# Analysis of gut microbiota-derived extracellular vesicles isolated from newly diagnosed multiple sclerosis patients

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Pro gradu

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Experimental work in this Pro gradu was performed in the Cancer and Translational Medicine Research unit and Biocenter Oulu in the research group of Docent Justus Reunanen who also supervised this work. Figure copyrights in this work belong to the corresponding author.

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## Abbreviations

ABC: ATP-binding cassette

ACN: Acetonitrile

AMP: Antimicrobial peptide

BBB: Blood-brain barrier

BMV: Bacterial membrane vesicle

CNS: Central nervous system

DC: Dendritic cell

DPBS: Dulbecco's phosphate buffered saline

EAE: Experimental autoimmune encephalomyelitis

EV: Extracellular vesicle

HLA: Human leukocyte antigen

HM: Homogenation medium

IL: Interleukin

INF-γ: Interferon gamma

LC-ESI-MS/MS: Liquid chromatography-electrospray ionization-tandem mass spectrometry

LPS: Lipopolysaccharide

MRI: Magnetic resonance imaging

MS: Multiple sclerosis

NRF2: Nuclear factor-erythroid 2 p45 related factor 2

NTA: Nanoparticle tracking analysis

OIMV: Outer-inner membrane vesicle

OMV: Outer membrane vesicle

PBS: Phosphate buffered saline

PPMS: Primary-progressive multiple sclerosis

PQS: *Pseudomonas* quinolone signal

PSA: Polysaccharide A

RRMS: Relapse-remitting multiple sclerosis

SCFA: Short chain fatty acid

SFB: Segmented filamentous bacteria

SIgA: Secreted immunoglobulin A

SPF: Specific pathogen-free

SPMS: Secondary-progressive multiple sclerosis

TEM: Transmission electron microscopy

Th: T-helper cell

TNF- α: Tumor necrosis factor-α

TNF: Tumor necrosis factor

TGF-β: Transforming growth factor β

Treg: T regulatory cell

UA: Uranyl acetate

WS: Working solution

WSB: Working solution buffer

# I LITERATURE SECTION

## 1. Introduction

The gut and the brain are in a bidirectional communication known as the gut-brain axis (Collins *et al.,* 2012). The gut-brain axis provides neuronal and humoral signals to orchestrate gut as well as brain function (Foster *et al.,* 2017). Microbial community of the human gastrointestinal tract, known as the gut microbiota, can utilize the routes of gut-brain axis to alter host physiology (Holzer & Farzi, 2014). During recent years, the gut microbiota has been recognized as an important factor in various aspects of health and disease, including gut health, behavior, neurological diseases, autoimmune diseases, immune system maturation, and brain development (Forsythe *et al.,* 2016; Sarkar & Banerjee, 2019; Wang & Wang, 2016).

Multiple sclerosis (MS) is one of the aspects of health and disease that are proposed to be regulated by gut microbiota (Sarkar & Banerjee, 2019). MS is an inflammatory autoimmune disorder in which the immune system damages myelin and axons of the central nervous system (CNS), leading to neurodegeneration and severe disability (Dendrou *et al.,* 2015, Legroux & Arbour, 2015; Reich *et al.,* 2018). Incidence of MS has increased worldwide (Browne *et al.,* 2014; Compston & Coles, 2002; Wallin *et al.,* 2019), being the most common cause of neurologic disability within young adults (Orton *et al.,* 2006).

So far, the role of gut microbiota in MS has majorly been studied with experimental autoimmune encephalomyelitis (EAE), a murine model of MS. Mice grown in germ-free conditions do not develop EAE, but colonization with microbes triggers the condition (Berer *et al.,* 2011; Lee *et al.,* 2011). Patients with MS have altered gut microbiota composition compared to healthy controls (Chen *et al.,* 2016; Jangi *et al.,* 2016; Miyake *et al.,* 2015). Chance that EAE susceptible mice will develop the disease is higher when they are colonized with gut microbes derived from MS patients compared to healthy controls (Berer *et al.,* 2017).

One possible mechanism how the gut microbiota can modulate immune system (Fábrega *et al.,* 2016), autoimmunity (Kimura *et al.,* 2018), and other host related functions is the production of extracellular vesicles (EVs). EVs are spherical and bilayered structures that contain various biomolecules such as cytosolic proteins, membrane proteins, lipids, polysaccharides, and nucleic acids. (Molina-Tijeras *et al.,* 2019), However, the role of gut microbial vesicles in health and disease is mostly unexplored. Therefore, the aim of this Pro gradu work was to observe, whether gut microbiota derived EVs of MS disease patients are

different from healthy controls by examining characteristics and the protein load of microbial EVs.

## 2. Review of the literature

### 2.1 Multiple sclerosis

Multiple sclerosis (MS), an inflammatory autoimmune disorder, is the most prevalent cause of nontraumatic neurologic disability among young adults, affecting more than 2 million people worldwide. Onset of the disease usually occurs between the age of 20 and 40, and is two-fold to three-fold more common among women than in men (Orton *et al.,* 2006). Occurrence of MS is higher in North America, Europe, and Australia compared to Africa, Asia, and South America, but the number of people inflicted with the disease has increased in both developed and developing countries (Browne *et al.,* 2014; Compston & Coles, 2002; Wallin *et al.,* 2019).

MS disease is characterized by inflammatory lesions that can appear anywhere in the CNS and the optic nerve due to infiltration of immune cells (Dendrou *et al.,* 2015). Lesions can be imaged with magnetic resonance imaging (MRI) and seen as white areas in the CNS. These lesions contain activated peripheral immune cells, activated glia cells, and astrocytes, which are involved in myelin degeneration, axonal and neuronal damage, and oligodendrocyte death (Compston & Coles, 2002; Legroux & Arbour, 2015; Miyake & Yamamura, 2019; Reich *et al.,* 2018).

Patients with MS disease typically experience neurological disability episodes, which can last from days to weeks. Symptoms and their severity vary, depending on the location of lesions (Fig. 1.) (Compston & Coles, 2008; Kearney *et al.,* 2015). Typical symptoms include loss of vision, double vision, impaired body balance and motility, limb weakness, sensory loss, and ataxia. At early disease stage, the neurons are mostly preserved but gradual neuroaxonal loss occurs as the disease progresses. Over time, usually in 10-20 years, the symptoms become persistent and lead to severe disability and cognitive impairment characterized by the loss of brain cells, deep grey matter and cortical demyelination, and ventricular enlargement (Dendrou *et al.,* 2015; Legroux & Arbour, 2015; Reich *et al.,* 2018).

MS disease is divided into different sub-types. The most common type is relapse-remitting MS (RRMS), that is characterized by periodic relapses and remissions. Relapses are neurologic disability episodes followed by remission, a partial recovery. Patients with RRMS can develop secondary-progressive MS (SPMS) over time, which involves constant progression

in disability. Another subtype is defined as primary-progressive MS (PPMS) in which the disease progression is constant from the onset (Thompson *et al.,* 2018).

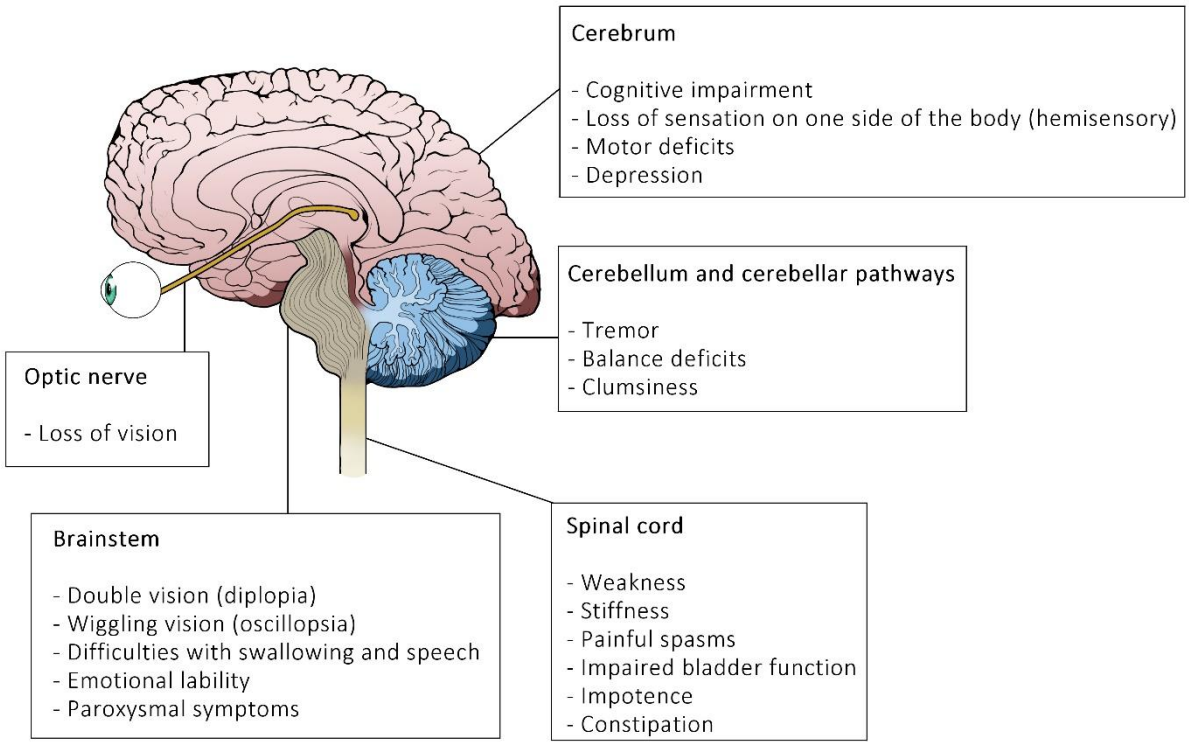


Figure 1. Symptoms of MS depend on the location of the lesion in the CNS and optic nerve.

Both genes and the environment are involved in the onset of MS (Compston & Coles; 2002). Known risk factors for MS are smoking, lack of vitamin D, Epstein-Barr virus infection during adulthood, and obesity (Alfredsson & Olsson, 2019). Genetic risk for MS involves ~100 regions in the human genome, and in overall, the genetics count for ~30 % of the overall risk. Majority of these regions are associated with the genes involving immune function, such as human leukocyte antigen (HLA) alleles (Ascherio *et al.,* 2012; Reich *et al.,* 2018). MS risk genes are also associated with other diseases involving immune dysfunction, such as ulcerative colitis, Crohn's disease, primary biliary cirrhosis, celiac disease, rheumatoid arthritis, psoriasis, and autoimmune thyroid disease (Beecham *et al.,* 2013).

CNS is considered isolated or immunoprivileged from the access of peripheral immune system due to the function of blood-brain barrier (BBB). BBB is a physical barrier between blood and brain tissue (Barret *et al.,* 2016), which protects neurons by limiting the access of peripheral components from the circulation (Banks *et al.,* 2015). Breakage of the BBB is considered to be a preclinical sign of MS before the onset of neurological symptoms (Sospedra & Martin, 2005). MS is an immune system driven disease that is primarily mediated

by pro-inflammatory T helper type (Th) 1 and Th17 cells (Kaskow & Baecher-Allan, 2018). MS manifests itself when self-reactive T cells migrate through the BBB and induce axonal damage and demyelination (Fletcher *et al.,* 2010). Demyelination is orchestrated by macrophages, microglia, cytotoxic $CD8^+$ cells (Dendrou *et al.,* 2015; Lassmann, 2014), and antibody secreting B cells (Reich *et al.,* 2018).

Self-reactive T cells are usually eliminated by negative selection in the thymus (Kappler *et al.,* 1987). Despite selection, part of the self-reactive cells escape into the periphery. However, self-reactive peripheral T-cells are not only present in people with autoimmune disease but are constantly generated among healthy individuals. People with no autoimmune disease are able to maintain unresponsiveness towards self-antigens, known as self-tolerance (Lohmann *et al.,* 1996; Semana *et al.,* 1999; Van Parijs & Abbas, 1998). Self-tolerance is mediated by T regulatory cells (Tregs) and absence of these cells has been shown to initiate autoimmune diseases (Sakaguchi *et al.,* 1995).

Two models, CNS-extrinsic and -intrinsic, exist to explain the pathogenesis of MS, since it is not known if the triggering action of MS occurs outside or inside the CNS. CNS-extrinsic model, or peripheral model, involves activation of T-cells outside the CNS, that leads to infiltration of CNS with T cells along with activated B cells and monocytes. CNS-intrinsic model suggests that infiltration of lymphocytes is a secondary response to currently unknown factors that could occur in the CNS (Dendrou *et al.,* 2015). Target antigens, that initiate MS episodes, are thought to be present only in the CNS, since it is the only place affected by infiltration (Thompson *et al.,* 2018).

Molecular mimicry and bystander activation are suggested to be possible CNS-extrinsic mechanisms how microbial agents could induce MS. Molecular mimicry mechanism involves self-reactive T and B cells that recognise microbial peptides or other antigens, presented by antigen presenting cells, that share molecular similarities with the self-antigens expressed in the CNS. These cells, that have recognized mimicking bacterial antigen, could infiltrate the CNS. If cells re-recognize antigens expressed by the CNS, they can initiate neurological damage. Bystander activation mechanism suggests that activation of self-reactive T cell reaction is initiated by cooperation of microbial antigens and cytokines. Alternatively, release of self-antigens and inflammation promoting viral particles from virus-infected cells could initiate autoimmune response (Sospedra & Martin, 2005).

### 2.3 Gut microbiota

Broad spectrum of microbes, termed as the gut microbiota, lives within the gastrointestinal tract of humans. While mainly consisting of bacteria, the human gut microbiota also harbors archaea, protists, yeasts, and viruses (Quigley, 2013). Estimated number of bacterial cells in the gut is 38 trillion, a number that is close to the number of cells in the human body. While the mass of microbiota is approximately 0.2 kg (Sender *et al.,* 2016), it has a gene reserve ~100-150 fold larger compared to human genome, providing functional potential to impact host physiology (Gill *et al.,* 2006; Qin *et al.,* 2010). Metabolic activity of the microbial community in the gut provides compounds that are beneficial to the host. Commonly known products include functional metabolites such as vitamin K, folic acid, short-chain fatty acids (SCFAs), and amino acids. Gut microbiota works as a barrier against pathogen colonization and promotes maturation and function of the immune system (Quigley, 2013). The role of microbiota in health and disease has been recognized as a potential factor, as the knowledge about the function of microbial community regarding the host well-being have increased over the last years (Quigley, 2013, Sarkar & Banerjee. 2019).

Establishment of gut colonization begins at birth when the infant is in first contact with maternal and environmental bacteria. Initial gut microbiota composition varies, depending whether the delivery is performed vaginally or by caesarean section (Dominguez-Bello *et al.,* 2010). Development of gut microbiota proceeds by breastfeeding, which provides a regular supply of bacteria, $8 \times 10^4$ - $8 \times 10^6$ bacteria cells per day (Heikkilä & Saris, 2003), to the infant gut (Fernández *et al.,* 2013). Starting from the age of one, the gut microbiota of an infant converts to an adult-like composition when solid foods are introduced to the diet (Stark & Lee, 1982). Adulthood microbiota is suggested to be stable (Zoetendal *et al.,* 1998), while the composition of gut microbiota can be shaped by various factors including diet (David *et al.,* 2014), intake of antibiotics (Ochoa-Repáraz *et al.,* 2009), environmental factors (Rothschild *et al.,* 2018) such as hygiene level (Adlerberth *et al.,* 1998), aging (Claesson *et al.,* 2011), function of immune system (Hooper & MacPherson, 2010), and stress (Karl *et al.,* 2017).

Most of the gut bacteria are anaerobes with a relation of 100 to 1000:1 compared to aerobic bacteria (Quigley, 2013). Mature human gut microbiota is dominated by a few bacteria phylum. Major contributors are *Bacteroidetes* and *Firmicutes,* and minor contributors belong to *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* (Arumugam *et al.,* 2011, Lozupone *et al.,* 2012; The Human Microbiome Project Consortium, 2012). Estimated number of species present in the gut is ~500-1000 (Xu & Gordon, 2003), while the core human

microbiota is suggested to involve ~40 partially stable species (Martínez *et al.,* 2013). Part of the gut microbiota involves transient microbes that more or less travel through the gastrointestinal tract (Sears *et al.,* 1950).

