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Flavonoids bridging the gut and the brain: natural supporters against neuroinflammation and neurodegeneration

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Outline

Abstract

In recent years, experimental evidence suggested a possible role of the gut microbiota in the onset and development of several neurodegenerative disorders, such as Alzheimer's (AD) and Parkinson's (PD) diseases, multiple sclerosis (MS) and pain. Flavonoids, including anthocyanins, epigallocatechin-3-gallate (EGCG), the flavonol quercetin, and isoflavones, are plant polyphenolic secondary metabolites that have shown therapeutic potential for the treatment of various pathological conditions, including neurodegenerative diseases, thanks to their antioxidant and anti-inflammatory properties, despite their low bioavailability which often limits their use in clinical practice. In more recent years it has been demonstrated that flavonoids are metabolized by specific bacterial strains in the gut to produce their active metabolites. On the other way round, both naturally-occurring flavonoids and their metabolites promote or limit the proliferation of specific bacterial strains, thus profoundly affecting the composition of the gut microbiota which in turn modifies its ability to further metabolize flavonoids. Thus, understanding the best way of acting on this virtuous circle is of utmost importance to develop innovative approaches to many brain disorders. In this review, we summarized some of the most recent advances in preclinical and clinical research on the neuroinflammatory and neuroprotective effects of flavonoids on AD, PD, MS and pain, with a specific focus on their mechanisms of action including possible interactions with the gut microbiota, to emphasize the potential exploitation of dietary flavonoids as adjuvants in the treatment of these pathological conditions.

Keywords: microbiota, anthocyanins, epigallocatechin-3-gallate (EGCG), quercetin, isoflavones, nutraceutical supplements.

1. The gut-brain axis and the role of the gut microbiota in brain physiology

The gut microbiota consists of a complex population of microorganisms (*i.e.* bacteria, viruses such as bacteriophages, and some eukaryotes including yeast) living in the digestive tract of humans and animals. *Bacteroidetes, Firmicutes, Proteobacteria, Fusobacteria, Tenericutes, Actinobacteria*, and *Verrucomicrobia* are the main classes of bacteria present in the human microbiota and make up approximately 90% of the total microbial population [1]. Several factors affect the composition of the gut microbiota, including host genetics, diet, age, and antibiotic intake [2].

The gut microbiota is responsible for several physiological functions: it protects against pathogens by colonizing mucosal surfaces and releasing antimicrobial substances, strengthens the immune system, plays a vital role in digestion, metabolism, and insulin resistance, and controls the proliferation and differentiation of epithelial cells. In addition, it influences the communication between the gut and the central nervous system (CNS) via the so-called gut-brain axis, defined as a two-way signaling system between the gut bacteria and the brain [1]. This network includes the enteric nervous system (ENS), both sympathetic and parasympathetic autonomic nervous system (ANS) branches, and neuroimmune and neuroendocrine signaling [3]. The mechanisms by which this communication occurs are not fully clear, but they could include neural, endocrine, immunological, and metabolic pathways [4].

In fact, recent studies have demonstrated that the gut microbiota can influence brain function and physiology by modulating serotoninergic, noradrenergic, dopaminergic, glutamatergic, and GABAergic neurotransmission, either by producing neuroactive substances by themselves (see Table 1), or by altering the levels of their precursors. For example, bacteria belonging to *Lactobacillus, Bifidobacteria*, *Enterococcus*, and *Streptococcus* species can produce acetylcholine, GABA, and serotonin, directly contributing to the communication between the gut and the brain [3,5]. Due to the presence of the blood-brain barrier (BBB), it is unlikely that neurotransmitters produced in the gut can reach the CNS and directly perform their function [6]; instead, it is more likely that they influence the brain indirectly by acting on the ENS, in particular via the *vagus* nerve, the primary afferent pathway that carries information about the immune system, gut microorganisms, and nutrients from the gut to the brain [7]. Another route of communication is represented by cytokines produced in the gut and in the brain: these molecules can travel through the bloodstream but, in physiological conditions, do not pass the BBB. However, evidence suggests that cytokines can reach the CNS, especially in those areas where BBB is not present, such as within

the median eminence of the hypothalamus or circumventricular organs, or in pathological conditions when the BBB is damaged [8].

BACTERIAL SPECIES	MEDIATORS	FUNCTIONS	REFERENCES
Candida, Escherichia, Enterococcus, Streptococcus	Tryptophan and serotonin	Release of cytokine-carrying exosomes binding to serotonin receptors on microglia, contributing to gut-induced modulation of neuroinflammation.	[4,9]
Bifidobacterium and Lactobacillus	GABA	Partially unknown; maybe the production of a GABA-associated analgesic lipopeptide facilitates the diffusion across the epithelial barrier, allowing the activation of GABA receptors on sensory neurons.	[4,10]
Lactobacillus	Dopamine and acetylcholine	It is unknown how catecholamines of bacterial origin influence host physiology, maybe via receptor-based mechanisms.	[4,10]
Escherichia and Saccharomyces	Norepinephrine		
All bacterial species	Metabolism of dietary fibers to SCFAs	Maintaining gut health by promoting intestinal barrier integrity and mucus production. SCFAs cross the BBB through transporters located on brain vascular epithelial cells, and exert multiple functions in the CNS.	[4,5]

Table 1. Microbiota-derived mediators and their functions.

The main pathways of communication between the gut and the brain are summarized in Figure 1.

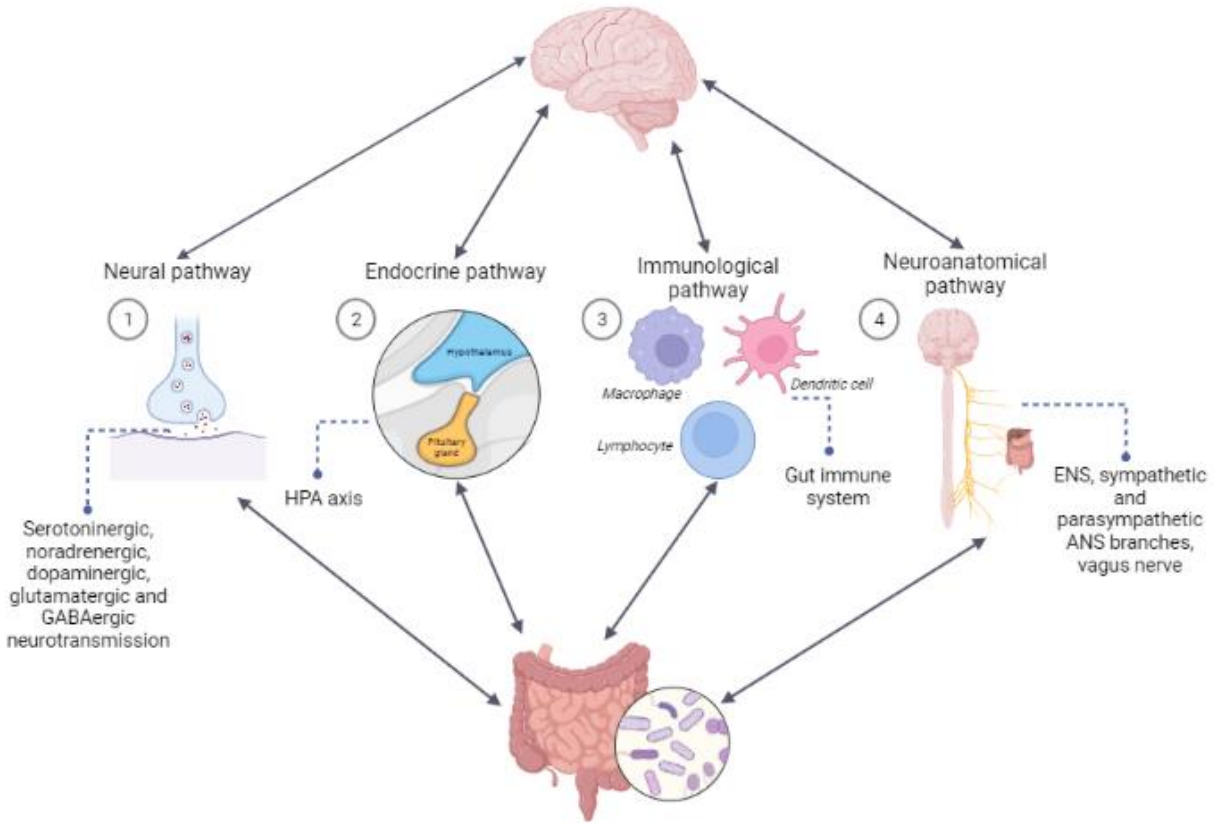

Figure 1. Pathways of communication between the gut and the brain.

Intestinal bacteria synthesize or metabolize various neuroactive substances that can influence brain function and physiology by modulating serotoninergic, noradrenergic, dopaminergic, glutamatergic and GABAergic neurotransmission (1) acting on the ENS, in particular via the *vagus* nerve (4). Moreover, mediators produced by the neuroendocrine-HPA (hypothalamic-pituitary-adrenal) axis (2) and the intestinal immune system (3) can enter the blood circulation through the intestinal mucosa barrier and reach the blood-brain barrier (BBB) and the brain. See text for details. Created with BioRender.com.

2. Pathological alterations of gut-brain communication: neurodegenerative disorders and pain

According to evidence correlating the gut microbiota with neurodegenerative diseases, the microbiota is able to activate the immune system as a consequence of alterations in the gut barrier (the so-called “leaky gut”), leading to systemic inflammation that causes damages to the BBB and promotes neuroinflammation and neuronal degeneration [11].

In this scenario, both age and diet play a key role. In fact, with increasing age the body undergoes changes in the microbiota, immune system, gut barrier, and BBB composition and functions. Moreover, in many cases aged people have a poor diet, which has been associated with

low gut bacterial biodiversity, inflammation and disability [12]. When considering the role of the gut-brain axis in brain pathologies, all these elements need to be taken into account, since many neurodegenerative diseases are correlated with aging.

Interestingly, the gut microbiota has been shown to significantly influence microglia, the major CNS-resident immune cells which are crucial for the response of the CNS against infection and injury, as well as for brain development and function. Microbiota-derived metabolites regulate the inflammation response mediated by microglia in the CNS, thus making microglia act as a critical mediator between the gut microbiota and CNS diseases [13].

In recent years, a number of experimental evidence suggested a possible role of the gut microbiota in the onset and development of several neurodegenerative disorders (see below; Figure 2). If confirmed by further studies, this hypothesis could pave the way for the development of new and more effective therapeutic approaches.

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by cognitive decline and disability, whose pathogenesis includes oxidative stress, neuroinflammation and accelerated apoptosis, accompanied by deposition of amyloid-β peptide plaques and Tau protein-based neurofibrillary tangles in the CNS [14].

The role of gut microbiota and its metabolites in the pathogenesis of neurodegenerative diseases, including AD, has been studied for years (for review see [14,15]).

