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THE ROLE OF GUT MICROBIOTA IN CEREBROVASCULAR DISEASE AND RELATED DEMENTIA

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Abstract

In recent years increasing evidence suggests that commensal microbiota may play an important role in health and disease, including cerebrovascular disease. Gut microbes impact physiology, at least in part by metabolizing dietary factors and also host-derived substrates and then generating active compounds including toxins. The purpose of the current review is to highlight the complex interplay between microbiota, their metabolites and essential functions for human health ranging from regulation of the metabolism and the immune system to modulation of brain development and function. We also discuss the role of gut dysbiosis in cerebrovascular disease, specifically in acute and chronic stroke phases and the possible implication of intestinal microbiota in post-stroke cognitive impairment and dementia, and we identify potential therapeutic opportunities of targeting microbiota in this context.

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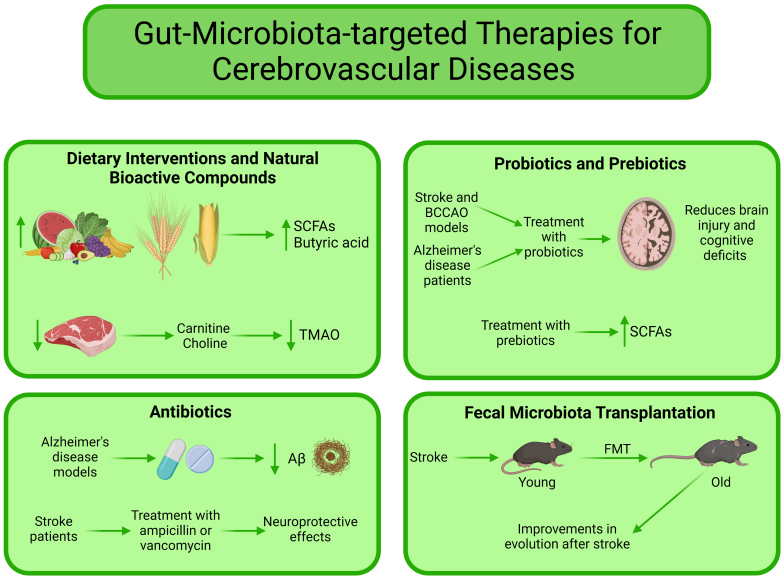

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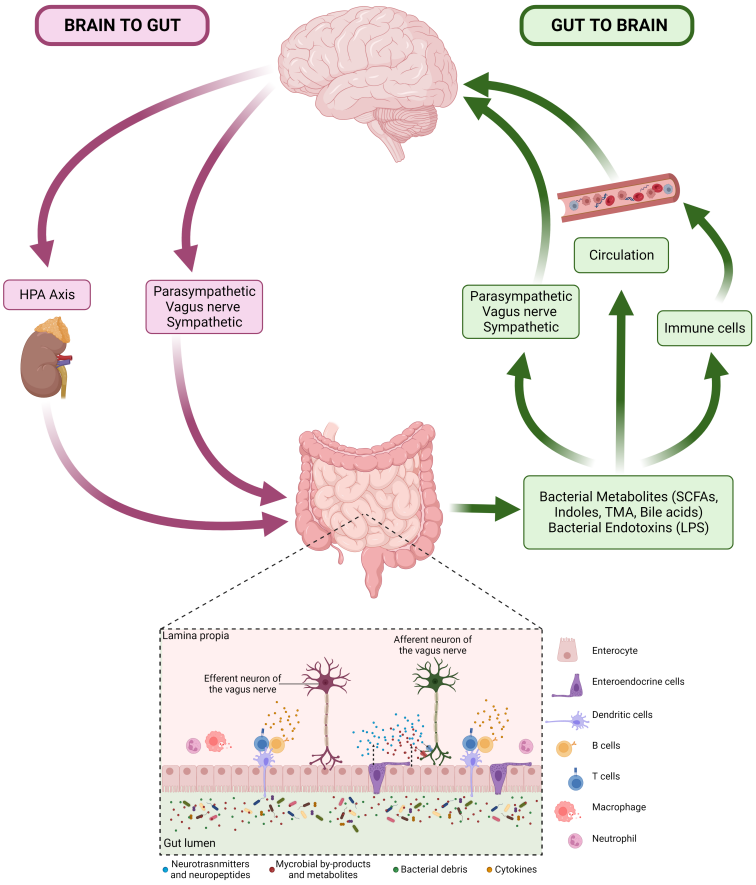

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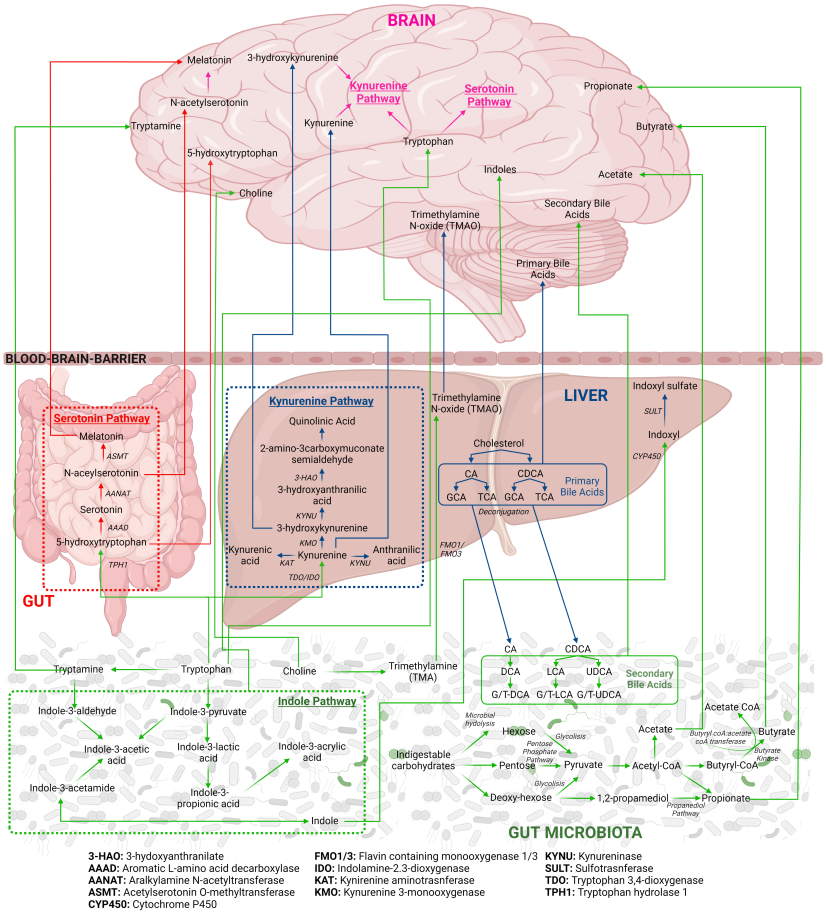

3-HAO: 3-hydoxyanthranilate
AAAD: Aromatic L-amino acid decarboxylase
AANAT: Aralkylamine N-acetyltransferase
ASMT: Acetylserotonin O-methyltransferase
CYP450: Cytochrome P450
FMO1/3: Flavin containing monooxygenase 1/3
IDO: Indolamine-2.3-dioxygenase
KAT: Kynirenine aminotrasnferase
KMO: Kynurenine 3-monooxygenase
KYNU: Kynureninase
SULT: Sulfotrasnferase
TDO: Tryptophan 3,4-dioxygenase
TPH1: Tryptophan hydrolase 1

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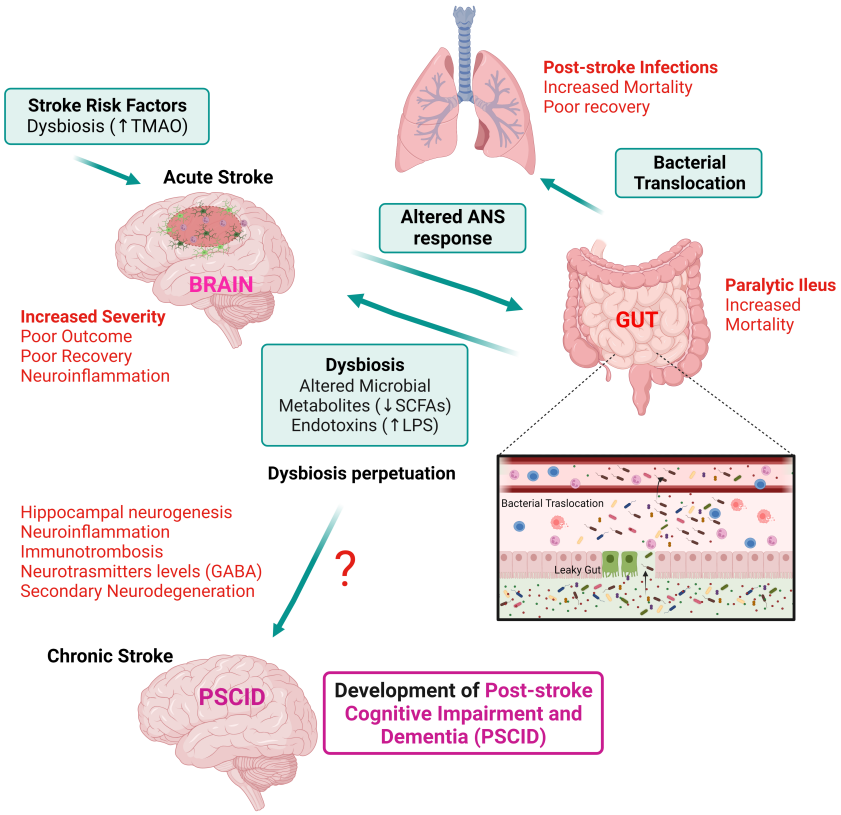

