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**From Blood to Gut: Exploring the Diagnostic and Prognostic
Potential of Extracellular Vesicles in Chronic Inflammatory and
Neuropsychiatric Diseases**

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*“L’unico modo giusto è il tuo.
Non esiste altra vittoria che essere sé
stessi.”*

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Summary

Extracellular vesicles (EVs) are double membrane lipid nanoparticles, released by all cell types in different biological samples. Their unique characteristic is represented by their cargo which contains several biomolecules such as metabolites, RNA, DNA and proteins, which reflect the physiological or pathological state of the cell of origin.

Recently, EVs gained interest as diagnostic, prognostic and predictive biomarkers for several human diseases.

This Ph.D. thesis aimed to explore the potential of EVs in reshaping diagnostic and prognostic processes in clinical settings by identifying disease-specific signatures. To achieve this, I evaluated different diseases, underscoring the versatility of EVs in reflecting complex biological mechanisms.

The first chapter of this thesis aims to assess the prognostic potential of the blood-derived EVs, particularly, platelets-derived EVs (PLT-EVs), in coronavirus disease 2019 (COVID-19) patients. As detailed in **paper 1** we employed a method validated in our lab for rapid EVs identification, starting from liquid biopsy (i.e blood or plasma) to evaluate the prognostic potential of PLT-EVs in COVID-19 patients. Notably, through this approach we found that COVID-19 patients with worse prognosis after being hospitalized for one week in intensive care unit (ICU), exhibited higher levels of PLT-EVs, highlighting their prognostic role.

After the third wave of SARS-CoV-2, our focus shifted to all those COVID-19 patients included in our cohort, who after months from the recovery and without any sign of infection, continued to experience a wide range of persistent symptoms. This new condition was named post-acute sequelae of SARS-CoV-2 infection (PASC) syndrome. Thus, in the second chapter, using advanced techniques such as activation induced marker (AIM) assay and dextramers, we identified in peripheral mononuclear blood (PBMC) of PASC patients an hyperactivation of the CD4⁺ compartment and high levels of SARS-CoV-2 specific CD8⁺ T cells compared to healthy controls (**paper 2** and **paper 3**).

In the third and last chapter, we investigated both blood-derived EVs and gut-derived EVs as mediators of communication within the gut-brain axis, particularly in the context of major depressive disorder (MDD) and multiple sclerosis (MS), by setting up and optimizing a fast and reliable method for the isolation of the gut-derived EVs. Our findings showed the existence of a specific fingerprint in both diseases.

Overall, this thesis advances the field by positioning EVs as innovative tools for disease diagnostics and prognosis, demonstrating their versatility across multiple human disorders. The new methodologies developed to detect and isolate EVs along the findings presented in this thesis provide a foundation for future translational research, with implications for personalized medicine and biomarker-driven disease management.

Sommario

Le vescicole extracellulari (VE) sono nanoparticelle lipidiche a doppio strato, rilasciate, in diversi campioni biologici, da tutte le tipologie cellulari. La loro caratteristica risiede nel contenuto di ciò che veicolano, costituito da una varietà di biomolecole, come metaboliti, RNA, DNA e proteine, che riflettono sia lo stato fisiologico che patologico della cellula di origine.

Recentemente, le VE hanno suscitato un crescente interesse come biomarcatori diagnostici, prognostici e predittivi in numerose patologie umane.

Questa tesi di dottorato ha avuto l'obiettivo di esplorare il potenziale delle VE nel trasformare i processi diagnostici e prognostici in ambito clinico, attraverso l'identificazione di firme molecolari specifiche per malattie-caso. Per raggiungere questo obiettivo, nei vari capitoli della tesi sono state valutate diverse malattie, mettendo in evidenza la versatilità delle VE nel riflettere meccanismi biologici complessi.

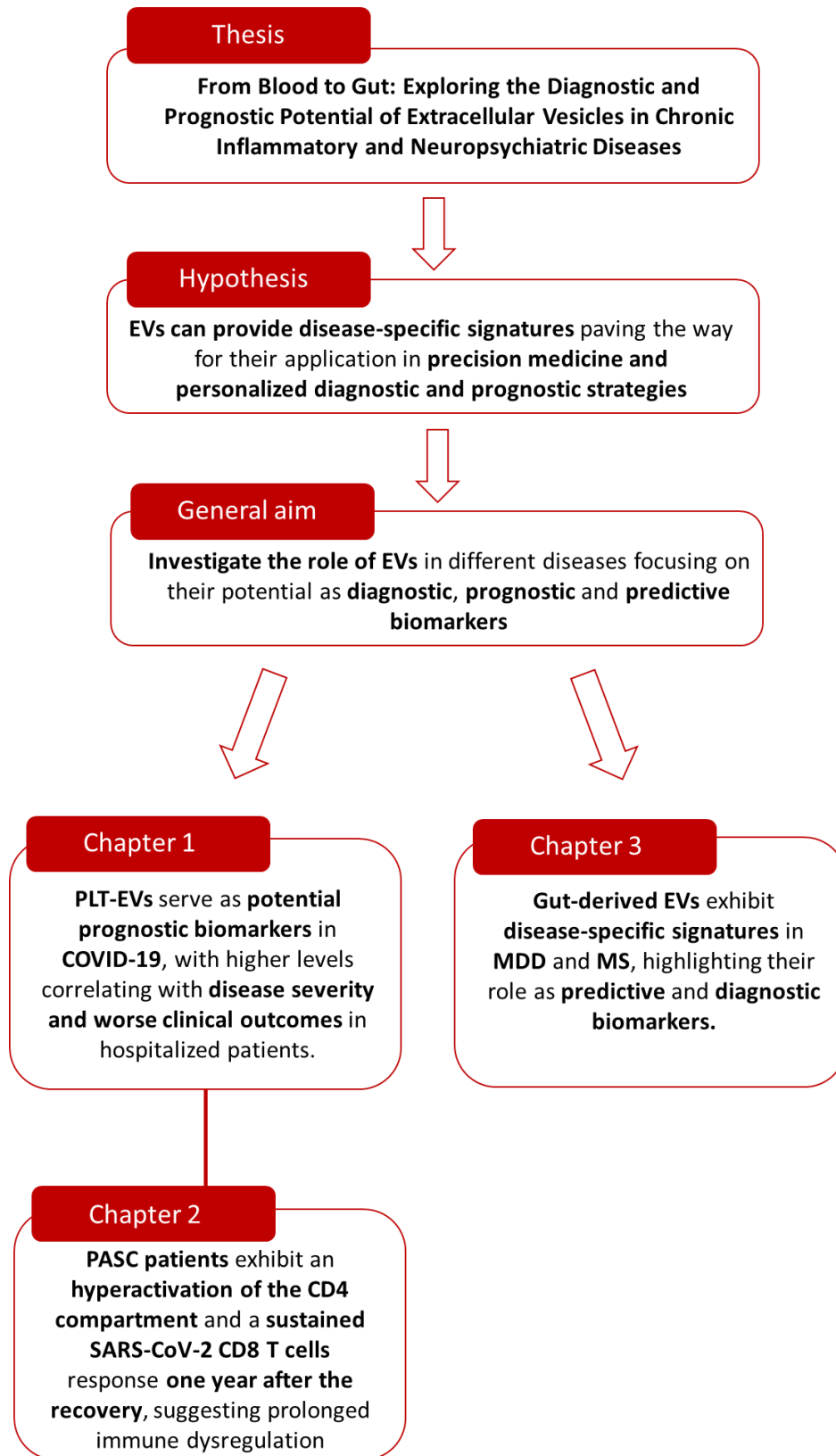
Nel primo capitolo, è stato valutato il potenziale prognostico delle VE derivate da cellule del sangue, in particolare delle VE di origine piastrinica (*PLT-EVs*), nei pazienti affetti da COVID-19. Come descritto nell'**articolo 1**, è stato utilizzato un metodo brevettato innovativo per l'identificazione rapida delle VE a partire da biopsie liquide (es. sangue o plasma), al fine di valutarne il ruolo prognostico. Da tale studio, è emerso che i pazienti COVID-19 con una prognosi peggiore, dopo una settimana in terapia intensiva, presentavano livelli più elevati di *PLT-EVs*, sottolineandone il ruolo come biomarcatori prognostici.

Dopo la terza ondata di SARS-CoV-2, l'attenzione si è spostata sui pazienti COVID-19 della coorte arruolata nello studio precedente che, a mesi di distanza dalla guarigione, continuavano a manifestare una vasta gamma di sintomi persistenti. Questa nuova condizione è stata denominata sindrome da sequele post-acute dell'infezione da SARS-CoV-2 (PASC). Nel secondo capitolo, mediante tecniche avanzate come il test di marker indotto dall'attivazione (*AIM assay*) e l'utilizzo di destrameri, è stata identificata nelle cellule mononucleate di sangue periferico un'iperattivazione del compartimento CD4⁺ e alti livelli di cellule CD8⁺ T specifiche per SARS-CoV-2 nei pazienti PASC rispetto ai controlli sani (**articoli 2 e 3**).

Nel terzo ed ultimo capitolo, la mia attenzione si è spostata dalle VE derivate da cellule del sangue a quelle originate dal microbiota intestinale, come mediatori di comunicazione dell'asse intestino-cervello, in particolare nel contesto del disturbo depressivo maggiore (DDM) e della sclerosi multipla (SM). Attraverso lo sviluppo e l'ottimizzazione di un metodo rapido e affidabile per l'isolamento delle VE di origine intestinale, è stato possibile identificare una *firma* specifica per entrambe le malattie.

Complessivamente, questa tesi contribuisce significativamente, posizionando le VE come strumenti innovativi nella diagnostica e prognosi delle malattie, dimostrando la loro versatilità in diverse condizioni patologiche. Le metodologie qui sviluppate per identificare ed isolare VE e i risultati ottenuti rappresentano una solida base per future ricerche traslazionali, con rilevanti implicazioni per la medicina personalizzata e la gestione delle malattie basata sui biomarcatori.

Flowchart of the thesis



Chapter 1: Extracellular vesicles as biomarkers in severe COVID-19 patients

Premise

At the beginning of 2020, Italy experienced the first wave of the SARS-CoV-2 outbreak, causing Coronavirus infectious disease 2019 (COVID-19), a pandemic burden with an unprecedented impact on the healthcare system worldwide. COVID-19 presented a complex clinical spectrum, that is characterized by a wide range of different symptoms such as fever, cough, dyspnea, muscle pain, headache, sore throat, chest pain, and abdominal pain. These symptoms caused by the virus can vary from mild to severe, with the development of acute respiratory distress syndrome (ARDS), requiring mechanical ventilation and intensive care unit (ICU) admission. The COVID-19 pandemic presented several challenges to clinicians because of its high spread and transmission, mortality rate and sequela leading to aftermath known as post-acute sequelae of SARS-CoV-2 infection (PASC). In that context, it was crucial to study the disease to get insights into the mechanisms driving these outcomes and to identify both diagnostic and prognostic biomarkers that allow clinicians to detect immediately the disease and stratify patients by risk and lastly to optimize treatment strategies.

At that time, the Immunomics lab, where I completed my PhD, chose to tackle the challenge of finding reliable and fast biomarkers for routine hospital use, focusing on extracellular vesicles (EVs) as promising candidates. EVs are double membrane nanoparticles resembling the status of their parental cell and are involved in the cell-to-cell communication in both physiological and pathological conditions. Several studies have shown that EVs can serve also as biomarkers for several possible diseases including COVID-19 and its sequelae. However, the drawbacks of EVs were, firstly, the time-consuming techniques used to isolate them, such as ultracentrifugation, which requires at least 1 hour and secondly, the sample manipulation, which frequently represents stressful conditions for cells, which alter the composition of the original EVs sample, potentially misrepresenting the real EVs profile.

We applied a new method using a patented kit to detect EVs directly in a few microliters of unmanipulated fresh blood samples within 1 hour. This approach, if proven to be reliable, will pave the way for using EVs as biomarkers in everyday clinical practice.

Background

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic outbreak Between the end of 2019 and the beginning of 2020, a novel coronavirus, called SARS-CoV-2, was identified for the first time in Wuhan, Hubei Province, China. SARS-CoV-2 was immediately recognized as a novel virus belonging to the Coronaviridae family, due to some shared similarities with other known coronaviruses as severe acute respiratory syndrome coronavirus (SARS-CoV) and middle east respiratory syndrome coronavirus (MERS-CoV), which were responsible of two different epidemics in 2003 and 2012 respectively (Microbe, 2023). Epidemiological evidence supports the zoonotic origin of SARS-CoV and MERS-CoV, and a similar mechanism was reported for SARS-CoV-2 (Hao et al., 2022). The clinical manifestation of the SARS-CoV-2 infection is the development of the coronavirus disease 19 (COVID-19), characterized by a wide range of different symptoms, from asymptomatic cases to severe pneumonia and multi-organ failure.

Despite the initial containment efforts, the rapid worldwide spread of the virus led to the declaration of COVID-19 pandemic by the World Health Organization (WHO) in March 2020 (**Figure 1**) (Pascarella et al., 2020).

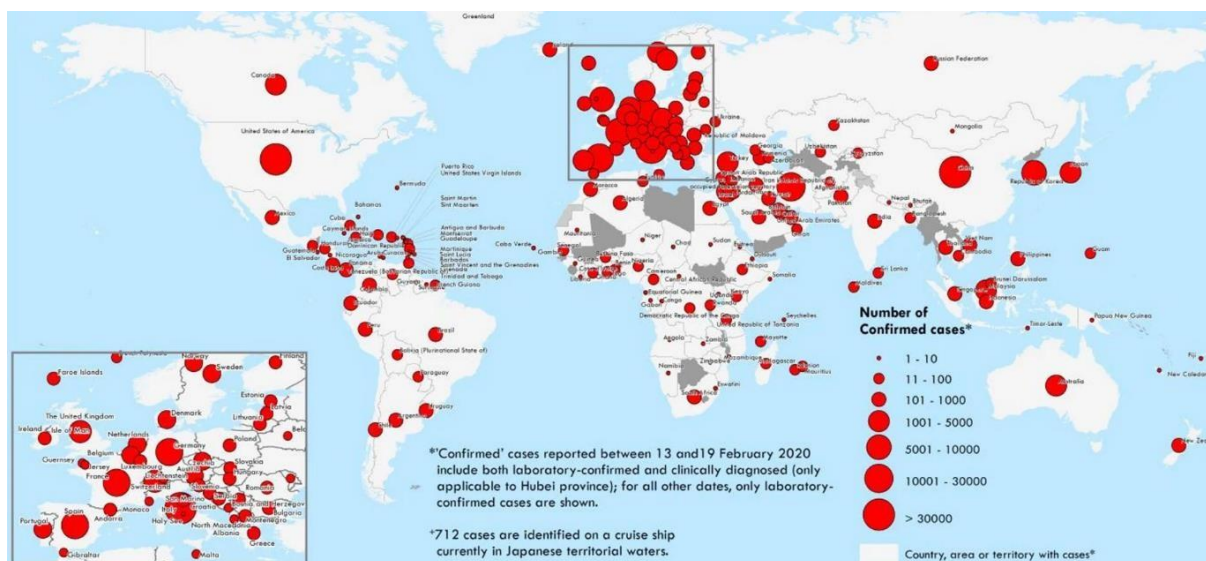


Figure 1. Cases of the COVID-19 global pandemic. (<https://www.who.int/>)

Coronaviridae family

The Coronaviridae are large lipid bilayer enveloped, positive-sense RNA viruses. They are the largest known RNA viruses, with genomes ranging from 25 to 32 kb and virions of 118–140 nm in diameter.

The Coronaviridae family comprises three sub-families: *Orthocoronavirinae*, *Letovirinae* and *Pitovirinae* (Payne, 2017).

The *Orthocoronavirinae* are divided into four genera: alphacoronavirus, betacoronavirus, gammacoronavirus and deltacoronavirus. Among them, alphacoronaviruses and betacoronaviruses have gained central attention because they are highly infectious and dangerous for mammalian species. Human and animal coronavirus infections mainly result in respiratory and enteric diseases (Lam et al., 2020)

Coronavirus virions are spherical and contain four different structural proteins: Spike (S), membrane (M), envelope (E) and nucleocapsid (N) proteins. The S protein is a large glycoprotein present on the surface of the virus. Homotrimers of the S protein form large structures called Spikes, which extrude from the surface of the virion, giving its distinctive corona-like shape. The S protein is also a fusion protein and mediates the binding to the host receptor to allow the virus to enter (Huang et al., 2020). The M glycoprotein is an integral type III membrane and is the most abundant structural protein in the coronavirus particles and plays a central role in virus assembly. It interacts with other viral proteins to help shape the virion (Li, 2016). The E protein is a transmembrane protein, and it is present in a small quantity in virions. The main functions of this protein are the assembly and the release of the virus. However, it seems also to be implicated in the virus's pathogenicity, as some of them lacking this protein are not lethal (Ruch and Machamer, 2012). The N protein is the only protein present in the nucleocapsid. It is a multi-domain RNA binding protein that interacts with the viral membrane protein during virion assembly and plays a critical role in enhancing the efficiency of virus transcription and assembly (Bai et al., 2021). A fifth structural protein, the hemagglutinin-esterase (HE), is present in a subset of β -coronaviruses. Its activity might be involved in the enhancing of the S protein during the binding and entry into the host (Zandi et al., 2021).

Life cycle of SARS-CoV-2

The life cycle of SARS-CoV-2 starts with the entry into the host cells through the binding of Spike proteins with the viral entry angiotensin-converting enzyme 2 (ACE2) receptor. This receptor is expressed by epithelial cells in the oral mucosa, liver, kidney, heart, intestine and lungs. Specifically in SARS-CoV-2 infection, S1 portion of the Spike can bind the ACE2 to the

host cells, while, the portion S2 can mediate and facilitate the fusion and the entry of the virus, due to the cleavage of the S1/S2 site by the host transmembrane serine proteases 2 (TMPRSS2) and also by cathepsin B and L (Pizzato et al., 2022). Subsequently, the viral genome is released in the cytoplasm of the infected cells, where the process of translation occurs. At the 5' end, the two open reading frames (ORF) 1a and 1b are directly translated into polyproteins 1a and 1ab that are then cleaved in functional proteins, including the RNA-dependent RNA polymerase (RdRp). On the contrary, the structural proteins S, envelope (E), membrane (M) and nucleocapsid (N), and accessory proteins are translated from subgenomic RNAs (sgRNAs) and used for the virion assembly. The sgRNAs are transcribed from the 3' end by the RdRp in a discontinuous way and then are fused together (Harrison et al., 2020; Sola et al., 2015). Upon translation, the new assembled virions exit from the cells via exocytosis and start to infect other cells (**Figure 2**).

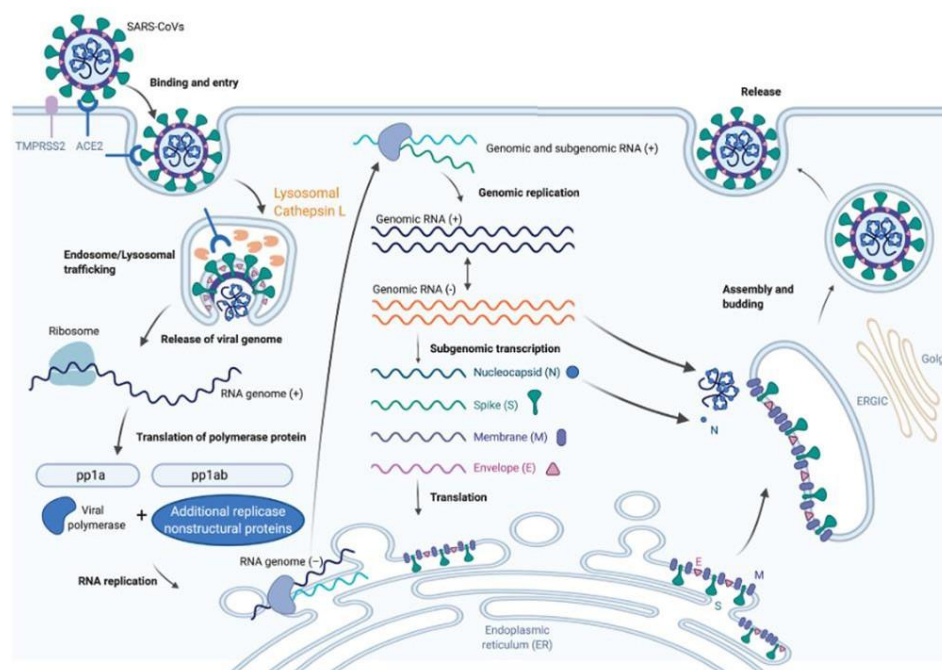


Figure 2. SARS-CoV-2 lifecycle. The SARS-CoV-2 spike protein binds the angiotensin-converting enzyme 2 (ACE2) receptor present on host cells, starting the mechanism of entry of the virus. The efficient viral entry depends on cleavage of the S1/S2 site by the surface transmembrane protease serine 2 (TMPRSS2); and/or by cathepsin L, which mediate virus–cell membrane fusion at the cell surface and in endosomal compartments. After the entry, the virus releases its RNA genome into the cytosol. The polyproteins (pp1a and pp1b) are cleaved by a virus-encoded protease into individual replicase complexes. Replication begins in the endoplasmic reticulum (ER). The subgenomic (sg) RNA transcription results in structural and accessory protein translation, which are ready, together with the positive-sense RNA genomes, for the virion assembly and the release outside the cell (Harrison et al., 2020).

Different clinical manifestation of COVID-19

The SARS CoV-2 route of transmission is airborne through droplets and aerosol (Bak et al., 2021).

SARS-CoV-2 infection caused unusual pneumonia, which was referred to as COVID-19 by the WHO. Manifestation of COVID-19 varied between individuals with different degrees of onset and severity ranging from asymptomatic infection and mild flu-like symptoms to ARDS, acute respiratory failure, pulmonary edema, sepsis, septic shock or multiple organ failure, and even death. The most typical symptoms of COVID-19 were fever, cough, shortness of breath, muscle aches, headache, diarrhea, sore throat and chest pain (Verity et al., 2020) (**Figure 3**).

The course of the disease ranged from being asymptomatic or mild and it became serious in about 10% of cases. Critical sequelae (respiratory failure, pneumonia, shock, and multiorgan failure) occurred in a 5% and required the admission to ICU (Bestetti et al., 2021; Pascarella et al., 2020). The damage of organs and tissues correlated to systemic inflammation caused by the so-called “cytokine release syndrome” (CRS) (Mohamed Khosroshahi et al., 2021). This phenomenon is caused by the host immune system, and it is characterized by an uncontrolled and huge release of pro-inflammatory cytokines due to infectious stimuli. There have been proposed different hypotheses underlying the establishment of the CRS. The first hypothesis implied the SARS-CoV-2 replication, which led to the pyroptosis, which is a massive inflammatory state of lytic-programmed cell death and the release of pro-inflammatory cytokines by lymphocytes and macrophages. Pyroptosis in virus-infected macrophages potentially acts as an immune attractant for other immune cells, which once in the infection site elicit an immune response. In addition, pro-inflammatory factors released from pyroptotic macrophages result in a cytokine storm (Hsu et al., 2022). This inflammatory milieu stimulates the proliferation and activation of myeloid-derived suppressor cells (MDSC), which inhibit the immune response through the release of nitric oxide synthase (NOS), arginase 1 (ARG1) (Wang et al., 2022). MDSCs have been hypothesized to play a dual role in severe COVID-19 cases: on one side by arresting SARS-CoV-2 replication and, on the other hand, by inhibiting T cell proliferation and interferon (IFN) release (Alsalman et al., 2022). This suppression ultimately delayed the immune response in these compromised patients.

The second hypothesis is based on the production of antibodies that can bind the S protein and trigger the inflammatory cascade, with the release of proinflammatory cytokines. In this

inflammatory environment, macrophages and lymphocytes start to accumulate into the lungs and drive acute respiratory distress (Jarlhelt et al., 2021).

According to the guide published by the WHO, the severity of the disease in adults can be classified as follows:

- **Mild disease:** characterized by common clinical symptoms, such as fever, cough, fatigue, and anorexia, without evidence of viral pneumonia or hypoxia.
- **Moderate disease:** characterized by clinical signs of pneumonia, including saturation of peripheral oxygen (SpO₂) ≥ 90% on room air, but no signs of severe pneumonia. Fever, cough, dyspnea, and fast breathing characterize this stage.
- **Severe disease:** characterized by clinical signs of severe pneumonia presenting one or more of the following features: respiratory rate > 30 breaths/min, severe respiratory distress, or SpO₂ < 90% on room air.
- **Critical disease:** characterized by acute respiratory distress syndrome (ARDS), ranging from mild ARDS to severe ARDS; sepsis, that leads to life-threatening organ dysfunction; septic shock (persistent hypotension despite volume resuscitation); and acute thrombosis (acute venous thromboembolism, acute coronary syndrome, and acute stroke).

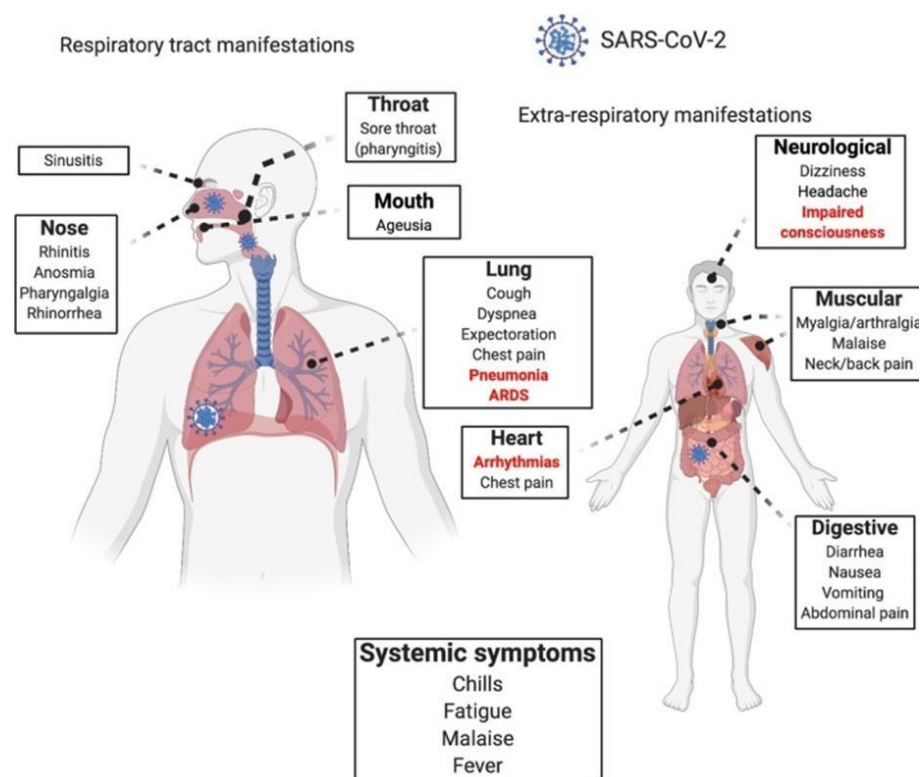


Figure 3. COVID-19 symptomatology. In red there are the symptoms that are prevalent in patients with severe COVID-19. (Harrison et al., 2020).

Mortality rate and comorbidities in COVID-19

Once ARDS progress, multiorgan failure and shock remained the main cause of death in COVID-19 patients. Generally, dyspnea onset requires 8 days from the beginning of first symptoms, ARDS requires 9 days, and intensive care unit (ICU) admission occurs in 10 to 14 days (Boechat et al., 2021)

However, the mortality rate is different among several studies (ranging from 1% to 3%), due to the variability among patients' features such as age (the rate is higher in elderly), comorbidities, and gender. Differences also depend on the rapid saturation of ICs and on the lack of sufficient support (Asselah et al., 2021; Fathizadeh et al., 2021; Pascarella et al., 2020). Tan and colleagues reported that mortality was higher in men than women. Indeed, men are more likely to be admitted in ICU and require mechanical ventilation. The different rate can be explained by a stronger inflammatory regulation and an enhanced immune response in women: female patients have shown a more robust T cell activation than males, who conversely have shown higher levels of pro-inflammatory cytokines. This could be explained by the distinct hormonal milieu between the two genders: testosterone can have immunosuppressive effects, as opposed to estradiol (Tan et al., 2021)

Besides old age (>75 years old) and gender, other concomitant factors that worsen the course of the disease can be obesity, hypertension, diabetes mellitus (DM), chronic kidney disease (CKD), and cardiovascular disorders (Bestetti et al., 2021). A higher risk of being hospitalized was indeed found in a multistate surveillance system, called COVID-NET, among people with obesity (Body Mass Index (BMI) $\geq 30 \text{ kg/m}^2$) and severe obesity (BMI $\geq 40 \text{ kg/m}^2$), CKDs, DM, hypertension and asthma (Ko et al., 2021).

Up to now, the mechanism by which hypertension predisposes to a severe course of the disease is still not clear, but the dysregulation of the renin–angiotensin–aldosterone system (RAAS) system, of the immune response, and gut dysfunction may contribute to a worse outcome (Muhamad et al., 2021).

Innate immune response in COVID-19

Innate immune response is the first line of defense against pathogens. It has three main roles: to restrict viral replication in infected cells, to create a local antiviral state, and to trigger the adaptive immune response (Sette and Crotty, 2021).

After the virus enters the cell, the viral RNA is immediately sensed by the pattern recognition receptors (PRRs) such as toll-like-receptors (TLRs), retinoic acid-inducible gene I (RIG-I) and the NOD-like receptors (NLRs). The PRRs that are mainly involved in the SARS-CoV-2 sensing are TLR-3 and TLR-7 in endosomes, RIG-I and the melanoma differentiation-associated gene 5 (MDA5) (Dai et al., 2022).

Upon viral RNA binding, both TLRs and RIG-I-like receptors (RLRs) engage adaptor proteins. TIR-domain-containing adapter-inducing interferon- β (TRIF) and myeloid differentiation factor 88 (MyD88) are recruited by TLRs, while RIG-1 and MDA5 activate mitochondrial antiviral-signaling protein (MAVS) and consequently recruit specific kinases, such as TANK-binding kinase 1 (TBK1). Then, phosphorylated interferon regulatory factors (IRFs), including IRF3 and IRF7 and transcriptional factors nuclear factor kappa light-chain-enhancer of activated B cells (NF- κ B) translocate in the nucleus, inducing the transcriptional activation of genes coding inflammatory cytokines and IFNs (Duncan et al., 2023).

Another pathway involved in the dsRNA sensing is the oligoadenylate-ribonuclease L (OAS-RNase L) activation. OAS3 synthesizes the activation of 2'-5'-linked oligoadenylates (2-5A), which leads to dimerization and activation of RNase L, resulting in viral degradation. An ultimate pathway implies the protein kinase R (PKR), that once activated, induces PKR autophosphorylation, resulting in the translation of the initiation factor eIF2 α , which results in protein synthesis shutdown and restriction of viral replication (Li et al., 2021) (**Figure 4**).

IFN I drives the release of other inflammatory molecules that possess antiviral functions. An example is shown by the Interferon-induced transmembrane proteins (IFITMs), which are proteins embedded in the lipid components of the plasma membrane of the cells and can interfere with the viral fusion mechanism, by changing the membrane curvature, thus reducing the membrane permeability. In contrast, SARS-CoV-2 can mount several strategies to overcome immune surveillance and inhibit the production of IFN type I and III (Prelli Bozzo et al., 2021). Several conserved *betacoronavirus* proteins, predominantly non-structural proteins (nsps), are known to exert direct IFN-antagonistic activities. Indeed, NSP1, NSP12, NSP13, NSP14, NSP15, ORF3b, and ORF6 have been reported to inhibit the nuclear translocation of IRF3 (Kumar et al., 2021). For example, the NSP12 inhibition of IRF3 nuclear translocation impairs downstream signaling of IFN I (Znaidia et al., 2022). By inhibiting the production of IFN I and III, SARS-CoV-2 can delay the innate immune response and continue to replicate.

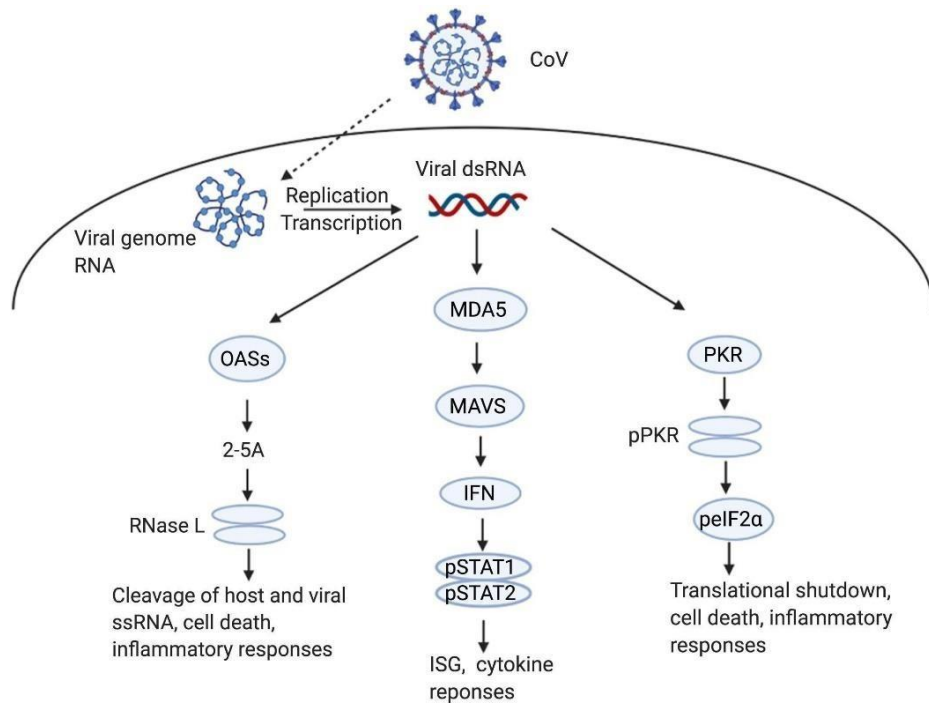


Figure 4. SARS-CoV-2 dsRNA sensed by the innate immune system. Type I and type III IFN production, followed by ISG transcription and cytokine responses, are caused by MDA5 signals via MAVS. RNase L is activated by OASs' production of 2-5A, which cleaves viral and host ssRNA to cause inflammation and death. PKR autophosphorylates before phosphorylating eIF2α, which leads to cell death (Li et al., 2021).

Adaptive immune response in COVID-19

Adaptive immunity comprises both humoral and cellular components. It includes SARS-CoV-2-specific helper T cells (CD4⁺) that closely regulate cytotoxic T cells (CD8⁺), that represent the effector components of cell-mediated immunity, and B cells, that produce virus-specific antibodies, among which also the neutralizing ones (Mohamed Khosroshahi et al., 2021; Sette and Crotty, 2021).

Antigen presenting cells (APC) have the task of exposing the antigen to their surfaces through the major histocompatibility complex (MHC) molecules. The MHC molecules of class I present the antigen to cytotoxic T cells, while the MHC molecules class II present the antigen to helper T cells. It has been proposed that different human leukocyte antigen (HLA) alleles could have a role in determining the genetic susceptibility to infections, including SARS-CoV-2 infection. Nevertheless, further studies are required to better understand this correlation (Mohamed Khosroshahi et al., 2021).

As mentioned before, high degrees of lymphopenia seem to be related to COVID-19 most severe cases. Low counts of lymphocytes are a common characteristic of viral infections, but

they typically resolve quickly. In the case of the novel coronavirus, however, it could become a long-lasting and defining characteristic (Gusev et al., 2022).