AD patients exhibit gut dysbiosis, an imbalance in the composition of the bacterial microflora, described as decreased microbial richness (number of *taxa*) and diversity (number of different *taxa*), a low relative abundance of beneficial bacteria with the consequent reduced potential to synthesize short chain fatty acids (SCFA: acetate, propionate, butyrate) as well as higher abundance of *taxa* that are known to promote or be associated with inflammation [16]. All these changes significantly impact on the development of CNS disorders, including AD. Additionally, bacterial nucleic acids have been found in *post mortem* brain tissue of AD patients, suggesting that both the gut epithelial barrier and the BBB become significantly more permeable in AD patients, and thereby facilitate cerebral colonization by microbes [17]. Based on the currently available data, whether dysbiosis is a consequence of AD or precedes the disease onset still needs to be clarified. However, it is known that altered microbiota composition towards a pro-inflammatory profile in AD patients can promote and sustain inflammation that in turn contributes to AD clinical progression, and evidence demonstrates that intestinal microbiota alterations are involved in molecular mechanisms driving

AD progression, including the deposition of amyloid-β protein in the brain and Tau phosphorylation (see Figure 2) [18].

Parkinson's disease (PD) is the world's second most prevalent neurodegenerative disorder, whose peculiar hallmark is an aberrant production of α-synuclein fibrils, known as Lewy bodies, in dopaminergic neurons in the *substantia nigra* leading to defective dopamine neurotransmission [19].

Several lines of evidence now demonstrate the involvement of the gut microbiota in the pathogenesis of PD, as reviewed elsewhere [20,21]. As for AD, it has been reported that changes in the gut microbiota composition can increase intestinal permeability and cause systemic exposure to bacterial endotoxins, which in turn leads to increased α-synuclein expression and supports its misfolding to generate Lewy bodies (see Figure 2). Intestinal Lewy bodies reach the CNS via the *vagus* nerve and destroy the *substantia nigra*, resulting in clinical signs of PD [22]. Moreover, in a PD model of α-synuclein-overexpressing mice, colonization with PD patients-derived gut microbiota exacerbates motor impairments, whereas germ-free mice displayed milder motor symptoms which were instead worsened, along with neuroinflammation, by the oral administration of specific microbial metabolites [22].

A number of studies have been also conducted on fecal samples from PD patients, as reviewed by Nandwana and colleagues [15], demonstrating a correlation between changes in the composition of the gut microbiota and motor/cognitive functions.

Interestingly, constipation is the most prevalent pre-motor sign in Parkinson's disease, involving more than 70% of individuals and preceding clinical symptoms of more than 10 years, thus being considered as a clinical biomarker for PD. A significant drop in numerous gut microbiota metabolites has been reported in PD patients, which might lead to constipation [23]. Moreover, a higher vulnerability to PD was reported in presence of intestinal infections, which are triggers of PD-like symptoms [24].

Multiple sclerosis (MS) is an autoimmune disorder of the CNS primarily characterized by demyelination and axonal loss, and represents a major cause of disability in young adults. The pathogenesis is complex and still poorly understood, but neuroinflammation and neurodegeneration are known to play a major role [25].

Gut bacteria are also involved in the pathogenesis of MS, as they are able to activate the host immune system through disruption of the gut barrier, leading to systemic inflammation that damages the BBB and promoting neuroinflammation and neuronal degeneration [26]. Whether pathogenic bacteria act as initiators of MS is not yet understood. However, since MS is an autoimmune disease and the gut microbiota regulates the immune system, it is likely that gut microorganisms contribute the generation of a pathological environment leading to the development of the disease.

The impact of the gut microbiota on the development of MS has been demonstrated in several preclinical studies on experimental autoimmune encephalomyelitis (EAE), the animal model of MS, where it appears to modulate the immune system by regulating BBB permeability, activating microglia and regulating myelin gene expression (see Figure 2) [27]. Moreover, regulatory T cells seem to have deficits in their function as a result of an aberrant gut microbiota composition [28] and EAE incidence in germ-free and antibiotic-treated mice is significantly lower compared to specific pathogen-free (SPF) animals [29]. Interestingly, evidence demonstrates that CNS-resident cells are affected by the commensal microbiota in the EAE model, as reviewed by [30].

After preclinical data clearly demonstrating the role of the gut microbiota in EAE pathogenesis, clinical research has focused on differences in commensal microbiota populations between MS patients and healthy controls. Analyses of fecal samples from MS patients showed the presence of gut dysbiosis [31], and clinical studies demonstrated that the gut microbiota acts as a pathogenic environmental risk factor by directing innate and adaptive immune responses towards characteristic pathogenic profiles of MS, thus being considered as a potential disease biomarker [30].

Recent evidence suggests a possible role of the gut microbiota in **pain** conditions, including visceral, inflammatory and neuropathic pain (for review see [32]).

A number of microbiota-derived signaling molecules act on their receptors thus regulating central and peripheral sensitization, which in turn mediate the development of chronic pain. In pain conditions, gut microbiota can modulate peripheral sensitization through multiple mediators, including microbial by-products (*e.g.* pathogen-associated molecular pattern, PAMPs), metabolites (*e.g.*, SCFAs), and the release of neurotransmitters or neuromodulators (*e.g.* GABA). Microbiota-derived mediators can in turn sensitize primary nociceptive neurons or decrease their excitability either directly or indirectly [33]. In the CNS, gut microbiota-derived mediators regulate

neuroinflammation, which involves the activation of cells in the BBB, microglia, and infiltrating immune cells, to modulate induction and maintenance of central sensitization (see Figure 2) [33].

Despite clinical studies are mostly observational, proofs for a causal role of gut microbiota alterations in pain conditions are provided by a wide number of preclinical studies, as comprehensively reviewed in [34].

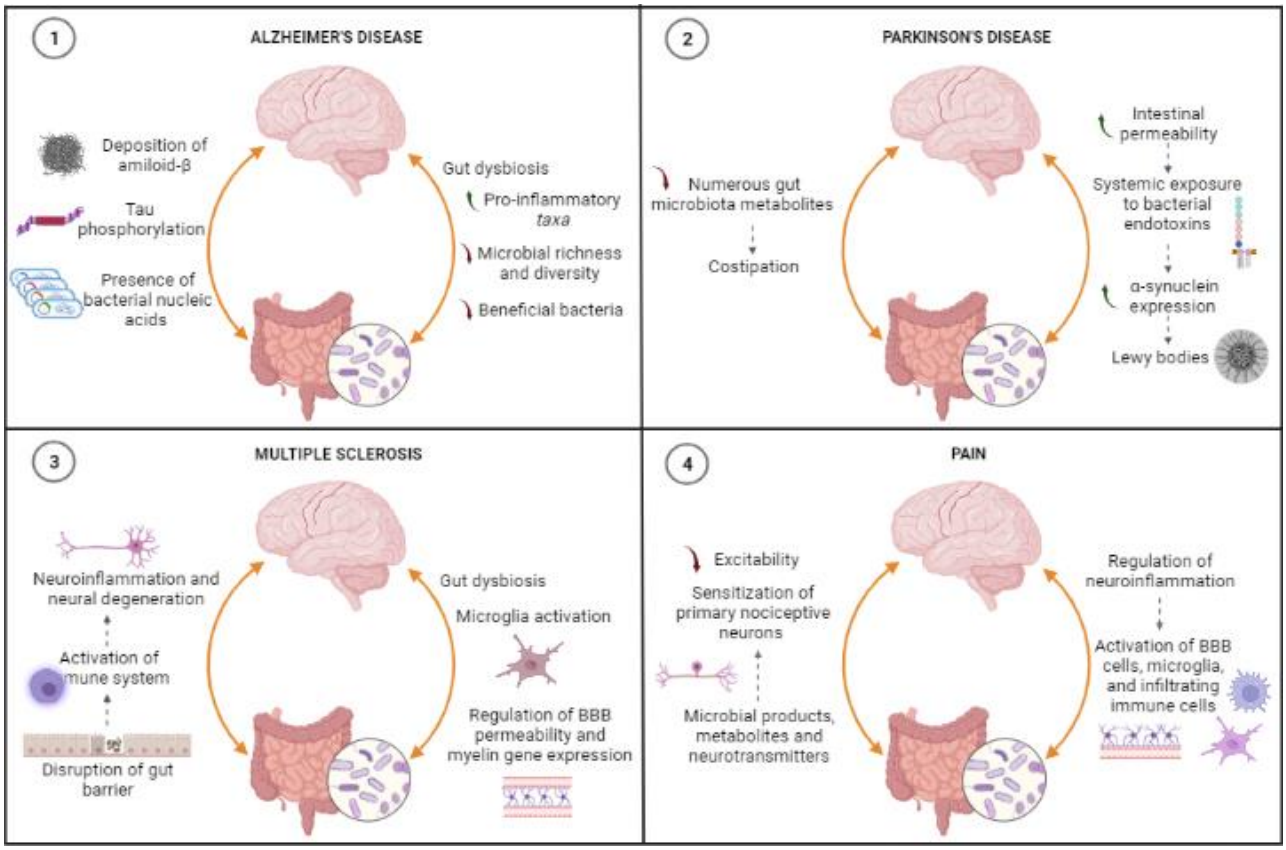

Figure 2. The gut-brain axis in neurodegenerative disorders and pain.

An imbalance in the gut microbiota composition and functions with alterations in the bidirectional cross-talk between the gut and the brain is involved in the pathogenesis of Alzheimer's disease (1), Parkinson's disease (2), multiple sclerosis (3) and pain (4). See text for details. Created with BioRender.com.

3. Naturally occurring flavonoids: their intestinal metabolic fate and their direct and indirect effects on the nervous system

Given the abundant preclinical and clinical evidence supporting the role of the gut microbiota in the pathogenesis of various CNS pathological conditions, the hypothesis of its manipulation as an innovative therapeutic approach is gaining momentum. To this purpose, the use of prebiotics (*i.e.*,

types of fibers or other naturally-occurring substances that feed healthy bacteria in the colon) and probiotics (*i.e.*, specific strains of beneficial bacteria as food supplements) is increasingly widespread, paralleled by numerous studies on fecal microbiota transplantation, which involves transplanting microorganisms derived from fecal samples of healthy subjects to patients with intestinal infections to restore the function of the microbiota [35].

Phytocompounds are the most commonly used nutraceuticals, and represent promising tools as preventive approach and supporting therapy for a number of chronic pathological conditions, such as cardiovascular diseases, diabetes, cancer, and osteoporosis. In most cases they are endowed with antioxidant or anti-inflammatory properties that are at the basis of their beneficial effects [36].

Flavonoids, including anthocyanins, proanthocyanidins, flavonols and isoflavones, are a family of polyphenolic secondary metabolites mainly found in edible plant parts, such as fruits, vegetables, and grains. Due to their known antibacterial, antiviral, antioxidant, anti-inflammatory, antimutagenic, and antitumoral properties, together with their minimal to no side effects, several studies have been conducted to explore their therapeutic potential to treat numerous diseases [37]. While investigating their therapeutic potential, it must be taken into account that flavonoids, and polyphenols in general, have a low bioavailability independently from their high or slow rate of absorption (see below). Thus, the parent compounds are barely found in plasma, where metabolites derived from phase I/phase II reactions and gut microbiota-derived metabolites (Figure 3) are instead highly abundant. Despite low oral bioavailability, most polyphenols, including flavonoids, proved significant biological effects as defined by the “low bioavailability/high bioactivity paradox” [38].

