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GUT MICROBIOTE DISTINCTIVE FEATURES IN PARKINSON DISEASE: FOCUS ON DUODOPA AND OTHER ANTIPARKINSONIAN DRUGS

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Marta

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INTRODUCTION

PARKINSON'S DISEASE

Parkinson's disease (PD) is a neurodegenerative disease first described in the nineteenth century by James Parkinson[1] and it is the second most common neurodegenerative disease, following Alzheimer's disease (AD). [2]

Parkinson's disease affects both sexes with a slight predominance among males and the estimated prevalence in Western countries is around 0.3 / 1000 in the general population. However, its frequency increases almost exponentially with age, reaching 9.5 / 1000 in people over the age of 65.[3] This frequency is destined to double in the near future, around 2030, due to the aging of the population. Nevertheess, PD may still occur even in young patients and up to 10% of patients experience symptoms before the age of 40.

PD is a progressively debilitating disease whose etiology is still unclear to date. From a histopathological point of view, PD is characterized by a distinctive depletion of dopaminergic neurons in the substantia nigra pars compacta (SNpc) with consequent dopamine deficiency, a process underlying the pathology typical motor symptoms (the typical triad: tremor, rigidity, bradykinesia). However, PD is also associated with numerous non-motor symptoms, some of which may precede motor dysfunction by over a decade, suggesting neurodegeneration in networks of neurotransmitters other than dopamine and outside the basal ganglia 2.

Thus, PD has been increasingly recognized as a slowly progressive neurodegenerative disorder that begins years before the onset of parkinsonian motor symptoms and involves the central nervous system, the autonomic and peripheral nervous systems.

The pathological sign of PD is the loss of dopaminergic neurons in the SNpc, more precisely in the ventrolateral level, where there are projections towards the dorsal putamen and the striatum, but also in the ventral tegmental area (VTA). Thus, the dysfunction in both nigro-striatal dopaminergic pathways from SNpc, as well as in the meso-limbic circuits originating from the VTA, leads to an imbalance of cortical and subcortical circuits implicated in motor control, but also in the modulation of emotion and cognition. Furthermore, the neuronal loss in PD occurs in many other areas, namely the locus coeruleus, Meynert's basal nucleus, pedunculopontine nucleus, raphe nucleus, dorsal nucleus of the vagus nerve, amygdala and hypothalamus and involves networks of neurotransmitters other than dopamine. [4]

The other peculiar histopathological characteristic of PD is the abnormally folded alpha-synuclein aggregation within the cell body and neuronal processes: the "Lewy bodies" [5]
The accumulation of Lewy bodies is not limited to the brain only, but can be found in the spinal cord and in the peripheral nervous system, as well as in the vagus nerve, the sympathetic ganglia, the cardiac plexus, the enteric nervous system, the salivary glands, the adrenal medulla, cutaneous nerves and the sciatic nerve [6 7]

To date, various hypotheses have been formulated on the progression of the neurodegenerative process in PD: Braak and colleagues [8] have suggested that neurodegeneration in PD propagates in the caudo-rostral direction in the nervous system, starting in the peripheral nervous system and progressively affecting the central nervous system within the brain. The Braak model attempts to explain the spatial and temporal progression of the neurodegenerative process and the consequent natural history of its clinical course. Six disease stages are commonly identified: stage 1 and stage 2 could occur in parallel with premotor symptoms, stage 3 could represent the beginning of the motor characteristics suggesting the depletion of dopaminergic neurons within the nigrostriatal circuits, stages 4 to 6 could represent an advanced stage of the disease.
More recently, Postuma and Berg have identified three phases of the MP progression: preclinical, prodromal and clinical. [9]
In the preclinical phase, the neurodegenerative process has already begun, yet the symptoms, both motor and non-motor, are absent. The diagnosis at this stage remains speculative due to the lack of validated biomarkers, such as blood, cerebrospinal fluid or neuroimaging markers. In prodromal PD, clinical symptoms or signs of neurodegeneration are evident, with predominance of non-motor symptoms and only mild motor signs, but the full diagnosis of PD cannot be made yet. Consequently, the prodromal stage implies the presence of neurodegeneration outside the substantia nigra or even outside the brain, at least 10 years before clinical PD can be diagnosed. Conversely, clinical PD implies a reduction of up to 80% of dopaminergic cells in the substantia nigra leading to the presence of complete parkinsonism, with progressive bradykinesia, in addition to tremor and/or rest stiffness. [9]

Clinical features.

Historically, PD has been regarded as a simple movement disorder, but is currently recognized as a multidimensional neurodegenerative disease characterized by motor and non-motor symptoms, including sleep disorders, cognitive and neuropsychiatric impairments, autonomic and gastrointestinal dysfunction,.

Motor symptoms

The cardinal motor characteristics of Parkinson's disease are bradykinesia, combined with resting tremor and rigidity.[10]

Bradykinesia is defined as the slowness of movement associated with the reduction of the amplitude and/or speed of the movement itself. Bradykinesia can influence various aspects of movement, such as voice, e face, axial frame, walking. However, it is the bradykinesia of the limbs that must be sought in the suspicion of PD.

Rigidity in PD is defined as resistance to passive movement, recalls the "lead tube" phenomenon and, unlike spastic rigidity, is independent of movement speed. The phenomenon known as "cogwheel" (or rack) is often present, but it is not sufficient to satisfy the minimum criteria for parkinsonian rigidity.

Finally, the tremor at rest is characterized by a frequency of 4-6 Hz, must be sought in a state of complete rest, and is suppressed at the movement start. Parkinsonian resting tremor may recur after prolonged posture: in this case it is termed re-emerging tremor. Other motor signs and symptoms are postural and gait disorders, such as camptocormia, festination, swallowing disorders, hypomimia and micrography. [11]

Patients with Parkinson's disease may experience various motor complications as the disease progresses, and is due to the chronic use of dopaminergic drugs, i.e. freezing of gait, dyskinesias,[12] motor and non-motor fluctuations, wearing-off phenomenon, morning akinesia, off-on phenomenon and dystonia. [13,14]

Non-motor symptoms

Numerous non-motor symptoms may appear during all oPD stages and may even precede the onset of the most classic motor symptoms by many years.[15] Non-motor symptoms are very common in the initial phase and the progression of the disease also involves these symptoms in the same way as the motor aspects, with a negative impact on patients' quality of life and institutionalization rates. [16]

Non-motor symptoms include sensory disturbances, visual disturbances, sleep disturbances, cognitive and neuropsychiatric deficits, autonomic and gastro-intestinal dysfunctions.

Sensitivity and sensory disturbances include hyposmia, pain and paresthesia.[17] Patients with PD may develop visual symptoms such as alteration of contrast sensitivity, color discrimination, speed of visual processing. [18]

Sleep disorders include insomnia, sleep fragmentation, excessive daytime sleepiness, obstructive sleep apnea syndrome (OSAS), restless leg syndrome (RLS) and REM sleep disorder (RBD)

Cognitive impairment in PD can affect executive and visuospatial functions, memory, attention and language. Furthermore, neuropsychiatric disorders are common and include symptoms such as apathy, depression, anxiety, hallucinations, psychosis and impulse control disorders.[19] True dementia typically occurs in later stages in the natural history of the disease and appears in nearly 83% of patients after 20 years of illness.

Autonomic dysfunctions in PD are constituted by orthostatic hypotension, excessive sweating, urinary and sexual disorders.

Gastro-intestinal disorders are the most frequent dysautonomic symptoms. These symptoms have an important incidence in the initial stages of PD and are an important marker for identifying a prodromal phase of the disease.

GASTROINTESTINAL SYMPTOMS IN PD

In the early stages of Parkinson's disease, gastrointestinal symptoms are the most frequent non-motor symptoms, with a frequency of about 60% - 80% and an important impact on the quality of life of patients.[20]

Furthermore, gastrointestinal symptoms are among the most common causes of access in emergency departments and often have serious consequences such as malnutrition (15% of patients), pulmonary aspiration (2.4%), megacolon (unknown incidence), bowel obstruction (rare), up to intestinal perforation (some reported cases; incidence unknown).[21]

Gastro-intestinal dysfunctions in PD consist of oro-dental disorders, swallowing disorders, malnutrition, gastric disorders, constipation. In in relation to their incidence, they are regarded as important symptoms in the progression of the disease and constitute a major cause of disability. Furthermore, not only is the gastrointestinal system in PD compromised both from a motor and disautonomic point of view, but it also plays an important role in the pathophysiology of motor fluctuations due to the impairment of absorption and metabolism of antiparkinsonian drugs. [22] To date, many studies endorse the theory of an important role of the gastro-intestinal tract in the pathogenesis of PD, indicating that it could represent a propagation

pathway of α-synuclein, allowing the characteristic diffusion of the degenerative process along the CNS.

Oro-dental disorders in patients with PD are mainly influenced by sialorrhea, which causes a change in the pH and salivary composition, and by poor oral hygiene, due to the patients' reduced ability to brush their teeth, with an increase in periodontal disease and a greater frequency of caries.[23] Sialorrhea is defined as excessive accumulation of saliva in the oral cavity due to an overproduction of saliva or a reduced salivary clearance. Sialorrhea has several negative effects for the patient with PD: social embarrassment, poor oral hygiene, bad breath, increased intraoral bacteria, difficulty eating and speaking, increased risk of aspiration pneumonia and an important impact on the quality of life.[24] Recent observations have shown that sialorrhea in PD is associated with the severity of motor symptoms and increases in the off phases (when the effects of antiparkinsonian drugs are exhausted and symptoms return), thus suggesting that altered swallowing could be the cause of sialorrhea, rather that the overproduction of saliva. Several experimental and clinical results also suggest that salivary production is indeed reduced in many patients with PD, probably due to dopamine depletion. As a result, dry mouth is present in more than 60% of patients with Parkinson's disease and could coexist with sialorrhea in 30% of them.[24]

Another interesting aspect of the function of the upper part of the digestive tract in patients with PD is the lack of taste and smell, which recently attracted the attention of the scientific world for its important potential correlations with the pathogenesis of the disease.[25,26]

Oropharyngeal and esophageal dysphagia are frequently reported in patients with PD, with a prevalence ranging from 9% to 77%. Dysphagia increases the risk of aspiration, thus contributing to the risk of infection of the upper respiratory tract and pneumonia.[27] Dysphagia typically occurs in advanced PD, but it may develop early, occasionally even as an onset symptom. Oropharyngeal dysphagia is often attributed to bradykinesia and rigidity secondary to dysfunction of the basal ganglia. However several authors have suggested a role also of the supplementary motor cortex, of the anterior cingulate cortex and of structures of the peripheral nervous system.[28]

The prevalence of malnutrition in Parkinson's disease ranges from 0% to 24%. Motor dysfunction (dysphagia), fear of off-motor phases, fasting associated with drug administration, drug-induced nausea and anorexia can affect food intake. In addition, dyskinesias also

contribute to weight loss by increasing energy expenditure.[29] Levodopa administration (normalized for body weight and adjusted for age, sex and severity of disease) has been associated with a dose-dependent altered nutritional status. In contrast, weight gain is associated with treatment with dopamine agonists and neurosurgical procedures, such as deep brain stimulation of the subthalamic nucleus, probably due to effects on the limbic areas.[30]

Stomach disorders are also very common in PD and consist of delayed gastric emptying and Helicobacter (H.) Pylori infection. In clinical practice, increasing attention is being paid to the role of gastric emptying in PD; in fact, the prevalence varies from 70% to 100%, although not all affected individuals show suchsymptoms.[31] Gastroparesis can be present both in the early and advanced stages of the disease. Nausea, vomiting, feeling of early satiety, excessive fullness, bloating and abdominal distension characterize gastroparesis and, since levodopa is absorbed in the small intestine, a symptom related to gastroparesis may consist of a delay or complete loss of the levodopa dose benefit (**Fig. 1**).[22]

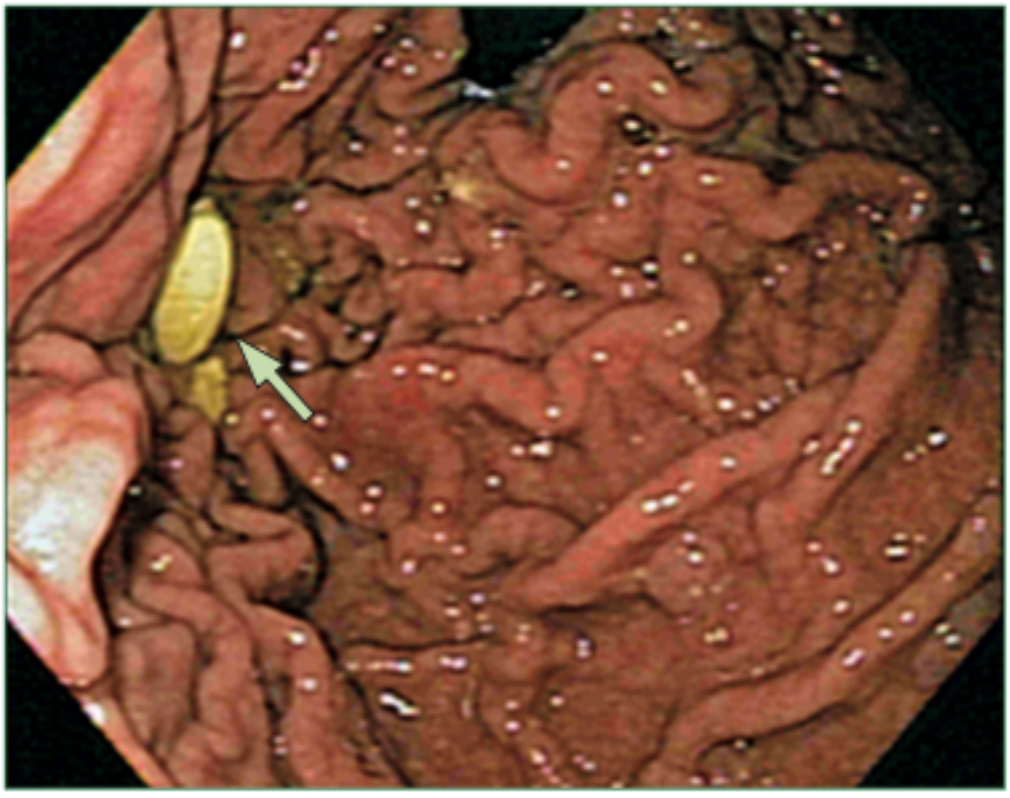


Fig.1 *Delay in gastric emptying, photograph taken during gastroscopy. Arrow points to a carbidopa tablet remaining intact in the patient's stomach.* [22]

The authors of a Danish population study reported that a diagnosis of Helicobacter Pylori gastritis was associated with an approximately 45% increase in the risk of Parkinson's disease.

[32] In this regard, a chronic systemic inflammatory response to H. pylori could be a common determinant factor in the onset, evolution and consequences of many neurological diseases, including Parkinson's disease. However, the relevance of this association is unclear because there are many shared risk factors for the development of H. pylori infection and PD and the prevalence of H. pylori in PD is similar to that in the general population. [22]

Constipation is the most common gastrointestinal symptom in PD, reported in 80-90% of patients . In view of its appearance long before the onset of motor symptoms, it is a particularly noteworthy gastrointestinal characteristic of the disease. A population study reported that the risk of developing Parkinson's disease increases with the severity of constipation.[33] Not only does the risk increase in subjects with constipation, but constipation is also emerging as one of the first features of autonomic dysfunction in Parkinson's disease, which already develop 15 years before motor symptoms.[34] Therefore, the detection of constipation could have a potential sensitivity as a clinical biomarker of prodromal Parkinson's disease. Constipation is a common adverse effect of many PD medications (especially anticholinergics and dopamine agonists). However, a delay in colon transit independently of drugs has been reported in PD. The mechanisms underlying the constipation in Parkinson's disease are not yet known: some studies have proposed that a reduced neuronal density in the myenteric ganglion could be a cause, but in patients with PD this has not been reported.[35] Other studies have shown the presence of phosphorylated α-synuclein in subjects with PD and concomitant chronic constipation, without obvious staining in those with PD or chronic constipation alone. [36]

Patients with PD may also eventually present dyssynergic defecation , characterized by an inability to relax or by the paradoxical increase in contraction of the pelvic floor muscles during the attempt at defecation. Although the dysfunction of the parasympathetic sacral nucleus and of the pelvic ganglia is implicated in Parkinson's disease, the basic pathogenesis of the dyssynergic defecation could be a consequence of a dopaminergic dysfunction, since the acute administration of apomorphine improves the disinergic defecation symptoms. [22]

The role of the gastro-intestinal system in the pathogenesis of PD

Nowadays more and more studies show that the gastro-intestinal tract can play an important role in the pathogenesis of PD as an initial site of its neurodegenerative process. [22] The main pathological process in PD is the abnormal accumulation of α-synuclein in the brain (Lewy bodies), and both Lewy bodies and neurodegenerative phenomena have been identified in the intestine of patients affected by PD.

The identification of Lewy disease in the intestines of patients with PD has been reported for the first time in an autopsy study where Qualman et al. identified myenteric Lewy bodies in one patient's colon and another's esophagus.[37] Later this data was replicated in numerous other case reports in various gastrointestinal districts, and more recently by Braak et. al in both myenteric and submucosal gastric plexus.[38]

As previously described, symptoms such as constipation appear on average 15 to 24 years before the diagnosis of PD, representing one of the earliest indicators of a pathological mechanism that only later leads to the overt disease. Given these results, the hypothesis that Parkinson's disease could derive from the intestine has received more and more attention. This idea was first presented about 10 years ago[39] and was followed by a heated debate in the scientific world because several studies at that time suggested that the pathological progression could be mediated by the prion properties of α-synuclein.[40]

The deposition of α-synuclein occurs in the myenteric and submucosal plexuses and in the nerve fibers of the mucosa, with a clear rostrocaudal gradient throughout the enteric nervous system. The highest concentrations of phosphorylated α-synuclein have been reported in the submandibular glands and lower esophagus, lower concentrations in the stomach and small intestine and lower concentrations in the colon and rectum. The causes of this distribution pattern are unknown, although deposition follows the innervation pattern of neurons in the visceromotor projection. These neurons originate in the dorsal motor nucleus of the vagus nerve (DMV) and innervate the longitudinal extension of the gastrointestinal tract, with predominant vagal innervation in the upper gastrointestinal tract and projections of the sympathetic system predominant in the most distal regions.[41] The vagal projection neurons are among the first cells of the CNS to present abnormal deposits of α-synuclein in PD. Therefore the DMV could provide a conduit for the abnormal forms of α-synuclein to access the central nervous system.

