as a biomarker in neurological disorders. *J Neurology Neurosurg Psychiatry*. (2019) 90:870–81.

21. Kappos L, Wolinsky JS, Giovannoni G, Arnold DL, Wang Q, Bernasconi C, et al. Contribution of relapse-independent progression vs relapse-associated worsening to overall confirmed disability accumulation in typical relapsing multiple sclerosis in a pooled analysis of 2 randomized clinical trials. *JAMA Neurology*. (2020) 77:1132–40.

22. Tur C, Carbonell-Mirabent P, Cobo-Calvo Á, Otero-Romero S, Arrambide G, Midaglia L, et al. Association of early progression independent of relapse activity with long-term disability after a first demyelinating event in multiple sclerosis. *JAMA Neurology*. (2023) 80:151–60.

23. Hamzaoui M, Garcia J, Boffa G, Lazzarotto A, Absinta M, Ricigliano VAG, et al. Positron emission tomography with [F]-DPA-714 unveils a smoldering component in most multiple sclerosis lesions which drives disease progression. *Ann Neurology*. (2023) 94:366–83.

24. Jäckle K, Zeis T, Schaeren-Wiemers N, Junker A, van der Meer F, Kramann N, et al. Molecular signature of slowly expanding lesions in progressive multiple sclerosis. *Brain*. (2020) 143:2073–88.

25. Hinsinger G, Galéotti N, Nabholz N, Urbach S, Rigau V, Demattei C, et al. Chitinase 3-like proteins as diagnostic and prognostic biomarkers of multiple sclerosis. *Multiple Sclerosis J*. (2015) 21:1251–61.

26. Cantó E, Tintoré M, Villar LM, Costa C, Nurtdinov R, Álvarez-Cermeño JC, et al. Chitinase 3-like 1: prognostic biomarker in clinically isolated syndromes. *Brain*. (2015) 138:918–31.

27. Steinacker P, Verde F, Fang L, Feneberg E, Oeckl P, Roeber S, et al. Chitotriosidase (CHIT1) is increased in microglia and macrophages in spinal cord of amyotrophic lateral sclerosis and cerebrospinal fluid levels correlate with disease severity and progression. *J Neurology Neurosurg Psychiatry*. (2018) 89:239–47.

28. Filipello F, Goldsbury C, You SF, Locca A, Karch CM, Piccio L. Soluble TREM2: Innocent bystander or active player in neurological diseases? *Neurobiol Dis*. (2022) 165:105630.

29. Högel H, Rissanen E, Barro C, Matilainen M, Nylund M, Kuhle J, et al. Serum glial fibrillary acidic protein correlates with multiple sclerosis disease severity. *Multiple Sclerosis J*. (2018) 26:210–9.

30. Abdelhak A, Foschi M, Abu-Rumeileh S, Yue JK, D'Anna L, Huss A, et al. Blood GFAP as an emerging biomarker in brain and spinal cord disorders. *Nat Rev Neurology*. (2022) 18:158–72.

31. Breij EC, Brink BP, Veerhuis R, Van den Berg C, Vloet R, Yan R, et al. Homogeneity of active demyelinating lesions in established multiple sclerosis. *Ann Neurology: Off J Am Neurological Assoc Child Neurol Society*. (2008) 63:16–25.

32. Ingram G, Loveless S, Howell OW, Hakobyan S, Dancey B, Harris CL, et al. Complement activation in multiple sclerosis plaques: an immunohistochemical analysis. *Acta neuropathologica Commun*. (2014) 2:1–15.

33. Pistono C, Osera C, Cuccia M, Bergamaschi R. Roles of extracellular vesicles in multiple sclerosis: from pathogenesis to potential tools as biomarkers and therapeutics. *Sclerosis*. (2023) 1:91–112.

34. Schnatz A, Müller C, Brahmer A, Krämer-Albers EM. Extracellular Vesicles in neural cell interaction and CNS homeostasis. *FASEB Bioadv*. (2021) 3:577–92.

35. Sáenz-Cuesta M, Osorio-Querejeta I, Otaegui D. Extracellular vesicles in multiple sclerosis: what are they telling us? *Front Cell Neurosci*. (2014) 8:100.

36. Barry OP, Kazanietz MG, Pratico D, FitzGerald GA. Arachidonic acid in platelet microparticles up-regulates cyclooxygenase-2-dependent prostaglandin formation via a protein kinase C/mitogen-activated protein kinase-dependent pathway. *J Biol Chem*. (1999) 274:7545–56.

37. Quandt J, Dorovini-Zis K. The beta chemokines CCL4 and CCL5 enhance adhesion of specific CD4+ T cell subsets to human brain endothelial cells. *J Neuropathology Exp Neurology*. (2004) 63:350–62.

38. Segura E, Nicco C, Lombard B, Véron P, Raposo G, Batteux F, et al. ICAM-1 on exosomes from mature dendritic cells is critical for efficient naive T-cell priming. *Blood*. (2005) 106:216–23.

39. Aires ID, Ribeiro-Rodrigues T, Boia R, Ferreira-Rodrigues M, Girão H, Ambrósio AF, et al. Microglial extracellular vesicles as vehicles for neurodegeneration spreading. *Biomolecules*. (2021) 11:770.

40. Agliardi C, Guerini FR, Zanzottera M, Bolognesi E, Picciolini S, Caputo D, et al. Myelin basic protein in oligodendrocyte-derived extracellular vesicles as a diagnostic and prognostic biomarker in multiple sclerosis: a pilot study. *Int J Mol Sci*. (2023) 24:894.

41. Anderhalten L, Wohlrab F, Paul F. Emerging MRI and biofluid biomarkers in the diagnosis and prognosis of multiple sclerosis. *Lancet Regional Health – Europe*. (2024) 44.

42. eBioMedicine. Blood biomarkers for multiple sclerosis: neurofilament light chain and beyond. *eBioMedicine*. (2024) 104.

43. Link H, Huang Y-M. Oligoclonal bands in multiple sclerosis cerebrospinal fluid: An update on methodology and clinical usefulness. *J Neuroimmunology*. (2006) 180:17–28.

44. McLean BN, Luxton RW, Thompson EJ. A study of immunoglobulin G in the cerebrospinal fluid of 1007 patients with suspected neurological disease using isoelectric focusing and the Log IgG-Index. A comparison and diagnostic applications. *Brain*. (1990) 113:1269–89.

45. Lunding J, Midgard R, Vedeler CA. Oligoclonal bands in cerebrospinal fluid:a comparative study of isoelectric focusing, agarose gel electrophoresis and IgG index. *Acta Neurologica Scandinavica*. (2000) 102:322–5.

46. Arrambide G, Espejo C, Carbonell-Mirabent P, Dieli-Crimi R, Rodríguez-Barranco M, Castillo M, et al. The kappa free light chain index and oligoclonal bands have a similar role in the McDonald criteria. *Brain*. (2022) 145:3931–42.

47. Rojas Juan I, Patrucco L, Cristiano E. Oligoclonal bands and MRI in clinically isolated syndromes: predicting conversion time to multiple sclerosis. *J Neurol*. (2010) 257:1188–91.

48. Berek K, Bsteh G, Auer M, Di Pauli F, Grams A, Milosavljevic D, et al. Kappa-free light chains in CSF predict early multiple sclerosis disease activity. *Neurol Neuroimmunology Neuroinflammation*. (2021) 8:e1005.

49. Dekeyser C, De Kesel P, Cambron M, Vanopdenbosch L, Van Hijfte L, Vercammen M, et al. Inter-assay diagnostic accuracy of cerebrospinal fluid kappa free light chains for the diagnosis of multiple sclerosis. *Front Immunol*. (2024).

50. Duell F, Evertsson B, Al Nimer F, Sandin Å, Olsson D, Olsson T, et al. Diagnostic accuracy of intrathecal kappa free light chains compared with OCBs in MS. *Neurol Neuroimmunology Neuroinflammation*. (2020) 7:e775.

51. Ning L, Wang B. Neurofilament light chain in blood as a diagnostic and predictive biomarker for multiple sclerosis: A systematic review and meta-analysis. *PloS One*. (2022) 17:e0274565.

52. Freedman MS, Gnanapavan S, Booth RA, Calabresi PA, Khalil M, Kuhle J, et al. Guidance for use of neurofilament light chain as a cerebrospinal fluid and blood biomarker in multiple sclerosis management. *EBioMedicine*. (2024) 101:104970.

53. Bäckström D, Linder J, Jakobson Mo S, Riklund K, Zetterberg H, Blennow K, et al. NfL as a biomarker for neurodegeneration and survival in Parkinson disease. *Neurology*. (2020) 95:e827–e38.

54. Sahrai H, Norouzi A, Hamzehzadeh S, Majdi A, Kahfi-Ghaneh R, Sadigh-Eteghad S. SIMOA-based analysis of plasma NFL levels in MCI and AD patients: a systematic review and meta-analysis. *BMC Neurology*. (2023) 23:331.

55. Urbano T, Maramotti R, Tondelli M, Gallingani C, Carbone C, Iacovino N, et al. Comparison of serum and cerebrospinal fluid neurofilament light chain concentrations measured by ella™ and lumipulse™ in patients with cognitive impairment. *Diagnostics*. (2024) 14:2408.

56. Vecchio D, Puricelli C, Malucchi S, Virgilio E, Martire S, Perga S, et al. Serum and cerebrospinal fluid neurofilament light chains measured by SIMOA™, Ella™, and Lumipulse™ in multiple sclerosis naïve patients. *Mult Scler Relat Disord*. (2024) 82:105412.

57. Magliozzi R, Mazziotti V, Montibeller L, Pisani AI, Marastoni D, Tamanti A, et al. Cerebrospinal fluid igM levels in association with inflammatory pathways in multiple sclerosis patients. *Front Cell Neurosci*. (2020) 14.

58. Mandrioli J, Sola P, Bedin R, Gambini M, Merelli E. A multifactorial prognostic index in multiple sclerosis. *J Neurology*. (2008) 255:1023–31.

59. Villar LM, Casanova B, Ouamara N, Comabella M, Jalili F, Leppert D, et al. Immunoglobulin M oligoclonal bands: biomarker of targetable inflammation in primary progressive multiple sclerosis. *Ann Neurol*. (2014) 76:231–40.

60. Villar LM, Sádaba MC, Roldán E, Masjuan J, González-Porqué P, Villarrubia N, et al. Intrathecal synthesis of oligoclonal IgM against myelin lipids predicts an aggressive disease course in MS. *J Clin Invest*. (2005) 115:187–94.

61. Villar LM, González-Porqué P, Masjuán J, Alvarez-Cermeño JC, Bootello A, Keir G. A sensitive and reproducible method for the detection of oligoclonal IgM bands. *J Immunol Methods*. (2001) 258:151–5.

62. Pike SC, Gilli F, Pachner AR. The CXCL13 index as a predictive biomarker for activity in clinically isolated syndrome. *Int J Mol Sci*. (2023) 24.

63. Lucchini M, De Arcangelis V, Piro G, Nociti V, Bianco A, De Fino C, et al. CSF CXCL13 and chitinase 3-like-1 levels predict disease course in relapsing multiple sclerosis. *Mol Neurobiology*. (2023) 60:36–50.

64. Khademi M, Kockum I, Andersson ML, Iacobaeus E, Brundin L, Sellebjerg F, et al. Cerebrospinal fluid CXCL13 in multiple sclerosis: a suggestive prognostic marker for the disease course. *Mult Scler*. (2011) 17:335–43.

65. DiSano KD, Gilli F, Pachner AR. Intrathecally produced CXCL13: A predictive biomarker in multiple sclerosis. *Mult Scler J Exp Transl Clin*. (2020) 6:2055217320981396.

66. Mohammed MS, Al-Rubae'i SHN, Rheima AM, Al-Kazazz FF. A novel sandwich ELISA method for quantifying CHI3L1 in blood serum and cerebrospinal fluid multiple sclerosis patients using sustainable photo-irradiated zero-valence gold nanoparticles. *Results Chem*. (2024) 11:101856.

67. Pérez-Miralles F, Prefasi D, García-Merino A, Gascón-Giménez F, Medrano N, Castillo-Villalba J, et al. CSF chitinase 3-like-1 association with disability of primary progressive MS. *Neurol Neuroimmunol Neuroinflamm*. (2020) 7.

68. Floro S, Carandini T, Pietroboni AM, De Riz MA, Scarpini E, Galimberti D. Role of chitinase 3–like 1 as a biomarker in multiple sclerosis. *Neurol Neuroimmunology Neuroinflammation*. (2022) 9:e1164.

69. Cantó E, Reverter F, Morcillo-Suárez C, Matesanz F, Fernández O, Izquierdo G, et al. Chitinase 3-like 1 plasma levels are increased in patients with progressive forms of multiple sclerosis. *Mult Scler*. (2012) 18:983–90.

70. Oldoni E, Smets I, Mallants K, Vandebergh M, Van Horebeek L, Poesen K, et al. CHIT1 at diagnosis reflects long-term multiple sclerosis disease activity. *Ann Neurol*. (2020) 87:633–45.

71. Beliën J, Swinnen S, D'hondt R, Verdú de Juan L, Dedoncker N, Matthys P, et al. CHIT1 at diagnosis predicts faster disability progression and reflects early microglial activation in multiple sclerosis. *Nat Commun*. (2024) 15:5013.

72. Rabin A, Bello E, Kumar S, Zeki DA, Afshari K, Deshpande M, et al. Targeted proteomics of cerebrospinal fluid in treatment naïve multiple sclerosis patients identifies immune biomarkers of clinical phenotypes. *Sci Rep*. (2024) 14:21793.

73. Comabella M, Fernández M, Martin R, Rivera-Vallvé S, Borrás E, Chiva C, et al. Cerebrospinal fluid chitinase 3-like 1 levels are associated with conversion to multiple sclerosis. *Brain*. (2010) 133:1082–93.

74. Ioannides ZA, Csurhes PA, Swayne A, Foubert P, Aftab BT, Pender MP. Correlations between macrophage/microglial activation marker sTREM-2 and measures of T-cell activation, neuroaxonal damage and disease severity in multiple sclerosis. *Mult Scler J Exp Transl Clin*. (2021) 7:20552173211019772.

75. Piccio L, Buonsanti C, Cella M, Tassi I, Schmidt RE, Fenoglio C, et al. Identification of soluble TREM-2 in the cerebrospinal fluid and its association with multiple sclerosis and CNS inflammation. *Brain*. (2008) 131:3081–91.

76. Öhrfelt A, Axelsson M, Malmeström C, Novakova L, Heslegrave A, Blennow K, et al. Soluble TREM-2 in cerebrospinal fluid from patients with multiple sclerosis treated with natalizumab or mitoxantrone. *Mult Scler*. (2016) 22:1587–95.

77. Ashton NJ, Suárez-Calvet M, Heslegrave A, Hye A, Razquin C, Pastor P, et al. Plasma levels of soluble TREM2 and neurofilament light chain in TREM2 rare variant carriers. *Alzheimer's Res Ther*. (2019) 11:94.

78. Cignarella F, Filipello F, Bollman B, Cantoni C, Locca A, Mikesell R, et al. TREM2 activation on microglia promotes myelin debris clearance and remyelination in a model of multiple sclerosis. *Acta Neuropathol*. (2020) 140:513–34.

79. Sun M, Liu N, Xie Q, Li X, Sun J, Wang H, et al. A candidate biomarker of glial fibrillary acidic protein in CSF and blood in differentiating multiple sclerosis and its subtypes: A systematic review and meta-analysis. *Mult Scler Relat Disord*. (2021) 51:102870.

80. Barro C, Healy BC, Liu Y, Saxena S, Paul A, Polgar-Turcsanyi M, et al. Serum GFAP and nfL levels differentiate subsequent progression and disease activity in patients with progressive multiple sclerosis. *Neurol Neuroimmunol Neuroinflamm*. (2023) 10.

81. Rosenstein I, Nordin A, Sabir H, Malmeström C, Blennow K, Axelsson M, et al. Association of serum glial fibrillary acidic protein with progression independent of relapse activity in multiple sclerosis. *J Neurol*. (2024) 271:4412–22.

82. Schindler P, Aktas O, Ringelstein M, Wildemann B, Jarius S, Paul F, et al. Glial fibrillary acidic protein as a biomarker in neuromyelitis optica spectrum disorder: a current review. *Expert Rev Clin Immunol*. (2023) 19:71–91.

83. Simrén J, Weninger H, Brum WS, Khalil S, Benedet AL, Blennow K, et al. Differences between blood and cerebrospinal fluid glial fibrillary Acidic protein levels: The effect of sample stability. *Alzheimers Dement*. (2022) 18:1988–92.

84. Sheremata WA, Jy W, Delgado S, Minagar A, McLarty J, Ahn Y. Interferon-beta1a reduces plasma CD31+ endothelial microparticles (CD31+EMP) in multiple sclerosis. *J Neuroinflammation*. (2006) 3:23.

85. Sáenz-Cuesta M, Haritz I, Tamara C-T, Maider M-C, Iñaki O-Q, Alvaro P, et al. Circulating microparticles reflect treatment effects and clinical status in multiple sclerosis. *Biomarkers Med*. (2014) 8:653–61.

86. Galazka G, Mycko MP, Selmaj I, Raine CS, Selmaj KW. Multiple sclerosis: Serum-derived exosomes express myelin proteins. *Mult Scler*. (2018) 24:449–58.

87. Moseley CE, Virupakshaiah A, Forsthuber TG, Steinman L, Waubant E, Zamvil SS. MOG CNS autoimmunity and MOGAD. *Neurol Neuroimmunol Neuroinflamm*. (2024) 11:e200275.

88. D'Anca M, Fenoglio C, Buccellato FR, Visconte C, Galimberti D, Scarpini E. Extracellular vesicles in multiple sclerosis: role in the pathogenesis and potential usefulness as biomarkers and therapeutic tools. *Cells*. (2021) 10.

89. Bhargava P, Nogueras-Ortiz C, Chawla S, Bæk R, Jørgensen MM, Kapogiannis D. Altered levels of toll-like receptors in circulating extracellular vesicles in multiple sclerosis. *Cells*. (2019) 8.

90. Bhargava P, Nogueras-Ortiz C, Kim S, Delgado-Peraza F, Calabresi PA, Kapogiannis D. Synaptic and complement markers in extracellular vesicles in multiple sclerosis. *Mult Scler*. (2021) 27:509–18.

91. Nogueras-Ortiz CJ, Eren E, Yao P, Calzada E, Dunn C, Volpert O, et al. Single-extracellular vesicle (EV) analyses validate the use of L1 Cell Adhesion Molecule (L1CAM) as a reliable biomarker of neuron-derived EVs. *J Extracell Vesicles*. (2024) 13:e12459.

92. Li D, Zou S, Huang Z, Sun C, Liu G. Isolation and quantification of L1CAM-positive extracellular vesicles on a chip as a potential biomarker for Parkinson's Disease. *J Extracell Vesicles*. (2024) 13:e12467.

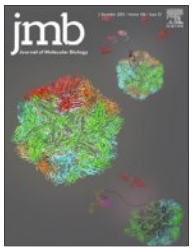
93. Mazzucco M, Mannheim W, Shetty SV, Linden JR. CNS endothelial derived extracellular vesicles are biomarkers of active disease in multiple sclerosis. *Fluids Barriers CNS*. (2022) 19:13.

94. Martinsen V, Kursula P. Multiple sclerosis and myelin basic protein: insights into protein disorder and disease. *Amino Acids*. (2022) 54:99–109.

95. Sáenz-Cuesta M, Alberro A, Muñoz-Culla M, Osorio-Querejeta I, Fernandez-Mercado M, Lopetegui I, et al. The first dose of fingolimod affects circulating extracellular vesicles in multiple sclerosis patients. *Int J Mol Sci*. (2018) 19.

96. Verderio C, Muzio L, Turola E, Bergami A, Novellino L, Ruffini F, et al. Myeloid microvesicles are a marker and therapeutic target for neuroinflammation. *Ann Neurol*. (2012) 72:610–24.

97. Geraci F, Ragonese P, Barreca MM, Aliotta E, Mazzola MA, Realmuto S, et al. 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*. (2018) 12:418.



Human B-cells can form Hetero-aggregates with Blood Platelets: A Novel Insight into Adaptive Immunity Regulation in Multiple Sclerosis

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Abstract

Multiple sclerosis (MS) is a chronic autoimmune disease characterized by inflammation and neurodegeneration. Our original study analyzes the interactions between blood platelets and leukocytes in MS, focused on their potential role in modulating immune responses. We demonstrated, for the first time, a significant increase in leukocyte migration towards platelets, indicating their higher chemotactic capabilities in MS. This novel finding is supported by microscopic imaging of platelet-leukocyte hetero-aggregates (PLAs). Our study included platelet activation status and platelet-lymphocyte cross-talk analysis distinguishing lymphocytic subpopulation in patients with relapsing-remitting (RRMS) and secondary progressive MS (SPMS) compared to healthy controls (HC). Flow cytometry method revealed an elevated expression of platelet activation typical markers i.e. PAC-1 and CD62P in both phenotypes of MS, especially in RRMS, and higher GPVI level in SPMS. Detailed immunophenotyping and confocal imaging showed an increased pool of platelet-lymphocyte aggregates (PLAs-Ly), particularly involving B-cells over T-cells across both MS phenotypes. The study also explored the involvement of the CD40-CD40L pathway, discovering significant correlations between platelet CD40L expression and lymphocytic antigen CD40, especially on B-cells in SPMS. This novel finding may indicate the special significance of platelet-B-cell cross-talk in progressive disease phenotype. Our research identified potential platelet-leukocyte interaction pathways that may influence the lymphocyte-mediated immune response in MS, highlighting the unexplored formation of platelet-B cell hetero-aggregates (PLAs-LyB).

Introduction

Multiple sclerosis (MS) is a chronic, inflammatory, and neurodegenerative disease of the central nervous system (CNS), affecting approximately 2.8 million people globally as of 2020.[1] According to the International Advisory Committee on Clinical Trials of MS there are four phenotypes of MS i.e. clinically isolated syndrome (CIS), relapsing-remitting MS (RRMS), primary-progressive MS (PPMS), and secondary-progressive MS (SPMS).[2] RRMS is the most prevalent form of the disease, impacting about 85% of patients. Traditionally, it has been primarily associated with inflammatory processes characterized by alternating immune-mediated acute episodes of neurological symptoms (relapses) and periods of remission, when symptoms partially or completely resolve. In turn, SPMS, typically developing after RRMS, has been predominantly linked to neurodegenerative processes, which involve a gradual and irreversible decline in neurological function.[3] However, more recent approaches indicate that both disease phases exhibit overlapping features of inflammation and neurodegeneration processes, which are not mutually exclusive and can coexist throughout the disease course, challenging this traditional distinction.[4]

Generally, MS pathophysiology is characterized by dysfunctional immune reactions driven mainly by antigen-presenting cells (APC), leading to neuroinflammation, demyelination, axonal damage, and progressive neurodegeneration. The inflammatory cascade in CNS is exacerbated by an increased permeability of the dysfunctional blood–brain barrier (BBB), making MS condition features associated not only with neuroinflammatory but also with vascular damage.[5]

In MS, the classic immune response is predominantly driven by the functioning of both autoreactive T helper (Th)1 and Th17 cells that promote the inflammation, and B-cells, which are responsible for producing antibodies against myelin basic protein (MBP).[6] Recent MS research have focused on the concept of 'smoldering inflammation', which refers to the chronic, non-relapsing, and immune-mediated mechanism of disease progression. This non-classical type of inflammation is likely shaped and sustained by many local factors in the CNS, such as cytokines, cellular cross-talk, and disbalance between active lymphocyte populations.[7] Although inflammation in MS has traditionally centered on T-cells, B-cells are now recognized as crucial mediators in the disease's pathogenesis. Some theories propose a two-stage development of MS, where T-cells dominate in the initial acute inflammation phase, while B-cells sustain chronic inflammation and tissue damage in later stages.[7] Growing evidence from neuropathological studies strongly suggests that B-cells may be pivotal in the pathophysiology of the disease[8] but their precise roles remains unclear. The fact that treatments targeting B-cells used in MS patients are effective, highlights their involvement in the pathophysiology of the disease.[9] Mechanisms through which B cells are thought to contribute to CNS autoimmunity are being investigated, but there are no studies on their cellular cross-talk in MS.

Interactions between platelets and leukocytes, including both T- and B-cells,[10] play a crucial role in linking vascular disturbing and inflammatory processes.[11] Generally, platelet-leukocyte aggregates (PLAs) were extensively studied in the context of monocytes and neutrophils,[12–16] as these cells show the highest affinity for platelet P-selectin (CD62P). However, it was also demonstrated that activated platelets can broadly regulate immune response by forming aggregates with lymphocytes, impacting their proliferation, production of immune-mediators, and infiltration in many pathologies.[17–21] In MS, platelets are hyperactive,[22–24] showing structural changes and increased adhesion capacity to endothelial cells.[25] Recent studies demonstrated that molecular changes in the cytoskeleton of blood platelets might enhance interactions with leukocytes.[26] Platelet-mediated pro-inflammatory activity is facilitated through direct receptor-ligand interactions as well as the release of biologically active compounds stored within platelet granules.[27]

PLAs exhibit exceptionally high adhesive properties, due to platelets playing a crucial role in mediating the adhesion of circulating immune cells to endothelium. Through bidirectional cellular interactions, the platelet-dependent leukocyte rolling phenomenon facilitates the recruitment of leukocytes to sites of endothelial damage, initiating diapedesis and promoting translocation of reactive cells across the compromised BBB into the CNS.[25,28] The massive size and rigid cellular structure of PLAs make them dysfunctional, leading to mechanical damage to the BBB rather than an effective immune response.[29]

Despite available studies on PLAs, the specific dynamics of platelet-leukocyte interactions in the course of MS remain elusive. By integrating advanced microscopy techniques, immunophenotyping, and cellular markers evaluation, this study aims to offer novel insights into how platelet-leukocyte interactions contribute to MS pathology, taking into account the hitherto unexplored platelet-B-cell cross-talk.

Results

Platelets exhibit an increased potential to attract leukocytes in MS patients

The Boyden chamber-based cell migration assay was used to evaluate the potential of platelets as chemoattractants for leukocytes (Figure 1A). The chemotactic potential of platelets was measured by simulating the *in vitro* migration of leukocytes towards platelets isolated from the same blood

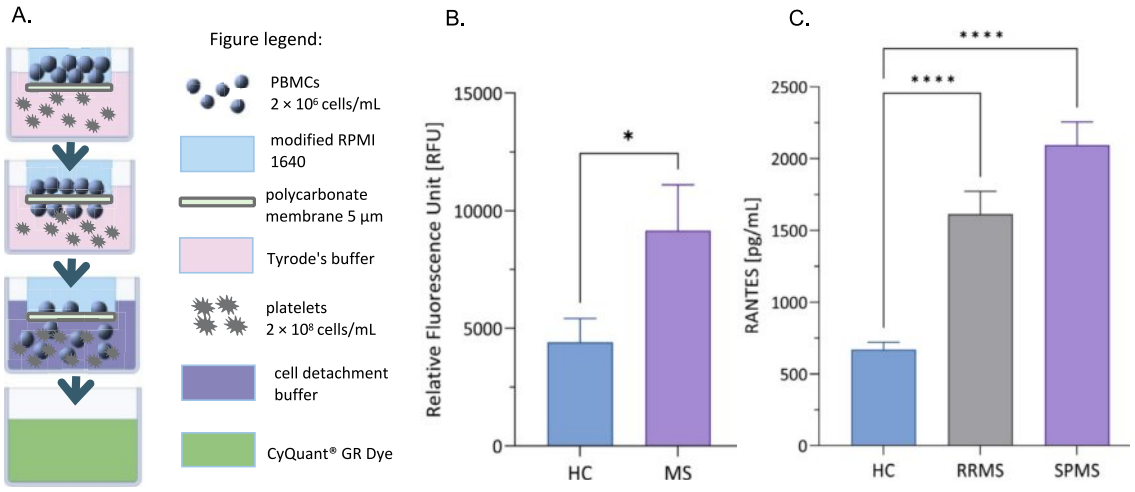


Figure 1. Chemotactic potential of platelets measured using the CytoSelect™ cell migration assay and by the level of RANTES chemokine using Bio-Plex Pro™ immunoassay (A) Flowchart of the Boyden chamber-based cell migration assay (CytoSelect™). (B) Quantification of fluorescence intensity of CyQuant® GR-stained leukocytes that migrated towards platelets in HC ($n = 12$) *vs.* MS patients ($n = 12$). Data are presented as mean RFU ± SEM. C) Plasma level of RANTES in HC ($n = 55$), RRMS ($n = 38$), and SPMS ($n = 55$). Data are presented as mean concentration [pg/mL] ± SEM. Statistical significance was determined using unpaired *t*-test with Welch's correction or the Kruskal-Wallis test followed by Dunn's multiple comparison test. Significant differences are indicated by $^{*}p < 0.05$ and $^{****}p < 0.0001$.

sample. The chemotactic potential of respective platelets population was quantified by fluorescence measurement and is shown in relative fluorescence units (RFU). The mean level of leukocytes migration towards platelets showed more than 2-fold increase in MS compared to healthy controls (HC) ($p = 0.046$) (Figure 1B).

Moreover, we measured the plasma concentration of Regulated upon Activation, Normal T-cell Expressed and Secreted (RANTES, also known as C–C motif ligand 5, CCL5), a critical platelet-derived chemokine involved in the recruitment of leukocytes to sites of inflammation. The mean concentration of RANTES was elevated in both RRMS and SPMS patients compared to HC (2.4 and 3.1-fold, respectively; $p < 0.0001$) (Figure 1C).

Analysis of PLAs morphology by SEM and confocal microscopy imaging

Scanning electron microscopy (SEM) and confocal microscopy imaging were utilized to analyze the morphology of PLAs and to verify the increased prevalence of these cellular aggregates in randomly selected MS samples compared to HC. These techniques also allowed us to reveal the structural changes of platelets during the formation of PLAs.

Figure 2 shows representative images illustrating evident differences in the distribution of platelets in the *peri*-leukocytic space in MS and HC, which corroborates the results obtained in the Boyden chamber experiment.

SEM microphotography (Figure 2A) revealed platelets in the HC group exhibiting a spherical shape with only slight signs of activation, such as short filopodia and small membrane protrusions, induced probably upon the contact with the poly-L-lysine surface and/or during other experimental procedures. PLAs were not observed in any of the HC images taken. Figure 2A reflects the background of physiological conditions, where platelets remain in a resting or minimally activated state without significant interaction with leukocytes.

Confocal microscopy imaging (Figure 2B) provided complementary insights to the SEM findings. In the HC group, platelets showed a resting or only slightly activated state. No homo- or hetero-aggregates between platelets and leukocytes were observed in any of the fields of view in random HC preparations. Fluorescence microscopy is the second independent technique that confirmed the physiological state of quiescent platelets in HC samples.

By contrast, the SEM image of MS blood samples (Figure 2C) displayed platelets exhibiting irregular shapes with clear signs of activation, such as elongated filopodia, multiple membrane protrusions, an increase in granularity and surface roughness. Platelets strive to form multiple contact points, forming massive homo-aggregates and PLAs. These dense clustering and complex structures significantly distinguish activated platelets from the control sample shown in Figure 2A.

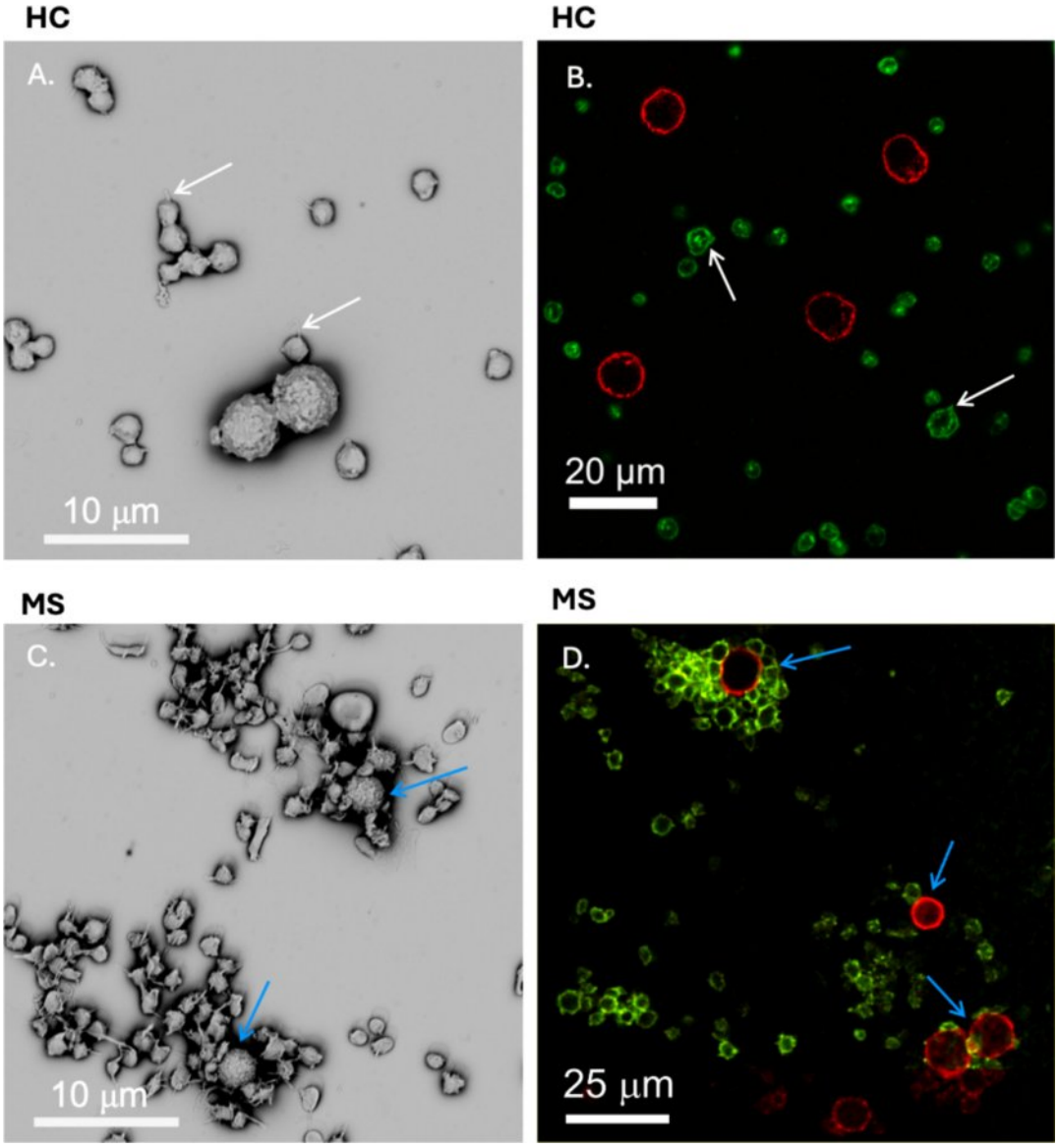


Figure 2. Representative microscopy images illustrating the differences in the distribution of platelets around leukocytes in HC group and MS patients. (A) SEM microphotography of HC blood sample showing resting platelets with a spherical shape and only a few cells with minor signs of activation (indicated with white arrows). (B) Confocal microscopy image of HC blood sample showing resting or slightly activated platelets (shown in green, stained with anti-CD61 antibody conjugated with BB515) and leukocytes (shown in red, stained with anti-CD45 antibody conjugated with PE). (C) SEM microphotography of MS blood sample with activated, irregularly shaped platelets, forming massive clusters of PLAs (indicated with blue arrows). (D) Confocal microscopy visualization of MS sample showing irregularly shaped, activated platelets, forming multiple PLAs (indicated with blue arrows).

Confocal microscopy images (Figure 2D) confirmed SEM findings. In MS sample, platelets exhibit highly activated state reflected in irregular shapes, membrane protrusions and surface roughness. Most of the leukocytes visible in the shown field of view are surrounded by highly adhesive platelets, forming PLAs. These images confirm the SEM findings and provide an overview of the complex and dense clusters of platelets in immediate proximity to leukocytes in MS blood samples, highlighting the contrast with the quiescent state observed in HC blood samples.

Figure 3 shows the accumulation of platelets on the surface of leukocytes and a detailed view of formed PLA. Interestingly, more intense CD45 PE staining is visible in platelet-leukocytes cross-talk sites indicating relocation of CD45 to these lipid domains in response to the interaction.

SEM microphotographs (Figure 4) of various MS blood samples demonstrate changes in platelet morphology and following stages of interactions between activated platelets and leukocytes in the process of PLAs forming. At the initial stage (Figure 4A), only a small cluster of blood platelets with their elongated filopodia extending on the membrane of leukocytes can be observed. The next stage of PLA formation (Figure 4B) shows an increasing accumulation of platelets around leukocytes. Platelets exhibit flattened-out lamellipodia sheets forming a so-called "fried egg" structure with ruffles and extend spiky filopodia, which form an extracellular trap-like construction surrounding leukocytes. This structure creates a scaffold that stabilizes PLAs, promoting firm adhesion. In the advanced stage of aggregation (Figure 4C), platelets form a dense clot with a fibrin coat clustering around the leukocyte.

Overall, the SEM analyses revealed clear examples of the progression in the formation and increase in complexity of PLAs formed in MS patients.

Increased platelet activation markers in MS

The median fluorescence intensity (MFI) of PAC-1 antibody (Figure 5A) directed against an activated form of GPIIb/IIIa demonstrates an elevated level of this marker of platelet activation in MS compared to HC (RRMS with 1.9-fold, $p = 0.017$ and SPMS with 1.65-fold, $p = 0.029$).

Similarly, the expression of platelet CD62P antigen (Figure 5B), which facilitates PLAs formation and adhesion to blood vessel walls through interactions with leukocytes and endothelial cells, is significantly elevated in both the RRMS (3.06-fold, $p = 0.0002$) and SPMS (2.9-fold, $p = 0.014$) groups compared to the HC group.

On the other hand, the expression of GPVI (Figure 5C), crucial for platelet-collagen interactions and thrombus formation, is significantly higher only in the SPMS group *vs.* HC (2.13-fold, $p = 0.034$).

Immunophenotyping and confocal microscopy imaging of platelet-lymphocyte complexes

After evaluation of the platelet activation status and chemotactic capabilities towards leukocytes, we conducted detailed immunophenotyping and confocal microscopy imaging analyses to quantify and visualize the aggregation patterns between platelets and lymphocyte subsets (B-cells, T-cells, helper (Th), cytotoxic (Tcyt), and regulatory (Treg) T-cells) across RRMS and SPMS phenotypes.

The gating strategy applied during the immunophenotyping analysis is presented in Figure 6, while Figure 7 summarize the percentage of formed PLAs-Ly. The calculation method is described in the experimental section.