In this scenario, the aim of this review is to highlight the potential exploitation of dietary flavonoids as adjuvants in the treatment of neuroinflammatory and neurodegenerative conditions, possibly through their actions on the gut microbiota and vice versa.

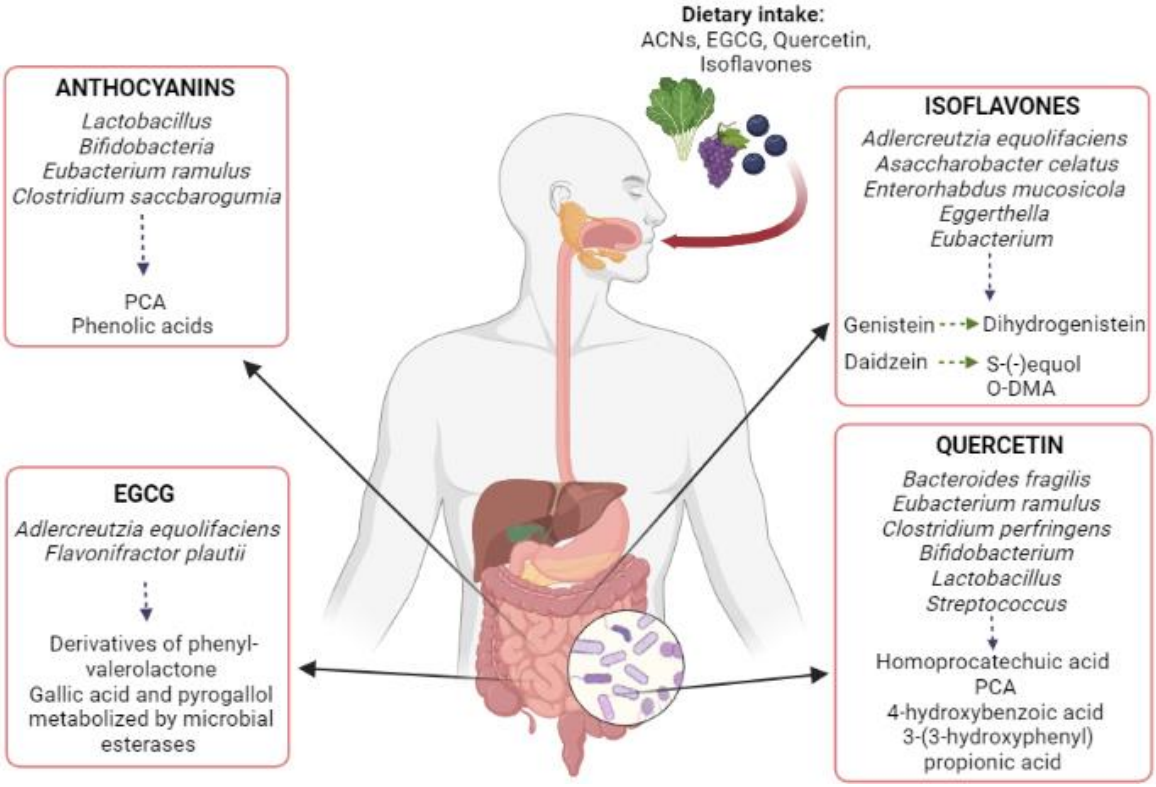

Figure 3. Metabolism of flavonoids by the gut microbiota

Anthocyanins, EGCG, isoflavones and quercetin are metabolized by specific bacterial strains in the gut to produce their active metabolites. See text for details. Created with BioRender.com.

3.1. ANTHOCYANINS

Anthocyanins (ACNs) are a class of flavonoids which confer red, purple, violet and blue pigmentation in certain varieties of cereals, and leafy and root vegetables (*e.g.* eggplant, red cabbage, black beans, red onions, purple carrots), but they are mostly abundant in fruits and berries [39–41]. ACNs can be classified into six types: pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin, which can be glycosylated and acylated, giving rise to more than 600 different types of ACNs [42,43]. The average dietary intake of ACNs ranges from 12,5 mg/day in the US population [44] to 18,4-64,9 mg/day in European countries with higher values in those of the Mediterranean area [45].

Several studies have reported the neuroprotective effect of ACNs in preclinical models of neurodegenerative diseases through multiple mechanisms of action [46,47]. The direct neuroprotective effect of ACNs is accomplished thanks to their rapid gastrointestinal absorption and their capacity to cross the BBB. Both in humans and animals, many ingested ACNs are absorbed intact, circulate in the plasma and pass into urine without undergoing metabolic changes [48]. ACNs

(about 25%) are rapidly absorbed in the rat stomach, appearing in the bloodstream after 6-20 minutes and reaching maximum levels after 60 minutes thanks to bilitranslocase, a membrane protein facilitating the uptake of bilirubin and flavonoids [49]. ACNs are also absorbed from the small intestine of rats and are then excreted into bile and urine. Absorption of glycosylated ACNs in the small intestine may occur through the extracellular hydrolysis of the glycoside via lactate phloridzin hydrolase (LPH) at the brush border or through the action of β-glucosidase, β-glucuronidase, and α-rhamnosidase, followed by passive diffusion of the aglycone [50] or through active transport of the intact molecule by bilintranslocase or a sodium-dependent glucose transporter (SGLT1) [51,52]. The concentration of native ACNs in plasma is in the range of nM to low μM [48], in accordance with human studies indicating that, despite their rapid absorption, the bioavailability of ACNs is very low (about 12%) [53]. Once absorbed, native ACNs are rapidly (*i.e.* 10 min) transported into the brain, where they can accumulate up to a concentration of 192 ng/g by bilitranslocase also located in the BBB [54].

Nonetheless, dietary ACNs mostly undergo a complex metabolism after ingestion and interact with endogenous enzymes, leading to the production of a large number of circulating and excreted metabolites and catabolic products [55]. ACNs are metabolized in the liver and then rapidly released in their intact form or as methylated and/or glucuronidated derivatives in urine and bile (20-25 min) and then reabsorbed in the enterohepatic circle, suggesting a prolonged persistence [56,57].

ACNs unabsorbed by the stomach and small intestine then reach the colon, where they firstly promote the proliferation of beneficial bacteria and the elimination of pathogenic ones, contributing to prevent the dysregulation of gut microbiota and the consequent systemic inflammation [58]. A number of *in vitro* and *in vivo* studies highlighted that ACNs promote the increase of *Lactobacilli* and *Bifidobacteria*, known to be beneficial and predominant in a healthy gut [59–61] and to reduce pathogenic *Staphylococcus aureus* and *Salmonella typhimurium* [59]. On the other hand, ACNs are extensively modified by the gut microbiota, increasing the number of possible metabolites produced [62,63]. Protocatechuic acid (PCA) is the major metabolite produced from cyanidin 3-glucoside (C3G), but phenolic acids, such as gallic, syringic, vanillic and p-coumaric acids, can be also formed [64,65]. Some of these ACN metabolites (*e.g.* PCA) have a high antioxidant capacity, can be efficiently absorbed and can significantly contribute to the protective effects of ACNs. As an alternative, they can directly exert their antioxidant activity in the gut [66,67].

To date, several preclinical studies have highlighted the existence of a possible neuroprotective role exerted by ACNs by reducing oxidative stress, neuroinflammation and excytotoxicity. Some *in*

vitro studies have shown that ACNs prevent the excessive increase of intracellular calcium, thus counteracting excitotoxicity [68,69]. Concerning their antioxidant mechanisms, ACNs exert a direct scavenger activity against reactive oxygen species (ROS) in the CNS, as their chemical nature allows them to cross the BBB, although this effect is limited by the low bioavailability of ACNs in their native form. More likely, the antioxidant activity of ACNs is mediated by promoting the activation and nuclear translocation of the transcription factor Nrf2 with the subsequent activation of antioxidant response genes [70] or by enhancing the activity of antioxidant enzymes, such as heme oxygenase-1 (HO-1), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) [71,72].

ACNs also exert their neuroprotective effect by reducing neuroinflammation at least in three ways. They can inhibit the nuclear translocation of the transcription factor NF-kB, thus preventing the synthesis of pro-inflammatory cytokines, such as IL-1β and TNF-α and of COX-2- and iNOS-related inflammatory mediators [73]. They can also act through the direct inactivation of COX-2 [74] or by interfering with the intracellular signaling mechanisms mediated by MAP kinases JNK and p-38, involved in the production of pro-inflammatory cytokines [75].

Acting on the three common mechanisms underlying neurodegenerative diseases, ACNs prevent the onset of neuronal apoptosis, but also directly modulate pro-and anti-apoptotic factors. The anti-apoptotic activity of ACNs is linked to the reduced release of the induction factor of apoptosis (AIF) and the reduced expression of pro-apoptotic proteins, such as Bax [76], but also to increasing levels of cell survival proteins, such as the anti-apoptotic proteins Bcl-2 and phosphorylated Akt kinase [77,78].

Despite the growing number of studies focusing on the potential interaction between ACNs and the gut–brain axis, only a few studies have linked specific metabolites generated through the influence of ACNs on microbiota to the prevention of neuroinflammation. Of note, an ACN-rich extract from blackberry was found to counteract high fat (HF)-diet induced neuroinflammation, by modulating gut microbiota with a consequent change on tryptophan metabolism and release of kyanurenic acid, a neuroprotective metabolite [79,80]. These studies suggest that administration of dietary ACNs can impact on microbiota composition and metabolism and may potentially result in prevention of onset and progression of neurodegenerative diseases.

3.2. EPIGALLOCATECHIN-3-GALLATE

Epigallocatechin-3-gallate (EGCG) belongs to the proanthocyanidin family of flavonoids. Overall, members of this group of molecules show highly complex structures composed of

condensed monomers of flavan-3-ols with various chemical structures which allow their classification into procyanidins ((epi)catechin units), propelargonidins ((epi)afzelechin units) and prodelphinidins ((epi)gallocatechin units) [38]. Proanthocyanidins are enriched in fruits (*e.g.*, apples, kiwi, berries, pears, grape seeds), beverages (such as green tea, black tea, red wine, and cocoa liquor), chocolate, cocoa products, and others. Thus, their mean daily intake is much higher in countries with a vegetable- and fruit-enriched diet, such as those in the Mediterranean area and in Asia, with respect to populations with a typical Western diet [38].

EGCG and other catechins are highly enriched in green tea, and their daily intake is around 90-300 mg/day [81]. The beneficial effects of green tea on health have been known for millennia especially in Far East countries and modern science has demonstrated that EGCG is its predominant active molecule [82]. As for most flavonoids and polyphenols, it is nevertheless worth mentioning that their beneficial effects are not only a matter of ingested quantity, but rather of bioavailability. Catechins are very long highly hydrophilic polymers, thus they cannot cross the cellular membrane lipid bilayer as such, and paracellular passive diffusion is believed to be their main mechanism of absorption. Additionally, they show low water solubility, which limits their solubilization in the gut, and are substrates of p-Glycoprotein (pGP), the transporter protein located on the intestinal epithelial cells that extrudes xenobiotics from the cellular cytoplasm to the intestinal lumen [83]. As a result of these characteristics, catechins have a very low oral bioavailability, with only a small percentage, predominantly represented by monomers and oligomers, absorbed in the small intestine and exposed to phase I and phase II metabolism, mostly glucuronidation, sulfonation and methylation, either in enterocytes or in hepatocytes [84]. Interestingly, glucuronic acid-conjugated ECGC metabolites generated in the liver can undergo an enterohepatic recycle through the biliary system, thus exerting additional beneficial actions in the gut [85].