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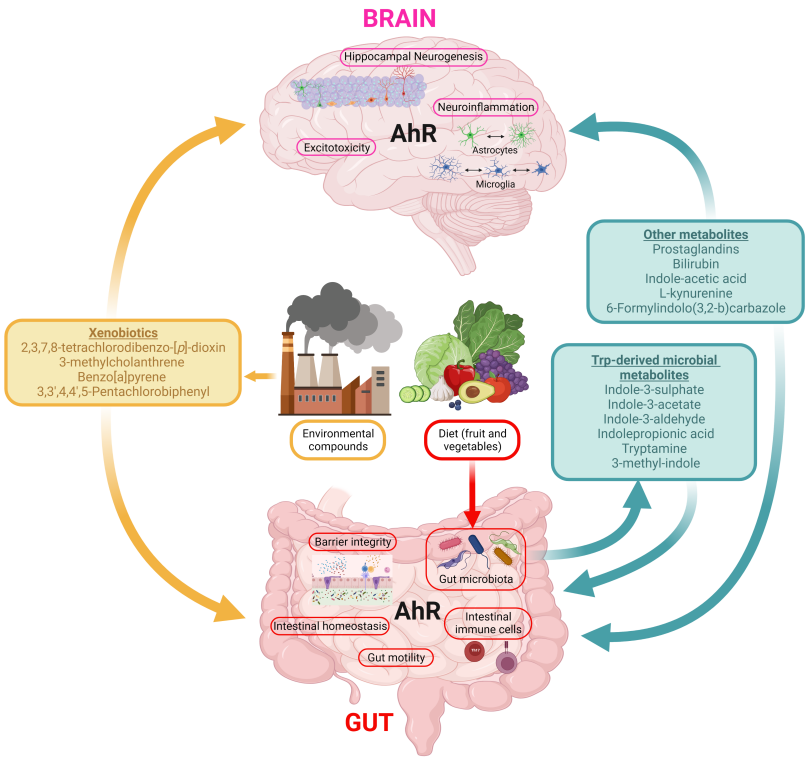

THE ROLE OF GUT MICROBIOTA IN CEREBROVASCULAR DISEASE AND RELATED DEMENTIA

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KEYWORDS: Stroke, Dementia, Microbiota, Gut, post-stroke cognitive impairment

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ABSTRACT

In recent years increasing evidence suggests that commensal microbiota may play an important role in health and disease, including cerebrovascular disease. Gut microbes impact physiology, at least in part by metabolizing dietary factors and also host-derived substrates and then generating active compounds including toxins. The purpose of the current review is to highlight the complex interplay between microbiota, their metabolites and essential functions for human health ranging from regulation of the metabolism and the immune system to modulation of brain development and function. We also discuss the role of gut dysbiosis in cerebrovascular disease, specifically in acute and chronic stroke phases and the possible implication of intestinal microbiota in post-stroke cognitive impairment and dementia, and we identify potential therapeutic opportunities of targeting microbiota in this context.

ABREVIATIONS

5-hydroxytryptamine receptor 1 (5HT1)
Adrenocorticotrophic hormone (ACTH)
Alzheimer's disease (AD)
Angiotensin-II (Ang-II)
Aminoacid (AA)
Aryl Hydrocarbon Receptor (AhR)
Autonomic nervous system (ANS)
Bile acids (BAs)
Blood-brain carrier (BBB)
Brain-derived neurotrophic factor (BDNF)
Central nervous system (CNS)
Corticotrophin receptor hormone (CRH)
Pathogen-associated molecular patterns (PAMPs)
Disease-associated microglia (DAM)
Dopamine (DA)
Enteric nervous system (ENS)
Enteroendocrine cells (EECs)
Faecal microbiota transplants (FMT)
γ- aminobutyric acid (GABA)
Gastrointestinal tract (GIT)
Germ-free (GF)
Glucagon-like peptide-1 (GLP-1)
Gut microbiota (GM)
Hypothalamic-pituitary-adrenal (HPA)
Irritable bowel syndrome (IBS)
Lipopolysaccharides (LPS)
Major depressive disorder (MDD)
MCAO (middle cerebral artery occlusion)
Modified Rankin Scale (mRS)
Multiple sclerosis (MS)
Noradrenaline (NA)
Nucleus tractus solaritarius (NTS)
Parkinson's disease (PD)
Pattern recognition receptors (PRR)
Post-stroke cognitive impairment and dementia (PSCID)
Postsynaptic density protein-95 (PSD-95)
Serotonin (5-HT)
Short-chain fatty acids (SCFAs)
Tryptophan (Trp)
Toll-like receptors (TLRs)
Trimethylamine N-oxide (TMAO)
Vagus nerve (VN)
Vascular dementia (VaD)

1. INTRODUCTION

Dementia is characterized by a deterioration in cognitive function beyond what might be expected from the usual consequences of biological ageing. This impairment in mental capacity supposes a dramatic reduction in quality of life and clearly compromises everyday tasks and independent living. Most dementias occur in individuals with advanced age. By 2050, this older age group is expected to increase by around 21% (WHO., 2017–2025). As a consequence of this population ageing and of the absence of efficient treatments, the number of affected individuals with dementia is estimated to rise from 50 million in 2018 to approximately 150 million in 2050 (WAR., 2018.; WHO., 2017–2025). This alarming scenario anticipates that dementia will become one of the major threats for public healthcare systems with a high socioeconomic impact worldwide. Therefore, there is a critical need for the development of therapies and strategies for achieving an optimal brain health and ageing process including preventing dementia and cognitive decline. Among the different types of dementia, vascular dementia (VaD) is the second leading type of dementia after the most prevalent one, Alzheimer's disease (AD) (WAR., 2018.; Alzheimers Dement. 2022). Importantly, cerebrovascular lesions are commonly found in the pathophysiology of AD patients (Iadecola, 2017; 2019; Loeb, 1993) up to the point that mixed VaD/AD pathology accounts for more than 50% of the demented subjects, reinforcing the role of vascular dysfunction as a critical component in the development of dementia (Azarpazhooh et al., 2018). Common vascular risk factors such as hypertension, atherosclerosis and cerebrovascular disease play key roles in the occurrence of dementia and cognitive decline (Iadecola, 2013; Iadecola et al., 2019). In this sense, stroke, a leading cause of death and disability worldwide (WSO., 2012) is a major risk factor for vascular dementia and AD (Rost et al., 2022). In the past few decades, advances in prevention, management and exhaustive healthcare resulted in reduced stroke mortality (Tsao et al., 2023); as a consequence, stroke is considered a chronically disabling disease, with many stroke survivors displaying a poor long-term functional outcome with motor, cognitive, and psychiatric impairments. Cognitive deficits are present in around 70% of stroke survivors, depending on stroke type, definition, and time point of assessment. Additionally, more than one-third of patients may develop post-stroke cognitive impairment and dementia (PSCID) later after stroke (Mijajlović et al., 2017; Rost et al., 2022). PSCID is defined by the presence of cognitive impairments manifesting in the 3 to 6 months after both ischemic or hemorrhagic stroke, and includes deficits specific to the lesion site, as those due to strategic infarcts in brain structures like the hippocampi, thalami, and key cortical regions, those that may have preceded the stroke, and those due to secondary process or neurodegeneration. The development of PSCID is likely caused by a combination of primary infarct size and location combined with the interplay of multiple factors that contribute to brain repair against those that may promote a secondary neurodegeneration (Mijajlović et al., 2017; Rost et al., 2022). Of note, accumulating evidence has revealed that microbiota-gut-brain axis plays an important role in the development and progression of different human pathologies affecting the CNS, including late-life cognitive impairment, and AD (Cryan, O'Riordan, Sandhu, Peterson, & Dinan, 2020; Morais, Schreiber, & Mazmanian, 2021). Changes in the gut microbiota are also seen in response to stroke, which may

worsen stroke severity and impair recovery after injury. Therefore, it is tempting to speculate that intestinal microbiota can also play role in the development of vascular cognitive decline, and especially in the development of PSCID as suggested for another types of dementias.

Hence, the main objective of the current review is to describe the existing associations between gut microbes and brain functioning after stroke, and to discuss the possible implication of intestinal microbiota in the development of PSCID. We first highlight the complex interplay between gut microbiota (GM), their metabolites and essential functions for human health, and describe the most important evidence that supports a direct role of GM in modulating brain functioning. We next summarize the bidirectional communication that exists between the brain and the gut including microbial metabolites. Finally, we analyse the role of gut dysbiosis during acute and chronic stroke phases, discuss the possible implication of GM in the development of PSCID and evaluate potential therapeutic opportunities that target microbiota in this context.

2. THE MICROBIOTA-GUT BRAIN AXIS IN PHYSIOLOGY

The microbiota-gut-brain axis represents a system that allows a bidirectional communication between brain and gut microbes. The gut microbiota (GM) includes trillions of symbiotic microorganisms like bacteria, archaea, viruses and fungi (Knight & Girling, 2003; Quigley, 2013), most of them commensal or mutualistic organisms, that colonize the digestive tract mostly at birth. Intestinal microbiota is generally stable over time, in part owing to the presence of a core microbiome although it can also display high temporal variability and personalization (L. Chen et al., 2021; Stewart et al., 2018; Valles-Colomer et al., 2023). Its relevance for the host is clearly reflected if we consider that microbiome, that is, all intestinal microbial genes, comprises more than one order of magnitude higher in genes than the human genome (Cryan et al., 2019; Quigley, 2013). Thus, the host microbiome influences not only the physiology of the gastrointestinal tract (GIT), like the mucosal immunity, protection against outside pathogens, etc., but also modulates the function of remote organs like the immune and the central nervous system (CNS) (Cryan et al., 2020; Fan & Pedersen, 2021; McCarville, Chen, Cuevas, Troha, & Ayres, 2020; Morais et al., 2021; Needham, Kaddurah-Daouk, & Mazmanian, 2020; Zheng, Liwinski, & Elinav, 2020). GM displays multiple metabolic actions, metabolizing essential substances like amino acids, vitamins, bile acids and different dietary compounds into a variety of metabolites, some of them with neuroactive properties, which are absorbed into the systemic circulation and serve as mediators of GM actions on distant tissues such as the brain (Quigley, 2013). All these microbiota functions depend on the fine balance between the relative abundance, diversity and composition of different microorganisms that colonize the intestine. In humans, GM is mainly composed by four categories of microbes, being the most prevalent, *Firmicutes, Bacteroidetes, Proteobacteria* and *Actinobacteria* (Consortium, 2012). Gut microorganisms show host specificity in their composition and function, so that the relative distribution of gut bacteria and archaea is unique to an individual and is influenced by factors like age or genetics but also by environmental ones such as diet, drugs, stress and lifestyle (Asnicar et al., 2021; Falony et al., 2016; Ghosh, Shanahan, & O'Toole,

2022; Valles-Colomer et al., 2023). In addition, bacterial load and diversity varies along the GIT, so intra-individual differences are found between the upper and lower GIT in both abundance and composition (Vuik et al., 2019) (Consortium, 2012). Given this heterogeneity, it is difficult to define a standard reference of GM from healthy people but it is believed that a healthy GM is characterized by a high taxa diversity, microbial gene richness and stable microbiome functional cores (L. Chen et al., 2021; Valles-Colomer et al., 2023). This is fundamental for claiming *dysbiosis*, that is, a term used to define a pathological dysregulation in the intestinal microbiome, which is associated with a variety of chronic diseases ranging from gastrointestinal disorders (like irritable bowel syndrome, IBS) to cardiovascular and CNS disease, making this condition a very attractive therapeutic target in pathological situations. The altered composition of microbiome determines the concentration of microbial metabolites, as well as neurotransmitters/neuromodulators which are released into circulation (Consortium, 2012; Fan & Pedersen, 2021; Honarpisheh, Bryan, & McCullough, 2022; Peh, O'Donnell, Broughton, & Marques, 2022; Tang, Kitai, & Hazen, 2017; Vogt et al., 2017). Thus, microbiota is a contributing factor to different diseases implicating, in some circumstances, the absence of normal metabolites generated by the healthy microbiota and, in others, the gain of high levels of metabolites with pathological actions that are generated by damage-associated microbiota.

