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**From Gut to Brain: Exploring the Role of
Extracellular Vesicles in Multiple Sclerosis**

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Abstract

Multiple sclerosis (MS) is an autoimmune disorder of the central nervous system (CNS) that affects nearly 2.8 million people worldwide. Despite its widespread prevalence, diagnosing MS remains a challenge due to its highly variable symptoms, clinical heterogeneity, and the persistent lack of reliable biomarkers. These complexities often lead to delays in diagnosis and treatment, underscoring the urgent need for novel, non-invasive diagnostic tools. In recent years, scientific attention has increasingly turned toward gut microbiota as a critical regulator of human health. Alterations in its composition, known as dysbiosis, have been implicated in the development and progression of several immune and neurological disorders, including MS. The gut and brain are known to communicate through the gut-brain axis, and among the emerging molecular messengers involved in this communication, extracellular vesicles (EVs) offer a potential mechanistic link between dysbiosis and neuroinflammation in MS. Stool samples, being non-invasive and easily accessible, offer a valuable platform not only for studying microbiota composition but also for profiling both bacterial and eukaryotic-derived EVs. This comprehensive approach provides insights into gut microbiota dynamics and their secreted mediators, shedding light on disease mechanisms and revealing potential therapeutic targets. Despite growing evidence linking gut dysbiosis, EVs, and MS, no studies to date have comprehensively characterized both eukaryotic (eEVs) and prokaryotic (bEVs) derived EVs from the gut in MS patients. The objective of this thesis is to explore their diagnostic potential, with a particular focus on identifying a distinct fingerprint of gut-derived EVs in MS. To achieve this, we collected stool samples from MS patients and healthy controls (HC). EVs were isolated using size exclusion chromatography (SEC) and ultracentrifugation (UC), while the characterization of isolated EVs was performed following the MISEV guidelines 2023 (Western blot, scanning electron microscopy (SEM), and proteomic analysis). bEVs were profiled by metaproteomic, while eEVs were characterized using the MACSPlex Exosome Kit. Western blot and SEM confirmed successful EV isolation from stool samples. Metaproteomic analysis of bEVs at the phylum level revealed that *Bacteroidetes* and *Firmicutes* were the most representative in both groups. However, our statistical analysis showed a significant decrease in *Firmicutes*-derived EVs in MS patients. At the species level, *Roseburia faecis*-derived EVs were higher in MS patients compared to HC, while *Bacteroides uniformis*-derived EVs were markedly reduced in MS patients. Furthermore, MS patients exhibited significantly fewer EVs expressing ROR1, CD24, and CD40, with a trend toward reduced CD44 expression. In conclusion, our findings suggest that bEVs and eEVs exhibit distinct compositional and molecular signatures in MS patients, supporting their potential role as non-invasive biomarkers and offering new insights into the gut-brain axis and its contribution to MS pathophysiology.

1. Introduction

1.1 Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic neurodegenerative autoimmune disease that affects the central nervous system (CNS), leading to inflammation and demyelination (1). The hallmark pathological feature of MS is the development of inflammatory plaques, which are areas of demyelination found in both the white and grey matter of the brain and spinal cord. These plaques result from an inflammatory response that leads to the destruction of myelin, produced by oligodendrocytes, and are often accompanied by axonal and neural damage (1,2). MS manifests through a broad spectrum of neurological deficits, such as vision loss, sensory impairment, motor function weakness, and cognitive dysfunction (1).

MS exhibits heterogeneous clinical courses classified into distinct disease phenotypes. The relapsing-remitting MS (RR-MS) is the most common form, in 70-80% of patients. RRMS is featured by relapsing neurological episodes with a duration of 24-48 hours over days to weeks. Primary progressive MS (PP-MS) occurs in 15–20% of patients and is characterized by progressive neurological deterioration from disease onset without relapse (3). Approximately 50% of patients with RRMS may develop secondary progressive MS (SP-MS) within 20 years following disease onset. SPMS is defined by relentless neurological worsening. Additionally, these three subtypes are sometimes included within the spectrum of MS: clinically isolated syndrome (a single demyelinating event with uncertain risk of progression), fulminant MS (characterized by rapid progression and early disability), and benign MS (marked by minimal disability and infrequent relapses) (3,4).

1.1.1 Epidemiology

MS primarily affects young adults, with a female-to-male ratio of approximately 3:1, a ratio that has been increasing over time. MS onset ranges between 25 and 40 years, though it varies depending on the MS subtype. In RR-MS, onset commonly occurs between 25 and 29 years, whereas in PP-MS, the average onset is later, around 39 to 41 years (5).

Geographically, MS is more prevalent in Western countries and relatively rare among Asian and African populations. Globally, the prevalence of MS is rising, with recent estimates indicating that over 2.8 million people are currently living with the disease. This increasing incidence may be attributed to improved diagnostic methods, greater public awareness, and shifting lifestyle factors, including decreased physical activity and the adoption of Westernized diets (6).

1.1.2 Etiology

MS is a multifactorial disease, arising from a complex interplay of genetic predispositions, epigenetic modifications, and environmental factors that collectively increase susceptibility to the disease (3).

The strongest and earliest identified genetic risk factor is the HLA-DRB1*15:01 allele, part of the MHC class II region, which increases MS risk approximately threefold. Other important susceptibility genes include interleukin-7, IL-2 receptor alpha, and tyrosine kinase 2 variant (TYK2) (7).

Epigenetic changes, which alter gene expression without modifying the underlying DNA sequence, may further modulate disease development. For instance, the majority of studies investigating miRNAs in inflammation and MS agree that dysregulated miRNA expression supports certain T-cell differentiation pathways and contributes to disease progression. For example, miR-326 enhances Th17 cell differentiation by suppressing the expression of Ets-1, a transcription factor that normally inhibits the development of naïve T cells into Th17 cells. Additionally, in the early stages of MS, an increased histone H3 deacetylation in oligodendrocytes has been observed, which is associated with impaired cell differentiation and may hinder remyelination (8).

Additionally, several environmental factors have been linked to MS risk. These include Epstein-Barr virus (EBV) infection, smoking, diet, and vitamin D deficiency. EBV is a herpesvirus that causes infectious mononucleosis and remains dormant in B cells after the initial infection. MS patients are characterized by an altered immune response to EBV antigens, such as EBV nuclear antigen-1 (EBNA-1). One proposed mechanism is molecular mimicry, where EBNA-1 resembles myelin proteins like myelin basic protein (MBP), triggering an autoimmune response that damages myelin in the CNS (9). Smoking is also a risk factor for MS by causing epigenetic alterations, such as DNA methylation and histone modifications, which dysregulate immune and CNS cell function. These changes lead to persistent overexpression of pro-inflammatory genes and reduced expression of protective genes, contributing to chronic inflammation and autoimmunity in MS (10). Additionally, Vitamin D plays a key role in myelin formation and its components, and its deficiency is a common feature in many neurological disorders. It is also crucial for immune regulation and inflammation control. The vitamin D receptor (VDR) is expressed on various immune cells, including B cells, T cells, macrophages, and dendritic cells. Through these receptors, vitamin D promotes the activation and differentiation of Tregs, which are essential for maintaining immune tolerance and preventing autoimmunity (11,12).

1.1.3 Diagnosis

The 2017 McDonald criteria use imaging and paraclinical evidence to demonstrate dissemination in space (DIS) and dissemination in time (DIT) of CNS lesions. DIS requires lesions in

≥2 different CNS locations (e.g., cortical, spinal, or infratentorial), while DIT confirms lesions at different time points. For patients with clinically isolated syndrome, cerebrospinal fluid oligoclonal bands can contribute to the diagnosis of MS, and symptomatic lesions can fulfill DIS/DIT criteria. Differential diagnoses include neuromyelitis optica spectrum disorders and systemic inflammatory or infectious diseases (13).

Magnetic resonance imaging (MRI) of the spinal cord and brain remains at the center of imaging lesions and disease evolution (14). Complementary neuroimaging and fluid biomarkers (e.g., examination of CSF) provide complementary diagnostic and prognostic data, particularly in the context of early or atypical cases (13).

1.1.4 Pathophysiology

The pathology of MS is defined by the formation of inflammatory demyelinating plaques within both white and gray matter of the CNS, particularly affecting the periventricular areas, cortex, spinal cord, optic nerves, cerebellum, and meninges.

The disease process begins with disruption of the BBB, which allows immune cells and especially autoreactive CD4⁺ and CD8⁺ T cells, that recognize myelin antigens, to infiltrate the CNS. This infiltration initiates an inflammatory cascade that damages myelin, oligodendrocytes, and eventually leads to axonal and neuronal loss (1,2).

Among the infiltrating immune cells, CD4⁺ T helper cells, particularly the Th1 and Th17 subsets, play a central role by secreting pro-inflammatory cytokines such as IFN- γ and IL-17, which further amplify inflammation and facilitate continued immune cell entry into the CNS. CD8⁺ cytotoxic T cells contribute directly to tissue injury by targeting and killing oligodendrocytes and neurons. B cells also play a key role by producing autoantibodies, presenting antigens to T cells, and releasing cytokines that sustain the inflammatory environment. Meanwhile, regulatory T cells (Tregs), which normally help suppress autoreactive immune responses, are functionally impaired in MS, allowing the immune attack to persist and progress. This dysregulated immune response is central to the chronic demyelination and neurodegeneration observed in the disease (**Figure 1**) (15,16).