An important contribution to the effects of catechins derives from their microbiota-dependent metabolism in the colon. EGCG is mostly metabolized to various derivatives of phenyl-valerolactone and by microbial esterases to gallic acid and pyrogallol; overall, these metabolites seem far more active than their parent compounds [86]. The individuation of the bacterial *taxa* that are predominantly responsible for EGCG metabolism is complicated by the high interindividual variability in the composition of microbiota and by the difficulties in performing reliable *in vitro* studies on human colonic bacteria that are not easy to cultivate. Nevertheless, both *Adlercreutzia equolifaciens* and *Flavonifractor plautii* have been identified in rat but also in humans as responsible for EGCG metabolism [87].

In vitro studies performed on fecal slurries from healthy volunteers supplemented with 200 mg/L of (+)catechins, to mimic the mean daily intake due to green tea drinking, has confirmed the high interindividual variability in the production of EGCG metabolites with the possibility to stratify subjects in low, medium and high metabolizers [88]. Interestingly, fast converters show higher microbiota variability and diversity in *taxa* composition, a sign of a healthy microbial environment [1], and more abundant production of SCFAs when compared to low and medium metabolizers [88]. This observation confirms that microbiota-produced EGCG metabolites positively influence the growth of beneficial bacterial *taxa* which, in turn, could be responsible of the overall anti-inflammatory and favorable effects of EGCG in various body districts.

On the other hand, catechins modify gut microbiota toward an overall beneficial composition with significant reduction of pathogenic *taxa*, such as *Listeria monocytogenes*, *Helicobacter pylori*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* [89], and a positive specific modulation of the growth of beneficial strains, *e.g.*, *Verrucomicrobia* and *Actinobacteria* by EGCG [90]. These data suggest that EGCG could prove beneficial in many pathologies characterized by dysbiosis, as recently emerging for many neurodegenerative disorders (see also below).

Apart from more general beneficial effects on health of green tea extracts that are shared with other classes of flavonoids, such as the antioxidant, anti-inflammatory, antitumoral, antiviral, hypoglycemic and antiaging activities as recently summarized in details elsewhere [82], EGCG is endowed with positive outcomes on specific pathological mechanisms involved in neurodegeneration. Despite the very different clinical characteristics, AD and PD share many common pathological features, including the presence of a pro-inflammatory environment which is sustained and promoted by activated microglia and astrocytes, oxidative stress, the aggregation/precipitation/deposition of misfolded proteins, namely amyloidogenic β-amyloid fragments ($A\beta_{1-42}$) and Tau protein in AD and α-synuclein in PD, and dysregulation of the autophagy process, as recently brilliantly reviewed in [91]. Thus, it is not surprising that molecules which interfere with any of these processes, such as EGCG, might prove beneficial in both neurodegenerative disorders. Along with a general anti-inflammatory, radical scavenging activity and with the ability to promote neuronal cell differentiation and survival *in vitro* [92], EGCG exhibits specific anti-amyloidogenic actions by inhibiting enzymes such as β-secretase1 (BACE1) whose activity is directly related to the generation of toxic protein fragments. Similar beneficial actions have been described in models of PD [91]. Additionally, *in vitro* studies on cell cultures

demonstrated that EGCG could induce autophagy, which in turn hinders the sustained release of pro-inflammatory mediators from reactive microglia and astrocytes [91].

Finally, ageing is often accompanied by alterations in lipid metabolism, with increased low-density lipoprotein (LDL)-cholesterol and triglyceride blood concentrations, and by the metabolic syndrome with associated clinical symptoms. Overall, these alterations may contribute to foster the progression of neurodegenerative diseases [93]. Thus, the well-known properties of green tea as lipid lowering beverage, with reduction of body weight and amelioration of liver functionality also through the involvement of gut microbiota, might prove useful in reducing the symptoms and progression of AD and PD as well [94].

3.3. FLAVONOLS

Flavonols are naturally present in the diet, especially in fruits, green vegetables, beverages, and certain medicinal plants and herbs [95]. They chemically consist of a central structure of 3-hydroxyflavone with various substituents.

Quercetin is the most common member of the flavonol subclass and represents 60-75% of total flavonoid dietary intake [96]. Its metabolic fate consists of an initial absorption by the small intestine, followed by deglycosylation to quercetin aglycone by lactase phloridzin hydrolase and by gut microbiota-derived beta-glucosidase [97,98]. Subsequently, quercetin aglycone undergoes phase II metabolism, forming glucuronidated, methylated, or sulfonated metabolites thanks to the involvement of enzymes such as uridine 5'-diphospho-glucuronosyltransferase (UGT), sulfotransferase (SULT) and catechol-O-methyltransferase (COMT) [99].

It has also been demonstrated that certain bacterial strains, including *Bacteroides fragilis, Eubacterium ramulus, Clostridium perfringens, Bacteroides JY-6, Bifidobacterium B-9, Lactobacillus L-2, and Streptococcus S-2*, are responsible for the transformation of quercetin into its metabolites. In particular, gut microbiota transforms quercetin into homoprocatechuic, protocatechuic, 4-hydroxybenzoic, and 3-(3-hydroxyphenyl)propionic acids [100]. In addition, quercetin metabolites can pass the BBB and are further metabolized by glial cells which remove conjugations, except for the methylated form, the only quercetin metabolite that has been found in the brain [101,102].

However, only a small percentage of ingested quercetin reaches the bloodstream and is available for tissues and organs due to its poor water solubility and permeability. Therefore, the use of quercetin has been limited because of its low bioavailability, which led to the use of high concentrations for long periods of treatment that may cause significant adverse effects [97]. In fact,

quercetin at high doses (1500 and 2000 mg/kg in mice) [103] reacts with free radicals and forms a toxic oxidation product called quercetin-quinine, which can react with sulfhydryl compounds such as protein-SH and cause toxic effects (*e.g.* cell injury by destroying the integrity of the cell membrane) if glutathione (GSH) concentration is so low to result ineffective in its sequestration [104]. Thus, to improve systemic bioavailability of lower doses of quercetin, several methods have been developed (*i.e.* quercetin-loaded nanoparticles) [105].

As mentioned, chronic microglial activation and neuroinflammation are closely associated with the pathogenesis of several neurodegenerative diseases, including AD, PD, MS, and amyotrophic lateral sclerosis (ALS) [106]. Recent studies have demonstrated that quercetin can protect against neuroinflammation. For example, quercetin suppressed neuroinflammation induced by chronic exposure to Zidovudine (azidothymidine, AZT), a nucleoside reverse transcriptase inhibitor (NRTI) that is commonly part of the highly active antiretroviral therapy (HAART) regimens. The administration of 50 mg/kg/day of quercetin inhibited the up-regulation of pro-inflammatory cytokines and microglial and astrocytic markers induced by AZT in the mouse cortex, hippocampus, and spinal cord [107].

In a more recent study, authors demonstrated that pre-treatment with quercetin significantly protected from 1-methyl-4-phenylpyridinium (MPP+)-induced mitochondrial dysfunction and lipopolysaccharide (LPS)-induced neuroinflammation *in vitro*. In addition, quercetin reduced the activation of microglia and astrocytes in the hippocampus and in the substantia nigra of LPS-injected mice, suggesting that it might be therapeutically effective for neuroinflammation-mediated neurodegeneration by alleviating mitochondrial damages [108].

At last, quercetin has been shown to significantly attenuate LPS-induced inflammatory factor production, cell proliferation and NF-κB activation in microglia. It also decreased the levels of the NLR family pyrin domain-containing 3 (NLRP3) inflammasome and of pyroptosis-related proteins. Its anti-inflammatory effect is related to the promotion of mitophagy, which enhanced damaged mitochondrial elimination, reduced mitochondrial ROS accumulation and alleviated NLRP3 inflammasome activation. In addition, treatment with quercetin protects primary neurons against LPS-induced microglial toxicity and alleviates neurodegeneration [109].

3.4. ISOFLAVONES

Isoflavone structure is composed of a planar basic ring with a benzene at C-3, which differentiate it from other flavonoids. The main dietary source of isoflavones is soy, and their

peculiar feature is the ability to bind to estrogen receptors, thus making them considered as natural phyto-estrogens [110].

After oral administration, almost all isoflavones, including genistein and daidzein, reach the colon unabsorbed as glycosides and are hydrolyzed by gut microbiota β-glucosidases to more bioavailable aglycones [111], which are either absorbed as such or are further metabolized by the gut microbiota [112]. It has been estimated that at least 30% of isoflavone metabolites have a bacterial origin [113].

Isoflavones are metabolized by different classes of gut bacteria, as reviewed elsewhere [114]. Genistein is first converted to dihydrogenistein, which binds to ERs and exert biological effects and has been detected in human urine and plasma at high concentrations. In turn, it is further metabolized to 6'-hydroxy-O-desmethylangolensin, with unknown effects, through absorption and enterohepatic circulation [115]. Other genistein metabolites, such as 4-hydroxyphenyl-2-propionic acid and 4-ethylphenol, have also been identified [116].

Daidzein is converted in two main metabolites by gut bacteria: a ring cleavage product, O-demethylangolensin (O-DMA) and, subsequently, equol by deoxygenation to a reduction product. Gut bacteria are enantioselective in metabolizing daidzein to exclusively S-(-)equol and not R-(+)equol. Interestingly, only 30–50% of the population produces equol, while 80–90% produces O-DMA [116]. An inverse relationship between equol and O-DMA excretion in various individuals has been observed, suggesting that the gut microbiota acts through two distinctive pathways to convert daidzein. Due to its nonplanar structure, equol has a good bioavailability and is able to inhibit oxidative stress, but it should be taken into account that the biotransformation capacity of isoflavones to form equol in humans is much lower than in animals [117].

Interestingly, studies conducted on the biotransformation kinetics of daidzein and genistein by a human intestinal bacterium indicated that the C-ring of genistein has a higher susceptibility to bacterial degradation than that of daidzein [118].

In a ROS absorbance capacity assay, when compared with parent isoflavones dihydrodaidzein and O-DMA showed the same or slightly lower antioxidant potency, while equol exhibited a higher antioxidant activity [119]. In another study on HepG2 cells, O-DMA, equol and daidzein all showed strong antioxidant properties by stimulating CAT and total SOD activity [120].

A recent paper employed a simulator of the human intestinal microbial ecosystem (SHIME) [121] to study the bioavailability of soy isoflavones and their interactions with the gut microbiota. Results confirmed that soy isoflavones are mainly digested in the colon, and their antioxidant

capacity changes with the progression of digestion. Moreover, isoflavones can modify the composition of the gut microbiota by inhibiting the growth of harmful bacteria, and promote the growth of probiotics and significantly improve their antibacterial ability *in vitro* [122].

