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# Peripheral Biomarkers of Parkinson’s Disease: focus on the Nrf2 Pathway, Oxidative Stress and Neuroinflammation

Biomarcatori periferici nella malattia di Parkinson: focus sul pathway di Nrf2, sullo stress ossidativo e sulla neuroinfiammazione

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## Abstract

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by interconnected pathogenic mechanisms, including α-synuclein misfolding and aggregation, neuroinflammation, and oxidative stress.

This thesis investigated potential peripheral biomarkers associated with these pathogenic processes in patients with sporadic Parkinson's disease across different disease stages, with particular attention to sex-related differences. Nrf2, SOD1, HO-1 and α-synuclein were evaluated in peripheral blood mononuclear cells (PBMCs), while NfL and other inflammatory markers, including TREM2, IL-6 and TNF-α, were assessed in plasma samples.

Nrf2, SOD1 and α-synuclein did not clearly distinguish PD patients from healthy controls, while HO-1 levels were reduced in PD patients, particularly in de novo and intermediate-stage patients. Plasma NfL was significantly increased in PD and was associated with more advanced disease stages. Most inflammatory markers showed no significant differences, although TNF-α was reduced in PD patients compared to healthy controls. Sex-related differences were observed for selected inflammatory markers, with higher TREM2 levels and lower IL-6 levels in females compared with male PD patients.

Overall, these preliminary findings do not support Nrf2, SOD1 or α-synuclein as reliable peripheral biomarkers of sporadic PD. Conversely, HO-1 may represent a candidate for further investigation, while NfL emerged as the marker most clearly associated with neuroaxonal damage and disease severity. Larger cohorts will be required to validate these findings.

# Table of contents

# Introduction

## Parkinson's disease

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by the degeneration of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) and by the accumulation of intraneuronal α-synuclein-positive inclusions known as Lewy bodies (Lastres-Becker et al., 2012).

### 1.1 Clinical Presentation

The clinical diagnosis of Parkinson's disease is primarily based on the identification of motor symptoms. According to the Movement Disorder Society clinical diagnostic criteria, parkinsonism is defined by the presence of bradykinesia in combination with either rest tremor, rigidity, or both (Postuma et al., 2015).

Once the motor symptoms emerge, it is estimated that approximately 50% of the dopaminergic neurons in the SNpc have already been lost (Pang et al., 2019).

Before the onset of the characteristic motor manifestations, Parkinson's disease is characterized by a prodromal phase, during which several pre-motor symptoms may appear even 10- or 20-years before the clinical diagnosis. These prodromal symptoms include constipation, REM sleep behavior disorders (RBD), anosmia, and depression. Other non-motor symptoms may also be observed such as anxiety, apathy, psychosis, and anhedonia (Silva et al., 2023).

#### 1.1.1 Clinical scales

To assess disease progression and better treat patients several clinical scales are used.

The Hoehn and Yahr (H&Y) scale, described for the first time in 1967, is still a useful clinical tool used to assess the progression of motor symptoms.

The H&Y scale defines five main stages based on the level of severity of the impairment:

- Stage 1: Unilateral involvement only usually with minimal or no functional disability
- Stage 2: Bilateral or midline involvement without impairment of balance
- Stage 3: Bilateral disease: mild to moderate disability with impaired postural reflexes; physically independent
- Stage 4: Severely disabling disease; still able to walk or stand unassisted
- Stage 5: Confinement to bed or wheelchair unless aided

(Hoehn et al., 1967)

Since the Hoehn and Yahr scale primarily reflects disease severity based on postural instability and functional disability, its ability to capture the full spectrum of motor impairment, particularly during the intermediate stages of PD, is limited.

The Modified Hoehn and Yahr scale defines five main stages and introduces two intermediate stages:

- Stage 1: Unilateral involvement only
- Stage 1.5: Unilateral and axial involvement
- Stage 2: Bilateral involvement without impairment of balance
- Stage 2.5: Mild bilateral disease with recovery on pull test
- Stage 3: Mild to moderate bilateral disease; some postural instability; physically independent
- Stage 4: Severe disability; still able to walk or stand unassisted
- Stage 5: Wheelchair bound or bedridden unless aided

(Goetz et al., 2004)

A more comprehensive scale is the Unified Parkinson's Disease Rating Scale (UPDRS), which considers both motor and non-motor symptoms. This scale was first developed in the 1980s, but in the early 2000s the Movement Disorder Society (MDS) identified some critical issues and introduced a revised version to incorporate new scientific developments and to remove ambiguities.

Once officially revised, it was termed MDS-UPDRS and it is now widely used in clinical trials and therapeutic monitoring.

The MDS-UPDRS is structured in four parts, divided into thematic domains, each part includes items scored using standardized clinical ratings that can be answered with commonly accepted clinical terms (0 = normal, 1 = slight, 2 = mild, 3 = moderate, and 4 = severe) (Goetz et al., 2008).

These standardized assessment methods allow an objective identification of clinical patterns across different patient populations, essential to obtain a more precise diagnosis and a more precisely targeted treatment.

### 1.1.2 The prodromal phase

As mentioned previously, RBD is considered one of the most characteristic manifestations of the prodromal phase of PD. Other commonly observed features include hyposmia, chronic constipation, and depression (Postuma et al., 2015).

These manifestations are consistent with Braak's hypothesis of an early ascending progression of synuclein pathology, which may originate from the enteric nervous system or the olfactory bulb, and only later progress to the midbrain (Braak et al., 2003).

A better characterization of these symptoms and this phase could be helpful in establishing earlier therapeutic interventions and developing strategies aimed at slowing disease progression.

### 1.1.3 Sex differences in Parkinson's disease

Parkinson's disease is characterized by marked heterogeneity, with important sex-related differences in its epidemiology and clinical manifestation.

Epidemiological studies have shown that the incidence of PD is approximately twice as high in men as in women (Baldereschi et al., 2000).

Even though men represent the majority of PD patients, some studies suggest that women may experience distinct patterns of disease progression and mortality.

Sex-related differences have also been reported in the clinical presentation of PD. For example, tremor is more frequently described as the initial motor manifestation in women (Cerri et al., 2019). Conversely, muscle rigidity tends to be more severe in men, while in women the postural instability tends to be more severe (Baba et al., 2005).

Sex-related differences have also been observed in non-motor symptoms. Women more frequently presented with anxiety, depression and gastrointestinal dysfunction, whereas men seem to be at higher risk of cognitive decline, impulse control disorders, and REM sleep behavior disorder (RBD) (Cerri et al., 2019).

## 1.2 Etiology

Parkinson's disease is the second most common neurodegenerative disorder after Alzheimer's disease and represents the most common movement disorder. Its prevalence increases with age, affecting approximately 1% of individuals over 60 years of age (Pang et al., 2019).

The development of this disease is not commonly related to a single genetic or environmental element, but its etiology is mostly considered multifactorial. Although several genetic forms of the disease have been identified, most cases are sporadic or idiopathic PD, with no clearly identifiable underlying cause (Kitada et al., 2012).

Age is considered a primary risk factor in the development of Parkinson's disease, with its incidence increasing with advancing age (Pang et al., 2019).

### 1.2.1 Familial Parkinson's disease and genetic risk factors in sporadic cases

Although most PD cases are sporadic, around 10% are associated with a family history of the disease, highlighting the contribution of genetic mutations to the etiology of Parkinson's disease (Thomas et al., 2007).

Several chromosomal loci and genes have been associated with PD, although only a limited number are known to cause monogenic forms of the disease.

Among these genes, mutations in *SNCA*, which encodes α-synuclein, and *LRRK2* are associated with autosomal-dominant PD forms, while mutations in *PRKN*, *PINK1* and *PARK7*/*DJ-1* are associated with autosomal-recessive forms (Klein et al., 2012).

Mutations in *PINK1* and *PRKN*, encoding PTEN-induced kinase 1 (PINK1) and Parkin respectively, are linked to mitochondrial quality control mechanisms. The encoded proteins regulate the recognition and removal of damaged mitochondria, connecting PD genetic mutations to mitochondrial dysfunction (Pickrell and Youle, 2015).

The genetic component is also relevant in sporadic PD, as demonstrated by the association between variants in the *GBA1* gene and the disease. *GBA1* encodes glucocerebrosidase, responsible for hydrolyzing glucosylceramide (Sidransky et al., 2009). This association supports the role of lysosomal dysfunction in PD pathogenesis.

## 1.3 Pathophysiology

### 1.3.1 α-synuclein: structure and pathological conditions

Human α-synuclein is a presynaptic neuronal protein composed of 140 amino acids and encoded by the *SNCA* gene. Structurally, α-synuclein can be divided into three main regions: an N-terminal domain, a central hydrophobic region with high aggregation propensity, and a C-terminal domain (Rocha et al., 2018). The central hydrophobic region is also referred to as the NAC domain (non-amyloid-β-Component domain), and it has a high amyloidogenic potential, contributing to aggregate formation, particularly in the context of C-terminal truncation (Koga et al., 2021).

Although the precise physiological function of α-synuclein is not fully understood, it is thought to play a role in neurotransmitter release, synaptic activity, and synaptic plasticity (Koga et al., 2021). In physiological conditions, in the human brain, this protein is predominantly found as a monomer; however, it can undergo misfolding due to overexpression of the SNCA gene, missense mutations, or oxidative stress. This misfolding results in the assembly of α-Synuclein monomers into β-sheet-rich oligomers and amyloid fibrils (Fauvet et al., 2012).

A-synuclein is a major component of Lewy bodies (LBs), which represent an important histopathological hallmark of Parkinson's disease and are characterized by the accumulation of aggregated α-synuclein (Wakabayashi et al., 2007).

### 1.3.2 Neuroanatomical context

To understand the motor manifestations of Parkinson's disease, it is necessary to consider the neuroanatomical structures involved in motor control. Motor symptoms become evident when pathological processes affect midbrain structures, particularly the *substantia nigra*, leading to

significant dopaminergic neuronal loss and the transition to the clinical phase of the disease (Braak et al., 2003).

The name "*substantia nigra*" derives from the dark pigmentation of this region, which is mainly due to the presence of neuromelanin in dopaminergic neurons (Martini et al., 2018). In PD, this pigmentation is progressively reduced because of the selective degeneration of these neurons (Felten et al., 2016) (Fig.1)

Morphologically and functionally, the *substantia nigra* is divided into two regions: the *pars compacta* (SNpc) and the *pars reticulata* (SNpr). The latter is primarily composed of GABAergic neurons (Sonne et al., 2026).