A general trend of increased platelet-lymphocyte hetero-aggregates (PLAs-Ly) is observed in both RRMS and SPMS groups compared to the HC group (Figure 7). The percentage of PLAs-Ly with

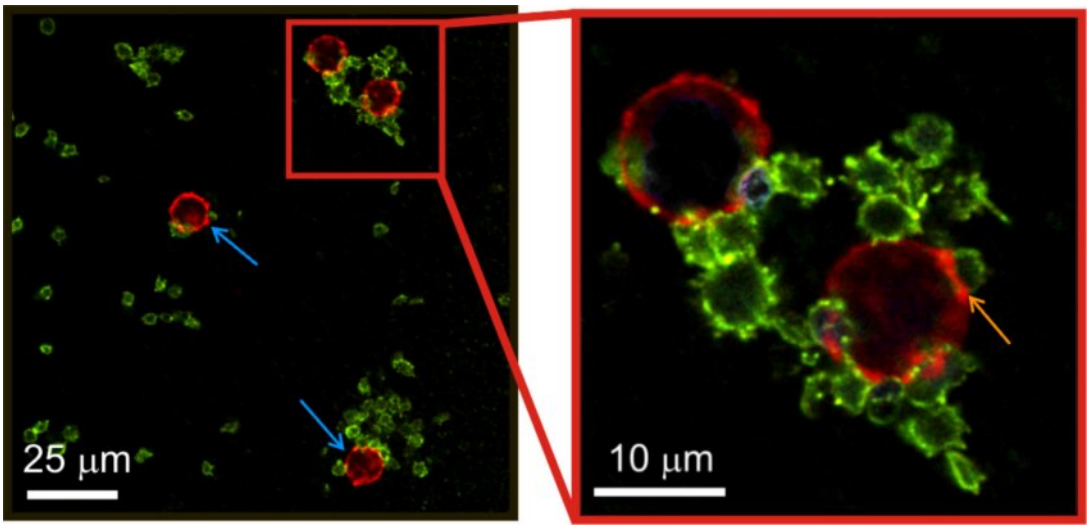
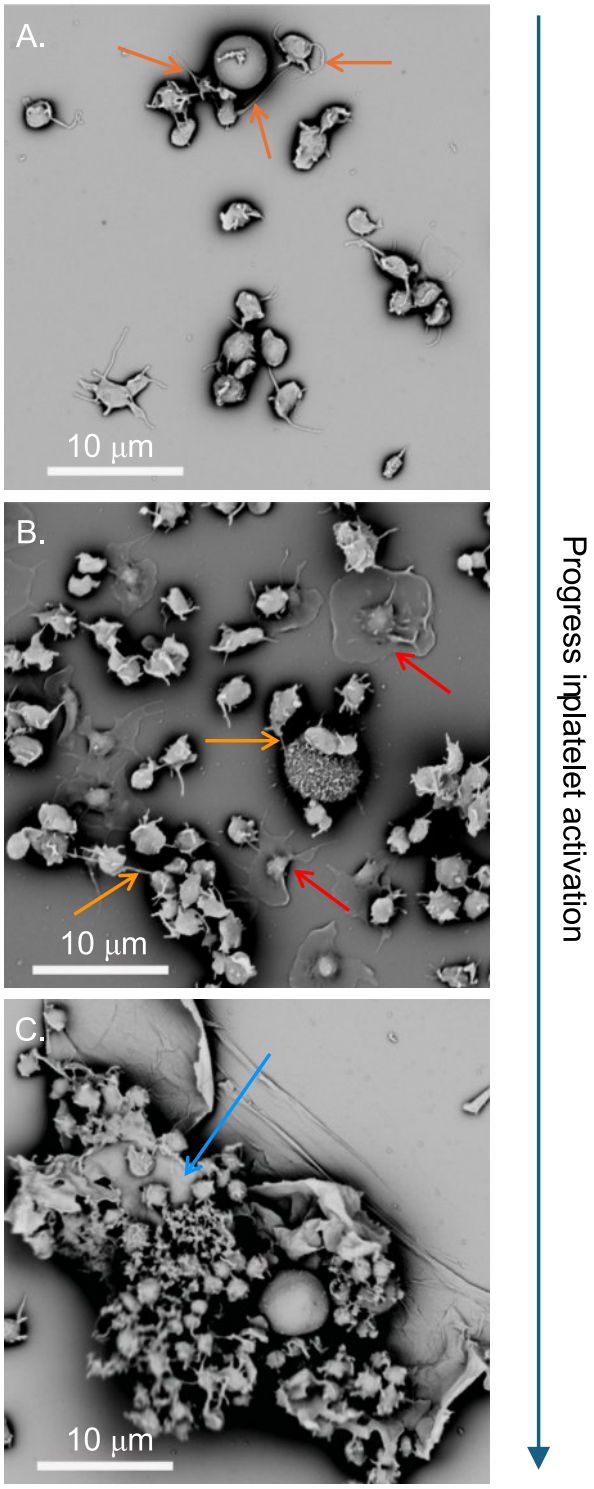


Figure 3. Representative confocal image of MS sample showing PLAs (blue arrows). Platelets were labelled with anti-CD61 BB515 antibody (shown in green), and leukocytes were labelled with anti-CD45 PE antibody (shown in red). Activated platelets interact with leukocytes, showing intense CD45 PE staining at the cellular contact points (orange arrow).



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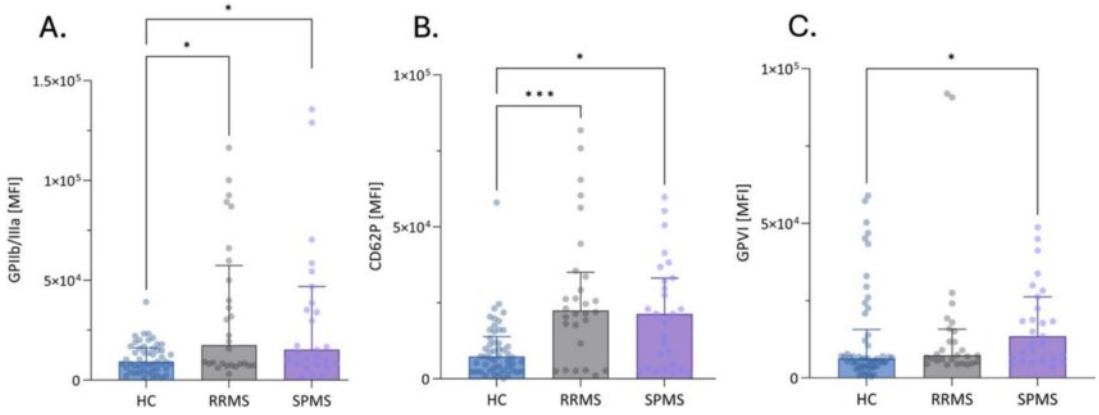


Figure 5. Expression level of platelet activation markers (A) GPIIb/IIIa, (B) CD62P and (C) GPVI analyzed by flow cytometry in HC ($n = 50$), RRMS ($n = 28$), and SPMS ($n = 27$) groups. Data shown is median of MFI ± IQR. Statistical analysis was performed using the Kruskal-Wallis test followed by Dunn's multiple comparison test. Significant differences between groups are indicated by $*p < 0.05$ and $***p < 0.001$.

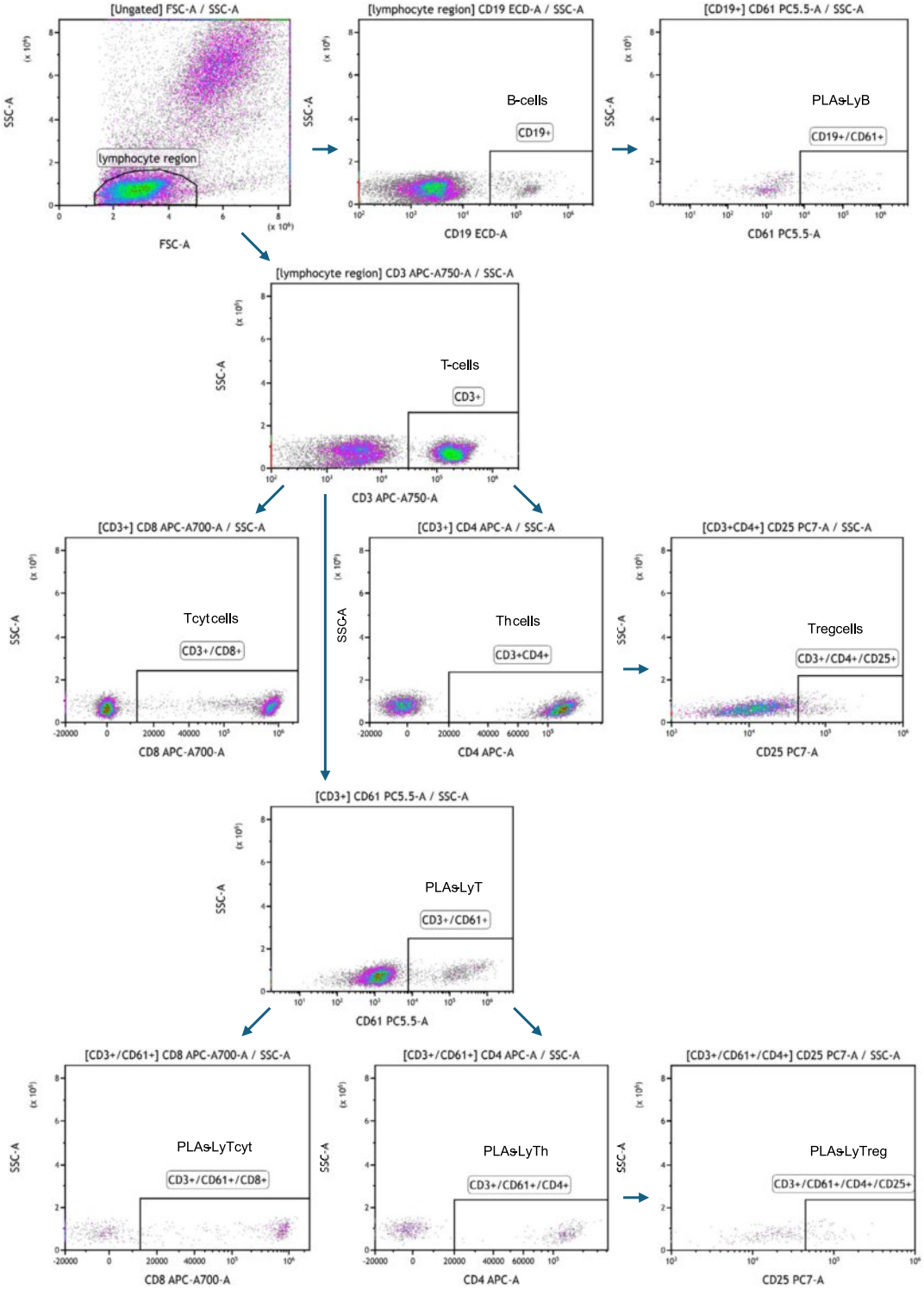
B-cells (PLAs-LyB; $CD61^+/CD19^+$) is higher in RRMS (1.55-fold) and SPMS (1.94-fold) patients compared to HC samples ($p = 0.009$ and $p < 0.0001$, respectively). While the percentage of PLAs-Ly with T-cells (PLAs-LyT; $CD61^+/CD3^+$) is significantly higher in SPMS compared to HC and RRMS (1.66-fold, $p = 0.005$ and 1.45-fold, $p = 0.0002$, respectively).

Immunophenotyping of T-cells subtypes showed that the percentage of PLAs-LyTh ($CD61^+/CD3^+CD4^+$) was similarly elevated in RRMS compared to HC and to SPMS (1.66-fold, $p = 0.006$ and 1.61-fold, $p = 0.017$, respectively). While the percentage of PLAs-LyTcyt ($CD61^+/CD3^+CD8^+$) and PLAs-LyTreg ($CD61^+/CD3^+CD4^+CD25^+$) were significantly higher only in SPMS: 1.65-fold SPMS *vs.* HC, $p = 0.015$ and 1.66-fold SPMS *vs.* HC, $p = 0.049$, respectively.

Next, we confirmed the formation of PLAs-LyB in MS blood samples using confocal microscopy imaging, an observation that has not been described previously. Figure 8A shows that platelets and B-cell in a representative field of view in HC blood sample. Platelets are not associated with B lymphocyte, there is no visible signs of interactions, nor PLAs formation. Non-activated platelets exhibit morphology typical for physiological conditions, smooth surface and regular outline, without substantial projections.

In contrast, confocal images of randomly selected MS blood samples, reveal examples of the PLAs-LyB formation (Figure 8B and C) involving substantially enlarged and activated platelets. In MS group platelets are in close proximity to or directly attached to B-cell, forming PLAs-LyB. Bright spots of fluorescence at the cell periphery are visible, highlighting the process of degranulation. The ruffled membrane of platelets is visible, and cells exhibit clearly increased size, as compared to HC sample (Figure 8B). In another field of view enlarged platelets form a cluster and interact with CD19-positive cell assembling PLAs-LyB (Figure 8C). In the three-dimensional visualization (Figure 8D) an example of spatial distribution of B lymphocyte and platelets of PLAs-LyB can be seen, showing the robust adhesion of platelets to B-cell, and confirming their interactions. Activated platelets having spherical or irregular shapes can also be seen. Platelets forming PLAs-LyB are tightly adhered to the surface of B-cell. Bright spots of accumulated fluorescence of $CD19^+$ markers on B-cell can be observed.

Figure 4. Representative SEM micrographs illustrating the morphology and various structural forms of PLAs in MS blood samples, highlighting different stages of platelets and leukocytes interactions. (A) The initial stage showing a small cluster of platelets with elongated filopodia (orange arrows) extending on leukocyte. (B) The next stage with increased platelet accumulation around leukocyte. Platelets exhibit flattened-out lamellipodia sheets with ruffles (red arrows) and spiky filopodia, forming a stabilizing scaffold (orange arrows). (C) Advanced stage with platelets forming a dense clot and a fibrin coat (blue arrow) clustering around the leukocyte.


[Ungated] FSC-A / SSC-A
lymphocyte region
FSC-A
SSC-A
[lymphocyte region] CD19 ECD-A / SSC-A
B-cells
CD19+
CD19 ECD-A
[CD19+] CD61 PC5.5-A / SSC-A
PLAs-LyB
CD19+/CD61+
CD61 PC5.5-A
[lymphocyte region] CD3 APC-A750-A / SSC-A
T-cells
CD3+
CD3 APC-A750-A
[CD3+] CD8 APC-A700-A / SSC-A
Tcytcells
CD3+/CD8+
CD8 APC-A700-A
[CD3+] CD4 APC-A / SSC-A
Thcells
CD3+CD4+
CD4 APC-A
[CD3+CD4+] CD25 PC7-A / SSC-A
Tregcells
CD3+/CD4+/CD25+
CD25 PC7-A
[CD3+] CD61 PC5.5-A / SSC-A
PLAs-LyT
CD3+/CD61+
CD61 PC5.5-A
[CD3+/CD61+] CD8 APC-A700-A / SSC-A
PLAs-LyTcyt
CD3+/CD61+/CD8+
CD8 APC-A700-A
[CD3+/CD61+] CD4 APC-A / SSC-A
PLAs-LyTh
CD3+/CD61+/CD4+
CD4 APC-A
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PLAs-LyTreg
CD3+/CD61+/CD4+/CD25+
CD25 PC7-A

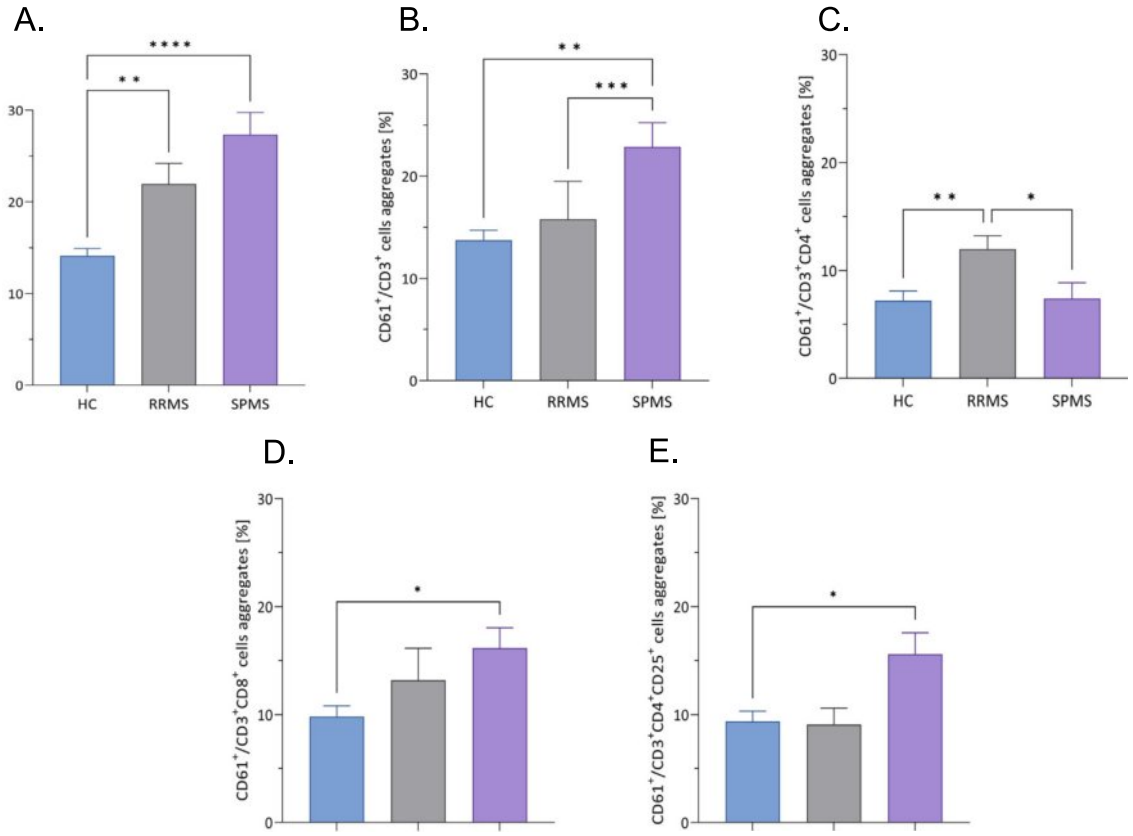


Figure 7. PLAs-Ly analyzed by flow cytometry. The mean percentage ± SEM of platelet ($CD61^+$) hetero-aggregates with lymphocyte subsets: (A) B-cells ($CD19^+$), (B) T-cells ($CD3^+$), (C) Th cells ($CD3^+CD4^+$), D) Tcyt cells ($CD3^+CD8^+$), and (E) Treg cells ($CD3^+CD4^+CD25^+$)) for: HC (n = 50), RRMS (n = 28), and SPMS (n = 27). Statistical differences between groups were analyzed using the Kruskal-Wallis test followed by Dunn's multiple comparison test, with significant differences indicated by $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

CD40-CD40L axis in platelet and lymphocyte co-activation

Continuing to define the phenomenon of platelet-lymphocyte co-activation in MS, we examined the antigens co-expression of the leading pro-inflammatory pathway CD40-CD40L.

The gating strategy applied during the CD40-CD40L analysis is presented in Figure 9, while Figure 10 summarizes the MFI of markers on platelets, T and B-cells. Figure 11 presents the correlation matrices for the expression of CD40 and CD40L on these cells.

Flow cytometry analysis showed a significant increase in expression of CD40 and CD40L on platelets in MS compared to HCs, indicating their readiness to participate in the CD40-CD40L-mediated activation (Figure 10). The MFI level of CD40 antigen on platelets was significantly elevated in RRMS compared to HC (3.3-fold,

Figure 6. Sequential gating applied to assess the percentage of lymphocyte subpopulations in PLAs. Whole blood was stained with anti-CD19 ECD (B-cells), anti-CD3 APC-Alexa Fluor 750 (T-cells), anti-CD4 APC (Th cells), CD8 Alexa Fluor 700 (Tcyt cells), CD25 PC7 (Treg cells), CD61 PC5.5 (platelets). The gating strategy is followed with arrows. PLAs-LyB – platelet-lymphocyte B hetero-aggregates; PLAs-LyT – platelet-lymphocyte T hetero-aggregates; PLAs-LyTh – platelet-T helper lymphocyte hetero-aggregates; PLAs-LyTcyt – platelet-T cytotoxic lymphocyte hetero-aggregates; PLAs-LyTreg – platelet-T regulatory lymphocyte hetero-aggregates.

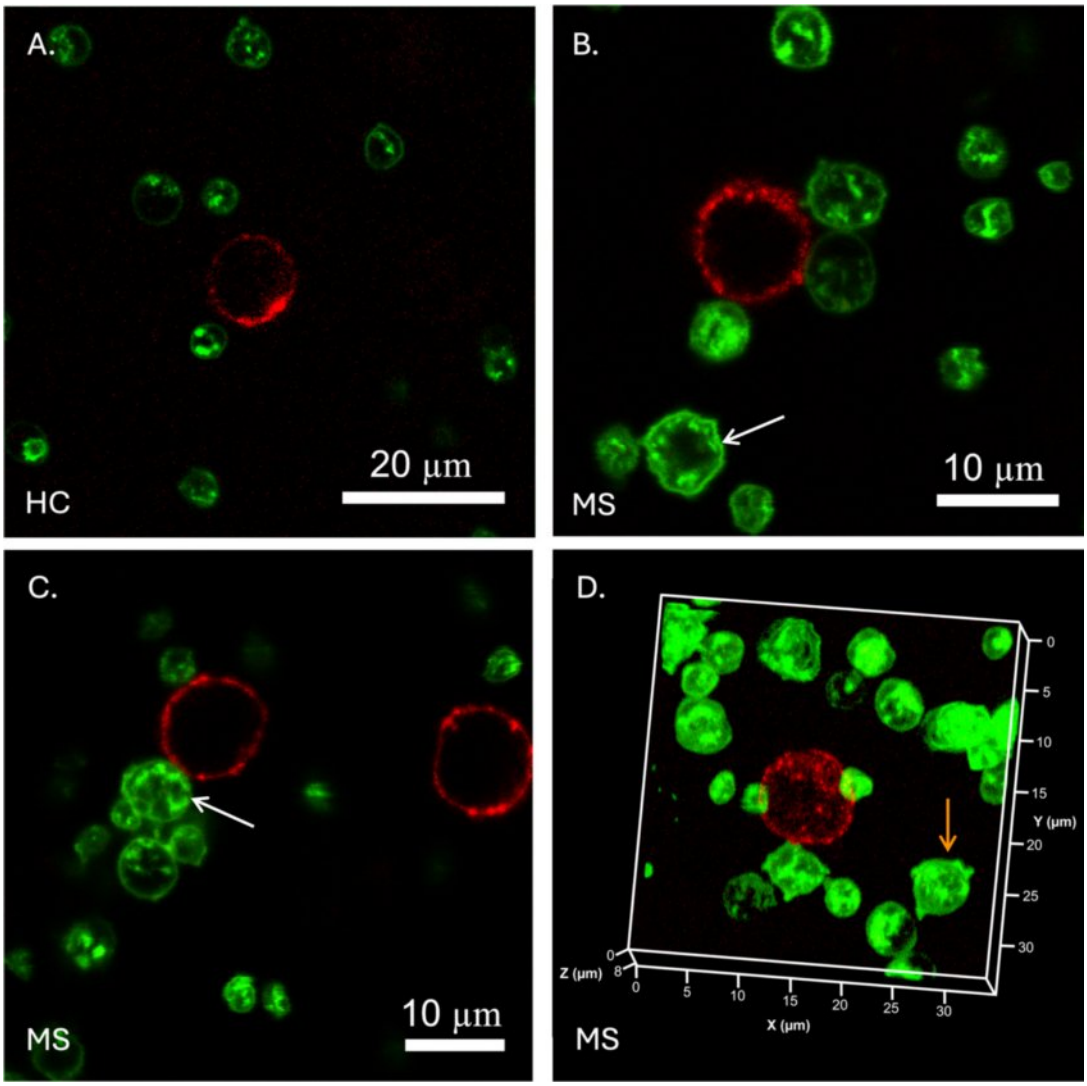


Figure 8. Representative confocal microscopy images of immunostained platelets and B-cells in whole blood of HC (A) and MS (B–D) samples. Platelets were labelled with anti-CD61 APC antibody (shown in green), and B-cells were labelled with anti-CD19 Alexa Fluor 488 antibody (shown in red). Enlarged and activated platelets are indicated with white arrows. An activated platelet with a spherical, irregular shape and numerous membrane protrusions is indicated with orange arrow.

p = 0.029). The expression of CD40L antigen on platelets was significantly increased in both RRMS and SPMS group compared to HC (5.87-fold and 5.22-fold, respectively with p < 0.0001) (Figure 10A). The second noteworthy finding, although not statistically significant, was a slightly higher expression of CD40 and CD40L on both T (Figure 10B) and B-cells (Figure 10C) in SPMS compared to RRMS.

Our intriguing finding is a statistically significant positive correlation between platelet CD40L expression and CD40 level on T-cells and B-cells both in RRMS and SPMS, as well as the HC group (Figure 11). In RRMS patients (Figure 11A), platelet CD40L expression showed a significant moderate correlation with T-cell CD40 level (r = 0.446, p = 0.017) and a significant strong correlation with B-cell CD40 expression (r = 0.567, p = 0.002). Similarly, in SPMS (Figure 11B), platelet CD40L level was significantly moderately correlated with T-cell CD40 (r = 0.402, p = 0.037) and strongly correlated with CD40 expression on B-cells (r = 0.640, p < 0.001). While, in the HC group (Figure 11C), the correlation between

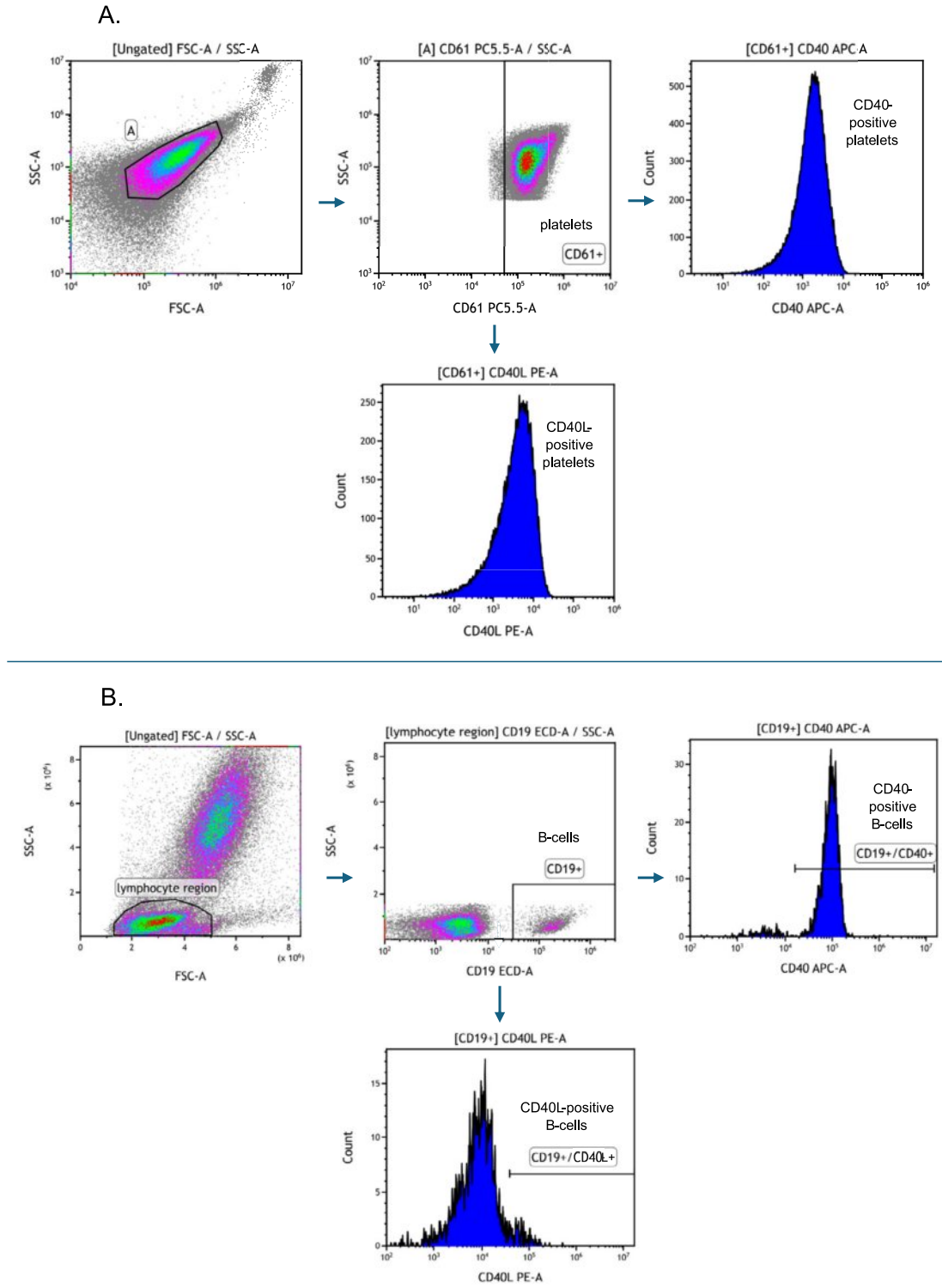


Figure 9. Sequential gating applied to assess the MFI levels of CD40/CD40L antigens on A) platelets and B) lymphocytes (exemplified by gating of B-cells). Whole blood stained with anti-CD40 APC, anti-CD40L PE, anti-CD61 PC5.5 (platelets), and anti-CD19 ECD (B-cells). The gating strategy is followed with arrows.

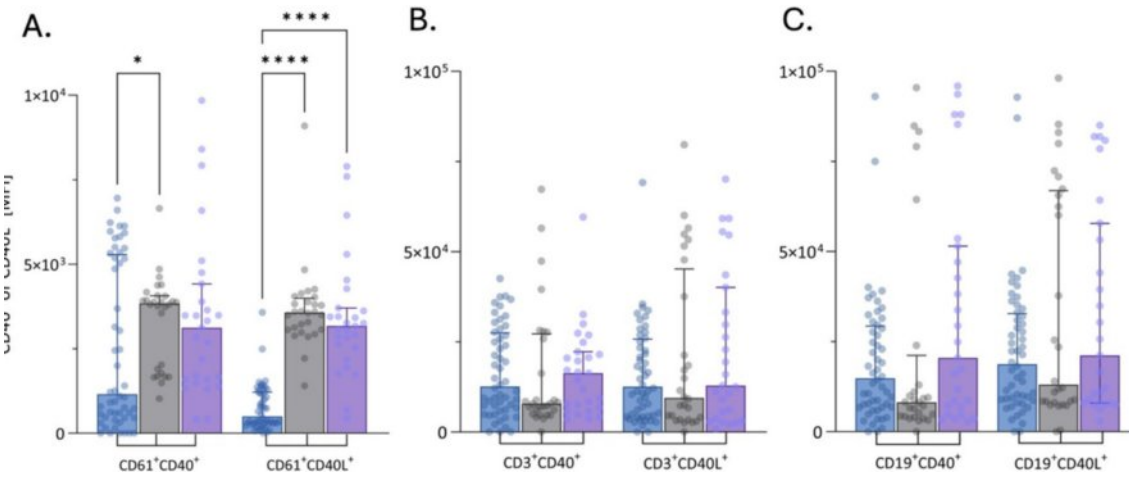


Figure 10. The expression levels of CD40 and CD40L on (A) platelets ($CD61^+$), (B) T-cells ($CD3^+$), and (C) B-cells ($CD19^+$) presented as median of MFI with IQR. Data are shown for HC ($n = 50$), RRMS ($n = 28$), and SPMS ($n = 27$) groups. Differences between groups were analyzed using the Kruskal-Wallis test followed by Dunn's multiple comparison test. Statistically significant differences are indicated by $^*p < 0.05$ and $^{****}p < 0.0001$.

platelet CD40L and CD40 on T-cells and B-cells was moderate ($r = 0.350$, $p = 0.009$) or weak ($r = 0.281$, $p = 0.038$), respectively. At once, no statistically significant correlations were found between platelet CD40 and lymphocyte CD40L. Notably, the especially strong correlation between platelet CD40L and B-cell CD40 in SPMS highlights the involvement of the B-cells in the platelet-lymphocyte cross-talk and indicates the activity of CD40-CD40L inflammatory pathway in the progressive stage of the disease.

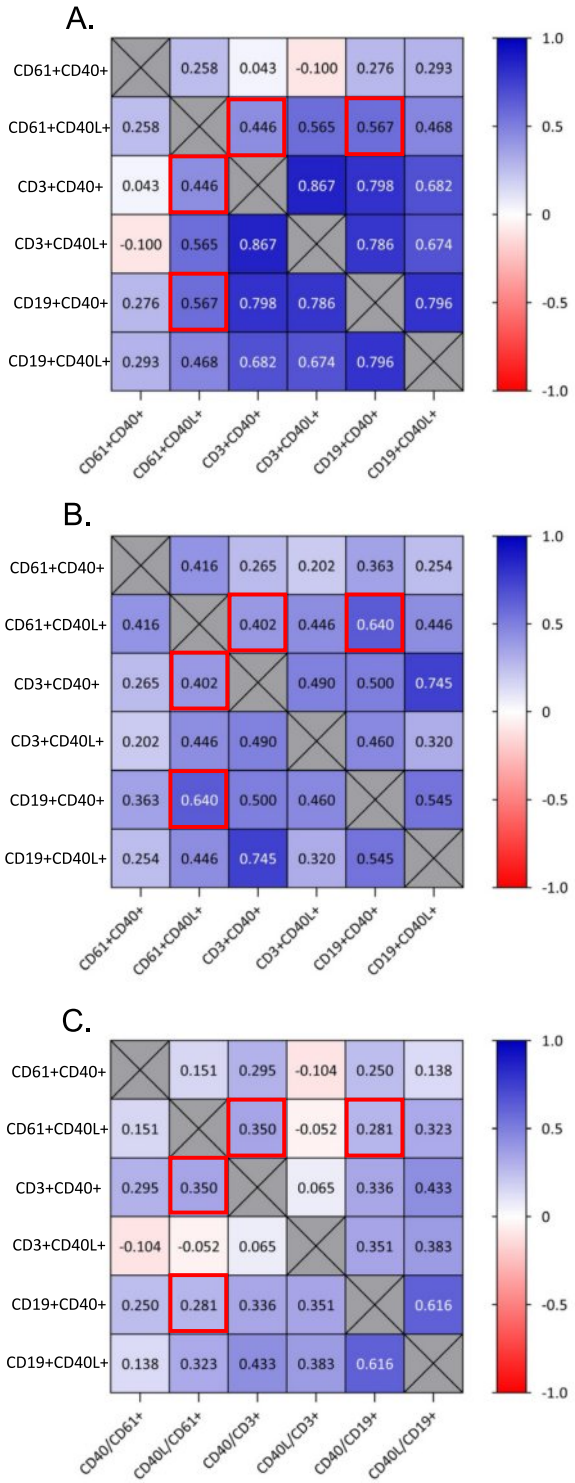
Discussion and conclusions

Recent studies have shown that excessive activation of platelets and lymphocytes in autoimmune diseases manifests as the formation of PLAs-Ly, characterized by unusually high adhesive abilities.[30] Platelets are believed to play a key role in mediating the adhesion of circulating lymphocytes to endothelial cells, facilitating their recruitment and initiating diapedesis.[31,32] However, these mechanisms have not yet been explored in MS.[19,33–35]

MS is a highly heterogeneous, neurodegenerative disorder characterized by an unpredictable clinical course and an elusive etiology. One of the hypotheses of MS pathogenesis suggests a vascular origin for the disease, related to the altered endothelium of intracerebral blood vessels caused by the pro-inflammatory milieu characteristic of MS.[36] Disrupting vascular wall integrity leads to the activation of blood platelets, which then adhere to inflamed endothelium and attract and form PLAs, promoting their infiltration across the permeable BBB into the CNS.[37] These cellular interactions may be crucial for the development of pathological events in the course of MS. The interactions between platelets, immune cells, and the endothelium are closely linked to the activation of all these cells, which can significantly impact blood vessel integrity and develop the local inflammation.[29]

Moreover, vascular lesions that lead to BBB disruption are critical for both the initiation and

Figure 11. The Spearman correlation matrices for the expression of CD40 and CD40L on platelets, T-cells, and B-cells across (A) RRMS ($n = 28$), (B) SPMS ($n = 27$), and (C) HC ($n = 50$) groups. The color gradient in the matrices represent the correlation coefficients (r) on a scale from -1 to 1. The numeric values within the boxes indicate the Spearman correlation coefficients. Results regarding the correlation of receptor-ligand co-expression of the CD40-CD40L pathway on platelets and T- or B-cells are highlighted with red outlines. The strength of the correlation is interpreted as follows: $|r| \leq 0.3$: weak correlation, indicating minimal relationship between the variables; $0.3 < |r| \leq 0.5$: moderate correlation, indicating a noticeable relationship; $0.5 < |r| \leq 0.7$: strong correlation, indicating a significant relationship; $|r| > 0.7$: very strong correlation, suggesting that one variable reflect most of the variation in the other variable.


A.
CD61+CD40+
CD61+CD40L+
CD3+CD40+
CD3+CD40L+
CD19+CD40+
CD19+CD40L+
0.258 0.043 -0.100 0.276 0.293
0.258 0.446 0.565 0.567 0.468
0.043 0.446 0.867 0.798 0.682
-0.100 0.565 0.867 0.786 0.674
0.276 0.567 0.798 0.786 0.796
0.293 0.468 0.682 0.674 0.796
CD61+CD40+ CD61+CD40L+ CD3+CD40+ CD3+CD40L+ CD19+CD40+ CD19+CD40L+
1.0 0.5 0 -0.5 -1.0
B.
CD61+CD40+
CD61+CD40L+
CD3+CD40+
CD3+CD40L+
CD19+CD40+
CD19+CD40L+
0.416 0.265 0.202 0.363 0.254
0.416 0.402 0.446 0.640 0.446
0.265 0.402 0.490 0.500 0.745
0.202 0.446 0.490 0.460 0.320
0.363 0.640 0.500 0.460 0.545
0.254 0.446 0.745 0.320 0.545
CD61+CD40+ CD61+CD40L+ CD3+CD40+ CD3+CD40L+ CD19+CD40+ CD19+CD40L+
1.0 0.5 0 -0.5 -1.0
C.
CD61+CD40+
CD61+CD40L+
CD3+CD40+
CD3+CD40L+
CD19+CD40+
CD19+CD40L+
0.151 0.295 -0.104 0.250 0.138
0.151 0.350 -0.052 0.281 0.323
0.295 0.350 0.065 0.336 0.433
-0.104 -0.052 0.065 0.351 0.383
0.250 0.281 0.336 0.351 0.616
0.138 0.323 0.433 0.383 0.616
CD40/CD61+ CD40L/CD61+ CD40/CD3+ CD40L/CD3+ CD40/CD19+ CD40L/CD19+
1.0 0.5 0 -0.5 -1.0

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progression of the disease. Epidemiological studies confirm an increased risk of ischemic events and other cardiovascular diseases in patients with MS, suggesting that MS is associated with vascular damage, primarily as a result of increased BBB permeability[38] and pro-thrombotic platelet activity.[39–45]

Cell-cell interactions serve as essential mechanisms through which platelets interconnect thrombosis and inflammation in MS. Platelet activation leads to the display of many receptors that are vital for their interactions with endothelial cells and leukocytes. This cellular cross-talk is mediated by the platelet GPIIb/IIIa receptor, which is essential for platelet aggregation,[46] CD62P, which bridges activated platelets and leukocytes,[47] and GPVI, which is responsible for cell adhesion to exposed extracellular matrix proteins,[48] facilitating their diapedesis through the vessel wall.[49] Our previous studies corroborated the notion of heightened platelet activity in MS,[23,50–52] along with capabilities of adhesion, aggregation, and complex formation with immune cells, as evidenced in this paper by increased expression of GPIIb/IIIa, GPVI and CD62P in MS patients.

The interaction between platelets and leukocytes is primarily mediated by the binding of platelet CD62P to its key ligand, P-selectin glycoprotein ligand-1 (PSGL-1), on the leukocyte surface. CD62P/PSGL-1 interaction has a functionally key role in leukocyte rolling and their adhesion to platelets and endothelium, which are critical steps in the process of leukocyte recruitment and extravasation.[53] Notably, while most lymphocytes, including all T-cell types, express PSGL-1, only 10–20% can bind CD62P due to the requirement for specific post-translational modifications.[54] Moreover, various subpopulations of leukocytes demonstrate a different affinity for CD62P present on platelets surface.[55] The CD62P/PSGL-1 connection also facilitates the interaction between PLAs and dysfunctional endothelium, a key event in blood vessels prone to inflammatory lesion development.[56]

Beyond direct physical receptor-mediated signaling, platelets engage in cell–cell cross-talk through chemotaxis *via* components of α-granule content, such as cytokines, chemokines, and lipid mediators.[57] By employing an *in vitro* cell migration assay, we confirmed the increased level of active leukocyte migration towards platelet isolated from MS patients *vs.* HC group. The Boyden chamber-based assay, a widely accepted method for studying cell migration, enabled us to assess the ability of platelets to attract leukocytes without physical contact.[58] According to the available literature, this phenomenon was demonstrated for the first time in MS in this work. However, the chemotactic abilities of blood platelets were previously described towards various cell types, including immune cells, stem cells, fibroblasts, and endothelial cells, not in MS condition.[59–63]

RANTES is known as one of the most crucial chemotactic proteins released from platelet α-granules during their activation.[64] In the presented work, we used the multi-antigen Bio-Plex assay, which assessed the concentration of this chemokine in plasma, allowing the results obtained from the Boyden chamber to be related to physiological conditions. The significantly increased RANTES plasma concentration in both RRMS and SPMS patients compared to controls confirmed the enhanced platelet chemotactic activity in MS. RANTES binds to chemokine receptors (CCR)1, CCR3, CCR4, and CCR5 on immune cells, promoting the activation and vascular migration of monocytes, dendritic cells, and neutrophils to injury sites, platelet aggregation, and PLAs formation,[57,65] contributing to atherosclerosis progression.[66–68] Additionally, RANTES plays a role in platelet-dependent lymphocyte differentiation[69] and facilitates immune cell transmigration across the BBB in autoimmune disorders, including MS.[64] T-cells in progressive MS show increased CCR5 expression and enhanced migration towards RANTES, predominantly exhibiting a Th1 phenotype.[70,71] Significantly elevated RANTES in progressive MS, also demonstrated in the current study, was suggested as a biomarker discriminating clinical phenotypes of the disease.[72] The level of this chemokine has been shown to be reduced in MS by interferon (IFN)-β-1b and 2-chlorodeoxyadenosine (cladribine) treatment.[73–75]

In this paper, we utilized two microscopic techniques, SEM and confocal microscopy, to complete our findings on increased platelet activation, aggregation readiness and chemotactic capabilities in MS. Our previous study documented significant platelet cytoskeletal alternations in MS patients, characterized by the formation of homo-aggregates and changes in platelet morphology and function.[26] Here, both imaging methods revealed the enhanced capacity of MS platelets to form extensive hetero-aggregates and intensify platelet structural changes in response to direct interactions with leukocytes (Figures 2–4).