The interaction between isoflavones and the gut microbiota has been recently highlighted as a potential regulator of several pathological conditions, including metabolic disorders such as obesity and diabetes [123–125], and disorders of the nervous system. For example, two recent papers demonstrated that soy isoflavone supplements ameliorated depression-like behavior in a rat model of chronic unpredictable mild stress by increasing monoamine neurotransmitter levels [126], by reducing the expression of pro-inflammatory factors and increasing the level of anti-inflammatory ones, by improving damaged colon tissue, and by enhancing BDNF and IκB-α and inhibiting NF-κB expression in the hippocampus [46]. These beneficial effects are related to their ability to reshape the gut microbiota composition. Similarly, authors reported that oral administration of a soybean meal extract rich in isoflavones modulated the composition of the gut microbiota and increases presynaptic function in healthy rats, which resulted in a better Morris water maze performance, meaning a better memory preservation [127].

Despite the direct role of the gut-brain axis still has to be fully elucidated, the protective effect of isoflavones, especially genistein, on neuroinflammation has been clearly demonstrated in a number of preclinical models, via mechanisms including inhibition of NF-κB, prostaglandins, pro-inflammatory cytokines, iNOS, ROS and free radical scavenging activity (for review see [128]). For example, isoflavones administration (80 mg/kg/day) protected from neuroinflammation in a rat model of haloperidol-induced dyskinesia, by reducing the expression of IL-1β and TNF-α [129]. Isoformononetin (IFN), a methoxyl isoflavone present in most of human dietary supplements, administered 20 mg/kg/day *per os*, significantly prevented streptozotocin (STZ)-induced inflammation in rats by decreasing the generation of ROS that in turn reduces the activation of NLRP3/ASC/IL-1 axis [130]. Genistein-3'-sodium sulfonate (0.5, 1, 2 mg/kg single dose), a derivative of genistein, proved able to protect cortical neurons against neuroinflammation in a rat model of cerebral ischemia/reperfusion injury, by reducing the expression of pro-inflammatory cytokines and inhibiting the phosphorylation of JAK2 and STAT3 via a mechanism that involves α7 nicotinic acetylcholine receptor [131]. An *in vitro* study mimicked the pathogenesis of PD by treating SH-SY5Y cell lines overexpressing A53T mutant α-synuclein with the environmental toxin rotenone. Results confirmed the neuroprotective effects of 20 μM genistein, which reduced oxidative stress damage and cell apoptosis by activating estrogen receptors and NFE2L2 channels [132]. A recent paper

reported that isoflavone-rich extracts from red clover flower and Tofu soybeans, as well as individual isoflavones daidzein and equol, rescued the loss of dopaminergic neurons and neurites shortening in primary mesencephalic cultures exposed to rotenone and an adenovirus encoding the A53T α-synuclein mutant, as *in vitro* PD models. Both the extracts and individual isoflavones also activated the Nrf2-mediated antioxidant pathway in astrocytes, and reduced deficits in mitochondrial respiration [133].

4. Can flavonoids be exploited as adjuvant in the therapy of nervous system pathologies? Pre-clinical and clinical evidence

4.1. ANTHOCYANINS

4.1.1. Anthocyanins in Alzheimer's (AD) and Parkinson's diseases (PD).

Beside *in vitro* studies defining the molecular mechanisms involved in the neuroprotective effect of ACNs, a number of preclinical *in vivo* studies have proved that ACNs can prevent the onset and progression of neurodegenerative diseases.

Concerning AD, initial studies on the APP/PSEN1 transgenic mouse model (Tg2576) carrying mutations in amyloid precursor protein (APP) and presenilin-1 (PSEN1), fed with blueberry extract from 4 months of age showed no deficits in Y-maze performance (at 12 months of age), despite no difference in Aβ plaques compared to non-transgenic mice [134]. A similar result was subsequently obtained in the APP/PS2 transgenic model fed with bilberry extract for 4-months starting at 1 month of age, showing retention of cognitive ability, not associated with a reduction in protein aggregate deposition in brains, but with an increase in insoluble deposits, suggesting that ACNs may promote an alternative non-toxic form of Aβ aggregation in these AD mouse models [135].

In other AD mouse models, supplementation with ACN-rich extracts promoted retention of cognitive abilities and was associated with a reduction of Aβ plaques. Dietary ACNs from mulberry extracts (26-130 mg/kg/day) supplied to the senescence-accelerated mouse prone 8 (SAMP8) model, widely used as a non-transgenic murine model for late-onset AD, reduced Aβ plaques and improved learning and memory, by activating the NRF2-dependent antioxidant defense system [136]. Similarly, when supplemented with bilberry and blackberry extracts (1,53 mg/g diet and 1,43 mg/g diet, respectively) the APdE9 mouse model overexpressing APP and PSEN1 mutated proteins showed a reduction in the deficit of spatial working memory associated to reduced APP levels, but no changes in the expression and phosphorylation of tau protein [137].

Different preclinical studies demonstrated that ACNs exert neuroprotective effects against AD by reducing oxidative damage and neuronal apoptosis and by promoting autophagy. Supplementation of ACNs (4 mg/Kg/day) from black soybean in an Aβ-induced rat model of AD was shown to reverse neuronal apoptosis by suppressing the intrinsic apoptosis pathway (*i.e.* Bax, cytochrome C, caspase-9 and caspase-3) [68]. Similar results were obtained with supplementation of ACNs (50 mg/Kg/day) from *Aronia melanocarpa*, showing an improvement in Morris water maze test and a reduction in cell loss and disorganization of pyramidal cells [138]. Furthermore, ACNs (12 mg/Kg/day) from Korean black beans supplemented to the APP/PSEN1 mice improved the performance in Morris water and Y-maze tests, prevented neuronal apoptosis by suppressing the activation of caspase-3 and reduced oxidative stress induced by Aβ aggregation through the activation of the p-PI3K/Akt/GSK3β pathway and, as a consequence, the NRF2-dependent antioxidant response *HO-1* and Glutamate-cysteine ligase modifier subunit of glutamate-cysteine ligase (*GCLM*) genes [70]. A similar activation of the p-PI3K/Akt/GSK3β pathway, prevention of apoptosis and hyperphosphorylation of tau were obtained with ACN-loaded gold nanoparticles supplied to Aβ-induced AD mice (12 μg/g diet for 14 days), which resulted to be significantly more effective than free ACNs at identical concentrations [139].

Both a short- and a long-term treatment (7 days *vs* 21-25 days) with ACNs (200 mg/Kg/day) from grape extract or *Hibiscus sabdariffa* attenuated memory deficits, protected against oxidative damage in the brain, restored acetylcholinesterase and ion pump activity and normalized PGE_2/cytokine production by modulating COX-2 pathway in a rat model of diabetes-associated AD, such as STZ-induced sporadic dementia of Alzheimer's type [72,140,141]. More recently, the expression of autophagy-related proteins were found to be promoted in APP/PSEN1 mice supplemented for 16 weeks with ACNs (150 mg/kg/day) from bilberry extract, with a major contribution in primary neurons given by PCA, the main microbiota-derived metabolite of C3G (see above) [142].

Some preclinical studies in LPS-treated mice suggest that dietary ACNs ([70]: 12 mg/Kg/day; [78,143]: 24 mg/Kg/day) may prevent neuroinflammation-induced Aβ deposition by increasing beneficial microbiota subpopulations and preventing gut dysbiosis. Dysbiosis is associated with circulation and entry of LPS into the brain, thus resulting in NF-kB activation and production of pro-inflammatory cytokines and BACE1 in the brain [70,78,143,144]. Some studies have highlighted that ACNs ([79]: 25 mg/Kg/day; [145]: 3.8 μmol/g) may increase beneficial microbial species, such as *Bifidobacterium* and *Lactobacillus* genera [65,79,145]. However, direct evidence of ACN-induced

microbiota populations involved in this protective effect in AD model mice is presently lacking. Despite many preclinical studies, only one community-based study is to date available, indicating that a higher consumption of pelargonidin (1.9 mg/day) from strawberries is associated to lower AD neuropathology [146].

A regular intake of ACNs, assumed by consuming strawberries and blueberries in combination with other antioxidant, such as vitamin E, has been associated to a lower PD risk [147], as confirmed by a recent dose–response meta-analysis on observational studies [148]. Importantly, ACNs from mulberry (0,008 mg/g diet) or bilberry extract (0,02-0,04 mg/g diet) supplemented to an MPTP-induced mouse model of PD significantly reduced hypokinesia, malondialdehyde (MDA) levels, loss of dopaminergic neurons and dopamine depletion, and increased SOD and GSH-Px activities [149,150]. Similarly, ACNs from grape extract (250 mg/Kg/day) improved motor function and prevented neuronal loss in a mouse model of PD induced by the injection of the neurotoxin 6-hydroxydopamine (6-OHDA) in the *corpus striatum* [151]. Based on cellular models of PD, this protective effect is achieved by reducing microglia activation and oxidative stress, thus preserving dopaminergic neurons from mitochondrial dysfunction and apoptosis [151,152].

4.1.2. Anthocyanins in multiple sclerosis (MS)

Only one study has highlighted that ACNs from grape skin (100 mg/kg/day) significantly reduced demyelination in a rat model of MS by restoring GSH level and SOD activity, suggesting that a possible NRF2-mediated antioxidant mechanism of protection may occur [153]. ACNs reduced infiltration of inflammatory cells, the expression of pro-inflammatory cytokines, such as IL-1β and TNF-α, increased the expression of anti-inflammatory cytokines, like IL-10, and of Na^+/K^+-ATPase and Ca^{2+}-ATPase ion pumps [153].

4.1.3. Anthocyanins in pain and other CNS disorders

Different types of ACNs at various dosages (30-100 mg/Kg/day) have been shown to have a pain-relieving effect in various rat models of acute inflammation-induced pain. High doses of (cyanidin-type) ACNs (400 mg/Kg/day) from tart cherry administered by oral gavage showed a similar reduction of carrageenan-induced paw edema and Complete Freund's Adjuvant (CFA)-induced pain as indomethacin (5 mg/Kg) [154]. Oral administration of an ACN-rich purple corn extract (approximately 95% cyanidin-type and 5% pelargonidin-type) also showed a significant

reduction in development of orofacial allodynia, in TG macrophage infiltration in an *in vivo* model of inflammatory trigeminal (TG) pain, and of microglial activation both *in vivo* and *in vitro*. The protective effect of purple corn was effective at 53 mg ACNs/kg/day and was comparable to the anti-inflammatory effects of acetyl salicylic acid (50 mg/kg), which nevertheless did not modify microglia activation, thus suggesting a possible application of ACN-rich dietary supplements as co-adjuvants in pain therapy, aimed at reducing drugs dosage and side effects [155]. Interestingly, a pilot study on patients affected by different types of chronic pain supplemented with a cherry juice (about 48 mg cyanidin-type ACNs per day) for 6 weeks highlighted a significant reduction in pain level from high to mild compared to placebo-treated patients [156]. Administration of ACNs from maqui-berry (83% delphinidin-type and 17% cyanidin-type) in a formalin-induced gastric pain rat model demonstrated a dose-dependent antinociceptive activity in both neurogenic and inflammatory phases of pain without gastric damage and demonstrated that parenteral intraperitoneal (i.p.) administration was effective at lower doses of maqui berry extract (62.5-125 mg/Kg) compared to oral administration which required higher doses (500-1000 mg/Kg). These concentrations are nonetheless achievable, corresponding to 2.84 g of maqui (47.02 mg of ACNs) [157].

