UNIVERSITÀ DEL PIEMONTE ORIENTALE

School of Medicine

Department of Health Sciences

Master degree in Medical Biotechnologies

Laboratory of Immunomics (CAAD-Translational Research Centre on Autoimmune and Allergic Disease)

Exploring biomarkers in major depressive disorder: a multi-OMICs approach on stool samples.

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Academic year: 2023-2024

Summer Session

Sommario

1.Abstract

Major depressive disorder (MDD) is a prevalent and debilitating mental disorder affecting millions of people with a prevalence of 3.8% worldwide and 5.4% in Italy, increasing with age. Despite its high prevalence, diagnosis of MDD is complex for several reasons: first, the symptoms are highly variable and can overlap with other conditions; second, its causes and response to treatment are quite heterogeneous; and third, the lack of reliable biomarkers able to predict disease outcome. Recent research highlights the significant role of the gut microbiota in human health. Its alteration, known as dysbiosis, seems to be involved in several diseases, including MDD. It has been recently shown that gut bacteria, as well as eukaryotic cells, release extracellular vesicles (EVs), that are nanoparticles involved in cell-cell communication. An innovative and non-invasive approach to study not only the microbiota composition, but also both eukaryotic and bacterial-derived EVs, is the analysis of stool samples. With these assumptions, this thesis aimed to: i) isolate fecal EVs (eukaryotic and prokaryotic), ii) analyze the association between bacterial abundance (by metagenomics on stool samples) and bacterial activation (by metaproteomic on fecal EVs) and iii) study the eukaryotic EVs (eEVs) released in the feces (by flow cytometry using the MACSPlex exosome kit). This led us to find a possible EVs fingerprint in MDD patients. To achieve this, we collected stool samples from 47 MDD patients, and both metagenomics and EVs isolation has been performed. Western blot and scanning electron microscopy (SEM) analysis confirmed the successful EVs isolation from stool samples. Metagenomic analysis revealed that, in our cohort, *Bacillota* phyla were the most abundant followed by *Bacteroidota*, *Pseudomonadota*, *Actinomycetota* and *Verrucomicrobiota*. On the other hand, metaproteomic performed on fecal EVs showed that the most active phyla were *Bacteroidota* followed by *Bacillota*, *Pseudomonadota*, *Actinomycetota* and *Verrucomicrobiota*. Linear regression model revealed that low levels of abundance of *Actinomycetota* and high levels of *Bacteroidota* at the time of enrolment were associated with higher improvement of depressive symptoms. On the contrary, high levels of CD1c+ and CD11c+ eEVs were associated with a lower response to treatment and lower improvement in social and working functioning and subjective quality of life, respectively. In conclusion, this thesis provides evidence that bacterial abundance and eEVs might have a significant role in the prognosis of MDD and could be used as potential biomarkers for the disease.

2.Introduction

2.1 Major depressive disorder

MDD is a complex psychiatric disorder affecting approximately 340 million people worldwide (Livia Chiriţă et al. 2015). 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 & 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 autoimmune disease (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 biomarkers that can serve as predictive or diagnostic tools. Many biomarkers have been tried in order to overcome this issue with techniques like 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 (Kang & Cho 2020).

One significant challenge in managing this disease is that its causes are multifactorial and only partially understood. Using the criteria in the Diagnostic and Statistical Manual of Mental Disorders (DSM), more than 200 combinations of symptoms can be applied to diagnose a MDD, underscoring the clinical diversity of the disorder, which results in a wide range of outcomes regarding response and remission over time (Østergaard et al. 2011; Belmaker & Agam 2008; Steinert et al. 2014) .

MDD patients typically suffer alterations in the metabolism of serine and serotonin. Serine is an important amino acid involved in various biological processes: cell membrane formation, energy production, antioxidant effect and it also plays a role in the production of important neurotransmitters in the brain, such as serotonin and dopamine. Studies have shown that changes in serine levels can affect the balance of neurotransmitters like serotonin, which plays a crucial role in regulating mood, sleep and appetite and it is implicated in MDD due to its impact on emotional well-being. The dysfunction of serotonin pathways can lead to disruptions in communication between brain cells, affecting mood regulation and potentially exacerbating depressive symptoms

in individuals with MDD (Du et al. 2020). Traumatic events, adverse life situations and psychological factors can also activate the immune system, by inducing the production of alarmins, which trigger proinflammatory cytokines from innate immune cells, promoting depressive-like behaviors in rodents. Susceptibility due to genetics, infection history, and ADs can also prime the immune system's response to stress. These factors influence both innate and adaptive immunity, affecting T helper cells, cytokine, and antibody production (Beurel et al. 2020). Thus, the dysregulation of the immune system in MDD might have a detrimental role in the progression of the disease.

2.2 Major depressive disorder and the immune system

MDD is intricately linked to immune system dysregulation, constituting a bidirectional relationship that influences both physical and mental health. Research has revealed that individuals with MDD often exhibit heightened levels of inflammatory markers such as C-reactive protein (CRP), interleukin-6 (IL-6), interleukin 1-beta (IL-1β) and tumor necrosis factor alpha (TNF-α), indicative of chronic inflammation. This inflammatory state not only exacerbates depressive symptoms but also increases vulnerability to various physical health conditions (Refisch et al. 2023).

The hypothalamic-pituitary-adrenal axis (HPA) regulates and integrates endocrine and neurological response, and it is responsible for establishing direct communication with the immune system through different receptors and ligands. The pituitary gland, after stimulation by the hypothalamus, secretes adrenocorticotropic hormone (ACTH) into the circulation. This is a key point because ACTH stimulates the adrenal cortex to release cortisol: in normal conditions the elevated level of cortisol exerts an inhibitory feedback mechanism on its receptors thereby stopping the stimulation of these structures to restore balance. When this balance is disrupted, hypercortisolemia directly stimulates the extrahepatic enzyme 2,3-indolimine dioxygenase (IDO). This enzyme is expressed in various tissues (intestine, liver and brain) but also in different cells of the immune system, as macrophages and dendritic cells (DCs) providing a direct link between brain and immune system (Ruiz et al. 2022). Even pro-inflammatory cytokines activate IDO, which results in a decrease in the concentration of tryptophan (Trp) and serotonin (5-HT). Under the influence of IDO, Trp is converted to kynurenine (KNY) which reduces 5-HT production in the nerve cells, potentially affecting 5-HT levels in the brain and thus neurological and psychiatric function. In addition, there is an increase in the concentration of toxic metabolites in the central nervous system (CNS) (Góralczyk-Bińkowska et al. 2022; Ruiz et al. 2022). Moreover, long-term inflammation ultimately leads to complete BBB destruction, with cytokines playing a pivotal role by increasing membrane permeability. Once in the brain, cytokines

can activate microglia, astrocytes, and oligodendrocytes through the nuclear factor-kappa B (NF-κB) pathway, leading to neuroinflammation(Ruiz et al. 2022) .

Significant interest has centered on targeting the altered stress-immune axis as a potential avenue for novel therapeutic strategies in depression (Pape et al. 2019). However, a recent development involves the emergence of the gut microbiota as a pivotal regulator of both stress and inflammation. Consequently, its role in depression is currently under investigation.

2.3 Gut microbiota

The human gut microbiota is composed by 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 involving metabolic pathways, oxidative stress and membrane barrier maintenance in the gut (Das & Ganesh 2023).

The gut microbiota plays a pivotal role in the immune response against pathogenic bacteria, by maintaining the integrity of the intestinal epithelium, nutrient metabolism and by modulating the immune system. The dominant gut microbial phyla include *Bacillota, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia*(Rinninella et al. 2019).

The formation of the gut microbiota begins at birth and continues to evolve throughout life, influenced by various factors such as diet, drugs, stress, and environmental factors. These factors can disrupt the delicate balance of the gut microbiota, leading to the expansion of pathogen microbes and inflammation state leading to dysbiosis (Thursby & Juge 2017).

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 & Vella 2022). As a result of the development of this syndrome, the translocation of multiple bacteria and their products causes activation of the immune system. This stimulation causes an increase in the concentration of pro-inflammatory cytokines, which if sustained over time can be detrimental for the host cells, including those of the CNS (Kinashi & Hase 2021).

Dysregulation of the immune system in the mucosa-associated lymphoid tissue (MALT), along with intestinal dysbiosis, can potentially lead to the development of various disorders, including cancers and diseases affecting organs distant from the gut, such as the brain. These conditions include multiple sclerosis (MS), ADs, and of course MDD.

For this reason, the study of the gut microbiota in various diseases is increasingly important nowadays. This approach could provide a very clear picture of the disease status as well as its prognosis. The study of the microbiota can be easily conducted through stool samples, as shown by Jandhyala et al (Jandhyala et al. 2015). By collecting a small stool sample, metagenomic analysis allows us to determine the genomic content of microbial communities and identify the most abundant microbes present. Metagenomic is able to sequence all the DNA fragments present in a sample and define the composition and diversity of the gut microbiota allowing to analyze the genetic diversity and composition of the gut microbiota, but it has some limitations. Firstly, metagenomic sequencing provides data on the genome as a whole, making it challenging to distinguish specific details about the bacterial state. Secondly, it also cannot distinguish DNA of live cells from DNA of dead cells, and DNA experiments are extremely sensitive (Rezasoltani et al. 2020). In this regard, to better understand the functions of the microbiota and its constituents, other meta-omics approaches, such as metaproteomics and metabolomics, have been developed. Metaproteomics is particularly useful for evaluating changes in protein expression within the microbes present in the human gut. It provides insights into the taxonomy, functions, and metabolic pathways that are undergoing changes in the gut microbiota, thus, could be used as a potential tool to depth characterize and have a complete overview of the microbiota (Wilmes et al. 2015). Recent advancements in proteomic techniques have enabled metaproteomic analysis on extracellular vesicles (EVs), revealing the most active species. A study by Maredia et al. demonstrated a significant connection between the production of EVs and bacterial activation (Maredia et al. 2012).

Recently, Ying et al. in their paper showed that the role of EVs in MDD, EVs can participate in neuro-inflammation and research highlighted their role as potential biomarkers (Li et al. 2023). In this context metagenomic but especially metaproteomics may represent two useful techniques for the study of EVs.