Upon platelet activation, their cytoskeleton undergoes rearrangement, leading to changes in cell shape that are crucial for the formation and stabilization of aggregates within a blood clot. The elongation of thin, finger-like filopodia, containing bundles of actin filaments, marks the initial morphological change during activation.[76] This elongation facilitates platelet adhesion and fibrin strand formation.[77] Representative SEM images in this study showed an increased formation of these cellular protrusions, which promotes the generation of PLAs in MS (Figure 4A). As cell–cell interactions progress (Figure 4B), platelets increasingly accumulate around leukocytes, displaying finger-like and spiky filopodia, partially remodelled to flattened lamellipodia, forming a „fried egg“ structure. This

spreading increases the platelet surface area, enhancing interaction and stabilizing the PLAs structure. Moreover, signs of platelet degranulation are evident. In the advanced platelet aggregation stage (Figure 4C) a dense fibrin-coated clot clustering around the leukocyte is observed, reflecting the culmination of the PLA formation process. The fibrin coating indicates the involvement of the coagulation cascade, frequently activated in chronic inflammatory conditions such as MS.[24,51] The dense platelet aggregation around leukocyte within a fibrin network suggests a dual role for platelets in promoting coagulation and providing a scaffold for immune cells retention within inflamed tissues.[78] Our microscopic images illustrate the dual role of blood platelets as a link between the thrombotic and inflammatory processes important in the course of MS.

Confocal microscopy imaging provided complementary insights to the SEM findings, reinforcing the evidence of heightened platelet activation and PLA formation in MS compared to the quiescent state in HC. In MS, blood platelets appear enlarged, and irregularly shaped, and form massive clusters, increasing the surface area for trapping leukocytes. Interestingly, more intense staining of leukocyte markers was observed at contact sites with platelets, though only in some of them, suggesting that CD45 may relocate and accumulate in response to specific cellular interaction. We speculate that this observation might indicate a distinction between stable and transient PLAs types, consistent with the so-called "kiss and fly" interaction, described in real-time videomicroscopy, where circulating platelets briefly attach to stationary neutrophils adjacent to the endothelium of cerebral vessels before detaching and reentering circulation.[79] However, the exact mechanisms underlying this interaction remain unexplored.

Due to many studies focused on the cross-talk between blood platelets and immune cells, monocytes and neutrophils were identified as leukocytes with the highest affinity for platelets, while available insights into platelet-lymphocyte interactions in the context of autoimmune response remain scarce. Thus, our main research task was to determine the capability of formation of PLAs-Ly in MS.

Early research on PLAs-Ly reported that the propensity for forming these cellular complexes varies between lymphocyte subpopulations and depends on cellular activation. For example, activation of T-cells increases their aggregation with platelets, while platelet activation significantly raises aggregation with natural killer (NK) cells and slightly with Tcyt cells. However, neither platelet nor B-cell activation increased PLAs-LyB formation.[30] In turn, our MS study unexpectedly reveals the highest percentage of B-cells in PLAs-Ly formation among all subsets measured. The study of Li et al.[30] focused on healthy subjects, whose platelets and lymphocyte subsets were activated by specific agonists. Still, it is known that the formation of PLAs can vary greatly depending on factors like pro-thrombotic and pro-inflammatory conditions, stress, temperature, and pH.[80] It might explain the discrepancies between our study, which focused on platelet activation under MS-related chronic conditions, and those mentioned previously,[30] involving *in vitro* stimulated cells.

Recently, the role of B-cells in MS has become a focus of intensive research.[81] Studies indicate the presence of intrathecal oligoclonal bands (OCBs) induced by mature B-cell-derived plasma cells in MS,[82] and antigen-specific memory B-cells have been detected in the CNS and MS lesions.[83] These findings have led to the implementation of high efficiency disease modifying therapies (DMTs) using rituximab, ocrelizumab and ofatumumab, which deplete B-cells by targeting the CD20 surface molecule.[84] Neuropathological evidence confirms the effectiveness of anti-CD20 therapy in reducing disease activity without altering OCB patterns or immunoglobulin levels.[8] The next novel MS therapies for both relapsing and progressive patients are currently in clinical trials.[85] These include inhibitors of Bruton's tyrosine kinase (BTK), an enzyme in the B-cell receptor (BCR) signaling pathway that aids in the development of autoreactive B-cells and plays a role in activating myeloid cells.[86] The success of these next-generation therapies especially underscores the importance of further exploring the specific contributions of B-cells to MS pathology and prompted us to focus on PLAs-LyB. Uncovering alternative B-cell involvement in disease mechanisms may help develop less intensive therapeutic options, potentially improving patient outcomes without the need for highly aggressive treatments.

Zamora et al.[87] found that platelet attachment to lymphocytes in systemic lupus erythematosus (SLE) patients may influence B-cell response and disease pathogenesis. Specifically, the percentage of B-cells with attached platelets correlated with soluble CD40L, with pre-switched memory B-cells showing the highest platelet attachment. In MS, memory B-cells exhibit increased proliferation after CD40 stimulation, while the absence of CD40 signaling severely impairs B-cell activation. Dysregulation of CD40-dependent nuclear factor (NF)-κB signaling is thought to lead to excessive B cell proliferation in MS.[88] In our study, we observed a significant correlation between platelet CD40L and lymphocytic CD40, particularly on B-cells in SPMS, which may underscore the role of platelets in B-cell activity and the engagement of platelet-B-cell interactions, especially in the progressive phenotype of

Table 1 Clinical characteristics of all HCs, RRMS, and SPMS patients recruited to the study. Parameters are presented as mean ± SD.

Clinical characteristics	HC (n = 55)	RRMS (n = 38)	SPMS (n = 55)
Age (years)	45 ± 12	48 ± 11	61 ± 9
Gender (number of female/male)	33/22	24/14	33/22
Leukocytes ($\times 10^3/\mu L$)	6.39 ± 1.5	6.64 ± 1.51	7.09 ± 2.13
Erythrocytes ($\times 10^6/\mu L$)	4.93 ± 0.45	4.47 ± 0.6	4.33 ± 0.58
Platelets ($\times 10^3/\mu L$)	266.65 ± 62.59	307.46 ± 114.4	305.15 ± 108.46
CRP[a] (mg/L)	2.29 ± 1.99	6.97 ± 9.45	8.66 ± 8
Time since last relapse (months)	N/A	3 ± 2	N/A
Disease duration (years)	N/A	14 ± 9	31 ± 8
EDSS[b]	N/A	5.5 ± 1.2	5.8 ± 0.6
mRS[c]	N/A	3.5 ± 0.8	3.8 ± 0.7
BDI[d]	N/A	9.3 ± 4.6	15.0 ± 8.9
MoCA[e]	N/A	25.3 ± 4.1	26.0 ± 3.3

[a] CRP, C-reactive protein.
[b] EDSS, Expanded Disability Status Scale.
[c] mRS, Modified Rankin Scale.
[d] BDI, Beck Depression Inventory.
[e] MoCA, Montreal Cognitive Assessment.

the disease. This CD40-CD40L pathway appears to be a crucial pathway by which platelets can contribute to the inflammatory cascade.[89]

Although our study offers valuable insights into platelet-lymphocyte interactions in MS, several limitations must be acknowledged. Understanding the mechanisms underlying PLAs-Ly formation is challenging due to the various environmental factors influencing this process in MS patients. This research provides an initial evaluation of the actual physical state of PLAs-Ly and their potential association with the CD40-CD40L activation pathway, but an in-depth investigation into the specific molecular mechanisms, including cell signaling pathways and animal models, was beyond the scope of this study. Given the exploratory nature of the present study, we prioritized broader trends over phenotype-specific differences to establish a foundation for future, more targeted research. We believe that a detailed investigation using the cell migration assay in RRMS and SPMS groups may provide valuable insights into the mechanisms underlying this phenomenon in acute and progressive condition of the disease. Additionally, excluding of MS patients receiving DMTs to minimize confounding factors limited our ability to assess the impact of these treatments on PLAs formation. Future studies should include cohorts of patients on different DMT regimens to more thoroughly explore the effects of therapy on PLA dynamics.

The excessive activation of platelets in MS, accompanied by their heightened adhesive and chemotactic abilities, fosters the formation of PLAs-Ly. Our findings suggest that B-cells, often overlooked in platelet-leukocyte cross-talk studies in MS, may play a pivotal role in this interaction. This is particularly relevant in light of emerging therapies targeting B-cells, such as anti-CD20 or BTK inhibitors treatments, which underscore the need for further exploration of B-cell-driven mechanisms in MS pathology. The observed correlations between platelet CD40L and lymphocytic CD40 in SPMS further point to the importance of the CD40-CD40L signaling pathway in platelet-B-cell interactions and the progressive stages of the disease.

To our knowledge, this is the first report of PLAs-LyB in MS, shedding new light on the adaptive immune response, particularly in progressive MS, and offering promise for further advances in B-cell-targeted therapies.

Material and Methods

Study groups

The study included 93 MS patients and 55 HCs, that clinical characteristics are presented in Table 1.

A total of 93 MS patients (38 RRMS and 55 SPMS) were qualified to the study. Participants were diagnosed based on the McDonald criteria (2017)[90] and disease phenotype ascertained as defined by Lublin et al.[91] The patients were recruited from the Department of Rehabilitation, Neurological Rehabilitation Division of III[rd] General Hospital in Lodz, Poland. All participants provided informed consent and completed a medical questionnaire before enrollment in the study. Prior to their inclusion, MS patients underwent medical investigation. Radiological assessments using magnetic resonance imaging (MRI) were conducted to determine the grey matter pathology and the volumes of white matter lesions. The disability status of MS patients was evaluated using the Expanded Disability Status Scale (EDSS)[92] and the modified Rankin scale (mRS).[93] Assessment with the Beck Depression Inventory (BDI)[94] was used to measure the severity of depression, while the Montreal Cognitive Assessment (MoCA)[95]

was used as tests for early detection of difficulties in spatial perception, naming, focus, language skills, memory, and orientation. Patients were excluded from the study based on the following criteria: use of medications affecting the biological activity of blood platelets; presence of additional neurological comorbidities and psychiatric disorders; treatment with disease-modifying drugs (such as interferon (IFN)-β, glatiramer acetate, natalizumab), hormones, corticosteroids, or immunomodulators; any infections within previous 4 weeks; diagnosis of diabetes mellitus or myocardial infarction; pregnancy, breastfeeding, or lack of the signed informed consent.

The study also included 55 HC participants, recruited from the Laboratory Diagnostics Center in Lodz. These individuals were not diagnosed with MS, were not pregnant or breastfeeding, and were free from autoimmune, neurodegenerative, and other CNS disorders, as well as chronic or acute inflammation. They were also medication-free. The HC group was matched to the MS group regarding age and gender. Each HC underwent a comprehensive health assessment, including basic morphology and inflammatory parameters, to confirm their health status.

Clinical material collection and preparation

The study material was blood samples drawn by venipuncture between 8 and 9 a.m. using Sarstedt® tubes (Nümbrecht, Germany) containing citrate phosphate dextrose adenine-1 (CPDA-1) as an anticoagulant. The freshly obtained blood samples were immediately processed according to the appropriate protocol for the analytical method applied.

Cell migration assay

The cell migration assay was conducted as a pilot study to refine and optimize the protocols for obtaining reliable results. Due to the exploratory nature of this phase, a reduced sample size was used, including MS patients (n = 12) and HC volunteers (n = 12).

Fresh whole blood samples were collected and immediately processed. The samples were centrifuged at 235g for 12 min. at 25 °C to separate the platelet-rich plasma (PRP). The PRP, forming the top layer after centrifugation, was carefully collected. Prostaglandin E1 (PGE1) (Sigma-Aldrich, USA) was added to the PRP at a final concentration of 1 μM to inhibit spontaneous platelet activation. To remove leukocyte and erythrocyte contamination, PRP was purified using MicroBeads conjugated to monoclonal human CD45 and CD235a antibodies, and MS MACS Columns (Miltenyi Biotec, USA), according to the manufacturer's protocol. Leukocyte presence after magnetic separation was evaluated using flow cytometry, employing a dual-staining strategy, with anti-CD61 BB515 (platelet marker) and anti-CD45 PE (leukocyte marker) antibodies, ensuring the effectiveness of the separation process. The platelet count was determined spectrophotometrically, and the concentration was adjusted to 2×10^8 platelets/mL using modified Tyrode's buffer for use in the cell migration assay. The concentration was selected based on the common recommendations to simulate physiological conditions and was further validated through the preliminary optimization tests.

PBMCs, which include lymphocytes (T cells, B cells, and NK cells) and monocytes, were isolated from whole blood by density gradient centrifugation using Gradisol G (1.115 g/mL, Aqua-Med, Poland). Further purification of leukocytes was performed using MicroBeads conjugated to monoclonal human CD45 antibodies and MS MACS Columns (Miltenyi Biotec, USA), according to the manufacturer's instructions. The cell pellet was resuspended in modified RPMI 1640 medium (Sigma-Aldrich, USA) containing 0.5% BSA, 2 mM $CaCl_2$, and 2 mM $MgCl_2$. The cell count was determined using a TC20 Automated Cell Counter (Bio-Rad Laboratories, Inc., USA), and the density was adjusted to 2×10^6 cells/mL for the cell migration assay. The selected cell density was based on standard protocols and was further validated through the preliminary optimization tests.

The *in vitro* cell migration assay was performed using the Boyden chamber,[96] specifically the CytoSelect™ 96-Well Cell Migration Assay kit (5 μm, Fluorometric Format) (Cell Biolabs, Inc., USA) according to the manufacturer's protocol. 150 μL of the platelet suspension (2×10^8 platelets/mL) was transferred in the lower chamber. 100 μL of the Peripheral blood mononuclear cells (PBMCs) suspension (2×10^6 cells/mL) was added to the upper chamber. The plate was incubated for 16 h at 37 °C in a 5% CO_2. No fetal calf serum was added to either chamber to maintain the integrity of the chemotactic gradient. After incubation, a mixture of cell Lysis Buffer with fluorescent CyQuant® GR Dye (this dye quantifies the migrated cells based on cellular nucleic acid content) was added to each well, and the relative fluorescence units (RFU) were measured using a microplate reader (BioTek Synergy H1). Results were adjusted for blank measurements to ensure accuracy. Cells in serum-free media migrating towards 10% fetal bovine serum (FBS) were applied as a positive control. A random migration control was cells in serum-free media without a specific chemoattractant to assess the baseline migration level.

Measurement of RANTES concentration by Bio-Plex system

The study included 93 MS patients (38 RRMS, 55 SPMS) along with 55 HC.

RANTES was concentration measured in plasma samples using the Luminex-based Bio-Plex Pro™ system (Bio-Rad Laboratories, Inc., USA), following the manufacturer's instructions. Before use, the instrument was calibrated to standardize the fluorescent signal and validated to ensure optimal performance of fluidics and optics systems.

The plasma samples were thawed, centrifugated and diluted with sample diluent buffer. Antibody-coupled beads were incubated with either the diluted plasma samples, standards or quality controls on shaker, followed by incubation with biotin-labeled detection antibodies and streptavidin-phycoerythrinAfter that, assay buffer was added and plate was shaken to re-suspend the beads for plate reading. Fluorescence was measured using the Bio-Plex® 200 System analyzer (Bio-Rad Laboratories, Inc., USA). The RANTES concentration were calculated using Bio-Plex Manager™ software (Bio-Rad Laboratories, Inc., USA).

Scanning electron microscopy (SEM)

The imaging of PLAs and the preparation of microscope slides were conducted following the protocol by Dziedzic et al.[26] Freshly isolated blood platelets were placed on 12 mm diameter coverslips precoated with poly-L-lysine (Merck, USA) and incubated at RT for 5 min. To fix the samples, a solution of 2.5% glutaraldehyde in 0.1 M phosphate buffered saline (PBS) (pH 7.4) was applied and incubated at RT for 10 min. After fixation, the coverslips were washed three times with PBS and incubated with 4% OsO4 in 0.1 M PBS (pH 7.4) at 4 °C for 16 h. Dehydration was performed using a Leica EM TP automatic tissue processor (Leica Microsystems, GmbH) with a series of ethanol and subsequent acetone washes (ethanol: 30%, 50%, 70%, 80%, 90%, 96%, 100%; acetone: 30%, 50%, 100%) for 10 min. each. The coverslips were then dried using a Leica EM CPD300 critical point dryer (Leica Microsystems, GmbH). Finally, they were mounted on stubs using carbon adhesives and coated with a 6 nm gold layer using a Leica EM ACE200 (Leica Microsystems, GmbH). The samples were examined with a Phenom ProX scanning electron microscope (ThermoFisher, USA).

Flow cytometry

Flow cytometry measurements require immediate processing of freshly collected blood to prevent cell degradation and activation, which could introduce variability into the results. For this reason, the flow cytometry analyses included carefully selected groups of MS patients (n = 55) (28 RRMS and 27 SPMS) and HC (n = 50).

For the assay, 20 μL of whole blood was directly stained with a prepared cocktail of fluorochrome-conjugated anti-human monoclonal antibodies (mAb) and incubated at RT for 30 min. in the dark. Subsequently, red blood cells were lysed using 1X BD FACS™ Lysing Solution (Becton Dickinson, USA) at RT for 20 min. and analyzed by flow cytometry.

Five distinct flow cytometry panels were employed to determine the platelet activation status (Panel 1 and 2); to immunophenotype lymphocytes subpopulations within PLAs-Ly (Panel 3); to assess CD40-CD40L pathway antigens exposure on platelets (Panel 4); to evaluate CD40-CD40L pathway antigens exposure on T-cells and B-cells (Panel 5). The details regarding each panel design are provided in Table 2.

Flow cytometry was performed using CYTOFlex System B5-R3-V5 (Beckman Coulter, USA) with CytExpert 2.0 software and BD FACSymphony A1 (Becton Dickinson, USA) with software BD FACSDiva 9.0.2. The compensation procedure was performed using VersaComp Antibody Capture Bead Kit (Beckman Coulter, Brea, CA, USA) as recommended by the manufacturer.

Analysis of the obtained data was performed using Beckman Coulter Kaluza Analysis Software. For Panels 1, 2 and 4, initial gating identified platelets based on forward scatter (FS) and side scatter (SS) characteristics and CD61 PC5.5 marker fluorescence. The expression levels of platelet surface antigens, including GPVI PE, PAC-1 FITC, CD62P PE, CD40 APC, and CD40L PE, were then measured based on their fluorescence on $CD61^+$ objects and reported as MFI. For Panel 3, T-cells and B-cells were similarly gated based on FS and SS characteristics and the fluorescence of CD3 APC-Alexa Fluor 750 and CD19 ECD markers. Within the $CD3^+$ population, subpopulations of T-cells were differentiated using the markers CD4 APC (Th cells), CD8 Alexa Fluor700 (Tcyt cells), CD25 PC7 (Treg cells). PLAs-Ly within each gated subpopulation were then identified based on CD61 PC5.5 fluorescent labeling and expressed as percentages of the total gated pool of each lymphocyte subpopulation. For Panel 5, T-cells and B-cells were gated based on FS and SS and the fluorescence of CD3 APC-Alexa Fluor 750 and CD19 ECD markers, respectively. The expression levels of CD40 APC and CD40L PE were subsequently measured on the $CD3^+$ or $CD19^+$ populations and presented as MFI.

Confocal microscopy

The aliquots of 20 μL of whole blood were directly incubated with a prepared cocktail of fluorochrome-conjugated anti-human mAbs (BD Biosciences) in three sets: anti-CD61 BB515 and anti-CD45 PE (set 1); anti-CD61 BB515 and anti-CD3 APC (set 2); anti-CD61 APC and anti-CD19 Alexa Fluor 488 (set 3). The samples were incubated with antibodies at RT for 30 min in the dark. Next, red blood cells were lysed with BD FACS™ Lysing

Solution at RT for 20 min. Samples prepared in this way were placed into thin-bottom 96-well microplate (SCREENSTAR, Greiner Bio-One, Kremsmünster, Austria) and visualized immediately with LSM 780 confocal microscope (Zeiss, Oberkochen, Germany) or with Leica TCS SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany).

While imaging with LSM 780 confocal microscope Plan-Apochromat 63x/1.40 objective was used. Samples were excited with 488 nm argon laser line (Alexa Fluor 488 or BB515 dyes), InTune tunable laser diode set to 550 nm (PE dye) or with 633 nm HeNe laser (APC dye). Fluorescence of each sample was imaged sequentially and separately in both channels. Fluorescence of

Table 2 Protocol of the panels design applied for flow cytometric analyses of platelet phenotype and activation status, immunophenotyping of lymphocytes forming aggregates with platelets, and CD40-CD40L axis antigens on platelet and B/T-cells.

mAb with fluorochrome	mAb working dilution	Role
Panel 1. Platelets activation		
anti-CD61 PC5.5[a] (Beckman Coulter, USA)	1:50	GPIIIa, major receptor for fibrinogen, marker of platelet identification
anti-GPVI[b] PE[c] (BD Biosciences, USA)	1:12.5	Receptor for collagen
Panel 2. Platelets activation status cont.		
anti-CD61 PC5.5 (Beckman Coulter, USA)	1:50	GPIIIa, major receptor for fibrinogen, marker of platelet identification
anti-PAC-1[d] FITC[e] (BD Biosciences, USA)	1:12.5	Receptor for GPIIb/IIIa, marker of platelet activation
anti-CD62P PE (BD Biosciences, USA)	1:12.5	P-selectin, marker of platelet activation
Panel 3. Immunophenotyping of lymphocytes in aggregates with platelets		
anti-CD61 PC5.5 (Beckman Coulter, USA)	1:50	GPIIIa, major receptor for fibrinogen, marker of platelet identification
anti-CD3 APC-Alexa Fluor 750 (Beckman Coulter, USA)	1:50	T-cell marker
anti-CD19 ECD (Beckman Coulter, USA)	1:50	B-cell marker
anti-CD4 APC (BD Biosciences, USA)	1:50	Th[f] cell marker
anti-CD8 APC-Alexa Fluor 700 (Beckman Coulter, USA)	1:50	Tcyt[g] cell marker
anti-CD25 PC7 (Beckman Coulter, USA)	1:50	Treg[h] cell marker
Panel 4. CD40-CD40L axis activation on platelets		
anti-CD61 PC5.5 (Beckman Coulter, USA)	1:50	GPIIIa, major receptor for fibrinogen, marker of platelet identification
anti-CD40 APC[i] (Beckman Coulter, USA)	1:50	Costimulatory protein in CD40-CD40L immune response axis
anti-CD154 (CD40L) PE (Beckman Coulter, USA)	1:12.5	CD40 ligand
Panel 5. CD40/CD40L axis activation on T-cells and B-cells		
anti-CD3 APC-Alexa Fluor 750 (Beckman Coulter, USA)	1:50	T-cell marker
anti-CD19 ECD[j] (Beckman Coulter, USA)	1:50	B-cell marker
anti-CD40 APC (Beckman Coulter, USA)	1:50	Costimulatory protein in CD40-CD40L immune response axis
anti-CD154 (CD40L) PE (Beckman Coulter, USA)	1:12.5	CD40 ligand

[a] PC, phycoerythrin cyanin.
[b] GP, glycoprotein.
[c] PE, phycoerythrin.
[d] PAC-1, first procaspase activating compound.
[e] FITC, fluorescein isothiocyanate.
[f] Th, T helper.
[g] Tcyt, T cytotoxic.
[h] Treg, T regulatory.
[i] APC, allophycocyanin.
[j] ECD, phycoerythrin-texas red.

Alexa Fluor 488 and BB515 was recorded in 495–550 nm range, fluorescence of PE was recorded in 553–600 nm range, and fluorescence of APC was recorded in 638–743 nm range. For 3D visualization, z-stack imaging of the sample was performed every 0.4 μm with 1 μm thick optical cross section, image rendering was done with ZEN 2012 SP5 software (Zeiss).

While imaging with TCS SP8 confocal microscope 63x/1.40 objective (HC PL APO CS2) was used. Samples were excited with 489 nm WLL laser (White Light Laser) for BB515 dye and 500 nm for PE. Fluorescence of each sample was imaged sequentially and separately in both channels. Fluorescence of BB515 was recorded in 500–535 nm range, and fluorescence of PE was recorded in 550–605 nm range. Cell visualization was performed using Leica Application Suite X (LASX; Leica Microsystems), while the results were processed with Leica LAS2.0.215022 software (Leica Microsystems, Wetzlar, Germany).

Statistical analysis

Statistical analysis and graphs generation were performed using GraphPad Prism 9.5.0. (GraphPad Software, Inc.). For each analysis, the group size was considered to ensure appropriate statistical power and accuracy. The Shapiro-Wilk test was employed to assess the normality of the distribution of the sample data. The Kruskal-Wallis test was used while assessing the statistical significance of differences between three independent groups for non-normally distributed data. Following the Kruskal-Wallis test, Dunn's post-hoc test was applied to perform pairwise comparisons between the groups. For comparisons between two groups, the unpaired *t*-test with Welch's correction was utilized. To analyze the strength and direction of the association between two ranked variables, the Spearman's rank correlation coefficient was used. A *p*-value of <0.05 was considered statistically significant for all tests.

CRediT authorship contribution statement

Karina Maciak: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Angela Dziedzic:** Writing – review & editing, Project administration, Methodology, Investigation. **Jacek Szymański:** Visualization, Methodology, Investigation, Formal analysis, Data curation. **Maciej Studzian:** Writing – review & editing, Visualization, Methodology, Investigation. **Justyna Redlicka:** Resources. **Elżbieta Miller:** Resources. **Sylwia Michlewska:** Visualization, Methodology. **Piotr Jóźwiak:** Visualization, Methodology. **Joanna Saluk:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

DATA AVAILABILITY

Data will be made available on request.

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.

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Ethics approval

The study was performed in accordance with the Declaration of Helsinki and approved by the University of Lodz Research Bioethics committee with resolution No. 3/KBBN- UŁ/IV/2018.

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References

1. Atlas of MS 2020 – Epidemiology report, MS International Federation (n.d.). https://www.msif.org/resource/atlas-of-ms-2020/ (accessed March 28, 2023).

2. Publications, ECTRIMS (n.d.). https://ectrims.eu/ms-clinical-trials-committee/publications/ (accessed July 27, 2024).
3. Lublin, F.D., Coetzee, T., Cohen, J.A., Marrie, R.A., Thompson, A.J., on behalf of the International Advisory Committee on Clinical Trials in MS, (2013). The 2013 clinical course descriptors for multiple sclerosis. *Neurology* **94** (2020), 1088–1092. https://doi.org/10.1212/WNL.0000000000009636.
4. Kuhlmann, T., Moccia, M., Coetzee, T., Cohen, J.A., Correale, J., Graves, J., Marrie, R.A., Montalban, X., Yong, V.W., Thompson, A.J., Reich, D.S., Amato, M.P., Banwell, B., Barkhof, F., Chataway, J., Chitnis, T., Comi, G., Derfuss, T., Finlayson, M., Goldman, M., Green, A., Hellwig, K., Kos, D., Miller, A., Mowry, E., Oh, J., Salter, A., Sormani, M.P., Tintore, M., Helen Tremlett, M., van der Trojano, A., Walt, S., Vukusic, E.W., (2023). Multiple sclerosis progression: time for a new mechanism-driven framework. *Lancet Neurol.*. **22**, 78–88. https://doi.org/10.1016/S1474-4422(22)00289-7.
5. Dobson, R., Giovannoni, G., (2019). Multiple sclerosis – a review. *Eur. J. Neurol.* **26**, 27–40. https://doi.org/10.1111/ene.13819.
6. Liu, R., Du, S., Zhao, L., Jain, S., Sahay, K., Rizvanov, A., Lezhnyova, V., Khaibullin, T., Martynova, E., Khaiboullina, S., Baranwal, M., (2022). Autoreactive lymphocytes in multiple sclerosis: pathogenesis and treatment target. *Front. Immunol.* **13**, 996469 https://doi.org/10.3389/fimmu.2022.996469.
7. Pukoli, D., Vécsei, L., (2023). Smouldering Lesion in MS: microglia, lymphocytes and pathobiochemical mechanisms. *Int. J. Mol. Sci.* **24**, 12631. https://doi.org/10.3390/ijms241612631.
8. Jain, R.W., Yong, V.W., (2022). B cells in central nervous system disease: diversity, locations and pathophysiology. *Nature Rev. Immunol.* **22**, 513–524. https://doi.org/10.1038/s41577-021-00652-6.
9. Cencioni, M.T., Mattoscio, M., Magliozzi, R., Bar-Or, A., Muraro, P.A., (2021). B cells in multiple sclerosis — from targeted depletion to immune reconstitution therapies. *Nature Rev. Neurol.* **17**, 399–414. https://doi.org/10.1038/s41582-021-00498-5.
10. Yan, C., Wu, H., Fang, X., He, J., Zhu, F., (2023). Platelet, a key regulator of innate and adaptive immunity. *Front Med (Lausanne)* **10**, 1074878 https://doi.org/10.3389/fmed.2023.1074878.
11. Dib, P.R.B., Quirino-Teixeira, A.C., Merij, L.B., Pinheiro, M. B.M., Rozini, S.V., Andrade, F.B., Hottz, E.D., (2020). Innate immune receptors in platelets and platelet-leukocyte interactions. *J. Leukoc. Biol.* **108**, 1157–1182. https://doi.org/10.1002/JLB.4MR0620-701R.
12. Zahran, A.M., El-Badawy, O., Mohamad, I.L., Tamer, D.M., Abdel-Aziz, S.M., Elsayh, K.I., (2018). Platelet activation and platelet-leukocyte aggregates in type I diabetes mellitus. *Clin. Appl. Thromb. Hemost.*. https://doi.org/10.1177/1076029618805861.
13. Loguinova, M., Pinegina, N., Kogan, V., Vagida, M., Arakelyan, A., Shpektor, A., Margolis, L., Vasilieva, E., (2018). Monocytes of different subsets in complexes with platelets in patients with myocardial infarction. *Thromb. Haemost.* **118**, 1969–1981. https://doi.org/10.1055/s-0038-1673342.
14. Popp, S.K., Vecchio, F., Brown, D.J., Fukuda, R., Suzuki, Y., Takeda, Y., Wakamatsu, R., Sarma, M.A., Garrett, J., Giovenzana, A., Bosi, E., Lafferty, A.R., Brown, K.J., Gardiner, E.E., Coupland, L.A., Thomas, H.E., Chong, B. H., Parish, C.R., Battaglia, M., Petrelli, A., Simeonovic, C. J., (2022). Circulating platelet-neutrophil aggregates characterize the development of type 1 diabetes in humans and NOD mice. *JCI Insight* **7**, e153993 https://doi.org/10.1172/jci.insight.153993.
15. Banka, A.L., Guevara, M.V., Brannon, E.R., Nguyen, N.Q., Song, S., Cady, G., Pinsky, D.J., Uhrich, K.E., Adili, R., Holinstat, M., Eniola-Adefeso, O., (2023). Cargo-free particles divert neutrophil-platelet aggregates to reduce thromboinflammation. *Nature Commun.* **14**, 2462. https://doi.org/10.1038/s41467-023-37990-z.
16. Srihirun, S., Sriwantana, T., Srichatrapimuk, S., Vivithanaporn, P., Kirdlarp, S., Sungkanuparph, S., Phusanti, S., Nanthatanti, N., Suwannalert, P., Sibmooh, N., (2023). Increased platelet activation and lower platelet-monocyte aggregates in COVID-19 patients with severe pneumonia. *PLoS One* **18**, e0282785 https://doi.org/10.1371/journal.pone.0282785.
17. Meikle, C.K., Meisler, A.J., Bird, C.M., Jeffries, J.A., Azeem, N., Garg, P., Crawford, E.L., Kelly, C.A., Gao, T. Z., Wuescher, L.M., Willey, J.C., Worth, R.G., (2020). Platelet-T cell aggregates in lung cancer patients: implications for thrombosis. *PLoS One* **15**, e0236966 https://doi.org/10.1371/journal.pone.0236966.
18. Maiorca, F., Lombardi, L., Marrapodi, R., Pallucci, D., Sabetta, A., Zingaropoli, M.A., Perri, V., Flego, D., Romiti, G.F., Corica, B., Miglionico, M., Russo, G., Pasculli, P., Ciardi, M.R., Mastroianni, C.M., Ruberto, F., Pugliese, F., Pulcinelli, F., Raparelli, V., Cangemi, R., Visentini, M., Basili, S., Stefanini, L., (2023). Breakthrough infections after COVID-19 vaccinations do not elicit platelet hyperactivation and are associated with high platelet-lymphocyte and low platelet-neutrophil aggregates. *Res Pract Thromb Haemost* **7**, 102262 https://doi.org/10.1016/j.rpth.2023.102262.
19. Albayati, S., Li, N., Unsworth, A.J., Liverani, E., (2022). Platelet-lymphocyte co-culture serves as an ex vivo platform of dynamic heterotypic cross-talk. *J Cell Commun Signal* **16**, 661–675. https://doi.org/10.1007/s12079-022-00676-0.
20. Dai, X.-P., Wu, F.-Y., Cui, C., Liao, X.-J., Jiao, Y.-M., Zhang, C., Song, J.-W., Fan, X., Zhang, J.-Y., He, Q., Wang, F.-S., (2021). Increased platelet-CD4+ T cell aggregates are correlated with HIV-1 permissiveness and CD4+ T cell loss. *Front. Immunol.* **12**, 799124 https://doi.org/10.3389/fimmu.2021.799124.
21. Carnaz Simões, A.M., Holmström, M.O., Aehnlich, P., Rahbech, A., Radziwon-Balicka, A., Zamora, C., Wirenfeldt Klausen, T., Skov, V., Kjær, L., Ellervik, C., Fassi, D.E., Vidal, S., Hasselbalch, H.C., Andersen, M.H., Thor Straten, P., (2022). Patients with myeloproliferative neoplasms harbor high frequencies of CD8 T cell-platelet aggregates associated with T cell suppression. *Front. Immunol.* **13**, 866610 https://doi.org/10.3389/fimmu.2022.866610.
22. Dziedzic, A., Morel, A., Miller, E., Bijak, M., Sliwinski, T., Synowiec, E., Ceremuga, M., Saluk-Bijak, J., (2020). Oxidative damage of blood platelets correlates with the degree of psychophysical disability in secondary progressive multiple sclerosis. *Oxid. Med. Cell. Longev.* **2020**, 2868014 https://doi.org/10.1155/2020/2868014.
23. Morel, A., Bijak, M., Miller, E., Rywaniak, J., Miller, S., Saluk, J., (2015). Relationship between the increased

haemostatic properties of blood platelets and oxidative stress level in multiple sclerosis patients with the secondary progressive stage. *Oxid. Med. Cell. Longev.* **2015**, 240918 https://doi.org/10.1155/2015/240918.
24. Bijak, M., Olejnik, A., Rokita, B., Morel, A., Dziedzic, A., Miller, E., Saluk-Bijak, J., (2019). Increased level of fibrinogen chains in the proteome of blood platelets in secondary progressive multiple sclerosis patients. *J. Cell Mol. Med.* **23**, 3476–3482. https://doi.org/10.1111/jcmm.14244.
25. Orian, J.M., D'Souza, C.S., Kocovski, P., Krippner, G., Hale, M.W., Wang, X., Peter, K., (2021). Platelets in multiple sclerosis: early and central mediators of inflammation and neurodegeneration and attractive targets for molecular imaging and site-directed therapy. *Front. Immunol.* **12**, 620963 https://doi.org/10.3389/fimmu.2021.620963.
26. Dziedzic, A., Michlewska, S., Jóźwiak, P., Dębski, J., Karbownik, M.S., Łaczmański, Ł., Kujawa, D., Glińska, S., Miller, E., Niwald, M., Kloc, M., Balcerzak, Ł., Saluk, J., (2024). Quantitative and structural changes of blood platelet cytoskeleton proteins in multiple sclerosis (MS). *J. Autoimmun.* **145**, 103204 https://doi.org/10.1016/j.jaut.2024.103204.
27. Parker, W.A.E., Storey, R.F., (2024). The role of platelet P2Y12 receptors in inflammation. *Br. J. Pharmacol.* **181**, 515–531. https://doi.org/10.1111/bph.16256.
28. Li, Z., Smyth, S.S., (2019). 16 – Interactions between platelets, leukocytes, and the endothelium. In: Michelson, A.D. (Ed.), *Platelets*. 4th ed. Academic Press, pp. 295–310. https://doi.org/10.1016/B978-0-12-813456-6.00016-3.
29. Lv, W., Jiang, X., Zhang, Y., (2024). The role of platelets in the blood-brain barrier during brain pathology. *Front. Cell. Neurosci.* **17**, 1298314 https://doi.org/10.3389/fncel.2023.1298314.
30. Li, N., Ji, Q., Hjemdahl, P., (2006). Platelet–lymphocyte conjugation differs between lymphocyte subpopulations. *J. Thromb. Haemost.* **4**, 874–881. https://doi.org/10.1111/j.1538-7836.2006.01817.x.
31. Diacovo, T.G., Catalina, M.D., Siegelman, M.H., von Andrian, U.H., (1998). Circulating activated platelets reconstitute lymphocyte homing and immunity in L-selectin-deficient mice. *J. Exp. Med.* **187**, 197–204. https://doi.org/10.1084/jem.187.2.197.
32. Diacovo, T.G., Puri, K.D., Warnock, R.A., Springer, T.A., von Andrian, U.H., (1996). Platelet-mediated lymphocyte delivery to high endothelial venules. *Science* **273**, 252–255. https://doi.org/10.1126/science.273.5272.252.
33. Kuznik, B.I., Vitkovsky, Y.A., Gvozdeva, O.V., Solpov, A. V., Magen, E., (2014). Lymphocyte-platelet crosstalk in Graves' disease. *Am. J. Med. Sci.* **347**, 206–210. https://doi.org/10.1097/MAJ.0b013e3182831726.
34. Hu, H., Zhu, L., Huang, Z., Ji, Q., Chatterjee, M., Zhang, W., Li, N., (2010). Platelets enhance lymphocyte adhesion and infiltration into arterial thrombus. *Thromb. Haemost.* **104**, 1184–1192. https://doi.org/10.1160/TH10-05-0308.
35. Lombardi, L., Maiorca, F., Marrapodi, R., Sabetta, A., Scafa, N., Pallucci, D., Miglionico, M., Romiti, G.F., Corica, B., Piconese, S., Polimeni, A., Pulcinelli, F., Cangemi, R., Visentini, M., Basili, S., Stefanini, L., (2023). Distinct platelet crosstalk with adaptive and innate immune cells after adenoviral and mRNA vaccination against SARS-CoV-2. *J. Thromb. Haemost.* **21**, 1636–1649. https://doi.org/10.1016/j.jtha.2023.03.003.
36. Mehdi-Alamdarlou, S., Ahmadi, F., Shahbazi, M.-A., Azadi, A., Ashrafi, H., (2023). Platelets and platelet-derived vesicles as an innovative cellular and subcellular platform for managing multiple sclerosis. *Mol. Biol. Rep.* **50**, 4675–4686. https://doi.org/10.1007/s11033-023-08322-7.
37. Dziedzic, A., Bijak, M., (2019). Interactions between platelets and leukocytes in pathogenesis of multiple sclerosis. *Adv. Clin. Exp. Med.* **28**, 277–285. https://doi.org/10.17219/acem/83588.
38. Cramer, S.P., Simonsen, H., Frederiksen, J.L., Rostrup, E., Larsson, H.B.W., (2014). Abnormal blood-brain barrier permeability in normal appearing white matter in multiple sclerosis investigated by MRI. *Neuroimage Clin.* **4**, 182–189. https://doi.org/10.1016/j.nicl.2013.12.001.
39. Christiansen, C.F., Christensen, S., Farkas, D.K., Miret, M., Sørensen, H.T., Pedersen, L., (2010). Risk of arterial cardiovascular diseases in patients with multiple sclerosis: a population-based cohort study. *Neuroepidemiology* **35**, 267–274. https://doi.org/10.1159/000320245.
40. Jadidi, E., Mohammadi, M., Moradi, T., (2013). High risk of cardiovascular diseases after diagnosis of multiple sclerosis. *Mult. Scler.* **19**, 1336–1340. https://doi.org/10.1177/1352458513475833.
41. Ramagopalan, S.V., Wotton, C.J., Handel, A.E., Yeates, D., Goldacre, M.J., (2011). Risk of venous thromboembolism in people admitted to hospital with selected immune-mediated diseases: record-linkage study. *BMC Med.* **9**, 1. https://doi.org/10.1186/1741-7015-9-1.
42. Christensen, S., Farkas, D.K., Pedersen, L., Miret, M., Christiansen, C.F., Sørensen, H.T., (2012). Multiple sclerosis and risk of venous thromboembolism: a population-based cohort study. *Neuroepidemiology* **38**, 76–83. https://doi.org/10.1159/000335496.
43. Zöller, B., Li, X., Sundquist, J., Sundquist, K., (2012). Risk of pulmonary embolism in patients with autoimmune disorders: a nationwide follow-up study from Sweden. *Lancet* **379**, 244–249. https://doi.org/10.1016/S0140-6736(11)61306-8.
44. Peeters, P.J.H.L., Bazelier, M.T., Uitdehaag, B.M.J., Leufkens, H.G.M., De Bruin, M.L., de Vries, F., (2014). The risk of venous thromboembolism in patients with multiple sclerosis: the Clinical Practice Research Datalink. *J. Thromb. Haemost.* **12**, 444–451. https://doi.org/10.1111/jth.12523.
45. Brønnum-Hansen, H., Koch-Henriksen, N., Stenager, E., (2004). Trends in survival and cause of death in Danish patients with multiple sclerosis. *Brain* **127**, 844–850. https://doi.org/10.1093/brain/awh104.
46. Tummala, R., Rai, M.P., (2024). Glycoprotein IIb/IIIa inhibitors. *StatPearls*. StatPearls Publishing, Treasure Island (FL) (accessed August 23, 2024) http://www.ncbi.nlm.nih.gov/books/NBK554376/.
47. Rinder, H., Bonan, J., Rinder, C., Ault, K., Smith, B., (1991). Activated and unactivated platelet adhesion to monocytes and neutrophils. *Blood* **78**, 1760–1769. https://doi.org/10.1182/blood.V78.7.1760.1760.
48. Perrella, G., Nagy, M., Watson, S.P., Heemskerk, J.W.M., (2021). Platelet GPVI (Glycoprotein VI) and thrombotic complications in the venous system. *Arterioscler. Thromb. Vasc. Biol.* **41**, 2681–2692. https://doi.org/10.1161/ATVBAHA.121.316108.
49. Kral-Pointner, J.B., Haider, P., Szabo, P.L., Salzmann, M., Brekalo, M., Schneider, K.H., Schrottmaier, W.C., Kaun,

C., Bleichert, S., Kiss, A., Sickha, R., Hengstenberg, C., Huber, K., Brostjan, C., Bergmeister, H., Assinger, A., Podesser, B.K., Wojta, J., Hohensinner, P., (2024). Reduced monocyte and neutrophil infiltration and activation by P-selectin/CD62P inhibition enhances thrombus resolution in mice. *Arterioscler. Thromb. Vasc. Biol.* **44**, 954–968. https://doi.org/10.1161/ATVBAHA.123.320016.

