UNIVERSITY OF PADOVA

Department of General Psychology

Department of Neuroscience

Master's degree in Cognitive Neuroscience and Clinical Neuropsychology

Final dissertation

Inflammatory markers and enteric nervous system pathology in the gastro-intestinal tract of Parkinson's Disease patients: a histopathological study

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Academic Year 2022-2023

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PART 1: Parkinson's Disease & Enteric Nervous System

Introduction

Aging brain

Aging refers to the complex mechanisms which lead to the damage of organs, cells, and molecules present in the body, the consequent loss of functionality and increased susceptibility to pathologies. Instead, from a clinical perspective an aging brain is characterized by a constant, relentless but slow cognitive decline which targets different cognitive domains such as memory, attention, working memory, or executive functions. Understanding aging is fundamental for understanding neurodegeneration. In fact, most of the neurodegenerative pathologies develop in elderly and age represents the first risk factor (Love et al., 2015; Schapira et al., 2017).

The complexity of the situation is enhanced by the fact that there is a high comorbidity of pathologies which incrementally increases with aging. In fact, mixed pathology is quite common in elderly population (Love et al., 2015; Schapira et al., 2017). The individuation and characterization of the specific traits which are associated with successful brain aging is of particular interest because they could be utilized as therapeutic strategies. The current knowledge about aging is that there are different changes in the structure and functionality of the brain, but being able to determine the exact extent is difficult. Alterations in myelination, neurons, axons, dendrites, synapses have been documented using animal models as well as in humans using neuroimaging techniques such as MRI, DTI, or fMRI (Love et al., 2015).

Nowadays, there is still not a widespread shared position about the role of aging in the brain and its implications in neurodegenerative diseases. For example, the key feature of neurodegeneration is, as the name itself says, the degeneration of neurons which lead to different clinical manifestations. All neurodegenerative pathologies such as Parkinson's disease (PD), Alzheimer's disease (AD), Multiple System Atrophy (MSA), are characterized by

neuronal loss. Nevertheless, even in this most basic point, there are divergent opinions. Researchers in the 1950s demonstrated how degeneration of neurons is an intrinsic characteristic of healthy aging. More recent studies demonstrated the opposite; the total number of neurons remain quite stable in the lifespan (Love et al., 2015).

It is possible to classify brain aging by macroscopic changes and microscopic changes. The main macroscopic changes include brain atrophy and lesions in the white matter. Older people show a decrease in cortical and subcortical structure and larger ventricles. This results in a reduction of brain weight and size compared to younger individuals. Some degree of cortical atrophy is present in healthy aging, but if excessive, it can be a clear sign of pathologies (*figure 1*).

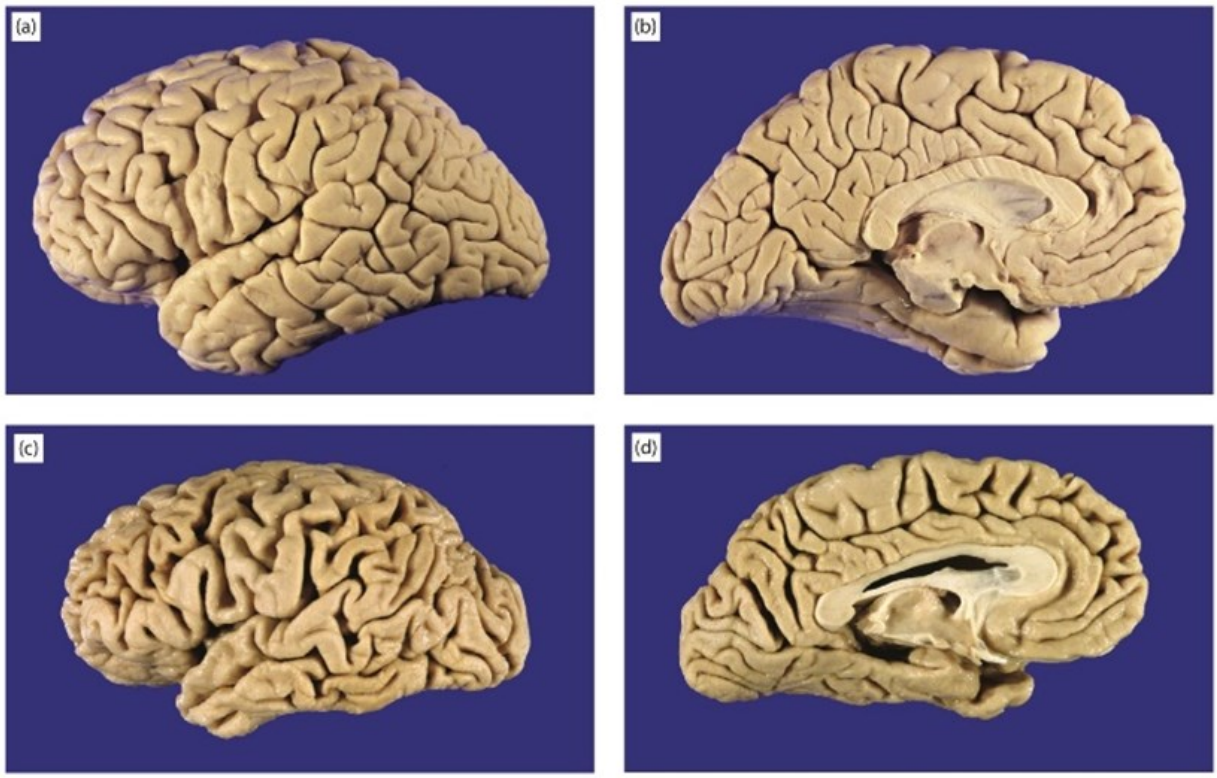


Figure 1. Cortical atrophy in Alzheimer's disease. (*a & b*) lateral and medial views of normal brain from a 73 year old man without neurological diseases (brain weight 1371 g). (*c & d*) lateral and medial views of a 71 year old man with Alzheimer's disease (brain weight 1063) (Love et al., 2015).

On the other hand, microscopic changes are more numerous than the macroscopic ones and they include: neuronal loss, dysfunction in the synapses, dendrites, axons, glial cells, and mitochondria. Regarding neuronal loss, as it was mentioned previously, the commonly accepted idea today is that the number of neurons does not change much in elderly (Love et al., 2015). This is a general notion which is valid for most of the brain regions but there are individual differences. A concrete example is the substantia nigra pars compacta (SNps) which appears to not follow this trend. The neurons in the substantia nigra appear to be particularly sensitive to aging. A study conducted by Ma et al. (1999) demonstrated, by using morphometric measurement in 26 different samples of SNps, that there is a statistically significant decrease of neurons in this brain region. The authors suggest that neuronal loss is highly selective during aging (Ma et al., 1999). This is particularly relevant to understand neurodegenerative diseases which target selectively the substantia nigra, such as PD. Another important aspect of microscopic changes in the aging brain which has to be taken in consideration is age-related glial changes. Glial cells, such as microglia and astrocytes, appear to contribute directly to exaggerated phenomena of neuroinflammation which can be detrimental for the healthy brain (Love et al., 2015).

Neurodegeneration

Neurodegeneration refers to different pathologies which target the nervous system characterized by the progressive degeneration of specific groups of neurons. Since the presence of this progressive neuronal loss, these pathologies are known as neurodegenerative disorders (Esiri & Perl, 2006).

The neurodegenerative diseases represent a serious problem in the current society because they are associated with a substantial decrease in quality for the patient and family, short life expectancy, and economic burden. The need for effective treatments is emphasized by the fact

that there is a rise in life-expectancy which is expected to increase in the next decades (Schapira et al., 2017), and age represents the major risk factor for neurodegeneration.

Neurodegenerative pathologies are characterized by the specific neuroanatomical systems involved, which will determine the clinical manifestation of the disease, and thus the prognosis of the pathology (Esiri & Perl, 2006). For example, AD is characterized pathologically by accumulation of ß amyloid and neurofibrillary tangles which target initially areas such as the limbic system and medial temporal lobe before spreading in all brain. The initial clinical manifestations are loss of episodic memory and language dysfunctions. A different example of neurodegenerative disease is PD, which is characterized pathologically by accumulation of Alpha synuclein (αSyn) which initially targets lower brain structures, then spreads to the substantia nigra (*figure 2*) and eventually involves the whole brain (Braak et al., 2006, Braak & Del Tredici, 2017). In PD, motor manifestations (such as tremor, bradykinesia, rigidity) constitute the cardinal criteria for the diagnosis, and occur when at least 40-60% of striatal dopamine nerve terminals are lost due to nigral degeneration. However, in the prodromal phase (the time between the onset of neurodegeneration and the occurrence of motor symptoms), several non-motor features such as gastrointestinal dysfunctions, orthostatic hypotension (OH), constipation, hyposmia, rapid-eye movement (REM) sleep behavior disorder (RBD), and neuropsychiatric symptoms (i.e. depression) have been frequently observed, sometimes preceding the motor involvement by up to 10 years (Tolosa et al., 2009; Koller, 1992; Hawkes, 2008).

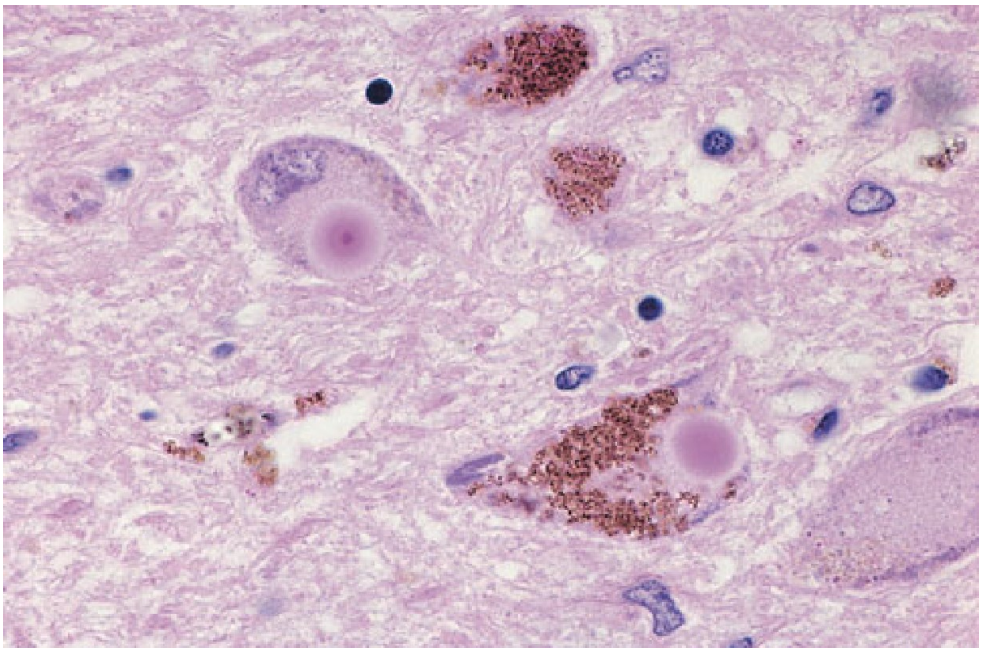


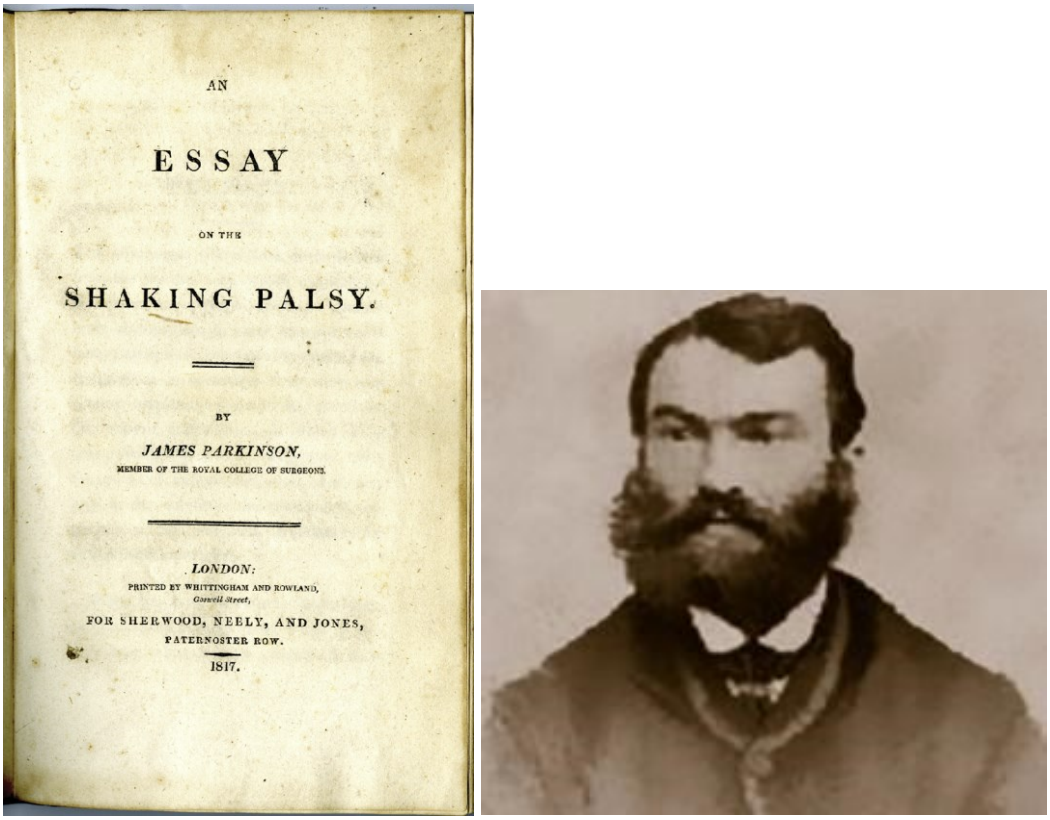
Figure 2. Substantia nigra pars compacta of a patient with Parkinson's disease, Hematoxylin & Eosin stain. The neurons can be recognized by their dark color since they are rich in neuromelanin, while Lewy bodies appear as eosinophilic cytoplasmic inclusions with a typical peripheral pale rim, known as "halo" (Esiri & Perl, 2006).

Even though the neurodegenerative pathologies have some distinct features, they share a common base characterized by the increase of incident with aging, synaptic loss, and neuronal loss. Additionally, human genetics has to be taken into consideration in neurodegeneration. Some genetic mutations, known as risk factors, are associated with an increase of incident of specific neurodegenerative pathologies. On the other hand, there are genetic mutations, known as genetic determinants, which are autosomal dominant meaning that the percentage of developing the disease later on in life is 100% (Schapira et al., 2017). Finally, it is only post-mortem which is possible to have the certainty of the diagnosis, through histopathology. This is mainly due to the fact that today, despite many advancements in the knowledge of neurodegenerative pathologies, it is still not possible to be sure of the diagnosis, nor effective and validated biomarkers have been discovered.

Neuropathology of Parkinson's Disease

Parkinson's disease is the second most common neurodegenerative disorder after Alzheimer's disease is characterized by core motor symptoms (rigidity, bradykinesia, and tremor), autonomic dysfunctions, and cognitive impairments. Parkinson's disease affects around 1-2% of the population above 65 years (Dinan & Cryan, 2017) and its global prevalence is increasing. In early stages, PD is characterized by impairment at the level of the midbrain, specifically the substantia nigra, in which there is the degeneration of a few thousands neurons which synthesize dopamine. The loss in dopaminergic neurons result in motor, autonomic dysfunction, and cognitive symptoms such as resting tremors, stiffness, muscular rigidity, slowness of movement, bradykinesia, postural instability, difficulty with balance and coordination, cognitive decline, depression, emotional changes, constipations, sleep problems, and difficulty with swallowing and chewing (National Institute of Neurological Disorders and Stroke, 2021; Esiri & Perl, 2006).

Parkinson's disease was named after James Parkinson published *an essay on the shaking palsy* in 1817 describing meticulously the neurodegenerative pathology (*Figure 3*).


AN

ESSAY

ON THE

SHAKING PALSY.

BY

JAMES PARKINSON,

MEMBER OF THE ROYAL COLLEGE OF SURGEONS.

LONDON:

PRINTED BY WHITTINGHAM AND ROWLAND,

Goswell Street,

FOR SHERWOOD, NEELY, AND JONES,

PATERNOSTER ROW.

1817.

Figure 3. The essay published by James Parkinson, which describes the pathology which today has its name: Parkinson's disease (Obeso et al., 2017).

At the time of James Parkinson, there was still no knowledge about the detailed characterization of PD that we have today. For example, James Parkinson did not know that PD initially is confined to the dopaminergic neurons in the substantia nigra, or that the pathology leads to cognitive dysfunctions. Nevertheless, James Parkinson was able to establish the neurodegenerative pathology as a clinical entity, individuating the main motor dysfunctions (Obeso et al., 2017). With the progression of PD and neuronal loss, there is a concomitant worsening of the symptoms which can lead to dramatic dyskinesias or frozen state. Additionally, also the non-motor symptoms get worse leading, in some cases, the patient to dementia. From a macroscopic point of view, the midbrain of a patient with Parkinson's disease shows a marked loss of pigmentation of the substantia nigra which appears pale (*Figure 4, 5 & 6*)(Obeso et al., 2017; Esiri & Perl, 2006).

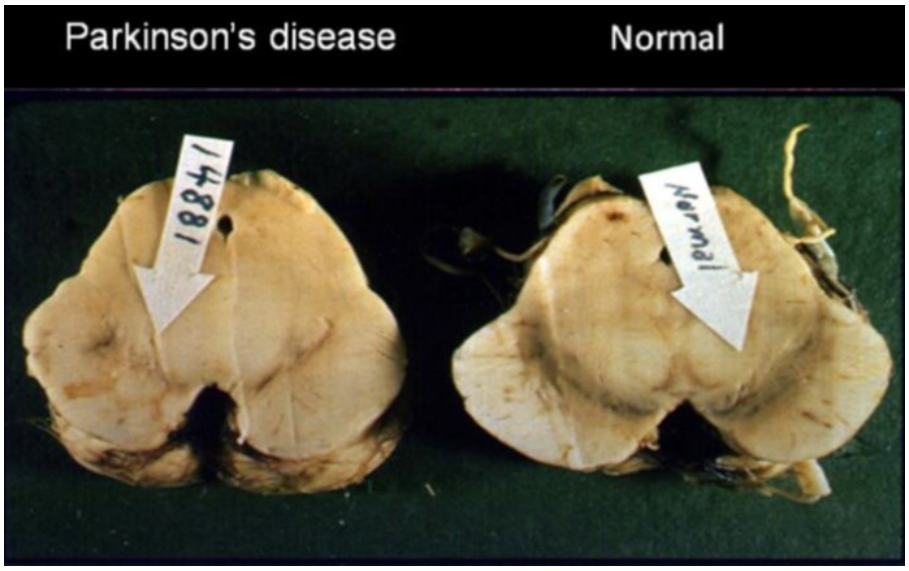


Figure 4. Two examples of a substantia nigra. On the left, there is a substantia nigra which belonged to a patient who had Parkinson's disease and it looks pale. In the right, a healthy substantia nigra characterized by the two black "strings" which represent the dopaminergic neurons rich in melanin (Mandel et al., 2009).

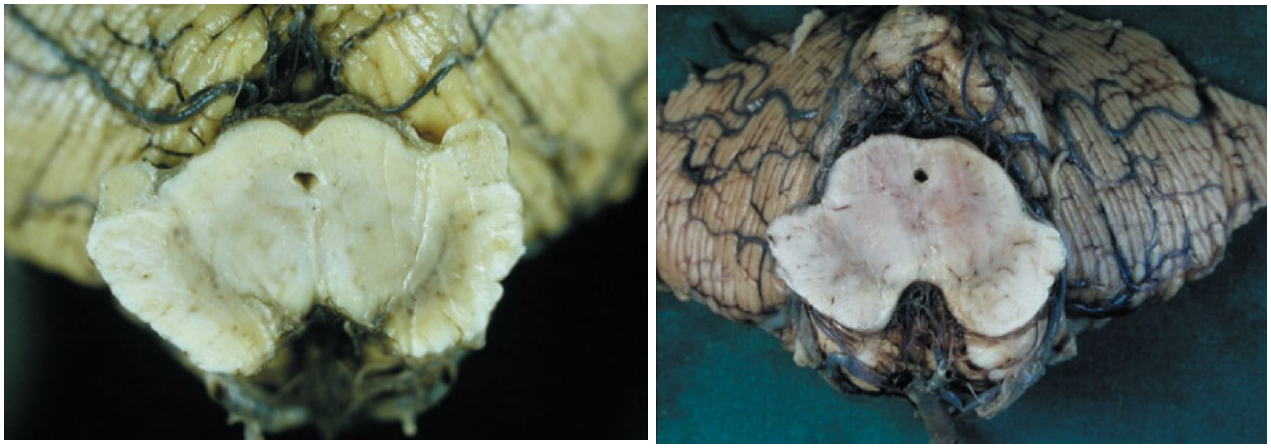


Figure 5 & 6. Two examples of the pale appearance of the substantia nigra in Parkinson's disease (Esiri & Perl, 2006).