Distribution of microbiota *i.e*. biogeography in the gastrointestinal tract varies, depending on the physiological properties of the gut at given region (Fig. 2). While providing variable microbial habitats, circumstances in the gut determine, which bacteria are able to flourish. *Lactobacillaceae* and *Enterobacbacteriaceae* dominate the small intestine. Due to harsh conditions in the small intestine caused by acidity, presence of oxygen, and antimicrobial agents, the resident microbes need to be fast growing and capable to compete with the host for the use of simple carbohydrates. Therefore, the number of microbes living in the small intestine is low compared to the lower parts of the intestine (Donaldson *et al.,* 2015; Swidsinski *et al.,* 2005).

Intestinal circumstances gradually grow more temperate and the bacterial load increases along the gut before reaching peak densities in the colon and cecum. In the colon, resident microbes use complex carbohydrates, compounds otherwise undigestable to the host, and mucus as an energy source. Transverse regions in the colon, such as outer and inner mucus and digested material, provide diverse habitats for bacterial growth. Microbes dominating the colon inner fold regions is composed by microbe families such as *Lachnospiraceae* and *Ruminococcaceae*. Outer colon mucus layer, or loose mucus, is inhabited by mucus-degrading bacteria including *Bacteroides acidifaciens*, *Bacteroides fragilis*, *Bifidobacteriaceae*, and *Akkermansia muciniphila*. Inner colon mucus, also known as attached mucus, is in close proximity of gut epithelial cell layer, and is inhabited by *B. fragilis* and *Acinetobacter spp.* Dominating microbial families, whom reside in the digested material, belong to *Bacteroidaceae*, *Prevotellaceae*, and *Rikenellaceae*. (Donaldson *et al.,* 2015; Frank *et al.,* 2007, Swidsinski *et al.,* 2005; Thursby & Juge, 2017).

Gut microbiota is suggested to have a role in neurodegenerative diseases due to its ability to affect CNS function via bidirectional communication system known as the microbiota-gut-brain axis (Fig. 3) (Sarkar & Banerjee, 2019). Communication routes of the microbiota-gut-brain axis include immune system, vagus nerve, enteric nervous system, gut hormones, and hypothalamus-pituitary-adrenal axis. Gut microbiota produce functional metabolites such as SCFAs, and tryptophan-derived compounds (Kelly *et al.,* 2017; Collins *et al.,* 2012) that are able to enter host circulation and cross BBB to affect *e.g.* the function of

astrocytes (Erny *et al.,* 2015) and microglia (Rothhammer *et al.,* 2016). Free fatty acid receptors 2 and 3 that recognize SCFAs are also present in the colon enteroendocrine cells (Lund *et al.,* 2018), ganglions of sympathetic (Kimura *et al.,* 2011), and parasympathetic nervous system, including the vagus nerve (Nøhr *et al.,* 2015).

Gut microbiota has been shown to affect BBB integrity by influencing the expression of claudin-5 and occludin, proteins that regulate permeability of BBB tight junctions. Bacteria that produce SCFAs, such as butyrate, propionate, and acetate promote BBB integrity (Braniste *et al.,* 2014). Additionally, butyrate producing bacteria have been reported to have neuroprotective properties in a mouse model of vascular dementia (Liu *et al.,* 2015). Propionate doesn't affect the expression of tight junction proteins, but protects BBB from lipopolysaccharide (LPS) induced inflammation by reducing oxidative stress (Hoyles *et al.,* 2018). LPS is a compound found on the outer membrane of gram-negative bacteria, and is known to reduce BBB integrity (Banks *et al.,* 2015). Propionate promotes the function of nuclear factor-erythroid 2 p45 related factor 2 (NRF2) (Hoyles *et al.,* 2018), that regulates the expression of genes involved in the protection of cells from oxidative stress (Dinkova-Kostova *et al.,* 2018). NRF2 is suggested to also have a protective role in neurogenerative diseases such as Parkinson's disease (Williamson *et al.,* 2012) and Alzheimer's disease (Fragoulis *et al.,* 2017). In other words, disruptions in BBB integrity can potentially promote development of neurodegenerative diseases (Sweeney *et al.,* 2018). Additionally with previously mentioned MS disease, breakage of BBB has been suggested to be an early biomarker in cognitive dysfunction (Nation *et al.,* 2019), and disruption of BBB is also associated with Parkinson's disease (Gray & Woulfe, 2015).

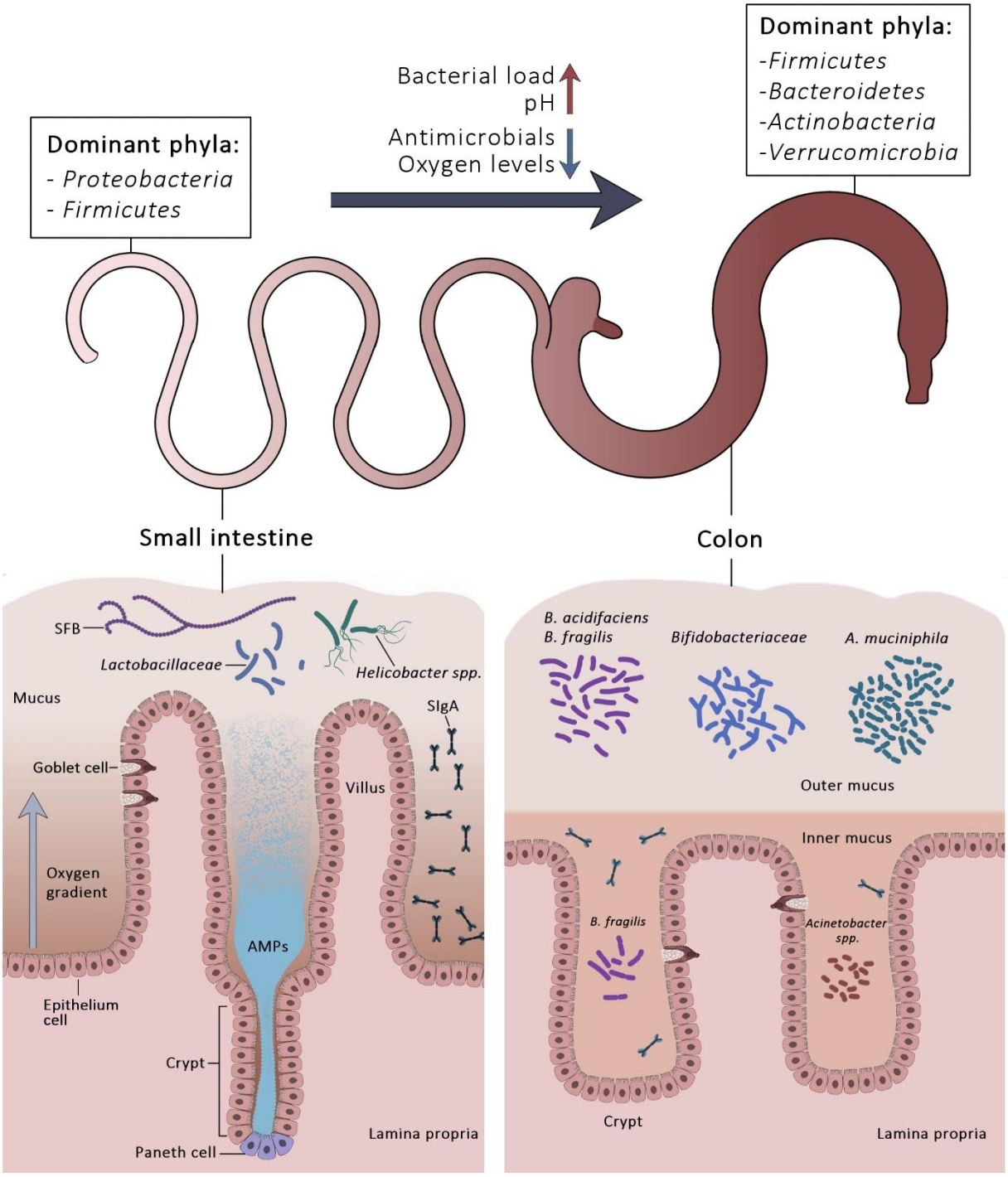


Figure 2. Gut biogeography of the small intestine and colon. Low pH, oxygen, antimicrobial peptides (AMPs) released by Paneth cells, and secreted immunoglobulin A (SIgA) limit bacterial growth in the small intestine. Goblet cells produce mucus that prevents the access of bacteria into host cells both in small intestine and colon. Bacterial diversity increases along the intestine where circumstances grow more favorable for bacterial survival. Outer mucus layer of the colon provides a growth habitat for various mucin-degrading bacteria whereas inner mucus is restricted for bacterial growth. SFB: Segmented filamentous bacteria.

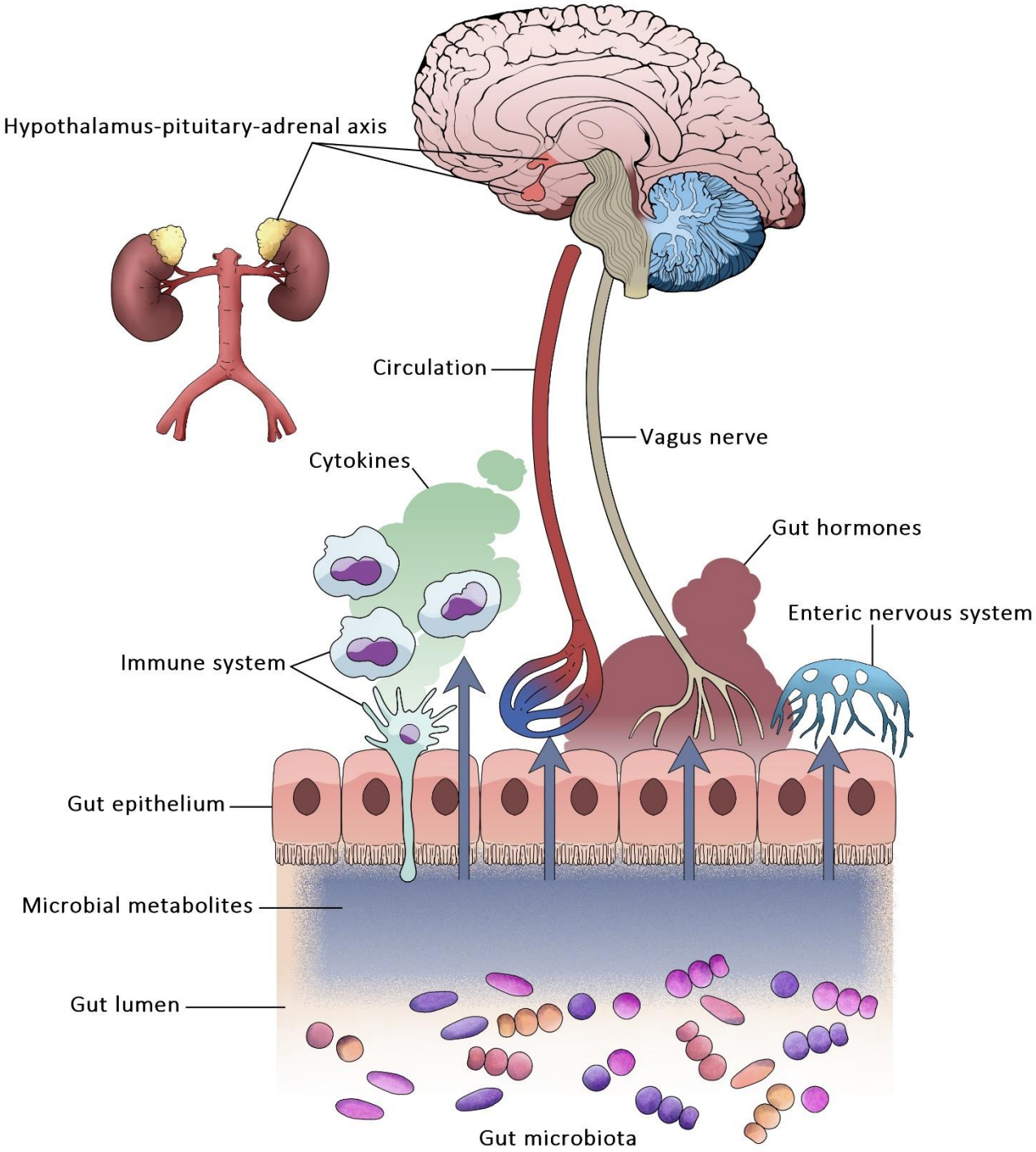


Figure 3. Communication routes of the microbiota-gut-brain axis. Metabolites produced by the gut microbiota influence function of the immune system *e.g.* by affecting cytokine production. Gut epithelial cells have a repertoire of receptors that recognize microbial products, which affects gut hormone release. Microbial products can pass gut barrier and influence vagus nerve and enteric nervous system. Products that enter circulation travel through the body and are even capable to enter the brain. Hypothalamus-pituitary-adrenal axis is a suggested route how the microbiota alters stress-related responses.

### 2.4 The role of gut microbiota in experimental autoimmune encephalomyelitis

Gut microbiota influences the function of the host immune system not only in the gut area but also in the periphery and in the CNS. The effect of gut microbiota can be both inflammatory and anti-inflammatory. Gut microbiota has been shown to promote innate immune system function against pathogens by releasing peptidoglycan into host circulation. (Clarke *et al.,* 2010). *Clostridium* clusters IV and XIVa are associated with accumulation of $Foxp3^+$ Tregs, cells that promote anti-inflammatory response, in the mice colon (Atarashi *et al.,* 2011). Segmented filamentous bacteria (SFB) can increase the amount of Th17 cells in mice lamina propria, leading to increased production of pro-inflammatory interleukin (IL)-17 and IL-21 from Th-17 cells. (Ivanov *et al.,* 2009). SCFAs have been shown to induce colonic Treg cell function and proliferation (Furusawa *et al.,* 2013; Smith *et al.,* 2013), as well as regulate microglia maturation and function in the CNS (Erny *et al.,* 2015). Gut bacteria can produce tryptophan-derived metabolites that function as a aryl hydrocarbon receptor antagonists to suppress neuroinflammation in the CNS (Rothhammer *et al.,* 2016).

The role of gut microbiota in the pathogenesis of MS can be studied with mice and rats that will develop a condition called experimental autoimmune encephalomyelitis (EAE). EAE is a Th-1/Th-17 cell mediated demyelinating disease of CNS and is commonly used as a MS model. EAE is induced by challenging the immune system with myelin proteins or with other myelin protein corresponding peptides in complete Freund's adjuvant, leading to autoimmune inflammation. Alternatively, induction can be done with activated neuroantigen-specific T cells via adoptive cell transfer. EAE can also occur spontaneously with certain transgenic mouse strains. EAE induced mice will develop progressive hind-limb paralysis. Depending on the used mouse strain, EAE can be chronic or take a relapse-remitting course. Severity of the EAE *i.e.* the clinical score is evaluated by using a five-point system (Table I). Scoring is used to monitor clinal stages of the disease such as remissions and relapses (Miller *et al.,* 2007).

In the onset of EAE peripheral T cells migrate across the BBB to CNS and cause inflammatory response (Furtado *et al.,* 2008). Th1 cells infiltrate CNS first and induce inflammation by recruiting Th17 cells (O'Connor *et al.,* 2008). Release of chemokines and pro-inflammatory cytokines including interferon gamma (INF-γ), tumor necrosis factor (TNF) and IL-17 damages CNS and promotes activation as well as chemoattraction of monocytes and macrophages, that assist in axonal demyelination (Miller *et al.,* 2007).

Table I. Evaluation of EAE severity in mice.

| Clinical score | Stage of paralysis |
|---|---|
| 0 | Normal, no signs of disease |
| 1 | Limp tail **or** hind limb weakness |
| 2 | Limp tail **and** hind limb weakness |
| 3 | Partial hind limb paralysis |
| 4 | Complete hind limb paralysis |
| 5 | Moribund |

Mice that would otherwise develop spontaneous EAE at the age of 3-5 months, stay healthy through their life when grown in germ-free conditions. However, colonization of germ-free mice with microbes returns the susceptibility to EAE and triggers autoimmunity. While EAE in germ-free mice can be induced, they show delayed disease onset, lower disease severity compared to specific pathogen-free (SPF)-mice, have increased amount of Tregs, and lower levels of pro-inflammatory cytokines IFN-γ, IL-17A in the intestine and spinal cord. Reduction in the amount of Th17 cells in germ-free mice lamina propria and Peyer's patches is also observed upon EAE induction (Berer *et al.,* 2011; Lee *et al.,* 2011). On the other hand, self-reactive Th17 cells were shown to accumulate in the intestine of spontaneous EAE resistant mice. Reduced amounts of these Th17 cells were observed in the periphery, suggesting the role of gut as a keeper of self-reactive immune cells (Berer *et al.,* 2014).