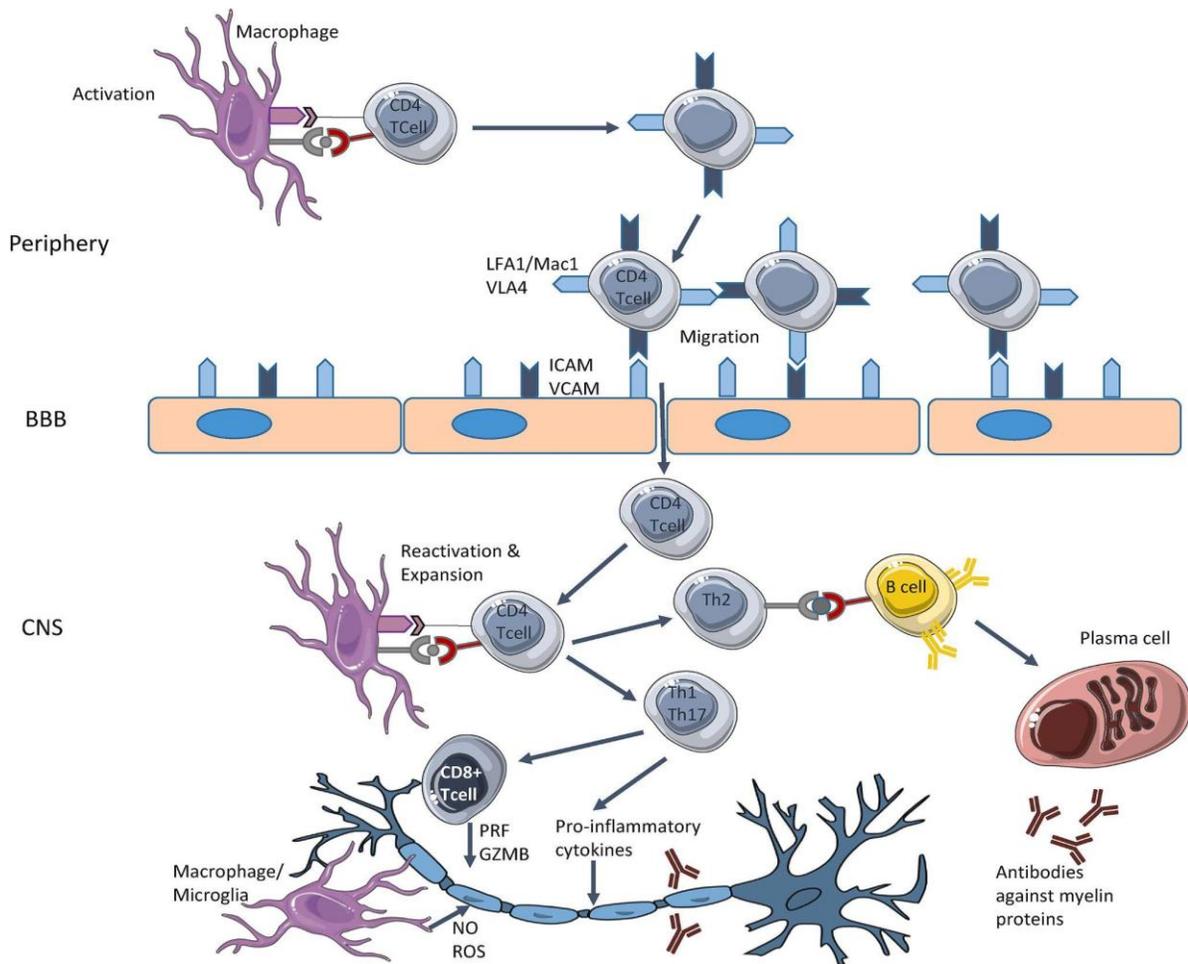


Figure 1: Schematic diagram of possible pathogenesis of multiple sclerosis. Lymphocytes activated in the periphery by a particular event will bypass the blood-brain barrier. Initially, they bind with the cell adhesion molecules present on the capillary endothelium and gain access into the brain. Once inside, the reactive cells activate the immune cell traffic (T-and B-cells) and mediate the devastating cascade. Cytotoxic T-cells release perforins and granzymes, and activated B-cells produce antibodies against the myelin sheath, thus mediating the demyelination process. Abbreviations: BBB, Blood-brain barrier, CNS, Central Nervous System, PRF, Perforin, GZMB, Granzyme B, NO, Nitric Oxide, ROS, Reactive Oxygen Species (15).

Histologically, MS lesions show perivenular mononuclear cell infiltration, with immune cells such as monocytes and macrophages promoting T-cell migration across the BBB and intensifying inflammation. Microglia, the CNS-resident immune cells, act as antigen-presenting cells and contribute to tissue injury by releasing cytotoxic molecules like nitric oxide and reactive oxygen species. As inflammation subsides, the affected areas often develop astrocytic scars, marking the chronic stage of the disease (3).

1.1.5 Treatment

Currently, there is no definitive cure for MS; therefore, existing therapies are aimed at relieving symptoms, reducing relapse frequency, and slowing disease progression. One of the primary first-line treatments for RRMS is recombinant interferon- β (IFN- β), which exerts anti-inflammatory effects by inhibiting the migration of the immune cells across the BBB and suppressing T cell activation. Additionally, it promotes the proliferation of oligodendrocytes, aiding in the repair of damaged CNS cells (17).

Another common therapy is glatiramer acetate, which mimics myelin basic protein and promotes a shift from pro-inflammatory Th1 cells to Th2 cells, whose cytokines exert anti-inflammatory effects within the CNS (18). Glatiramer acetate is administered subcutaneously and is generally well-tolerated, but it is ineffective for progressive forms of MS (13).

Among the newer immunomodulatory therapies are ofatumumab, a monoclonal antibody targeting CD20 that effectively depletes B cells (19), and teriflunomide, which inhibits pyrimidine synthesis, thereby suppressing the proliferation of both T and B lymphocytes (20).

For more severe or treatment-resistant cases, second-line therapies are employed. Fingolimod acts on sphingosine-1-phosphate (S1P) receptors to sequester lymphocytes in lymph nodes, preventing their migration to the CNS (21). Natalizumab is a humanized monoclonal antibody that blocks leukocyte entry into the CNS by targeting α 4-integrin, though long-term use may result in the formation of neutralizing antibodies (13,22).

Mitoxantrone is a synthetic anthracenedione derivative that is an antineoplastic and immunomodulator. Mitoxantrone acts by macrophage-mediated suppression of B cell, T helper and T cytotoxic lymphocyte function, inhibition of myelin degradation in a dose-dependent manner (23). However, its use is limited due to the adverse effects that have been documented, including amenorrhea and alopecia (13).

Disease-modifying therapies show varying effectiveness, ranging from possible benefits to limited impact on disease progression. Typically, younger patients with a shorter duration of progression tend to benefit more from these therapies (13).

Despite the fact that most current treatments for MS primarily target various components of the immune system to reduce inflammation and disease progression, recent advances have begun to highlight the pivotal role of the intestinal microbiota in MS pathogenesis. Beyond its role in nutrient processing, the gut microbiota is a key modulator of immune homeostasis, contributing to the maintenance of tolerance and the regulation of systemic inflammation. In MS, changes in the gut microbiota (dysbiosis) can influence the immune system, disrupt the BBB, and contribute to autoimmune demyelination (2,24).

1.2 Gut Microbiota

The human gut microbiota comprises a complex consortium of bacteria, archaea, eukarya, viruses, and parasites, dominated by bacterial divisions such as *Firmicutes* and *Bacteroidetes* (collectively >90% of the population), alongside *Actinobacteria*, *Fusobacteria*, *Proteobacteria*, *Verrucomicrobia*, and *Cyanobacteria*. Feedback mechanisms help maintain homeostasis in this system. Positive feedback can disrupt microbial cooperation, for example, in mutualistic relationships where two species enhance each other's growth, leading to reduced community diversity. In contrast, three negative feedback mechanisms help restore balance: (1) the host immune system selectively shapes the microbial community, (2) spatial separation limits direct interactions between species, and (3) the availability of alternative carbon sources reduces the mutual dependence between cooperating microbes. (25).

The gut microbiota exerts critical immune functions, inhibiting pathogenic colonization through nutrient competition, bacteriocin production, and maintenance of epithelial integrity. These protective roles are mediated via pH modulation, antimicrobial peptide secretion, and signaling pathway regulation (26).

Gut microbiota colonization begins in infancy, with emerging evidence suggesting that the microbial gut colonisation process may be initiated already prenatally by a distinct microbiota in the placenta and amniotic fluid (27). However, most microbial establishments occur during birth and postnatal exposure to maternal and environmental sources. Over the first 2–3 years, microbiota composition matures into an adult-like profile, shaped by delivery mode (vaginal vs. cesarean), diet (breast milk vs. formula), geography, and antibiotic use (28).

These factors can disrupt the delicate balance of the gut microbiota, leading to the expansion of pathogen microbes and inflammation state leading to dysbiosis (29). Dysbiosis is frequently associated with the so called “leaky gut” syndrome (LGS), which is characterized by a reduced barrier function and an increased intestinal permeability (30). As a result of the development of this syndrome, the translocation of multiple bacteria and their products causes activation of the immune system. This stimulation causes an increase in the concentration of pro-inflammatory cytokines, which if sustained over time can be detrimental for the host cells, including those of the CNS (31).

Under normal conditions, the gut barrier remains intact, allowing immune molecules such as immunoglobulin A (IgA) and antimicrobial peptides (AMPs) to perform their protective functions by inhibiting the proliferation and spread of pathogens (32). The gut-associated lymphoid tissue (GALT) plays a central role in maintaining immune homeostasis by regulating peripheral tolerance. This mechanism prevents immune responses against harmless dietary components and bacterial self-antigens.

Also, in a balanced gut environment, epithelial cells contribute to immune regulation by producing transforming growth factor- β (TGF- β), which promotes the differentiation of immune cells into anti-inflammatory phenotypes.

However, intestinal dysbiosis can disrupt this balance and lead to immune system dysregulation. This affects mucosa-associated lymphoid tissue (MALT), particularly the GALT. When GALT is impaired, it loses its ability to eliminate autoreactive T lymphocytes through negative selection. As a result, autoreactive T and B cells may emerge, contributing to systemic immune dysfunction and chronic inflammation (33).

An increasing number of diseases have been associated with dysbiosis in the gut microbiota composition. While these changes have been characterized in detail in diseases such as inflammatory bowel disease and metabolic syndrome, more recent data have suggested alteration of the gut microbiota composition as a part of the pathogenesis of neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease. However, it is still unclear how the changes in the gut microbiota relate to the pathological processes observed in the brain. It has been suggested that microbiota and the CNS could communicate with each other in a bi-directional way through the so-called gut-brain axis (GBA) (34).

The study of the microbiota can be conducted through stool samples. Metagenomic analysis of stool samples enables profiling of microbial genomic content, revealing taxonomic diversity and abundance. However, it has some limitations. Firstly, metagenomic sequencing provides data on the genome as a whole, making it challenging to distinguish specific details about the bacterial state. Secondly, it also cannot distinguish the DNA of live cells from the DNA of dead cells, and DNA experiments are extremely sensitive (35). To address these gaps, multi-omics approaches like metaproteomics (assessing microbial protein expression) and metabolomics (profiling metabolic outputs) provide functional insights into microbiota activity and pathways. Metaproteomics identifies active microbial species and their roles in health and disease. Recent advances have extended its application to EVs, revealing the most active species. A study by Maredia et al. demonstrated a significant connection between the production of EVs and bacterial activation (36). These tools offer transformative potential for characterizing dysbiosis, predicting disease trajectories, and guiding therapeutic interventions (37).