2.4 Extracellular Vesicles

Extracellular vesicles 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 (30-150 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 μm 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 & 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 capacity to act as carriers of different macromolecules, facilitating cell-to-cell interactions. Moreover, emerging evidence shows that EVs can be used as therapeutic tools in different fields such as cancer immunotherapy and regenerative medicine (Xu et al. 2016). It is known that EVs can be released either by both eukaryotic and prokaryotic. (Yáñez-Mó et al. 2015).

2.5 Eukaryotic Extracellular vesicles (eEVs)

The biogenesis of eukaryotic extracellular vesicles (eEVs) is a critical cellular mechanism that facilitates the transport and distribution of proteins, lipids, and other molecules within the cell, playing a vital role in maintaining cellular organization. In particular, this process is different between exosomes and microvesicles. The biogenesis of exosomes depends on several cell stages: first, the inward budding of a portion of the cellular plasma membrane leads to the formation of an endosome. Small vesicles can be formed by further inward budding of the limiting membrane inside an endosome, leading to the formation of a multivesicular body (MVBs) (Shao et al. 2018).

Second, MVBs are transported to the plasma membrane, where they fuse to release exosomes into the extracellular space (Fyfe et al. 2023). Considering all the factors that participate in this process, one of the most important is the endosomal sorting complex (ESCRT), a family of proteins, that is associated with MVBs and is involved in budding and membrane scission of plasma membrane (Mathieu et al. 2019).

In contrast, MVs biogenesis occurs via the direct outward blebbing and pinching of the plasma membrane, releasing nascent MVs into the extracellular space. This process is accompanied by changes in plasma membrane and lipid components (Tricarico et al. 2017) .

Finally, apoptotic bodies, which are released spontaneously by all cell types during apoptosis might play an important role in immune regulation, with many implications in different diseases (Kakarla et al. 2020).

eEVs orchestrate complex communication between cells. Specifically, proteins and lipids on the eEVs surface act as ligands, binding to receptors on recipient cells. After binding, eEVs can release their cargo (including proteins, lipids, DNA, and RNA) into the cytoplasm through endocytosis or fusing directly with the recipient cell membrane (Colombo et al. 2014).

The release of eEVs content is strictly regulated by different members of the protein superfamily of small guanosine triphosphatases known as GTPases, including members of ADP-ribosylation factor (ARF) family, Rab and Rho (Mathieu et al. 2019).

The effect on recipient cells can be different, in some cases it can induce cell survival, while in others, it can induce apoptosis or immunomodulation. Different molecules, including proteins (i.e.: tetraspanins (TSPANs) and integrins) and lipids are known to mediate these biological effects.

TSPANs are a superfamily of small proteins present in every cell type. The 33 human TSPANs have different functions including cell adhesion, tissue differentiation and immune cell maturations (Broadbent et al. 2024). They are characterized by four transmembrane domains containing conserved polar residues, a small extracellular loop (SEL), large extracellular loop (LEL), and short cytoplasmic tail (Mulcahy et al. 2014).

TSPANs are highly abundant on the EVs surface, suggesting they may have a role in EVs function. Due to their abundant content of TSPANs such as CD63, CD9 and CD81 in EVs, they are commonly used as specific EVs markers .

Recent advancements in EVs research have revealed their exciting potential as biomarkers for disease detection, targets for new therapies, and innovative drug delivery systems.

Using EVs as biomarkers has several advantages for different reasons: first of all, EVs are released by all cell types and are present in various biological fluids, thus allowing for their isolation and analysis using *"noninvasive*" approaches (Tricarico et al. 2017); secondly, EVs cargo contains information related to the cells of origin, reflecting its current metabolic/pathological state, that could be relevant to understand pathophysiology of the investigated condition (Ciferri et al. 2021) .

EVs are very well studied in different fields such as cancer, cardiovascular disease and ADs. In cancer, EVs have a crucial role in remodeling the tumor microenvironment, regulating cancer progression and metastasis (Huang et al. 2022). Besides, in cardiovascular disease, EVs have been investigated as prognostic indicators of endothelial dysfunction and myocardial injury (Zhang et al. 2022). In ADs, EVs might have a dual role, acting both as pro-inflammatory or anti-inflammatory mediators: EVs released from activated immune cells, such as macrophages or DCs, can carry pro-inflammatory cytokines such as IL-1β and TNF-α. On the contrary, EVs derived from regulatory T cells (Tregs) can

carry anti-inflammatory cytokines (e.g.,TGF-β, IL-10); these EVs suppress inflammation by inhibiting pro-inflammatory cytokine production and promoting Treg differentiation(Arteaga-Blanco & Bou-Habib 2021).

EVs seem to be implicated also in the pathogenesis of neurodegenerative diseases (NDs). NDs are characterized by progressive degeneration or death of neurons leading to the decline in cognitive ability. Despite the distinct characteristics of each disorder, NDs share common features, including the pathological accumulation of proteins in the brain, like tau and amyloid-β in Alzheimer's disease or α-synuclein in Parkinson's disease. In this regard, EVs play a role in the pathological accumulation of these proteins, as they can cross the blood-brain barrier (BBB) and release their contents into the brain. In NDs, aggregation-prone proteins can be packaged into EVs, which are then released and transfer their toxic cargo to other brain cells (Reed & Escayg 2021).

Studying the role of eEVs is very important in various physiological and pathological contexts. However, isolating and characterizing only the eEVs results in a significant loss of information because, as previously mentioned, prokaryotes can also produce this type of nanoparticle. Like eEVs, bacterial EVs (bEVs) can also play a crucial role in both physiological and pathological conditions.

2.6 Bacterial extracellular vesicles (bEVs)

bEVs are small, spherical structures released by various bacterial species into their surrounding environment. Recent work has shown that bEVs diverge in structure and composition and are mainly divided in four types: Gram-negative-derived single-layered outer-membrane vesicles (OMVs) or double-layered outer-inner membrane vesicles (O-IMV); cytoplasmic membrane vesicles (CMVs); and tube-shaped membranous structure (TSMSs) (Toyofuku et al. 2019).

OMVs and O-IMVs can be formed via explosive cell lysis, in which cryptic prophage endolysin activity results in cell wall degradation or DNA damage, giving rise to bEVs formation. CMVs are also released by Gram-negative bacteria through the budding of the outer membrane. TSMSs, also known as nanotubes, can be produced by both Gram-positive and Gram-negative bacteria and play a role in the exchange of components by acting as a bridge between different cell types (Hosseini-Giv et al. 2022) .

OMVs range in size from 20 to 250 nm and commonly contain lipopolysaccharide (LPS), phospholipids, membrane-associated proteins, and peptidoglycan, as well as cytoplasmic content like enzymes, DNA, and RNA. Instead, CMVs range from 20-200 nm in diameter and are derived

directly from the cytoplasmic membrane. Finally, TSMSs range from 50 to 70 nm and connect different cells enhancing their activity (Hosseini-Giv et al. 2022).

bEVs serve as key mediators of intercellular communication within microbial communities and between bacteria and the host environments. bEVs can influence different biological processes, such as modulation of the host's immune responses and regulation of microbial community dynamics through the transfer of their cargo. The latter includes a variety of signaling molecules like lipid mediators, proteins, and genetic material. The precise mechanisms by which bEVs exert their biological effects on recipient cells are not well characterized but it is known that, thanks to their stable membrane-bound structure, bEVs are shielded from degradation by extracellular enzymes. Furthermore, bEVs can serve also as vehicles for the delivery of virulence factors, toxins, and antimicrobial resistance determinants, influencing bacterial pathogenesis and antibiotic resistance (Bitto & Kaparakis-Liaskos 2017). bEVs can penetrate different barriers, such as the intestinal epithelium and the vascular endothelium, to reach distant locations inside the host. bEVs can cross the intestinal barrier using two different pathways: the paracellular (between cells) and transcellular (through cells) pathways (Sultan et al. 2021).

Studies have shown that bEVs can directly interact with immune cells such as macrophages, DCs, and T cells, influencing their function and activation state (Buzas 2023). bEVs enclose multiple copies of microorganism-associated molecular patterns, including periplasmic proteins, DNA, RNA, LPS and peptidoglycan, which interact with pattern recognition receptors such as NOD-like receptor 1 (NLR), NLR 2 and Toll-Like Receptors (TLR) on immune cells to start a cascade of immune signaling (Sultan et al. 2021)

Immune signaling is a complex and dynamic process through which cells of the immune system communicate with each other and can be divided into pro-inflammatory signaling and anti-inflammatory signaling. The former involves the activation of inflammation, characterized by a significant release of pro-inflammatory cytokines and the recruitment of specific cells like T helper and cytotoxic T cells. The latter refers to a process that deactivates the immune response and induces immune tolerance, preventing an immune reaction against a particular antigen (i.e.: antigens derived from commensal bacteria).

Recent studies have demonstrated the role of bEVs in different systems such as respiratory, nervous and gastrointestinal. Within the intestinal microenvironment bEVs aid in the colonization and proliferation of bacteria by transporting adhesion factors that promote bacterial attachment (Liang et al. 2022) suggesting a detrimental role of bEVs in modulating the composition and the function

of the gut microbiota. On the other hand, it has been demonstrated by different authors that bEVs can communicate with the CNS using the so-called gut-brain-axis. This axis consists of a bidirectional communication network between the GI tract and the CNS, linking gut, microbiota, intestinal function, and mental health. The CNS has the capability to control both intestinal movement and coordinate local immune responses using neuromodulators through the vagus nerve and HPA. Conversely, the digestive system can influence aspects of the nervous one like appetite or mood. These exchanges primarily occur through neuroendocrine pathways, which include cytokines, neurotransmitters, neuropeptides and also bEVs (***Figure 1***). Moreover, the immune system, in collaboration with the gut microbiota, significantly contributes to these communication processes (Boussamet et al. 2022).