In contrast to the previous findings, other studies reported that an excessive T cell response could be related to severe disease. It is not clear the reason why some patients develop a poor response while others have a massive one (Chen and John Wherry, 2020a). One study, conducted among 125 hospitalized patients highlighted two different immunophenotypes, associated with severe diseases: the first characterized by a robust CD4⁺ T cells response with few circulating T follicular helper cells (cTfh), hyperactivated or exhausted CD8⁺ T cells, activated CD8⁺ terminally differentiated effector memory (TEMRA) cells, and plasmablast response; the second characterized by low T and B cells. A third immunotype associated with mild disease was instead characterized by less helper T cells activation, T-box transcription factor TBX21 (Tbet)⁺ effector CD4⁺ and CD8⁺ T cells, and proliferating memory B cells (Mathew et al., 2020). The rapid induction of virus-specific T helper cells is associated with mild disease and also accelerated viral clearance (Sette and Crotty, 2021). In detail, a higher proportion of IFN γ -producing T helper 1 (Th1)-like cells with antiviral functions was described in mild cases compared to severe cases. It has been suggested that Th2 and Th17 responses play a role in the worsening of the disease: Th2 response is associated with lung immunopathology, while Th17 responses are associated with ARDS. On the contrary, cTfh may play a key role for an effective immunity and their frequency increases in parallel to SARS-CoV-2 clearance. cTfh cells are essential for the development of virus-specific neutralizing antibodies (Chen and John Wherry, 2020a; Neidleman et al., 2020). A cytotoxic T cell response has been found both in patients with acute disease and in about 70% of convalescent patients with COVID-19. Jamal and colleagues reported that CD28⁺, CD8⁺ and CD3⁺ cytotoxic suppressor T cells are generally decreased in people with poor outcomes (Jamal et al., 2021). Indeed, the presence of SARS-CoV-2-specific CD8⁺ T cells is associated with a low level of disease severity (Rha and Shin, 2021). Schreibing and colleagues demonstrated that severe COVID-19 display hyperactivated viral-specific CD8⁺ T cells, while mild cases display a more functional, terminally differentiated CD8⁺ effector T cell phenotype. Within the group that recovered from severe COVID-19, they also observed a population of exhausted T cells, characterized by the expression of T Cell Immunoreceptor With Ig And ITIM Domains (TIGIT), Cytotoxic T-Lymphocyte Antigen 4 (CTLA4), Programmed cell death protein 1 (PD-1) and T-cell immunoglobulin and mucin-domain containing-3 (Tim-3) (Schreibing et al., 2021). However, another study demonstrated

that cytotoxic T cells expressing PD-1 are not exhausted, but functional since they are able to secrete IFN- γ . Indeed, further research are needed to understand if exhausted T cells are really exhausted or just highly activated (Chen and John Wherry, 2020a; Rha and Shin, 2021)

After primary viral infection, usually the number of effector T cells decreases in favor of different subsets of memory T cells once activated upon reinfection with the same pathogen. To develop a robust recall response, the primary response should be effective in viral clearance in order to allow progenitor memory T cells to differentiate in fully formed populations. Regarding SARS-CoV-2 infection, the

development of a memory response may vary depending on the age of the individual or the severity of the disease (Jarjour et al., 2021).

Even concerning the B cell response, lymphopenia is unevenly reported in patients with poor outcomes. SARS-CoV-2 specific antibodies start to appear within 5-15 days of post-symptoms onset, and they are directed mostly against S and N antigens. Usually, in viral infection, a higher antigen load correlates with higher antibody titers. Also concerning the novel coronavirus, high neutralizing antibody titers were associated with severe COVID-19 and with a potential extrafollicular B cell response (Melenotte et al., 2020; Sette and Crotty, 2021). Conclusively, these data suggest that different compositions in peripheral blood immune cells may be indicative of different severity-degrees of COVID-19 (**Figure 5**).

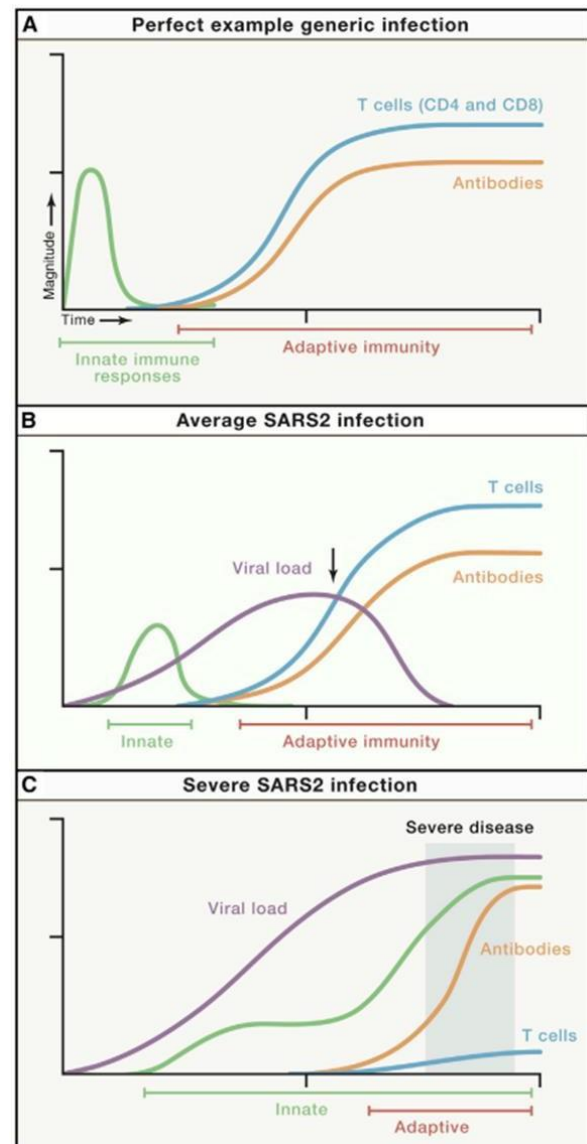


Figure 5. Models of possible kinetics of immune responses to SARS-CoV-2 infection. The picture shows the timing and the duration of innate and adaptive immune responses in **A**) Immune response during a generic viral infection **B**) Immune response during an average of SARS-CoV-2 infection **C**) Immune response during a severe SARS-CoV-2 infection (Sette and Crotty, 2021).

Biomarkers in COVID-19

The emergence of the COVID-19 pandemic, due to a novel coronavirus, presented significant challenges because of its unclear etiopathogenesis and high hospitalization and mortality rate. In this context, clinicians dealt with faced a significant gap in the availability of fast, reliable, and disease-specific diagnostic and prognostic biomarkers, which were essential for effective patient management in this unexpected scenario.

At the time of virus spreading overworld few biomarkers were identified, but unfortunately, they were poorly specific. Among them, cardiac troponin T (cTnT), and D-dimer, were employed as an index of myocardial injury, but these markers were shown to be not specific for SARS-CoV-2 infection (Battaglini et al., 2022; Rasmi et al., 2022).

In addition to these biochemical markers, imaging such as computed tomography (CT) scans played also a critical role for the evaluation of the extent of pulmonary involvement, though it overlapped significantly with features of other forms of pneumonia making not so easy a differential diagnosis of Sars-CoV-2 from other lung infections. Thus, these gaps highlighted the unmet need for searching for more specific and reliable biomarkers.

In recent years, membrane-bound nanoparticles, known as extracellular vesicles (EVs), gained a lot of attention as potential biomarkers for several human diseases. The significance of EVs lies in their cargo, which consists of different biomolecules (i.e. DNA, metabolites, proteins, RNA) that resemble both the physiological and pathological states of their cell of origin. Since they are released by all the cell types in several biofluids, this feature makes them a suitable accessible biomarker. EVs can cross and enter in all the organs in the body, both near and distant, exerting their functions as mediators in cell-to-cell communication. Moreover, they can remain stable over time in biological samples and simplify the identification of rare biomolecules in biofluids (Boukouris and Mathivanan, 2015). For instance, in patients with focal segmental glomerulosclerosis (FSGS) or steroid-sensitive nephrotic syndrome (SSNS), the urinary exosomal WT-1 (Wilms' tumor-1) has been suggested as a promising non-invasive biomarker with podocyte specificity that can identify early progression and treatment-induced regression of podocyte injury (Zhou et al., 2013).

Similarly, EVs have been described as biomarkers for infectious diseases. For example, they were suggested as biomarkers for predicting the risk of Hepatitis B virus (HBV) reactivation and detection of early fibrosis in hepatitis B and C. According to a recent study, patients with undetectable plasma HBV-DNA, showed an increase of plasma-derived EVs. This study

demonstrated that HBV-DNA-derived EVs are uptaken by hepatocytes, highlighting their role in HBV reactivation (Sukriti et al., 2019). Based on these findings, the authors suggested that these EVs could serve as a diagnostic or predictive biomarker of early HBV reactivation. In other studies, different authors demonstrated that the characterization of EVs cargo, focusing on microRNA (miR) i.e. miR-15a, miR-30d-5p, miR-30a- 5p, miR-192-5p, miR-26a-5p, miR-23a-5p, and miR-191-5p may discriminate between septic patients and those with non-infectious inflammation (Caserta et al., 2016).

All this evidence paves the way for further exploration of the ability of EVs to address the unmet clinical need for disease-specific diagnostic, prognostic, and predictive tools, improving the early detection, monitoring, and management of severe COVID-19 cases.

Extracellular vesicles (EVs)

EVs are nanoparticles with a lipid bilayer membrane, released by all cell types into extracellular space. They play an important role in the regulation of many biological processes and intracellular communication, leading to their capacity to transfer proteins, lipids, and nucleic acids (Yáñez-Mó et al., 2015).

EVs can be differentiated based on their size, contents, and function into exosomes, microvesicles (MVs) and apoptotic bodies.

Exosomes (30-100 nm in diameter) are formed from the inward budding of the endosomal membrane and are secreted when multivesicular bodies (MVBs) fuse with the plasma membrane; instead, MVs (100 nm to 1 mm in diameter) are formed directly by budding of the cell's plasma membrane; lastly, apoptotic bodies (50 nm up to 5000 nm in diameter) are released into the extracellular space when the cell dies (Doyle and Wang, 2019).

EVs have been shown to play a role as mediators of physiological and pathological processes. In this regard, EVs have gained significant attention as potential predictive and prognostic biomarkers in different diseases, due to their nature to act as carriers of different macromolecules, facilitating cell-to-cell interactions.

EVs biogenesis and uptake

EVs originate through different mechanisms either as an inward-budding vesicle within the endocytic system (exosomes) or as an outward-budding vesicle at the plasma membrane (microvesicles) (**Figure 6**).

The biogenesis of exosomes begins with the inward budding of small parts of the plasma membrane, containing several molecules such as nucleic acids, lipids, and proteins. These small intracellular vesicles form the early endosome. Then, the early endosome transforms into the late endosome. The late endosomes accumulate intraluminal vesicles (ILVs) formed by inward budding of the endosomal membrane. During this process, cytosolic proteins, nucleic acids, and lipids are sorted into these small vesicles. Late endosomes containing a lot of small vesicles, that are termed MVBs. These MVBs can either fuse with the lysosome if the content is fated for degradation or fuse with the cellular membrane releasing the ILVs as exosomes into the extracellular space (Rooks and Garrett, 2016). The exosome generation pathway is regulated by the endosomal sorting complex required for transport (ESCRT). The ESCRT complex is composed by four proteins: ESCRT-0, -I -II -III and accessory proteins (Alix, VPS4, and VTA-1), that sequentially act to bind future exosome cargoes and form intraluminal vesicles (ILVs) incorporating those cargoes. The ESCRT machinery is required for MVB formation because it sorts ubiquitinated intracellular cargos that are destined for lysosomal degradation into MVBs (Maas et al., 2017a).

In the case of EVs that are formed by direct budding from the plasma membrane (microvesicles and apoptotic blebs), there are different molecular mechanisms of biogenesis, less characterised. One mechanism of their synthesis, involves the recruitment of the same ESCRT machinery and of the subunits Tumor Susceptibility Gene 101 (TSG101) and vacuolar protein sorting-associated protein 4 (VPS4) to the plasma membrane by adaptor protein arrestin domain-containing protein 1 (ARRDC1), thereby promoting the generation of MVs (Teng and Fussenegger, 2021).

EVs can release their content and interact with target cells through endocytosis or fusion with the recipient cells (Tricarico et al., 2016). The release of EVs content is strictly regulated by different GTPases, among which members of ADP-ribosylation factor (ARF) family, Rab and Rho. Moreover, their entrance is mediated by the interaction with surface receptors present on the plasma membrane (Mulcahy et al., 2014). Different molecules, among which integrins, tetraspanins, lipids and components of the extracellular matrix, are known to be the mediators of these interactions. Tetraspanins are a superfamily of small transmembrane proteins that are expressed in almost all eukaryotic cells, and in mammals the tetraspanin protein family includes more than 30 members (Jankovičová et al., 2020). The tetraspanin family comprises different proteins characterized by their common specific molecular structure, represented

by four 5 transmembrane domains containing conserved polar residues, a small extracellular loop (SEL) and large extracellular loop (LEL), and short cytoplasmic tails. Among tetraspanin molecules, CD9, CD63 and CD81 are the main markers

of EVs for eukaryotic cells. Exosomes have a high expression of these three molecules; however, since tetraspanin expression has also been detected on cell surfaces, they can be used also as general markers for all the types of EVs (Jankovičová et al., 2020).

Several factors may determine the likelihood of EV-cell fusion occurring: the first is due to ligand-receptor binding or glycoprotein interactions, and the second is the lipid composition of EVs and cellular membranes, which is likely to affect their propensity to fuse, because high lipid raft content in EV may facilitate their fusion with cells (Maas et al., 2017b).

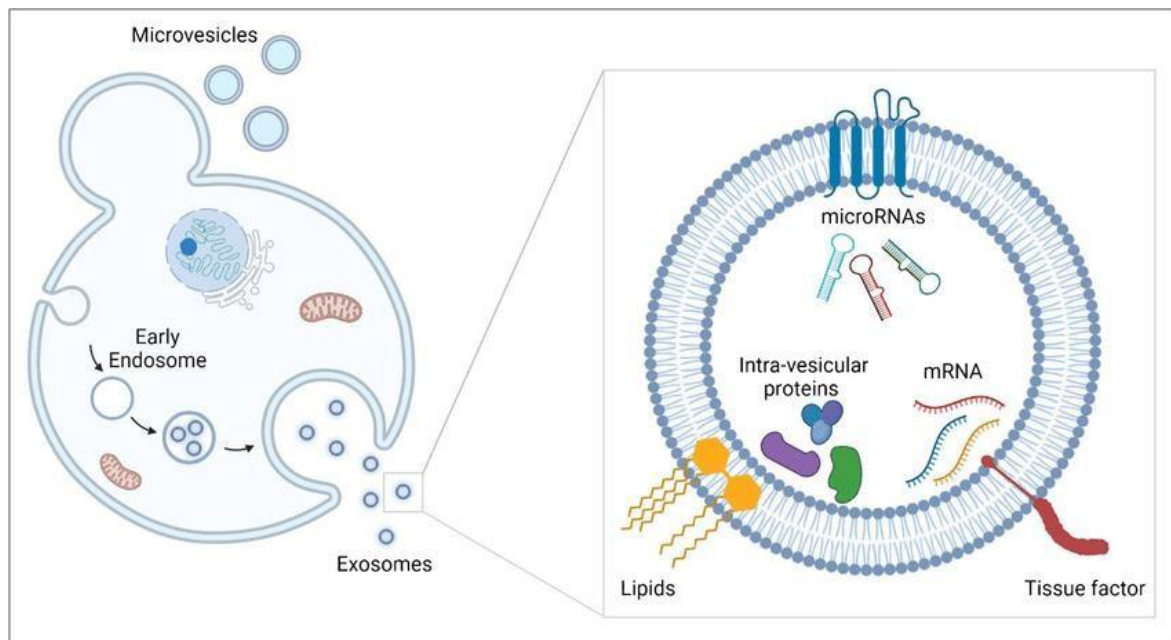


Figure 6. Microvesicles and exosome formation with their cargo. On the left, exosomes originate from the inward budding of early endosomes into multivesicular bodies (MVBs), which later fuse with the plasma membrane to release exosomes, alongside microvesicles budding directly from the cell membrane. On the right, a zoomed-in view reveals exosomes' lipid bilayer structure, embedded proteins, and internal cargo, including microRNAs, mRNAs, and intra-vesicular proteins, highlighting their role in intercellular communication and signaling. (Eckhardt et al., 2022)

EVs in immune regulation

EVs play a multifaceted role among immune cells and in the immune response's regulations. The immune system is a complex network of cells, organs and molecules that protect the organism against foreign pathogens. In this regard, EVs are implicated in the regulation of inflammation, antigen presentation and immune cell activation.

In the innate immune system, EVs derived from monocytes contain different miRNAs and also

alarmins, that are chemotactic proteins that can bind TLRs and heat shock proteins, causing immune activation (Chen et al., 2019). They carry inflammatory cytokines such as interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α), which sustain inflammatory signals and recruit other cells (Kalluri, 2024). Neutrophils derived-EVs can transport antimicrobial peptides and reactive oxygen species (ROS), which can help in the erasure of the infection (Chen et al., 2019). Additionally, natural killer (NK) derived-EVs carry inside cytotoxic molecules as granzymes and perforin, which can start the apoptosis pathways in target cells (S. He et al., 2024).

In adaptive immunity, EVs are involved in the antigen presentation. This process is carried on by the APCs (i.e., B cells, macrophages, and dendritic cells (DCs)) that are able to capture, process and present antigens, through the MHC, to CD4⁺ and CD8⁺ T cells directly as well as indirectly (Robbins and Morelli, 2014). In the direct way, EVs released by APCs carry surface MHC class I and class II molecules and, therefore, can potentially directly present the antigen to CD8⁺ and CD4⁺ T cells, respectively. Instead, in the indirect antigen presentation, EVs released by mature DCs carry on their surface different adhesion molecules such as MHC class II, CD86 and Intercellular Adhesion Molecule 1 (ICAM1), that can enhance the binding of the vesicles to APCs or the binding and the activation of T cell during APC–T cell interactions. Indeed, EVs released by mature DCs are able to transfer the ability to activate T cells to non-professional APCs. In the last antigen presentation mechanism, peptide-MHC complexes of EVs fuse to APCs' surfaces and can be directly presented to T cells without the need for the peptide-MHC to be reprocessed. This occurs through a mechanism known as cross-dressing (Robbins and Morelli, 2014).

EVs act also as carriers of antigens, indeed, microbial molecules can be present inside the EVs, as for example, in the case of *Mycobacterium bovis*, that infect macrophages, which release EVs loaded with mycobacterial antigens that activate T-cells response (Mehanny et al., 2021). Similarly, tumor derived EVs can transport tumor antigens that are taken up by DCs, which can process them to cytotoxic T lymphocytes (CTLs) (Menay et al., 2024).

Besides these active roles in mounting and sustaining the immune response against pathogens, EVs are involved also in immunosuppressive and anti-inflammatory mechanisms. For instance, EVs derived from mesenchymal stem cells (MSCs) can suppress inflammation and promote tissue regeneration, representing a promising treatment for different diseases (Lou et al., 2024).

Role of EVs in inflammation

Inflammation is the non-specific response to conserved peptides, motifs, and signals, which may be pathogens or damage-associated molecular patterns. Under non-pathological conditions, inflammatory responses are self-limiting and very controlled.

EVs are involved in the initiation, propagation and resolution of inflammatory responses. They exert a dual role in inflammation. Indeed, they can transport a wide range of different molecules, such as pro-inflammatory and anti-inflammatory cytokines, chemokines, proteases and damaged associated molecular patterns (DAMPs). In inflammatory conditions, EVs communicate with the immune cells, by influencing the systemic inflammatory response. For instance, EVs released by macrophages carry cytokines, like IL-6 and TNF- α , which are mediators in the recruitment and activation of immune cells. Moreover, these cytokines loaded-EVs beyond enhancing inflammation, they can also generate a feedback loop that sustains the inflammatory stimuli and recruit immune cells.

Emerging evidence highlights the involvement of the immune cells-derived EVs in inflammatory lung diseases as ARDS.

In chronic inflammatory conditions, EVs exacerbate and sustain inflammation through different mechanisms which comprise immune cells cross-talk and the delivery of autoantigens. An example are autoimmune diseases (ADs), particularly, in rheumatoid arthritis (RA), EVs derived from synovial fibroblasts carry pro-inflammatory molecules that exacerbate joint inflammation and tissue damage, driving the progression of the disease.

Therefore, EVs act as both markers and active participants in inflammatory processes. They can initiate, sustain, or resolve inflammation, depending on their molecular cargo and cellular origin, illustrating their complex and influential role in shaping the inflammatory landscape within the body. The functional diversity of EVs, spanning pro-inflammatory, immunoregulatory, and tissue repair roles, underscores their potential as both diagnostic biomarkers and therapeutic targets in inflammatory diseases.

EVs in viral infections

EVs have recently emerged as a novel mode of viral propagation exploited by both enveloped and non-enveloped viruses (Nolte-'t Hoen et al., 2016).

Current studies have revealed that EVs derived from infected cells contain viral nucleic acids

and viral proteins that may play a dual role: on one side, these EVs can contribute to alert the immune system from an ongoing infection; on the other, they may co-participate in the infection with other cells, by “boosting” infectivity (Urbanelli et al., 2019). EVs derived from the human immunodeficiency virus (HIV) have shown the capability to vehiculate HIV proteins, such as Nef, in the host cells. EVs containing Nef are involved in different pathogenic effects such as the induction of T-cell apoptosis, the down-modulation of cell surface molecules (i.e., MHC-I and CD4) to favor immune evasion, and the restoration of the infectivity of HIV particles defective in Nef protein. Moreover, Nef-EVs incorporate tumor necrosis factor- α - converting enzyme (TACE), which is a metalloproteinase responsible for the activation and release of TNF α . Therefore Nef-EVs produce a huge amount of active TNF α , that leads to the activation and proliferation of CD4⁺ T cells, rendering them competent for HIV expression and replication. This process seems to be correlated with the HIV-associated immune pathogenesis (Tang et al., 2024). Another mechanism that renders cells more susceptible to infections is the ability of EVs to deliver viral receptors to cells, which are deprived of them. For instance, in HIV infection, platelet (PLT)-derived EVs can transfer HIV co-receptors C-X-C chemokine receptor type 4 (CXCR4) and C-C chemokine receptor type 5 (CCR5) to endothelial cells that normally do not express them. Hence, this transferring process causes the establishment of a chronic inflammatory status and the unsuitability in eradicating HIV-1 infection and probably increasing the tropism *in vivo* and favoring its dissemination (Navarrete-Muñoz et al., 2022). EVs derived from Epstein-Barr virus (EBV) retain the latent membrane protein 1 (LMP1), which is a pro-oncogenic protein, that deregulate cellular transduction pathways and promote transformation and immortalization of B cells (Rastelli et al., 2008). EVs can carry inside entire non-enveloped viruses, such as the hepatitis A virus (HAV). HAV takes advantage of being transported inside the EVs, because, when inside it, EVs act as an envelope for the virus. In this way, the HAV encapsulated in EVs is recognized as a component of the self and so, these EV-enveloped viruses can camouflage themselves and elude the immune system (Feng et al., 2013). Moreover, EVs released by infected cells are also able to act as “garbage disposal”. They eliminate host proteins that have a pivotal role in prompting the immune response, thus avoiding and downregulating the immune system. Upon EBV infection, cells release, through EVs, to the extracellular environment the γ - interferon-inducible protein 16 (IFI16), that is able to sense the EBV genome, and by doing this, infected cells are able to escape to the control of the innate immune system (Ansari et al., 2013). The level of EVs that are released by the cells

increases when cells undergo viral infections. EVs are not only involved in favoring viral spreading, but also in promoting antiviral mechanisms. In fact, EVs released by HIV-infected cells, transport the host antiviral apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G), a cytidine deaminase which restricts viral replication, by changing the base sequence (from guanine to adenosine) of the translated viral DNA. This transition causes the loss of the replicative capacity of the virus, resulting in the production of nonviable virions. Other soluble host factors present in EVs cargo, able to counteract HIV infection, include guanosine monophosphate (GMP) and adenosine monophosphate (AMP), which triggers the host immune response mediated by interferons. In case of EBV infection, a mechanism has been found, through which EVs derived from EBV-infected cells release deoxyuridine triphosphatase (dUTPase), which is an intercellular signaling molecule, that prompts NF- κ B activation and cytokines secretion in DCs and peripheral mononuclear blood cells (PBMCs). By activating aforementioned processes there is a fine-tuning of the immune response against EBV (Giannessi et al., 2020). EVs released by cytomegalovirus (CMV) infected cells carry the glycoprotein B, that can activate CD4⁺ T cells, thereby inducing an adaptive immune response and helping to maintain a pool of T cells implicated in the control of CMV infection. The level of EVs that are released by the cells is associated with the increase during the viral infections. This happens for *Plasmodium falciparum*, which is sequestered in erythrocytes and activates endothelial cells (EC) to release EVs (Combes et al., 2004). Moreover, viruses such as the dengue virus and H1N1 influenza virus induce the release of EV from PLTs (Ostermeier et al., 2022).

EVs in COVID-19

EVs have been suggested as key players in the pathogenesis of Sars-CoV-2 infection, since a hallmark of COVID-19 is excessively prolonged and persistent inflammatory state as most patients exhibit elevated levels of pro-inflammatory cytokines, which have also been detected in EVs (Yan et al., 2021). Moreover, EVs are also involved in the promotion of blood coagulation. This pro-coagulant activity is primarily attributed to the abundant presence of negatively charged phospholipids, such as phosphatidylserine, on the outer surface of EV membranes. These phospholipids provide a catalytic platform for coagulation factor assembly, facilitating clot formation. Additionally, EVs express active tissue factor (TF), a key initiator of

the coagulation cascade, further enhancing their prothrombotic potential (Barrion et al., 2024). The combined effects of these factors underscore the possible role of EVs in COVID-19-associated coagulopathy, contributing to the increased risk of thromboembolic events observed in infected individuals.

EVs serving as carriers of inflammatory mediators, may exacerbate immune responses and contribute to the cytokine storm observed in severe cases. Indeed, alveolar macrophage-derived EVs play a pivotal role in worsening the inflammatory response by facilitating the influx of pro-inflammatory molecules and immune cells into the lungs. This process amplifies lung injury and respiratory distress, making EVs critical components in COVID-19 pathophysiology.

Notably, EVs expressing CD31 and P-selectin glycoprotein ligand-1 (PSGL-1) were found to be abundant in COVID-19 patients, highlighting endothelial damage and dysfunction, which are crucial factors in disease severity (Barrion et al., 2024).

Understanding their role can provide new insights into therapeutic strategies aimed at mitigating severe inflammatory responses in affected patients.

Challenges in EVs applications

The clinical translation of EVs-based technologies is challenging, and stringent regulatory and quality standards must be met to ensure safety, efficacy, and consistencies. One of the key criteria is the development of standardized protocols for their isolation and characterization, which is crucial to ensure reproducibility and comparability among different studies. This standardization is essential for their reliable use as biomarkers. To address this, guidelines like the Minimal Information for Studies of Extracellular Vesicles (MISEV) support such attempt. Moreover, good manufacturing practice (GMP) compliance is important for ensuring the quality, purity, potency, and stability of EVs-based products, which is highlighted by regulatory agencies such as the U.S. Food and Drug Administration (FDA) (Wang et al., 2024).

Like other biological therapies, these products are governed by established regulatory frameworks that demand extensive preclinical studies and well-designed clinical trials to establish safety and therapeutic potential.

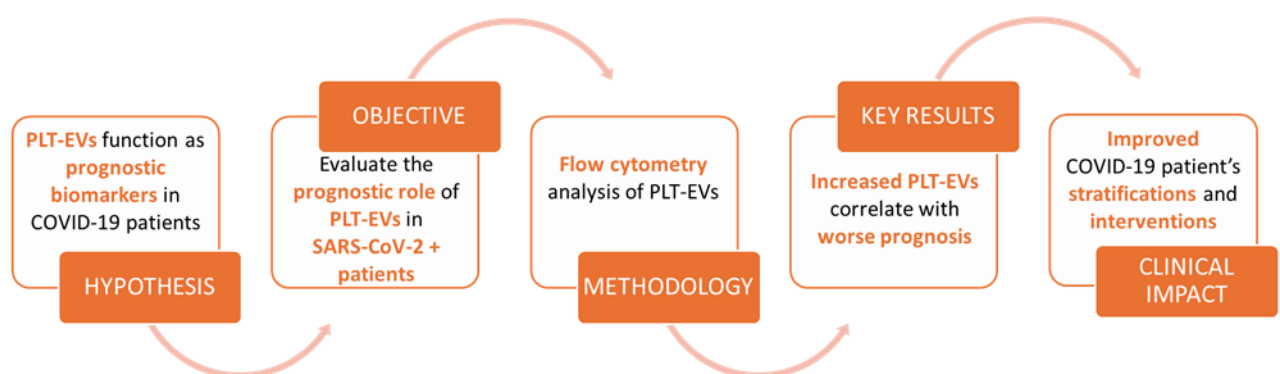
In addition, the successful placement of EVs-based technologies in clinical workflows relies on the establishment of stringent quality control parameters, scalable manufacturing processes, and the endorsement of agencies such as the FDA and European Medicines Agency (EMA) (Nieuwland

et al., 2023).

EVs-based technologies demonstrate great potential as tools for diagnostics and therapeutics to overcome these challenges and bring innovation in precision medicine and biomarker-based disease management.

AIM 1

During the SARS-CoV-2 pandemic, researchers worldwide shifted their focus to studying COVID-19. Likewise, our lab redirected its efforts toward COVID-19 research too. Particularly, we focused on searching for easy and reliable diagnostic and prognostic biomarkers for COVID-19, hopefully translatable in the hospital's routine. Previous findings from our lab identified in platelets- derived EVs (PLT-EVs), a diagnostic biomarker of SARS-CoV-2 infection. Based on these previous findings, we hypothesized that PLT-EVs could also serve as prognostic tools, helping clinicians distinguish between moderate and severe COVID-19 cases. Despite the promising role of PLT-EVs in diagnosis, their prognostic value remains largely unexplored, representing a critical knowledge gap. The general aim of **Chapter 1** of this thesis was to evaluate whether PLT-EV levels in hospitalized COVID-19 patients—measured after one week of ICU admission—could predict severe clinical outcomes. By addressing this question, we sought to determine whether PLT-EVs could function as early warning biomarkers, allowing for improved patient stratification and timely intervention strategies. However, challenges remain, including the standardization of EV quantification methods, the influence of confounding clinical variables, and the need for validation in larger, multi-center cohorts. These limitations highlight the necessity for further research to establish PLT-EVs as a robust prognostic tool for COVID-19 management.



Flowchart of Chapter 1.

Summary PAPER 1

PLT-EVs are the most abundant circulating vesicles, have been shown to increase in various human diseases, including viral infections such as influenza and HIV. In the case of influenza, it has been proposed that the H1N virus activates platelets, leading to the release of EVs, while in HIV, PLT-EVs may serve as shuttles facilitating viral propagation.

Our previous findings demonstrated that PLT-EVs represented a diagnostic marker for SARS-CoV-2 infection, detected by employing a fast and reliable method in flow cytometry (Cappellano et al., 2021).

The objectives of this paper were: (i) to validate our previous findings regarding the use of PLT-EVs as a biomarker in a third cohort of COVID-19 patients and (ii) to evaluate their prognostic significance in hospitalized patients, after one week from the admission in the ICU (**paper 1**). We collected peripheral blood from COVID-19 patients at two different time points: at the time of admission to the emergency room (T0) and after one week of hospitalization (T7) and we respectively counted PLT-EVs levels, by flow cytometry-based method. When stratifying COVID-19 patients based on the mortality score at T0, we identified two distinct patterns of patients: one group showed a decrease in the PLT-EVs count, during the first week of hospitalization, while the second displayed increased levels. The latter group showed an unfavorable outcome during hospitalization. This observation strongly supports the possibility of a potential role of PLT-EVs as prognostic biomarkers in severe COVID-19.

Research Article

Worse Disease Prognosis Is Associated to an Increase of Platelet-Derived Extracellular Vesicles in Hospitalized SARS-CoV-2 Patients

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Platelet-derived extracellular vesicles (PLT-EVs), the most abundant circulating EVs, have been found to be increased in several human diseases, including viral infections. Recently, we documented that PLT-EV counts are higher in SARS-CoV-2+ patients, enrolled during the first two waves of COVID-19, occurred in Italy last year, and we suggested PLT-EVs as a biomarker of SARS-CoV-2 infection. The present study is aimed at testing the ability of PLT-EV levels, measured at hospital admission and within one week of hospitalization, to predict patient's outcome. We applied an easy, fast, and reliable method, based on flow cytometry, for the detection of PLT-EVs in unmanipulated blood samples. In a cohort of SARS-CoV-2 patients, enrolled during the third wave of COVID-19 in Italy, we confirmed that PLT-EV counts are higher in comparison to healthy controls. Moreover, their number is not affected by prehospitalization treatment neither with heparin nor with steroids that are recommended by WHO guidelines. Noteworthy, we identified two pattern of patients, those who increased their PLT-EV level during first week and those reducing it. The former group represented more compromised patients, with higher 4C score, and unfavorable outcome. In conclusion, our new findings would suggest that a worse evolution of the disease is linked with increasing PLT-EV levels in the week after hospital admission.

1. Introduction

Extracellular vesicles (EVs) are microparticles that bud from all cells' surface and are transported in body fluids. EVs have been classified by size, biogenesis, and cell type of origin; currently, EVs are categorized into three main types: (1) microvesicles (MVs) (100–1000 nm in diameter), (2) exosomes (20–150 nm), and (3) apoptotic blebs (1000–5000 nm) [1, 2]. EVs circulate and act in the extracellular environment and can resist to the enzymatic digestion due to the presence of their lipid membrane, which is highly

enriched in cholesterol, sphingomyelin, annexin, phosphatidylserine, and glycosphingolipids [3]. EVs are deeply involved in the mechanisms of cell-to-cell communication, which is based on different processes including horizontal transfer of several molecules [4].

EVs are stable and can be detected in several body fluids such as blood, saliva, urine, and breast milk [5]. By originating from the parental cells, they may resemble the current state of disease because they carry the same molecules (e.g., miRNA, mRNA, and lipid). Therefore, EVs have attracted attention as they represent an easily obtainable object of

study, through liquid biopsies. Considering the minimally invasive nature of the sampling process and their easy accessibility, EVs are emerging as diagnostic/prognostic biomarker for several human diseases [6].

According to the International Society for Extracellular Vesicles (ISEV) guidelines, several methods have been recommended to identify, characterize, and isolate EVs [7]. These include ultracentrifugation, size-exclusion chromatography, immunoaffinity capture, and microfluidics. These methods involve different steps of centrifugation, precipitation, and ultracentrifugation, and they require manipulation of the samples (from blood to plasma) which would represent a stress condition for the cells which in turn might increase EVs release per se; thus, the amount of EVs present in a sample may be distorted. In our recent publication, we established a quick method (1 hr) for the quantification of platelet- (PLT-) derived EVs in fresh blood without sample's manipulation using flow cytometry [8].

At the beginning of 2020, coronavirus disease-19 (COVID-19) was declared a pandemic by the World Health Organization (WHO). Nowadays, it is well recognized as a complex disease involving high levels of inflammation and thrombosis, and PLT hyperactivation correlates with disease severity [9]. Though many efforts have been done, few biomarkers for COVID-19 have been identified; however, these are individually poorly specific, and novel biomarkers are needed to better predict patient outcome.

We recently showed that PLT-EV counts were increased in two independent cohorts of SARS-CoV-2 patients hospitalized during the first two waves of COVID-19 pandemic, occurring in Italy between April 2020 and December 2020. Therefore, we suggested that PLT-EVs could be used as a biomarker of SARS-CoV-2 infection [8]. Given that we applied a fast and reliable method for EV count and easy to implement in hospital clinical laboratories, in this study, we aimed at testing the predictive value of PLT-EVs in COVID-19 evolution during the first week of COVID-19 patient hospitalization.

2. Methods

2.1. Patients. During the third wave of COVID-19 pandemic occurred between December 2020 and April 2021, we enrolled all SARS-CoV-2+ patients hospitalized at University Hospital "Maggiore della Carità" (Novara, Italy) with blood samples available and who signed the informed consent to participate in the study. SARS-CoV-2 infection was confirmed by reverse-transcriptase polymerase chain reaction (RT-PCR). Blood was withdrawn into sodium citrate collection tubes at two different time points: at the time of admission at the emergency room (T0) and after one week of hospitalization (T7). Blood parameters were measured by using the Sysmex XN-2000™ Hematology System (Sysmex, Kobe, Japan) at Hospital "Maggiore della Carità" at the time of enrollment, while EV analysis was performed within few hours after blood withdrawal at the indicated time points above. Moreover, information on gender, age, comorbidities, and prehospitalization treatment with steroids and heparin was collected. The 4C score was also calculated

to evaluate the mortality risk at T0 of the enrolled patients.

A sample of healthy controls (HC), identified among hospital workers, was also included in the study.

The study was approved by local ethic committee (CE67/20); written informed consent was obtained from the patients or their legal representative.

2.2. Flow Cytometry. EVs were quantified by flow cytometry from the whole blood of SARS-CoV-2+ patients, as previously described by us [8]. Briefly, we used a custom kit (Becton and Dickinson, NJ, USA) containing a cationic probe which stains lipophilic membrane of EVs and a viability dye (i.e., phalloidin) which identifies intact and viable circulating EVs. Intact EVs were then stained with a combination of three monoclonal antibodies (Becton and Dickinson, NJ, USA) to detect EVs released by the most abundant cell populations in the blood: leukocyte-derived EVs (CD45+), endothelial-derived EVs (CD31+), and PLT-derived EVs (CD31+ CD41a+). Samples were acquired using FACSymphony A5 (Becton and Dickinson, NJ, USA), and flow cytometry data were analyzed using the FACSDiva software (Becton and Dickinson, NJ, USA). The gating strategy is shown in [8]. The count of EVs/ μ L was obtained using the following formula:

$$\text{EVs}/\mu\text{L} = \frac{\text{No. of EV events for a given population} * \text{dilution factor}}{\text{Acquired volume}} \quad (1)$$

2.3. Statistical Analysis. Descriptive statistics were used to summarize patients' characteristics. Categorical variables were reported as absolute frequencies and percentages while numerical variables as median and first (Q1) and third quartiles (Q3) since not normally distributed. D'Agostino and Pearson test was used to assess the normality distribution of numerical variables. Mann-Whitney test was used to compare the average platelets' EV count between patients with SARS-CoV-2 and HC and 4C score between subjects increasing or decreasing platelets' EVs between T0 and T7, while Wilcoxon sum rank test was used to compare average platelets' EV count between T0 and T7. Finally, Kruskal-Wallis test was used to compare average platelets' EV count in patients assuming corticosteroid and heparin alone or in combination. All tests performed were two tailed, and the type one error was set to 0.05. All analyses were performed using Prism version 8.4.3.