In parallel to dysbiosis, a *leaky gut* (that is, a reduction of intestinal barrier integrity or increased permeability) can be observed in different pathological contexts. The mammalian intestine has a single epithelial layer that physically separates the microbiota, which are located in the lumen, from the rest of the body (**Figure 1**). This epithelial barrier is fundamental for maintaining gastrointestinal health, since it avoids gut microbes from entering into the circulation. An increase in gut permeability may lead to *bacterial translocation*, promoting the passage of bacteria and of excessive microbial metabolites into blood which may reach peripheral tissues such as liver, spleen, kidney, and lung. In fact, bacterial translocation has been observed after stroke and it is believed to contribute to post-stroke infections (Caso et al., 2009; Stanley et al., 2016; Tuz, Hasenberg, Hermann, Gunzer, & Singh, 2022; Wen et al., 2019). But even in the absence of translocation, the leaky gut may result in an increase of microbial metabolites in the blood such as trimethylamine N-oxide (TMAO), short-chain fatty acids (SCFAs), indoles, kynurenines, and different neurotransmitters which cannot be efficiently removed by the liver and then might directly affect the CNS. Most of these metabolites cannot cross the blood-brain carrier (BBB) and accumulate in the blood, some others increase BBB permeability, facilitating the entrance of neuroactive microbial compounds. In addition, this accumulation of neuroactive microbial metabolites in the blood is especially relevant in those situations wherein alterations in the BBB function occur, like aging and PSCID, mixed dementia and even AD (Connell et al., 2022; Honarpisheh et al., 2022; Mijajlović et al., 2017; Morais et al., 2021; Rost et al., 2022). A dysfunctional BBB facilitates that microbial metabolites reach the CNS and act on different neural substrates mediating both beneficial and pathological responses.

3. A ROLE FOR GUT MICROBIOTA ON BRAIN FUNCTION

Different animal models and interventions are commonly used to interrogate the role of the GM in physiological host functions. Among these, germ-free mice, antibiotic usage, faecal microbiota transplants and probiotics/prebiotics administration are the most common. These strategies are also especially relevant for manipulating GM under CNS pathological contexts and have been also widely used for exploring the role of gut microbes on cognition. At this regard, the use of *germ-free (GF) mice*, i.e., mice that have not been exposed to microorganisms since birth, demonstrated that the CNS is altered at multiple levels in the absence of microbiota, supporting the existence of a functional microbiota-gut-brain axis. GF mice display deficits in different cognitive domains including anxiety, locomotion, exploratory and social behaviors, learning and memory affecting mainly, although not exclusively, the hippocampus, the amygdala and the striatum (Connell et al., 2022; Cryan et al., 2019; Cryan et al., 2020). This selectivity for specific regions suggests that microbial influence might differ among brain regions. The neurochemistry is also different in GF mice with changes in neurotransmitters like serotonin (5-HT), noradrenaline (NA) and dopamine (DA) (Bercik et al., 2011) and in synaptic plasticity proteins such as postsynaptic density protein-95 (PSD-95), synaptophysin, 5-hydroxytryptamine receptor 1 (5HT1), brain-derived neurotrophic factor (BDNF) and c-fos (Bercik et al., 2011; Clarke et al., 2013). In addition, animals lacking microbiota also show important alterations in physiological processes including neurogenesis, myelination, dendritic growth, BBB permeability and even display a reduced microglial response compared to those animals hosting commensal bacteria (Diaz Heijtz et al., 2011; Gareau et al., 2011; Morais et al., 2021). A great advantage of GF mice is that they allow for specific bacterial colonization, making then a commonly used strategy for studying whether one or a few known bacteria alter brain functioning. In addition, GF mice are also used for the generation of humanized microbiota mice, that is, a GF mouse transplanted with human microbiota that allows to investigate in mice the contribution of the specific human GM to brain diseases (Park & Im, 2020). A second common approach used for investigating how GM modulates cognition is *the use of antibiotics* (Cryan et al., 2019; Desbonnet et al., 2015; Fröhlich et al., 2016; Winek et al., 2016). A critical aspect when using antibiotics is whether they are absorbable or not. If they are, they may enter into the circulation, cross BBB and exert direct effects on the CNS function and behavior (as metronidazole or minocycline acting on microglial cells). By contrast, non-absorbable antibiotics such as vancomycin do not cross BBB and become concentrated in the GIT, excluding direct effects of antibiotics on the brain or other distant tissues (Cryan et al., 2019). Chronic antibiotic administration for depleting microbiota has been shown to exert effects on different paradigms such as sociability, memory and anxiety-like behaviors in mice (Cryan et al., 2020; Desbonnet et al., 2015; Fröhlich et al., 2016). Importantly, microbiota elimination produces changes in some tryptophan-derived metabolites with neuroactive properties like serotonin and L-kynurenine. Again, as previously shown for GF mice, antibiotics also alter some synaptic proteins like BDNF, serotonin transporter and neuropeptide Y (Cryan et al., 2019; Cryan et al., 2020; Desbonnet et al., 2015). A great advantage of antibiotics is that they are the perfect tool to mimic the clinical scenario in humans. In fact, antibiotic administration has

been demonstrated to promote behavioral changes not only in animals but also in humans (Morais et al., 2021). A third strategy, used in clinics for treating *Clostridium difficile* infection (van Nood et al., 2013), is gut bacterial colonization by *faecal microbiota transplants (FMT)* which basically consists of the transfer of the GM from one subject to another. This procedure can be done in rodents by oral administration of faecal material, wherein the donor microbiome colonizes the recipient GIT. This colonization process is facilitated by using as recipient a GF mouse or antibiotic-treated mouse although, in some cases, a passive GM transfer is used. As a result, different studies have demonstrated that behaviors like depression and anxiety can be transferred from the host to the faecal microbiota recipient (Bruce-Keller et al., 2015; Kelly et al., 2016). For instance, the study of Bercik and collaborators (Bercik et al., 2011) took advantage of the well documented differences in both behaviour and GM composition of two common laboratory mice strains. They observed that when BALB/C mice, a strain which displays high anxiety-like behavior, were colonized with gut microbes from Swiss mice (a very calm mice strain), colonized BALB/C mice exhibited a decreased anxiety supporting the role of microbiota in brain functions (Bercik et al., 2011). Finally, administration of *prebiotic, probiotics, and dietary substrates* has also provided important cues about the existence of microbiota-gut-brain axis. Administering prebiotics such as some dietary fibers and resistant starches as well as probiotics modulates behavior in both rodents and humans and promotes changes in learning, depression, anxiety, general hypothalamic neuronal activity and stress, alongside changes in immune markers, hippocampal synaptic efficacy and tryptophan metabolism (Cryan et al., 2019; Koh, De Vadder, Kovatcheva-Datchary, & Bäckhed, 2016; Markowiak & Śliżewska, 2017; Quigley, 2013). In addition, probiotics, that is, live bacteria that, when ingested in adequate amounts, are beneficial for the host health, have also been employed in humans demonstrating that administration of specific strains has beneficial effects on cognitive performance. In this regard, the administration of *Lactobacillus*, the most widely used strain was capable of preventing memory deficits in GF mice (Hsiao et al., 2013; Markowiak & Śliżewska, 2017; Quigley, 2013). Finally, diet contents and quantity have a major role in shaping the gut microbiota composition, microbial derived metabolites and thereby how gut microbes modulate host functions and hence brain and behavior. As they say, “we are what we eat”, linking dietary signals with the microbiota-gut-brain axis.

4. PATHWAYS OF COMMUNICATION BETWEEN GUT MICROBIOTA AND CNS

All previous evidence widely supports that the resident intestinal microbiota can exert considerable influence over host behavior by modulating brain function through different pathways. Of course, this communication system is bidirectional, that is, the brain can influence basic gastrointestinal and immune-related functions. A clearly example of this complex interaction between the gut and the brain is how the prognosis of different chronic gastrointestinal illnesses is directly influenced by factors like stress and depressive behavior. These emotional factors may also modify the microbiota composition by influencing the integrity of the gut epithelial barrier and altering gut motility, then potentially contributing to dysbiosis, which highlights the intricate mechanisms that control this bidirectional modulation. This may explain, for instance, that patients

with IBS, an intestinal disease characterized by low gut bacterial diversity, are also frequently comorbid with different psychiatric illness like depression (Cryan et al., 2020; Morais et al., 2021; Needham et al., 2020). Gastrointestinal dysfunction such as nausea, dysphagia and defecatory problems are also common symptoms in different neurodegenerative disorders like Parkinson's disease (PD) and multiple sclerosis (MS), (Morais et al., 2021). Post-stroke intestinal ileus is one of the complications observed in stroke patients (Tuz et al., 2022). A recent study provided genetic insight into the gut-brain relationship, implicating shared but non-causal genetic susceptibility of disorders affecting GIT with AD's risk (Adewuyi, O'Brien, Nyholt, Porter, & Laws, 2022). Therefore, there is a clear pattern of co-occurrence of neurological diseases including dementia, with GIT disorders or dysfunction probably suggesting that shared genetics and common biological pathways may explain the association. The microbiota-gut-brain axis allows intestinal microbiota to communicate with the brain and the brain with the gut, and involves the autonomic nervous system (ANS), specifically the enteric nervous system (ENS) and the vagus nerve (VN), the neuroendocrine system, the hypothalamic-pituitary-adrenal (HPA) axis, the immune system and, finally, metabolic pathways and microbial metabolites (Bonaz, Bazin, & Pellissier, 2018; Carabotti, Scirocco, Maselli, & Severi, 2015; Cryan et al., 2019) (see **Figure 1**).