As a further demonstration of the role of vagal projections in PD, a reduced incidence of PD has been registrered in vagotomy individuals[42], contributing to the development of the intestine-brain theory of the pathophysiological process of PD, where the vagus nerve would have a critical role in its diffusion. According to some authors, the spread of pathological α-synuclein could occur through its disintegrated and soluble type in the gastrointestinal tract, subsequently infiltrating the CNS.[22]

The fact of an early impairment of the enteric nervous system in regions that are much more

accessible than the brain, has led to evaluate enteric α-synuclein as a disease biomarker. Many groups have exploited the accessibility of the colonic mucosa, practicing colon biopsies as a source of tissue. The results, however, concluded that colic α-synuclein does not provide a high specificity for the diagnosis of Parkinson's disease, since the presence of α-synuclein was detected in the colon even in healthy subjects.

Although there is good sensitivity for detecting α-synuclein in the colon, the clear rostrocaudal gradient in α-synuclein deposition in the enteric nervous system suggests that tissues from more proximal regions of the gastrointestinal tract may provide greater sensitivity and specificity for the diagnosis.[43]

Furthermore, although there is a clear model demonstrating the presence of synucleinopathy in the regions of the gastrointestinal tract affected by PD, no study has shown a causal link between this pathological abnormality and the corresponding gastrointestinal symptoms.

Further studies are still needed to improve our understanding of the association between enteric synucleinopathy and gastrointestinal dysfunction, which could either help develop treatments targeting gastrointestinal symptoms, or provide an important contribution to the understanding of the PD pathogenesis.

INTESTINAL MICROBIOTE

The term "microbiome" literally means "small biome", that is the ecosystem that includes all the micro-organisms of a particular environment, together with their genes and their environmental interactions. The set of microorganisms is called microbiota or microbial community and can include bacteria, archaea, viruses, fungi and other eukaryotic microorganisms (protozoa).[44]

It is estimated that the human body is composed of over one hundred trillion bacteria, comprising more than 1000 different species and over 7000 strains counting more than 10 million genes.[45]

The greatest microbial density is observed in the gastrointestinal tract: it is estimated that 70% of all the microorganisms present in the body reside in the large intestine, ie up to 10^{13}-10^{14} bacteria per gram of content at the level of the colon.[46] Although to date more than 50 bacterial phyla have been detected in the human intestine, most are *Bacteroidetes* and *Firmicutes*, while *Proteobacteria*, *Verrucomicrobia*, *Actinobacteria*, *Fusobacteria* and *Cyanobacteria* are present in minor proportions.[47]

The microbial colonization of the human intestine begins even before birth. It has long been believed that it was sterile before birth, but recent studies have shown that intestinal colonization can occur in utero. After birth, the intestinal microbiota develops rapidly and undergoes a progressive change closely correlated to the type of nourishment, characterized by the passage from an exclusively milky diet (maternal / artificial milk which also determines a change) to a solid diet. [48]

Similarly the development of the the microbiota is also influenced by numerous external and internal factors related to the host. External factors include the microbial load of the surrounding environment, the diet, as well as the composition of the maternal microbiota; internal factors include intestinal pH, microbial interactions, environmental temperature, physiological factors such as peristalsis, bile acids, host secretions, immune responses, drug therapy and bacterial receptors of intestinal mucosa.[48]

With advancing age, and especially with the degeneration process of the organism functional capacities, the proportions of *Bifidobacterium*, *Faecalibacterium prausnitzii* and multiple members of the *Firmicutes* typically decrease, while the proportions of Escherichia coli and others members of the Proteobacteria and Staphylococcus increase.[49,50]

The gastrointestinal microbiome of the elderly may also differ from that of younger adults based on the reduced potential of vitamin B12 biosynthesis and as a result of the activity of microbial reductases, as well as an increased potential for DNA damage, stress response and compromise of the immune system. [51] These results suggest that the microbiota of the elderly may represent a proinflammatory phenotype. Aging and inflammation are combined processes and the key features of aging include the reduction of gastrointestinal function and host immune response along with the development of low grade chronic inflammation. Advanced age (65-100 years) brings further important transitions in the composition, function and stability of the GI microbiota [52] with a greater inter-individual variation than that of younger adults. [53]Much of this variation appears to be diet-driven and strongly correlates with relative health indicators, including markers of fragility and inflammation. [52]

Main functions of intestinal microbiota

Since the intestinal microbiota encodes a significantly higher number of genes than the human host, it is not surprising that it is capable of activating a variety of metabolic functions that are absent or limited in humans.

The intestinal microbiota is considered a real "metabolic organ" which, in addition to its nutritional functions, contributes to immune regulation and systemic inflammation. [54] *(**Fig.2**)*. The knowledge of the main functional contributions emerged thanks to studies conducted on germ-free animals (GF), in which an abnormal body development, atrophy of the intestinal wall, a reduced weight of heart, lungs and liver and of the immune system were observed. [55]

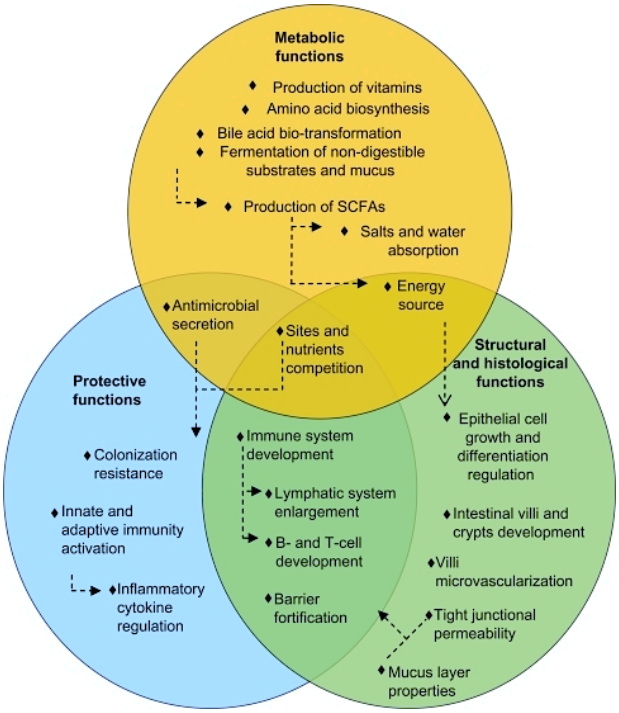


Fig. 2 *Main functions of intestinal microbiota*

The substrates metabolised by the intestinal microbiota include dietary residues, macromolecules of the mucosa (for example mucins), endogenous metabolites (in particular bile acids) and xenobiotic chemicals. The main classes of food substrates include carbohydrates, amino acids, some lipids (for example polyunsaturated fatty acids) and phytochemicals. [56, 57]

Human enzymes cannot degrade more complex carbohydrates and polysaccharides including cellulose, xylans, resistant starch and inulin; they are metabolized by the colon-resident microbiota in oligogasarides and monosaccharides and subsequently fermented in final products such as short chain fatty acids (SCFAs), mainly acetate, propionate and butyrate. SCFAs are absorbed into the colon, where butyrate provides energy for colon epithelial cells

and acetate and propionate reach the liver and peripheral organs, where they act as substrates for gluconeogenesis and lipogenesis. [58]

Used locally as a source of energy by colon epithelial cells, SCFAs are essential to maintain the integrity of the intestinal barrier as they regulate the expression of tight-junction proteins. Add to this, SCFAs have profound effects on the health of the intestine not only as a source of energy but also as modulators of inflammation, vasodilation, wound healing, also contributing to intestinal motility.

Gut bacteria also play a fundamental role in the synthesis of a variety of vitamins, such as B12 and K, and in the absorption of calcium, magnesium and iron ions. [59]

Furthermore, the gastro-intestinal microbiota can catabolize proteins into amino acids and participate in the luminal conversion of amino acids into biogenic amines, immunomodulatory compounds and other signaling molecules. (i.e. the conversion of glutamate to γ-aminobutyric acid, tyrosine in tyramine and phenylalanine to β-phenylethylamine)

The intestinal microbiome plays an important role in the regulation of bile acids. The latter cholesterol-derived metabolites facilitate intestinal absorption of dietary lipids and fat-soluble vitamins, influence systemic cholesterol levels and inhibit colonization by intestinal pathogens such as Clostridium difficile. [60]

Commensal organisms play a protective role as they prevent pathogenic colonization by competing for the sites of adhesion to the intestinal mucosa and for nutrients and through the production and secretion of antimicrobial substances (antimicrobial peptides, AMP), such as defensins, catelicidins and type C lectins. Commensal microbiota contributes to the body's physiological protection as it is essential for the development of the immune system. GF animals have been shown to have a deficit in the number of different types of immune cells and immunoglobulins as well as local and systemic lymphoid structures.

While the condition of intestinal eubiosis is essential to promote the health and well-being of the host, the overgrowth of some bacterial populations generates a variety of harmful conditions, which the host tries to cope with different mechanisms.

The immune system of intestinal mucosa must therefore satisfy two functions, sometimes apparently conflicting. It must be tolerant of the overlying microbiota to prevent the induction of an excessive and harmful systemic immune response and at the same time must be able to contain the excessive growth of the intestinal microbiota and prevent its systemic translocation. The result can be a protective response to commensal bacteria, an immune response to

pathogenic organisms or a trigger of apoptosis. Therefore, the commensal bacteria of the gastrointestinal tract play an active role in the immune system development and homeostasis.

Gut-brain-microbiota axis

The central nervous system (CNS) and the gastro-intestinal system (GIS) modulate their functions reciprocally, both in health and disease conditions, through a bi-directional communication pathway called gut-brain axis.

Recently, the role of the enteric microbiota has been recognized as an integral part of this signaling pathway, so much so that the definition " gut-brain-microbiota axis "was coined consequently. [61,62]

Growing evidence suggests that bacteria residing in the intestine may have an impact on the CNS. Bacterial colonization of the intestine plays an important role in the development and postnatal maturation of the immune, endocrine and even neural systems.

The dysfunction of the microbiota-brain-gut axis has been implicated in various neuronal disorders, such as Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, autism, stroke, depression and drug addiction. to the point of considering it as a potential diagnostic and therapeutic target. [63]

The impact of the intestinal microbiota on the regulation of the brain-intestine axis involves direct neurological, immunological and neuroendocrine mechanisms [64] which occur through the neurons of the sympathetic and parasympathetic nervous system, as well as through neuroendocrine signaling of hormones and other neuromodulatory molecules (**Fig.3**). An afferent and an efferent signaling path is given. The afferent path allows enteroendocrine signaling and is carried out both by both the vagus nerve, which has both afferent and efferent divisions and by the immune system. [65] The main efferent path instead is represented by the hypothalamic-pituitary-adrenal axis (HPA). When this latter axis is activated, the resulting secretion of cortisol (in humans) or corticosterone (in rodents) influences the activity of immune cells, both locally in the intestine and systemically. Neuronal efferent activation also includes the efferent branch of the vagus nerve which, when activated, induces the release of acetylcholine, in turn influencing the levels of cytokines. [66]

Increasing evidence suggests that the intestinal microbiota acts directly through the production of neuroendocrine metabolites (hormonal metabolites such as SCFA, neurotransmitters and neuromodulators such as γ-aminobutyric acid, serotonin, dopamine, gastrointestinal hormones,

precursors of neuroactive compounds such as tryptophan and quinurenine) and, indirectly, as a modulator of inflammatory responses, immune responses and hormonal secretion. [64, 67]

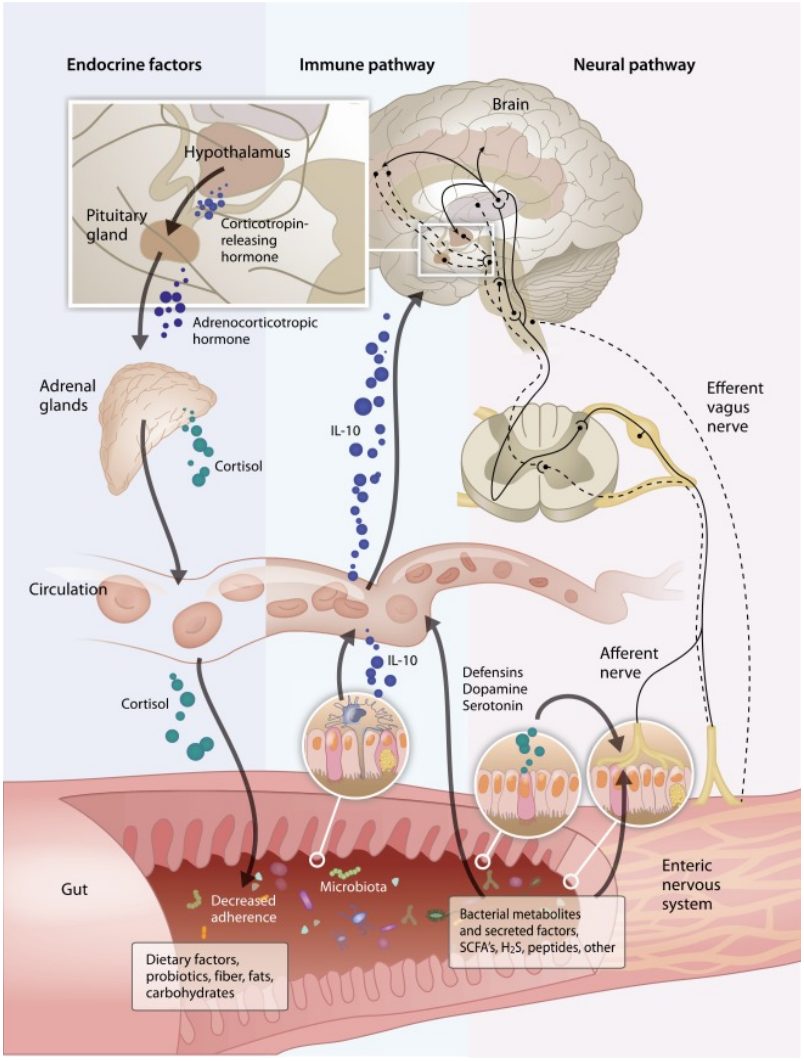


Fig.3 *Microbiota-brain-intestine axis. Bidirectional signaling between the gastrointestinal tract (GIT) and the central nervous system (CNS) occurs through neurons of the sympathetic and parasympathetic nervous system, as well as through neuroendocrine signaling of hormones and other neuromodulatory molecules.*

SCFA-mediated microbiota signaling .The role of SCFAs as the main energy substrate and regulators of gene expression in anti-inflammatory processes has been extensively discussed above. SCFAs also act as direct mediators between the intestine and the brain, carrying out their functions far from their production site, using monocarboxylic-acids transporters, which are also expressed on the blood-brain barrier (BBB). [68, 69]

Increasing evidence suggests that SCFAs can directly influence brain function and behavior.

It is interesting to note that both butyric acid and propionic acid influence the synthesis of dopamine and noradrenaline by increasing the gene expression of tyrosine hydroxylase [70,71] Furthermore, it is shown that propionic acid is able to modulate serotonergic neurotransmission [69] and lower levels of GABA, serotonin and in vivo dopamine. [70]

Additionally, it has been shown that SCFAs derived from the gut influence the maturation and function of microglia, the macrophages resident in the CNS. [72]

It is important to underline how SCFAs are also directly implicated in the release of hormones and neuropeptides, such as the peptide glucagon (GLP-1) and the peptide YY (PYY) from intestinal enteroendocrine cells. [73]

However, SCFAs can also exert pathological effects in cases of increased early exposure to these bacterial metabolites during key neurodevelopmental periods. [74]

Afferent signaling of the intestinal microbiota mediated by neurotransmitters. The intestinal microbiota produces several neurotransmitters such as dopamine (DA), noradrenaline (NA) and GABA which can reach distal sites through circulation. [75] For example, members of the genera Escherichia and Bacillus synthesize dopamine and / or noradrenaline and members of the genera Lactobacillus and Bifidobacterium produce GABA. [67, 76 , 77]

NA and DA are crucial neurotransmitters regulating many physiological processes in the brain and body and it has been shown that the levels of both neurotransmitters are reduced in the cecum of GF mice compared to normal mice: [78] This suggests that the intestinal microbiota represents a potential source of catecholamines. These results would suggest a correlation between intestinal bacteria and dopamine levels in conditions such as Parkinson's disease. However, although the microbiota appears to be able to modulate central catecholaminergic neurotransmission, it is unlikely that these micro-derived catecholamines will have a central effect, as they are unable to cross the BBB.

Some intestinal bacteria are even able to produce GABA, the main inhibitory neurotransmitter in the CNS which is also associated with such pathological conditions as depression and anxiety. Lactobacillus and Bifidobacterium strains derived from the human intestine have been shown to produce GABA. [79] GABA transporters are located on the blood-brain barrier (BBB), a mechanism through which GABA derived from microbes reaches the SNC. [80] Furthermore, the administration of Lactobacilli to mice altered the expression of GABA receptors in different regions of the brain, resulting in a decrease in anxiety and depressive behavior. [81, 65]

Several studies have indicated tryptophan metabolism (TRP) as another mechanism involved in brain-gut communication: TRP is an essential amino acid, the precursor of many biologically active agents, such as 5-HT (serotonin) [81] TRP as essential amino acid must be provided by the diet: once absorbed by the intestine and made available in the circulation, it can cross the BBB to participate in the synthesis of 5-HT in the SNC. [81]

The microbiota is able to metabolize TRP, resulting in the production of 5-HT. This has been amply demonstrated in GF mice, which have lower plasma levels of 5-HT than conventional mice. [82, 83]However, most of the tryptophan is not metabolized in 5-HT, but, in reality, the dominant physiological pathway is found along the path of the chineurin. It has been observed that the destruction of this metabolic pathway is linked to gastrointestinal and cerebral disorders. [84]

The correlation between microbiota-derived neurotransmitters and brain function awaits further studies that can more clearly define the contribution of peripheral-level alterations of these neurotransmitters to the neuroendocrine function.