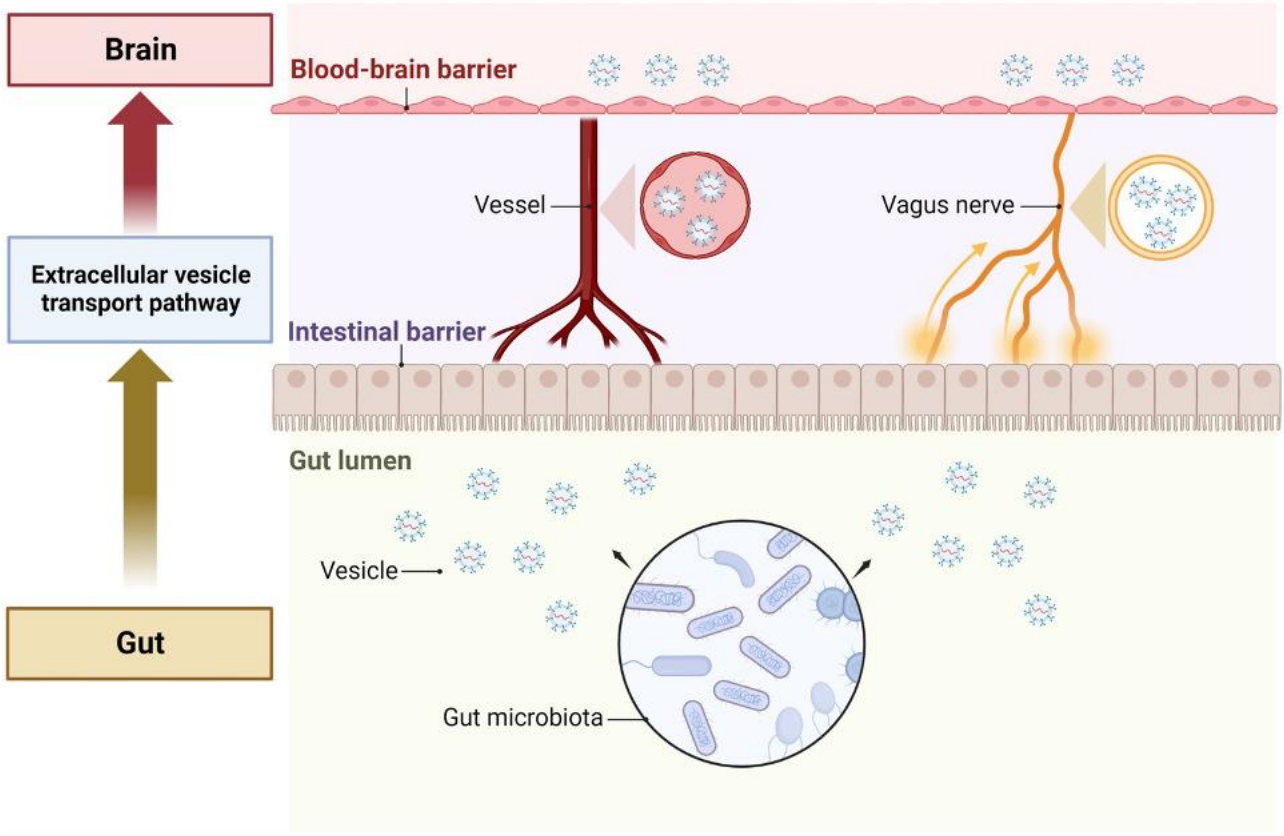


Figure 1 *The role of EVs in mediating gut-brain communication. The gut microbiota release EVs in the gut lumen. The vesicles pass through the cells of the gut epithelial layer lining the lumen and enter the blood vessels to be transported through the circulation. The vesicles then pass through the blood–brain barrier and enter the brain cells to exert their effects. In addition, vesicles can also be transported to the brain via the vagus nerves. EVs, gut microbiota-released extracellular vesicles.*

The significant involvement of the gut-brain axis in MDD pathogenesis has been increasingly recognized and it can now be considered a potential target to provide new treatment strategies (Góralczyk-Bińkowska et al. 2022; Boussamet et al. 2022). In this context, eEVs and bEVs have emerged as promising biomarkers for MDD.

2.7 Major depressive disorder and EVs

There are mounting evidence supporting the involvement of EVs in many different diseases, including MDD: indeed, vesicles carry and deliver many different bioactive molecules, which may confer genotypic and phenotypic changes in the target cells. These vesicles, which include exosomes and microvesicles, act as carriers of various molecular signals such as miRNAs, proteins, and lipids between cells. In the context of MDD, 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 EVs derived from the gut microbiota and the mechanisms underlying the MDD. Notably, Li et al. show that microRNAs (miRNAs) in EVs play a key role in the physiological and pathological processes of MDD and could also be a diagnostic and prognostic biomarker for depression. Specifically, miRNAs are transferred from neurons to microglia through EVs leading to the release of proinflammatory factors and the promotion of neuroinflammation. Also, mitochondrial DNA (mtDNA) released from damaged mitochondria can be packaged into EVs generated by mitochondria for transfer and activate NF-κB (Li et al. 2023). A recent study from Choi et al. demonstrated that, in mice, bEVs deriving from specific bacteria can interact with gut and brain. Particularly, they discovered 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, the exact role of bEVS or eEVs in MDD is not fully elucidated, giving the necessity of further studies specific in this field. As a response to outer stress sources, the brain can modulate the 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).

3.Objective of the thesis

Recent research highlights the significant role of the gut microbiota in human health, influencing the development of various diseases, including MDD. EVs from gut microbiota and other peripheral cells can interact with the CNS via the gut-brain axis, underscoring the microbiota's impact on mental health. Stool samples present a promising and non-invasive method to study the gut microbiota, offering a valuable biological resource for examining both the microbiota itself and its secreted products, such as bEVs.

Despite the correlation between gut dysbiosis, MDD and EVs, limited research has been conducted to comprehensively characterize both eEVs and bEVs deriving from the gut of patients with MDD.

The general aim of this thesis was to evaluate the diagnostic/prognostic potential of fecal EVs, both bEVs and eEVs, in MDD patients. Specifically, we focused on:

1. To set up a new and reliable method to isolate fecal EVs and characterize them.
2. To evaluate bacterial abundance through metagenomic and their activation through metaproteomic on bEVs.
3. To correlate the biological data (bEVs and eEVs) at the time of enrolment with the clinical parameters after three months.

4. Material and methods

4.1 Patient recruitment

Patients were recruited from “Città della Salute e della Scienza di Torino”, Turin, Italy. Prior to the enrollment, 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 major depressive episode (MDE), necessitating the initiation or modification of an antidepressant treatment. The severity of depressive symptoms was evaluated with the Montgomery Åsberg Rating Scale (MADRS) (Montgomery), where higher scores indicate greater severity (Montgomery & Åsberg 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, response to treatment, improvement of functioning and subjective quality of life were assessed. Response to treatment 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).

4.2 Metagenomic analysis

This procedure was carried out by the company Biodiversa (Padova, Italy). Briefly, DNA extraction was performed on a Qiagen HT instrument starting from 200 mg of stool. For the extraction was used the Qiagen DNeasy 96 Power Soil kit following the manufacturer instructions. Libraries were prepared from DNA according to manufacturer instructions with Illumina DNA Prep kit. Libraries quality were evaluated by size analysis on 2100 Bioanalyzer (Chip DNA HS) and concentrations were determined using Qubit DNA HS assay kit (Thermo Fisher).

4.3 Fecal EVs isolation

Half a gram of frozen feces (-80°C) were dissolved in 10 ml filtered phosphate buffered saline (PBS 1X) and two rounds of centrifugation were applied for 15 minutes at 5 000 rpm at 4°C to remove solid debris. After these two centrifugations, supernatant was filtered using filters with different sizes: 1.2, 0.45 and 0.22 µm. EVs were then separated from small molecules by loading them in a flow-through filter with a molecular weight cut-off of 100 kDa (Amicon Ultra filter unit) and concentrated to 250 µl through a centrifugation at 4 000 rpm for 45 minutes at 4°C. Two hundred and fifty µL of filtered PBS 1X were used to wash the filter to have the highest EVs recovery (with a final volume of 500 µL). Subsequently, free proteins were removed through size exclusion chromatography (SEC) using a specific protocol optimized in our laboratory. Specifically, 10 mL of CL-2B Sepharose columns were prepared by adding 2,5 mL of PBS 1X to 7,5 mL of Sepharose Gel CL-2B, the sample was loaded and the first 33 drops were discarded then, the next 99 were collected. Subsequently, a step of ultracentrifugation (100 000 x *g* for 1h at 4°C) was performed. After ultracentrifugation, the pellet was resuspended in 200 µL of PBS 1X and stored at -80°C for further analyses.

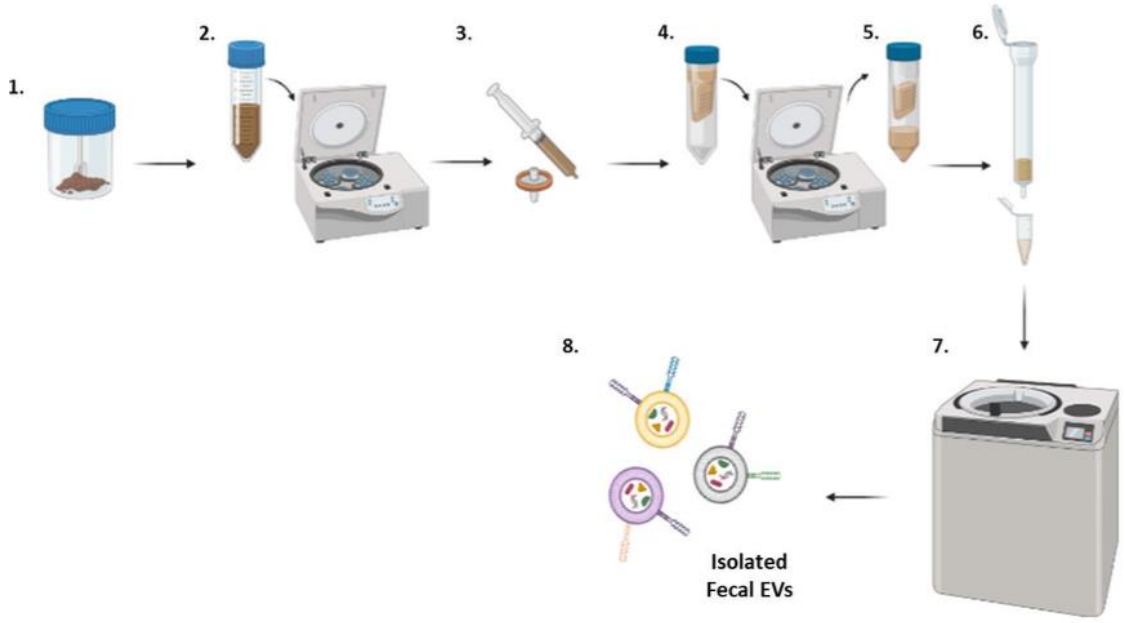


Figure 2: ***Experimental workflow for fecal EVs isolation****. Half a gram of feces was dissolved in 10 mL of filtered PBS 1X (1), and two centrifugation steps were performed (2). Then, the supernatant was filtered through four filters of different sizes: 1.2, 0.45 and 0.2 µm (3). After the filtration steps, the sample was loaded into an Amicon Ultra filter of 100 kDa (4) to concentrate it (5). In order to purify the sample, SEC was performed. (6). Subsequently, a step of ultracentrifugation was applied to pool the fraction of the fecal EVs (7,8).*

4.4 Western blot

Proteins in fecal 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).