1.3 The gut-brain axis (GBA)

The GBA is mediated through immune, neuronal, and endocrine pathways. Different plausible mechanisms for crosstalk between the brain and the gut microbiota have been suggested. The CNS can regulate intestinal motility as well as orchestrate local immunity via neuromediators involving the vagus nerve and the hypothalamic-pituitary-adrenal axis. While gut microbiota influence neurodevelopment through several mechanisms, including the production of microbial metabolites such as short-chain fatty acids (SCFAs) which can cross the BBB and modulate brain function. It also affects neurodevelopment by regulating the synthesis of neurotransmitters (e.g., serotonin,

dopamine, and GABA), and by modulating immune signaling through cytokines (**Figure 2**) (34,38).

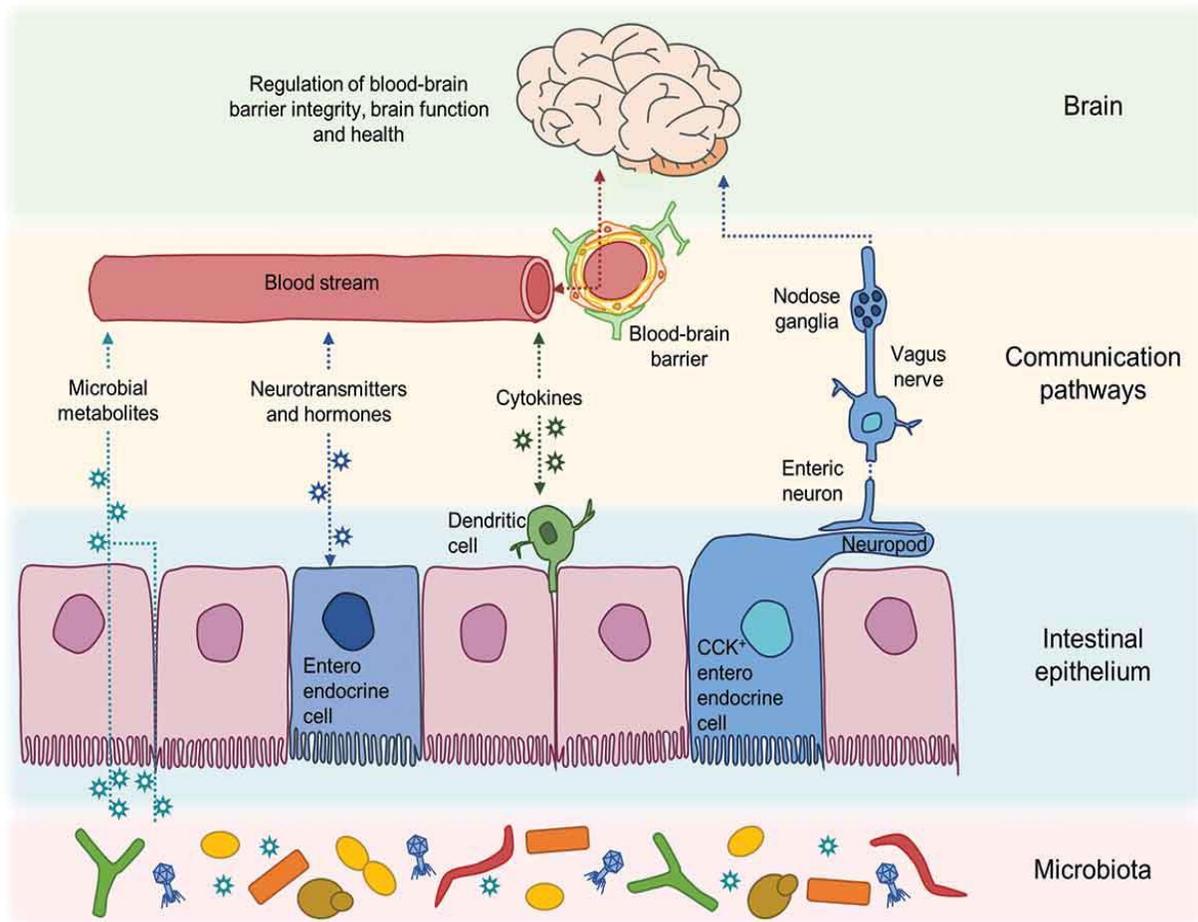


Figure 2. Pathways of communication along the gut-microbiota-brain axis. A complex interplay of epithelial, immune and neural cell signalling networks is involved in sensing and communicating changes in microbial metabolites in the gut and the brain involving both circulatory and neural routes (39).

Besides the pathways described above, researchers proposed an additional mechanism through which the gut microbiota can communicate with and influence the brain. A key mechanism involves the release of extracellular vesicles (EVs), which are produced by both microorganisms and host cells in the gut. These vesicles, thanks to their small size and specialized composition, can cross physiological barriers that are typically hard to access, such as the intestinal barrier and the BBB. Once in the bloodstream, they can travel to distant organs, including the CNS, and even reach the brain via the vagus nerve (40,41).

1.4 Extracellular vesicles (EVs)

EVs are double-layer lipid nanoparticles, and they carry a diverse cargo of proteins, metabolites, and nucleic acids (both DNA and RNA), reflecting the identity and functional state of their cells

of origin (41,42). This enables EVs to play significant roles in intercellular communication, which is a fundamental mechanism in both physiological and pathological contexts. Increasingly, EVs are being recognized as valuable biomarkers for a wide range of diseases because they are easily accessible since they are present in all biological fluids, such as blood, urine, and feces. Their surface markers and contents provide critical insight into the condition of the source cells, making them highly promising tools for early diagnosis, monitoring, and prognosis of diseases (41,43,44).

EVs are classified into three major types based on their size and biogenesis: apoptotic bodies (ApoBDs), which are relatively large (1–5 μm) and heterogeneous in structure and composition; microvesicles (MVs), ranging from 150 nm to 1 μm in diameter; and exosomes (EXOs), the smallest subtype (30–150 nm) with a sucrose density of 1.13–1.19 g/mL (**Figure 3**) (45).

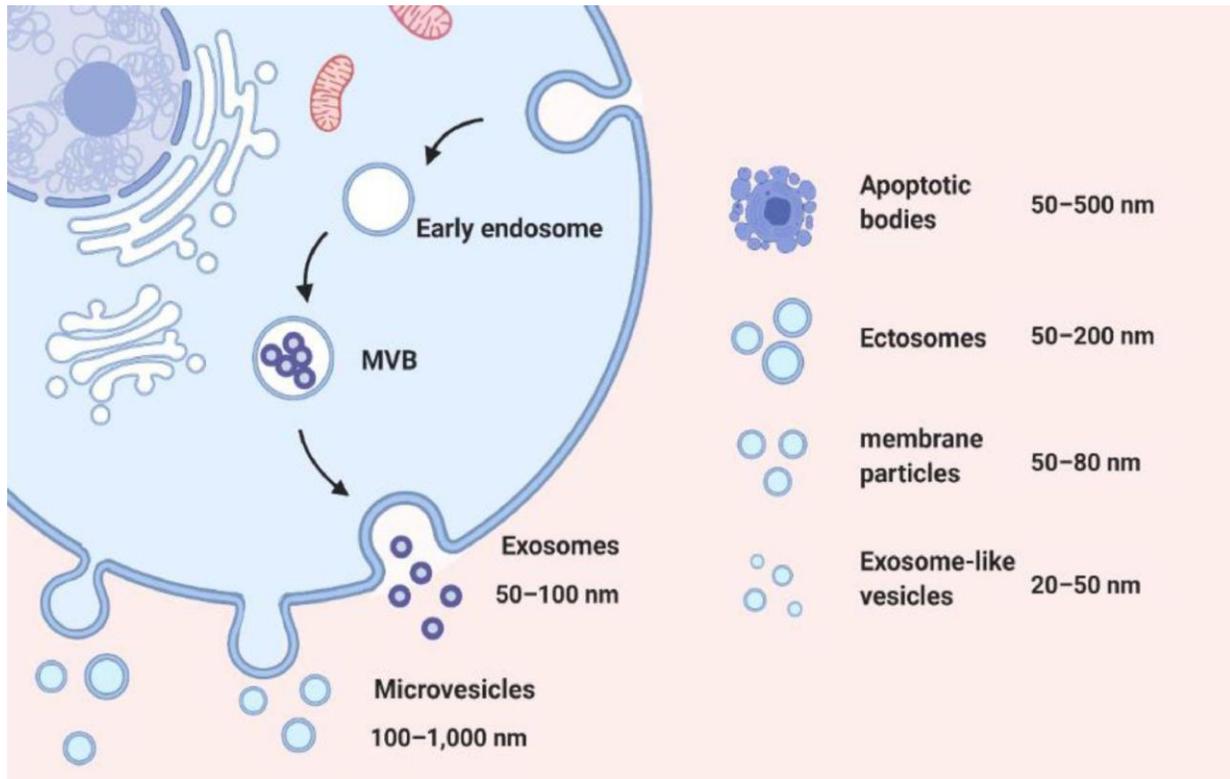


Figure 3: Biogenesis of exosomes and microvesicles. Exosomes are packaged in the late endosome and generated by the fusion of multivesicular bodies (MVBs) with plasma membrane. Microvesicles (MVs) usually refer to vesicles budding/blebbing from the plasma membrane. In addition, ectosomes, membrane particles, exosome-like vesicles, and apoptotic vesicles are also components of extracellular vesicles (EVs) with different size (46).

EXOs originate from the inward budding of endosomal membranes, forming intraluminal vesicles (ILVs) within MVBs. MVBs either fuse with lysosomes for degradation or release ILVs as EXOs via plasma membrane fusion, a process regulated by ESCRT-dependent pathways (involving ALIX, TSG101, and VPS4) or ESCRT-independent mechanisms (tetraspanins like CD9/CD81/CD63 and lipid rafts). Recent studies emphasize roles for endoplasmic reticulum contact sites, Rab GTPases, and SNARE proteins (e.g., Vamp7, YKT6) in exosome maturation and release. In contrast, MVBs form via outward plasma membrane budding, driven by

cytoskeletal rearrangements, lipid-anchored protein clustering, and regulatory proteins (e.g., ARF, ESCRT-I). Their scission involves Ca^{2+} -mediated cytoskeletal disassembly and ESCRT-III. Other particles include ApoBDs, released during apoptosis and carrying DNA fragments and histones, and smaller nonvesicular nanoparticles like exomeres (unique protein profiles) and supermeres (RNA-enriched), which exhibit distinct biodistribution patterns (47)

EVs have been shown to play versatile roles in biology and medicine. They are involved in numerous physiological and pathological processes, including reproductive biology, oncology, regenerative medicine, and inflammatory diseases (43,44,48). Beyond their diagnostic value, already widely recognized, EVs are also being explored as therapeutic agents and innovative drug delivery platforms, owing to their intrinsic biocompatibility and ability to target specific cells and tissues (48).