The substantia nigra is a group of neurons located in the midbrain responsible for the synthesis of dopamine and for the dopaminergic innervation of the basal ganglia. The substantia nigra is indeed functionally implicated in the basal ganglia circuits, which play a key role in the

regulation of movement, learning, and motivation. They consist of the neostriatum, i.e. caudate nucleus and putamen, the paleostriatum, i.e. the globus pallidus, and are connected to several other diencephalic and mesencephalic structures. The substantia nigra is particularly affected in PD, while the globus pallidus, caudate nucleus and putamen appear spared.

The primary functions performed by the substantia nigra include regulation of dopamine levels, regulation of movement and reinforcement learning. Dopamine has a key role in Parkinson's disease. Dopamine is a neurotransmitter that is involved in the regulation of movement, as well as other functions such as motivation and reward. It is important to mention that even if the main neurotransmitter involved in Parkinson's disease is dopamine, it is not the only one. With the progression of the pathology, other brain regions get affected interfering with the synthesis of other neurotransmitters. For example, Parkinson's could eventually degenerate the locus coeruleus causing a deficiency of the neurotransmitter norepinephrine. Another instance is the degeneration of the nucleus of Meynert with a consequent shortage of cholinergic neurons and then the neurotransmitter they synthesize, acetylcholine (Esiri & Perl, 2006).

Diagnosis & Symptomatology

Diagnosis of Parkinson's disease is typically made through a combination of a medical history, neurological examination, and some additional test to rule out different pathologies especially imaging of the dopaminergic system (i.e. Datscan). Additional tests are blood and laboratory testing, as well as genetic screening in case of familial history and/or young onset.

Even though the hallmarks of Parkinson's disease are considered to be the motor dysfunctions, they do not represent the initial stage of the pathology. Parkinson's disease is characterized by a long prodromal phase that can last 5 to 10 years where the initial symptoms experienced by the patient are non-motor symptoms. In fact, the first symptoms of PD in the preclinical stage are gastrointestinal dysfunctions, autonomic dysfunctions, olfactory loss, sleep behavior disorder, hyposmia, constipation, depression, pain, and anhedonia/apathy. The official diagnosis of PD is made upon the evaluation of four motor symptoms which usually emerge later on compared to others non-motor symptoms: tremor, muscular rigidity, bradykinesia, and postural instability. Importantly, research has demonstrated that when the initial symptoms appear, the substantia nigra has already lost from 60 to 80 percent of dopaminergic neurons (National Institute of Neurological Disorders and Stroke, 2021). This highlights the presence of the wide prodromal phase which goes undetected for years. The four main motor symptoms which constitute the hallmarks of PD comprise:

- Tremor: the shaking usually starts unilaterally in one hand, but it is not the only region of the body which can be initially affected. There is evidence that the shaking could start in the jaw or in the foot. The shaking in the hand is characterized by a rhythmic motion which targets the thumb and forefinger mimicking the gesture of counting money. The tremor is visible when the hand is in a resting condition, or the patient is going through a stressful situation. The shaking disappears with goal-directed behavior or when the subject is sleeping.

- Muscular rigidity: condition characterized by a constant muscular contraction and tension, and resistance to movement. A feature which characterized muscular rigidity is the so-called "cogwheel" rigidity, which refers to short, jerky movements when the doctor tries to move the patient's arms.
- Bradykinesia: general and widespread slowing down of goal-directed and automatic movements. Also the face's muscles appear affected leading to change in facial expressions, a situation known as "masked face".
- Postural instability: the increased probability of falling due to mutated sense of balance and coordination.

Even though Parkinson's disease has some characterizing symptoms, it does not affect each individual in the same way. Intraindividual differences in PD regard the symptoms and the velocity of progression of the pathology. In addition to these four primary symptoms, there is a great number of other symptoms which are most often less evident, but affect people with PD. For example, the parkinsonian gait which is characterized by festination (tendency to make small steps), start hesitation (difficulty in initiating movements), freezing (abrupt stop when walking), and reduced swinging of the arms. Particularly interesting for the aim of this project are the symptoms related to the gastrointestinal dysfunctions such as reflux, vomiting, nausea, constipation, and bowel incontinence (National Institute of Neurological Disorders and Stroke, 2021; Warnecke et al., 2022).

The gastrointestinal pathologies are gaining particular attention in current scientific research. This is because the gastrointestinal system includes what is known as the enteric nervous system, a highly specialized neural network which has some important implications in PD (Warnecke et al., 2022) as we will see more in detail later on.

Etiology & Lewy bodies

The cause of Parkinson's disease is not known, but the main risk factors for the onset of PD seem to be age, sex (men seem to be more prone to get PD than women), genetic heritage, and exposure to pesticides (National Institute of Neurological Disorders and Stroke, 2021). Most of the cases are sporadic, but there are also familial instances of patients who have specific genetic mutations which determine the onset of PD. There are around 25 genes which are correlated with PD. Some genes which have been implicated in the development of PD are the SNCA, LRRK2, DJ-1, PRKN, PINK1, and GBA (Franco et al., 2021; National Institute of Neurological Disorders and Stroke, 2021). Other genes that have been implicated in the pathogenesis of PD are related to the gene for αSyn: A30P, A53T, E46K, H50Q, and G51D.

Even though the implications related to genetics and environment in the pathogenesis of PD are not understood yet, different key elements in the progression of the disorder have been localized such as over-inflammatory response, oxidative stress, and αSyn misfolding (Rietdijk et al., 2017). An important factor that needs to be considered in the etiology of Parkinson's disease is the abnormal accumulation of the misfolded αSyn forming Lewy bodies (Franco et al., 2021; National Institute of Neurological Disorders and Stroke, 2021). The scientist who first described these inclusions was Friedreich Lewy in 1912. The brain regions in which Lewy Bodies were first identified were the Substantia Nigra and dorsal motor nucleus of the vagus. Lewy bodies have a key role in Parkinson's disease because their presence at the level of the substantia nigra have been correlated with the degeneration of the dopaminergic neurons. However, it is important to specify that the presence of Lewy bodies is not a condition which is exclusively present in Parkinson's disease. In fact, Lewy bodies are encountered also in other pathologies and also in the brains of elderly healthy subjects who do not have neurological dysfunctions (Esiri & Perl, 2006).

Alpha synuclein

The hallmark of PD is the degeneration of dopaminergic neurons at the level of the substantia nigra which has been correlated by a consistent presence of Lewy bodies in the surviving neurons. Lewy bodies are constituted mainly of misfolded and aggregated αSyn. Therefore, understanding the role of the αSyn has fundamental implications in understanding PD. Alpha synuclein is a protein which was initially discovered for the first time in a Torpedo fish in 1988. The abundant presence of the protein at the level of the synapses and nucleus of the neurons in the CNS, made the scientist choose the name "synuclein", in which "sy" stands for synapse, and "nuclein" for nucleus. Further research demonstrated the presence of αSyn also in humans. Since then, research has been focused in determining the exact role and function of αSyn, without much success. The pathogenic mechanism which leads to the misfolding, abnormal aggregation, and accumulation of αSyn remains a mystery which needs to be unveiled in order to find effective treatments to pathologies which concern this protein. Even though the CNS contains most of the αSyn, it has been demonstrated recently that αSyn is not exclusively present in the brain (Mehra et al., 2019; Ghosh et al., 2017). According to Emmi et al. (2023), αSyn is present also in peripheral structures such as the enteric nervous system in the gastrointestinal tract. This discovery has important implications in understanding the pathogenesis of certain diseases in which αSyn has a primary role, such as PD.

The current knowledge related to αSyn is that it is a transmembrane protein made by 140 amino acids and molecular mass of around 14 kDa which is found mainly at the level of the neurons' synapses, but also nucleus, axons, cytoplasm, endoplasmic reticulum, and Golgi apparatus. Three different functions have been attributed to αSyn. First, the presence of αSyn in the pre-synapses could indicate a role in vesicular trafficking of neurotransmitters. Second, αSyn appears to play a role in transport of fatty acids to various cellular membranes. Third,

αSyn seems to have a primary role in the SNARE complex which is a specialized protein complex that mediates membrane fusion in cells, and they are directly involved in neurotransmitter release. αSyn can assume two forms: it can be soluble and unstructured, or bound to the neurons' membrane and adopting a helix shape. For some reason which is not completely understood yet, in some pathologies the αSyn assumes an abnormal behavior in which it begins to misfold and aggregate, with toxic consequences for the neurons. However, how the misfolded and aggregated αSyn create damage to the neuron is another key point which has not been elucidated yet (Mehra et al., 2019; Ghosh et al., 2017).

Prion-like mechanism to explain spreading of Lewy bodies

Another point which remains still not entirely clear is how the spreading of αSyn takes place. Nowadays, research has been able to individuate the ability of the Lewy pathology "to jump" from one neuron to the other, but without providing an exact explanation for how this process occurs. The most influential theory which has been proposed to explain the mechanism of propagation of abnormally aggregated αSyn is based on a prion-like mechanism (Natale et al., 2021; Dinan & Cryan, 2017).

Prions are misfolded proteins that can cause other proteins of the same type to also misfold, leading to the formation of aggregates. Similarly, αSyn aggregates can spread from one neuron to another by inducing the misfolding of normal αSyn in the recipient neuron, resulting in the formation of Lewy bodies in new regions. The process of αSyn spreading is thought to occur in four steps:

1) αSyn aggregates are released from neurons into the extracellular space.

2) Once in the extracellular space, the αSyn aggregates can be taken up by neighboring neurons.
3) Once inside the recipient neuron, the αSyn aggregates induce the misfolding of normal αSyn, leading to the formation of new aggregates.
4) These new aggregates can then be released from the neuron and taken up by other neighboring neurons, leading to the spread of αSyn throughout the brain (Liu et al., 2019).

Treatments

The variety of treatments

A variety of treatment options are available to help manage symptoms and improve quality of life, even if a definitive cure is not available yet. There are two main classes of treatments: medication and surgery. Medications are usually of three types: drugs that increase the level of dopamine such as levodopa, drugs which target different neurotransmitters than dopamine such as anticholinergic drugs to increase the level of cholinergic neurotransmitters in the brain, and medications which target cognitive symptoms and autonomic dysfunctions such as antidepressants. A detailed list of available medication prescribed for treating the motor symptoms of Parkinson's disease can be found in *table 1*.

Medications to treat motor symptoms of PD

Category	Generic	Brand Name
Drugs that increase brain levels of dopamine	Levodopa/carbidopa	Parcopa, Sinemet
Drugs that mimic dopamine (dopamine agonists)	Apomorphine Pramipexole Ropinirole Rotigotine	Apokyn Mirapex Requip Neupro
Drugs that inhibit dopamine breakdown (MAO-B inhibitors)	Rasagiline Selegiline (deprenyl)	Azilect Eldepryl, Zelapar
Drugs that inhibit dopamine breakdown (COMT inhibitors)	Entacapone Tolcapone	Comtan Tasmar
Drugs that decrease the action of acetylcholine (anticholinergics)	Benztropine Ethopropazine Trihexyphenidyl	Cogentin Parsidol Artane

Table 1. Main medications to treat motor symptoms of Parkinson's disease (National Institute of Neurological Disorders and Stroke, 2021).

Surgery is another option available to manage the symptoms of PD. Nowadays, the great improvements in the surgical techniques have led this practice to be considered a good alternative in case other medications are not effective. Initially, brain surgery was aimed to destroy specific groups of neurons which were considered to be responsible for the movement symptoms. There are two main types of this kind of brain surgery: thalamotomy and pallidotomy. Thalamotomy refers to the destruction of part of the thalamus to reduce tremors. Pallidotomy refers to the destruction of part of the globus pallidus to reduce tremors, bradykinesia, and rigidity. The main drawback against these kinds of surgery is that they concern permanent destruction of brain tissue, and they do not treat the non-motor symptoms (National Institute of Neurological Disorders and Stroke, 2021). Therefore, new techniques such as deep brain stimulation (DBS), thermocoagulation, and radiosurgery, have been developed to overcome this major issue (Church, 2021).

Importantly, physical therapy can also play an important role in helping individuals with Parkinson's disease maintain their mobility and independence. Research has shown that long-term physiotherapy is fundamental to manage motor symptoms and reduce the intake of antiparkinsonian medication such as L-dopa. According to the work of Okada et al., (2021), interventions aimed to enhance the aerobic ability, cognitive capacities, balance, mobility, coordination, and physical exercise for more than 6 months can significantly reduce PD symptoms in off medications patients compared to control (Okada et al., 2021).

Finally, there is also growing interest in developing cell-based therapies to replace lost dopamine-producing neurons in the substantia nigra and potentially restore normal motor function. An experiment conducted by Kordower et al., (2008) aimed to transplant fetal cells in the brain of a person with PD which produce proteins. The subject was a 61-year-old woman with PD. The idea was to create a small factory inside the brain which produces dopamine. The fetal cells were taken from brains of children of mothers who decided to have abortion. This actually replaced the dopamine in the patient with PD, and she survived for more than 14 years. When the patient died for a cardiac arrest in 2007, her brain was analyzed and indeed it was found that there were dopaminergic neurons in the substantia nigra, but also Lewy bodies. The implanted cells were just 1 month old (*figure 7*) (Kordower et al., 2008).

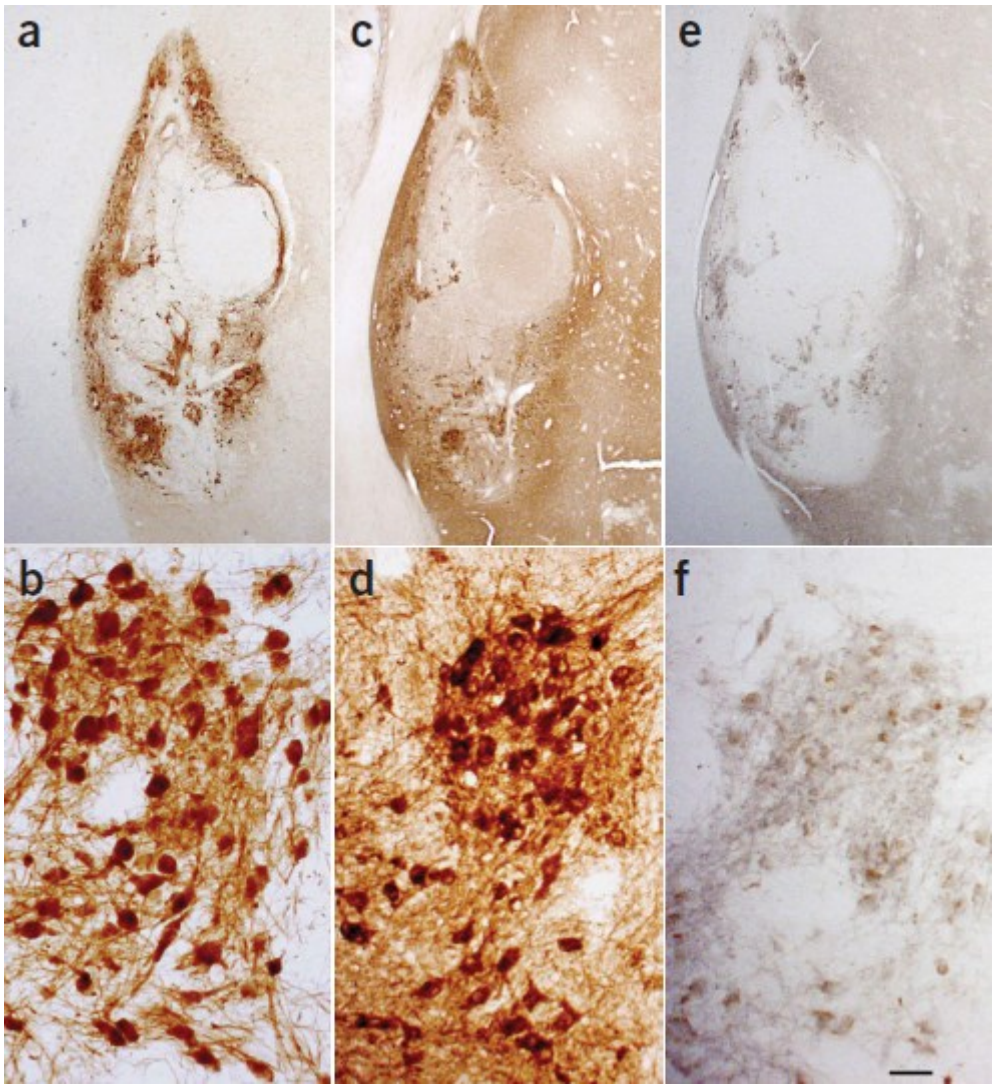


Figure 7. Long-term result of transplantation of embryonic cells in the substantia nigra. The image is made by three image couples (*a-b, c-d, & e-f*) in which different staining have been used for the same brain region. Tyrosine hydroxylase in *a-b*. VMAT2 in *c-d*. DAT in *e-f*. The three couples of images show a repopulation of dopaminergic neurons in the substantia nigra demonstrating the great potential of staminal cells in treating PD (Kordower et al., 2008).

Levodopa or L-dopa

The first-line medication and main prescribed drug to deal with Parkinson's disease is Levodopa. Levodopa, or also called L-dopa, belongs to the group of medication which rise the level of dopamine in the brain, and it can replace temporarily the missing dopamine in the brain and then reducing dramatically the clinical symptoms (Poewe et al., 2010; National Institute of Neurological Disorders and Stroke, 2021; Yang et al., 2022). In addition to improving the motor symptom, the intake of L-dopa also improves the cognitive and emotional frame of the patient who show fewer negative thoughts, less crying and depressive episodes (Marsh, 2013).

The discovery of L-dopa was a breakthrough in the scientific research against PD. Arvid Carlson, the scientist who first was able to demonstrate the extraordinary efficacy of L-dopa against PD, was awarded with the Nobel prize for Medicine in 2000. Arvid Carlson demonstrated that by injecting rabbits with Reserpine, a medication which depletes catecholamine (dopamine is a catecholamine), the rabbits become parkinsonian and unable to move. However, after the same rabbits were given a precursor of dopamine, the L-dopa, they were back to normal function. Arvid Carlson got the Nobel prize for medicine because he demonstrated that symptoms of Parkinson could be triggered by medications depleting dopamine from the brain and that you could replace the dopamine by injecting an amino acid which can be later transformed in dopamine (Carlsson et al., 1957). In fact, the L-dopa is a large neutral aminoacid which crosses the blood brain barrier, and then to be converted to dopamine. Dopamine does not cross the blood brain barrier as L-dopa does (*figure 8*).

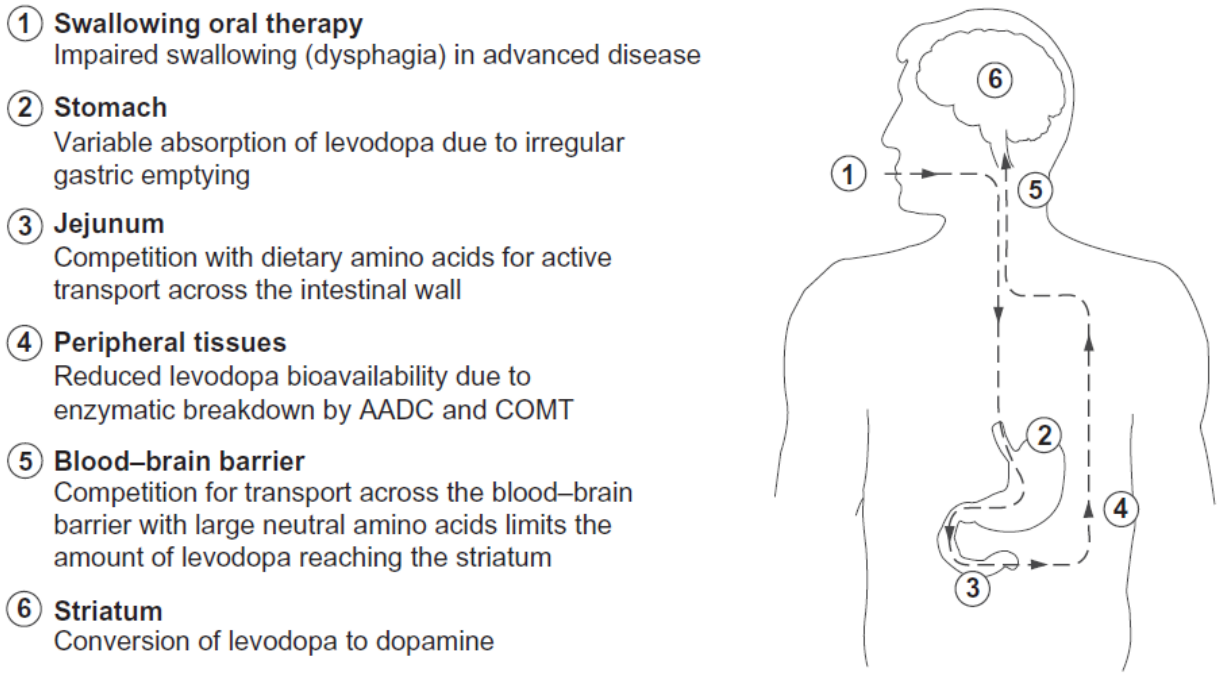


Figure 8. The route of levodopa from the mouth where the patient orally ingested the medication, until the brain in which levodopa is converted in dopamine in the striatum (Poewe et al., 2010).