4.5 Scanning electron microscopy (SEM)

A small volume (10 μL) of sample was placed on a glass coverslip in a 24-well plate. After the sample dried, the plate was incubated for 20 minutes with 10 μL of 2.5% glutaraldehyde solution diluted in water (Electron Microscopy Sciences, Hatfield, PA, USA). Subsequently, following the evaporation of the glutaraldehyde, an ethanol dehydration scale was performed: the sample was incubated with drops of increasing ethanol concentrations (70% and 90%) for 30 minutes each, followed by a final incubation with 100% ethanol for 1 hour. Then, we added a small volume of hexamethyldisilazane (Electron Microscopy Sciences, Hatfield, PA, USA) and 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 Scanning Electron Microscope (SEM) (JSM-IT500, JEOL, Italy).

4.6 Metaproteomic analysis

Fecal 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 μL of ammonium bicarbonate, 15 μL of trifluoroethanol (Sigma-Aldrich Inc., US) and 2.5 μL of dithiothreitol 200 mM (Sigma-Aldrich Inc., US) were added to the sample that were incubated at 60°C for 45 min. Alkylation was performed adding 10 μL of iodoacetamide IAM 200 mM and the sample was incubated at RT, in the dark, for 1 h. Then, 2.5 μL of DTT were added to the sample and incubated for another hour. Finally, after the addition of 100 μL of ammonium bicarbonate, 200 μL of water and after reaching a pH of 7.8, 10 μL of trypsin 0.2 μg/μL (Sigma-Aldrich Inc., US) were added to the sample and incubated at 37°C overnight. Digestion was then stopped with 2 μL of formic acid. The mixture of peptides was then desalted through the Discovery DCS-18 solid phase extraction. The sample was evaporated through the SpeedVac (ScanVac, LaboGene, Denmark) and reconstituted with 20 μL of water with 0.1% formic acid for the LC-MS analysis.

4.7 MACSPlex exosomes kit

MACSPlex exosome kit (Miltenyi Biotec) allows the detection of 37 exosomal surface epitopes plus two isotype controls (***table 1 and figure 3***). This kit comprises a cocktail of different fluorescently-labeled 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 Dicknson, San Jose, CA, USA).

CDs markers of MACSPlex kit	
CD3	CD24
CD4	CD81
CD19	HLA-ABC
CD105	CD63
CD56	CD40
HLA-DRDPDQ	MCSP
CD8	CD86
CD2	CD69
CD209	CD20
CD42a	CD142
CD29	CD44
REA control	CD146
CD62P	CD41b
CD9	CD326
CD1c	CD133-1
CD25	CD31
CD49e	CD14CD45
ROR1	mIgG1 control
SSEA-4	
CD11c	

Table 1 *Markers of the bead's populations*

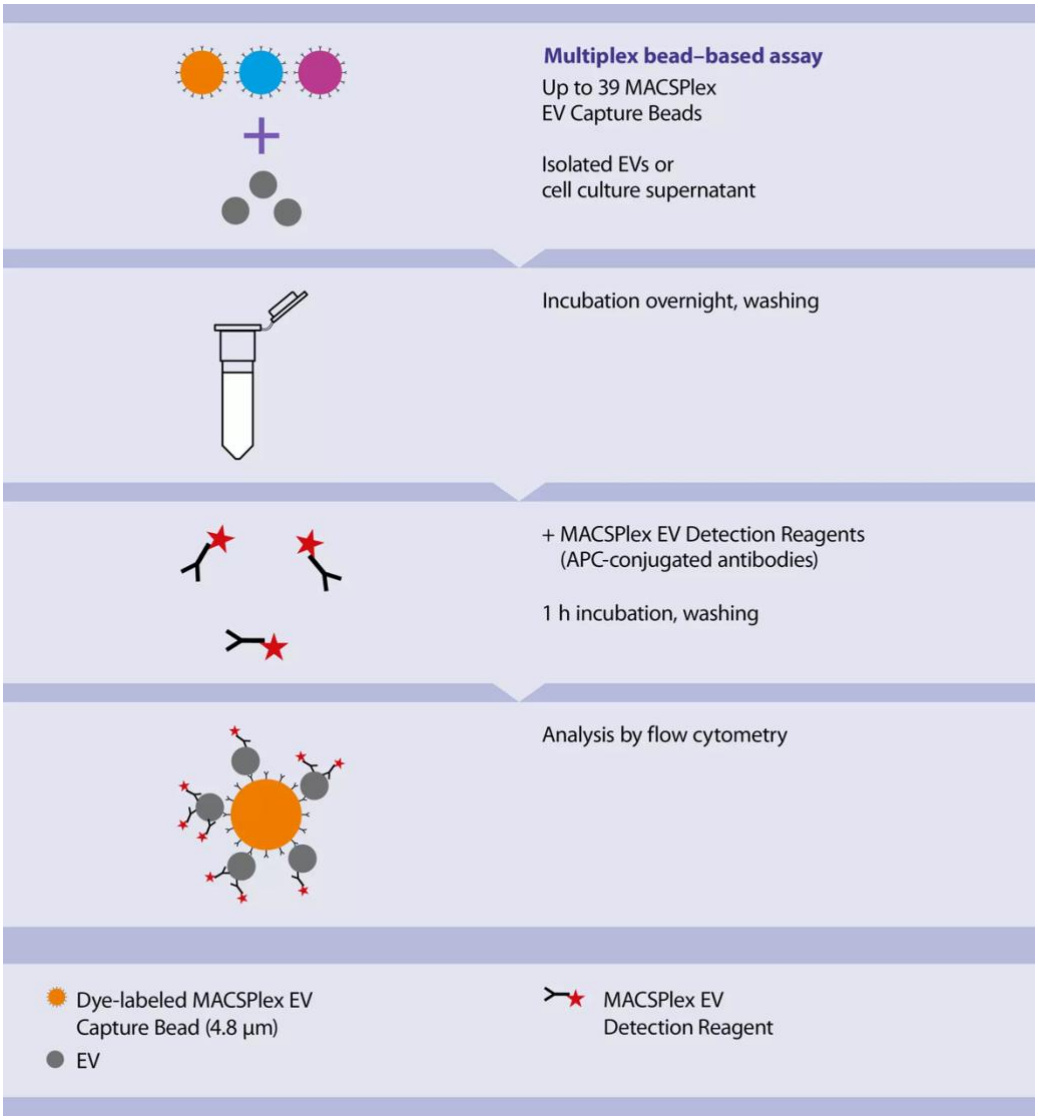


Figure 3 Principle of MACSPlex Exosome Kit *MACSPlex Capture Beads kit contains a cocktail of various fluorescently-labeled bead populations, each coated with a specific antibody. Bound EVs on the beads are stained by a detection reagent and will generate a signal that is detectable by flow cytometry. (https://www.miltenyibiotec.com)*

4.8 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 abundance, 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).

5.Results

5.1 General features of the patients cohort

47 MDD patients have been enrolled in the study as reported in ***Table 2***. The sociodemographic characteristic 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 (baseline) to T1 (the three-month follow-up), all patients were treated with antidepressants, which led to a substantial clinical improvement in their depressive symptoms (***Table 2***). 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 2 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.*

5.2 Characterization of fecal eEVs and bEVs by western blot and Scanning Electron Microscopy

Both eukaryotic and prokaryotic cells release EVs inside the feces. To discriminate them, specific markers must be assessed: tetraspanins for eEVs and LPS for bEVs. Therefore, western blot analysis was conducted to detect tetraspanins (CD9, CD63, and CD81) and LPS. Peripheral blood mononuclear cells (PBMCs) served as positive control for tetraspanins, while gram-negative cells (E. Coli) were used as positive control for LPS.

Tetraspanins CD63 and CD9 were detected in the sample, with CD63 showing a smear from 30 kDa to 60 kDa and CD9 exhibiting a signal around 25 kDa (***Figure 4A-4B***). Surprisingly, tetraspanin CD81 was not detected in our sample (***Figure 4C***). As expected, LPS showed a signal around 10 kDa (***Figure 4D***).

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 4E***).

Finally, we characterized EVs also with SEM, showing spheroid and cup-shaped 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.

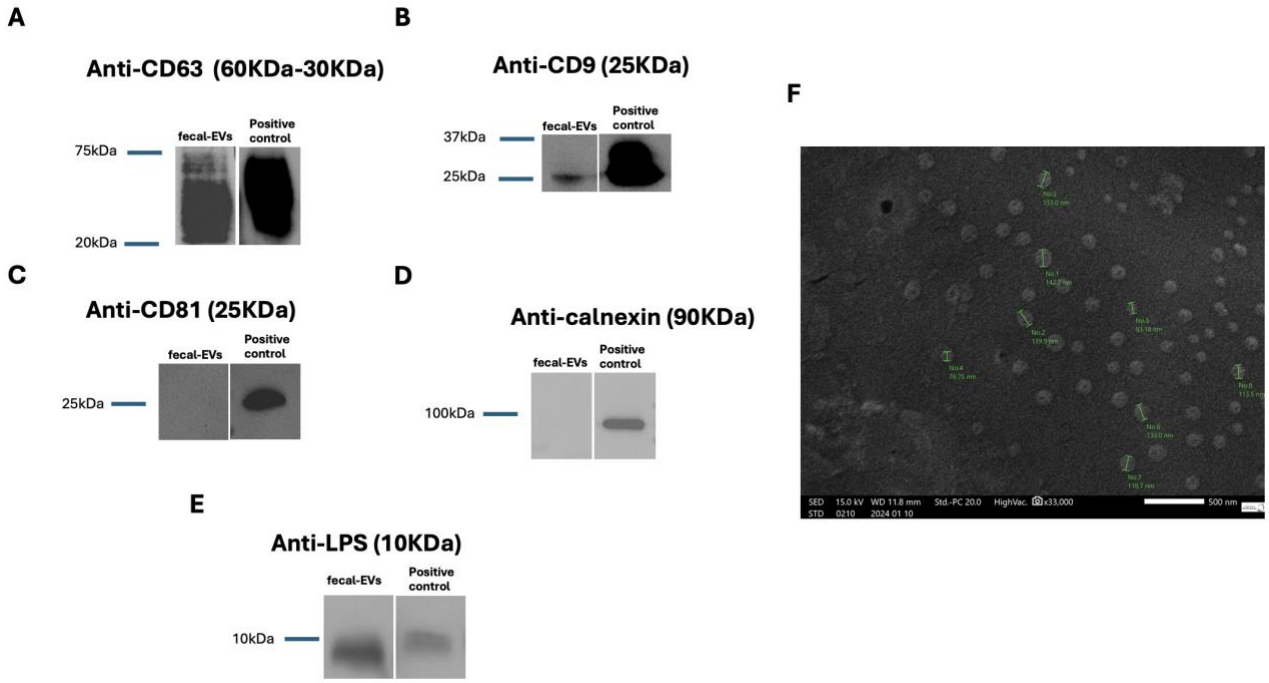


Figure 4: *Characterization of EVs by western blot.* ***A****) Representative western blot of CD63 in fecal EVs sample and positive control* ***B****) Representative western blot of CD9 in fecal EVs sample and positive control* ***C****) Representative western blot of CD81 in fecal EVs sample and positive control* ***D****) Representative western blot of Calnexin in fecal EVs sample and positive control* ***E****) Representative western blot of LPS in fecal EVs sample and positive control* ***F****) Representative images of EVs from stool samples obtained by scanning electron microscopy (SEM).*