50. Dziedzic, A., Miller, E., Saluk-Bijak, J., Niwald, M., Bijak, M., (2021). The molecular aspects of disturbed platelet activation through ADP/P2Y12 pathway in multiple sclerosis. *Int. J. Mol. Sci.* **22** https://doi.org/10.3390/ijms22126572.
51. Dziedzic, A., Miller, E., Bijak, M., Przyslo, L., Saluk-Bijak, J., (2020). Increased pro-thrombotic platelet activity associated with thrombin/PAR1-dependent pathway disorder in patients with secondary progressive multiple sclerosis. *Int. J. Mol. Sci.* **21**, 7722. https://doi.org/10.3390/ijms21207722.
52. Morel, A., Rywaniak, J., Bijak, M., Miller, E., Niwald, M., Saluk, J., (2017). Flow cytometric analysis reveals the high levels of platelet activation parameters in circulation of multiple sclerosis patients. *Mol. Cell. Biochem.* **430**, 69–80. https://doi.org/10.1007/s11010-017-2955-7.
53. Lam, F.W., Vijayan, K.V., Rumbaut, R.E., (2015). Platelets and their interactions with other immune cells. *Compr. Physiol.* **5**, 1265–1280. https://doi.org/10.1002/cphy.c140074.
54. Frenette, P.S., Denis, C.V., Weiss, L., Jurk, K., Subbarao, S., Kehrel, B., Hartwig, J.H., Vestweber, D., Wagner, D.D., (2000). P-Selectin glycoprotein ligand 1 (PSGL-1) is expressed on platelets and can mediate platelet-endothelial interactions in vivo. *J. Exp. Med.* **191**, 1413–1422. https://doi.org/10.1084/jem.191.8.1413.
55. Starossom, S.C., Veremeyko, T., Yung, A.W.Y., Dukhinova, M., Au, C., Lau, A.Y., Weiner, H.L., Ponomarev, E.D., (2015). Platelets play differential role during the initiation and progression of autoimmune neuroinflammation. *Circ. Res.* **117**, 779–792. https://doi.org/10.1161/CIRCRESAHA.115.306847.
56. Kuenz, B., Lutterotti, A., Khalil, M., Ehling, R., Gneiss, C., Deisenhammer, F., Reindl, M., Berger, T., (2005). Plasma levels of soluble adhesion molecules sPECAM-1, sP-selectin and sE-selectin are associated with relapsing-remitting disease course of multiple sclerosis. *J. Neuroimmunol.* **167**, 143–149. https://doi.org/10.1016/j.jneuroim.2005.06.019.
57. Ludwig, N., Hilger, A., Zarbock, A., Rossaint, J., (2022). Platelets at the crossroads of pro-inflammatory and resolution pathways during inflammation. *Cells* **11**, 1957. https://doi.org/10.3390/cells11121957.
58. Chen, H.-C., (2005). Boyden chamber assay. *Methods Mol. Biol.* **294**, 15–22. https://doi.org/10.1385/1-59259-860-9:015.
59. Chahal, A.S., Gómez-Florit, M., Domingues, R.M.A., Gomes, M.E., Tiainen, H., (2021). Human platelet lysate-loaded poly(ethylene glycol) hydrogels induce stem cell chemotaxis in vitro. *Biomacromolecules* **22**, 3486–3496. https://doi.org/10.1021/acs.biomac.1c00573.
60. Suess, P.M., Smith, S.A., Morrissey, J.H., (2020). Platelet polyphosphate induces fibroblast chemotaxis and myofibroblast differentiation. *J. Thromb. Haemost.* **18**, 3043–3052. https://doi.org/10.1111/jth.15066.
61. Hayashi, N., Takehara, K., Soma, Y., (1995). Differential chemotactic responses mediated by platelet-derived growth factor alpha- and beta-receptors. *Arch. Biochem. Biophys.* **322**, 423–428. https://doi.org/10.1006/abbi.1995.1484.
62. Witte, A., Chatterjee, M., Lang, F., Gawaz, M., (2017). Platelets as a novel source of pro-inflammatory chemokine CXCL14. *Cell. Physiol. Biochem.* **41**, 1684–1696. https://doi.org/10.1159/000471821.
63. Ribeiro, L.S., Migliari Branco, L., Franklin, B.S., (2019). Regulation of innate immune responses by platelets. *Front. Immunol.* **10** https://doi.org/10.3389/fimmu.2019.01320.
64. Guerrero, S., Sánchez-Tirado, E., Agüí, L., González-Cortés, A., Yáñez-Sedeño, P., Pingarrón, J.M., (2022). Development of an electrochemical CCL5 chemokine immunoplatform for rapid diagnosis of multiple sclerosis. *Biosensors* **12**, 610. https://doi.org/10.3390/bios12080610.
65. Ueba, T., Nomura, S., Inami, N., Yokoi, T., Inoue, T., (2014). Elevated RANTES level is associated with metabolic syndrome and correlated with activated platelets associated markers in healthy younger men. *Clin. Appl. Thromb. Hemost.* **20**, 813–818. https://doi.org/10.1177/1076029612467845.
66. von Hundelshausen, P., Schmitt, M.M.N., (2014). Platelets and their chemokines in atherosclerosis—clinical applications. *Front. Physiol.* **5** https://doi.org/10.3389/fphys.2014.00294.
67. von Hundelshausen, P., Koenen, R.R., Sack, M., Mause, S.F., Adriaens, W., Proudfoot, A.E.I., Hackeng, T.M., Weber, C., (2005). Heterophilic interactions of platelet factor 4 and RANTES promote monocyte arrest on endothelium. *Blood* **105**, 924–930. https://doi.org/10.1182/blood-2004-06-2475.
68. von Hundelshausen, P., Weber, K.S., Huo, Y., Proudfoot, A.E., Nelson, P.J., Ley, K., Weber, C., (2001). RANTES deposition by platelets triggers monocyte arrest on inflamed and atherosclerotic endothelium. *Circulation* **103**, 1772–1777. https://doi.org/10.1161/01.cir.103.13.1772.
69. Gerdes, N., Zhu, L., Ersoy, M., Hermansson, A., Hjemdahl, P., Hu, H., Hansson, G.K., Li, N., (2011). Platelets regulate CD4+ T-cell differentiation via multiple chemokines in humans. *Thromb. Haemost.* **106**, 353–362. https://doi.org/10.1160/TH11-01-0020.
70. Zang, Y.C.Q., Samanta, A.K., Halder, J.B., Hong, J., Tejada-Simon, M.V., Rivera, V.M., Zhang, J.Z., (2000). Aberrant T cell migration toward RANTES and MIP-1α in patients with multiple sclerosis: overexpression of chemokine receptor CCR5. *Brain* **123**, 1874–1882. https://doi.org/10.1093/brain/123.9.1874.
71. Balashov, K.E., Rottman, J.B., Weiner, H.L., Hancock, W. W., (1999). CCR5+ and CXCR3+ T cells are increased in multiple sclerosis and their ligands MIP-1α and IP-10 are expressed in demyelinating brain lesions. *PNAS* **96**, 6873–6878. https://doi.org/10.1073/pnas.96.12.6873.
72. Tejera-Alhambra, M., Casrouge, A., de Andrés, C., Seyfferth, A., Ramos-Medina, R., Alonso, B., Vega, J., Fernández-Paredes, L., Albert, M.L., Sánchez-Ramón, S., (2015). Plasma biomarkers discriminate clinical forms of multiple sclerosis. *PLoS One* **10**, e0128952 https://doi.org/10.1371/journal.pone.0128952.
73. Iarlori, C., Reale, M., Lugaresi, A., De Luca, G., Bonanni, L., Di Iorio, A., Feliciani, C., Conti, P., Gambi, D., (2000). RANTES production and expression is reduced in

relapsing-remitting multiple sclerosis patients treated with interferon-β-1b. *J. Neuroimmunol.* **107**, 100–107. https://doi.org/10.1016/S0165-5728(00)00261-7.
74. Lechner, J., von Baehr, V., Schick, F., (2021). RANTES/CCL5 signaling from jawbone cavitations to epistemology of multiple sclerosis – research and case studies. *DNND* **11**, 41–50. https://doi.org/10.2147/DNND.S315321.
75. Bartosik-Psujek, H., Belniak, E., Mitosek-Szewczyk, K., Dobosz, B., Stelmasiak, Z., (2004). Interleukin-8 and RANTES levels in patients with relapsing–remitting multiple sclerosis (RR-MS) treated with cladribine. *Acta Neurol. Scand.* **109**, 390–392. https://doi.org/10.1111/j.1600-0404.2004.00259.x.
76. Sandmann, R., Köster, S., (2016). Topographic cues reveal two distinct spreading mechanisms in blood platelets. *Sci. Rep.* **6**, 22357. https://doi.org/10.1038/srep22357.
77. Allen, R.D., Zacharski, L.R., Widirstky, S.T., Rosenstein, R., Zaitlin, L.M., Burgess, D.R., (1979). Transformation and motility of human platelets: details of the shape change and release reaction observed by optical and electron microscopy. *J. Cell Biol.* **83**, 126–142. https://doi.org/10.1083/jcb.83.1.126.
78. Wilhelm, G., Mertowska, P., Mertowski, S., Przysucha, A., Strużyna, J., Grywalska, E., Torres, K., (2023). The crossroads of the coagulation system and the immune system: interactions and connections. *Int. J. Mol. Sci.* **24**, 12563. https://doi.org/10.3390/ijms241612563.
79. Laroche, A., Soulet, D., Bazin, M., Levesque, T., Allaeys, I., Vallières, N., Gunzer, M., Flamand, L., Lacroix, S., Boilard, E., (2022). Live imaging of platelets and neutrophils during antibody-mediated neurovascular thrombosis. *Blood Adv* **6**, 3697–3702. https://doi.org/10.1182/bloodadvances.2021006728.
80. Pluta, K., Porębska, K., Urbanowicz, T., Gąsecka, A., Olasińska-Wiśniewska, A., Targoński, R., Krasińska, A., Filipiak, K.J., Jemielity, M., Krasiński, Z., (2022). Platelet-leucocyte aggregates as novel biomarkers in cardiovascular diseases. *Biology* **11**, 224. https://doi.org/10.3390/biology11020224.
81. Comi, G., Bar-Or, A., Lassmann, H., Uccelli, A., Hartung, H.-P., Montalban, X., Sørensen, P.S., Hohlfeld, R., Hauser, S.L., E.P. of the 27th A.M. of the E.C. Foundation, (2021). Role of B cells in multiple sclerosis and related disorders. *Ann. Neurol.* **89**, 13–23. https://doi.org/10.1002/ana.25927.
82. Miyazaki, Y., Niino, M., (2022). B-cell depletion therapy for multiple sclerosis. *Immunol. Med.* **45**, 54–62. https://doi.org/10.1080/25785826.2021.1952543.
83. Baker, D., Marta, M., Pryce, G., Giovannoni, G., Schmierer, K., (2017). Memory B cells are major targets for effective immunotherapy in relapsing multiple sclerosis. *EBioMedicine* **16**, 41–50. https://doi.org/10.1016/j.ebiom.2017.01.042.
84. Sellebjerg, F., Blinkenberg, M., Sorensen, P.S., (2020). Anti-CD20 monoclonal antibodies for relapsing and progressive multiple sclerosis. *CNS Drugs* **34**, 269–280. https://doi.org/10.1007/s40263-020-00704-w.
85. Krämer, J., Bar-Or, A., Turner, T.J., Wiendl, H., (2023). Bruton tyrosine kinase inhibitors for multiple sclerosis. *Nature Rev. Neurol.* **19**, 289–304. https://doi.org/10.1038/s41582-023-00800-7.
86. Dybowski, S., Torke, S., Weber, M.S., (2023). Targeting B cells and microglia in multiple sclerosis with bruton tyrosine kinase inhibitors: a review. *JAMA Neurol.* **80**, 404–414. https://doi.org/10.1001/jamaneurol.2022.5332.
87. Zamora, C., Toniolo, E., Diaz-Torné, C., Cantó, E., Magallares, B., Ortiz, M.A., Perea, L., Corominas, H., Vidal, S., (2019). Association of platelet binding to lymphocytes with B cell abnormalities and clinical manifestations in systemic lupus erythematosus. *Mediators Inflamm.* **2019**, 2473164 https://doi.org/10.1155/2019/2473164.
88. Chen, D., Ireland, S.J., Remington, G., Alvarez, E., Racke, M.K., Greenberg, B., Frohman, E.M., Monson, N.L., (2016). CD40-mediated NF-κB activation in B cells is increased in multiple sclerosis and modulated by therapeutics. *J. Immunol.* **197**, 4257–4265. https://doi.org/10.4049/jimmunol.1600782.
89. Cognasse, F., Duchez, A.C., Audoux, E., Ebermeyer, T., Arthaud, C.A., Prier, A., Eyraud, M.A., Mismetti, P., Garraud, O., Bertoletti, L., Hamzeh-Cognasse, H., (2022). Platelets as key factors in inflammation: focus on CD40L/CD40. *Front. Immunol.* **13**, 825892 https://doi.org/10.3389/fimmu.2022.825892.
90. 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**, 162–173. https://doi.org/10.1016/S1474-4422(17)30470-2.
91. Lublin, F.D., Reingold, S.C., Cohen, J.A., Cutter, G.R., Sørensen, P.S., Thompson, A.J., Wolinsky, J.S., Balcer, L. J., Banwell, B., Barkhof, F., Bebo, B., Calabresi, P.A., Clanet, M., Comi, G., Fox, R.J., Freedman, M.S., Goodman, A.D., Inglese, M., Kappos, L., Kieseier, B.C., Lincoln, J.A., Lubetzki, C., Miller, A.E., Montalban, X., O'Connor, P.W., Petkau, J., Pozzilli, C., Rudick, R.A., Sormani, M.P., Stüve, O., Waubant, E., Polman, C.H., (2014). Defining the clinical course of multiple sclerosis. *Neurology* **83**, 278–286. https://doi.org/10.1212/WNL.0000000000000560.
92. Kurtzke, J.F., (1983). Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). *Neurology* **33**, 1444–1452. https://doi.org/10.1212/wnl.33.11.1444.
93. Modified Rankin Scale (MRS) – Strokengine, (n.d.). https://strokengine.ca/en/assessments/modified-rankin-scale-mrs/ (accessed May 19, 2024).
94. Sullivan, M.J., Weinshenker, B., Mikail, S., Bishop, S.R., (1995). Screening for major depression in the early stages of multiple sclerosis. *Can. J. Neurol. Sci.* **22**, 228–231. https://doi.org/10.1017/s0317167100039895.
95. Freitas, S., Batista, S., Afonso, A.C., Simões, M.R., de Sousa, L., Cunha, L., Santana, I., (2018). The Montreal Cognitive Assessment (MoCA) as a screening test for cognitive dysfunction in multiple sclerosis. *Appl. Neuropsychol. Adult* **25**, 57–70. https://doi.org/10.1080/23279095.2016.1243108.
96. Boyden, S., (1962). The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. *J. Exp. Med.* **115**, 453–466. https://doi.org/10.1084/jem.115.3.453.

MANUSKRYPT ARTYKUŁU DOŚWIADCZALNEGO

Extracellular vesicle-derived miR-760 as a novel candidate marker differentiating stable RRMS from SPMS

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Abstract.

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system with heterogenous clinical course, lacking non-invasive biomarkers for phenotype differentiation. This study aimed to explore circulating extracellular vesicle (EV)-derived miRNA signatures and related molecular profiles capable of distinguishing stable relapsing-remitting MS (RRMS) from secondary progressive MS (SPMS).

Plasma samples were collected from stable RRMS (n = 30), SPMS (n = 30), and healthy controls (HC) (n = 30), followed by total EVs isolation and characterization using transmission electron microscopy, dynamic light scattering, and flow cytometry. RNA was extracted from EVs, and miRNA profiles were analyzed via RNA sequencing and RT-qPCR. Cytokine and neurodegeneration markers were quantified using the BioPlex® system and ELISA. Functional enrichment and network analyses of miRNA targets were performed, alongside logistic regression modeling to explore potential distinguishing features.

Four EV-derived miRNAs (miR-760, miR-98-5p, miR-301a-3p, miR-223-3p) showed significant differences ($p < 0.05$) between stable RRMS and SPMS. An integrative model combining miRNAs with FGF basic protein enabled accurate phenotypes differentiation (AUC = 0.97). miR-760 showed the strongest distinctive capacity for stable RRMS. Additionally, miR-98-5p was markedly up-regulated in both stable RRMS and SPMS compared to HC. Network analysis of miRNA targets suggested distinct immunoregulatory patterns across MS phenotypes.

Plasma EV-derived miRNAs – particularly miR-760, and miR-98-5p – showed strong potential as molecular indicators associated with disease phenotype in MS. Integrating EV-miRNA profiling with protein markers support efforts toward more precise stratification of MS patients. Further studies in independent cohorts and functional validation are warranted before clinical translation.

Keywords: Multiple Sclerosis, Extracellular Vesicles, miRNA, miR-760, Biomarkers, Neuroinflammation, Neurodegeneration

1 Introduction

Multiple sclerosis (MS) is a chronic immune-mediated disorder affecting approximately 2.8 million people worldwide as of 2020 [1]. It primarily targets the central nervous system (CNS), leading to focal and diffuse neuroinflammatory damage in the brain and spinal cord. The disease is driven by a sustained inflammatory

response involving $CD4^+$ and $CD8^+$ T cells, B cells, and other immune mediators reactive against myelin antigens [2].

Clinically, MS manifests in heterogeneous phenotypes. Approximately 85% of patients initially present with a clinically isolated syndrome (CIS) that progresses to a relapsing-remitting course (RRMS), while a subset eventually transitions to secondary progressive MS (SPMS). A smaller group exhibits primary progressive MS (PPMS), characterized by insidious neurological decline from onset [3]. The variability in radiological, histopathological, and clinical presentation, along with differential drug responsiveness, complicates both diagnosis and disease monitoring [4].

Despite advancements in MS diagnostics over the past decade, it still relies heavily on clinical assessment, necessitating careful differentiation from alternative conditions. The introduction of AQP4-IgG and MOG-IgG assays has improved the distinction between neuromyelitis optica spectrum disorder (NMOSD) and myelin oligodendrocyte glycoprotein antibody-associated disease (MOGAD) from MS. However, the absence of MS-specific biomarkers remains a major clinical challenge [5]. Routine blood tests, such as neurofilament light chain (NfL), which is not entirely disease-specific, have limited diagnostic value, increasing the risk of misdiagnosis [5,6].

Emerging evidence suggests that extracellular vesicles (EVs) play a potential role in the clinical medicine, as reservoirs of biomarkers that reflect the pathological state in immune and neurodegenerative disease [7,8]. EVs, as defined by the International Society of Extracellular Vesicles (ISEV), encompass two major types of vesicles – exosomes and ectosomes (microvesicles) [9]. Released by parental cells, EVs mediate intercellular communication by transferring bioactive molecules, including nucleic acids, lipids, and proteins. In MS, EVs are implicated in antigen presentation, blood-brain barrier (BBB) disruption, lymphocyte activation, and CNS infiltration, reflecting disease pathology [10]. Among EV-associated candidates for biomarkers, microRNAs (miRNAs) are of particular interest due to their stability in body fluids and their regulatory role in post-transcriptional gene expression [11]. Several miRNAs, including miR-155, miR-146a, and miR-181c, have been identified as potential MS biomarkers, correlating with disease activity, relapse risk, and expanded disability status scale (EDSS) scores [12–15]. However, the clinical applicability of EV-miRNA signatures in MS remains uncertain due to methodological heterogeneity, emphasizing the need for further validation, which may ultimately lead to the development of standardized analytical approaches [16]. Further robust and validated data are essential before implementation of standarized biomarkers to complement clinical approaches and support accurate MS diagnosis or phenotype stratification.

Therefore, based on previously published data and our novel RNA-seq findings, we aim to refine the candidate pool of EV-associated miRNAs potentially relevant to MS pathophysiology in stable RRMS and SPMS. Using a multi-omic approach combining miRNA expression profiling, protein marker quantification, and integrative bioinformatics, we aimed to identify molecular signatures associated with disease activity and explore their functional relevance in the context of MS progression.

2 Materials and methods

2.1 Sample collection and preparation

Peripheral blood samples were collected via venipuncture between 8:00 and 9:00 a.m. in Sarstedt® tubes (Nümbrecht, Germany) containing citrate phosphate dextrose adenine (CPDA)-1 as an anticoagulant. Plasma was separated by centrifugation at 4500 RPM for 12 min. at 25°C, aliquoted, and stored at -80°C until further analysis. A total of 60 patients with MS were recruited from the Department of Rehabilitation, Neurological Rehabilitation Division, III General Hospital in Lodz, Poland. MS diagnosis was confirmed based on the 2017 McDonald criteria [17], and disease phenotype classification followed Lublin et al. [18]. All participants provided written informed consent (Research Bioethics committee with resolution No. 3/KBBN- UŁ/IV/2018) and completed a detailed medical questionnaire. Clinical assessments included neurological examinations, magnetic resonance imaging (MRI) to evaluate grey matter pathology and white matter lesion volume, and disability assessment using the EDSS.

Exclusion criteria for MS patients included the use of medications affecting platelet biology, disease-modifying therapies (e.g., interferon-β, glatiramer acetate, natalizumab), hormones, corticosteroids, or immunomodulators; recent infections (≤ 4 weeks); comorbid neurological or psychiatric disorders; diabetes mellitus; myocardial infarction; pregnancy; or breastfeeding.

A healthy control group (HC, n = 30) was recruited from the Laboratory Diagnostics Center in Lodz, Poland. HC participants were age- and sex-matched to the MS cohort and confirmed to be free of MS, autoimmune diseases, neurodegenerative disorders, and acute or chronic inflammatory conditions. Additional exclusion criteria included pregnancy, breastfeeding, and medication use. Health status was verified through comprehensive medical evaluation, including routine hematological and inflammatory marker assessments.

2.2 Bio-Plex multiplex immunoassay

Cytokine profiling was performed using the Bio-Plex Pro™ multiplex assay kit (Bio-Rad Laboratories, Inc., USA) following the manufacturer's instructions. This assay enabled the simultaneous quantification of the following 27 cytokines in a single sample: fibroblast growth factor (FGF) basic, eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, interleukin (IL)-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 , IL-13, IL-15, IL-17A, interferon gamma-induced protein (IP)-10, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet-derived growth factor (PDGF)-BB, regulated on activation, normal T expressed and secreted (RANTES), tumor necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF). Prior the analysis, the instrument was calibrated and validated to ensure optimal performance of fluidics and optics systems.

Plasma samples were thawed at 4°C and kept on ice until use. Reagents were equilibrated to room temperature (RT) before the assay. To remove precipitates, samples were centrifuged at 10,000 × *g* for 10 min at 4°C and diluted 1:4 with the provided sample diluent buffer. Antibody-coupled beads were prepared, added to a 96-well microplate, and incubated with diluted plasma samples, standards, or quality controls for 1 h at RT on a shaker. After washing, biotin-labeled detection antibodies were added and incubated for 30 min at RT, followed by streptavidin-phycoerythrin conjugates for 10 min at RT, with washing steps between incubations. Finally, assay buffer was added, the plate was shaken to resuspend the beads, and fluorescence was measured using the Bio-Plex® 200 System analyzer (Bio-Rad Laboratories, Inc., USA). Cytokine concentrations were determined using Bio-Plex Manager™ software (Bio-Rad Laboratories, Inc., USA) with standard curve interpolation.

2.3 Measurement of neurodegeneration markers concentration

Human enzyme-linked immunosorbent assay (ELISA) kits were applied to measure the plasma concentrations of NfL (Cloud-Clone Corp., USA) and glial fibrillary acidic protein (GFAP) (Elabscience, USA). All measurements were performed in duplicate using the UV-Vis microplate reader SPECTROstar Nano system (BMG Labtech GmbH, Germany). Protein concentrations were calculated by comparing the optical density (OD) values of the samples to standard curves.

2.4 Extracellular vesicles isolation and characterization

Prior to EVs isolation, plasma samples (1 ml) were treated with RNase A (100 ng/ml) for 10 min. at 37°C to remove unprotected circulating RNA [19]. EVs were then isolated using the Total Exosome Isolation Kit (plasma) (Invitrogen, USA) following the manufacturer's protocol. The resulting pellet was resuspended in 200 µl of 1× phosphate-buffered saline (PBS) and stored at -20°C for short-term preservation.

To assess the purity and morphology of the isolated EVs, nine randomly selected samples (three from each MS group and the HC group) were analyzed using transmission electron microscopy (TEM). EV samples were fixed with 2.5% glutaraldehyde and placed on 200-mesh carbon-coated copper grids (Polysciences, USA). The samples were then stained with 2% uranyl acetate (ACS Reagent, USA) and examined using a JEM-1010 transmission electron microscope (JEOL Ltd., Tokyo, Japan). Developed films were scanned using the Perfection V700 PHOTO scanner (Epson, Japan).

Dynamic light scattering (DLS) was used to assess the size distribution and homogeneity of extracellular vesicle population. The hydrodynamic diameter and polydispersity index (PDI) were measured using a Zetasizer Nano-ZS (Malvern Instruments Ltd., UK), 633 nm laser, 173° detection angle, in PBS (pH 7.4), 25°C. EVs size was determined from three independent replicates, with the average calculated from five runs per sample.

To assess EV characteristics, 18 randomly selected samples (6 from each RRMS, SPMS, and HC group) were analyzed for EV surface markers using flow cytometry. EV-associated proteins CD63-FITC and CD81-Pacific Blue (Beckman Coulter, Brea, CA, USA) were detected by direct staining with fluorochrome-conjugated anti-human monoclonal antibodies. Samples were incubated at RT for 30 min in the dark before analysis on a BD FACSymphony A1 flow cytometer (Becton Dickinson, USA). Data were acquired and processed using BD FACSDiva software (v9.0.2).

2.5 Total RNA isolation

Total RNA, including miRNA, was extracted from plasma-derived EVs using the Total Exosome RNA & Protein Isolation Kit (Invitrogen, USA) employing acid-phenol:chloroform for organic extraction according to the manufacturer's protocol. Briefly, ethanol was added to the aqueous phase obtained from acid-phenol:chloroform extraction and passed through a glass-fiber filter cartridge, which immobilized the RNA. The filter was subsequently washed, and RNA, including the small RNA fraction, was eluted using a low ionic-strength buffer.

To monitor RNA recovery and reverse transcription efficiency, 10 pM of synthetic cel-miRNA-39 (5′-UCACCGGGUGUAAAUCAGCUUG-3′) was added to each sample before RNA isolation.

RNA concentration was assessed using the Agilent RNA 6000 Pico Kit and Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Extracted RNA was stored at -80°C until further processing for RNA sequencing.

2.6 RNA sequencing

The screening RNA expression analysis was performed for 9 randomly selected samples (3 from each RRMS, SPMS, and HC group). RNA sequencing libraries were prepared using the QIAseq miRNA Library Kit (Qiagen) following the manufacturer's protocol. Libraries were sequenced on the Illumina NextSeq 500/550 platform using Mid Output kits (v2.5). Image processing, base calling, and demultiplexing were performed with NextSeq Control Software (Illumina, San Diego, CA, USA).

2.7 Selected miRNA expression analysis

Total RNA was reverse transcribed into complementary DNA (cDNA) using the TaqMan™ Advanced miRNA cDNA Synthesis Kit (Applied Biosystems™, USA) according to the manufacturer's protocol. Undiluted cDNA was stored at -80 °C prior to quantitative real-time PCR (RT-qPCR) analysis.

TaqMan™ Advanced miRNA Assays (Applied Biosystems™, USA) were applied to quantify each miRNA expression (Table 1). qPCR reactions were carried out using TaqMan™ Fast Advanced Master Mix (Applied Biosystems™) following the manufacturer's protocol. cDNA was diluted 1:10 in 0.1× TE buffer, and 5 µl of the diluted sample was used as the template in a total reaction volume of 20 µl.

Endogenous control genes for RT-qPCR were selected using the RefGenes tool within the Genevestigator database and validated experimentally to identify the most stably expressed reference gene under the study conditions.

RT-qPCR was performed on the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., USA) under the following conditions: polymerase activation (20 s., 95°C), denaturation (3 s., 95°C), and extension (30 s., 60°C) for 49 cycles. Fluorescence detection was recorded as cycle threshold (Ct) values.

miRNA expression levels were quantified using the comparative ΔΔCt method. For each sample, the ΔCt value was determined as the difference between the Ct value of the target miRNA and that of the endogenous reference gene (miR-451a). The mean ΔCt of the HC group was used as the reference baseline. The ΔΔCt value for each sample in the RRMS and SPMS groups was calculated as the difference between its ΔCt and the mean ΔCt of the HC group. Fold-change (FC) values were determined using the $2^{-\Delta\Delta Ct}$ and $\log_2$-transformed for better clarity of results presentation.

Table 1. miRNA assays selected for RT-qPCR analysis.

Assay name	Mature miRNA Sequence	Assay ID
hsa-miR-451a	AAACCGUUACCAUUACUGAGUU	478107_mir
hsa-miR-223-3p	UGUCAGUUUGUCAAAUACCCCA	477983_mir
hsa-miR-98-5p	UGAGGUAGUAAGUUGUAUUGUU	478590_mir
hsa-miR-760	CGGCUCUGGGUCUGUGGGGA	483112_mir
hsa-miR-16-5p	UAGCAGCACGUAAAUAUUGGCG	477860_mir
hsa-miR-23a-3p	AUCACAUUGCCAGGGAUUUCC	478532_mir
hsa-miR-146a-5p	UGAGAACUGAAUUCCAUGGGUU	478399_mir
hsa-miR-181c-5p	AACAUUCAACCUGUCGGUGAGU	477934_mir
hsa-miR-155-5p	UUAAUGCUAAUCGUGAUAGGGGUU	483064_mir
hsa-miR-326	CCUCUGGGCCCUUCCUCCAG	478027_mir
hsa-miR-301a-3p	CAGUGCAAUAGUAUUGUCAAAGC	477815_mir
hsa-miR-191-5p	CAACGGAAUCCCAAAAGCAGCUG	477952_mir

2.8 miRNA target genes and functional enrichment analysis

To identify functionally relevant target genes of the differentially expressed miRNAs (RRMS vs SPMS), we filtered experimentally validated interactions from miRecords, miRTarBase, and TarBase databases using the multiMiR package and org.Hs.eg.db database in R (v4.4.2) [20].

Gene ontology (GO) enrichment analysis was performed to identify associated biological processes (BP), cellular components (CC), and molecular functions (MF). Pathway enrichment analysis was conducted using Kyoto encyclopedia of genes and genomes (KEGG) pathway database [21,22]. For both overrepresentation analysis, the clusterProfiler package was used, applying Benjamini-Hochberg correction and a significance threshold of $p < 0.05$. Pathways were ranked based on GeneRatio (the proportion of input genes mapping to a given pathway) and adjusted p-values, and visualizations were generated using the enrichplot package [23].

To investigate molecular differences between RRMS and SPMS, we integrated publicly available disease-associated genes with experimentally validated mRNA targets of miR-98-5p, miR-760, miR-301a-3p, and miR-223-3p. Disease-related genes were retrieved from the DisGeNET (v24.4) database [24,25] by querying "secondary progressive multiple sclerosis" for SPMS and "multiple sclerosis relapse" and "multiple sclerosis exacerbation" for RRMS. The top 30 genes for each disease phenotype were selected from DisGeNET based on the highest disease-gene association (GDA) scores, which quantify the strength of the gene-disease relationship.

To identify group-specific regulatory interactions, we cross-referenced these disease-associated gene lists with our miRNA-target dataset. To enhance the specificity of target, genes common to both RRMS and SPMS were excluded, focusing on distinct molecular signatures differentiating the two phenotypes. The remaining miRNA-mRNA pairs were used to construct interaction networks for RRMS and SPMS in Cytoscape (v3.10.3).

For data visualizations ggplot2 package was applied [26]. Figures were edited using GIMP (v2.10.38).

2.9 Statistical analysis

Statistical analyses were performed using STATISTICA Software (v13.3) (StatSoft; Tulsa, OK, USA). A p-value < 0.05 was considered statistically significant for all tests.

The Shapiro-Wilk test was employed to assess the normality of the distribution of the data. For non-normally distributed data, the U Mann-Whitney test was used for assessing the statistical significance of differences between two independent groups. The Kruskal-Wallis test was used while assessing the statistical significance of differences between three independent groups for non-normally distributed data. Following the Kruskal-Wallis test, false discovery rate (FDR) method of Benjamini and Hochberg was applied to perform multiple comparisons between the groups.

To identify statistically significant variables, univariate statistical analysis was conducted using logistic regression. Variables identified as significant in the univariate analysis ($p < 0.05$) were included in a multivariate

logistic regression model to assess the probability of specific clinical outcome (RRMS vs SPMS). The model was developed using the stepwise forward method, excluding variables with high collinearity based on correlation analysis performed using Spearman's rank correlation coefficient for non-normally distributed data. Sigma-restricted parameterization was applied in the analysis to stabilize coefficient estimates and ensure interpretable reference categories. The Hosmer-Lemeshow test was applied to assess the goodness-of-fit of the logistic regression model. A p-value > 0.05 in this test indicated adequate model fit, demonstrating consistency between predicted probabilities and observed outcomes.

Factor analysis was applied to identify clusters of interrelated variables. The principal component extraction method with Varimax rotation was used to reduce the number of variables to key factors, ensuring interpretability of the results.

Receiver operating characteristic (ROC) curve analysis was performed for selected variables to evaluate their ability to distinguish between clinical groups. The area under the curve (AUC) was calculated as a measure of sensitivity and specificity, with 95% confidence intervals (CI).

3 Results

3.1 Study groups

A total of 60 MS patients (30 RRMS and 30 SPMS) and 30 HC were included in the study. Basic clinical characteristics, including age, gender, C-reactive protein (CRP) levels, erythrocyte sedimentation rate (ESR), disease duration, and EDSS scores, were collected. A summary of participant characteristics is presented in Table 2.

Table 2. Clinical features of study groups. Parameters presented as mean ± SD. [a)] CRP, C-reactive protein; [b)] ESR, erythrocyte sedimentation rate; [c)] EDSS, Expanded Disability Status Scale.

Clinical characteristics	HC (n = 30)	RRMS (n = 30)	SPMS (n = 30)
Age (years)	46.7 ± 11.88	43.9 ± 10.5	62.7 ± 7.7
Gender, female/male (%)	18/12 (60/40)	19/11 (63.3/36.7)	17/13 (56.7/43.3)
CRP [a)] (mg/L)	2.26 ± 2.46	10.21 ± 10.99	15.64 ± 28.89
ESR [b)] (mm/h)	9.0 ± 9.8	29.37 ± 15.11	33.3 ± 12.7
Disease duration (years)	N/A	10.9 ± 6.3	30.6 ± 8.7
EDSS [c)]	N/A	5.0 ± 1.3	5.8 ± 0.5

3.2 Differentiated profile of inflammatory and neurodegeneration markers in MS patients

Of the 27 cytokines measured using the multiplex immunoassay, eight (IL2, IL-5, IL-6, IL-7, IL-12, IL-15, PDGF-BB, VEGF) were excluded from further analysis due to concentrations falling below the detection limit. Additionally, two neurodegeneration markers (NfL and GFAP) were analyzed.

Table 3 presents the median (± interquartile range, IQR) values for remaining 19 cytokines and two neurodegeneration markers. The analysis revealed significant alterations in markers concentration in both RRMS and SPMS patients compared to HC. The most pronounced increase was observed for GM-CSF, with a 5.92-fold elevation in RRMS and a 10.83-fold increase in SPMS. Among the most dysregulated cytokines, IL-1ra exhibited a 3.81-fold rise in RRMS and a 2.73-fold increase in SPMS. Similarly, MIP-1β (2.30-fold in RRMS, 2.65-fold in SPMS) and MIP-1α (2.54-fold in SPMS) showed marked elevation. Pro-inflammatory cytokines such as TNF-α (2.49-fold in RRMS, 2.50-fold in SPMS) and IL-8 (3.74-fold in RRMS) also demonstrated substantial up-regulation. Notably, the overall trend suggested a more pronounced increase in SPMS.

A total of 16 cytokines and one neurodegeneration marker (NfL) showed statistically significant differences across groups in the Kruskal-Wallis test and were further analyzed using the FDR method by Benjamini and Hochberg for RRMS and SPMS subgroups.

Ultimately, three markers exhibited significant differences in concentration between the analyzed subgroups, with all showing increased mean concentrations in SPMS patients compared to RRMS (Fig. 1). IL-4, a cytokine regulating the immune response, promoting the differentiation of T helper (Th)2 cells was 1.27-fold elevated ($p = 0.0044$). IL-17, which contributes to inflammation by promoting the activation of Th17 cells, showed a 1.26-fold increase ($p = 0.0331$). FGF basic, the growth factor involved in neuroprotection and myelination, exhibited a 1.33-fold increase ($p = 0.0101$).

Table 3. Plasma cytokine profiles in RRMS and SPMS patients and HC. Bolded and framed values are the top three with the highest fold change within each patient group compared to HC.