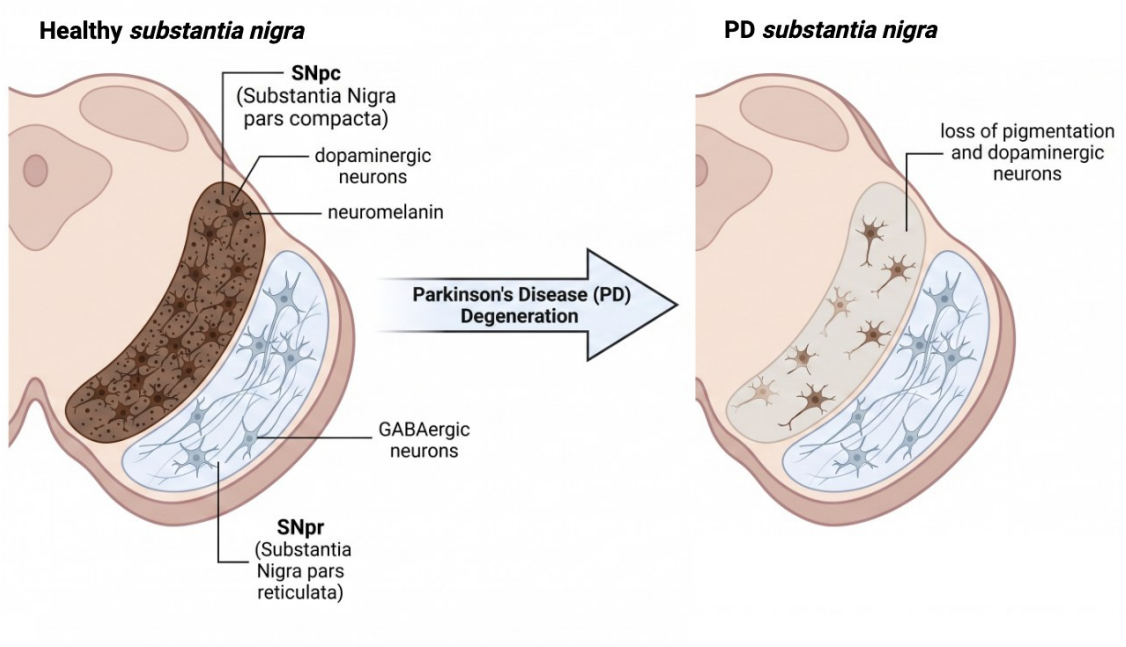


**Figure 1. Degeneration of dopaminergic neurons in the *substantia nigra* in Parkinson's Disease (created with BioRender).** In PD, the characteristic pigmentation of *substantia nigra* is progressively reduced because of the selective degeneration of dopaminergic neurons.

The *substantia nigra* is an important component of the basal ganglia, a group of subcortical nuclei involved in the selection, initiation, and modulation of movement (Felten et al., 2016).

In the motor circuit, the striatum represents the main input structure of the basal ganglia. It receives excitatory glutamatergic projections from the cerebral cortex and dopaminergic projections from the SNpc.

The main output nuclei of the basal ganglia are the internal segment of the *globus pallidus* (GPi) and the *substantia nigra pars reticulata* (SNpr). They consist of inhibitory GABAergic neurons, and they project primarily to the thalamus (Lanciego et al., 2012).

According to the canonical model for the motor circuit, motor output is mainly regulated through two functionally opposite systems: the direct and indirect pathways.

Dopaminergic input from SNpc exerts a facilitatory effect on direct pathway neurons in the striatum, and an inhibitory effect on indirect pathway neurons.

The direct pathway originates from D1 dopamine receptors (D1R)-containing striatal medium spiny neurons (MSNs), and it projects to the GPi and the SNpr. The activation of the direct pathway facilitates movement by inhibiting the basal ganglia output nuclei, reducing their inhibitory influence on the thalamus.

The indirect pathway originates from D2 dopamine receptor (D2R)-expressing striatal MSNs, and it reaches the GPi/SNpr through intermediate relay nuclei, the external segment of the *globus pallidus* (GPe) and the subthalamic nucleus (STN). The activation of this pathway suppresses movement through increased inhibitory influence of the output nuclei on the thalamus.

Therefore, dopamine exerts a dual effect on striatal MSNs, facilitating movement by promoting activity in the direct pathway while suppressing activity in the indirect pathway (Lanciego et al., 2012).

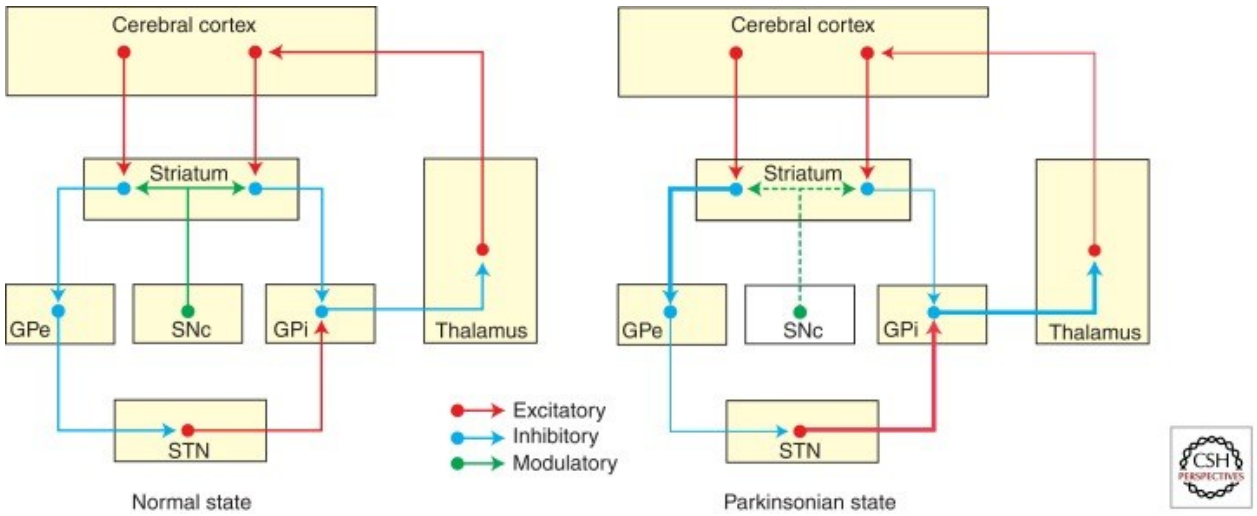


**Figure 2. Schematic comparisons between normal state and Parkinsonian state in the basal ganglia circuitry (Lanciego et al., 2012).** Dopaminergic projections from the SNpc regulate the balance between the direct and indirect pathways. In Parkinson's disease, loss of nigrostriatal dopaminergic input disrupts this balance, increasing inhibitory basal ganglia output to the thalamus and reducing thalamocortical motor activation.

For a correct motor coordination these projections are fundamental, and their degeneration contributes to the motor dysfunctions characteristic of Parkinson's disease and other movement disorders (Felten et al., 2016).

The reduced dopaminergic input decreases direct pathway facilitation and increases indirect pathway activity, leading to excessive inhibitory output from the basal ganglia to the thalamus. Consequently, thalamocortical motor activation is reduced, contributing to the development of motor symptoms such as bradykinesia and rigidity (Obeso and Lanciego, 2011).

### 1.3.3 Prion-like hypothesis

Regarding the progression of Parkinson's disease pathology, the prion-like hypothesis suggests that misfolded α-synuclein species may act as seeds promoting the spread of pathology. These pathological species can induce the misfolding and aggregation of normally folded α-synuclein in neighboring neurons, leading to a self-propagating mechanism (Goedert, 2015).

The propagation of these aggregates depends on a cycle of seeded nucleation, in which α-synuclein fibrils promote the formation of new pathological species and their subsequent propagation through fragmentation and generation of new active seeds (Marrero-Winkens et al., 2020).

A better understanding of α-synuclein spreading may support the development of new therapeutic strategies targeting protein misfolding, aggregation, and propagation (Walker et al., 2016). Overall, a better understanding of both neuroanatomical and molecular aspects is essential for characterizing disease progression and may contribute to the development of therapeutic approaches aimed at both symptom management and targeting the underlying pathological mechanisms.

### 1.3.4 Pathogenic mechanisms

Parkinson's disease pathogenesis is multifactorial and involves interconnected mechanisms including mitochondrial dysfunction, oxidative stress, neuroinflammation, autophagy-lysosomal dysfunction, unfolded protein response, and apoptotic pathways (Rocha et al., 2018) (Fig. 3).

