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Mini-Reviews in Medicinal Chemistry, **xxxx,** *xx,* **000-000**

MINI-REVIEW ARTICLE

Microbiota-Gut-Brain Axis in Neurological Disorders: From Leaky Barriers Microanatomical Changes to Biochemical Processes

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ARTICLE HISTORY

Received: April 11, 2022
Revised: April 19, 2022
Accepted: April 29, 2022

DOI:
10.2174/1389557522666220622111501

Abstract: ***Background*:** The gastrointestinal tract and the central nervous system are distinct because of evident morpho-functional features. Nonetheless, evidence indicates that these systems are bidirectionally connected through the gut-brain axis, defined as the signaling that takes place between the gastrointestinal tract and central nervous system, which plays in concert with the gut microbiota, *i.e.*, the myriad of microorganisms residing in the lumen of the human intestine. In particular, it has been described that gut microbiota abnormalities, referred to as dysbiosis, may affect both central nervous system development and physiology.

***Objective*:** Starting from the possible mechanisms through which gut microbiota variations were found to impact several central nervous system disorders, including Autism Spectrum Disorder and Alzheimer's Disease, we will focus on intriguing, although poorly investigated, aspects such as the epithelial and vascular barrier integrity. Indeed, several studies suggest a pivotal role of gut microbiota in maintaining the efficiency of both the intestinal barrier and blood-brain barrier. In particular, we report evidence indicating an impact of gut microbiota on intestinal barrier and blood-brain barrier homeostasis and discuss the differences and the similarities between the two barriers. Moreover, to stimulate further research, we review various tests and biochemical markers that can be used to assess intestinal and blood-brain barrier permeability.

***Conclusion*:** We suggest that the evaluation of intestinal and blood-brain barrier permeability in neurological patients may not only help to better understand central nervous system disorders but also pave the way for finding new molecular targets to treat patients with neurological impairment.

Keywords: Barrier permeability, blood-brain barrier, central nervous system, gut microbiota, intestinal barrier, vascular barrier.

1. INTRODUCTION

The symbiotic relationship between the human body and its microbiome, that is, the microbial community mainly populating the intestine, represents a critical interplay in the body's homeostasis [1]. Any change to this balance may cause or contribute to the disease status of the host. In recent years, the characterization of the gut microbiota and the mechanisms by which it interacts with the human body has become the target of extensive investigation. The gut microbiota is composed of an estimated number of 10^{13}-10^{14} bacteria belonging to one thousand different species [2], in addition to fungi and viruses [3-5]. Each species can potentially engage in a biochemical relationship with the host. If the interaction is supportive, it is named eubiosis, since it is proven to exert a beneficial impact on Gastrointestinal (GI) physiology by helping digestive processes, harvesting energy [6], protecting against pathogens [7], shaping the immune response by regulating T cells maturation and activation [8], interacting with the enteric nervous system [9] and, last but not least, modulating the intestinal epithelial and vascular barrier [10, 11]. The disruption of eubiosis, elicited by a number of factors including inflammation, unhealthy dietary style, drugs, ageing, and metabolic disorders, causes dysbiosis, which is linked to various clinical phenotypes such as inflammatory bowel disease [12], diabetes [13] and obesity

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1389-5575/xx $65.00+.00

[14]. Initially, it was assumed that microbiota abnormalities could be involved only in GI diseases. However, in recent years, several studies indicate that the range of gut microbiota impacts on the host can be widened by the possibility of an action on distal organs, especially on CNS. Notably, GI symptoms can precede or co-exist in CNS disorders, such as in children with neurodevelopmental disorders [15, 16] or in patients suffering from cognitive impairment due to neurodegenerative diseases [17]. Recently, dysbiosis has been linked to several neurological diseases [18, 19], supporting the bidirectional communication between microbiota and the gut-brain axis. Thus, it is possible to refer to this system as the microbiota-gut-brain axis. Mechanisms involving the immune system, neural pathways (*e.g.*, the vagus nerve), the hypothalamic-pituitary-adrenal interplay and tryptophan metabolism promoted by microbial metabolites can be major regulators/activators of the microbiota-gut-brain axis [20-23]. During dysbiosis, microbiota-derived pro-inflammatory factors can reach the CNS, causing neuroinflammation [24-26]. Moreover, it has been demonstrated that a dysbiotic gut enhances mucosal T-helper 1 and T-helper 17 cell activation [27, 28]. This process may trigger systemic inflammation that can involve the CNS. However, the cascade of events that leads the microbial-derived molecules to exert an actual effect on the brain represents the crucial question that still remains unanswered. Emerging evidence indicate that a key factor in this cascade might be the alteration of both the Gastrointestinal Epithelial-Vascular Barrier (here referred to as Intestinal Barrier, IB) and the Blood-Brain Barrier (BBB), thus leading scientists to focus their research interest to barrier permeability.

2. DYSBIOSIS AND CNS DISORDERS

To understand the cross-talk between IB and BBB, it is necessary to clarify the so far obtained evidence that links dysbiosis to CNS disorders.