Importantly, EVs can be released by all cell types, encompassing both eukaryotic and prokaryotic origins. This universal capacity highlights the evolutionary conservation and fundamental importance of EV-mediated communication across domains of life. The following two sections will explore in greater detail the characteristics, functions, and biomedical relevance of EVs derived from eukaryotic (eEVs) and prokaryotic (bEVs) cells, respectively.

1.4.1 Eukaryotic Extracellular Vesicles (eEVs)

Eukaryotic Extracellular Vesicles (eEVs) are membrane-bound particles released by diverse eukaryotic cell types, including mast cells, epithelial and endothelial cells, dendritic cells, and astrocytes. These vesicles can be isolated from cell culture media and body fluids such as blood plasma, saliva, amniotic fluid, milk, and urine (49).

The lipid bilayer of EVs shields their cargo (proteins, lipids, nucleic acids) from enzymatic degradation, enabling long-distance transfer of physiological or pathological signals (50). Upon release, EVs mediate intercellular communication via diverse uptake mechanisms: direct membrane fusion, influenced by lipid composition and pH; clathrin-dependent endocytosis, requiring dynamin for vesicle scission; caveolin-mediated uptake via cholesterol-rich caveolae; macropinocytosis, an actin-dependent bulk engulfment process; phagocytosis, mediated by phosphatidylserine receptors in immune cells; and lipid raft-dependent internalization, independent of clathrin or caveolin but reliant on cholesterol (47,51).

Functionally, eEVs exerts a wide range of functions across different tissues and body fluids, ranging from local cellular interactions to systemic effects on organs.

For example, platelet-derived extracellular vesicles (pEVs) have traditionally been considered procoagulant agents due to their role in clot formation. However, recent studies using improved isolation techniques that minimize platelet contamination have revealed that pEVs can also exhibit fibrinolytic activity, suggesting a more heterogeneous and context-dependent function. Clinical observations further support this complexity; in Scott syndrome, where pEV levels are reduced, and in Stormorken syndrome, where pEVs are elevated, patients typically present with only mild

bleeding symptoms. These findings indicate that although pEVs can modulate coagulation, their overall contribution to hemostasis appears to be limited and highly dependent on the physiological context (52).

Another example, different subtypes of eEVs play roles in renal physiology and disease processes. Under physiological conditions, Exosomes from renal tubular cells help regulate fluid and electrolyte balance by transferring key proteins like glyceraldehyde 3-phosphate dehydrogenase and aquaporins between nephron segments. This exosomal communication enhances sodium and water transport. In pathological conditions, eEVs can promote fibrosis and inflammation. EVs secreted by damaged kidney cells can be transferred to other normal kidney cells, changing their phenotype and activating fibroblasts. However, some eEVs, especially mesenchymal stem cell-derived EVs (MSC-derived EVs), aid in tissue repair and have protective effects. Renal EVs are also promising non-invasive biomarkers, particularly urinary EVs, which reflect specific kidney damage and may assist in diagnosing various renal diseases (53).

EVs are also critically involved in modulating immune responses. Within the innate immune system, monocyte-derived EVs carry various microRNAs and alarmins which are chemotactic proteins that activate toll-like receptors (TLRs) and heat shock proteins and initiate immune activation. These EVs can transport inflammatory cytokines such as IL-1 β and TNF- α , sustaining immune signaling and recruiting other immune cells (42,54). Also, neutrophil-derived EVs deliver antimicrobial peptides and reactive oxygen species (ROS), helping to eradicate pathogens (54).

In the adaptive immune system, EVs may facilitate antigen presentation. Antigen-presenting cells (APCs) can release EVs displaying MHC class I and II molecules. These EVs are capable of directly stimulating CD8⁺ and CD4⁺ T cells. Furthermore, EVs from mature dendritic cells express adhesion molecules such as CD86 and ICAM-1, enhancing T cell activation either directly or by transferring stimulatory capacity to other, non-professional APCs (55).

EVs also act as antigen carriers. For instance, macrophages infected with *Mycobacterium bovis* release EVs loaded with bacterial antigens that can activate T cells (56). Similarly, tumor-derived EVs transport tumor antigens to dendritic cells, which then process and present them to cytotoxic T lymphocytes (CTLs) (57).

In addition to their pro-inflammatory roles, EVs contribute to immunosuppression and resolution of inflammation. For example, MSC-derived EVs are known for their ability to suppress immune responses and promote tissue repair, making them promising therapeutic tools (58).

Thus, the functional diversity of eEVs underscores their potential as both diagnostic biomarkers and therapeutic targets in a variety of inflammatory and immune-related diseases.

1.4.2 Bacterial Extracellular Vesicles (bEVs)

Bacterial extracellular vesicles (bEVs) are secreted by both Gram-positive and Gram-negative bacteria, encapsulating diverse molecular cargo such as proteins, lipids, lipoproteins, DNA, RNA and virulence factors. These vesicles follow distinct biogenesis pathways and cargo-sorting mechanisms, enabling roles in bacterial physiology and host-pathogen interactions (59).

Gram-negative bEVs, termed outer membrane vesicles (OMVs), derive from the outer membrane and are rich in diverse molecular cargo. Early protein analyses using electrophoresis and Western blotting identified abundant outer membrane proteins (e.g., OmpA, OmpC, OmpF), periplasmic proteins, and virulence-related enzymes. In recent years, mass spectrometry-based proteomics has expanded this understanding, identifying over 3,500 vesicular proteins (25,60).

Lipidomic studies have revealed that the vesicle membranes are primarily composed of glycerophospholipids, including phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin. Additionally, Gram-negative EVs carry both surface-bound and luminal DNA, with luminal DNA shown to be resistant to DNase digestion (25).

Gram-positive bEVs, identified more recently than OMVs, are spherical, bilayered structures (20–100 nm) with subtypes ranging from empty vesicles to those laden with toxins, proteins, or nucleic acids. Though their biogenesis remains less defined, it is hypothesized to be evolutionarily conserved.

Lipidomic studies have shown that EVs from species like *Bacillus anthracis* and *Streptococcus pneumoniae* are enriched in short-chain saturated fatty acids (C12–C16), such as myristic and palmitic acids, in contrast to their cell membranes (61,62).

These EVs also carry DNA, including 16S rRNA and toxin genes like alpha-toxin and perfringolysin O in *Clostridium perfringens*, as well as extracellular DNA that contributes to biofilm formation in *Streptococcus mutans*. This DNA enhances biofilm integrity and stability, especially during growth in biofilm conditions (63,64).

Proteomic analyses across multiple Gram-positive species, including *Staphylococcus aureus*, *Bacillus anthracis*, *Listeria monocytogenes*, and *Streptococcus pneumoniae*, have identified cytoplasmic, membrane, and extracellular proteins within EVs. Notably, *Staphylococcus aureus* EVs are particularly enriched in virulence factors, such as superantigens, hemolysins, Staphopain A, lipase, β -lactamase, and IgG-binding proteins, which play key roles in host interaction and bacterial competition.

These findings suggest that Gram-positive bacteria use EVs not only for cargo secretion but also as a mechanism for virulence delivery, with selective cargo sorting involved in their biogenesis (25).

Functionally, Gram-positive bEVs play multifaceted roles in host-pathogen interactions, virulence, and bacterial communication. In *Staphylococcus aureus*, EVs deliver α -hemolysin, exacerbating skin inflammation via keratinocyte necrosis and/or up-regulation of pro-inflammatory mediator production from keratinocytes (65). Similarly, EVs from pathogens like *Streptococcus pneumoniae* and *Clostridium perfringens* exhibit immunomodulatory properties, altering host immune signaling. *Streptococcus pneumoniae* secretes lipoprotein-enriched EVs that, when used to immunize mice, elicit a protective immune response against subsequent infection with a virulent strain by priming innate and adaptive immune cells toward bacterial antigens (25).

bEVs influence disease pathogenesis, implicated in colitis, sepsis, pulmonary fibrosis, diabetes, cardiovascular disorders, and neurological conditions. Their ability to transfer bioactive molecules across biological barriers positions them as key mediators of both physiological and pathological processes (57).

1.5 MS and EVs

EVs have emerged as pivotal mediators in the complex pathophysiology of MS. A major hallmark of MS is the autoimmune response mediated by autoreactive B and T cells targeting myelin components. This immune attack disrupts the communication between the immune system and the CNS. Within this altered context, EVs have emerged as key players, potentially contributing to both pathological and reparative processes (66).

The detrimental role of EVs in MS pathogenesis extends beyond direct myelin damage. EVs have been shown to modulate immune responses, promote cellular activation, enhance inflammation, and contribute to BBB disruption (67).

During BBB dysfunction, a pro-inflammatory microenvironment triggers endothelial cells to release cytokines and metalloproteinases via eEVs, leading to the disintegration of tight junctions. This results in increased paracellular leakage of soluble mediators, leukocyte infiltration into the CNS, sustained inflammation, and elevated levels of platelet-derived EVs in MS patients (68).

Several studies have underscored the pathological significance of EVs in MS. For instance, D'Anca et al. showed that injecting microglia-derived EVs into mice with experimental autoimmune encephalomyelitis (EAE), the animal model of MS, exacerbated brain inflammation and disease severity (66). Similarly, Blonda et al. reported that endothelial-derived EVs facilitate their passage through the BBB during MS relapses, further intensifying CNS inflammation (69).

Also, Sáenz-Cuesta et al. found that CD61⁺ PEVs, CD45⁺ leukocyte-derived EVs (LEVs), and CD14⁺ monocyte-derived EVs (MEVs) are significantly elevated in the plasma of RRMS patients compared to healthy controls, suggesting that these eEV subtypes actively contribute to the

propagation of peripheral inflammation, disruption of the BBB, and subsequent demyelinating pathology in MS (67).

In addition, EAAT2 which is a glutamate transporter enriched in plasma- and CSF-derived EVs, has been found to correlate with MS severity (70). Moreover, Mohammadinasr et al. identified differentially expressed exosomal microRNAs (miRNAs) in the CSF and serum of RRMS patients, supporting their potential as noninvasive biomarkers (71).

Emerging evidence highlights the involvement of gut-brain axis dysregulation in MS. Several studies have demonstrated that individuals with MS exhibit gut microbiota dysbiosis compared to healthy controls. This includes a depletion of beneficial bacteria such as *Bacteroides fragilis* and an enrichment of potentially pro-inflammatory taxa like *Methanobrevibacter*. These microbial imbalances are associated with immune system dysregulation, increased intestinal permeability, and heightened inflammatory responses. All of this may contribute to the onset and progression of MS. Despite these findings, the relationship between gut and MS disease activity, clinical course, or disability progression remains unclear. Understanding this connection is crucial, as it may offer novel targets for MS treatment and prevention strategies (72,73).