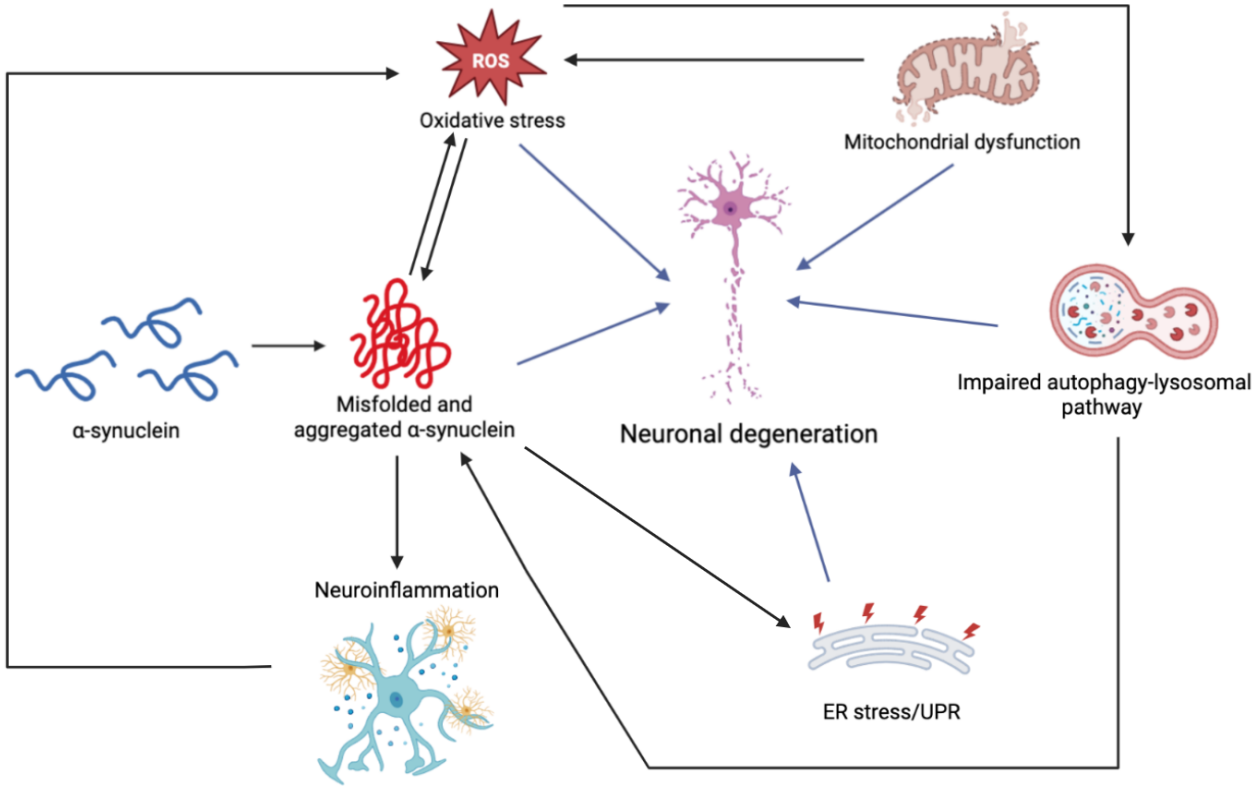


**Figure 3. Schematic representation of the interconnected pathogenic mechanisms in PD (created with BioRender).** Mitochondrial dysfunction, oxidative stress, α-synuclein aggregation, neuroinflammation, autophagy-

lysosomal dysfunction, ER stress/UPR activation, and apoptotic pathways interact in a self-amplifying network that contributes to dopaminergic neuronal degeneration in PD.

Under physiological conditions, mitochondria are involved in cellular processes, such as the regulation of calcium homeostasis, stress response and cell death pathways (Winklhofer and Haass, 2009).

Considering the important role that mitochondria play in maintaining cellular homeostasis, strict mitochondrial quality control mechanisms are required. This is supported by the involvement of PTEN-induced kinase 1 (PINK1) and Parkin, an E3 ubiquitin ligase, two proteins that mediate the removal of damaged mitochondria through mitophagy (Pickrell and Youle, 2015).

When mitochondrial quality control is impaired, the clearance of damaged organelles is reduced, and their accumulation may lead to neuronal damage in PD, with dopaminergic neurons being particularly vulnerable to mitochondrial dysfunction (Narendra et al., 2010) (Pacelli et al., 2015).

Mitochondria represent an important source of oxidative stress, as the electron transport chain located in their inner membrane can leak electrons, resulting in the formation of endogenous reactive oxygen species (ROS) (Dias et al., 2013).

Oxidative stress represents another important mechanism in PD pathogenesis. It occurs when ROS production exceeds the antioxidant capacity of the cell, damaging cellular macromolecules and possibly leading to cell death (Hauser and Hastings, 2012).

In normal cellular conditions, ROS production is regulated by antioxidant systems, including molecules and enzymes, that limit their accumulation, preventing oxidative damage (Gulcin, 2020).

The selective vulnerability of dopaminergic neurons of *substantia nigra pars compacta* may be partly explained by their unusually large axonal arborization and number of axon terminals, features that increase their energetic demand and may consequently increase their vulnerability to oxidative stress (Pacelli et al., 2015).

Oxidative stress is also associated with the promotion of α-synuclein misfolding and aggregation, which in turn increases ROS production, creating a vicious cycle that may further advance neurodegeneration (Dias et al., 2013).

Oxidative stress plays an indirect role in the formation of α-synuclein aggregates by shifting the ratio of ferrous ($Fe^{2+}$) to ferric ($Fe3^{+}$) iron towards the ferric oxidation state. This state is necessary for the generation and maintenance of α-synuclein oligomers (Levin et al., 2011).

The misfolding and aggregation of α-synuclein may also activate microglia, promoting neuroinflammatory responses and the release of pro-inflammatory cytokines, contributing to neurotoxicity (Rocha et al., 2018).

Aggregated or extracellular α-synuclein can act as a damage-associated molecular pattern (DAMP), activating inflammatory cascades via the nuclear factor kappa-light-chain enhancer of activated B

cells (NF-κB) pathway, which can contribute to the priming and subsequent activation of the NLRP3 inflammasome (Ferreira and Romero-Ramos, 2018).

Under physiological conditions, microglia maintain a homeostatic state, surveying the CNS and becoming activated in the presence of infectious agents and pathological stimuli. In the context of PD, the initial microglial response to α-synuclein aggregates can be considered protective, supporting neuronal survival (Araújo et al., 2022).

However, chronic microglial activation may contribute to neurodegeneration through the overproduction of pro-inflammatory cytokines and oxidative stress (Ferreira and Romero-Ramos, 2018).

Beyond activating neuroinflammatory responses, α-synuclein aggregation is also associated with disruption of proteostasis (Du et al., 2025).

One of the main systems responsible for α-synuclein degradation is the autophagy-lysosomal pathway. When this system is impaired, the pathological α-synuclein aggregations may accumulate, contributing to PD pathogenesis (Rocha et al., 2018).

In healthy cells, this pathway mediates the sequestration of damaged cellular components into autophagosomes, which then fuse with lysosomes to form autolysosomes. Within these structures, the sequestered substrates are degraded by lysosomal hydrolytic enzymes, allowing the recycling of cellular components (Nixon and Rubinsztein, 2024).

When either autophagic flux or lysosomal function is disrupted, accumulation of misfolded or aggregated proteins may occur. This alteration in proteostasis may contribute to disease-specific neurodegenerative mechanisms (Du et al., 2025).

In addition, the accumulation of misfolded proteins can induce endoplasmic reticulum (ER) stress and activate the unfolded protein response (UPR), which may contribute to apoptotic cell death when ER stress is chronic or unresolved (Cacabelos, 2017).

The ER is an important cellular organelle that contributes to protein synthesis, folding and post-translational modifications, calcium homeostasis, and lipid metabolism. Impairment of ER function may result in the accumulation of misfolded proteins, triggering ER stress (Costa et al., 2020).

To restore ER homeostasis, the UPR is activated as an adaptive mechanism aimed at reducing protein synthesis, promoting correct protein folding, and enhancing the degradation of misfolded proteins (Costa et al., 2020)

In PD pathogenesis, prolonged ER stress and UPR activation may become maladaptive mechanisms, promoting neuronal dysfunction and ultimately neurodegeneration (Colla, 2019).

Apoptotic pathways include intrinsic and extrinsic mechanisms, with the intrinsic mitochondrial pathway being particularly relevant in PD-related neuronal loss. In this pathway, persistent cellular stress can promote mitochondrial outer membrane permeabilization, cytochrome c release, and

activation of caspase-dependent signalling, ultimately leading to programmed cell death (Tatton et al., 2003).

Overall, these mechanisms interact with each other and contribute to the progressive degeneration of dopaminergic neurons in Parkinson's disease.

### 1.4 Therapeutic strategies

Treatments for Parkinson's disease range from conventional pharmacological approaches to more advanced therapeutic interventions. Conventional pharmacological treatment mainly relies on dopamine (DA) replacement strategies, including dopamine precursors such as levodopa (L-DOPA), dopamine agonists, monoamine oxidase B (MAO-B) inhibitors, and catechol-O-methyltransferase (COMT) inhibitors (Cacabelos et al., 2017).

Since as already mentioned, DA is fundamental to the proper functioning of the motor nervous system, its synthesis and degradation must be tightly regulated. On these grounds, one of the primary therapeutic approaches is represented by L-3,4-dihydroxyphenylalanine (L-DOPA), which is then converted into dopamine by aromatic L-amino acid decarboxylase (AADC) (Tai et al., 2024).

This treatment increases DA levels in the brain temporarily. It is usually effective in the management of motor symptoms but also comes with its adverse effects. Specifically, L-DOPA pharmacokinetics are highly variable between individuals, and this leads to increased doses for patients who could develop dyskinesias as a long-term complication of treatment over the years (Willemsen t al., 2010).

Dopamine agonists represent a possible alternative or adjunct to levodopa therapy, since they directly stimulate dopamine receptors and may be associated with a lower risk of some motor complications (Kulisevsky et al., 2018).

They act by stimulating DA receptors in the striatum, in particular D2 and D3 receptors are the main therapeutic targets (Jing et al., 2023).

Monoamine oxidases exist as two isoforms, MAO-A and MAO-B, both involved in the metabolism of monoamine neurotransmitters, including dopamine (Tan et al., 2022).

In PD, MAO-B inhibitors reduce dopamine degradation, thereby increasing dopamine availability in the synaptic space (Chen et al., 2005).

Specifically in Parkinson's disease MAO-B inhibitors like selegiline and rasagiline seem to have a good efficacy in the treatment of PD patients in both early and advanced stages of the disease (Binde et al., 2018).

COMT catalyzes the conversion of L-DOPA into 3-O-methyl-DOPA (3-OMD) both in the periphery and in the CNS. Its activity leads to decreased levels of levodopa that will also have to compete with the product of this reaction during the transportation across the blood-brain barrier (BBB), worsening the motor functions of the patients (Regensburger et al., 2023).

COMT inhibitors like tolcapone, entacapone, and opicapone act by increasing the amount of levodopa crossing the BBB and by reducing the competition between L-DOPA and 3-OMD (Bonifacio et al., 2012).

Over the past decades, deep brain stimulation (DBS) has become an established advanced therapy for selected patients with PD. DBS consists of delivering controlled electrical stimulation to specific brain targets in order to modulate dysfunctional neural circuits (Jourdain et al., 2014).
The subthalamic nucleus is one of the most commonly targeted brain structures in DBS for Parkinson's disease because of its central role in basal ganglia motor circuits. DBS is traditionally considered suitable for a selected patients with advanced PD, that show motor fluctuations, dyskinesias secondary to chronic levodopa and marked tremor.
However, more recent studies have demonstrated that carefully selected patients in earlier stages of PD may also benefit from DBS (Lozano et al., 2019).