Cytokine concentration	HC (n = 30) [median ± IQR]	RRMS (n = 30) [median ± IQR]	SPMS (n = 30) [median ± IQR]	p-value (Kruskal-Wallis)
IL-1β [pg/ml]	0.33 ± 0.35	0.57 ± 1.01	0.74 ± 1.27	0.0138
IL-1ra [pg/ml]	62.87 ± 19.96	**239.30 ± 324.7**	**171.6 ± 226.2**	< 0.0001
IL-4 [pg/ml]	1.33 ± 0.64	2.05 ± 0.91	2.54 ± 1.12	< 0.0001
IL-8 [pg/ml]	0.94 ± 0.8	**3.52 ± 4.66**	4.44 ± 3.98	< 0.0001
Il-9 [pg/ml]	62.97 ± 38.95	140.2 ± 38.7	151.8 ± 83.6	< 0.0001
IL-10 [pg/ml]	1.21 ± 0.79	1.38 ± 0.87	1.97 ± 2.4	0.0173
IL-13 [pg/ml]	1.2 ± 1.35	2.17 ± 2.56	2.01 ± 1.93	0.0458
IL-17 [pg/ml]	8.54 ± 3.39	17 ± 7.42	21.46 ± 8.73	< 0.0001
RANTES [pg/ml]	640.5 ± 399.2	1318 ± 1097.5	1285 ± 1229.5	< 0.0001
Eotaxin [pg/ml]	79.81 ± 54.62	73.81 ± 39.48	84.41 ± 41.06	> 0.05
FGF basic [pg/ml]	4.97 ± 4.42	7.81 ± 4.54	11.24 ± 4.4	< 0.0001
G-CSF [pg/ml]	52.14 ± 13.93	103.5 ± 55.35	139 ± 93.6	< 0.0001
IFN-γ [pg/ml]	2.68 ± 1.23	5.67 ± 6.1	4.7 ± 6.78	< 0.0001
IP-10 [pg/ml]	322.1 ± 223.9	411.9 ± 444.8	563.8 ± 501.3	> 0.05
MCP-1 [pg/ml]	17.65 ± 8.69	16.65 ± 10.94	16.28 ± 11.15	> 0.05
MIP-1α [pg/ml]	0.52 ± 0.31	1.1 ± 1.16	1.32 ± 0.93	< 0.0001
MIP-1β [pg/ml]	22.26 ± 12.23	51.25 ± 22.27	**58.97 ± 31.99**	< 0.0001
TNF-α [pg/ml]	23.45 ± 24.53	58.43 ± 22.03	58.53 ± 29.78	< 0.0001
GM-CSF [pg/ml]	0.12 ± 0.43	**0.71 ± 1.11**	**1.18 ± 1.89**	0.0005
GFAP [pg/ml]	145.8 ± 70.9	170 ± 108.2	190.4 ± 114.1	> 0.05
NfL [pg/ml]	18.44 ± 17.6	34.38 ± 30.6	39.11 ± 38.2	< 0.0001

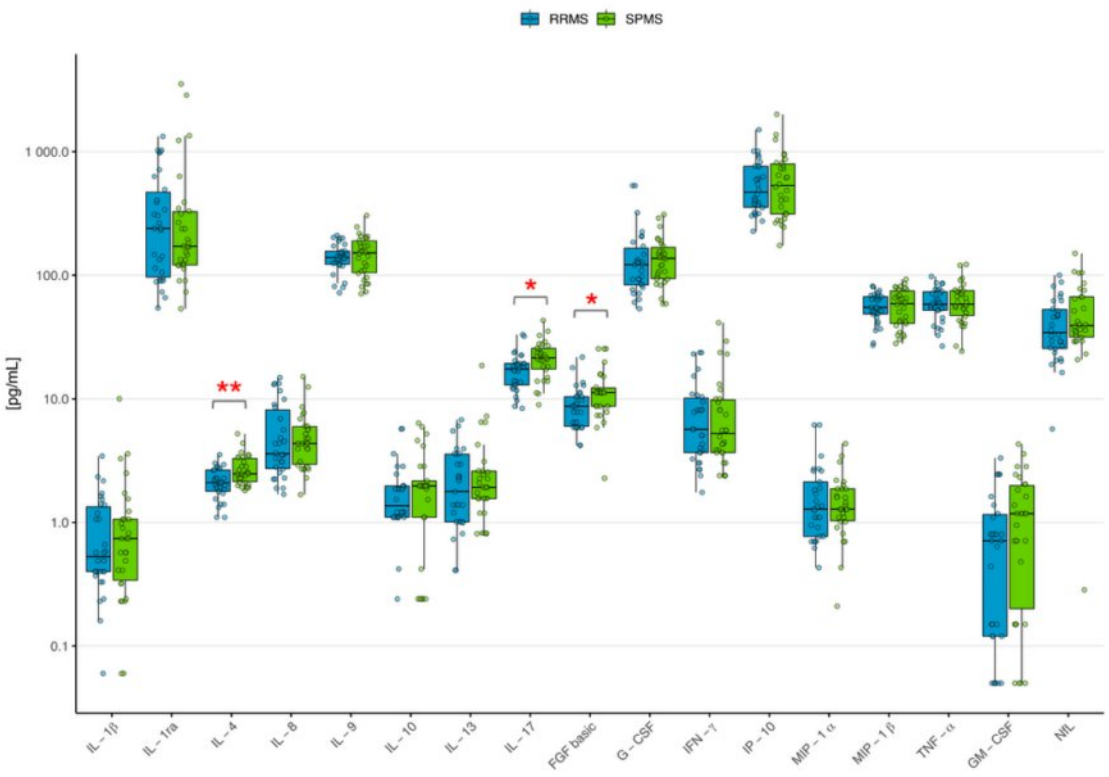

Fig. 1. Differential concentration of selected plasma-derived cytokines in RRMS (n = 30) and SPMS (n = 30) patients. Data presented as median ± IQR. Statistical significance was determined using the post-hoc FDR method by Benjamini and Hochberg. Significant differences between groups are indicated by *p < 0.05 and **p < 0.01.

3.3 Characterization of plasma-derived EVs

TEM images revealed a heterogeneous population of vesicles with predominantly spherical morphology and well-defined membrane boundaries (Fig. 2A). The DLS analysis further supported the structural integrity and purity of the sample, confirming a z-Average diameter of 45 nm and a PDI of 0.33, indicating a fairly uniform vesicle population (moderate monodispersity) (Fig. 2B).

Flow cytometry analysis of 18 randomly selected samples (six from each study group) confirmed the presence of CD61 and CD83 antigens on EVs membrane (~14% of double positive CD63 FITC/CD81 PB objects) (Fig. 2C).

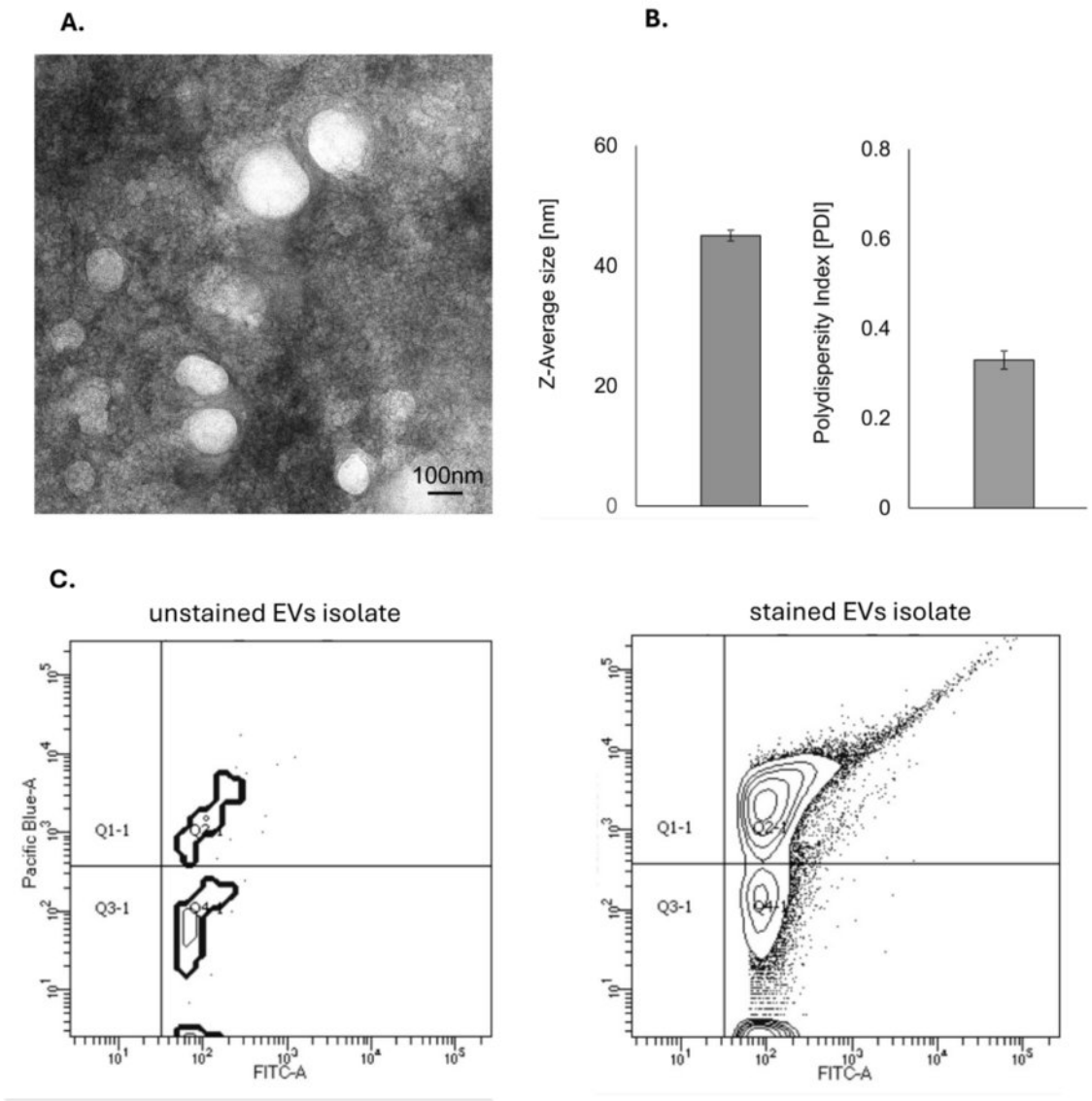

Fig. 2. Identification and characterization of plasma extracellular vesicles (EVs). (A) Representative electron microscopic image of EVs. (B) Average size distribution and polydispersity index (PDI) of EVs measured by dynamic light scattering (DLS). (C) Contour plot of EVs isolates stained with anti-CD63 FITC and anti-CD81 Pacific Blue and analyzed by flow cytometry. For the negative control unstained EVs isolates were used.

3.4 miRNA expression profiling

Raw sequencing data obtained from RNA sequencing underwent a comprehensive quality control assessment using FastQC (v0.12.1). Reads were subsequently filtered, trimmed, and evaluated for quality using fastp (v0.23.4). Additional miRNA-specific quality assessment was conducted with miRTrace (v1.0.1) to evaluate miRNA composition and potential biases. Processed reads were aligned to the human reference genome (GRCh38.p14/hg38) using Bowtie (v1.1.1) aligner, optimized for short reads.

Differential miRNA expression analysis was conducted using DESeq2 (v1.44.0) in R (v4.4.1) to identify statistically significant differences in miRNA expression between sample groups. To enhance miRNA identification, the miRTop (v0.4.25) tool was utilized, enabling classification and annotation of miRNA isoforms based on sequence variations.

Reference genome sequences were retrieved from the NCBI database, while miRNA sequences and annotations were obtained from the miRBase. The mature.fa and hairpin.fa files from miRBase were used as references for miRNA and hairpin structures, respectively.

Based on RNA sequencing analysis, two miRNAs (miR-98-5p and miR-760) were selected for RT-qPCR validation. Additionally, nine miRNAs (miR-155-5p, miR-326, miR-301a-3p, miR-191-5p, miR-223-3p, miR-181c-5p, miR-146a-5p, miR-23a-3p, and miR-16-5p) were chosen based on their established and/or predicted roles in regulating inflammatory markers and neurodegeneration, as determined through a comprehensive literature review and the miRDB database of predicted miRNA-target interactions.

RT-qPCR differential miRNA expression was calculated with the comparative ΔΔCt method, normalized to hsa-miR-451a, for RRMS and SPMS with HC as the reference population. The resulting $-\Delta\Delta Ct$ values, representing $\log_2$-transformed fold-change estimates, were analyzed for RRMS and SPMS differences. ΔCt values were used to present the differential expression between HC group and RRMS and SPMS.

Table 4 presents the ΔCt median ± IQR values for RT-qPCR analyzed miRNAs. The analysis revealed significant alterations in expression in both RRMS and SPMS patients compared to HC. Statistically significant differences were observed for miR-301a-3p, miR-181c-5p, miR-98-5p, and miR-760 (Kruskal–Wallis test, $p < 0.05$). miR-98-5p was significantly up-regulated in both RRMS and SPMS relative to HC, with greater up-regulation observed in SPMS. miR-301a-3p exhibited strong down-regulation in both disease groups, most prominently in RRMS. miR-760 was markedly down-regulated in RRMS but displayed a return toward baseline levels in SPMS. miR-181c-5p showed a consistent up-regulation in both RRMS and SPMS compared to HC.

Table 4. Differential EV-derived miRNA expression in RRMS and SPMS patients and HC. Arrows indicate the direction of miRNA regulation in disease groups relative to HC. ↑: miRNA up-regulated relative to HC; ↓: miRNA down-regulated relative to HC; ∼: no meaningful change in miRNA expression relative to HC.

ΔCt value	HC (n = 30) [median ± IQR]	RRMS (n = 30) [median ± IQR]	SPMS (n = 30) [median ± IQR]	p-value (Kruskal-Wallis)
miR-155-5p	9.12 ± 6.06	10.89 ± 3.43 ↓	8.93 ± 5.68 ∼	> 0.05
miR-326	15.64 ± 10.7	12.74 ± 12.09 ↑	11.33 ± 6.73 ↑	> 0.05
miR-301a-3p	11.71 ± 6.8	23.92 ± 12.1 ↓	13.91 ± 12.5 ↓	**< 0.0001**
miR-191-5p	6.12 ± 1.68	5.7 ± 1.16 ↑	5.88 ± 3.52 ↑	> 0.05
miR-223-3p	4.24 ± 2.23	5.215 ± 1.81 ↓	4.18 ± 2.41 ∼	> 0.05
miR-181c-5p	14.71 ± 5.27	10.21 ± 7.06 ↑	9.2 ± 5.12 ↑	**0.0037**
miR-146a-5p	8.78 ± 9.13	8.01 ± 5.58 ↑	6.96 ± 4.74 ↑	> 0.05
miR-23a-3p	4.54 ± 1.96	4.26 ± 2.66 ↑	3.92 ± 2.41 ↑	> 0.05
miR-16-5p	6.71 ± 3.53	5.08 ± 4.79 ↑	5.39 ± 2.44 ↑	> 0.05
miR-98-5p	2.9 ± 4.87	-2.27 ± 3.19 ↑	-4.65 ± 4.5 ↑	**< 0.0001**
miR-760	-2.485 ± 5.01	1.96 ± 5.21 ↓	-1.73 ± 5.45 ∼	**< 0.0001**

Given the observed significant differences in both miRNA expression and protein marker levels, we next assessed their potential associations using Spearman correlation analysis (Fig. 3). The resulting correlation matrices revealed group-specific associations. In SPMS, miR-98-5p showed significant negative correlation with IL-17 ($p = 0.013$, $r = -0.447$); miR-760 showed significant negative correlation with IL-4 ($p = 0.008$, $r = -0.472$) and IL-17 ($p = 0.003$, $r = -0.520$). In contrast, no statistically significant correlations were observed in the RRMS group.

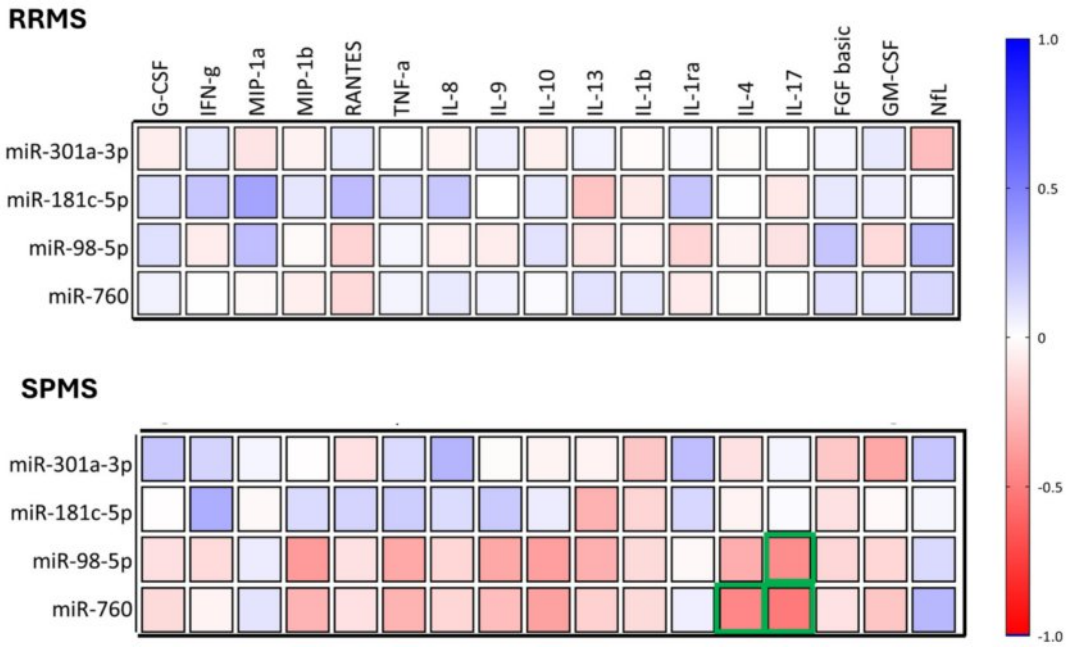

Fig. 3. Spearman correlation matrices between miRNA expression levels and protein marker concentrations. Correlation analyses were performed for RRMS and SPMS groups. The color gradient scale represents the correlation coefficients (r) reflecting the strength and direction of the correlation. Statistically significant correlations ($p < 0.05$) are outlined in green.

Among miRNAs analyzed by RT-qPCR, four demonstrated statistically significant differences in $-\Delta\Delta Ct$ values between RRMS and SPMS: miR-98-5p ($p = 0.0014$), miR-760 ($p < 0.0001$), miR-301a-3p ($p = 0.0095$), and miR-223-3p ($p = 0.0215$), exhibiting the most pronounced statistical significance (Fig. 4). Of these, miR-98-5p showed marked up-regulation in both RRMS and SPMS relative to HC ($log_2FC = 5.09$ and $log_2FC = 8.01$). In contrast, miR-223-3p was slightly down-regulated in RRMS ($log_2FC = -0.46$) and slightly up-regulated in SPMS ($log_2FC = 0.67$). miR-301a-3p demonstrated a substantial down-regulation in both RRMS and SPMS ($log_2FC = -8.79$ and $log_2FC = -4.72$, respectively). miR-760 was markedly down-regulated ($log_2FC = -4.34$) and slightly up-regulated in SPMS ($log_2FC = 0.65$) compared to HC.

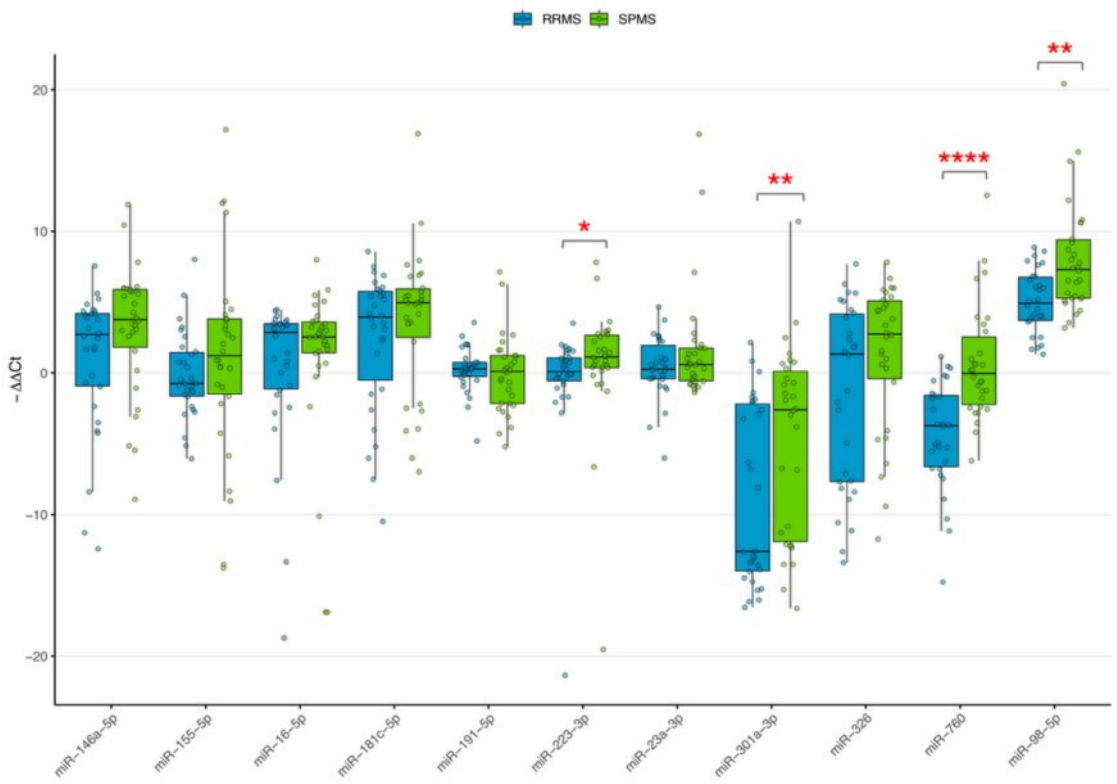

Fig. 4. Differential miRNA expression between RRMS (n = 30) and SPMS (n = 30). Data presented as median ± IQR of $\log_2$-transformed fold-change (−ΔΔCt). Statistical analysis was performed using the U Mann-Whitney test. Significant differences between groups are indicated by *p < 0.05, **p < 0.01, and ****p < 0.0001.

3.5 Statistical modeling for stable RRMS vs SPMS differentiation

A logistic regression model was used to assess the probability of classifying patients into the RRMS group based on miRNA expression levels. Among the ten analyzed ΔCt values, three miRNAs showed a statistically significant association with RRMS classification. Specifically, each one-unit increase in the ΔCt value of miR-301a-3p corresponded to a 17.8% increase in the odds of RRMS classification, whereas miR-146a-5p was associated with a 26.8% increase. The strongest effect was observed for miR-760, where a unit increase in ΔCt led to a 107.5% increase in the odds of RRMS. Statistical details are summarized in Table 5.

Table 5. Logistic regression analysis of miRNA predictors for RRMS classification.

Predictor	p-value	Odds ratio (OR)	95% confidence interval (CI)
miR-760	< 0.0001	2.075	1.397 – 3.081
miR-301a-3p	0.022	1.178	1.024 – 1.355
miR-146a-5p	0.029	1.268	1.024 – 1.571

A linear regression analysis further revealed significant associations between specific miRNAs and immunological markers. A progressive stepwise regression approach identified a significant inverse relationship between miR-760 expression and IL-4 levels in SPMS ($p = 0.0071$, $r = -0.4815$, $R^2 = 0.2319$), while miR-98-5p was significantly inversely associated with IL-17 levels ($p = 0.0181$, $r = -0.4287$, $R^2 = 0.1838$) (Fig. 5). Additionally, miR-16-5p showed a significant relationship with neurodegeneration markers, influencing GFAP

levels in RRMS ($p = 0.0450$, $R^2 = 0.1359$) and NfL levels in the overall analysis ($p = 0.0355$). However, upon stratification by MS subtype, the association of NfL lost statistical significance, indicating potential disease-phase-specific effects.

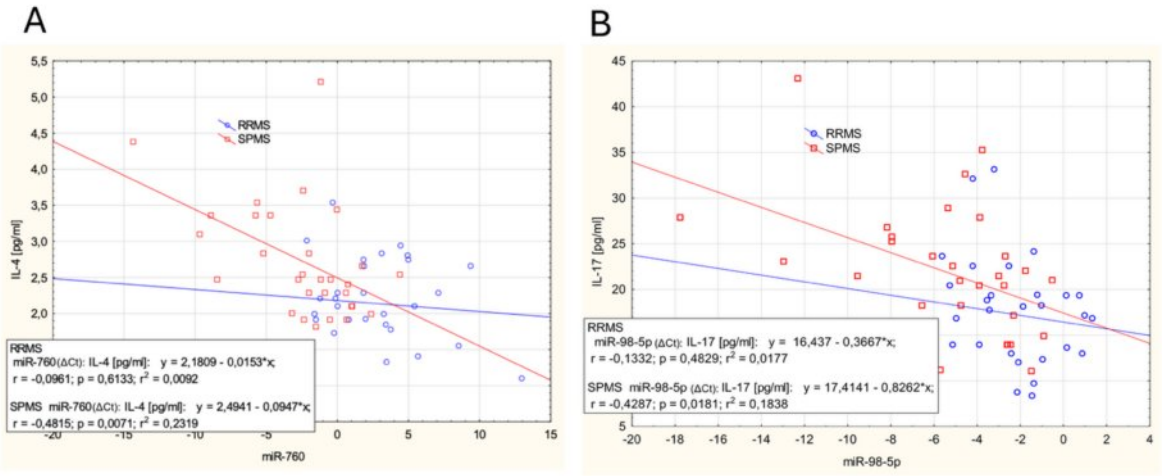

Fig. 5. Linear regression analysis between miRNA expression (ΔCt) and cytokine levels in RRMS and SPMS patients. (A) Relationship between miR-760 and IL-4 levels. (B) Relationship between miR-98-5p and IL-17 levels. Regression lines and corresponding equations, correlation coefficients (r), p-values, and R^2 values are shown for each group.

Based on univariate logistic regression results, a multivariate logistic regression model was developed using a forward stepwise approach. The multivariate logistic regression model, adjusted for potential collinearity, identified miR-760 as the strongest predictor of RRMS classification, followed by miR-301a-3p, and miR-146a-5p. Conversely, higher levels of FGF basic and miR-191-5p were associated with decreased odds of RRMS classification. Statistical details are summarized in Table 6.

Table 6. Multivariate logistic regression model for RRMS classification.

Predictor	p-value	Odds ratio (OR)	95% confidence interval (CI)
miR-760	0.003	3.417	1.533 – 7.618
miR-301a-3p	0.009	1.436	1.093 – 1.888
FGF-basic	0.044	0.687	0.477 – 0.99
miR-191-5p	0.012	0.391	0.188 – 0.817
miR-146a-5p	0.011	1.667	1.126 – 2.468

ROC curve analysis demonstrated strong discriminatory power of the final model, yielding an AUC (95% CI) of 0.973 (0.94 – 1) (Fig. 6), confirming its robustness in distinguishing RRMS from SPMS. The model showed a sensitivity of 93.33%, and a specificity of 90%. Goodness-of-fit analyses further supported the model's reliability (Table 7). The Hosmer-Lemeshow (HL) test yielded a non-significant result (HL-statistic = 5.542, $p = 0.6984$), indicating an adequate fit to the data. The global null hypothesis ($\beta = 0$) was tested using three methods: the likelihood ratio test ($\chi^2 = 58.12$, $df = 5$, $p < 0.001$), the score test ($\chi^2 = 31.17$, $df = 5$, $p < 0.0001$), and the Wald test ($\chi^2 = 11.91$, $df = 5$, $p = 0.036$), all of which confirmed that at least one predictor significantly contributed to the model.

Collectively, these findings underscore the utility of miRNA expression profiles in distinguishing RRMS from SPMS, with miR-760 emerging as a particularly strong classifier.

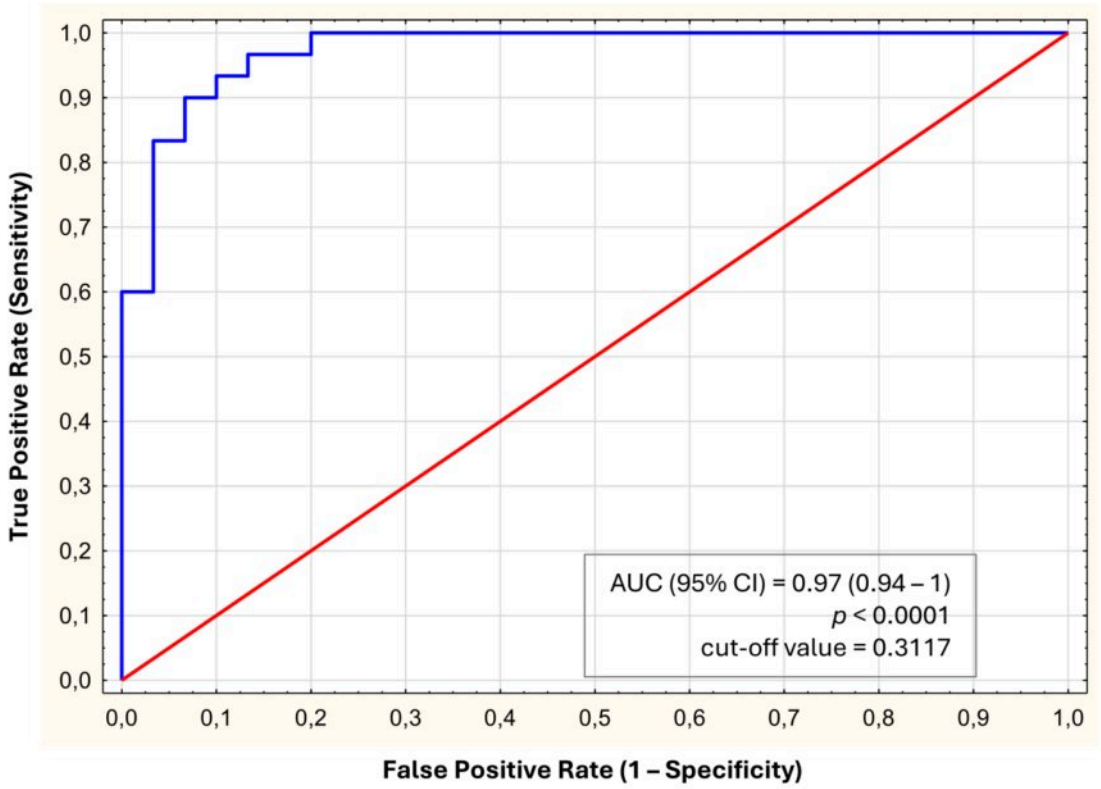

Fig. 6. Receiver operating characteristic (ROC) curve for model differentiating RRMS patients from SPMS based on FGF basic, miR-760, miR-301a-3p, miR-191-5p, and miR-146a-5p. The quality of the model was measured by the area under the curve (AUC) with 95% confidence interval (CI) and optimal cut-off providing a balanced trade-off between test sensitivity and specificity.

Table 7. Summary of goodness-of-fit measures.

Measure	Df	Statistic	Statistic/Df
Deviance	54	25.058003	0.464037
Scaled deviance	54	25.058003	0.464037
Pearson's Chi^2	54	31.143738	0.576736
Scaled Pearson's Chi^2	54	31.143738	0.576736
AIC	-	37.058003	-
AICC	-	38.642908	-
BIC	-	49.62407	-
Cox-Snell R^2	-	0.620409	-
Nagelkerke R^2	-	0.827212	-
Log-likelihood	-	-12.529001	-

3.6 Target gene prediction and functional enrichment

Human miRNA targets were retrieved from experimentally validated datasets using the multiMiR package, yielding 16 085 genes, with most targeted by miR-98-5p (Fig. 7).

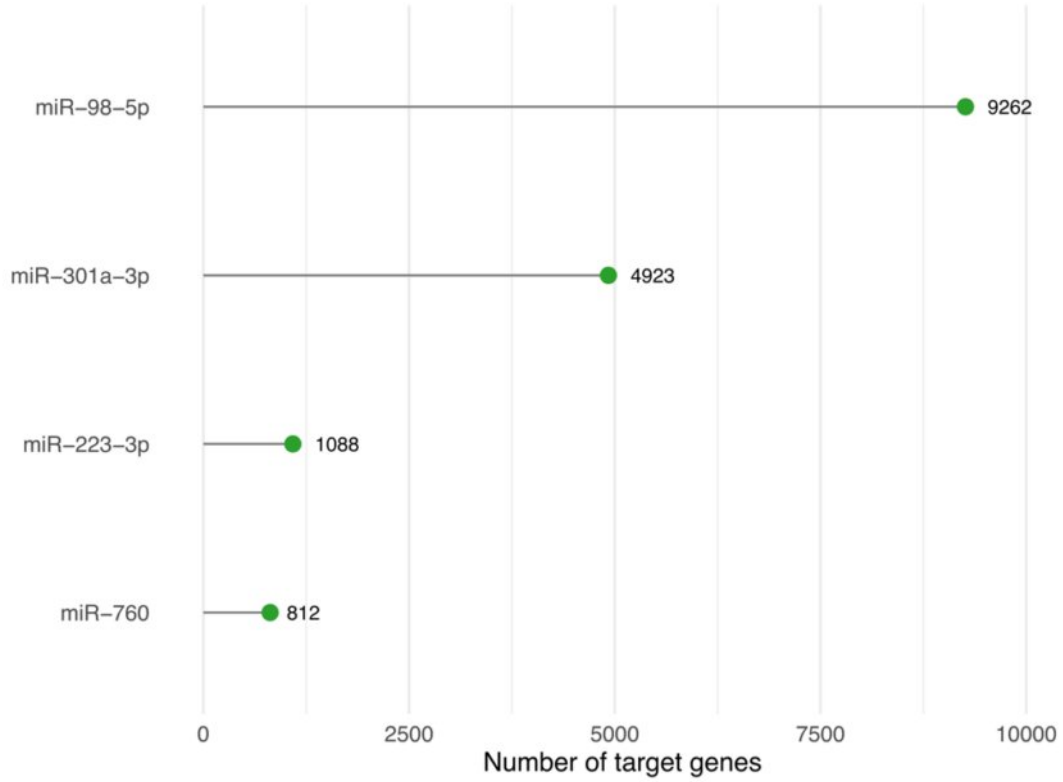

Fig. 7. Analysis of targets of selected miRNAs. The bar plot shows the total number of experimentally validated miRNA-mRNA interaction.

In GO functional enrichment analysis, 2 899 results were associated with differentially expressed miRNA (miR-98-5p, miR-760, miR-301a-3p, and miR-223-3p) target genes ($p < 0.05$), including 2 214 BP, 351 CC, and 334 MF. The top 10 overrepresented terms for each category are shown in Fig. 8.

The most significantly enriched BP included small GTPase-mediated signal transduction, regulation of cellular catabolic processes, neuron projection development, mitotic cell cycle phase transition, and proteasome-mediated protein catabolic processes. Additionally, pathways related to autophagy (macroautophagy, regulation of autophagy) and Golgi vesicle transport were overrepresented, indicating a strong association with intracellular signaling and degradation mechanisms. The CC analysis revealed enrichment in membrane-associated structures, including vacuolar membrane, lysosomal membrane, and cell-substrate junction, as well as synaptic components such as neuron-to-neuron synapse, postsynaptic specialization, and asymmetric synapse. Nuclear structures, including nuclear speck and chromosomal region, were also significantly represented. Significantly enriched MF included DNA-binding transcription factor binding, GTPase regulator activity, nucleoside-triphosphatase regulator activity, and histone modifying activity. Additionally, pathways related to ubiquitin-protein ligase binding, small GTPase binding, and protein serine/threonine kinase activity were identified, highlighting the involvement of post-translational modification and intracellular signaling mechanisms.

The KEGG[21] pathway enrichment showed that the analyzed miRNA target genes play a role in 144 pathways. Of these, the top 30 with are shown in Fig. 9. The analysis identified significant enrichment in neurodegeneration, intracellular signaling, and cellular homeostasis. Several neurodegenerative disease pathways, including Alzheimer's disease, amyotrophic lateral sclerosis (ALS), and spinocerebellar ataxia, were overrepresented. Moreover, key intracellular signaling pathways, including MAPK, AMPK, FoxO, Hippo, and ErbB, were enriched, highlighting their roles in inflammation, cell survival, proliferation, and oxidative stress. Enrichment in autophagy, mitophagy, ubiquitin-mediated proteolysis, and protein processing in the endoplasmic

reticulum indicates impaired proteostasis, a key feature in neuroinflammation. Additionally, pathways related to cellular senescence, adherens junctions, and nucleocytoplasmic transport highlight disruptions in cellular integrity and signaling.

To investigate molecular differences between stable RRMS and SPMS, a network analysis of miRNA-mRNA interactions was performed. The validated mRNA targets of miR-98-5p, miR-760, miR-301a-3p, and miR-223-3p were integrated with disease-associated genes retrieved from the DisGeNET database. The constructed networks present distinct regulatory patterns in stable RRMS and SPMS, with an assigned disease specificity index (DSI), whose value is inversely proportional to the number of diseases associated with a particular gene (Fig. 10).

In RRMS, functional categories of selected genes included immune signaling (*FOXP3*, DSI = 0.37), apoptosis (*FAS*, DSI = 0.33), sphingolipid signaling (*S1PR1*, DSI = 0.47; *S1PR5*, DSI = 0.64), and stress response marker *MAP2K7* (DSI = 0.38). The neurodegeneration marker gene *GFAP* (DSI = 0.37) was also identified. In SPMS, interactions were mainly linked to immune regulation and were targeted by miR-98-5p and miR-301a-3p: *IL10* (DSI = 0.27), *CSF1* (DSI = 0.34), *CD8A* (DSI = 0.33), and *IL7* (DSI = 0.42). Additionally, chemokine receptors involved in signal transduction, *CCR7* (DSI = 0.43) and *CCR5* (DSI = 0.4), were targeted. *SDC1* (DSI = 0.4), associated with extracellular matrix organization and hemostasis, was also identified.

These findings indicate immune-dominant regulation in SPMS, while stable RRMS exhibits additional pathways related to transcriptional control, apoptosis, and cellular stress response. miR-98-5p exhibited the highest number of interactions across both conditions, predominantly regulating immune-related genes in SPMS and extending to transcriptional and stress response pathways in stable RRMS.

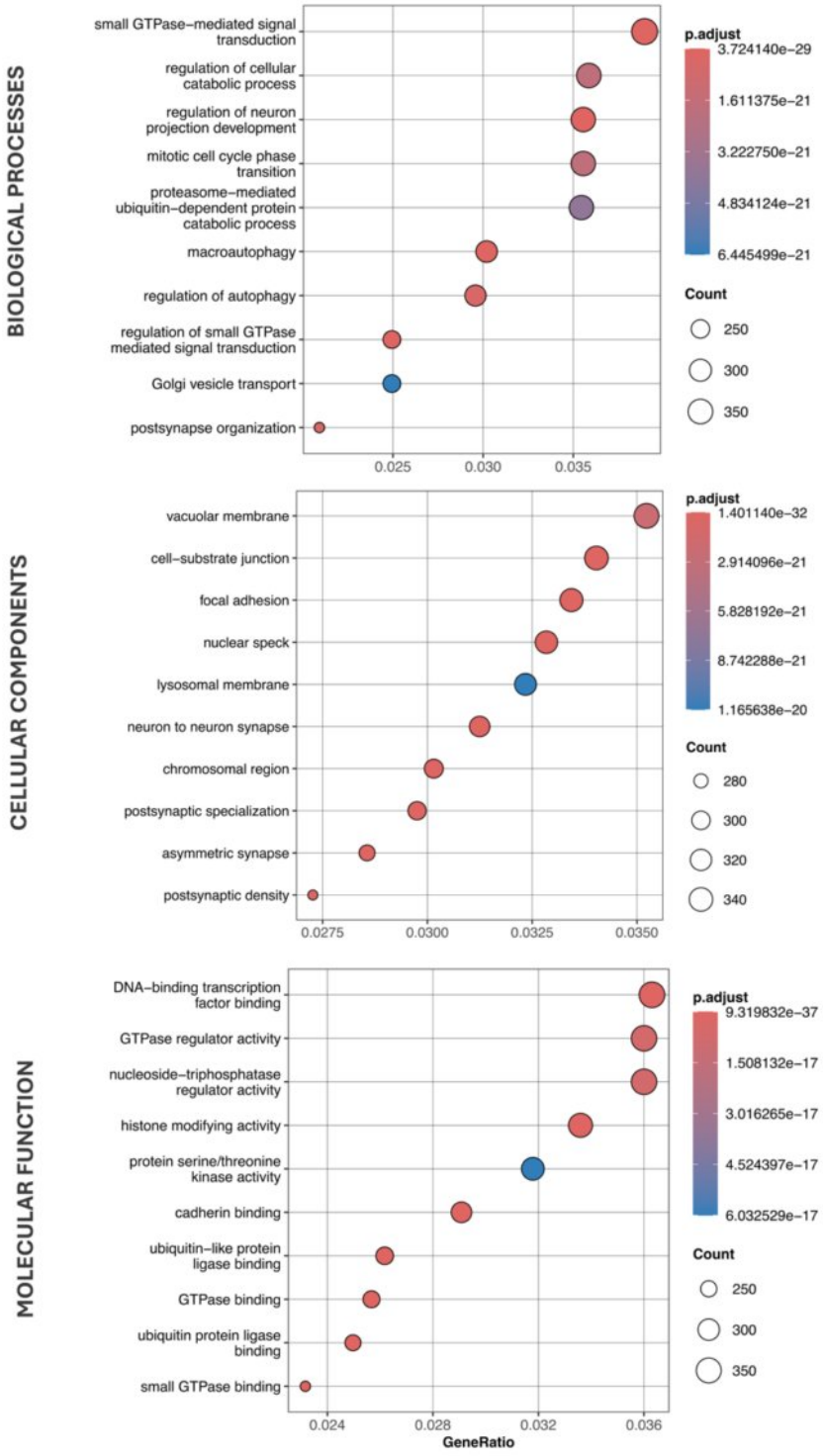

Fig. 8. Gene ontology (GO) functional enrichment analysis for miR-98-5p, miR-760, miR-301a-3p, and miR-223-3p.