3. Results

We enrolled 78 SARS-CoV-2+ patients hospitalized at University Hospital "Maggiore della Carità" (Novara, Italy). Table 1 summarizes the demographic and clinical data of the cohort of hospitalized SARS-CoV-2+ patients. PLT-EVs were quantified in unmanipulated blood, as previously described by us [8].

We found that PLT-EV counts were higher in SARS-CoV-2 patients compared to HC, regardless of heparin or steroid prehospitalization treatments (Figure 1). These

TABLE 1: Demographic and clinical features of hospitalized SARS-CoV-2+ patients.

	Median (Q1-Q3)
Age (years)	67 (58-80)
Gender (M/F), N (%)	55/23 (71%)
WBC ¹ ($\times 10^3/\mu\text{L}$)	8.61 (6.36-11.26)
Lymphocytes ($\times 10^3/\mu\text{L}$)	0.81 (0.59-1.13)
Creatinine (mg/dL)	0.86 (0.74-1.16)
AST ² (mU/mL)	39 (30-49)
ALT ³ (mU/mL)	34.5 (20-47.5)
CRP ⁴ (mg/dL)	6.44 (2.48-13.95)
PCT ⁵ (ng/mL)	0.13 (0.05-0.28)
PLTs ($\times 10^3/\mu\text{L}$)	216 (173-300)
PT-INR ⁶	1.02 (0.96-1.08)
Ferritin (ng/mL)	564 (171-1106)
LDH ⁷ (U/L)	587.5 (495-752)
D-dimer ($\mu\text{g/L}$)	1070 (609-1471)
IL ⁸⁻⁶	14.75 (6.4-27)
SpO ₂ /FiO ₂	427.5 (400-447)
PaO ₂ /FiO ₂	247 (214-295)
SpO ₂	89 (85-94)
FiO ₂	21 (21-21)
Respiratory rate	25 (15-30)

¹White blood cell, ²aspartate aminotransferase, ³alanine aminotransferase, ⁴C-reactive protein, ⁵procalcitonin, ⁶prothrombin time-international normalized ratio, ⁷lactate dehydrogenase, and ⁸interleukin.

results strongly confirmed our previous findings showing PLT-EVs as biomarker of SARS-CoV-2 infection [8].

To investigate variation over time in PLT-EV counts, we monitored the kinetic of their absolute counts at the time of admission to the emergency room (T0) and one week after hospitalization (T7). 38/78 patients dropped out during the follow-up because of hospital discharge or death, and only 40/78 (black dots shown in Figure 1(a)) completed the study. 67.5% of the patients within this group were males (mean age 63.5 years (SD 14.1 years)), and the 4C mortality score [10], evaluated at T0, was 9.4 (SD 4.4); the median duration of symptoms before hospital admission corresponded to 7 days (Q1-Q3 4-9.5); percentage of SARS-CoV-2+ patients treated with steroids and heparin before hospital admission was 60% and 27.5%, respectively; 45% of patients were admitted to intensive care unit while 55% were admitted to other wards with intermediate intensity of care; the 30-day mortality was 17.5%.

With regard to the absolute count of PLT-EVs measured over time, we identified two different patterns in our population: 23 out of 40 patients (subgroup A) showed a statistically significant increase of PLT-EVs from T0 to T7, while 17 out of 40 patients (subgroup B) showed a significant decrease (Figure 2).

Interestingly, by stratifying patients based on their 4C mortality score [4] calculated at T0, we found that more compromised patients presented a significant increase of PLT-EV count during the hospital stay (subgroup A) (Figure 2).

4. Discussion

PLT-EVs, the most abundant circulating EVs, have been found to be increased in several human diseases, including viral infections, such as influenza and HIV [11–13]. In the former, it has been suggested that influenza H1N virus would activate PLTs which in turn release EVs [11]; in the latter, PLT-EVs might act as shuttle in the propagation of the virus [13]. PLT-EVs have been shown to contain SARS-CoV-2 RNA [14], as well as the exosomal cargo, and it was suggested that the virus might use the endocytosis route to spread infection [15].

Recently, we showed that the PLT-EV count is higher in SARS-CoV-2+ patients admitted to the emergency room, in comparison with SARS-CoV-2- patients and HC. We demonstrated that PLT-EVs also have a good performance as a diagnostic biomarker in discriminating SARS-CoV-2+ from SARS-CoV-2 patients, and we hypothesized that they might be involved in thromboembolism and vascular leakage, which are clinical hallmarks of SARS-CoV-2 infection [8].

The involvement of PLT-EVs in COVID-19 has been also shown by four independent groups [14, 16–18]. Zaid et al. evaluated PLT-EVs in PLT-free plasma of SARS-CoV-2+ patients. They found that PLT-EV levels were increased in the nonsevere SARS-CoV-2+ group in comparison with severe one; however, upon normalization on PLT number, they found that PLT-EVs were also increased in severe patients [14]. Guervilly et al. showed that tissue factor bearing PLTs and EVs were higher in COVID-19 patients who require mechanical ventilation [17]. Another study showed that half of EV population in COVID-19 patients was of PLT origin, and their counts were increased in comparison with HC [18]. PLT-EV counts were also found to be even more increased 30-day postdischarge, after COVID-19 remission [16].

In this study, we confirmed our previous findings [8] and evaluated for the first time, the kinetic of PLT-EVs within the acute phase, during the hospital stay.

As a detection/quantification method for EVs, we applied flow cytometry combined to patented probes, which is a technique, among others, recommended by the International Society for Extracellular Vesicles (ISEV) [7]. We used a protocol already validated by other groups [19–22] and, recently, by us in COVID-19 [8]. This protocol shows the advantage to probe directly and quickly PLT-EVs in fresh unmanipulated blood, since platelets—especially when under stress conditions (i.e., caused by ultracentrifugation)—may become activated, thus releasing EVs *per se*. Also, PLT-EVs might be partially lost when plasma is double centrifuged to remove platelets or due to repeated freeze/thaw cycles on stored plasma specimens [23].

Considering that those stressful conditions could interfere with the biological significance of the results obtained, the use of unmanipulated blood would be preferable and may provide more reliable data, as we did in our work.

We identified two subgroups of patients showing either an increase or a decrease of PLT-EV counts after one week of hospitalization compared to PLT-EV counts assessed at the time of admission to the emergency room. Since the

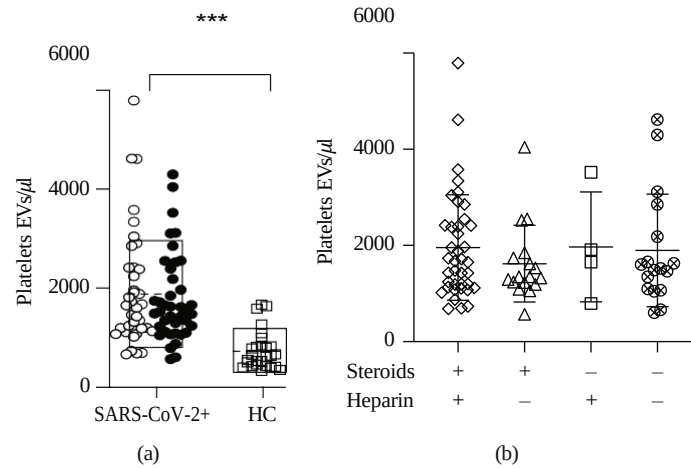


FIGURE 1: PLT-EV counts are higher in SARS-CoV-2+ patients regardless of heparin and steroid treatments. (a) Dot plot showing the absolute count of PLT-EVs in SARS-CoV-2+ patients enrolled during the 3rd wave ($n = 78$) and HC ($n = 27$); black dots indicate patients hospitalized for one week; empty boxes show mean $\pm$ SD; (b) PLT-EV counts at T0, stratified accordingly to prehospital treatments with/without heparin and steroids. For statistical analysis, D'Agostino and Pearson normality test was used before to perform Mann–Whitney and Kruskal–Wallis test. *** $p < 0.001$.

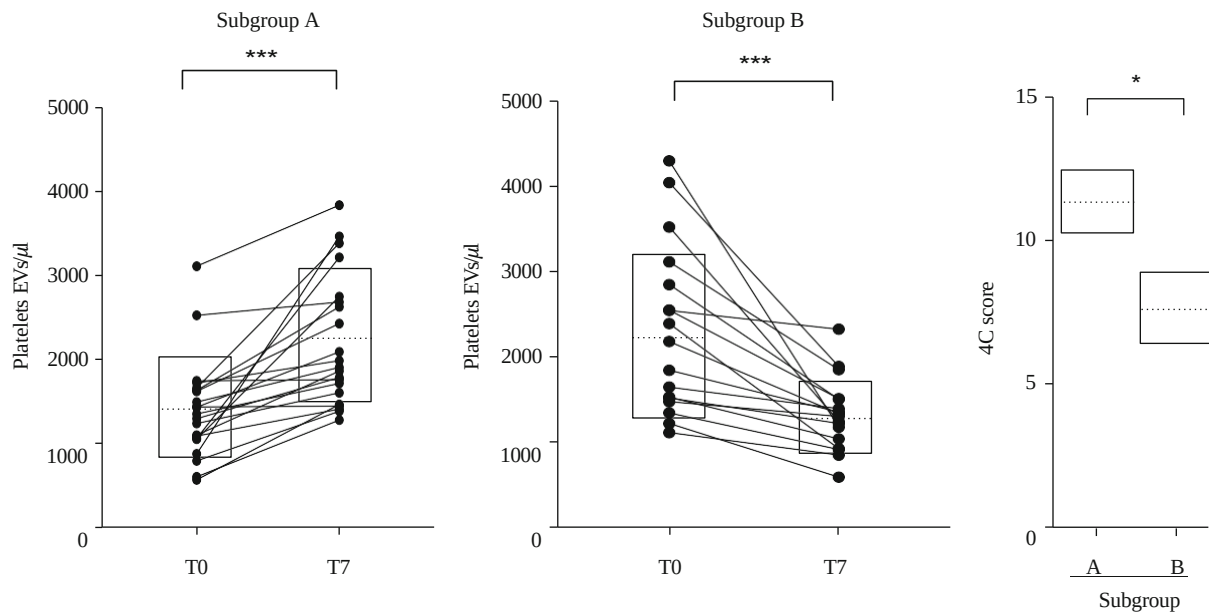


FIGURE 2: PLT-EV count is higher in more compromised SARS-CoV-2+ patients within one week of hospitalization. Line graphs identifying the two subgroups of patients (A and B, previously shown in Figure 1, black dots) showing either an increase or a decrease of PLT-EV counts at T7 and the 4C score evaluated at T0. For statistical analyses, D'Agostino and Pearson normality test was used before performing Wilcoxon sum rank and Mann–Whitney tests. *** $p < 0.001$ and * $p < 0.05$.

recent guidelines for COVID-19 suggested to consider the 4C mortality score [10], which was not evaluated by Zaid et al. or by us [14] previously, we applied it to this study. This score refers to patient demographics, clinical observations, and blood parameters that are commonly available at the time of hospital admission by accurately characterizing the population of patients at high risk of death in hospital [10]. Interestingly, we found that more compromised patients (with an increased 4C mortality score) presented a significant increase of PLT-EV counts during the hospital stay.

During the COVID-19 pandemic, the use of the anticoagulant heparin and the anti-inflammatory dexamethasone has been shown as promising tools for the management of

severe COVID-19 patients' symptoms [24]. We documented that prehospital treatment with steroids or heparin did not interfere with PLT-EV counts. Since we did not find in our cohort any correlation between absolute PLT-EV and platelet counts or d-dimer values (not shown), our findings lead us to hypothesize that the increase in PLT-EVs was related to the inflammatory response triggered by SARS-CoV-2 infection, rather than to PLTs or coagulation activation, though the role of PLTs in thromboinflammation is well documented [25].

Antiphospholipid antibodies (aPL) are detected in nearly half of SARS-CoV-2+ patients, and their prevalence was shown to be even higher in severe ones; but it is still debated

if aPL positivity is just an epiphenomenon of an upregulated inflammatory state triggered by COVID-19 or a true player in the thrombotic storm of severe forms [26]. Potentially, aPL may target PLT-EVs and thus contribute to inflammatory state in COVID-19.

A recent study reported a higher expression of platelet CD142 marker (i.e., tissue factor) onto surface of serum-derived EVs in SARS-CoV-2+ patients compared with SARS-CoV-2-, both developed pneumonia. Interestingly, CD142 displayed higher biological activity only in SARS-CoV-2+ patients [27]. These findings might suggest that PLT-EVs initiate the extrinsic pathway of coagulation and, thereby, directly contribute to the high thrombotic risk in COVID-19. Lastly, since PLT-EVs are negatively charged, they may sustain the propagation of coagulation [28].

5. Conclusions

Our findings suggest a relationship between prognosis of SARS-CoV-2 and absolute count of PLT-EV absolute count and kinetics: these may be seen as biomarkers to monitor severity of SARS-CoV-2 and/or pathogenetic actors. By applying our fast and reliable method for EV count, which could easily be implemented in hospital clinical laboratories, PTL-EV counts could be translated into clinical practice. Of note, our method is not intended to replace PCR for viral quantification but it can rather represent a tool for clinicians to predict worsening of COVID-19 condition. Nevertheless, we firmly believe that further studies, in different and bigger cohorts, are still needed to confirm our data and clarify the potential pathophysiological role of PLT-EVs in the development of COVID-19.

Data Availability

The data that support the findings of this study are available upon request from the corresponding author.

Ethical Approval

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of Ospedale Maggiore della Carità di Novara (protocol code 325/CE and date of approval 23-03-2020).

Consent

Informed consent was obtained from all subjects involved in this study.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

G.C., D.R., M.M., C.V., and E.C. performed the experiments and analyzed the data. M.G.C. and F.G. collected the laboratory data. L.S., G.C., and L.C. performed the statistical

analyses. F.G., M.G.C., R.V., and L.C. provided the patient samples and collected the clinical data. R.V. and A.C. conceived the study. G.C. and A.C. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Chapter 2: Involvement of the T cells compartment in post-Acute Sequelae of SARS-CoV-2 (PASC) patients

Premise

The SARS-CoV-2 pandemic continues to profoundly affect global health, not only through the acute phase of infection but also via its long-term consequences. A substantial number of individuals who recover from COVID-19 faced lingering symptoms that persisted for months after the initial illness, significantly impacting their quality of life. The symptoms included mainly fever, dyspnea, fatigue etc., and although they were not dangerous for the patient's life, they had a huge impact on work, home activities and social life. This syndrome was defined post-Acute Sequelae of SARS-CoV-2 (PASC). Moreover, neither vaccination nor infection generated lasting immunity, so reinfections were occurring much more rapidly and having a more severe impact than many expected. Vaccines were far from conferring full protection against PASC. Although some reports indicate that a smaller percentage of people develop PASC from Omicron compared to earlier variants, the number of people infected and reinfected means a lot more people will experience PASC syndrome going forward. Over time, some PASC patients recovered, but a large study reported that in those still experiencing symptoms two months after the acute illness, 85% were still not well after one year.

Our laboratory, together with other partners, won a project named Technomed-hub, that allowed us to shed light on this new emerging phenomenon. The general aim of this project was to build a technological platform 2.0 to support medical research, based on the integration of data derived from multi-omic platforms with self-learning algorithms, data mining and big data analytics.

Thanks to the Maggiore della Carità hospital, we were able to contact all individuals who had recovered from COVID-19 but continued to report similar symptoms one year later.

In this context, our general aim was to characterize and study the adaptive immune system of these patients, to find a specific immune signature, that will allow us to understand the causes of the presence of this syndrome. Our initial approach focused on studying the most abundant circulating EVs, through the method previously applied for our COVID-19 research. However, despite the number of patients included in the study, we did not find significant results. Consequently, we decided to shift our analysis and focus on peripheral blood mononuclear cells (PBMCs) from these patients to gain deeper insights into their adaptive immune system.

We performed activation induced marker (AIM) test and dextramer analysis to identify potential immune signatures, aiming to better understand the adaptive immune response in these patients and its role in the persistence of symptoms.

Background

Post-acute sequelae of SARS-CoV-2 infection (PASC) syndrome

Among patients who recovered from COVID-19, unfortunately, there was a large portion that experienced a wide range of persistent symptoms that lasted months after the acute phase of the illness (Andrade et al., 2021). The WHO estimates that 10-20% of those recovering from the SARS-CoV-2 infection manifest at least one symptom after several months. This condition has been dubbed as PASC syndrome (Hastie et al., 2023).

Post-acute sequelae of SARS-CoV-2 (PASC) syndrome is a multi-systemic disorder characterized by pulmonary, immunological, cardiovascular, neuropsychiatric, gastroenterological, hepatic, renal, endocrine, and dermatological sequelae (**Figure 7**).

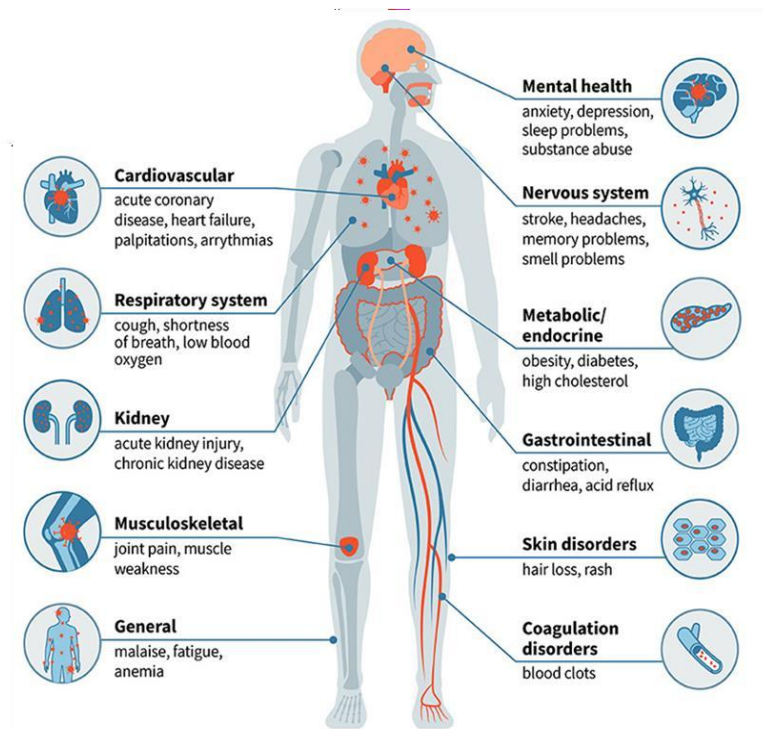


Figure 7. PASC symptomatology. (<https://www.esanum.it>)

While it is not typically life-threatening, it can significantly affect a patient's daily activities and social life (Bellan et al., 2021; Rivas-Vazquez et al., 2022). This syndrome is not related to the severity of the acute illness, in fact it can affect all types of COVID-19 patients (from mild to severe), even the asymptomatic ones.

Even though PASC is a relatively new syndrome, the concept of persistent, chronic symptoms following a viral illness is not entirely new. In fact, similar conditions were already described in the past and were known as “post viral syndromes”. This latter was characterized by the

persistence of a wide range of chronic symptoms such as fatigue, muscle weakness, excessive sweating, mental exhaustion and poor concentration with the lack of a recognizable precipitating cause (Bannister, 1988). An example of virus, that after the primary infection, can lead to the development of the post viral syndrome, is the Ebola virus. Indeed, there is a portion of patients that survived from Ebola virus disease (EVD) but then dealt with physical and psychological symptoms like extreme fatigue, menstrual cessation, clouded vision, hair loss and deafness, which can last for 12 months or more (Wilson et al., 2018).

Since lungs were the target organ of SARS-CoV-2 infection, one of the main hallmarks of PASC is the reduction of diffusing capacity of the lung for carbon monoxide (DLCO). Indeed, some patients, who recovered from COVID-19, had ventilator dependence and difficulty in oxygen removal, accompanied by lung function abnormalities, and other manifestations of pulmonary fibrosis (Han et al., 2024). Moreover, at 6 months the majority of PASC patients suffered from a reduction of DLCO, followed by a reduction of total lung capacity (Parker et al., 2021). One year after hospitalization, a reduction in DLCO was also noted independently from the severity of acute illness among 196 patients, a DLCO <80% was observed in 96 (49.0%), and a DLCO <60% was observed in 20 (10.2%) (Bellan et al., 2021). Others common symptoms were the presence of residual symptoms, which comprise fever, cough, dyspnea, asthenia, diarrhea, dysgeusia, anosmia, arthralgia and myalgia and the alteration of the 2 minutes walking test (2MWT). A meta-analysis revealed that the most frequent symptoms between 3 and 6 months after the infection's recovery, were fatigue, dyspnea, sleep disorder and concentration difficulty. Between 6 to 9 months were effort intolerance, fatigue and sleep disorder. In addition, between 9 and 12 months there were fatigue, dyspnea and loss of taste. Considering several studies, sweating, chest pain, anxiety and depression were also described with a prevalence lower than 20% (Michelen et al., 2021). Notably, the risk of developing PASC has been found to be twice prevalent in women compared to men, but only until 60 years of age, when the risk becomes similar. In fact, old age was also associated with the development of this condition. The difference among genders is consistent with the different immunological responses described in the acute stage of the illness; indeed, females have a stronger immunological response that may be one of the major causes of PASC, as it happens for autoimmune diseases (Ortona and Malorni, 2022). In addition, the presence of more than 5 symptoms during the acute disease and the presence of comorbidities seem to be associated with an increased risk of long covid (Raveendran et al., 2021).

Adaptive immune system in PASC syndrome

Several mechanisms have been pointed out as possible causes underlying PASC syndrome. The first proposed mechanism is immune dysregulation due to Spike protein's ability to act as superantigen, triggering an unspecific activation of the T cells. Activated T cells can trigger broad B cells activation with the consequent production of autoantibodies. The second regards the formation of neutralizing antibodies targeting IFN I, potentially triggering an autoimmune response. The last one regards the viral persistence inside the host's organism, which causes a continuous inflammatory status that could lead to autoimmunity (Brodin et al., 2022).

These mechanisms are interconnected as they all share a common factor: the adaptive immune system. This system seems to serve as the central link between these diverse pathological processes, implying that immune dysregulation plays a key role in the development of PASC. Some studies have been conducted to analyze the adaptive immunity in patients with persistent symptoms, however using distinct techniques, considering the response to different epitopes, at various time points, and again comparing different groups of patients with different features. Therefore, the low quantity and the high variability in research make it difficult to fully understand whether alterations in the adaptive immune system, and especially in the T cells response, are involved in this condition.

Recent studies have investigated the differences in the adaptive immune responses between healthy controls (HC) and PASC patients. A study by Littlefield et al., demonstrated that PASC patients with pulmonary impairment had higher frequency of IFN- γ and TNF- α producing SARS-CoV-2 specific T cells in peripheral blood compared to patients without PASC. Notably, these TNF- α producing SARS-CoV-2 specific T cells, had the highest levels of Ki67, indicating their elevated proliferation capacity (Littlefield et al., 2022). Further investigations by *Phetsouphanh et al.* and *Su et al.* highlighted a T cell dysregulation in PASC patients. Specifically, *Phetsouphanh et al.* found that activated myeloid cells, monocytes and CD8 T cells were higher in PASC patients after 8 months after recovery from COVID-19. Moreover, they found in the plasma of those patients increased and persistent levels of IFN I and III and IL-6, suggesting a sustained organism's inflammation (Phetsouphanh et al., 2022). Similarly, *Su et al.* through a multi-omics approach discovered that PASC patients after 3 months after the acute illness, had antibodies against the SARS-CoV-2 receptor-binding domain (RBD), indicating the resolution of the infection. Furthermore, the authors found that these patients

had new expanded clonotypes of cytotoxic CD8 and cytotoxic CD4 cells. Transcriptomic analysis further revealed that genes associated with immune cells, previously found to be enriched in COVID-19 patients, were still high in PASC patients. For instance, they exploited increased cytotoxic CD4 T cells, proliferative exhausted T cells and MDSCs, which usually are predictors of mortality in COVID-19 patients (Su et al., 2022). These alterations in the immune cell profile are believed to contribute directly to the chronic inflammatory environment seen in PASC, exacerbating symptoms and prolonging recovery.

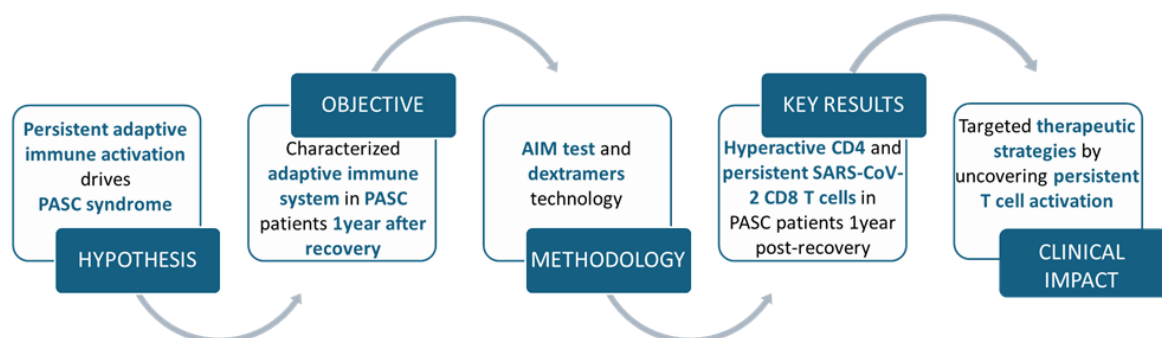
A study by Guo *et al.*, highlighted the crucial role of SARS-CoV-2-specific T-cell responses in protecting against re-infection and providing long-term immunological memory. Additionally, various research efforts have evaluated these T-cell responses, examining their persistence and effectiveness 6 to 10 months after viral clearance (Guo et al., 2024).

In their work, Opsteen *et al.* evaluated SARS-CoV-2 specific T-cell response through the activation induced marker (AIM) assay, 6 months post-infection. They found that PASC patients had an increased and sustained SARS-CoV-2-specific CD4⁺ T-cell response overtime, with respect to those who recover from the infection without any symptoms (Opsteen et al., 2023). These findings, taken together with other data, point to the persistence of viral antigen stimulation that continuously triggers the adaptive immune system.

AIM 2

The second objective of this thesis was to characterize the adaptive immune system of PASC patients, one year after the recovery from COVID-19. We used the AIM test and dextramers technologies to perform an in-depth analysis of the T cell-mediated immune response. Specifically, with the AIM test approach we stratified PASC patients and HC (i.e. COVID patients enrolled at the study with the PASC one, that after 12 months did not display residual symptoms) in “*responders*” and “*non responders*” based on their ability to mount an immune response against SARS-CoV- 2 spike peptides. *Responders*, characterized by activation upon encountering spike peptides, exhibited a well-functioning memory T cell compartment. Conversely, *non-responders* displayed impaired memory T cell functionality, failing to elicit an adequate immune response. Beyond the functionality of the immune memory, we also evaluated the magnitude and the multifunctionality of this response, to deeply describe it (**Paper 2**).

Moreover, we also investigated specific SARS-CoV-2 CD8⁺ T cells in PASC patients one year after the recovery. It is well established that antigens-specific CD8⁺ T cells have a crucial role in fighting viral infections. Indeed, they proliferate and activate during viral infections and decrease consequently with the resolution of the infection. However, evidence suggested that SARS-CoV-2 CD8⁺ T cells remain activated in the organism of these patients, leading to the establishment of a persistent immune activation, which can result in the formations of the symptoms that characterized PASC syndrome. We investigated them through dextramer technology which allows us to quantify and characterize SARS-CoV-2 CD8⁺ T cells and their subsets in PASC patients (**Paper 3 in preparation**). This study introduces innovative methodologies by combining the AIM test and dextramer technology to provide a comprehensive analysis of the adaptive immune response in PASC patients.



Flowchart of Chapter 2.

Summary PAPER 2: accepted for publication

PASC is a multisystemic disorder affecting pulmonary, immunological, cardiovascular, neuropsychiatric, gastroenterological, hepatic, renal, endocrine, and dermatological systems. Although not typically life-threatening, it can significantly impact on a patient's daily activities and social life. The pathological mechanisms underlying PASC remain poorly understood, with hypotheses suggesting viral persistence, immune system activation by SARS-CoV-2 superantigens, and autoimmune responses, all involving the adaptive immune system. Adaptive immunity plays a crucial role in protecting against reinfections and recent evidence suggests the involvement of T cells in PASC syndrome. This work aimed to evaluate the cellular memory in PASC patients one year after the hospital discharge and correlate it with different clinical parameters, to find a possible fingerprint associated with PASC syndrome.

We enrolled 215 patients who had recovered from COVID-19, 365 days post-hospital discharge. PBMCs from PASC patients (n=64) and HC (n=24) were *in vitro* stimulated with a pool of spike peptides. Specific CD4⁺ and CD8⁺ T cell responses were evaluated by performing the AIM assay through flow cytometry, which allows determination of long-term protective immunity. Our results indicated a significant activation of the CD4⁺ T cell compartment, with a higher proportion of responders in PASC patients (78%) compared to HC (54%). Within the responder group, central memory (CM) T cells expressing IL-2 and TNF- α were increased in PASC patients. Clinical correlations revealed that patients with reduced diffuse lung capacity for carbon monoxide (DLCO) and those with residual symptoms exhibited higher levels of SARS-CoV-2-specific T cell responses.

One main limitation of the study is the lack of tracking immune dynamics overtime, since we have only data on the immune system one year after the recovery from the infections, with no intermediate time points.

Overall, our study contributes to existing research in the field that persistent immune activation may be a driver of PASC syndrome. The hyperactivation of CD4⁺ compartment, one year post infection would suggest that immune dysregulation does not resolve with viral clearance, but continues, potentially leading to chronic inflammation and tissue damage.

Additionally, understanding the potential role of residual viral antigens in sustaining immune activation could open new therapeutic avenues for managing PASC-related immune dysregulation.

Post-COVID-19 sequelae are associated with sustained SARS-CoV-2-specific CD4+ immune responses.

Running head: “CD4⁺ response in PASC”

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Abstract

Background: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has led to widespread post-acute sequelae of COVID-19 (PASC), affecting multiple body systems. Despite its prevalence, PASC's pathogenesis remains unclear, with hypotheses suggesting viral persistence, immune activation, and autoimmune responses among the pathogenetic mechanism. This study aimed to evaluate T cell memory response in PASC patients, one year post-hospital discharge and correlate it with clinical parameters to identify a potential PASC-associated fingerprint.

Methods: Peripheral blood mononuclear cells (PBMCs) from PASC patients and healthy controls (HC) were stimulated with a pool of spike peptides. CD4⁺ and CD8⁺ T cell responses were evaluated by flow cytometry using the activation-induced markers assay (AIM).

Results: Findings showed significant activation of the CD4⁺ T cell compartment, with a higher proportion of responders among PASC patients. Central memory (CM) T cells expressing pro-inflammatory cytokines were more prevalent in *responders*. Clinical correlations revealed higher SARS-CoV-2-specific T cell responses in patients with reduced diffuse lung capacity for carbon monoxide (DLCO) and residual symptoms.

Conclusion: These immune changes, especially in CM T cells, could play a pivotal role in PASC's development and persistence, impacting patients' daily lives.

Keywords: post-acute sequelae (PASC); CD4⁺ T-cell response; activation-induced markers (AIM); viral persistence; cytokine production; multifunctionality; immune fingerprint.

Highlights:

- The research revealed a defective CD8⁺ T cell response compared to the CD4⁺ subset one year after primary infection. PASC subjects showed an increased proportion of persistent memory response in the CD4⁺ subset, particularly in central memory (CM) CD4⁺ AIM⁺ cells producing cytokines.
- Subjects with impaired lung function (DLCO <80%) and persistent symptoms showed increased magnitude of immune activation. The study found hyperactivation of CD4⁺ T-cells at different maturation stages and with diverse multifunctionality features in these individuals.

Authorship: Conceptualization A.C., methodology C.V., C.B.M., D.R.; software, A.G.; formal analysis, D.R., C.V., C.B.M.; data curation, C.V., D.R.; writing—original draft preparation, C.V., D.R., C.B.M.; writing—review and editing,

Post-acute sequelae of COVID-19 (PASC); CORonaVirus Disease-2019 (COVID-19); Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2); Activation induced marker (AIM) assay; Diffuse lung capacity for carbon monoxide (DLCO); Central memory (CM); Follicular helper T cells (Tfh); Staphylococcal enterotoxin B (SEB); 2 minutes walking test (2MW); Effector memory (EM); terminal effector memory re-expressing CD45RA (TEMRA).

C.V., C.B.M., D.R., G.C. and A.C.; patients' recruitment: M.B. funding acquisition, A.C., M.B. All authors have read and agreed to the published version of the manuscript.

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Introduction

The population worldwide has experienced increased mortality and morbidity due to the pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Among patients who recovered from CORonaVirus Disease-2019 (COVID-19), unfortunately, there is still a portion that experiences a wide range of persistent symptoms that last months after the acute phase of the illness [1]. The World Health Organization (WHO) estimates that the 10-20% of those recovering from the SARS-CoV-2 infection manifest at least one symptom after several months. This condition has been dubbed as post-acute sequelae of SARS-CoV-2 infection (PASC) syndrome [2].

PASC syndrome is a multi-systemic disorder characterised by pulmonary, immunological, cardiovascular, neuropsychiatric, gastroenterological, hepatic, renal, endocrine, and dermatological sequelae. While it is not typically life-threatening, it can significantly affect patient's daily activities and social life [3], [4]. Pulmonary involvement is a hallmark of PASC syndrome, and includes impaired lung function, that can be assessed through the diffusing capacity of the lung for carbon monoxide (DLCO). DLCO is a key parameter that measures the ability of gases to diffuse from the alveoli into the bloodstream, reflecting the integrity of both the alveolar-capillary membrane and pulmonary blood flow. A reduction in this parameter is frequently shown by PASC patients. A value below 80% of expected indicates a respiratory impairment, that is more severe in the case of a value below 60% [5]. Evidence suggests that the underlying mechanisms of pulmonary complications during COVID-19 are closely linked to immune system dysregulation. In particular, immune cells drive alveolar endothelium inflammation leading to long term consequences such as pulmonary fibrosis [6], [7] [8]. Given the critical role of immune cells, particularly T cells, in mediating these processes, evaluating T cells subsets in PASC patients with reduced DLCO may provide valuable insight into the adaptive immune mechanisms driving pulmonary dysfunction in PASC syndrome.

Despite the prevalence and growing awareness of PASC, the possible pathological mechanisms underlying this syndrome remain poorly defined. The struggle is related to the wide array of symptoms associated with PASC and the complexities of COVID-19 pathogenesis. Nevertheless, different hypotheses have been proposed including viral persistence, immune system activation triggered by SARS-CoV-2 superantigens, and autoimmune responses [9], [10]. Overall, a common feature is the involvement of the adaptive immune system [9].

Adaptive immunity comprises circulating antibodies, B and T cells, which protect the organism against re-infections. Among them, Guo *et al.*, have highlighted the crucial role of SARS-CoV-2-specific T-cell responses in protecting against re-infection and providing long-term immunological memory [11]. Additionally, various research efforts have evaluated these T-cell responses, examining their persistence and effectiveness 6 to 10 months after viral clearance. In a study by Hou *et al.*, the authors performed an ELISPOT assay in PASC patients' serum collected one year after the primary infection. They demonstrated that specific T-cell response can last several months after the infection [12]. In their work, Opsteen *et al.* evaluated SARS-CoV-2 specific T-cell response through the activation induced marker (AIM) assay, 6 months post-infection. They found that PASC patients had an increased and sustained SARS-CoV-2-specific CD4⁺ T-cell response overtime, with respect to those who recover from the infection without any symptoms [13]. These findings, taken together with other data, point to the persistence of viral antigen stimulation, that continuously triggers the adaptive immune system. All these studies share a common limitation: they did not account for correlations between the biological data obtained and the specific clinical parameters evaluated in patients who recovered from COVID-19.