Overall, the current therapeutic strategies can improve motor symptoms and the quality of life, but they mainly act at the symptomatic level without directly preventing the progression of neurodegeneration. For this reason, new studies are focusing on exploring more the biological mechanisms underlying Parkinson's disease progression, including neuroinflammation and oxidative stress. A better understanding of these processes could help identify new therapeutic targets.

### 1.5 Biomarkers

Today, considerable efforts are being made to identify reliable biomarkers that can complement the clinical assessment of motor and non-motor symptoms. These biomarkers may help support the diagnosis of Parkinson's disease, enable its earliest possible detection, monitor disease progression, and evaluate the effects of therapeutic interventions (Parnetti et al., 2019).
Among the most extensively studied potential biomarkers for PD, α-synuclein is of particular interest, since it reflects a key pathological feature of the disease. Different α-synuclein species, including misfolded, oligomeric, phosphorylated α-synuclein at residue Ser129, and aggregated forms, can be investigated in biological fluids such as cerebrospinal fluid (CSF) and blood, even in early stages of the disease (Parnetti et al., 2019).
Neurofilament light chain (NfL) is a well-established biomarker of neurodegeneration and has been investigated as a prognostic biomarker in PD. NfL is a neuronal cytoskeletal protein released following axonal damage and is useful to assess the presence of neurodegeneration. However, since it is not specific to PD, its role is mainly associated with prognosis, disease monitoring, and differential diagnosis (Gaetani et al., 2019).

# 2. Neuroinflammation

## 2.1 Neuroimmunology

For decades, the brain was considered an immunologically privileged organ, meaning that it was considered isolated from immune surveillance and protected from peripheral immune activity. However, this view has been revised, and the central nervous system (CNS) is now considered immunologically distinct, with its own finely regulated immune environment (Glass et al., 2010).

The primary innate immune response in the CNS is carried out by microglia, the resident immune cells of the brain and spinal cord. Under physiological conditions, microglia remain in a homeostatic state and are involved in the surveillance of the surrounding microenvironment, as well as in synaptic pruning. In contrast, under pathological conditions, microglia can become activated and release cytotoxic molecules, including pro-inflammatory cytokines and reactive oxygen species (ROS).

Astrocytes also contribute to this process. Under physiological conditions, they support neuronal survival and help maintain CNS homeostasis, whereas under pathological conditions they may become reactive and lose some of their neuroprotective functions (Kędzia et al., 2026).

In addition to the innate immune response of glia cells, the brain also interacts with the peripheral adaptive immune system.

The blood-brain barrier (BBB) prevents large molecules or cells from entering the CNS, protecting the brain, but in pathological conditions BBB permeability can be increased, allowing activated lymphocytes to infiltrate the brain parenchyma (Wraith et al., 2012).

Once these adaptive immune cells enter the CNS, they can interact with resident cells, such as microglia and astrocytes, either amplifying or regulating the neuroinflammatory response depending on the immune cell subtype involved (Almolda et al., 2015).

Regulatory T cells (Tregs), a subtype of adaptive immune cells, play a role in the resolution of neuroinflammation in CNS by inhibiting of the activation of the microglia (microgliosis) and by suppressing the reactive state of astrocytes (astrogliosis) through the secretion of specific anti-inflammatory soluble factors or through direct contact-dependent mechanisms (Harkins et al., 2022).

Cytokines are key mediators of neuroimmune communication: they are small signalling proteins that can have a pro-inflammatory role or an anti-inflammatory role.

Major pro-inflammatory cytokines include tumor necrosis factor-α (TNF-α), interleukin-1beta (IL-1β) and IL-6, that help the defensive response, but that could also become part of the pathogenesis of the disease.

Conversely, anti-inflammatory cytokines include IL-10 and transforming growth factor-beta (TGF-β), which contribute to neuroprotection within the CNS (Khan et al., 2023).

In addition to cytokines, neuroinflammatory processes can also be characterized through molecules related to glial activation and neuron–glia communication, including Fractalkine, also known as chemokine C-X3-C motif ligand 1 (CX3CL1), triggering receptor expressed on myeloid cells 2 (TREM2) and S100B.

Fractalkine is a chemokine expressed primarily by neurons in the CNS, and its receptor CX3CR1 is expressed by microglia. The interaction between CX3CL1 and its receptor is involved in the regulation of microglial activity (Chu et al., 2025).

TREM2 is also expressed by microglia, and it's an immunoglobulin-like receptor that contributes to microglia proliferation, survival, activation, and phagocytosis. TREM2 deficiency has been associated with enhanced NLRP3 inflammasome activation (Huang et al., 2024).

S100B is an astrocytic calcium-binding protein, and its high levels have been associated with neurodegenerative and neuroinflammatory diseases (Sathe et al., 2012).

### 2.1.1 Neuroinflammation and its implications in Parkinson's disease

As previously stated, microglia play a primary role in neuroinflammatory responses, and this is relevant also in Parkinson's disease, where the microglia activation can be triggered by the presence of misfolded α-synuclein aggregates.

Initially, microglia promote neuronal survival, but when this activation becomes chronic or excessive, it can contribute to the conditions leading to neuronal dysfunction and degeneration.

Activated microglia can be polarized into two different phenotypes: M1-like and M2-like (Araújo et al., 2022).

The M1-like phenotype is associated with the release of neurotoxic factors, usually pro-inflammatory cytokines such as TNF-α, IL-1β, IL-2 and IL-6, but also reactive oxygen species (ROS), which contribute to oxidative stress.

Differently, the M2-like subtype has an anti-inflammatory role, which supports neuroprotection and tissue repair (Araújo et al., 2022).

In the context of Parkinson's disease, chronic microglial activation tends to shift toward a predominantly pro-inflammatory phenotype. This imbalance can lead to increased dopaminergic neuronal damage, contributing to disease progression. Conversely, the promotion of the M2-like subtype has been associated with increased neuronal survival (Castro et al., 2022).

The balance between these activation states is regulated by a network of transcription factors that modulate microglia responses to microenvironmental cues.

The pro-inflammatory M1-like state is associated with the activation of the nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB), which, following activation by inflammatory stimuli, including signals induced by misfolded α-synuclein aggregates, translocates into the nucleus, where it induces the expression of target genes involved in inflammation, including pro-inflammatory cytokines and enzymes involved in inflammatory processes, such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) (Alrouji et al., 2023). (Lawrence, 2009).

Activation of the transcription factor NF-κB has been observed in the central nervous system in animal models and patients affected by neurodegenerative diseases, supporting its involvement in Parkinson's disease pathogenesis (Bassani et al., 2015; Ghosh et al., 2007).

Signal transducer and activator of transcription 1 (STAT1) and activator protein-1 (AP-1) also contribute to the inflammatory signalling process (Kim et al., 2013).

Following the transcriptional induction of pro-inflammatory mediators, a critical step in the M1-like response is the assembly of the NLRP3 inflammasome, an intracellular multiprotein complex of the innate immune system, that mediates caspase-1 activation and the secretion of pro-inflammatory cytokines IL-1β and IL-18, sustaining the pro-inflammatory signals (Kelley et al., 2019).

NLRP3 inflammasome activation generally requires two steps: priming and activation. The priming phase involves the engagement of inflammatory receptors, such as Toll-like receptors, TNF receptors, or IL-1 receptors, leading to NF-κB-dependent transcription of inflammasome-related components. The activation step can be triggered by several stimuli, including potassium ($K^+$) efflux from the cell, mitochondrial dysfunction, or lysosomal damage (Pike et al., 2022).

Conversely, the neuroprotective M2-like response is associated with the activation of transcriptional regulators, such as signal transducer and activator of transcription 6 (STAT6) and peroxisome proliferator-activated receptor gamma (PPARγ) (Szanto et al., 2010).

In addition to immune-mediated responses, cells of the CNS can activate endogenous protective mechanisms in response to oxidative and metabolic stress. Among these, the nuclear factor-erythroid 2-related factor 2 (Nrf2) pathway plays a crucial role in maintaining cellular redox homeostasis and protecting cells from oxidative stress damages (Johnson et al., 2008).

### 2.2 Nrf2

Nrf2 is a cap 'n' collar (CNC) basic leucine zipper (bZIP) transcription factor that plays a central role in the cellular response to oxidative and electrophilic stress. It is widely described as a major regulator of the antioxidant response, since oxidative and electrophilic stress promote Nrf2 stabilization, nuclear translocation, and transcriptional activity (Bellezza et al., 2018).

Human Nrf2 consists of 605 amino acids and contains seven highly conserved functional domains known as Nrf2-ECH homology domains, or Neh domains, numbered from Neh1 to Neh7 (Saha et al., 2020) (Fig. 4).


**Figure 4. Schematic representation of the Nrf2 domain structure (modified from Saha et al., 2020).** Nrf2 contains seven conserved Neh domains, each involved in specific aspects of protein regulation, DNA binding, transcriptional activation, or degradation.

The Neh1 domain contains the CNC and bZIP region, which is required for DNA binding and heterodimerization with small Maf (musculoaponeurotic fibrosarcoma oncogene homolog) proteins, as well as nuclear localization signals involved in Nrf2 nuclear translocation (Yamamoto et al., 2018).

The Neh2 domain is crucial for the regulation of Nrf2. This N-terminal domain contains two peptide binding motifs (ETGE and DLG), which interact with Kelch-like ECH- associated protein 1 (Keap1), the main intracellular regulator of Nrf2.

The C-terminal Neh3 domain contributes to Nrf2 transcriptional activity by interacting with transcriptional co-activators (Saha et al., 2020).

Neh4 and Neh5 domains have a role in the control of transcriptional transactivation, interacting with cAMP Responsive Element Binding protein (CREB)-Binding Protein (CBP) (Katoh et al., 2001).

The Neh6 domain is a Keap1-independent regulatory region containing a degradation signal for Nrf2. Under oxidative stress conditions, phosphorylation by glycogen synthase kinase 3 (GSK-3) facilitates Nrf2 degradation (Yamamoto et al., 2018).