Nowadays, the administration of L-dopa is supplemented with additional drugs such as carbidopa or DDC and COMT inhibitors. Carbidopa has the ability of allowing the conversion of Levodopa in dopamine only in the brain, increasing the efficacy of L-dopa in treating parkinsonian symptoms (National Institute of Neurological Disorders and Stroke, 2021). A specific technique, which is called Levodopa Carbidopa Intestinal Gel (LCIG), involves the combination of levodopa and carbidopa. LCIG is usually used in advanced PD patients, and it consists in surgical procedure to implant a small tube (called a PEG-J tube) that passes through the abdominal wall and into the small intestine. The tube is used to deliver the LCIG medication directly into the intestine. DDC and COMT are inhibitors of the enzyme which metabolize the L-dopa, in this way there is more availability in the brain.

Usually, the first two years people experience relatively good motility thanks to L-dopa, a period called “honeymoon”. Unfortunately, with time, the compensatory mechanism through the supplement of dopamine is not functioning anymore, and patients also feel the side effects of the medication such as involuntary movements and the cognitive profiles declines. Examples of side effects due to the intake of L-dopa are hallucinations, psychosis, dyskinesia, nausea, and low blood pressure (National Institute of Neurological Disorders and Stroke, 2021).

Even though L-dopa is effective, it doesn’t stop the progression of Parkinson’s disease. People taking levodopa feel better and they don’t have the perception that the disease is progressing, and this led to the idea that L-dopa blocks the development of PD. Today, it is known that it does not work in that way. At some point, after 5-6 years from the PD diagnosis, the patient reaches the disease milestone called development of motor complications. This is the moment in which people cannot be stabilized by medications anymore. At this point, patients can experience more often “off periods” in which the medications seem not working (Aradi & Hauser, 2020; National Institute of Neurological Disorders and Stroke, 2021). For the first year we have the “honeymoon”, and then at some point we have the development of motor complications (Aradi & Hauser, 2020).

Evolution of Parkinson's disease

Parkinson's disease is characterized by a preclinical stage, an early treatable stage, and advanced stage. The preclinical stage goes often unnoticed to doctors and it is characterized by olfactory loss, constipation, anxiety, and depression. The early treatable stage includes the development of the motor symptoms which allow the doctor to make the official diagnosis of PD; rest tremor, bradykinesia, muscular rigidity, and non-motor symptoms. In this stage, medications can alleviate the symptoms. Finally, in the advanced stage, medications are less effective and there are motor complications, gait and balance, cognitive decline which lead to dementia, autonomic dysfunctions. The three stages just described, can be associated with the progressive spreading of Lewy bodies from the gastrointestinal system to the brain of PD patients, a process which was described meticulously by Braak, and today widely known as Braak's staging (Braak et al., 2006; Braak & Del Tredici, 2007; Rietdijk et al., 2017).

Heiko Braak is a German medical doctor who is specialized in neuroanatomy and, before his retirement, was a professor of clinical neuroanatomy at the university of Johann Wolfgang Goethe University, Frankfurt/Main, Germany (Braak & Del Tredici, 2007; The Michael J. Fox foundation, 2022). The theory of Braak and the role of the enteric nervous system in the gastrointestinal tract in the pathology of Parkinson's disease represent the main pivotal points on which this research project is built, and for this reason they will be described and analyzed in detail.

Enteric Nervous System

Gastrointestinal (GI) system

Before discussing the Braak's hypothesis, the enteric Nervous System (ENS), and their implications in PD, a general overview is necessary to introduce in which context the ENS is situated, and it operates. The ENS is embedded in the gastrointestinal tract which is a complex system of organs and structures that work together to convert food into energy and nutrients that can be absorbed and utilized by the body (*figure 9*).

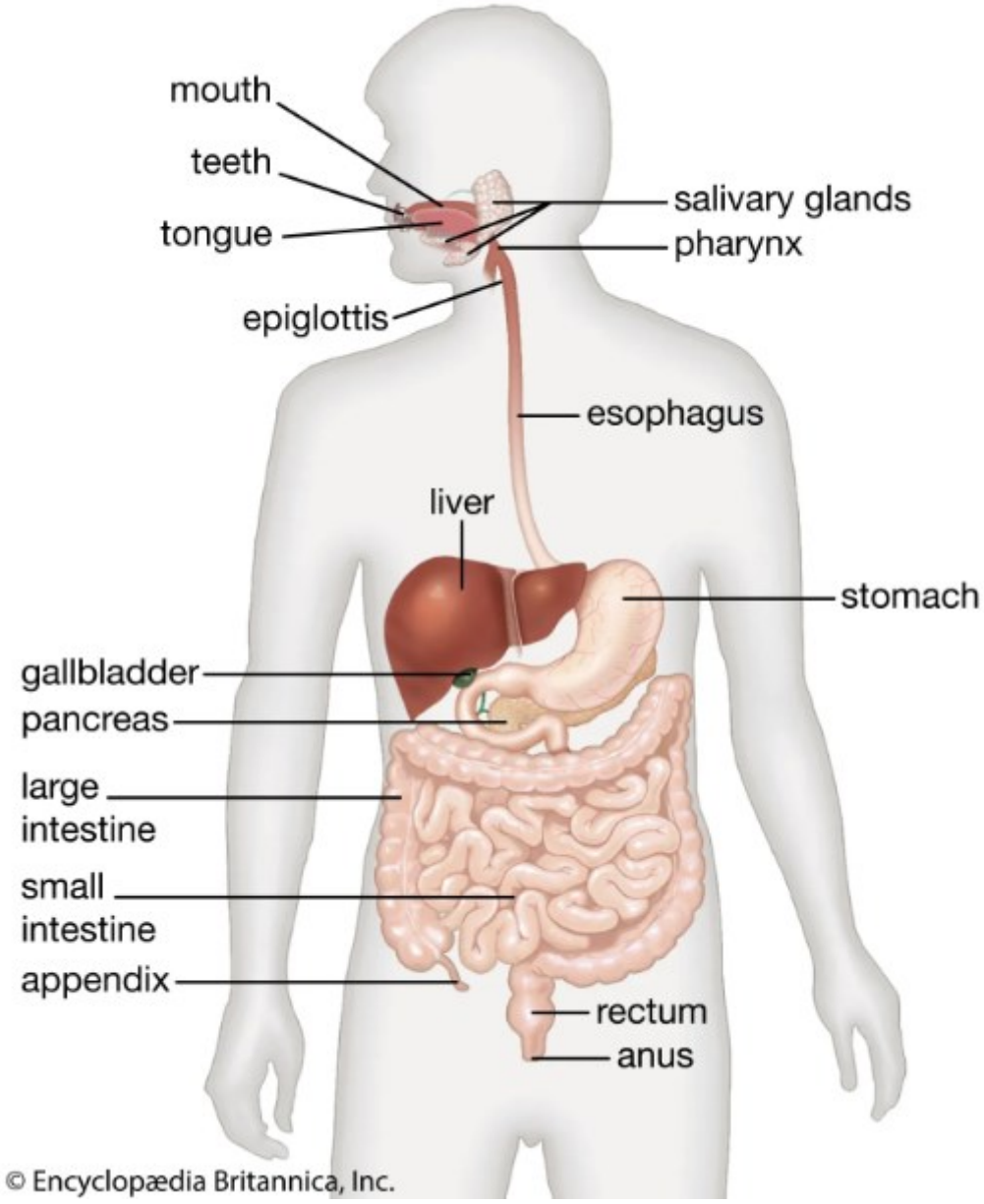


Figure 9. Visual representation of the gastrointestinal (GI) tract (Encyclopedia Britannica, 2022).

The gastrointestinal tract is made up of the mouth, esophagus, stomach, small intestine, large intestine, rectum, and anus. The process of digestion begins in the mouth, where food is mechanically broken down by chewing and mixed with saliva to form a bolus. The bolus is then transported down the esophagus to the stomach through the process of peristalsis, which is the rhythmic contraction of smooth muscle in the wall of the esophagus. In the stomach, the bolus is mixed with gastric juices, which contain digestive enzymes and acid, to form a homogenized mixture called chyme. The term "chyme" comes from the Greek word "chymos," which means "juice." The chyme is then slowly released into the small intestine, where it is mixed with bile and pancreatic juice to aid in the digestion of carbohydrates, proteins, and fats. The small intestine is lined with tiny finger-like projections called villi, which increase the surface area for the absorption of nutrients. The remaining chyme is then transported to the large intestine, where most of the remaining water and electrolytes are absorbed. The large intestine is also home to a diverse population of bacteria, which play a role in the fermentation of undigested carbohydrates and the production of short-chain fatty acids. Finally, the feces are eliminated from the body through the rectum and anus (National institute of Diabetes and Digestive and Kidney Diseases, 2022).

A fundamental component of the gastrointestinal system which has been shown to have fundamental implications in different pathology such as Parkinson's disease, Alzheimer's disease, autism, and multiple sclerosis is the intestinal barrier (Pellegrini et al., 2023). The intestinal barrier is made by multiple factors such as epithelial layer, immune system, and mucus which create a dynamic environment that is able to react to different stimuli. Starting from the lumen and moving to the gut:

- Lumen: the cavity in which there is the transition of food, liquids, and digestive enzymes such as bile and gastric acid which are essential for the degradation and absorption of nutrients. The lumen present in the GI is one of the largest in the human body.
- Mucus layer: a physical barrier composed of water, mucus, microbiota.
- Epithelial cells: specialized cells which have different functions such as the transport of luminal content, responding to harmful inputs by releasing specific substances, and they form an internal barrier to prevent noxious elements from entering the gut. Specifically, a protein called Tight Junction (TJ) connects the epithelium cells forming the barrier. Vancamelbeke & Vermeire (2017) consider the epithelial cells the most important determinant of the intestinal barrier.
- The lamina propria: a layer of immune cells such as cytokines, chemokines, T cells, B cells, dendritic cells, and macrophages (Camilleri, 2019; Vancamelbeke & Vermeire, 2017).
- The wall of the gut: it is composed of different layers strictly connected to each other such as the Muscularis Mucosae, longitudinal muscle, circular muscles, and submucosa. Here, it is where the ENS lies (Furness et al., 2014) (*figure 10*).

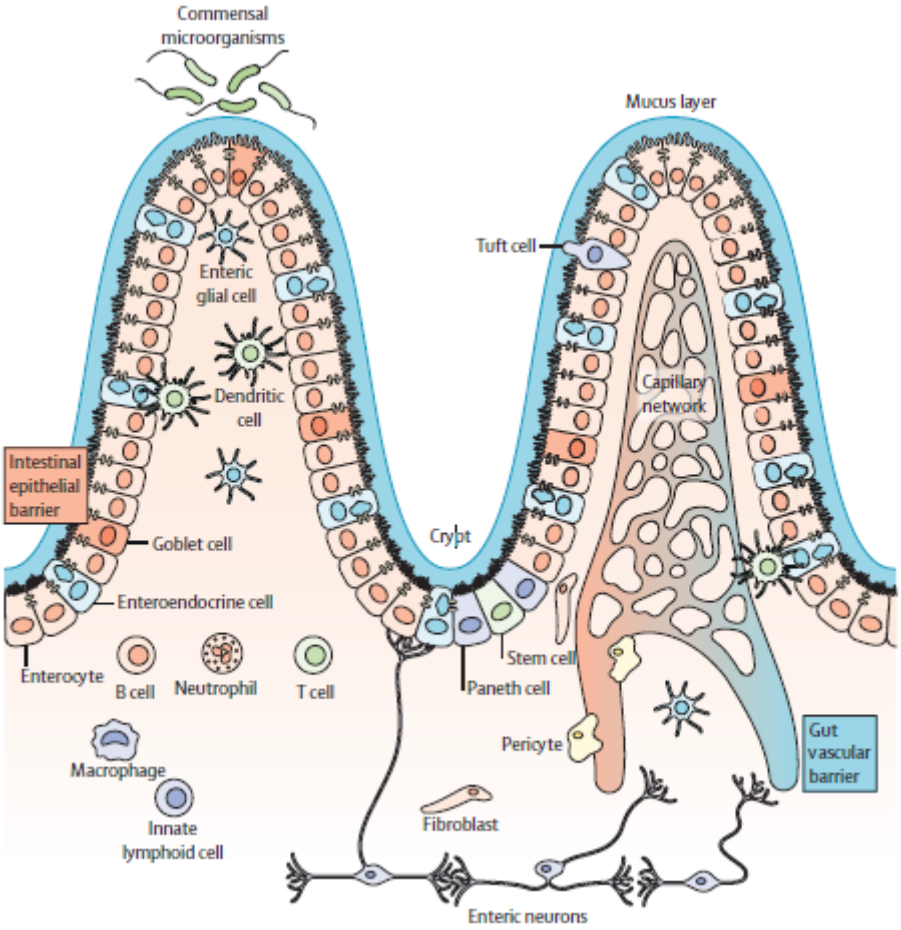


Figure 10. A representation of the complexity of the intestinal barrier. The image refers to a small section of the intestinal barrier which includes two protrusions (villi), the invagination between them (crypt), and their main components. At the top, there are in green the commensal microorganism which refers to the microbiota. Additionally, there are some specific components not described above such as the goblet cells, enteroendocrine cells, enterocytes, Paneth cells, and tuft cells. Goblet cells secrete mucus, while enteroendocrine cells secrete hormones. Enterocytes are involved in the secretion and absorption of different substances such as peptides. Paneth cells secrete substances to ensure homeostasis and protect the neighboring stem cells. Tuft cells behave as a vessel between the CNS and the gut (Pellegrini et al., 2023).

The Enteric Nervous System (ENS)

In order to perform all the sophisticated activities related to the digestion and absorption of nutrients, the gastrointestinal tract is provided with a proper kind of nervous system, known as enteric nervous system (ENS). The ENS is a complex network of neurons and glial cells that is embedded in the wall of the gastrointestinal tract, including the esophagus, stomach, small intestine, large intestine, and rectum. The enteric nervous system includes a great quantity of neurons. It is estimated that the number of neurons present in the ENS vary between 200 and 600 million which are organized in different small groups of neurons (Furness et al., 2014). According to Purves et al. (2001), there are more neurons in the gut than in the spinal cord. A big chunk of the ENS neurons is found within the gut wall (Natale et al., 2021). The majority of these neurons are organized in two different plexus: myenteric and submucosal plexus (Furness et al., 2014) (*figure 11*).

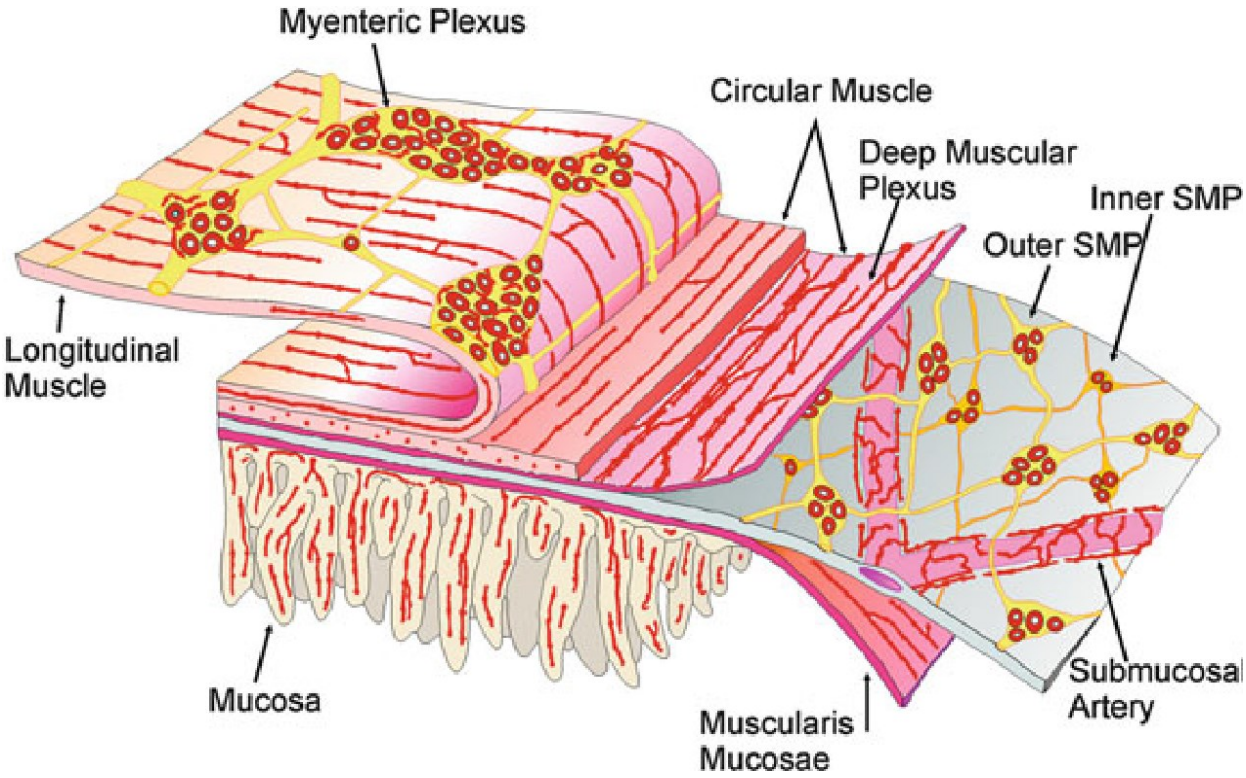


Figure 11. The structure of the ENS. The picture shows a representation of the human small intestine. The main two plexuses of neurons are represented: the myenteric and submucosal (SMP). The SMP is made of two elements: the inner SMP and the outer SMP. The fibers coming from the plexuses innervate the muscularis mucosae, longitudinal and circular muscles (Furness et al., 2014).

In addition to the elevated presence of neurons, the gastrointestinal system is characterized by heterogeneous neuronal phenotypes. In fact, 20 functional classes of neurons have been identified (Natale et al., 2021). The main types of neurons which are found in the wall of the gut are sensory, local, and motor. The sensory neurons are responsible for controlling the status of the gut. The local circuit neurons integrate the information from the sensory neurons. The motor neurons have a primary role in controlling the smooth muscles in the gut and the secretions of enzymes such as bile and gastric acid which are essential for the digestion and absorption of nutrients (Purves et al., 2001). Additionally, the ENS neurons can be divided into other two subgroups. The first way is the ganglionated plexus in which there are clusters of neurons. The second way is the non-ganglionated plexus in which there are fibers made by axons and glial cells (Natale et al., 2021).

Even the way in which the neurons in the ENS communicate to each other is highly heterogeneous. More than 50 distinct neurotransmitters have been identified such as acetylcholine, dopamine, noradrenaline, serotonin, and glutamate. What is surprising is that dopamine is the only catecholaminergic neurotransmitter present in the ENS, and this could particularly be relevant in understanding pathologies which involve the progressive loss of this neurotransmitter such as Parkinson's disease (Natale et al., 2021).

Neurons are not the only kind of cells present in the ENS. Glial cells are also present and of considerable importance (*figure 11*). The population of glial cells in the ENS is different

from other peripheral cells and they resemble astrocytes in the CNS. Indeed, dysfunction of glial cells are associated with gut pathologies (Natale et al., 2021).

The ENS can function independently of the central nervous system (a characteristic which makes the ENS unique between the other peripheral organs) and has the ability to control digestive functions, including motility, secretion, and blood flow (Natale et al., 2021; Furness et al., 2014). This is achieved through the dense network of neurons and their ability to communicate with each other through a variety of neurotransmitters and signaling molecules.

The autonomous route is not the only way in which the ENS can function. The ENS can also receive direct inputs from the CNS, allowing for the coordination of digestive functions with other physiological processes (Natale et al., 2021). The three main kinds of pathways which put in communication the gut with the brain are the vagal, spinal thoracolumbar, and spinal lumbosacral. Each of these ways of communication possess afferent sensory information and efferent motor information (Furness et al., 2014). A fundamental role in this bidirectional communication is performed by the hypothalamus, pituitary gland, and adrenal gland. Nowadays, this bidirectional interaction is known as gut-brain axis. All of this creates a wide, integrated, and well-organized distinct neural network which has resulted in the attribution of the name “second brain” or also “neurological brain” (Natale et al., 2021).

Microbiome

Recently, another important finding has been made regarding the GI system and ENS. The presence of the wide and heterogenous intestinal microbiota have been demonstrated to be actively involved in intriguing interactions among hormonal, epithelial, hormonal, and immune responses (*figure 11*). The term microbiome refers to bacteria, microbes, and viruses that reside in the human body and have an important impact in health and in disease.

The microbiome is actively involved in the functioning of the human body performing functions such as aiding metabolic activities, defense against pathogens, and immune system regulation. For example, one of the main activities of the microbiome is related to the digestion of food. Microbiomes are able to digest some kind of food that our body alone would not be able to digest, and it can also extract nutrients from them. This is the example of the xyloglucans which are found in vegetables. A specific kind of microbiome, known as Bacteroides, are made of ad-hoc traits which are able to digest xyloglucans which would otherwise not be digested by our body. This demonstrates the mutually beneficial relationships between the human body and gut microbiota. Another important function performed by the microbiota is to affect the production of mucus at the level of the mucus layer. In fact, there are microbiota which can stimulate the release of mucus (Yang et al., 2022).