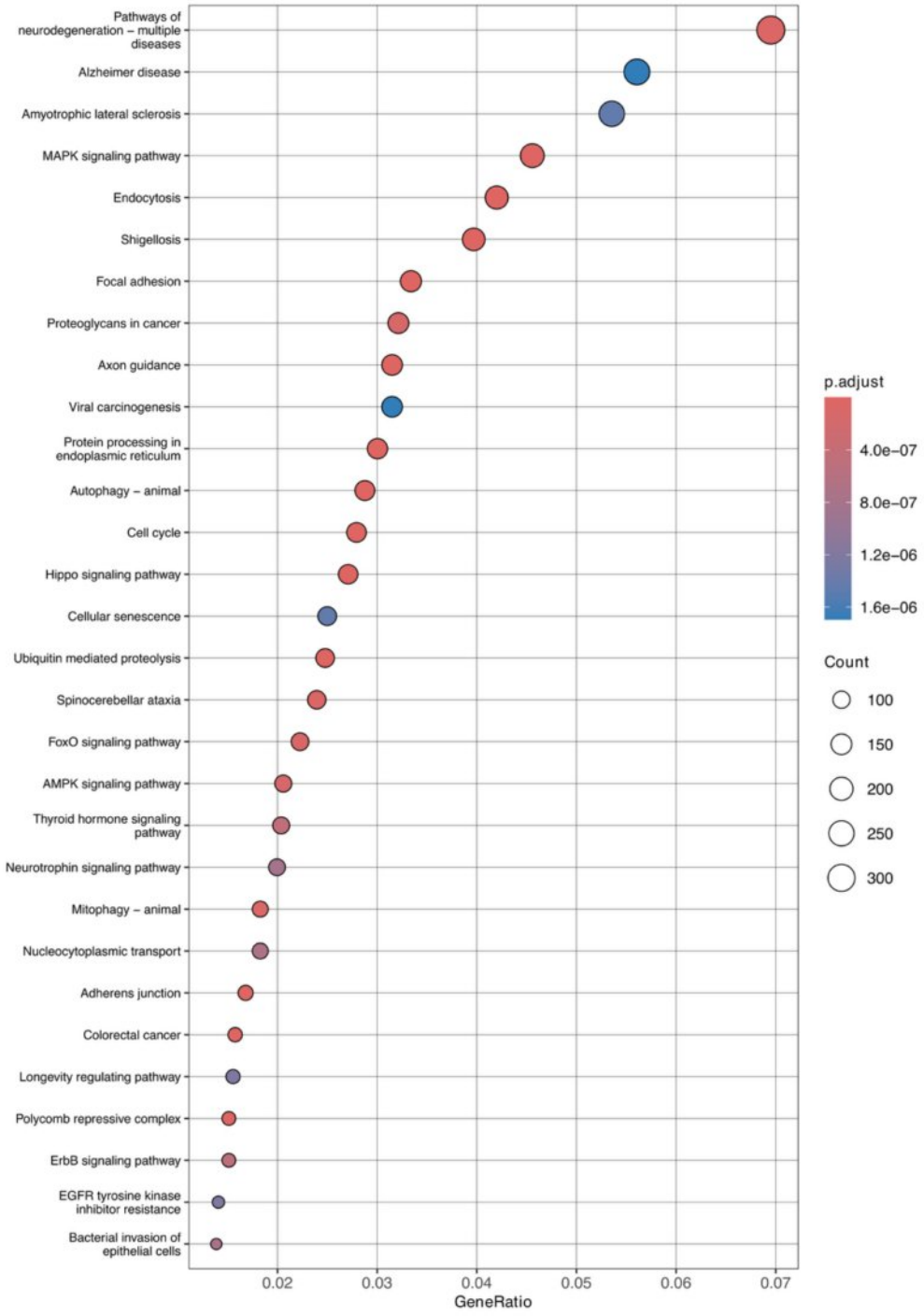

Fig. 9. Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis for miR-98-5p, miR-760, miR-301a-3p, and miR-223-3p

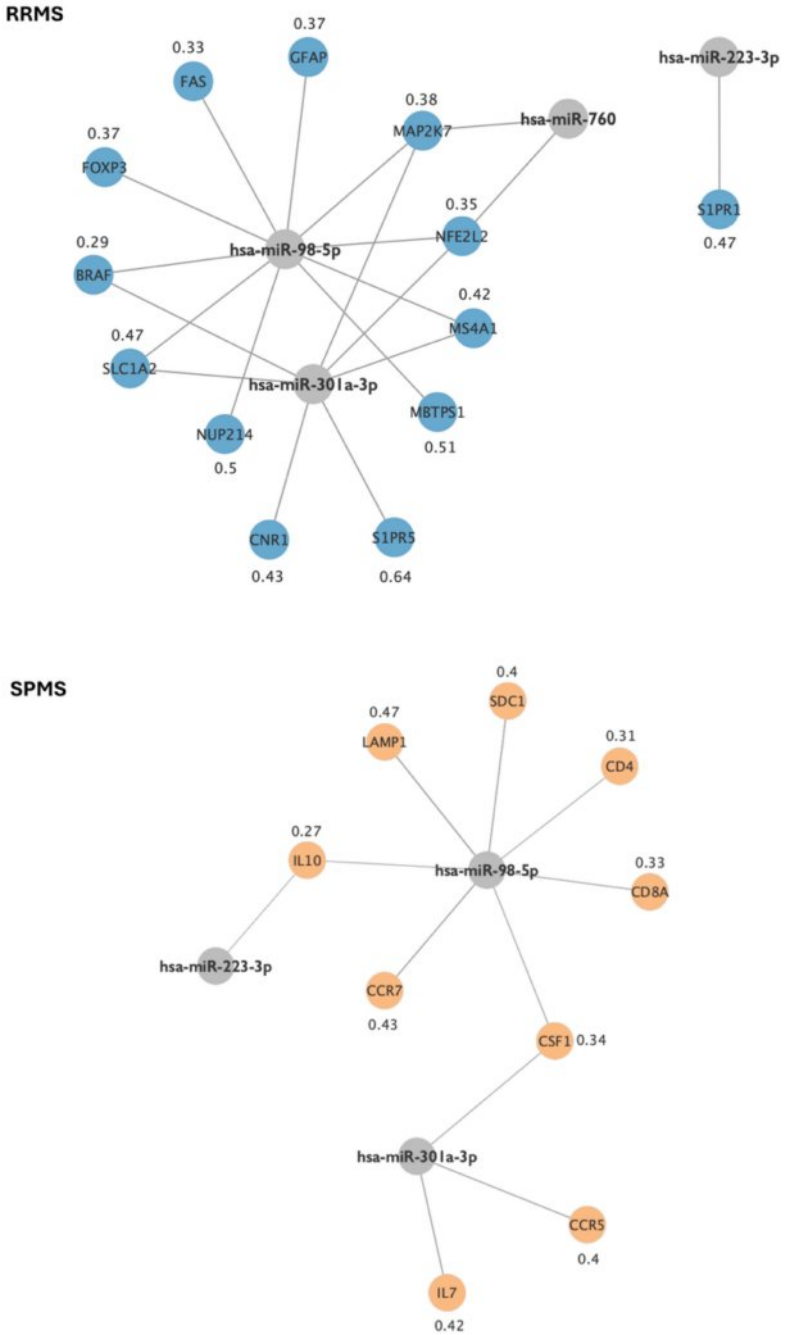

Fig. 10. miRNA-mRNA interaction networks in stable RRMS and SPMS. Nodes represent miRNAs (sources) and their target genes, with edges indicating regulatory interactions. The values assigned to each gene indicate the disease specificity index (DSI).

4 Discussion

MS is a neurodegenerative, incurable disease manifested by the destruction of myelin sheaths in result of the autoimmune mechanism, driven by infiltration of T and B-cells into the CNS [4]. Early differentiation between clinical phenotypes, particularly RRMS and SPMS, is crucial for prognosis and treatment planning, highlighting the need for reliable and easily accessible biomarkers. Although MRI of the brain and spinal cord plays a pivotal role in the diagnostic process, it does not fully reflect the picture of disease pathophysiology. To complement neuroimaging and enhance clinical decision-making, fluid-based biomarkers are extensively studied [6]. Among available biofluids, CSF stands out due to its direct relationship with the CNS, however, its collection requires an invasive procedure of lumbar puncture. Recent advances enabling the detection of brain-derived proteins, such as GFAP, CHIT1, CHI3L1, sTREM2, and NfL, in peripheral blood have opened promising avenues for non-invasive monitoring of MS pathophysiology using blood-based biomarkers [27–31]. Thus, profiling circulating EV-derived miRNAs may provide greater specificity than peripheral blood sample analysis as they are physically separated from body fluids, reflect the current molecular condition of the origin cell, and readily cross the BBB into the circulation.

In this exploratory study, we applied multi-omics approach, including cytokine profiling, neuronal and glial damage biomarkers measurement, EV-derived miRNA expression analysis, and integrative bioinformatics, to identify peripheral signatures that differentiate stable RRMS and SPMS phenotypes.

Among investigated EV-associated miRNAs, four candidates (miR-98-5p, miR-760, miR-301a-3p, and miR-223-3p) exhibited significantly higher expression in SPMS comparing to stable RRMS ($-\Delta\Delta Ct$). Importantly, logistic regression analyses underscored miR-760 as the strongest predictive marker for distinguishing stable RRMS from SPMS. This finding highlights miR-760 as a promising candidate for stratifying patients according to disease phenotypes. To the best of our knowledge, this observation is novel in the MS context, considering limited previous explorations specifically addressing the clinical utility of miR-760. Nevertheless, miR-760 was previously shown to mediate the improvement of remyelination via inhibiting G protein-coupled receptor (GPR)17, which pathological overexpression limits late-stage myelin maturation in oligodendrocyte precursor cells [32]. Moreover, delivering exosome-encapsulated miR-760-3p to the brain has shown an anti-ferroptotic effect on neurons after ischemic brain injury [33]. This suggests that miR-760 may play a broader neuroprotective role beyond remyelination, potentially by modulating oxidative stress pathways and cell death mechanisms. Recent findings also indicate that reduction of miR-760-3p results in up-regulation of MAPK3K8, leading to activation of pro-inflammatory NF-κB pathway, which may support the survival and activation of autoreactive B-cells within the CNS [34]. In our analysis, both linear regression and Spearman correlation demonstrated a statistically significant inverse relationship between miR-760 expression and IL-4 levels in SPMS ($p = 0.0071$, $R^2 = 0.2319$; $p = 0.008$ $r = -0.472$, respectively), suggesting a potential immunoregulatory role of miR-760 in the progressive disease stage. This association was not observed in RRMS, indicating that the functional relevance of miR-760 may be phenotype-specific. Moreover, an inverse correlation between miR-760 and IL-17 levels ($p = 0.003$, $r = -0.520$), a key effector of Th17-driven inflammation, was observed exclusively in SPMS group.

Notably, a recent immunophenotyping study reported that while a range of Th17-lineage subsets is expanded in RRMS, only Th17 cells show increased frequencies in SPMS, supporting the involvement of Th17-driven inflammation in progressive disease stages [35].

Given the central role of Th17 cells in MS immunopathology [36], other miRNAs associated with this T-cell subset are of particular interest. Among them, miR-98-5p, exerts a protective effect by preventing BBB dysfunction and inhibiting neuroinflammation in the CNS [37–40]. Dysregulation of miR-98-5p has been reported in several MS studies [41–43]. Moreover, its significant correlation with IL-17 levels supports emerging evidence that identifies miR-98-5p as a crucial regulator of inflammatory pathways, particularly via modulation of Th17 responses [44,45]. In our study, stepwise linear regression revealed a significant inverse association between miR-98-5p and IL-17 levels in SPMS ($p = 0.0181$, $R^2 = 0.1938$). This finding was confirmed by Spearman correlation analysis ($p = 0.013$, $r = -0.447$), with the association again observed exclusively in SPMS patients. These results suggest a stage-specific role for miR-98-5p in modulating Th17-related inflammation. Our findings are consistent with previous studies showing that miR-98-5p inhibits Th17 cells differentiation in experimental autoimmune encephalomyelitis (EAE) – likely through the direct or indirect down-regulation of RAR-related orphan receptor gamma t (RORγt), the key transcription factor driving Th17 cell development [44].

As demonstrated in our study, the significant differential expression of miR-301a-3p – a Th17 subset-associated miRNA – between stable RRMS and SPMS, supports its possible functional role in immune alterations, autoimmune demyelination, and neurodegeneration [46–49]. In other study, exosomal miR-301a-3p was decreased in RRMS patients during relapse and proposed as a potential relapse biomarker [50]. Nevertheless, research results are not consistent in this matter – peripheral blood mononuclear cells (PBMCs)-derived miR-301a expression was found up-regulated in RRMS patients in relapse [51] and higher in post-acute vs stable phase of remission [52]. This inconsistency underscores also the need for further studies to clarify the temporal and cellular context of miR-301a-3p expression during disease activity and also highlights the importance of sample origin in interpreting molecular signatures in MS. A recent MS study suggested that miR-223-3p could be a therapeutic target for chronic inflammation by improving the immunosuppressive function of myeloid-derived suppressor cells via a STAT3-dependent mechanism [53]. Here, we found its expression to be significantly up-regulated in SPMS compared to stable RRMS, extending recent findings on its potential as a differential biomarker of neurodegenerative conditions, a regulator of MS-related processes, including Th1 differentiation, macrophage M2 polarization, and myelin debris clearance, and biomarker distinguishing MS phenotypes [54–58]. Importantly, miR-223-3p has been found up-regulated at sites of myelin damage in both MS and experimental model of demyelination and identified as an endogenous regulator of the NLRP3 (NBD-, LRR- and pyrin domain-containing protein 3) inflammasome [59]. By targeting NLRP3, miR-223-3p may help limit the production of pro-inflammatory cytokines such as IL-1β and IL-18, thereby reducing chronic microglial activation and subsequent tissue damage. Moreover, miR-223-3p expression was negatively correlated with T1 lesion volumes in SPMS and PPMS, and its temporal variability was associated with relapse phases [60]. The inverse correlation with lesion burden implies a potential neuroprotective or inflammation-limiting role of miR-223-3p, particularly in progressive MS forms. Furthermore, its temporal fluctuations during relapse may support its use in monitoring disease activity or therapeutic response over time.

Our multivariate logistic regression model integrating FGF basic, miR-760, miR-301a-3p, miR-191-5p, and miR-146a-5p demonstrated excellent discriminatory power in differentiating stable RRMS from SPMS. The ROC curve analysis yielded an AUC of 0.97 (95% CI: 0.94 – 1; sensitivity 93.33% and specificity 90%) (Fig. 4). Studies have shown the high expression of FGF basic withing the neuroinflammatory lesions and positive correlation with macrophages and microglia activation. In contrast, in the myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$-induced EAE, a commonly used animal model of MS, the level of FGF basic was reduced, suggesting its involvement in promoting remyelination. This effect was mediated through ERK/Akt phosphorylation, brain-derived neurotrophic factor (BDNF), and the down-regulation of remyelination inhibitors [57]. miR-191-5p, though less studied in MS, was found up-regulated both in SPMS and PPMS [62]. Evidence regarding the diagnostic or phenotypic utility of miR-191-5p in MS remains inconsistent. Some studies have reported no significant differences in its serum levels between MS patients and healthy individuals, nor between RRMS and progressive MS subtypes [63]; while others observed its overexpression in RRMS and PPMS, with no differences between the subtypes [64]. Interestingly, a negative correlation was observed between miR-191-5p expression and disease duration, suggesting a possible link to long-term disease progression [63]. This finding may indicate a role for miR-191-5p in the late stage of MS, consistent with its proposed value as a predictive biomarker for Alzheimer's disease development [65]. In contrast to the ambiguous role of miR-191-5p, miR-146a-5p is one of the most extensively studied and well-characterized inflammation-related miRNAs in MS. Acting as a suppressor of innate immune activation it has been consistently implicated in diagnostic and prognostic contexts, showing strong potential as a biomarker for disease activity and progression [66].

Functional enrichment analysis of the predicted miRNA targets revealed several key biological processes and signaling pathways that may underlie the molecular divergence between stable RRMS and SPMS. Among the most significantly overrepresented were small GTPase-mediated signaling, autophagy, cellular senescence, and pathways implicated in major neurodegenerative diseases, including Alzheimer's and ALS. These findings align with recent data suggesting impaired autophagic flux and defective protein homeostasis as critical drivers of neurodegeneration [67,68]. Furthermore, the marked enrichment of key intracellular signaling pathways (such as MAPK, AMPK, FoxO, Hippo, and ErbB) known to influence neuroinflammation, oxidative stress responses, and neuronal survival suggests that miRNA-regulated disruption of cellular homeostasis may play a pivotal role in SPMS progression [69–77].

Network analyses revealed distinct miRNA-mRNA regulatory architectures associated with each MS phenotype, reflecting divergent underlying mechanisms of the disease. In SPMS, regulatory networks were dominated by immune-related genes primarily targeted by miR-98-5p and miR-301a-3p, including *IL10*, *CSF1*,

CD8A, and chemokine receptors *CCR5* and *CCR7* – previously implicated in chronic neuroinflammation, impaired immune resolution, and sustained microglial activation [78–84]. This supports growing evidence that progressive MS is driven by compartmentalized, smoldering inflammation within the CNS [85]. In turn, the stable-specific network revealed a broader functional diversity, involving pro-apoptotic signaling (*FAS*), lipid-mediated immunoregulation (*S1PR1*, *S1PR5*), and stress-response pathways (*MAP2K7*) [86–88].

Despite the promising findings, several limitations of this study should be acknowledged. First, the relatively moderate sample size may limit the generalizability of the results and warrants validation in external larger independent cohorts. Second, although EVs isolation and characterization protocols were employed, heterogeneity in EV populations remains inherent to current methodological standards, potentially influencing biomarker specificity and sensitivity. Future methodological advances allowing subtype-specific EV isolation could substantially improve biomarker precision.

Collectively, our findings extend the current understanding of MS phenotype differentiation, underscoring plasma-derived EV-miRNAs as putative markers with translational relevance. miR-760, miR-98-5p, miR-301a-3p, and miR-223-3p emerge as compelling candidates reflecting underlying neuroinflammatory and neurodegenerative processes specific to stable RRMS and SPMS. Notably, miR-760 and miR-98-5p represent particularly important discoveries within this study – identified initially through unbiased RNA-sequencing and subsequently validated by RT-qPCR, both miRNAs demonstrated statistically significant associations with molecular disease parameters, including IL-4 and IL-17 levels. These findings were further supported by group-specific correlation matrices, which revealed that selected miRNA–cytokine relationships were present exclusively in the SPMS group. Their robust expression profiles and integration into predictive logistic regression models point to their potential relevance as indicators of immune activity of MS subtypes. Nevertheless, these findings warrant validation in independent, longitudinal cohorts and functional studies to establish their clinical applicability and mechanistic significance.

Abbreviations

ALS: Amyotrophic lateral sclerosis

AUC: Area under the curve

BBB: Blood-brain barrier

BDNF: Brain-derived neurotrophic factor

BP: Biological processes

CC: Cellular components

cDNA: Complementary DNA

CI: Confidence interval

CIS: Clinically isolated syndrome

CNS: Central nervous system

CPDA: Citrate phosphate dextrose adenine

CRP: C-reactive protein

CSF: Cerebrospinal fluid

DLS: Dynamic light scattering

DSI: Disease specificity index

EAE: Experimental autoimmune encephalomyelitis

EDSS: Expanded disability status scale

ELISA: Enzyme-linked immunosorbent assay

ESR: Erythrocyte sedimentation rate

EV: Extracellular vesicle

FC: Fold-change

FDR: False discovery rate

FGF: Fibroblast growth factor

G-CSF: Granulocyte colony-stimulating factor

GDA: Disease-gene association

GFAP: Glial fibrillary acidic protein

GM-CSF: Granulocyte macrophage colony-stimulating factor

GO: Gene ontology

GPR: G protein-coupled receptor

HC: Healthy control

HL: Hosmer-Lemeshow

IFN: Interferon

IL: Interleukin

IP: Interferon gamma-induced protein

IQR: Interquatrile range

ISEV: International society of extracellular vesicles

KEGG: Kyoto encyclopedia of genes and genomes

MCP: Monocyte chemoattractant protein

MF: Molecular functions

MIP: Macrophage inflammatory protein

miRNA: microRNA

MOG: Myelin oligodendrocyte glycoprotein

MOGAD: Myelin oligodendrocyte glycoprotein antibody-associated disease

MRI: Magnetic resonance imaging

MS: Multiple sclerosis

NfL: Neurofilament light chain

NLRP3: NBD-, LRR- and pyrin domain-containing protein 3

NMOSD: Neuromyelitis optica spectrum disorder

OD: Optical density

PBMCs: Peripheral blood mononuclear cells

PBS: Phosphate-buffered saline

PDGF: Platelet-derived growth factor

PDI: Polydispersity index

PPMS: Primary progressive multiple sclerosis

RANTES: Regulated on activation, normal T expressed and secreted

ROC: Receiver operating characteristic

RORγt: RAR-related orphan receptor gamma t

RRMS: Relapsing-remitting multiple sclerosis

RT: Room temperature

RT-qPCR: Quantitative real-time PCR

SPMS: Secondary progressive multiple sclerosis

TEM: Transmission electron microscopy

Th: T helper

TNF: Tumor necrosis factor

VEGF: Vascular endothelial growth factor

Data availability statement

The raw and processed RNA sequencing data generated in this study have been deposited in the Gene Expression Omnibus (GEO) under accession number GSE303912.
To review GEO accession GSE303912:
Go to https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE303912
Enter token oxufugusxnobngf into the box

References

1. Atlas of MS 2020 - Epidemiology report. *MS International Federation* https://www.msif.org/resource/atlas-of-ms-2020/.
2. Kasper, L. H. & Shoemaker, J. Multiple sclerosis immunology: The healthy immune system vs the MS immune system. *Neurology* **74 Suppl 1**, S2-8 (2010).
3. Publications. *ECTRIMS* https://ectrims.eu/ms-clinical-trials-committee/publications/.
4. Filippi, M. *et al.* Multiple sclerosis. *Nat Rev Dis Primers* **4**, 1–27 (2018).
5. Solomon, A. J. *et al.* Differential diagnosis of suspected multiple sclerosis: an updated consensus approach. *The Lancet Neurology* **22**, 750–768 (2023).
6. Di Filippo, M. *et al.* Fluid biomarkers in multiple sclerosis: from current to future applications. *Lancet Reg Health Eur* **44**, 101009 (2024).
7. Sáenz-Cuesta, M., Osorio-Querejeta, I. & Otaegui, D. Extracellular Vesicles in Multiple Sclerosis: What are They Telling Us? *Front Cell Neurosci* **8**, 100 (2014).
8. Soleymani, T., Chen, T.-Y., Gonzalez-Kozlova, E. & Dogra, N. The human neurosecretome: extracellular vesicles and particles (EVPs) of the brain for intercellular communication, therapy, and liquid-biopsy applications. *Front. Mol. Biosci.* **10**, (2023).
9. Welsh, J. A. *et al.* Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches. *Journal of Extracellular Vesicles* **13**, e12404 (2024).
10. Mycko, M. P. & Baranzini, S. E. microRNA and exosome profiling in multiple sclerosis. *Mult Scler* **26**, 599–604 (2020).
11. Ho, P. T. B., Clark, I. M. & Le, L. T. T. MicroRNA-Based Diagnosis and Therapy. *Int J Mol Sci* **23**, 7167 (2022).
12. McCoy, C. E. miR-155 Dysregulation and Therapeutic Intervention in Multiple Sclerosis. *Adv Exp Med Biol* **1024**, 111–131 (2017).
13. Otaegui, D. *et al.* Differential Micro RNA Expression in PBMC from Multiple Sclerosis Patients. *PLOS ONE* **4**, e6309 (2009).
14. Regev, K. *et al.* Comprehensive evaluation of serum microRNAs as biomarkers in multiple sclerosis. *Neurol Neuroimmunol Neuroinflamm* **3**, e267 (2016).
15. Baulina, N. M. *et al.* MicroRNAs: The Role in Autoimmune Inflammation. *Acta Naturae* **8**, 21–33 (2016).
16. Piket, E., Zheleznyakova, G. Y., Kular, L. & Jagodic, M. Small non-coding RNAs as important players, biomarkers and therapeutic targets in multiple sclerosis: A comprehensive overview. *J Autoimmun* **101**, 17–25 (2019).
17. Thompson, A. J. *et al.* Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. *The Lancet Neurology* **17**, 162–173 (2018).
18. Lublin, F. D. *et al.* Defining the clinical course of multiple sclerosis. *Neurology* **83**, 278–286 (2014).
19. Ebrahimkhani, S. *et al.* Exosomal microRNA signatures in multiple sclerosis reflect disease status. *Sci Rep* **7**, 14293 (2017).
20. Ru, Y. *et al.* The multiMiR R package and database: integration of microRNA–target interactions along with their disease and drug associations. *Nucleic Acids Research* **42**, e133 (2014).
21. Kanehisa, M. & Goto, S. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* **28**, 27–30 (2000).
22. Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y. & Morishima, K. KEGG: new perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Res* **45**, D353–D361 (2017).
23. Yu, G., Wang, L.-G., Han, Y. & He, Q.-Y. clusterProfiler: an R Package for Comparing Biological Themes Among Gene Clusters. *OMICS* **16**, 284–287 (2012).
24. DISGENET: Genomics Platform for Precision Medicine. https://www.disgenet.com.
25. Piñero, J. *et al.* The DisGeNET knowledge platform for disease genomics: 2019 update. *Nucleic Acids Research* **48**, D845–D855 (2020).
26. Wickham, H. *Ggplot2: Elegant Graphics for Data Analysis*. (Springer-Verlag New York, Cham, 2016). doi:10.1007/978-3-319-24277-4.
27. Simrén, J. *et al.* Differences between blood and cerebrospinal fluid glial fibrillary Acidic protein levels: The effect of sample stability. *Alzheimer's & Dementia* **18**, 1988–1992 (2022).
28. Freedman, M. S. *et al.* Guidance for use of neurofilament light chain as a cerebrospinal fluid and blood

biomarker in multiple sclerosis management. *eBioMedicine* **101**, 104970 (2024).
29. Ashton, N. J. *et al.* Plasma levels of soluble TREM2 and neurofilament light chain in TREM2 rare variant carriers. *Alzheimer's Research & Therapy* **11**, 94 (2019).
30. Beliën, J. *et al.* CHIT1 at diagnosis predicts faster disability progression and reflects early microglial activation in multiple sclerosis. *Nat Commun* **15**, 5013 (2024).
31. Mohammed, M. S., Al-Rubae'i, S. H. N., Rheima, A. M. & Al-Kazazz, F. F. A novel sandwich ELISA method for quantifying CHI3L1 in blood serum and cerebrospinal fluid multiple sclerosis patients using sustainable photo-irradiated zero-valence gold nanoparticles. *Results in Chemistry* **11**, 101856 (2024).
32. Wang, Y. *et al.* Buyang huanwu decoction promotes remyelination via miR-760-3p/GPR17 axis after intracerebral hemorrhage. *Journal of Ethnopharmacology* **328**, 118126 (2024).
33. Wang, Y. *et al.* Anti-CHAC1 exosomes for nose-to-brain delivery of miR-760-3p in cerebral ischemia/reperfusion injury mice inhibiting neuron ferroptosis. *J Nanobiotechnol* **21**, 109 (2023).
34. Zhang, H. *et al.* circNup188/miR-760–3p/Map3k8 axis regulates inflammation in cerebral ischemia. *Molecular and Cellular Probes* **64**, 101830 (2022).
35. Kalra, S. *et al.* Th17 cells increase in RRMS as well as in SPMS, whereas various other phenotypes of Th17 increase in RRMS only. *Mult Scler J Exp Transl Clin* **6**, 2055217319899695 (2020).
36. Moser, T., Akgün, K., Proschmann, U., Sellner, J. & Ziemssen, T. The role of TH17 cells in multiple sclerosis: Therapeutic implications. *Autoimmun Rev* **19**, 102647 (2020).
37. Huang, X., Tan ,Yixin, Wu ,Ruifang, Li ,Qianwen & and Luo, S. MicroRNA-98-5p Inhibits IFI44L-Mediated Differentiation of Dendritic Cells and Activation of Interferon Pathway in Systemic Lupus Erythematosus. *Immunological Investigations* **53**, 475–489 (2024).
38. Yang, J. *et al.* Neuronal extracellular vesicle derived miR-98 prevents salvageable neurons from microglial phagocytosis in acute ischemic stroke. *Cell Death Dis* **12**, 1–16 (2021).
39. Song, C. *et al.* Post-transcriptional regulation of α7 nAChR expression by miR-98-5p modulates cognition and neuroinflammation in an animal model of Alzheimer's disease. *The FASEB Journal* **35**, e21658 (2021).
40. Rom, S., Dykstra, H., Zuluaga-Ramirez, V., Reichenbach, N. L. & Persidsky, Y. miR-98 and let-7g* Protect the Blood-Brain Barrier Under Neuroinflammatory Conditions. *J Cereb Blood Flow Metab* **35**, 1957–1965 (2015).
41. Identification of miRNAs and their potential effects on multiple sclerosis related pathways using ın silico analysis. *Multiple Sclerosis and Related Disorders* **59**, 103642 (2022).
42. Hadi, N. *et al.* Altered Expression of Circulating miR-377 and miR-98 in Relapsing-remitting Multiple Sclerosis. *International Journal of Medical Laboratory* **7**, 1–8 (2020).
43. Çam, B. E., Elgün, T., Köprülü, T. K., Çoban, A. & Korkut, Ş. V. miR-98 and miR-629 can be used as a potential biomarker on relapsing-remitting multiple sclerosis patients. *Gene Reports* **37**, 102041 (2024).
44. Mohammadi-Kordkhayli, M. *et al.* Vitamins A and D Enhance the Expression of Ror-γ-Targeting miRNAs in a Mouse Model of Multiple Sclerosis. *Mol Neurobiol* **60**, 5853–5865 (2023).
45. Xu, Q.-F. *et al.* Oleanolic acid regulates the Treg/Th17 imbalance in gastric cancer by targeting IL-6 with miR-98-5p. *Cytokine* **148**, 155656 (2021).
46. Mycko, M. *et al.* Mir-301a Regulation of the Development of a Th17 Response Controls Autoimmune Demyelination (P07.085). *Neurology* **78**, P07.085-P07.085 (2012).
47. He, C. *et al.* miR-301a promotes intestinal mucosal inflammation through induction of IL-17A and TNF-α in IBD. *Gut* **65**, 1938–1950 (2016).
48. Martinez, B. & Peplow, P. V. MicroRNA biomarkers in frontotemporal dementia and to distinguish from Alzheimer's disease and amyotrophic lateral sclerosis. *Neural Regeneration Research* **17**, 1412 (2022).
49. Mou, L., Xin, M. & Li, E. Dysregulation of miR-301a-3p serves as a non-invasive biomarker and is associated with inflammatory responses in traumatic spinal cord injury. *Acta Biochim Pol* **69**, 725–730 (2022).
50. Selmaj, I. *et al.* Global exosome transcriptome profiling reveals biomarkers for multiple sclerosis. *Ann Neurol* **81**, 703–717 (2017).
51. Tavakolpour, V. *et al.* Increased expression of mir-301a in PBMCs of patients with relapsing-remitting multiple sclerosis is associated with reduced NKRF and PIAS3 expression levels and disease activity. *Journal of Neuroimmunology* **325**, 79–86 (2018).
52. Niwald, M., Migdalska-Sęk, M., Brzeziańska-Lasota, E. & Miller, E. Evaluation of Selected MicroRNAs Expression in Remission Phase of Multiple Sclerosis and Their Potential Link to Cognition, Depression, and Disability. *J Mol Neurosci* **63**, 275–282 (2017).
53. Cantoni, C., Ghezzi, L., Choi, J., Cross, A. H. & Piccio, L. Targeting miR-223 enhances myeloid-derived

suppressor cell suppressive activities in multiple sclerosis patients. *Multiple Sclerosis and Related Disorders* **76**, 104839 (2023).
54. Mancuso, R. *et al.* Circulatory miR-223-3p Discriminates Between Parkinson's and Alzheimer's Patients. *Sci Rep* **9**, 9393 (2019).
55. Citterio, L. A., Mancuso, R., Agostini, S., Meloni, M. & Clerici, M. Serum and Exosomal miR-7-1-5p and miR-223-3p as Possible Biomarkers for Parkinson's Disease. *Biomolecules* **13**, 865 (2023).
56. Safari, A. *et al.* MicroRNAs and their Implications in CD4+ T-cells, Oligodendrocytes and Dendritic Cells in Multiple Sclerosis Pathogenesis. *Current Molecular Medicine* **23**, 630–647 (2023).
57. Maciak, K., Dziedzic, A. & Saluk, J. Remyelination in multiple sclerosis from the miRNA perspective. *Front. Mol. Neurosci.* **16**, (2023).
58. Pietrasik, S., Dziedzic, A., Miller, E., Starosta, M. & Saluk-Bijak, J. Circulating miRNAs as Potential Biomarkers Distinguishing Relapsing–Remitting from Secondary Progressive Multiple Sclerosis. A Review. *International Journal of Molecular Sciences* **22**, 11887 (2021).
59. Galloway, D. A. *et al.* Investigating the NLRP3 inflammasome and its regulator miR-223-3p in multiple sclerosis and experimental demyelination. *Journal of Neurochemistry* **163**, 94–112 (2022).
60. Vistbakka, J., Sumelahti, M.-L., Lehtimäki, T. & Hagman, S. Temporal variability of serum miR-191, miR-223, miR-128, and miR-24 in multiple sclerosis: A 4-year follow-up study. *Journal of the Neurological Sciences* **442**, 120395 (2022).
61. Rajendran, R., Böttiger, G., Stadelmann, C., Karnati, S. & Berghoff, M. FGF/FGFR Pathways in Multiple Sclerosis and in Its Disease Models. *Cells* **10**, 884 (2021).
62. Vistbakka, J., Elovaara, I., Lehtimäki, T. & Hagman, S. Circulating microRNAs as biomarkers in progressive multiple sclerosis. *Mult Scler* **23**, 403–412 (2017).
63. Shaheen, N. M. H. *et al.* The significance of miRNA-191-5P and miRNA-24-3P as novel biomarkers for multiple sclerosis: a case–control study. *Egyptian Journal of Medical Human Genetics* **25**, 2 (2024).
64. Vistbakka, J., Sumelahti, M.-L., Lehtimäki, T., Elovaara, I. & Hagman, S. Evaluation of serum miR-191-5p, miR-24-3p, miR-128-3p, and miR-376c-3 in multiple sclerosis patients. *Acta Neurologica Scandinavica* **138**, 130–136 (2018).
65. Kumar, P. *et al.* Circulating miRNA Biomarkers for Alzheimer's Disease. *PLOS ONE* **8**, e69807 (2013).
66. Rajabi, S., Sadegi, K., Hajisobhani, S., Kaveh, M. & Taghizadeh, E. miR-146a and miR-155 as promising biomarkers for prognosis and diagnosis of multiple sclerosis: systematic review. *Egypt J Med Hum Genet* **25**, 73 (2024).
67. Feng, X., Hou, H., Zou, Y. & Guo, L. Defective autophagy is associated with neuronal injury in a mouse model of multiple sclerosis. *Bosn J Basic Med Sci* **17**, 95–103 (2017).
68. Di Domenico, F. & Lanzillotta, C. The disturbance of protein synthesis/degradation homeostasis is a common trait of age-related neurodegenerative disorders. *Adv Protein Chem Struct Biol* **132**, 49–87 (2022).
69. Ahmed, T. *et al.* Map kinase signaling as therapeutic target for neurodegeneration. *Pharmacol Res* **160**, 105090 (2020).
70. Askari, H. *et al.* AMP-activated protein kinase as a mediator of mitochondrial dysfunction of multiple sclerosis in animal models: A systematic review. *J Cell Physiol* **239**, e31230 (2024).
71. Bierhansl, L. *et al.* Thinking outside the box: non-canonical targets in multiple sclerosis. *Nat Rev Drug Discov* **21**, 578–600 (2022).
72. Ten Bosch, G. J. A., Bolk, J., 't Hart, B. A. & Laman, J. D. Multiple sclerosis is linked to MAPKERK overactivity in microglia. *J Mol Med (Berl)* **99**, 1033–1042 (2021).
73. Marziali, L. N. *et al.* p38γ MAPK delays myelination and remyelination and is abundant in multiple sclerosis lesions. *Brain* **147**, 1871–1886 (2024).
74. Gökdoğan Edgünlü, T. *et al.* The effect of FOXO gene family variants and global DNA metylation on RRMS disease. *Gene* **726**, 144172 (2020).
75. Hwang, I. *et al.* FOXO protects against age-progressive axonal degeneration. *Aging Cell* **17**, e12701 (2018).
76. Sahu, M. R. & Mondal, A. C. The emerging role of Hippo signaling in neurodegeneration. *J Neurosci Res* **98**, 796–814 (2020).
77. Ou, G.-Y., Lin, W.-W. & Zhao, W.-J. Neuregulins in Neurodegenerative Diseases. *Front Aging Neurosci* **13**, 662474 (2021).
78. Festa, B. P. *et al.* Microglial-to-neuronal CCR5 signaling regulates autophagy in neurodegeneration. *Neuron* **111**, 2021-2037.e12 (2023).

79. Flores-Montoya, G. *et al.* The C–C chemokine receptor 7: An immune molecule that modulates central nervous system function in homeostasis and disease. *Brain, Behavior, & Immunity - Health* **29**, 100610 (2023).
80. Han, J. *et al.* Inhibition of colony stimulating factor-1 receptor (CSF-1R) as a potential therapeutic strategy for neurodegenerative diseases: opportunities and challenges. *Cell Mol Life Sci* **79**, 219 (2022).
81. Hagan, N. *et al.* CSF1R signaling is a regulator of pathogenesis in progressive MS. *Cell Death Dis* **11**, 1–25 (2020).
82. Hwang, D. *et al.* CSF-1 maintains pathogenic but not homeostatic myeloid cells in the central nervous system during autoimmune neuroinflammation. *Proc Natl Acad Sci U S A* **119**, e2111804119 (2022).
83. Cui, L.-Y., Chu, S.-F. & Chen, N.-H. The role of chemokines and chemokine receptors in multiple sclerosis. *Int Immunopharmacol* **83**, 106314 (2020).
84. Iyer, S. S. & Cheng, G. Role of Interleukin 10 Transcriptional Regulation in Inflammation and Autoimmune Disease. *Crit Rev Immunol* **32**, 23–63 (2012).
85. Niedziela, N. *et al.* Clinical and therapeutic challenges of smouldering multiple sclerosis. *Neurologia i Neurochirurgia Polska* **58**, 245–255 (2024).
86. Bilinska, M., Frydecka, I., Podemski, R. & Gruszka, E. Fas expression on T cells and sFas in relapsing-remitting multiple sclerosis. *Acta Neurol Scand* **107**, 387–393 (2003).
87. Dumitrescu, L. *et al.* An update on the use of sphingosine 1-phosphate receptor modulators for the treatment of relapsing multiple sclerosis. *Expert Opin Pharmacother* **24**, 495–509.
88. Lacorazza, H. D. Pharmacological inhibition of the MAP2K7 kinase in human disease. *Front Oncol* **14**, 1486756 (2024).

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Karina Wasilewska: Methodology, Investigation, Data analysis and interpretation, Writing – original draft and editing, Visualization, Project administration. **Angela Dziedzic**: Methodology, Writing – review and editing, Supervision. **Shamundeeswari Anandan**: Writing – review and editing. **Elżbieta Miller**: Resources. **Łukasz Łaczmański**: Methodology, Data analysis and interpretation. **Radosław Zajdel**: Statistical analysis. **Sylwia Michlewska**: Methodology, Visualization. **Dorota Kujawa**: Investigation. **Marta Gancarek**: Data analysis and interpretation. **Justyna Raczkowska**: Investigation. **Lidia Włodarczyk**: Resources. **Patrycja Nowak**: Investigation. **Joanna Saluk**: Conceptualization, Funding acquisition, Methodology, Project administration, Writing – review and editing, Supervision.

Additional information

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Ethics approval

The study was performed in accordance with the Declaration of Helsinki and approved by the University of Lodz Research Bioethics committee with resolution No. 3/KBBN- UŁ/IV/2018.

Competing interests

The authors declare no competing interests.