4.A) HOW BRAIN CONTROLS GUT FUNCTION: The CNS may directly modulate gut function through the innervation of the gut wall by the ANS (both sympathetic and parasympathetic) and the ENS, a specialized independent nervous system of the GIT that is structured into the submucosal and myenteric plexus. The ENS is responsible for the coordination of different gut functions like, for instance, gut motility. Different factors like brain neurotransmitters, hormones and cytokines may activate the ENS, the efferent fibers of the VN and also some sympathetic innervation that, in turn, may influence gut motility and permeability, microbiota composition, and mucosal immune response (Bonaz et al., 2018; Carabotti et al., 2015; Cryan et al., 2019; Mayer, Tillisch, & Gupta, 2015). Neurotransmitters can also act directly on gut bacteria influencing bacterial metabolism, proliferation, and virulence. In addition, the HPA is implicated in controlling gut barrier integrity. Stress responses activate the HPA axis by acting on hypothalamic neurons and making them to secrete corticotrophin receptor hormone (CRH) which produces the release of adrenocorticotrophic hormone (ACTH). The adrenal gland is then stimulated for the synthesis and release of cortisol which acts, for instance, on neuroimmune signaling responses affecting intestinal barrier integrity (Bonaz et al., 2018; Carabotti et al., 2015; Cryan et al., 2019; Mayer et al., 2015).

4.B) HOW GUT AND MICROBIOME CONTROL THE CNS: In the other direction, that is, the gut controlling the CNS, 3 main pathways have been described: *1)* Direct neural mechanisms, *2)* Cellular immune function and *3)* Systemic circulatory factors and microbial metabolites.

Communication through direct neural mechanisms: One of the main neuronal communication pathways by which the gut controls the CNS involves the afferent fibers of VN. The VN is able to sense gut microbiota, to transfer this information to the CNS where it is integrated and then, to

generate an adapted or inappropriate response in the gut (Bonaz et al., 2018; Cryan et al., 2019; Cryan et al., 2020). The VN is the most important component of the parasympathetic nervous system and is composed of both afferent (80%; from the gut to the CNS) and efferent (20%; from the CNS to the gut) fibers (Bonaz et al., 2018). Vagal afferent fibers are located along the gut wall innervating the muscle and mucosa layers but they are not in direct contact with the microbiota, located in the lumen. Then, vagal fibers can sense microbiota signals only indirectly, either through the diffusion of bacterial compounds or metabolites or by means of other cells located in the epithelium that relay luminal signals. The afferent fibers can be stimulated directly by microbiota metabolites and/or components like the vagal activation mediated by butyrate or even by bacterial products like lipopolysaccharides (LPS), which are sensed by Toll-like receptor 4 (TLR4) receptor located on vagal nerves (Bonaz et al., 2018; Carabotti et al., 2015; Cryan et al., 2019; Mayer et al., 2015). In addition, vagal afferents can also be stimulated indirectly by hormones released by the enteroendocrine cells (EECs) (which are also stimulated directly by microbiota) such as serotonin, glucagon-like peptide-1 (GLP-1), cholecystokinin, ghrelin and neuropeptide Y. These afferent vagal projections act mainly through the activation of the *nucleus tractus solaritarius* (NTS) located in the *medulla oblongata*. Projections from the NTS to the hypothalamus are clearly involved in regulating hormone release through hypothalamic neurons of the HPA axis (Bonaz et al., 2018; Bravo et al., 2011; Cryan et al., 2019). In addition, NTS projections can also reach the hypothalamus and the limbic forebrain (mainly hippocampus, amygdala and limbic cortex) and affect the way in which these regions influence appetite and food intake behavior. This communication may provide the neural network underlying the link between behavior and gut function in health (the so-called “stomach butterflies”, for instance) and disease (like IBS and depression). Vagal communication has been demonstrated to play a key role in modulating host behavior in different studies. In this sense, administration of *Lactobacillus rhamnosus* modulates anxiety-like behavior in mice and changes the expression of GABA receptors in brain areas associated with fear and emotions, such as amygdala and hippocampus (Bravo et al., 2011). Importantly, most effects of *L. rhamnosus* were abrogated in vagotomised mice, suggesting that the effects of the bacteria depend on neuronal communication to the brain. Moreover, the VN is also critical for mediating the beneficial effects of *Lactobacillus reuteri* in promoting social behavior in animal models of autism spectrum disorders (ASD) (Sgritta et al., 2019). In addition to the communication through the VN, a recent study using neuronal tracing techniques demonstrated that GF mice display increased activation of gut extrinsic neurons connecting the brainstem sensory nuclei and gut sympathetic neurons when compared to conventional mice (Muller et al., 2020), suggesting that microbiota may have an inhibitory effect on pathways implicated in the regulation of gut motility. In agreement, administration of SCFA-synthetizing bacteria also inhibits this neuronal pathway, supporting that GM can modulate neuronal pathways of the gut–brain axis through the production of specific microbial metabolites.

Immunological mechanisms for gut-brain communication: The GM is a critical factor for the development and function of the peripheral immune system and for the maturation of the intestinal mucosal immune system (Zheng et al., 2020). Signals from the GM also play important roles in

modulating the proper maturation and activity of microglia, the primary innate immune cells in the CNS. GM contributes to microglia homeostasis, probably through SCFAs actions. In fact, as commented before, GF and antibiotic-treated mice displayed important defects in microglial maturation which lead to impaired innate immune responses, showing increased numbers of immature microglial cells (determined by both transcriptional signature and morphological features of microglia) (Erny et al., 2015; Matcovitch-Natan et al., 2016). This provides a link of GM-mediated microglial control which might be of special relevance in dementias like in AD wherein dramatic changes in the molecular signatures of microglia have been described (the so-called *disease-associated microglia* -DAM- phenotype) (Butovsky & Weiner, 2018). Another important immune pathway, specially under pathological circumstances, involves either the activation of peripheral immune cells or the interaction of host mucosal surface cells by different microbiota products such as LPS and peptidoglycans. Pattern recognition receptors (PRR) present in host cells such as TLRs (Bryant C, 2019) recognize pathogen-associated molecular patterns (PAMPs), which then may stimulate and instruct the host immune response promoting the release of circulating cytokines and chemokines (Hsiao et al., 2013). Changes to systemic immunity drive altered immune signaling inducing directly neuroinflammation or promoting the migration of different types of immune cells form the periphery into the brain like T cells, monocytes and neutrophils (Benakis et al., 2016; Singh et al., 2016; Singh et al., 2018).

Communication through microbial synthesized-metabolites: Many GM-mediated effects in the CNS depend on hundreds of metabolites and bioactive molecules such as neurotransmitters, SCFAs, indoles, and bile acids that are produced by gut microbes and derived from the transformation of host or dietary products (Connell et al., 2022; McCarville et al., 2020; Peh et al., 2022). These metabolites may enter into the systemic circulation, travel to the brain and there, may influence the function of most part of neural populations including neurons, microglia and astrocytes or even different cellular components of the BBB (**Figure 2**).

Products of bacterial fermentation: SCFAs are the most-studied GM metabolites (Connell et al., 2022; McCarville et al., 2020; Peh et al., 2022). The most common SCFAs are acetate, propionate, and butyrate, which are produced by fermentation of resistant starch and dietary fibers. Since mammals are not able to generate enzymes that digest these polysaccharides, they pass undigested through the gut, where microbiota use them as an energy source and generate SCFAs as end products (Koh et al., 2016). SCFAs mediate the control of both mucosal and systemic immunity and exert important vasoactive actions (Corrêa-Oliveira, Fachi, Vieira, Sato, & Vinolo, 2016). In addition, SCFAs influence host cells through a variety of mechanisms like activation of G-protein coupled receptors, histone acetylation and cell proliferation. Loss of SCFA-producing bacteria has been described in several pathological models, including stroke, hypertension, obesity, and diabetes mellitus wherein SCFAs supplementation seem to exert a beneficial effect (Connell et al., 2022; McCarville et al., 2020; Peh et al., 2022; Roager & Licht, 2018).

Amino Acid (AA) Metabolism: The best studied AA in the context of host-microbe interactions are tryptophan and L-carnitine (Connell et al., 2022; McCarville et al., 2020; Peh et

al., 2022). **Tryptophan (Trp)** is an essential AA that cannot be synthesized by animal cells and is abundant in high protein content foods, such as meats, nuts, fish and eggs (Agus, Planchais, & Sokol, 2018). Trp is metabolized in the gut by three main pathways. The first is the direct transformation of Trp by gut bacteria into several molecules including indoles and its derivatives. These microbe-derived metabolites directly produced by dietary Trp include different indoles such as indole-3-sulphate, indole-3-acetate, indole-3-aldehyde, indolepropionic acid, tryptamine, and 3-methyl-indole, among others (Agus et al., 2018; Hubbard, Murray, & Perdew, 2015). Interestingly, all these microbial metabolites activate the Aryl Hydrocarbon Receptor (AhR) ("Aryl hydrocarbon receptor: Aryl hydrocarbon receptor.," Last modified on 23/02/2021. Accessed on 06/02/2023) (see Section 6). The metabolism of Trp by microbes, by limiting the availability of host Trp, can indirectly modulate the other two major Trp metabolic pathways, that is, the serotonin pathway and the kynurenine pathway (KP). Thus, gut microbes may affect the levels of various neuroactive metabolites and neurotransmitters, including L-kynurenine, that has also been demonstrated to activate AhR, again reinforcing the fundamental role of AhR in the gut-brain axis. The GM is a major actor in intestinal serotonin production. Hence, GF mice exhibited impaired serotonin production in the gut and low concentrations in the blood (Agus et al., 2018). Trp metabolism is therefore critical for proper cognition. Indeed, numerous studies have identified abnormal Trp metabolism, with alterations either in Trp-derived microbial metabolites or in the serotonin and KP products, in patients with cognitive decline (Connell et al., 2022; Cryan et al., 2020; Cuartero et al., 2015; Morais et al., 2021; Sun et al., 2022). The other AA metabolized by GM is **L-carnitine.** This AA is mainly present in red meat and, once in the gut, is converted to trimethylamine (TMA) by intestinal bacteria. Then, TMA is oxidized in the liver by hepatic flavin monooxygenases to finally form TMAO, an indirect product of bacteria metabolism that has been demonstrated to contribute in atherosclerosis pathogenesis (Koeth et al., 2013) modulating platelet hyper responsiveness and thrombosis potential (W. Zhu et al., 2016). Due to TMAO's high association with atherosclerosis and cardiovascular disease, TMAO has been considered a risk factor of vascular dementia and PSCID (Koeth et al., 2013; Morais et al., 2021; Needham et al., 2020; Tang et al., 2017).