Enteroendocrine signaling. Enteroendocrine cells (EEC) are widely distributed in the gastrointestinal tract and exert regulatory effects on bidirectional communication between the intestine and the brain. The products secreted by the EEC influence a variety of physiological functions in the host such as the control of intestinal secretion and motility, the regulation of food intake and metabolism. [85] and the main hormones secreted by CEE are cholecystokinin (CCK), peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) and mainly secreted in response to the ingestion of fats, proteins and carbohydrates.

It is interesting note that there is a body of evidence suggesting a function of intestinal microbes in modulating the secretion of these hormones, directly or indirectly through the production of SCFA. [86]

Immune signalling The contribution of the intestinal microbiota as a trigger of the immune system is widely studied: intestinal bacteria can induce alterations in the levels of circulating proinflammatory and anti-inflammatory cytokines, which directly affect brain function, especially in areas such as the hypothalamus, where IL-1 and IL-6 promote a powerful release of CRF (Corticotropin-releasing factor). [87]

LPS, activator of the Toll-like receptor 4 (TLR4), is able to cross the intestinal epithelial barrier in response to certain conditions such as stress or to a high-fat diet leading to immune activation of the HPA axis. [88]

Intenstinal microbiota and Parkinson Disease

Growing evidence leaves no doubt that a diversified and balanced composition of intestinal microbiota (eubiosis) is fundamental for the well-being of the organism. Faced with all the functions performed by the intestinal microbiota it is clear how it actively influences the intestine functions and the individual's health state. The composition of the intestinal microbiota is constantly influenced by factors such as diet, drugs, intestinal mucosal integrity, the immune system and mutual interactions between the components of the microbiota itself. When the aforementioned factors rapidly reduce microbial diversity and promote the expansion of specific bacterial *taxa*, a condition known as "intestinal dysbiosis" occurs. Often, a single factor is not sufficient to induce dysbiosis because the intestinal microbiota has an inherent resilience capacity, i.e. an ability to adapt to changes in nutrient availability and in environmental conditions. The combined actions of different factors, on the contrary, can change the microbial groups towards a point of no return, even into dysbiotic alterations of pathological significance.

An increasing number of diseases are associated with intestinal dysbiosis, which in some cases contributes to the development or severity of the disease: it has been shown to be associated with inflammatory intestinal diseases (IBD) such as ulcerative colitis and Crohn's disease [89] , autoimmune diseases [90] and neurological disorders. [91]

As previously described, the early appearance of non-motor symptoms in Parkinson's disease, such as hyposmia and gastro-intestinal (GI) symptoms, has led to the hypothesis that the disease may start outside the CNS. Thanks to the identification of Lewy bodies in the mucosa of the GI tract in the most initial phases of PD, the GI tract was identified as a potential access site for the pathological process. Changes in the intestinal microbiota associated with intestinal inflammation could contribute to the initiation of misfolding of α-synuclein . [92]

Intestinal dysbiosis can cause changes in barrier function and permeability of the intestinal epithelium, promoting both effects on the GI immune system and CNS. [93] On the one hand, there is a consequent over-stimulation of the innate immune system which can produce

systemic and CNS inflammation. [94] On the other hand the intestinal-inflammation-associated dysbiosis may contribute to the initiation of misfolding of α-synuclein. [95](**Fig.4**).

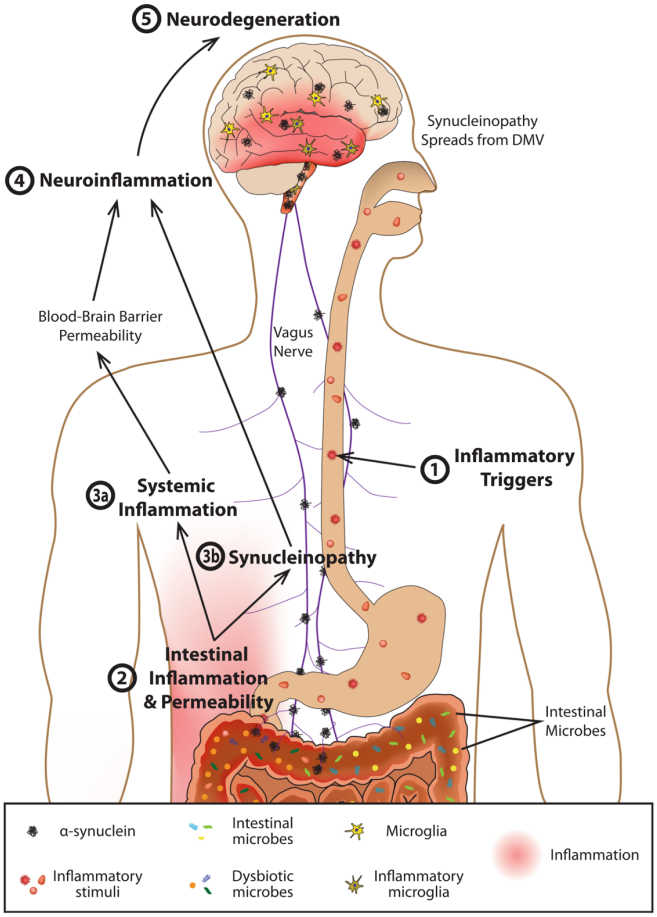


Fig.4 *Pathogenesis model of PD induced by inflammation and intestine. In a susceptible individual, inflammatory triggers (1) initiate immune responses in the gut that adversely affect the microbiota, increase intestinal permeability and induce greater expression and aggregation of αSYN (2). Synucleinopathy can be transmitted from the intestine to the brain via the vagus nerve (3b). Chronic inflammation and intestinal permeability promote systemic inflammation, which can increase the permeability of the blood-brain barrier (3a). Intestinal inflammation, systemic inflammation and synucleinopathy promote neuroinflammation (4) which drives the neurodegeneration characterizing PD (5).*

More in detail, neuroinflammation is associated with the upregulation of signaling by TLR2 receptors and proinflammatory cytokines such as TNF-α, IL-1β and IL8 in serum, inducing blood-brain barrier rupture and promoting inflammation and microglia-mediated neurotoxicity. [96]

A recent study confirmed that intestinal permeability in subjects with PD had markedly increased compared to healthy controls (HC) and this was associated with a greater presence of Escherichia coli in the intestinal mucosa and systemic exposure to Lipopolysaccharide (LPS). [97]

LPS is a proinflammatory bacterial endotoxin that can cause delayed and progressive nigral pathology when systemically administered. [98] Its intraperitoneal administration induces hyperpermeability of the large intestine and abnormal aggregation of α-syn phosphorylated. [99]

In this regard, while the researches that studied the nasal microbiota in PD did not reveal significant differences between patients and controls, [100, 101] to date, on the contrary, many studies have shown a significant difference between the intestinal microbiota of patients affected by PD and healthy controls, with important variability among the individual taxa reported.

The first study on the relationship between intestinal dysbiosis and PD was conducted by Scheperjans et al. These researchers observed a reduction in *Prevotellaceae* and an increase in *Enterobacteriaceae* in patients with PD. [102]

Subsequent studies showed that less abundant populations of *Prevotella*, *Lactobacillus*, *Peptostreptococcus*, and *Butyricicoccus* spp. resided in PD patients with an increase in *Proteus* and *Enterobacter* spp compared to healthy controls. [103]

The studies conducted in patients with PD are currently not completely comparable to each other due to the multiple methodological differences used, as well as the different geographical and ethnic characteristics of the study populations. However, some consistent models seem to emerge: potentially "anti-inflammatory" bacteria have been found to be less abundant in PD, while a greater concentration of "pro-inflammatory" bacteria has been demonstrated compared to controls. [104]

These changes in the composition of the intestinal microbiota in PD seem to be associated above all with a reduced production of SCFA and resulting in a proinflammatory intestinal environment [105] Unger et al. confirm this hypothesis, identifying a significant reduction of SCFA in faecal samples of patients with PD compared to the control group. [106]
Butyrate acts locally on the mucosa of the colon but can also exert remote effects through the ENS and through the inhibition of histone deacetylase, protecting dopaminergic neurons. Furthermore, the SCFA seem to exert an important effect on peristalsis, supporting the hypothesis that reduced butyrate concentrations in the faeces of patients with PD could exert significant effects on the ENS and also contribute to gastrointestinal dysmotility. Unger and Keshavarzian agree on the results: the first shows a reduction in SCFA and *Faecalibacterium prausnitzii*, the second a reduction in butyrate-producing bacteria belonging to the genera

Blautia, *Coprococus* and *Roseburia*. Also the relative abundance of the *Lachnospiraceae* family - also a producer of SCFA - was reduced in patients with PD and in mice that received a fecal transplant from patients with PD. [104]

A recent study has attracted increasing attention with regard to the pathogenesis of Parkinson's disease. [107] This study was carried out in a transgenic mouse model (ASO) of PD overexpressing α-synuclein. Transgenic animals, maintained in normal conditions with their typical microbiota, rapidly developed neuroinflammation, α-synucleinopathy and motor dysfunction. However, when ASO mice were bred in a germ-free environment, the development of PD-related neuropathology was significantly limited. This effect was reversed when ASO germ-free (GF) mice were colonized with the feces of wild-type mice or fed orally with SCFA and the development of PD-related neuropathology was significantly increased. Interestingly, the colonization of GF transgenic mice with microbiota from patients with PD has promoted greater motor dysfunction than that of the microbiota resulting from healthy controls. Based on these observations, the authors concluded that the signals of intestinal microbes are necessary for the neuroinflammatory response and the development of characteristic motor and non-motor signs in the preclinical model of PD.
Furthermore, the authors proposed a possible mechanism by which intestinal microbiota influences disease development in mice, indicating SCFA as the main modulators of glial activation and inducers of disease status. In the study, the SCFA were the main factor in microglial activation and acceleration of α-synucleinopathy, thus not confirming the hypothesis of SCFA depletion as an influential factor in the pathogenesis of PD. Therefore, the question on the explicit role of SCFA in neuroinflammation and neurodegeneration processes would seem to be still open.

On the basis of these observations the strong connection between intestinal dysbiosis, intestinal permeability and neurological dysfunction in Parkinson's disease appears clear.
The literature has now reported a great deal of results and information on the intestinal microbiota associated with PD, but these results are however contrasting and show consistency only at higher taxonomic levels. (**Tab.1**)
At phylum level, for example, statistically significant changes were observed only in the phyla *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Verrucomicrobia* and *Proteobacteria*.
Keshavarzian observed a significant reduction in the abundance of the phylum Firmicutes and a significant increase in the abundance of *Bacteroidetes* in the faeces of PD patients compared to healthy controls. [104]

These data are in contrast with the results subsequently obtained by Unger [106] in 2016 and by Li W. [108] in 2017 and Bedarf et al[109] These differences could derive from inhomogeneity of the study populations in the two works, which differ in average age, duration of the disease and therapy.

A.ltereted microbiota	***References***
Lactobacillus ↑ *Clostridium coccoides, Bacteroides fragilis* ↓	*Hasegawa et al 2015*
Ralstonia,Verrucomicrobiaceae ↑ *Prevotella, Blautia, Coprococcus, Fecalibasterium, Roseburia* ↓	*Keshavarzian et al 2015*
Enterobacteriaceae,Verrucomicrobiaceae ↑ *Prevotellaceae* ↓	*Scheperjans et al 2015*
Enterobacteriaceae,,Bifidobacterium ↑ , *Lactobacillaceae, Enterococcaceae, Fecalibacterium prausnizii , Prevotellaceae* ↓	*Unger et al 2016*
Verrucomicrobiaceae,Firmicutes ↑, *Erysypelotrichaceae , Prevotellaceae , Coprococcus* ↓	*Bedarf et al 2017*
Akkermansia , Ruminococcaceae, Lactobacillus,Bifidobacterium,Verrucomicrobiaceae, Veillonella ↑ *Lachnospiraceae, Roseburia, Coprococcus* ↓	*Hill-Burns et al 2017*
Escherichia-Shigella, Streptococcus, Proteus , Actinobacteria, Enterococcus, Firmicutes Veillonella ↑ *Blautia, Fecalibacterium, Ruminococcus* ↓	*Li et al 2017*
Christensenella,Catabacter, Lactobacillus, Oscillospira, Bifidobacterium Christensenella minuta, Catabacter hongkongensis, Lactobacillus mucosae, Ruminococcusbromii,Papillibacter,cinnamivorans ↑, *Dorea, Dorea longicatena, Bacteroides, Bacteroides coprocola, Bacteroides dorei Bacteroides plebeus, Bacteroides massiliensis, Prevotella, Prevotella copri, Faecalibacterium, Stoquefichus massiliensis, Blautia glucerasea, Coprococcus eutactus Ruminococcus callidus* ↓	*Petrov et al 2017*
Akkermansia, Verrucomicrobiaceae , Verrucomicrobiales , Verrucomicrobia ↑	*Heintz-Buschart et al 2017*
Barnesiellaceae, Enterococcaceae, Lactobacillaceae ↑	*Hopfner et al 2017*
Eubacteriaceae, Bifidobacteriaceae, Aerococcaceae, Desulfovibrionaceae ↑ *Firmicutes, Tenericutes, Euryarchaeota. Streptococcaceae, Methylobacteriaceae, Comamonadaceae, Halmonadaceae, Hyphomonadaceae, Brucellaceae, Xanthomonadaceae, Lachnospiraceae, Actinomycetaceae, Sphingomonadaceae, Pasteurellaceae, Micrococcaceae, Intrasporangiacea, Methanobacteriacea, Idiomarinaceae, Brevibacteriaceae, Gemellaceae.* ↓	*Lin et al 2017*
Clostridium IV, Aquabacterium, Holdemania, Sphingomonas, Clostridium XVIII, Anaerotruncus,Butyricicoccus ↑ *Lactobacillus , Sediminibacterium* ↓	*Quian et al 2018*
Proteobacteria, Verrucomicrobia. Bifidobacteriaceae, Christensenellaceae, Coriobacteriaceae, Lactobacillaceae, Enterobacteriaceae, Verrucomicrobiaceae ↑ *Lachnospiraceae* ↓	*Barrichella et al 2019*

Tab.1

Keshavarzian also observed a significant increase in the phylum *Verrucomicrobia* in the MP samples, while LI W. reported an increase in the relative abundance of *Actinobacteria* and *Proteobacteria*.

There are contrasting observations in the litterature regarding the composition of the intestinal microbiota in PD patients also at the taxonomic level of the family; in general, such the studies would seem to be in agreement with a reduction in the abundance of *Lachnospiraceae* [110, 104, 109] and the increase in *Enterobacteriaceae* [108, 106] and *Verrucomicrobiaceae* [110,104,109,111]

Furthermore, a recent study has shown abnormalities related to *Enterobacteriaceae* in PD, or the translocation of the species Escherichia coli into the mucous membrane of the colon, which supports the relevance of these bacteria in the pathology. [112]

The *Lactobacillaceae* and *Prevotellaceae* families are the most frequently identified and discussed families: overall there is a significant prevalence of the *Lactobacillaceae* family [110, 104,109] and a reduction of the *Prevotellaceae* in PD. [113, 109, 102] The decrease in the relative abundance of *Prevotellaceae* and the increase in *Lactobacillaceae* seem to be associated with reduced levels of the hormone ghrelin. [103] Bowel hormones such as ghrelin regulate the function of dopamine in the nigrostriatal pathway and may limit neurodegeneration. [114]

In most of the works, the *Prevotellaceae* family is reduced in patients with PD, although, in some, this data did not show statistical significance [108, 104].

This family includes commensal bacteria involved in the synthesis of mucin in the intestinal mucous layer and in the production of SCFA through the fermentation of fibers. [115]

The reduced abundance of *Prevotellaceae* could lead to a decrease in mucin synthesis and an increase in intestinal permeability. Increased permeability of the mucosa could cause greater local and systemic exposure to antigens and bacterial endotoxins, which in turn would trigger or maintain an excessive expression of α-synuclein in the colon or even promote misfolding. [112]

Furthermore, the genus Prevotella belonging to this family is associated with a high biosynthesis capacity of thiamine and folate. Therefore, a reduced prevalence of Prevotella would be in line with the decreased levels of these vitamins in patients with PD.

At a higher taxonomic resolution, such as that of gender, the data relating to the composition of the intestinal microbiota are rather concordant. All the authors who find a significant change in the abundances of Bifidobacterium, Lactobacillus, Oscillospira and Akkermansia are in favor of their increase in subjects with PD in relation to controls; on the contrary, several authors report a significant decrease in the same patients in the genera Blautia, Dorea, Roseburia,

Prevotella and Faecalibacterium. Although these genera are significantly reduced, they are all SCFA producers, whose reduction can interrupt the barrier function and promote inflammation.

Bedarf and Petrov, more than others, have characterized the fecal microbiota in PD up to the species level: the metagenomic analysis carried out has used NGS techniques. In detail, Bedarf reports ten microbial species that are significantly increased or decreased in PD [109] against the fifteen bacterial species reported in Petrov's work. [116] These are original observations, not comparable in the two studies, in line only with a significant reduction of the bacterial species Prevotella in PD subjects.

However, the causal relationship between the alterations in the composition of the microbiota and the pathogenesis of PD as well as the identification of a microbial pattern associated with the pathology remain unclear and further investigation is needed.

Confirming the important role of intestinal dysbiosis in PD, some bacteria have been associated with more severe forms of the disease or some specific phenotypes. For example, *Enterobacteriaceae* have shown a significant correlation with postural instability and gait disturbances, suggesting that a greater abundance of this family in the fecal microbiota may be associated with a more severe α-synucleinopathy fat the level of the ENS of these patients. [102] However, Unger et al., according to a greater abundance of *Enterobacteriaceae* in faecal samples of patients with PD, did not detect any significant difference between the different phenotypes (classified as tremor dominant, hypokinetic-rigid or mixed). [103]

Studies on the correlations between intestinal dysbiosis and disease progression have also shown conflicting results [117, 118] Clostridium coccoides was associated with the early stages of PD, while Lactobacillus gasseri with the more advanced stages. However all studies conducted in this regard suggest the need for longer follow-up and more numerous samples.