The composition of microbiomes is different between individuals, but it is estimated to be made up by around 1000 different species of bacteria, with the Bacteroidetes and Firmicutes being the dominant ones (Shreiner et al., 2015). Specifically related to the human adult gut, it was shown that the gastrointestinal system alone contains more than 1 kg of bacteria which is around the same weight of the brain. Additionally, the number of bacteria in the gut exceeds the total number of cells in the human body and they contain more than 100 times as many genes as in the human genome. Surprisingly, the complexity of the gut-microbiome overcomes the complexity of the human brain (Dinan & Cryan, 2017).

Overall, the technological advancement and multiple efforts of scientists are beginning to elucidate the mechanism in which the host and the microbiome influence each other. These findings could eventually lead to new opportunities in dealing with different human pathologies (Shreiner et al., 2015).

Microbiome-gut-brain axis

Nowadays, the complex system of interactions between the microbiome, gut and brain is called the microbiome-gut-brain axis. The way in which the bidirectional communication between these three entities takes place is not entirely clear yet, but involves the immune system, the endocrine pathways, neural networks, and metabolic activities. The vagus nerve remains the main pathway of multiple bidirectional interaction in the microbiome-gut-brain axis. An interesting fact about the gut microbiota is the ability of regulating and releasing neurotransmitters which are commonly found in the CNS. For example, specific groups of microbiomes influence the levels of serotonin precursor which will eventually transform in serotonin in the brain. Other gut microbiome populations can directly release GABA, noradrenaline, serotonin, dopamine, and acetylcholine (Dinan & Cryan et al., 2017) (*figure 12*).

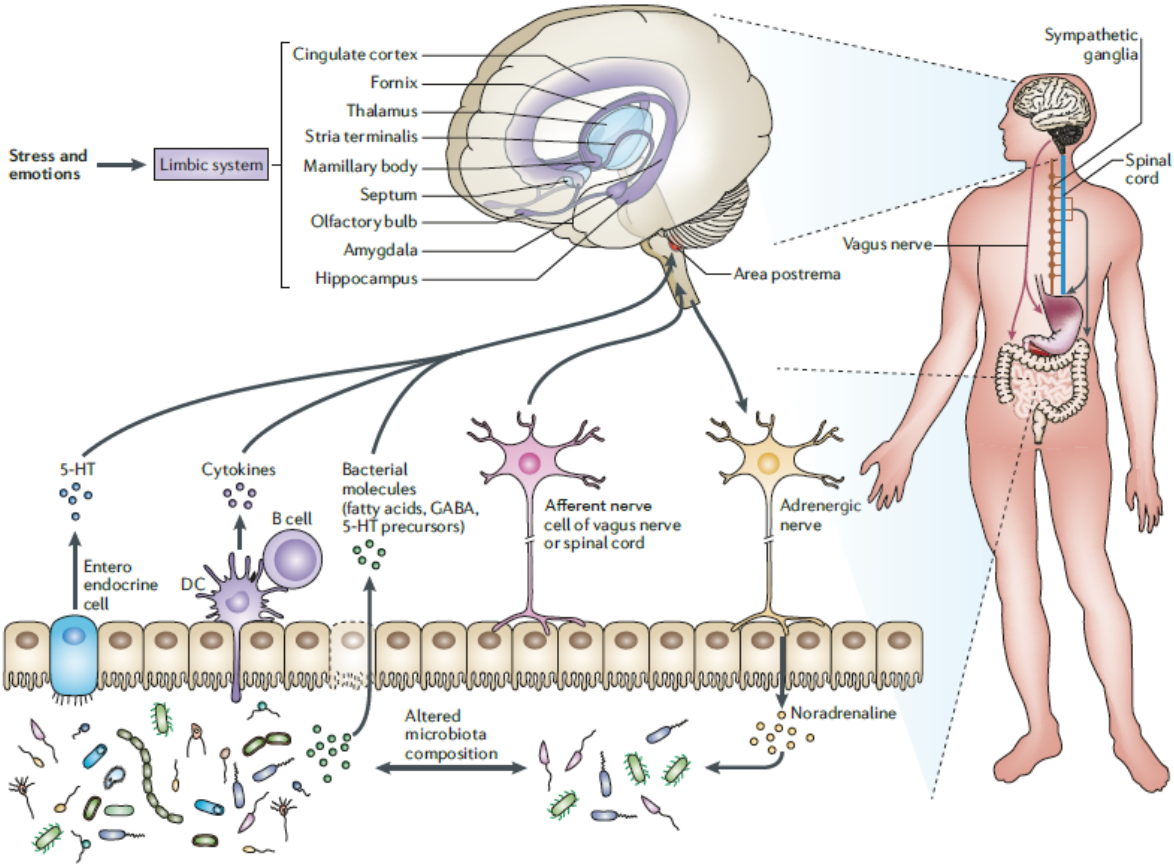


Figure 12. The microbiota-gut-brain axis. The image depicts different key players in the bidirectional communication between microbiota, gut, and brain. The way in which the multiple interactions take place are through the immune system, endocrine system, neural networks, metabolic pathways (Collins et al., 2012).

The gut microbiota and its relationship with the CNS appears to be implicated in several pathologies such as Parkinson's disease. Feces of people with PD were analyzed and it was found that the presence of a specific kind of microbiota, called Prevotellace, was reduced by around 80% compared with healthy subjects. Additionally, it was found an extensive presence of another species of microbiota, called Enterobacteriaceae which was correlated with motor disorders. These discoveries highlight the importance of the microbiota when considering pathologies such as Parkinson's disease (Dinan & Cryan, 2017).

Additionally, alteration in the microbiome can have an important impact on the intestinal permeability (Yang et al., 2022). The concept of intestinal permeability and its implications on PD will be widely described later on.

Braak staging - Enteric nervous system & Parkinson's disease

Heiko Braak's theory

The motor symptoms which characterize Parkinson's disease are most often anticipated by autonomic system dysfunctions, targeting areas such as the gastrointestinal system. The common clinical manifestation that an impairment in the gastrointestinal system can lead are hypersalivation, pharyngeal and esophageal dysphagia, gastroparesis, constipation, small intestine, anorectal dysfunction, and colon dysmotility (Rietdijk et al., 2017). Other autonomic dysfunctions related to the gastrointestinal system are weight loss, reflux, and loss of appetite (Dinan & Cryan, 2017). Constipation remains the most recognized symptom of PD linked to the gastrointestinal system because it significantly impairs the quality of life of people. Therefore, a deeper knowledge of the enteric nervous system and gastrointestinal system are essential in order to shed some light about the pathogenesis of Parkinson's disease.

Heiko Braak is one of the pioneers of the gut-brain hypothesis in the pathogenesis of PD. Braak was able to analyze a numerous quantities of brains of people who had Parkinson's disease and, based on an extensive analysis aimed to individuate the presence of Lewy bodies, he created a hypothesis about the development of PD. Following Braak's hypothesis, sporadic PD is caused by pathogens which from the environment enter the body, reach the gut, and initiate the Lewy body pathology (Braak et al., 2006; Rietdijk et al., 2017). At the level of the gastrointestinal system, the pathogens enter the mucosa, and trigger the initial misfolding and aggregation of αSyn in the ENS neurons (Braak et al., 2006; Natale et al., 2021) (*figure 13*).

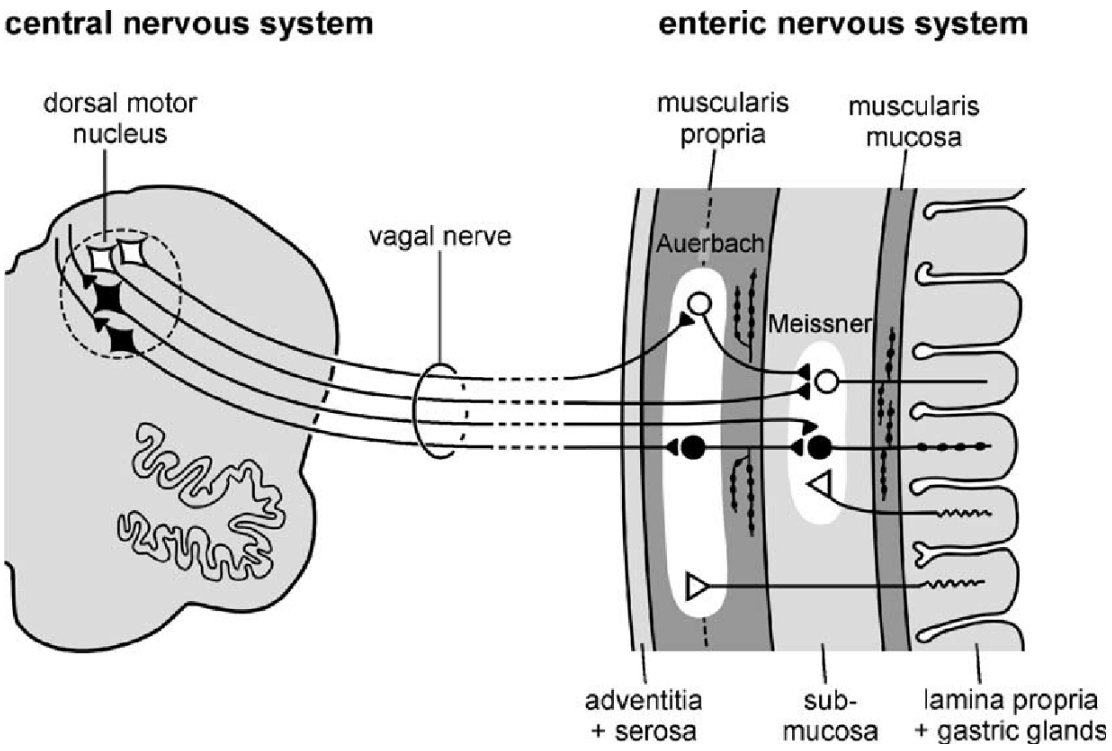


Figure 13. Schematic representation of Braak's hypothesis regarding the pathogenesis and development of PD. A pathogenic agent is able to cross the intestinal barrier affecting the neurons in the submucosal Meissner plexus also known as submucosal plexus, which in turn affects the Auerbach plexus also known as myenteric plexus. From there and through the vagus nerve, the pathology reaches the CNS (Braak et al., 2006).

Braak also postulated a staging system which describes the spreading of the Lewy bodies from the ENS to the CNS (Braak et al., 2006; Rietdijk et al., 2017). According to Braak staging, the pathology will begin to spread by climbing the vagus nerve, reaching the dorsal motor nucleus of the vagus nerve (DMNV) in the medulla oblongata, and from there, gradually to all CNS (Braak et al., 2006; Natale et al., 2021; Rietdijk et al., 2017). Specifically, Braak described six stages which correspond to incrementally different levels of Lewy bodies spreading in the CNS:

- Stages 1-2: they correspond to the preclinical stage of PD in which the patient shows symptoms such as sleep behavioral disorder characterized by agitated dreams, hyposmia, constipation, depression, pain, anhedonia, and apathy. Here, the brain regions affected by the Lewy pathology

are: enteric plexus of the gastrointestinal tract, medulla oblongata, olfactory bulb, pontine tegmentum, locus coeruleus, and sympathetic nerve fiber in the heart.

- Stages 3-4: they correspond to the clinical stage of PD in which the patient has the core symptoms of PD such as rest tremor, bradykinesia, muscular rigidity, and mild cognitive impairment (MCI). Here, the brain regions affected by the Lewy pathology are: substantia nigra, basal forebrain, medial temporal cortex, and amygdala.
- Stages 5-6: they correspond to the advanced stage of PD in which the patient shows symptoms such as postural instability, fall, dyskinesia, dysphagia, hallucinations, and delusions. Here, the brain regions affected by the Lewy pathology are: higher order association cortices (temporal and frontal), and primary cortices (Braak & Del Tredici, 2017).

Braak's hypothesis has been confirmed by clinical evidence, in vitro and in vivo research (*figure 14*).

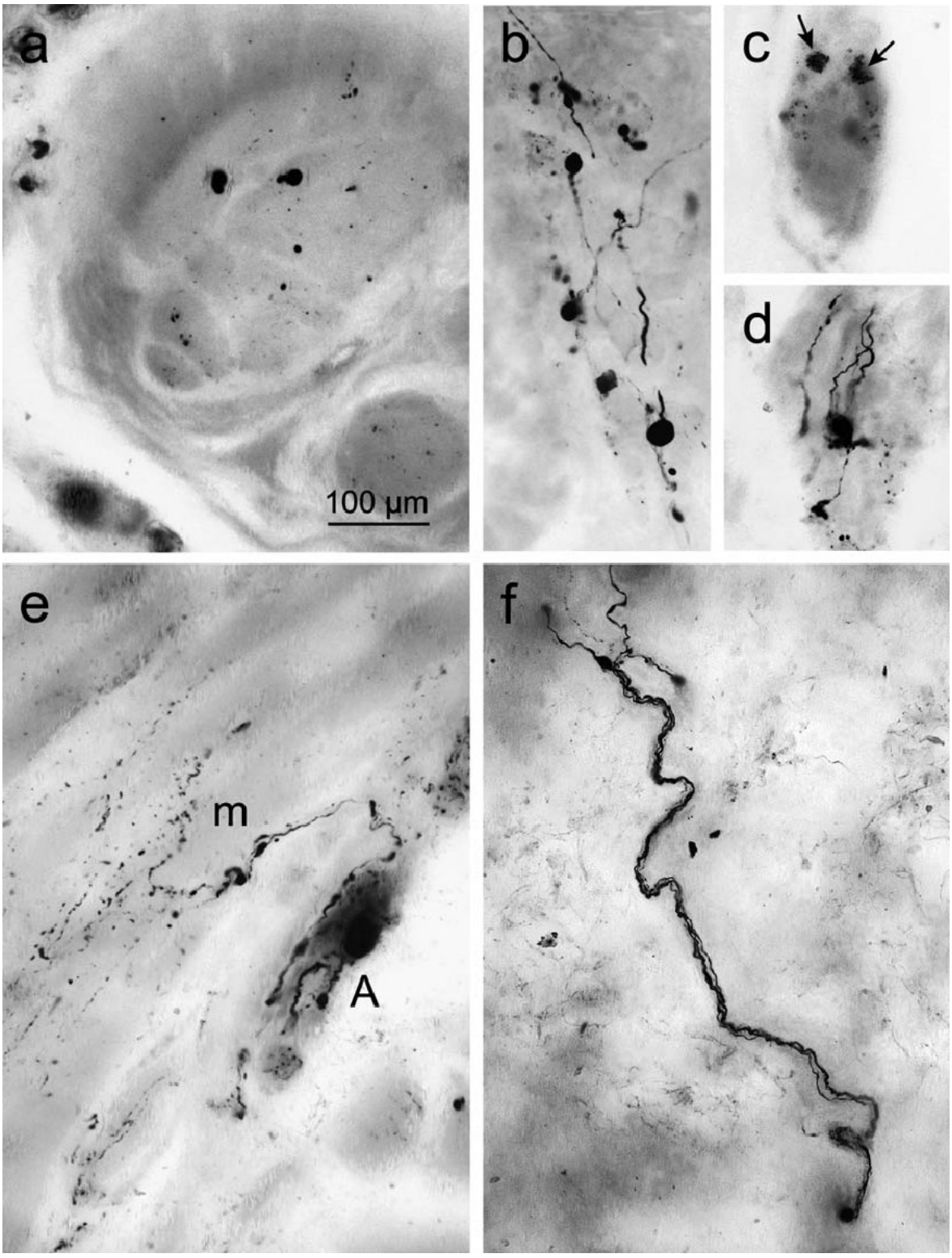


Figure 14. Abnormal αSyn aggregation in the ENS. The images are representation of the submucosal and myenteric plexuses in which the misfolding of αSyn is stained in black. (*e*) Smooth muscle cells (*m*) enter in contact with the αSyn stained fibers generated by the Myenteric plexus (*A*) (Braak et al., 2006).

From a clinical perspective, research has shown as the presence of Lewy bodies in the gastrointestinal tract anticipates the diagnosis of PD. The same clinical evidence is supported by the utilization of animal models (Rietdijk et al., 2017). According to Dinan & Cryan (2017), in the initial phase of PD, Lewy bodies are present at the level of the submucosal and myenteric plexuses of the ENS before the brain is affected by the pathology (Dinan & Cryon, 2017). Additionally, Yang et al. (2022) agree on the idea that the gastrointestinal system is the initial site of the pathogenesis of PD which then spreads in the brain. Other evidence comes from the fact that Lewy pathology and consequent neuronal loss have been individuated in the vagus nerve and DMNV in patients with PD before the disease actually reached the CNS. This evidence is confirmed also by using animal models (Rietdijk et al., 2017).

Parkinson's disease can be classified in two distinct subgroups. The first classification refers to a top-down brain first type, in which the trigger for the initial accumulation of the αSyn is in the CNS and subsequently spread in the peripheral nervous system including the enteric nervous system. The second classification refers to a bottom-up body first type, in which the αSyn begins to accumulate in the ENS and subsequently spread in the CNS (Natale et al., 2021) (*Figure 15*).

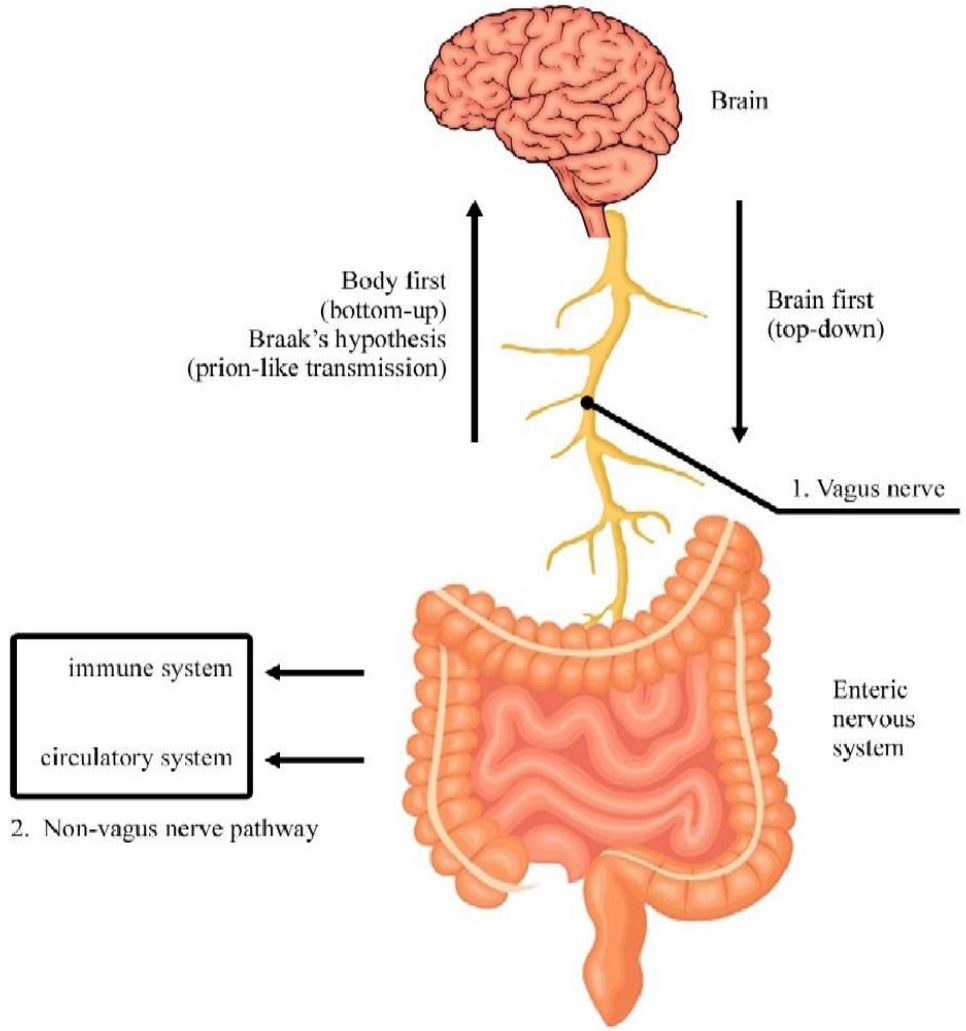


Figure 15. The two kinds of pathogenic hypothesis underlying Parkinson's disease (bottom-up and top-down) which are characterized by the bidirectional communication between CNS and ENS. There are two ways in which this bidirectional communication takes place: vagus nerve, or non vagus nerve pathway through the immune system and circulatory system (Natale et al., 2021).

Not only is there a bidirectional link which puts in direct communication the ENS and the CNS, but they can both undergo very similar stages of neurodegeneration. For example, it has been shown that both systems can share a selective loss of dopaminergic neurons, αSyn accumulation, and Lewy bodies (Natale et al., 2021).

Criticism to Braak's hypothesis

Despite the large quantities of evidence above describing the validity of Braak's hypothesis, there is still uncertainty whether it correctly explains the pathogenesis of PD in all patients. Even though a big chunk of PD patients follows the Braak staging, there is also a smaller subset of patients who do not follow the staging. In fact, they do not have Lewy bodies In the DMNV even though they have PD.