Bile Acid Metabolism: Bile acids (BAs) are compounds produced by the host that upon modification by gut bacteria form bile salts. BAs regulate a variety of psychological functions but also are associated with cognitive decline in particular, in AD pathology (Connell et al., 2022; Morais et al., 2021; Needham et al., 2020). The primary bile acids cholate and chenodeoxycholate are produced in the liver through oxidation of cholesterol and then, are conjugated to amino acids taurine or glycine, forming bile salts. Conjugated bile salts that reach at the intestinal tract are metabolized by gut bacteria into secondary bile acids. Secondary bile acids can further be manipulated by the GM by dihydroxylation producing deoxycholic acid and lithocholic acid (Connell et al., 2022; McCarville et al., 2020; Peh et al., 2022). Different BAs has been linked to cognition and dementia, for instance serum levels of secondary deoxycholic acid levels in AD.

Gut microbiota-dependent synthesis of neurotransmitters: Gut microbes are able to synthesize different neurotransmitters by themselves and even may promote the generation of

neurotransmitters by the hosts. One of the most representative examples is the neurotransmitter γ- aminobutyric acid (GABA) which is produced by *Bacteroides, Bifidobacterium, Parabacteroides* and *Escherichia* (Lyte, 2013; Strandwitz et al., 2019). In fact, mice treated with antibiotic displayed altered faecal GABA levels, suggesting that microbiota is contributing to circulating levels of GABA. This is of interest since multiple diseases are associated with an altered GABAergic profile, such as depression, stroke and even PSCID (Strandwitz et al., 2019; Torres-López C & ME, 2022). In the context of major depressive disorder (MDD) (Strandwitz et al., 2019), by coupling microbiome sequencing with functional magnetic resonance imaging in patients with MDD and altered GABA pattern, a recent study found that the relative abundance of faecal *Bacteroides* negatively correlates with brain signatures of depression (Strandwitz et al., 2019). Bacteria are also important in the production of another types of neurotransmitters such as serotonin, NA, DA and acetylcholine (Lyte, 2013). In the case of serotonin, *Bifidobacterium infantis* has been shown to increase the circulating levels of Trp and thus influence central serotonin transmission. In physiological conditions, although these microbial-synthesized neurotransmitters can cross the intestinal barriers, their influence on the brain is likely to be indirect, probably acting on the ENS since they cannot cross BBB and reach the brain. However, under pathological contexts wherein the BBB is compromised, microbial-synthesized neurotransmitters might probably exert direct brain effects.

5.) THE MICROBIOTA-GUT-BRAIN AXIS IN THE ACUTE AND CHRONIC STROKE PHASES

In cerebrovascular disease and specifically in stroke, increasing evidence indicates that targeting gut microbiota might be considered a therapeutic strategy. The relationship between microbiota and stroke is very complex and involves vascular predisposing factors such as atherosclerosis, and the stroke phase, ranging from the acute stroke to the most chronic phase wherein the development PSCID takes place (Benakis et al., 2016; Durgan, Lee, McCullough, & Bryan, 2019; Honarpisheh et al., 2022; Lee, d'Aigle, et al., 2020; Lee, Venna, et al., 2020; Peh et al., 2022; Singh et al., 2018; Spychala et al., 2018). The different changes related to the microbiota-gut-brain axis in stroke are summarized in **Figure 3** and will be described in the next sections.

5.A) RISK FACTORS AND GUT MICROBIOTA

Atherosclerosis is one the major vascular risk factors for stroke, dementia and PSCID (Iadecola, 2013; Iadecola et al., 2019). Recent studies have found that bacterial DNA is found around atherosclerotic plaques, probably altering plaque stability. Importantly the bacterial taxa observed in the atherosclerotic plaques was also present in the gut of the same subjects (Koren et al., 2011). These results might indicate a bacterial translocation process wherein the origin of bacteria located in the plaque would be the gut. In addition, the metabolite TMAO has also been implicated in atherosclerosis (Koeth et al., 2013; Yin et al., 2015). Indeed, by using GF mice, antibiotics treatment and ApoE$^{-/-}$ mice, TMA/TMAO generated from metabolism of dietary nutrients has been demonstrated to have pro-atherogenic effect contributing to the development of atherosclerotic plaques (Koeth et al., 2013). TMAO is linked to a reduction in cholesterol transport,

alteration in tissue cholesterol and sterol metabolism, and changes in the composition and transport of BAs in both the liver and gut, altering lipids levels and also producing dyslipidemia (Peh et al., 2022; Tang et al., 2017). Indeed, patients with *hyperlipidemia* also showed abnormal GM composition which, in its turn, would aggravate dyslipidemia, while regulating GM could alleviate the abnormality of serum lipid in animal models (Peh et al., 2022; Tang et al., 2017). These findings demonstrate that GM might be an important regulator of the prognosis of hyperlipidemic stroke and its consequences. In this sense, a recent study also demonstrated that GM signature in hyperlipidemic patients is a predictor of adverse outcomes after acute ischemic stroke determined by modified Rankin Scale (mRS) scores at 3 months after admission (J. Chen et al., 2022). *Hypertension* is the most prevalent modifiable risk factor for stroke and dementia (van der Flier et al., 2018). Gut dysbiosis has been associated with hypertension in both animals and humans. In this context, dysbiosis has been found in models of hypertension in rats, including the genetic SHR model and the one generated by angiotensin-II (Ang-II) infusion. Hypertensive rats displayed a decrease in microbial diversity and increased *Firmicutes/Bacteroidetes* ratio. The Ang-II model has also been used in GF mice, which did not show any sign of hypertension, indicating that microbiota is necessary for AngII-induced hypertension (Lau et al., 2017; Li et al., 2017; Tang et al., 2017). Furthermore, by using FMT wherein normotensive rats were colonized with microbiota of hypertensive rats, GM transfer was enough for elevating blood pressure in normotensive rats (Lau et al., 2017; Li et al., 2017; Tang et al., 2017). In humans, the composition of gut microbiota found in pre-hypertensive patients is the same that is observed in hypertensive patients and its quite different to control patients, suggesting that dysbiosis precedes hypertension rather than being a consequence of it (Lau et al., 2017; Li et al., 2017). In hypertensive patients, dysbiosis is reflected by a decreased diversity of the intestinal microbiota and a higher *Firmicutes/Bacteriodetes* ratio, as observed in animal models of hypertension (Li et al., 2017). *Age* is the predominant risk factor for cognitive decline, dementia and stroke. Microbiota composition, richness and function changes with aging (Connell et al., 2022; Ghosh et al., 2022; Honarpisheh et al., 2022; Lee, d'Aigle, et al., 2020). In humans, these changes have been associated with a decrease in species diversity, with a reduction in *Clostridiales* and *Bifidobacterium* and an increase in *Proteobacteria* and *Pathobionts* (O'Toole & Jeffery, 2015; Odamaki et al., 2016). Importantly, these changes have been suggested to play a role in the low-grade inflammation which is observed in aging, so-called *"inflammaging"*. The microbial metabolite profile has been also demonstrated to be completely different with aging (O'Toole & Jeffery, 2015; Odamaki et al., 2016). This, together with the alteration in the BBB as we age, may facilitate the ability of microbial metabolites to penetrate into the brain having a direct impact on cognition. In this sense, different bacterial metabolites such as SCFAs, nitrites, TMAO and indoles, exert direct effects on BBB permeability, integrity and vascular function (Connell et al., 2022; Ghosh et al., 2022; Lee, d'Aigle, et al., 2020; Lee, Venna, et al., 2020). The GM also varies among stroke patients in different age groups. In experimental stroke in mice, age-related changes in the GM were shown to influence stroke outcome (Spychala et al., 2018). First, authors corroborated that microbiota is altered after stroke in young mice and is similar to microbiota found

in control aged mice (with an increase *Firmicutes/Bacteroidetes* ratio). Accordingly, FMT from aged donor to young recipient, increased mortality following MCAO and decreased performance in behavioral testing. Conversely, young microbiota colonization of aged mice increased survival and improved recovery following MCAO.

5.B) GUT MICROBIOTA COMPOSITION PRIOR ISCHEMIA PREDISPOSE FOR STROKE AND DETERMINE ITS SEVERITY

The composition of GM prior to ischemia influences stroke severity and it is associated with stroke outcome and primary lesion size. As commented in the previous section, predisposing and risk factors wherein GM composition is altered are good examples. In addition, multiple studies have shown that the absence of microbiota in GF mice promotes a higher infarct size after stroke compared to conventional mice (Benakis et al., 2016; Benakis et al., 2020; Singh et al., 2016; Singh et al., 2018). Importantly, infarct volume in GF mice is completely normal in size when they are colonized with a healthy GM indicating therefore, that gut microbes have a profound impact on stroke outcome and in the primary lesion size. Furthermore, Singh et al., (Singh et al., 2016) by using the proximal Middle Cerebral Artery Occlusion (MCAO) model demonstrated that GF mice that received faecal microbes from ischemic donor mice had larger infarct volumes and display functional deficits compared to the recipients of FMT from sham mice. Among the mechanisms that mediate microbiota-dependent changes in stroke lesion, these authors demonstrated the implication of a pro-inflammatory T-cell polarization (Th1 and Th17 cells) in the Peyer patches and also in the ischemic brain. The study by Benakis and collaborators (Benakis et al., 2016) also reflected that GM can be manipulated to either improve or worsen stroke outcomes. By administering antibiotics in mice prior stroke, they showed that antibiotic-induced alterations in commensal microbiota reduces ischemic brain injury and, remarkably, that this neuroprotective effect can be transferred by faecal microbiota transplants. Changes in bacterial gut composition alters immune homeostasis in the small intestine leading to an increase in the neuroprotective/anti-inflammatory regulatory T (Treg) cells and a reduction in IL-17+ γδ T cells that by opposite contribute to ischemic damage and exert a pro-inflammatory effect suggesting that GM might be a key regulator in priming the neuroinflammatory response to brain injury (Benakis et al., 2016). In addition, as commented previously, colonization of young mice by aged control microbiota, although did not promote higher infarct volumes, had a profound negative effect, increasing stroke mortality and impairing recovery (Singh et al., 2016).