2.1. Autism Spectrum Disorder (ASD)

ASD is a heterogeneous neurodevelopmental disorder that affects approximately 1 in 60 subjects [29]. ASD is probably the first and, so far, most discussed neurological disorder in which dysbiosis has been observed [30]. Almost half of the ASD patients exhibit GI symptoms, such as diarrhea and chronic abdominal pain, along with the "classical" behavioral and emotional manifestations [31, 32]. Different studies indicate a change in the stability and composition of gut microbiota in ASD patients [33-35] and a higher presence of pro-inflammatory cytokines in ASD patients with GI manifestations compared to those without digestive symptoms [36]. Moreover, it is documented that probiotic therapy not only improved GI dysfunction, leading to a switch in the microbiota composition from a predominance of pro-inflammatory bacteria to one of the anti-inflammatory bacteria but also reduced the severity of behavioral and emotional symptoms [37]. Even though no study conducted so far investigated the mechanisms that could eventually link dysbiosis to ASD, indirect evidence from studies on gut microbiota analysis and the effects of probiotic treatment in ASD children provide support to the concept that dysbiosis may play a role in the development or maintenance of ASD. Clearly, further studies on this possible pathogenetic interplay are eagerly awaited.

2.2. Attention-Deficit/Hyperactivity Disorder (ADHD)

ADHD is a neurodevelopmental disorder that globally affects 7.2% of children below 18 years [38]. Two studies compared the gut microbiota of ADHD children to the gut microbiota of age-related healthy individuals and found a decreased amount of *Faecalibacteria* [39, 40], commensal bacteria that are documented to produce anti-inflammatory factors [41]. Therefore, its decreased levels might favor intestinal inflammation. In this regard, another study that reviewed the possible involvement of pro-inflammatory cytokines in ADHD found that affected children display higher levels of IL-6 and IL-10 cytokines compared to healthy children [42]. Thus, inflammation caused by gut dysbiosis may have an important role in ADHD pathophysiology.

2.3. Mental Disorders

Mental disorders include anxiety, depression, bipolar disorder, schizophrenia, and other psychotic conditions. The incidence of these psychopathological forms is dramatically increasing, and, to date, about 20% of the worldwide population is estimated to be affected [43]. The first evidence that probiotic treatment can improve such conditions date back to the early 20th century [44, 45]. Moreover, studies performed on germ-free mice demonstrated that microbiota transfer from psychiatric patients results in the development of a pathological behavioral profile [46-48]. A recent meta-analysis has revealed that almost all studies that addressed the comprehension of the gut microbiota composition in psychiatric patients found dysbiosis, with significant differences between patients and controls in terms of phylum, family or genus level. Furthermore, this meta-analysis pointed out that patients with anxiety, depression, bipolar disorder, and schizophrenia showed an imbalance between anti- and pro-inflammatory bacteria, with a decrease in the first and an increase in the latter [17]. These findings support the concept that a dysbiotic gut plays a role in inflammation and therefore influences the mechanisms leading to psychiatric disorders.

2.4. Multiple Sclerosis (MS)

MS is a neuroinflammatory disease characterized by immune-mediated demyelination of neural axons. MS prevalence is about 40 people per 100.000 [49]. Most patients display a relapsing-remitting form of the disease leading to a worsening of the neuroanatomical lesions and severity of neurological manifestations over time [50]. The progression of the CNS abnormalities causes death in more than 50% of the patients [51]. Notably, two-thirds of MS patients experience GI symptoms, such as constipation and/or fecal incontinence [52]. Several studies addressed the involvement of gut microbiota in MS. Dysbiosis was found in MS patients and animal models, along with a decrease in anti-inflammatory and an increase in pro-inflammatory factors

[53-55]. A difference in the composition of gut microbiota was also identified in patients with active disease compared to those in remission, who displayed a gut microbiota similar to that of healthy controls [56-58]. Moreover, gut microbiota from MS patients transplanted to a transgenic mouse model of spontaneous brain autoimmunity induced a significantly higher incidence of autoimmunity than mice receiving healthy control-derived microbiota [59]. Remarkably, a research group that decided to treat the GI symptoms of three MS patients with severe constipation using multiple fecal transplants observed that not only the patients displayed an improvement in intestinal function but also in CNS symptoms [60]. In addition, MS patients given probiotic supplementation showed an improvement in motor symptoms and inflammatory parameters [61]. Thus, these results support the hypothesis that suggests dysbiosis as a pivotal factor for MS onset and progression.

2.5. Alzheimer's Disease (AD)

AD is the most common cause of progressive dementia, affecting nearly 50 million people globally [62]. Previous studies indicated that AD might be associated with peripheral infection, which can then spread systemically and cause neuroinflammation [63-65]. Indeed, gut microbiota metabolites were detected in cerebrospinal fluid of AD patients along with AD biomarkers phosphorylated Tau protein and β amyloid (1-42) [66]. Moreover, dysbiosis has been found in a β amyloid precursor protein (APP) transgenic mouse model compared to wild-type [67]. A remarkable observation was that 5xFAD mice, overexpressing human APP and presenilin 1 transgenes with a total of five AD-linked mutations, recapitulated significant fecal microbial variations that change with age. The same study also reported that a shift from anti-inflammatory to pro-inflammatory factors accompanied the gut microbiota age-related variations. This pattern was absent in wild-type mice, which displayed a more stable gut microbiota composition throughout the lifespan [68]. These findings are consistent with what is observed in humans since dysbiosis has been associated with age-related inflammation, named *inflammaging* [69, 70], and that aging is the most relevant risk factor for the sporadic form of AD, which is also the most common one [71]. Taken together, these results indicate that the microbiota-gut-brain axis can be involved in AD pathological mechanisms and that *inflammaging* might be a pivotal factor for dysbiosis.