Additionally, there are still debates related to if the Lewy bodies have a causative role in PD or not. In fact, the presence of widespread Lewy body pathologies is documented in healthy individuals. This demonstrates that the development of PD can be not strictly linked to the Braak staging hypothesis.

Another criticism refers to the experimental model used by Braak to find his results. In fact, there is a suspicion of selection bias where Braak systematically excluded cases which didn't have Lewy bodies at the level of the DMNV. Moreover, Braak's hypothesis does not explain how the Lewy bodies could be directly implicated in neural loss which leads to the symptomatology of PD (Rietdijk et al., 2017).

The intestinal permeability

The intestinal barrier is a complex system made by different layers which interact with the food in the lumen to extract nutrients. The GI tract is characterized by a semipermeable barrier which decides what should enter or what should not enter. The protein which directly affects the permeability of the intestinal barrier is the Tight Junctions (TJ), which is found at the level of the epithelial layer, and it forms a sort of bond between epithelial cells which does not allow pathogens to enter the barrier passing through the space between epithelial cells (Yang et al., 2022). Intestinal permeability can be measured by a simple urine test which detects how, a previous ingested probe, is filtered by the human body (Camilleri, 2019).

The permeability of the intestinal barrier assumes crucial importance because it could trigger PD (Yang et al., 2022) and other pathologies such as multiple sclerosis, autism, asthma, depression, and obesity (Camilleri, 2019). The first person to hypothesize an involvement of the intestinal permeability in PD pathogenesis was Braak in his famous theory, mentioned in the previous paragraph (Yang et al., 2022). For this reason, the intestinal permeability can be considered an extension of the Braak's hypothesis. According to Emmi et al., (2023), Yang et al. (2022), & Braak's theory, dysfunctions at the level of the intestinal permeability could create the precondition in which there would be the first misfolding of αSyn in the ENS. Indeed, dysfunctions that target the TJ, such as the lower expression of transmembrane proteins which make up the protein such as occluding, cause problems of intestinal permeability (*figure 16*).

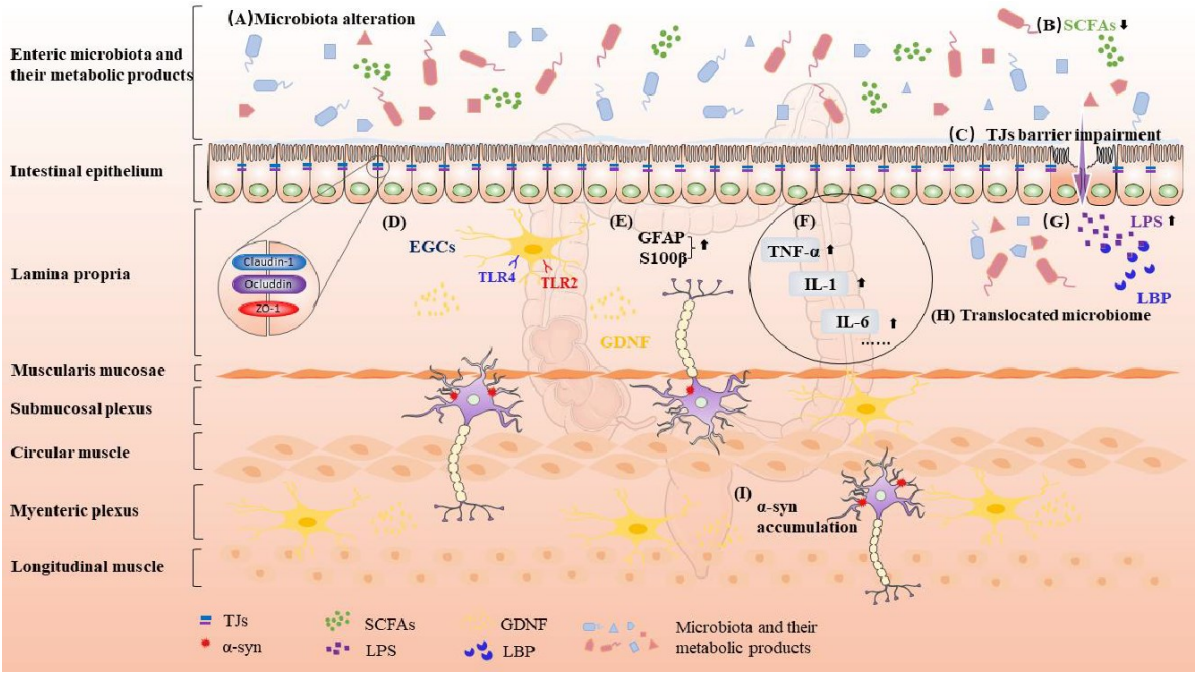


Figure 16. Factors which could determine dysfunction in the intestinal permeability which can eventually degenerate in the first Lewy bodies found in the ENS. (*A*) Microbiota dysfunctions can alter the intestinal permeability status. (*B*) According to Rietdijk et al., 2017, lower levels of short-chain fatty acids (SCFA) are associated with pro-inflammatory effects. (*C*) Dysfunctions at the level of the TJ increase intestinal permeability. (*D-E*) Enteric Glial cells abnormal activation and overexpression of glial markers. (*F*) Release of inflammatory

cytokines. (*G*) increased level of lipopolysaccharide which have been demonstrated to be implicated in αSyn misfolding. (*H*) bacteria and LPS pass the intestinal barrier. (*I*) αSyn begins to accumulate (Yang et al., 2022).

The intestinal permeability hypothesis as a possible explanation of the initial stage of PD is intriguing but there are also drawbacks which need to be considered. First, intestinal permeability is affected by a great number of different elements making it difficult to draw causal conclusions. For example, two factors which are usually not considered in research, but they influence intestinal permeability are sleep and stress (Camilleri, 2019). Second, some research which investigated the relation between PD patients and intestinal permeability led to inconclusive results (Yang et al., 2022).

Inflammatory response in the ENS and its implication in PD

ENS & CNS communicate through the immune system

The immune system can be considered a channel who puts in communication the gut and the CNS. This pathway of communication takes place through the release of cytokine molecules at the level of the gut which travel through blood vessels reaching the brain. Nevertheless, the brain is provided with the Blood Brain Barrier (BBB) which, in normal situations, does not allow cytokines from the peripheral nervous system to directly affect the brain. However, the BBB does not have the same consistency in all parts of the brain, and some regions could be more exposed to cytokines coming from the peripheral system. This is the example of the hypothalamus in which cytokines such as the interleukin (IL)-1 and IL-6 can trigger the hypothalamic-pituitary-adrenal axis (HPA), leading to the production of cortisol. This mechanism represents the most powerful triggering of the stress response. Therefore,

dysregulation at the HPA axis can cause complications to the microbiome-gut-brain axis or vice versa, which eventually will lead to pathological conditions (Dinan & Cryan, 2017).

Inflammation – a key player in PD

Inflammation is a hallmark of Parkinson's disease. Inflammatory response in the ENS is implicated in the pathogenesis of Parkinson's disease, but the exact role in which the immune system participates in the development and spreading of the pathology in the CNS is not entirely understood yet.

The first Lewy bodies are found in the enteric nervous system. At the level of the stomach, there is significant pathology which contributes to trigger an inflammatory environment that later leads to the development of PD (Emmi et al., 2023; Mulak & Bonaz, 2015). The reason which explains how the chronic inflammation can lead to the misfolding of αSyn is unknown. A possible explanation could be that fragments of αSyn can trigger the chronic inflammation. In turn, the chronic inflammation would target the αSyn protein which is commonly found in the membrane of neurons causing damage to them. This will aggravate the pathology. Therefore, inflammation is a very important factor.

Inflammation can impair the barrier integrity and result in leaky gut. Many different diseases have been associated with dysfunction in the intestinal permeability such as Parkinson's disease (Camilleri, 2019). PD is characterized by intestinal inflammation which is correlated with an increased permeability of the intestinal barrier and higher presence of oxidative stress and αSyn at the level of the mucosa (Rietdijk et al., 2017). At the level of the gastrointestinal system, the intestinal epithelium forms a controlled barrier between the bloodstream and the gut contents. Tight junctions (TJs), also known as zona occludens, play a crucial role in maintaining the integrity of the intestinal barrier, and a malfunction of TJs can lead to increased permeability in the intestinal epithelium (*figure 16*). Many different pro-

inflammatory cytokines which are released by the immune cells present in the ENS have an effect on the TJ to increase barrier permeability (Yang et al., 2022). Therefore, it could be plausible that an increased inflammation could lead to an increment of the oxidative stress which will end up in the initial misfolding of αSyn, creating the preconditions for the development of Parkinson's disease (Rietdijk et al., 2017) (*figure 16-17*).

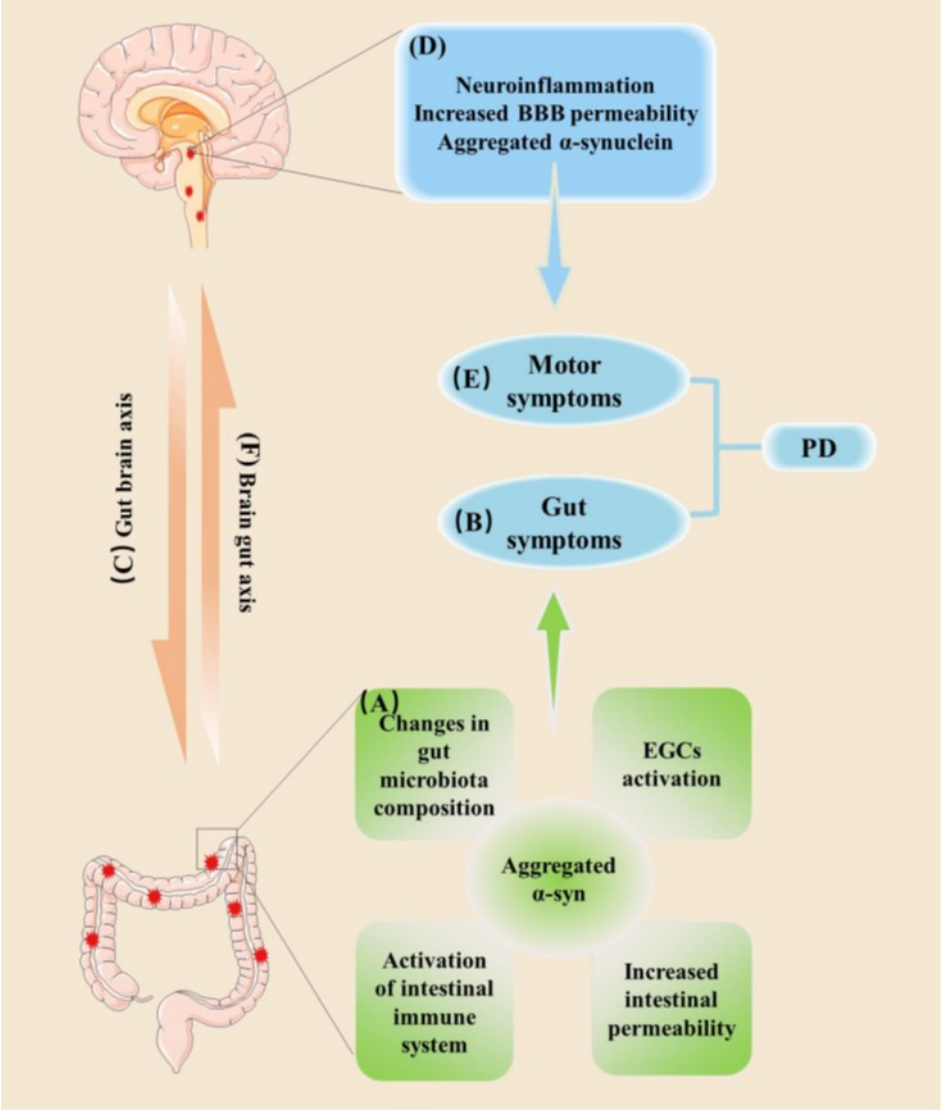


Figure 17. The "pathological loop" which promotes a situation of chronic inflammation which in turn leads to the pathogenesis of PD. (*A*) Elements which are responsible for the trigger of a pro-inflammatory environment which will end up in the first misfolding of αSyn in the ENS. (*B*) The consequence of *A* is gut symptoms. (*C*) The misfolded αSyn and the consequent chronic inflammation spreads to the vagal nerve reaching the CNS. (*D*) At the level of the CNS, the

chronic inflammation and αSyn promote neuroinflammation and neurodegeneration. (*E*) The consequence of D is motor symptoms (Yang et al., 2022).

Microbiota-immune system-PD interactions

The gut-microbiome plays an important role in gut inflammation which could directly impact PD. The relations between the immune system and microbiome are numerous, difficult, and bidirectional. The immune system needs to learn to recognize and tolerate the host-microbiome, while the host-microbiome needs "to educate" the immune system; this leads to complex interactions between the two. This microbiome-immune system interaction is gaining great attention from scientific research because it could be a key factor in elucidating the pathogenesis of inflammatory disorders (Shreiner et al., 2015).

The food that people ingest have a direct impact on the formation of gut-microbiome. In turn, specific gut-microbiome can release short-chain fatty acids (SCFA) which have anti-inflammatory effects and are associated with a general well-being of the human gut. This is an example of a diet rich in fibers. On the contrary, diets rich in refined carbohydrates and saturated fat can result in a pro-inflammatory environment which could eventually lead to the pathogenesis of PD (Rietdijk et al., 2017). Additionally, lower levels of two kinds of microbiota called Faecalibacterium Prausnitzii and Prevotellace in the GI system are correlated to an increase in inflammation. On the other hand, an abundance of Faecalibacterium Prausnitzii is correlated with anti-inflammatory effects (Yang et al., 2022).

Enteric Glial cells (EGC) in Parkinson's disease

The EGCs represent a distinctive type of peripheral glial which are found at the level of the intestinal barrier and ENS (Yang et al., 2022). The EGCs could be a key player in understanding the pathogenesis of PD. In fact, dysfunctions in the EGCs can lead to intestinal inflammation which leads to PD.

At the level of the ENS, the number of EGC appears to exceed the number of neurons. It seems like that each neuron of the ENS is equipped with a glia cell, which has a variety of fundamental functions in helping neurons such as protection, trophic role, supportive activity, facilitate neurotransmission, and they can represent a bridge between the neural network and the immune system (Natale et al., 2021). Traditionally, it was believed that the EGCs participated only in supervising and aiding neurons by providing nutrients. Nevertheless, recent evidence has shown that EGCs are characterized by more complex patterns of behaviors including important implications in the immune response. In fact, EGCs are characterized by the ability to modulate the immunological response and glial-derived factors which influence the intestinal barrier permeability. This evidence is shown also in mouse models, where the removal of the EGCs end up in an alteration of the intestinal barrier permeability. The release of glial-derived factors can have a direct influence on the inflammatory cytokine production or repair after the inflammatory damage (Yang et al., 2022) (*figure 18*).

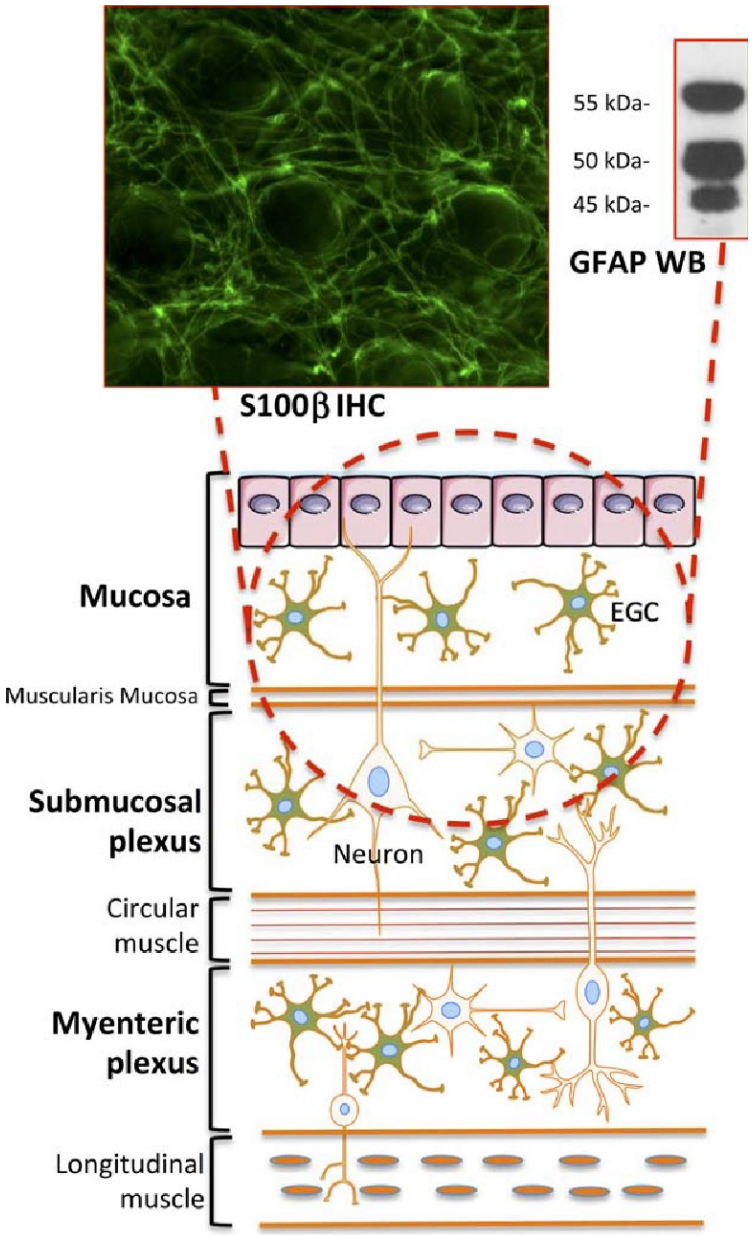


Figure 18. EGCs at the level of the ENS. The EGCs are represented in green, and they are present at the level of the mucosa, submucosa plexus, and myenteric plexus. The red dashed circle represents the portion of the GI tract which can be routinely analyzed through biopsies, found at the level of the mucosa and submucosa plexus. At the top we see two techniques used to analyze EGCs. In the top left, S100B IHC refers to the immunohistochemical (IHC) detection of the protein S100B which is a calcium-binding protein primarily expressed in glial cells. In the top right, GFAP WB refers to a Western blot analysis of the protein GFAP (glial fibrillary acidic protein). Western blotting is a laboratory technique used to detect specific

proteins in a sample using antibodies. GFAP is primarily expressed in glial cells. Additionally, 55-50-45 kDa refers to the size of a protein, specifically its molecular weight, which is measured in kilodaltons (kDa) (Clairembault et al., 2015).

From the ENS to the CNS: Microglia implication in neurodegeneration

The chronic inflammatory environment promoted by the EGC at the level of the ENS could be the trigger of αSyn aggregation which, in turn, aggravates even more the extent of the inflammatory response. The αSyn in the ENS starts to accumulate and there is the onset of the first Lewy bodies which begin to spread reaching the CNS. Here, the spreading of the Lewy pathology continues to progress creating a chronic neuroinflammation which leads to synaptic dysfunction, a key feature of all neurodegenerative diseases (Yang et al., 2022). However, there are no EGC in the CNS because they are a unique kind of glial cells found in the gut. In the CNS, we find another kind of glial cells, called microglia, which are directly involved in the neuroinflammation and synaptic dysfunction.

Microglia are sensitive immune cells characterized by a spider-like shape in the CNS which constantly survey the environment with their motile ramifications and can react quickly to damages by adopting distinct phenotypes. The fact that microglia constantly survey the CNS environment attributed them the nickname of "versatile watchdogs" that tightly control tissue homeostasis (Fixemer et al., 2021; Schwabenland et al., 2021).

In the past decades, the common idea was that microglia had a modulator role in neurodegeneration mainly related to neuroinflammation and release of neurotoxins, but not directly related to the process of degeneration of the neurons (Rajendran & Paolicelli, 2018). Nowadays, it has been demonstrated that in homeostatic conditions, microglia have a small soma, many thin processes and they monitor synapses. However, when the homeostasis of the

system is under threat (such as αSyn accumulation in PD), they rapidly change their morphology assuming a bigger soma and arms, and they migrate in group toward the specific location (Schwabenland et al., 2021; DeTure et al., 2019). This makes microglia an early and easily detectable biomarker of any kind of pathologies which concern the central nervous system such as neurodegeneration and neuroinflammation (Schwabenland et al., 2021). Microglia represent the main type of phagocytes in the brain (Rajendran & Paolicelli, 2018). In addition to phagocytosis, other important roles of microglia reduce proinflammatory cytokine secretion and minimize tissue injury.

Microglia could be a two-edged sword for the central nervous system; they can be both beneficial and detrimental to brain homeostasis and brain health. In fact, microglia are involved in the pathogenesis of many diseases such as Alzheimer's disease and Parkinson's (Schwabenland et al., 2021). The fact that microglia play a fundamental role in neurodegenerative diseases is given by the fact that microglia are involved in the process of damaging synaptic structures by the means of phagocytosis (*figure 19*). This ability of microglia of interacting with synapses does not relate only to pathological conditions. In fact, microglia are the main character of the synaptic pruning activity during early ages which is fundamental for healthy brain development (Rajendran & Paolicelli, 2018).