5.C) STROKE ALTERS GUT MICROBIOTA COMPOSITION

Analysis of GM composition in ischemic and hemorrhagic stroke patients has revealed that stroke promotes gut dysbiosis and, in the most cases, the degree of GM changes correlates with stroke severity. So far, several clinical studies have explored changes in GM composition in patients with stroke compared to control. Overall, these studies identified 62 upregulated and 29 downregulated microbial taxa (Peh et al., 2022). From all these studies, just a few tried to associate microbiome to stroke severity. Among these, one of the first clinical studies demonstrating dysbiosis after stroke showed that the GM of stroke and transient ischemic attack

patients was clearly different from that of control group (Yin et al., 2015), with more opportunistic pathogens, such as *Enterobacter, Megasphaera, Oscillibacter*, and *Desulfovibrio*, and fewer commensal or beneficial genera including *Bacteroides, Prevotella*, and *Faecalibacterium*. Importantly, this dysbiosis correlated with the severity of the disease. Consistently, Xu and cols., (K. Xu et al., 2021) performed two clinical cohort studies in stroke patients and brain ischemia in mice to capture the gut dysbiosis dynamics after stroke and their relationship with stroke prognosis. They demonstrated that ischemic stroke rapidly triggers GM dysbiosis with *Enterobacteriaceae* overgrowth that, in turn, exacerbates brain infarction demonstrating therefore, a bidirectional interaction between stroke and GM. Chang and cols. (Chang et al., 2021) also detected an apparent dysbiosis of blood microbiota in patients with acute ischemic stroke compared to healthy people, showing that *Ruminococcaceae* and *Prevotella* were elevated in blood samples of stroke patients with poor functional outcome. The study by Yamashiro (Yamashiro et al., 2017) analyzed faecal GM and metabolites in a Japanese cohort of stroke and control subjects. They found that the abundance of *Lactobacillus ruminis* was higher in stroke patients and correlated with inflammatory markers as interleukin-6. In addition, a decrease in microbial metabolites such as SCFAs, acetic and valeric acids was also detected, supporting multiple alterations in GM after stroke. The study by Tan et al., (Tan et al., 2021), with a cohort of 40 acute ischemic patients and 92 controls, showed that the intestinal microbiota was different in stroke patients from healthy controls, especially those with increased stroke severity, in which SCFAs levels, especially acetate, were associated with an increased risk of 90-day poor functional outcomes. A recent clinical study also demonstrated that gut dysbiosis takes place after hemorrhagic stroke (L. Chen et al., 2022): by using RNA 16S sequencing, macrogenomics sequencing and untargeted metabolomics to explore the differences in gut microbial-metabolome interactions between patients with intracerebral hemorrhage and healthy control populations, they found a significant decrease in the phylum of *Firmicutes* and a significant increase of *Bacteroidetes* in hemorrhagic stroke patients which is accompanied by changes in serum microbial metabolites and correlates with the severity of intracerebral hemorrhage (L. Chen et al., 2022). In addition, a role of GM in post-stroke prognosis and early stroke outcome has been demonstrated in a study including 104 patients with acute ischemic stroke and 90 healthy individual showing that the stroke dysbiosis index (SDI) correlated not only with brain injury but also with early unfavorable outcome (Xia et al., 2019). Authors also performed experimental stroke models where they corroborated that mice receiving faecal transplants from patients with higher SDI (i.e., higher dysbiosis) developed a more severe brain injury than mice receiving transplants from low SDI patients, therefore reinforcing the causal relationship between gut dysbiosis and stroke outcome and severity (Xia et al., 2019).

The impact of stroke on the GM composition, dysbiosis and the mechanisms through which stroke affects the gut have been evaluated in experimental cerebral ischemia models (Houlden et al., 2016; Singh et al., 2016; Singh et al., 2018; K. Xu et al., 2021). In this sense, a study in cynomolgus monkeys found intestinal dysbiosis after stroke with an increase in *Bacteroidetes* and a reduction *Firmicutes* and *Faecalibacterium* (Y. Chen et al., 2019). An interesting finding in the

study of Singh et al. (Singh et al., 2016) is that gut dysbiosis after stroke in mice may depend on lesions size. By using the proximal MCAO filament model which produces large hemispheric lesions, sequencing of GM composition revealed that ischemic mice displayed gut dysbiosis reflected by a reduction in bacterial diversity and an increase in bacterial *Firmicutes, Bacteroidetes, and Actinobacteria*; on the contrary, when stroke was induced by distal MCAO with the permanent electrocoagulation model, which causes smaller lesions, no significant change in the microbiota composition and species diversity was observed, suggesting that infarct size has a role in stroke-induced dysbiosis. This was also observed in the study by Houlden (Houlden et al., 2016) wherein intestinal dysbiosis correlated with the extent of injury in both experimental stroke and traumatic brain injury. Finally, the study by Spychala and cols., (Spychala et al., 2018) provided evidence of dysbiosis after experimental stroke, although in this case major changes were detected in two main bacterial phyla in GM *Firmicutes* and *Bacteroidetes*, with an increased ratio of *Firmicutes* to *Bacteroidetes* (Spychala et al., 2018). The gut virome has also been demonstrated to change after experimental stroke, where it might play a crucial role in disease progression and recovery (Chelluboina, Kieft, Breister, Anantharaman, & Vemuganti, 2022).

Apart from gut dysbiosis, some clinical studies also support for alterations on microbial metabolites. For instance, the studies in humans by Yamashiro and by Tan showed a reduction in faecal SCFAs (Tan et al., 2021; Yamashiro et al., 2017). In agreement, SCFAs are also reduced in different experimental stroke models (Benakis et al., 2020; R. Chen et al., 2019; Lee, d'Aigle, et al., 2020). Of note, Sadler and cols. (Sadler et al., 2020) demonstrated that SCFA supplementation in the drinking water of mice significantly improved recovery of affected limb motor function after stroke promoting synaptic plasticity processes at different levels, and systemic and brain resident immune cells were demonstrated as the main effectors (Sadler et al., 2020). While SCFAs seem to play a beneficial and protective role after stroke, some exceptions have been reported (Henry et al., 2021). In this sense, the recent study by Zhu et al., (W. Zhu et al., 2021) in experimental stroke model showed that gut microbes, through dietary choline and TMAO generation, directly impact cerebral infarct size resulting in adverse outcomes following stroke. In addition, they also demonstrated that either dietary choline supplementation, which raises plasma TMAO, or direct TMAO feeding prior stroke ischemia impacted negatively stroke severity.

5.D) GUT MICROBIOTA AND POST-STROKE COMPLICATIONS

Extensive brain injury impairs gastrointestinal function: Several studies have demonstrated gastrointestinal disturbances in stroke patients such as dysphagia, gastrointestinal bleeding, or constipation (Tuz et al., 2022). A common post-stroke complication affecting the gut, which is a major contributor to stroke outcome, disability, and mortality, is the so-called paralytic intestinal ileus or post-stroke ileus, which is characterized by abdominal distension and absent bowel sounds causing a reduced gastrointestinal motility, associated with overgrowth of intestinal bacteria and subsequent dysbiosis. As we commented above, microbiota gut function is under

the control of CNS by innervation of the gut wall with both the ANS and the ENS. In addition, HPA axis may also play a role after stress responses. In this sense, previous reports have identified that brain impairment by stroke promotes a dysregulation of the ANS and a pronounced stress response that participates in the inflammatory post-stroke response (Chamorro et al., 2012; Dorrance & Fink, 2015; Meisel, Schwab, Prass, Meisel, & Dirnagl, 2005; Mracsko et al., 2014). In fact, post-stroke intestinal dysfunction and associated dysbiosis are probably a consequence of the catecholaminergic stress response generated after stroke (Houlden et al., 2016; Singh et al., 2016), although additional altered signaling through the ANS (for instance, the loss of cholinergic signaling in the ileum in favor of adrenergic one) or circulating factors may also contribute to this stroke complication.

Bacterial translocation and post-stroke infection: Post-stroke infections are the most common problems of stroke patients, affecting around a 30% of stroke survivals and associated with higher mortality and poor stroke outcome. Urinary tract infection and pneumonia are the most common types of infection, but pneumonia has a greater impact on clinical outcomes (Elkind, Boehme, Smith, Meisel, & Buckwalter, 2020). As we previously delineated, the integrity of the epithelial gut barrier is fundamental for maintaining intestinal homeostasis avoiding gut microbes access to the circulation or distant organs. In this regard, it has been proposed that post-stroke infections may be due to the loss of integrity of the gut epithelial barrier after stroke (Crapser et al., 2016). As a consequence, GM can translocate into the circulation and from there, disseminate into inappropriate tissues, for instance, the lung being therefore potentially pathogenic and contributing to post-stroke pneumonia, as demonstrated by Stanley and cols. (Stanley et al., 2016). In fact, the analysis of stroke human samples demonstrated that more than 70% of bacteria found in the lungs, were bacteria commonly found in the gut (and in the oral cavity) such as *Enterococcus spp*., *Escherichia coli*, and *Morganella morgana*. Haak and cols. (Haak et al., 2021) also demonstrated that alterations in gut bacteria producing trimethylamine and butyrate are associated with stroke-associated infections. In addition, alteration of GM in the aged mice increased the risk and the severity of post-stroke lung infection (Crapser et al., 2016; Stanley et al., 2016; Wen et al., 2019). In agreement with bacterial translocation to the lungs contributing to post-stroke infection, GF mice did not develop spontaneous pneumonia after stroke (Stanley et al., 2016).