The Neh7 domain inhibits Nrf2-ARE signalling pathway by promoting the binding of Nrf2 to the retinoic X receptor α (RXRα) (Saha et al., 2020).

### 2.2.1 Nrf2 pathway and regulation

Under basal conditions, Nrf2 is sequestered in the cytoplasm by its main intracellular regulator, Keap1, which acts as an adaptor protein for the Cullin 3/RBX1 E3 ubiquitin ligase complex, promoting Nrf2 ubiquitination and subsequent proteasomal degradation (Kobayashi et al., 2004).

Keap1 binds the ETGE and DLG motifs within the Neh2 domain of Nrf2 through its two glycine repeat domains/Kelch (DGR), with the ETGE motif displaying a higher binding affinity than the DLG motif (Bellezza et al., 2018).

Under oxidative or electrophilic stress, modification of reactive cysteine residues in Keap1 disrupts the low-affinity DLG interaction while the ETGE interaction is maintained, thereby preventing Nrf2 ubiquitination and degradation (Taguchi et al., 2011) (Kansanen et al., 2012) (Fig. 5).

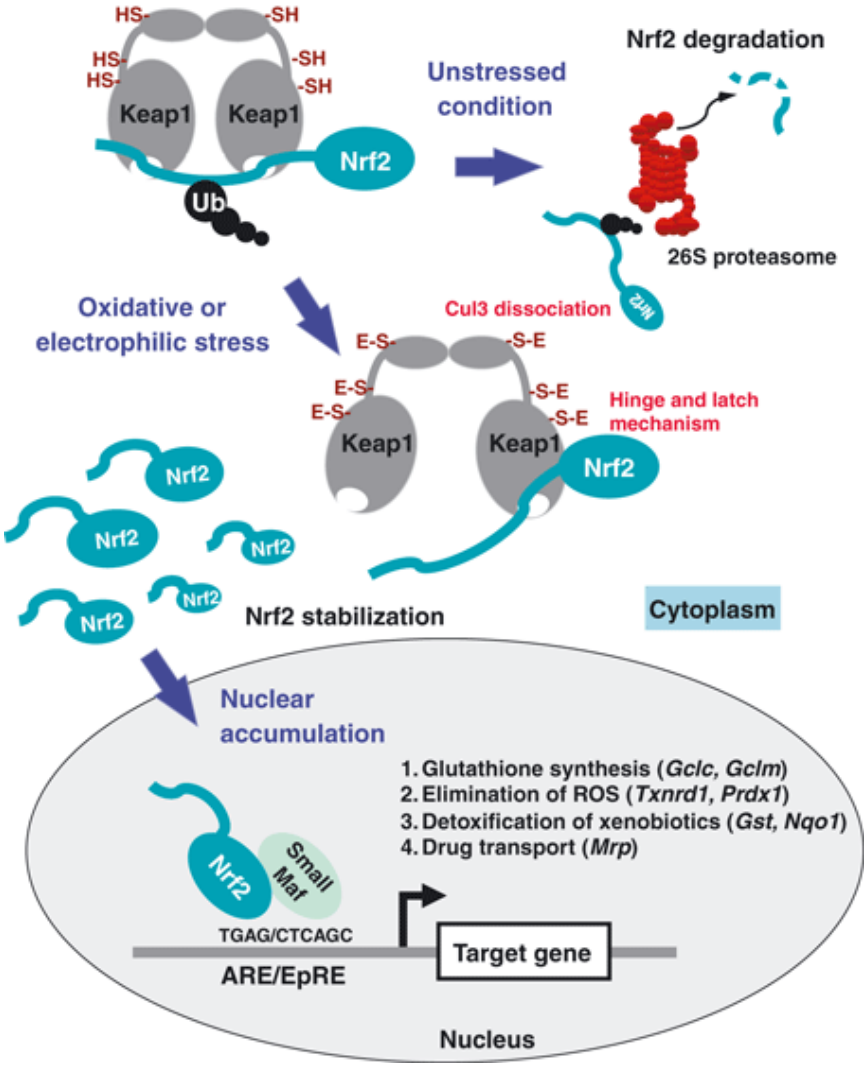


**Figure 5. Schematic representation of the Keap1–Nrf2 pathway.** (Taguchi et al., 2011) Under oxidative or electrophilic stress, modification of reactive cysteine residues in Keap1 prevents Nrf2 ubiquitination and degradation, inducing the expression of cytoprotective genes involved in antioxidant defense.

As a result, Nrf2 escapes proteasomal degradation, translocates into the nucleus, and heterodimerizes with small Maf proteins. The heterodimers bind antioxidant response elements (AREs) within the promoter regions of target genes involved in the response to oxidative stress, thereby maintaining redox homeostasis in cells and tissues.

Activation of this pathway induces the expression of several cytoprotective genes, including heme oxygenase-1 (HO-1), one of the major antioxidant and anti-inflammatory downstream proteins of the Nrf2 pathway (Saha et al., 2020).

Another relevant downstream effector is superoxide dismutase-1 (SOD1), an antioxidant enzyme involved in the detoxification of superoxide radicals, and in the maintenance of redox homeostasis (Zhuang et al., 2019).

Moreover, Nrf2 can negatively regulate NF-κB signaling, a major pathway involved in the regulation of inflammatory responses, through multiple mechanisms, including the reduction of reactive oxygen species (ROS) levels and the inhibition of NF-κB nuclear translocation and downstream inflammatory responses. Therefore, a reciprocal regulatory interaction between these two transcription factors has been proposed (van der Horst et al., 2022).

### 2.3 Anti-inflammatory therapeutic strategies

Targeting neuroinflammatory mechanisms involved in dopaminergic neuronal dysfunction and degeneration represents a potential therapeutic approach in Parkinson's disease.

#### 2.3.1 Therapies targeting Nrf2: DMF

Dimethyl fumarate (DMF), an FDA-approved drug for multiple sclerosis and psoriasis (Valencia-Sanchez et al., 2019) (Fig. 6), is a methyl ester derivative of fumaric acid that can activate Nrf2 signaling, promoting the expression of antioxidant, cytoprotective, and anti-inflammatory genes (Campolo et al., 2017).

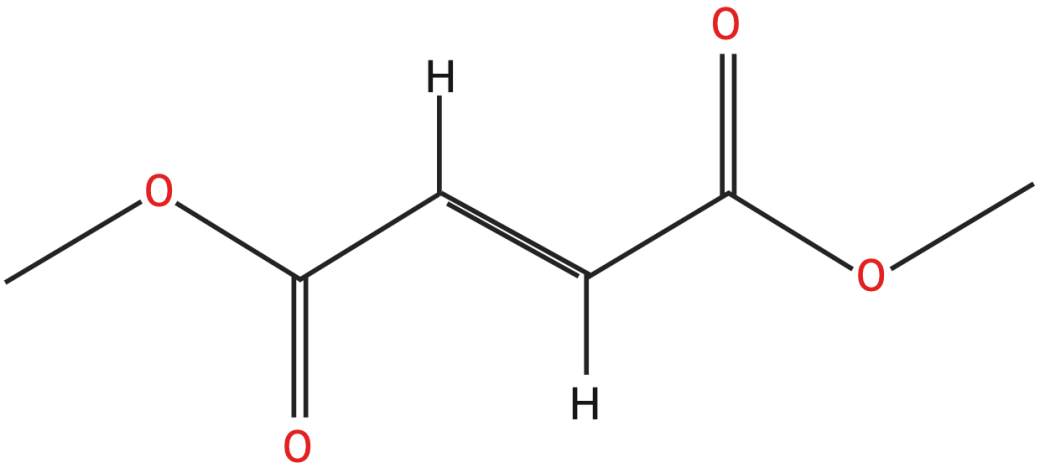


**Figure 6. Chemical structure of dimethyl fumarate (created with BioRender)**

DMF-mediated Nrf2 activation may occur through Keap1-dependent mechanisms and Keap1-independent pathways involving glycogen synthase kinase 3 beta (GSK-3β) signaling (Rosito et al., 2020).

In the first scenario, upon administration, DMF acts as an electrophilic compound that covalently modifies reactive cysteine residues in Keap1, increasing Nrf2 accumulation and transcriptional pathway activation. Through this mechanism, DMF contributes to the regulation of oxidative stress and neuroinflammation in the CNS.

In a neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-mouse model of PD, DMF treatment was shown to reduce microglia activation and neuroinflammatory markers while enhancing Nrf2 signaling (Campolo et al., 2017).

Furthermore, mitochondrial dysfunction is a key mechanism involved in PD pathogenesis. Preclinical studies suggest that DMF may exert mitochondrial protective effects by promoting mitophagy and reducing mitochondrial oxidative stress (Salvadè and Gardoni, 2025).

DMF may represent a promising therapeutic strategy in PD by modulating oxidative stress and neuroinflammatory pathways, although further rigorously designed clinical studies are required. Future research will be essential to clarify its potential as a disease-modifying strategy and to evaluate the role of drug repurposing approaches in the development of novel therapeutic interventions for neurodegenerative disorders (Salvadè and Gardoni, 2025).

### 2.3.2 Therapies targeting NF-κB

Since NF-κB activation contributes to the priming phase of NLRP3 inflammasome assembly by inducing the transcription of inflammasome-related components, modulation of this pathway may represent a possible therapeutic approach to reduce neuroinflammatory processes associated with PD progression (Kelley et al., 2019).

Moreover, NF-κB signaling pathway promotes the transcription of several inflammatory mediators, including cytokines such as TNF-α, IL-6 and the precursor form of IL-1β (Mussbacher et al., 2023).

In the canonical NF-κB pathway, activation of I*κ*B kinase (IKK) complex, composed of the catalytic subunits IKKα and IKKβ and the regulatory subunit IKKγ, also known as NF-κB essential modulator (NEMO), phosphorylates the inhibitory protein I*κ*B*α*. This process promotes IκBα ubiquitination and subsequent proteasomal degradation, allowing NF-κB translocation into the nucleus and activation of inflammatory gene transcription (Flood et al., 2011).

Given the implication of IKKα and IKKβ in inflammatory signaling, selective inhibitors targeting these kinases have been proposed as potential therapeutic strategies to limit NF-κB-mediated neuroinflammation and protect dopaminergic neurons (Zhang et al., 2010).