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In-Depth Characterization of L1CAM$^+$ Extracellular Vesicles as Potential Biomarkers for Anti-CD20 Therapy Response in Relapsing–Remitting Multiple Sclerosis

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Abstract

The effective suppression of inflammation using disease-modifying therapies is essential in the treatment of multiple sclerosis (MS). Anti-CD20 monoclonal antibodies are commonly used long-term as maintenance therapies, largely due to the lack of reliable biomarkers to guide dosing and evaluate treatment response. However, prolonged use increases the risk of infections and other immune-mediated side effects. The unique ability of brain-derived blood extracellular vesicles (EVs) to cross the blood–brain barrier and reflect the central nervous system (CNS) immune status has sparked interest in their potential as biomarkers. This study aimed to assess whether blood-derived L1CAM$^+$ EVs could serve as biomarkers of treatment response to rituximab (RTX) in patients with relapsing-remitting MS (RRMS). Serum samples (n = 25) from the baseline (month 0) and after 6 months were analyzed from the RTX arm of the ongoing randomized clinical trial OVERLORD-MS (comparing anti-CD20 therapies in RRMS patients) and were compared with serum samples from healthy controls (n = 15). Baseline cerebrospinal fluid (CSF) samples from the same study cohort were also included. EVs from both serum and CSF samples were characterized, considering morphology, size, and concentration, using transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA). The immunophenotyping of EV surface receptors was performed using flow cytometry with the MACSPlex exosome kit, while label-free quantitative proteomics of EV protein cargo was conducted using a proximity extension assay (PEA). TEM confirmed the presence of EVs with the expected round morphology with a diameter of 50–150 nm. NTA showed significantly higher concentrations of L1CAM$^+$ EVs ($p < 0.0001$) in serum total EVs and EBNA1$^+$ EVs ($p < 0.01$) in serum L1CAM$^+$ EVs at baseline (untreated) compared to in healthy controls. After six months of RTX therapy, there was a significant reduction in L1CAM$^+$ EV concentration ($p < 0.0001$) and the downregulation of TNFRSF13B ($p = 0.0004$; FC = -0.49) in serum total EVs. Additionally, non-significant changes were observed in CD79B and CCL2 levels in serum L1CAM$^+$ EVs at baseline compared to in controls and after six months of RTX therapy. In conclusion, L1CAM$^+$

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EVs in serum showed distinct immunological profiles before and after rituximab treatment, underscoring their potential as dynamic biomarkers for individualized anti-CD20 therapy in MS.

Keywords: multiple sclerosis (MS); brain-derived blood exosomes; anti-CD20 therapy; rituximab; treatment-response biomarkers; characterization of L1CAM$^+$ EVs

1. Introduction

Almost 3 million people worldwide are affected by multiple sclerosis (MS), an inflammatory and degenerative disease of the central nervous system (CNS) [1]. Initially, most patients (85–90%) experience a relapsing-remitting course (RRMS) marked by episodic inflammation and, if not effectively treated, followed by a secondary progressive (SPMS) phase, associated with gradual increasing disability. A primary progressive course (PPMS) is seen in approximately 10–15% of cases [2].

Strong epidemiological evidence suggests that Epstein–Barr Virus (EBV) is a prerequisite for developing MS, although the exact underlying pathogenic mechanisms remain unclear [3,4]. EBV is transmitted through saliva and, upon infecting B-cells, enters a state of "deep" latency, where it transfers its DNA to the B-cell nucleus, with the possibility to still be replicated along with the cellular DNA and be reactivated following B-cell activation [3,5].

The suppression of inflammatory activity is the cornerstone of MS treatment with disease-modifying therapies [6,7]. Evolving clinical experience from anti-CD20 therapies has shown their high efficacy in relapsing forms of the disease. Anti-CD20 therapy is also the first treatment approach proven to modify disability worsening in PPMS [8]. It reduces inflammatory activity, usually shown by the almost complete prevention of new clinical relapses and new brain magnetic resonance imaging (MRI) lesions in treated patients [9]. Anti-CD20 therapies in current practice internationally include rituximab, ocrelizumab, ofatumumab, and most recently ublituximab [8,10].

Anti-CD20 therapies are typically initiated with an induction dose, followed by regular fixed maintenance dosing at regular intervals. While convenient, these fixed regimens may result in overtreatment, as B-cell reconstitution often occurs long after B-cell depletion, with considerable interindividual variability. B-cell counts frequently remain suppressed at the time of scheduled redosing, with immune reconstitution ranging from 27 to 125 weeks post-depletion (median: 72 weeks) [11]. Notably, approximately 6% of total circulating T-cells express CD20, and transient drops in T-cell numbers occur for 3–6 months following anti-CD20 treatment, possibly related to the depletion of CD20-positive T-cells [11–14].

Together, these data suggest that a reprogramming of immunity from an activated to a resting state may occur following therapy and could account for the high efficacy of treatment [8].

In MS treatment, CD19 (B-cell marker) is frequently used as a surrogate measure to quantify B-cells during treatment. A recent study showed that the use of memory B-cell counts as treatment monitoring biomarkers in rituximab reinfusion protocols can reduce the mean number of infusions with a persistent reduction in disease activity [15]. However, existing data are insufficient for establishing a therapeutic cut-off of B-cells in RRMS [16]; as anti-CD20 therapies deplete pre-B-cells to late plasma blasts (including CD20$^+$ T-cells), using only CD19 counts as biomarkers might therefore be oversimplistic, compelling the need for more robust biomarkers [16].

Extracellular vesicles (EVs) are membrane-bound particles, released by virtually all cell types. They can exert their action locally or migrate to distant locations via biological fluids

(urine, cerebrospinal fluid (CSF), peripheral blood, saliva, breast milk, tears) [17]. EVs process a sophisticated cargo-sorting mechanism, carrying lipids, proteins, nucleic acids, and specific membrane proteins that largely reflect their cell of origin. These characteristics, combined with their ability to cross the blood–brain barrier (BBB) and increased stability, highlight brain-derived blood EVs as promising biomarkers for CNS diseases, including MS [18–21].

Recent research on brain-derived blood EVs in MS has particularly focused on L1 cell adhesion molecule (L1CAM), in addition to other markers like glutamate aspartate transporter (GLAST) and myelin oligodendrocyte glycoprotein (MOG). These studies primarily investigate the potential of these biomarkers related to disease activity and progression [3,22–27]. Despite this cumulative evidence, the utility of L1CAM as a marker of brain/neuron-derived EVs is still under debate as it is also expressed at comparable RNA levels by Schwann cells of the peripheral nervous system, skin epithelial cells, and kidney tubule epithelia and may occur as free L1CAM peptides [28–32]. This study is the first to comprehensively characterize L1CAM$^+$ EVs, assessing size, concentration, morphology, protein cargo, and surface immunophenotype, in both serum and CSF, while also evaluating their utility as dynamic biomarkers for anti-CD20 treatment response in RRMS.

We hypothesize that L1CAM$^+$ EVs reflect central immune activity and could serve as serum biomarkers to monitor treatment response to anti-CD20 therapy.

2. Results

2.1. Patient Characteristics and EV Characterization: Size, Morphology, and Tetraspanin Profiling

Total EVs and L1CAM-enriched EVs (L1CAM$^+$ EVs) were analyzed in serum samples from 25 newly diagnosed, treatment-naïve RRMS patients at baseline (month 0) and after six months (month 6) of rituximab therapy, who all showed no signs of new disease activity. In addition, diagnostic CSF samples from the same cohort (month 0) and serum control samples from 15 healthy individuals were included (see Figure 1).

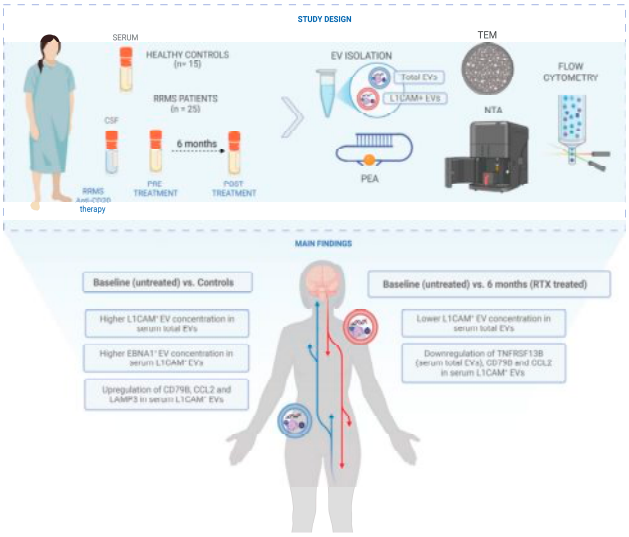


Figure 1. Schematic overview of the study design, analysis set-up, and main findings: Significantly higher concentrations of L1CAM$^+$ EVs ($p < 0.0001$) in serum total EVs and EBNA1$^+$ EVs ($p < 0.01$) in serum L1CAM$^+$ EVs at baseline (untreated) compared to in healthy controls were observed. After six months of RTX therapy, there was a significant reduction in L1CAM$^+$ EV concentration ($p < 0.0001$) and the downregulation of TNFRSF13B ($p = 0.0004$; FC = −0.49) in serum total EVs.

The cohort predominately consisted of women (80%), with a mean age of 39.8 (SD = 10.36) years and stable disease. TEM imaging revealed the expected double-membraned, rounded morphology of EVs with a diameter range of 50–150 nm (see Figures 2a and S1c: pilot experiment results; $n = 5$). The profiling of tetraspanins (CD9, CD63, CD81), which are common EV transmembrane markers, showed the highest expression of CD9 in serum total EVs, CD63 in serum L1CAM$^+$ EVs, and CD81 in both total and L1CAM$^+$ CSF EVs (see Figures 2b and S1b).

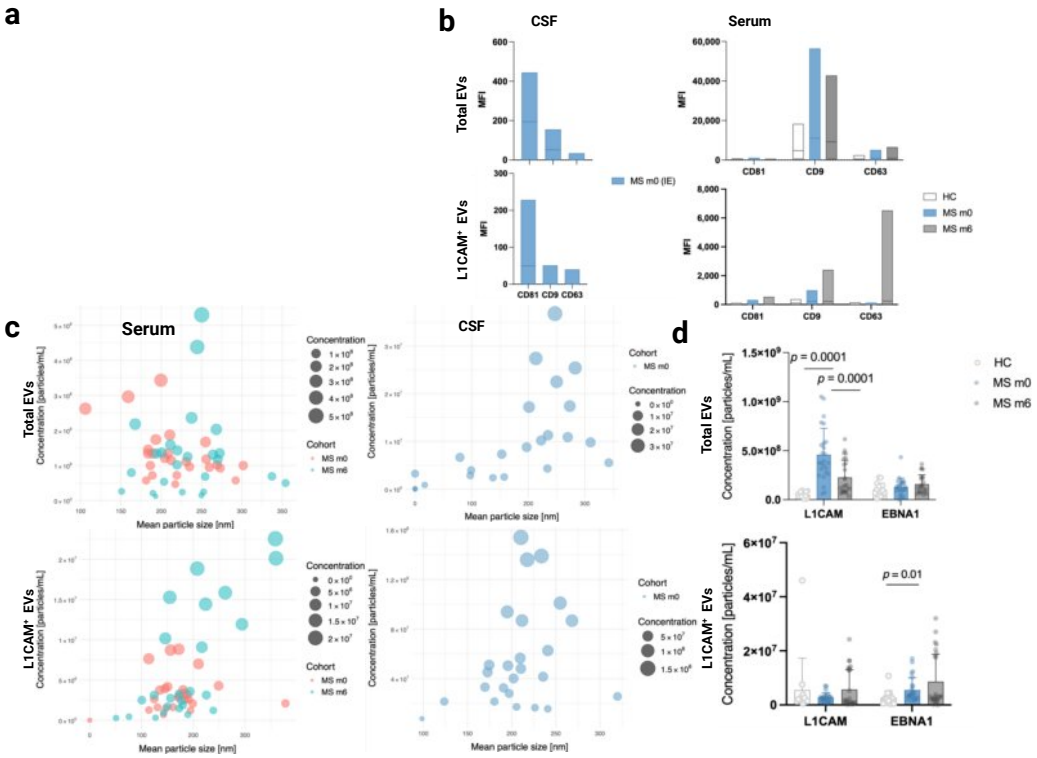
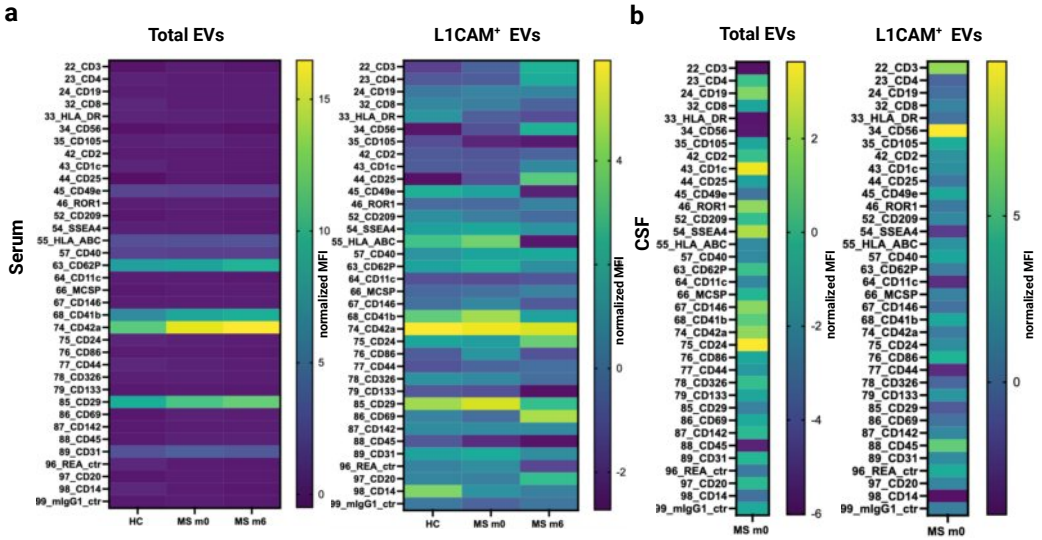


Figure 2. Characteristics of serum and CSF EVs: (**a**) Morphology detected with TEM imaging (scale bar: 200 nm) and (**b**) tetraspanin (CD81, CD9, and CD63) (bead population numbers: 65, 53, and 56, respectively—flow cytometry analysis) expression profile of MS patients (month 0—m0 and month 6—m6) and healthy controls, with total EVs and L1CAM$^+$ EVs expressed in mean fluorescence intensities (MFIs) as floating bar plots with min. and max. ranges. NTA: (**c**). Bubble plots show the relationship between mean EV particle size and the concentration of total EVs and L1CAM$^+$ EVs. Each point represents an individual sample, with bubble size proportional to EV concentration. (**d**). L1CAM and EBNA concentration in serum total EVs and L1CAM$^+$ EVs. Significantly higher concentrations of L1CAM$^+$ EVs ($p < 0.0001$) in serum total EVs and EBNA1$^+$ EVs ($p < 0.01$) in serum L1CAM$^+$ EVs at baseline (untreated) compared to in healthy controls were observed. After six months of RTX therapy, there was a significant reduction in L1CAM$^+$ EV concentration ($p < 0.0001$). Statistical significance between HC and m0 was assessed using the U Mann–Whitney test. Statistical significance between m0 and m6 was determined using the Wilcoxon signed-rank test.

2.2. Significant Changes in L1CAM$^+$ EV Concentrations Before and After Rituximab-CD20 Treatment in Serum Total EVs

NTA revealed a significantly increased concentration of L1CAM$^+$ EVs ($p < 0.0001$) in serum total EVs at baseline (untreated) compared to in healthy controls (see Figure 2d). The expression of 21 immune and inflammation-related proteins (see Supplementary Table S1—PEA

analysis) were assessed in serum L1CAM⁺ EVs; CD79B (p = 0.03; fold change (FC) = 0.24) and CCL2 (p = 0.02; fold change (FC) = 0.50), demonstrated differential expression at baseline prior to rituximab therapy compared to controls, although these differences were non-significant after adjustments (Figure 3c and Supplementary Table S2).



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Total EVs

Protein	term	est.log2	p.value	p.value.adj.fdr
IL6	M0vsM6	-0.3772596	0.030385514	0.3190479
TNFRSF13B	HCvsM0	-0.413829995	0.037324110	0.5456926
TNFRSF13B	M0vsM6	-0.4955192	0.000462144	0.00970504

L1CAM⁺ EVs

Protein	term	est.log2	p.value	p.value.adj.fdr
CCL2	HCvsM0	0.506825712	0.029332362	0.256803366138
LAMP3	HCvsM0	0.137778939	0.032037121	0.256803366138
CD79B	HCvsM0	0.243402026	0.036686195	0.256803366138

Figure 3. (**a**,**b**). Surface immune profiling of total and L1CAM⁺ EVs shown as heatmaps (EV markers' geometric mean fluorescence intensity (MFI) was normalized to the mean MFI for specific EV markers (CD9, CD63, and CD81), obtaining normalized MFI values) from CSF and serum samples comparing MS patients (m0, m6) and healthy controls. Statistical significance between HC and m0 was assessed using the U Mann–Whitney test and Wilcoxon signed-rank test (m0 and m6) with no significant findings. (**c**). Linear regression models adjusted for sex and age were used to estimate differences over time (MS = m0 and m6) and between sample groups (HC and MS) for the 21-flex panel proteins. Differentially expressed proteins with significant changes in both serum total and L1CAM⁺ EVs are presented. After six months of RTX therapy, there was a significant downregulation of TNFRSF13B (p = 0.0004; FC = −0.49) in serum total EVs. The models were adjusted for the covariates 'age' and 'sex', and inferential tests were two-tailed with a nominal alpha level of 0.05. Raw p-values were adjusted for multiple testing by controlling the false discovery rate with the Benjamini and Hochberg method, and the critical value (q-value) was set to $\leq$0.01.

Interestingly, matched CSF total EVs also showed elevated concentrations of CCL2 (FC = 9.35) and TREM2 (FC = 10.71) at baseline (see Supplementary Table S4). The immunophenotyping of 37 surface markers though flow cytometric analysis indicated the elevated (ns) expression of CD41b, CD42a, and CD29 in serum L1CAM⁺ EVs at baseline compared to healthy serum controls.Additionally, the increased expression of CD1c and CD24 was observed in CSF total EVs at baseline (see Figure 3a,b and Supplementary Table S6).

The concentration of L1CAM⁺ EVs in serum total EVs significantly decreased (p < 0.0001) after rituximab treatment at month 6 compared to baseline, as indicated by NTA, (see Figure 2d). The serum total EV cargo exhibited the significant downregulation

of TNFRSF13B ($p = 0.0004$; FC = −0.49) and non-significant downregulation of CD79B (ns; FC = −0.02), based on PEA analysis (see Figure 3 and Supplementary Table S3).

Additionally, the serum L1CAM$^+$ EV cargo showed a non-significant downregulation of CCL2 (ns; FC = −0.14) and the upregulation of TNF (ns; FC = 0.11), IL-4 (ns; FC = 1.70), and VSNL1 (ns; FC = 0.09) at month 6 after rituximab therapy compared to at baseline (see Figure 3c and Supplementary Tables S2 and S3). Matched CSF L1CAM$^+$ EVs also demonstrated increased concentrations of IL-4 (FC = 9.35) and TREM2 (FC = 3.2) at baseline (see Supplementary Table S5).

Surface immune receptor profiling revealed the elevated (ns) expression of CD3 and CD56 in CSF L1CAM$^+$ EVs at baseline, as well as CD42a, CD24, CD69, and CD25 in serum L1CAM$^+$ EVs at month 6 compared to at baseline (Figure 3 and Supplementary Table S6).

2.3. Significantly Elevated EBNA1$^+$ EV Concentration in Serum L1CAM$^+$ EVs at Baseline Before Rituximab Treatment Compared to HC

EBV is considered as a strong prerequisite for developing MS and Epstein–Barr nuclear antigen 1 (EBNA1), which is known to be expressed in all known latent types of EBV infection. The NTA revealed a significantly increased concentration of EBNA1$^+$ EVs ($p < 0.01$) in serum L1CAM$^+$ EVs at baseline compared to in controls (see Figures 2d and S1). Additionally, an upregulated expression of LAMP3 ($p = 0.03$; FC = 0.13) was observed in L1CAM$^+$ EVs at baseline compared to in controls, although this was non-significant after adjustment (PEA analysis—see Figure 3c and Supplementary Table S2).

The upregulation of LAMP3 (ns; FC = 0.11) was also noted in serum total EVs at month 6 compared to at baseline (Supplementary Table S3). The immunophenotyping of the surface receptors in serum total EVs showed the elevated (ns) expression of CD29, CD42a, CD41b, and CD62p at month 6 compared to at baseline (see Figure 3a).

3. Discussion

In this study, we assessed whether L1CAM$^+$ blood-derived EVs could serve as biomarkers for guiding personalized rituximab (anti-CD20) therapy in RRMS. As demonstrated in Figures 2 and 3, L1CAM, EBNA1, and selected immune-related proteins (CD79B, CCL2, TNFRSF13B) were dynamically regulated in response to rituximab therapy, particularly in L1CAM$^+$ EVs.

During the course of MS, immune cells release EVs, which provide important information regarding ongoing pathological processes [33–36]. EVs have been studied in the context of MS for their roles in inflammation and T-cell activation. However, previous research has primarily focused on total circulating EVs, without discriminating between specific cell subpopulations [20,37].

Currently available methods to examine brain processes in MS include clinical and MRI assessments, as well as CSF, which involve the invasive procedure of lumbar puncture and serum markers of axonal damage or astrocyte activation [38]. Recent advances in serum EV analysis have expanded the possibilities to study subpopulations of EVs from specific cellular origins, creating new opportunities to assess processes in the brain and the involvement of the immune system in the disease [34,39]. Research on brain-derived blood EV research in MS, particularly focusing on L1CAM, alongside other subpopulations such as GLAST and MOG, has gained attention recently, primarily examining the potential of these EVs as biomarkers in relation to disease status and treatment response [3,22–27].

B and T lymphocytes play crucial roles in the pathogenesis of MS. Recent studies have shown that increased levels of CCR5 in Th1-derived EVs and CCR3 in Th2-derived EVs are strong indicators of disease activity, particularly in the presence of gadolinium-enhancing lesions in the brain and spinal cord [39]. Additionally, increased levels of CD19$^+$ B-cell-

derived EVs have been observed in patients during clinical relapses compared to periods of remission [39].

Similarly, our findings indicate a significant increase ($p < 0.0001$) in the concentration of L1CAM$^+$ EVs within total EVs and the upregulation of CD79B ($p = 0.03$; FC = 0.24; ns after adjustment), a protein subunit of the B-cell receptor, as well as CCL2 ($p = 0.02$; FC = 0.50; ns after adjustment), specifically at baseline prior to rituximab therapy compared to in controls in both serum total and L1CAM$^+$ EVs. Furthermore, we noted the downregulation of these proteins in L1CAM$^+$ EVs along with TNFRSF13B ($p = 0.0004$; FC = -0.49)—a gene that produces TACI, a B-cell-specific member of the TNF receptor superfamily. A significant decrease ($p < 0.0001$) in L1CAM$^+$ EV concentration in total EVs was also observed at month 6 compared to at baseline in total serum EVs.

Another study indicated that higher levels of T-cell-derived EVs and smaller sizes of neuron-derived EVs were associated with clinical relapses [22]. CCR2 is the major receptor for CCL2 and functions as a potent chemoattractant for monocytes and T-cells. Chemokines and their receptors are vital for the bidirectional trafficking of leucocytes across the BBB. Several studies have explored the significance of CCL2 and CCR2 in MS, revealing that CCL2 levels are consistently low in the CSF, despite being abundantly expressed within the CNS lesions. These studies suggest that CCL2 is consumed by migrating inflammatory cells, which downregulate CCR2, as they cross the BBB [40].

In line with our findings, CSF total EVs showed the upregulation of CCL2 (FC = 9.35) at baseline prior to rituximab therapy. Interestingly serum L1CAM$^+$ (brain-derived) EVs exhibited higher CCL2 expression ($p = 0.02$; ns after adjustment; FC = 0.50) compared to serum total EVs (ns; FC = 0.28) at baseline. Additionally, Iglesias et al. investigated B-cell-derived EVs from the blood and CSF for their myelin antibody content from 136 MS patients, 23 white matter brain lesion controls, and 39 healthy controls. They found autoreactive myelin antibodies in EVs released by peripheral B-cells but not by populations of B-cells resident in CSF [23].

Furthermore, another study examined antibody titers against nuclear (anti-EBNA1) and capsid (anti-VCA) EBV antigens in EVs and in plasma, also evaluating the content of myelin antibodies in EVs [3]. Patients with active disease showed higher levels of anti-EBNA1 in EVs than patients with inactive disease [3]. Correspondingly, our findings revealed increased EBNA1 ($p < 0.01$) concentrations and the upregulation of LAMP3 ($p = 0.03$; ns after adjustment; FC = 0.13) in serum L1CAM$^+$ EVs at baseline, indicating higher disease activity in newly diagnosed patients prior to initiation of rituximab therapy compared to HC.

This study has several strengths, including the availability of paired CSF and serum samples at baseline, in addition to a well-characterized cohort of newly diagnosed, treatment-naïve patients who all received the same disease-modifying therapy, rituximab. However, the study also has limitations, most notably its limited sample size ($n = 25$), the absence of long-term clinical outcomes beyond six months, and a lack of replication in an independent cohort with a critical comparison including other disease-modifying therapies or patients with active disease activity. Additionally, as mentioned before, while L1CAM is commonly used to enrich CNS-derived EVs, its expression can also be detected in other peripheral tissues; hence, further recommended validations of neuronal origin with additional markers such as GAP43, β-III-tubulin and VAMP2 based on new published findings should be rigorously followed up [29,32]. Nevertheless, our findings suggest that L1CAM$^+$ serum EVs may serve as promising biomarker for treatment response biomarkers in RRMS. Validation in larger, longitudinal cohorts with clinical outcome data is essential. If validated, L1CAM$^+$ EVs could facilitate more flexible and individualized dosing sched-

ules for anti-CD20 therapy, potentially reducing side effects associated with unnecessary immune suppression.

4. Materials and Methods

4.1. Patient Cohorts and Control Samples

Diagnostic CSF (n = 25; month 0) and serum samples (n = 25) from baseline (month 0) and after six months of treatment were obtained from RRMS patients receiving rituximab as a part of an ongoing randomized clinical trial (OVERLORD-MS: https://clinicaltrials.gov/ct2/show/NCT04578639 (accessed on 1 September 2024)) at Haukeland University Hospital, Bergen, Norway. Clinical evaluations included a history of relapses and disability assessment using the Expanded Disability Status Scale (EDSS). The OVERLORD-MS study was approved by the Regional Committee for Medical and Health Research Ethics, Western Norway—REC West ID: 66391. All participating patients provided informed consent for treatment response biomarker research.

Serum control samples (n = 15) were collected from heathy volunteers with informed consent approved under REC West ID: 74985. Clinical samples from all cohorts were collected and handled in accordance with relevant guidelines and regulations. Pilot experiments for the initial set-up of various analyses (Supplementary Figures S1 and S2) also included serum samples from the OVERLORD-MS study.

In briefly, 2 × 6–8 mL blood was collected without anticoagulant. Following centrifugation at 1400× g for 12 minutes (min) at room temperature, the blood clotted, and serum was frozen at −80 °C without any additives. CSF samples, upon collection, were centrifuged first at 300× g for 15 min at 4 °C, followed by a second centrifugation at 680× g for 10 min at 4 °C, before being frozen at −80 °C without any additives.

4.2. Isolation of EVs from CSF and Serum Samples

Frozen serum and CSF samples (−80 °C) were uniformly thawed using a thermomixer (Eppendorf, Hamburg, Germany) at 10 °C for 20 min. Total EVs were isolated according to the manufacturer's instructions. The total exosome isolation kit for other body fluid kits was used for CSF samples, while the total exosomes isolation serum kit (Invitrogen—life technologies, Thermo Fischer Scientific, Carlsbad, CA, USA) was utilized for serum samples.

From the total EVs, L1CAM$^+$ EVs were further isolated using the exosome–streptavidin isolation/detection kit (Invitrogen—life technologies, Thermo Fischer Scientific, Carlsbad, CA, USA). The initial step involved coupling of dynabeads®, magnetic beads with a biotinylated antibody, CD171 (CD171/L1CAM monoclonal antibody clone eBio5G3 (5G3), Biotin, eBioscience, Thermo Fischer Scientific, Carlsbad, CA, USA), following the manufacturer's protocol. These total and immuno-purified L1CAM$^+$ EVs were subsequently characterized as explained below (Section 4.3).

4.3. Characterization of EVs

4.3.1. Transmission Electron Microscopy (TEM) Imaging

A droplet of intact EVs (10 µL resuspended in water; total serum EVs required an additional dilution of 1:30) was placed on a glow-discharged 200 mesh formvar carbon coated copper grid to be absorbed for 1 min. Excess sample was then removed using blotting paper, and the grids were washed once with milli-Q water before being stained for 30 sec in 2% uranyl acetate. The grids were allowed to dry for 30 min before imaging was conducted using a Hitachi HT7800 transmission electron microscope (Minato-ku, Tokyo, Japan).

4.3.2. Nanoparticle Tracking (NTA) Analysis

EV size, concentration, L1CAM content, and EBNA1 content were determined using nanoparticle tracking analysis (NTA) with the NanoSight NS500 nanoparticle analyzer (Malvern Instruments, Malvern, Worcestershire, UK). Frozen EV samples (both total and L1CAM$^+$ EVs) were thawed at 4 °C and immediately prepared for staining. For each sample, 5 µL of EV suspension was incubated on ice for at least 30 min with the following reagents: CellMask™ Green dye (1:53; Thermo Fisher Scientific, Waltham, MA, USA) to assess the total quantity of EVs in the sample; L1CAM antibody conjugated to Alexa Fluor® (1:2; Santa Cruz Biotechnology, Dallas, TX, USA) to identify L1CAM content in EVs; and EBNA1 antibody conjugated to phycoerythrin (PE) (1:36; Bio-Techne R&D Systems, Minneapolis, MN, USA) to quantify EBNA1 content in EVs. All staining procedures were performed on ice to preserve vesicle integrity.

For L1CAM$^+$ EVs, an aliquot of the EV suspension was diluted 1:50 in sterile PBS, while for total EV quantification, samples were diluted to a final volume of 1:500 in sterile PBS. Measurements were performed using laser excitation of 405 nm for EBNA-1 detection using the PE-conjugated antibody, 535 nm for CellMask™ Green, and 488 nm for L1CAM detection using the Alexa Fluor®-conjugated antibody. For each patient sample, three 60 s videos were recorded, capturing 30 frames per position at a detection threshold of 5. The mean particle diameter and concentration were calculated for each run, and the average of the triplicate measurements was used for further analysis.

For pilot experiments (Supplementary Figure S1), frozen EV samples were thawed at 4 °C. A total of 20 µL of prediluted total EVs (1:100) were stained for a minimum of 30 min with 1 µL CellMask™ Green-CMG dye Plasma Membrane Stain (Thermo Fisher Scientific, pre-diluted 1:20). Total EVs were additionally stained with 1 µL of 1:50 pre-diluted EBV EBNA-1 antibody (clone 1EB12) conjugated to Phycoerythrin-PE dye (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) on ice.

The stained samples were further diluted to a final volume of 500 µL in phosphate-buffered saline (PBS, Gibco, Waltham, MA, USA), resulting in final dilution factors of 1:2500 for total EVs and 1:25 for L1CAM$^+$ EVs. These samples were then analyzed using a ZetaView® (Particle Metrix, Ammersee, Germany). The manufacturer's default software settings were employed for EV analysis. For each measurement, three cycles were performed by scanning 11 cell positions and capturing 30 frames per position. For measuring EBNA1- or CMG-stained particles, a 550/25 nm long-pass (LP) fluorescence filter with a sensitivity of 96 and a trace length of 7 (for PE-conjugated antibody) or 10 (for CMG) was used. Data analysis was performed with built-in ZetaView Software version 8.05.14 SP7.

4.3.3. Flow Cytometry

The MACSPlex Human Exosome Kit (Miltenyi, Bergisch Gladbach, Germany) was utilized to examine the surface immune profile of both the total and immuno-purified L1CAM$^+$ EVs following the staining procedure as per the manufacturer's instructions (short protocol for the assay using 1.5 mL tubes). The analyses were performed using a conventional BD Fortessa flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) with the standard set-up recommended from the kit (Supplementary Table S6).

Briefly, EVs were incubated with antibody-coated MACSPlex exosome capture beads and then labeled with the MACSPlex exosome detection reagents, which included CD9, CD63, and CD81. These complexes were subsequently analyzed based on the fluorescence characteristics of both the MACSPlex Exosome Capture Bead and the detection reagents.

4.3.4. Proximity Extension Assay (PEA)

CSF and serum samples were sent in one batch to Olink Proteomics (Bevital AS, Bergen, Norway) for the quantification of 21 immune and inflammation-related proteins included in the Olink Flex panel (see Supplementary Table S1) using proximity extension assay (PEA).

In brief, the PEA utilizes single-stranded DNA (ssDNA) oligonucleotides covalently attached to pairs of protein-specific antibodies. When both antibodies bind in close proximity to their target protein, their ssDNA oligos hybridize to form a double-stranded DNA (dsDNA) sequence. The Olink Flex procedure requires a minimal sample volume of 1 µL and no replicates and consists of three core steps: incubation (antibody binding), extension/amplification (DNA reporter generation), and detection (qPCR quantification using the Olink Signature Q100 instrument, Waltham, MA, USA).

Internal controls, incubation, extension, and detection controls monitor assay performance in each step, with the extension control normalizing technical variation between samples. NPX (Normalized Protein eXpression), a log2-scale relative quantification unit, is calculated by adjusting cycle threshold (Ct) values using the extension control, a pre-determined bridging factor, and triplicate calibrator. Absolute quantification in pg/mL is achieved by fitting NPX values to protein-specific 4PL model standard curves derived during validation. For details, see the service provider's homepage (https://olink.com/products/olink-flex (accessed on 1 December 2024)).

4.4. Data Analysis

Following the initial gating strategy of the flow data (as per MACSPlex Human Exosome Kit—data analysis protocol), using FlowJo™ v10 software (BD Biosciences, Franklin Lakes, NJ, USA), an equal number of events across both timepoints (m0 and m6) and controls within each sample subpopulation (serum L1CAM$^+$ EVs = 15,290 events; serum total EVs = 6028 events; CSF L1CAM$^+$ EVs = 19,571 events; CSF total EVs = 10,680 events) was used for further analysis. All downstream analyses were based on normalized geometric mean fluorescence intensity (nMFI) values. In short, a blank control composed of only MACSPlex buffer, incubated with beads and MACSPlex exosome detection reagents (CD9, CD63, and CD81), was used to measure the background signal. Each EV marker's geometric mean fluorescence intensity (MFI) was normalized to the mean MFI for specific EV markers (CD9, CD63, and CD81) obtaining the normalized MFI (Supplementary Figure S3).

ZetaView Software version 8.05.14 SP7 was utilized for both acquisition and analysis. In PEA analysis, between-group differences (MS vs. HC) in protein levels at baseline (m0) and follow-up (m6) were analyzed by linear regression (generalized least squares) adjusting for heterogeneity in variance between groups using the gls function in the R package nlme v3.1-159. Change scores from baseline to follow-up in the group of MS patients were analyzed by the same linear model that included an unstructured covariance matrix and a variance function structure allowing for different variance per stratum of 'time'. The models were adjusted for the covariates 'age' and 'sex', and inferential tests were two-tailed with a nominal alpha level of 0.05. Raw *p*-values were adjusted for multiple testing by controlling the false discovery rate with the Benjamini and Hochberg method, and the critical value (q-value) was set to $\leq$0.01. In general, statistical analysis was performed using Microsoft Excel (Redmond, WA, USA) and GraphPad Prism 10 (San Diego, CA, USA). Data are expressed as arithmetic mean $\pm$ standard deviation (SD); if other statistical tests were used, the statistical significance per experiment is shown in figure legends.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms26157213/s1.

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Institutional Review Board Statement: The OVERLORD-MS study was approved on 02.11.2020 by the Regional Committee for Medical and Health Research Ethics, Western Norway—REC West ID: 66391. All participating patients provided informed consent for treatment response biomarker research.

Informed Consent Statement: Informed consent was obtained from all individuals involved in this study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to ethical restrictions.

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Conflicts of Interest: K.-M.M. has served on a scientific advisory board for Alexion, received speaker honoraria from Biogen, Lundbeck, Novartis, and Roche, and has participated in clinical trials organized by Biogen, Merck, Novartis, Roche, and Sanofi. O.T. has participated in advisory boards and received speaker honoraria from Biogen, Merck, Novartis, Teva, Roche, Sanofi, and Bristol Myers Squibb and has participated in clinical trials organized by Merck, Novartis, Roche, and Sanofi. The other authors declare no conflicts of interest.