Given that EVs are known carriers of bacterial metabolites and nucleic acids, and can cross biological barriers including the BBB, Gut-derived EVs may play a critical role in interkingdom communication between the gut and CNS and can influence both physiological and pathological processes (74). Despite the growing recognition of EVs, no studies to date have comprehensively characterized both eEVs and bEVs derived from the gut of MS patients.

2. Objective of the thesis

MS is a chronic and debilitating inflammatory disease of CNS that affects millions of people worldwide, with a global prevalence of approximately 2.8 million cases (75). Despite its prevalence, diagnosis remains a complex process due to several factors: i) the symptoms are highly variable and may overlap with other neurological conditions; ii) the clinical and molecular heterogeneity of the disease; and iii) the lack of robust and reliable prognostic biomarkers.

Emerging research underscores the pivotal role of the gut microbiota in shaping human health and influencing disease pathogenesis. EVs derived from gut microbiota and peripheral cells mediate bidirectional communication with the central nervous system through the gut-brain axis, highlighting their involvement in neurological disorders. Stool samples, as a non-invasive and accessible biological matrix, offer a critical platform for analyzing microbial composition and their bioactive products, including EVs. This approach enables a comprehensive profiling of gut microbiota dynamics and their secreted mediators, providing insights into disease mechanisms and potential therapeutic targets.

Despite growing evidence linking gut dysbiosis, EVs, and MS, limited studies have comprehensively characterized both eukaryotic and prokaryotic EVs derived from the gut of MS patients.

The objective of this thesis is to evaluate their diagnostic potential, with a particular focus on identifying a distinct fingerprint of gut-derived EVs in MS. This could pave the way for the development of non-invasive diagnostic tools or early biomarkers for MS diagnosis.

To address this, we first isolated and characterized fecal EVs, both eEVs and bEVs, using a combination of techniques including Western blotting, scanning electron microscopy (SEM), and proteomic analysis. Bacterial activity was assessed through metaproteomic profiling of bEVs isolated from MS patients. eEVs were characterized using the MACSPlex Exosome Kit, which allows for the detection of 37 different exosomal surface epitopeantigens through fluorescent-conjugated antibodies.

3. Materials and methods

3.1 Patients' recruitment

MS patients and healthy subjects (HC) were recruited from the neurology department of the Maggiore della Carità Hospital in Novara. Fecal samples were collected at diagnosis (T0) using standardized methods. The fecal sample was aliquoted within 2-3 hours of collection and stored at -80°C for future analyses. Prior to recruitment, each participant signed an informed consent statement, and the study was approved by the ethics committee (CE 117/19).

3.2 Samples Isolation

Half grams of frozen feces (-80°C) were dissolved in 10 ml filtered phosphate-buffered saline (PBS), and two rounds of centrifugation were applied for 15 minutes at 5000 rpm (4°C) to remove solid debris. After these two centrifugations, the supernatant was filtered using a filter of 5.0, 1.2, 0.45, and $0.22\ \mu\text{m}$. EVs were then separated from small molecules by loading the flow onto a filter with a molecular weight cut-off of 100 kDa (Amicon Ultra filter unit) and concentrating it to $250\ \mu\text{l}$ through centrifugation at 4000 rpm for 45 minutes (4°C). Two hundred fifty μl of filtered PBS were used to wash the filter to have the highest recovery of EVs (with a final volume of $500\ \mu\text{l}$). Subsequently, free proteins were removed through SEC. Ten ml of CL-2B Sepharose columns were prepared by adding 2,5 ml of PBS to 7,5 ml of Sepharose Gel CL-2B, the sample was loaded, and different fractions were collected. Then, a step of ultracentrifugation ($100'000\text{g}$ for 1h at 4°C) was performed to pool the fractions. After ultracentrifugation, pellets were resuspended in $200\ \mu\text{l}$ of PBS and stored at -80°C for further analyses (**Figure 4**).

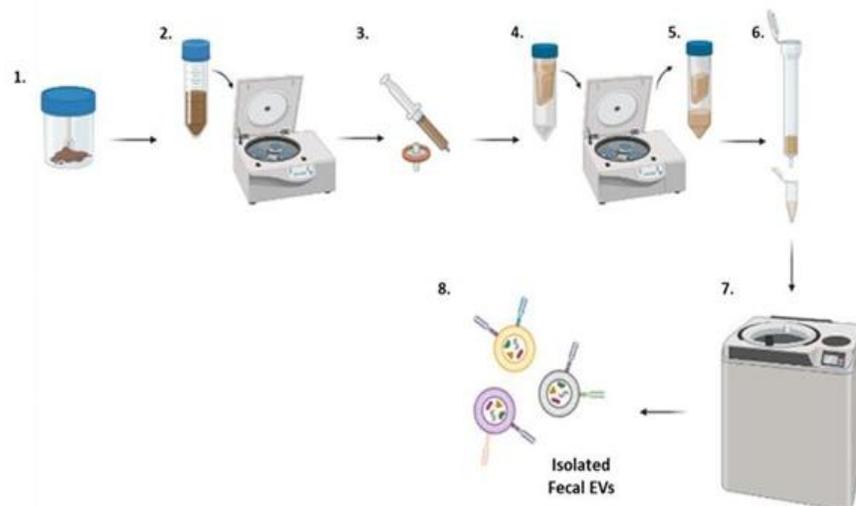


Figure 4. Experimental workflow for gut-derived EVs isolation. Half grams of feces were dissolved in 10 ml of filtered PBS (1), and two steps of centrifugation were applied (2). Then, supernatant was filtered through four filters of different sizes: 5.0, 1.2, 0.45, and 0.2 μm (3). After the filtration steps, the sample was loaded into an Amicon 94 Ultra filter of 100kDa (4) to concentrate it (5). To purify the sample, SEC was performed, and 24 fractions were collected (6). Subsequently, a step of ultracentrifugation was applied to pool the fraction of the gut-derived EVs (7,8).

3.3 Nanoparticle Tracking Analysis (NTA)

Nanoparticle tracking analysis (NTA) is a commonly used technique for the rapid visualization and quantification of EVs in a sample. Moreover, NTA enables the dynamic detection of EVs in a liquid solution, providing an accurate and real-time count of the EV concentration within the sample. The collected fractions of gut-derived EVs isolated by SEC were analyzed with NTA using NS300. The samples were diluted 1:80 in filtered PBS 1X. For each sample, three videos of 60 seconds were recorded and analyzed with camera level 12/13 and detection threshold 4. Recorded videos were analyzed with NTA 2.3 Software (Malvern Panalytical).

3.4 Western blot analysis

Proteins present in gut-derived EVs were quantified using the Qubit Assay (Invitrogen). A total of 15 μg of protein was separated on a 12% SDS-PAGE gel, and the proteins were transferred to a nitrocellulose membrane. The membrane was blocked for 1 hour with BSA 3% and incubated overnight at 4°C with the following antibodies: anti-CD63 (Invitrogen, diluted 1:500), anti-CD9 (Invitrogen, diluted 1:500) anti-CD81 (Invitrogen, diluted 1:500) and calnexin (Invitrogen, diluted 1:5000), anti-LPS (Abcam, diluted 1:1000) in BSA 3%. After incubation with primary antibodies, the membrane was washed with 0.1% Tween-20 in Tris-buffered saline (TBST) three times for 10 min. Incubation with a secondary antibody was performed at room temperature (RT) with goat anti-mouse IgG secondary antibody HRP conjugated (Invitrogen) for 1 hour. The membrane was washed three times with TBST for 10 min, and it was then exposed to an ECL chemiluminescence kit (Western Nova 2.0, Cyanagen) in a 1:1 ratio and incubated for 2 minutes. The images were acquired using the Chemidoc (BIO-RAD Chemidoc imaging system).

3.5 Scanning electron microscopy (SEM)

SEM allows to have ulterior proof of the isolation of EVs in a sample. A small volume (10 μl) of EVs sample was placed on a glass coverslip in a 24-well plate. After the sample dried, the plate was incubated for 20 minutes with 10 μl of 2.5% glutaraldehyde solution diluted in water (Electron Microscopy Sciences, Hatfield, PA, USA). Subsequently, following the evaporation of the glutaraldehyde, an ethanol dehydration scale was performed: the sample was incubated with drops of increasing ethanol concentrations (70% and 90%) for 30 minutes each, followed by a final incubation with 100% ethanol for 1 hour. Then, we added a small volume of hexamethyldisilazane (Electron Microscopy Sciences, Hatfield, PA, USA) and incubated it for 20 minutes until it was completely dry. Lastly, we coated a layer of gold on the glass coverslip with a sputter coater

machine (DII-29030SCTR Smart Coater, JEOL, Italy) and observed by using the bench SEM (JSM-IT500, JEOL, Italy).

3.6 Metaproteomic analysis

Metaproteomic analysis is a unique method that allows us to identify specific bacterial phyla and species responsible for producing EVs. Gut-derived EVs were lysed with cold Lysis Buffer (50 mM Tris HCl, pH 7.2, 0.05% SDS) and by sonication. The sample was then put on a rotator for 15 min at 4°C, and then cold acetone was added to precipitate proteins. The sample was centrifuged at 14,000 x g, for 10 min, at 4°C, and the pellet was resuspended in Urea Buffer (8 M Urea, 100 mM Tris HCl, pH 8) and ammonium bicarbonate 100 mM. The extracted proteins were quantified using the Bradford protein assay according to manufacturers' instructions (Bio-Rad Laboratories), and they were subjected to enzymatic digestion. Briefly, for the reduction of proteins, 25 µl of ammonium bicarbonate, 15 µl of trifluoroethanol (Sigma-Aldrich Inc., US), and 2.5 µl of dithiothreitol 200 mM (Sigma-Aldrich Inc., US) were added to the samples, which were incubated at 60°C for 45 min. Alkylation was performed by adding 10 µl of iodoacetamide IAM 200 mM, and the sample was incubated at RT, in the dark, for 1 h. Then, 2.5 µl of DTT was added to the sample and incubated for another hour. Finally, after the addition of 100 µl of ammonium bicarbonate, 200 µl of water, and after reaching a pH of 7.8, 10 µl of trypsin 0.2 µg/µl (Sigma-Aldrich Inc., US) were added to the sample and incubated at 37°C overnight. Digestion was then stopped with 2 µl of formic acid. The mixture of peptides was then desalted through the Discovery DCS-18 solid phase extraction. The sample was evaporated through the SpeedVac (ScanVac, LaboGene, Denmark) and reconstituted with 20 µl of water with 0.1% formic acid for the LC-MS analysis.