5.3 Discordant gut microbiota features between bacterial abundance and bacterial activation.

To provide insights about the composition of the microbial community of MDD patients, we performed a metagenomic analysis. Metagenomic is one of the main methods employed to measure and characterize the gut microbiota, aiding in the classification of the bacteria according to the phyla, classes, orders, families, genera, and species.

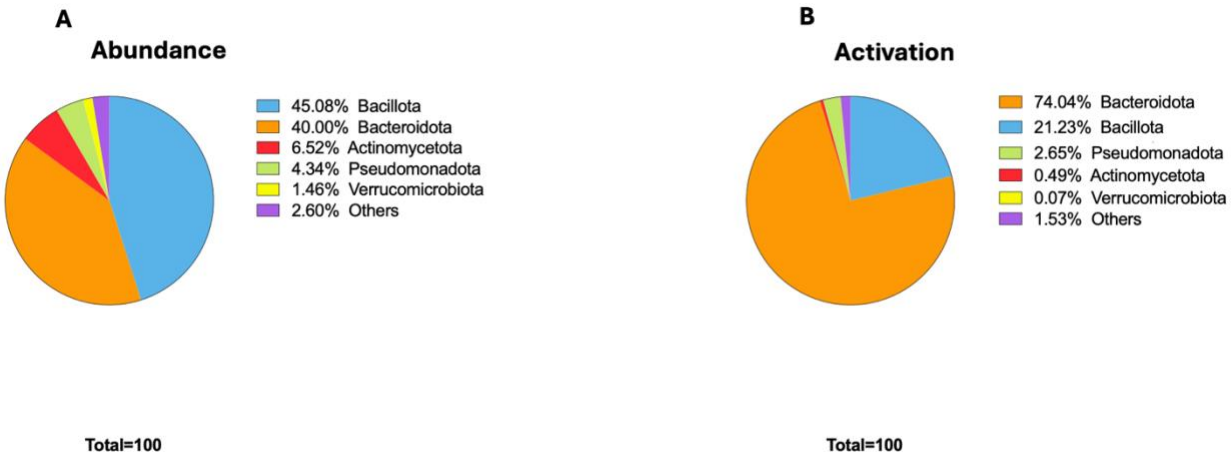
Stool samples of 47 MDD patients at the time of MDE (T0) were analyzed through metagenomic. At phylum level, the most prevalent genes were consistently assigned to five major phyla: *Bacillota* (45.08%), *Bacteroidota* (40%), *Actynomicetota* (6.52%), *Pseudomonadota* (4.34%) and *Verrucomicrobiota* (1.46%), while other phyla add up to 2,60% (**Figure 5A**).

Metagenomic provides an overview of the gut bacteria abundance but does not offer any information on their activation status. 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 to five phyla: *Bacteroidota* (74.04%), *Bacillota* (21.23%), *Pseudomonadota* (2.65%), *Actynomicetota* (0.49%), *Verrucomicrobiota* (0.07%) and others (1.53%) (***Figure 5B***).

Despite the overall consistency, we observed significant discrepancy between bacterial abundance and bacterial activation.

Thus, we performed a comparison in the five most representative phyla. Specifically, *Bacteroidota* was significantly higher in activation compared to abundance (***Figure 5C***); while *Bacillota, Actynomycetota*, *Verrucomicrobiota* and *Pseudomonadota* showed a strong decrease in activation compared to their abundance (***Figure 5D-G***).



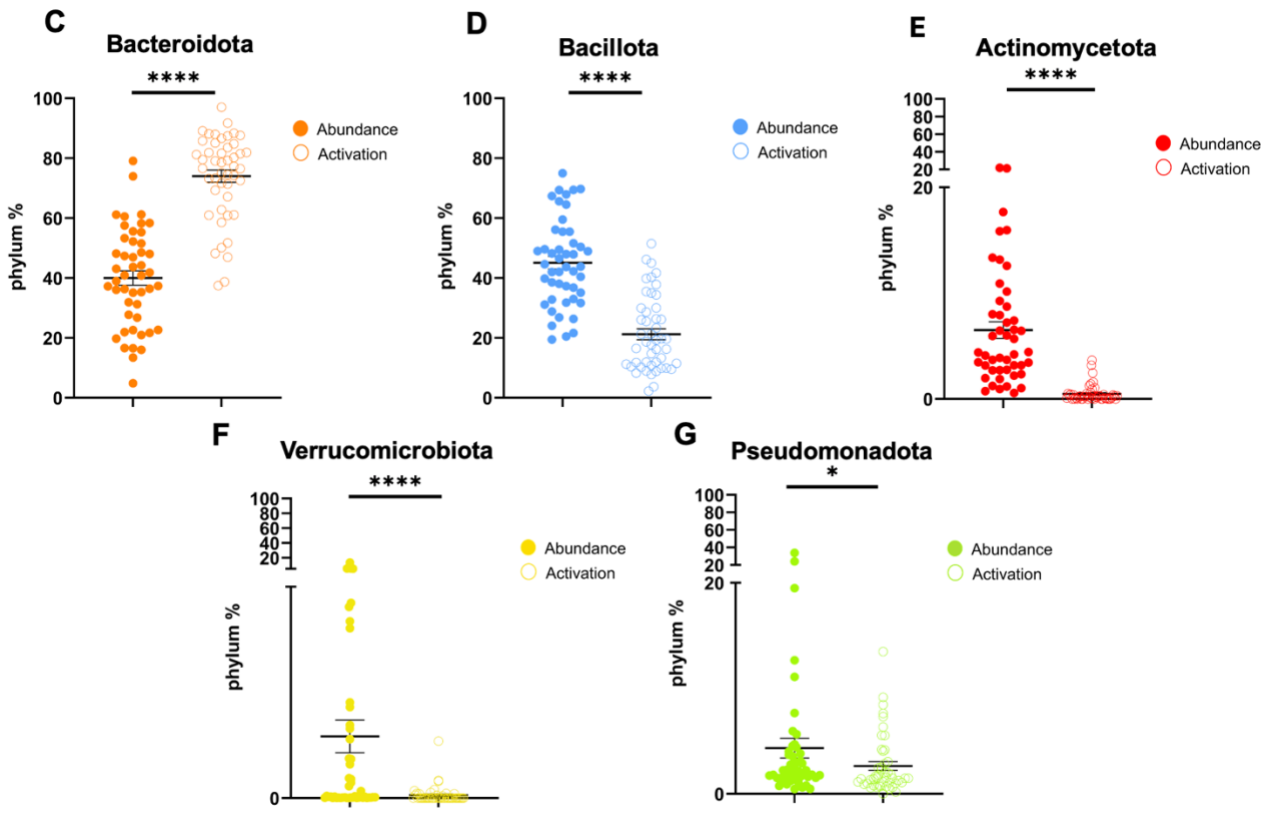


Figure 5: Discordant of gut microbiome in metagenomic and metaproteomic analysis. ***A****) Taxonomic distribution at the phylum level in bacterial abundance.* ***B****) Taxonomic distribution at the phylum level in bacterial activation.* ***C****) Graph depicts the percentage of Bacteroidota abundance and activation.* ***D****) Graph depicts the percentage of Bacillota abundance and activation.* ***E****) Graph depicts the percentage of Actinomycetota abundance and activation.* ***F****) Graph depicts the percentage of Verrucomicrobiota abundance and activation.* ***G****) Graph depicts the percentage of Pseudomonadota abundance and activation. Mann-Whitney t test was used. *p<0.05 , ****p<0.0001.*

5.4 *Acinomycetota* and *Bacteroidota* abundance, evaluated at the baseline, are associated with the clinical outcome 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, response to treatment, improvement of functioning and subjective quality of life were assessed. In particular, response to treatment was evaluated with the variation (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 variation of the Recovery Index (Δ RI T1-T0). We correlated the five most abundant and active phyla with the Δ RI and Δ MADRS. Indeed, the recovery index (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 bacterial abundance and Δ-RI did not yield statistically significant results. However, the correlation analysis between abundance and Δ-MADRS indicated that a decrease in *Actinomycetota* abundance and an increase in *Bacteroidota* abundance were associated with a poorer prognosis (r=-0.3462; r= 0.3335) (***Figure 6A-B)***.