As the PD progresses clinically, as described above, the GI system malfunctions progress as well, with a greater slowing of gastrointestinal motility, which in turn predisposes patients to the overgrowth of small intestine bacteria (SIBO, small intestinal bacterial overgrowth) . The role of SIBO has been studied by Fasano et al, [119] which showed that the prevalence of SIBO is greater in patients with PD, compared to controls and that the occurrence of SIBO was associated with more severe motor fluctuations (in particular delayed-on and no-ON). These motor fluctuations can be attributed to peripheral factors, such as anomalies of the bioavailability of levodopa from the gastrointestinal tract and the malabsorption associated with

SIBO, also due to inflammation of the intestinal mucosa and to the altered metabolism of levodopa by intraluminal bacteria.

Finally, even "external modifiers" such as lifestyle habits, diet, drugs and antiparkinsonian therapies could play a role in the composition of the intestinal microbiota. Epidemiological evidence suggests that smokers and coffee drinkers have a lower risk of PD and it has been suggested that these factors could reduce the release of pro-inflammatory cytokines from the intestine to the blood stream, reducing CNS degeneration or promoting microbial species resulting in intestinal dysbiosis.

Still in the context of the "external modifiers", differences have been reported according to the geographical site, which can therefore reflect the environmental, lifestyle and dietary peculiarities of the regions studied. [110] The diet itself has been shown to modify the intestinal microbiota. [120]

Furthermore, numerous studies have shown a different composition and stability of the intestinal microbiota in patients with constipation compared to healthy controls with a reduction of *Lactobacillus*, *Bifidobacterium*, and *Bacteroides* spp, *Prevotella* and an increase in potentially pathogenic bacteria such as *Pseudomonas aeruginosa* and *Campylobacter jejuni* and butyrate-producing bacteria such as *Firmicutes*, *Coprococcus*, *Roseburia* and *Faecalibacterium*. [121] These alterations can affect intestinal motility and secretory functions by changing the amount of physiologically active substances available and the metabolic environment of the intestine.
Since constipation is one of the main non-motor disabling symptoms in PD, although the precise mechanism of regulation of intestinal motility by the microbiota is still partly unclear, the correlation between microbiota and constipation could be important in PD.
Regarding drugs, in PD some studies suggest a modulating role on the microbiota composition of some antiparkinsonian drugs, namely COMT inhibitors and anticholinergics with borderline significance for carbidopa / levodopa. [102, 110]
An interaction between drugs and the microbiome is not surprising and it can be argued that alterations in the composition of the intestinal microbiome can provide information to evaluate the efficacy or toxicity of the drugs themselves.
Concerning this, Rekdal et al characterized an interspecies pathway for gut bacterial L-dopa metabolism and demonstrated its relevance in human gut microbiotas. They showed that the enzymes that degrade L-dopa occur in microbiomes from human stool samples and that L-dopa

degradation occurs with considerable variation in people with and without Parkinson's disease. They showed that L-dopa degradation can be predicted predominantly by microbial tyrosine decarboxylase (tdc) gene expression and Enterococcus faecalis abundance in stool samples, leading to dopamine production. Moreover, they showed that Eggerthella lenta further metabolizes the dopamine produced by L-dopa decarboxylation to m-tyramine, using a distinctly microbial reaction, catechol dehydroxylation. [122] It is possible that microbial *tdc* limits L-dopa effect by its transformation to dopamine and that the transformation of dopamine to m-tyramine influences the multiple side effects of L-dopa administration. Furthermore, recent studies show that higher amounts of *tdc* in stool from Parkinson's disease patients correlate with increasing L-dopa dosage and disease duration.

An interaction between PD medications and the microbiome is not surprising, and it may be argued that alterations in the composition of the gut microbiome may give information to assess efficacy or toxicity of PD medications. [123]

These discoveries raise questions about the biological consequences of gut microbial metabolism of PD drugs and and have been linked to PD phenotypes, to the heterogeneous responses to L-dopa observed among patients, including decreased efficacy and harmful side effects, and to endogenous dopamine metabolism, influencing gut motility and pathogen colonization.

AIMS OF THE STUDY

The contribution of the intestinal microbiota as a possible factor or co-causal factor of various pathologies is supported by growing evidence showing that during the course of the pathology we are witnessing the rupture of the intestinal eubiotic balance, which leads to a condition known as intestinal dysbiosis. This is reflected in an excessive stimulation of the innate immune system, in the dysregulation of intestinal permeability and in the induction of a systemic inflammatory response typically observable during pathology.
In Parkinson's disease recent studies have highlighted the close association between the alterations of the intestinal microbiota and the disease itself, affecting the causal relationship between intestinal dysbiosis and the pathogenesis and the identification of the most implicated bacterial species. Currently the development of NGS sequencing techniques for the analysis of complex microbial communities has allowed us to overcome the biggest obstacle deriving from the analytical techniques of standard microbiology, stemming from the fact that the majority of the microorganisms that make up the microbiome are not cultivable. Therefore, the use of these modern techniques is an important tool to identify the pathophysiological implications of microorganisms in the body.

All the data discussed above suggest that a complex interplay interaction between gut microbiome and disease progression, motor and non-motor phenotype and antiparkinsonian medications exists and still need to be clarified.

The present thesis aims to confirm whether the faecal microbiome of a large cohort of PD patients differs from that of control subjects, reflecting differences that might support a role of the gut microbiota in the PD pathophysiology.
This study is articulated into two different parts. In the first part we studied the abundance of several bacterial taxa and definite functional pathways in a cohort of PD patients compared to healthy subjects with the purpose of widening the knowledge on the role of gut microbiota and its distinct metabolic expression patterns in the pathogenesis of Parkinson’s disease.
Subsequently, in the second part, we focus on the evaluation of a possible specific independent effect of PD antiparkinsonian medications on gut microbiota.

Levodopa-carbidopa intestinal gel (LCIG) is a gel containing Levodopa (LD) and the decarboxylase inhibitor carbidopa that is infused continuously through the abdominal wall, by means of a percutaneous endoscopic gastrostomy, up to the upper jejunal part of the small

intestine. LCIG leads to a significant improvement of LD-motor complications, but can have a positive effect also on non-motor symptoms (NMS), including the GI ones, as assessed by clinical scales. To date, no study has assessed the effect of LD infused directly in the intestine on gut microbiome. Thus we aimed at identifying the possible effect of LD intraduodenal infusion in modifying gut microbiome composition.

METHODS

Patients and samples

The Institutional Review Boards and Human Subject Committees at participating institutions approved the present study (Prot. PG/2017/17817).

Patients. 115 patients with diagnosed PD and 51 healthy controls were recruited. Subjects characteristics are shown in **Tab.2.**

Patient inclusion criteria were: diagnosis of idiopathic PD according to the UK Brain Bank criteria. The exclusion criteria were atypical or secondary parkinsonism; the use of probiotic or antibiotic supplements in the three months before enrollment; the presence of a primary gastrointestinal disease; the concomitant presence of an internal medicine, neurological, or unstable psychiatric illness together with severe cognitive impairment**.** Our patients were evaluated by MDS-UPDRS III and IV (motor part and motor fluctuations / dyskinesias) and by NonMotor Symptom Scale (NMSS).

All patients were classified in different onset-phenotype cathegories in agreement with the algorithm of Stebbins and co-workers: Group 1 as tremor dominant (TD),Group 2 patients with postural instability and gait difficulty (PIGD), and Group 3 dyskinetic patients. Moreover, they were classified in 3 different treatment groups: patients assuming Levodopa (LD-Group), patients with LCIG (Duodopa-Group), patients who have not assumed any medicaments containing Levodopa at the moment of recruitment. (Naïve-Group).

Since, as explained above, PD patients during the natural history of the disease show motor fluctuations due to the progressive loss of dopamineicirgic neurons and their striatal and cortical connections. These complications are observed in 50% of patients after 5 years of disease and in 80% of patients after 10 years of treatment. [124]. However , other authors defined the turnpoint of advanced disease at around 10-15 years of disease. Consequently, patients were divided in three groups concerning disease duration : patients with <3 year disease duration, 3-13 years and patients with >13 years (we elaborate a mean of previously defined turnpoints of advanced disease).

The control group was composed of healthy participants matched by age, BMI, and selected among the people accompanying PD patients at the outpatient cinic.

Stool samples from each subject were collected at outpatient facilities of the Neurology Department AO Brotzu (Cagliari, Italy) and the Department of Neuroscience "Rita Levi Montalcini", University of Torino (Turin, Italy) and delivered to the laboratory within 3 hours or stored in – 80 freezer.

DNA purification and quantification. DNA extraction, purification and quantification by real-time PCR were performed as previously described (Santoru et al., 2017). In particular, quantitative PCR was performed using the primers pair 5'-CCTACGGGNGGCWGCAG-3' (forward) and 5'-GACTACHVGGGTATCTAATCC-3' (reverse), using genomic DNA from E. coli ATCC25922 as reference to prepare the standard curve.

Library preparation and sequencing. Degenerate primers 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' and 5'GTCTCGTGGGCTCCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3' were used for amplification, with ligated overhang Illumina adapter consensus sequences. Amplification profile was as follows: initial denaturing step at 95°C for 3 min, 35 cycles denaturation at 95°C for 30 sec, annealing at 52°C per 30 sec, extension at 72°C for 30 sec. After 35 cycles, the reaction was completed with a final extension of 7 min at 72°C.

The 550 bp amplicons were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter) according to the manufacturer's instructions. For the multiplexing barcode procedure, the Illumina Nextera XT Index kit with dual 8 bases indices were used. PCR reactions containing 25 µL of KAPA HiFi HotStart Ready Mix 2x, 5 µL of Nextera XT i5 and i7 Index primer (Illumina) each, 5 µL of purified amplicons and nuclease-free PCR Grade - water to a final volume of 50 µL, were carried out on an Applied Biosystem 9700 thermal cycler. PCR profile was as follows: one cycle of 95°C for 3 min, eight cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, followed by a final extension cycle of 72°C for 5 min. The barcoded amplicons were then purified using Agencourt AMPure XT magnetic beads. Afterwards, the barcoded libraries were quantified using the Agilent High Sensitive DNA Kit (Agilent Technologies), and normalized to ensure an equal representation of the samples. The quality and the size of the pooled libraries were verified using Agilent DNA 1000 Analysis kit (Agilent Technologies) on the Agilent 2100 Bioanalyzer system (Agilent Technologies), and

finally sequenced on the MiSeq platform using MiSeq v3 Reagent Kit (Illumina), using PhiX v3 as a control.

Data analysis. Analysis of the data generated on the Miseq System was carried out using the BaseSpace 16S Metagenomics App (Illumina), whereas operational taxonomic units (OTUs) mapping to the Greengenes database (V.13.8) was performed using the Quantitative Insights Into Microbial Ecology (QIIME) platform (V.1.8.0). Alpha- (Shannon, Simpson, Fisher, Chao1, and ACE) and beta-diversity analysis was performed on the Microbiome Analyst tool [30].

Statistical analysis. Linear discriminant analysis Effect Size (LEfSE, http://huttenhower.sph.harvard.edu/galaxy/) was employed to identify distinguishing taxa between the two groups at multiple levels and to visualize the results using taxonomic bar charts and cladograms. Algorithm was performed on Galaxy computational tool to identify bacterial taxa that were statistically different among PD patients and controls. Only bacteria present in at least 25% of our samples and with a relative abundance ≥0.1% in cases and/or controls were considered. The significance of differential abundances with respect to the class of interest was evaluated by the non-parametric factorial Kruskal-Wallis (KW) sum rank test. Biological consistency was investigated using a set of pairwise tests among subclasses using the (unpaired) Wilcoxon rank-sum test. LDA was used to estimate the effect size of each differentially abundant feature and provide a visualization of differential features ranked by effect size and a representation of these features on a taxonomic tree. Results were then re-analyzed by the Benjamini and Hochberg false discovery rate (FDR) correction test for multiple comparisons. Heat maps of gut microbiota composition were generated using STAMP software.

To test for confounding, General Linear Model (GLM) or Multivariate Association with Linear Models (MaAsLin) were performed followed by Bonferroni or FDR correction for multiple comparisons using Statistical Package for the Social Sciences version (SPSS) 25.0 for Windows [31] and Galaxy computational tool (Version 1.0.1), respectively. Only bacteria that were found to be significant at the univariate level after FDR correction were considered. No normally distributed variables had been normalized using their logarithmic value prior to perform GLM. The differences of microbiota composition between cases and controls were adjusted for sex, age, BMI, coffee consumption, smoking status covariates.

For pharmacological treatment analysis, the data were corrected for sex, age, BMI, physical activity, constipation, coffee consumption, smoking status, diet (assessed by the Adherence-

to-Mediterranean-diet questionnaire, (*Francesco Sofi et al Validation of a literature-based adherence score to Mediterranean diet: the MEDI-LITE score, International Journal of Food Sciences and Nutrition, 2017 http://dx.doi.org/10.1080/09637486.2017.1287884*), NMSS, UPDR-III and UPDRS-IV scales, disease length and phenotype covariates. Box plots were created using Galaxy computational tool to represent the significant relative bacterial abundance in the different type groups of PD patients .

Metabolomics

Frozen feces (150 mg) were mixed with 800 µL of methanol and 200 µL of Milli-Q water and then vortexed for 1minute. After 10 minutes of sonication in water with ice (Digital ultrasonic Cleaner, DU-32, Argo-Lab, Italy), samples were kept at -20°C for 20 minutes and then centrifuged at 14000 rpm for 10 min at 4°C. For each sample, the supernatant (650 µL) was dried under vacuum with vacuum concentrator overnight and were derivatised with 50 µL of methoxyamine dissolved in pyridine (10 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA). After 1 h at 70°C, 100 µL of N-Methyl-N-(trimethylsilyl)-trifluoroacetamide, (MSTFA, Sigma-Aldrich, St. Louis, MO, USA) were added and samples were left at room temperature for one hour. Successively, samples were re-suspended in 400 µL of hexane containing the internal standard (Sigma-Aldrich, St. Louis, MO, USA) and filtered with Acrodisc Syringe Filters with 0.45 mm PTFE Membrane (SIGMA, St. Louis, MO, USA). An aliquot (20µl) from each sample was used to create a pool for quality control (QC). A QC sample was injected every 10 samples to check the goodness of the analysis.

GC-MS analysis

One microliter of derivatised sample was injected splitless into a 7890A gas chromatograph coupled with a 5975C Network mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with a 30 m ×0.25 mm ID, fused silica capillary column, with a 0.25 µM TG-5MS stationary phase (Thermo Fisher Scientific, Waltham, MA, USA). The injector and transfer line temperatures were at 250°C and 280°C, respectively. The gas flow rate through the column was 1 ml/min. The column initial temperature was kept at 60 °C for 3 min, then increased to 140°C at 7°C/min, held at 140°C for 4 min, increased to 300°C at 5°C/min and kept for 1 min. Identification of metabolites was performed using the standard NIST 08, and GMD mass spectra libraries and, when available, by comparison with authentic standards. Data processing was performed by using a pipeline in KNIME (1). In brief, peak detection and deconvolution were performed in a R-XCMS package, filtering was performed using blank

samples and keeping features present in ≥50% of the samples. Missing value imputation was conducted by using random forest algorithm. Relative concentrations of the discriminant metabolites were obtained by the chromatogram area and then normalized by median fold change.

Statistical analysis

The multivariate statistical analysis was performed by using SIMCA-P software (ver. 14.0, Umetrics, Sweden). The variables were UV scaled and a Principal components analysis (PCA) was used to identify the presence of outliers. PCA is an unsupervised analysis that allows estimating and visualizing the distribution of the samples. Partial Least Square-Discriminant Analysis (PLS-DA) and Orthogonal Partial Least Square-Discriminant Analysis (OPLS-DA) were performed in order to classify samples and elucidate metabolites able to differentiate the classes. GraphPad Prism software (version 7.01, GraphPad Software, Inc., CA, USA) was used to perform the univariate statistical analysis of the data and Spearman correlations between the microbiome and the metabolome. To verify the significance of metabolites obtained using multivariate statistical analysis, a Mann-Whitney-U test with Holm-Bonferroni sequential corrected p-values was used.

RESULTS

Overall composition of gut bacterial microbiome. Characteristics of the 107 patients with PD and 51 healthy controls (HC) are shown in **Tab.2.**

TABLE 2: Subjects characteristics			
Variable	**PD patients (107)**	**Healthy subjects (Ctrls=51)**	**p value**
Age, mean ± SD	70.20 ± 10.25	51.67 ± 13.42	0.001
BMI, mean ± SD	25.48 ± 4.24	23.70 ± 3.47	0.365
Sex, n (%)			
Male	69 (64.49)	31 (60.78)	0.644
Female	38 (35.51)	20 (39.22)	
Coffee consumption, n (%)			
Yes	68 (66.66)	44 (86.27)	0.015
No	34 (33.33)	7 (13.73)	
Missing	5	0	
Smoking status, n(%)			
Yes	5 (5.00)	18 (35.29)	0.000
No	95 (95.00)	33 (64.71)	
Missing	7		

Tab.2 Characteristics associated to Parkinson and Healthy subjects. Values in the groups were presented as: M= Mean, SD= Standard deviation, n= number, % = Percentage. Abbreviation: BMI= Body Mass Index;.p values were calculated with Student t-test for Age and BMI. For the other variables Chi Square test were performed.

The age distribution in cases (mean 70,2) and controls (mean 51,6) was higher in cases (pChi2 = 0.001). The sex ratio was identical between cases and controls. Nicotine use was more frequent in controls (18/ 35%) than patients (5/5%) (pChi2 = 0.000) and caffeine consumption was more frequent in controls (44/86%) than in patients 68/66%) (pChi2 = 0.0015).