3.7 MACSPlex exosomes kit

MACSPlex exosomes kit is a fast and reliable technique that allows the detection of a wide range of different immune markers present on eEVs. The MACSPlex exosome kit (Miltenyi Biotec) allows the detection of 37 exosomal surface epitopes plus two isotype controls (**Figure 5**). This kit comprises a cocktail of different fluorescently labelled bead populations, each coated with a specific antibody binding the respective surface epitope. Sixty µl of MACSPlex Buffer were added to 60 µl of samples. Then, 15 µl of MACSPlex Exosome Capture Beads were added to each tube, and the tubes were incubated overnight at RT, protected from light, using a tube rotator on permanent run (12 rpm). Subsequently, beads were washed with 500 µl of MACSPlex buffer at 3,000 x g for 5 minutes, and EVs bound to capture beads were stained for 1 hour at RT with 5 µl of CD9-, CD63- and CD81-APC conjugated antibodies (MACSPlex Exosome Detection Reagents). Finally, the sample was washed with MACSPlex buffer for 15 minutes on the rotator, and then it was acquired using FACSymphony A5 (Becton and Dickinson, San Jose, CA, USA).

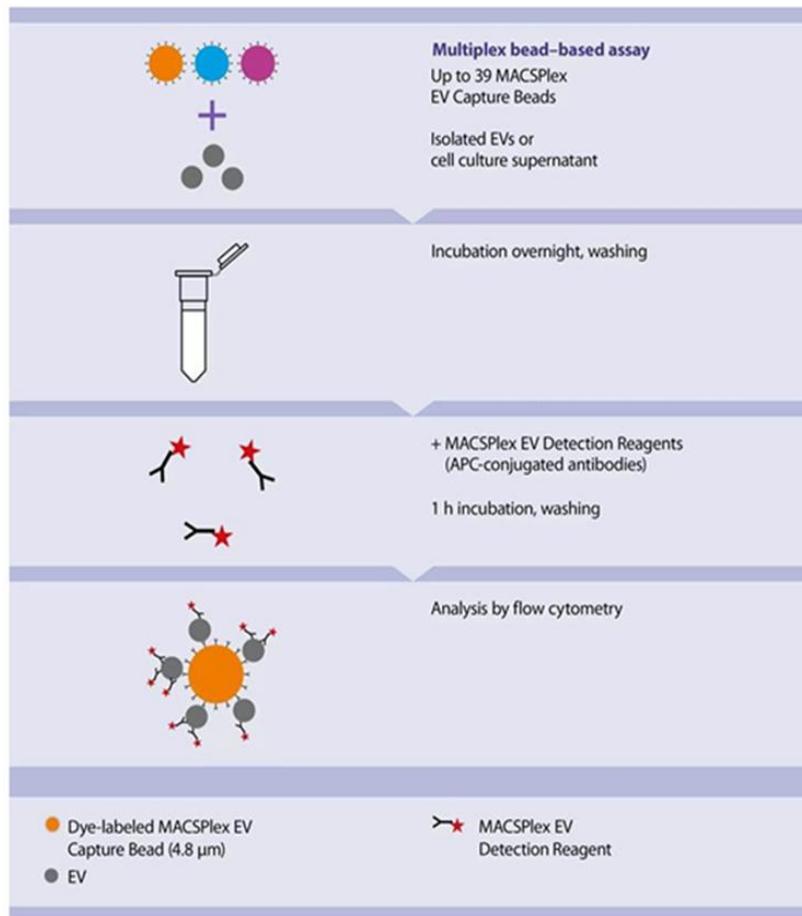


Figure 5. Principle of the MACSplex Exosome Kit. The MACSplex Capture Beads kit contains a cocktail of various fluorescently labelled bead populations, each coated with a specific antibody. Bound EVs on the beads are stained by a detection reagent and will generate a signal that is detectable by flow cytometry.

3.8 Statistical analysis

The differences between HC and MS for eEVs and bEVs populations were analyzed using Mann-Whitney T test. Data are expressed as mean \pm standard error. P value below 0,05 was considered statistically significant. The statistical analyses were performed with GraphPad InStat software (Prism 8 version 8.4.3) (GraphPad Software, San Diego, CA, USA).

4. Results

4.1 Validation of the isolation method through NTA analysis

The first step was to optimize and validate our method for the isolation of gut-derived EVs from stool samples. Stool samples were processed through different centrifugation and filtration steps, resulting in a mixture of EVs and free proteins. To purify the EVs, we employed SEC, and with this procedure, we collected 24 different fractions. NTA was performed to identify EVs-enriched fractions. As shown in **Figure 6 A**, the concentration of EVs (particles/ml) starts to increase from the 4th fraction, with the highest peak in the 5th (5.48×10^6 particles/ml), and then notably declines after the 8th fraction. This result was in line with Kameli and colleagues, showing that the central fractions (up to 12th) of SEC are the most enriched ones (76). Moreover, the EVs' size was also evaluated, and the results show that all fractions range from 100 nm to 200 nm (**Figure 6 B**). While SEC provides highly purified samples, it also results in a bigger volume, and too diluted EVs are difficult to use in other applications. For this reason, we pooled the fractions 1-3, 4-12, and 13-24, and we analysed them by NTA (**Figure 6 C-D**). At the end, we decided to use the 4-12 pool for further analysis, because it was the one with the highest EVs concentration.

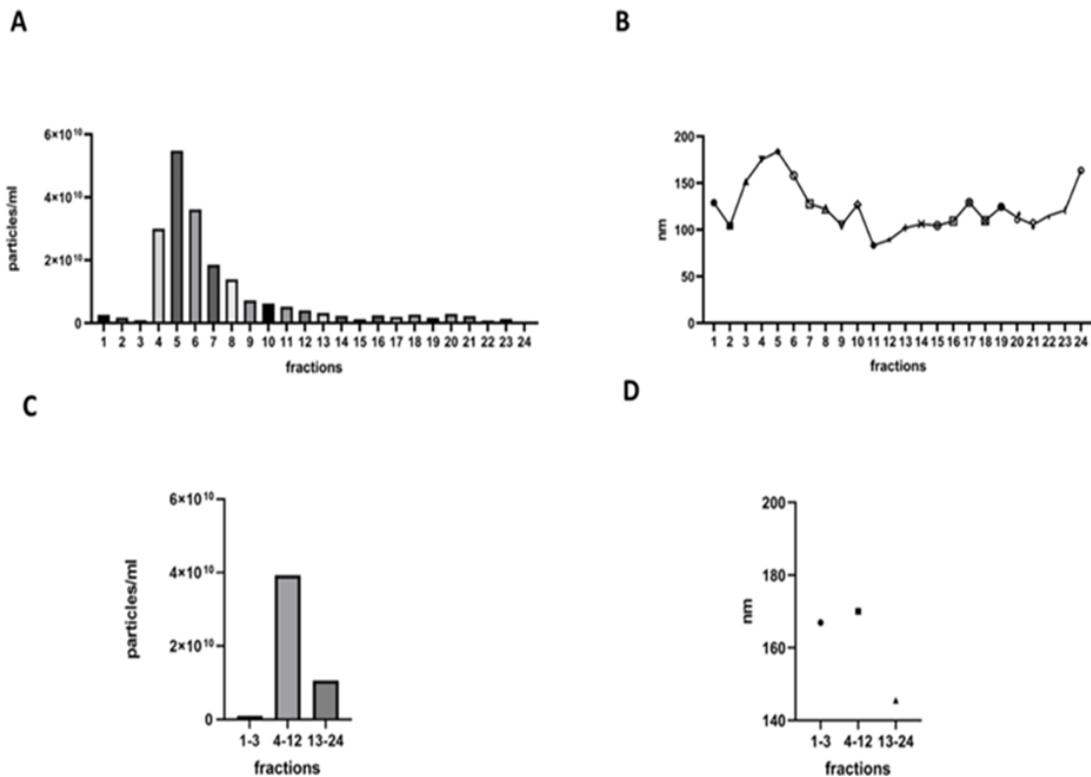


Figure 6. Validation of isolated gut-derived EVs through NTA. A) Concentration of gut-derived EVs in the 24 fractions analyzed by NTA B) Size of gut-derived EVs in the 24 fractions analyzed by NTA C) Concentration of gut-derived EVs in the combined samples. D) dimension of gut-derived EVs in the combined samples.

4.2 Characterization of gut-derived EVs by western blot and SEM

After NTA analysis, the chosen 4-12 pool was further characterised through western blot and SEM analysis. Stool samples contain both eukaryotic and prokaryotic cells, as well as their respective EVs. To discriminate them, we used specific markers: tetraspanins for eEVs and LPS for bEVs. Therefore, western blot analysis was conducted to detect tetraspanins (CD9, CD63, and CD81) and LPS. PBMCs served as positive control for tetraspanins, while EVs derived from gram-negative bacteria (*E. Coli*) were used as positive control for LPS. Tetraspanins CD63 and CD9 were detected in the 4-12 pool, with CD63 showing a smear from 30 kDa to 60 kDa and CD9 exhibiting a signal around 25 kDa (**Figure 7 A-B**). Surprisingly, tetraspanin CD81 was not detected in our sample (**Figure 7 C**). As expected, LPS showed a signal around 10 kDa (**Figure 7 D**). Calnexin is a well-characterized marker of the endoplasmic reticulum (ER), and it is typically not present in EVs, which are derived from the plasma membrane and endosomal system. EVs were negative for the expression of calnexin, which instead was detected in PBMCs at around 90 kDa. (**Figure 7 E**). Finally, we characterized gut-derived EVs also with SEM, showing spherical morphologies, with an average diameter of approximately 130 nm. These results suggest that both eEVs and bEVs were successfully isolated from stool samples with our approach.