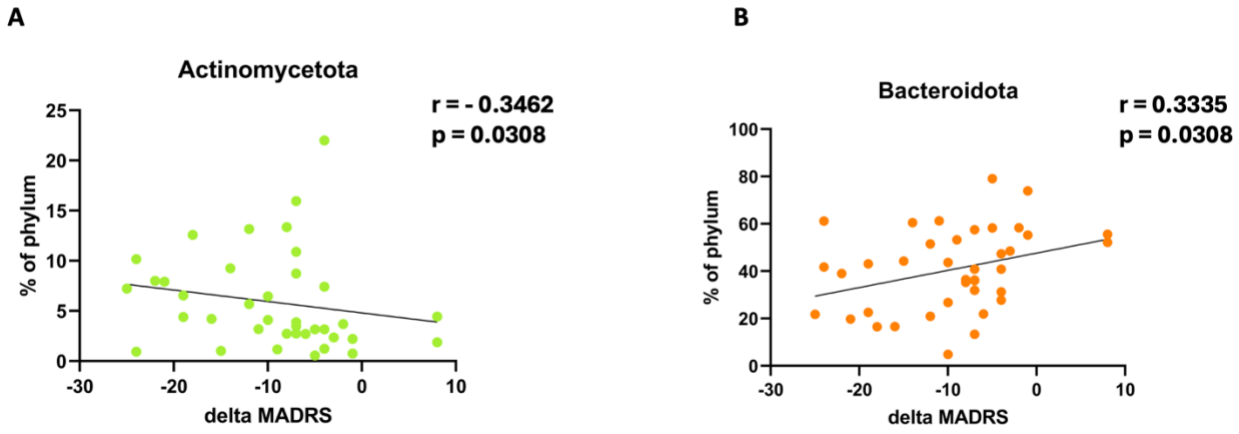


Figure 6. Linear regression between bacterial abundance and Δ MADRS. A) *Linear regression showing the relationship between Δ-MADRS and Actinomycetota. r= -0.3462 and p=0.0308.* ***B)*** *Linear regression showing the relationship between Δ-MADRS and Bacteroidota. r= 0.3335 and p=0.0308.*

5.5 CD1c+ and CD11c+ EVs are associated with the clinical outcome after three months.

Western blot analysis revealed that the isolation protocol successfully isolated EVs also of eukaryotic origin which can be released by epithelial and immune cells in the gut. In MDD patients, dysbiosis can lead to a 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 used the MACSPlex exosome kit with flow cytometry. This kit enables the simultaneous detection of 37 surface epitopes known to be present on eEVs (***Table 1***).

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 fecal EVs from MDD patients (***Figure 7***).

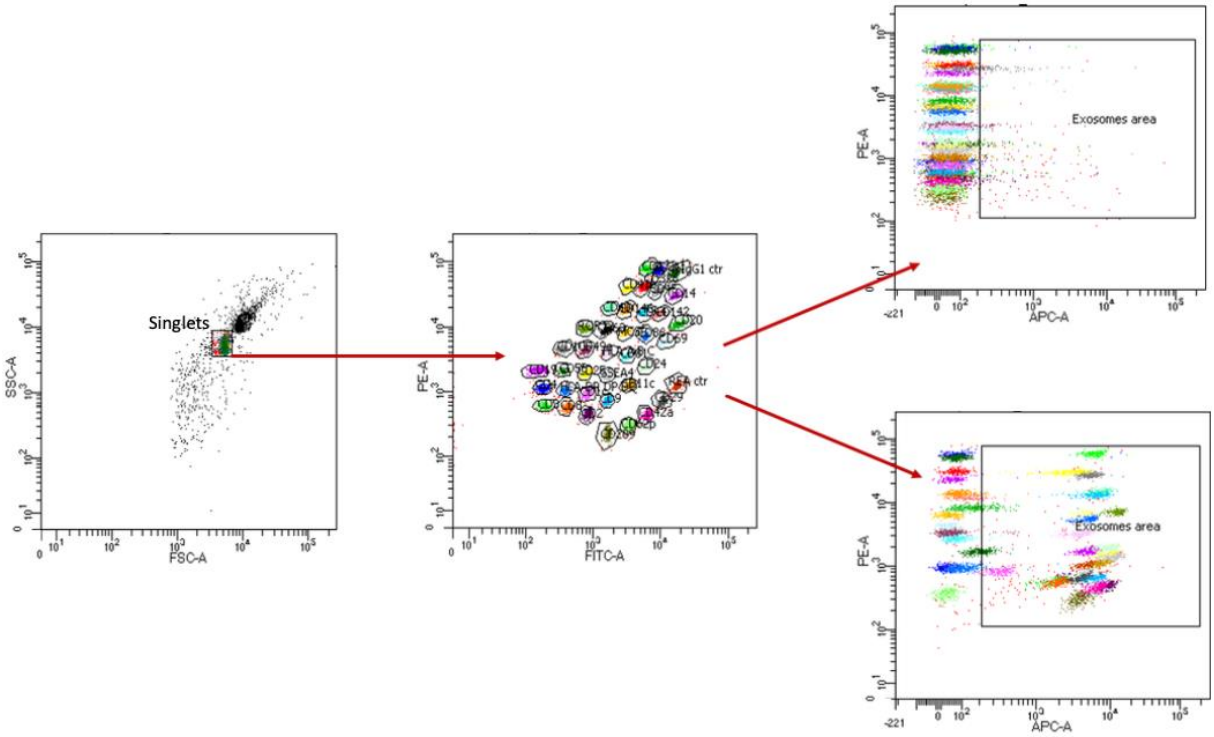


Figure 7. Gating strategy for the detection of different subtypes of eEVs. *After the discrimination of bead singlets (FSC vs SSC), the different subtypes of EVs were discriminated according to their fluorescent intensity (PE vs FITC). Finally, the signal of the detection antibodies (anti CD9 and CD63) in the APC channel defined the EVs area where the complexes beads-EVs-detection antibodies specific for each subtype of EVs are present.*

Thus, we correlated eEVs with the Δ-MADRS and Δ-RI. Among all the 37 different subtypes of EVs that can be detected using the MACSPLEX kit, two populations (CD1c and CD11c) were associated with a worse prognosis. The results showed that the increase of CD1c is correlated with a poor response to treatments (***Figure 8A***) and the increase of CD11c is correlated with a worse quality of life (***Figure 8B***).

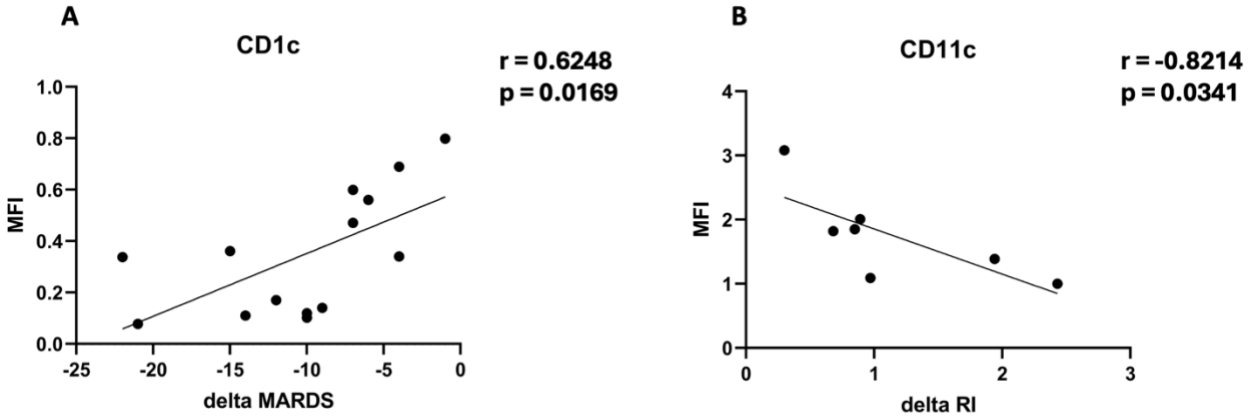


Figure 8. Linear regression between eEVs and Δ MADRS and Δ RI. A) *Linear regression showing the relationship between Δ-MADRS and CD1c. r= -0.6248 and p=0.0169.* ***B)*** *Linear regression showing the relationship between Δ-RI and CD11c. r= -0.8214 and p=0.0341.*

6. Discussion

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. As a consequence, diagnosing and determining a suitable therapy before the patient's condition deteriorates is challenging. 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 the microbiota can be easily performed through stool samples as already demonstrated (Jandhyala et al. 2015). Indeed, by collecting a small stool sample it is possible to understand, by metagenomic analysis, the genomic content of microbial communities and have information on the most abundant microbes present. Despite providing an overview of gut bacteria abundance, it does not offer any information on their activation status. However, recent advancements in proteomic methodologies have led to the development of metaproteomic directly on bEVs, which can highlight bacterial interactions and identify the most active species (Maredia et al. 2012). Although a correlation between gut dysbiosis, MDD and EVs is recognized, limited research has been conducted to comprehensively characterize both bEVs and eEVs originating from the gut microbiota of patients with MDD. Several evidence underscores the interrelationship among an imbalanced gut microbiota, ADs, and EVs (Louka & 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 in the lumen of toxins, antigens and bacteria, through the junctions of the epithelium. The released harmful agents can then reach the bloodstream, affecting different organs and triggering the immune system of the host.

In this scenario, the hypothesis underlying this thesis is that both bEVs and eEVs isolated from feces may have 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, fecal EVs isolated from the available cohort were characterized in order to assess the presence of both bEVs and eEVs.

International society for EVs (ISEV) indicates different methods to characterize EVs, and western blot is one of the most used (Royo et al. 2020). 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 4A, figure 4B***), confirming the presence of eEVs despite the lack of the band for CD81 (***Figure 4C***). 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 manuscript, 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 fall in the MVs area (100 nm to 1 μm in diameter) or in the exosomes area (30-150 nm in diameter). Our SEM results showed the presence of EVs with a mean size of 130 nm (***Figure 4F***) 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 4D)*** (Hyun Jeong Oh a n.d.)

After confirming the presence of bEVs within the fecal EVs by western blot (***Figure 4E***), we dived in deeper, by performing a metagenomic and metaproteomic analysis to find a bEVs-specific fingerprint in MDD patients. The results obtained from metagenomic analysis made on stool samples of MDD patients indicate that *Bacillota*, *Bacteroidota*, *Actynomicetota*, *Pseudomonadota* and *Verrucomicrobiota* were present in the highest percentage, suggesting that these phyla of bacteria were the most abundant in the gut microbiota of MDD patients (***Figure 5***). Our metagenomic data are in line with data present in the literature where it has been shown that the five most abundant phyla are *Bacilotta* and *Bacteroidote*s (around 90% of gut microbiota), followed by *Pseudomonadota*, *Actinobacteria and Verrucomicrobia* that are less abundant compared to the others (Rinninella et al. 2019);(Jandhyala et al. 2015).

Our results are also consistent with Zhong and colleagues which reported that *Bacillotta*, *Bacteroidota* and *Actinobacteriota* are the main phyla present in MDD patients and HC with some difference in the genera (Zhong et al. 2022).