During adolescence/early adulthood (5-10 weeks), the disturbance of the gut microbiota, known as gut dysbiosis, have been shown to promote EAE pathogenesis by impairing self-tolerance in mice (Yadav *et al.,* 2017). Whether the EAE establishes as chronic progressing or relapse-remitting, is suggested to be regulated by the gut microbiota (Gandy *et al.,* 2019). Risk for EAE is higher when spontaneous EAE susceptible mice are colonized with gut microbes derived from MS patients (Berer *et al.,* 2017). Similarly, colonization of germ-free mice with MS patient derived microbiota, before EAE induction, resulted more severe disease compared to colonization with microbiota from healthy controls. (Cekanaviciute *et al.,* 2017). Mice treated with MS patient derived microbiota showed reduced production of anti-inflammatory IL-10 from splenocytes (Berer *et al.,* 2017) and mesenteric lymph nodes (Cekanaviciute *et al.,* 2017). Interestingly, oral fecal administration from EAE mice during peak-disease, *i.e.* when clinical score of EAE is at its highest, could reduce EAE severity when administered to mice before EAE induction (Liu *et al.,* 2019). Transfer of gut microbiota from

albino oxford rats, that are highly resistant to EAE, could also ameliorate the disease in dark agouti rats (Stanisavljević *et al.,* 2018).

Use of antibiotics have been shown to alter EAE. Perturbation of gut microbiota with orally administered antibiotics prior to EAE induction has been shown to impair EAE development and reduce disease severity in mice. Reduced EAE severity is associated with decreased production of pro-inflammatory cytokines and, on the other hand, increased production of anti-inflammatory IL-10 (Gödel *et al.,* 2020; Ochoa-Repáraz *et al.,* 2009; Seifert *et al.,* 2018; Yokote *et al.,* 2008; Zeraati *et al.,* 2019). Opposite results are observed in rats when antibiotics are orally administered after birth (Stanisavljević *et al.,* 2018). Early life antibiotic treatment exacerbated EAE severity in rats when the disease was induced several weeks after the antibiotics were excluded from the drinking water (Stanisavljević *et al.,* 2019). Interestingly, antibiotic treatment didn't affect EAE significantly if the disease was already manifested and ongoing (Gödel *et al.,* 2020).

Ameliorating effects on EAE have also been achieved with probiotics, that are administered prior/after EAE induction or/and during the ongoing disease (Abdurasulova *et al.,* 2017; Chen. *et al.,* 2019; Consonni *et al.,* 2018; Ezendam *et al.,* 2008; He *et al.,* 2019; Kwon *et al.,* 2013; Lavasani *et al.,* 2010; Libbey *et al.,* 2018; Liu *et al.,* 2019; Maassen *et al.,* 1998; Mangalam *et al.,* 2017; Ochoa-Repáraz *et al.,* 2010; Rezende *et al.,* 2012; Salehipour *et al.,* 2017; Takata *et al.,* 2011), while no effect or increased EAE severity have also been reported (Baken *et al.,* 2006; Ezendam & van Loveren, 2008; Ezendam *et al.,* 2008; Kobayashi *et al.,* 2010; Maassen *et al.,* 1998). Results of these studies are listed in Table II.

Table II. Effect of probiotic administration on EAE. Continued on next pages.

| Used probiotic | Probiotic administration | Used model | Results | Reference |
|---|---|---|---|---|
| ***L. paracasei* DSM 13434, *L. plantarum* DSM 15312, *L. plantarum* DSM 15313** | Probiotics were administered *ad libitum* in water for 2 weeks prior EAE induction. After induction probiotic administration continued via gavage on alternate days until the end of the experiment. | Induced EAE in conventional mice. | Reduced EAE severity, reduced CNS infiltration by $CD4^+$ T cells, decreased myelin oligodendrocyte glycoprotein-reactive T-cell proliferation, decreased IFN-γ and TNF-α production, and increased IL-4, Il-10 and transforming growth factor β (TGF-β)1 expression. | Lavasani *et al.,* 2010 |

| Used probiotic | Probiotic administration | Used model | Results | Reference |
|---|---|---|---|---|
| ***B. fragilis*** | Mice were treated with antibiotics, followed by a single *B. fragilis* administration via gavage. | Induced EAE in SPF-mice. | Reduced EAE severity. Increased levels of IFN-γ, IL-10, IL-12, GATA-3 and SMAD-3. Decreased levels of IL-17 and IL-6. | Ochoa-Repáraz *et al.,* 2010 |
| ***P. acidilactici* R037** | Administration started 2 weeks before EAE induction with probiotics served *ad libitum* in water **OR** with daily gavage until the end of the experiment. | Induced EAE in SPF-mice. | Reduced EAE severity, decreased CNS infiltration of mononuclear cells, reduced Il-17 production. Increased Il-10 expression. | Takata *et al.,* 2011 |
| **Hsp65 producing *L. lactis*** | Probiotics were offered *ad libitum* in a medium. Administration was done for 4 days. Immunization was done after 10 days. | Induced EAE in conventional mice. | Reduced EAE severity and onset, reduced CNS inflammatory cell infiltration, absent white matter lesions, reduced production of IFN-γ and IL-17 and increased expression of IL-10. | Rezende *et al.,* 2012 |
| **Mixture of *L. casei, L. acidophilus, L. reuteni, B. bifidum* & *S. thermophilus*** | Probiotics were given via gavage. Administration started 3 weeks before EAE induction and continued until the end of the experiment. | Induced EAE in SPF-mice. | Reduced EAE severity and incidence, decreased lymphocyte infiltration to CNS. Reduced production of IFN-γ, TNF-α and IL-17. Increased IL-10 expression. | Kwon *et al.,* 2013 |
| ***E. faecium* L3** | Probiotics were given via gavage. Administration started 2 days after disease induction and lasted for two weeks. | Induced EAE in conventional rats. | Reduced EAE severity. Shift in gut microbiota composition. | Abdurasulova *et al.,* 2017 |
| ***P. histicola*** | Probiotics were given via gavage. Administration started 7 days after disease induction and included 7 doses on alternate days. | Induced EAE in SPF-mice. | EAE incidence reduced by 75%. Reduced CNS inflammation and demyelination. Decreased IFN-γ and IL-17 production. Increased Il-10 production. Reduced BBB and gut permeability. Decreased number of Th1 and Th17 cells in the CNS. Increased number of $CD4^+$ $CD25^+$ Tregs and tolerogenic dendritic cells. Shift in gut microbiota to pre-EAE state. | Mangalam *et al.,* 2017 |

| Used probiotic | Probiotic administration | Used model | Results | Reference |
|---|---|---|---|---|
| ***L. plantarum* A7, *B. animalis* PTCC 1631 or a mixture of these.** | Probiotics were given via gavage. Administration started during the day of disease induction and continued for 22 days. | Induced EAE in SPF-mice. | Reduced EAE severity and delayed onset. Reduction was higher with probiotic mixture. Reduced CNS infiltration and demyelination. Reduced self-reactive T-cell proliferation, and Th1/Th17 polarization. Increased number of Foxp3 Tregs. Reduced levels of IFN-γ, IL-17 and IL-6. Increased levels of IL-4, IL-10 and TGF-β. | Salehipour *et al.,* 2017 |
| ***L. crispatus* LMG P-23257 + *L. rhamnosus* ATCC 53103 / *B. animalis* subsp. *Lactis* BB12 + *B. animalis* subsp. *Lactis* LMG S-28195** | Probiotics were given via gavage. Administration started one week before disease induction (5 doses) and continued for two weeks after induction (10 doses). | Induced EAE in conventional rats. | Reduced EAE severity. Decreased CNS infiltration by T cells. Decreased astrocytosis. Reduced levels of IFN-γ, IL-17 and TNF-α (bifidobacterial treated rats). Increased levels of IL-6 and TGF-β in spinal cord. | Consonni *et al.,* 2018 |
| ***L. paracasei*** | Probiotics were given via gavage. Administration started 14 days prior EAE induction and continued for 19 days. | Induced EAE in conventional mice. | Reduced EAE severity, incidence and weight loss. | Libbey *et al.,* 2018 |
| ***C. butyricum*** | Probiotics were given via gavage. Administration was done 3 weeks prior induction. | Induced EAE in SPF-mice. | Reduced EAE severity. Decreased CNS lymphocyte infiltration, and demyelination. Shifted gut microbiota composition. Decreased number of Th17 and Th1 cells. Increased proportion of Foxp3 Tregs. | Chen *et al.,* 2019 |
| ***A. muciniphila*** | Probiotics were given via gavage. Administration started during established EAE and lasted for 7 days. | Induced EAE in SPF-mice. | Reduced EAE severity. Reduced demyelination and axonal loss. Increased number of myelin oligodendrocyte glycoprotein-specific Foxp3+ Tregs and total Tregs. | Liu *et al.,* 2019 |
| ***L. reuteri* DSM 17938** | Probiotics were given via gavage. Administration started during the day of disease induction and continued for 20 days. | Induced EAE in SPF-mice. | Delayed onset and reduced EAE severity. Reduced CNS infiltration by inflammatory cells. Decreased number of $CD3^+$ T cells, Th17 cells and Th1 cells. Reduced levels of IFN-γ and IL-17. | He *et al.,* 2019 |

| Used probiotic | Probiotic administration | Used model | Results | Reference |
|---|---|---|---|---|
| ***L. reuteri* ML1, *L.casei* 393, *L.plantarum* NCIB 8826, *L. murines* CNRZ** | Probiotics were given via gavage. 5 doses on alternate days prior EAE induction. | Induced EAE in conventional mice. | *L. reuteri* increased EAE severity, *L.casei* and *L.plantarum* had no or little effect on EAE severity. *L. murines* reduced EAE severity. | Maassen *et al.,* 1998 |
| ***L. casei* Shirota** | Probiotics were given via gavage. Administration started 8 days prior EAE induction and continued for 28 days until the end of the experiment. | Induced EAE in conventional rats. | Increased EAE severity. | Baken *et al.,* 2006 |
| ***B. animalis*** | Probiotics were given via gavage. Administration started 5 weeks before induction and continued for 27 days until the end of the experiment. | Induced EAE in SPF-rats. | Shortened duration of clinical symptoms. Slight but unsignificant reduction in EAE severity. | Ezendam *et al.,* 2008 |
| ***L. casei* Shirota** | Probiotics were given via gavage. Administration started 5 weeks before induction and continued for 27 days until the end of the experiment. | Induced EAE in conventional rats. | Increased disease duration in female rats. No significant changes in EAE severity. | Ezendam & van Loveren, 2008 |
| ***L. casei* Shirota, B. *breve* Yakult** | Probiotics were given via gavage. Administration started one week before induction and continued 28 days until the end of the experiment. **OR** Administration started 5 weeks before induction and continued for 4 weeks until the end of the experiment. | Induced EAE in conventional rats | Slight but unsignificant reduction in EAE severity with *L. casei* Shirota. *C. breve* Yakult had no effect regarding EAE. | Kobayashi *et al.,* 2010 |

Majority of the listed studies report that probiotic administration ameliorates EAE and is associated with reduced inflammatory (Th-17/Th-1 cells, IFN-γ, TNF-α and IL-17) and increased regulatory response (Tregs, IL-10). However, mechanisms how gut microbiota is able to regulate EAE remains largely unknown, while several factors such as capsular

polysaccharide A (PSA) of *Bacteroides fragilis* (Ochoa-Repáraz *et al.,* 2010; Wang *et al.,* 2014), SCFAs (Melbye *et al.,* 2019), and tryptophan metabolites (Rothhammer *et al.,* 2018; Sonner *et al.,* 2019) have been suggested to play a role in EAE regulation.

Interestingly, one EAE ameliorating mechanism is suggested to involve host derived fecal micro-RNA miR-30d, that is enriched in EAE mice as well as untreated MS patients. Fecal transfer from EAE mice during peak-disease ameliorated the disease in EAE induced mice. Similar results were achieved with feces-derived RNA and synthetic miR-30d. MiR-30d, upregulated by colon dendritic cells (DCs), was found to expanse *Akkermansia muciniphila* growth by inducing β-galactosidase production. *A. muciniphila* was shown to affect cytokine release of DCs by inducing the expression of transforming growth factor β (TGF-β) and decreasing the expression IL-6 and IL-1β. This in turn, promoted naive $CD4^+$ T cell differentiation to anti-inflammatory $Foxp3^+$ Tregs (Liu *et al.,* 2019). Additionally, increased amount of *A. muciniphila* have also been reported among MS patients (Berer *et al.,* 2017; Cekanaviciute *et al.,* 2017; Jangi *et al.,* 2016; Tremlett *et al.,* 2016).

Probiotics administration (Abdurasulova *et al.,* 2017; Chen *et al.,* 2019; He *et al.,* 2019) and changes with diet (Cignarella *et al.,* 2018; Libbey *et al.,* 2018) are associated with both changes in gut microbiota composition and EAE amelioration. Administration of *Lactobacillus reuteri* to EAE mice increased the proportion of *Bacteroides* and decreased the abundance of *Proteobacteria* and *Deferribacteres*. Alterations in gut microbiota composition were also associated with EAE severity scores. 16 genera, such as *Clostridium*, *Anaeroplasma*, *Ruminococcus,* and *Rikenellaceae,* were associated with more severe disease whereas 17 genera, including *Bifidobacterium*, *Prevotella*, *Lactobacillus*, and S24-7 were involved with reduced EAE severity (He *et al.,* 2019). Administration of *Clostridium butyricum* was shown to ameliorate EAE and affect the abundancy and diversity of the microbiota. *C. butyricum* increased *Bacteroides*/*Firmicutes* ratio and the amount of *Prevotella*. Decrease in the amount of *Desulfovibroneceae* and *Ruminococcus* was also observed (Chen. *et al.,* 2019). Administration of *Enterococcus faecium* also reduced EAE severity and was associated with reduction in *Bifidobacterium*, *E.coli*, and *Bacteroides fragilis,* and with increased levels of *Faecalibacterium prausnitzii* (Abdurasulova *et al.,* 2017). Studies regarding changes in diet showed that increased abundance of *Lactobacillaceae* was observed together with reduced EAE severity in mice, that received Teklad sterilizable rodent diet compared to mice receiving Teklad global soy protein-free extruded rodent diet (Libbey *et al.,* 2018). Intermittent fasting

was also shown to reduce EAE severity and increase the abundance of *Bacteroidaceae, Lactobacillaceae,* and *Prevotellaceae* (Cignarella *et al.,* 2018).

### 2.5 Gut microbiota in multiple sclerosis

Studies including MS patients, regarding the role of gut microbiota, have been limited so far. Alterations in gut microbiota composition in MS patients compared to healthy controls have been reported (Chen *et al.,* 2016; Jangi *et al.,* 2016; Miyake *et al.,* 2015). Preliminary results also indicate that the gut microbiota composition affects the relapse risk in pediatric RRMS patients (Tremlett *et al.,* 2016). Diversity of the gut microbiota seems to be similar between MS patients and healthy controls (Mirza *et al.,* 2020; Reynders *et al.,* 2020). However, there is microbial heterogeneity between different MS subtypes and differences in microbial taxa (Reynders *et al.,* 2020). MS patients seem to have lover amount of *Prevotella, Bacteroides coprophilus,* and *Bacteroides fragilis,* and higher amounts of archaea *Methanobrevibacter*, *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* (Mirza *et al.,* 2020). One case-study reported the presence of epsilon toxin producing type B *Clostridium perfringens* in MS patients. Epsilon toxin can enter host circulation and travel to the brain where the toxin initiates symptoms typical to MS disease (Rumah *et al.,* 2013). Few studies are published so far regarding the effect of probiotic intake in MS patients. 12 week long intake of probiotic mixture containing *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum* and *Lactobacillus fermentum* had favorable effect on disability score and mental health of MS patients (Kouchaki *et al.,* 2017), and increased expression of IL-8 and TNF-α (Tamtaji *et al.,* 2017). Anti-inflammatory response and reduced expression of MS related risk alleles were observed with MS patients who were given a probiotic mixture containing *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* for 2 months (Tankou *et al.,* 2018).

### 2.6 Microbial extracellular vesicles – multifunctional voyagers

Eukaryotes and prokaryotes alike, including the gut microbiota, release extracellular vesicles (EVs) (Fig. 4.) into their environment. EVs are a heterogenous group of spherical bilayered structures that vary in size and origin (Molina-Tijeras *et al.,* 2019). Role of microbial EVs is suggested to be involved in bacterial survival, nutrient sensing, transportation, eradication of competing bacteria, virulence against the host, and host immune modulation (Macia *et al.,* 2020). Interestingly, bacterial EVs are also proposed to be one mechanism how gut microbiota is able to communicate with the host organism (Fábrega *et al.,* 2016; Kimura *et al.,* 2018; Molina-Tijeras *et al.,* 2019).