TABLE PD subjects characteristics

Variable	PD patients (Naive=23)	PD patients (Levodopa=46)	PD patients (Duodopa=38)	p value
Age, mean ± SD	67,57 ± 9,37	69,85 ± 11,26	72,38 ± 9,36	0.473 (N-L) 0.670 (N-D) 0.224 (D-L)
BMI, mean ± SD	25,20 ± 3,25	26,79 ± 4,27	24,15 ± 4,37	0.187 (N-L) 0.366 (N-D) 0.764 (D-L)
Sex, n (%)				
Male	13 (56.52)	36 (78.30)	20 (52.60)	**0.037 (N-L)**
Female	10 (43.47)	10 (21.70)	18 (47.40)	0.906 (N-D) 0.013 (D-L)
Constipation, n (%)				
Yes	5 (21.74)	19 (44.20)	27 (71.10)	0.071 (N-L)
No	18 (78.26)	24 (55.80)	11 (28.90)	**0.000 (N-D)**
Missing	0	3	0	0.015 (D-L)
Coffee consumption, n (%)				
Yes	17 (73.91)	29 (67.40)	22 (61.10)	0.586 (N-L)
No	6 (26.08)	14 (32.60)	14 (38.90)	0.311 (N-D) 0.558 (D-L)
Smoking status, n(%)				
Yes	1 (4.35)	4(9.30)	0 (0)	0.469 (N-L)
No	22 (95.65)	39 (90.70)	34 (100)	0.220 (N-D)
Missing	0	3	4	0.068 (D-L)
Diet, n (%)				
Omnivorous	17 (94.40)	26(96.30)	33 (97.10)	0.768 (N-L)
Gluten free	0 (0)	0 (0)	1 (2.90)	0.297 (N-D)
No Milk and milk derivatives	1 (5.60)	1 (3.70)	0 (100)	0.358 (D-L)
Missing	5	19	4	
Whole food, n (%)				
Yes	8(44.40)	11(40.70)	7 (20.60)	0.805 (N-L)
No	10 (55.60)	16 (59.30)	27(79.40)	0.071 (N-D)
Missing	5	19	4	0.087 (D-L)
Oil consumption, n (%)				
8g	2(12.50)	4 (14.80)	4(11.80)	0.857 (N-L)
24g	2 (12.50)	2 (7.40)	3(8.80)	0.152 (N-D)
32g	1 (6.30)	2 (7.40)	4(11.80)	0.184 (D-L)
64g	0 (0)	0(0)	1(2.90)	

Supplements, n (%)				
Yes	2 (11.01)	0(0)	3 (8.80)	0.082 (N-L)
No	16 (88.90)	26 (100)	31 (91.2)	0.790 (N-D)
Missing	5	20	4	0.120 (D-L)
Balanced nutrition, n (%)				
Adequate	12(66.70)	21(77.80)	22 (64.70)	0.409 (N-L)
No adequate	6(33.3)	6 (22.20)	12(35.30)	0.888 (N-D)
Missing	5	19	4	0.266 (D-L)
Physical activity, n(%)				
No activity	5(22.70)	13(30.20)	18 (50.00)	0.368 (N-L)
Moderate	7 (31.80)	18 (41.90)	15 (41.70)	**0.000 (N-D)**
Regular	10 (45.50)	12 (27.90)	3 (8.30)	**0.052 (D-L)**
Missing	1	3	2	
Phenotype, n (%)				
Tremor-dominant	19 (79.20)	20 (43.50)	4 (10.05)	**0.005 (N-L)**
Akinetic-rigid	4 (16.70)	18 (39.10)	15 (39.50)	**0.000 (N-D)**
Dyskinetic	0 (0)	8 (17.40)	19(50.45)	**0.001 (D-L)**
NMSS, n(%)				
0-39	20(90.90)	2(40.00)	4 (14.30)	**0.008 (N-L)**
40-80	2 (9.10)	3 (60.00)	14 (50.00)	**0.000 (N-D)**
>80	0 (0)	0(0)	10 (35.70)	0.182 (D-L)
Missing	1	41	10	
UPDRS-III, n(%)				
Mild (0-32)	20(90.90)	21(65.60)	12 (36.40)	0.077 (N-L)
Moderate (33-58)	2 (9.10)	7 (21.90)	16 (48.50)	**0.000 (N-D)**
Severe (>58)	0(0)	4 (12.50)	5 (15.20)	0.048 (D-L)
Missing	1	14	5	
UPDRS-IV, n(%)				
Mild (0-4)	22(100)	20(64.50)	12 (32.40)	**0.007 (N-L)**
Moderate (5-12)	0 (0)	9 (29.00)	21 (56.80)	**0.000 (N-D)**
Severe (>12)	0(0)	2 (6.50)	4 (10.80)	**0.030 (D-L)**
Missing	1	15	1	
Duration of disease, n (%)				
0-3 years	17 (73.90)	11 (29.70)	0 (0)	**0.004 (N-L)**
4-13 years	5 (21.70)	21 (56.80)	13 (34.20)	**0.000 (N-D)**
>13 years	1 (4.30)	5 (13.5)	25(65.80)	**0.000 (D-L)**
Missing	0	9	0	

Tab.3 *Characteristics associated to Parkinson subjects. Values in the groups were presented as: M= Mean, SD= Standard deviation, n= number, % = Percentage. Abbreviation: BMI= Body Mass Index; N= Naive; L= Levodopa; D= Duodopa. p values were calculated with Student t-test for Age and BMI. For the other variables Chi Square test were performed.*

Considering the three different treatment groups, LD-Group, Duodopa-Group, Naïve-Group, no significant difference was shown with regards to age, BMI, coffee and smoking, diet and food habits.

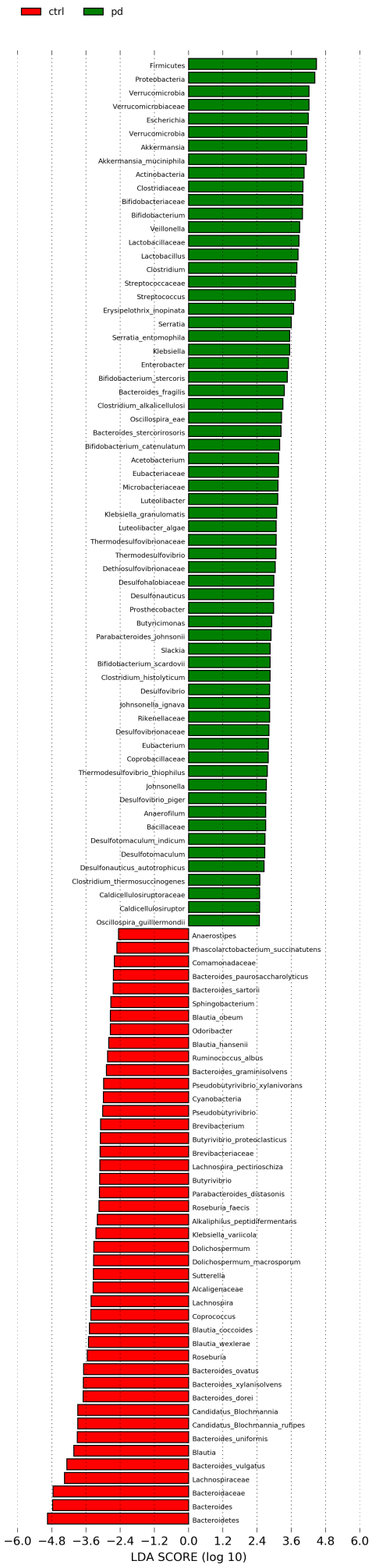
In the LD-Group sex ratio was significantly different compared to the Naïve-Group; more patients were constipated in the Naïve-Group compared to the Duodopa-Group. The Naïve-Group patients were significantly more active compared to the Duodopa-Group, while the LD-Group patients were more active than the Duodopa-Group. For disease features, phenotypes and duration see **Tab.3.**

Parkinson Disease (PD) versus Control-Group

To identify the distinguishing taxa within the groups, the linear discriminant analysis (LDA) effect size (LEfSe) method was implemented (**Fig.5**). At the phylum level, *Firmicutes*, *Proteobacteria*, *Verrucomicrobia, Actinobacteria*, were significantly enriched in diseased samples, while *Bacteroidetes* and *Cyanobacteria* were significantly decreased.

The abundance of 17 families was significantly modified between PD and Control-Group: *Bifidobacteriaceae, Microbacteriaceae, Rikenellaceae, Bacillaceae, Caldicellulosiruptoraceae, Clostridiaceae, Coprobacillaceae, Dethiosulfovibrionaceae, Lactobacillaceae, Thermodesulfovibrionaceae, Streptococcaceae, Desulfohalobiaceae, Desulfovibrionaceae,* and *Verrucomicrobiaceae* are increased in PD, while *Brevibacteriaceae, Bacteroidaceae*, *Lachnospiraceae*, *Comamonadaceae* and *Alcaligenaceae* are reduced in the same patients.

29 genera resulted significantly prevalent in PD: *Bifidobacterium, Slackia, Butyricimonas, Caldicellulosiruptor, Acetobacterium, Clostridium, Actobacterium, Eubacterium, Akkermansia, Escherichia, Ferrimicrobium, Streptococcus, Veillonella, Anaerofilum, Johnsonella, Lactobacillus, Desulfotomaculum, Streptococcus, Vellonella, Thermodesulfovibrio, Desulfonauticus, Desulfovibrio, Enterobacter, Escherichia, Klebsiella, Serratia, Akkermansia, Leuteolibacter, Prosthecobacter*. Instead, 14 genera were significantly reduced in PD: *Bacteroides*, *Brevibacterium, Bacteroides*, *Blautia*, *Odoribacter*, *Sphingobacterium, Dolichospermum, Anaerostipes, Butyrivibrio Coprococcus, Lachnospira, Pseudobutyrivibrio, Roseburia, Candidatus Blochmanna, and Sutterella*.



21 species were significantly increased in PD: *Bifidobacterium catenulatum*, *Bifidobacterium scartovi, Bifidobacterium stercoris, Bacteroides fragilis, Bacteroides stercoridoris, Parabacteroides johnsonii, Clostridium alkalicellulose, Clostridium histolyticum, Clostridium thermosuccinogenes, Erysipelothrix inopinata, Johnsonella ignava , Desulfotomaculum indicum, Oscillospira eaae, Oscillospira guilliermondii, Thermodesulfovibrio thiophilus, Desulfonauticus autotrophicus, Desulfovibrio piger,Klebsiella granulomatis, Serratia entomophila, Akkermansia muciniphila, Luteolibacter algae.* 23 were, conversely, the species significantly reduced in PD: *Bacteroides dorei Bacteroides ovatus, Bacteroides graminisolvens, Bacteroides paurosaccharolyticus, Bacteroides sartori, Bacteroides uniformis, Bacteroides vulgatus, Bacteroides xylanisolvens Parabacteroides distasonis, Dolichospermum macrosporum, Phascolarctobacterium succinatutens, Alkaliphilus peptidifermentans, Lachnospira pectinoschiza, Blautia coccoides, Blautia hansenii, Blautia obeum, Blautia wexlerae, Butyrivibrio proteoclasticus, Pseudobutyrivibrio xylanivorans, Roseburia faecis, Ruminococcus albus, Candidatus Blochmannia rufipes and Klebsiella variicola.*

Interestingly, within the phylum *Firmicutes*, the major differences concerned the *Lachnospiraceae* family, within which different genera and species were significantly reduced in the patients group; in particular, *Blautia wexlerae*, *Blautia coccoides*, *Lachnospira pectinoschiza*. As for the phylum Bacteroidetes, the major alterations concerned the *Bacteroidaceae* family, within which 8 species showed a significant reduction in PD.

Fig.5a *LEfSe PD vs Control-Group*

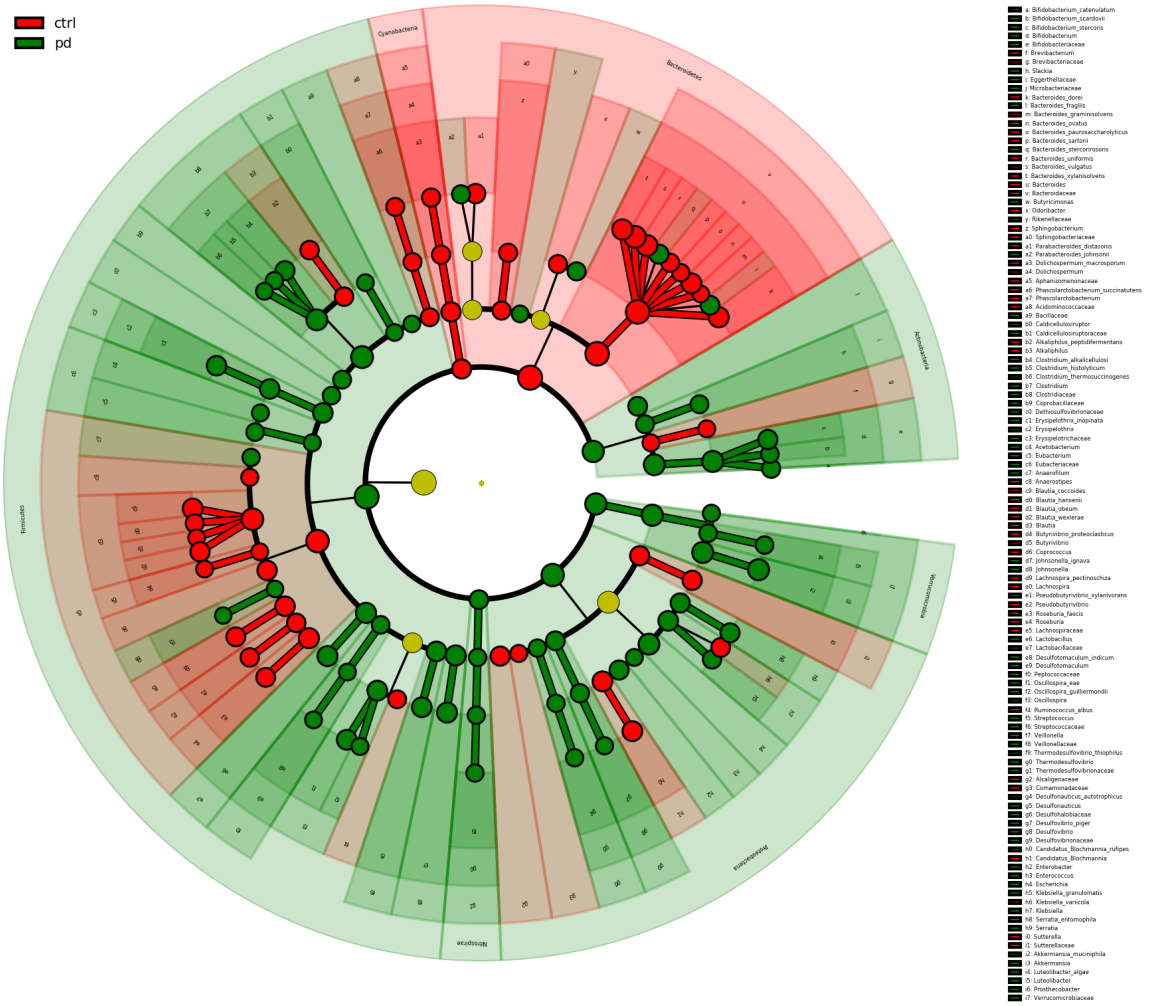


Fig.5b *Cladigram PD vs Control-Group*

Composition of gut microbiome adjusted for potential confounders

The analysis for confounders was performed using the Generalized Linear Model (GLM) adjusted for sex, age, BMI, Diet, constipation, coffee consumption and smoking status covariates showed significant changes in the composition of the intestinal microbiota in PD compared to Control-Group at various taxonomic levels (**Tab.4**).

The major changes affected bacteria of the *Lachnospiraceae* family from *Firmicutes phylum*. Accordingly, the genera *Blautia*, and *Anaerostipes* were significantly reduced in the PD group Furthermore, the species *Lachnospira pectinoschiza*, *Blautia coccoides*, *Blautia wexlerae* and *Blautia obeum* were significantly reduced in PD patients while *Oscillospira guilliermondii* was significanlty more abbundant in PD. Also from *Firmicutes phylum* there was an increase of *Veillonella* genus in PD group.

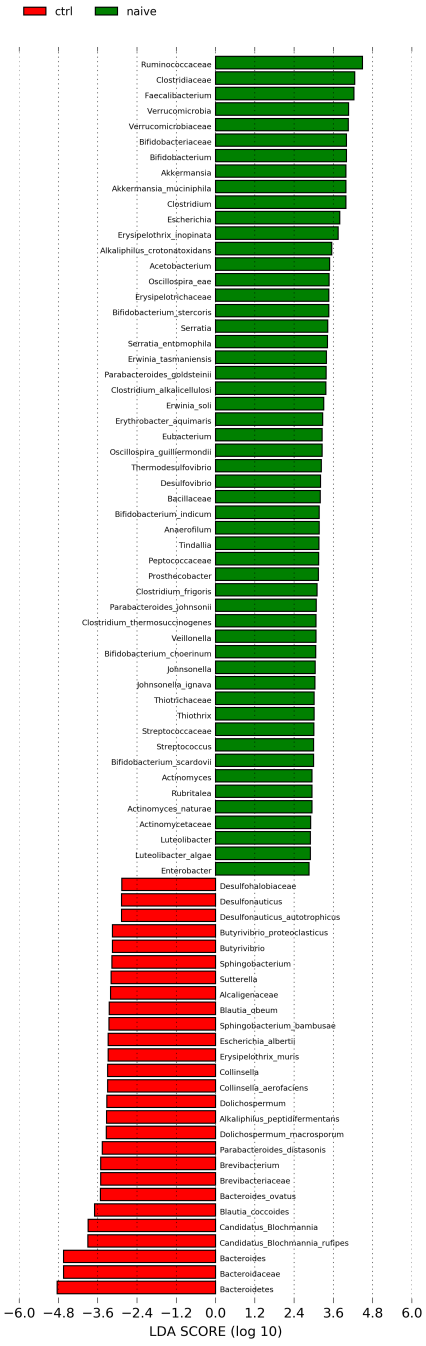
Regarding the *Proteobacteria* phylum, the *Candidatus Blochmannia* genus and the *Candidatus Blochmannia rufipes* species of the *Enterobacteriaceae* family were significantly reduced in PD patients, with an increase of *Serratia entomophila* in the same group. Also, the *Brevibacteriaceae* family belonging to the phylum *Actinobacteria,* as well as the *Dolichospermum* genus belonging to the *Cyanobacteria* phylum*,* were significantly reduced in PD.