It has been widely established that a persistent and uncontrolled inflammation, together with a dysregulated immune response, can lead to the development of a long-term sequelae following viral infections [14], such as those caused by Ebola, Lassa and Influenza viruses. This uncontrolled inflammation and immune system activation might be implicated in the development and persistence of severe symptoms [15]. In this context, the assessment of the SARS-CoV-2-specific T-cell response and its relationship with clinical data becomes crucial to better characterize the disease and provide a potential diagnostic tool.

There are different strategies to measure humoral immunity and its efficiency (e.g. ELISA and neutralizing antibody assays), but very few allow for the assessment of the entire spectrum of the cellular immunity. Traditional methods for quantifying antigen-specific T-cell responses include proliferation assays, cytokine production detection (ELISPOT), and peptide-MHC multimer staining. However, these methods have limitations such as lack of information on T-cell phenotype, failure to detect low abundant cells like follicular helper T cells (Tfh), and complexity in capturing the complete range of cytokine production. In contrast, the AIM test is an *in*

vitro assay, which allows to detect: i) T-cell phenotype, ii) different cytokine production (interferon- γ (IFN- γ), interleukin 2 (IL-2) and tumor necrosis factor (TNF- α)) and iii) the maturation curve [16].

The primary objective of our work was to evaluate the SARS-CoV-2-specific T-cell response in patients one year after hospital discharge and to correlate these findings with various clinical parameters. The novelty of our work relies in the comprehensive approach, which extends beyond immune profiling. We specifically correlated the dysregulated immune response—encompassing both CD4+ and CD8+ T cells—with distinct clinical symptoms in PASC patients. We performed the AIM assay, which provides a wider depiction of the overall antigen-specific T-cell response and increases the comprehension in both quantitative and qualitative terms of these responses.

Materials and methods

Study participants

We enrolled 92 patients who have been hospitalized due to COVID-19 between March 1st, 2020, and June 29th, 2020. The follow-up occurred with an average of 366 days after recovery and hospital discharge. Different clinical parameters associated with PASC including the presence of residual symptoms, pulmonary function tests and 2MWT have been performed as previously described by *Bellan et al.* [4]

Activation induced marker (AIM) assay

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by standard procedure using Ficoll-paque density gradient centrifugation. After washing, PBMCs were cryopreserved in FBS 10% DMSO for further analysis.

Cryopreserved PBMCs were thawed in complete RPMI 1640 medium. PBMCs were resuspended in RPMI supplemented with heat-inactivated human serum from male AB plasma (Merck, Darmstadt, Germany). Cells were then stimulated with PepTivator® SARS-CoV-2 Prot_S complete spike peptide pool (1 μ g/mL) (Miltenyi Biotec, Germany) in presence of 1 μ g/mL anti-CD28 (LifeTechnoloiges, Carlsbad, CA, USA). Cells that were cultured in RPMI medium supplemented only with heat-inactivated human serum in presence of 1 μ g/mL anti-CD28 antibody were used as negative control, while cells that were cultured in presence of 1 μ g/mL anti-CD28 antibody and 1 μ g/mL Staphylococcal enterotoxin B (SEB) (Merck, Darmstadt, Germany) were used as positive control.

For each sample and each condition (stimulated, unstimulated and SEB stimulation), 1×10^6 cells were plated in 96-well round bottom plates and incubated at 37°C, 5% CO₂ for 4h. After 4h incubation, samples were supplemented with 10 μ g/mL of brefeldin A (Becton and Dickinson, Franklin Lakes, NJ, USA) and incubated for the following 12 hours.

Flow cytometry

BD Horizon™ Fixed Viability Stain 575V (Becton and Dickinson, Franklin Lakes, NJ, USA) was used to assess the cell viability. Surface staining of the cells was performed with titrated concentrations of the following combinations of antibodies: anti-CD4 APC-H7 (clone SK3), anti-CD8 V500C (clone SK1), anti-CD197 PE (clone 2-L1-A), anti-CD45RA BV786 (clone HI100), and anti-CXCR5 in BV421 (clone RF8B2). After adding the surface markers, samples were incubated for 20 minutes at 37°C. After incubation and washing, cells were fixed and permeabilized with Fixation/Permeabilization Kit (Becton and Dickinson, NJ, USA) for intracellular staining.

Intracellular staining was carried out for 30 min at 4°C with titrated concentrations of the following combinations of antibodies: anti-CD3 PerCP-Cy5.5 (clone SK7) anti-CD137 APC (clone), anti-CD69 PE-Cy7 (clone FN50) anti-IL-2 BV711 (clone 5344.111), anti- IFN- γ FITC (clone B27), anti- TNF- α Alexa Fluor 700 (clone MAb11). All the antibodies were purchased from Becton and Dickinson (Franklin Lakes, NJ, USA).

Acquisition of samples was performed on FACSymphony A5, while analysis was done using FACS Diva software (Version 9.0.0, Becton and Dickinson, Franklin Lakes, NJ, USA).

The percentage of SARS-CoV-2-specific subpopulations was calculated subtracting the negative control for each sample to its spike-specific activation.

The gating strategies were shown in supplementary figure S1, S2, S3, S4 and S5.

Statistical analysis

Mann–Whitney U test was used to detect any significant differences in antigen-specific T-cell response between healthy control (HC) and PASC patients. p values r below 0.05 were considered statistically significant. GraphPad Instat software was used (Version 8.3, GraphPad Software, San Diego, CA, USA). The effect size (Cohen's d) was

calculated based on the observed means and standard deviations in each group, following the pooled standard deviation approach.

Results

Study cohort.

We recruited 92 subjects one year after the recovery from the first wave of COVID-19 infection (March-June 2020). The demographics of participants are listed in Table 1.

Briefly, out of the 92 participants, 71% were males and 29% females, with an average age of 60 years. In this cohort, average half of the subjects displayed a reduced DLCO (47.8%) and more than 60%, residual symptoms (which include fever, cough, dyspnea, asthenia, diarrhea, dysgeusia, anosmia, arthralgia and myalgia) with 70% alteration of the 2MWT. In contrast, the HC group included in this study, although they had also been hospitalized during the first wave of COVID-19, showed no signs of PASC 12 months post-infection. Specifically, they did not exhibit any residual symptoms, DLCO impairment, or abnormalities in the 2MWT. Overall, the cohort consisted of 70% (n=64) PASC patients and 30% (n=28) HC.

Table 1. General features of the study participants. Qualitative variables are shown as frequencies (%)

Demographic characteristics	
Age, years	60 [50-70]
Sex M/F	65 (70.6)/27(29.3)
Clinical parameters at 12 months	
DLCO, <80%/>80%	44 (47.8)/(48(52.1)
Residual symptoms, N/Y	61 (66.3)/31 (33.6)
Altered 2MWT, N/Y	70 (76)/22 (23.9)
PASC, N/Y	28 (30.4)/64 (69.5)

Association of demographic factors with PASC.

To explore the relationship between demographic characteristics (i.e., gender and age) and PASC, we conducted chi-square tests with a twofold approach. First, we analyzed the association between gender and PASC and found a statistically significant relationship ($p = 0.0120$) (Table 2). Although, due to sample size constraints, this finding should be taken carefully, this result indicates that male patients are more likely to experience PASC compared to female patients. Second, we assessed the relationship between age, categorized as ≤ 60 years and >60 years, and PASC. We observed that PASC is significantly associated ($p = 0.0246$), to patients older than 60 years (Table 3).

Table 2. Distribution of PASC and HC patients by gender. A chi-square test revealed a statistically significant association between gender and PASC ($p=0.0120$).

	PASC	HC	Total	p value
Male	40	25	65	0.0120
Female	24	3	28	
Total	64	28	92	

Table 3. Distribution of PASC and HC patients by age. A chi-square test revealed a statistically significant association between gender and PASC ($p=0.0246$).

	PASC	HC	Total	p value
≤ 60 years	29	20	49	0.0246
> 60 years	35	8	43	
Total	64	28	92	

Increased CD4⁺ memory T-cell response to SARS-CoV-2 peptides in PASC patients.

To investigate the extent of a SARS-CoV-2 T-cell memory response one year after the primary infection, we performed an in vitro T-cell activation method (the AIM test) capable to phenotype T cells, assess their functionality (cytokine expression) and maturation stage, thorough quantitative and qualitative examination of T cell-memory response against spike peptides. We classified our subjects into responders and non-responders based on the presence or absence of T-cell response against the SARS-CoV-2 peptides. As expected from a 1 year far activation to spike peptides, we found non-responders i.e subject lacking a memory response, in both the helper and the cytotoxic compartment. Interestingly, the portion of responders were higher in the CD4⁺ AIM⁺ compartment (71%) compared to the CD8⁺ AIM⁺ one (43%) (Figure 1). We subsequently analysed the data of each T-cell compartment by stratifying PASC and HC. Surprisingly, we found that the majority of *responders* of the CD4⁺ AIM⁺ compartment was mainly represented by PASC patients, 78% vs 53% of HC (p=0.025) (Table 4.). Instead, in the CD8⁺ AIM⁺ compartment there were no differences between the two groups (Figure 2).

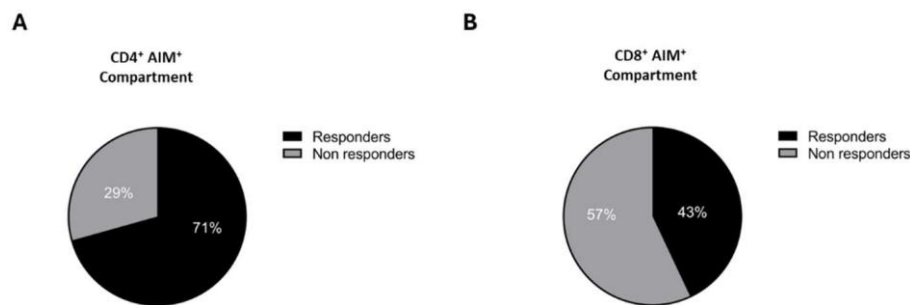


Figure 1. Portion of *responders* and *non-responders* among the enrolled subjects. (A) In the CD4⁺ AIM⁺ compartment, the percentage of *responders* (71%) was higher compared to the *non-responders* group (29%). (B) In the CD8⁺ AIM⁺ compartment, the percentage of *responders* (43%) among all the participants was lower respectively to the *non-responders* (57%).

Table 4. Differences of the percentage of *responders* between HC and PASC in the CD4⁺ AIM⁺ and CD8⁺ AIM⁺ compartment. *p<0.05

	CD4 ⁺ AIM ⁺ responders	CD8 ⁺ AIM ⁺ responders
HC	15/28 (54%)	10/28 (36%)
PASC	50/64 (78%)	30/64 (47%)
p value	0.025*	0.3667

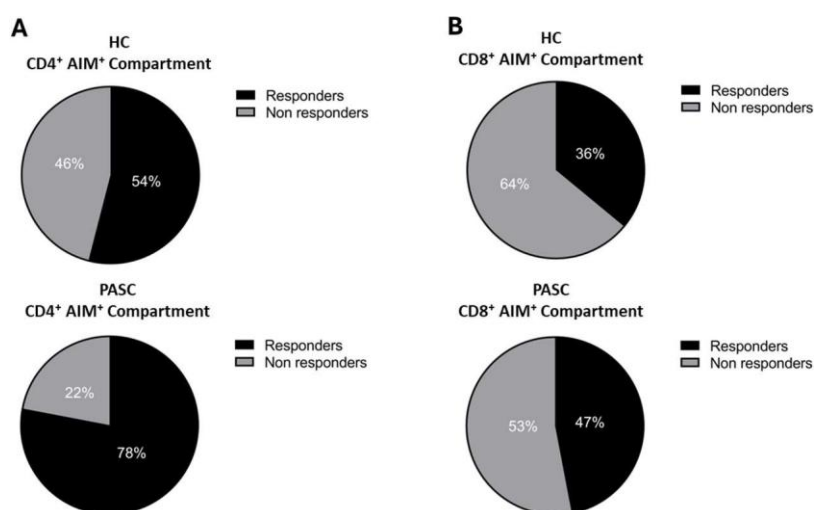


Figure 2. Portion of *responders* and *non-responders* between HC and PASC patients, in the CD4⁺ AIM⁺ and CD8⁺ AIM⁺ compartment. (A) The portion of *responders* was higher compared to *non-responders* in both HC and PASC patients for the CD4⁺ AIM⁺ compartment. (B) For the CD8 AIM⁺ compartment, the portion of *responders* was higher in comparison to *non-responders* in HC, instead for PASC patients the percentage of *responders* was lower compared to *non-responders*.

The magnitude of multifunctional CD4⁺ CM T cells is higher in HC and PASC patients.

The AIM test allows to deeply characterize the phenotype of the T cells that respond to the SARS-CoV-2 peptides, evaluating both the magnitude of the SARS-CoV-2-specific T cells and their multifunctionality, i.e. cells secreting one or more pro-inflammatory cytokines simultaneously.

We found significant differences between HC and PASC patients, regarding mainly the CD4⁺ AIM⁺ compartment. Indeed, the subset of central memory (CM) T cells (identified as CD45RA⁻, CD197⁺) that secrete cytokines were higher in PASC patients. In contrast, evaluation of the magnitude and the multifunctionality of the CD8⁺ AIM⁺ compartment did not reveal any difference between HC and PASC patients (Figure 3).

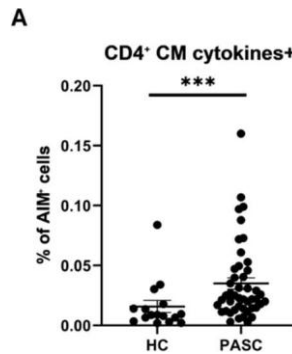


Figure 3. Evaluation of the magnitude of the CD4⁺ AIM⁺ compartment between HC and PASC patients. (A) Dot plot shows the percentage of CD4⁺ CM T cells that secrete pro-inflammatory cytokines. Mann-Whitney U test was used *** $p < 0.001$.

High magnitude of SARS-CoV-2 memory T cells is linked to PASC patients with reduced pulmonary function.

PASC patients were divided into two distinct groups: those with a DLCO < 80% and those with a DLCO $\geq$ 80%, using this cut off to identify patients with a certain degree of respiratory impairment. Recent studies reveal that a large proportion of PASC patients exhibit a reduction of the DLCO < 80%, indicating a persistent damage in the alveolar-capillary space and possible fibrosis [17].

Considering both the magnitude and the multifunctionality, the total CD4⁺ AIM⁺ cytokine⁺ population, as well as all the maturation curve e phases (CD4⁺ naïve, CM, EM CD4⁺ and TEMRA) AIM⁺ cytokines, were higher in patients with a DLCO < 80% (Figure 4A-G).

Additionally, also the CD4⁺ AIM⁺ Tfh were higher in patients with a reduced DLCO (Figure 4H).

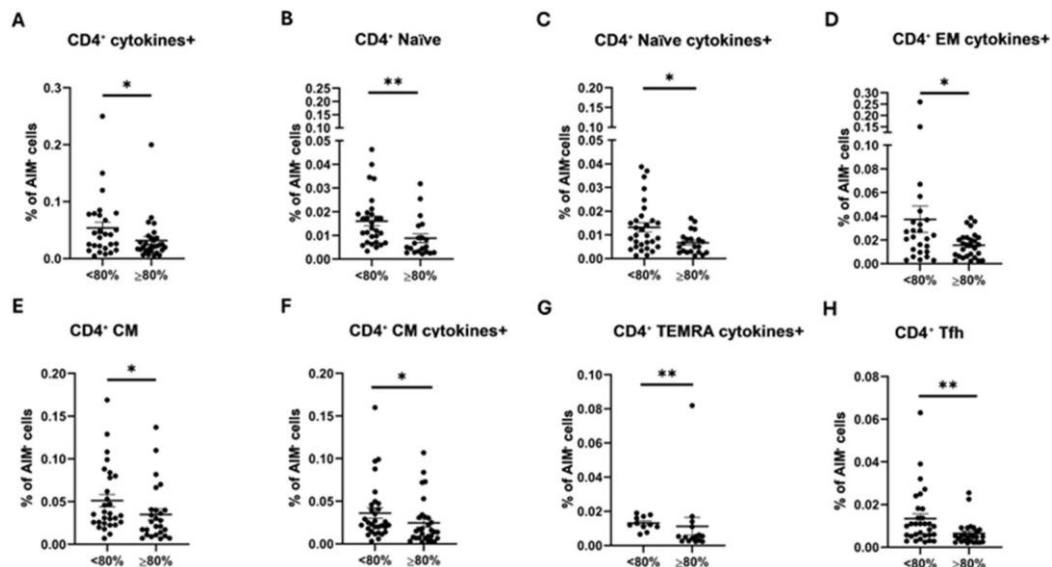


Figure 4. Evaluation of the magnitude of the CD4⁺ AIM⁺ compartment between patients with a DLCO < 80% and $\geq$ 80%. (A) Dot plot shows the percentage of CD4⁺ T cells that secrete the pro-inflammatory cytokines. (B) Dot plot shows the percentage of CD4⁺ naïve T cells. (C) Dot plot shows the percentage of CD4⁺ naïve T cells that secrete pro-inflammatory cytokine. (D) Dot plot shows the percentage of CD4⁺ EM that secrete pro-inflammatory cytokines. (E) Dot plot shows the percentage of CD4⁺ CM T cells (F) Dot plot shows the percentage of CD4⁺ CM AIM⁺ T cells. (G) Dot plot shows the percentage of CD4⁺ TEMRA T cells that secrete pro-inflammatory cytokines. (H) Dot plot shows the percentage of CD4⁺ Tfh cells. Mann Whitney U test was used * $p < 0.05$; ** $p < 0.01$.

High magnitude of SARS-CoV-2 T cells of the CD4⁺ AIM⁺ compartment is linked to residual symptoms in PASC patients.

PASC patients were stratified based on the presence or absence of residual symptoms and the magnitude and the multifunctionality of the T-cell subsets was evaluated.

Notably, in the CD4⁺ AIM⁺ compartment, we found an increase of the CD4⁺ cytokines, CD4⁺ EM cytokines, CD4⁺ Tfh cytokines in those patients with residual symptoms (Figure 5A, B and C).

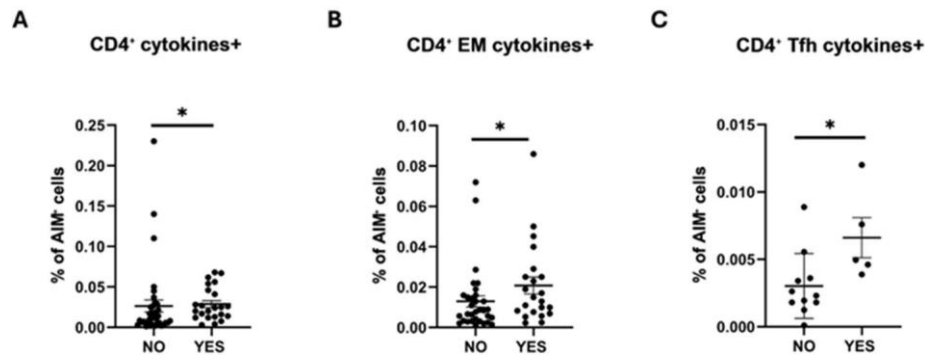


Figure 5. Evaluation of the magnitude of the CD4⁺ AIM⁺ compartment between patients with and without residual symptoms. (A) Dot plot shows the percentage of CD4⁺ T cells that secrete pro-inflammatory cytokines. (B) Dot plot shows the percentage of CD4⁺ EM T that secrete pro-inflammatory cytokines. (C) Dot plot shows the percentage of CD4⁺ Tfh cells that secrete pro-inflammatory cytokines. Mann Whitney U test was used *p<0.05.

Discussion

PASC syndrome encompasses a range of non-specific health problems whose onset, persistence or worsening follow an acute SARS-CoV-2 infection. Its treatment and pathophysiology represent a major challenges [18], and current evidence indicates the possible role of T cells and cellular immunity in disease pathogenesis [19]. Our results highlighted that PASC syndrome was more frequent in males compared to females. These findings contrast with the existing, although limited data in the literature, which suggest that females are predominantly affected by PASC and are more prone to develop the syndrome. Stewart et al. and others reported that hormones and menopause may contribute to the increase of pro-inflammatory cytokines such as IL-6, IL-1 and TNF- α , resulting in the establishment of inflammatory status and persistence of diseases [20]. We can not exclude the possibility that this discrepancy is due to the gender imbalance in our cohort. Nevertheless, on the opposite, and in line with literature we found that individuals older than 60 years were more prone to develop PASC syndrome. Since older patients are more susceptible to comorbid conditions, which can impact their overall health and weaken the immune system's ability to effectively respond to subsequent illnesses [21].

In our study, we inquire about the potential correlation between PASC symptoms and immune perturbation by adopting the AIM test, an *in vitro* T-cell activation method that allows for the investigation of long-term protective immunity following spike peptide stimulation [22]. Being capable to phenotype T cells, assessing their functionality (cytokine expression) and maturation stage, this technique offers a thorough quantitative and qualitative examination of cell-mediated memory against spike peptide in post-acute SARS-CoV-2 infection, which may help in dissecting the intricate immunological background of its sequelae. Based on the immunological T-cell response against the SARS-CoV-2 peptides, we were able to discriminate, among enrolled subjects those who showed an activation, either in CD4⁺ or CD8⁺ compartment. Our data highlighted a defective CD8⁺ response compared to the CD4⁺ subset, one after year the primary infection. Given their ability to eliminate virus-infected cells and produce effector cytokines, CD8⁺ T cells are crucial in the regulation of viral infection; in the contraction phase that follows antigen clearance, a small proportion of effector CD8⁺ T cells differentiate into memory CD8⁺ T cells [23]. However, previous studies indicated that, in cases of viral persistence or cancer, the formation of memory CD8⁺ T cells is compromised. This may explain the huge impairment of CD8⁺ activation observed in our cohort [24], [25]. Moreover, it has been evaluated that, among COVID-19 survivors, the proliferative capacity of memory CD8⁺ T cells was not as robust as that of CD4⁺ memory T cells, even if SARS-CoV-2-specific cells remained functionally responsive, demonstrating enhanced effector abilities including activation, proliferation, and

secretion of immune mediators [26]. Longitudinal studies have revealed sustained dysregulation of immune responses in subjects with PASC [27]; accordingly, the stratification of our cohort in PASC and non-PASC subjects, revealed an increased proportion of subjects with a persistent memory response in the CD4⁺ subset in the symptomatic group, while no significant differences emerged in the CD8⁺ compartment, even though the number of *responders* among symptomatic subjects outnumbered those of asymptomatic ones. This result may provide a deeper insight in the immune regulation in recovered patients, since CD8⁺ response seems to be generally more hampered, and it does not discriminate subjects according to post-infection repercussions. On the other hand, a more efficient response against spike peptides, in terms of magnitude, emerged to be restricted to CD4⁺ compartment in PASC subjects. Significant differences came out in CM CD4⁺ AIM⁺ cells, producing at least one of the evaluated cytokines. Our data are in line with a previous study that found a higher proportion of CD4⁺ CM cells in PASC compared to recovered individuals, potentially supporting a persistent reservoir [19]. Indeed, CD4⁺ CM cells are essential for the establishment of immunological memory and to provide long-lasting protection against pathogens, by activating and orchestrating the functions of other immune cells through the secretion of cytokines (IL-2, TNF- α , IFN- γ) [27]. Our results support previous evidence, suggesting a persistent activation of CD4⁺ T cells after severe SARS-CoV-2 infection [28]. To investigate more specifically the association between immune activation and post-infection manifestations, we stratified our cohort according to DLCO, a common clinical predictor of residual lung function, and residual symptoms (fever, arthromyalgia, asthenia, cough, dyspnea, diarrhea, dysgeusia and anosmia). We found an increased magnitude of the immune activation in subjects with impaired lung function (DLCO <80%) and persistent symptomatology. The association between reduced DLCO resulting from severe COVID-19 and increased immune activation is not entirely clear-cut and may involve multiple determinants, since DLCO reduction after severe lung infection can be ascribed to various factors, including inflammation, fibrosis, microvascular thrombosis, and damage to the alveolar-capillary membrane [29]. Both acute and post-acute COVID-19 cases show a deregulated immune response, which include cytokine storms and excessive inflammation [27], which contribute to lung damage and impaired gas exchange [30]. Similarly, other post-infection symptoms may result from underlying increased inflammation, that may also be explained by antigenemia and/or viral persistence in various anatomical locations [13]. To our knowledge, the deep phenotyping of spike-specific activated T cells and the magnitude of activation in PASC subjects with impaired DLCO or residual symptoms has never been characterized before. Our results confirm the clear hyperactivation of CD4⁺ T-cells at different maturation stages and with diverse multifunctionality features, already suggested by previous studies [22], [31]. The concomitant activation of subsets with different maturation stage, may be counterintuitive, but not unexpected, given the complexity of immune system recovery following severe COVID-19 infections. The persistent activation of naïve T cells may be bystander-driven, and this hypothesis is supported by the elevated levels of T-cell-related cytokines found in the plasma of severe patients even after recovery [29]. CD4⁺ Tfh AIM⁺ cells resulted to be significantly increased in subjects with reduced lung function, while multifunctional AIM⁺ Tfh cells were significantly increased in patients showing residual symptoms. These cells play a crucial role in adaptive immune regulation. A previous study reported that after SARS-CoV-2 infection and/or vaccination, Tfh cells are recalled by antigen re-exposure [32]. We can so speculate that enduring reservoirs of circulating Tfh cells and memory CD4⁺ T cells, developed during primary infection, may be effectively mobilized upon encountering the antigen again, potentially playing a pivotal role in inducing a persistent immune response. Whether the immune activation state is a cause or consequence of post-infection symptoms remains an unclear matter. Multiple studies suggest an underlying autoimmune pathology dealing with this constant immune hyperactivation [33], [34], but the topic is still debated. The AIM assay stands out for its sensitivity and ability to characterize T-cell subpopulations and their responses and permitted a deep characterization of the complex immunological mechanisms associated to post-acute infection sequelae. Additionally, examining different T-cell subpopulations can help to assess whether the prevalence of a particular subset correlates with a higher risk of other specific infections, such as herpes viruses, hepatitis B, or cytomegalovirus reactivation. Our work represents a step forward into understanding the complexity of the immunological picture related to this and multi-faced condition. However, it presents some limitations, specifically, i) AIM test is an in-vitro test and as such it does not fully replicate the complexity of the in-vivo microenvironment. Indeed, in a physiological setting, interactions between immune cells, cytokines, and tissues create a dynamic environment that can influence T cell activation and response. ii) the limit size of our cohort especially the discrepancy among HC and PASC patients. Therefore, further investigations are needed to confirm and expand upon our findings.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the University of Eastern Piedmont (protocol 117/20; NO-MORE COVID study)

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The authors declare that the data supporting the findings of this study are included within the article or [Supplementary Materials](#) and are available from the corresponding author upon reasonable request.

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Figures caption

Figure 1. Portion of *responders* and *non-responders* among the enrolled subjects. (A) In the CD4⁺ AIM⁺ compartment, the percentage of *responders* (71%) was higher compared to the *non-responders* group (29%). (B) In the CD8⁺ AIM⁺ compartment, the percentage of *responders* (43%) among all the participants was lower respectively to the *non-responders* (57%).

Figure 2. Portion of *responders* and *non-responders* between HC and PASC patients, in the CD4⁺ AIM⁺ and CD8⁺ AIM⁺ compartment. (A) The portion of *responders* was higher compared to *non-responders* in both HC and PASC patients for the CD4⁺ AIM⁺ compartment. (B) For the CD8 AIM⁺ compartment, the portion of *responders* was higher in comparison to *non-responders* in HC, instead for PASC patients the percentage of *responders* was lower compared to *non-responders*.

Figure 3. Evaluation of the magnitude of the CD4⁺ AIM⁺ compartment between HC and PASC patients. (A) Dot plot shows the percentage of CD4⁺ CM T cells that secrete pro-inflammatory cytokines. Mann-Whitney U test was used *** p<0.001.

Figure 4. Evaluation of the magnitude of the CD4⁺ AIM⁺ compartment between patients with a DLCO < 80% and ≥ 80%. (A) Dot plot shows the percentage of CD4⁺ T cells that secrete the pro-inflammatory cytokines. (B) Dot plot shows the percentage of CD4⁺ naïve T cells. (C) Dot plot shows the percentage of CD4⁺ naïve T cells that secrete pro-inflammatory cytokine. (D) Dot plot shows the percentage of CD4⁺ EM that secrete pro-inflammatory cytokines. (E) Dot plot shows the percentage of CD4⁺ CM T cells (F) Dot plot shows the percentage of CD4⁺ CM AIM⁺ T cells. (G) Dot plot shows the percentage of CD4⁺ TEMRA T cells that secrete pro-inflammatory cytokines. (H) Dot plot shows the percentage of CD4⁺ Tfh cells. Mann Whitney U test was used *p<0.05; **p<0.01.

Figure 5. Evaluation of the magnitude of the CD4⁺ AIM⁺ compartment between patients with and without residual symptoms. (A) Dot plot shows the percentage of CD4⁺ T cells that secrete pro-inflammatory cytokines. (B) Dot plot shows the percentage of CD4⁺ EM T that secrete pro-inflammatory cytokines. (C) Dot plot shows the percentage of CD4⁺ Tfh cells that secrete pro-inflammatory cytokines. Mann Whitney U test was used *p<0.05.

S1. Gating strategy CD4/CD8. Dot plots showing total CD4⁺ and CD8⁺ along with their maturation curve and gate positioning.

S2. Gating strategy of activated T cells with measurements of cytokines (Negative, Spike and SEB). Dot plots showing a negative, Spike and SEB samples for every compartment (AIM⁺ CD4⁺ and CD8⁺) with the evaluation of multifunctionality and gate positioning.

S3. Gating strategy of T follicular cells. Dot plot shows the gating strategy for the identification of T follicular helper (Tfh) cells

S4. Hierarchy of the CD4⁺ AIM⁺ populations. Table shows the Boolean gate used for the identification of AIM⁺ CD4⁺ cell subsets

S5. Hierarchy of the CD8⁺ AIM⁺ populations. Table shows the Boolean gate used for the identification of AIM⁺ CD8⁺ cell subsets

Summary PAPER 3 (in preparation)

Under normal conditions, antigen-specific T cells increase their proliferation during viral infections and then decline as the infection resolves. In PASC patients, these cells appear to persist and remain active even after the resolution of the SARS-CoV-2 infection leading to an impairment of the immune response and to the development of symptomatology typical of PASC syndrome.

In this paper we aimed to investigate the role of SARS-CoV-2 specific CD8⁺ T cells in PASC patients one year after the recovery from coronavirus disease 2019 (COVID-19).

To achieve this, we employed dextramers, a specialised technology designed to study antigen-specific T cells. Dextramers are composed of dextran-based sugar polymer backbone, which serve as a scaffold to attach major histocompatibility complex (MHC) class I molecules loaded with different Spike peptides, which are recognised by the TCR receptor on antigen-specific T cells. Moreover, dextramers have also fluorophores on their backbone, enabling the detection of specific T cells that recognize and bind the Spike peptide, through flow cytometry (a schematic representation of this technology is shown in **Figure 1** of the manuscript).

Our results showed that these patients have higher levels of the SARS-CoV-2 CD8⁺ DEX⁺ T cells compared to HC. Notably, phenotype analysis revealed that percentages SARS-CoV-2 CD8⁺ DEX⁺ effector memory cells re-expressing CD45RA (TEMRA) T cells and activated SARS-CoV-2 CD8⁺ DEX⁺ T cells (identified as CD38⁺) were higher in PASC respect to HC, suggesting a persistent immune activation.

Despite the significant results present in our work, we recognize some limitations. One is that we did not evaluate the SARS-CoV-2-specific CD4⁺ T cells. Studying and evaluating their role could offer a more comprehensive understanding of immune dysregulation in PASC patients one year after the recovery. Moreover, another one regards the small sample size, which should be increased to validate our approach and results.

Overall, our work brings attention to the potential role of persistent viral antigens in maintaining immune activation. Adding this in clinical settings could be useful to develop therapeutic interventions aimed at controlling chronic immune activation in PASC as well as in other chronic post-viral syndromes. Furthermore, our study paves the way for the use of immune profiling as diagnostic and prognostic tools in chronic disorders.

Persistent activation of SARS-CoV-2 specific CD8+ T cells in PASC patients one year after recovery

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Abstract

Persistent immune activation following SARS-CoV-2 infection is a key area of study, particularly in understanding the role of CD8⁺ T cells in post-acute sequelae of SARS-CoV-2 infection (PASC). While CD8⁺ T cells are crucial for viral clearance and immune memory, their dysregulation may contribute to immunopathology, as observed in coronavirus disease (COVID-19) associated acute respiratory distress syndrome (ARDS). This study focuses on characterizing SARS-CoV-2-specific CD8⁺ T cells in PASC patients one year after recovery using dextramers technology. We evaluated 39 HLA-A02 positive patients, 33 of whom had PASC, defined by residual symptoms, impaired diffuse lungs capacity for carbon monoxide (DLCO), reduced 2-minute walking test, and psychological symptoms, while six served as healthy controls (HC). Using flow cytometry, SARS-CoV-2-specific CD8⁺ T cells were identified and categorized into memory subsets: Naïve, central memory (CM), effector memory (EM), and terminal effector memory (TEMRA). TEMRA and activated (CD38⁺) subsets were significantly elevated in PASC patients compared to HC.

These findings highlight sustained CD8⁺ T cell activation and in PASC, shedding light on the long-term immunological impact of COVID-19.

Introduction

Persistent activation of the immune system following (SARS-CoV-2) infection remains a critical area of investigation, particularly in understanding the role of CD8⁺ T cells in PASC syndrome.

Despite their lower frequency in lymphoid organs than CD4⁺ T cells, these cytotoxic T cells are essential for eradicating viral infections (L. Sun et al., 2023).

Typically, respiratory virus infection in mouse models showed higher levels of antigen-specific CD8⁺ T cells in the lungs and within the airways (Schmidt and Varga, 2018). They become activated after the encounter between the T cell receptor (TCR) and viral peptides, which are bound to an major histocompatibility complex (MHC) class I molecule. This interaction, to prime an optimal CD8 activation and proliferation, requires other costimulatory signals. Following the expansion, antigen-specific CD8 T cells become activated and start to produce inflammatory cytokines as interferon-gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α). Moreover, they acquire effector functions with the production and release of granzyme B and perforin, which have a cytolytic activity (Lewis and Ly, 2021).

The kinetic of antigen-specific CD8⁺ T cells depends on the type of virus, with usually the main peak of expansion immediately after the clearance of the virus. After the resolution of the infection, levels of antigen-specific CD8 T cells decrease and start the development of a subset, which remains in the host to establish long-lasting memory, that will confer protection against re-infections (Schmidt and Varga, 2018).

Despite their beneficial role in mediating viral clearance and protecting against secondary infections, excessive and persistent levels of CD8⁺ T cells can contribute to the induction of immunopathology. For instance, in COVID-19, the development of the acute respiratory distress syndrome (ARDS) can be addressed to the persistent hyperactivation of antigen-specific CD8⁺ T cells, which release high concentrations of pro-inflammatory cytokines and chemokines, leading to a series of adverse events in COVID-19 patients (Chen and John Wherry, 2020b). However, despite the presence of data about the role of antigen-specific CD8 T cells in COVID-19, we lack the detailed characterisation of SARS-CoV-2 specific CD8 T cells in patients with PASC syndrome. A recent study reported that the virus-specific T cells response in convalescent patients at different time points. After 6 months from mild and severe acute COVID-19, a progressive switch of SARS-CoV-2-specific CD8⁺ T cells from T effector memory (EM) to a (TEMRA) phenotype was described. At 10 months after the post-symptom onset, among SARS-CoV-2-specific CD8 T cells, EM and TEMRA cells were increased, while T central memory (CM) cells were decreased (Jung et al., 2021). Another study evaluated the response after 12 months by showing that almost all CD8⁺ T cells acquired a TEMRA phenotype (Adamo et al., 2022). Some studies have been conducted to analyse antigen-specific T cells in PASC

patients, but high variability, due to the heterogeneity of techniques and time-points, make it difficult to develop a straight way to study them. Quantifying and studying antigen-specific T cells can be performed through the ELISpot assay, by lymphocyte proliferation assays in response to antigen or by tetramers. Even if these technologies are reliable and frequently used, they have some limitations. These include a very limited phenotypic analysis, a prior knowledge of the cytokines that you want to investigate, and the in vitro stimulation, that does not reflect the exact physiopathology (Janetzki, 2015). In this regard, dextramers technology allows a precise snapshot of what is going on in the immune system of PASC patients, one year after the hospital discharge, without any external stimulation. The advantage of the dextramers technology is that they have more MHC molecules and fluorophores alongside the sugar backbone, resulting in increased detectable fluorescence intensity, which enables the measurement of low affinity TCRs, otherwise undetected by other classes of multimer reagents (Tario et al., 2015). Based on this, we evaluated the presence of SARS-CoV-2 CD8 T cells in PASC patients one year after the recovery from COVID-19, through dextramers staining. This approach allowed us to deeply study the cytotoxic T cells compartment and its activation status, understanding the long-term immunological consequences of COVID-19 1 year after the recovery.