Metagenomic provides an overview of the gut bacteria abundance but does not offer any information on their activation status. Recent advancements in proteomic methodologies have led to the development of metaproteomic directly on bEVs, which can highlight the most active species. Indeed, in a study of Maredia et al. the authors describe that the release of EVs from different cell types can reflect the activation status of those cells (Maredia et al. 2012).

Interestingly, our comparative analysis of metagenomics and metaproteomics at the phylum level highlighted a discrepancy between the most abundant phyla and most active ones in our cohort of MDD patients. Indeed, *Bacteroidetes* were found to be the most active compared to all the other phyla (**Figure 5C**). There is evidence that the genera of *Bacteroides*, belonging to the phylum of *Bacteroidetes*, are able to release large amounts of bEVs (Zafar & Saier 2021).

Moreover, Xu et al. 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 *Bacteroides* 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. n.d.).

These data derived from a metagenomic analysis could be aligned to our data, suggesting together that a higher presence of bEVs of that phylum could represent a specific fingerprint in patients with MDD.

It is well known that some molecules metabolized by the bacteria in the gut can affect the gut-brain axis, with Trp being one of the most important. Trp metabolism plays a crucial role in a host's health due to the fact that it can synthesize two essential neuro active compounds: 5-HT and 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. (Luo et al. 2020)detected the presence of Trp inside EVs, supporting this idea.

Our main hypothesis is that bEVs, particularly those produced by *Bacteroidotes* 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 the brain. In line with this, 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* 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). For this reason, 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 higher reduction of depressive symptoms showed, at the MDE (T0), low levels of *Actinomycetota* and high levels of *Bacteroidota*. The former (*Actinomycetota)* is one of the most important phyla in maintaining the homeostasis and its decrease could lead to a dysbiosis which is known to be linked to depressive symptoms (Binda et al. 2018) (Liu et al. 2023). Conversely, the latter (*Bacteroidota*) seems to be connected to a slight worsening of the depressive symptoms after three months. This could be ascribed to an imbalance in the phyla present in the gut of MDD patients. In a recent study, Olvera-Rosales et al. found that the microbial abundance and diversity are significantly reduced in MDD patients. They highlighted that in those patients, the presence of *Lachnospiraceae* and *Ruminococcaceae* families, which belonged to the *Bacillota* phylum, are decreased causing dysbiosis and inflammation in both gut and brain. We can speculate that *Bacteroidota* may proliferate at the expense of other phyla, resulting in an imbalance and exacerbation of depressive symptoms as shown by our results (***Figure 6***) (Olvera-Rosales et al. 2021).

Our data on the bacterial abundance and activation are preliminary since they only concern the phyla, which is one of the first taxonomic classes. For this reason, further analyses are needed to investigate more in depth, analyzing also other taxonomic classes, to better understand the role of the gut microbiota in this disease.

This is one of the limitations of this study. Moreover, we did not consider the 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 (Thursby & Juge 2017). Then, since *Bacillota* and *Bacteroidetes* constitute

90% of all phyla in the human gut, few data are present in literature regarding the less representative phylum found in our analysis (metagenomic and metaproteomic) (Jandhyala et al. 2015).

After confirming the presence of eEVs within fecal EVs, we decided to phenotype the eEVs using the MACSPlex exosome kit. This analysis of multiple eukaryotic surface markers of fecal EVs have a high diagnostic potential because of the advantage of the simultaneous profiling of several EVs subpopulations. The flow cytometry analysis demonstrated that a higher production of CD1c+ EVs in the gut correlated with a worsening of depressive symptoms (Δ MADRS), while a higher production of CD11c+ EVs correlated with a declining of social and working functions and a subjective quality of life (Δ RI) (***Figure 8 A-B***).

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 class I major histocompatibility complex (MHC) 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 interleukin-17 (IL-17), interleukin-21 (IL-21), interferon-gamma (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 in our patient cohort, some individuals 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 2***). In general, antidepressant drugs like Selective Serotonin Reuptake Inhibitors (SSRIs) and Serotonin-Norepinephrine Reuptake Inhibitors (SNRIs) might exert different immunomodulatory effects. Moreover, DCs express the serotonin transporter (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 proinflammatory cytokines such as IFN-γ, TNF-alpha and IL-1β, 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 increase in noradrenaline 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 fecal 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 fecal 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 & 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.

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 insights are needed to thoroughly investigate and understand the potential of both bEVs and eEVs as prognostic factors in MDD.

7. References

Agus A, Planchais J & Sokol H (2018) Gut Microbiota Regulation of Tryptophan Metabolism in Health and Disease. *Cell Host Microbe* 23, 716–724.

Arteaga-Blanco LA & Bou-Habib DC (2021) The role of extracellular vesicles from human macrophages on host-pathogen interaction. *Int J Mol Sci* 22.

Barranco I, Padilla L, Parrilla I, Álvarez-Barrientos A, Pérez-Patiño C, Peña FJ, Martínez EA, Rodriguez-Martínez H & Roca J (2019) Extracellular vesicles isolated from porcine seminal plasma exhibit different tetraspanin expression profiles. *Sci Rep* 9.

Belmaker RH & Agam G (2008) Major Depressive Disorder. *New England Journal of Medicine* 358, 55–68.

Beurel E, Toups M & Nemeroff CB (2020) The Bidirectional Relationship of Depression and Inflammation: Double Trouble. *Neuron* 107, 234–256.

Binda C, Lopetuso LR, Rizzatti G, Gibiino G, Cennamo V & Gasbarrini A (2018) Actinobacteria: A relevant minority for the maintenance of gut homeostasis. *Digestive and Liver Disease* 50, 421–428.

Bitto NJ & Kaparakis-Liaskos M (2017) The therapeutic benefit of bacterial membrane vesicles. *Int J Mol Sci* 18.

Boussamet L, Rajoka MSR & Berthelot L (2022) Microbiota, IgA and Multiple Sclerosis. *Microorganisms* 10.

Chen CY, Yeh YW, Kuo SC, Liang CS, Ho PS, Huang CC, Yen CH, Shyu JF, Lu RB & Huang SY (2018) Differences in immunomodulatory properties between venlafaxine and paroxetine in patients with major depressive disorder. *Psychoneuroendocrinology* 87, 108–118.

Choi J, Kwon H, Kim YK & Han PL (2022) Extracellular Vesicles from Gram-positive and Gram-negative Probiotics Remediate Stress-Induced Depressive Behavior in Mice. *Mol Neurobiol* 59, 2715–2728.

Ciferri MC, Quarto R, Tasso R & Bernardi S (2021) biology Extracellular Vesicles as Biomarkers and Therapeutic Tools: From Pre-Clinical to Clinical Applications. Available at: https://doi.org/10.3390/biology10050359.

Colle R, Masson P, Verstuyft † Céline, Fève B, Werner E, Boursier-Neyret C, Walther B, David DJ, Boniface B, Falissard B, Chanson P, Corruble E & Becquemont L (2020) Peripheral tryptophan, serotonin, kynurenine, and their metabolites in major depression: A case-control study PCN Psychiatry and Clinical Neurosciences. *Psychiatry Clin Neurosci* 74, 112–117. Available at: http://onlinelibrary.wiley.com/doi/10.1111/pcn.12944/full.

Colombo M, Raposo G & Théry C (2014) Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol* 30, 255–289.

Das TK & Ganesh BP (2023) Interlink between the gut microbiota and inflammation in the context of oxidative stress in Alzheimer's disease progression. *Gut Microbes* 15.

Doyle LM & Wang MZ (2019) Overview of extracellular vesicles, their origin, composition, purpose, and methods for exosome isolation and analysis. *Cells* 8.

Du Y, Gao XR, Peng L & Ge JF (2020) Crosstalk between the microbiota-gut-brain axis and depression. *Heliyon* 6.

Endicott J, Nee J, Harrison W & Blumenthal R (1993) Quality of Life Enjoyment and Satisfaction Questionnaire: a new measure. *Psychopharmacol Bull* 29, 321–6.

Fava M & Kendler KS (2000) *Review Major Depressive Disorder tions in "first-world" countries such as the United States*,

Fyfe J, Casari I, Manfredi M & Falasca M (2023) Role of Lipid Signalling in Extracellular Vesicles-mediated Cell-to-cell Communication. *Cytokine Growth Factor Rev* 73, 20–26.

Ganguly D, Haak S, Sisirak V & Reizis B (2013) The role of dendritic cells in autoimmunity. *Nat Rev Immunol* 13, 566–577.

Gao K, Mu CL, Farzi A & Zhu WY (2020) metaboliti come la kynurenine. *Advances in Nutrition* 11, 709–723.

Góralczyk-Bińkowska A, Szmajda-Krygier D & Kozłowska E (2022) The Microbiota–Gut–Brain Axis in Psychiatric Disorders. *Int J Mol Sci* 23.

Herr N, Bode C & Duerschmied D (2017) The Effects of Serotonin in Immune Cells. *Front Cardiovasc Med* 4.

Hosseini-Giv N, Basas A, Hicks C, El-Omar E, El-Assaad F & Hosseini-Beheshti E (2022) Bacterial extracellular vesicles and their novel therapeutic applications in health and cancer. *Front Cell Infect Microbiol* 12.

Huang D, Rao D, Xi X, Zhang Z & Zhong T (2022) Application of extracellular vesicles proteins in cancer diagnosis. *Front Cell Dev Biol* 10.

Hyun Jeong Oh a b, c, YS c, SC c, DWH a, DSL a, Convective exosome-tracing microfluidics for analysis of cell-non- autonomous neurogenesis.

IsHak WW, Bonifay W, Collison K, Reid M, Youssef H, Parisi T, Cohen RM & Cai L (2017) The recovery index: A novel approach to measuring recovery and predicting remission in major depressive disorder. *J Affect Disord* 208, 369–374.

Jacobsen JPR, Medvedev IO & Caron MG (2012) The 5-HT deficiency theory of depression: perspectives from a naturalistic 5-HT deficiency model, the tryptophan hydroxylase 2 ^{Arg} 439 ^{His} knockin mouse. *Philosophical Transactions of the Royal Society B: Biological Sciences* 367, 2444–2459.

Jandhyala SM, Talukdar R, Subramanyam C, Vuyyuru H, Sasikala M & Reddy DN (2015) Role of the normal gut microbiota. *World J Gastroenterol* 21, 8836–8847.