Tab. - Statistically significant differences of gut microbiota in PD patients vs Ctrls

Phylum	Family	Genus	Species	Type		p value	Bonferroni corr p value
Actinobacteria	Brevibacteriaceae			PD-CTRL	↓	0,000	0,000
Firmicutes	Dethiosulfovibrionaceae			PD-CTRL	↑	0,005	0,006
	Lachnospiraceae	Anaerostipes		PD-CTRL	↓	0,004	0,156
		Blautia		PD-CTRL	↓	0,023	0,027
			Blautia_coccoides	PD-CTRL	↓	0,003	0,018
			Blautia_obeum	PD-CTRL	↓	0,001	0,002
			Blautia_wexlerae	PD-CTRL	↓	0,012	0,055
		Johnsonella	Johnsonella_ignava	PD-CTRL	↑	0,017	0,044
		Lachnospira	Lachnospira_pectinoschiza	PD-CTRL	↓	0,021	0,025
	Ruminococcaceae	Oscillospira	Oscillospira_guilliermondii	PD-CTRL	↑	0,008	0,206
	Veillonellaceae	Veillonella		PD-CTRL	↑	0,027	0,004
Proteobacteria	Enterobacteriaceae	Candidatus_Blochmannia		PD-CTRL	↓	0,000	0,000
			Candidatus_Blochmannia rufipes	PD-CTRL	↓	0,000	0,000
		Serratia	Serratia_entomophila	PD-CTRL	↑	0,004	0,006
Cyanobacteria	Aphanizomenonaceae	Dolichospermum		PD-CTRL	↓	0,000	0,000

Tab.4 *Generalized Linear Model (GLM) followed by Bonferroni correction for multiple comparisons in Statistical Package for the Social Sciences Version (SPSS) 25.0 for Windows. The differences of microbiota composition between PD patients vs Ctrls subjects were adjusted for sex, age, BMI, Diet, constipation, coffee consumption and smoking status covariates. Bacteria with a relative abundance ≥ 0.1% in at least 25% of the population of one of the two study groups were considered. MD: Mean difference between logarithmic value of relative abundance in the two groups; Bonferroni corrected p values: P <0.05*

PD drugs versus controls



Considering the subgroups of different treatment (Duodopa-Group, Levodopa-Group, Naïve-Group), we found different relative abundances of bacteria population in comparison to CTRL-Group, as showed in **Fig. 6 .**

After correction for confounders such as sex. age. BMI. constipation. coffee consumption. smoking status and diet **Naïve-Group (NG)** showed significant difference compared to CTRL Group. The major changes affected bacteria from *Candidatus Blochmannia* genus and *Candidatus Blochmannia* rufipes and *Escherichia alberti* species (*Proteobacteria phylum*), *Brevibacteriaceae family* and *Brevibacterium genus* (*Actinobacteria phylum*) and *Dilichospermum genus* and *Dolichospermum macrosporum* species (*Cyanobacteria*) and *Erysipelothrix muris* species (*Firmicutes*), that were significantly reduced in NG, while *Erwinia soli* and *Erwinia tasmaniensis* species (from *Erwiniaceae* family in *Proteobacteria* Phylum) were significantly increased. (**Tab. 5**)

Fig.6a *LEfSe Naïve-Group vs Control-Group*

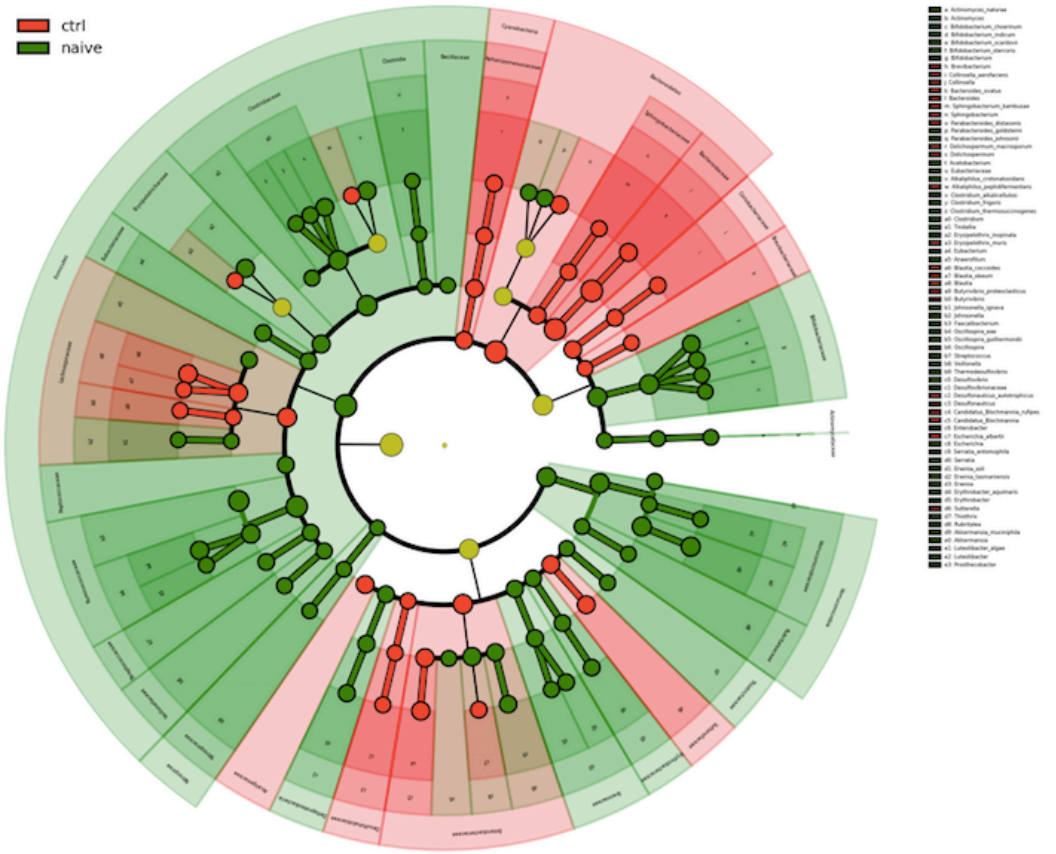
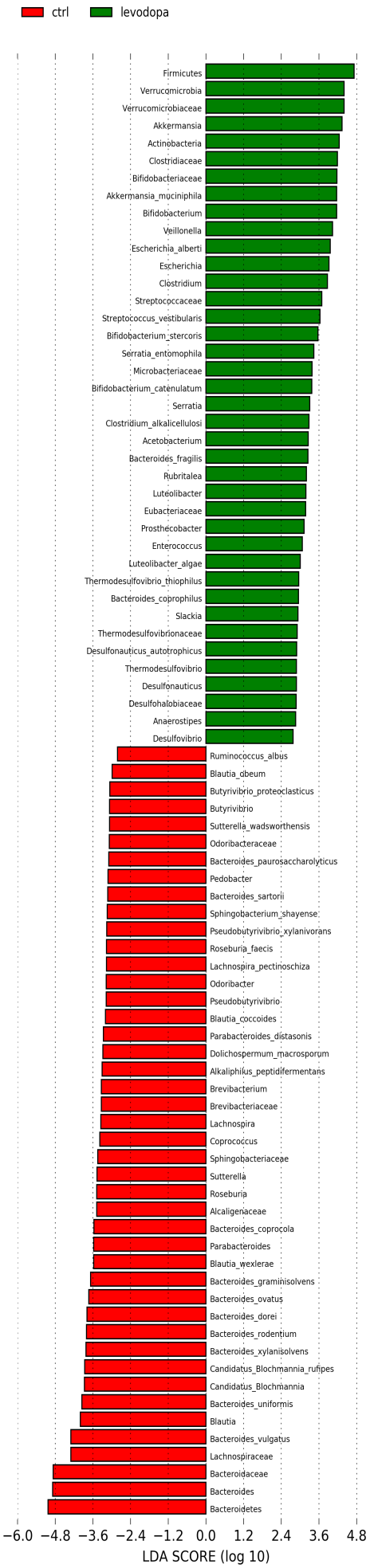


Fig.6b *Cladigram Naïve-Group vs Control-Group*

Tab. - Statistically significant differences of gut microbiota in Naïve-Group (NG) patients vs Control-Group (Ctrl)

Phylum	Family	Genus	Species	Type	Coefficient		corr. p value
Actinobacteria	Brevibacteriaceae			N-CTRL	-0.014	↓	0.000
		Brevibacterium		N-CTRL	-0.014	↓	0.000
Cyanobacteria	Aphanizomenonaceae	Dolichospermum		N-CTRL	-0.015	↓	0.000
			Dolichospermum macrosporum	N-CTRL	-0.025	↓	0.000
Firmicutes	Erysipelotrichaceae	Erysipelothrix	Erysipelothrix muris	N-CTRL	-0.030	↓	0.000
Proteobacteria	Enterobacteriaceae	Candidatus_Blochmannia		N-CTRL	-0.098	↓	0.000
			Candidatus_Blochmannia rufipes	N-CTRL	-0.113	↓	0.000
		Escherichia	Escherichia albertii	N-CTRL	-0.019	↓	0.041
	Erwiniaceaea	Erwinia	Erwinia soli	N-CTRL	0.033	↑	0.000
			Erwinia tasmaniensis	N-CTRL	0.046	↑	0.000

Tab.5 *Multivariate Analysis by Linear Model (MaAsLin) performed on Galaxy tool (Version 1.0.1). The differences of microbiota composition between Naive PD patients vs Ctrls were adjusted for sex. age. BMI. constipation. coffee consumption. smoking status and diet covariates. Bacteria with a relative abundance ≥ 0.1% in at least 25% of the population of one of the two study groups were considered; p values and FDR corrected p values (q-value): < 0.05; N=Naive; Ctrl=Controls;*



After correction for the same confounders **Levodopa-Group (LD)** showed major changing in *Bacteroides* and *Firmicutes* phyla with a reduction of *Bacteroides raminisolvens*, *Bacteroides paurosaccharolyticus*, *Bacteroides sartorii, Bacteroides uniformis*, *Bacteroides xylanisolvens, Blautia obeum, Blautia wexlerae Lachnospira pectinoschiza, Roseburia faecis*. Also *Candidatus Blochmannia* genus and *Candidatus Blochmannia rufipes* species and *Brevibacteriaceae* family were reduced in LD compared to Control-Group, while *Veillonella* genus (*Firmicutes*) and *Serratia entomophila* (*Proteobacteria*) were increased in the same group . (**Fig.7**, **Tab. 6)**

Also **Duodopa-Group** showed significant difference compared to CTRL group in the multivariate analysis. The most important difference was found at phylum level with an increase in the abbundance in *Proteobacteria*, specifically with an increse of *Enterobacter*, *Tolumonas*, *Escherichia*, *Klebsiella* and *Serratia* geni and with *Tolumonas auensis*, *Klebsiella granulomatis* and *Serratia entomophil*a species. Moreover, significant difference at family level was found in the reduction in Duodopa Group of *Brevibacteriaceae* and *Brevibacterium* genus. Also *Blautia* genus (*Firmicutes* phylum) showed a significant reduction, confirmed in different species such as *Blautia coccoides*, *Blautia hansenii*, *Blautia obeum*, *Blautia wexlerae*. More significant difference at species level were found with an increase of *Erysipelothrix inopinata* (*Firmicutes*) and a reduction of *Candidatus Blochamnnia rufipes* and at its genus *Candidatus Blochmannia*.(**Fig.8**, **Tab. 7)**

Fig.7a *LEfSe LD-Group vs Control-Group*

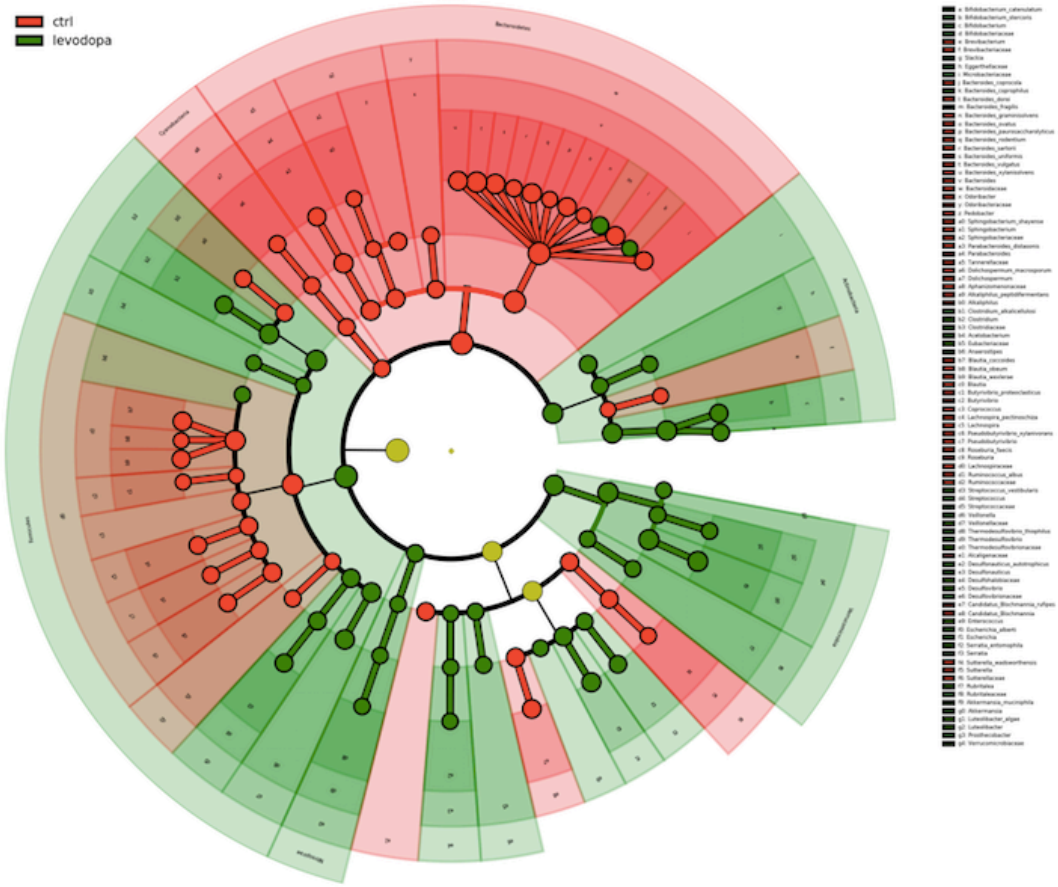


Fig.7b *Cladrigram LD-Group vs Control-Group*

Phylum	Family	Genus	Species	Type		Bonferroni corrected p value
Actinobacteria	Brevibacteriaceae			L-CTRL	↓	0,005
Bacteroidetes	Bacteroidaceae	Bacteroides	Bacteroides_graminisolvens	L-CTRL	↓	0,003
			Bacteroides_paurosaccharolyticus	L-CTRL	↓	0,021
			Bacteroides_sartorii	L-CTRL	↓	0,005
			Bacteroides_uniformis	L-CTRL	↓	0,046
			Bacteroides_xylanisolvens	L-CTRL	↓	0,015
Firmicutes	Lachnospiraceae	Blautia	Blautia_obeum	L-CTRL	↓	0,008
			Blautia_wexlerae	L-CTRL	↓	0,024
		Lachnospira	Lachnospira_pectinoschiza	L-CTRL	↓	0,043
		Roseburia	Roseburia_faecis	L-CTRL	↓	0,046
	Veillonellaceae	Veillonella		L-CTRL	↑	0,000
Proteobacteria	Enterobacteriaceae	Candidatus_Blochmannia		L-CTRL	↓	0,000
			Candidatus_Blochmannia_rufipes	L-CTRL	↓	0,000
		Serratia	Serratia_entomophila	L-CTRL	↑	0,051

Tab.6 *Generalized Linear Model (GLM) followed by Bonferroni correction for multiple comparisons in Statistical Package for the Social Sciences Version (SPSS) 25.0 for Windows. The differences of microbiota composition between PD patients treated with Levodopa vs Ctrls subjects were adjusted for sex, age, BMI, Diet, constipation, coffee consumption and smoking status covariates. Bacteria with a relative abundance ≥ 0.1% in at least 25% of the population of one of the two study groups were considered. MD: Mean difference between logarithmic value of relative abundance in the two groups; Bonferroni corrected p values: P <0.05.*