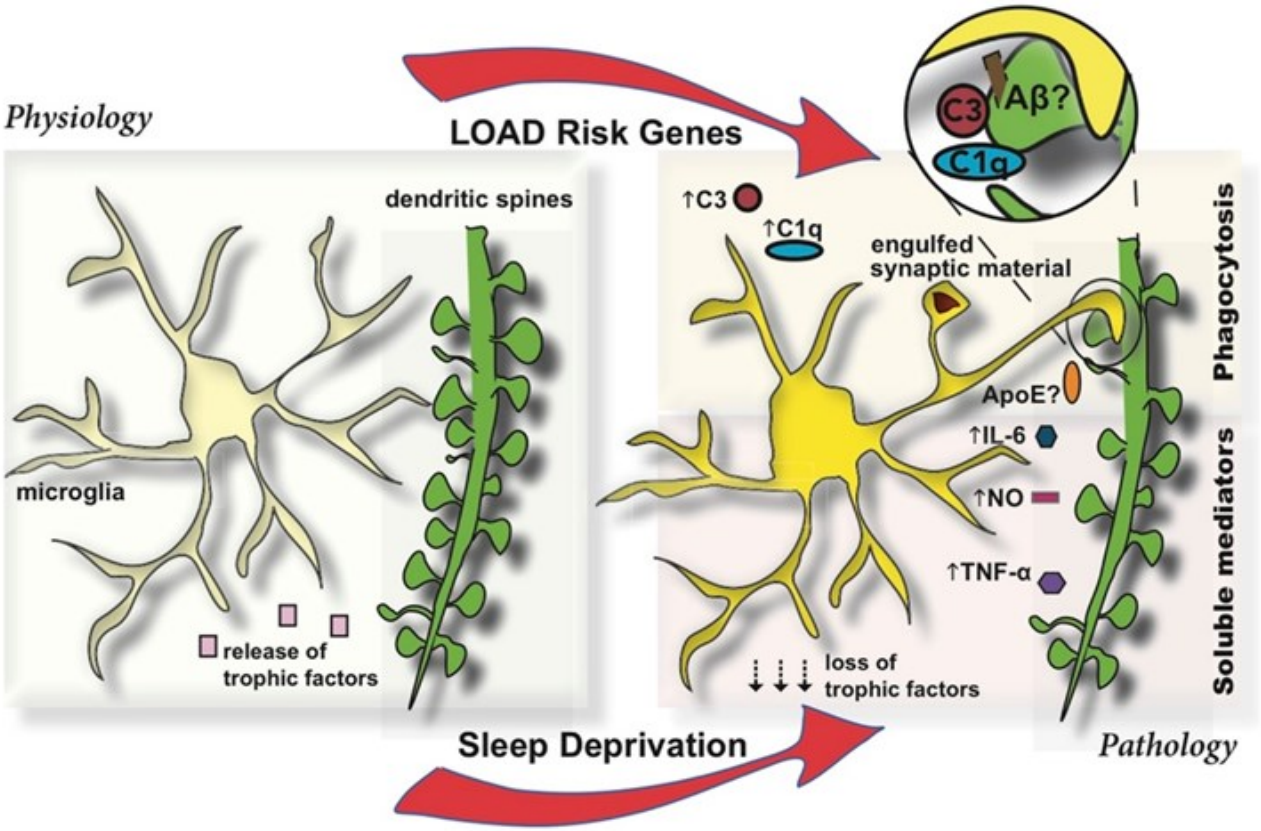


Figure 19. On the left, the role of microglia in supporting homeostasis during healthy conditions. On the right, the pathological role of microglia. Top right, phagocytosis of synapses by macroglia aided by complement molecules C1q and C3 (Rajendran & Paolicelli, 2018).

Covid-19-inflammation-PD

COVID-19, also known as coronavirus, emerged as a global health crisis in late 2019. The first cases were reported in Wuhan, China, and the virus quickly spread to become a pandemic, affecting millions of people worldwide (Jones, 2020). COVID-19 is characterized by neurological symptoms, but it is still not entirely clear why; they could be the result of viral invasion, neuroinflammation, or other pathologies.

An important finding about Covid-19 infection has been made recently which has important implications in the understanding of inflammation and PD. According to Emmi et al. (2023), COVID-19 has the ability of spreading in the CNS targeting specific brain regions such as the substantia nigra and the DMNV. Because of the importance of the substantia nigra in PD, COVID-19 could predispose or aggravate PD. Indeed, COVID-19 patients are characterized by upregulations of different genes related to inflammation, microglia activation, neurodegeneration, and antiviral response. Activation of microglia in COVID-19 patients was reported to be statistically significantly higher than control, indicating a situation of neuroinflammation which can be detrimental for the neurons' wellbeing (Emmi et al., 2023). To make the situation worse, the dopaminergic neurons in the substantia nigra appear to be particularly vulnerable to chronic inflammation (Brundin et al., 2020). Additionally, COVID-19 patients demonstrated a generalized brain atrophy compared to control (Emmi et al., 2023) which could be related to an exaggerated inflammatory response because of COVID-19, which triggers aggressive microglia that damage synapses leading to neuronal loss.

The infection of COVID-19 is not limited to the CNS, but it can also affect the GI system (Brundin et al., 2020). As it was widely described previously, a pathological condition of the GI system and ENS can spread to the CNS through the vagus nerve or vice versa (Natale et al., 2021). Therefore, the vagus nerve can constitute a key viral entry point for the COVID-19 infection (Brundin et al., 2020).

Research related to COVID-19 and PD reveal another intriguing aspect which can elucidate the role of αSyn. Evidence demonstrated an antiviral and protective role of the αSyn in response to virus infection in the CNS. Mouse models without αSyn, showed an abnormal viral presence in the brain. While a right amount of αSyn is beneficial, an overexpression due to excessive viral infections could end up in an abnormal accumulation and misfolding of the protein becoming a threat for the brain's health (Emmi et al., 2023; Brundin et al., 2020). The idea that pathogens could trigger a cascade of events which lead to neurodegeneration is in line with the Braak's hypothesis (Braak et al., 2066; Rietdijk et al., 2017), and supported also by clinical evidence in which people who have been infected by the Spanish flu were subject to post-encephalic parkinsonism (Emmi et al., 2023) (*figure 20*).

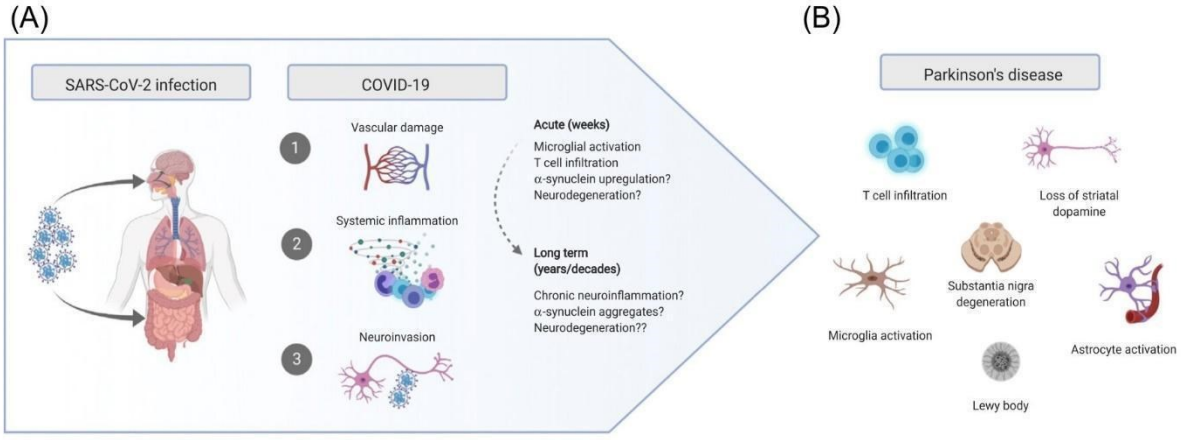


Figure 20. Illustration of how COVID-19 could lead to PD risk. (*A-B*) The COVID-19 infection targets the CNS or the GI system (including ENS). The COVID-19 infection will cause vascular complications, chronic inflammation, and neural damage which will result in T-cells infiltration, microglia and astrocytes activation, αSyn misfolding, substantia nigra alteration, and neurodegeneration (Brundin et al., 2020).

PART II: Experimental Procedures

Background

Despite significant progress in elucidating PD pathophysiology, with particular regard to biochemical and genetic causes, there are no currently known reliable biomarkers that allow for accurate diagnosis of the disease, nor effective treatments. Current diagnostic criteria rely on clinical features, such as motor and non-motor symptoms (NMS) but require post-mortem confirmation for definitive diagnosis. In this context, post-mortem pathological assessment represents the current gold-standard for the diagnosis of PD and is based on the knowledge of occurring accumulation of abnormal proteins that characterizes disease development and progression. While clinical diagnostic criteria are being reworked to allow for more accurate clinical characterization of PD even prior to the onset of motor symptoms, there is still no biological diagnostic criterion (i.e. biomarker), aside from post-mortem confirmation, to reach a definitive and conclusive diagnosis. Furthermore, hypokinetic motor symptoms, which were traditionally regarded as the clinical hallmark of the disorder, are now known to represent a late symptom of an already ongoing disease, with their manifestation occurring only after a great majority of dopaminergic neurons of the substantia nigra (SN) have degenerated, greatly reducing treatment options and efficacy. Hence, there is an urgent but unmet need for sensitive PD biomarkers to allow early and conclusive diagnosis (possibly at a prodromal stage) and predict disease progression, both critical elements to develop disease-modifying treatments. Current studies for the identification of PD biomarkers have been investigating both clinical, genetic, neuroimaging and biochemical markers, with the latter representing the most reliable candidates. On the other hand, the treatments available today to counteract the advancement of PD (L-dopa, brain surgery, physical therapy, stem cell transplantation) represent valid methods to mitigate the pathology, but they fail in providing a definitive cure.

In this context, the role of the gut-brain axis in PD has been greatly highlighted by recent developments in both clinical and preclinical research. Indeed, PD is characterized by a long prodromal stage in which patients are affected by autonomic dysfunctions at the level of the GI system, such as constipation and gastro-intestinal dysmotility, suggesting a potential early involvement of the GI tract (Mulak & Bonaz, 2015; Rietdijk et al., 2017).

There is growing interest in the detection of αSyn aggregates, the histological hallmark of PD, in peripheral tissues including the gastrointestinal (GI) tract. Recent animal models suggested a possible bidirectional transmission of αSyn pathology, that may originate either in the enteric nervous system and spread towards the brain or begin in the brain and then spread towards the periphery. These findings have been supported by studies on human post-mortem samples, where Lewy Body Pathology has been detected in the Enteric Nervous System (Dinan & Cryon, 2017). In-vivo, αSyn pathology has been detected in both duodenal and colonic mucosa biopsies in early and advanced PD patients (Emmi et al., 2023; Skorvanek et al., 2018). These findings suggest that the ENS may represent an early site of αSyn pathology, and that gastro-intestinal sampling may represent a safe and feasible tool for the detection of αSyn aggregates, thus representing a promising biomarker of the pathology. Nevertheless, the mechanisms underlying αSyn aggregation in peripheral tissues, and eventual spreading from the ENS towards the CNS and vice-versa, remain to be elucidated.

In this context, the immune system and the inflammatory response may represent a relevant factor in mediating, or even determining, αSyn pathology. Inflammation in the ENS could lead to the initial abnormal aggregation of αSyn, and eventual spreading towards the CNS (Mulak & Bonaz, 2015). Increased levels of proinflammatory cytokines and activated T cells have also been reported in the serum and CFS of PD patients (Mogi et al., 1994; Brodackj et al., 2008; Bas et al., 2001). Inflammation can increase the intestinal barrier permeability leading to a

cascade of events such as the production of pro-inflammatory cytokines, oxidative stress, and potentially αSyn misfolding (Camilleri, 2019; Yang et al., 2022; Rietdijk et al., 2017).

Considerable attention has also been drawn to EGCs, a distinct type of glial cells found uniquely in the ENS, which may play a critical role in the crosstalk between inflammation, protein aggregation and neurodegeneration (Natale et al., 2021; Yang et al., 2022). Indeed, EGCs have recently emerged as critical players in regulating GI function in PD, as higher levels of expression for enteric glial markers (such as Glial Fibrillary Acidic Protein – GFAP – and SOX10), were reported in the GI tract of PD patients, while histopathological evaluation of GI biopsies revealed a pattern of enteric gliosis in PD (Emmi et al., 2023). Nevertheless, despite the prominent role played by the inflammation in PD, the exact role of the immune system in the onset, development, and spreading of PD remains to be elucidated (Mulak & Bonaz, 2015).

The aim of this study was to investigate the histopathological changes in the enteric nervous system by characterizing both αSyn aggregates, enteric glial responses and evaluating immune-cell populations in duodenum biopsies of PD patients with extensive clinical and demographical documentation.

Material and Methods

Subjects

Eighteen (18) patients (12 males, 6 females; mean age 65.2 years, 95% CI 61.4 to 69.0; mean disease duration 11.3 years, 95% CI 9.0 to 13.6) with advanced PD who required initiation of Levodopa Carbidopa Intestinal Gel (LCIG) infusion were enrolled for the study.

All patients underwent Percutaneous Endoscopic Gastrostomy with Jejunal extension (PEG-J) placement; an average of four 3 mm^3 duodenal-wall biopsies were sampled in a topographically unrelated district to PEG-J placement. Along with the routine clinical assessment (MDS- UPDRS I- II- III, IV, Hoehn and Yahr scales), the Wexner Constipation Score (WCS) was also calculated. In addition, we also investigated 4 early untreated PD subjects (3 males and 1 female; mean age 63.2 years, 95% CI 50.5 to 76.0; mean disease duration 2.7 years, 95% CI -0.5 to 6.0) with disease duration <5 years. Early PD patients voluntarily underwent screening diagnostic endoscopy with biopsy collection. The clinical data of the PD cohort is reported in *Table 2*.

Duodenal biopsies from 18 subjects comparable for age- and sex- (9 males, 9 females; mean age 68.6 years, 95% CI 63.8 to 73.4) undergoing screening diagnostic endoscopy were included as healthy controls (HCs). Of note, control subjects were further evaluated clinically and interviewed to exclude any manifestation suggestive of PD or any other neurological disorder.

The study protocol received approval by the ethical committee for clinical experimentation of Padua Province (Prot. n. 0034435, 08/06/2020). Informed consent for the use of biological samples was obtained from all patients. All procedures on human tissue samples were carried out in accordance with the Declaration of Helsinki.

	Healthy controls N=18		Advanced PD N=18		Early PD N=4	
	Median	*2.5 - 97.5 P*	*Median*	*2.5 - 97.5 P*	*Median*	*2.5 - 97.5 P*
AGE AT BIOPSY	68.5	54.0 - 86.0	66	48.0 - 79.0	64	53.0 - 70.0
SEX (M%)	50%		66.70%		75.00%	
AGE AT DIAGNOSIS	-	-	55.5	34.0 - 67.0	61.5	51.0 - 66.0
AGE AT LCIG INITIATION	-	-	65	45.0 - 76.0	-	-
YEARS OF DISEASE	-	-	12.5	5.0 - 21.0	3.5	1.0 - 6.0
LEDD AT LCIG INITIATION	-	-	1246.5	750.0 - 2588.0	425.0	400.0 - 450.0
MDS-UPDRS part I	-	-	10	6.0 - 24.0	1.0	1.0 -1.0
MDS-UPDRS part II	-	-	17	2.0 - 37.0	5.0	4.0 - 10.0
MDS-UPDRS part III	-	-	32.5	10.0 - 58.0	20.0	19.0 -31.0
MDS-UPDRS part IV	-	-	8	3.0 - 13.0	0	0.0 - 2.0
H&Y>2	-		43.80%		0%	
PDQ-8	-	-	12	0.0 - 21.0	-	-
ADL	-	-	5	2.0 - 6.0	-	-
IADL	-	-	5	2.0 - 8.0	-	-
PD-CFRS	-	-	1	0.0 - 9.0	-	-
MMSE (corrected score)	-	-	26.2	22.0 - 30.0	-	-
MoCA (corrected score)	-	-	24.52	17.1 - 30.0	25.2	23.6 - 27.9

*Table 2***.** Demographic and clinical characteristics of Parkinson's Disease and healthy controls patients.

Tissue processing and staining

Tissue samples were fixed in phosphate-buffered 4% paraformaldehyde, embedded in paraffin, and sectioned at the microtome (5µm slices). Single and double-marker immunoperoxidase staining for aggregated αSyn (Monoclonal Mouse, Clone 5G4, Millipore), Glial Fibrillary Acidic Protein (GFAP, Monoclonal Rabbit, Dako Omnis), β-III-Tubulin (Rabbit Polyclonal, BioLegend), CD3 (Polyclonal Rabbit Anti-Human, Citrate Buffer HIER, dilution 1:200, Dako Omnis, Code Number: GA503), CD20 (Monoclonal Mouse Anti-Human, Citrate Buffer HIER, dilution 1:200 Clone KP1, Dako Omnis, Code Number: M0814) and

CD68 (Monoclonal Mouse Anti-Human, EDTA Buffer HIER, IHC dilution 1:5000, IF dilution 1:500, Clone L26, Dako Omnis, Code Number: M0756), HLA-DR Antibody (Monoclonal Rabbit Anti-Human, Citrate Buffer HIER, dilution 1:50 Clone: LN-3, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was performed as previously reported (Emmi et al., 2023a;2023b). Antigen retrieval was performed on a PT-Link Dako Antigen retrieval station using Citrate-buffer at pH 6 solution at 96° for 15 minutes, followed by 1 minute 95% formic acid for the αSyn-5G4 antibody.

Immunoperoxidase staining was repeated at least three times to assure reaction consistency and was independently evaluated by three morphologists blind to the clinical findings. Controversies were resolved by consensus.

Morphometrical quantification

Photomicrographs were acquired under a Leica DM4500B microscope (Leica Microsystems) connected to a Leica DFC320 high-resolution digital camera (Leica Microsystems) and a computer equipped with software for image acquisition (QWin, Leica Microsystems) and analysis (ImageJ). Specimens were digitally scanned at 20x magnification and an average of 3±1 non-overlapping counting fields were defined and loaded into ImageJ software for semi-automatic immunoreactivity quantification.

A Maximum Entropy Threshold was applied and manually adjusted for each section to discern immunopositivity elements from background and negative tissue. Quality control of the applied threshold was performed by an expert morphologist by overlying the images to the original photomicrographs. Particle analysis was employed with an 8-infinity μm threshold to define immunoreactive elements quantity and total area occupied within the digital image.

Counting fields for each available sample were treated as repeated measures and averaged per single subject.

Immunofluorescence and confocal microscopy

Fluorescent immunohistochemistry was performed manually. Antigen retrieval was performed on deparaffinized tissue as in immunoperoxidase staining methods. Following autofluorescence was quenched with a 50 mM NH_4Cl solution for 10 minutes. Sections were treated with permeabilization and blocking solution (15% vol/vol Goat Serum, 2% wt/vol BSA, 0.25% wt/vol gelatin, 0.2% wt/vol glycine in PBS) containing 0.5% Triton X100 for 90 minutes before primary antibodies incubation. Primary antibodies were diluted in blocking solution and incubated at 4°C overnight. Alexa-Fluor plus 488 Goat anti-Mouse secondary antibody (A32723, Thermo Fisher Scientific) and Alexa-Fluor plus 568 anti-Rabbit secondary antibody (A-11011, Thermo Fisher Scientific) were diluted 1:200 in blocking solution as above and incubated for 60 minutes at room temperature. Hoechst 33258 were used for nuclear staining (Invitrogen, dilution: 1:10000 in PBS) for 10 minutes. Slides were mounted and cover slipped with Mowiol solution (Novabiochem). Confocal immunofluorescence z-stack images were acquired on a Leica SP5 Laser Scanning Confocal Microscope using a HC PL FLUOTAR 20x/0.50 Dry or HCX PL APO lambda blue 40X/1.40 Oil objectives. Images were acquired at a 16-bit intensity resolution over 2048 × 2048 pixels. Z-stacks images were converted into digital maximum intensity z-projections, processed, and analyzed using ImageJ software.

The antibodies used for IF were the following: mouse anti-aggregated αSyn clone 5G4 (MABN389, Sigma-Aldrich, 1:1000); rabbit Glial Fibrillary Acidic Protein (GFAP, Dako Omnis, 1:1000); mouse Glial Fibrillary Acidic Protein (GFAP, Genetex, 1:1000); rabbit β-III Tubulin(Poly18020; BioLegend, 1:10000).

Statistical Analyses

Statistical analyses and visualizations were performed using GraphPad Prism v.9. Nonparametric data were analyzed with Mann–Whitney U-test. Pearson's correlation analysis has been employed to assess possible correlations between αSyn expression in duodenum and clinical characteristics, including motor and non-motor scales, cognitive assessments and main non-motor symptoms of PD. Values are indicated as the median, with significance as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.