Kakarla R, Hur J, Kim YJ, Kim J & Chwae YJ (2020) Apoptotic cell-derived exosomes: messages from dying cells. *Exp Mol Med* 52.

Kang SG & Cho SE (2020) Neuroimaging biomarkers for predicting treatment response and recurrence of major depressive disorder. *Int J Mol Sci* 21.

Kinashi Y & Hase K (2021) Partners in Leaky Gut Syndrome: Intestinal Dysbiosis and Autoimmunity. *Front Immunol* 12.

Leal Rojas IM, Mok WH, Pearson FE, Minoda Y, Kenna TJ, Barnard RT & Radford KJ (2017) Human blood cD1c+ dendritic cells promote Th1 and Th17 effector function in memory cD4+ T cells. *Front Immunol* 8.

Li Y, Gui Y, Zhao M, Chen X, Li H, Tian C, Zhao H, Jiang C, Xu P, Zhang S, Ye S & Huang M (2023) The roles of extracellular vesicles in major depressive disorder. *Front Psychiatry* 14.

Liang X, Dai N, Sheng K, Lu H, Wang J, Chen L & Wang Y (2022) Gut bacterial extracellular vesicles: important players in regulating intestinal microenvironment. *Gut Microbes* 14.

Liu L, Wang H, Chen X, Zhang Y, Zhang H & Xie P (2023) Gut microbiota and its metabolites in depression: from pathogenesis to treatment. *EBioMedicine* 90.

Livia Chiriţă A, Gheorman V, Bondari D & Rogoveanu I (2015) Current understanding of the neurobiology of major depressive disorder. *Rom J Morphol Embryol* 56, 651–658. Available at: http://www.rjme.ro/.

Longo S, Rizza S & Federici M (2023) Microbiota-gut-brain axis: relationships among the vagus nerve, gut microbiota, obesity, and diabetes. *Acta Diabetol* 60, 1007–1017.

Louka E & Koumandou VL (2024) The Emerging Role of Human Gut Bacteria Extracellular Vesicles in Mental Disorders and Developing New Pharmaceuticals. *Curr Issues Mol Biol* 46, 4751–4767.

Luo P, Mao K, Xu J, Wu F, Wang X, Wang S, Zhou M, Duan L, Tan Q, Ma G, Yang G, Du R, Huang H, Huang Q, Li Y, Guo M & Jin Y (2020) Metabolic characteristics of large and small extracellular vesicles from pleural effusion reveal biomarker candidates for the diagnosis of tuberculosis and malignancy. *J Extracell Vesicles* 9.

Marano G, Mazza M, Lisci FM, Ciliberto M, Traversi G, Kotzalidis GD, De Berardis D, Laterza L, Sani G, Gasbarrini A & Gaetani E (2023) The Microbiota–Gut–Brain Axis: Psychoneuroimmunological Insights. *Nutrients* 15.

Maredia R, Devineni N, Lentz P, Dallo SF, Yu J, Guentzel N, Chambers J, Arulanandam B, Haskins WE & Weitao T (2012) Vesiculation from Pseudomonas aeruginosa under SOS. *The Scientific World Journal* 2012.

Mathieu M, Martin-Jaular L, Lavieu G & Théry C (2019) Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. *Nat Cell Biol* 21, 9–17.

Montgomery SA & Åsberg M (1979) A New Depression Scale Designed to be Sensitive to Change. *British Journal of Psychiatry* 134, 382–389.

Mulcahy LA, Pink RC & Carter DRF (2014) Routes and mechanisms of extracellular vesicle uptake. *J Extracell Vesicles* 3.

Mundt JC, Marks IM, Shear MK & Greist JH (2002) The Work and Social Adjustment Scale: A simple measure of impairment in functioning. *British Journal of Psychiatry* 180, 461–464.

Olvera-Rosales LB, Cruz-Guerrero AE, Ramírez-Moreno E, Quintero-Lira A, Contreras-López E, Jaimez-Ordaz J, Castañeda-Ovando A, Añorve-Morga J, Calderón-Ramos ZG, Arias-Rico J & González-Olivares LG (2021) Impact of the gut microbiota balance on the health-disease relationship: The importance of consuming probiotics and prebiotics. *Foods* 10.

Østergaard SD, Jensen SOW & Bech P (2011) The heterogeneity of the depressive syndrome: When numbers get serious. *Acta Psychiatr Scand* 124, 495–496.

Pape K, Tamouza R, Leboyer M & Zipp F (2019) Immunoneuropsychiatry — novel perspectives on brain disorders. *Nat Rev Neurol* 15, 317–328.

Platten M, Nollen EAA, Röhrig UF, Fallarino F & Opitz CA (2019) Tryptophan metabolism as a common therapeutic target in cancer, neurodegeneration and beyond. *Nat Rev Drug Discov* 18, 379–401.

Reed SL & Escayg A (2021) Extracellular vesicles in the treatment of neurological disorders. *Neurobiol Dis* 157.

Refisch A, Sen ZD, Klassert TE, Busch A, Besteher B, Danyeli LV, Helbing D, Schulze-Späte U, Stallmach A, Bauer M, Panagiotou G, Jacobsen ID, Slevogt H, Opel N & Walter M (2023) Microbiome and immuno-metabolic dysregulation in patients with major depressive disorder with atypical clinical presentation. *Neuropharmacology* 235.

Rezasoltani S, Bashirzadeh DA, Mojarad EN, Aghdaei HA, Norouzinia M & Shahrokh S (2020) Signature of gut microbiome by conventional and advanced analysis techniques: Advantages and disadvantages. *Middle East J Dig Dis* 12, 249–255.

Rinninella E, Raoul P, Cintoni M, Franceschi F, Miggiano GAD, Gasbarrini A & Mele MC (2019) What is the healthy gut microbiota composition? A changing ecosystem across age, environment, diet, and diseases. *Microorganisms* 7.

Royo F, Théry C, Falcón-Pérez JM, Nieuwland R & Witwer KW (2020) Methods for Separation and Characterization of Extracellular Vesicles: Results of a Worldwide Survey Performed by the ISEV Rigor and Standardization Subcommittee. *Cells* 9.

Ruiz NAL, Del Ángel DS, Brizuela NO, Peraza AV, Olguín HJ, Soto MP & Guzmán DC (2022) Inflammatory Process and Immune System in Major Depressive Disorder. *International Journal of Neuropsychopharmacology* 25, 46–53.

Shao H, Im H, Castro CM, Breakefield X, Weissleder R & Lee H (2018) New Technologies for Analysis of Extracellular Vesicles. *Chem Rev* 118, 1917–1950.

Steinert C, Hofmann M, Kruse J & Leichsenring F (2014) The prospective long-term course of adult depression in general practice and the community. A systematic literature review. *J Affect Disord* 152–154, 65–75.

Sultan S, Mottawea W, Yeo J & Hammami R (2021) Gut microbiota extracellular vesicles as signaling molecules mediating host-microbiota communications. *Int J Mol Sci* 22.

Takenaka MC & Quintana FJ (2017) Tolerogenic dendritic cells. *Semin Immunopathol* 39, 113–120.

Thursby E & Juge N (2017) Introduction to the human gut microbiota. *Biochemical Journal* 474, 1823–1836.

Toyofuku M, Nomura N & Eberl L (2019) Types and origins of bacterial membrane vesicles. *Nat Rev Microbiol* 17, 13–24.

Tricarico C, Clancy J & D'Souza-Schorey C (2017) Biology and biogenesis of shed microvesicles. *Small GTPases* 8, 220–232.

Wilmes P, Heintz-Buschart A & Bond PL (2015) A decade of metaproteomics: Where we stand and what the future holds. *Proteomics* 15, 3409–3417.

Wiredu Ocansey DK, Hang S, Yuan X, Qian H, Zhou M, Valerie Olovo C, Zhang X & Mao F (2023) The diagnostic and prognostic potential of gut bacteria in inflammatory bowel disease. *Gut Microbes* 15.

Xu J, Xu H-M, Peng Y, Zhao C, Zhao H-L, Huang W, Huang H-L, He J, Du Y-L, Zhou Y-J, Zhou Y-L & Nie Y-Q The effect of different combinations of antibiotic cocktails on mice and selection of animal models for further microbiota research. *APPLIED MICROBIAL AND CELL PHYSIOLOGY*. Available at: https://doi.org/10.1007/s00253-021-11131-2.

Xu R, Greening DW, Zhu HJ, Takahashi N & Simpson RJ (2016) Extracellular vesicle isolation and characterization: Toward clinical application. *Journal of Clinical Investigation* 126, 1152–1162.

Yáñez-Mó M, Siljander PRM, Andreu Z, Zavec AB, Borràs FE, Buzas EI, Buzas K, Casal E, Cappello F, Carvalho J, Colás E, Cordeiro-Da Silva A, Fais S, Falcon-Perez JM, Ghobrial IM, Giebel B, Gimona M, Graner M, Gursel I, Gursel M, Heegaard NHH, Hendrix A, Kierulf P, Kokubun K, Kosanovic M, Kralj-Iglic V, Krämer-Albers EM, Laitinen S, Lässer C, Lener T, Ligeti E, Line A, Lipps G, Llorente A, Lötvall J, Manček-Keber M, Marcilla A, Mittelbrunn M, Nazarenko I, Nolte-'t Hoen ENM, Nyman TA, O'Driscoll L, Olivan M, Oliveira C, Pállinger É, Del Portillo HA, Reventós J, Rigau M, Rohde E, Sammar M, Sánchez-Madrid F, Santarém N, Schallmoser K, Ostenfeld MS, Stoorvogel W, Stukelj R, Van Der Grein SG, Helena Vasconcelos M, Wauben MHM & De Wever O (2015) Biological properties of extracellular vesicles and their physiological functions. *J Extracell Vesicles* 4, 1–60.

Zafar H & Saier MH (2021) Gut Bacteroides species in health and disease. *Gut Microbes* 13, 1–20.

Zhang X, Wu Y, Cheng Q, Bai L, Huang S & Gao J (2022) Extracellular Vesicles in Cardiovascular Diseases: Diagnosis and Therapy. *Front Cell Dev Biol* 10.

Zhong Q, Chen JJ, Wang Y, Shao WH, Zhou CJ & Xie P (2022) Differential Gut Microbiota Compositions Related With the Severity of Major Depressive Disorder. *Front Cell Infect Microbiol* 12.