5.E) THE VICIOUS CIRCLE OF INJURED BRAIN AND DYSBIOTIC GUT MICROBIOTA IN POST-STROKE RECOVERY

Stroke alters gut motility, increases gut permeability, activates resident immune cells, and changes the gut microbiome to a dysbiotic one. Subsequently, this dysbiotic GM in turn communicates to the brain having detrimental effects after stroke, by increasing lesion size and stroke severity. The mechanisms by which dysbiosis further exacerbates stroke damage probably involve local neuroinflammation, migration of immune cells into the brain, bacterial endotoxins and/or metabolites that can cross the disrupted BBB exerting neurotoxic actions. Therefore, the

brain participates in gut dysbiosis and, subsequently, the gut dysbiosis feeds back to promote neuroinflammation following cerebral ischemia. This vicious circle hinders the recovery during the sub-acute stroke phase. As commented before, different studies have demonstrated that FMT, antibiotics or specific supplementation with microbial metabolites (like SCFAs) prior and/or at the time of ischemia may have a positive or a negative impact in stroke recovery (Sadler et al., 2020; Singh et al., 2016; Spychala et al., 2018). However, for being considered as a viable therapy for stroke treatment, the GM should be amenable to manipulation after stroke onset in order to contribute to post-stroke recovery. In this sense, it has been demonstrated that "bacteriotherapy" is a viable post-stroke treatment option in the aged (Lee, d'Aigle, et al., 2020): FMT from young donor to recipient ischemic aged mice three days after stroke, improved behavioral recovery and gut integrity and conferred a protective phenotype in both gut and brain T cells. In addition, they demonstrated that a reduction of microbial SCFAs is implicated in dysbiosis-mediated injury and showed that restoring SCFAs levels after stroke through probiotics and prebiotics was enough for improving outcomes in stroke aged mice (Lee, d'Aigle, et al., 2020).

A plethora of research studies show that dietary modifications influence the GM and that these modifications are associated with pro- or anti-inflammatory responses. In this sense, a recent study demonstrated that changes in the diet after stroke can be used for restoring GM: specifically, they observed that faecal dysbiosis after stroke in mice was reversed by protein restriction and improved influenced stroke outcome. Therefore, the modification of dietary protein content may represent an efficient and easy strategy for promoting stroke recovery and targeting the microbiota (Silva de Carvalho et al., 2022) once stroke has occurred.

5.F) ASSOCIATION OF GUT MICROBIOTA WITH POST-STROKE COGNITIVE IMPAIRMENT AND DEMENTIA

Despite the immense differences in neuropathology of the most common dementias, that is, AD or those vascular-driven including PSCID, they are associated with shared and disease-specific abnormalities in the composition and function of the GM (Association, Alzheimer's Disease Facts and Figures; Connell et al., 2022; Cryan et al., 2019; Cryan et al., 2020; Honarpisheh et al., 2022; Jung et al., 2022; Z. Zhu et al., 2022). However, whether aberrant microbiota in this context is causal (that is, implicated in predisposition, initiation or progression) or, on the contrary, a secondary epiphenomenon of the disease is still under debate. Of note, GM composition is known to be significantly altered in patients with mild cognitive impairment, that is, a preclinical stage that precedes dementia in AD, suggesting therefore that changes in microbial composition may occur during the early period of cognitive deterioration (Z. Zhu et al., 2022).

Most evidences on the implication of GM in dementia arise from studies in demented patients in general or from studies focused in AD. Gut dysbiosis and alterations in microbiota composition in both AD patients and animal models is very well documented. In this context, changes in GM composition in AD patients include a decrease in the relative abundance of *Firmicutes* and *Bifidobacterium spp*., and an increase in *Bacteroidetes, Shigella and Escherichia spp*., which have been correlated with inflammation and amyloid aggregates (Cattaneo et al., 2017; Verhaar

et al., 2021; Vogt et al., 2017; Zhuang et al., 2018). The role of the microbiota in AD has been studied in different AD animal models including 5XFAD transgenic mice and the APP/PS1 line, which display important changes in the GM and microbial metabolites. In these mice, microbiota depletion by using antibiotics reduced brain amyloid deposition and inflammatory profile suggesting that the GM exacerbates the AD pathology (Colombo et al., 2021; Dodiya et al., 2022; Minter et al., 2016; X. Wang et al., 2019; Zhuang et al., 2018), however, the exact roles and the molecular mechanisms through GM mediate neurodegeneration in AD are still unknown.

PSCID, which develops in the months following stroke, is likely caused by a combination of stroke lesion size and location combined with a plethora of molecular mechanisms that may include a prolonged inflammatory response and immunothrombosis, a secondary neurodegeneration in remote areas, defects on myelin removal and phagocytosis, changes in neurotransmitters like GABA, alterations in physiological process like neurogenesis, malfunctioning of the glymphatic system, among others (Cuartero et al., 2019; Doyle & Buckwalter, 2020; Rost et al., 2022). Interestingly, a great deal of these endogenous processes may be modulated by the GM and their metabolites, making gut microbes a very attractive target for chronic stroke. So far, most evidences that associate GM with the development of PSCID arise from very recent clinical studies. In this regard the study by Xia and cols. not only demonstrated that the GM plays a role in post-stroke prognosis and early outcome, but also, that dysbiosis persists long-term after stroke (Xia et al., 2019). This persistent dysbiosis was confirmed also by a recent study including 12 stroke patients, 18 control participants with stroke risk factors for stroke, and 12 healthy participants (Hammond et al., 2022), where GM and its association with leaky gut markers, dietary intake, and functional recovery measures were evaluated the first three weeks after stroke. Although sample size is limited, data support that dysbiosis is still observed 3 weeks after stroke, with significantly lower abundances of butyrate producers, secondary BAs producers, and sulfate reducers in the stroke group. It is plausible that this persistent dysbiosis which is detected in patients long-term after stroke onset contributes to the development of PSCID. Indeed, first associations between microbiota and PSCID arise from the studies carried out by Ling and collaborators. (Ling, Gong, et al., 2020; Ling, Gu, et al., 2020) who characterized GM in faecal samples from ischemic stroke patients. Patients were divided into two different groups, a PSCID group and the non-impaired, non-PSCID group, according to their MoCA scores 3 months after stroke onset. In both studies, quite similar results were found regarding bacterial composition. At the phylum level, *Proteobacteria* was highly increased in the PSCID group compared with the non-impaired. In addition, after age adjusting, a decrease in the abundance of Firmicutes was also observed in the impaired stroke group. The study of Liu and cols.(Liu et al., 2020) tried to find an association between of PSCID and GM metabolites. Again, stroke patients were classified in PSCID and non-PSCID based on their MoCA scores. Main findings of this study show that PSCID patients displayed a decrease in alpha-diversity and disturbed microbial composition compared with non-PSCID patients. In addition, increased *Fusobacterium* and deficiency of microbial metabolized SCFAs were significantly associated with PSCID. A recent study by Wang

(H. Wang et al., 2022) analysed the role of microbiota in the development of PSCID in both stroke patients and experimental stroke models in mice. The study includes a cohort of 83 stroke patients that were classified as PSCID and non-PSCID (34 and 49 stroke patients respectively) by MoCA scores 3 months after stroke. By analyzing GM composition, microbial metabolites and peripheral inflammatory factors levels, PSCID patients showed significantly higher levels of *Enterobacteriaceae*, lipopolysaccharide (LPS) and peripheral inflammatory markers. For corroborating these data, ischemic mice were colonized by FMT with GM from PSCID and non-PSCID patients. Consistently with data from stroke patients, ischemic mice that received microbiota from PSCID patients displayed a higher level of *Enterobacteriaceae*, an increased expression of intestinal TLR4, increased levels of circulating LPS and inflammatory cytokines and a reduction in faecal SCFA butyrate. Finally, authors demonstrated that supplementation with sodium butyrate via drinking water rescued detrimental changes caused by colonization of ischemic mice with microbiota from PSCID patients.

In summary, although further studies are necessary for establishing the role of GM in the development of PSCID, the studies so far are consistent, revealing differences in the relative abundance of several taxa such as *Gammaproteobacteria, Proteobacteriaa* and *Enterobacteriaceae* between PSCID and non-impaired patients, therefore suggesting that a persistent gut dysbiosis may contribute to cognitive decline and dementia long-term after stroke.

6. POTENTIAL THERAPEUTIC OPTIONS FOR TARGETING GUT MICROBIOTA IN CEREBROVASCULAR DISEASE

So far, different therapeutic approaches have been tested in pathological diseases affecting the CNS such as PD, epilepsy, MS and AD (Connell et al., 2022; Cryan et al., 2019; Cryan et al., 2020; Morais et al., 2021). Owing to the exponentially growing knowledge gained from clinical and experimental studies about the impact of the GM in cerebrovascular disease including acute stroke and PSCID, there is an increased interest in testing the effect of microbiome interventions specifically in stroke patients. These include trials testing, among others, probiotics/prebiotics, dietary interventions, antibiotics, heterologous and autologous faecal microbiota transplantation and others such as vagal stimulation or modulation of different receptors like AhR **(Figure 4).**

Probiotics/prebiotics and dietary interventions: So far, most common probiotics include *Bifidobacterium, Lactobacillus* and *Saccharomyces spp*. which have a long history as safe probiotics. In addition, potential next-generation probiotics include *Faecalibacterium prausnitzii* 185, *Akkermansia muciniphila* and several *Clostridia spp*. (Fan & Pedersen, 2021). Different studies carried out in stroke animal models have shown that treatment with probiotics reduces brain damage and cognitive decline after stroke (Akhoundzadeh, Vakili, Shadnoush, & Sadeghzadeh, 2018; Sun et al., 2016). Consistently, clinical studies also demonstrated some positive effects (X. Chen, Hu, Yuan, Yang, & Ka Li, 2022). This beneficial effect of probiotics has also been observed in a hypoperfusion model caused by the occlusion of bilateral common carotid artery, where probiotic treatment reduced hippocampal injury and vascular cognitive impairment (Rahmati et al., 2019). Treatment with probiotics has not only been beneficial in cerebrovascular

diseases but also in AD patients wherein probiotic administration resulted in some favorable effects (Leblhuber, Steiner, Schuetz, Fuchs, & Gostner, 2018; Tamtaji et al., 2019).