However, NF-κB also regulates several physiological cellular functions, including immune responses and cell survival. Therefore, therapeutic strategies targeting NF- κB should aim at the controlled modulation of NF-κB signaling rather than its complete inhibition, in order to preserve its physiological functions (Liu T et al., 2017).

## 3. Oxidative stress

Oxidative stress is defined as an imbalance between oxidant production and antioxidant defense mechanisms, resulting in alterations in redox signaling and, when excessive or prolonged, in molecular damage (Sies, 2015).

Oxidative damages can affect proteins, membrane lipids, and nucleic acids, compromising cellular functions. Therefore, efficient antioxidant and repair systems are essential for maintaining cellular homeostasis, particularly in tissues with high metabolic demands such as the central nervous system (Davies, 2000).

### 3.1 Reactive oxygen and nitrogen species

Reactive oxygen species and reactive nitrogen species (RNS) are chemically reactive molecules generated during cellular metabolism. They include both free radicals, which contain unpaired electrons, and non-radical molecules with oxidizing properties. When their production exceeds the capacity of antioxidant systems to control them, their accumulation can induce oxidative damage to biological structures and contribute to cellular dysfunction (Weidinger and Kozlov, 2015).

The main ROS include the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide ($H_2O_2$) and hydroxyl radicals ($OH^{\bullet}$) (Zhang et al., 2022).

$O_2^{\bullet -}$ is one of the primary ROS generated in biological systems and can arise from the one-electron reduction of molecular oxygen, particularly during mitochondrial electron transport (Brand, 2016). In mammalian cells, superoxide is mainly converted by superoxide dismutase (SOD), which catalyzes its dismutation into $H_2O_2$ (Palma et al., 2020).

$H_2O_2$ is generated mainly through the dismutation of superoxide, either spontaneously or through SOD activity. Although less reactive than radical species, $H_2O_2$ is more stable and can diffuse across membranes, allowing it to act both as a signaling molecule and as a precursor of more reactive species (Veal et al., 2007).

Hydroxyl radicals ($OH^{\bullet}$) are among the most reactive ROS and can be generated from hydrogen peroxide through metal-catalyzed reactions, such as the Fenton reaction. Because of their high reactivity, they can rapidly damage nearby biomolecules, including proteins, lipids and nucleic acids (Michiels et al., 2004).

Among RNS, nitric oxide (NO) is particularly relevant in the nervous system. Under physiological conditions, NO acts as a signaling molecule and participates in the regulation of neuronal and vascular functions. However, during oxidative stress and inflammation, NO can react with superoxide anion to form peroxynitrite ($ONOO^-$), a highly reactive molecule associated with nitrosative stress (Pacher et al., 2007). Peroxynitrite can promote protein nitration, mitochondrial dysfunction, and neuronal damage, processes that are particularly relevant in Parkinson's disease pathogenesis (Stykel and Ryan, 2022).

### 3.2 Sources of ROS generation

Reactive oxygen species can be generated from both endogenous and exogenous sources. Endogenous ROS production is particularly relevant in cells with high metabolic demands, such as neurons, because these cells strongly depend on mitochondrial oxidative phosphorylation for ATP production (Lushchack et al., 2021).

#### 3.2.1 Mitochondrial ROS production

One of the main endogenous sources of ROS is the mitochondrial electron transport chain (ETC). During cellular respiration, electrons are transferred through the ETC to generate the proton gradient required for oxidative phosphorylation. However, a small fraction of electrons can prematurely react with molecular oxygen, leading to the formation of superoxide anion ($O_2^{\bullet -}$) (Teleanu et al., 2022).

The ETC is composed of four transmembrane protein complexes (complexes I –IV), together with two mobile electron carriers, ubiquinone and cytochrome c. Complex V, also known as $F_1F_0$ ATP synthase, uses the proton gradient generated by electron transport to produce ATP (Zhang et al., 2022).

In Parkinson's disease, defects in mitochondrial quality control, including PINK1/Parkin-mediated mitophagy, can promote the accumulation of dysfunctional mitochondria. In the context of oxidative stress, this is relevant because damaged mitochondria can increase electron leakage from the ETC and promote ROS production (Pickrell and Youle, 2015; Blesa et al., 2015).

Exogenous sources, including radiation, environmental toxins, and some drugs, can further contribute to ROS production, although endogenous sources are particularly relevant in neurodegenerative diseases (Kim et al., 2015).

Under physiological conditions, ROS production is controlled by antioxidant systems. However, mitochondrial dysfunction can increase electron leakage can increase, promoting oxidative stress and cellular dysfunction (Brand, 2016).

In Parkinson's disease, mitochondrial ROS production has particular relevance because mitochondrial dysfunction strongly contributes to the selective vulnerability of dopaminergic neurons. Increased ROS generation may promote oxidative damage, α-synuclein aggregation and activation of neuroinflammatory pathways, creating a vicious cycle in which mitochondrial impairment and oxidative stress amplify each other (Blesa et al., 2015).

#### 3.2.2 Dopamine metabolism and neuroinflammation-derived ROS

Although mitochondria represent a major source of ROS, additional mechanisms contribute to oxidative stress in Parkinson's disease.

Dopamine metabolism represents another important contributor, as dopamine degradation is associated with the generation of ROS that may contribute to the loss of dopaminergic neurons. Monoamine oxidase-mediated dopamine metabolism produces hydrogen peroxide, while dopamine oxidation can lead to the formation of dopamine quinones. All these processes can disrupt neuronal redox homeostasis and contribute to pathophysiology (Meiser et al., 2013).

Neuroinflammation can also contribute to oxidative and nitrosative stress. Activated microglia can release various pro-inflammatory cytokines and free radicals, amplifying neurodegeneration (Arimoto and Bing, 2003).

In particular, the simultaneous production of superoxide and nitric oxide can promote the formation of peroxynitrite, a highly reactive species that can induce protein nitration. This mechanism is relevant in Parkinson's disease, since peroxynitrite-mediated nitration has been associated with α-synuclein aggregation (Souza et al., 2000).

Consistently, studies observed that nitrated α-synuclein could be detected in Lewy bodies found in PD patients' *substantia nigra*, suggesting a potential link between inflammatory processes, oxidative damage, and α-synuclein pathology (Murray et al., 2003).

### 3.3 Antioxidant defence mechanisms

Antioxidant molecules and enzymes contribute to the maintenance of cellular redox homeostasis by limiting the accumulation of reactive species and preventing oxidative damage (Gulcin, 2020).

Among the major antioxidant enzymes, superoxide dismutase (SOD) plays a central role by catalyzing the conversion of superoxide anion into hydrogen peroxide.

SOD metalloenzymes are present in different cellular compartments: Cu, Zn-SOD (or SOD1) is mainly localized in the cytosol and in the mitochondrial intermembrane space, Mn-SOD (or SOD2) is located in the mitochondrial matrix, and the inner membrane, and Cu,Zn-SOD (or SOD3) is found in the extracellular compartment (Fridovich et al., 1995).

Since $H_2O_2$ can become toxic when accumulated, it must be further detoxified by antioxidant enzymes such as catalase and glutathione peroxidase (GPx). Catalase breaks it down into water and molecular oxygen (Ighodaro and Akinloye, 2018).

GPx reduces $H_2O_2$ using reduced glutathione (GSH) as an electron donor, generating oxidized glutathione disulfide (GSSG), which can be regenerated by glutathione reductase to maintain the cellular antioxidant capacity (Dringen, 2000).

In addition to antioxidant enzymes directly involved in ROS detoxification, such as SOD and GPx, other cytoprotective systems contribute to the cellular response to oxidative stress (Ighodaro and Akinloye, 2018)(Saha et al., 2020).

Among these, heme oxygenases (HO) act as rate-limiting enzymes in the degradation of heme. Heme degradation generates carbon monoxide (CO), biliverdin (BV) and free ferrous ions ($Fe^{2+}$) (Wu and Hsieh, 2022).

CO acts as signalling molecule and may play a role in anti-inflammatory processes, while biliverdin is converted to bilirubin that can play a role as antioxidant (Wunder and Potter, 2003).

Since $Fe^{2+}$ may promote the generation of free radicals within the mitochondrial and other cellular compartments promoting oxidative damage, the cytoprotective role of the HO system also depends on efficient iron sequestration by ferritin (Zukor et al., 2009) (Loboda et al., 2016).

Among the mammalian HO isoforms, HO-1 is the inducible isoform and one of the major downstream targets of the Nrf2 pathway under oxidative stress conditions (Saha et al., 2020).

### 3.4 Oxidative stress in Parkinson's disease

Oxidative stress plays an important role in neurodegenerative diseases. Although numerous studies reported that ROS may not represent the initial trigger of neurodegeneration, their accumulation could aggravate disease progression by promoting cellular damage and affecting multiple pathological pathways. Due to their high oxygen consumption and limited antioxidant defence, neurons are particularly vulnerable to oxidative stress (Liu Z et al., 2017).

In Parkinson's disease patients, dopaminergic neurons of the *substantia nigra pars compacta* are particularly susceptible to oxidative damage due to the combined effects of mitochondrial dysfunction, dopamine metabolism, and neuroinflammatory responses (Blesa et al., 2015).

Maintaining redox homeostasis is therefore essential for neuronal survival, whereas its disruption may contribute to neurodegeneration in Parkinson's disease (Dias et al., 2013).

Moreover, oxidative stress is also interconnected with other pathological mechanisms involved in Parkinson's disease. Reactive species can promote α-synuclein misfolding and aggregation, which in turn may further impair mitochondrial function and exacerbate neuroinflammatory responses. Overall, the interaction between these mechanisms creates a self-amplifying cycle that contributes to neuronal dysfunction and loss.

### 3.5 Antioxidant therapeutic strategies

Given the central role of oxidative in Parkinson's disease pathogenesis, several therapeutic approaches have been investigated to reduce oxidative damage and preserve neuronal survival.

Among the molecules investigated, coenzyme Q10 (CoQ10), also known as ubiquinone, has attracted considerable interest because of its dual role in mitochondrial bioenergetics and antioxidant defense (Garrido-Maraver et al., 2014).

CoQ10 is an essential component of the mitochondrial electron transport chain, where it participates in oxidative phosphorylation and ATP production. In addition, it exerts antioxidant activity by limiting lipid peroxidation and protecting cellular membranes from oxidative damage (Crane, 2001). Preclinical studies have observed neuroprotective capacity of CoQ10 in both in vitro and in vivo models of Parkinson's disease (Garrido-Maraver et al., 2014).