Materials and methods

Patients' enrollment

In this thesis, 39 patients were included after being discharged between March 1 and June 29, 2020, from the Azienda Ospedaliero Universitaria Maggiore della Carità university hospital in Novara, Italy, where they had been admitted for COVID-19. Written informed consent was obtained from the patient or their legal representative.

We defined the presence of PASC syndrome based on the presence of several parameters like presence of residual symptoms, assessment of diffusing capacity of the lungs for carbon monoxide (DLCO) and 2 minutes walking test (2MWT), 1 year after the recovery from COVID-19.

HLA-A02 typing

In order to evaluate T cell response against the SARS-CoV-2 using the dextramers technology, which requires a specific MHC class I molecule, patients were tested for the presence of the

HLA-A02 allele. This HLA type was selected because it is the most widely distributed in the population.

For the HLA-A02, 20 µl of the whole blood were added to 80 µl of PBS with 2 mM EDTA, and then stained with 2 µl of FITC-conjugated anti-HLA-A02 monoclonal antibody. After 20 minutes of incubation at 4°C, 2 ml of lysing buffer 1X were added in order to lyse red blood cells. After 20 minutes of incubation at room temperature (RT) in the dark, samples were washed by adding 2 ml of PBS with 2 mM EDTA and were centrifuged at 1500 rpm for 5 minutes. Then, samples were resuspended in 200 µl of PBS with 2 mM EDTA and acquired using FACSymphony™ A5 (Becton Dickinson) flow cytometer and analyzed using FACSDiva software (Becton Dickinson). Of the 141 patients recruited, 39 were identified as HLA-A02 carriers.

PBMCs isolation

Patients' peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood using Lympholyte®-H Cell Separation Media (company). The whole blood, mixed to PBS 1X, was layered over top of the same quantity of Lympholyte. Then, samples were centrifuged at RT at 1800 rpm for 20 minutes without break. After centrifuge the PBMCs layer, formed following density gradient, was removed, and transferred in a new tube for washing. Subsequently, the cells were frozen, adding 500 µl of FBS and 500 µl of FBS with 20% DMSO, and stored at -80°C, until the use.

SARS-CoV-2-specific cytotoxic T cells evaluation

To identify and evaluate SARS-CoV-2-specific CD8+ T cells in HC and PASC patients, the MHC I dextramers and the antibodies staining were performed on PBMCs. MHC I dextramers technology allows detection with high specificity of the population of CD8+ T cells that recognize different epitopes of the spike protein. In **Figure 1** is shown the schematic representation of a Dextramer®. Specifically, it is composed of a dextran backbone loaded with MHC molecules presenting one peptide antigen, and fluorochromes to detect cells by flow cytometer.

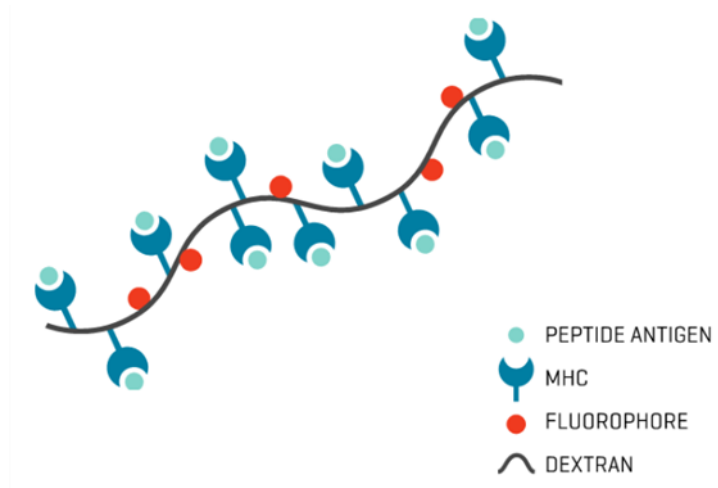


Figure 1. (<https://www.immudex.com/>).

6 different dextramers were used, each of them composed of MHC I molecules HLA-A*02 type presenting one epitope of the spike protein (**table 1**).

Dextramers	Allele	SARS-CoV-2 antigen	Epitope
Dextramer 1	A*0201	Spike	RLNEVAKNL
Dextramer 2	A*0201	Spike	VLNDILSRL
Dextramer 3	A*0201	Spike	NLNESLIDL
Dextramer 4	A*0201	Spike	TLDSKTQSL
Dextramer 5	A*0201	Spike	FIAGLIAIV
Dextramer 6	A*0201	Spike	YLQPRTFLL

Table 1. The allele, the SARS-CoV-2 antigen, and the specific epitope that compose each dextramer.

PBMCs were thawed and, after counting, $1-3 \times 10^6$ cells were used for the staining. The Live & Dead staining was performed to discriminate between living and dead cells. After an incubation of 20 minutes at 4°C, the samples were washed and stained using 67,2 µl of the dextramers mix (**table 2**). Biotin was added to the mix to block any free binding sites on the Dextramer backbone. Before using, the mix was centrifuged for 1 minute at 10.000 x g to pellet the possible aggregate. As positive and negative controls, other two mixes were prepared: the positive one using dextramers with MHC I molecules presenting antigens of the influenza virus, and the negative one using MHC I molecules with a non-sense peptide.

After 10 minutes of incubation at RT in the dark, samples were stained adding 50 µl of the antibodies mix per million cells (**table 3**). After 20 minutes of incubation at RT in the dark, samples were washed twice and resuspended in 300 µl of PBS with 2 mM EDTA for the acquisition. All samples were acquired using FACSymphony™ A5 (Becton Dickinson) and analyzed using FACSDiva software (Becton Dickinson).

Dextramers mix	Fluorochrome	Volume (µl) for each sample
Dextramer 1	APC	10 µl
Dextramer 2	APC	10 µl
Dextramer 3	APC	10 µl
Dextramer 4	APC	10 µl
Dextramer 5	APC	10 µl
Dextramer 6	APC	10 µl
PBS 10X		6 µl
Biotin 100 µM		1,2 µl
TOTAL:		67,2 µl

Table 2. The fluorochrome and the volume (µl) of each single component required to obtain the dextramers mix for one sample.

Antibodies mix	Fluorochrome	Volume (µl) for 1*10 ⁶ cells
STAIN BUFFER		17,5 µl
BRILLIANT STAIN BUFFER (BSB)		10 µl
CD3	BUV496	5 µl
CD56	FITC	5 µl
CD8	BUV805	2,5 µl
CD197	BV711	2,5 µl
CD95	Bv480	2,5 µl
CD38	BB700	2 µl
CD45	BUV395	1 µl
CD4	BUV737	0,75 µl

CD16	PE-Cy7	0,5 µl
CD14	PE	0,5 µl
CD45RA	BUV563	0,25 µl
TOTAL:		50 µl

Table 3. The fluorochrome and the volume (µl) of each single component required to obtain the antibodies mix per million cells.

Statistical analysis

Any differences between SARS-CoV-2-specific cytotoxic T cell response in HC and in PASC patients were detected by using Mann–Whitney *t*-test, where *p* values below 0.05 were considered statistically significant. The statistical analyses were performed with GraphPad Instat software (Prism 8 version 8.4.3) (GraphPad Software, San Diego, CA, USA).

Results

Evaluation of SARS-CoV-2 immune memory CD8⁺ T cells subset by multiparametric analysis

SARS-CoV-2-specific cytotoxic T cells were evaluated in 39 HLA-A*2 positive patients, among them, 33 were considered PASC patients (presence of residual symptoms, DLCO < 80%, negative 2 minutes walking test (2 MWT), anxiety and psychological symptoms) while the remaining 6 were healthy.

Firstly, the gating strategy (**figure 1A**) was designed to identify CD8⁺ T cells that recognize specifically six different epitopes of the viral spike protein.

Moreover, to further assess the CD8⁺ DEX⁺ T memory cells subsets, Naïve, CM, EM, and TEMRA were evaluated. Indeed, memory CD8 T cells subtypes differ for their phenotype, function and protective capacity. Indeed, CM cells are early differentiated progenitors that can handle systemic infections due to their localization in secondary lymphoid organs. Instead, EM cells are characterised by rapid effector function, that can allow them to fight infections arising within peripheral organs. TEMRA cells are a terminally differentiated subset that express CD45RA; they express markers of senescence and have low proliferative and functional capacity (Mahnke et al., 2013; Martin and Badovinac, 2018). The surface marker CD38 that is a NADase implicated in cell differentiation during inflammation, cytokines release, and it is also expressed on T cells during the activation was used to evaluate how many of these CD8⁺

DEX⁺ remained active (Piedra-Quintero et al., 2020; Sandoval-Montes and Santos-Argumedo, 2005). In addition, CD38 seems to be involved in COVID-19 as mediators of lung damage and fibrosis (Horenstein et al., 2021).

A

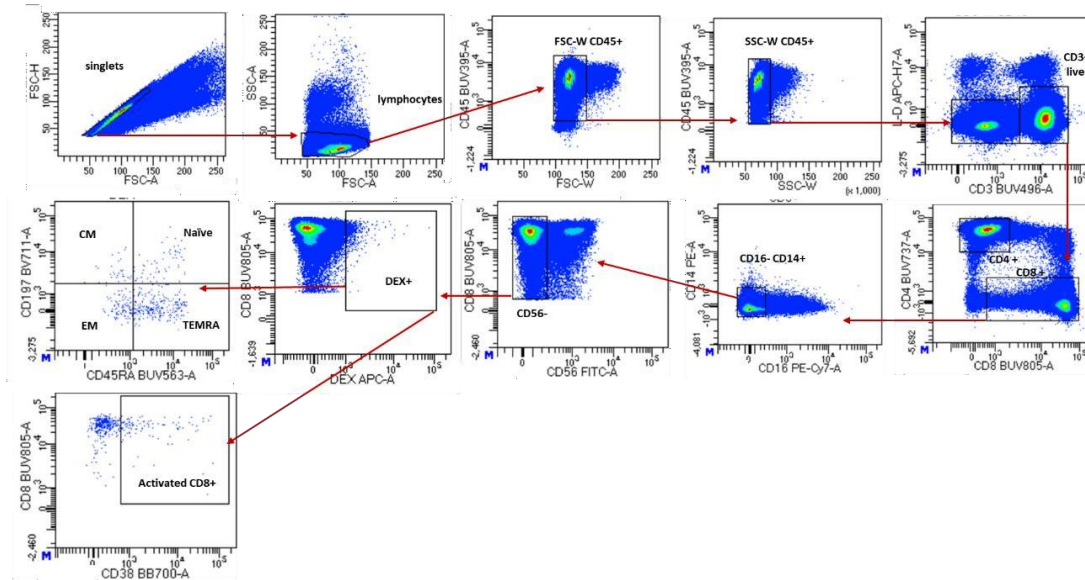


Figure 1. SARS-CoV-2-specific cytotoxic T cells gating strategy. A) Singlets were identified (FSC-H vs FSC-A) and lymphocytes were then gated through the evaluation of their morphology (FSC-A vs SSC-A). A further method to discriminate singlets was applied assessing the width (FSC-W and SSC-W) of lymphocytes that express CD45. Subsequently, live T cells (CD3⁺ and negative for the live/dead dye) are identified. Cytotoxic T cells (CD8⁺) were discriminated from helper T cells (CD4⁺). CD14⁻/CD16⁻ cells and subsequently CD56⁻ cells were gated in order to exclude the NK cells that can give a non-specific signal because they are able to bind the MHC class I molecule without any peptide. Finally, the dextramer positive (DEX⁺) T cells were identified and then further classified. CD8⁺ DEX⁺ cells were divided in four different memory subsets: Naïve T cells (CD45RA⁺ and CD197⁺), CM T cells (CD45RA⁻ and CD197⁺), EM T cells (CD45RA⁻ and CD197⁻), and TEMRA T cells (CD45RA⁺ and CD197⁻). Moreover, among CD8⁺ DEX⁺ cells, activated T cells were discriminated (CD38⁺).

Activated CD8⁺ DEX⁺ T cells and TEMRA are increased in PASC patients

The differences of the percentage of total CD8⁺ DEX⁺ T cells and its subsets (EM, CM, TEMRA, Naïve and activated T cells which are all expressed out of CD8⁺ DEX⁺ T cells) were then evaluated between HC and PASC patients. Subjects were stratified in HC and PASC according to the presence of a wide range of symptoms typical of PASC syndrome one year after the hospital discharge. We found that specific SARS-CoV-2 cytotoxic T cells were higher in PASC compared to HC (**Figure 2A**). Among T cell subsets, we found that the percentage of TEMRA

SARS-CoV-2 cytotoxic T cells and activated SARS-CoV-2 cytotoxic T cells were significantly increased in PASC patients (**Figure 2B, C**).

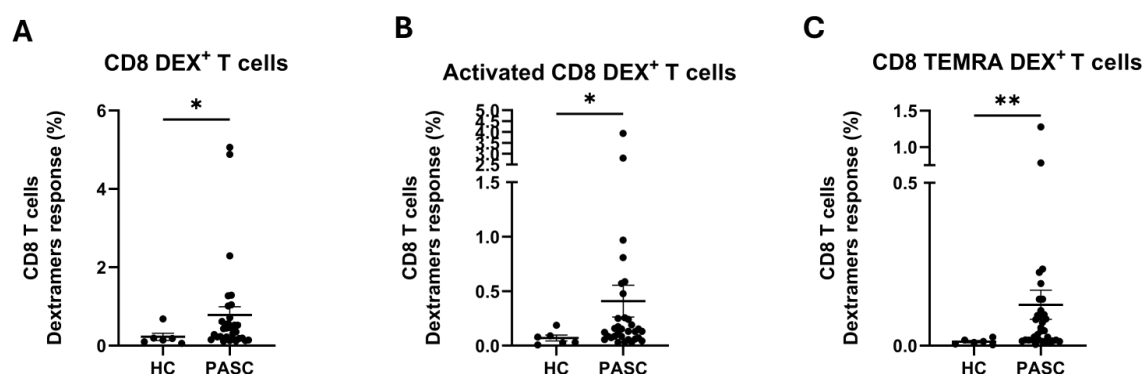


Figure 2. Differences in SARS-CoV-2 cytotoxic T cells in HC and PASC patients. Graphs show the percentage of A) CD8 DEX⁺ T cells B) activated CD8 DEX⁺ T cells C) CD8 TEMRA DEX⁺ T cells within HC and PASC patients. Mann–Whitney t-test was used. * $p < 0.05$, ** $p < 0.005$

Conclusions

CD8⁺ T cells are crucial for viral clearance and immune memory, but their dysregulation may contribute to immunopathology, as observed in COVID-19-associated acute respiratory distress syndrome (ARDS). This opens to the necessity to continue in characterising the adaptive immune system of PASC patients, in order to elucidate the possible immune alterations of adaptive immunity that might be at the basis of the PASC syndrome. Findings on antigen-specific T cells in COVID-19 patients reported their dual role: while they can be protective, they may also be detrimental and induce the development of the cytokine storm (Moderbacher et al., 2020). Other studies emphasized that in order to exert a protective function, a coordinated response between CD4⁺ and CD8⁺ T cells is crucial, as any imbalance in these responses may lead to adverse outcomes. Despite research on antigen-specific T cells responses in COVID-19 patients, there is limited knowledge regarding the detailed characterization of this specialized T cells responses in PASC patients one year after the hospital discharge. Few studies have explored the role of antigen-specific T cells in PASC syndrome months after the resolution of the acute illness, with a deep insight on the CD8⁺ T cell subsets, which are generally considered to be more important players in viral infections. Moreover, several studies revealed their critical role in the formation of long-

lasting memory, recognizing viral epitopes deriving from both highly variable and conserved proteins, providing robust immune protection (Moss, 2022). In addition to the limited number of studies on antigen-specific CD8⁺ T cells in PASC syndrome, we have also to consider the high variability among these studies due to the application of different techniques to evaluate this specific type of cells.

For instance, to investigate antigen-specific T cells one can use the ELISpot, or AIM test or tetramers technology. All these methods are highly heterogeneous and have some caveats, as for example, a very limited phenotypic analysis, a prior knowledge of the cytokines one wants to investigate, and the *in vitro* stimulation, that does not reflect the exact physiopathology (Amara, 2009; Tippalagama et al., 2023).

A novel technology in the field is represented by dextramers, which are composed of a dextran sugar backbone conjugated with numerous MHC class I molecules and fluorophores. Dextramers allow us to have a precise snapshot of the status of the immune cells of PASC patients in the peripheral blood.

We firstly tested all the patients for the HLA-A*2 and then only those that resulted to be positive (n=39) were stained with the dextramers. We compared the percentage of SARS-CoV-2 specific cytotoxic T cells between HC and PASC patients, analyzing the activated ones and the different memory subsets. Notably, our results showed that the percentage of SARS-CoV-2 CD8 DEX T cells were higher in PASC patients. Further phenotypic analysis showed an increase in TEMRA CD8⁺ DEX T cells and activated CD8⁺ DEX T cells in PASC patients. Our findings would suggest a prolonged immune response to the virus, possibly due to the ongoing antigenic stimulation, due to the viral persistence. The work of Gaebler et al, demonstrated that memory B cells display clonal turnover after 6.2 months, and the antibodies that they expressed, had greater somatic hypermutation, indicative of continued evolution of the humoral response. They concluded that memory B cell response to SARS-CoV-2 evolved between 1.3 and 6.2 months after infection in a manner that is consistent with antigen persistence (Gaebler et al., 2021). Subsequently, they evaluated the presence of the virus in various districts and intestinal biopsies revealed the persistence of SARS-CoV-2 nucleic acids and immunoreactivity in the gut. Over time, several works have been published where the phenomenon of the viral persistence was demonstrated in various organs such as liver, CNS and lungs. Viral persistence can cause continuous stimulation of the immune system causing chronic inflammation that can lead to PASC syndrome. Based on these findings, we can

speculate that our result, demonstrating higher levels of SARS-CoV-2 CD8⁺ DEX⁺ T cells may reflect an ongoing viral persistence in the organism of these patients compared to HC. Specifically, the TEMRA phenotype, observed in our study, is typical of inflammatory environments, with high levels of pro-inflammatory cytokines such as IL-6, IL-15 and IL-2. A study by *Woodruff et al.* highlighted elevated inflammatory markers in PASC patients, which could promote the differentiation of CD8⁺ T cells into TEMRA subsets (Woodruff et al., 2023). Consistently, our phenotypic characterization revealed significantly higher frequencies of SARS-CoV-2 CD8⁺ DEX⁺ TEMRA T cells in PASC patients compared to HC. Moreover, a study from *Loyal et al.* demonstrated that SARS-CoV-2 reactive T cells with low TCR avidity have been shown to be cross-reactive, whereas SARS-CoV-2 T cells with high functional TCR avidity have been linked to improved functional activity and specificity for the SARS-CoV-2 antigens (Loyal et al., 2021). Supporting this hypothesis, Paniskaki and colleagues found an increased count of CD8⁺ TEMRA T cells in PASC patients, known to have a strong effector activity but limited proliferate capacity. Additionally, these cells can be activated via cytokines, independently from TCR. This uncontrolled and unspecific activation provides and exacerbates tissue damage (Paniskaki et al., 2023). These data were in accordance with our results that showed increased SARS-CoV-2 TEMRA CD8⁺ DEX⁺ T cells in patients with PASC syndrome.

Another important finding was the increased levels of SARS-CoV-2 activated CD8⁺ DEX⁺ T cells observed in PASC patients. A study from *Zhan et al.* demonstrated that persistent activated CD8⁺ T cells produce high levels of TNF- α and induce fibrosis (Zhang and Zhang, 2020). This mechanism might be responsible for lung fibrosis and so impair the lung functionality (e.g., DLCO<80%), which is one of the main symptoms of PASC, possibly explaining the higher levels of activated SARS-CoV-2 CD8⁺ T cells in these patients. Moreover, Cheon and colleagues reported that, although during the acute illness CD8⁺ T cells mainly exert a protective function, a prolonged and/or dysregulated CD8⁺ T cell activity can be responsible for the pulmonary damage. Their and our findings are in line with this hypothesis, indeed they characterized tissue-resident T cells and found that CD69⁺ and CD103⁻ T cells exhibited more inflammatory and cytopathic features in lungs (Cheon et al., 2021).

These findings all together provide new insights into the immunopathology of PASC, emphasizing the role of persistent activation and dysregulation of SARS-CoV-2-specific CD8⁺ T cells in driving chronic inflammation and tissue damage.

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Chapter 3: Gut-derived extracellular vesicles: unveiling their role in the gut-brain axis in MS and MDD

Premise

In the first chapter the focus was on blood-derived EVs as biomarker in COVID-19, while in this last chapter of my thesis we focused on the role of gut-derived EVs as mediators of communication within the gut-brain axis, particularly in the context of major depressive disorder (MDD) and multiple sclerosis (MS); the first one represent a mental health disorder affecting mood and behavior, while the second disease is a neurological autoimmune disease affecting the central nervous system. In both conditions, disruption of the gut-brain axis may play a role in inflammation and neurological symptoms.

Despite the awareness in prevention and personalized treatments, current diagnostic and prognostic tools for MDD and MS lack of specificity and sensitivity, leading to delayed diagnosis and ineffective treatment for both conditions.

A growing body of evidence demonstrated the value of EVs as disease specific markers, able to evaluate and understand differences among diseases. Based on this evidence, this chapter proposes a novel approach for diagnosis and prognosis by analyzing gut-derived EVs as functional carriers of disease-specific molecular fingerprints.

By studying gut-derived EVs, this chapter aims also to shed light on the role of gut-brain axis in the context of MDD and MS. To achieve this, we established a method for isolating EVs from stool samples, serving as a mirror of EVs in the gut. Using MACSPlex technology for eukaryotic EVs (eEVs) profiling and metaproteomic analysis for bacterial EVs (bEVs) characterization, we identified disease-specific EV signatures that could serve as diagnostic, prognostic, and predictive biomarkers for MDD and MS, providing novel insights in understanding and managing these complex disorders.

This chapter presents a non-invasive biomarker approach for early disease detection, prognosis, and a deeper understanding of gut-brain communication. Moreover, it will pave the way for EVs-driven diagnostic and therapeutic advancements in neuroimmunology and mental health

Background

Gut microbiota

The human gut microbiota is composed of various commensal and symbiotic species mainly located in the gastrointestinal (GI) tract (Marano et al., 2023). The GI tract hosts nearly 1,000 different bacterial species, totaling trillions of individual organisms. These bacteria are involved in several regulative processes concerning metabolic pathways, oxidative stress and membrane barrier maintenance in the gut (Das and Ganesh, n.d.).

The important roles of the gut microbiota include the modulation of the immune response against external pathogens, the maintenance of the intestinal epithelium's integrity and the metabolism of the nutrients. The bacteria that inhabit the intestine are classified according to the *phyla*, *classes*, *orders*, *families*, *genera*, and *species*. The most represented *phyla* of the gut microbiota are *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and *Verrucomicrobia* among which *Firmicutes* and *Bacteroidetes* are the major types (Rinninella et al., 2019).

The gut microbiota is formed within the first five years of life and in adulthood is influenced by various factors such as diet, drugs, stress, and environment. A healthy and balanced gut microbiota exhibits stability, high taxonomic diversity, resilience, and symbiotic interactions with the host. When disruptions among these factors arise, they also impact and alter the balance of the microbiota community, causing the establishment of a phenomenon called dysbiosis (Thursby and Juge, 2017a). Dysbiosis can be generally described as the loss of the gut microbial diversity and richness. It has some typical features such as the loss of the commensals, which are the common residing strain of the gut and the expansion of pathobionts, like the bacterial family of Enterobacteriaceae, which levels increase during enteric infections and inflammation (Petersen and Round, 2014).

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 (Camilleri and Vella, 2022). The latter causes the translocation of gut bacteria and their products from the intestine to the circulatory system, triggering the release of pro-inflammatory cytokines, leading to the activation of the immune system (**Figure 8**).

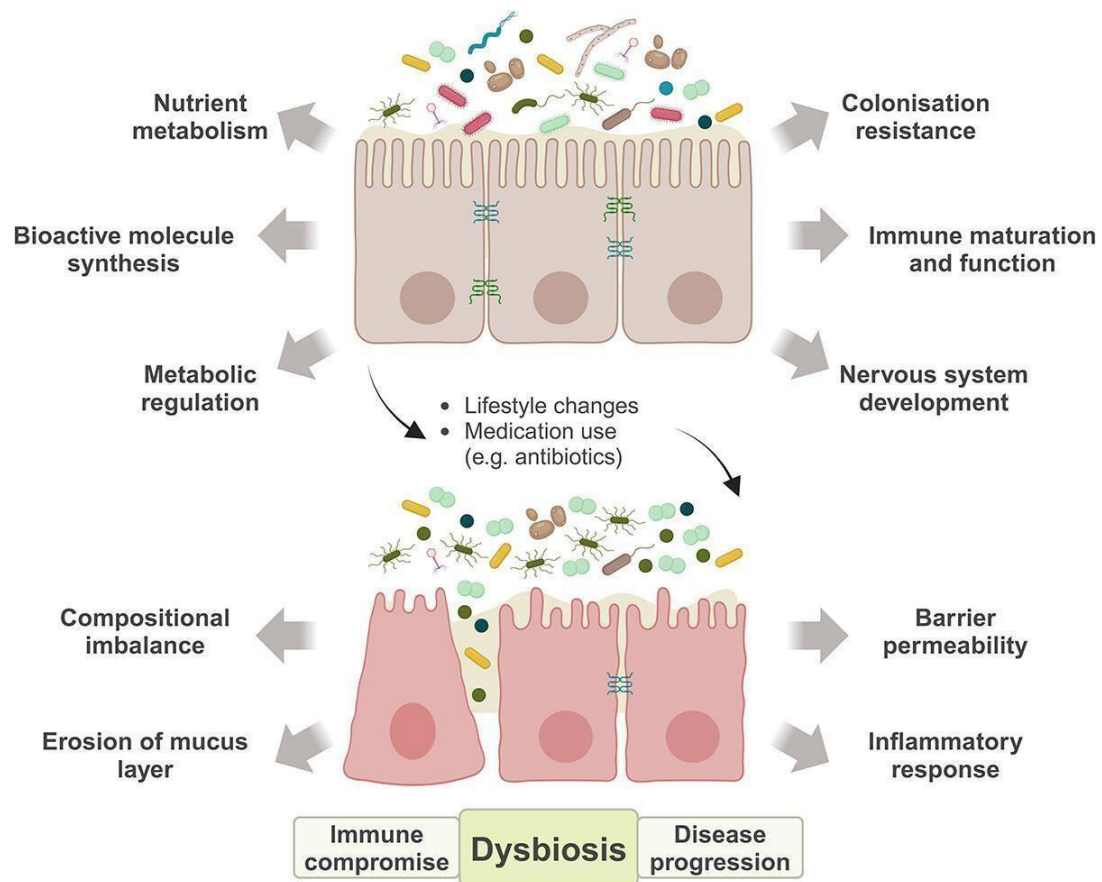


Figure 8. Microbiota in health and dysbiosis. Different external factors can influence the gut microbiota composition, leading to dysbiosis. Dysbiosis is characterized by a predominance of harmful bacteria that can compromise the physiological functions. This can result in gastrointestinal, metabolic and neurological disorders accompanied by immune dysregulation (Melo-Marques et al., 2024)

In normal conditions, the gut barrier remains intact and immune molecules, such as IgA and antimicrobial peptides (AMPs) exert their functions by inhibiting the proliferation and spreading of pathogens (Muniz et al., 2012). The gut-associated lymphoid tissues (GALT) maintain homeostasis, controlling the mechanism of peripheral tolerance, resulting in the prevention of the immune response to dietary or bacterial self-antigens. Moreover, epithelial cells, when they are in a balanced gut environment, produce a transforming growth factor- β (TGF- β), which induces the differentiation of the immune cells into the anti-inflammatory phenotypes (Round and Mazmanian, 2009).

Conversely, during chronic dysbiosis, exaggerated levels of enterotoxins such as lipopolysaccharide (LPS) can cause the disruption of the gut barriers, with the entry of pathogens and their harmful products like pathogen associated molecular patterns (PAMPs) into the bloodstream. PAMPs are sensed by TLRs, whose interaction results in the activation

of intracellular signaling pathways, leading to NF- κ B translocation to the nucleus. This activation drives the transcriptions of genes involved in the pro-inflammatory cytokines production, fueling systemic inflammatory states. The presence of bacteria's metabolites outside the intestine further influences the immune responses, by enhancing the differentiation of Treg cells into pathogenic Th17 cells. For instance, high levels of segmented filamentous bacteria (SFB) induce the transcription of stress response gene such as serum amyloid (SAA), which promote IL-22 and IL-6 secretion and promote the Th17 cells differentiation (Sano et al., 2015). Abundant levels of LPS can impact macrophage polarization, inducing the proliferation of M1 phenotype. High frequencies of M1 macrophage led to the production of pro-inflammatory cytokines, which lead to chronic inflammation and contribute to autoimmune disease formation (Zhang et al., 2019).

Dysregulation of the immune system as a consequence of the intestinal dysbiosis can also affect the mucosa-associated lymphoid tissue (MALT), in particular the GALT. Once disrupted, GALT loses its ability to perform the negative selection of T lymphocytes, potentially leading to the formation of autoreactive T and B cells. The extensive effects of intestinal dysbiosis on systemic immunity and chronic inflammation are highlighted by this disruption in immune control (Takiishi et al., 2017).

Recently, multifactorial diseases, such as Inflammatory Bowel Diseases (IBD), metabolic disorders, neurological disorders and autoimmune diseases, show an increasing incidence and a strong association with dysbiosis. For these reasons, the study of the gut microbiota and its interplay with other organs is becoming increasingly important.

Gut-derived EVs

Microorganisms and cells within the gut microbiota release EVs, which mediate different functions both physiological and pathological. Gut-derived EVs fill an essential role in the interkingdom interactions, indeed, due to their nature, they can cross physiological barriers, that are typically not easily accessible, such as the blood-brain barrier (BBB) and intestinal barrier, enabling them to reach the central nervous system (CNS) or host cells at the intestinal mucosal surface (Díaz-Garrido et al., 2021). Once in the target organ, these EVs can release

their cargo, composed of miRNA, proteins, metabolites and other bioactive molecules, influencing cell function and mediating cell-to-cell communication.

There are different types of EVs in the gut, including the eukaryotic EVs (eEVs), that derived from intestinal or epithelial cells and the prokaryotic ones, as archaea (aEVs) and particularly those deriving from bacteria (bEVs) (Melo-Marques et al., 2024)(**Figure 9**).

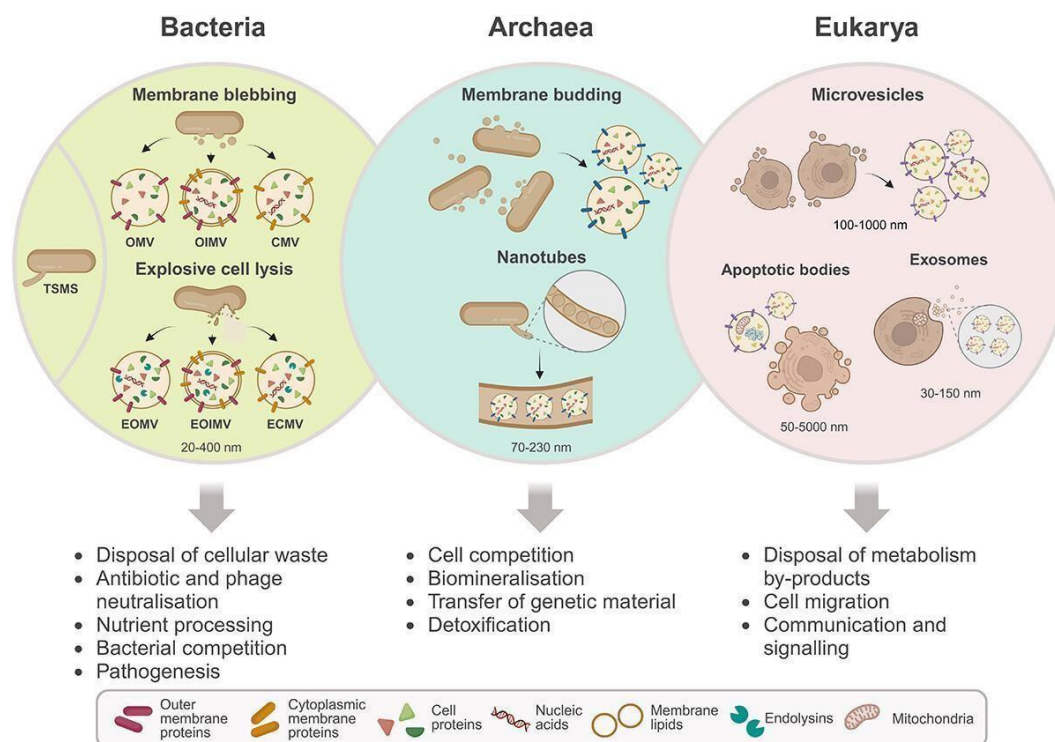


Figure 9. Different types of gut-derived EVs. Bacterial EVs can originate from membrane blebbing and give rise to outer-membrane vesicles (OMV) and outer-inner membrane vesicles (OIMV), produced by Gram-negative bacteria, and cytoplasmic membrane vesicles (CMV), produced by Gram-positive strains. Bacterial EVs can also originate from cell lysis, forming EMOV, EOIMV and ECMV, respectively. Bacteria can also produce EVs in the form of tube-shaped membranous structures (TSMS). Archaea produced EVs through membrane budding or nanotubes. Eukaryotic cells can produce different types of EVs: microvesicles from the membrane budding; apoptotic bodies from apoptosis and exosomes from the endocytic pathway (Melo-Marques et al., 2024).

bEVs are divided mainly into four different categories: i) outer-membrane vesicles (OMVs), ii) outer-inner membrane vesicles (O-IMV), iii) cytoplasmic membrane vesicles (CMVs) and iv) tube-shaped membranous structures (TSMS) (Hosseini-Giv et al., 2022). OMVs are Gram-negative derived and can be single or double layer. Therefore, they can be formed via cell lysis, in which cryptic prophage endolysin activity results in cell wall degradation or DNA damage giving rise to bEV formation. Instead, CMVs are released by Gram-positive bacteria and can be formed by the budding of the cytoplasmic membrane, followed by the crossing through the

cell wall. Finally, TSMS can be produced by both Gram-positive or Gram-negative bacteria and play a role in the component exchange by acting as a bridge between different cells.

It has been demonstrated that Gram-negative OMVs are released from the bacterial envelope, and they are released for several purposes including host-microbe interactions, and their size varies between 20-400 nm (Volgers et al., 2018). The interaction between host and microbes is mediated by OMV cargoes, including lipids, LPS, different types of proteins and nucleic acids. They can also affect the biofilm formation and host cell function modulations. CMVs range from 20-200 nm in diameter and are derived directly from the cytoplasmic membrane. TSMS have an average dimension of 50-70 nm (Magaña et al., 2023).

Generally, the composition and content of bEVs originate in the producer strain and differ depending on the growth phase and conditions such as the target cells and the microenvironment (Dauros Singorenko et al., 2017). Within the intestinal microenvironment, bEVs contribute to the colonization and proliferation of bacteria by carrying adhesion factors that facilitate bacterial attachment. By actively participating in regulating the balance of gut bacteria, bEVs have the potential to facilitate enhanced colonization of probiotics, which is crucial for the therapeutic benefits they offer. Alternatively, targeted disruption of bEVs could be employed to diminish the colonization of pathogenic bacteria (Liang et al., n.d.).

bEVs can cross the intestinal barrier using two different pathways: i) the paracellular (between cells) and ii) transcellular (through cells) pathways (Sultan et al., 2021). Indeed, bEVs can alter the composition of tight junctions: for example, vesicles from commensal bacteria increase the expression of tight junction proteins to limit paracellular transport. Instead, vesicles from *Campylobacter jejuni* break down the junction proteins E-cadherin and occludin lead to the leaky gut (Elmi et al., 2016).