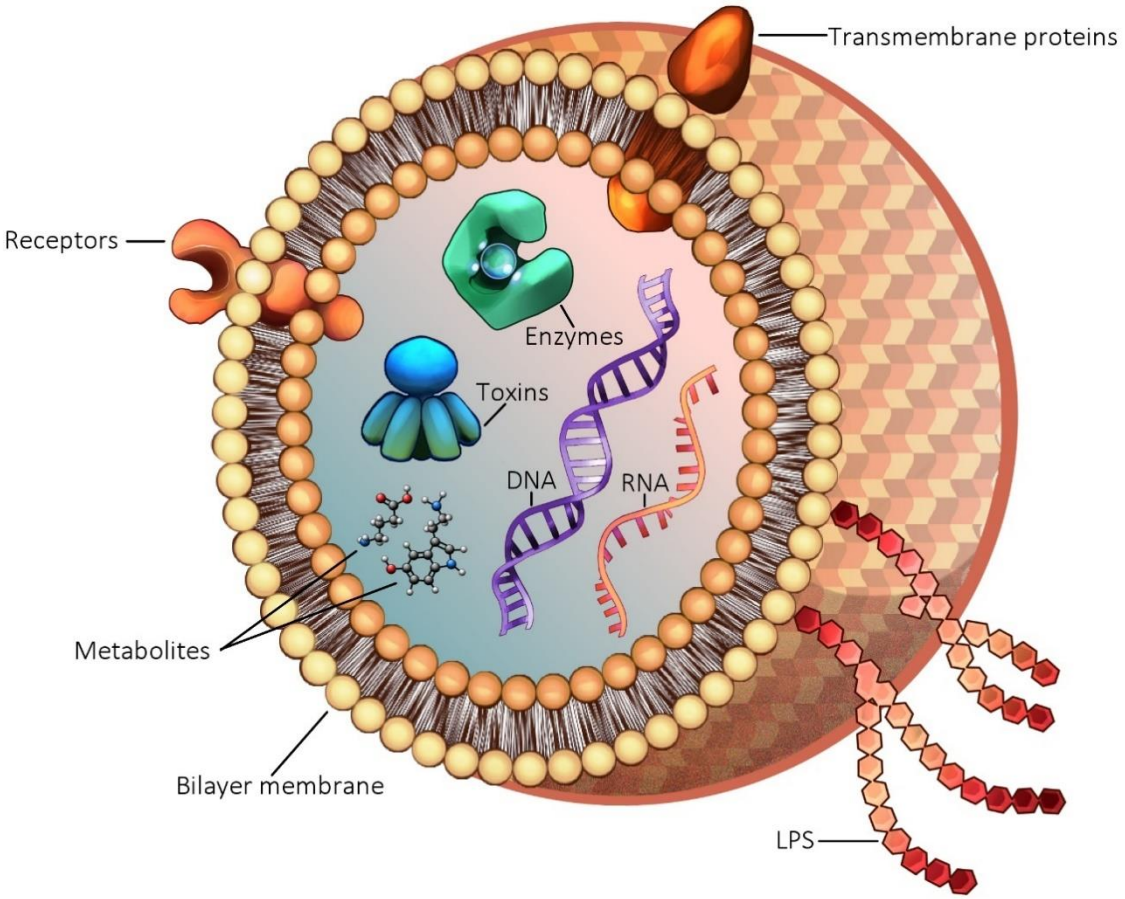


Figure 4. Structure and components of bacterial EV. LPS: lipopolysaccharide (only in gram-negative bacteria EVs).

Microbial EVs contain a cargo that resembles contents of the parental bacteria (Dorward *et al.,* 1989; Kadurugamuwa & Beveridge, 1995; Kolling & Matthews, 1999; Molina-Tijeras *et al.,* 2019; Patrick *et al.,* 1996), though bacteria are also able to selectively sort cargo into EVs (Haurat *et al.,* 2011). Biomolecules present in EVs include cytosolic and membrane proteins, lipids, polysaccharides, LPS, toxins, and nucleic acids, such as linear and circular DNA, and RNA (Dorward *et al.,* 1989; Kadurugamuwa & Beveridge, 1995; Kolling & Matthews, 1999; Molina-Tijeras *et al.,* 2019; Patrick *et al.,* 1996).

EVs released by gram-negative bacteria are known as outer membrane vesicles (OMV), if EVs bled from the outer membrane of the bacterial cells. Other type of EV released by gram-negative bacteria is called outer-inner membrane vesicle (OIMV), which includes both inner membrane and outer membrane of the parental cell. EVs released by gram-positive bacteria are called bacterial membrane vesicles (BMV), which bled through the thick peptidoglycan wall (Liu *et al.,* 2018; Macia *et al.,* 2020; Toyofuku *et al.,* 2019). Size of microbial EVs varies, but is usually between 25-250 nm (Caruana & Walper, 2020).

Production of OMVs is a common feature among gram-negative bacteria (Kulp & Kuehn, 2010). However, biogenesis of OMVs is still largely unknown but is thought to involve several mechanisms including either blebbing or cell lysis (Fig. 5.) (Pathirana & Kaparakis-Liaskos, 2016; Toyofuku *et al.,* 2019). One OMV blebbing mechanism is based on accumulation of phospholipids to the outer leaflet of outer membrane. This is due to reduced or deleted expression of genes *vacJ* and/or *yrb*, that express ATP-binding cassette (ABC) transport system (Roier *et al.,* 2016).

Outer membrane of gram-negative bacteria is asymmetric, having LPS at the outer leaflet and phospholipids at the inner leaflet. One function of ABC transporters is to traffic phospholipids out from the outer leaflet to prevent phospholipid accumulation (Malinverni & Silhavy, 2009). Iron limitation and possibly other environmental factors could downregulate *vacJ* and *yrb*, resulting increased accumulation of phospholipids to outer leaflet and induced OMV production. *VacJ* and *yrb* gene deletion increases OMV production of distinct bacteria species, leaving a suggestion that such OMV production mechanism is potentially conserved among gram-negative bacteria (Roier *et al.,* 2016).

Other suggested OMV production mechanism, known as bilayer-couple model, is based on the function of *Pseudomonas* quinolone signal (PQS). PQS is a hydrophobic molecule (Pesci *et al.,* 1999) that functions as a signal molecule for bacterial communication and is required for vesicle formation in *Pseudomonas aeruginosa* (Mashburn & Whiteley, 2005). Similarly with previous model, it is suggested that accumulation of PQS to outer leaflet and interaction with LPS induces OMV formation. PQS is suggested to bind LPS lipid A, which results low-rate asymmetric flip-flop action, leading to outer leaflet expansion and OMV formation (Schertzer & Whiteley, 2012).

Another OMV secretion mechanism involves turgor pressure against the outer membrane caused by the accumulation of peptidoglycan, misfolded proteins or LPS into bacterial periplasmic space. Turgor pressure could bend the outer membrane, leading to OMV production (Haurat *et al.,* 2015; McBroom & Kuehn, 2007). OMV production can be also altered by changes in the outer membrane structure, presumably in the structure and metabolism of peptidoglycans, and their interaction with lipoproteins (Schwechheimer *et al.,* 2014).

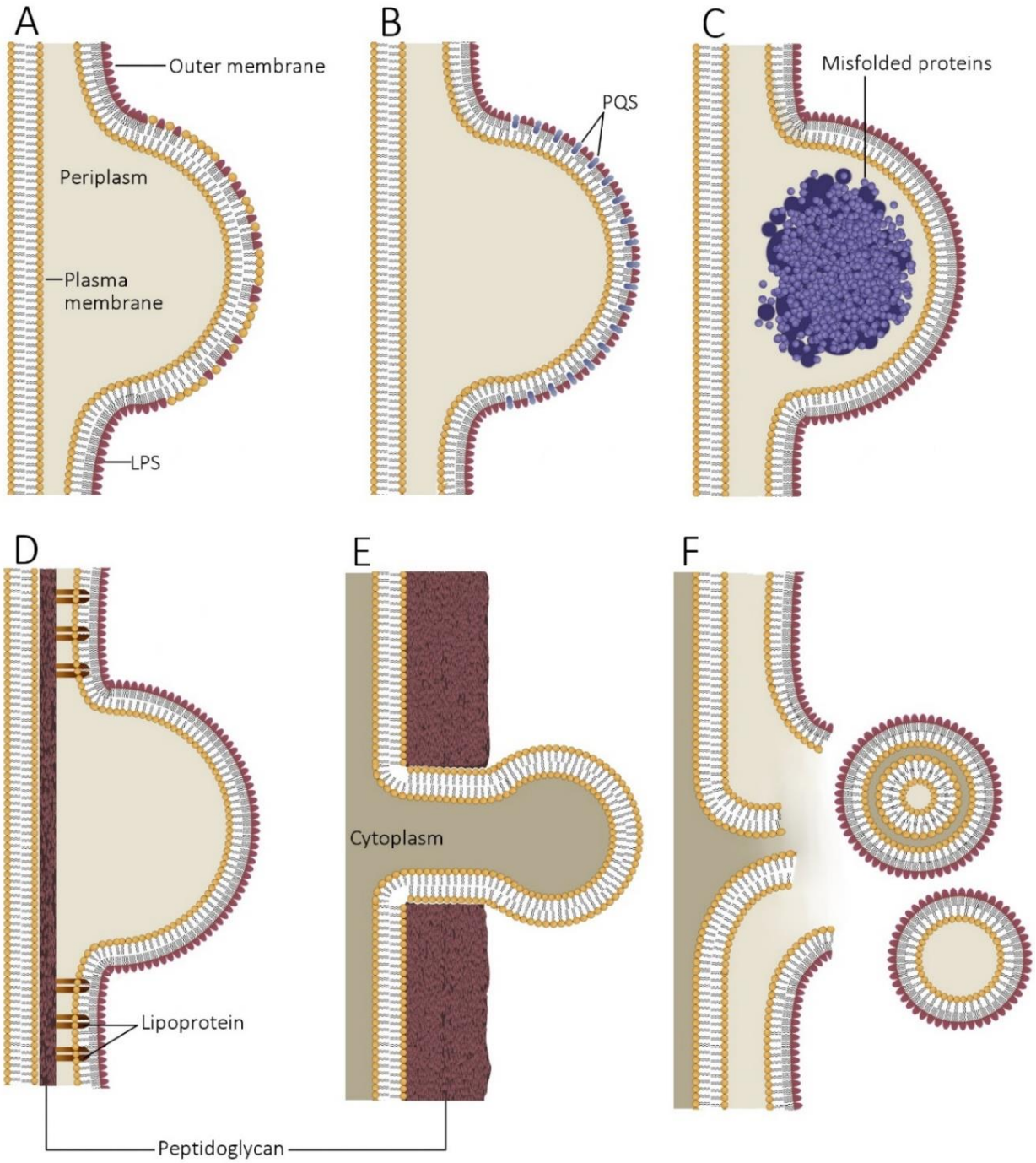


Figure 5. Proposed EV biogenesis mechanisms in gram-negative (**A**-**D**, **F**) and gram-positive bacteria (**E**). **A**: Excess accumulation of phospholipids to the outer leaflet of outer membrane of gram-negative bacteria induces EV formation. **B**: Accumulation of *Pseudomonas* quinolone signal (PQS) to outer leaflet of outer membrane and interaction with LPS induces EV formation in *P. aeruginosa*. **C**: Misfolded proteins accumulate into the periplasmic space of gram-negative bacteria, inflicting turgor pressure against outer membrane, which leads to EV formation. **D**: Modulation of peptidoglycan-lipoprotein-outer membrane structures induce EV formation. **E**: Opening of thick peptidoglycan wall in gram-positive bacteria causes plasma membrane to bleb out from the cell, leading to EV formation. **F**: EVs form as a result of explosive cell lysis or "bubbling" cell death. LPS: lipopolysaccharide. Peptidoglycan is omitted from **A**-**C** and **F** for clarity.

Mechanism how gram-positive bacteria release BMVs despite the thick peptidoglycan cell wall have been suggested to involve degradation of peptidoglycans that allows the release of BMVs (Liu *et al.,* 2018). It was demonstrated that the expression of prophage-encoded endolysin in *Bacillus subtilis* caused holes in the cell wall, which allowed plasma membrane to bleb out from the bacterial cell. BMV production also induced vesiculation from adjacent bacterial cells. However, BMV production was shown to be lethal for the bacterial cells (Toyofuku *et al.,* 2017). Prophage-independent BMV production mechanism is suggested to involve phenol-soluble modulins (a family of surfactant-like proteins) and peptidoglycan cross-linking that resulted BMV production from *Staphylococcus aureus* (Wang *et al.,* 2018).

EVs are resistant to proteases (Kesty & Kuehn, 2004). Therefore, they can transport otherwise protease-susceptible molecules to further destinations than these cargo molecules would travel without protecting membrane. Packing cargo in EVs could ensure that proteins are delivered in high concentrations to distant targets where high effector concentration is required to achieve an effect (Kulp & Kuehn, 2010). Indeed, bacterial EVs have been shown to recognize different bacterial cells and harbor specificity for their targets (Toyofuku *et al.,* 2017).

In bacteria-bacteria interaction, EVs can contain signal molecules, which are used to communicate and coordinate social activities between bacterial cells (Mashburn & Whiteley, 2005), known as quorum sensing (Parsek & Greenberg, 2000). Bacterial EVs can fuse to other bacteria and release their cargo, including proteins (Vasilyeva *et al.,* 2008) and genetic material into recipient cells (Kahn *et al.,* 1983; Kolling & Matthews, 1999; Renelli *et al.,* 2004; Yaron *et al.,* 2000). EV fusion can provide a repertory of foreign antigens, that recipient bacteria can represent on their outer membrane (Kadurugamuwa & Beveridge, 1996; Kadurugamuwa & Beveridge, 1999). EVs have shown to traffic phage receptors to otherwise resistant bacterial cells and make them susceptible for phage infection (Tzipilevich *et al.,* 2017). Bacteria can pack misfolded proteins and toxic compounds and remove them via, vesiculation that increases bacterial survival (McBroom & Kuehn, 2007). EVs are also capable to bind (Schooling & Beveridge, 2006) and degrade (Ciofu *et al.,* 2000) antibiotics to prevent antimicrobial action.

### 2.7 Pathogenic and beneficial properties of microbial extracellular vesicles

Microbial EVs are thought to be potential pathogenic factors due to their capability to carry toxins (Grenier & Mayrand, 1987; Kolling & Matthews, 1999) and other virulence factors (Ellis

& Kuehn, 2010). Studies regarding EVs of potentially harmful bacteria have shown that EVs contribute to pathogenic actions. *Staphylococcus aureus*, a common opportunistic pathogen, releases EVs that contain several toxins, such as α-hemolysin, δ-hemolysin, γ-hemolysin, leukotoxin D, exfoliative toxin C, and exfoliative toxin A. (Gurung *et al.,* 2011; Jeon *et al.,* 2016; Thay *et al.,* 2013). *S. aureus* EVs are cytotoxic (Gurung *et al.,* 2011; Thay *et al.,* 2013) and promote inflammation when inhaled (Kim *et al.,* 2012). *Escherichia coli* EVs are capable to lyse red blood cells (Balsalobre *et al.,* 2006) and induce systemic inflammatory response syndrome or lethal sepsis (Park *et al.,* 2010). EVs from certain strains of *E. coli* contain heat-labile enterotoxin (Wai *et al.,* 1995), that is known to cause diarrhea (Spangler. 1992). Enterotoxigenic *E. coli* EVs were able to target and release enterotoxin into host adrenal gland and intestinal epithelial cells, whereas EVs from non-pathogenic strain didn't show cell specificity (Kesty *et al.,* 2004). EVs from different *E .coli* strains cause distinct immunological responses, meaning that host responses to microbial EVs are strain specific (Diaz-Garrido *et al.,* 2019).

Interestingly, EVs can promote the growth of pathogenic bacteria. Such is case with *Mycobacterium tuberculosis*, which was shown to proliferate faster in lungs and spleens of mice that were injected with *M. tuberculosis* EVs beforehand (Prados-Rosales *et al.,* 2011). *M. tuberculosis* EVs induced T cell anergy, which was suggested to be caused by EV delivered lipoarabinomannan, a phagosome maturation inhibitor present in mycobacterial membrane (Athman *et al.,* 2017).