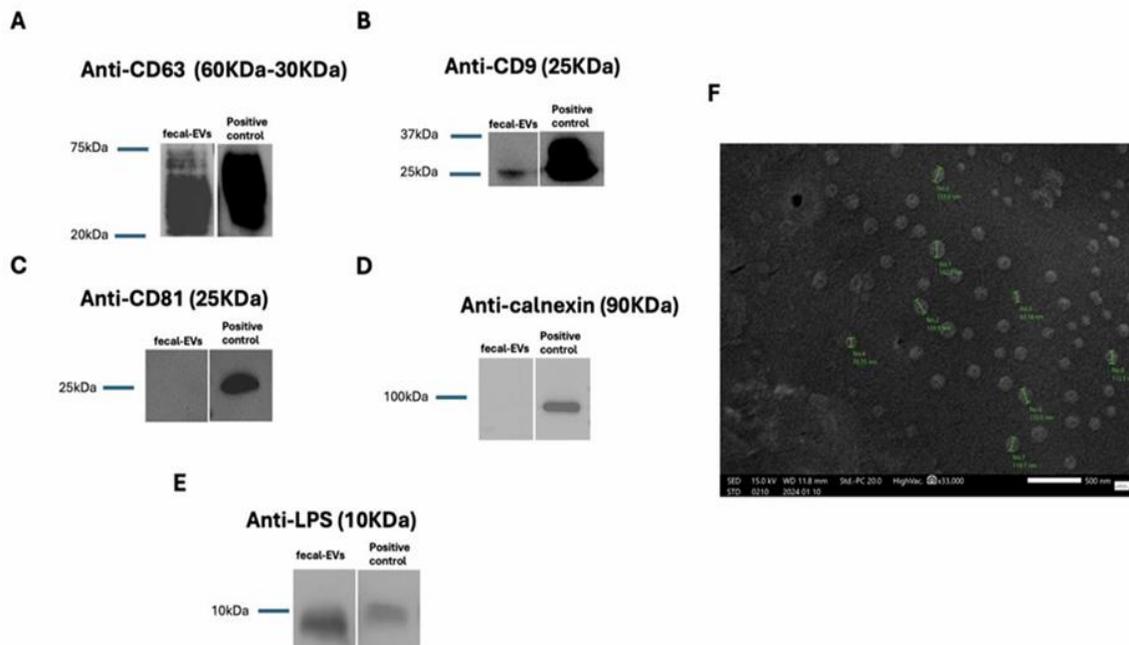


Figure 7. Characterization of EVs by Western blot and SEM. A) Representative western blot of CD63 in gut-derived EVs sample and positive control B) Representative western blot of CD9 in gut-derived EVs sample and positive control C) Representative western blot of CD81 in gut-derived EVs sample and positive control D) Representative western blot of calnexin in gut-derived

EVs sample and positive control E) Representative western blot of LPS in gut-derived EVs sample and positive control F) Representative images of EVs from stool samples obtained by scanning electron microscopy (SEM).

4.3 Metaproteomic analysis

We performed metaproteomic analysis on the bEVS to have a specific pattern of bacterial activation associated with MS. At phyla level, the meta-proteins found in bEVs samples of HC and MS patients were assigned mainly to five phyla. HC showed a predominance of *Bacteroidetes* (51.59%), *Firmicutes* (47.67%), *Proteobacteria* (0.48%), *Chlamydiae* (0.07%), and *Actinomycetota* (0.18%) (**Figure 8 A**). Instead, for MS patients, *Bacteroidetes* (64.23%), *Firmicutes* (33.64%), *Proteobacteria* (1.14%), *Chlamydiae* (0.83%), and *Actinomycetota* (0.13%) were the top five (**Figure 8 B**). The bacteria most active at the phyla level were similar between HC and MS, with *Bacteroidetes* and *Firmicutes* maintaining equal proportions. At the species level, we found predominantly these following five species: *Roseburia faecis* (19.46%), *Phocaeicola vulgatus* (17.83%), *Faecalibacterium prausnitzii* (8.26%), *Bacteroides uniformis* (6.48%), *Roseburia intestinalis* (5.50%) (**Figure 9 A**). On the other hand, in MS patients, the most active were *Roseburia faecis* (40.35%), *Prevotella copri* (14.52%), *Phocaeicola vulgatus* (10.63%), *Phocaeicola coprophilus* (4.93%), *Faecalibacterium prausnitzii* (3.13%) (**Figure 9 B**).

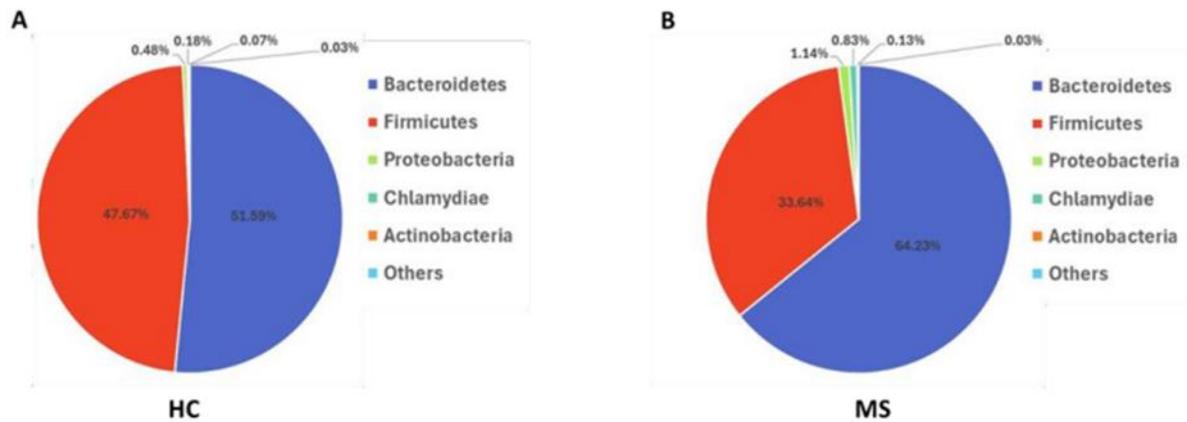


Figure 8. Taxonomic distribution at the Phyla level in bacterial activation. A) The pie chart shows the percentages of the five most active Phyla in HC, B) The Pie chart shows the percentages of the five most active Phyla in MS T0.

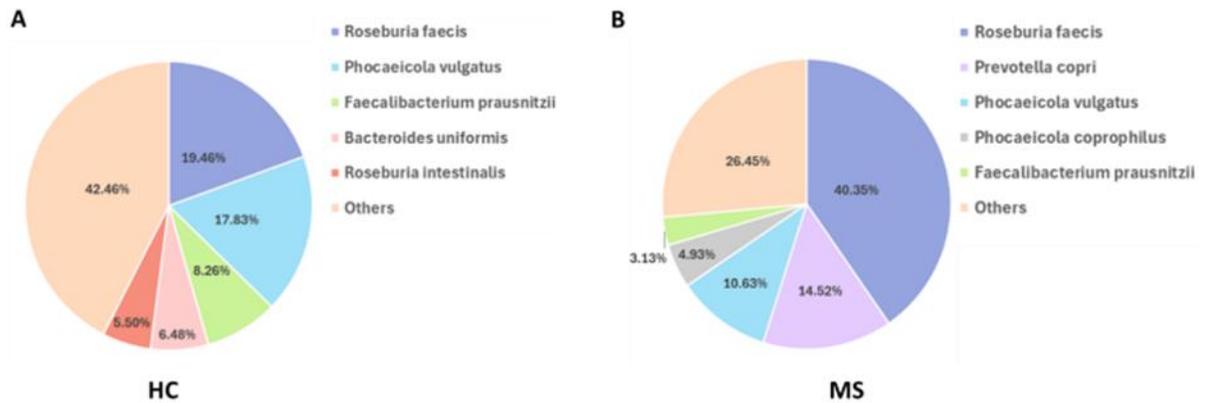


Figure 9. Taxonomic distribution at the species level in bacterial activation. A) The pie chart shows the percentages of the five most active species in HC, B) The Pie chart shows the percentages of the five most active species in MS T0.

4.3.1 Differences in phyla activation between HC and MS

The pie charts (**Figure 8 A, B**) showed different percentages of phyla between HC and MS. Statistical analysis revealed a significant difference in *Firmicutes*. Indeed, our results showed that bEVs produced by *Firmicutes* were elevated in HC with respect to MS patients (**Figure 10 A**).

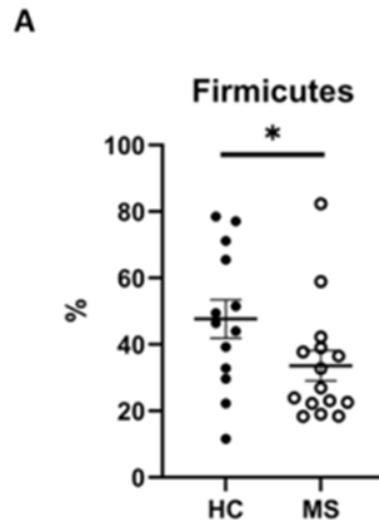


Figure 10. Differences in the Phyla activation between HC and MS. A) Dot plot depicts the percentage in the activation of *Firmicutes* between HC and MS. * $p < 0.05$.

4.3.2 Differences in species activation between HC and MS

To explore the species thoroughly and find a specific MS bEVs fingerprint, we performed a comparison among the four most active species between HC and MS. Our findings highlighted a trend in the activation of *Roseburia faecis*, which resulted to be more active in MS patients compared to the HC (Figure 11 A). Conversely, *Bacteroides uniformis* showed higher activity in HC (Figure 11 B).

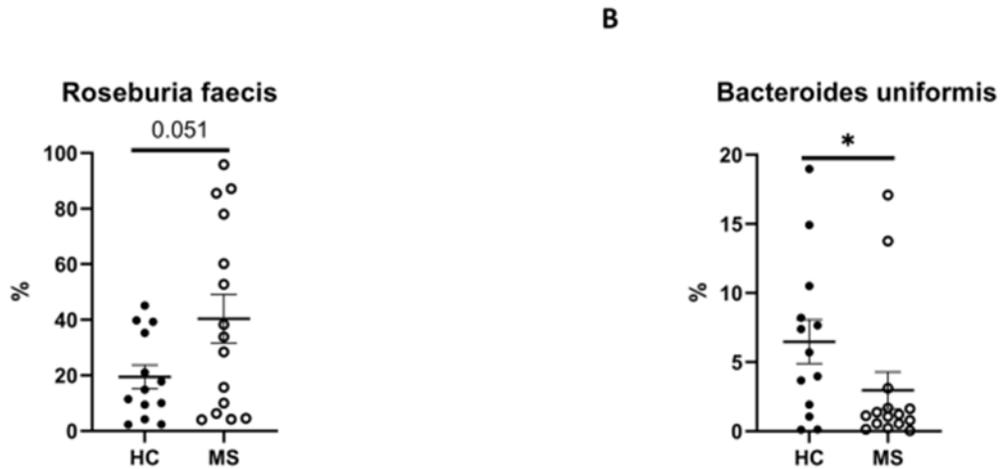


Figure 11. Differences in the species activation between HC and MS. A) Dot plot depicts the percentage in the activation of *Roseburia faecis* between HC and MS. B) Dot plot depicts the percentage in the activation of *Bacteroides uniformis* between HC and MS. * $p < 0.05$.