2.6. Parkinson's Disease (PD)

PD is the second most common neurodegenerative disorder to date affecting about 10 million people worldwide [72]. Evidence suggests that α-synucleinopathy, one of the principal PD pathological signs, is initiated in the ENS before it occurs in the CNS [73]. This is supported by the fact that gastrointestinal symptoms are associated with the early stages of PD in humans and animal models [74-76]. As observed in all the aforementioned neurological disorders, PD patients exhibit dysbiosis, too [77-79]. Furthermore, a study found that PD patients not only display a different gut microbiota composition compared to healthy individuals but also have a higher abundance of pro-inflammatory bacteria [80]. Finally, PD patients were found to harbor an intestinal flora depleted in short-chain fatty acids [81-83] that are enlisted in the group of bacteria metabolites with a pivotal and possibly protective role in the microbiota-gut-brain axis [84]. Together, these studies suggest a pro-inflammatory gut milieu in PD that might have a fundamental role in the early stages of the disease.

Further studies are needed to clarify the effective role of dysbiosis in the pathophysiology of CNS disorders and to define if dysbiosis is a cause, a consequence or a co-morbidity of neurological disorders. However, a recurring element in all the studies that associated neurological disorder with microbiota alterations is the observation of higher levels of pro-inflammatory factors. The striking fact about this *leitmotiv* is that augmentation of both IB and BBB permeability is linked to the presence of inflammation [85, 86]. Therefore, examining barrier permeability might clarify the connection between dysbiosis and CNS diseases.

3. MICROBIOTA AND IB PERMEABILITY

The IB, shown in Fig. (**1**), is a dynamic entity that interacts with various stimuli [11]. Indeed, the IB consists of two different barriers that co-operate with each other to allow only certain molecules to reach the systemic circulation: the outer Intestinal Epithelial Barrier (IEB) and the inner Intestinal Vascular Barrier (IVB) [11]. Starting from the intestinal lumen, the IEB is composed of two layers of mucus, a monolayer of absorptive enterocytes and other specialized cells (goblet cells, enteroendocrine cells and Paneth cells) and, underneath this monolayer, the *lamina propria* where resident immunocytes are located [87-89]. On the other hand, IVB has been less investigated, as the first study to address it was performed in 2015 [90]. In this study, C57BL/6J mice were injected at the level of the intestinal loop with fluorescein isothiocyanate (FITC)-dextran of different molecular sizes and the leakage of the dye into the serum was examined. As a result, 4kDa molecules were detected in the serum, while 70kDa molecules were not. These findings indicated the existence of an intestinal-vascular barrier that allows the selective access of specific intestinal luminal contents to the circulatory system [90]. Furthermore, authors observed that the introduction of *Salmonella typhimurium* infection, a leakage-inducing factor, caused the passage of 70kDa molecules too, confirming the presence of the IVB and the possibility to induce its permeabilization. Moreover, they found that leakage was probably linked to an inflammatory reaction that caused a down-regulation of the endothelial Wingless-related integration site signaling, which evoked an increase in Plasmalemma Vesicle Associated Protein (PVAP) expression [90]. PVAP is a molecular component of the endothelium involved in the fenestrae formation. It forms homodimers that create a mesh in the middle of the fenestrae diaphragms. The higher is PVAP expression, the more fenestrae are present. Therefore, PVAP is considered an important