As with beneficial bacteria, EVs can also have favorable properties. *Lactobacillus casei* EVs were found to contain P40 and P75 hydrolases (Rubio *et al.,* 2017), proteins that have been associated with intestinal cell proliferation and survival (Yan *et al.,* 2007). EVs of *Bifidobacterium longum* were able to alleviate food allergy symptoms in mice by inducing mast cell apoptosis, and reduce the occurrence of diarrhea in dose-dependent manner (Kim *et al.,* 2016). EVs from *Lactobacillus rhamnosus* GG were reported to have anti-proliferative effect on liver cancer cells (Behzadi *et al.,* 2017), and *Lactobacillus plantarum* WCFS1 EVs were able to increase *Caenorhabditis elegans* survival against vancomycin-resistant *Enterococcus faecium* infection (Li *et al.,* 2017).

EVs derived from vaginal *Lactobacillus* were reported to protect human T cells, cervico-vaginal and tonsillar tissues from HIV-1 replication and inhibit viral attachment as well as viral entry to susceptible cells (Ñahui Palomino *et al.,* 2019). Previously described

*Bacteroides fragilis,* bacteria, that produce PSA with anti-inflammatory properties, have shown to release PSA containing EVs. *B. fragilis* EVs were able to protect mice from experimental colitis by suppressing TNF-α production and Th-17 development. PSA is recognized by DCs via TLR2 dependent manner, thus enhancing Tregs and production of anti-inflammatory cytokines (Shen *et al.,* 2012).

*Akkermansia muciniphila* EVs were also shown to reduce the severity of experimental colitis in mice (Kang *et al.,* 2013). *A. muciniphila* EVs, when administered to mice with high-fat diet, decreased the permeability of gut tight junctions, reduced weight gain and improved glucose tolerance (Chelakkot *et al.,* 2018). Beneficial impact on reduced weight gain of *A. muciniphila* EVs was shown to be greater compared to actual bacterial cells (Ashrafian *et al.,* 2019). Intestinal barrier reinforcing function (Alvarez *et al.,* 2016) and ameliorating effect in experimental colitis (Fábrega *et al.,* 2017) have also been observed with *E. coli* Nissle 1917 EVs.

Interesting about bacterial EVs in the gut is, that EVs have been shown to cross intestinal epithelium, enter host circulation, locate to organs (Jones *et al.,* 2020), and cross BBB to CNS (Wei *et al.,* 2020). Gut microbiota are suggested to contribute to brain development, behavior, and neurodegenerative diseases (Cryan & Dinan, 2012; Forsythe *et al.,* 2012; Foster *et al.,* 2016; Sarkar & Banerjee, 2019) but the mechanisms behind these actions are largely unknown. Few recent studies have reported that microbial EVs potentially affect brain function and behavior. One study reported that administration of gut microbiota derived EVs from Alzheimer's disease patients induced neuroinflammation, tau protein hyperphosphorylation, and cognitive impairment in mice (Wei *et al.,* 2020). Another study showed that EVs of *L. plantarum* affected the expression of neurotropic factors in mouse hippocampus and had anti-depressant-like effect in stress-induced depression (Choi *et al.,* 2019).

# II EXPERIMENTAL PART

## 3. Aim of the project

Aim of the project was to determine, whether gut-microbiota-derived EVs from fecal samples are different between MS patients and healthy controls. Our hypothesis is that EVs produced by the gut microbiota are a major host-modulating factor in health and disease, including MS. According to our knowledge, no literature has been published regarding the role of gut microbial EVs in MS.

## 4. Materials and Methods

### 4.1 Samples

Fecal samples from sixteen (16) recently diagnosed MS patients (< 5 years from diagnosis) and 18 healthy controls were kindly provided by Prof. Anne Remes. Both sample groups represented mixed sex and age. Two in-house healthy control fecal samples were used to verify EV isolation protocol suitability. All samples were stored at -80 C° prior use. 1x modified Dulbecco's phosphate buffered saline (DPBS) was used as a negative control.

### 4.2 Isolation of extracellular vesicles

Experimental workflow is summarized in Fig. 6.

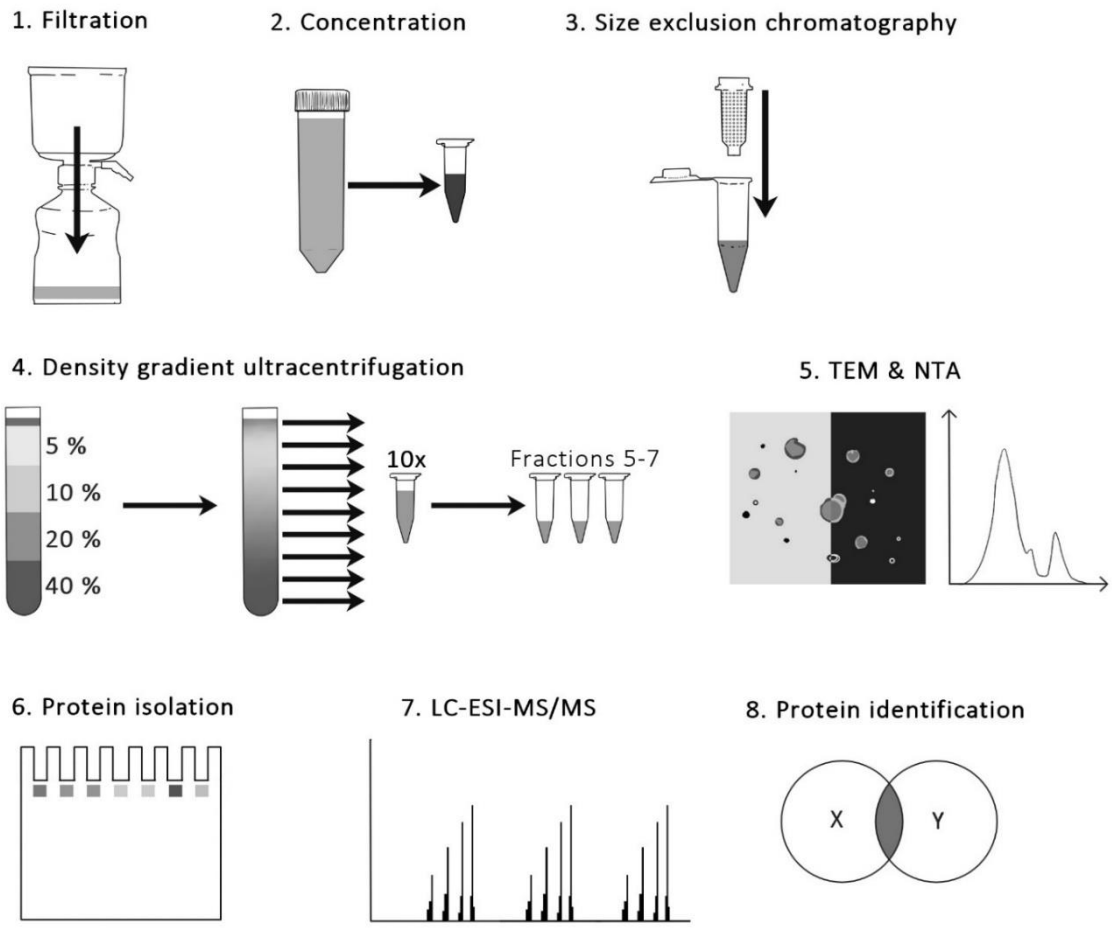


Figure 6. Experimental workflow. EVs were isolated and purified from fecal material (steps 1 - 4). Isolated EVs were qualified with transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA) (step 5). Proteins were isolated from EVs (step 6). Isolated proteins were analyzed by using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS), PEAKS bioinformatics software, and in-house developed bioinformatics tools (steps 7 - 8).

### *4.2.1 Filtration*

100-1000 mg of thawed fecal material was suspended in sterile-filtered 1x DPBS (Sigma-Aldrich). Suspended samples were centrifuged at 14 000 g for 30 min at +4 C° with a fixed angle rotor. Supernatant was collected and centrifuged again. After centrifugation, supernatant was filtered with 40 µm nylon filter (Falcon Cell strainer) and sterile-filtered with 0,45 µm PES-filter (Biofil 1000ml Vacuum bottle filter). Filtered samples were stored at +4 C° Same 0,45 µm PES-filters were used to prepare all other sterile-filtered solutions.

### *4.2.2 Concentration*

Filtered samples were concentrated by using Centricon® Plus-70 PL-100 centrifugal filter devices (Merck Millipore) according to manufacturer's manual. Due to lack of suitable rotor adapters, Centricons were wrapped with bubble wrap to fit them into rotor adapters. Centricons

were washed with sterile-filtered 1x DPBS by centrifugation with a swinging bucket rotor at 3500 g for 5 min at +4 C° and liquid left in filters was removed by centrifugation at 1000 g for 2 min at +4 C°. Samples were concentrated by centrifugation at 3500 g for 45 min at +4 C°. Concentrated samples were retrieved by centrifugation at 1000 g for 2 min at +4 C°. Samples were stored at +4 C°.

### *4.2.3 Size exclusion chromatography*

EVs (30-300 nm in diameter) were isolated from concentrated samples by using Exo-Spin™ Mini-Columns (Cell guidance systems). Storage liquid in the column was removed by spinning at 50 g for 10 sec with a swinging bucket rotor. The column was washed with sterile-filtered 1x DPBS by spinning at 50 g for 10 sec. Sample was added to the column and spinned at 50 g for 1 min. EVs were eluted from the column by adding sterile-filtered 1 x DPBS and by spinning at 50 g for 1 min. Isolated EVs were stored at 4 °C.

### *4.2.4 Density gradient ultracentrifugation*

EVs were purified by using OptiPrep™ density gradient ultracentrifugation. Gradient solutions were done by using sterile-filtered homogenization medium (HM; 0,25M Sucrose, 10mM Tris-HCl, 1mM EDTA, pH 7,4) and sterile-filtered working solution buffer (WSB; 0.25M Sucrose, 60mM Tris-HCl, 6mM EDTA, pH 7,4). Working solution (WS) was done by mixing Optiprep™ gradient media (60% w/v iodixanol in water) and WSB in 1:5 ratio. 40%, 20%, 10%, and 5% OptiPrep™ gradient solutions were freshly prepared by mixing WS and HM. Density gradient was prepared by adjusting 40 %, 20 %, 10 %, and 5% solutions, respectively, into ultracentrifugation tubes (14 x 89 mm Ultra-Clear, Beckman Coulter). Sample was put on the top of the gradient and centrifuged at 100 000 g ≥ 15 hours at 4C° with a swinging bucket rotor. After centrifugation, gradient-sample mixture was divided into 10 fractions. Sample fractions were stored at +4 °C.

### *4.2.5 Washing of extracellular vesicles*

Frequently, fractions 5-7 included purest and highest amount of EVs. EVs appeared in fractions 4 and 8 as well. However, fraction 4 was usually empty and fraction 8 had often excess amount of impurities, as analyzed by TEM. To save time, fractions 5-7 were usually selected for washing. Fractions were mixed with sterile-filtered 1x DPBS and washed by ultracentrifugation at 100 000 g for 2,5 hours at 4 °C. 14 x 89 mm Polypropylene Centrifugation Tubes (Beckman Coulter) were used for sample washing. Supernatant was removed and the pellet was suspended in sterile-filtered 1x DPBS. Washed samples were stored at 4 °C.

## 4.3 Qualification of extracellular vesicles

### *4.3.1 Negative staining*

Negative staining was performed by the Biocenter Oulu electron microscopy core facility service. Washed EV fractions were negatively stained. Drop of EV sample was placed on carbon coated glow-discharged copper grid (400 mesh). Sample was incubated on the grid for 20 min. Grids were washed twice with a drop of PBS for 1 min. Samples were fixed with 1 % glutaraldehyde in PBS for 5 min. Grids were washed 8x1 min in a drop of water, stained with neutral 2 % uranyl acetate (UA), washed again with water, and coated with a droplet of 2 % methylcellulose-UA for 10 min. Grids were removed from the droplet and excess fluid was blotted with Whatman no. 1 filter paper. Grids were dried ≥10 min and stored in a grid storage box at RT.

### *4.3.2 Transmission electron microscopy*

Transmission electron microscopy (TEM) was used to check the purity and presence of EVs in samples by using Tecnai G2 Spirit microscope. Since EVs tend to accumulate on the edge of the grid due to one-sided blotting, the grids were imaged in a five-point manner, starting from the middle and moving through four corners in square-shaped form. Images were taken with 23 000x or 18 500x magnification.

### *4.3.3 Fraction combination*

Fractions of the same sample, which were considered to have pure EVs, were combined. Fractions selected for combination had clearly less amount of non-EV material (flagella and fibrils) compared to the amount of EVs present in the sample. Combined fractions were stored at 4 °C.

### *4.3.4 Nanoparticle tracking analysis*

EV concentration and size distribution was determined with nanoparticle tracking analysis (NTA). Used NTA instrument was NanoSight NS300 (Malvern) coupled with NanoSight NTA Software. NS300 is a laser-based light scattering system that illuminates and tracks nanosized particles (10-2000 nm in diameter). According to manufacturer's manual, accuracy of NS300 is at highest when measured particle concentrations are between $10^7$-$10^9$ particles/ml. Therefore, EV sample aliquots were diluted in sterile-filtered 1x DPBS to ensure good reliability. Used mixture ratio was 1:24. EVs from one sample were measured with 4 consecutive 1 min long imaging sessions and results from each measurement were combined. Significancy of the results was tested with Student's T-test.

## 4.4 Protein analysis of extracellular vesicles

### *4.4.1 Methanol precipitation of extracellular proteins*

Extracellular proteins were precipitated by mixing sterile water, methanol, and chloroform to washed samples. Samples were vortexed and centrifuged at 14 000 g for 1 min by using a fixed angle rotor. Methanol-water-mix and chloroform formed a layer where precipitated proteins located. Methanol-water mix was removed from the top of the interface. Protein precipitate was washed by adding more methanol. Samples were vortexed and centrifuged at 16 000 g for 5 min to pellet precipitated proteins. Supernatant was removed and pellets were dried until no liquid was seen in the tubes. Samples were suspended in 1x Laemly loading buffer, boiled at 95 °C for 5 min, and cooled.

### *4.4.2 Protein loading*

Precipitated protein samples were loaded on pre-casted 12 % separating gels (Mini-Protean TGX, Bio-Rad) and run at 10-15 min at 110 V until the Laemly loading buffer had moved ~1 cm. Gel run was performed to store precipitated proteins in the gel. The gel was fixed in 50 % ethanol and 10 % acetic acid for 30 min in a shaker at RT and rinsed with sterile water. The gel was stained with 1x Sypro Ruby protein stain in a shaker for overnight at RT. The gel was de-stained with 5 % acetic acid for 5 min in a shaker. ~1 x 1cm gel pieces, that contained sample, were cut under the UV light with a scalpel. Gel pieces were dried with absolute ethanol by 2 x 5 min incubation at RT until the gel pieces became white and hard. Ethanol was removed and dried gels were stored at -20 °C.

### *4.4.3 Sample submission*

Dried gels were sent at ambient temperature to Turku Bioscience Proteomics Facility for protein identification analysis.

### *4.4.4 Reduction*

Samples were processed at Turku Bioscience Proteomics Facility according to their standard protocols. Dried gels were rehydrated and washed 3 x 10 min with water. Proteins present in gels were reduced with 20 mM 1,4-dithiotreitol for 30 min. Gels were dehydrated with 100 % acetonitrile (ACN).

### *4.4.5 Alkylation*

Gels were alkylated with 55mM iodoacetamide for 20 min in dark at RT and washed twice with 100 mM ammonium bicarbonate. Gels were dehydrated with ACN and dried with vacuum centrifugation for 5 min.

#### *4.4.6 Trypsin digestion*

Gels were digested with 0,02 µg/µl trypsin solution for 20 min. Gels were covered with 10% ammonium bicarbonate solution and incubated overnight at 37 °C.

#### *4.4.7 Extraction of peptides*

100% ACN was added to samples. Samples were vortexed and incubated for 15 min at 37 °C. Supernatant was collected. Extraction was repeated with 50 % ACN/5 % formic acid solution. Solution was dried with vacuum centrifugation. Dried samples were stored at -20 °C.

#### *4.4.8 Liquid chromatography-electrospray ionization-tandem mass spectrometry*

For mass spectrometry, samples were suspended in formic acid. Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) analysis was done with nanoflow HPLC system (Easy-nLC1200, Thermo Fisher Scientific) and coupled with Q Exactive HF mass spectrometer (Thermo Fisher Scientific). Mass spectrometer was equipped with a nano-electrospray ionization source. Samples were loaded on a trapping column and separated on a 15 cm C18 column (75µm x 15 cm, ReproSil-Pur 5µm 200 Å C18-AQ, Dr. Maisch HPLC GmbH). The mobile phase included water with 0,1 % formic acid (solvent A) and ACN/water (80:20 v/v) with 0,1 % formic acid (solvent B). Peptides were eluted with linear gradient (8 % to 43 %) of solution B for 10 min. Data was received by using Thermo Xcalibur 4.1 software (Thermo Fisher Scientific).