A pain-relieving effect was also obtained with some ACN aglycones, such as malvidin and delphinidin. In a rat model of monosodium iodoacetate (MIA)-induced osteoarthritis, malvidin supplementation (10-20 mg/Kg/day) was effective in reducing apoptosis of chondrocytes, pro-inflammatory interleukins (Il-1β, IL-6 and TNF-α) by suppressing NF-kB nuclear translocation in an IkBα-independent manner, and matrix metalloproteinases (*i.e.* MMP-3, -9 and -13), which are responsible for cartilage degradation [158]. In a rat model of CFA-induced paw edema i.p. administration of cyclodextrin-stabilized delphinidin aglycone (50 mg) reduced mechanical and heat hyperalgesia, while its local application dose-dependently reduced mechanical hyperalgesia, paw volume, formation of the lipid peroxidation product 4-hydroxy-2-nonenal (4-HNE), and macrophages infiltration [159].

Interestingly, two studies determined that ACNs reduce onset and progression of ALS. Oral administration of an ACN-rich extract from strawberries (mostly pelargonidin-type at 2 mg/Kg/day) in the hSOD1^{G93A} mouse model of ALS significantly delayed the onset of the disease (about 17 days), extended the survival (about 11 days), significantly preserved skeletal muscle strength and neuromuscular junctions (NMJs) in gastrocnemius muscle compared to untreated hSOD1^{G93A} mice.

A significant reduction of astrogliosis, but not of motor neuron loss, was observed in spinal cord [160]. Similar results were obtained by administrating PCA, the main ACN metabolite, in the same ALS mouse model, but at higher dose (100 mg/Kg/day) and at the onset of disease, with the additional benefit of preserving motor neurons from apoptosis [66].

4.2. EGCG

4.2.1. EGCG in Alzheimer's (AD) and Parkinson's diseases (PD)

Studies have accumulated in preclinical models of various inflammation-based pathologies and results allow to identify possible mechanisms that might prove useful in neurodegenerative disorders as well (see above; [91]). Additionally, catechin metabolites have been found in brain tissue, thus confirming that they can cross the BBB and directly exert their anti-neurodegenerative protective effects [161] .

For example, some preclinical studies have shown that a 200 mg/kg/day of ethylacetate extract of grape fruits, enriched in various catechins including EGCG, can reduce toxic $A\beta_{1-42}$ protein deposition in the brain and improve cognitive functions in an animal model of AD [161]. Additionally, 100-300 mg/kg/day of EGCG prior to rotenone daily injections prevented the development of Parkinsonism-like symptoms in rats, through a reduction of neuroinflammation and lipid peroxidation and the stimulation of metabolic activity in the brain, along with increased catecholamine concentrations in the *corpus striatum* [162]. These studies and others, reviewed in [91], clearly highlight the need for high daily EGCG doses to achieve any significant therapeutic effect, based on the chemical instability of EGCG, its extensive metabolism and its low bioavailability. Regretfully, these dosages appear to be very closed to the toxic ones. According to the European Food Safety Agency (EFSA) panel on Food Additives and Nutrient Sources added to Food, EGCG dosages above or equivalent to 800 mg/day caused acute liver inflammation, with increased transaminase level and other side effects such as anemia, hypoglycemia, dizziness, and kidney complications both in animals and in humans [81,82].

This problem emerged also from the PROMESA (Progression Rate of Multiple System Atrophy under EGCG supplementation as anti-aggregation-approach) clinical study, a prospective, randomized, double-blind, placebo-controlled parallel group phase II/III, multicenter study comparing placebo versus EGCG as a potential neuroprotective agent in patients suffering from Multiple System Atrophy (ClinicalTrials.gov #NCT02008721; EudraCT #2012-000928-18). This is a neurodegenerative disease characterized by the aggregation and deposition of α-synuclein, with a

much faster progression with respect to PD. Although no beneficial neuroprotective outcomes have been observed in the intervention group after a 48-week (+ 4 weeks of washout) administration of 800-1200 mg/day of EGCG, some cases of hepatic toxicity clearly indicated that the toxic dosage has been reached [163].

Thus, the United State Pharmacopoeia (UPS) has conducted an extensive review of all available literature data on reported toxicity of green tea extracts, enriched with EGCG. In fact, the use of highly concentrated extracts poses different concerns with respect to the traditional and long-lasting consumption of green tea as beverage, spanning from the high concentrations of active ingredients, the unwanted presence of residues of contaminants (*i.e.*, solvents, pesticides and other impurities) to more specific pharmacokinetics and pharmacodynamic issues. Based on the reviewed preclinical and clinical data, the UPS has decided to include a label cautionary statement in the Powdered Decaffeinated Green Tea Extract (PDGTE) monograph, warning to take with food and the recommendation to avoid use in patients suffering from liver diseases and to immediately discontinue use in the case of any side effects [164].

Several chemical strategies have been developed or are currently under development to improve EGCG bioavailability and stability, with the generation of prodrugs (*e.g.*, ester, glycosylated and methylated EGCG derivatives), in addition to innovative formulation approaches through the use of lipid nanocarriers, nanoparticles and others, as recently reviewed in [82]. It is worth mentioning that if new studies confirm that microbiota metabolites are mostly responsible for EGCG effects, these novel chemical and technological approaches aimed at promoting EGCG absorption in the gut might, paradoxically, limit the availability of EGCG for microbiota-mediated metabolism, thus hindering the overall beneficial therapeutic outcome. On the other hand, promoting EGCG bioavailability will increase the circulating concentrations of EGCG phase I and phase II metabolites, which are endowed with significant pharmacological effects as well (see above).

Despite a wealth of *in vitro* and preclinical promising data, the failure of the PROMESA clinical trial has greatly challenged the hypothesis of utilizing EGCG and other polyphenols as neuroprotective agents in proteinopathies, not only for safety concerns related to the very high dosages, but also to the very limited effect on only a small subset of patients with reduced striatal loss following EGCG treatment [165]. Several hypotheses might be raised to explain this failure in patients, including the already highly compromised condition of brain tissues at the time of intervention and the low brain bioavailability of EGCG and of its active derivatives [165]. No considerations on the role of microbiota metabolism of EGCG have been raised, which might further

clarify why patients did not respond to the treatment. Additional insights into the mechanism of action of EGCG in neurodegeneration are still needed to design better therapeutic strategies.

Based on the www.Clinicaltrials.gov website, 1 clinical study is registered for the use of EGCG in newly diagnosed PD patients (#NCT00461942) and 3 for the use of EGCG in AD patients (#NCT03978052, # NCT00951834, # NCT01699711); all studies are completed with no published results.

4.2.2. EGCG in multiple sclerosis (MS)

As mentioned above, the pathological trigger of MS is autoimmunity, which is later sustained and expanded by neuroinflammation and generation of ROS [25]. Thus, the well-known anti-inflammatory and antioxidant properties of EGCG appear as more than reasonable weapons towards MS progression. Preclinical studies in EAE models have shown neuroprotective effects of EGCG, also through the downregulation of NF-κB in T cells, which led to a significant reduction of the motor symptoms of the disease even when EGCG was administered after the onset of the pathology [166,167].

Based on encouraging *in vitro* and preclinical evidence, EGCG has been tested at a dose of 800 mg/day for 18 months as add-on therapy in patients with Relapsing-Remitting Multiple Sclerosis (RRMS) taking glatiramer acetate, a disease-modifying drug. Unfortunately, no significant differences have been observed at the end of the treatment with respect to the placebo arm both in terms of demyelinating lesions at MRI analysis and clinical disease activity. No side effects have been highlighted [168].

An additional interesting aim of a phytochemical intervention with EGCG in MS regards the treatment of the progressive anthropometric changes in patients, with the loss of muscle and lean mass, leading to muscle weakness and fatigue, and the parallel increase in body fat and overweight. The latter not only contributes to movement impairment, but increases the overall inflammatory status of the patient, which in turn might accelerate disease progression [169]. A former randomized, double-blind, placebo-controlled, crossover clinical trial tested the ability of EGCG to improve energy metabolism and substrate utilization in 18 MS patients with RRMS under glatiramer acetate therapy (Clinicaltrial.gov #NCT NCT01417312). All patients received 600 mg/day of EGCG or placebo over 12 weeks, with 4-week washout in between. Results showed that EGCG improved muscle metabolism by shifting from fat to carbohydrate oxidation both at rest and during exercise,

with an overall increased working efficiency. Results were more evident in men than in women, thus suggesting sex-specific effects on autonomic and endocrine control of metabolism [170].

More recently, based on the known anti-inflammatory actions of EGCG, a combined dietary approach including EGCG and coconut oil has been developed, with the latter known to promote the generation of the ketone body β-hydroxybutyrate which is endowed with anti-neuroinflammatory activities. The results of this pilot clinical trial (Clinicaltrial.gov #NCT03740295) on 27 patients with RRMS or Secondary Progressive MS (SPMS), whose diet was supplemented for 4 months with extra virgin coconut oil and 800 mg/day EGCG, showed significant reduction in markers of inflammation (*i.e.*, IL-6) and a reduced body fat percentage with respect to 24 patients assuming an isocaloric diet + placebo [171]. Five additional clinical trials are currently registered for the use of EGCG in MS patients.

4.2.3. EGCG in pain and other CNS disorders

Based on the antioxidant and anti-inflammatory properties of EGCG, its role in the prevention and/or reduction of neuropathic pain has been tested in different animal models, as reviewed in [172]. EGCG was found effective in various types of neuropathic pain, including spinal nerve ligation (SNL) and chronic constriction injury (CCI) of the sciatic nerve and diabetic neuropathy, by addressing several intracellular pathways spanning from a reduction of ROS and MDA formation to the inhibition of the Toll-like receptor 4 (TLR4)/NF-κB pathway [172]. Interestingly, the antiallodynic effect of EGCG has been also directly connected to the down-regulation of the chemokine fractalkine (CX3CL1), which is known to be involved in the pathological cross-talk between neurons and activated glial cells in the spinal cord after CCI of the sciatic nerve [173]. These latter data demonstrate that, apart from its general effects which are common to several classes of polyphenols, EGCG interacts with specific pathways that are dysregulated in chronic pain conditions.

The potential beneficial effects of EGCG in ALS has been initially hypothesized stemming from its anti-inflammatory and neuroprotective activities, and confirmed in transgenic mouse models of the disease in which EGCG supplementation (5.8 μg/g/day orally for 60 days after birth) modulated glutamate neurotransmission, reduced protein misfolding, increased BDNF release, leading to an overall increased life expectancy [174]. However, the recent demonstration that ALS is accompanied by significant dysbiosis in both animal models and in patients, leading to altered

production of beneficial metabolites, such as tryptophan and SCFAs, has prompted researchers to reconsider the mechanisms at the basis of the beneficial effects of EGCG, and of other polyphenols, in ALS with a specific focus on the beneficial influence on gut microbiota composition (see also above) [174].