After passing the epithelial barrier and entering in the submucosa, bEVs engage with diverse immune cells, influencing the host's immune system. This interaction involves direct engagement with distinct populations of immune cells such as neutrophils, macrophages, and DCs (Kaparakis-Liaskos and Ferrero, 2015). bEVs carry multiple copies of metabolism-associated molecular patterns (MAMPs), including DNA, RNA, LPS and peptidoglycan: these molecules interact with receptors such as NOD1, NOD2 and TLRs on immune cells, leading to activation of a cascade of immune signaling (Sultan et al., 2021). Immune signaling can be divided into pro-inflammatory signaling and anti-inflammatory signaling. The former leads to the activation of inflammation with a strong release of pro-inflammatory cytokines and the

recruitment of specific cells such as T helper and cytotoxic T cells, while the latter refers to a process that induces immune tolerance, a mechanism that prevents the immune response against a particular antigen (i.e. self-antigens). Once the tolerance is lost, disorders like autoimmune diseases or food allergies can occur. BEVs represent two sides of the same coin, as they have the potential to both drive dysbiosis and inflammation and contribute to their treatment and resolution. In the context of gut dysbiosis, bEVs produced and released by harmful bacteria can contribute to the maintenance of pathological states. For example, vesicles deriving from *P. aeruginosa* contain esterase A (EstA), a bacterial virulence factor, which induces the production of nitric oxide and pro-inflammatory cytokines (Ge et al., 2023). In contrast, bEVs derived from probiotics can modulate and actively shape the gut microbiota, restoring the initial homeostasis. For example, in a study from *Dean et al.*, they demonstrated that bEVs from *Latobacillus acidophilus* are enriched with bacteriocins, which target the opportunist *Lactobacillus delbrueckii* (Dean et al., 2020). Based on this evidence, bEVs seems to take part in the establishment of different diseases that are linked to gut dysbiosis, such as metabolic disorders, neurological disorders and ADs (D. Sun et al., 2023).

Gut-brain axis

The gut-brain axis was first demonstrated by Pavlov in 1904, who showed that the release of gastric and pancreatic secretion was due to some sensory signals such as the smell and the sight of the food (Filaretova and Bagaeva, 2016).

This bidirectional communication is composed of the CNS, the autonomic nervous system (ANS), the enteric nervous system (ENS) and the hypothalamic pituitary-adrenal axis (HPA). The autonomic system, elaborate both afferent signals, arising from the lumen and transmitted to CNS, and on the contrary, efferent signals from the CNS to the intestine (Carabotti et al., 2015). Neuronal cells and hormones can influence immune cells, epithelial cells, enteric neurons, smooth muscle cells, interstitial cells of Cajal and enterochromaffin cells, as well as microorganisms present within the gut. On the other side, resident cells of the gut, together with different microorganisms, influence neuronal cells and hormones (Chaudhry et al., 2023). The communications from the brain to the gut is mainly driven by two distinct mechanisms: the neural pathway and the neuroendocrine pathway.

The first one involved the ENS, which is composed of neurons and glial cells in the intestinal submucosa and muscularis propria. The ENS control autonomously the gastrointestinal physiology and functions and interacts with the CNS through the vagus nerve (VN), microbiota-derived metabolites, and neuroendocrine pathways. ENS afferent neurons can be influenced by different signals derived from the gut bacteria, modulating brain activity through VN. Indeed, bacteria products present in the ENS can be transmitted to the CNS to the VN (Y. He et al., 2024).

The second one is characterized by the HPA axis, which represents a mechanism of communication between the brain and the gut. The host brain can communicate bidirectionally with gut microbiota through neurotransmitters. There are four main neurotransmitters that play a role in the gut-brain communication: serotonin (5-HT), dopamine (DA), noradrenaline (NE), and gamma-aminobutyric acid (GABA). These molecules are also synthesized in enteroendocrine cells, which also release other hormones in the gut like gastric inhibitory peptide, ghrelin, serotonin, somatostatin and cholecystokinin (Zhao et al., 2023) (**Figure 10**).

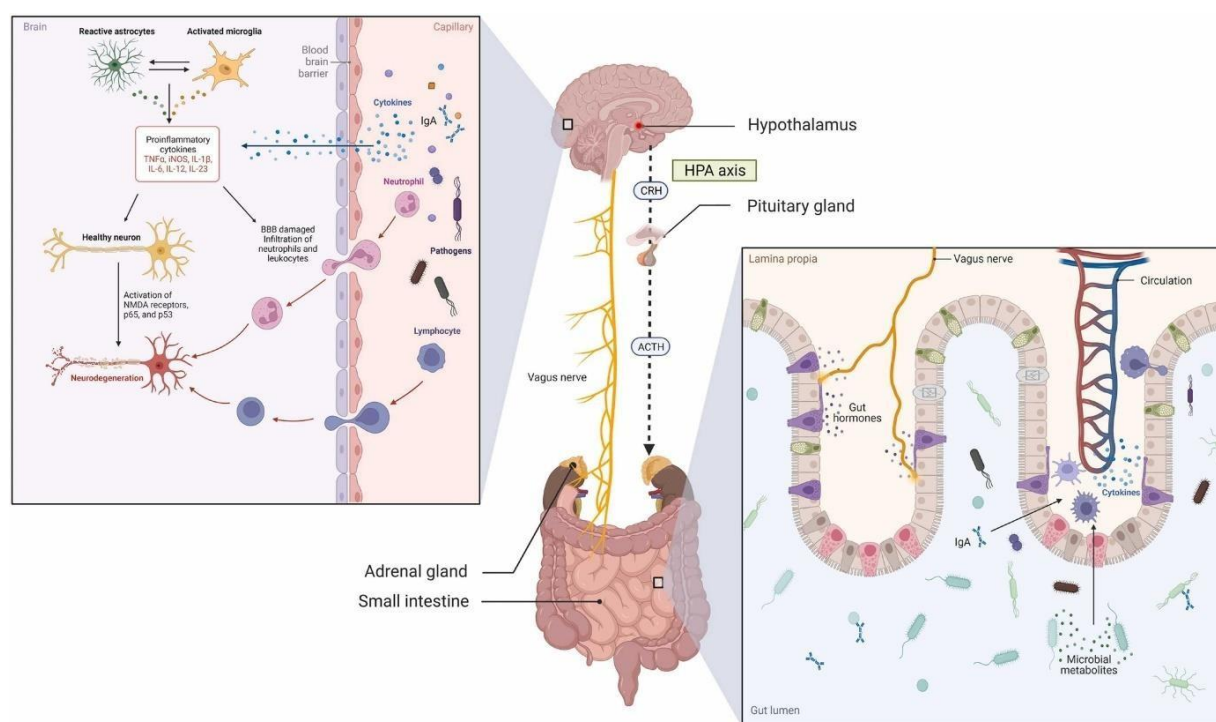


Figure 10. Mechanisms of gut-brain axis. This is a bidirectional communication through the vagus nerve, HPA axis, and immune pathways. Gut microbiota influences brain function through metabolites and immune signaling, while neuroinflammation impacts the gut via systemic responses (Zhao et al., 2023)

Besides these pathways, there are also some mechanisms through which gut microbiota

communicate and influence the brain. These mechanisms are the releasing of active substances, the generation of quorum sensing, which is a communication process in bacteria that allows them to detect and respond to cell population density by regulating gene expression, and the metabolization of dietary nutrients to produce secondary metabolites. Recent studies revealed that microorganisms in the gut can release vesicles which contain different active substances. These vesicles, thanks to their size and composition, can cross the intestinal barriers, enter the bloodstream and reach the target organs. They can even get easy access to the BBB through the vessels or VN and communicate directly with neuronal cells and vice versa (**Figure 11**). Microbial quorum sensing plays a critical role in shaping the gut ecosystem. Quorum sensing, when a threshold is reached, starts to influence gene expression. By giving some bacterial *species* a competitive edge through the synthesis of enzymes, it also influences the composition of microbiota, and consequently also the functions of the intestine, the immune response and the communications with distal organs. The gut microbiota can produce secondary metabolites that can influence brain functions. For instance, the short-chain fatty acids (SCFAs) such as butyric acid, propionic acid and acetic acid that are able to stimulate sympathetic nervous system, mucosal serotonin release and to influence memory and learning process (Kasarello et al., 2023).

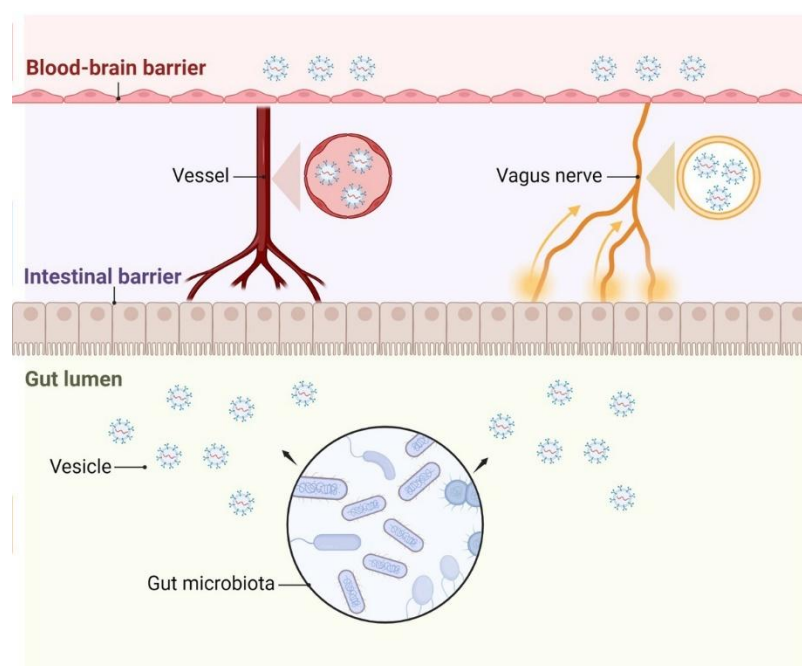


Figure 11. EVs in gut-brain axis. EVs from gut microbiota reach the intestinal barrier and through the vagus nerve or vessels can cross the blood brain barrier and reach the brain and also the contrary (Sun et al., 2023).

Major Depressive Disorder (MDD)

MDD is a complex psychiatric disorder affecting approximately 340 million people worldwide (Chiriță et al., n.d.). It is characterized by various abnormalities impacting mood, cognition, neurovegetative functions and psychomotor activities with symptoms like persistent feeling of sadness, loss of interest or pleasure in activities once enjoyed, and changes in sleep and appetite. These symptoms must be present for at least two weeks and significantly interfere with daily life to be diagnosed as MDD (Fava and Kendler, 2000). MDD is linked to a reduction in patients' lifespan, primarily due to an elevated risk of suicide, and it also increases their susceptibility to many medical conditions such as cardiovascular disease, cancer and ADs to which usually patients show poorer outcomes and response to treatment (Beurel et al., 2020). Despite the effectiveness of antidepressant drugs that exert immunomodulatory effects, and the extensive literature on the neuroanatomic, neuroendocrinological, and neurophysiologic aspects of MDD, there remains an unmet clinical need for reliable diagnostic and predictive biomarkers. Many biomarkers have been proposed to overcome this issue, for example magnetic resonance imaging (MRI), positron emission tomography (PET) scans and electroencephalogram (EEG) along with proteomic, transcriptomic and metabolomic studies but an exhaustive answer has yet to be found (Kennis et al., 2020). One significant challenge in managing this disease is that its causes are multifactorial and only partially understood.

Etiology of MDD

Genetic factors

MDD seems to have a genetic component. Recent family studies demonstrated a 3-fold increased risk for MDD in first-degree relatives (Rhee et al., 2025). Genome Wide Association Studies (GWAS) discovered 178 genetic risk loci, including those associated with presynaptic vesicle trafficking (PCLO), dopaminergic neurotransmission (a primary target of antipsychotics), glutamate ionotropic receptor kainate type subunit 5 (GRIK5), and metabotropic glutamate receptor 5 (GRM5), and neuronal calcium signaling such as calcium voltage-gated channel subunit alpha1 E (CACNA1E) and calcium voltage-gated channel auxiliary subunit alpha2 delta1 (CACNA2D1), are found to be associated with an increased risk of MDD (Lohoff, 2010). Transcriptomic studies on MDD patients revealed deficits in the transcription of somatostatin (SST) in the subgenual anterior cingulate cortex and the

amygdala of MDD patients. SST appears to be involved in cortical local inhibitory circuit, so a deficit in this neuropeptide can cause abnormal corticolimbic network activity and clinical mood symptoms across neurological disorders (Lin and Sibille, 2013)

Neurobiological factors

The first major hypothesis of the cause of MDD was postulated about 50 years ago and attributed the development of the disease to a deficiency of the brain monoaminergic transmitters 5-HT, NE and DA (Jiang et al., 2022).

5-HT spreads from all the brain areas and it is responsible for different physiological processes such as regulation of emotions, appetite and sleep.

NE influences the function of the prefrontal cortex, the processing of the working memory, and regulates behavior. DA modulates reward and motivation functions, memory, and attention (Azizi, 2022).

The monoamines hypothesis is based on the increased activity of the monoamine's oxidase, an enzyme, which degrades the amines, influencing their availability in the brain. Moreover, a functional deficiency of monoamine neurotransmitters also impacts on the proteins' transporter functions. In the brain, the transport of proteins is involved in the pre-synaptic reuptake of neurotransmitters, enhancing it and making possible a continuous neurotransmission. Thus, a decrease in the transport of proteins could contribute to lower monoamine levels (Boku et al., 2018).

Environmental factors

One important environmental factor associated with the development of MDD is stress. Several studies suggested that adverse life events can lead to MDD. A traumatic or stressed event always precedes a major depressive episode (MDE), such as job loss or a relative's death. The mechanisms underlying MDD caused by social adverse events and stress are still unknown. However, there was evidence that chronic stress can cause changes in the structure and function of neurons (Richter-Levin and Xu, 2018). Moreover, MDD patients exhibit high levels of cortisol, the so-called "stress hormone", which causes dysregulation of the HPA axis. Sustained cortisol levels have been linked to neuronal damage, particularly in the hippocampus, and the suppression of neurogenesis, further exacerbating depressive symptoms. Dysregulation of the HPA axis influences the immune system, contributing to pro-

inflammatory responses often observed in MDD patients. For example, elevated levels of pro-inflammatory cytokines, such as IL-6 and TNF- α , are commonly reported in individuals with MDD (Arinami et al., 2023).

Epigenetic modifications

Epigenetics refers to changes in gene expression which can be heritable, but environmentally modifiable. Epigenetic regulation is involved in both physiological and pathological processes. Increasing evidence supports that there are epigenetic components in the development of MDD. The epigenetic mechanisms that drive MDD are: DNA methylation, histone modifications and nucleosome positioning (Penner-Goeke and Binder, 2019). DNA methylation is the addition of a methyl group to cytosine residues by DNA methyltransferases (DNMTs). Research revealed different DNA methylation patterns in MDD. One study demonstrated the presence of hypomethylation of the synapsin gene (SYN2) in MDD patients. Instead, another showed a hypermethylation of TESC gene correlated with the disruption of the right parahippocampal cingulum and, for that reason, involved in MDD (Li et al., 2019).

Histones regulate gene expression both positively and negatively, thanks to posttranslational modifications catalyzed by enzymes that exert their function on amino acid residues located on histones. The posttranslational modifications that can occur are: methylation, acetylation, phosphorylation, ubiquitination, SUMOylation, crotonylation, citrullination, and ADP-ribosylation. The consequences of histones modifications are the alterations of the interactions between histones and gene expression. As an example, decreased levels of histone 3's acetylation was present in the hippocampus of depression-like behaviors in mice (Covington et al., 2011).

MDD diagnosis

Despite its prevalence worldwide, MDD diagnosis still represents a clinical challenge. The currently used criteria are based on clinical symptoms without an objective testing available. This is the reason why MDD has a higher rate of missed and incorrect diagnoses. Studies estimate that nearly 30-50% of MDD cases may go undetected or be incorrectly diagnosed (Cui et al., 2024). Misdiagnosis is common in individuals with other comorbidities, like chronic pain or fatigue, which share overlapping symptoms with MDD. There are several scales that allow clinicians to have a more objective assessment of the disease and classify symptom's

severity as Hamilton Depression Rating Scale (HDRS), Montgomery-Asberg Depression Rating Scale (MADRS), and Beck Depression Inventory (BDI). Suicidality, in terms of suicidal ideation and attempts, is among the stronger predictors of suicide and should be assessed with validated scales like the Columbia-Suicide Severity Rating Scale (CSSRS) (Zheng et al., 2019).

MDD treatment

There are different antidepressant treatments which can follow psychotherapies.

Tricyclic antidepressants (TCA) are the most used drugs for the treatment of MDD. The mechanism of action is based on the changes in the neurotransmitter's availability in the brain. Indeed, TCAs inhibit the reuptake of neurotransmitters exerting their antidepressant effects (Gillman, 2007). Other treatments used in everyday practice are the selective serotonin reuptake inhibitor (SSRIs) and serotonin/norepinephrine reuptake inhibitors (SNRIs). They inhibit serotonin transporters (SERT) and norepinephrine transporter (NET), which prevent the reuptake of 5-HT and NE into presynaptic neurons, resulting in an increased concentration of those neurotransmitters (Fanelli et al., 2021). An example of SSRIs is represented by fluoxetine, which can act as an agonist of the astrocytic 5-HT_{2b} receptors. After the stimulation of the 5-HT receptors, epidermal growth factor receptor (EGFR) is transactivated and subsequently activates the mitogen-activated protein kinases/extracellular signal-regulated kinases (MAPK/ERK) and phosphoinositide 3-kinase/serine/threonine kinase (PI3K/AKT) signalling cascades, which control the expression of mRNA or proteins that may be linked to mood disorders, such as SERT (Peng et al., 2014). Instead, SNRIs drugs are venlafaxine and duloxetine, their dual effect makes them effective for symptoms like social anxiety and pain (Bymaster et al., 2001).

The involvement of the immune system in MDD

Nowadays, there is emerging evidence that refers to MDD as an immune inflammatory disorder, highlighting the active role of the immune system (Kouba et al., 2024). Different findings assess that MDD includes the involvement of cell-mediated immune activation and a chronic inflammatory response, with an enhanced production of pro-inflammatory cytokines. Consistent with these findings, there is the fact that immune-inflammatory pathways influence the neuro progression mechanism in MDD, causing neuronal apoptosis and neurotoxic effects.

Different studies revealed that the administration of LPS, provokes inflammation and the release of pro-inflammatory cytokines such as IL-6, TNF- α and IL-1 β , lead to the establishment of the so-called “sickness behaviour”. This phenomenon is characterised, along with typical immune response to sickness as fever, also by the presence of reduced psychomotor, physical and social activities, fatigue, lethargy, reduced food intake, sleep abnormalities and altered cognition (Liu et al., 2018).

A possible mechanism through which TNF- α induces depression-like symptoms is the activation of the release of other cytokines, with the consequence of neuroinflammation. Moreover, the hyperproduction of these cytokines activates the Indoleamine-2,3-dioxygenase (IDO) production at the expense of 5-HT (Berthold-Losleben and Himmerich, 2008).

Another evidence linking MDD to inflammation is that MDD patients showed a higher increase of IL-6 and IL-10, in comparison with those experiencing few depressive symptoms. Recently, *Serafini et al.* found that suicidal ideation/attempts in MDD was associated with differences in inflammatory cytokines profile, characterized by altered levels of IL2, IL-6, IL-8, TNF- α , and vascular endothelial growth factor (VEGF) (Serafini et al., 2013). As confirmation of this, antidepressant drugs seem to have a therapeutic potential in treating also immune inflammatory conditions, suggesting that a reduction in activated immune inflammatory response might be associated with responsiveness to MDD treatment. Several studies have been conducted, both in animals and humans, to elucidate this dual effect of antidepressant drugs. In the animal model, fluoxetine or paroxetine reduced LPS-induced inflammation and depressive-like behaviors (Ohgi et al., 2013). In MDD patients it has been shown that drugs like SSRIs and TCAs inhibit the release of IL-6, IL1 β , TNF- α and IFN- γ (Hannestad et al., 2011). The evaluation of the proinflammatory cytokine levels in MDD patients might serve as predictors of antidepressant responses. Indeed, patients with poor responses to duloxetine show high levels of IL-1 β , IL-12, IFN- γ , IL-1 β /IL-10 and TNF- α (Fornaro et al., 2013).

On the other hand, the use of anti-inflammatory drugs alone or coupled with antidepressants has emerged as a possible therapeutic target for MDD. For instance, celecoxib, that is a cyclooxygenase (COX)-2 inhibitors, significantly improved depressive symptoms compared to the placebo group in a 6-week randomized controlled trial (RCT) (Müller et al., 2006). The association of celecoxib and fluoxetine also seems superior to fluoxetine monotherapy (Akhondzadeh et al., 2009). Another anti-inflammatory drug, the acetylsalicylic acid, seems to accelerate the response to antidepressants when combined with SSRIs and when used as add-

on presented additional improvement compared to antidepressant alone (Gałecki et al., 2009).

MDD and EVs

There are mounting evidence supporting the potential involvement of EVs in MDD.

In fact, EVs have been shown to influence neuroinflammatory pathways, which are significantly implicated in the development and progression of depressive symptoms.

Current studies suggest a possible link between the gut-derived EVs and the mechanisms underlying the MDD. Notably, Li et al. show that miRNAs containing EV cargo play a key role in physiological and pathological processes; specifically, miRNAs are transferred from neurons to microglia through EVs, leading to the release of proinflammatory factors responsible of neuroinflammation.

A recent study from Choi et al. demonstrated that, in mice, bEVs deriving from specific bacteria can interact with the gut and brain. Particularly, they found that bEVs from *Akkermansia muciniphila* (phylum *Verrucomicrobiota*) protect against LPS-induced intestinal permeability changes and also that bEVs *Lactobacillo plantarum* is important for brain health and function (Choi et al. 2022). Despite this, it remains unclear, highlighting the need for further specific studies in this field.. As a response to outer stress sources, the brain can modulate microbiota in a maladaptive way, leading to depressive disorders and neuropsychiatric impairments through various pathways including the autonomic nervous system, enteric nervous system, spinal nerves, immune cells, endocrine cells and bacterial metabolites (Longo et al. 2023).

Multiple sclerosis (MS)

MS is an autoimmune disease affecting the central nervous system (Schepici et al., 2019). Typically, MS is diagnosed around the age of 30 years and is characterized by the progressive accumulation of neurological impairments, which make everyday activities difficult during the advanced stage of the disease (Zeydan and Kantarci, 2020). The primary pathological feature of MS is the formation of inflammatory plaques (areas of demyelination) that can be found in white and grey matter of the brain and spinal cord. The plaques arise from an inflammatory process that causes the destruction of myelin, formed by the oligodendrocytes, with consequent neuronal loss (**Figure 12**).

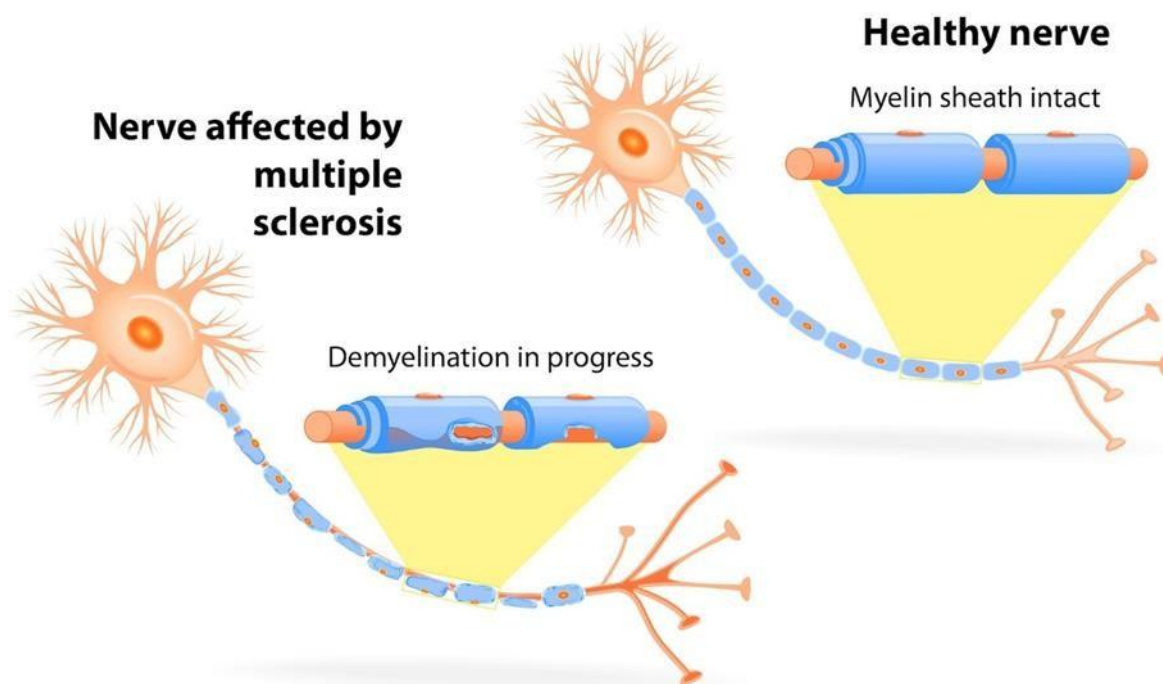


Figure 12. Axonal damage in MS.(<https://www.istockphoto.com/it/illustrazioni/multiple-sclerosis>)

The demyelination process is triggered by an abnormal permeability of the BBB, which permits the infiltration of peripheral pathogenic T and B cells, antibodies, monocytes, and inflammatory mediators in the CNS (Schepici et al., 2019) .

MS can be classified into four distinct subtypes: relapsing-remitting (RR) is present in the 70-80% of patients and is characterised by unpredictable acute attacks followed by a period of remission, primary progressive (PP) usually these patients have fewer brain lesions, secondary progressive (SP) is considered the second phase of the disease and the 65% of RR patients will develop it and lastly progressive-relapsing (PR) is the less common subtypes, it occurs only in 5% of patients and is associated with symptoms such as eye pain and double vision, along with sexual, intestinal and urinary system dysfunction, dizziness, and depression (Klineova and Lublin, 2018). Epidemiological studies have revealed that genetic and environmental factors are involved in the development of MS (Noto and Miyake, 2022). Among the environmental factors, drugs, diet and stress have a detrimental role in the development of the disease. These factors are known to play a role in the perturbation of the gut microbiota and it has already been demonstrated by different studies the association of gut microbiota, dysbiosis and MS development (Alfredsson and Olsson, 2019; Noto and Miyake, 2022).

Etiology of MS

Genetic factors

The main genes associated with the risk of MS are present in the HLA region of chromosomes 6. Among them, HLA-DR2+, HLA-DQ6, DQA 0102 and DQB1 0602, HLA-DRB1, DR15, DRB1*1501, and DRB1*1503 are alleles susceptible to the onset of MS. In addition, IL-7 and IL-2 receptor alpha are other sensitive genes associated with MS (Ferrè et al., 2020; Parnell and Booth, 2017).

Environmental factors

There are several different environmental factors that may act many years before MS onset. These include: EBV infection, smoking, diet and vitamin D deficiency. Specifically, EBV is a herpesvirus that infects 95% of people, causing infectious mononucleosis. With the resolution of the primary infection, EBV remains in a quiescent state in B cell's host, where it can be reactivated. MS patients are characterised by an altered immune response to EBV antigens, such as EBV nuclear antigen-1 (EBNA-1). One mechanism by which EBV might trigger MS is the molecular mimicry that occurs when viral proteins are so like host proteins that the immune responses start to attack the host's own tissues. Specifically, EBNA-1 protein shares structural similarities with several myelin proteins, as myelin basic protein (MBP) (Hedström, 2023). This situation leads to a cross-reaction by the immune cells, which will destroy, beyond the EBNA-1 proteins, also the myelin present in the CNS, causing MS. Smoking is also a risk factor for MS, with an OR of 1.5 (Handel et al., 2011). It causes alterations to the immune system that contribute to the formation of antibodies against myelin and also against drugs used in MS such as natalizumab. Moreover, cigarette smoke contains a lot of toxic agents, among them ROS. High levels of ROS cause oxidative stress, which induces the apoptosis of oligodendrocytes, resulting in neuroinflammation. Another mechanism through which smoking increases susceptibility to MS is epigenetic alteration. Indeed, smoke is associated with DNA methylation and histone modifications, which will alter the immune and CNS cells. These modifications contribute to an overexpression of pro-inflammatory genes or inhibition of genes involved in the prevention of autoimmunity. These epigenetic changes can persist over time and become stable, resulting in a sustained inflammatory state that will increase the probability of MS (Wingerchuk, 2012). Vitamin D has a role in the formation of myelin and its components, in fact, it lacks a common hallmark of all neurological diseases. Vitamin D is

important in immune modulation and the reduction of inflammation. The vitamin D receptor (VDR) is present on immune cells such as B cells, T cells, macrophages and DCs. Therefore, vitamin D can activate and enhance the differentiation of regulatory T cells (Tregs), which are essential for avoiding autoimmune reactions (Sintzel et al., 2017). MS patients are characterised by vitamin D deficiency, which leads to low levels of Tregs, resulting in inability to eliminate autoreactive T cells against myelin. Moreover, vitamin D suppresses Th1 and Th17 cells and the production of IL-6 and IL-17, which are pro-inflammatory and enhance the proliferation of Th2 and the production of IL-10, that exert anti-inflammatory functions (Roffe-Vazquez et al., 2019). Vitamin D also plays a role in MS progression. Indeed, low levels of vitamin D have been associated with higher relapse rates. Some studies suggest that vitamin D supplementation may reduce relapse rates and slow disease progression in individuals with MS (Alharbi, 2015).

Epidemiology

MS is an autoimmune disease that mainly affects young adults with a predominance of 3-fold more in females than males. The onset of the disease is usually between the ages of 25 and 40 years old but based on two types of MS there are some differences. For RR MS the mean age is 25 to 29, while for the PP MS is 39 to 41 (Harbo et al., 2013).

MS is predominantly a western disease; indeed, it is relatively rare in Asian and African populations. In general, Europeans are more commonly affected compared to other ethnic groups. The global prevalence of MS has been increasing, with estimates suggesting over 2.8 million people are affected worldwide. The incidence of MS appears to be rising in some regions, potentially due to improved diagnostic techniques, increased awareness, and lifestyle changes, such as reduced physical activity and westernized diets (Walton et al., 2020).

MS diagnosis

For MS diagnosis there are no specific tests, but clinicians use a combination of medical history and neurological evaluation employing MRI, lumbar punctures (LP) for cerebrospinal fluids (CSF) analysis, optical coherence tomography, evoked potential tests and blood analysis (Ömerhoca et al., 2018). MRI can highlight the presence of abnormal brain areas that are typical in patients with an established MS. Moreover, MRI can also exclude the presence of other neuropathologies. Usually, MRI alone is sufficient to diagnose MS, but in patients who have atypical clinical manifestations, CSF may represent an efficient tool (Hemond and Bakshi, 2018). CSF can be used to test the presence of normal white cell counts and autoantibodies linked to MS. Frequently, MS patients experience optic neuritis, that is a condition that causes changes in the retina's composition. Thus, a common test used is optical coherence tomography, which can detect damage to retinal nerve fibers (Deisenhammer et al., 2019). Another tool used to support diagnosis is evoked potential tests, through which there is the recording of electrical signals produced by the patient's nervous system in response to stimuli. Electrodes measure the speed through which the information passes in the nerves (Calugaru et al., 2016). To efficiently treat MS, an early diagnosis is required.

MS treatment

Up to date, there is no definitive cure for MS. In this regard, the available treatments are aimed to target symptomatology, reduce MS attacks and modify the disease's progression. The first line MS treatment is the recombinant IFN- β , which can elicit an anti-inflammatory response through different mechanisms, such as the prevention of the immune cell's migration in the brain through the BBB and the suppression of T cells activation. Moreover, IFN- β also induces the proliferation of oligodendrocytes, which repair the damaged cells in the CNS. It is widely used in the case of RR MS due to its efficacy and long-term safety profile (Cohan et al., 2021). An immunomodulatory drug used as a disease modifier is glatiramer acetate, which consists of a pool of synthetic peptides that have similar sequences to MBP. The mode of action of this drug is not completely elucidated, but the beneficial effect is related to the anti-inflammatory properties that promote Th2 cells development. These cells accumulate in the CNS, where they exert their functions, suppressing inflammation. Glatiramer is administered subcutaneously once a day and usually is well tolerated by patients. The only side effects interest the site of injection, with the formation of erythema, swelling and pruritus. Glatiramer

is very effective in treating RRMS (Lalive et al., 2011).

Ofatumumab is an anti-CD20 monoclonal antibody that is administered to patients with MS, but mainly in those with the RRMS. This drug depletes B cells, leading to fewer relapses and improvements in MS symptomatology (Kang and Blair, 2022).

Another immunomodulatory drug is teriflunomide, that selectively and reversibly inhibits the mitochondrial enzyme dihydroorotate dehydrogenase, implied in de novo synthesis of pyrimidine, resulting in the reduction of the proliferation of T and B cells. In this way, it can block the inflammatory processes involved in the MS pathogenesis (Miller, 2021).

Dimethyl fumarate is an immunomodulatory agent, with anti-inflammatory properties. The mechanism of action is based on the activation of the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) transcriptional pathway, which is responsible for the upregulation of antioxidant genes (Bresciani et al., 2023).

The second line treatments included: fingolimod, natalizumab, alemtuzumab and mitoxantrone. Fingolimod is a sphingosine 1-phosphate receptor (S1PR) modulator that, after its phosphorylation, binds with high affinity the S1PR, leading to the internalization and degradation of the receptor in lymphocytes. As a result, autoreactive lymphocytes cannot exit the lymph nodes and go into CNS (Mehling et al., 2011). Natalizumab is a monoclonal antibody against $\alpha 4$ -integrin which is involved in the adhesion of activated leukocytes. By inhibiting the $\alpha 4$ -integrin, activated leukocytes are no longer able to bind the inflamed endothelium and so inflammatory cells cannot migrate into the endothelium. An important concern in the use of natalizumab is that after 12 months of administration, there is the possibility of developing antibodies against the drug, which makes the treatment ineffective (Khoy et al., 2020).

Alemtuzumab is a recombinant monoclonal antibody against the CD52, which is present on the surface of T and B cells. Alemtuzumab acts through antibody-dependent cellular cytotoxicity and complement-mediated lysis following cell surface binding. Consequently, it causes T and B cells depletion and induce repopulation (Li et al., 2018).

Mitoxantrone is a synthetic anthracenedione derivative that interacts with the DNA, exerting its immunosuppressive functions. Mitoxantrone can inhibit proliferation and induce the apoptosis of T and B cells, macrophages and APCs (Fox, 2004).

MS and EVs

The major hallmark in MS is the autoimmune response led by autoreactive B and T cells against

myelin components. In MS the communication between the immune system and CNS has been altered, and in this context EVs could play a strategic part in both pathological and reparative mechanisms (D'anca et al., 2021).

The detrimental role of EVs in MS pathogenesis is not only limited to myelin damage. EVs have also the potential to affect the immune response, promote cellular activation, amplify inflammation, and contribute to the BBB disruption (Sáenz-Cuesta et al., 2014). During BBB dysfunction, a pro-inflammatory microenvironment induces the endothelium to release cytokines and metalloproteinases through eEVs, promoting disruption of the endothelial tight junctions (Dolcetti et al., 2020). This process increases paracellular leakage of soluble mediators, leading to the activation of leucocytes into the CNS, pro-inflammatory microenvironment and a significant increase of platelets derived-EVs in MS patients.

Notably, a study by D'Anca et al. demonstrated that injecting microglia-derived EVs into mice with experimental autoimmune encephalomyelitis (EAE), the mouse model of MS, resulted in increased brain inflammation and worsened disease progression (D'Anca et al., 2021). EEVs also play a negative role during MS relapses by facilitating their passage through the BBB, leading to increased inflammation within the CNS (Blonda et al., 2018). Furthermore, Verderio et al. observed an excessive production of eEVs in the cerebrospinal fluid (CSF) of individuals diagnosed with MS compared to healthy controls (Verderio et al., 2012).

Several independent studies reported increased levels of eEVs in MS patients compared to healthy donors, and these eEVs especially those of endothelial origin were associated to the relapsing phase of MS. For example, Marcos-Ramiro et al. demonstrated a relevant increase in plasma of EVs deriving from platelets and endothelium in all clinical forms of MS, while Sáenz-Cuesta et al. demonstrated the involvement of platelets, lymphocytes and monocytes-derived eEVs in MS development (Marcos-Ramiro et al., 2014), (Sáenz-Cuesta et al., 2014).

Lastly, histopathological features of MS, as well as genetic risk alleles, suggest that the disease is driven by an aberrant peripheral immune response targeting CNS antigens and there are different evidences that suggest the implication of EVs as key mediators of crosstalk in CNS autoimmunity (Palacio et al., 2023).

AIM 3

MDD and MS are complex neurological diseases, both characterized by a disruption in the gut-brain axis. This complex bidirectional relationship includes distinct players, such as immune cells, neurotransmitters, hormones, bacterial metabolites and EVs. New emerging evidence suggests that gut-derived EVs may play a central role in modulating immune responses, neurotransmitter signaling, and systemic inflammation, potentially influencing diseases onset and progression. Despite the knowledge on gut-derived EVs, the lack of a standardized method for their isolation and characterization limits the understanding of their specific role and their use.