The study protocol received approval by the ethical committee for clinical experimentation of Padua Province (Prot. n. 0034435, 08/06/2020). Informed consent for the use of biological samples was obtained from all patients. All procedures on human tissue samples were carried out in accordance with the Declaration of Helsinki.

Results

Alpha-Synuclein Pathology

αSyn-5G4 immunoreactive elements detected in duodenal specimens were classified according to morphological criteria into compact and globular immunoreactivities, granular cellular immune reactivities, and dot-like immunoreactivity (*figure 21*). These three morphologies were found in both PD and HC. The fourth morphology was observed as thread-like immunoreactivities, which represented the most reliable immunoreactivity type to discern PD from controls (*figure 21*). Double immunoperoxidase staining confirmed the colocalization between αSyn-5G4 threaded immunoreactivities and pan-neuronal markers such as Beta-III-Tubulin and PGP9.5, confirming aggregated αSyn deposits in duodenal nerve fibers of the mucosa and submucosa as previously reported (Emmi et al., 2023). Similar thread-like immunoreactivities were also found in the duodenal mucosa and submucosa of early PD subjects.

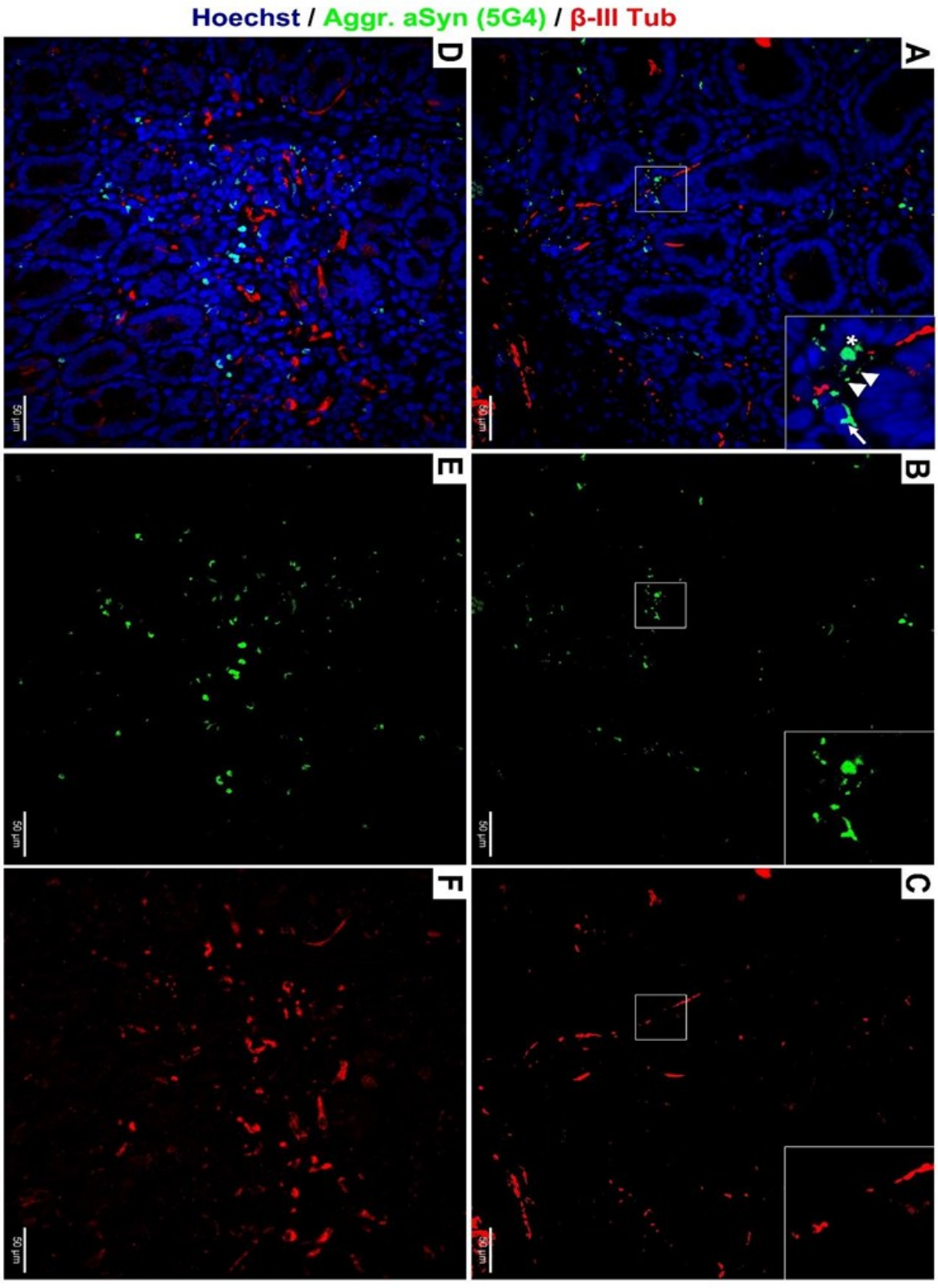


Figure 21. Immunofluorescence images of Globular (asterisk), Cellular (arrow) and Dot-Like (arrowhead) aggregated αSyn (Clone 5G4) immunoreactivity (green) not colocalizing with neuronal marker β-III-Tubulin (red).

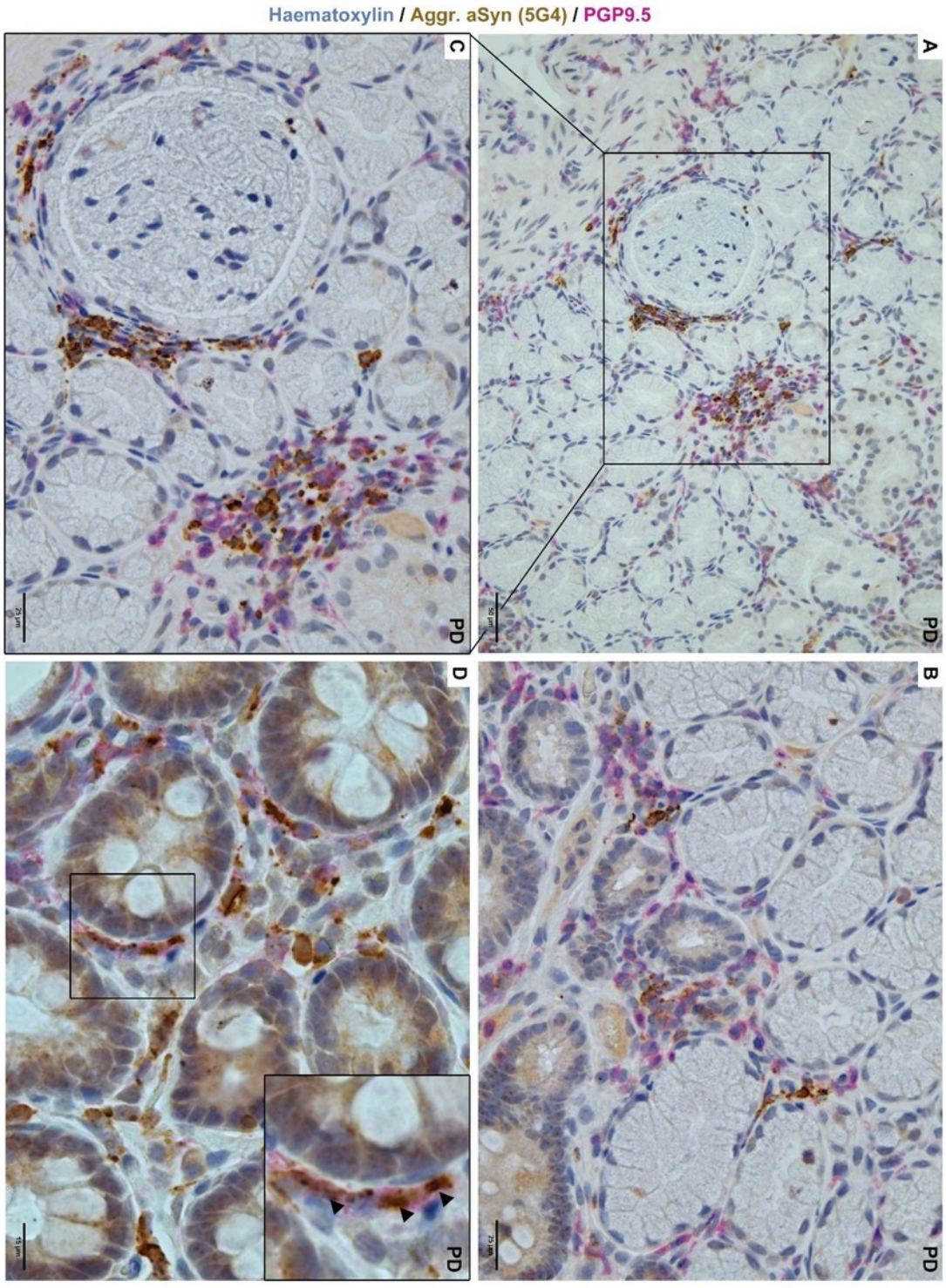


Figure 22. Aggregated αSyn (Clone 5G4) (brown) and neuronal marker PGP9.5 (magenta) double immunoperoxidase staining (A-D) reveals colocalization between nerve fibers of the mucosa and aggregated αSyn in PD patients (D, black arrows), similarly to 5G4 / β-III-Tubulin double staining (*figure 22*).

Detection of 5G4 immunoreactive threads in mucosal and submucosal nerve revealed a sensitivity of 100.00% (95% CI=81.47-100.00%), specificity of 94.44% (95% CI=72.71-99.86%), Positive Predictive Value of 94.74% (95%CI=72.82-99.18%) and Negative Predictive Value of 100.00% for discerning manifest PD from HC. The calculated accuracy was thus equal to 97.22% (95%CI=85.47-99.93%), as reported in *Table 3*. Hence, the presence of 5G4 immunoreactive threads in the nerves of the mucosa and submucosa is highly accurate in differentiating PD from control subjects.

Diagnostic method	Detection of Thread-like 5G4 IR
Sensitivity	1.00 (0.81-1.00)
Specificity	0.94 (0.73-0.99)
Positive likelihood ratio	18.00 (32.92-67.08)
Negative likelihood ratio	0.00
Accuracy	0.97 (0.86-0.99)
Diagnostic odds ratio	431.67 (16.46-11320.50)

Table 3. 5G4 immunohistochemistry as a diagnostic marker for PD in duodenal biopsies using thread-like immunoreactivities (IR) as indicator. Confidence intervals are represented as 95 % CI.

Enteric gliosis

GFAP immunoreactive elements presented as discrete, round immunoreactive cells localized predominantly within the duodenal mucosa and submucosa (*Figure 23A-D* & High-Magnification Inserts). Morphometrical analyses revealed both increased EGC density (PD mean: 95.2±32.6; CTRL mean: 45.8±14.9; *Figure 23F*) and increased cell size (PD mean: 28.2±1.4 μm^2; CTRL mean: 25.1±1.2 μm^2; *Figure 23E*) in advanced PD patients compared to HCs (****$p<0.0001$ for both cell density and size), suggesting for local reactive gliosis. Spearman's rank correlation analysis between αSyn-5G4 immunoreactive area, EGC density and EGC cell size in the duodenum in advanced PD patients revealed a strong correlation ($R_s=0.751$, $p<0.0001$ and $R_s=0.741$, ****$p<0.0001$, $n=36$, respectively).

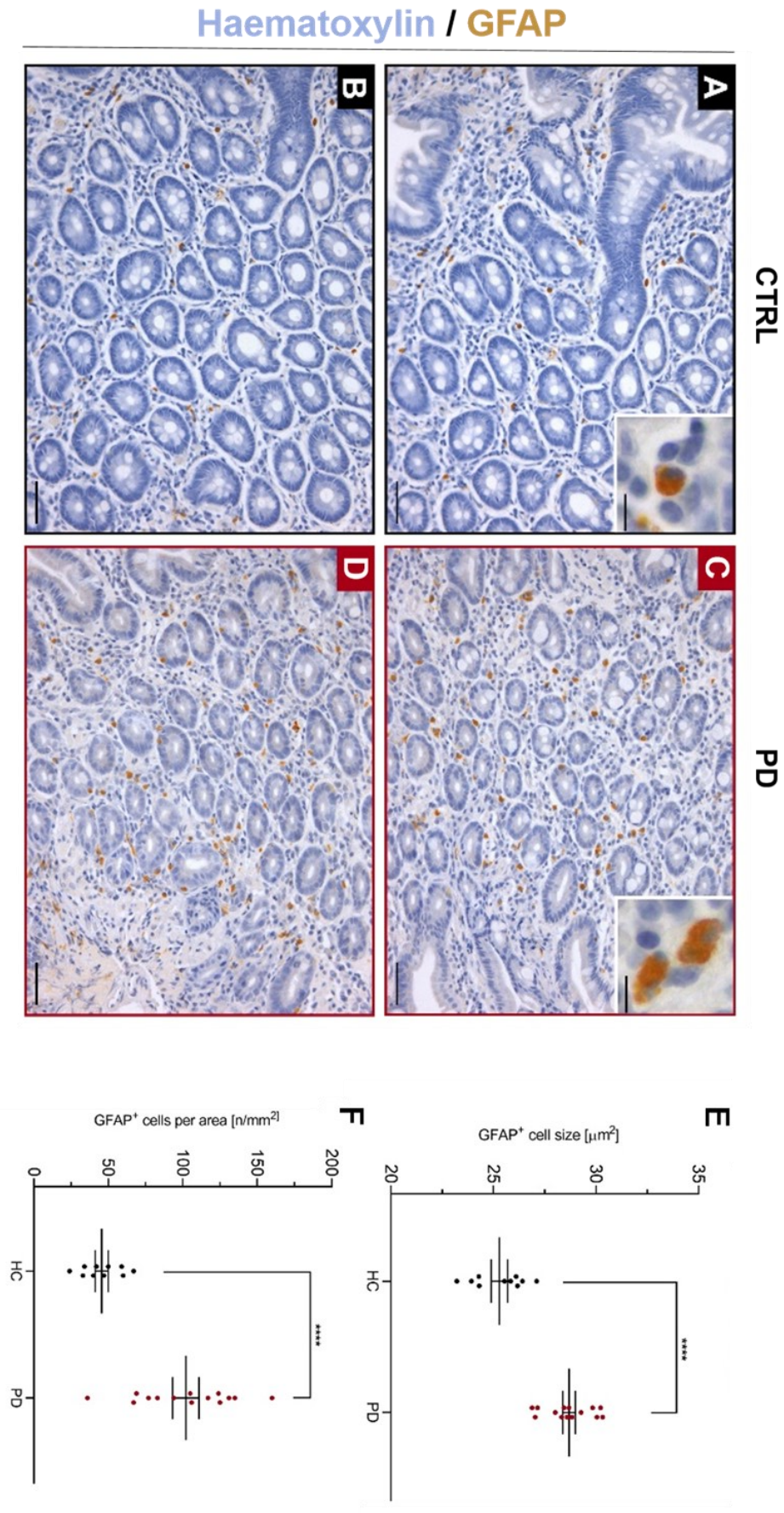

Haematoxylin / GFAP
A
B
C
D
CTRL
PD
E
GFAP+ cell size [µm2]
20
25
30
35
HC
PD
****
F
GFAP+ cells per area [n/mm2]
0
50
100
150
200
HC
PD
****

Figure 23. A-C) Enteric glial cells (GFAP antibody) in the duodenal biopsies of representative patients with advanced PD (PD) and non-PD patients (healthy control, HC). E) Quantitative analysis of duodenal GFAP+ cell size in PD (n= 18) group and control group (n = 18). F) Quantitative analysis of duodenal GFAP+ cell density in PD (n = 18) group and control group (n = 18). All data have been analyzed by comparing the two groups with Mann-Whitney test, **** $p<0.0001$.

Immune cell populations and inflammatory markers

Lymphocytic and phagocytic cells were evaluated in the context of the duodenal mucosa. The lymphocytic population is known to circulate throughout lymphoid tissues and blood, and reside in non-lymphoid organs, mainly in barrier tissues, such as the intestines (Ma et al., 2019). Moreover, intestinal lymphocytes are constantly exposed to food and microbial antigens. Gastro-intestinal alterations and in particular dysbiosis, as recently detected in PD (Huang et al., 2021), trigger immune responses via the activation of resident lymphocytes. T-lymphocytes were marked by means of anti-CD3 immunohistochemistry, while B-Lymphocytes were detected with an anti-CD20 antibody. Phagocytic cells, represented in the duodenum by intestinal macrophages, constitute the largest populations of macrophages in the body (Wang et al., 2019). CD68 antigen is expressed by macrophages and monocytes and is a well-established phagocytic marker, indicating increased lysosomal activity. Furthermore, the expression of MHC-II molecules (HLA-DR) was evaluated. HLA-DR is a cell surface receptor and constitutes a ligand for the T-cell receptor (TCR) with purpose to present foreign antigens (*figure 23*). It is present on so called antigen-presenting cells (macrophages, B-cells, and dendritic cells). Increased abundance of this on the cell surface is a marker for immune stimulation (Schamboeck et al., 1983).

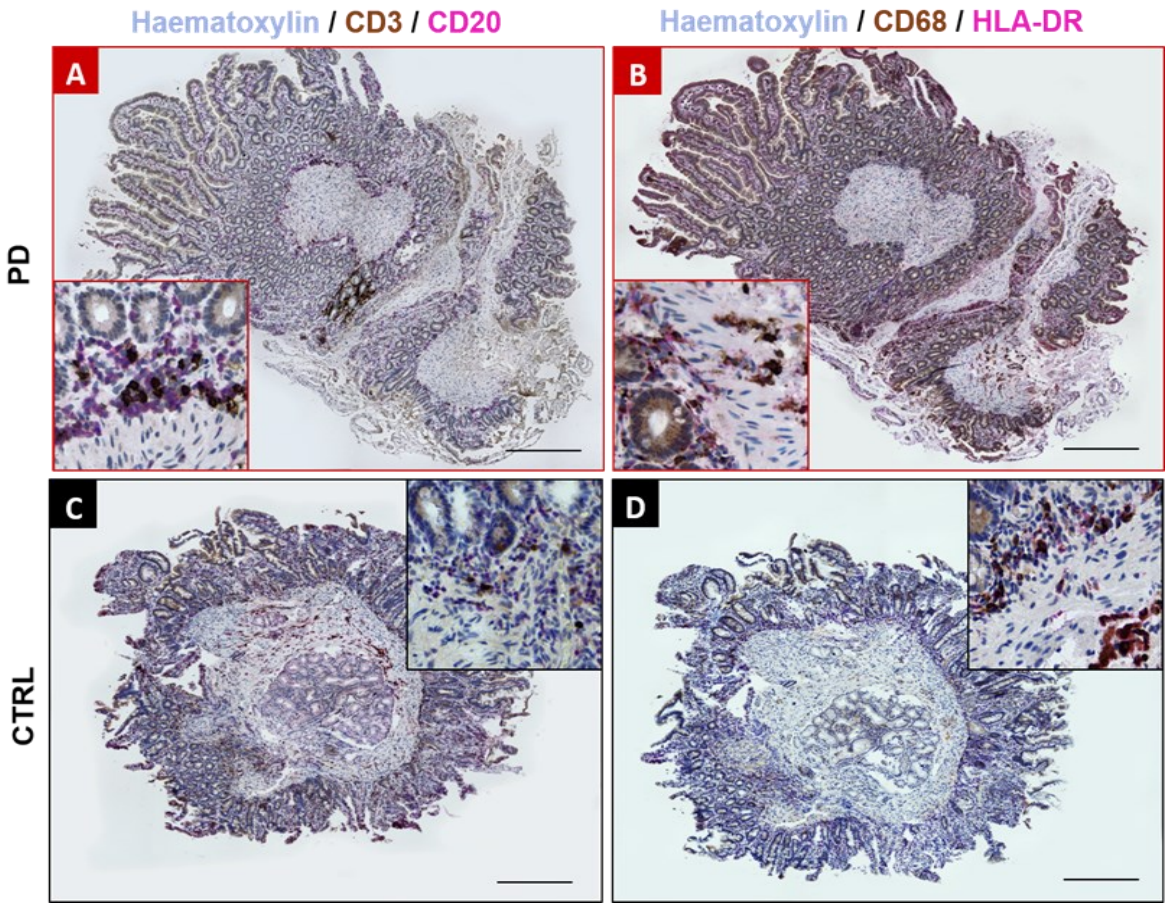


Figure 23. Investigation of immune cell populations via double-label immunoperoxidase staining in PD (*A-B*) and Healthy Control (*C-D*) subjects. *A,C*) CD3 (brown) and CD20 (magenta) antibodies in the mucosa and submucosa reveal distinct cellular immune reactivities. *B,D*) CD68 (brown) and HLA-DR (magenta) antibodies in the mucosa and submucosa reveal phagocytic and antigen-presenting cells, respectively.