References

1. Walton, C.; King, R.; Rechtman, L.; Kaye, W.; Leray, E.; Marrie, R.A.; Robertson, N.; La Rocca, N.; Uitdehaag, B.; van der Mei, I.; et al. Rising prevalence of multiple sclerosis worldwide: Insights from the Atlas of MS, third edition. *Mult. Scler.* **2020**, *26*, 1816–1821. [CrossRef]
2. Thompson, A.J.; Baranzini, S.E.; Geurts, J.; Hemmer, B.; Ciccarelli, O. Multiple sclerosis. *Lancet* **2018**, *391*, 1622–1636. [CrossRef]
3. Fernández-Fournier, M.; López-Molina, M.; Torres Iglesias, G.; Botella, L.; Chamorro, B.; Laso-García, F.; Puertas, I.; Tallón Barranco, A.; Otero-Ortega, L.; Frank-García, A.; et al. Antibody Content against Epstein–Barr Virus in Blood Extracellular Vesicles Correlates with Disease Activity and Brain Volume in Patients with Relapsing–Remitting Multiple Sclerosis. *Int. J. Mol. Sci.* **2023**, *24*, 14192. [CrossRef]
4. Mrad, M.F.; Saba, E.S.; Nakib, L.; Khoury, S.J. Exosomes From Subjects With Multiple Sclerosis Express EBV-Derived Proteins and Activate Monocyte-Derived Macrophages. *Neurol. Neuroimmunol. Amp Neuroinflamm.* **2021**, *8*, e1004. [CrossRef]
5. Bjornevik, K.; Cortese, M.; Healy, B.C.; Kuhle, J.; Mina, M.J.; Leng, Y.; Elledge, S.J.; Niebuhr, D.W.; Scher, A.I.; Munger, K.L.; et al. Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis. *Science* **2022**, *375*, 296–301. [CrossRef]
6. Torkildsen, Ø.; Myhr, K.M.; Bø, L. Disease-modifying treatments for multiple sclerosis—A review of approved medications. *Eur. J. Neurol.* **2016**, *23* (Suppl. 1), 18–27. [CrossRef]
7. Myhr, K.M.; Torkildsen, Ø.; Lossius, A.; Bø, L.; Holmøy, T. B cell depletion in the treatment of multiple sclerosis. *Expert. Opin. Biol. Ther.* **2019**, *19*, 261–271. [CrossRef]
8. Greenfield, A.L.; Hauser, S.L. B-cell Therapy for Multiple Sclerosis: Entering an era. *Ann. Neurol.* **2018**, *83*, 13–26. [CrossRef]

9. Lehmann-Horn, K.; Kinzel, S.; Weber, M.S. Deciphering the Role of B Cells in Multiple Sclerosis—Towards Specific Targeting of Pathogenic Function. *Int. J. Mol. Sci.* **2017**, *18*, 2048. [CrossRef]
10. Steinman, L.; Fox, E.; Hartung, H.P.; Alvarez, E.; Qian, P.; Wray, S.; Robertson, D.; Huang, D.; Selmaj, K.; Wynn, D.; et al. Ublituximab versus Teriflunomide in Relapsing Multiple Sclerosis. *N. Engl. J. Med.* **2022**, *387*, 704–714. [CrossRef]
11. Palanichamy, A.; Jahn, S.; Nickles, D.; Derstine, M.; Abounasr, A.; Hauser, S.L.; Baranzini, S.E.; Leppert, D.; von Büdingen, H.C. Rituximab efficiently depletes increased CD20-expressing T cells in multiple sclerosis patients. *J. Immunol.* **2014**, *193*, 580–586. [CrossRef]
12. Holley, J.E.; Bremer, E.; Kendall, A.C.; de Bruyn, M.; Helfrich, W.; Tarr, J.M.; Newcombe, J.; Gutowski, N.J.; Eggleton, P. CD20+inflammatory T-cells are present in blood and brain of multiple sclerosis patients and can be selectively targeted for apoptotic elimination. *Mult. Scler. Relat. Disord.* **2014**, *3*, 650–658. [CrossRef]
13. Graves, J.; Vinayagasundaram, U.; Mowry, E.M.; Matthews, I.R.; Marino, J.A.; Cheng, J.; Waubant, E. Effects of rituximab on lymphocytes in multiple sclerosis and neuromyelitis optica. *Mult. Scler. Relat. Disord.* **2014**, *3*, 244–252. [CrossRef]
14. Sabatino, J.J.; Wilson, M.R.; Calabresi, P.A.; Hauser, S.L.; Schneck, J.P.; Zamvil, S.S. Anti-CD20 therapy depletes activated myelin-specific $CD8^+$ T cells in multiple sclerosis. *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 25800–25807. [CrossRef]
15. Novi, G.; Bovis, F.; Fabbri, S.; Tazza, F.; Gazzola, P.; Maietta, I.; Currò, D.; Bruschi, N.; Roccatagliata, L.; Boffa, G.; et al. Tailoring B cell depletion therapy in MS according to memory B cell monitoring. *Neurol.—Neuroimmunol. Neuroinflamm.* **2020**, *7*, e845. [CrossRef]
16. van Kempen, Z.L.; Toorop, A.A.; Sellebjerg, F.; Giovannoni, G.; Killestein, J. Extended dosing of monoclonal antibodies in multiple sclerosis. *Mult. Scler.* **2021**, *28*, 13524585211065711. [CrossRef]
17. Lötvall, J.; Hill, A.F.; Hochberg, F.; Buzás, E.I.; Di Vizio, D.; Gardiner, C.; Gho, Y.S.; Kurochkin, I.V.; Mathivanan, S.; Quesenberry, P.; et al. Minimal experimental requirements for definition of extracellular vesicles and their functions: A position statement from the International Society for Extracellular Vesicles. *J. Extracell. Vesicles* **2014**, *3*, 26913. [CrossRef]
18. Gurunathan, S.; Kang, M.H.; Jeyaraj, M.; Qasim, M.; Kim, J.H. Review of the Isolation, Characterization, Biological Function, and Multifarious Therapeutic Approaches of Exosomes. *Cells* **2019**, *8*, 307. [CrossRef]
19. Huo, L.; Du, X.; Li, X.; Liu, S.; Xu, Y. The Emerging Role of Neural Cell-Derived Exosomes in Intercellular Communication in Health and Neurodegenerative Diseases. *Front. Neurosci.* **2021**, *15*, 738442. [CrossRef]
20. Mycko, M.P.; Baranzini, S.E. microRNA and exosome profiling in multiple sclerosis. *Mult. Scler.* **2020**, *26*, 599–604. [CrossRef]
21. Hornung, S.; Dutta, S.; Bitan, G. CNS-Derived Blood Exosomes as a Promising Source of Biomarkers: Opportunities and Challenges. *Front. Mol. Neurosci.* **2020**, *13*, 38. [CrossRef]
22. Torres Iglesias, G.; Fernández-Fournier, M.; Botella, L.; Piniella, D.; Laso-García, F.; Carmen Gómez-de Frutos, M.; Chamorro, B.; Puertas, I.; Tallón Barranco, A.; Fuentes, B.; et al. Brain and immune system-derived extracellular vesicles mediate regulation of complement system, extracellular matrix remodeling, brain repair and antigen tolerance in Multiple sclerosis. *Brain Behav. Immun.* **2023**, *113*, 44–55. [CrossRef]
23. Torres Iglesias, G.; Fernández-Fournier, M.; López-Molina, M.; Piniella, D.; Laso-García, F.; Gómez-de Frutos, M.C.; Alonso-López, E.; Botella, L.; Chamorro, B.; Sánchez-Velasco, S.; et al. Dual role of peripheral B cells in multiple sclerosis: Emerging remote players in demyelination and novel diagnostic biomarkers. *Front. Immunol.* **2023**, *14*, 1224217. [CrossRef]
24. Mazzucco, M.; Mannheim, W.; Shetty, S.V.; Linden, J.R. CNS endothelial derived extracellular vesicles are biomarkers of active disease in multiple sclerosis. *Fluids Barriers CNS* **2022**, *19*, 13. [CrossRef]
25. Bravo-Miana, R.D.C.; Arizaga-Echebarria, J.K.; Sabas-Ortega, V.; Crespillo-Velasco, H.; Prada, A.; Castillo-Triviño, T.; Otaegui, D. Tetraspanins, GLAST and L1CAM Quantification in Single Extracellular Vesicles from Cerebrospinal Fluid and Serum of People with Multiple Sclerosis. *Biomedicines* **2024**, *12*, 2245. [CrossRef]
26. Torres Iglesias, G.; López-Molina, M.; Botella, L.; Laso-García, F.; Chamorro, B.; Fernández-Fournier, M.; Puertas, I.; Bravo, S.B.; Alonso-López, E.; Díez-Tejedor, E.; et al. Differential Protein Expression in Extracellular Vesicles Defines Treatment Responders and Non-Responders in Multiple Sclerosis. *Int. J. Mol. Sci.* **2024**, *25*, 10761. [CrossRef]
27. Lim Falk, V.; Mueller-Wirth, N.; Karathanasis, D.; Evangelopoulos, M.E.; Maleska Maceski, A.; Zadic, A.; Kuhle, J.; Schlup, C.; Marti, S.; Guse, K.; et al. Extracellular Vesicle Marker Changes Associated With Disease Activity in Relapsing-Remitting Multiple Sclerosis. *Neurol. Neuroimmunol. Neuroinflamm.* **2025**, *12*, e200404. [CrossRef]
28. Fowler, C.D. NeuroEVs: Characterizing Extracellular Vesicles Generated in the Neural Domain. *J. Neurosci.* **2019**, *39*, 9262–9268. [CrossRef]
29. Gomes, D.E.; Witwer, K.W. L1CAM-associated extracellular vesicles: A systematic review of nomenclature, sources, separation, and characterization. *J. Extracell. Biol.* **2022**, *1*, e35. [CrossRef]
30. Hill, A.F. Extracellular Vesicles and Neurodegenerative Diseases. *J. Neurosci.* **2019**, *39*, 9269–9273. [CrossRef]
31. Norman, M.; Ter-Ovanesyan, D.; Trieu, W.; Lazarovits, R.; Kowal, E.J.K.; Lee, J.H.; Chen-Plotkin, A.S.; Regev, A.; Church, G.M.; Walt, D.R. L1CAM is not associated with extracellular vesicles in human cerebrospinal fluid or plasma. *Nat. Methods* **2021**, *18*, 631–634. [CrossRef]

32. Nogueras-Ortiz, C.J.; Eren, E.; Yao, P.; Calzada, E.; Dunn, C.; Volpert, O.; Delgado-Peraza, F.; Mustapic, M.; Lyashkov, A.; Rubio, F.J.; et al. Single-extracellular vesicle (EV) analyses validate the use of L1 Cell Adhesion Molecule (L1CAM) as a reliable biomarker of neuron-derived EVs. *J. Extracell. Vesicles* **2024**, *13*, e12459. [CrossRef]
33. Marostica, G.; Gelibter, S.; Gironi, M.; Nigro, A.; Furlan, R. Extracellular Vesicles in Neuroinflammation. *Front. Cell Dev. Biol.* **2020**, *8*, 623039. [CrossRef]
34. Mustapic, M.; Eitan, E.; Werner, J.K., Jr.; Berkowitz, S.T.; Lazaropoulos, M.P.; Tran, J.; Goetzl, E.J.; Kapogiannis, D. Plasma Extracellular Vesicles Enriched for Neuronal Origin: A Potential Window into Brain Pathologic Processes. *Front. Neurosci.* **2017**, *11*, 278. [CrossRef]
35. Vandendriessche, C.; Bruggeman, A.; Van Cauwenberghe, C.; Vandenbroucke, R.E. Extracellular Vesicles in Alzheimer's and Parkinson's Disease: Small Entities with Large Consequences. *Cells* **2020**, *9*, 2485. [CrossRef]
36. Dolcetti, E.; Bruno, A.; Guadalupi, L.; Rizzo, F.R.; Musella, A.; Gentile, A.; De Vito, F.; Caioli, S.; Bullitta, S.; Fresegna, D.; et al. Emerging Role of Extracellular Vesicles in the Pathophysiology of Multiple Sclerosis. *Int. J. Mol. Sci.* **2020**, *21*, 7336. [CrossRef]
37. Raghav, A.; Singh, M.; Jeong, G.B.; Giri, R.; Agarwal, S.; Kala, S.; Gautam, K.A. Extracellular vesicles in neurodegenerative diseases: A systematic review. *Front. Mol. Neurosci.* **2022**, *15*, 1061076. [CrossRef]
38. Barro, C.; Healy, B.C.; Liu, Y.; Saxena, S.; Paul, A.; Polgar-Turcsanyi, M.; Guttmann, C.R.G.; Bakshi, R.; Kropshofer, H.; Weiner, H.L.; et al. Serum GFAP and NfL Levels Differentiate Subsequent Progression and Disease Activity in Patients With Progressive Multiple Sclerosis. *Neurol. Neuroimmunol. Neuroinflamm.* **2023**, *10*, 1. [CrossRef]
39. 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. Potential Roles of Extracellular Vesicles as Biomarkers and a Novel Treatment Approach in Multiple Sclerosis. *Int. J. Mol. Sci.* **2021**, *22*, 9011. [CrossRef]
40. Mahad, D.; Callahan, M.K.; Williams, K.A.; Ubogu, E.E.; Kivisäkk, P.; Tucky, B.; Kidd, G.; Kingsbury, G.A.; Chang, A.; Fox, R.J.; et al. Modulating CCR2 and CCL2 at the blood-brain barrier: Relevance for multiple sclerosis pathogenesis. *Brain* **2006**, *129 Pt 1*, 212–223. [CrossRef]

Supplementary information

In-depth characterization of L1CAM^{+} blood extracellular vesicles as potential biomarkers for anti-CD20 therapy response in relapsing–remitting multiple sclerosis

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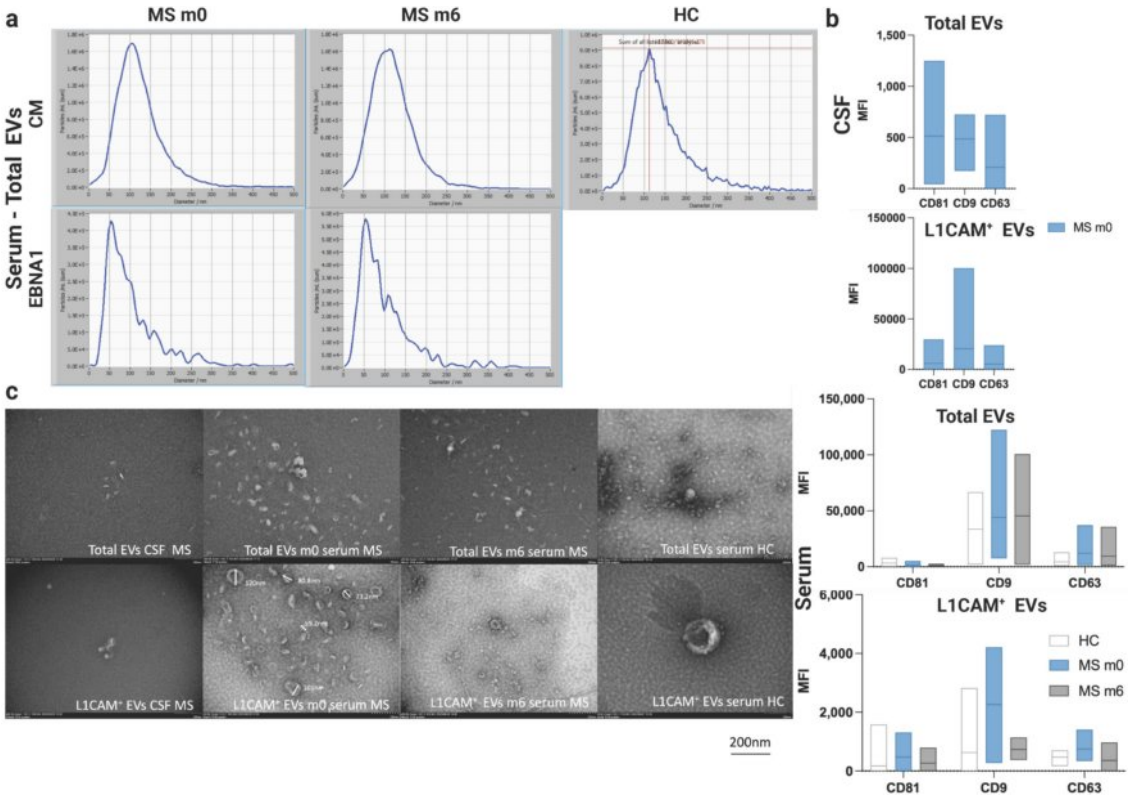
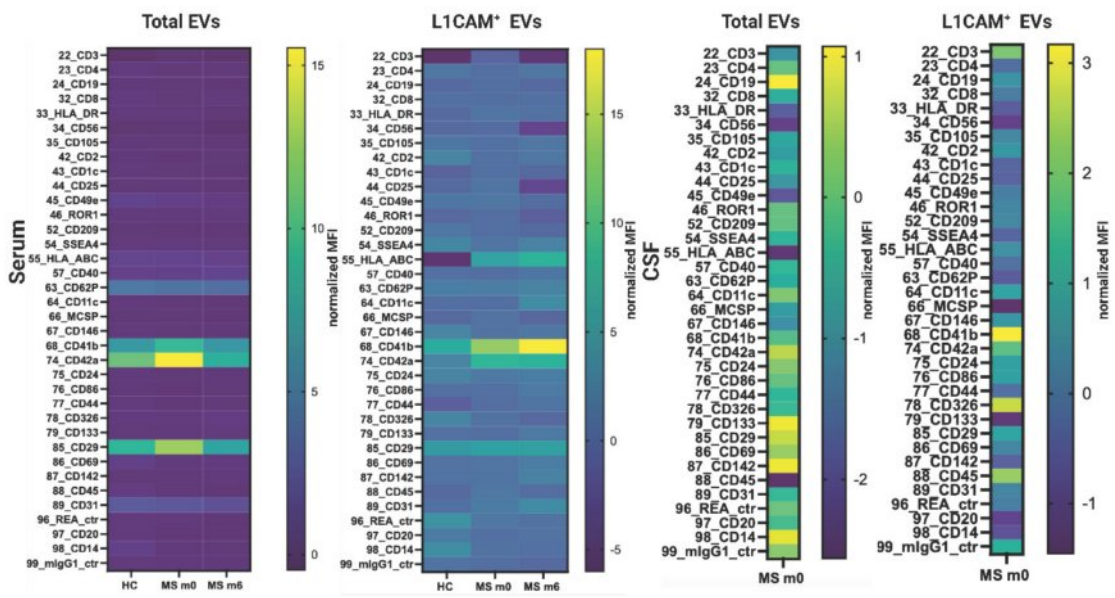
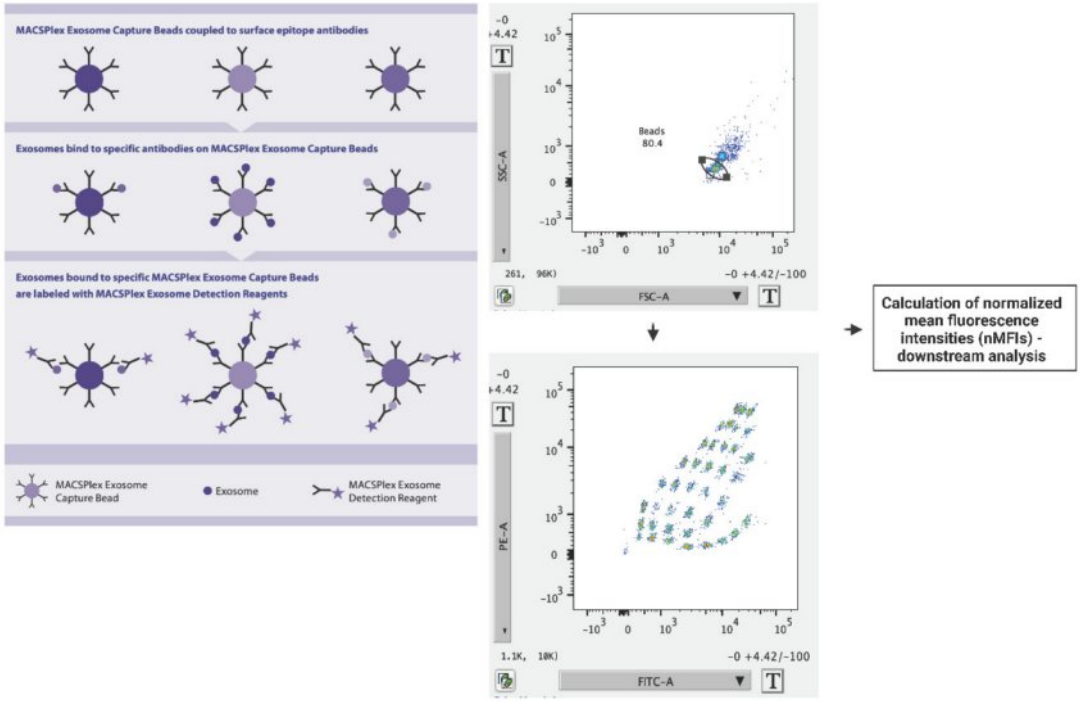


Supplementary Figure S1. Characteristics of serum and CSF EVs (pilot experiments; *n=5*): (a) NTA analysis—total EV concentration and EBNA1$^+$ EV concentration in serum total EVs and (b) tetraspanin (CD81, CD9, and CD63) (bead population numbers: 65, 53, and 56 respectively—flow cytometry analysis) expression profile of MS patients (month 0—m0 and month 6—m6) and healthy controls, with total EVs and L1CAM$^+$ EVs expressed in mean fluorescence intensities (MFIs), as floating bar plots with min. and max. ranges.



Supplementary Figure S2. Pilot experiments (*n=5*): Surface immune profiling of total and L1CAM^{+} EVs shown as heatmaps (EV markers' geometric mean fluorescence intensity (MFI) was normalized to the mean MFI for specific EV markers (CD9, CD63, and CD81), obtaining normalized MFI values) from CSF and serum samples comparing MS patients (m0. m6) and healthy controls.



Supplementary Figure S3. General principle and data analysis procedure (MACSPlex Exosome Kit—flow cytometry analysis)—initial gating strategy for the detection of MACSPlex Exosome Capture Bead populations (Supplementary Table 6). Following initial gating strategy, all downstream analyses were based on normalized geometric mean fluorescence intensity (normalized MFI) values. In short, a blank control composed of only MACSPlex buffer, incubated with beads and MACSPlex exosome detection reagents (CD9, CD63, and CD81), was used to measure the background signal. Each EV marker's geometric mean fluorescence intensity (MFI) was normalized to the mean MFI for specific EV markers (CD9, CD63, and CD81), obtaining normalized MFIs.

Supplementary Table S1: Overview of proximity extension assay (PEA) flex panel (21 proteins).

Protein name (gene name)	UniProt No
Transforming growth factor beta-1 proprotein (TGFB1)	P01137
Tumor necrosis factor (TNF)	P01375
Interferon gamma (IFNG)	P01579
C-X-C motif chemokine 10 (CXCL10)	P02778
Interleukin-6 (IL6)	P05231
Granzyme B (GZMB)	P10144
Interleukin-8 (CXCL8)	P10145
C-C motif chemokine 3 (CCL3)	P10147
T-cell-specific surface glycoprotein CD28 (CD28)	P10747
C-C motif chemokine 2 (CCL2)	P13500
Interleukin-10 (IL10)	P22301
B-cell antigen receptor complex-associated protein beta chain (CD79B)	P40259
Interleukin-17A (IL17A)	Q16552
Triggering receptor expressed on myeloid cells 2 (TREM2)	Q9NZC2
Interleukin-4 (IL4)	P05112
Tumor necrosis factor receptor superfamily member 13B (TNFRSF13B)	O14836
Lysosome-associated membrane glycoprotein 3 (LAMP3)	Q9UQV4
Calbindin (CALB1)	P05937
Neurotrophin-3 (NTF3)	P20783
Glial cell line-derived neurotrophic factor (GDNF)	P39905
Visinin-like protein 1 (VSNL1)	P62760

Supplementary Table S2: Differential expression of proteins in serum L1CAM^{+} EVs (PEA analysis).

Protein	**term**	**est.log2**	**p.value**	**p.value.adj.fdr**
GDNF	HCvsM0	-0.0941196	0.23268804	0.678346865
GDNF	M0vsM6	-0.0147704	0.7526507	0.939593942
IFNG	HCvsM0	0.02144309	0.75380304	0.994986221
IFNG	M0vsM6	0.02475317	0.48272582	0.939593942
CCL2	HCvsM0	0.50682571	0.02933236	0.256803366
CCL2	M0vsM6	-0.1466272	0.39091575	0.939593942
TNF	HCvsM0	0.09869598	0.35532455	0.678346865
TNF	M0vsM6	0.1113644	0.17661566	0.939593942
CALB1	HCvsM0	-0.0075376	0.8552049	0.994986221
CALB1	M0vsM6	-0.0058484	0.89434305	0.939593942
IL6	HCvsM0	0.06726025	0.28230612	0.678346865
IL6	M0vsM6	0.02886178	0.42863951	0.939593942

LAMP3	HCvsM0	0.13777894	0.03203712	0.256803366
LAMP3	M0vsM6	-0.0582832	0.25771699	0.939593942
TNFRSF13B	HCvsM0	0.05441479	0.65223156	0.994986221
TNFRSF13B	M0vsM6	-0.070706	0.37293476	0.939593942
IL4	HCvsM0	-0.0371312	0.73546297	0.994986221
IL4	M0vsM6	0.0830568	0.3616405	0.939593942
TREM2	HCvsM0	0.01371952	0.9327282	0.994986221
TREM2	M0vsM6	0.0429716	0.69364372	0.939593942
GZMB	HCvsM0	0.15907193	0.29042179	0.678346865
GZMB	M0vsM6	-0.0175532	0.89489016	0.939593942
TGFB1	HCvsM0	-0.1073167	0.17789739	0.678346865
TGFB1	M0vsM6	0.0259024	0.68116695	0.939593942
IL10	HCvsM0	-0.0048669	0.95544899	0.994986221
IL10	M0vsM6	0.0066916	0.93959394	0.939593942
VSNL1	HCvsM0	0.00096556	0.99498622	0.994986221
VSNL1	M0vsM6	0.099796	0.40447806	0.939593942
CCL3	HCvsM0	0.10574815	0.16856023	0.678346865
CCL3	M0vsM6	-0.0313888	0.52947453	0.939593942
CXCL8	HCvsM0	0.0336432	0.81410246	0.994986221
CXCL8	M0vsM6	0.0717256	0.74055353	0.939593942
CXCL10	HCvsM0	-0.1116862	0.31067856	0.678346865
CXCL10	M0vsM6	0.0227836	0.78171444	0.939593942
IL17A	HCvsM0	0.04010414	0.76339122	0.994986221
IL17A	M0vsM6	-0.0101408	0.93360394	0.939593942
NTF3	HCvsM0	-0.111682	0.40707273	0.712377278
NTF3	M0vsM6	0.0527444	0.40991603	0.939593942
CD28	HCvsM0	0.1073404	0.33766679	0.678346865
CD28	M0vsM6	0.0450104	0.67133409	0.939593942
CD79B	HCvsM0	0.24340203	0.0366862	0.256803366
CD79B	M0vsM6	-0.0273176	0.78811724	0.939593942

Supplementary Table S3: Differential expression of proteins in serum total EVs (PEAanalysis).

Protein	term	est.log2	p.value	p.value.adj.fdr
GDNF	HCvsM0	-0.0737981	0.45424027	0.55065247
GDNF	M0vsM6	0.0674264	0.25346082	0.59140857
IFNG	HCvsM0	0.26435437	0.23541861	0.5456926
IFNG	M0vsM6	-0.2821888	0.07567835	0.38435591
CCL2	HCvsM0	0.28044315	0.3378097	0.5456926
CCL2	M0vsM6	-0.1588544	0.10810283	0.38435591
TNF	HCvsM0	0.33050819	0.06738182	0.5456926

TNF	M0vsM6	-0.0916212	0.3532373	0.67436212
CALB1	HCvsM0	-0.4075336	0.25467234	0.5456926
CALB1	M0vsM6	0.0010628	0.98180841	0.99376386
IL6	HCvsM0	0.58613001	0.08395289	0.5456926
IL6	M0vsM6	-0.3772596	0.03038551	0.3190479
LAMP3	HCvsM0	-0.1333377	0.25570295	0.5456926
LAMP3	M0vsM6	0.1169996	0.14330118	0.42990354
TNFRSF13B	HCvsM0	-0.41383	0.03732411	0.5456926
TNFRSF13B	M0vsM6	-0.4955192	0.00046214	0.00970504
IL4	HCvsM0	0.12211743	0.32594406	0.5456926
IL4	M0vsM6	-0.08491	0.33601792	0.67436212
TREM2	HCvsM0	0.17862929	0.45437005	0.55065247
TREM2	M0vsM6	-0.1203196	0.16822516	0.44159106
GZMB	HCvsM0	-0.1521611	0.49918702	0.55065247
GZMB	M0vsM6	0.082164	0.57186559	0.80061182
TGFB1	HCvsM0	0.1794297	0.31240653	0.5456926
TGFB1	M0vsM6	0.0604112	0.54545638	0.80061182
IL10	HCvsM0	-0.3168211	0.2615744	0.5456926
IL10	M0vsM6	-0.062948	0.56091222	0.80061182
VSNL1	HCvsM0	0.37644975	0.20355735	0.5456926
VSNL1	M0vsM6	-0.1660452	0.0595543	0.38435591
CCL3	HCvsM0	-0.0603316	0.7828962	0.7828962
CCL3	M0vsM6	-0.0872548	0.44137415	0.77240476
CXCL8	HCvsM0	-0.1446685	0.45968243	0.55065247
CXCL8	M0vsM6	-0.04623	0.6903423	0.83195444
CXCL10	HCvsM0	0.22963381	0.28755122	0.5456926
CXCL10	M0vsM6	0.0078872	0.9541804	0.99376386
IL17A	HCvsM0	0.29800818	0.10895403	0.5456926
IL17A	M0vsM6	-0.21633	0.10981597	0.38435591
NTF3	HCvsM0	-0.2459383	0.45269616	0.55065247
NTF3	M0vsM6	-0.0456296	0.67513991	0.83195444
CD28	HCvsM0	-0.2994642	0.52443092	0.55065247
CD28	M0vsM6	-0.0009476	0.99376386	0.99376386
CD79B	HCvsM0	-0.15161	0.51094789	0.55065247
CD79B	M0vsM6	-0.0451852	0.7131038	0.83195444

Supplementary Table S4: Assessment of protein expression in CSF total EVs at baseline (PEA analysis).

Protein	**term**	**mean NPX (est. log2)**
GDNF	M0	0.26
IFNG	M0	0.38
CCL2	M0	9.35

TNF	M0	-0.08
CALB1	M0	5.71
IL6	M0	3.15
LAMP3	M0	-0.07
TNFRSF13B	M0	4.60
IL4	M0	1.23
TREM2	M0	10.71
GZMB	M0	0.21
TGFB1	M0	1.10
IL10	M0	0.92
VSNL1	M0	0.65
CCL3	M0	0.86
CXCL8	M0	6.47
CXCL10	M0	5.90
IL17A	M0	0.39
NTF3	M0	0.06
CD28	M0	0.24
CD79B	M0	1.97

Supplementary Table S5: Differential expression of proteins in CSF L1CAM^{+} EVs at baseline (PEA analysis).

Protein	**term**	**mean NPX (est. log2)**
GDNF	M0	-0.15
IFNG	M0	0.36
CCL2	M0	0.24
TNF	M0	-0.27
CALB1	M0	-1.22
IL6	M0	1.26
LAMP3	M0	0.04
TNFRSF13B	M0	0.15
IL4	M0	1.70
TREM2	M0	3.26
GZMB	M0	-1.29
TGFB1	M0	0.65
IL10	M0	0.92

VSNL1	M0	-1.82
CCL3	M0	-0.40
CXCL8	M0	0.08
CXCL10	M0	-0.11
IL17A	M0	0.39
NTF3	M0	0.06
CD28	M0	0.39
CD79B	M0	0.19

Supplementary Table S6: Overview of surface marker antibodies used for the MACSPlex Exosome Kit.

No.	**Antibody**	**Isotype**
22	CD3	mIgG2a
23	CD4	mIgG2a
24	CD19	mIgG1
32	CD8	mIgG2a
33	HLA-DRDPDQ	recombinant human IgG1
34	CD56	recombinant human IgG1
35	CD105	recombinant human IgG1
42	CD2	mIgG2b
43	CD1c	mIgG2a
44	CD25	mIgG1
45	CD49e	recombinant human IgG1
46	ROR1	mIgG1κ
52	CD209	mIgG1
53	CD9	mIgG1
54	SSEA-4	recombinant human IgG1
55	HLA-ABC	recombinant human IgG1
56	CD63	mIgG1κ
57	CD40	mIgG1κ
63	CD62P	recombinant human IgG1
64	CD11c	mIgG2b
65	CD81	recombinant human IgG1
66	MCSP	mIgG1
67	CD146	mIgG1
68	CD41b	recombinant human IgG1
74	CD42a	recombinant human IgG1
75	CD24	mIgG1
76	CD86	mIgG1
77	CD44	mIgG1

78	CD326	mIgG1
79	CD133/1	mIgG1κ
85	CD29	mIgG1κ
86	CD69	mIgG1κ
87	CD142	mIgG1κ
88	CD45	mIgG2a
89	CD31	mIgG1
96	REA control	recombinant human IgG1
97	CD20	mIgG1
98	CD14	mIgG2a
99	mIgG1 control	mIgG1

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OŚWIADCZENIE

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Maciak K, Dziedzic A, Miller E, Saluk-Bijak J. "miR-155 as an Important Regulator of Multiple Sclerosis Pathogenesis. A Review". *Int. J. Mol. Sci.* 2021, 22(9), 4332
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głównej roli w opracowaniu koncepcji pracy przeglądowej; zebraniu materiałów literaturowych; współtworzeniu podstawowej wersji manuskryptu, w tym na samodzielnym opracowaniu figury; współredagowaniu końcowej wersji manuskryptu na podstawie uzyskanej recenzji oraz przygotowaniu odpowiedzi dla recenzentów.

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głównej roli w opracowaniu koncepcji pracy przeglądowej; zebraniu źródłowych materiałów literaturowych; współtworzeniu podstawowej wersji manuskryptu, wraz z samodzielnym opracowaniem figury; współredagowaniu końcowej wersji manuskryptu poprzez wprowadzenie poprawek merytorycznych na podstawie uzyskanej recenzji, wraz z uzasadnieniem dla recenzentów oraz pełnieniu funkcji autora korespondencyjnego.

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współplanowaniu metodyki i optymalizacji protokołów prac doświadczalnych; preparatyce części materiału biologicznego i zabezpieczeniu go do dalszych eksperymentów; izolacji płytek krwi oraz leukocytów; przeprowadzeniu testu migracji komórek w komorze Boydena; barwieniu immunofluorescencyjnym preparatów

kompleksów płytkowo-leukocytarnych celem przeprowadzenia obrazowania na mikroskopie konfokalnym; ilościowej analizie białka przy użyciu systemu Bio-Plex; przygotowaniu próbek do analizy na cytometrze przepływowym oraz opracowaniu uzyskanych z tej analizy danych surowych; analizie statystycznej wyników badań, ich interpretacji i wizualizacji; współtworzeniu podstawowej wersji manuskryptu wraz z samodzielnym opracowaniem abstraktu graficznego; współredagowaniu końcowej wersji manuskryptu poprzez wprowadzenie poprawek merytorycznych na podstawie uzyskanej recenzji, wraz z uzasadnieniem dla recenzentów, oraz pełnieniu funkcji autora korespondencyjnego.

Oświadczam, że w opublikowanej pracy

Anandan S, Maciak K, Breinbauer R, Mostafavi S, Kvistad CE, Torkildsen O, Myhr KM. "Brain-derived blood biomarkers in multiple sclerosis-current trends and beyond". ***Front Immunol.*** **2025, 16:1569503**

mój udział polegał na:

współtworzeniu koncepcji pracy przeglądowej; zebraniu materiałów literaturowych; opracowaniu tabeli przedstawiającej charakterystykę biomarkerów; przygotowaniu figury oraz wprowadzeniu poprawek merytorycznych na podstawie uzyskanej recenzji.

Oświadczam, że w opublikowanej pracy

Anandan S, Maciak K, Breinbauer R, Otero-Ortega L, Feliciello G, Stojanović Gužvić N, Torkildsen O, Myhr KM. "In-Depth Characterization of L1CAM^{+} Extracellular Vesicles as Potential Biomarkers for Anti-CD20 Therapy Response in Relapsing–Remitting Multiple Sclerosis". ***Int J Mol Sci.*** **2025, 26(15), 7213**

mój udział polegał na:

przeprowadzeniu przeglądu literatury dotyczącego markerów umożliwiających określenie pochodzenia komórkowego pęcherzyków zewnątrzkomórkowych izolowanych z surowicy krwi; przeglądzie, ocenie i selekcji metod badawczych optymalnych dla osiągnięcia zaplanowanych celów projektu; uczestnictwie w wyborze metodyki adekwatnej do analizy proteomicznej zawartości pęcherzyków zewnątrzkomórkowych oraz zaprojektowaniu panelu markerów do identyfikacji ich pochodzenia komórkowego; współpracy w uzgodnieniu zakresu i harmonogramu prac związanych z izolacją pęcherzyków zewnątrzkomórkowych oraz analizą metodą PEA (Proximity Extension Assay) w komercyjnym laboratorium; współtworzeniu protokołu analizy materiału biologicznego i pozyskiwania danych; analizie statystycznej, interpretacji i wizualizacji wyników uzyskanych metodą NTA (Nanoparticle Tracking Analysis); przygotowaniu abstraktu graficznego; edycji tekstu manuskryptu oraz wprowadzeniu poprawek na podstawie uzyskanej recenzji.

Oświadczam, że w manuskrypcie artykułu

Maciak K, Dziedzic A, Anandan S, Miller E, Łaczmański Ł, Zajdel R, Michlewska S, Kujawa D, Gancarek M, Raczkowska J, Włodarczyk L, Nowak P, Saluk J. "Extracellular vesicle-derived miR-760 as a novel candidate marker differentiating stable RRMS from SPMS"

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Maciak K.

podpis

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Łódź, 16.09.2025 r.

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Maciak K, Dziedzic A, Miller E, Saluk-Bijak J. "miR-155 as an Important Regulator of Multiple Sclerosis Pathogenesis. A Review". *Int. J. Mol. Sci.* 2021, 22(9), 4332
mój udział polegał na:
współtworzeniu zarówno koncepcji pracy przeglądowej, jak i końcowej wersji manuskryptu poprzez wprowadzenie merytorycznych poprawek po recenzji i odpowiedzi dla recenzentów; a także na pełnieniu funkcji autora korespondencyjnego.

Oświadczam, że w opublikowanej pracy
Maciak K, Dziedzic A, Saluk J. "Remyelination from the miRNA perspective". *Front Mol Neurosci.* 2023, 16:1199313
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Oświadczam, że w manuskrypcie artykułu

Maciak K, Dziedzic A, Anandan S, Miller E, Łaczmański Ł, Zajdel R, Michlewska S, Kujawa D, Gancarek M, Raczkowska J, Włodarczyk L, Nowak P, Saluk J. "Extracellular vesicle-derived miR-760 as a novel candidate marker differentiating stable RRMS from SPMS"

mój udział polegał na:

współuczestniczeniu w ustaleniu metodyki badań eksperymentalnych oraz w optymalizacji protokołów; konsultacjach merytorycznych w zakresie realizacji prac badawczych; redagowaniu roboczej wersji manuskryptu i korekcie merytorycznej; zatwierdzeniu ostatecznej wersji manuskryptu.

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mój udział polegał na:

współopracowaniu koncepcji pracy przeglądowej; sprawowaniu nadzoru nad przygotowaniem pracy; zatwierdzeniu ostatecznej wersji manuskryptu.

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mój udział polegał na:

zgromadzeniu kohort pacjentów z RRMS i SPMS; prowadzeniu rejestru pacjentów; dostarczeniu danych o parametrach klinicznych i wynikach badań laboratoryjnych pacjentów; zapewnieniu materiału biologicznego od pacjentów; konsultacjach merytorycznych; współuczestnictwie w pozyskaniu środków finansowych na realizację badań; zatwierdzeniu ostatecznej wersji manuskryptu.

Oświadczam, że w manuskrypcie artykułu

Maciak K, Dziedzic A, Anandan S, Miller E, Łaczmański Ł, Zajdel R, Michlewska S, Kujawa D, Gancarek M, Raczkowska J, Włodarczyk L, Nowak P, Saluk J. "Extracellular vesicle-derived miR-760 as a novel candidate marker differentiating stable RRMS from SPMS"

mój udział polegał na:

współopracowaniu koncepcji badań, zgromadzeniu kohort pacjentów z RRMS i SPMS, prowadzeniu rejestru pacjentów, dostarczeniu danych o parametrach klinicznych i wynikach badań laboratoryjnych pacjentów, zapewnieniu materiału biologicznego od pacjentów, konsultacjach merytorycznych, zatwierdzeniu ostatecznej wersji manuskryptu.

Signed by / Podpisano przez: Elżbieta Dorota Miller

podpis

Date / Data: 2025-09-17 08:21

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Pracownia Obrazowania Mikroskopowego
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Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki

Łódź, 16.09.2025 r.

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mój udział polegał na:

konsultacjach merytorycznych w zakresie obrazowania mikroskopowego; preparatyce materiału biologicznego i przygotowaniu wizualizacji mikroskopowych przy użyciu SEM oraz obrazowania konfokalnego; korekcie merytorycznej roboczej wersji manuskryptu.

Oświadczam, że w manuskrypcie artykułu

Maciak K, Dziedzic A, Anandan S, Miller E, Łaczmański Ł, Zajdel R, Michlewska S, Kujawa D, Gancarek M, Raczkowska J, Włodarczyk L, Nowak P, Saluk J. "Extracellular vesicle-derived miR-760 as a novel candidate marker differentiating stable RRMS from SPMS"

mój udział polegał na:

konsultacjach merytorycznych; preparatyce materiału biologicznego i przygotowaniu wizualizacji mikroskopowych przy użyciu TEM; przeprowadzeniu analiz pęcherzyków zewnątrzkomórkowych metodą DLS; korekcie merytorycznej roboczej wersji manuskryptu.

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Łódź, 16.09.2025 r.

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współtworzeniu koncepcji pracy przeglądowej oraz roboczej wersji manuskryptu; wprowadzeniu poprawek merytorycznych na podstawie uzyskanej recenzji; sprawowaniu nadzoru nad przygotowaniem końcowej wersji pracy oraz odpowiedzi dla recenzentów; zatwierdzeniu ostatecznej wersji manuskryptu.

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mój udział polegał na:
współtworzeniu koncepcji i metodyki badań; konsultacjach merytorycznych dotyczących interpretacji wyników i redagowania roboczej wersji manuskryptu; sprawowaniu nadzoru nad naniesieniem poprawek i przygotowaniem końcowej wersji zrecenzowanego manuskryptu oraz opracowaniem odpowiedzi dla recenzentów; zatwierdzeniu ostatecznej wersji manuskryptu; pozyskaniu środków finansowych na realizację badań.

Oświadczam, że w manuskrypcie artykułu

Maciak K, Dziedzic A, Anandan S, Miller E, Łaczmański Ł, Zajdel R, Michlewska S, Kujawa D, Gancarek M, Raczkowska J, Włodarczyk L, Nowak P, Saluk J. "Extracellular vesicle-derived miR-760 as a novel candidate marker differentiating stable RRMS from SPMS"

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współtworzeniu koncepcji i metodyki badań; konsultacjach merytorycznych dotyczących interpretacji wyników; współredagowaniu roboczej i końcowej wersji manuskryptu; pozyskaniu środków finansowych na realizację badań.

podpis

Shamundeeswari Anandan | Bergen, Norway, 04.09.25

First name and surname | **Place and date**

Department of Clinical Medicine, University of Bergen

Affiliation

STATEMENT

I hereby declare that in the work

Publication title: In-Depth Characterization of L1CAM+ Extracellular Vesicles as Potential Biomarkers for Anti-CD20 Therapy Response in Relapsing–Remitting Multiple Sclerosis

Authors: Shamundeeswari Anandan*, Karina Maciak, Regina Breinbauer, Laura Otero-Ortega, Giancarlo Feliciello, Nataša Stojanovic Gužvic, Oivind Torkildsen and Kjell-Morten Myhr

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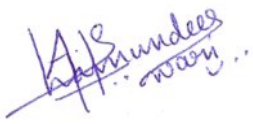
my contribution consisted of:

Conceptualization, clinical data/sample collection and retrieval, experimental data collection/curation, formal analysis, funding acquisition, writing (original draft), writing (review and editing).



Shamundeeswari Anandan

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STATEMENT

I hereby declare that in the work

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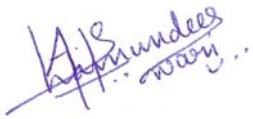
Authors: Shamundeeswari Anandan*, Karina Maciak, Regina Breinbauer, Sepideh Mostafavi, Christopher Elnan Kvistad, Oivind Torkildsen and Kjell-Morten Myhr

Year of Publication*:* 2025

Journal: Front. Immunol. 16:1569503. doi: 10.3389/fimmu.2025.1569503

my contribution consisted of:

Conceptualization, formal Analysis, funding acquisition, investigation, project administration, resources, supervision, writing – original draft, writing– review & editing.



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Year of Publication*:* 2025

Journal: Int. J. Mol. Sci. 2025, 26, 7213

my contribution consisted of: Experimental data collection/curation, writing (review and editing).

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