4.3. FLAVONOLS

4.3.1. Flavonols in Alzheimer's (AD) and Parkinson's diseases (PD)

Several studies have shown that quercetin plays a neuroprotective role against AD [175]; in particular, this compound enhances cognitive functions and strengthens learning and memory abilities in different AD animal models through multiple mechanisms [95].

First, it has been demonstrated that quercetin ameliorated brain deficits in a triple transgenic AD mouse model (3xTg-AD mice): intraperitoneal injections of quercetin 25 mg/kg every 48 hours for 3 consecutive months reversed brain levels of β-amyloidosis and tauopathy, ameliorated astroglia and microglia reactivity in the hippocampus and in the amygdala, and improved cognitive and emotional behavior performance [176]. In a more recent study, an oral preventive treatment with quercetin for 12 months had a remarkable effect on β-amyloidosis and tauopathy reduction in the hippocampus and amygdala of 3xTg-AD mice, positively affecting cognitive functional recovery [177].

In the SAMP8 mouse model, the oral administration of quercetin-loaded zein nanoparticles (25mg/kg every other day for two consecutive months) improved the cognition and memory impairments, related with a reduction of astrogliosis in the hippocampus. In addition, a significant amount of quercetin has been found in the brain, thus indicating the potential of zein nanoparticles to promote its oral absorption [175].

In $A\beta_{1-42}$-induced AD-like mice, the oral administration of 50 mg/kg/day quercetin-3-O-glucuronide (Q3G) protected against Aβ accumulation, Tau phosphorylation and cognitive dysfunctions, and triggered a beneficial effect on synaptic plasticity. Moreover, Q3G protected against neuroinflammation and brain insulin resistance (IR). In addition, the study showed that $A\beta_{1-42}$ injection induced gut dysbiosis, and the treatment with Q3G changed the composition of the gut microbiota and restored the production of SCFAs, suggesting that Q3G might also reverse brain IR through the gut-brain axis [178].

Treatment with quercetin-conjugated superparamagnetic iron oxide nanoparticles at the dose of 25 mg/kg/day for 42 consecutive days protected against $AlCl_3$-induced neurotoxicity in a rat

model of AD, and improved learning and memory impairment caused by oxidative stress by inhibiting acetylcholinesterase, enhancing the expression of antioxidant enzymes, and reducing the expression of NOS [179].

In the same animal model, quercetin has been shown to attenuate behavioral deficits, to improve cholinergic and dopaminergic dysfunctions, and to reduce Aβ plaques aggregation in the hippocampus. These effects have been associated with the downregulation of *APP*, *BACE1*, *APH1*, and *PSEN1* and the upregulation of *ADAM10* and *ADAM17* gene expression levels [179].

Overall, these studies show the neuroprotective role of the flavonol quercetin in different models of AD through the improvement of cognitive functions, the reduction of inflammatory markers, oxidative stress, and the aggregation of soluble and insoluble Aβ [95].

Several animal models have been used to study the neuroprotective effects of quercetin in PD. In rotenone-induced PD, the pre- and post-administration of quercetin at the dose of 50 mg/kg/day restored motor and non-motor deficits, such as depression and cognitive impairments, enhanced antioxidant enzyme activities, and attenuated neurotransmitter alterations. In particular, both preventive and therapeutic administration prevented the decrease in dopamine and serotonin levels through an antioxidant mechanism. However, pre-supplementation produced more significant results than post-supplementation, suggesting that quercetin can be a potential preventive agent to reduce the risk and progression of PD [180]. In the same animal model, authors investigated the effect of quercetin in combination with piperine, a major alkaloid responsible for the pungency of black pepper which is able to enhance the bioavailability of various compounds, including quercetin, by inhibiting glucuronyl transferase, P-glycoprotein, and drug-metabolizing enzymes. Results showed that quercetin treatment attenuated motor deficits and biochemical and neurotransmitter alterations, and prevented degeneration of dopaminergic neurons by controlling redox potential through anti-inflammatory and mitochondrial energy restoration mechanisms. Moreover, the combination of quercetin (25 mg/kg/day) with piperine (2.5 mg/kg/day) significantly enhanced its neuroprotective effect [181].

By employing the 6-OHDA-induced PD model in rats, which shows some of the pathological, biochemical, and behavioral hallmarks of the disease, a study demonstrated that the daily administration of 10 mg/kg and 25 mg/kg of quercetin by oral gavage for one month significantly improved cognitive impairment, as indicated by the decrease of the mean escape latency and time needed for discovering the hidden platform area, and increased hippocampal BDNF level by

improving neuronal firing rate, modulating neuronal oxidative stress and maintaining the antioxidant status [182].

In another study, the administration of 10 or 30 mg/kg/day of quercetin over 14 days by oral gavage relieved 6-OHDA-induced progressive PD-like motor behaviors, mitigated neuronal death and reduced mitochondrial damage and α-synuclein accumulation. In addition, authors showed for the first time that the neuroprotective effect of quercetin is suppressed by the knockdown of the *PINK1* or *Parkin* genes, suggesting that its effect is directly associated with the PINK1-Parkin mitophagy pathway that leads to the elimination of α-synuclein aggregates and relieves behavior phenotypes [183].

Lastly, in the same animal model, the daily administration of 10 and 25 mg/kg quercetin nanocrystals by gavage for 4 weeks prevented memory loss, increased antioxidant enzyme activities, and reduced MDA levels in the hippocampus. In addition, the study demonstrated that quercetin nanocrystals have a greater bioavailability in comparison with quercetin alone [184].

In conclusion, all these studies show that quercetin has a neuroprotective role in two main animal models of PD by improving behavioral deficits, together with a preventive effect on inflammatory markers and oxidative stress parameters and the reduction of α-synuclein aggregation [95].

4.3.2. Flavonols in multiple sclerosis (MS)

To date, only a few studies have investigated the effect of quercetin in MS, and none of these have been published recently. However, one study demonstrated that intraperitoneal injection of SJL/J mice with 50 or 100 μg of quercetin for 25 days ameliorated experimental allergic encephalomyelitis in association with the inhibition of IL-12 production and neural antigen-specific Th1 cells differentiation, and prevented IL-12-induced phosphorylation of STAT3, STAT4, TYK2, and JAK2, which leads to the reduction of Th1 cell differentiation and T cell proliferation [185].

4.3.3. Flavonols in pain and other CNS disorders

In the previous paragraphs, we have shown that quercetin exerts strong antioxidant, anti-inflammatory, and neuroprotective effects. Recently, quercetin has been shown to have also analgesic effects in animal models of pain.

In particular, the effect of quercetin on diabetes-induced neuropathy has been recently demonstrated in two studies. In the first one, the administration of 100 mg/kg quercetin alleviated

thermal hyperalgesia in *db/db* mice and significantly reduced the total dendritic length, the number of dendritic branches, and the dendritic spine density in the spinal dorsal horn neurons. In addition, this compound decreased the up-regulated expression of synaptic plasticity-associated proteins in the spinal cord dorsal horn, a characteristic of neuronal sensitization leading to hyperalgesic and allodynic responses [186]. In the second study, the intraperitoneal administration of 50 mg/kg/day quercetin for 14 days in a rat model of type 2 diabetes had proven to enhance both the mechanical withdrawal threshold (MWT) and the thermal withdrawal latency (TWL). Moreover, quercetin treatment also reduced the expression of purinergic P2X4 receptor and glial fibrillary acidic protein (GFAP), a marker of activated satellite glial cells (SGCs), and decreased the phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK) in the dorsal root ganglia (DRG) of diabetic rats, thus suggesting that this might be the mechanism behind the relief from mechanical and thermal hyperalgesia [187].

Regarding neuropathic pain, the effect of quercetin has been investigated in a spared nerve injury (SNI) rat model, and results showed that oral administration of 800 mg/kg/day before SNI surgery attenuated mechanical allodynia. In addition, quercetin inhibited GFAP expression in SGCs of the ipsilateral L5 DRG compared to the control group. These findings suggest that the mechanism behind the suppression of neuropathic pain by quercetin involves the inhibition of SGCs activation [188]. Moreover, quercetin administration to CCI rats (30, 60 and 120 mg/kg for 28 days) increased the MWT, improved the plantar TWL and reduced the number of inflammatory cells at the ligation site of the sciatic nerve. Also, quercetin reduced the levels of TNF-α, IL-6, and IL-1β and alleviated neuralgia by activating the AMPK pathway and inhibiting the MAPK pathways (p-38, p-ERK, and p-JNK) [189].

Quercetin was also investigated in a few studies on ALS models, which is associated with the deposition of aggregates of SOD1. It has been demonstrated that treatment of SOD1 wild-type fibrils with quercetin (90 μM) at different phases of aggregation inhibited fibrillation as quercetin binds to the SOD1 dimer, blocked its fibrilization and reduced the cytotoxicity of SOD1 fibrils. In addition, quercetin arrested the elongation of fibrils by blocking the fibrillar core regions on the intermediate species formed during the aggregation of SOD1. Experimental data also showed the anti-amyloidogenic potential of this flavonol against A4V SOD1 mutant fibrillation [190,191].

4.4. ISOFLAVONES

4.4.1. Isoflavones in Alzheimer's (AD) and Parkinson's diseases (PD)

To date, the most studied isoflavone in age-related disorders, including AD, is genistein, as reviewed by [192–194].

A recent paper investigated the effects of genistein in a rat model of AD induced by bilateral intracerebroventricular infusion of $A\beta_{1-42}$, characterized by significant memory impairment and neurochemical alterations in the hippocampus, including reduced levels of the synaptic proteins synaptophysin and postsynaptic density protein 95 (PSD-95), hyperphosphorylation of Tau with increased activation of glycogen synthase kinase-3β (GSK-3β) and JNK, and inactivation of ERK. Oral administration of genistein (10 mg/kg for 10 days) improved Aβ-induced cognitive impairment by reducing synaptotoxicity and Tau hyperphosphorylation, and promoting ERK activation [195].

Ipriflavone, a semi-synthetic isoflavone derived from soybeans and employed as therapeutic agent in postmenopausal osteoporosis [196], is also known to protect against mutagenicity, cytotoxicity, and chromosomal damage [197], and showed antioxidant properties against H_2O_2-induced ROS production and cell damage [198]. The neuroprotective effect of 50 mg/kg/day oral ipriflavone against memory dysfunction and AD has been demonstrated, via a mechanism that involves the activation of MAPK/ERK1/2 pathway, AChE inhibition, and oxidative stress reduction [199]. A recent paper investigated the effect of ipriflavone in a model of AD induced by a mixture of aluminum, cadmium and fluoride in rats. Results showed that ipriflavone, administrated *per os* at the dose of 50 mg/kg/day, significantly improved neurobehavioral deficits and cognitive dysfunctions via antioxidant/anti-inflammatory mechanism. Moreover, reduced mRNA expression of APP and tau protein, preventing amyloid plaques and neurofibrillary tangle aggregation, was also shown [200].