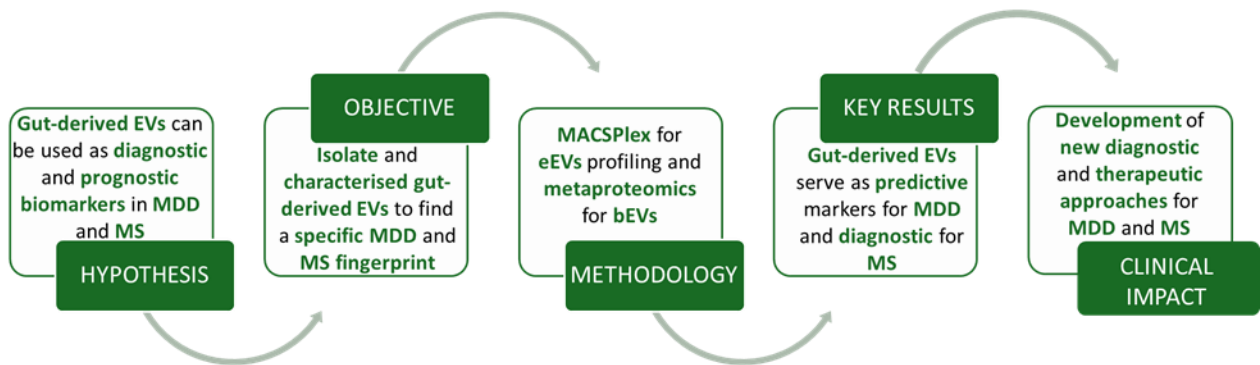
Both MDD and MS are multifactorial disorders with diagnoses that are not always easy and reliable, making the demand for high specific and sensitive biomarkers very high.

Given the potential role of EVs as diagnostic, prognostic and predictive biomarkers in several human diseases, including their potential demonstrated in the previous chapter on COVID-19, the third objective of my thesis was to unveil the role of gut-derived EVs in MDD and MS as disease-specific biomarkers and mechanistic mediators of gut-brain communication, with the goal of developing innovative diagnostic and prognostic tools that improve disease stratification and clinical decision-making.

To achieve this, we focus on two innovative approaches. First, we developed a reliable method for isolating gut-derived EVs from stool samples, ensuring the preservation of their structural and functional integrity for downstream analyses. This standardized methodology would enhance clinical and translational research applications. Secondly, the identification of disease-specific EVs signatures in MDD and MS by analyzing both eEVs and bEVs. This involved the use of MACSPlex technology for eEVs profiling and metaproteomics for bEVs characterization. Our aim was to generate a disease-specific EVs fingerprint through this study in order to uncover novel mechanisms of gut-brain interactions in neurological disorders.

This chapter introduces a novel class of biomarkers with higher specificity and sensitivity than current diagnostic methods, enabling earlier and more precise disease detection.

Our findings have the potential to transform patient care by facilitating personalized treatment strategies, improving disease monitoring, and paving the way for microbiome-based therapeutics, ultimately advancing precision medicine in neuroimmunology and mental health.



Flowchart Chapter 3.

Materials and methods

MDD patients' recruitment and samples collection

Patients were enrolled at “Città della Salute e della Scienza di Torino”, Turin, Italy. Prior to the enrolment, an informed consent statement was signed by each participant. Inclusion criteria were age between 18 and 70 years old and diagnosis of MDD with an ongoing MDE, necessitating the initiation or modification of an antidepressant treatment. The severity of depressive symptoms was evaluated with the MADRS, where higher scores indicate greater severity (Montgomery and Asberg, 1979). Functioning was assessed using the Work and Social Adjustment Scale (W-SAS), where higher scores indicate a more significant functioning impairment (Mundt et al., 2002). The Quality-of-Life Enjoyment Satisfaction Questionnaire—Short Form (Q-LES-Q-SF, 16-item version) was employed to assess patients' quality of life and satisfaction with life (Endicott et al., 1993). It is a self-rated questionnaire; higher scores correspond to higher subjective quality of life and satisfaction. The W-SAS and Q-LES-Q-SF total scores were integrated into the recovery index (RI), which provides a standardized measure of working and social functioning and subjective quality of life. It ranges from -1.643 to 4.242. Higher scores indicate better functioning and quality of life. The RI was calculated using an online tool (<http://tinyurl.com/RecoveryIndex>) proposed in the index validation study (IsHak et al., 2017). In this study, stool samples were collected at time of MDE (T0) using standardized methods. The stool sample was processed and aliquoted within 2-3 hours, and stored at -80°C.

During the three-month follow-up (T1) visit, improvement of depressive symptoms and functioning and subjective quality of life were assessed. Improvement of depressive symptoms was evaluated with the variation (Δ) of the MADRS total score from baseline to the follow-up visit (Δ MADRS T1-T0).

The improvement in functioning and subjective quality of life was evaluated with the variation of the Recovery Index (Δ RI T1-T0).

MS patients' recruitment and samples collection

Prior to enrolment, an informed consent statement was signed by each participant. In this thesis, stool samples were collected from MS patients at diagnosis (T0) and from HC, using

standardized methods. The stool sample was processed and aliquoted within 2-3 hours, and stored at -80°C.

Gut-derived EVs 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, supernatant was filtered using filter with different sizes: 5.0, 1.2, 0.45 and 0.22 µm. EVs were then separated from small molecules by loading the flow through onto a filter with a molecular weight cut-off of 100 kDa (Amicon Ultra filter unit) and concentrating it to 250 µl through a 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 µl). Subsequently, free proteins were removed through size exclusion chromatography (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 24 different fractions were collected. Then, a step of ultracentrifugation (100'000g for 1h at 4°C) was performed to pool the fractions. After ultracentrifugation, pellets were resuspended in 200 µl of PBS and stored at -80°C for further analyses (**Figure 13**).

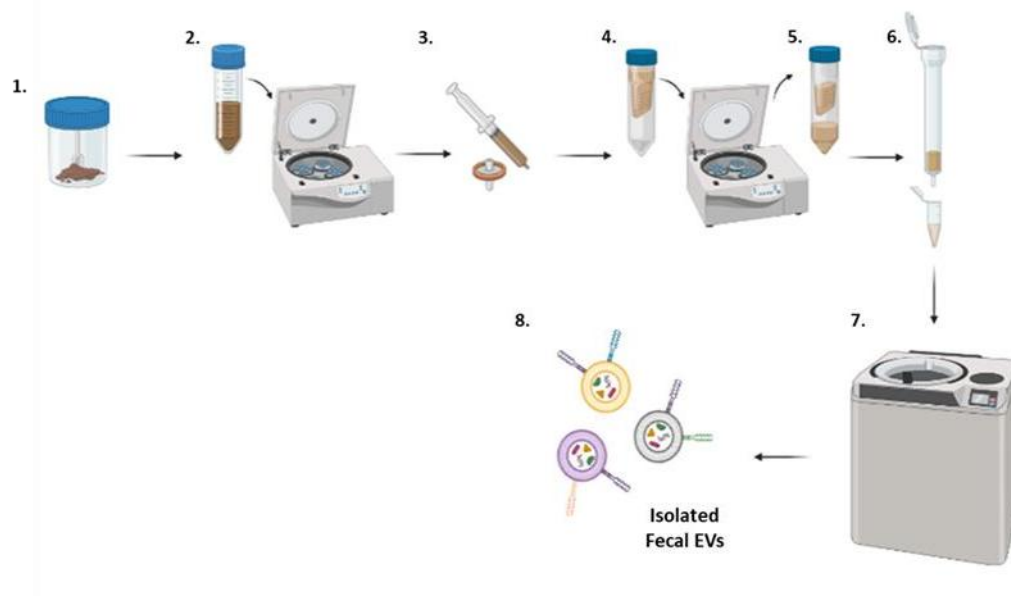


Figure 13. Experimental workflow for gut-derived EVs isolation. Half gram of feces were dissolved in 10 ml of filtered PBS (1), and two steps of centrifugations were applied (2). Then, supernatant was filtered through four filter of different size 5.0, 1.2, 0.45 and 0.2 µm (3). After the filtration steps, the sample was loaded into Amicon

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).

Nanoparticle Tracking Analysis (NTA)

Nanoparticles tracking analysis (NTA) is a widely used technique to visualize and count rapidly the EVs that are present 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).

Western blot analysis

Western blot is the gold standard for the characterization of EVs, as it allows the identifications of markers that are specifically present in EVs. Thus, this technique ensured the presence of EVs in a sample and serves as a validation step to confirm the effectiveness of the isolation method. Proteins present in gut-derived EVs were quantified using the Qubit Assay (Invitrogen). A total of 15 µg of protein were separated on a 12% SDS-PAGE gel and the proteins were transferred into 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 ECL chemiluminescence kit (Western Nova 2.0, Cyanagen) in 1:1 ratio and incubated for 2 minutes. The images were acquired using the Chemidoc (BIO-RAD Chemidoc imaging system).

Scanning electron microscopy (SEM)

SEM allows to have ulterior proof on the isolations of EVs in a sample. A small volume (10 μ l) 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 μ l 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 we incubated it for 20 minutes until it was completely dry. Lastly, we coated a layer of gold on the glass coverslip with sputter coater machine (DII-29030SCTR Smart Coater, JEOL, Italy) and observed by using the bench SEM (JSM-IT500, JEOL, Italy).

Metaproteomic analysis

Metaproteomic analysis is a unique method that allows us to identify specific bacteria 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. 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 μ l of ammonium bicarbonate, 15 μ l of trifluoroethanol (Sigma-Aldrich Inc., US) and 2.5 μ l of dithiothreitol 200 mM (Sigma-Aldrich Inc., US) were added to the sample that were incubated at 60°C for 45 min. Alkylation was performed adding 10 μ l of iodoacetamide IAM 200 mM and the sample was incubated at RT, in the dark, for 1 h. Then, 2.5 μ l of DTT were added to the sample and incubated for another hour. Finally, after the addition of 100 μ l of ammonium bicarbonate, 200 μ l of water and after reaching a pH of 7.8, 10 μ l of trypsin 0.2 μ g/ μ l (Sigma-Aldrich Inc., US) were added to the sample and incubated at 37°C overnight. Digestion was then stopped with 2 μ l 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.

MACSPlex exosomes kit

MACSPlex exosomes kit is a fast and reliable technique that allows to detect a wide range of different immune markers present on eEVs. MACSPlex exosome kit (Miltenyi Biotec) allows the detection of 37 exosomal surface epitopes plus two isotype controls (**Figure 14**). 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 rotator and then it was acquired using FACSymphony A5 (Becton and Dickinson, San Jose, CA, USA).

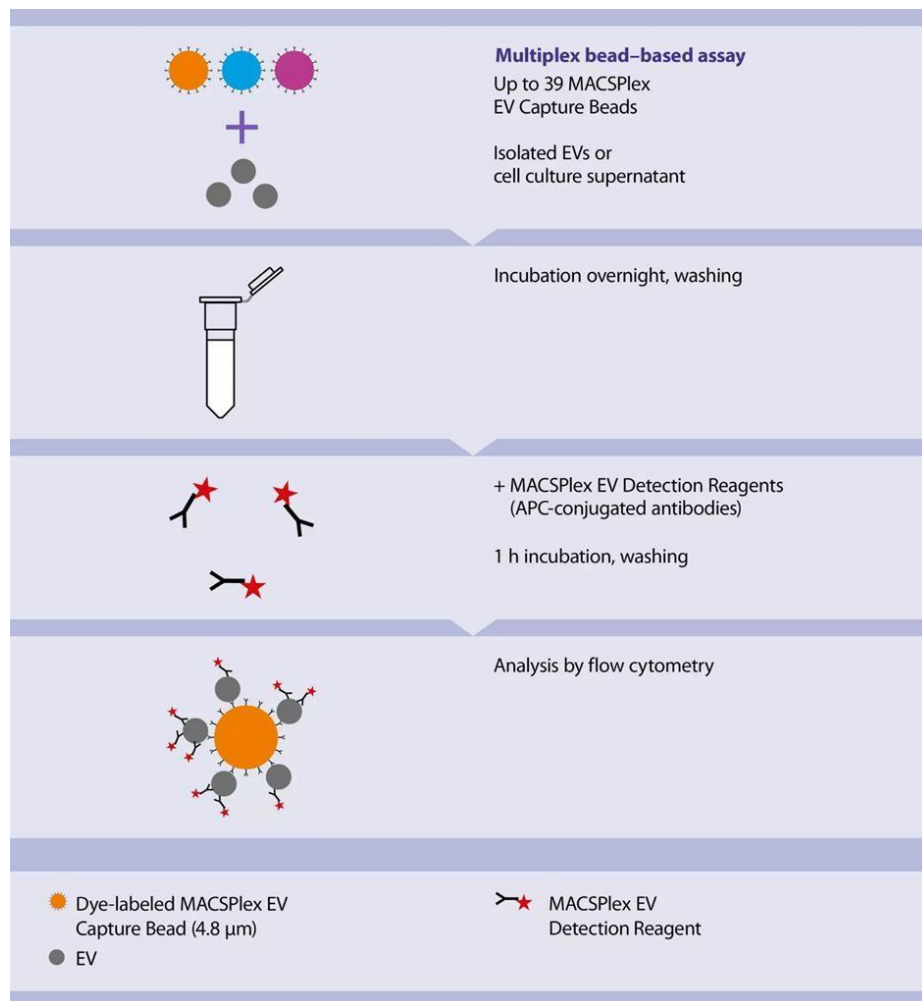


Figure 14. Principle of MACSPlex Exosome Kit. MACSPlex Capture Beads kit contains a cocktail of various fluorescently labelled bead populations, each coated with a specific antibody. Bound EVs on the beads are stained by a detection reagent and will generate a signal that is detectable by flow cytometry. (<https://www.miltenyibiotec.com>)

Statistical analysis

Descriptive analyses were initially conducted to characterize the participants' sociodemographic and clinical profiles. Next, a t-test for paired samples was used to compare baseline symptoms, functioning, and subjective quality of life. Differences in bacterial abundance and activation were analyzed using the Mann-Whitney test, with p-values below 0.05 considered statistically significant. Additionally, a linear regression analysis was performed, with the independent variables being the relative frequencies of bacterial activation and the 37 populations of eEVs identified. The dependent variables were the Δ MADRS T1-T0 and Δ RI T1-T0 over the three-month follow-up period. Statistical analyses were conducted using GraphPad Instat software (Prism 8 version 8.4.3) (GraphPad Software, San

Diego, CA, USA). The differences between HC and MS at the *phyla*, *species* levels and for eEVs 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).

Results

Validation and optimization of the isolation method of gut-derived EVs through NTA analysis

The first step was to optimize and to 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 15 A**, the concentration of EVs (particles/ml) starts to increase from the 4th fraction, with the highest peak in the 5th (5.48x10⁶ 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 (Kameli et al., 2021). Moreover, the EVs size was also evaluated, and the results show that all fractions range from 100 nm to 200 nm (**Figure 15 B**).

While SEC provides highly purified samples, it also results in 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 15 C-D**). In the end, we decided to use the 4- 12 pool for further analysis, because it was the one with the highest EVs concentration.

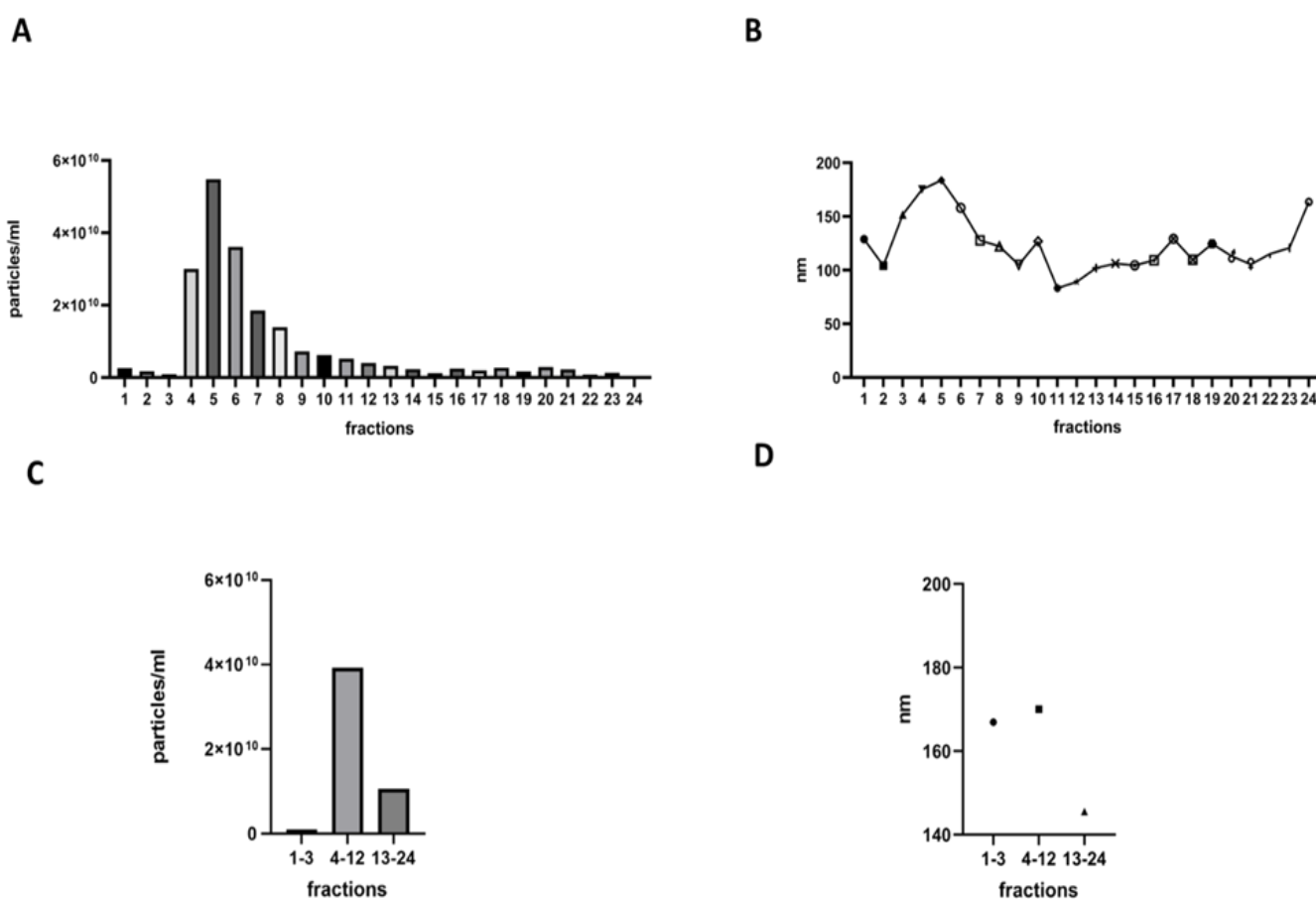


Figure 15. Validation of isolated gut-derived EVs through NTA. **A)** Concentration of gut-derived EVs in the 24 fractions analyzed by NTA **B)** Size of gut-derived EVs in the 24 fractions analyzed by NTA **C)** Concentration of gut-derived EVs in the combined samples. **D)** dimension of gut-derived EVs in the combined samples.

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 16 A-B**). Surprisingly, tetraspanin CD81 was not detected in our sample (**Figure 16 C**). As expected, LPS showed a signal around 10 kDa (**Figure 16 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 around 90 kDa. (**Figure 16 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.

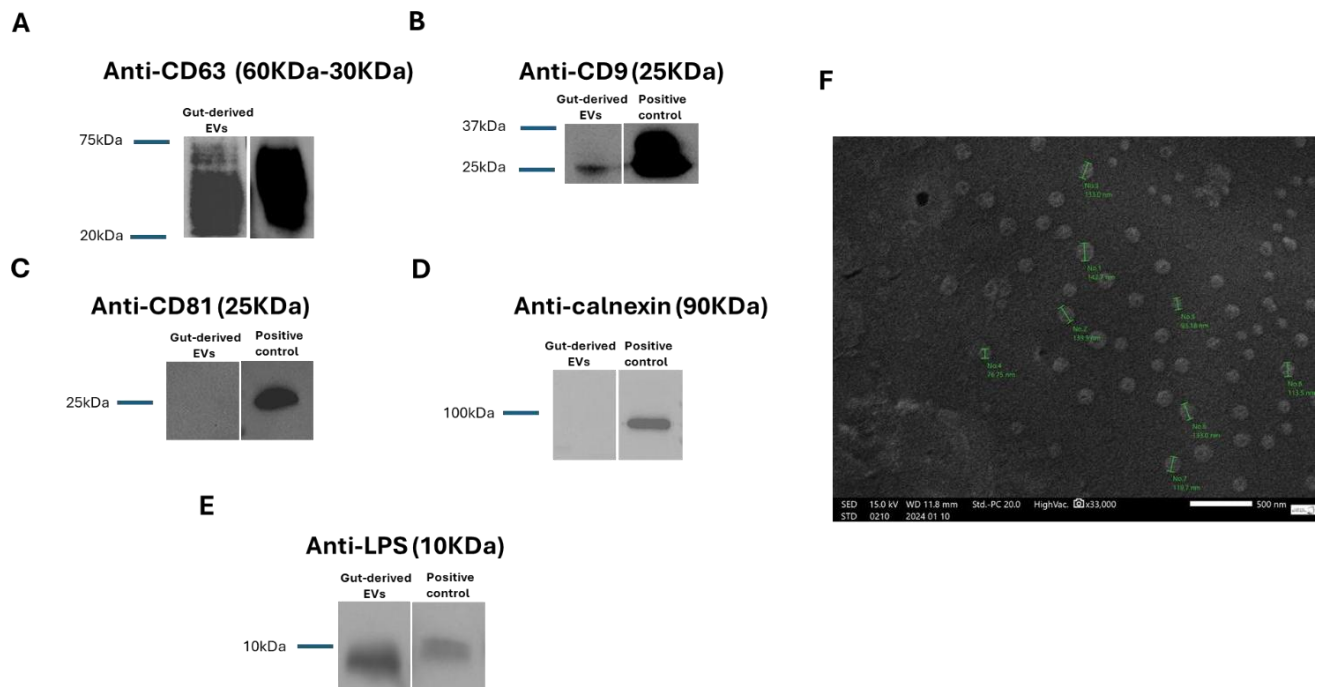


Figure 16. Characterization of EVs by western blot and SEM. A) Representative western blot of CD63 in gut-derived EVs sample and positive control B) Representative western blot of CD9 in gut-derived EVs sample and positive control C) Representative western blot of CD81 in gut-derived EVs sample and positive 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).

Results of gut-derived EVs in MDD patients

General features of the patient's cohort

47 MDD patients were enrolled in the study as reported in **Table 1**. The sociodemographic characteristics of the patients were: mean age 53, with a higher prevalence of women (76.59%). The duration of illness and the number of previous MDEs were heterogeneous, ranging from the first episode to 15 episodes with a history of MDD longer than 40 years. With the onset of MDE, it was decided to implement a new treatment regimen, ensuring that all patients would be placed under therapy. Consequently, from T0 to T1, all patients were treated with antidepressants, which led to a substantial clinical improvement in their depressive symptoms (**Table 1**). In our cohort, at T0, 11 patients were initiating antidepressant therapy while 36 had their treatment modified.

	Baseline (T0)	Follow up (T1)	p value
Age years	53y.o.		
Gender M/F	11(23.40%)/36(76.59%)		
Number of previous MDE	3.4		
Depressive symptoms, MADRS total score	25.8	15.15	<0.0001
Impairment in working and social functioning, W-SAS total score	19.63	14	<0.0001
Quality of life enjoyment and satisfaction, Q-LES-Q-SF % max score	34.84	47.52	0.0002
Recovery Index (RI), standardized score	0.56	1.52	<0.0001

Table 1. Sociodemographic and clinical characteristics (n=47). Data are reported as mean for continuous variables and as absolute and relative frequencies (%) for categorical variables. MDE: major depressive episode; MADRS: Montgomery-Åsberg Depression Rating Scale; W-SAS: Work and Social Adjustment Scale; Q-LES-Q-SF: Quality of Life Enjoyment Satisfaction Questionnaire—Short Form.

Metaproteomic analysis on bEVs reveal activation patterns in MDD patients

Recent advancements in proteomic methodologies have led to the development of metaproteomic directly on bEVs, which can highlight the most active species. Indeed, a study by Maredia et al. showed that there is a strong link between bEVs production and bacterial activation (Maredia et al. 2012). In this regard, we performed metaproteomics analysis on the bEVS to have a specific pattern of bacterial activation associated with MDD.

At *phylum* level, the meta-proteins found in bEVs samples of MDD patients were assigned mainly to five *phyla*: *Bacteroidetes* (74.38%), *Firmicutes* (21.33%), *Pseudomonadota* (2.66%), *Actinomycetota* (0.49%), *Cyanobacteriota* (0.22%) and others (0.91%) (**Figure 17 A**). Instead at *species* level, we found predominantly these five *species*: *Bacteroides uniformis* (18.63%), *Segatella copri* (14.77%), *Roseburia faecis* (7.59%), *Parabacteroides distasonis* (6.55%), *Phocaeicola massiliensis* (3.54%) and others (48.93%) (**Figure 17 B**).

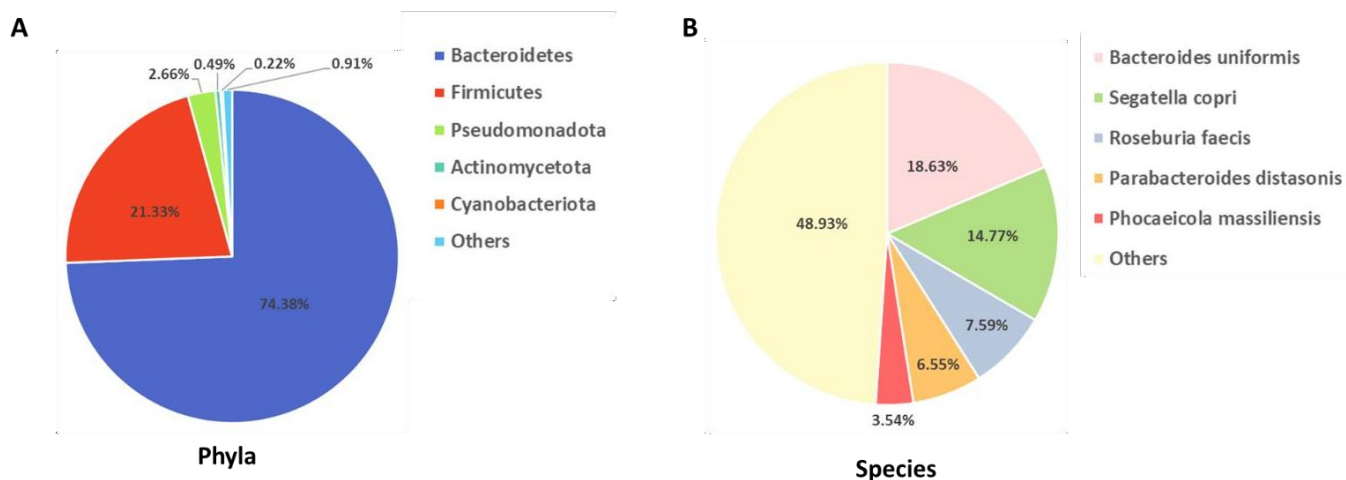


Figure 17. Taxonomic distribution of bEVs in MDD patients. A) Pie chart depicts the five most abundant bEVs at phyla level in MDD patients, B) Pie chart depicts the five most abundant bEVs at species level in MDD patients.

BEVs derived from *Bacteroides uniformis* are associated with clinical outcomes after three months

The use of gut microbiota as prognostic markers for diseases is gaining significant attention, providing new insights into disease progression and treatment response. Based on that, during the three-month follow-up visit, depressive symptoms, improvement of functioning and subjective quality of life were assessed. In particular, improvement of depressive symptoms was evaluated with the Δ of the MADRS total score from baseline to the follow-up visit (Δ MADRS T1-T0) while the improvement in functioning and subjective quality of life was evaluated with the Δ RI T1-T0. We correlated the five most active *phyla* and *species* with the Δ RI and Δ MADRS. Indeed, the RI provides a standardized measure of working and social functioning and subjective quality of life while the severity of depressive symptoms was evaluated with the MADRS. The correlation analysis between bEVs deriving from the five *phyla* and both Δ -RI and Δ MADRS did not yield statistically significant results. On the other hand, the correlation between bEVs deriving from the five most active *species* and Δ MADRS were not significative, while we found a statistical significance in the correlation between bEVs derived from *B. uniformis* and Δ -RI ($r=-0.3178$ $p=0.0487$). Notably, an increase in bEVs derived from *B. uniformis* correlated with a less improvement in the quality of life (**Figure 18**).

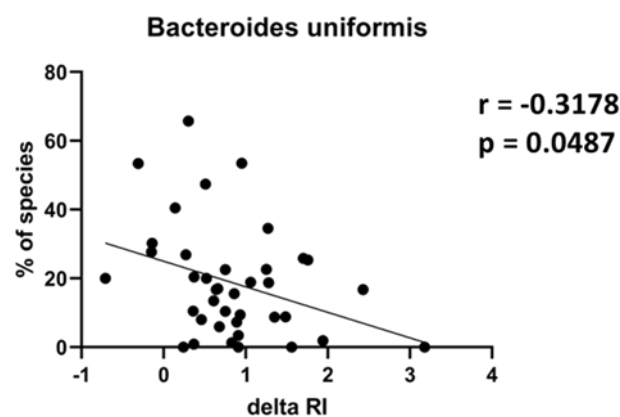


Figure 18. Linear regression between bEVs from *Bacteroides uniformis* and Δ RI. A) Linear regression showing the relationship between Δ -RI and *Bacteroides uniformis*. $r = -0.3178$ and $p = 0.0487$.

CD1c⁺ and CD11c⁺ EVs are associated with the clinical outcome after three months. Western blot analysis revealed that the isolation protocol successfully isolated also EVs of eukaryotic origin which can be released by epithelial and immune cells located in the gut. In MDD patients, dysbiosis can lead to LGS, significantly increasing the number of cells releasing EVs into the intestinal lumen and feces. Therefore, eEVs can be very useful in identifying a reliable and non-invasive disease biomarker. To quantify and characterize the origin of eEVs, we employed the MACSPlex exosome kit with flow cytometry. This kit enables the simultaneous detection of 37 surface epitopes known to be present on eEVs.

The multiplex assay includes 39 bead populations distinguishable by flow cytometry. Each bead is coupled with an antibody that targets one of the 37 EVs surface epitopes and two isotype negative controls. A semi-quantitative analysis of EVs surface markers was then performed using flow cytometry, resulting in a characteristic surface profile of gut-derived EVs from MDD patients (**Figure 19 A**).

Thus, we correlated eEVs with the Δ -MADRS and Δ -RI. Among all the 37 different subtypes of EVs detected using the MACSPLEX kit, we found that CD1c-EVs and CD11c-EVs were associated with a worse prognosis. The results showed that the increase of CD1c was correlated with a lower improvement in depressive symptoms (**Figure 19 B**) and the increase of CD11c was correlated with a worse quality of life (**Figure 19 C**).

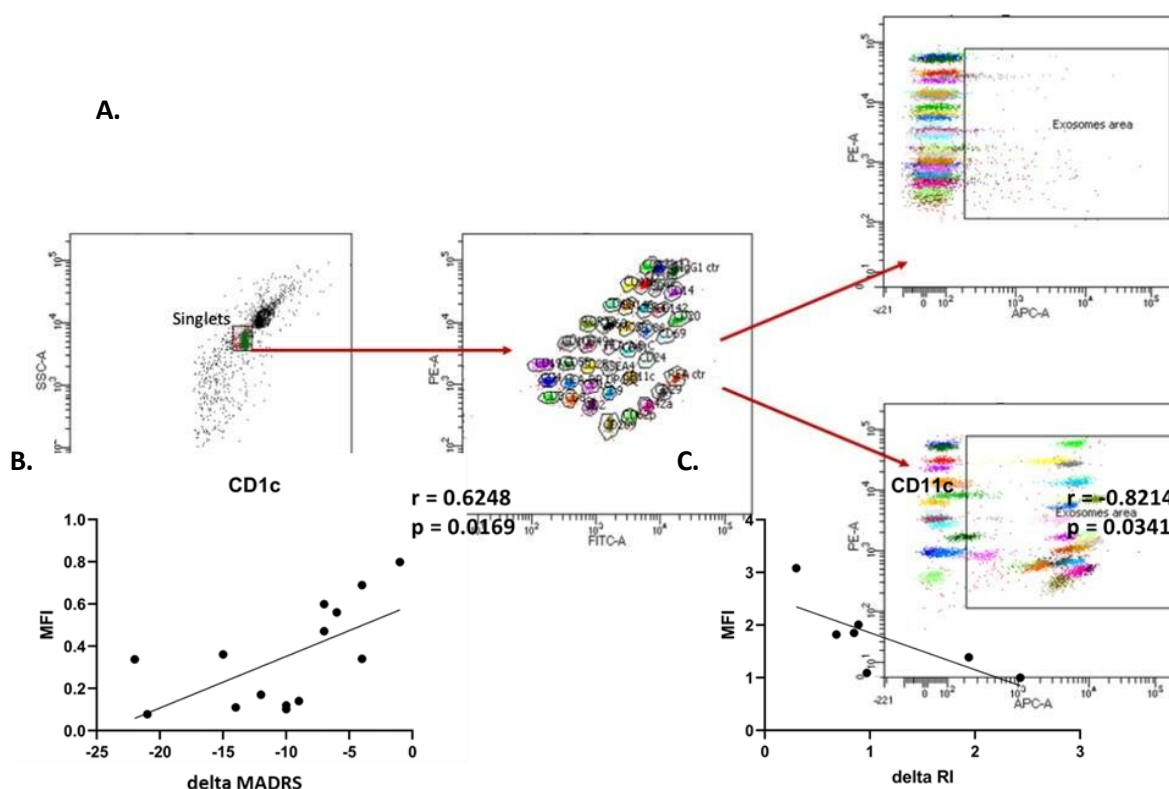


Figure 19. Gating strategy and for the detection of different subtypes of eEVs and linear regression between eEVs and Δ MADRS and Δ RI. A) After the discrimination of bead singlets (FSC vs SSC), the different subtypes of EVs were discriminated according to their fluorescent intensity (PE vs FITC). Finally, the signal of the detection antibodies (anti CD9 and CD63) in the APC channel defined the EVs area where the complex beads-EVs-detection antibodies specific for each subtype of EVs are present. **B)** Linear regression showing the relationship between Δ -MADRS and CD1c. $r = -0.6248$ and $p = 0.0169$. **C)** Linear regression showing the relationship between Δ -RI and CD11c. $r = -0.8214$ and $p = 0.0341$.

Elevated EVs Parameters in MDD

The analysis of EVs in MDD patients revealed a significant upregulation of specific EVs parameters. *Bacteroides uniformis*-derived EVs, along with EVs expressing CD1c and CD11c markers, were consistently elevated in MDD patients, particularly those who experience less improvement in depressive symptoms and quality of life. These findings highlight the potential of these EVs parameters as predictive biomarkers for identifying MDD patients with poorer outcomes.

EVs parameters	Overexpressed in poor-response MDD patients
<i>Bacteroides uniformis</i> derived EVs	↑
CD1c derived EVs	↑
CD11c derived EVs	↑

Table 2. Listed of EVs parameters expressed in MDD patients with poorer outcomes. Table shows EVs parameters, specifically, *Bacteroides uniformis*-derived EVs, CD1c-derived EVs and CD11c-derived EVs are over expressed in MDD patients with poorer outcomes.

Conclusions of gut-derived EVs in MDD patients

MDD is a common and debilitating mental disorder that affects millions of people around the world with a prevalence of 3.8%; in Italy, the prevalence of this disorder is about 5.4%, increasing with age (<https://www.istat.it>, <https://www.who.int>). Despite its prevalence, the diagnosis is quite complex because the symptoms are highly variable, and there is a lack of specific tests and reliable biomarkers, able to predict the clinical outcome. The gut microbiota is now recognized as a significant feature in human health, as it greatly impacts the functioning of the host's body and plays a crucial role in various diseases, including MDD. MDD patients commonly display signs of impaired gut barriers and disrupted gut homeostasis, which may result in immune exposure to gut bacteria and exogenous molecules, causing an improper activation of the immune system (Góralczyk-Bińkowska et al., 2022). The study of microbiota can be easily performed through stool samples as already demonstrated (Jandhyala et al., 2015).

Although a correlation between gut dysbiosis, MDD, and EVs is recognized, more research is needed to comprehensively characterize both bEVs and eEVs from the gut microbiota of patients with MDD. Several pieces of evidence underscore the interrelationship among an imbalanced gut microbiota, autoimmune diseases, and EVs (Louka and Koumandou, 2024). Specifically, gut dysbiosis can lead to the establishment of the LGS, a condition in which the intestinal barrier is disrupted, allowing the entry into the lumen of toxins, antigens and bacteria, through the junctions of the epithelium. The released harmful products can then reach the bloodstream, affecting different organs, triggering and activating the immune system of the host.

In this scenario, the hypothesis underlying this work is that both bEVs and eEVs, isolated from stool samples, might play a role in the establishment and maintenance of MDD. Moreover, due to their nature, they could be used as a powerful biomarker for MDD's diagnosis and prognosis, in the setting of precision medicine. Based on that, gut-derived EVs were isolated from the MDD patient's cohort and characterized in order to study both bEVs and eEVs.