Another possible therapeutic approach aims to restore glutathione homeostasis.

Studies have reported significant reduced glutathione (GSH) depletion in PD patients. Consequently, glutathione replacement has been investigated as a potential therapeutic strategy to preserve dopaminergic function by limiting oxidative stress and mitochondrial dysfunction (Pearce et al., 1997).

Overall, antioxidant-based approaches remain promising, but their clinical efficacy in Parkinson's disease is not fully established. Further studies are required to determine whether targeting oxidative stress can provide meaningful disease-modifying benefits in patients with Parkinson's disease.

# Aim of the work

Parkinson's disease is characterized by interconnected pathogenic mechanisms, including α-synuclein misfolding and aggregation, neuroinflammation and oxidative stress, which may also be reflected by peripheral molecular alterations.

Therefore, the aim of this thesis was to investigate potential peripheral biomarkers associated with these pathogenic processes in patients with sporadic Parkinson's disease across different disease stages, with particular attention to sex-related differences.

Particular attention was paid to the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), a master regulator of cellular antioxidant and cytoprotective responses that has emerged as a promising pharmacological target and a potential candidate for drug repurposing strategies.

The study included healthy controls and patients with sporadic Parkinson's disease. Peripheral blood mononuclear cells (PBMCs) and plasma were isolated from peripheral blood samples and used for molecular analyses.

Specifically, the protein expression of Nrf2 and its downstream antioxidant targets, superoxide dismutase 1 (SOD1) and heme oxygenase-1 (HO-1), was evaluated in PBMCs.

In parallel, circulating inflammatory and neurodegeneration-related proteins were analyzed in plasma samples to identify peripheral molecular signatures associated with Parkinson's disease across different clinical stages.

Overall, this work aimed to explore the potential relevance of Nrf2 and its downstream targets as candidate disease-associated biomarkers.

# Materials and Methods

## 1. Subjects

This study aims at covering different stages of disease progression, from the very early phase, in which the recruitment of drug-naïve patients allows to rule out a possible bias due to dopaminergic treatment, to the advanced stage, which is featured by the appearance of motor fluctuations and dyskinesias.

The study recruited in total 150 subjects, specifically 109 PD patients and 41 healthy controls (CTR). The participants were all recruited at the IRCCS Mondino Foundation, National Institute of Neurology (Pavia, Italy).

Age- and gender-matched healthy subjects without history or evidence of neurological disorders will be recruited among non-blood relatives of PD patients.

Clinically established PD patients were diagnosed according to the Movement Disorder Society (MDS) diagnostic criteria and were divided into three subgroups according to their modified Hoehn and Yahr stage:

1. **Early stage**: newly diagnosed ("de novo") disease, no history of dopaminergic treatment, and H&Y stage = 1;
2. **Moderate stage**: H&Y stage ≤ 2;
3. **Advanced stage**: H&Y stage ranging from 2.5 to 3.5, and evidence of motor fluctuation.

Patients with genetic PD forms (associated with PD-linked genes or risk factors), essential tremor, atypical or secondary parkinsonian syndromes, H&Y ≥ 4 or dementia diagnosed according to the DSM-IV criteria were excluded.

Other exclusion criteria were applied to both patients and healthy controls to minimize potential confounding factors that could affect peripheral inflammatory and oxidative stress markers. These included: diabetes; cancer and chemotherapy (past and ongoing); hematological diseases; alcoholism; main renal, respiratory, hepatic and endocrine diseases; Body Mass Index (BMI) > 29; acute infectious, inflammatory or cardiovascular diseases, other chronic metabolic, inflammatory or cardiovascular diseases; current smokers; current treatment with N-acetylcysteine and/or antioxidants; dietary restrictions.

## 2. Isolation of PBMCs

Peripheral whole blood samples were collected from both patients and healthy controls in EDTA blood collection tubes and processed on the same day of collection.

The separation between plasma and the cellular fraction was obtained through centrifugation at 1000xg at room temperature for 20 minutes.

The plasma was then collected, and cellular debris was removed by centrifugation at 1600xg at room temperature for 20 minutes. The supernatant was then transferred into fresh tubes and stored at -80°C.

The cellular fraction were resuspended in 1X phosphate-buffered saline (PBS), to restore the original aqueous volume of whole blood, and carefully layered onto Ficoll solution (Histopaque®-1077, Sigma-Aldrich, Merck KGaA). Ficoll is a polysaccharide-based density gradient medium that allows the separation of blood components according to their density.

PBMCs were isolated by density-gradient centrifugation at 800xg at room temperature for 20 minutes, with minimum acceleration and deceleration. At the end of centrifugation, PBMCs and platelets were within the layer between the PBS (upper layer) and the Ficoll solution, commonly referred to as the buffy coat, whereas granulocytes and erythrocytes were found in the lower fraction (Fig. 7).

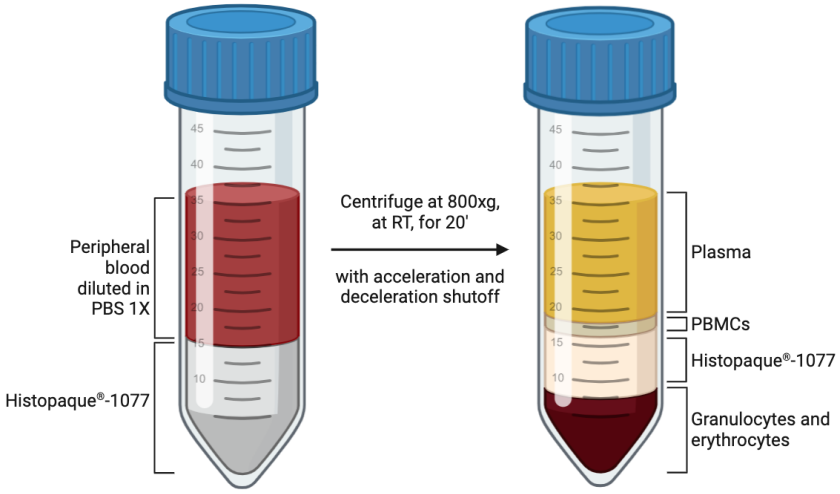


**Figure 7. Schematic representation of the isolation of PBMCs via density gradient centrifugation (created with BioRender).** Peripheral blood diluted in 1X PBS is layered over Histopaque®-1077. After centrifugation at 800xg for 20 minutes at room temperature (with acceleration and deceleration brakes disabled), distinct layers are formed based on density. The resulting stratification includes an upper layer of plasma, a "buffy coat" interface containing the PBMCs, the Histopaque®-1077 medium, and a bottom pellet consisting of granulocytes and erythrocytes.

The buffy coat was collected and transferred into a fresh tube.

To separate PBMCs from platelets, the buffy coat was centrifuged at 300xg at room temperature for 15 minutes. To improve both the yield and purity of the PBMC preparation, the supernatant was

transferred into a fresh tube, while the PBMC pellet was resuspended in 5 mL of PBS. Both tubes were centrifuged again at 300xg at room temperature for 15 minutes. At this point the platelet-containing supernatant was discarded, and the cell pellets from both tubes were combined into a single tube and resuspended.

PBMCs concentration and viability were determined using a CellDrop Automated Cell Counter (DeNovix Inc., Wilmington, DE, USA). Cell viability was assessed using dual-color fluorescence after staining with Acridine Orange (AO) and Propidium Iodide (PI). AO penetrates both viable and non-viable nucleated cells and stains nucleic acids, whereas PI only enters cells with compromised plasma membranes, allowing the discrimination between viable and non-viable cells.

According to the concentration of viable cells, the suspension was adjusted with 1X PBS in order to obtain a final concentration of $10^6$ cells/mL. The final volume was divided into 1 mL aliquots.

The aliquots were centrifuged at 845xg for 15 minutes to pellet the cells. The supernatant was discarded, and the cell pellet was stored at -80°C.

## 3. Preparation of lysates from PBMCs and protein quantification (BCA)

The frozen cell pellets were resuspended in 80 µL of CelLytic™ M (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) lysis buffer supplemented with Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), Phosphatase Inhibitor Cocktail 3 (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and Protease Inhibitor Cocktail (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). Cell lysis was obtained by mechanical resuspension and vortex mixing.

The samples were then kept on ice and incubated under shaking for a minimum of 30 minutes.

Cellular debris was removed by centrifugation at 16000xg at 4°C for 20 minutes.

The supernatant was then transferred into fresh tubes and stored frozen until downstream analyses.

Protein concentrations of the lysates were determined by the bicinchoninic acid (BCA) assay, which is based on the reaction of reduction of $Cu^{2+}$ to $Cu^{+}$ in the presence of proteins. The resulting BCA–copper complex is water soluble, and its absorbance at 562 nm allows the determination of protein concentration based on a standard curve generated using known concentrations of bovine serum albumin (BSA).

This procedure was performed following these steps:

| | |
|---|---|
| **Sample Preparation** | For each sample, 5 µL of lysate were diluted 10-fold by adding 45 µL of $ddH_2O$. |
| **Standard preparation** | • An 8-point serial dilution of BSA was prepared to generate the standard curve (1000, 800, 600, 400, 200, |

| | |
|---|---|
| | 100, 50 and 0 µg/mL (blank)). A volume of 50 µL of each standard point was transferred into a new tube. |
| **Reaction and incubation** | • A working BCA solution was prepared in a 50:1 ratio;<br>• 500 µL of BCA solution were added to each tube and thoroughly mixed by vortex;<br>• Once vortexed, the tubes were incubated in a dry heat block at 37°C for 30 minutes. |
| **Measurements** | • Following incubation, 200 µL of each reaction mixture was transferred in duplicate to a transparent 96-well plate;<br>• Absorbance was measured at 562 nm using a CLARIOstarPlus (BMG Labtech) plate reader. |

## 4. ELISA

The protein levels of total and aggregate α-synuclein in PBMC lysates were quantified using sandwich enzyme-linked immunosorbent assay (ELISA).

This technique is based on the specific recognition of the target protein by a capture antibody immobilized on the assay plate and a detection antibody binding to a different epitope of the same target protein. The signal generated by the enzyme-conjugated detection system is proportional to the amount of target protein present in the sample.