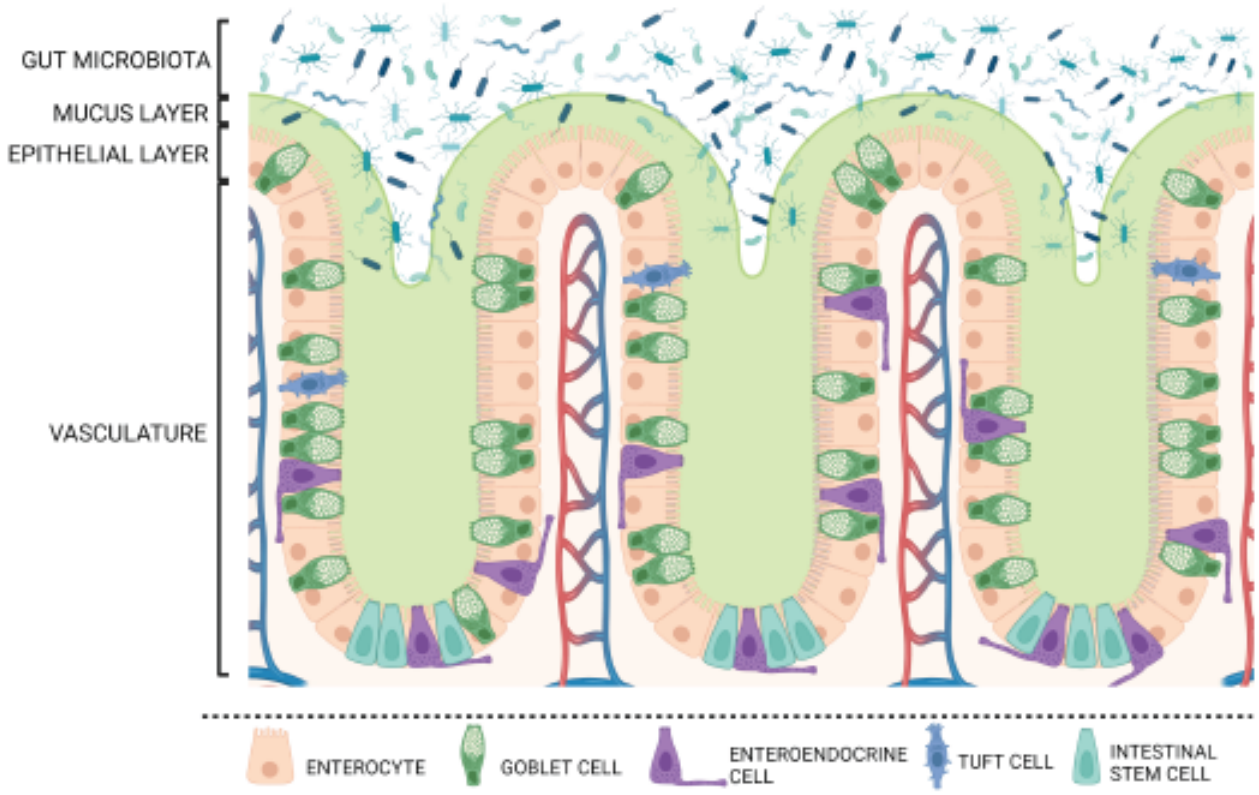

Fig. (1). Intestinal Barrier representation. The intestinal barrier is composed of two parts: an outer Intestinal Epithelial Barrier and an inner Intestinal Vascular Barrier. Starting from the intestinal lumen where the microbiota resides (top), the Intestinal Epithelial Barrier is made of a mucus layer (green) in contact with the mucosal epithelium and of a monolayer of enterocytes and specialized intestinal cells, *i.e.*, goblet cells, enteroendocrine cells, tuft cells, and intestinal stem cells. Whereas the Intestinal Vascular Barrier, found underneath, is made of the endothelial cells' monolayer of the intestinal capillaries. Image created with Biorender.com. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

regulator of vascular permeability [91]. In a modern perspective, The IB can be viewed as a set of two distinct barriers that work synergically to control molecules getting across the system. In inflammatory state, the IB permeability is increased, thereby causing the spread of inflammatory mediators systemically with possible involvement of other tissues and organs, including the CNS.

The regulation of IB permeability is mediated by endogenous and exogenous factors, such as cytokines, toxins, and drugs [87]. Since its pivotal impact on intestinal function, the gut microbiota has also been suggested to be a key modulator of IB permeability [92]; on the other hand, dysbiosis has been linked to GI diseases, in which an altered IB permeability has been demonstrated. Studies comparing germ-free and conventional mice have shown that the lack of gut microbiota impacts intestinal cell morphology and architecture [93, 94]. Compared to controls, germ-free mice display a significantly decreased total intestinal surface area, shorter ileal villi, smaller intestinal crypts, and a thinner mucus layer [95-97]. The ability of gut microbiota to affect IB permeability has also been supported by probiotics, *i.e.*, the live microorganisms providing a beneficial effect on the host when administered in adequate amounts. For example, oral administration of *Lactobacilli* prevented stress-induced IB changes [98]. Additionally, treatment with VSL#3, a high-concentration probiotic preparation of eight live freeze-dried bacterial species, protected IB after dextran sodium sulfate (DSS)-induced colitis in mice [99]. Remarkably, it has been demonstrated that short-chain fatty acids, that are specifically produced by indigestible polysaccharide metabolizing commensal bacteria in the gut [100] can exhibit beneficial effects on the IB [101-103]. Concerning pathological implications, dysbiosis and dysfunctional IB have been identified to various diseases such as obesity, diabetes mellitus, and inflammatory bowel disease [104]. These diseases are characterized by the presence of chronic intestinal inflammation that alters IB permeability by changing tight junction protein expression [104]. Given this evidence, it is possible that dysbiosis has a role in establishing or exacerbating the inflammatory state that characterizes these conditions and, therefore, IB abnormalities. Indeed, more studies are required to clarify how gut microbiota impacts IB permeability.