4.4 Differences in eEVs subtypes between HC and MS patients

Among all the 37 different sub-types of EVs that can be detected using the MACSPLEX kit, four populations were differentially modulated in MS patients compared to HC. Precisely, MS patients showed a statistically significant reduction of ROR1, CD24, and CD40-expressing EVs and a trend in CD44-expressing EVs. (Figure 12 A-D).

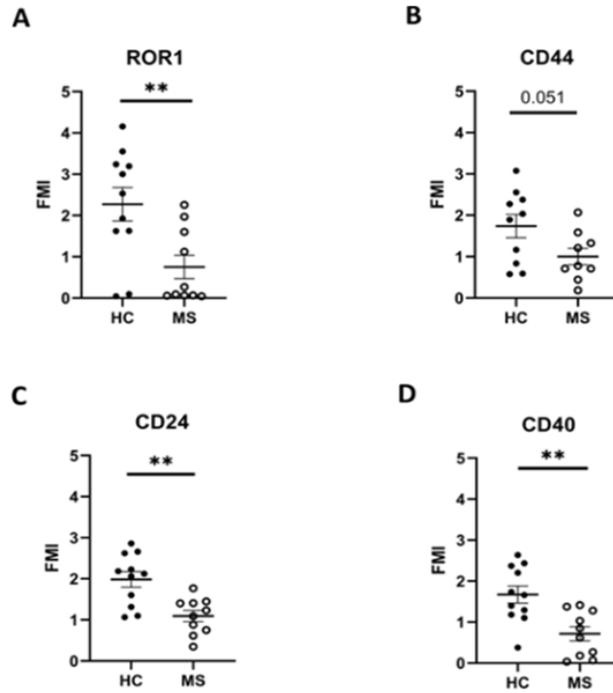


Figure 12. eEVs differences between HC and MS T0 patients. Graphs depict the normalized MFI of the different subtypes of eEVs that are modulated in MS patients expressing A) ROR1, B) CD44, C) CD24, and D) CD40. Mann-Whitney *t* test was used. * $p < 0.05$, ** $p < 0.01$

5. Discussion

This study is the first to comprehensively profile gut-derived extracellular vesicles in multiple sclerosis, directly comparing both eEVs. and bEVs between MS patients and HC.

We successfully established and validated a reliable method for isolating gut-derived EVs from stool samples using a combination of ultracentrifugation and SEC, followed by NTA and western blotting to improve purity and yield.

While ultracentrifugation alone is effective for EV concentration, it may also isolate protein contaminants (77). SEC offers a gentler purification step that separates EVs from free proteins and other impurities, improving sample purity (78). Given the complexity of stool samples highly enriched in free proteins, this combined SEC-UC method was particularly suitable for obtaining high-quality EVs (SEC) and sufficient concentration (UC) for downstream metaproteomic and phenotypic analyses.

Our approach yielded EVs that ranged from 100 to 200 nm in size, were consistent with size range reported for EVs in various biofluids (79), and were free from contamination by cellular debris, as confirmed by the absence of calnexin.

Interestingly, in our study, WB analysis confirmed the presence of CD9 and CD63, indicating successful isolation of EVs, despite the absence of a detectable CD81 band. These findings align with those of Barranco et al. (2019), who demonstrated that different EV subtypes exhibit variable expression of tetraspanins. Specifically, their quantitative analysis revealed that CD63 and CD9 are more abundantly expressed in MVs, while CD81 is more prominent in exosomes (80). This suggests that the detectability of specific tetraspanins may vary depending on the predominant EV subtype in a given sample. Based on this, we investigated whether our EVs fell within the size range of MVs (100–1000 nm) or exosomes (≤ 100 nm). SEM analysis revealed a mean EV diameter of approximately 130 nm, supporting the notion that our sample may contain a larger proportion of MVs, which could explain the lack of CD81 expression.

Our metaproteomic analysis of bEVs revealed distinct microbial activity patterns between HC and MS patients. At the phylum level, both groups were dominated by *Bacteroidetes* and *Firmicutes*, consistent with the core human gut microbiota (81). However, MS patients exhibited a significant reduction in *Firmicutes*-derived EVs, corroborating previous microbiome studies that reported decreased *Firmicutes* abundance in MS patients, which is linked to reduced production of anti-inflammatory SCFAs such as butyrate (82,83), and aligning with previous studies, which described less *Firmicutes* in the gut microbiota of MS patients (84).

At the species level, *Roseburia faecis* was markedly more active in MS patients. *Roseburia* species are known butyrate producers and generally considered beneficial for gut health and immune modulation (85).

Despite its positive effects, the excessive activity of *Roseburia faecis* at the disadvantage of other species could be detrimental to other hosts' microbiota members. Indeed, higher levels of *Roseburia faecis* may result in decreased microbial diversity, highlighting a lower richness in MS patients.

However, *Roseburia faecis* increased activity in MS may reflect a compensatory response to inflammation or dysbiosis if other microbes are depleted rather than a purely protective role. Also, its abnormal activation may be due to stress; indeed, it is known that bacteria often release more EVs under stress. In Gram-positive gut symbionts, abiotic stresses (temperature shifts, osmotic changes) and rapid growth phases enhance EV formation (86). In MS, gut bacteria face inflammatory stress (cytokines, reactive oxygen species) and altered milieu (e.g. shifts in pH, bile acids or medication), any of which could trigger *Roseburia faecis* to shed more EVs.

Conversely, *Bacteroides uniformis* showed reduced activity in MS patients. A specific human-derived strain, *Bacteroides uniformis* CECT 7771, is well known for its anti-inflammatory properties. In a landmark study, researchers administered daily doses of this strain to obese mice on a high-fat diet and observed a profound shift in their inflammatory profile. The high-fat diet had elevated levels of pro-inflammatory cytokines (IL-1 α , TNF- α , and IFN γ) in both gut and systemic tissues. However, treatment with *Bacteroides uniformis* CECT 7771 reversed these increases and restored anti-inflammatory cytokines, including IL-10 and IL-33, thereby re-establishing a more balanced, homeostatic immune environment. Moreover, the same probiotic intervention exerted profound effects on the cellular arm of immunity. The abundance of regulatory T cells (Tregs) increased significantly in treated animals. At the same time, the expression of Toll-like receptor 5 (TLR5) in Peyer's patches and adipose tissue normalized, dampening the overactive innate immune signaling characteristic of diet-induced obesity (87). Taken together, these findings underscore how loss of *Bacteroides uniformis* in MS patients could exacerbate both gut-driven and central inflammation in MS.

Our characterization of host-derived EVs (eEVs) revealed significant alterations in MS patients, notably a reduction in EV populations expressing ROR1, CD24, CD44 and CD40.

ROR1 is involved in Wnt signaling pathways, and its activation leads to stimulation of the NF- κ B pathway, which in turn drives the transcription of genes promoting T cell survival and proliferation. Notably, the NF- κ B pathway plays paradoxical roles in autoimmunity and inflammation. While it is essential for initiating immune responses, it is also critical for establishing immune tolerance. Impaired NF- κ B signaling is linked to defects in central tolerance, and it is also required for maintaining Treg function, highlighting its role in peripheral tolerance

(88,89). Given these dual functions, dysregulated ROR1/NF- κ B signaling may contribute to the breakdown of immune tolerance observed in MS, suggesting a potential role in MS pathogenesis.

CD24 is a glycoprotein expressed on dendritic cells as well as on T and B lymphocytes. It is essential for T cell proliferation and for the regulation of APC function. It has been shown that CD24 can inhibit Treg; indeed, Tregs derived from CD24 knockout mice exhibit enhanced suppressive activity compared to their wild-type counterparts (90).

Unfortunately, these findings may be in contrast with our results. In fact, CD24 downregulation could be interpreted as a consequence of increased Treg activity, which is typically reduced in patients with MS.

One possible explanation is that CD24 downregulation on EVs does not reflect a lower density of the molecule on the EV surface per se, but rather a reduced number of Tregs secreting these vesicles into the intestinal lumen. This hypothesis is supported by evidence showing the presence of autoreactive T lymphocytes (91) and Tregs (92) in the gut-associated lymphoid tissue (GALT) of mice with experimental EAE, where a decreased number of Tregs has been shown to impair disease remission. CD40 is a membrane-bound costimulatory protein belonging to the tumor necrosis factor receptor (TNFR) family. It is constitutively expressed on B cells and DCs, and upon activation, its expression extends to a broad range of hematopoietic cells, including T cells, monocytes, and macrophages. DCs play a central role in maintaining immune tolerance, which is essential to prevent immune responses against self-antigens. When this mechanism fails, autoimmune diseases such as MS may arise. CD40 is also expressed by regulatory DC, which are involved in tolerogenic activity and promote the production of IL-10, an anti-inflammatory cytokine (93). Conversely, there is evidence that CD40 can also promote the proliferation of Th1 lymphocytes and B cells (94).

EVs expressing CD40 may exert dual and opposing immunomodulatory effects upon interaction with CD40L-expressing cells: (i) they may induce signaling in CD40L⁺ T cells, thereby promoting a pro-inflammatory response, or (ii) they may inhibit T cell activation by interfering with the CD40–CD40L interaction between DCs and T cells. The latter mechanism may explain our findings, which revealed a reduction in CD40-expressing EVs in MS patients. This decrease could reflect a loss of EV-mediated immunosuppressive activity, ultimately favoring a pro-inflammatory milieu. Given the conflicting data in the literature regarding the role of CD40, and the absence of studies on CD40-derived EVs in fecal samples, further experiments are needed to clarify the specific role of these EVs in MS.

CD44 is a transmembrane glycoprotein that mediates cellular responses to the extracellular microenvironment. It plays a key role in lymphocyte adhesion and migration across the blood–brain barrier, processes that are critical for immune cell infiltration into the CNS (95). Therefore,

altered expression of CD44 on EVs may influence immune cell trafficking and contribute to MS pathogenesis.

Taken all together, MS is a heterogeneous disease, with each patient exhibiting a unique constellation of immune and gut–brain signaling disturbances. In our small cohort, this variability likely amplified the differences we observed compared to earlier reports. Moreover, most prior studies examined the whole cells or soluble factors rather than focusing on EVs.

Functional assays, using BBB models, would clarify whether these vesicles actively modulate inflammation or barrier function. Integrating metaproteomic EV profiles with metabolomic data may uncover key bioactive cargos that drive immune changes. Finally, expanding cohort sizes and including multiple MS subtypes will strengthen the validity of EVs as biomarkers or therapeutic targets.

In conclusion, our findings emphasize the complex interplay between gut microbes, their EVs, eEVs and the host immune system in MS. By illuminating both bacterial-derived pro-inflammatory EVs and host-derived regulatory vesicles, we lay the groundwork for novel microbiota-targeted therapies and EV-based diagnostics for MS.

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