In a recently published paper, authors performed a systematic investigation of four prenylated isoflavones, philippinone A-D (compounds 1-4), together with six known analogues (compounds 5-10), obtained from the roots of *Flemingia philippinensis*. All compounds were evaluated for their inhibitory effect on Aβ aggregation, and compound 2 and 5 showed significant inhibitory activity, thus representing promising candidates in the treatment of AD [201].

Very few clinical data are available so far. In a study from 2015, a 6-month administration of 100 mg/day soy isoflavones did not prove effective on cognitive parameters in men and women over the age of 60 with AD [202]. Other studies have investigated the effect of isoflavones treatment on cognitive functions, displaying with controversial results [203].

Both *in vitro* and *in vivo* pharmacological evidence supports the beneficial effects of isoflavones in the prevention and treatment of PD, as reviewed elsewhere [204,205]. In particular, the neuroprotective properties of genistein in preclinical models of PD have been extensively demonstrated [206], for example in 6-OHDA-lesioned rats in which an isoflavone-enriched soy extract reduced motor dysfunctions [133].

A very recent study employed *Drosophila melanogaster*, which is a widely accepted paraquat (PQ)-induced *in vivo* PD model, to investigate the neuroprotective efficacy of calycosin. This is an isoflavonoid phytoestrogen extracted from *Astragalus membranaceus* which already proved neuroprotective on cerebral ischemia/reperfusion-induced neurological injury, intracerebral hemorrhage and high glucose-induced oxidative stress and neuroinflammation, as well as neuronal apoptosis. Previous studies reported that calycosin (i.p. administration for 7 days at the doses of 15 or 30 mg/kg/day) mitigated MPTP-induced PD-like conditions in mice by inhibiting the activation of TLR-NF-κβ and MAPK signaling-mediated inflammatory responses [207], and through its antioxidant properties which limited α-synuclein-induced neurotoxicity [208]. In this study, 100 μM calycosin for 48h showed protective effect against PQ-induced neurodegeneration, as revealed by better locomotor performance and increased fly survival, by modulating oxidative stress signaling and neuronal cell death, improving mitochondrial functions, and restoring autophagy [209].

Despite the abundance of promising results in both *in vitro* and *in vivo* preclinical studies, no clinical data employing isoflavones in PD patients are available so far.

4.4.2. Isoflavones in multiple sclerosis (MS)

The effect of isoflavones administration in preclinical models of MS has been investigated in the last few years [210].

Recently, a paper demonstrated the protective role of genistein in the treatment of gray matter lesions in MS by using the mouse model of cuprizone-induced demyelination. Genistein-treated (30 mg/kg/day) mice showed recovered myelination and reduced loss of mature oligodendrocytes. Moreover, genistein reduced the cuprizone-induced increase in the expression of genes related to phagocytosis, such as CD68 and lysosomal-associated membrane protein 1 (LAMP1) in sorted microglia, and increased the expression of myelin-related genes, such as myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG), in the hippocampus [211]. In the same model, another very recent study investigated the protective effects of Biochanin A (BCA), an O-methylated isoflavone which showed antioxidant, anti-inflammatory and neuroprotective activities, on memory

decline. Authors observed that 40 mg/kg/day BCA administration significantly increased the grip strength, improved spatial memory in the Y-maze test and recognition memory in the novel object recognition task and novel arm discrimination task, compared with the cuprizone group. Moreover, 5 weeks of BCA administration reduced neuronal damage in the prefrontal cortex and hippocampus [212].

In addition to the cuprizone-induced demyelination model, the vast majority of preclinical studies on MS take advance of the EAE model, which, although controversial, reproduces several features of the human disease (see also above). In this model, authors demonstrated that genistein (200 mg/kg/day administered starting from 2 days before EAE induction until day 6 post-immunization) delayed the onset of the disease and reduced inflammatory infiltration and demyelination. Moreover, genistein treatment increased the expression of TLR3, TLR9 and IFN-β, decreased transcription factors for Th1 and Th17 cells and up-regulated Treg lymphocytes in the spinal cord [213].

Interestingly, it has been reported that MS patients have reduced abundance of isoflavones-metabolizing gut bacteria, suggesting that the inability to digest these compounds could represent a risk factor for disease development. Moreover, MS prevalence is lower in countries where high amounts of isoflavones are consumed (10 to 30 mg/day), such as China and Japan, compared to Western countries where consumption of isoflavones is much lower (0.1 to 1 mg/day) [214]. In this respect, a very recent paper showed that an isoflavone-rich diet provided protection in a mouse model of EAE, as demonstrated by lower motor score, reduced cellular infiltration in the CNS and CD4+ T cell activation and proliferation, and that this effect was dependent on the presence of bacteria able to metabolize isoflavones and of their metabolite equol [215].

As for PD, no clinical data supporting the protective role of isoflavones in MS patients are available.

4.4.3. Isoflavones in pain and other CNS disorders

The first report that consumption of soy-containing diet protects from the development of partial sciatic nerve ligation-induced neuropathic pain dates back to 2001 [216]. From then on, the beneficial effects of isoflavones in preclinical models of neuropathic pain have been confirmed by several authors, as reviewed by Shen and colleagues [217].

It is known that calcium channel dysfunctions are associated with a wide range of neurological disorders, including neuropathic pain. In a recent paper, by using electrophysiology techniques

authors showed that 100 μM genistein reduces the activity of the human $CaV_{3.3}$ channel in a concentration-dependent manner [218]. A modulatory role for genistein has also been demonstrated on voltage-gated Na^+ channels in the trigeminal ganglion *in vitro* [219]. A recent work showed that local genistein injection (0.1-10 mM) in adult rats reduced nociceptive wide-dynamic range neuronal excitability in the spinal-trigeminal *nucleus caudalis*, by inhibiting neuronal firing rates in a reversible and dose-dependent manner and showing the same potency of inhibition of 1 % lidocaine [220].

Emerging evidence shows genistein's potential as neuroprotective agent in the development of neuropathic pain, due to its anti–inflammatory and antioxidant properties, and estrogen receptor binding activities. In this respect, authors recently demonstrated that pre-treatment with genistein (7.5, 15, and 30 mg/kg/day) antagonized 17β-estradiol potentiation in experimental occlusal interference-induced masseter hyperalgesia, and blocked the effect of 17β-estradiol by downregulating TRPV1 protein expression thus reducing the percentage of TRPV1-positive neurons in the trigeminal ganglion [221].

Moreover, preclinical studies suggested a potential role for soy isoflavones, in particular genistein, in the management of fibromyalgia-related pain syndrome via different mechanisms, as reviewed in [222].

The efficacy of isoflavones in the treatment of different pain syndromes has been confirmed by clinical evidence. A double-blind randomized control trial including placebo, 40 mg or 80 mg isoflavones/day was conducted on 18 women suffering from cyclical mastalgia, a diffused condition in Western populations which is believed to have an hormonal basis. Nine out of the 12 women on treatment had a worthwhile improvement in their pain compared to only 2 out of 6 on placebo, with pain reduction of 13% for placebo, 44% for 40 mg isoflavone and 31% for 80 mg per day [223].

Due to their estrogenic effect, isoflavones have been shown to improve various pre-menstrual syndrome symptoms, including pain [224]. For the same reason, isoflavones proved effective also in the treatment of menopausal symptoms [225], including headache pain [226]. A very recent study investigated the relationship between headache and dietary consumption of a variety of nutrients in middle-aged women, showing that isoflavones intake significantly reduced headache frequency in peri- and post-menopausal subjects [227]. Moreover, 10 mg/day S-equol appeared to be as effective as soy isoflavones in reducing hot flash frequency and more effective for relieving muscle and joint pain in post-menopausal women [228]. An interesting study investigated the clinical effect of a 4-week application of a vaginal gel formulation containing isoflavones compared with no topical

treatment in women with post-menopausal vaginal dystrophy, characterized by symptoms that include itch and burning pain. All patients also received daily oral isoflavones, and results showed that the combination of oral and topical isoflavones was more effective than oral treatment alone in reducing vaginal dystrophy symptoms [229].

Moreover, a study on 42 patients with prostate cancer subjected to radiation therapy evaluated the effect of 200 mg soy isoflavones administration against radiation-induced sides effect, including pain. At 6 months of treatment, soy-treated patients reported to have less pain with bowel movements (0% vs. 14.8%) than placebo-treated patients [230].

An early study showed that genistein exhibited both estrogen-independent and estrogen-dependent neuroprotective effects in a male murine model of ALS [231]. More recent data in a transgenic mouse model of ALS showed that genistein (16 mg/kg/day) suppressed the inflammatory response, restored the autophagic activities and improved the viability of motor neurons, thus improving ALS symptoms and prolonging the lifespan of ALS mice [232].

Interestingly, genistein showed promising results as therapeutic agent for mucopolysaccharidosis (MPS) type III, also known as Sanfilippo syndrome, a neurodegenerative metabolic disorder which primarily affects the brain and spinal cord and is characterized by behavioral disturbance, loss of mobility, progressive intellectual disability, and death in the second decade of life [233]. As a consequence of promising preclinical data, several clinical trials have been conducted to explore the role of genistein in MPS III patients, as reviewed by [194], that brought to the conclusion that genistein treatment at doses of 150 mg/kg/day appears to be safe and effective in MPS patients.

Despite promising preclinical evidence, no clinical trials have been conducted so far to evaluate the therapeutic potential of isoflavones in neuroinflammation.

5. Conclusions and future perspectives

To date, experimental evidence suggests that flavonoids intake as dietary supplements represents a promising approach for the prevention and treatment of pathological conditions characterized by neuroinflammation and neurodegeneration. However, despite very promising preclinical data, their use in clinical practice still presents many issues.

First, while the role of the bidirectional communication between the gut and the brain in the pathogenesis of CNS disorders has been widely demonstrated, as well as the protective role of

flavonoids metabolites obtained from gut bacteria (see Figure 3), whether there is a direct role of the gut microbiota is not entirely clear. In fact, available data are often contradictory, and do not clarify whether flavonoids beneficial properties are due to their gut absorption followed by the anti-inflammatory and antioxidant activities of their metabolites, or if they exert a direct effect on the gut microbiota composition and function which are in turn responsible for the observed improvements. Moreover, results obtained from clinical trials are presently limited for some classes of flavonoids (*e.g.* anthocyanins and flavonols), whereas for others (*e.g.* isoflavones) are often not satisfactory, possibly due to high individual variability in patients' microbiota, whose composition and function is easily influenced by several individual factors.

In addition, the choice of the dose to be administered to patients is still an open question, as in some cases (*e.g.* EGCG and quercetin) the dose employed in animal studies is converted to very high doses for humans, which present a high risk of toxicity. What is still missing, and urgently needed, is more uniformity in the description of the dosages used in preclinical studies, which could help understanding whether the currently used doses of extracts/pure compounds in animal models are translatable to humans, whether they are economically viable to conduct a clinical trial, and most importantly, in case of a successful clinical trial, to use them in clinical practice.

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Authors declare the absence of conflict of interest.

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