4. MICROBIOTA AND BBB PERMEABILITY

The BBB, shown in Fig. (**2**), is a complex multicellular structure that separates the CNS from the systemic

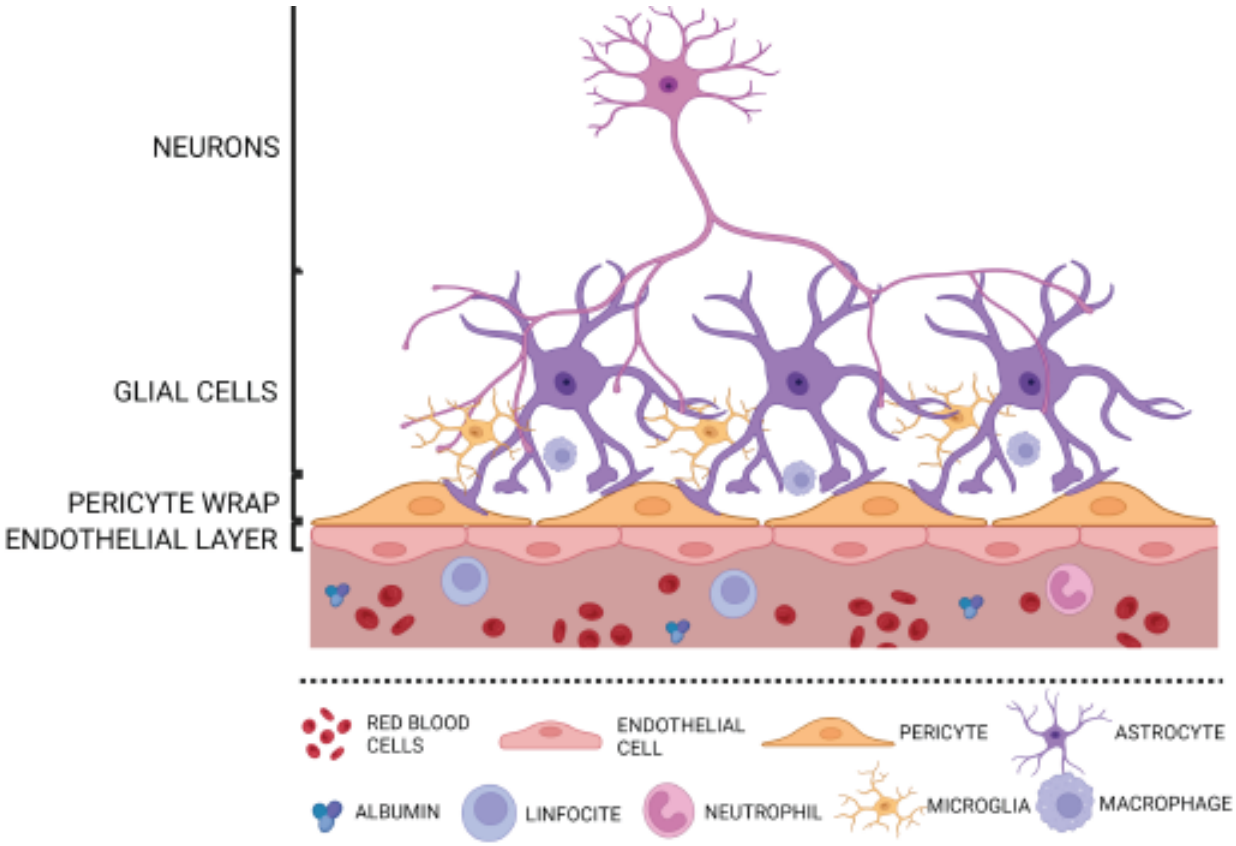

Fig. (2). Blood-Brain Barrier representation. The Blood-Brain Barrier is composed of a monolayer of endothelial cells (bottom), the pericytes that wrap up the brain capillaries and the astrocytes' end feet that communicate with them. The neurons participate in Blood Brain Barrier regulation by synapsing with astrocytes. The resident immune cells (*i.e.*, microglia and macrophages) are also implicated in Blood-Brain Barrier modulation by influencing astrocytes', pericytes' and neurons' activity. Image created with Biorender.com. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

circulation [105]. The BBB is now considered to be part of the neurovascular unit (NVU), a group of closely related cells and extracellular matrix components that synergically regulate cerebral blood flow and molecule exchange between the systemic circulation and the brain [106]. The NVU includes the endothelial cells of the brain capillaries, the pericytes that wrap the capillaries around, the astrocytic end-feet, and the neurons that synapse with them [106]. The BBB is a selective membrane that is permeable only to specific molecules. However, the concept of a static and rigid barrier is being overcome by a more dynamic view. Indeed, the BBB is starting to be considered a finely tuned communication center that manages not only the molecule exchange from the blood to the brain but also the cross-talk between the CNS and the rest of the organism [105]. When BBB function is altered, the effects are detrimental: BBB breakdown leads to edema, neuroinflammation, and neurodegeneration [107]. BBB alterations have been found in a number of neurological conditions, including ASD [108], mental disorders [109], and neurodegenerative diseases, such as MS, AD, and PD [110]. Moreover, recent studies have suggested that BBB dysfunction may be an early biomarker of neurodegenerative disorders, including MS, AD, and PD, since evidence of higher BBB permeability was found in patients before the occurrence of neurodegenerative symptoms [111]. The precise mechanisms that lead to an increased BBB permeability in neurological disorders are still unclear. However, it is likely that high levels of pro-inflammatory factors in the blood might be a possible cause [112-115].