#### *4.4.9 PEAKS analysis*

Raw proteomics data from LC-ESI-MS/MS was provided by Turku Bioscience Proteomics Facility. Raw data was processed with PEAKS software (Bioinformatics Solutions Inc.). PEAKS software was used to identify peptides/proteins of a protein mixture based on database and spectral library searching and *de novo* sequencing.

#### *4.4.10 Protein analysis*

Protein content analysis of MS patient and healthy control EVs was performed with in-house developed bioinformatic algorithm based on R tool.

## 5. Results

### 5.1 Sufficient isolation of extracellular vesicles was possible with ≤1000 mg of fecal material

In the original protocol 4000 mg of fecal material was used for isolation. However, provided samples were smaller ~ 400 mg. Therefore, suitability of EV isolation protocol was first tested

with variable amount of fecal starting material from reserve control samples. First EV isolation test was done with 100, 200, 400, 600, 800, and 1000 mg of fecal material from the same fecal sample. First isolation round included EV isolation, EV quantification, methanol precipitation of extracellular proteins and protein loading to gel. EV isolation test was repeated with another control fecal sample using 100, 200, 400, and 600 mg of fecal starting material. Methanol precipitation of extracellular proteins and protein loading to gel were excluded from the second test since point of interest was to verify the presence of EVs.

EVs were isolated from both test samples and fractions 5-8 were selected for washing. Fractions were negatively stained according to the described protocol and imaged with TEM. TEM showed that EVs were present in all tested samples (Fig. 7. and Fig. 8.). Observed EVs varied by their structure and size (Fig. 9.). Part of the EVs showed wrinkled, round or tube-like structure. Surface of the EVs appeared smooth, granular or fibrous. EVs sometimes formed aggregates where multiple EVs are stuck together. EVs that resembled multilamellar bodies were also present in samples. Flagella-like structures were common in fraction 8. Therefore, fraction 8 from all samples were excluded from the NTA analysis.

Fractions 5-7 per sample were combined. EV concentration of the samples were measured with NTA (Fig. 10.) and compared to results from 4000 mg of fecal material (Check attachments, Supplement Fig. 1). Magnitude of EV concentration per ml increased along with the amount of used fecal material and varied between the $9^{th}$ power and $10^{th}$ power.

Proteins from the first test sample were isolated and loaded into a gel (Fig. 11.). The gel was imaged and cut under the UV light. Three bands were visible in the gel corresponding to 600, 800 and 1000 mg of fecal starting material. The well corresponding to 400 mg sample looked shady but due to image background it was difficult to interpret.

Overall, presented data showed that EV isolation was reasonable with smaller amount of fecal starting material compared to previously used 4000 mg.

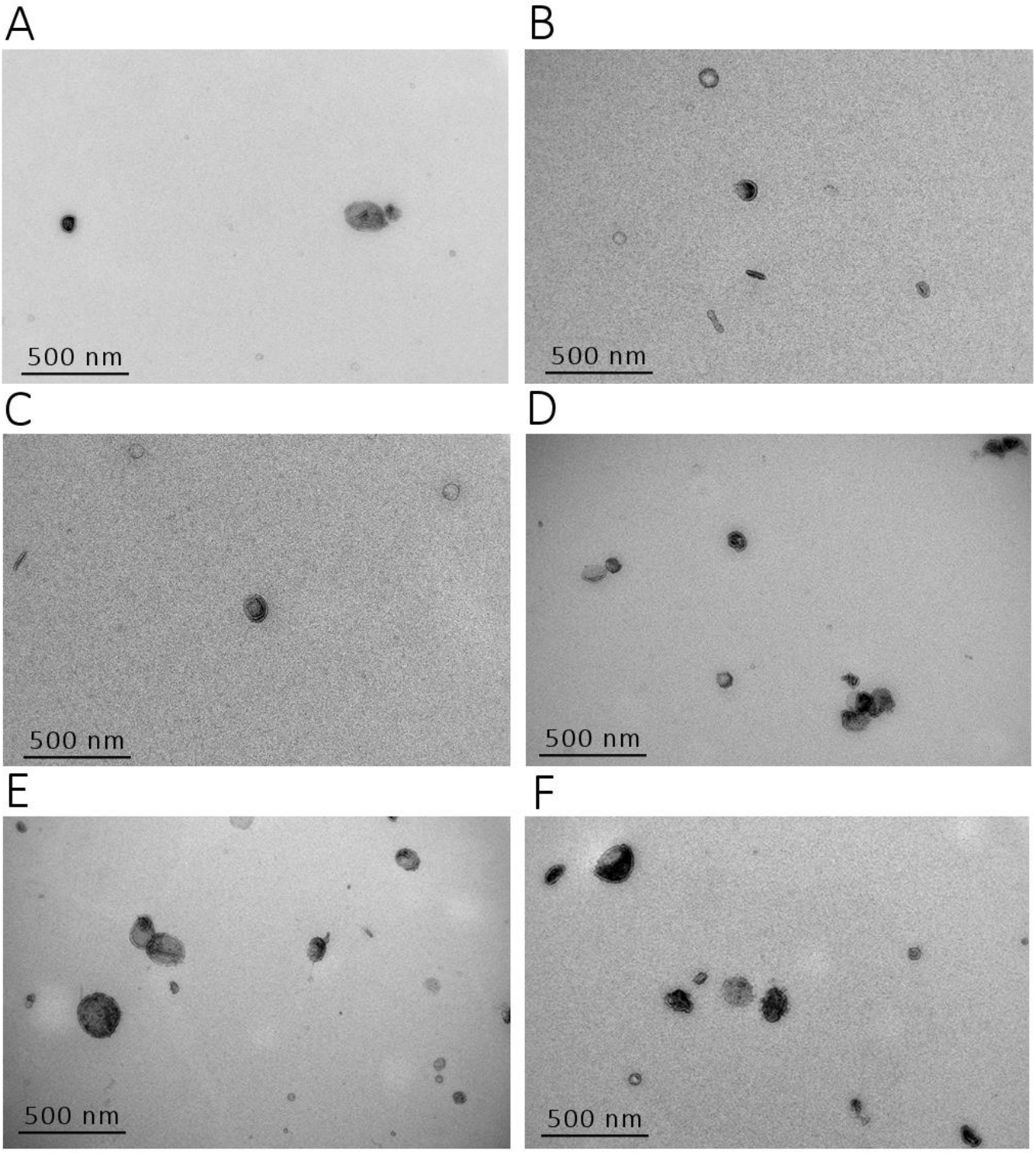


Figure 7. Transmission electron microscopy results of EVs isolated with variable amount of fecal material from the first test sample. EVs showed variation in size and structure. Purity and quantity of EVs was usually highest in fractions 5-7 (data not shown). EVs appeared with a single bilayer or with a double bilayered structure. Magnification 23 000x. **A**: 100 mg of fecal material, fraction 7. **B**: 200 mg of fecal material, fraction 7. **C**: 400 mg of fecal material, fraction 5. **D**: 600 mg of fecal material, fraction 7. **E**: 800 mg of fecal material, fraction 6. **F**: 1000 mg of fecal material, fraction 6.

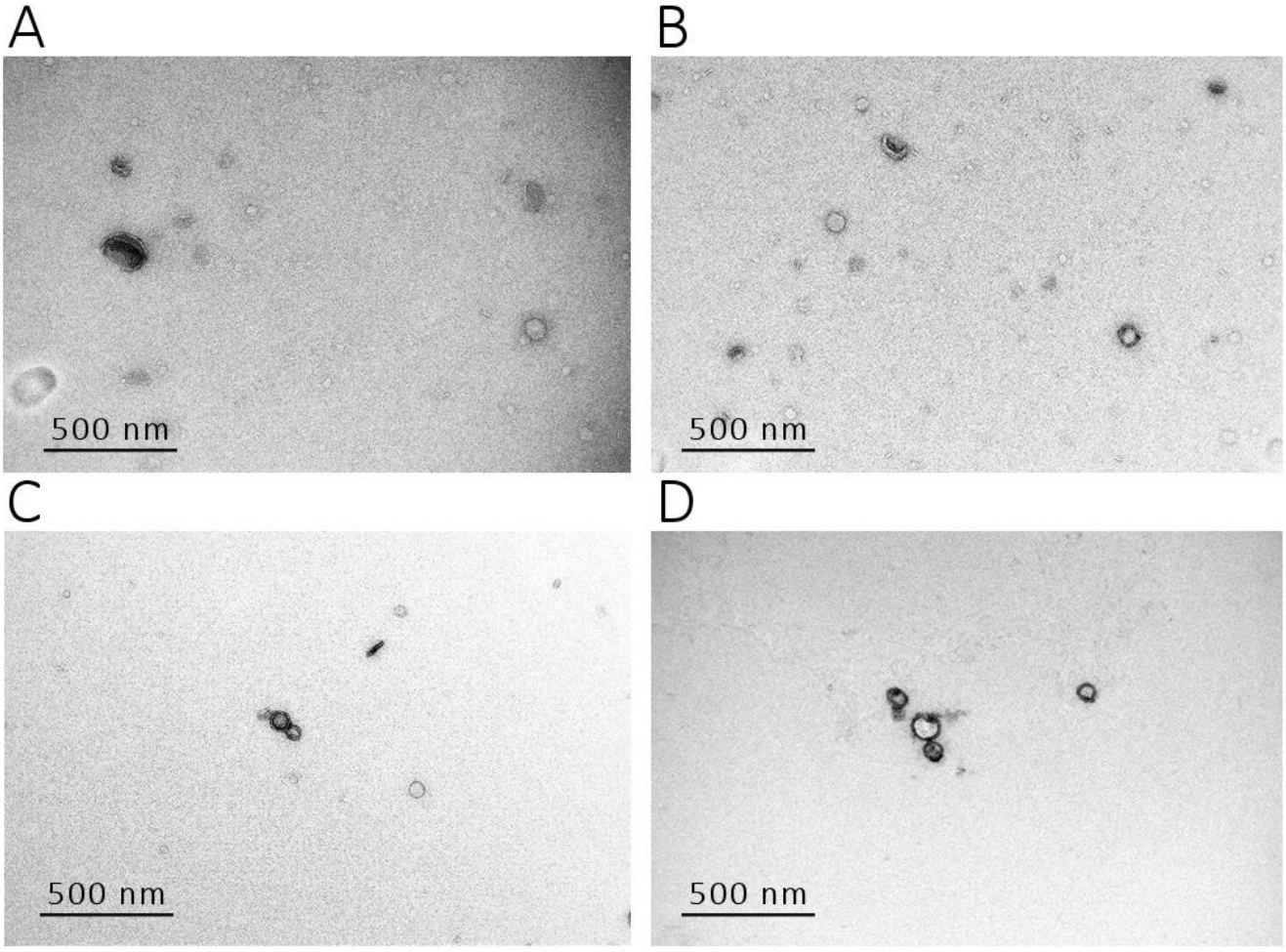


Figure 8. Transmission electron microscopy results of EVs isolated with variable amount of fecal material from the second test sample. Magnification 23 000x. **A**: 100 mg of fecal material, fraction 6. **B**: 200 mg of fecal material, fraction 6. **C**: 400 mg of fecal material, fraction 7. **D**: 600 mg of fecal material, fraction 7.

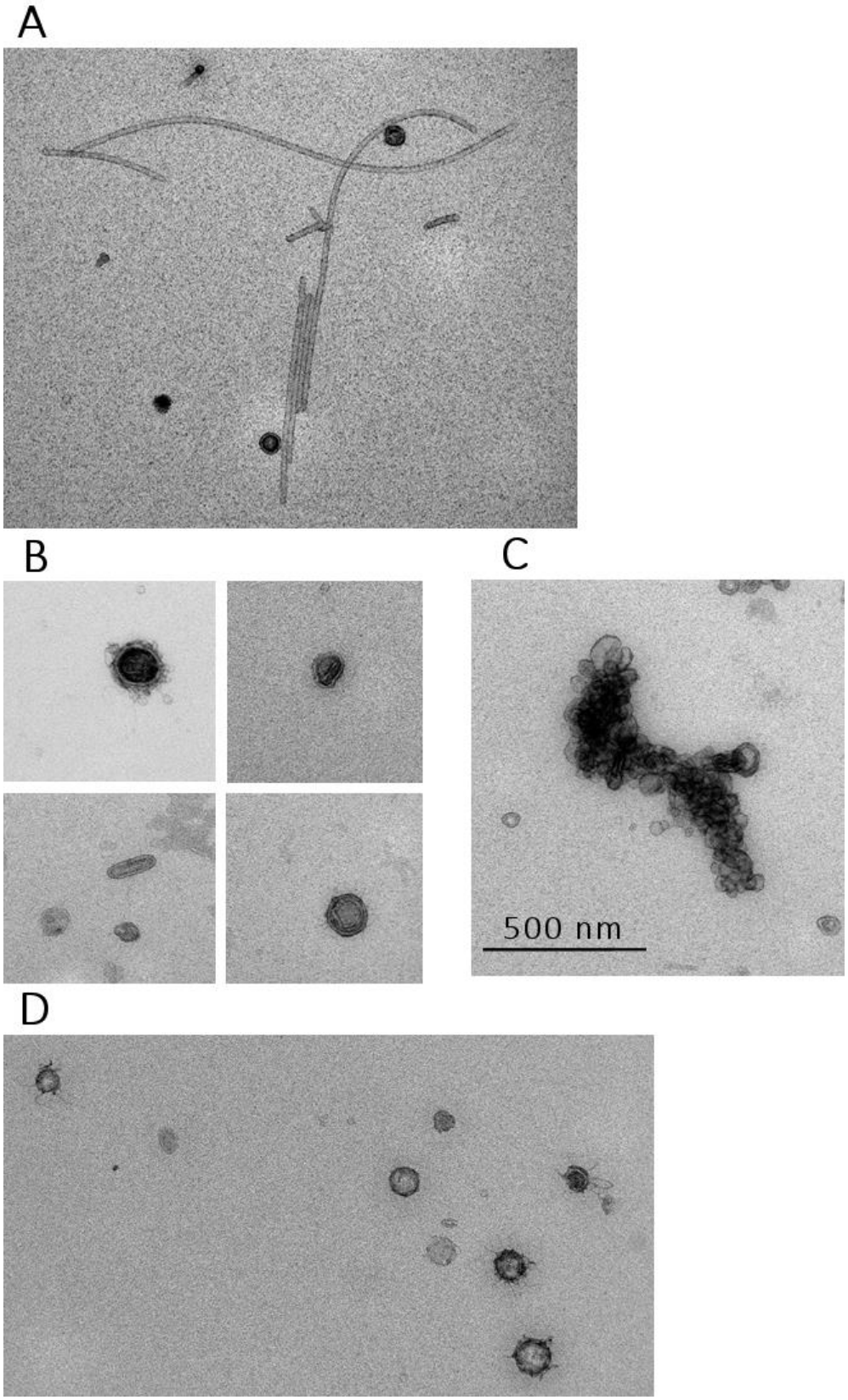


Figure 9. TEM images of different EV-like particles and other structures present in samples. 23000 x magnification. **A**: Flagella-like structures are often present in fraction 8. **B**: Collation of EV-like structures. Upper left; EV-particle with fibrous-like surface. Upper right; wrinkled EV-particle. Lower left; tube-shaped EV particle. Lower right: Multilamellar-like EV-particle. **C**: EV aggregate. **D**: EV-particles with granular and fibrous-like surface.

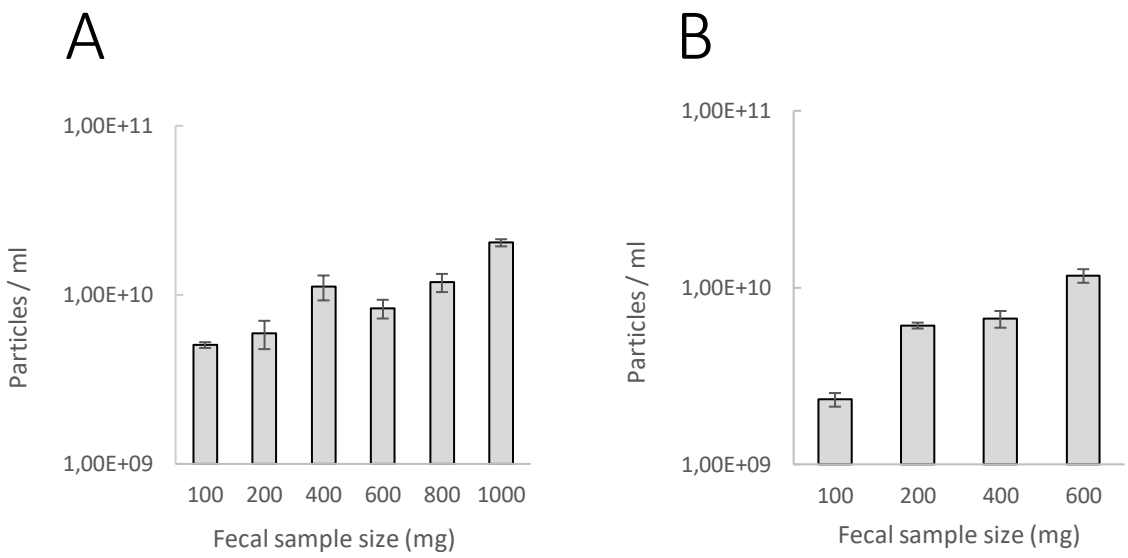


Figure 10. Logarithmic EV concentrations per ml (Y-axis) of test samples. NTA was used to determine EV concentration. Variable amount of fecal material (X-axis) was used for EV isolation. **A**: EV concentrations of the first test sample. **B**: EV concentrations of the second test sample.