Prebiotics are components of food that cannot be digested by the digestive tract enzymatically (Markowiak & Śliżewska, 2017). Thus, they are fermented by microbiota to generate metabolites such as SCFAs that, as commented above, have a beneficial effect in the ischemic stroke (Sadler et al., 2020). Then, increasing the endogenous production of SCFAs is an amenable strategy that can be achieved either by prebiotic administration or by dietary modifications. In this sense, a diet rich in fruits and vegetables and also in resistant starches (such as whole grains and legumes) might promote an increase in the levels of SCFAs, the neuroprotective microbial metabolites reduced after stroke (Lee, d'Aigle, et al., 2020; Markowiak & Śliżewska, 2017; Sadler et al., 2020; Tan et al., 2021). In addition, treatment with prebiotics/probiotics reduced the incidence and severity of pneumonia in hospitalized patients, one of the main complications after stroke (Barraud, Bollaert, & Gibot, 2013). While increasing, for instance, dietary fiber consumption may be helpful for recovery after stroke, the consumption of red meat is not recommended since it increases the levels of the pro-thrombotic TMAO, a detrimental factor associated with poor prognosis and stroke severity (Rexidamu, Li, Jin, & Huang, 2019; Yin et al., 2015; Zhai et al., 2019).

Antibiotics: The use of antibiotics for the treatment of stroke and dementias is so far controversial. Although it has been demonstrated to be beneficial in animal models of AD(Colombo et al., 2021; Dodiya et al., 2022; Minter et al., 2016) results from clinical trials with AD patients either had no effect on AD progression or even caused some neuropsychiatric side effects (Bravo et al., 2011; Molloy, Standish, Zhou, Guyatt, & Group, 2013). Similar results were observed in stroke patients (Westendorp et al., 2021) or in animal stroke models (Benakis et al., 2016; Winek et al., 2016; Xia et al., 2019). In current clinical practice, stroke patients are often treated with antibiotics due to post-stroke infections. However, not only bacteria in the target organ (like the lungs or the urinary tract) but also the gut bacterial populations are influenced by antibiotics treatment. Therefore, although antibiotics are a useful tool to manipulate the GM, antibiotic administration can have off-target effects and even promote microbiota independent changes in host metabolites that makes difficult their use as therapeutic tool.

Faecal Microbiota Transplantation (FMT): Transplantation with healthy bacteria may be a potential approach for restoring microbiota in stroke patients. Colonization with GM by FMT from healthy donors has been established in the treatment of patients with *C. difficile* colitis and also has proved safe and successful in patients with IBD, refractory bronchiolitis and pseudomembranous colitis (van Nood et al., 2013). FMT therapy has already been tested in CNS diseases such as PD and ASD, reducing the symptoms in both cases (Morais et al., 2021; H. M. Xu et al., 2021). Normalization of brain lesion-induced dysbiosis via FMT improved stroke outcome in experimental stroke models (Lee, d'Aigle, et al., 2020; Lee, Venna, et al., 2020; Spychala et al., 2018; Yamashiro et al., 2017). Although beneficial effects have been observed in animal stroke models, FMT trials conducted in patients who suffered stroke so far do not provide

evidence of a beneficial effect (H. Wang et al., 2022; H. M. Xu et al., 2021). Further studies are necessary to stablish the effects of microbial colonization for stroke treatment.

Other strategies for targeting stroke dysbiosis: Vagotomy, which is a surgical procedure that severs the VN and disrupts signaling from various peripheral organs to the brain, has been used to establish a casual role between the VN and the GM in different disorders affecting the CNS like PD, epilepsy and depression. For example, VN stimulation is an approved therapy for resistant epilepsy and depression (Aaronson et al., 2013; Morais et al., 2021). The role of the VN in stroke has been widely studied and involves both afferent and efferent fibers (Dorrance & Fink, 2015), pointing to the possibility that *activating the VN is a method of treating stroke*. In fact, stimulation of the VN has been shown to improve motor function in stroke patients. However, the function of the VN in stroke which might involve other mechanisms beyond targeting GM, still requires further investigation.

As we commented previously, Trp-microbe-derived metabolites produced different indoles like indole-3-acetate and indole-3-aldehyde that activate the *Aryl Hydrocarbon Receptor (AhR).* AhR is also activated by L-Kyn, reinforcing the fundamental role of AhR in the gut-brain axis. AhR is a ligand activated transcription factor mainly known by mediating the toxic and carcinogenic effects of xenobiotic compounds like dioxins but with important roles in homeostasis (Agus et al., 2018; Barroso, Mahler, Fonseca-Castro, & Quintana, 2021; Hubbard et al., 2015; Rothhammer & Quintana, 2019). In addition, many AhR ligands are processed and inactivated by cytochrome p450 family proteins, such as Cyp1A1, which is a direct AhR transcriptional target constituting a feedback loop for AhR signaling (Schiering et al., 2017). AhR is a key modulator of important physiological functions, including modulation of the immune system, metabolism, behavior, and lifespan. In addition, AhR has been implicated in pathological disorders affecting the CNS as stroke, AD and multiple sclerosis (Cuartero et al., 2014; Rothhammer et al., 2016; Sun et al., 2022). Mounting evidence indicates that reduced blood and faecal levels of gut microbiota–derived AhR ligands are associated with many human diseases, such as IBDs, obesity, hypertension and even AD. The ability of AhR to interact with multiple microbial metabolites and its ubiquitous expression in the immune system, in the gut and the CNS enables AhR to regulate physiological processes including brain function in response to microbial and metabolic signals. AhR is fundamental for intestinal homeostasis by acting on epithelial renewal, barrier integrity, and affecting the maintenance of intestinal immune cells, including innate lymphoid cells (ILCs), Th17 and Treg cells. In addition, activation of AhR may also regulate gut motility through effects on neurons from the ENS (Agus et al., 2018; Barroso et al., 2021; Hubbard et al., 2015; Schiering et al., 2017) demonstrating that AhR is a key component of GIT homeostasis. Furthermore, AhR activation by directly or indirectly gut-derived metabolites was shown to modulate neuroinflammation in both AD and MS (Barroso et al., 2021; Hubbard et al., 2015; Rothhammer et al., 2016; Sun et al., 2022) and also in physiological processes like hippocampal neurogenesis, aging (Bravo-Ferrer et al., 2019; de la Parra et al., 2018; Wei et al., 2021) and, importantly, in stroke (W. C. Chen et al., 2019; Cuartero et al., 2014). Therefore, the AhR pathway interacts with the microbiota-gut brain axis at multiple levels, altering for instance microbiota composition by

modulating GIT barrier, integrity and motility but also acting as an effector of microbial metabolites in the brain. Then, AhR might be amenable receptor for modulating the microbiota-gut-brain axis at therapeutic level in stroke **(Figure 5).**

CONCLUSION AND PERSPECTIVES

Mounting information from humans and animals indicates that gut microbiota is fundamental in controlling cognition and brain function. Therefore, GM dysregulation is implicated in the development and progression of multiple pathologies and neurodegenerative disorders affecting the CNS such as PD, MS, ASD and AD. There is also no doubt that gut microbiota is associated with cerebrovascular disease and stroke at multiple levels, clearly participating in the acute stroke etiopathogenesis and having important effects in stroke severity, outcome and recovery. Importantly, persistent dysbiotic microbiota is also observed long-term affecter stroke onset, suggesting the implication of GM in the development of cognitive decline and dementia after stroke. Since vascular dementia is the second cause of dementia after the most prevalent one AD, to establish a causal relationship between specific bacteria and PSCID pathology might have important repercussions and would be particularly relevant for the development of therapeutic strategies directed just to target disease associated microbiota while maintaining intact the good one. This fascinating perspective, that of course requires further investigation might combine dietary and lifestyles interventions with for instance directed probiotics or prebiotics and even pharmacological targeting as suggested for AhR. Therefore, GM provides a new promising avenue to modulate cerebrovascular disease and, specifically, stroke outcome in both acute and chronic stroke.

FIGURE LEGENDS

Figure 1. Routes of bidirectional communication in the microbiota-gut-brain axis. The routes of communication involve the autonomic nervous system, the neuroendocrine system, the hypothalamic–pituitary–adrenal (HPA) axis, the immune system and metabolic pathways. Left (in purple) represents pathways through which the brain controls gut. On the right (in green), main pathways for gut to brain signalling including neuronal, immunological and microbial metabolites-induced pathways. Inset reflects the epithelial gut barrier with gut bacteria located in the lumen and with the main cell types implicated in controlling gut function, intestinal mucosal immunity and, subsequently, gut homeostasis. (created with BioRender.com).

Figure 2. Main microbial derived metabolites implicated in gut microbiota signalling, and their synthesis pathways. Metabolic pathways implicated directly or indirectly in the microbiota-gut-brain axis. Microbial derived metabolites include indoles which derive from Trp metabolism, biles acids (BAs), TMAO and SCFAs. Notice than some of these compounds can reach the brain. In addition, the serotonin and the kynurenine pathways are also shown. (created with BioRender.com).

Figure 3. Association of the gut-microbiota–brain axis with stroke. Different vascular risk factors like hypertension and atherosclerosis, and age are associated to dysbiosis and changes in microbiota composition and microbial metabolites like SCFAs or increased TMAO. In this sense, changes in microbiota composition prior to stroke are associated with increased stroke severity and poor stroke outcome. After stroke, the injured brain causes an alteration in the communication pathways that control the gut, promoting epithelial barrier breakdown, a leaky gut, translocation of bacteria and bacterial toxins, gut dysbiosis and paralytic ileus. Dysbiotic gut bacteria after stroke further exacerbate ischemic damage and impair post-stroke recovery by mechanisms that included reduced SCFAs production and increased neuroinflammation. Bacterial translocation contributes to post-stroke infection impairing recovery and increasing mortality after stroke. Finally, persistent dysbiosis is observed long-term after stroke and likely contributes to the development of post-stroke cognitive impairment and dementia. (created with BioRender.com).

Figure 4. Therapeutic options for gut microbiota targeting. Gut microbiota-targeted strategies for ischemic stroke may include dietary interventions, probiotics and prebiotics supplementation, faecal microbial transplantation, and rationalization of antibiotic use. (created with BioRender.com).

Figure 5. The Aryl hydrocarbon receptor (AhR) and its relation to the microbiota-gut-brain axis. AhR is a ligand activated transcription factor that is activated for multiples environmental ligands like dioxins but also for microbial Trp-derived metabolites**.** The ability of AhR to interact with multiple microbial metabolites, and even with some metabolites of the kynurenine pathway, and its ubiquitous expression in the gut and the CNS, enable this receptor to regulate physiological processes in brain function and also to maintain proper gut function. (created with BioRender.com).

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