Recently, the microbiota has been indicated by evidence as a factor capable of interfering with the BBB. Since BBB integrity is negatively affected by inflammation, and dysbiosis can lead to a systemic inflammatory reaction, the idea of a potential impact of gut microbiota on BBB permeability is recently taking hold. Remarkably, several studies have shown that gut microbiota derived-factors can modulate BBB permeability. For instance, SCFA has been shown to up-regulate brain endothelial cells' tight junction expression, therefore decreasing BBB permeability [116]. Moreover, SCFA was found to have a protective effect on BBB integrity by acting on brain endothelial cells and microglia [117, 118] and improving neuroinflammation [119]. Other factors that seem to have a protective effect on BBB are indoles that some bacterial species produce from tryptophan and vitamins [120]. Conversely, other factors like pro-inflammatory cytokines, LPS, and quinolinic acid, a microbial metabolite

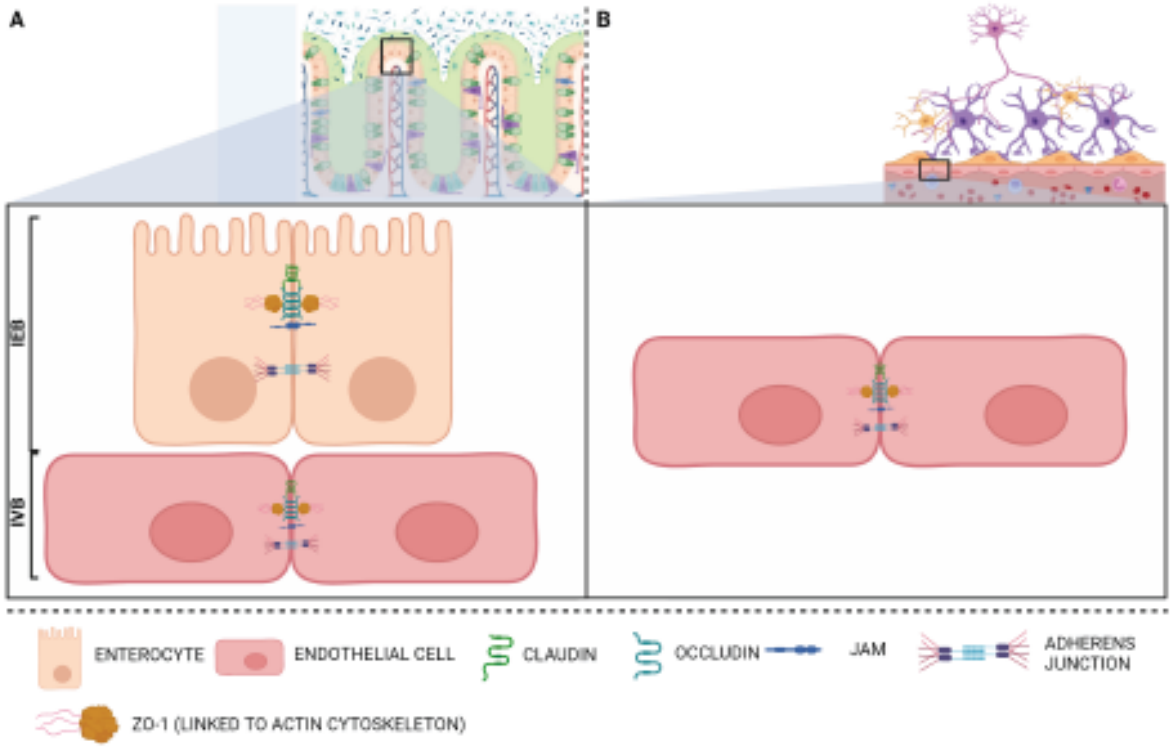

Fig. (3). Junctional systems in Intestinal Barrier and Blood-Brain Barrier. Starting from the apical region of the Intestinal Barrier (panel A) and Blood-Brain Barrier (panel B) cells, first the tight junctions and then the adherens junctions are found. Both IB and BBB share the same junctional proteins, *i.e.*, claudins, occludin, Junctional Adhesion Molecules (JAM), and zonula occludens families (ZO-1 is represented) for tight junctions and cadherins, α- and β-catenin, plakoglobin and other adaptor proteins for adherence junctions. IEB = Intestinal Epithelial Barrier; IVB = Intestinal Vascular Barrier. Image created with Biorender.com. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

derived from tryptophan, were linked to BBB breakdown [121, 122]. Therefore, different microbial species may have different impacts on BBB permeability. Additionally, factors that have a protective effect on BBB, like short-chain fatty acids, are often found depleted in CNS disorders where dysbiosis has been observed, whereas factors that induce BBB breakdown were augmented [123]. Further studies are necessary to clarify the impact of gut microbiota on BBB, but the rising evidence that links dysbiosis to augmented BBB permeability offers a new and fascinating perspective on how the microbiota-gut-brain axis can affect CNS disorders.