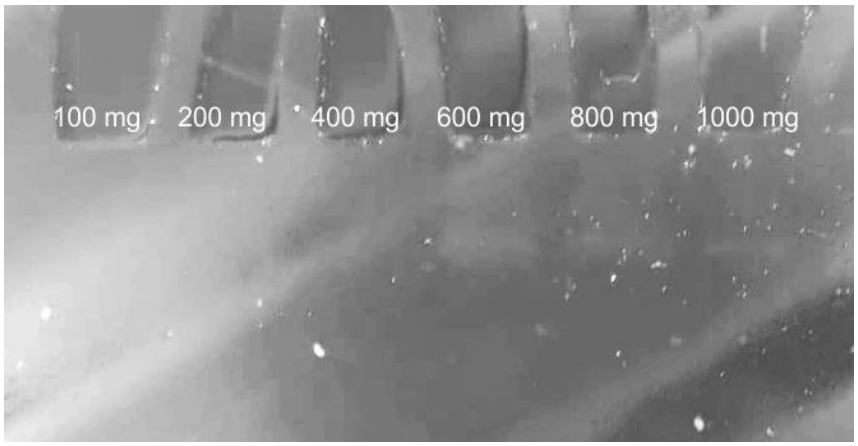


Figure 11. Isolated proteins of the first test sample in a gel. Three bands corresponding to 600, 800 and 1000 mg of fecal material used were visible in the gel.

### 5.2 Extracellular vesicles were similar in size and concentration between multiple sclerosis patients and healthy controls

EVs were isolated from MS disease and healthy control fecal samples by using 400 mg of fecal material. Fractions 5-7 were washed, negatively stained and imaged with TEM. Unfortunately, coating of grids was thicker than usual due to evaporation of the coating stock solution. Thick coating made samples more difficult to image and interpret (Fig. 11). Quantity of EVs in TEM samples varied from abundant to almost empty.

Fractions 5-7 were combined from each sample and measured with NTA. In MS group mean concentration of EVs was 1,14E+10 ±8,59x$10^9$ particles/ml, whereas EV concentration in healthy controls was 6,51x$10^9$ ±5,52x$10^9$ particles/ml. Differences in concentration did not reach statistical significance (Fig. 14.). Distribution of EVs by concentration and size varied in both groups (Fig. 12. & 13.) where majority of EVs were either centered or dispersed along X-axis. Mean size of EVs in MS group was 202,0±31,8 nm and modal average was 162,3±22,9 nm. Mean EV size in healthy controls was 191,3±27,4 nm and modal average 153,1±26,1 nm. Distribution of EVs was evaluated with D10, D50 and D90 values, which represents a threshold value where a certain percentage of EVs (10%, 50% or 90%) are smaller than the given threshold size. For MS group mean D10, D50 and D90 were 132,8±15,6 nm, 184,4±28,3 nm, and 291,8±55,1 nm, respectively. For healthy controls distribution values in respected order were 122,9±17,2 nm, 173,5±25,9 nm and 280,7±50,7 nm. While mean, modal averages, and mean values of D10, D50 and D90 were higher in MS group compared to healthy controls, the results weren't statistically significant. Overall, results suggest that size and concentration of EVs were similar between groups.

Proteins were isolated from EV samples of both groups and loaded into gels (Fig. 15.) where few bands were visible under the UV light. Gel pieces were cut from the gel, dried with ethanol, and send to Turku Bioscience proteomics facility for protein sequencing. Samples were analyzed with LC-ESI-MS/MS and raw data from the measurement was received for protein identification and analysis. Unfortunately, acquiring results from protein analysis was delayed due to hinderances caused by the SARS-CoV-2 procedures during spring 2020. Therefore, protein identification results weren't presented in this work.

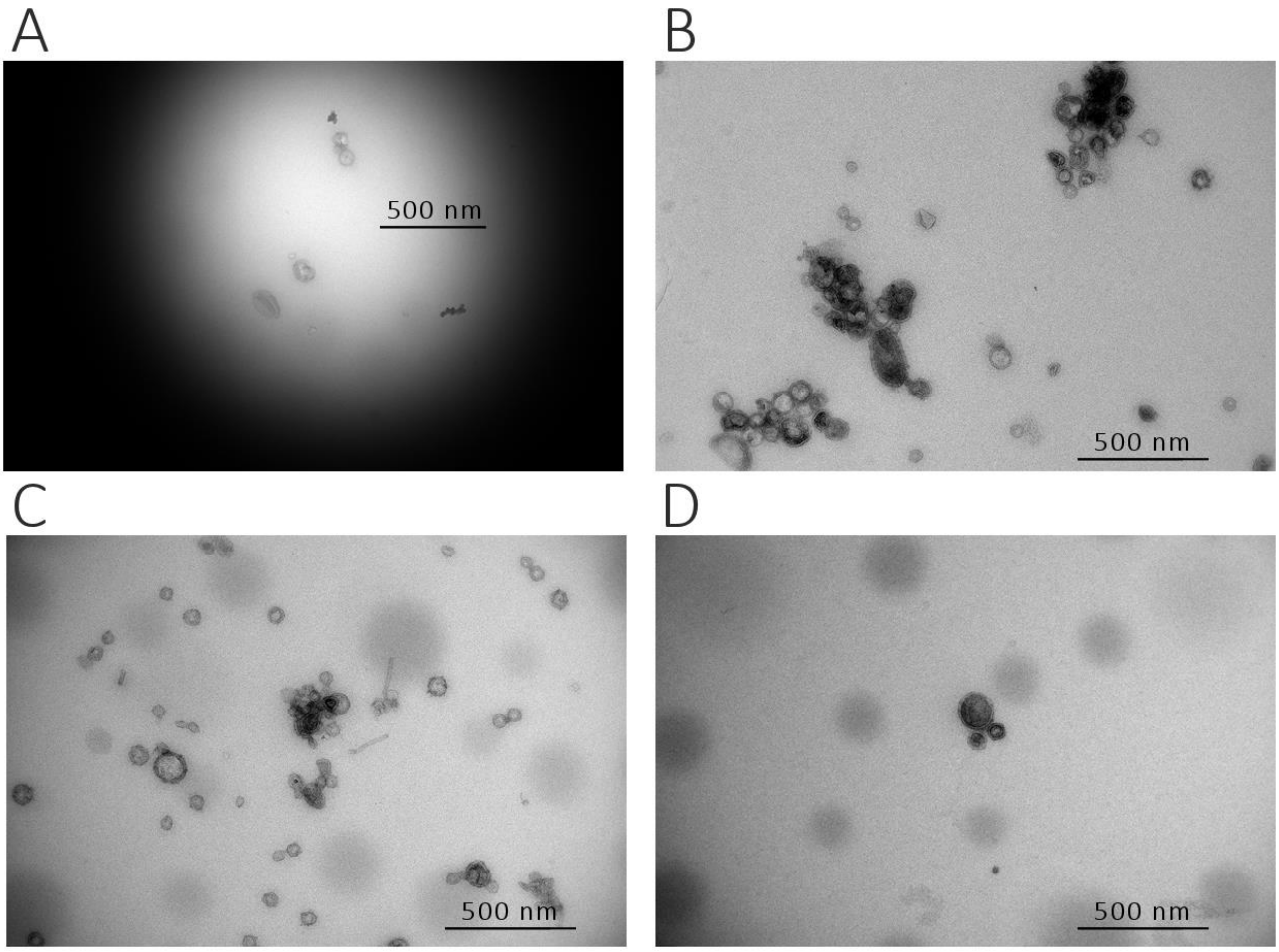


Figure 11. TEM image examples of isolated EVs from MS patients and healthy controls. Amount of EVs present in TEM samples varied between samples in both sample groups. **A**: MS patient sample, fraction 5, 18500x magnification. Thick coating made samples difficult to see through. Electron beam was centered on a smaller area to visualize EVs. **B**: MS patient sample, fraction 7, 23000x magnification. **C**: Healthy control EVs with few fibril-like structures, fraction 7, 23000x magnification. **D**: Healthy control EVs, fraction 6, 23000 x magnification.

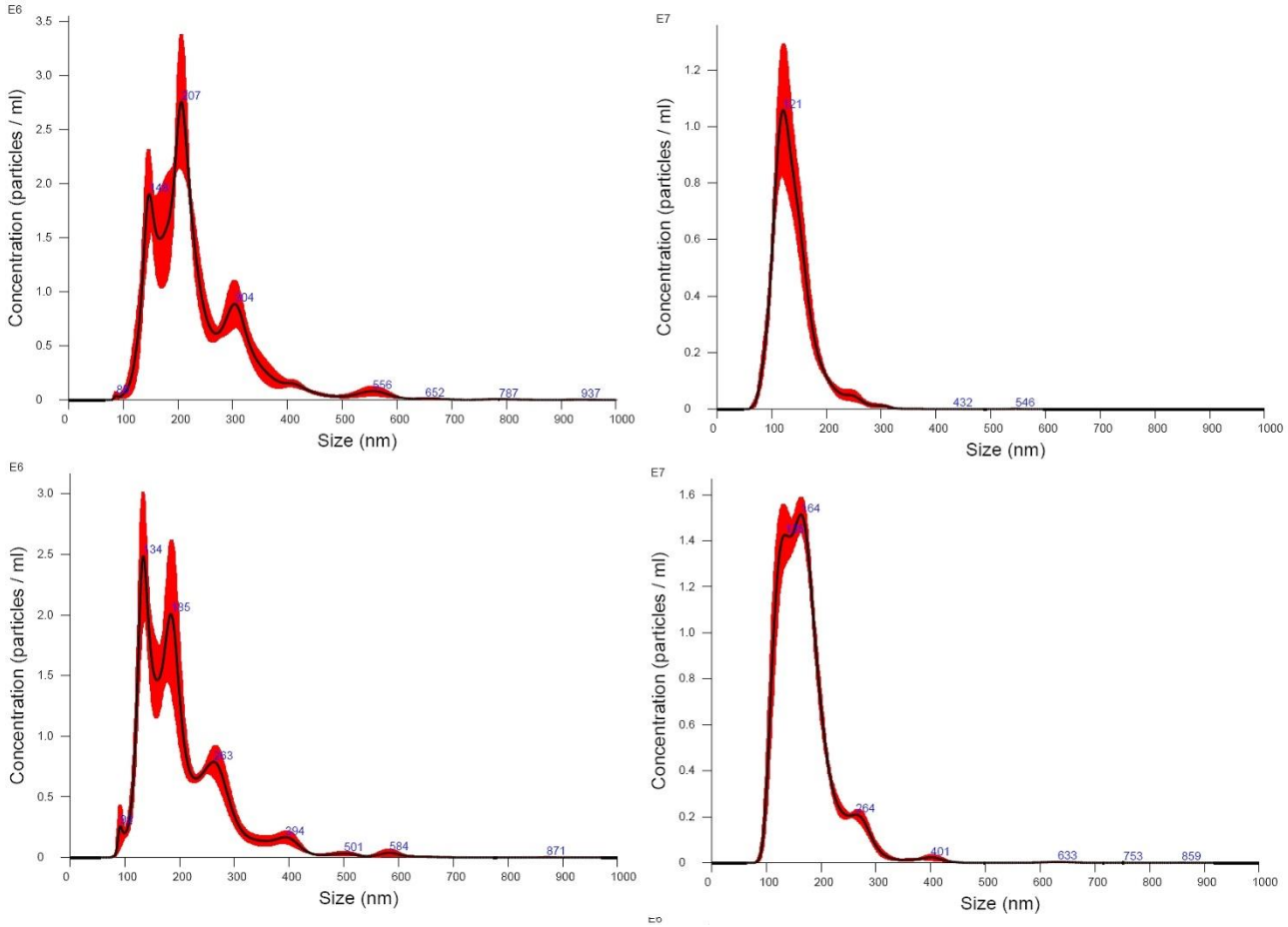


Figure 12. Disperse (left column) and centered (right column) EV distribution examples from MS patients. EV concentration and size was measured with NTA. Y-axis: concentration (particles / ml). X-axis: Size (nm). Black curve represents mean trend from merged data. Red area represents deviation from 4 consecutive measurements.

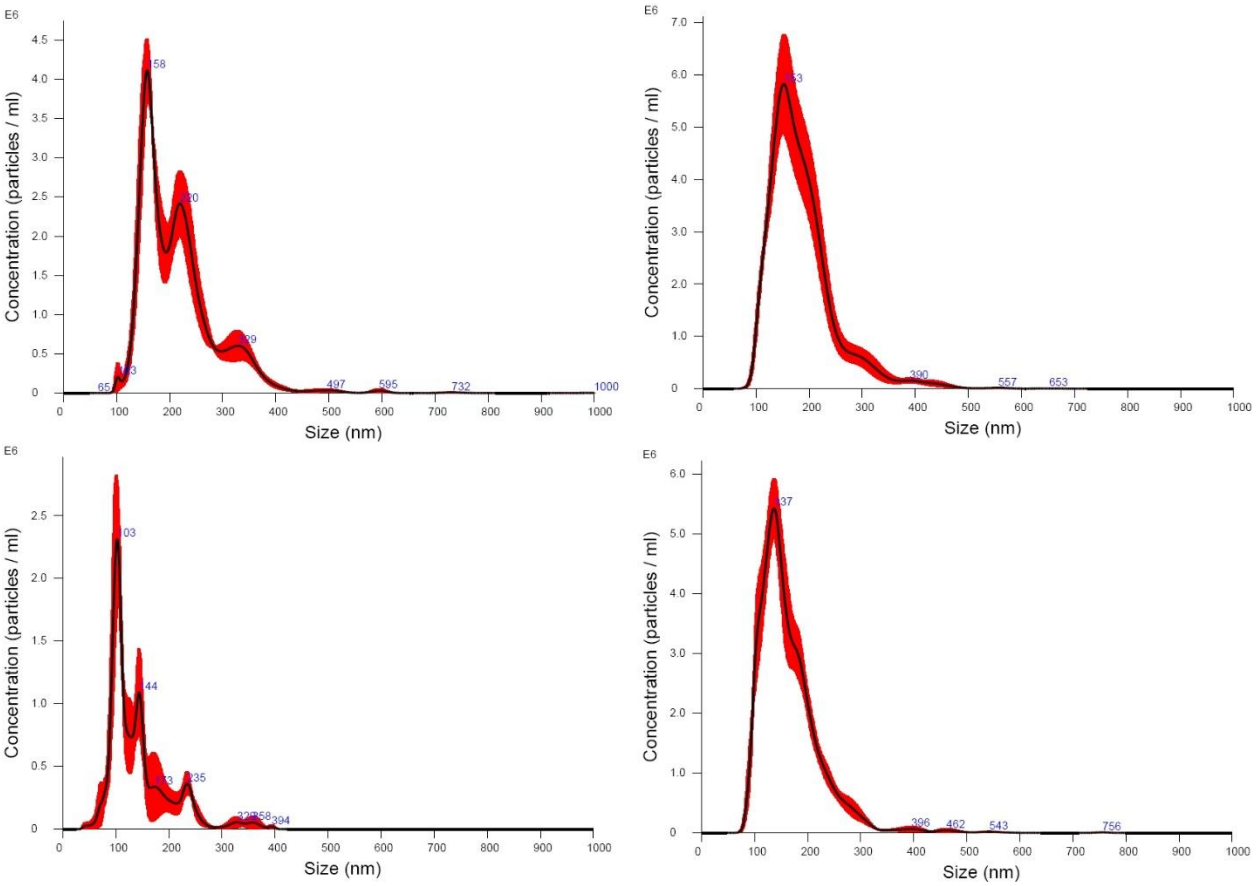


Figure 13. Disperse (left column) and centered (right column) EV distribution examples from healthy controls. EV concentration and size was measured with NTA. Y-axis: concentration (particles / ml). X-axis: Size (nm). Black curve represents mean trend. Red area represents deviation from 4 consecutive measurements.