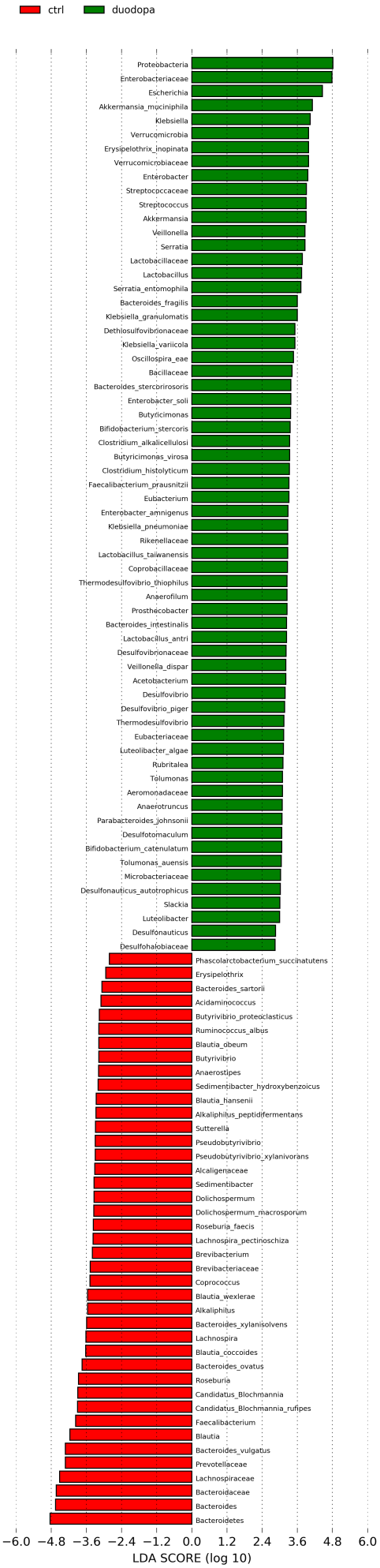


Fig.8a *LEfSe Duodopa-Group vs Control-Group*

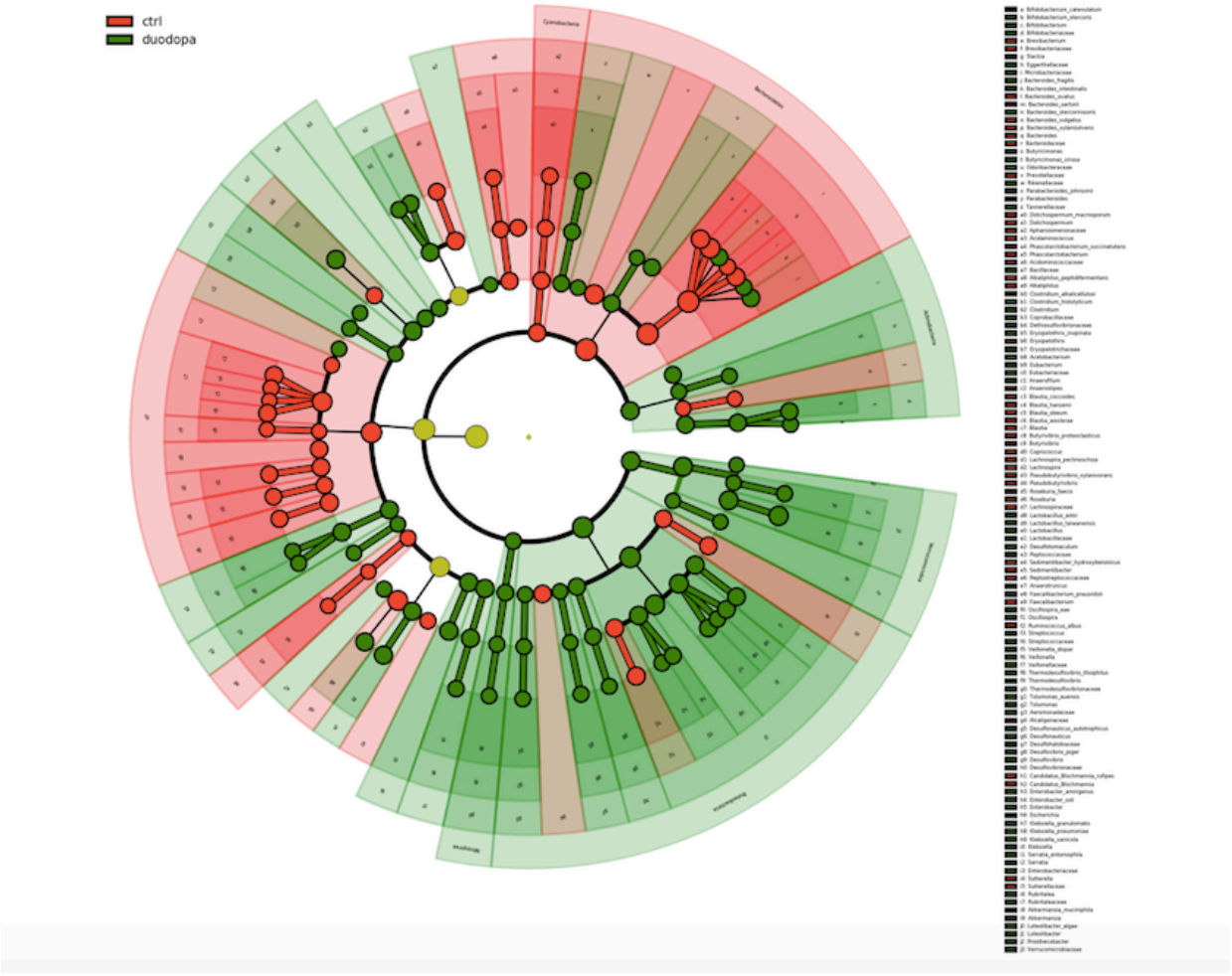


Fig.8b *Cladrigram Duodopa-Group vs Control-Group*

Phylum	Family	Genus	Species	Type		Bonferroni corrected p value
Actinobacteria	Brevibacteriaceae			D-CTRL	↓	0,000
		Brevibacterium		D-CTRL	↓	0,000
Cyanobacteria	Aphanizomenonaceae	Dolichospermum		D-CTRL	↓	0,002
Firmicutes	Erysipelotrichaceae	Erysipelothrix	Erysipelothrix inopinata	D-CTRL	↑	0,003
		Blautia		D-CTRL	↓	0,050
			Blautia coccoides	D-CTRL	↓	0,012
			Blautia hansenii	D-CTRL	↓	0,011
			Blautia obeum	D-CTRL	↓	0,010
			Blautia wexlerae	D-CTRL	↓	0,043
Proteobacteria				D-CTRL	↑	0,007
	Aeromonadaceae	Tolumonas		D-CTRL	↑	0,004
			Tolumonas auensis	D-CTRL	↑	0,020
	Enterobacteriaceae			D-CTRL	↑	0,007
		Candidatus Blochmannia		D-CTRL	↓	0,000
			Candidatus Blochmannia rufipes	D-CTRL	↓	0,000
		Enterobacter		D-CTRL	↑	0,043
		Escherichia		D-CTRL	↑	0,001
		Klebsiella		D-CTRL	↑	0,014
			Klebsiella granulomatis	D-CTRL	↑	0,000
		Serratia		D-CTRL	↑	0,000
			Serratia entomophila	D-CTRL	↑	0,000

Tab.7 *Generalized Linear Model (GLM) followed by Bonferroni correction for multiple comparisons in Statistical Package for the Social Sciences Version (SPSS) 25.0 for Windows. The differences of microbiota composition between PD patients treated with Duodopa vs Ctrls subjects were adjusted for sex, age, BMI, Diet, constipation, coffee consumption and smoking status covariates. Bacteria with a relative abundance ≥ 0.1% in at least 25% of the population of one of the two study groups were considered. MD: Mean difference between logarithmic value of relative abundance in the two groups; Bonferroni corrected p values: P <0.05;*

To identify the specific effect of the different drugs in PD we compared **Levodopa-Group (LD)** and **Duodopa-Group** with the **Naïve-Group (NG)**.

Considering the comparison between the **Levodopa-Group** and the **Naïve-Group** **(Fig.9)** LEFSE analysis showed different bacterial distributions among the two groups with the most important contribution given by bacteria from *Proteobacteria* phylum in the LD-Group and *Ruminococcaceae* and *Erysipelotrichaceae* family for *Firmicutes* phylum in NG.

onsidering the comparison between **Duodopa-Group** and **Naïve-Group (NG)** *Proteobacteria* phylum and *Enterobacteriaceae* species with *Klebsiella*, *Escherichia* and *Enterobacter* genera were more abundant in Duodopa Group, while *Firmicutes* and *Lachnospiraceae* and *Blautia* were more abbundnat in NG.

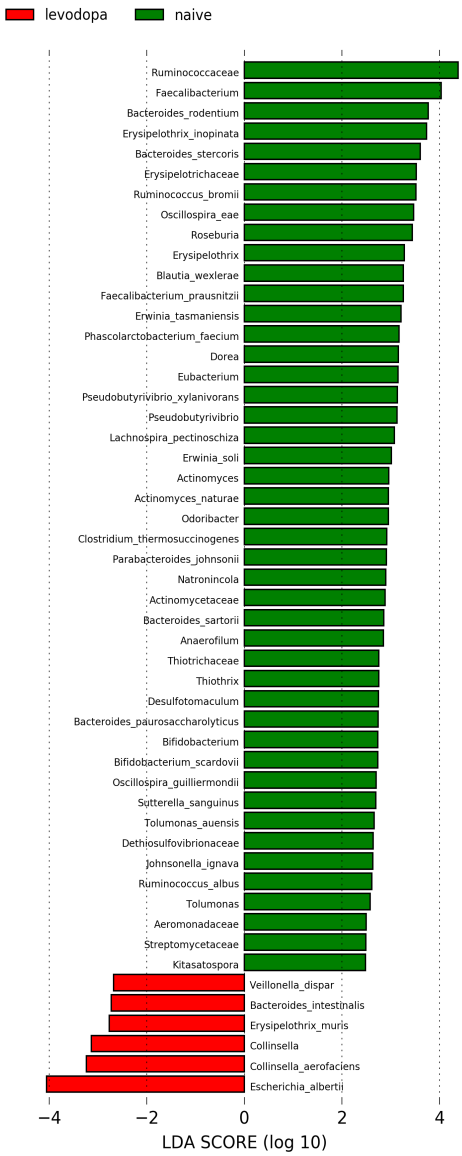


Fig.9 *LEfSe LD-Group vs Naïve-Group*

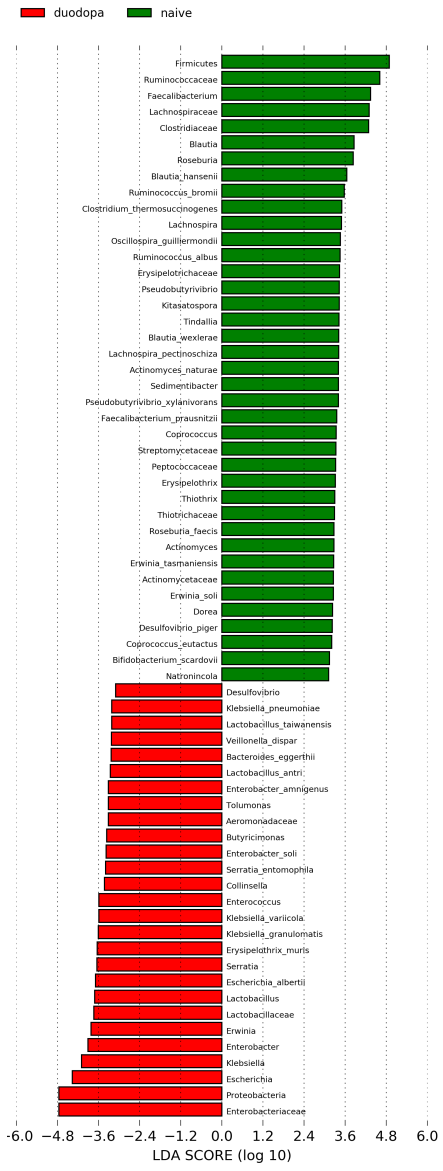
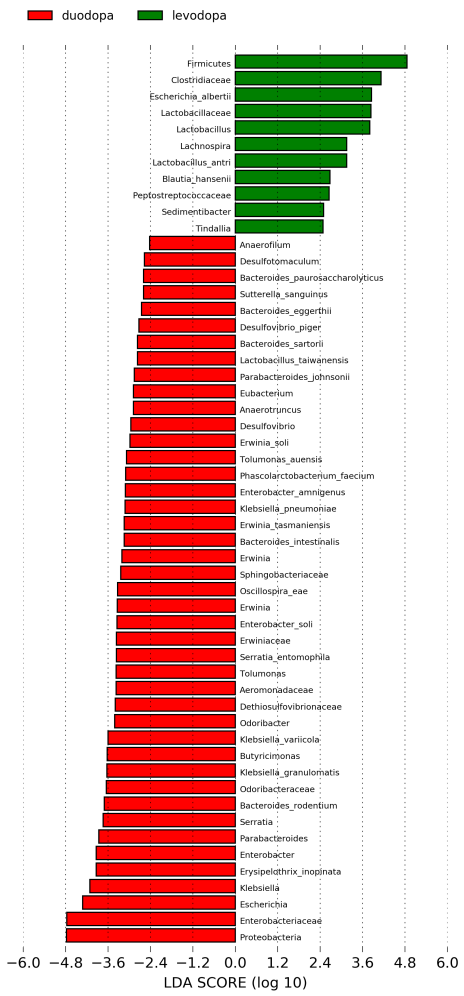


Fig.10 *LEfSe Duodopa vs Naïve-Group*



Moreover, considering the comparison between **Levodopa-Group** and **Duodopa-Group** bacteria from *Proteobacteria* phylum, *Enterobacteriaceae*, *Odoribacteriaceae* and *Aereomondaceae* families and species within them were more abundant in Duodopa-Group, while *Firmicutes* phylum and its families such as *Lactobacillaceae*, *Clostridiaceae*, *Peptostreptococcaceae* and species within them were significantly higher in Levodopa group. **(Fig.11)**

After correction of confounders such as sex, age, BMI, physical activity, constipation, phenotype, coffee consumption, smoking status, diet, oil consumption, supplement, balanced nutrition, NMSS UPDR-III and UPDRS-IV scales covariates, Duodopa Group showed a significant higher abbundance of *Enterobacteriaceae* family, *Escherichia* and *Serratia* genera compared to Levodopa Group and a lower abbundance of *Erwinia soli* species compared to Naïve Group. These results remained significant also after adding disease duration as a confounder, showing a true effect lead by the different drugs. (**Tab.8**)

Fig.11 *LEfSe Duodopa-Group vs Levodopa-Group*

Tab. Statistically significant differences in LD-Group and Duodopa-Group

Phylum	Family	Genus	Species	Type		p value	q value
Proteobacteria	Enterobacteriaceae			L-D	↓	0.000	0.010
		Escherichia		L-D	↓	0.000	0.035
		Serratia		L-D	↓	0.000	0.002
	Erwiniaceae	Erwinia	Erwinia soli	N-D	↓	0.000	0.000

Tab.8 *Multivariate Analysis by Linear Model (MaAsLin) performed on Galaxy tool (Version 1.0.1). The differences of microbiota composition between PD patients untreated (Naive group) and treated with Levodopa and Duodopa were adjusted for sex, age, BMI, physical activity, constipation, phenotype, coffee consumption, smoking status, diet, oil consumption, supplement, balanced nutrition, NMSS UPDR-III and UPDRS-IV scales covariates. Bacteria with a relative abundance ≥ 0.1% in at least 25% of the population of one of the three study groups were considered; p values and FDR corrected p values (q-value): < 0.05; N=Naive; L=Levodopa; D=Duodopa*

Metabolomic

Considering **Naïve-Group (NG)** versus **Control-Group** we identify several metabolites that were distributed significantly differently in the two groups. 2-Aminobutyric acid, butyric acid, glyceric acid, leucine, serine, tetradecanoic acid, threonine, hydroxipropionic acid were more abbundant in NG while alanine, aspartic acid, glutamic acid, glutamine, methionine, phenylalanine, putrescine and succinic acid were more abbundant in Control-Group. (**Fig.12**)

Considering **Levodopa-Group (LD)** in comparison with **NG** metabolites significantly more abbundant in LD were 4-hydroxyphenylproprionic acid, alanine, cadaverine, glutamine, glycine, hydroxicaproic acid, methionine, phenylalanine, proline, threonine, valine while butyric acid, glyceric acid, hydrocinnamic acid, isoleucine, leucine, nicotinic acid and serine were significantly more abbundant in NG. (**Fig.13**)

Considering **Duodopa-Group** compared to **NG** only 4-hydroxyphenylpropionic acid cadaverine and tetradecanoid acid were significantly higher in Duodopa-Group. (**Fig.14**)

Finally considering the comparison between **LD-Group** and **Duodopa Group** 11 metabolites were significantly higher in Duodopa-Group: 2 amynobutyric acid, acetic acid, butyric acid, caffeic acid, coprostanol, coprostanone, fumaric acid, leucine, pentadecanoic acid, tetradecanoic acid, threonine. Metabolis more abbundant in LD-Group were: alanine, amynobutyric acid, aminovaleric acid, aspartic acid, galacturonic acid, glutamic acid, glutaminehydroxycaproic acid, isoleucine, methionine, phenylalanine, proline, putrescine, thiamine, valine. (**Fig.15**)

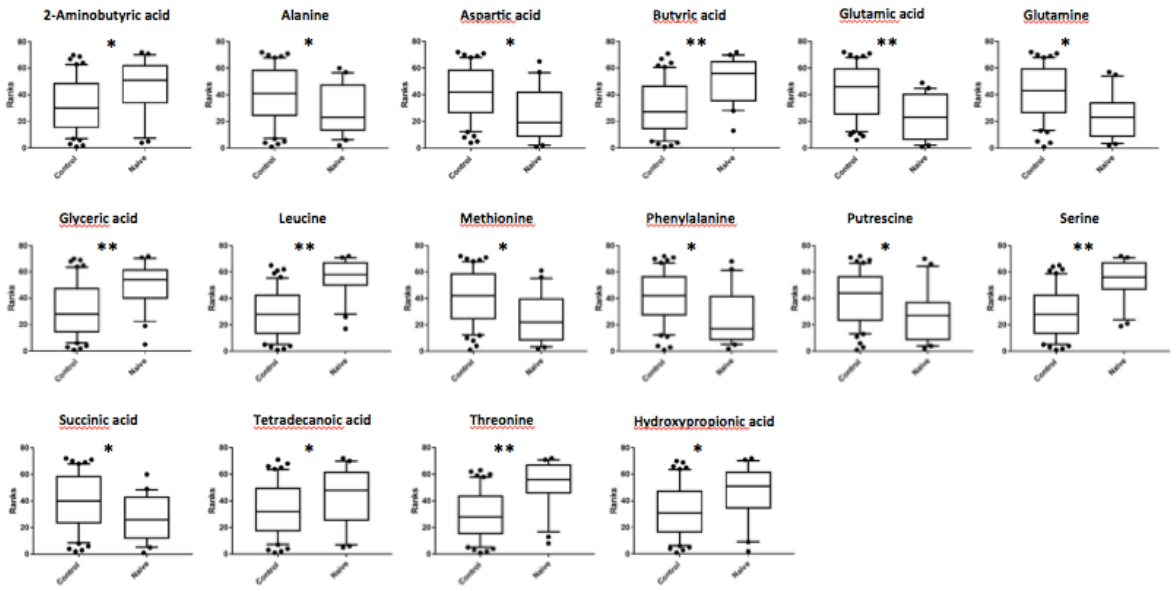


Fig.12 *Metabolomic (Control vs Naive-Group) Mann-Whitney-U tests with Holm-Bonferroni sequential corrected p-values. * p<0,05, ** p<0,01*