Our isolation method represents significant advancement over conventional approaches by combining multiple techniques, including ultrafiltration, molecular weight centrifugation, SEC, and ultracentrifugation. These integrated techniques allow us to achieve a high yield rate of EVs, with a mean of 4×10^{10} particles/ml per sample. Moreover, thanks to SEC we obtained pure samples of EVs. All these provide us with an optimized platform for downstream applications

such as biomarker discovery and mechanistic studies. Despite the improvements achieved with our EVs isolation method, some limitations must be acknowledged. Indeed, the combination of multiple techniques, while highly effective, is very time-consuming, making it challenging to translate into everyday clinical practice and for scalability in large clinical studies. Future efforts should address these limitations by accelerating the isolation method and enhancing scalability. After setting and optimizing the method for the gut-derived EVs isolation, we characterized them. The international society for EVs (ISEV) suggests different methods to characterize EVs, and western blot is one of the most used (Welsh et al., 2024). Indeed, with this technique, it is easy to detect the presence of tetraspanins on eEVs (CD9, CD63 and CD81), and LPS on bEVs. In our study, western blot analysis revealed the presence of the bands of two out of three. Tetraspanins, namely CD9 and CD63 (**Figure 16 A, B**), confirming the presence of eEVs despite the lack of the band for CD81 (**Figure 16 C**). Our results were consistent with the study of Barranco and colleagues, which reported that different EV subtypes can express tetraspanins in different proportions (Barranco et al., 2019). Thus, it's possible that some of these tetraspanins are more detectable in some samples than others. In their work, they described that in a comparative quantitative analysis of tetraspanins expression, the levels of CD63 and CD9 were higher in MVs compared to exosomes, while the expression of CD81 was higher in exosomes than in MVs (Barranco et al., 2019). Thus, we asked if the dimension of the purified eEVs falls in the MVs area (100 nm to 1 µm in diameter) or in the exosomes area (30-100 nm in diameter). Our SEM results showed the presence of EVs with a mean size of 130 nm (**Figure 16 F**) justifying the absence of CD81 in our sample. Lastly, calnexin was used to assess the effective isolation of EVs from cell debris. Since calnexin is an integral ER membrane protein, it is not typically present in EVs. This was also demonstrated by Hyun Jeong Oh who investigated and confirmed its absence in EVs (**Figure 16 D**) (Oh et al., 2017).

After confirming the presence of bEVs within the gut-derived EVs by western blot (**Figure 16 E**), we dived in deep, by performing a metaproteomic analysis to find a bEVs-specific fingerprint in MDD patients. The results obtained from the bEVs exhibited two distinct patterns, one for the *phyla* and the other for the *species*. The bEVs derived from the most active five *phyla* of gut bacteria were: *Bacteroidetes* (74.38%), *Firmicutes* (21.33%), *Pseudomonadota* (2.66%), *Actynomicetota* (0.49%), *Cyanobacteriota* (0.22%) and others (0.91%). While for the *species*, the five most active were: *Bacteroides uniformis* (18.63%), *Segatella copri* (14.77%), *Roseburia faecis* (7.59%), *Parabacteroides distasonis* (6.55%),

Phocaeicola massiliensis (3.54%) and others (48.93%).

Interestingly, data obtained from *phyla* bEVs and the one obtained from the *species* bEVs were in accordance. Indeed, almost all the five most active *species* belonged to the *Bacteroidetes phyla*, which resulted in being the most active among all the others (**Figure 17 A**). Importantly, our findings aligned with the literature, where there is evidence that the genera of *Bacteroides*, belonging to the *phylum* of *Bacteroidetes*, can release large amounts of bEVs (Zafar and Saier Jr, 2021). Notably, analyzing the active species, we found that the most active one in MDD patients was the *Bacteroides uniformis* (**Figure 17 B**). This was in line with the study of Xu et al., in which they discovered that the *species* *Bacteroides uniformis*, *B. fragilis*, and *B. caccae*, all belonging to the *Bacteroidetes phylum*, are abundant in the gut microbiota of MDD patients and are implicated in neurogenesis inhibition. Indeed, they discovered that these *species* were able to increase the susceptibility to depression-like behavior, neurogenesis inhibition and metabolic disturbance in antibiotic cocktail (ABX) treated mice (Xu et al., 2021). It is well known that some molecules metabolized by the bacteria in the gut can affect the gut-brain axis, with tryptophan (trp) being one of the most important. Trp metabolism plays a crucial role in a host's health since it can synthesize two essential neuro active compounds: 5-HT and kynurenine (KYN) (Colle et al., 2020). Ninety percent of the 5-HT present in humans originates from trp by enterochromaffin cells and it remains stored in the gut. The 5-HT produced in the periphery cannot cross the BBB and it cannot reach the CNS (Gao et al., 2020). However, due to the nature and size of EVs, which are known to be able to pass the BBB and potentially act in the CNS, these could be natural transporters of neurotransmitters or their precursors like Trp to the brain. In fact, Luo et al. detected the presence of trp inside EVs, supporting this idea (Luo et al., 2020).

Our main hypothesis is that bEVs, particularly those produced by *Bacteroides uniformis*, may transport trp in the brain, where it is then metabolized in 5-HT. Thus, we can speculate that in MDD, this transport might be impaired, resulting in a lower production of 5-HT. In line with that, it has been already demonstrated that MDD patients have decreased levels of 5-HT in the brain and plasma, suggesting a reduction of its synthesis (Jacobsen et al., 2012). Another possible explanation could implicate trp metabolism that it is known to have two distinct metabolites: KYN and 5-HT, one at the expense of the other. Indeed, if the reaction is extensively diverted to the production of KYN, there is a strong reduction of 5-HT, which can subsequently lead to depression (Agus et al., 2018).

These two hypotheses may potentially explain the mechanism by which *Bacteroidetes uniformis* bEVs can contribute to the persistence of MDD. Microbial metabolism contributes to the establishment and maintenance of a healthy host by producing metabolites from dietary substrates and modifying host molecules (i.e.: Trp metabolism); signals from these microbial metabolites influence the host's energy metabolism, preserve mucosal integrity, and support immune maturation and homeostasis. These observations suggest that gut bacteria are closely linked to human health and disease. Thus, its characterization using OMICs technologies could be used to find possible diagnosis, prognosis, and treatment biomarkers (Wiredu Ocansey et al., 2023). Thus, we decided to correlate the biological data collected during an MDE and the clinical evaluation after three months. Our findings reported that patients with a lower improvement of quality of life at the MDE (T0) exhibited high levels of the bEVs derived from the *Bacteroides uniformis* (**Figure 18 A**). This finding robust the hypothesis that this specific bacterium might be involved in the transport of 5-HT and influence the trp metabolism. Both these mechanisms result in a reduction of 5-HT levels, exacerbating the symptomatology of MDD.

Moreover, we did not consider any correlation with diet and the lifestyle of our MDD patients, even though it is well known that these aspects influence the composition and function of gut microbiota, thus representing a limit of the study (Thursby and Juge, 2017b).

After confirming the presence of eEVs within gut-derived EVs, we decided to phenotype them using the MACSPlex exosome kit. This kit allows the analysis of simultaneous profiling of several eEVs. We found that CD1c⁺ EVs enriched in the gut correlated with a worsening of depressive symptoms (Δ MADRS), while a higher production of CD11c⁺ EVs correlated with a decline of social and working functions and a subjective quality of life (Δ RI) (**Figure 19 B, C**).

Based on our results, **Table 2**. summarizes all the EVs parameters that should be up expressed in MDD patients with poorer outcomes.

CD1c, part of the CD1 family glycoproteins, is expressed on the cell surface of DCs, B cells and some T cells. This family is structurally similar to the MHC class I molecules, but instead of presenting peptides, CD1 molecules present lipids and glycolipids to T cells. Moreover, Leal Rojas et al demonstrate that CD1c⁺ DC promotes Th1 and Th17 responses, so CD1c⁺ DC shows significantly higher production of IL-17, IL-21, IFN- γ and IL-6. So, also this marker may have a significant role in autoimmune inflammation (Leal Rojas et al., 2017).

CD11c is an integrin, expressed on DCs, macrophages and monocytes. It plays a key role in

antigen presentation, helping to activate T cells. Additionally, it is involved in leukocyte adhesion and phagocytosis (Ganguly et al., 2013).

Our results highlighted a correlation between these markers and the clinical outcome (Δ MADRS and Δ RI). At the time of enrollment, some patients had been receiving therapy while others had not. With the onset of MDE, it was decided to implement a new treatment regimen, ensuring that all patients would be placed under therapy. Consequently, from T0 to T1, all patients were treated with antidepressants, which led to a substantial clinical improvement in their depressive symptoms (**Table 1**). In general, antidepressant drugs like SSRIs and SNRIs might exert different immunomodulatory effects. Moreover, DCs express the SERT, that is functionally equivalent to the one present in the CNS (Herr et al., 2017). SSRI and SNRI are known to block SERT activity, resulting in higher levels of 5-HT in both CNS and periphery. SSRIs seem to decrease the level of pro-inflammatory cytokines such as IFN- γ , TNF- α and IL-1 β , may restore the negative feedback by cortisol on the HPA axis and may promote a more balanced immune response by reducing inflammation. Instead, SNRIs have a greater anti-inflammatory effect through an increase in NE levels, which may have immunosuppressive effects on DCs and monocytes (Chen et al., 2018). Based on this evidence, we can speculate that if, at the time of MDE, the levels of gut-derived eEVs CD1c⁺ and CD11c⁺ were high, the patient responded less to the treatment. This could be ascribed basically by two different hypotheses. First, the drug, instead of primarily acting on the CNS, was acting on the SERT expressed by immune cells, which appear to be the most active in these patients, as indicated by their gut-derived eEV levels. Second, patients with high levels of CD11c⁺ eEVs may respond less to treatment because they have low levels of 5-HT, due to altered synthesis of this neurotransmitter. Indeed, CD11c could also be expressed by tolerogenic DCs (tDCs). These cells are recognized for inducing Treg expansion, which suppresses the immune system by releasing anti-inflammatory cytokines and upregulating IDO expression (Takenaka and Quintana, 2017). IDO degrades trp into KYN, further promoting Treg expansion (Platten et al., 2019).

Consequently, increasing the metabolism of trp towards KYN thus promotes a significant reduction in 5-HT.

One of the limitations of this study is the samples heterogeneity and a lack of standardization in stool collection. Since stool contents varies within different sections of the stool, the specific area from which the sample is collected can influence the detected EV profile, potentially affecting the consistency and reproducibility of the results. Another limitation relies in the

small patient cohort size and lack of multiple controls, which could enhance the superior function of EVs in diagnosing different stages of the diseases.

In conclusion, these preliminary findings suggest a significant interconnection between gut microbiota, bEVs, eEVs, and MDD, which could revolutionize our understanding of the mechanisms underlying this condition. However, further researchers are needed to improve and implement standardization of EVs approaches to enable their translation into clinical practice

Results of gut-derived EVs in MS patients

Metaproteomics analysis on bEVs reveal differences in activation patterns between HC and MS T0 patients

We performed metaproteomics 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*. For the HC: *Bacteroidetes* (51.59%), *Firmicutes* (47.67%), *Proteobacteria* (0.48%), *Chlamydiae* (0.07%), *Actynomicetota* (0.18%) and others (0.03%) (**Figure 20 A**). Instead for the MS T0: *Bacteroidetes* (64.23%), *Firmicutes* (33.64%), *Proteobacteria* (1.14%), *Chlamydiae* (0.83%), *Actynomicetota* (0.13%) and others (0.03%) (**Figure 20 B**). The bacteria most active at the *phyla* level were similar between HC and MS T0, with *Bacteroidetes* and *Firmicutes* maintaining equal proportion.

At *species* level, we found predominantly these five *species* in HC: *Roseburia faecis* (19.46%), *Phocaeicola vulgatus* (17.83%), *Faecalibacterium prausnitzii* (8.26%), *Bacteroides uniformis* (6.48%) *Roseburia intestinalis* (5.50%), and others (42.46%) (**Figure 21 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%) and others (26.45%) (**Figure 21 B**). At first sight it was clear that there were some differences among the most active *species* between HC and MS T0. Indeed, beside *Roseburia faecis*, *Phocaeicola vulgatus* and *Faecalibacterium prausnitzii* all the other *species* are different. Moreover, we found that in MS patients there was less richness and diversity due to the high levels of *Roseburia faecis*, which predominate over the others.

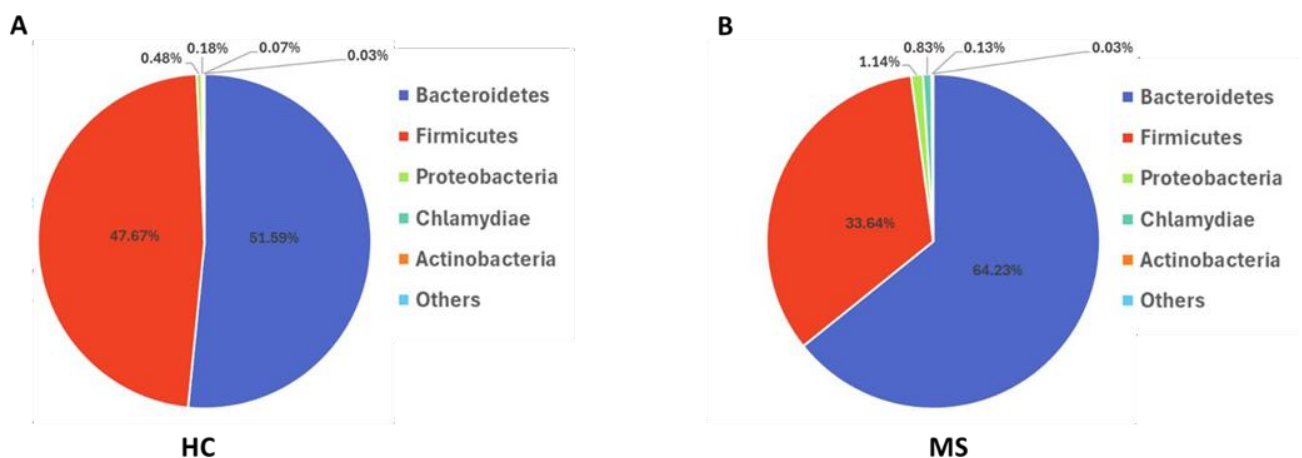


Figure 20. Taxonomic distribution at the Phyla level in bacterial activation. A) Pie chart shows the percentages of the five most active Phyla in HC, B) Pie chart shows the percentages of five most active Phyla in MS T0.

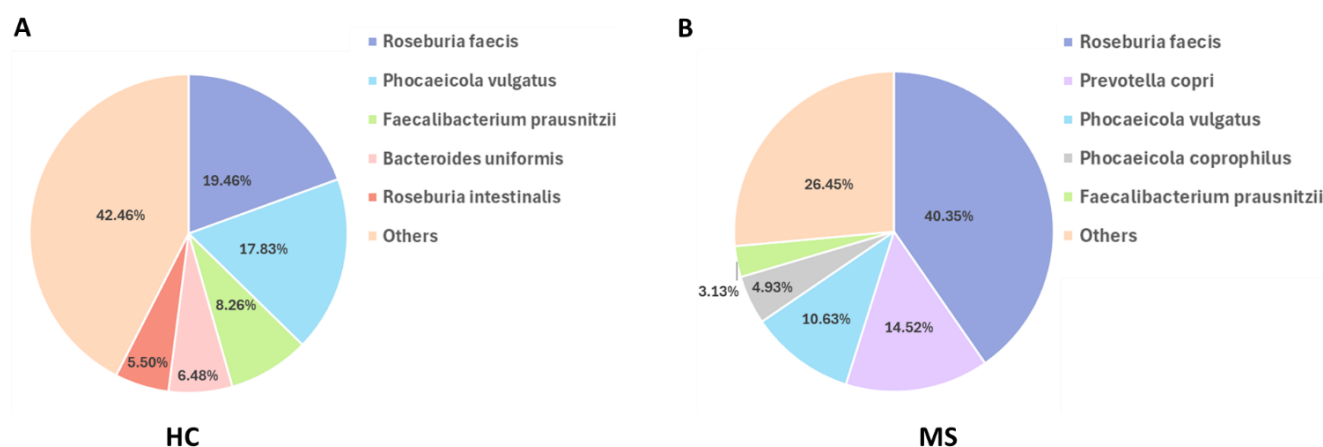


Figure 21. Taxonomic distribution at the species level in bacterial activation. A) Pie chart shows the percentages of the five most active species in HC, B) Pie chart shows the percentages of five most active species in MS T0.

Differences in *phyla* activation between HC and MS

The pie charts (**Figure 20 A, B**) showed different percentages of *phyla* between HC and MS. Statistical analysis revealed a significant difference in *Firmicutes* activation. Indeed, our results showed that bEVs produced by *Firmicutes* were elevated in HC respect to MS patients (**Figure 22 A**).

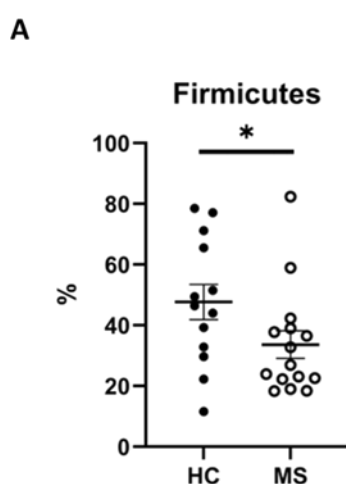


Figure 22. Differences in the Phyla activation between HC and MS. A) Dot plot depicts the percentage in the activation of *Firmicutes* between HC and MS. *p<0.05

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 five 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 23 A**). Conversely, *Bacteroides uniformis* showed higher activity in HC (**Figure 23 B**)

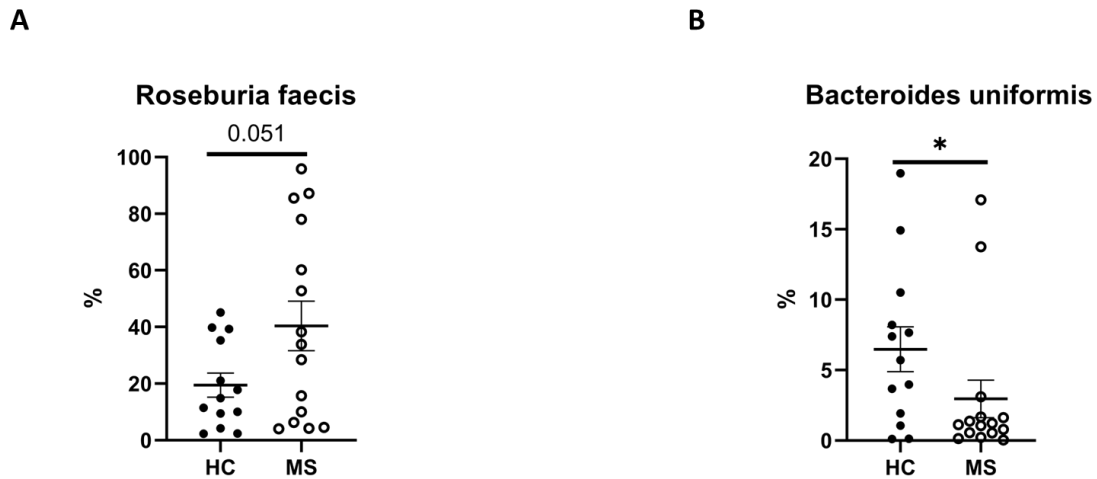


Figure 23. Differences in the species activation between HC and MS. A) Dot plot depicts the percentage in the activation of *Roseburia faecis* between HC and MS. **B)** Dot plot depicts the percentage in the activation of *Bacteroides uniformis* between HC and MS. * $p < 0.05$.

Differences in eEVs subtypes between HC and MS T0 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, CD40-expressing EVs and a trend in CD44-expressing EVs. (**Figure 24 A-D**).

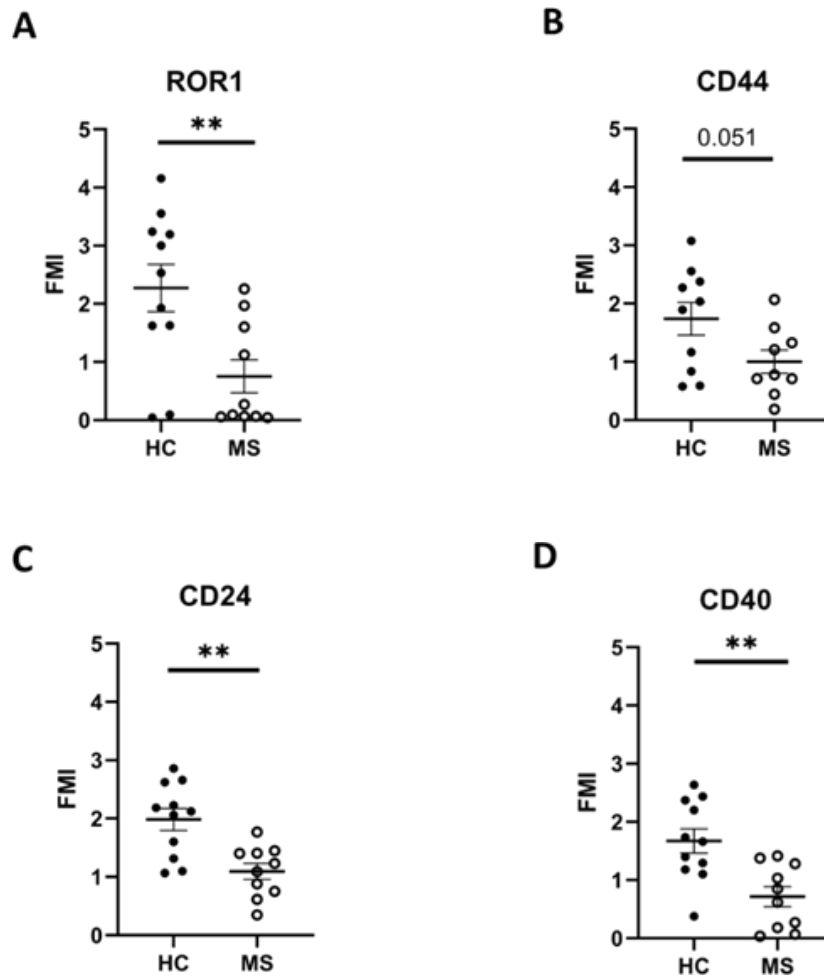


Figura 24. EEVs differences between HC and MS T0 patients. Graphs depict the normalized MFI of the different sub-types of eEVs that are modulated in MS patients expressing **A)** ROR1, **B)** CD44, **C)** CD24 and **D)** CD40). Mann-Whitney t test was used. * $p < 0.05$, ** $p < 0.01$.

Potential diagnostic EVs parameters in MS

Our previous results provided in MS the existence of distinct patterns of EVs derived from specific cell types or bacteria, which could serve as potential diagnostic biomarkers. Specifically, *Bacteroides uniformis*-derived EVs, ROR1-derived EVs, CD44-derived EVs, CD24-derived EVs, and CD40-derived EVs were found to be significantly down expressed in MS, highlighting a consistent reduction of these bacteria and immune cells markers. Conversely, *Roseburia faecis*-derived EVs were uniquely overexpressed in MS. These findings indicate that these EVs parameters may provide valuable insights into the pathophysiology of MS and serve as non-invasive biomarkers for early diagnosis and disease monitoring (**Table 2**).

EVs parameters	Up/Down in MS
<i>Bacteroides uniformis</i> derived EVs	↓
<i>Roseburia faecis</i> derived EVs	↑
<i>ROR1</i> derived EVs	↓
<i>CD44</i> derived EVs	↓
<i>CD24</i> derived EVs	↓
<i>CD40</i> derived EVs	↓

Table 3. Listed of EVs parameters down or up expressed in MS. The table shows EVs pattern present in MS patients, specifically, *Bacteroides uniformis*-derived EVs, *ROR1*-derived EVs, *CD44*-derived EVs, *CD24*-derived EVs, and *CD40*-derived EVs are significantly down expressed in MS, instead *Roseburia faecis*-derived EVs are over expressed in MS patients.

Conclusions of gut-derived EVs in MS patients

Gut microbiota is widely recognized as a significant key factor in human health, as it greatly impacts the functioning of the host's body and plays a crucial role in various diseases, including ADs such as MS. MS patients commonly display signs of impaired gut barriers and disrupted gut homeostasis, which may result in immune exposure to gut bacteria causing an improper activation of the immune system.

To investigate the relationship of gut microbiota and MS, the analysis of EVs has gained significant interest due to their role as messengers in cellular communication. Although a correlation between gut dysbiosis, MS, and EVs is known, comprehensive characterization of bEVs and eEVs from the gut microbiota of MS patients has been limited.

Several evidence underscores the relationship among an imbalanced gut microbiota, ADs, and EVs. Specifically, gut dysbiosis can profoundly influence the immune response, often manifesting alongside the phenomenon of leaky gut. This condition permits the entry of exogenous molecules, notably bacterial products and metabolites, which can disrupt tissue homeostasis, trigger inflammation, and stimulate immune responses (Parodi and Kerlero de Rosbo, 2021).

By conducting a metaproteomic analysis on bEVs, we were able to detect a specific pattern of bacterial activation in HC and MS patients. The results obtained indicated no significant differences in the five most active *phyla* detected between MS patients and HC. Indeed, the predominant *phyla* in both MS and HC were *Bacteroidetes* and *Firmicutes*, which share similar percentages (**Figure 20 A, B**). Statistical analysis showed that *Firmicutes* were higher in HC respect to MS (**Figure 22 A**), aligning with previous studies, which described less *Firmicutes* in the gut microbiota of MS patients (Chen et al., 2016). *Firmicutes* are primary producers of SCFAs from dietary fibers, which have anti-inflammatory properties. Their reduced activity in MS patients can be attributed to the chronic inflammatory state characteristic of the disease (Fusco et al., 2023).

To have a precise and specific MS bEVs fingerprint, that serves as a possible diagnostic biomarker, we further analyzed the most active *species* of gut bacteria present in HC and MS. Looking at the pie chart (**Figure 21 A, B**), at species level, *Roseburia faecis* resulted to be the most active bacterium in both HC and MS, with a higher percentage in MS. Statistical analysis revealed a trend, indicating a slightly increased activity of *Roseburia faecis* in MS patients (**Figure 23 A**). This result is discordant with the existing literature, which highlights its

beneficial functions as a commensal (Tamanai-Shacoori et al., 2017). However, despite its positive effects, the excessive activity of *Roseburia faecis* at the disadvantage of other *species* could be detrimental for other host's microbiota members. Indeed, higher levels of *Roseburia faecis* may result in decreased microbial diversity, highlighting a lower richness in MS patients. Its hyperproliferation could also result in limited nutrients for other bacterial *species* and due to its abnormal activation, perceived as a threat trigger the immune system. In addition, *Roseburia faecis* is responsible for the production of the butyrate, a SCFA with anti-inflammatory properties, beneficial for the gut's host. However, a study by Ploger et al described that chronic high levels of butyrate cause a decrease of the intestinal pH in ruminants, resulting in alterations of the epithelium composition and barrier structure, leading to the development of pathological conditions. Based on this, we can assume that an increased activity of *Roseburia faecis* in MS patients, may result in excessive butyrate production, which might become harmful and lead to the establishment of pathological states (Plöger et al., 2012). Moreover, *Roseburia faecis*, upon activation, produces high levels of lactate, that is an intermediate of butyrate. This compound resulted to be very high in MS patients (Bhargava and Anthony, 2020). Thus, we can hypothesise that increased *Roseburia faecis* activity might drive higher levels of this metabolite in MS patients.

Prevotella copri, was present only among the five most active *species* in MS patients, with a high percentage (26.45%). Different studies revealed that this specific bacterium is responsible for an increase of pro-inflammatory cytokines, which may explain its elevated activity in MS patients (Scher et al., 2013). However, statistical analysis did not reveal significant differences in *Prevotella copri* activation between HC and MS.

Bacteroides uniformis was detected among the five most active *species* of HC and a comparison between HC and MS of its activity levels revealed that this bacterium was more active in HC. Indeed, a specific strain, *Bacteroides uniformis* CECT 7771, is known to have probiotic functions. It reduces acyl carrier protein expression, a fundamental component required for LPS biosynthesis (Galloway and Raetz, 1990; Masoudi et al., 2014). In this way, it can inhibit LPS formation along with the consequent expression of pro-inflammatory cytokines (Wang et al., 2021). Based on our results, **Table 3.** summarizes all the EVs parameters that should be up or down expressed in MS patients.

On the other hand, the analysis of multiple eukaryotic surface markers of gut-derived EVs have a high diagnostic potential because of the advantage of the simultaneous profiling of several

EVs subpopulations. The flow cytometry analysis demonstrated that four markers (ROR1, CD44, CD24 and CD40) were downregulated in MS patients compared to HC (**Figure 24 A-D**). CD24 is a marker of T and B cells, and our results suggest that MS patients might have a less production of this type of eEVs or a less expression of this marker per single eEVs. On the other side, CD40 is a marker of APCs like B cells and DCs, and also this marker resulted down modulated in MS patients.

CD24 is expressed on hematopoietic cells, including B cells, T cells, neutrophils, eosinophils and DCs, and different immunological functions for CD24 have been reported. While CD24 expressed in B and T cells is associated with their expansion, interestingly, on DCs, CD24 is a negative regulator for T-cell homeostatic proliferation (Fang et al., 2010). It has been demonstrated that CD24 has a detrimental role also in the Treg function. Indeed, Treg derived from CD24 knock-out mice has an increase in suppression activity compared to wild-type groups (Shi et al., 2022). These data are in contrast with our results, indeed, a downregulation of CD24 could be ascribed to an increase of Treg activity usually absent in MS patients. One possible explanation is that CD24 down-modulation on EVs does not reflect fewer molecules on the surface of EVs but rather reflects fewer Tregs that secrete CD24 EVs into the intestinal lumen. This is in line with the data describing the presence of autoreactive T cells and Tregs in the GALT of mice with EAE where it was observed that a reduced number of Tregs impaired the remission phase of the disease (Kadowaki et al., 2016; Ochoa-Repáraz et al., 2016).

The APCs have a detrimental role in maintaining the immune tolerance that is fundamental to avoid immune response against self-antigen. When this mechanism fails, there is the onset of the ADs such as MS. Thus, the downregulation of CD40 and CD24 in our data might be explained by an impairment of immune tolerance. Indeed, CD40 can be expressed in regulatory DCs (regDCs) which are specialized cells involved in the tolerogenic activity inducing the production of the anti-inflammatory cytokines IL-10 (Dawicki et al., 2021). On the contrary, there are evidence suggesting that CD40 may also play a role in promoting the proliferation of Th1 and B cells (Ma and Clark, 2009). In line with this and since CD40-derived EVs can interact with CD40L positive cells, we can speculate these eEVs can mask the CD40L on T cells in the intestinal tissue, reducing their activation. Thus, MS patients might have more CD40L positive cells able to interact with CD40 on APC cells, increasing their activation state and leading to the disease. As long as there are conflicting data on the function of CD40 in the literature, and there are no studies about CD40-derived EVs in stool samples, further experiments are needed

in order to better elucidate the specific role of these eEVs in the MS. CD44 is a cell adhesion protein involved in regulating cell migration and immune response. Previous studies have suggested that CD44 may play a role in the migration of T lymphocytes across the blood-brain barrier, a critical process in the pathogenesis of MS. Consequently, the reduction of CD44 in gut-derived EVs could indicate a disruption in T lymphocyte migration to the CNS, potentially affecting the neurogenic inflammation characteristic of MS (Dzwonek and Wilczynski, 2015). Additionally, elevated concentrations of CD44 have been observed in astrocytic plaques of MS patients, further emphasizing that CD44 is more highly expressed at the disease site than in the periphery (Girgah et al., 1991).

Although the brain and gut are anatomically distant, they can influence each other through the gut-brain axis. EVs may travel between the CNS and the gut in both directions. Our hypothesis is that, through this axis, CD44⁺ EVs originating in the gut may migrate to the brain, exacerbating the disease, which could explain the observed decrease of CD44⁺ EVs in the gut of MS patients.

Finally, ROR1 is a receptor tyrosine kinase that serves as a Wnt5a receptor and is widely expressed in embryonic development and multiple human cancers: in particular; ROR1 has been identified as a signature gene in B-cell chronic lymphocytic leukemia (B-CLL) (Balakrishnan et al., 2017). Interestingly, active ROR1 signaling leads to the activation of NF- κ B, that once activated, leads to the transcription of genes that promote T cell survival and proliferation. Moreover, the NF- κ B pathway has paradoxical roles in the regulation of autoimmunity and inflammation: particularly, NF- κ B is crucial for induction of immune tolerance, and impairment of the NF- κ B pathway is associated with defects in central tolerance. In addition, Treg function is maintained by NF- κ B, confirming a role of NF- κ B also in peripheral tolerance maintenance (Barnabei et al., 2021). According to these data, we can speculate a possible role of ROR1 on EVs in MS because of its implication in NF- κ B pathway activation related to tolerance. This can be confirmed by our results from flow cytometry where we assessed a significantly lower amount of ROR1 in eEVs of MS patients compared to HC, but further evidence is necessary to have an overwhelming proof of ROR1 importance in development of MS.

In conclusion, these findings on eEVs together with the ones of bEVs, might contribute to creating a specific MS EVs fingerprint. By evaluating gut-derived EVs, we were able to find different EVs that can be suggested as diagnostic biomarkers in the identification of this

complex disease.

Concluding remarks

This thesis provides novel insights into the potential of both eukaryotic and prokaryotic EVs as disease-specific signatures, highlighting their role in early diagnosis, disease progression monitoring, and the personalization of therapeutic strategies. The lack of accessible and reliable biomarkers remains one of the most significant challenges in precision medicine, and the delay in identifying the right drug contributes to disease progression and the occurrence of severe, often irreversible, damage.

The findings obtained in the context of the SARS-CoV-2 pandemic, as well as in patients later affected by PASC, emphasize the importance of EVs and the adaptive immune system in shaping new diagnostic and therapeutic perspectives. PLT-EVs may serve as a potential diagnostic/prognostic biomarker for disease severity and prognosis in critical COVID-19 cases, highlighting the importance of monitoring these levels during ICU management. PASC patients were characterized by enhanced levels of activated SARS-CoV-2 specific CD8⁺ T cells, highlighting the constant activation of their T cells, resulting in the persistent symptoms of the PASC syndrome. The identification of specific epitopes recognized by CD8⁺ T cells in SARS-CoV-2, including immunodominant regions derived from viral protein, might allow the development of new therapeutics that could be tailored to target these specific epitopes, potentially alleviating persistent activation of CD8⁺ T cells.

However, these results also underscore the need for further cytomic studies to deepen our understanding of T-cell involvement in the persistence of PASC symptoms. This is crucial for a better comprehension of the immunological mechanisms underlying post-infectious syndromes and the development of targeted interventions. Additionally, integrating multi-omics approaches, such as metabolomics, would represent a critical step towards identifying diagnostic, prognostic, and predictive biomarkers with unprecedented precision. These combined strategies will not only enhance the scientific understanding of disease pathogenesis but also facilitate the translation of research findings into practical applications in precision medicine.

Beyond the pandemic context, the exploration of EVs in non-communicable diseases, such as MDD and MS, unveiled promising opportunities. The gut-brain axis emerges as a pivotal element, with EVs of bacterial and eukaryotic released in the gut acting as potential mediators of this intricate interaction. Our results showed disease-specific EV signatures that would offer an innovative perspective to unravel the underlying dynamics of these disorders and to develop more targeted therapeutic approaches.

A significant future direction involves improving EV detection techniques to enable rapid and user-friendly diagnostics, suitable for routine clinical practice. The method of EV detection we validated, starting from few μ l fresh blood, could be easily implemented in hospitals as it requires only a basic flow cytometer, a widely available and cost-effective instrument commonly used in clinical laboratories.

Despite the significant findings present in this thesis, there are several limitations that must be acknowledged. First, the small size of patient cohort; expanding the study cohort will be necessary to validate the results obtained and ensure their applicability across different populations. Secondly, the lack of information on EVs cargo limits a comprehensive understanding of their functional role in disease pathogenesis.

Looking ahead, further research in this field should be focused on integrating multi-omics approaches, including in the study transcriptomics and metabolomics, in order to have a complete overview of the EVs role in different diseases and have more specific and sensitive biomarkers. Another important future direction will be conducting functional assays to evaluate the biological effects and test toxicity and immunomodulatory properties of these EVs. These studies would be crucial in determining whether EVs can be safely and effectively employed, being diagnostic/prognostic biomarkers, as therapeutic agents, in order to develop new personalized EVs driven medicine that will greatly improve patients' outcomes.

In conclusion, the insights gained from this thesis constitute a critical foundation for harnessing the full potential of EVs as indispensable tools in modern medicine.

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