5. BBB AND IB: COMPARISON OF THE TWO BARRIERS

Although their function and anatomical location are obviously different, BBB and IB share some common molecular features (Fig. **3**). As mentioned before, the components of the BBB are the brain endothelial cells, pericytes, and the astrocytic end-feet. During development, pericytes are recruited directly by endothelial cells, forming the vascular unit of BBB. On the other hand, astrocytes connect the vascular unit to neurons, thus forming the NVU. Notably, the perivascular space between the vascular unit and the astrocytes, occupied by the basement membrane secreted by endothelial cells and pericytes, contains resident immune cells, such as microglia and macrophages. Constant communication between the NVU and the other cells in the brain assures the correct BBB function [124-127]. Likewise, the IB has an NVU of blood capillaries associated with pericytes and the enteric glial cells, likely influencing the endothelial barrier functions similarly to astrocytes. Enteric glia and astrocytes are spatially distinct, yet their function appears to be overlapping. In this respect, transplanting the enteric glia into the damaged spinal cord of rats was demonstrated to accelerate the repair of the damage and BBB reconstitution [128]. In contrast to CNS, however, the intestinal immune cells do not reside in the neurovascular unit of IB but are found in the *lamina propria* and infiltrate the NVU only in case of inflammation [129]. Another difference between BBB and IB is the size of the molecules that can diffuse through them: 4 kDa molecules get across the IB, whereas BBB can allow passage of eight-fold larger molecules (400–600 kDa). This difference between the two barriers depends on distinct functions. Indeed, the IB not only acts as a defense mechanism but is also involved in the adsorption of nutrients. Whereas, the main role of BBB is to maintain the specific CNS milieu, protecting it from the frequent changes of the bloodstream and ensuring nutrient supply to the brain. A remarkable observation is that gut and brain epithelial/endothelial cell seal-

ing is similar in terms of junctional components [130, 131]. In both IB and BBB, starting from the apical region of cells, tight junctions are found. Tight junctions include various proteins, such as occludin, claudins, *zonula occludens*, tricellulin, cingulin, and junctional adhesion molecules. These proteins interact with each other and the cytoskeleton, forming a dynamic entity programmed to rapidly open or seal the endothelial monolayer [132]. Below the tight junctions, the adherens junctions are found. Adherens junctions are composed of cadherins, α- and β-catenin, plakoglobin and other adaptor proteins connected to the cytoskeleton and play a key role in the mechanical coupling between neighboring cells [133]. Recent transcriptome analysis showed that the gene expression levels of tight junctions and adherens junction components, such as junctional adhesion molecule-A, junctional adhesion molecule-B, endothelial cell adhesion molecule, zonula occludens-1 and zonula occludens-2, VE-cadherin, α-catenin, β-catenin, p120, and plakoglobin, were similar in the endothelial cells of brain, colon, and small intestine [131]. This similarity has great translational interest with the pathophysiology of permeability-associated disorders of CNS, as agents that can permeabilize the IB and could potentially affect the BBB.

6. METHODS TO ASSESS IB AND BBB PERMEABILITY

The similarities between IB and BBB at structural and molecular levels suggest that whenever damage occurs to one, the other might also be involved. Moreover, since dysbiosis is starting to be observed in many CNS pathologies, the speculation that disruption of IB could precede BBB breakdown and therefore be the cause or, at least, a risk factor for developing CNS disorders is starting to rise too. This hypothesis is called the "leaky gut-leaky brain" theory. Thus, we suggest that assessing both IB and BBB permeability in patients with neurological disorders is, on the one hand, necessary to clarify the "leaky gut-leaky brain" theory and, on the other hand, might represent a new frontier in understanding CNS pathologies. Hence, we report the current techniques to measure IB and BBB, respectively.

6.1. Methods to Assess IB Permeability

IB permeability can be evaluated by orally administrated tracer molecules or by appraising the proposed IB permeability biomarkers so far. The most frequent method to assess IB permeability is through the lactulose and mannitol test [134], which involves the simultaneous oral administration of these two non-digestible sugars and the measurement of their excretion in urine over a defined period of time. Intestinal permeability is measured as the ratio of excretion percentage of lactulose/mannitol in the urine. The higher this ratio is, the more permeable the membrane results. This method is based on the concept that under normal conditions, due to its large size, LAC absorption is restricted. Other used probes are ^{51}chromium-EDTA, 1 kDa PEG, and other sugars, such as the multi-sugar test. Even though these tests are considered accurate, they are time-consuming, mostly complicated for patients, cannot be performed retrospectively and still have limited validity since reference values are not well defined [135, 136]. For this reason, various endogenous factors have been proposed as IB permeability biomarkers. Among these, much interest is being driven by zonulin. Zonulin belongs to the haptoglobin family of acute-phase reaction proteins and has been proposed to modulate intestinal permeability by disassembling tight junctions. Plasma and especially fecal levels of zonulin have been suggested to mirror intestinal permeability and, to date, several gastrointestinal conditions show increased zonulin levels [137]. Another proposed biomarker for IB permeability is fecal albumin. Albumin has a very large molecular weight (67 kDa) and, therefore, can pass IB and reach the gut lumen only in the occurrence of IB damage. For this reason, fecal albumin is starting to be used as an IB biomarker, especially in animal models [138]. Other proteins suggested as potential IB biomarkers are lipopolysaccharide-binding protein, intestinal fatty acid-binding protein, calprotectin, citrulline, glucagon-like peptide 2, α-1-antitrypsin, and lipocalin 2. However, further studies are required to validate the data so far obtained on these factors [136-139]. Even though any of the proposed biomarkers can be considered specific and sensible enough to determine IB integrity on its own, their evaluation can offer an important supplement to the test based on orally administrated tracers.