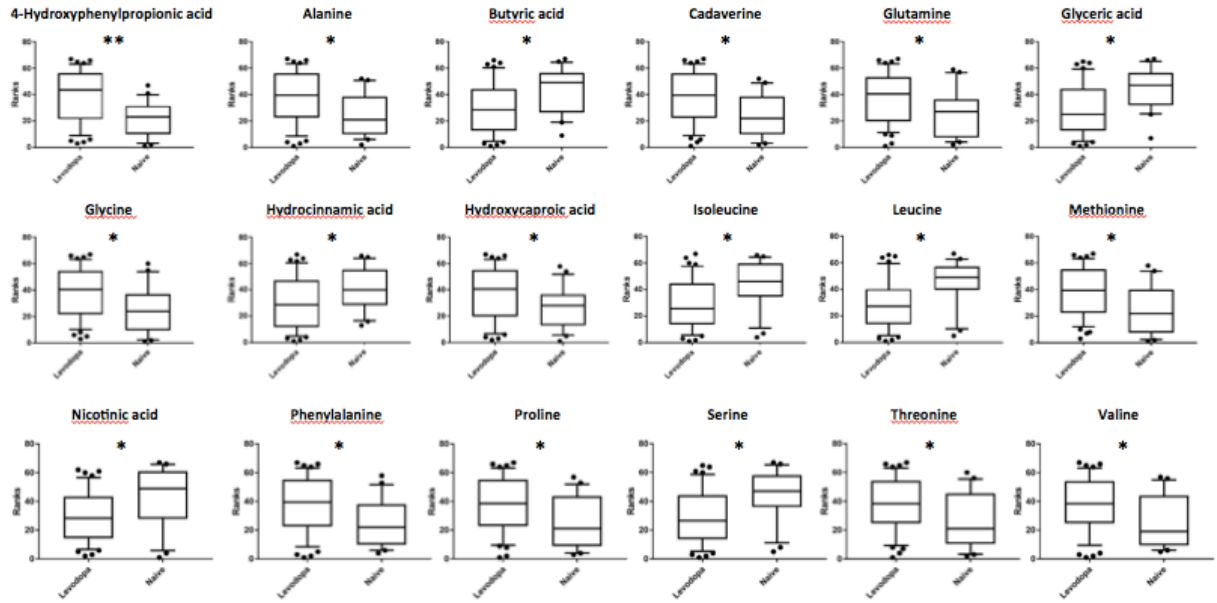


Fig.13 *Metabolomic (Levodopa-Group vs Naive-Group) Mann-Whitney-U tests with Holm-Bonferroni sequential corrected p-values. * p<0,05, ** p<0,01*

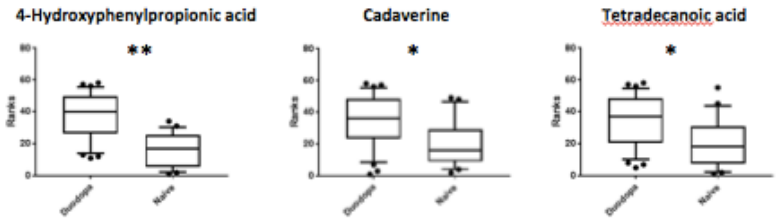


Fig.14 *Metabolomic (Duodopa-Group vs Naive-Group) Mann-Whitney-U tests with Holm-Bonferroni sequential corrected p-values. * p<0,05, ** p<0,01*

Fig.15 *Metabolomic (Duodopa-Group vs Levodopa-Group) Mann-Whitney-U tests with Holm-Bonferroni sequential corrected p-values. * p<0,05, ** p<0,01*

DISCUSSION

The aims of this study were to confirm whether the faecal microbiome of PD patients differs from that of the control subjects, and to evaluate the role of PD medicaments, specifically intestinal infusion therapy with LCIG, as modifying factors in the microbiota composition.

Comparison between PD-Group and Control-Group

In the first part of the study, we evaluated the different composition of fecal microbiota in PD patients and in a control group of patients without PD or other neurodegenerative diseases. Many studies conducted on microbiota in PD patients are not directly comparable because of major methodological differences and especially because most studies did not consider the many confounders that could affect the gut microbiota. As previously described above and as already suggested by other authors,[125] we have performed a GLM method adjusted for sex, age, BMI, constipation, coffee consumption and smoking status covariates in the comparison of PD and the Control-Group. The most important results were the reduction of genera and species of the *Lachnospiraceae* family from *Firmicutes phylum, such as Blautia* and *Anaerostipes, Lachnospira pectinoschiza,* of the *Candidatus Blochmannia* genus and the *Candidatus Blochmannia rufipes* species from the *Enterobacteriaceae* family (*Proteobacteria phylum*). Also, the *Brevibacteriaceae* family, belonging to the phylum *Actinobacteria,* as well as the *Dolichospermum* genus, belonging to the *Cyanobacteria* phylum*,* were significantly reduced in the PD-Group, while the *Lachnospira pecticoschiza*, *Oscillospira guilliermondii* species and *Veillonella* genus were increased in the PD-Group.

These findings are confirmative of previous studies on PD: an increase in the proinflammatory species (*Veillonella*), and the reduction of antiinflammatory species, such as SCFAs producing bacteria *(Lachnospiraceae).*

In this first part of the study, in an attempt to better understand the correlation between Parkinson disease and gut microbiote, and in order to avoid the confounding effects led by several treatments, we selected a subgroup of patients (N=23) who had never undertaken any Levodopa treatment, i.e. the Naive-Group (NG).

NG showed similar abundance to what we found in all PD patients, with a significant decrease of bacteria from *Proteobacteria phylum,* such as *Candidatus Blochmannia* and

Escherichia alberti, the *Brevibacteriaceae family* (*Actinobacteria phylum*), the *Dolichospermum genus* and *Dolichospermum macrosporum* species (*Cyanobacteria*). *Erwinia soli* and *Erwinia tasmaniensis* species were (from the *Erwiniaceae* family in *Proteobacteria* phylum) significantly increased in the NG.

Erwinia soli and *Erwinia tasmaniensis* belong to *Proteobacteria* phylum which has been consistently reported as increased in PD patients with a proinflammatory effect. *Actinobacteria Brevibacteriaceae* [108] was reduced in NG: this has been already reported in PD, but its mechanisms has not been clearly identified so far. However, this has been so far the first report for *Cyanobacteria* and for *Proteobacteria Candidatus Blochmannia* in PD. [126] The members of *Cyanobacteria* phylum, which belongs to the blue-green algae, produce a series of neurotoxins, among which beta-N-Methylamino-L-alanine (BMAA) is the most common. A link between cyanobacterial derived BMAA and neurodegenerative diseases was shown already more than 10 years ago by Cox et al and high levels of *Cyanobacteria* neurotoxin have been associated with AD, ALS and Huntington's disease. However none of the examined studies detected an altered relative abundance in *Cyanobacteria* in PD or in other neurological diseases.[126] The reduction of *Cyanobacteria-Dolichospermum* shown in our paper might suggest a protective role of these bacteria, enhancing what already suggested in PD animal models where *Spirulina*, another bacteria from *Cyanobacteria* phylum, might have anti-inflammatory and antioxidant actions. [127] *Blochmannia* bacteria are found in carpenter ants. To our knowledge, they have never been reported in human microbiota until now. These bacteria have an important role in synthesizing essential and non-essential amino acids, including tyrosine, helping the ant to process nitrogen. Considering the important role of tyrosine metabolism in PD and PD drugs, we can speculate, even if this result should be confirmed by further studies, that the abundance of *Candidatus Blochmannia* might be influenced by this mechanism.

Our analysis of the population sub-groups allowed us to show that the reduction of SCFA producing bacteria was not meaningful in the NGGroup, suggesting a more important influence of drugs on the relative abundance of these bacteria than the effect of the disease itself. This effect will be discussed in detail in the following paragraph about subgroups comparison.

Nonetheless, we found no change, in the NG, in *Lactobacillaceae*, *Verrucomicrobia* and *Prevotellaceae*, while the early publications proposed these bacteria as significant in the PD-microbiota analysis. [102,110,125] We believe that we can explain this heterogeneity of results with,

first of all, the small sample size of some studies and the lack of control for the multiple confounders, as only a few studies have addressed this issue before. [102,110,125, 128]For example the debated results concerning the genus *Prevotella,* found as the most important bacteria influenced by PD in the first studies on this topic, [108,109,116] were not confirmed by the studies mentioned above, as *Prevotella* is extremely sensitive to the different dietary and lifestyle habits. Moreover, not all authors considered the same confounders (in this analysis we included BMI, and generic information about diet). In the GLM analysis we only considered bacteria with a relative abundance ≥ 0.1% in at least 25% of the population in one of the two study groups.

Distinctive features of microbiota in the comparison between Duodopa-Group, Levodopa-Group, Naïve-group.

Given the potential important influence that drugs could exert on the intestinal microbiota, we focused our analysis on PD subjects in pharmacological treatment. We specifically evaluated the effects of Duodopa compared to the Levodopa treatment .

Previously published papers evaluated the effect of COMT inhibitors (Catechol-O-methyltransferase), anticholinergics, and carbidopa/levodopa. These results were not consistent: COMT inhibitors were found to increase *Enterobacteriaceae* composition and to reduce *Firmicutes*, while carbidopa/levodopa was found to influence gut microbiota to the limit of statistical significance. [102,106,110]

However, the literature does not report studies specifically focused on the Levodopa effect on gut microbiota. Our study, for the first time, also evaluated LCIG therapy in patients with PD, with LCIG used in more than 35% of the study cohort under examination.

LCIG therapy involves continuous intraduodenal infusion, using a percutaneous endoscopic gastrostomy, of Levodopa and Carbidopa, the latter a Dopa decarboxylase (DDC) inhibitor. This treatment leads to a significant improvement in complications due to long-term Levodopa oral administration, but to date no specific research on the relationship between intestinal therapy and microbiome has been found in literature.

After correction for confounders, **LD-Group** showed major changing compared to the Control-Group in bacteria from *Bacteroidetes* and *Firmicutes* phyla with a reduction of specific genera and species compared to the Control-Group (mostly *Bacteroides* and *Blautia*).

Candidatus Blochmannia genus, *Candidatus Blochmannia rufipes* species, and *Brevibacteriaceae* family were reduced, while *Veillonella* (*Firmicutes*) and *Serratia entomophila* (*Proteobacteria*) were increased in the same group.

The comparison of **Duodopa-Group** with Control-Group showed similar results in the multivariate analysis: reduction of *Brevibacteriaceae,* of *Blautia* genus and of *Candidatus Blochamnnia* confirmed in the different species, together with an increase of *Erysipelothrix inopinata* (*Firmicutes*). However, the most important difference for the Duodopa-Group (**Fig. 9-10-11**), not identified in the Levodopa-Group evaluation, was found at the phylum level, with an increase in the abundance in *Proteobacteria* phylum. Specifically, we showed an increase of *Enterobacter*, *Tolumonas*, *Escherichia*, *Klebsiella* and *Serratia* genera and of *Tolumonas auensis*, *Klebsiella granulomatis* and *Serratia entomophil*a species.

Since the reduction of *Candidatus Blochmannia* and of *Brevibacterium* has already been shown in both the PD-Group and Naïve-Group, these bacteria may have no correlation with the treatments themselves.

However, our results suggest that some of the previously-reported distinctive traits of microbiota in PD, such as the reduction of bacteria producing fecal SCFA (*Lachnospiraceae* family and *Blautia* and *Anaerostipes* genera), could mostly be linked to the effect of treatments, since they were found reduced in both the LD-Group and Duodopa-Group, but not in the Naïve Group of our cohort. The reduction of SCFA producing bacteria results in a proinflammatory gut environment prone to the development of processes relevant to degenerative phenomena that might contribute to the PD neurodegenerative process. Enteric SCFAs, including acetic acid (AA), propionic acid (PPA), and butyric acid (BA) are a main class of signaling molecules produced from gut bacteria fermentation of dietary carbohydrates, odd-chain fatty acids, and some proteins.[129,108, 105]

Moreover, bacteria from the *Bacteroidetes phylum (Bacteroidaceae and species from Bacteroides genus)* had specifically decreased in the LD-Group. These bacteria have already been described as reduced in PD patients [126, 106, 108, 116], and they were found to be SCFAs (butyrate) productive bacteria, with antiinflammatory properties. Consequently, their reduction in the LD-Group might be an additional contribution to the creation of a pro-inflammatory intestinal environment.

Furthermore, the putative proinflammatory effect of PD drugs is further supported by the

finding that both the Levodopa and Duodopa groups showed an increase in the abundance of bacteria defined putative pathobionts: *Erysipelothrix muri*, *Veillonella*, *Serratia entomophil.* The pathogenic effect of these bacteria is due to the production of neurotoxins, probably contribuiting to the evolution of the neurodegenerative processes.

This proinflammatory effect idientified in the Levodopa-Group seems more intense for the Duodopa-Group, where a significant difference was found at the phylum level for *Proteobacteria* (already described in PD [104,108,125] , and for specific genus that have previously shown specific neurotoxic potential *Enterobacter*, *Escherichia*, *Klebsiella* and *Serratia* geni).

A multivariate analysis was performed for a direct comparison among the three different treatment subgroups, correcting for sex, age, BMI, physical activity, constipation, phenotype, coffee consumption, smoking status, diet, oil consumption, supplement, balanced nutrition, NMSS UPDR-III and UPDRS-IV scales covariates. In this analysis, the Duodopa-Group showed again a significant higher abundance of *Enterobacteriaceae* family, *Escherichia* and *Serratia* genera compared to the LD-Group, and a lower abbundance of *Erwinia soli* species compared to the Naïve-Group. These results remained significant also after adding disease duration as a confounder, showing a true effect led by the Duodopa treatment.

Enterobacteriaceae increase has already been reported in treated PD patients, [126] and they were found to be positively correlated with UPDRS score, PD duration and postural instability.[108, 106] Even though our study should be confirmed by further analysis on different populations, since we performed a multivariate analysis considering all these features as confounders, we can conclude that Duodopa leads to an augmentation of proinflammatory bacteria from *Proteobacteria* and *Eneterobacteriaceae* family.

Therefore, these results lead us to the important finding that Levodopa modulates gut microbiota in PD, with a potential pro-inflammatory effect, and with higher effect given by LCIG, probably due to its direct infusion in the GI tract.

Our study has some limitations, as well as previously reported papers on this topic, We recognize that the analysis of gut microbiota was evaluated without sigmoid mucosal biopsies, because we chose to provide a large population of patients. However, although most of the studies on this topic limited the analysis to an indirect functional prediction of the bacterial metabolic pathways [126], our results are confirmed by the direct evaluation of intestinal metabolits, thanks to the metabolomic analysis. This analysis gives a strong support to the

identification of the different bacterial taxa, with a reduction of butyric and nicotinic acid in the LD-Group and an increase in potentially toxic metabolites, such as cadaverine both in the LD-Group and Duodopa-Group. To enable us to investigate a true cause–effect relationship, further evaluations are needed, together with specifically statistical correlations and subgroup analyses.

Most studies conducted on microbiota in PD patients are not always directly comparable because of major methodological differences. Consequently, since many papers on this topic have been published so far and we expect confirmation for this trend in the future, we suggest, in agreement with other authors, that adopting homogeneity in the methods is of the utmost importance.

However, we believe that our quantitative results, in accordance with the metabolomics analysis, suggest that Levodopa (and LCIG) may also influence the microbiote-intenstinal-brain axis with an increase in the excessive stimulation of the innate immune system, dysregulation of intestinal permeability and the induction of systemic inflammatory responses.

CONCLUSIONS AND FUTURE PERSPECTIVES

The present results support the potential role of gut microbiota in the pathophysiology of PD and suggests, for the first time, that Levodopa and mostly its intraudodenal injection (Duodopa) might significantly influence its composition.

We confirmed some potential distinctive features already described by other authors in PD: an increase of proinflammatory species and the reduction of antiinflammatory species in gut microbiota.
Most of these bacteria have already been described (*Proteobacteria*). However, this is the first report so far of a reduction of *Cyanobacteria* and of *Proteobacteria Candidatus Blochmannia* in PD. As mentioned above, these bacteria might be linked to a potential protective role (antioxidant or aminoacid metabolisms), but such results need to be confirmed in further studies.

In addition, as a major finding of our work, we suggest a specific potential pro-inflammatory and harmful effect of Levodopa on gut microbiota, with higher effect given by LCIG (Duodopa). Compared to the Control-Group, patients under Levodopa (LD-Group) and LCIG (Duodopa-Group) showed a further significant increase compared to the NG-Group in putative pathobionts bacteria (*Erysipelothrix muri*, *Veillonella*, *Serratia entomophily),* with

the potential production of neurotoxins. Moreover, we found a reduction of *Blautia* in both groups, while we reported no change for these bacteria in the NG-group. Consequently, we observed that one of the previously-reported distinctive traits of microbiota in PD, the significant reduction of bacteria producing SCFAs, seems to be linked more probably to the influence of PD drugs than to the effects of the disease itself. This result is confirmed also in the metabolomic study.

LCIG showed the highest potential pro-inflammatory effect, since we found a significant increase at phylum level for *Proteobacteria*, a result not identified in the Levodopa evaluation. Moreover, the *Enterobacteriaceae* family, *Escherichia* and *Serratia* genii (family and species from Proteobacteria) were more abundant in the Duodopa-Group, in direct comparison with the Levodopa-Group, after correction for major confounders.

Consistently, we can propose that Duodopa leads to an augmentation of proinflammatory bacteria compared not only to controls, but also to Levodopa.

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