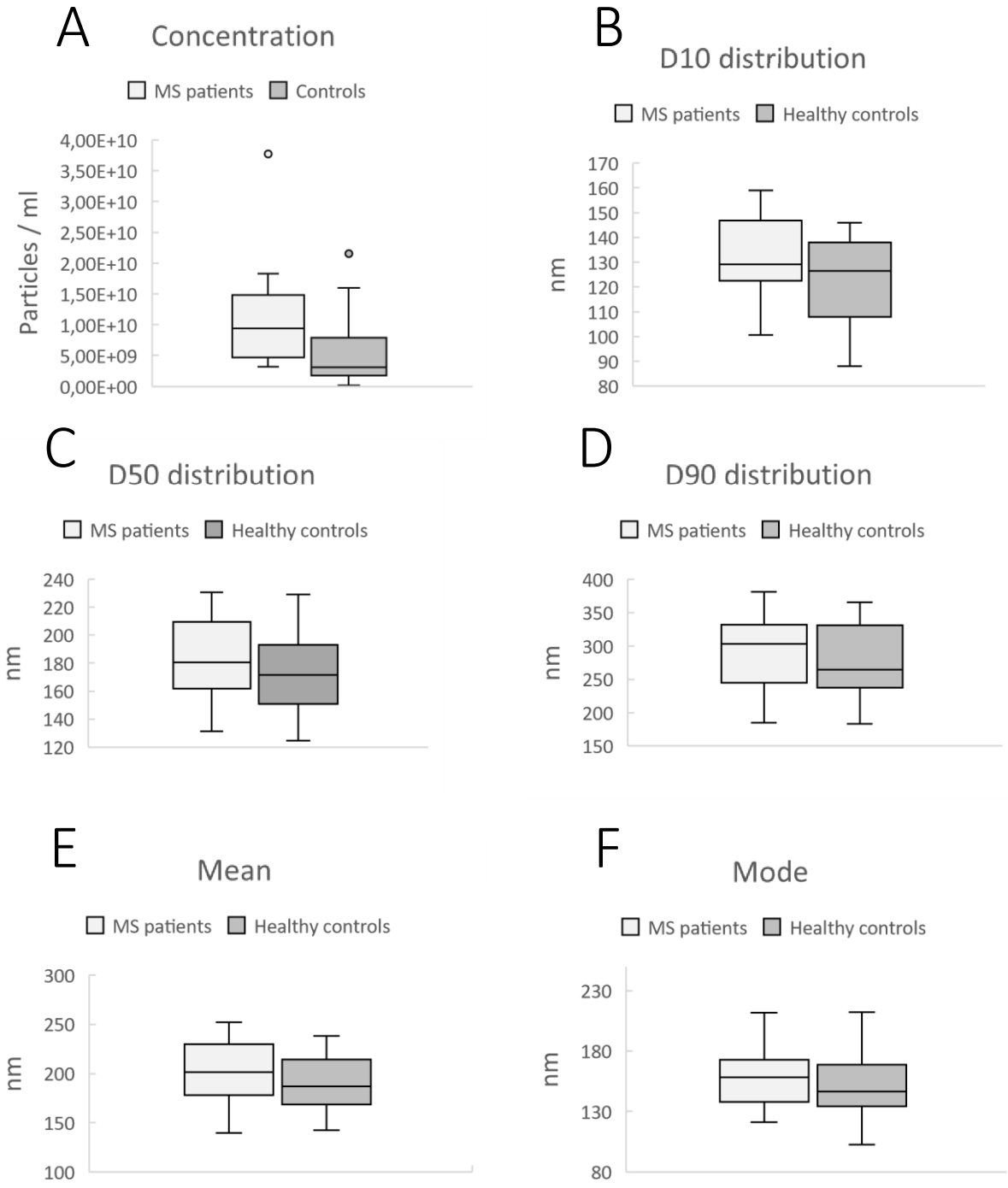


Figure 14. Comparison of EV concentration and size between MS patients and healthy controls. **A**: EV concentration (particles / ml) of MS patients and healthy controls. **B-D**: D10, D50 and D90 size distribution of EVs between MS patients and healthy controls. D10, D50 and D90 describes the EV size where 10 %, 50%, and 90 % of vesicles are smaller than the threshold value (Y-axis). **E**: Mean EV size of MS patients and healthy controls. **F**: Mode EV size of MS patients and healthy controls. nm: nanometer.

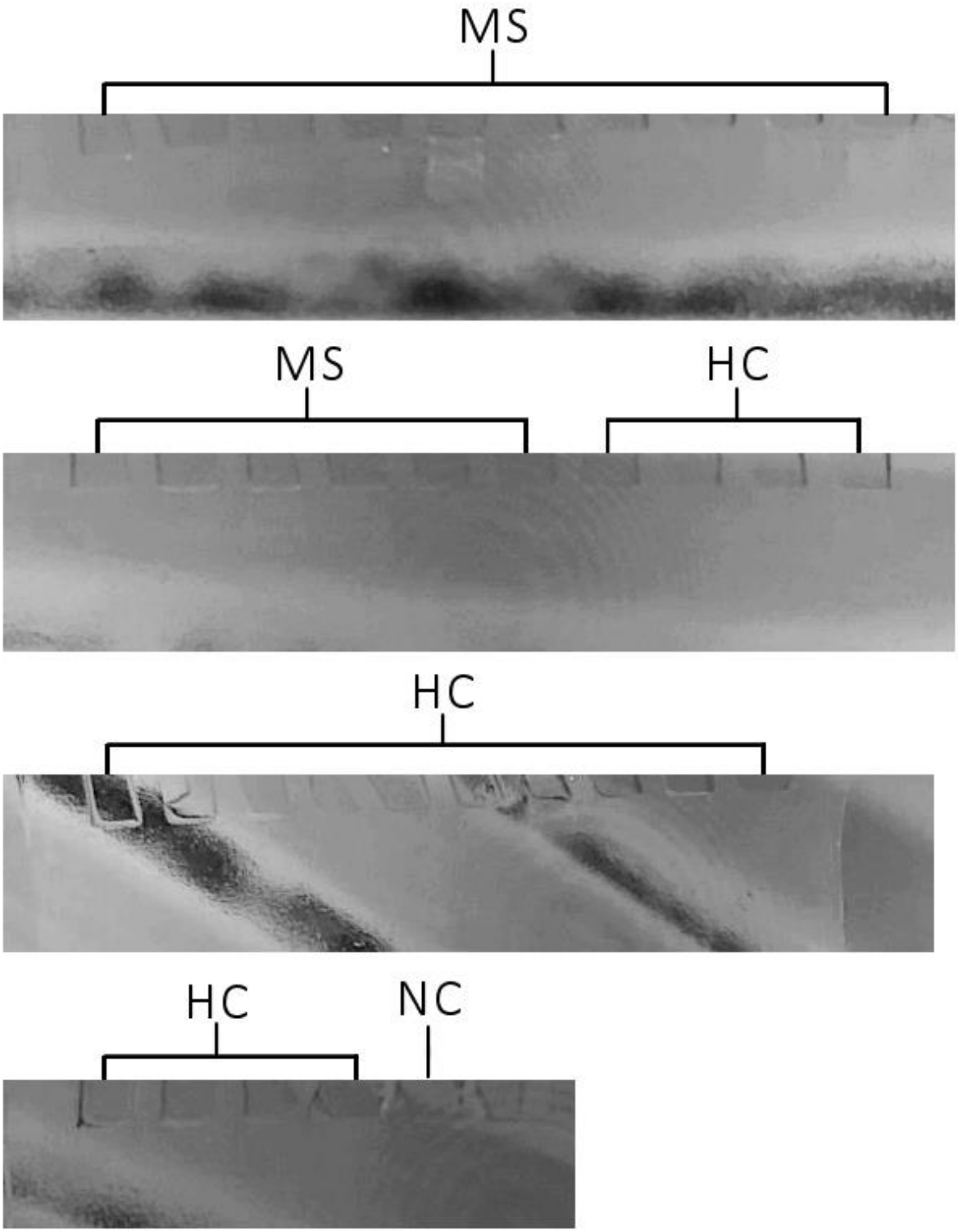


Figure 15. Gel picture of proteins isolated from MS patients and healthy control EVs. MS: MS patients. HC: healthy controls. NC: Negative control (DPBS).

## 6. Discussion

EVs produced by the gut microbiota offer an intriguing mechanism how the gut microbiota can alter host function. Role of gut microbiota derived EVs is a novel field of research and largely unknown factor regarding host-microbiota interaction in health and disease. This work aimed to study microbial EVs isolated from fecal samples of MS patients and healthy controls, and according to our knowledge, is the first time such analysis was done with MS samples.

Provided fecal samples for the experimental work were smaller than anticipated. Therefore, suitability of samples for EV isolation was tested with in-house fecal control samples that were 100-1000 mg in weight. EVs were successfully isolated from all tested samples. Using smaller amount of fecal material also prevented clogging of used filters and concentrators, which was a frequent problem when 4000 mg of fecal material was used. Smaller sample size for future EV isolations could provide practical benefit, regarding space limitations in sample storage and use of equipment, as less amount of plastics and reagents are required for isolation, potentially making EV isolation from fecal samples faster and more affordable.

Concentration and size of isolated EVs were similar between sample groups while great variation was observed between individuals. NTA graphs showed that EV populations were either centered between certain size range, seen as single peak, or distributed with multiple peaks, possibly representing subgroups within EV population. The gut microbiota composition have been observed to be similar in phylum level among humans, but similar to fingerprints, the gut microbiota composition of each individual is unique (Lozupone *et al.,* 2012; Quigley. 2013), which is probably also interlinked to EV profile. Indeed, next objectives are to recognize, which bacteria release the most of EVs based on EV 16S sequencing, determine gut microbiota composition from whole fecal sample, and compare results to known gut microbiome data regarding MS disease and healthy controls.

Even though size exclusion chromatography was used to collect EVs that were 30-300 nm in diameter, particles larger than 300 nm were recorded in NTA. This could be explained by the formation of EV aggregates seen in TEM images. Formation of EV aggregates presumably occurs during density gradient ultracentrifugation and sample washing, since centrifugation at 100 000 g has been shown to generate EV aggregates (Linares *et al.,* 2015), which can potentially affect concentration and biological properties of EVs.

Despite collected EVs were within bacterial vesiculation range, isolated EVs represent a mixed population of EVs that can originate from sources other than gut microbiota,

including the host and ingested food. Currently, microbial EVs cannot be separated from EVs of other source. Mixed EV population does not impede protein analysis since identification of proteins from other sources can be excluded from the analysis. However, multi-source nature of EV population should not be ignored when assessing the role of microbial EVs if functional experiments are performed with feces derived EVs.

Since EVs were isolated based on their size and density, virions with similar properties may also be present in samples (Momen-Heravi *et al.,* 2013). Careful interpretation of TEM images is required as spherical shaped virions can be mistaken for EVs, potentially causing false positive observations. However, distinguishing EVs from virions can be challenging due to variation in the appearance of bacterial EVs, which can e.g. appear smooth or granular (Dorward *et al.,* 1989). Consequently, presence of virions can cause bias in NTA analysis regarding EV concentration and size distribution but evaluating the impact of virions in EV characterization is currently inaccessible.

Another problem is uncertainties in EV preservation. Proteins from MS samples and few control samples were isolated before closing of the university due to SARS-CoV-2 pandemics. Proteins from the rest of the control samples were isolated two months later. For the whole time EVs were kept at 4 °C, which could potentially affect EV properties. Regarding proteins however, stability of enzymatic activity is preserved in EVs better compared to free enzyme over wide scale of temperatures ranging from -80 to 37 °C. It was reported that ~80 % enzyme activity was maintained when EVs were stored for 14 days at 4 °C or at -80 °C (Alves *et al.,* 2016), suggesting that EVs provide a microenvironment that is favorable for protein preservation. On the other hand, long-term preservation of RNA in EVs is suggested to require -80 °C storage conditions (Mendt *et al.,* 2018).

Amount of isolated protein, based on visual interpretation of gels, varied between samples in both groups. Sadly, results of protein analysis were excluded from this work due to unfortunate global situation. Originally, protein data was scheduled to be ready latest at 2020 spring. However, practical work halted during early spring for two months due to closing of the university of Oulu as an attempt to slow down the spreading of SARS-CoV-2. Experimental work continued after re-opening of the university and protein samples were sent in June 2020 to Turku Bioscience proteomics facility for protein sequencing. Analysis of protein data required a bioinformatics specialist who had a scheduled vacation at the time, and subsequently, went on a maternity leave during summer, which hindered data analysis.

As previously described studies indicate that colonization of mice with MS patient microbiota changes the course of EAE, it would be interesting to test whether the administration of feces-derived EVs from MS patients affects EAE severity, population of immune cells and cytokine profile. Practically, role of EVs in MS could be observed by repeating similar experimental settings what were used in previously discussed studies regarding the role of gut microbiota in EAE.

## 7. Conclusions

Studying gut microbiota derived EVs is a thrilling but challenging field of research, partly due to its novel nature. The role of gut microbiota in health and disease has received sky-rocketing interest and certainly increased attention regarding the role of gut microbial EVs will follow. This work aimed to study gut microbial derived EVs from MS patients and healthy controls. Size and concentration of EVs were statistically similar between groups while all mean values were higher in MS group compared to healthy controls. This work failed to represent the main objective, which was the protein content analysis of gut microbiota derived EVs in MS patients and healthy controls. However, this work accomplished to show that sample size as low as 100 mg is enough for EV isolation, which can be used to improve EV isolation practice.

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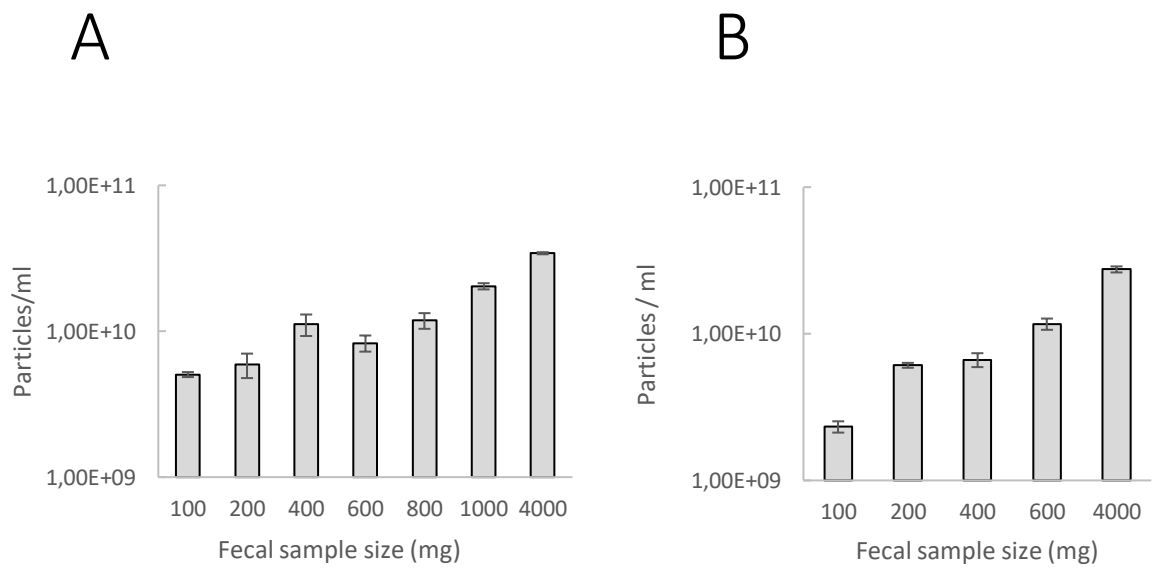
## 9. Attachments




Supplement figure 1. EV concentration in logarithmic scale of the first test sample combined with previously observed EV concentration with 4000 mg of fecal starting material. **A**: Concentrations of the first test sample combined with 4000 mg data from other measurement. Concentration of the 4000 mg sample is not totally comparable since 2,5x volume of EV eluant from Exo-Spin EV isolation step was used in density gradient ultracentrifugation compared to other samples. **B**: Concentrations of the second test sample combined with 4000 mg data from other measurement. Concentration of the 4000 mg sample is not totally comparable due to uncertainties in the used volume (either 1-2,5x) of EV eluant from Exo-Spin EV isolation step compared to other samples.