6.2. Methods to Assess BBB Permeability

BBB permeability can be investigated by quantitatively or qualitatively evaluating exogenous tracers or endogenous factors and imaging methods. Unfortunately, the methods that are currently considered the most sensitive must be performed *ex-vivo*. For instance, the most used assay is Evans blue tracer intravenous injection. Evans blue is an azo dye that has a high affinity for serum albumin. Since albumin cannot normally cross the BBB, detecting Evans blue in the brain parenchyma by fluorescence microscopy indicates BBB breakdown [140]. Other exogenous tracers that are currently used are radiolabeled sucrose, sodium fluorescein, horseradish peroxidase, and dextran. Another method to assess BBB permeability is by analyzing tight junction protein expression [141, 142], but this is only possible by evaluating brain samples *ex vivo*. Unfortunately, the BBB permeability assays that can be performed *in vivo* are significantly less sensitive and need further validation studies. The endogenous proteins that have been proposed as BBB permeability biomarkers *in vivo* are albumin, immunoglobulin G (IgG), and S100β [143]. Since albumin and IgG cannot cross the BBB in normal conditions, their detection in the cerebrospinal fluid indicates BBB leakage. Conversely, S100β is a protein secreted by glial cells that is plentiful in CNS but almost undetectable in serum without BBB dysfunction. Therefore, increased S100β serum levels have been associated with BBB breakdown [144, 145]. Lastly, BBB leakage can be detected *in vivo* through imaging assays, such as Computed Tomography (CT), Near-Infrared Spectroscopy (NIRS), and Magnetic Resonance Imaging (MRI). However, only a few studies support imaging techniques to assess BBB permeability due to instrument cost, complicated procedures, and limited distinguishability [143].

It is notable that, compared to the variety of assays and biomarkers being investigated to assess IB permeability, the methods currently proposed to evaluate BBB permeability

are few and, most importantly, are, for the majority, not performable *in vivo*. For this reason, we believe it is necessary to spend further resources and energy to find new strategies to assess BBB integrity.

CONCLUSION

The clinical and scientific evidence supporting the existence of a bidirectional axis linking IB and BBB is constantly increasing. However, the proper molecular mechanisms linking these two systems have not been elucidated yet. Indeed, microbiota homeostasis seems to play an important role in maintaining both IB and BBB functions. Nevertheless, the studies that attempt to clearly demonstrate this hypothesis are still arguable and need further confirmation. This review suggests that drawing attention to barrier permeability might help clarify these dynamics. Indeed, IB and BBB share similar morphological characteristics, especially at the vascular level, and the same mechanisms, *i.e.*, inflammation and cytokine stress, seem to alter both barriers by affecting endothelial junctional proteins. Therefore, an inflammatory status may affect the IB and then spread to the brain by altering the BBB as well. In many neurological diseases, a possible linking factor is thought to be gut microbial dysbiosis. However, whether dysbiosis is the cause or a consequence of inflammation affecting both IB and BBB remains to be established. In particular, the molecular processes that are activated directly and indirectly by bacteria promoting barrier leakage or strengthening represent targets of investigation of crucial importance for future therapeutic implications. The role of gut microbiota in neurological diseases should be clarified to decipher the relationship between dysbiosis, barriers, and neurological diseases. In this context, new therapeutic approaches, such as fecal microbiota transplantation which is already used to treat several GI diseases, may be applicable to treat neurological disorders. Additionally, it could be relevant to assess IB permeability in large cohort studies of neurological patients to verify the occurrence of a leaky gut. All in all, it is crucial to identify and validate at least one common strategy/biomarker to assess BBB permeability *in vivo* in order to verify if neurological patients display a leaky brain. This possibility may not only improve the understanding of neurological diseases but also help in finding new therapeutical targets.

LIST OF ABBREVIATIONS

FITC	=	fluorescein isothiocyanate
IEB	=	Intestinal Epithelial Barrier
IVB	=	Intestinal Vascular Barrier
PVAP	=	Plasmalemma Vesicle Associated Protein

CONSENT FOR PUBLICATION

Not applicable.

FUNDING

This work was financially supported by the Italian PRIN-MIUR grants (to M.Y.F., L.M.), by Intesa S. Paolo Foundation grant (to L.C.), Fondazione Cassa di Risparmio di Bologna (to S.R.), and Fondazione del Monte di Bologna e Ravenna grant (to L.C.).

CONFLICT OF INTEREST

Dr. Matilde Follo is the editorial advisory board member of the journal Mini-Reviews in Medicinal Chemistry.

ACKNOWLEDGEMENTS

The Cellular Signalling Laboratory (DIBINEM) of the University of Bologna is supported by Ministero dell'Istruzione, dell'Università e della Ricerca – Dipartimenti eccellenti on the project Personalized medicine.

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