T-Lymphocytes (CD3+)

Statistical analyses performed using a Mann–Whitney U-test revealed statistically significant differences (**p =0.0374*) between CD3+ cell densities (CD3+ Cells / mm^2) between PD and control (*Figure 24*). 12 Control and 11 PD. Control: minimum 1.000, 25% percentile 34.25, median 90.67, 75% percentile 165.8, maximum 201.0, mean 100.2, Std. deviation 69.71, 95% CI 55.90 to 144.5. PD: minimum 72.33, 25% percentile 81.5, median 221.0, 75% percentile 610.0, maximum 852.3, mean 329.0, Std. deviation 283.3, 95% CI 138.7 to 519.3.

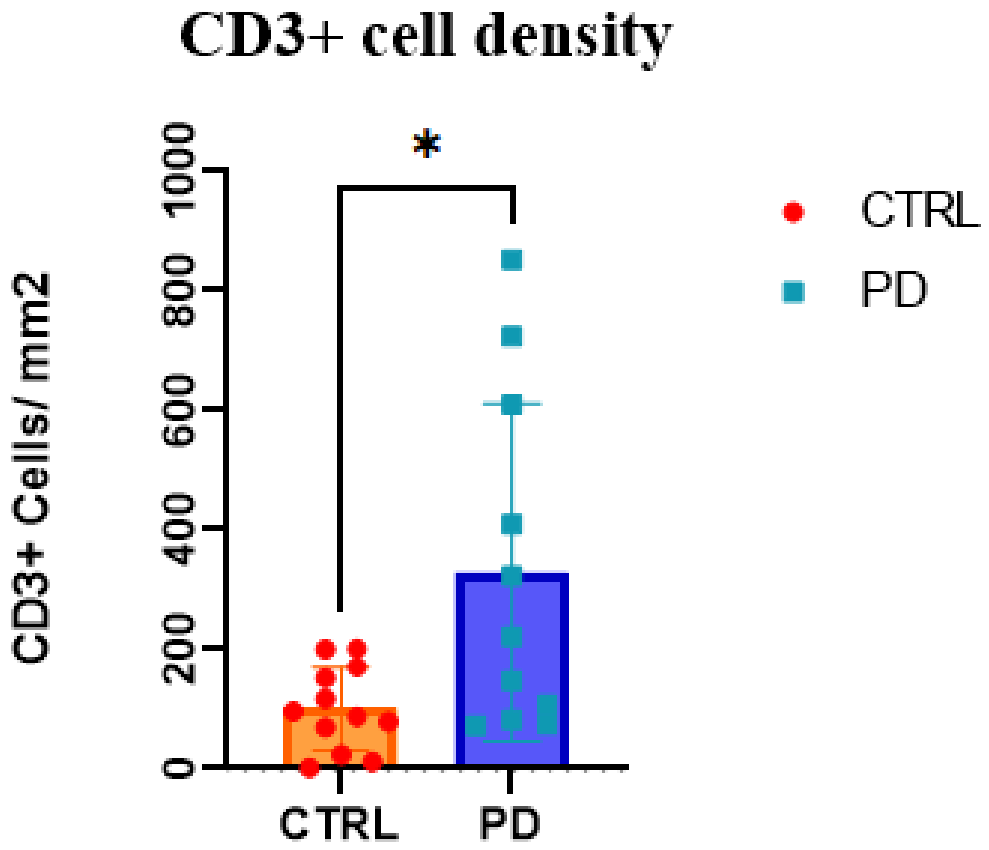


Figure 24. Quantitative analysis of CD3+ cell densities (CD3+ Cells / mm^2), Mann-Whitney U-test. *p<0.05 (**p =0.0374*).

B-Lymphocytes (CD20+)

Statistical analyses performed using a Mann–Whitney U-test revealed statistically significant differences (**p =0.0188)* between CD20+ cell densities (CD3+ Cells / mm^2) between PD and control (*Figure 25*). 12 Control and 11 PD. Control: minimum 117.0, 25% percentile 294.7, median 429.7, 75% percentile 881.2, maximum 1313, mean 559.9, Std. deviation 367.5, 95% CI 326.4 to 793.4. PD: minimum 378.5, 25% percentile 558.0, median 991.3, 75% percentile 1287, maximum 1708, mean 1008, Std. deviation 444.8, 95% CI 709.5 to 1307.

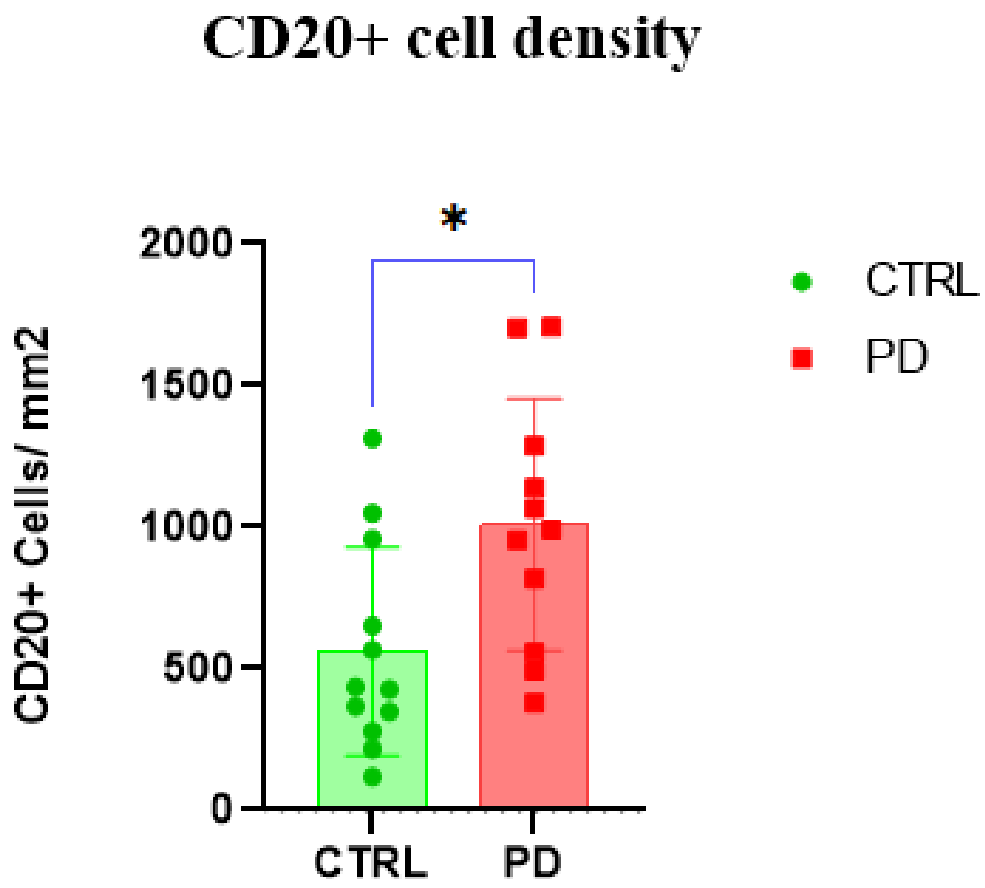


Figure 25. Quantitative analysis of CD20+ cell densities (CD3+ Cells / mm^2), Mann-Whitney U-test. *p<0.05 (**p =0.0188*).

CD68+

Statistical analyses performed using a Mann–Whitney U-test revealed a non-statistically significant differences (**p =0.6392)* between CD68+ cell densities (CD3+ Cells / mm^2) between PD and control (*Figure 26*). 12 Control and 11 PD. Control: minimum 309.7, 25% percentile 625.7, median 949.8, 75% percentile 1365, maximum 2735, mean 1111, Std. deviation 754.7, 95% CI 631.9 to 1591. PD: minimum 390.7, 25% percentile 649.0, median 955.3, 75% percentile 2085, maximum 3243, mean 1294, Std. deviation 906.5, 95% CI 684.8 to 1903.

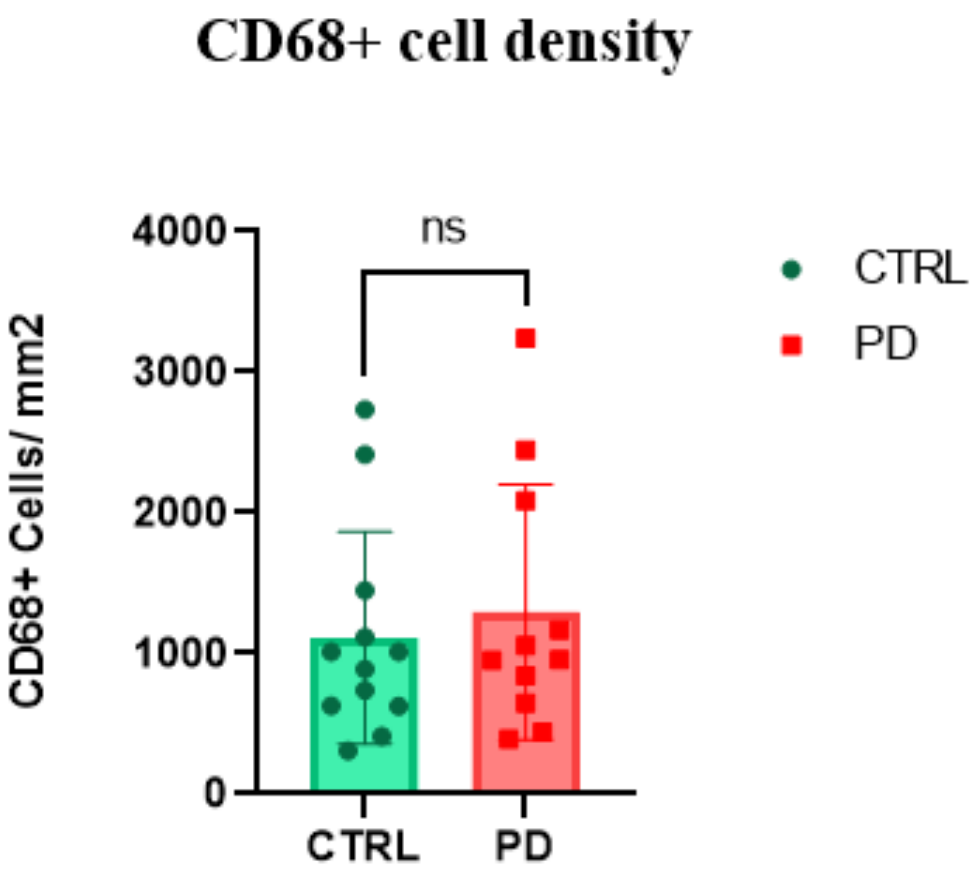


Figure 26. Quantitative analysis of CD68+ cell densities (CD3+ Cells / mm^2), Mann-Whitney U-test. p>0.05 (*p=0.6392*).

HLA-DR+

Statistical analyses performed using a Mann–Whitney U-test revealed a statistically significant differences (**p =0.0388)* between HLA-DR+ % area between PD and control (*Figure 27*). 12 Control and 11 PD. Control: minimum 0.14, 25% 0.4625, median 1.3, 75% percentile 1.368, maximum 1.950, mean 1.053, Std. deviation 0.5594, 95% CI 0.6971 to 1.408. PD: minimum 0.03, 25% percentile 0.79, median 2.08, 75% percentile 3.42, maximum 4.36, mean 2.099, Std. deviation 1.441, 95% CI 1.131 to 3.067.

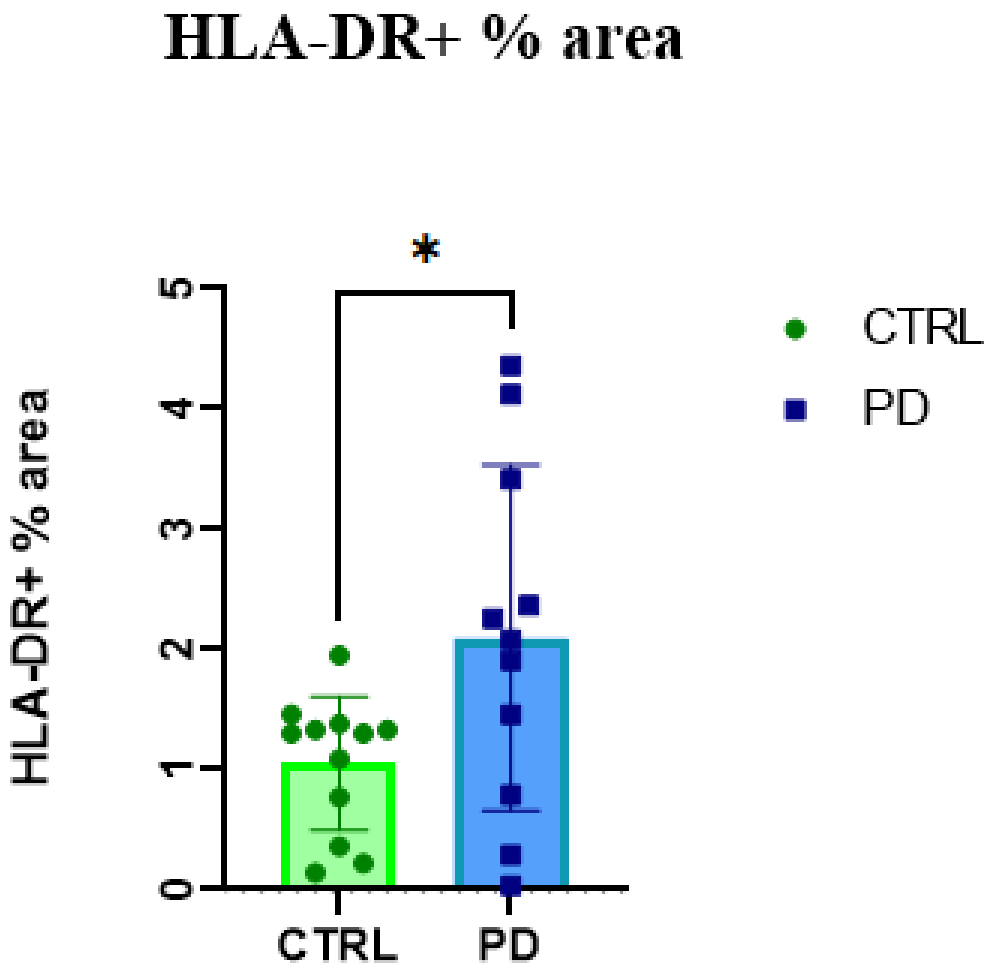


Figure 27. Quantitative analysis of HLA-DR+ % area, Mann-Whitney U-test. *p<0.05 (**p =0.0388*).

Discussion

The experimental study demonstrates three main factors in PD patients. First, PD patients present thread-like aggregated αSyn in the nerve fibers of the GI mucosa, unlike controls. Second, PD patients are characterized by higher densities of EGCs with hypertrophic features, indicating enteric gliosis. Third, PD patients show the presence of a chronic inflammatory response defined by higher densities of T and B lymphocytes, as well as MHC-II complexes, in the ENS. The information provided by the research project broadens our understanding of how the ENS is involved in PD and proposes that the duodenum may be a potential area to investigate for identifying the disease in its early stages. Indeed, the combination of aggregated αSyn, enteric gliosis, and chronic inflammation in the GI tract suggest prominent involvement in PD.

While inflammation may play a potential role in protein misfolding and aggregation, recent evidence also supports the direct involvement of αSyn in mediating antiviral and inflammatory responses (Tulisiak et al., 2019). Indeed, upregulation of αSyn upon infection mediates interferon signaling and contributes to inhibit viral replication (Beatman et al., 2015; Monogue et al., 2022). While this may represent a protective factor during infection, viral-induced overexpression of αSyn can potentially lead to protein misfolding, aggregation and Lewy-body pathology, as recently shown in-vitro (Wu et al., 2022). Indeed, exposure to specific viral proteins, such as the nucleocapsid protein of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2, the causative agent of the global COVID-19 pandemic), may trigger αSyn misfolding and Lewy Body pathology due to the innate αSyn upregulation occurring upon infection. This is particularly relevant due to the current COVID-19 pandemic (Emmi et al., 2023b), with recent evidence confirming higher incidence of neurodegenerative diseases following viral infection (Levine et al., 2023).

It remains unclear whether αSyn misfolding and aggregation arises predominantly at the level of the ENS, and eventually spreads towards the brain according to the previously discussed Braak's hypothesis, or if protein misfolding occurs throughout the body, including the brain, at different stages of the pathology; indeed, systemic involvement of PD, regardless of the brain- or body-first hypothesis, appears to be increasingly supported by rapidly growing evidence (Emmi et al., 2023a; Braak et al., 2006; Natale et al., 2021; Rietdijk et al., 2017; Dinan & Cryon, 2017; Yang et al. 2022).

Nevertheless, at the level of the ENS, aggregated αSyn may elicit an inflammatory response characterized by enteric gliosis, lymphocytic infiltration, and expression of MHC-II molecules. Chronic inflammation may further trigger and facilitate αSyn misfolding and aggregation, giving rise to a vicious cycle that further exacerbates ENS pathology and the GI manifestations of PD. This is further supported by the detection of differences in lymphocytic cell populations, but not in monocyte-derived cells, suggesting a prominent role of the adaptive immune response.

T cells have been shown to recognize epitopes derived from αSyn and altered populations of T cells have been found in PD and MSA patients, providing evidence that these cells can be key to the pathogenesis of the disease (George et al., 2021). In the central nervous system, recent evidence showed that compared to wildtype mice, immunocompromised mice had an 8-fold increase in phosphorylated αSyn pathology in the substantia nigra. Reconstituting the T cell population decreased the accumulation of phosphorylated αSyn pathology but led to an increase in microglial activation (George et al., 2021).

On the other hand, antigen presenting cells (APC), and B cell populations in general, also appear to be prominently involved in PD (Scott, 2022), even though most studies suggest an impairment or reduction of this immune population in PD. Moreover, B cells are not only involved in antibody production, but also mediate T cell responses, act as antigen-presenting cells, and are

involved in cytokine production. Different types and subpopulations, with distinct functions, of B cells have been characterized in humans; furthermore, B cell populations may be influenced by disease duration, treatment, and in the case of our cohort, by PEG-J placement. Hence, while we employed broad markers for T and B cell populations suggesting functional alterations, more accurate phenotyping is required to further elucidate the role played by these cells in PD inflammation.

Conclusion

This research project investigated the implication of the enteric nervous system in Parkinson's disease. The structure of the study has a funnel shape, divided in two main parts. The first part was dedicated to the explanation of the two main factors of the project: Parkinson's disease and enteric nervous system. The second part was dedicated to the explanation of the experiment which was conducted to advance the knowledge related to the inflammation of the enteric nervous system in Parkinson's disease.

In the initial part of the thesis, Parkinson's disease was examined in multiple aspects: neuropathology, diagnosis, symptomatology, etiology, available treatments, and the progression of the disease. Regarding the explanation of the etiology of PD, a topic which has important implications for this thesis has been discussed: Alpha-synuclein. Additionally, the first part includes an explanation of the enteric nervous system and its implications on Parkinson's disease. The enteric nervous system was explained considering its structure and composition, the context in which it is situated (the gastrointestinal system), and the microbiome. Instead, the implications of the enteric nervous system in PD were analyzed by taking in account Braak's theory, intestinal permeability, and the immune response. The topic of the immune system response in the ENS in PD was addressed by analyzing the interaction between CNS and ENS mediated by the immune system, the inflammatory response, the microbiota-immune system-PD axis, the role of the enteric glial cells in the ENS, and the role of the microglia in the CNS. At this point, the funnel shape structure of the project reaches the bottom, focusing specifically on the inflammation in the ENS, αSyn pathology, enteric glial response, and how they interact with each other in PD. This represents the topic on which the research idea and project discussed in the second main part of the research is built on.

The second main part of the study concerns the investigation of αSyn pathology, enteric glial response, and four inflammatory markers CD3, CD20, CD68, and HLA-DR through

immunohistochemistry in the ENS of two groups of people: patients with PD, and healthy control (HC). The results of this thesis highlighted three major findings which differentiate PD patients from HCs. First, in the duodenum of PD patients, including those in early stages of the disease, thread-like pattern of aggregated αSyn immunoreactivity were detected, indicating αSyn deposits in peripheral nerves of the GI tract. Second, significant changes in the structure of enteric glial cells (EGC), indicative of reactive gliosis, were detected. Third, the results prove a significant increase in lymphocyte populations (T and B cells) and MHC-II molecules.

Indeed, this research project paves the way for further research. The demonstration and characterization of aggregated αSyn, enteric gliosis, and chronic inflammation in the enteric nervous system in people with Parkinson's disease could be applied as a starting point for new evidence. New studies could elucidate the mechanism that links inflammation and first abnormal misfolding and aggregation of αSyn. Otherwise, this research could be a good starting point to find a reliable biomarker based on a distinctive inflammatory response in the enteric nervous system.

In conclusion, our findings indicate that there is a notable presence of αSyn pathology, enteric gliosis, and inflammatory response in the duodenum of Parkinson's disease patients. Hence, the enteric nervous system is a key player in the pathogenesis of Parkinson's disease. Specifically, the inflammatory response found by the research project is characterized by an elevated number of B and T lymphocytes in the duodenum of PD patients. Instead, the monocyte lineage does not appear to be prominently involved. Further studies are required to assess lymphocyte subpopulations, with particular regard to T and B cells, and their potential role in PD inflammation.

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