Total α-synuclein levels were measured using the LEGEND MAX™ Human α-Synuclein (Colorimetric) ELISA Kit (BioLegend), while aggregate α-synuclein levels were assessed using the LEGEND MAX ™ Human α-Synuclein Aggregate ELISA Kit (BioLegend).

The assay was performed according to the manufacturer's instructions (Fig. 8):

1. The samples were diluted 1:4000 and 1:50 for total and aggregate α-synuclein measurements, respectively, in 1X Reagent Diluent.
2. The α-synuclein standard curve was prepared by serial dilution of an α-synuclein standard of known concentration.
3. The pre-coated ELISA plate with anti-α-synuclein capture antibodies was washed 4 times with 1X Wash Buffer.
4. 50 µL of Reagent Diluent (1X) were added to both the standard wells and the sample wells.
5. 50 µL of samples, standards and blank were loaded into the plate in duplicate.
6. The plate was incubated for 2 hours at room temperature to allow α-synuclein present in the samples to bind to the capture antibodies immobilized on the plate.
7. After incubation, the plate was washed 4 times with Wash Buffer.

8. The plate was incubated for 1 hour at room temperature with a biotinylated detection antibody against α-synuclein.
9. After incubation, the plate was washed 4 times with Wash Buffer.
10. The plate was incubated for 30 minutes at room temperature with horseradish peroxidase (HRP)-conjugated with avidin.
11. After incubation, the plate was washed 5 times with Wash Buffer
12. The plate was incubated for 15 minutes at room temperature in the dark with Substrate Solution F. The intensity of the colorimetric signal generated in each well was proportional to the amount of protein present in the sample.
13. The enzymatic reaction was stopped by adding Stop Solution, resulting in a color change from blue to yellow due to the acidic pH.
14. Finally, absorbance was measured at 450 nm using a CLARIOstar$^{Plus}$ plate reader.

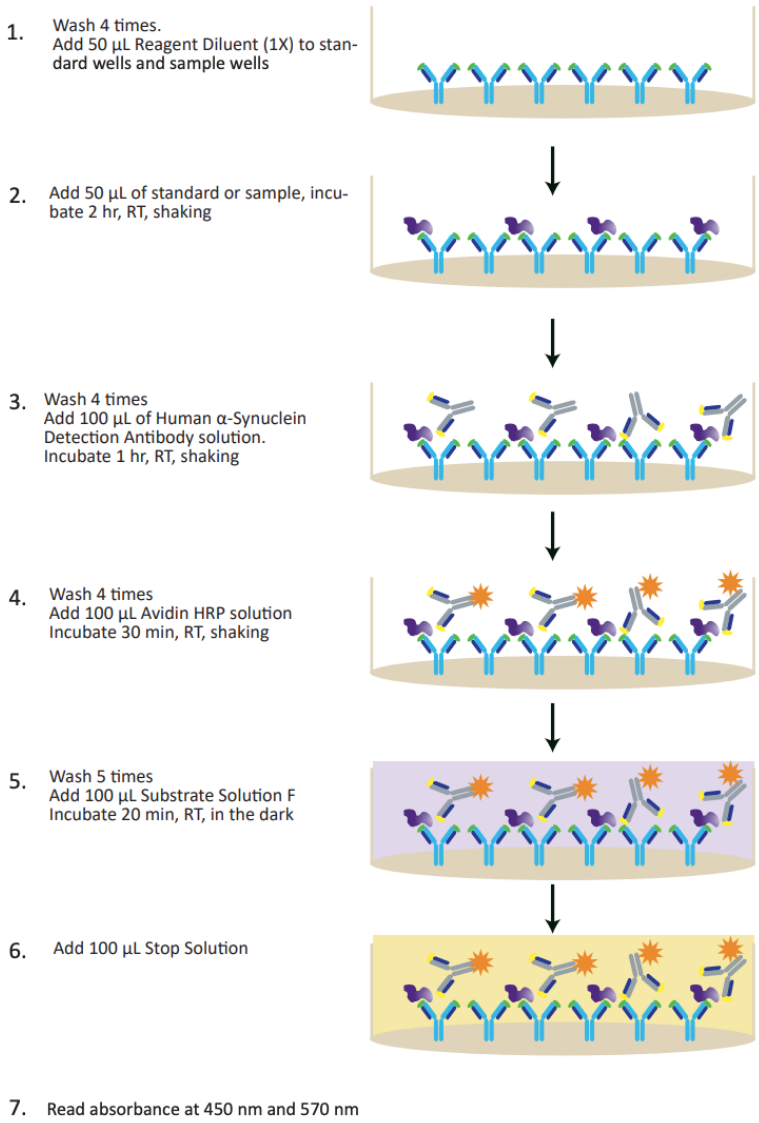

**Figure 8. Schematic representation of the LEGEND MAX™ Human α-Synuclein (Colorimetric) ELISA Kit (BioLegend) procedure.**

The standard curve was generated from the absorbance values of the α-synuclein standards, and sample concentrations were calculated by interpolation from the standard curve.

## 5. ELLA™

ELLA™ (Bio-Techne Corporation, Minneapolis, MN, USA) is an automated, cartridge-based immunoassay platform based on the principles of traditional ELISA.

The system relies on Simple Plex™ microfluidic cartridges, in which samples, reagents and assay-specific calibration parameters are processed through microfluidic channels.

Each channel contains three Glass Nano Reactors (GNRs) coated with specific capture antibodies, allowing the target protein to be captured and quantified in technical triplicates (Fig. 9).

This automated approach allows standardized analysis, reducing operator-dependent variability and improving assay reproducibility.

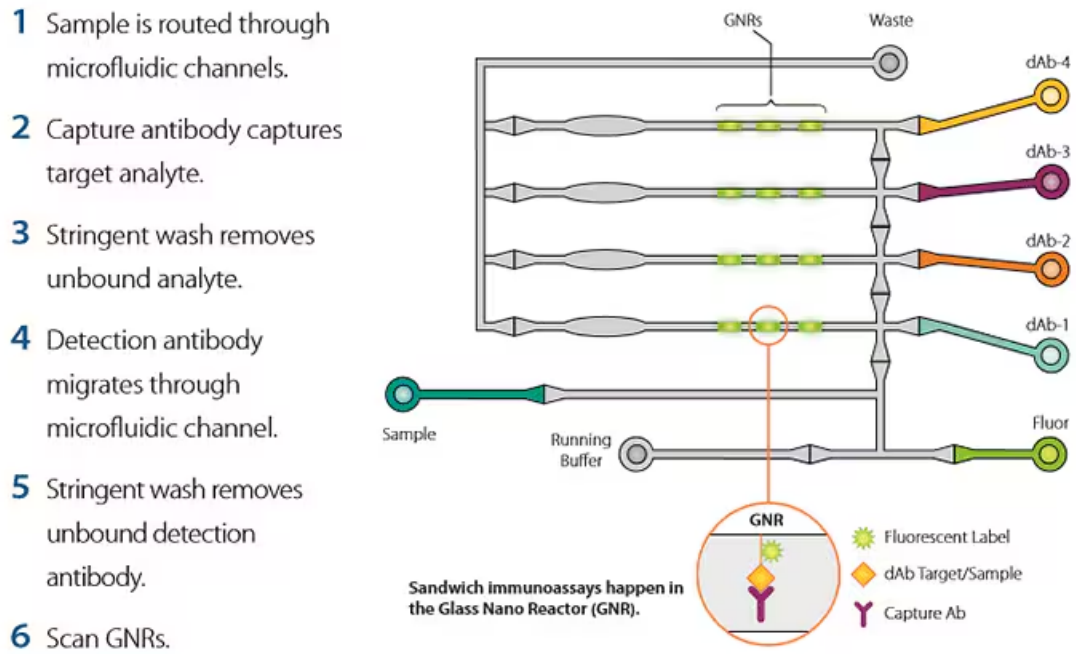


**Figure 9. Schematic representation of ELLA™ microfluidic cartridges workflow (Bio-Techne Corporation, Minneapolis, MN, USA).**

According to the manufacturer's instructions, the steps listed below were followed:

| | |
|---|---|
| **Sample preparation** | The samples were thawed and then centrifuged at 300xg at room temperature for 10 minutes |

| | |
|---|---|
| **Dilution** | The samples were diluted in Sample Diluent according to the dilution factor specified for each analyte. |
| **Cartridge Loading** | • 1000 µL of Wash Buffer were loaded into the wash buffer cartridge wells.<br>• 50 µL of each sample were loaded into the sample cartridge wells |
| **Run** | The cartridge was loaded into the instrument, and the assay run was started using the Simple Plex Runner software (Bio-Techne) |

## 6. Western Blot

Western Blot is an immunochemical technique used for detection and semi-quantitative analysis of specific proteins in biological samples.

Proteins are first separated according to their molecular weight by Sodium Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene difluoride (PVDF) membrane. Target proteins are detected through specific primary antibodies and HRP-conjugated secondary antibodies.

Chemiluminescence detection is achieved through the enzymatic reaction between HRP and the enhanced chemiluminescence (ECL) substrate.

This procedure was performed as described below:

**Sample preparation for SDS-PAGE electrophoresis**

| **Reagent** | **Volume** |
|---|---|
| Sample (30 µg) | X µL |
| Loading buffer (2X) | 10 µL |
| Deionized water | to 20 µL |
| **Total volume** | **20 µL** |

- Samples were prepared in fresh 1.5 mL Eppendorf tubes to a final volume of 20 µL
- Samples were heated at 100°C for 2 minutes before loading onto the gel.
- Protein separation was performed using PageRuler™ Plus Prestained Protein Ladder (5 µL) as molecular weight marker.

**1X running buffer preparation**

1X running buffer solution was prepared by diluting 50 mL of 10× Tris-Glycine-SDS Buffer with 450 mL of deionized water to a final volume of 500 mL.

**Electrophoretic run**

Protein samples were loaded onto Any kD™ Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad Laboratories, Hercules, CA, USA) and separated by SDS-PAGE at a constant voltage of 120 V.

**Transfer**

Following electrophoretic separation, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes using the Trans-Blot® Turbo Transfer System (Bio-Rad Laboratories, Hercules, CA, USA) at 2.5A and 25V for 3 minutes.

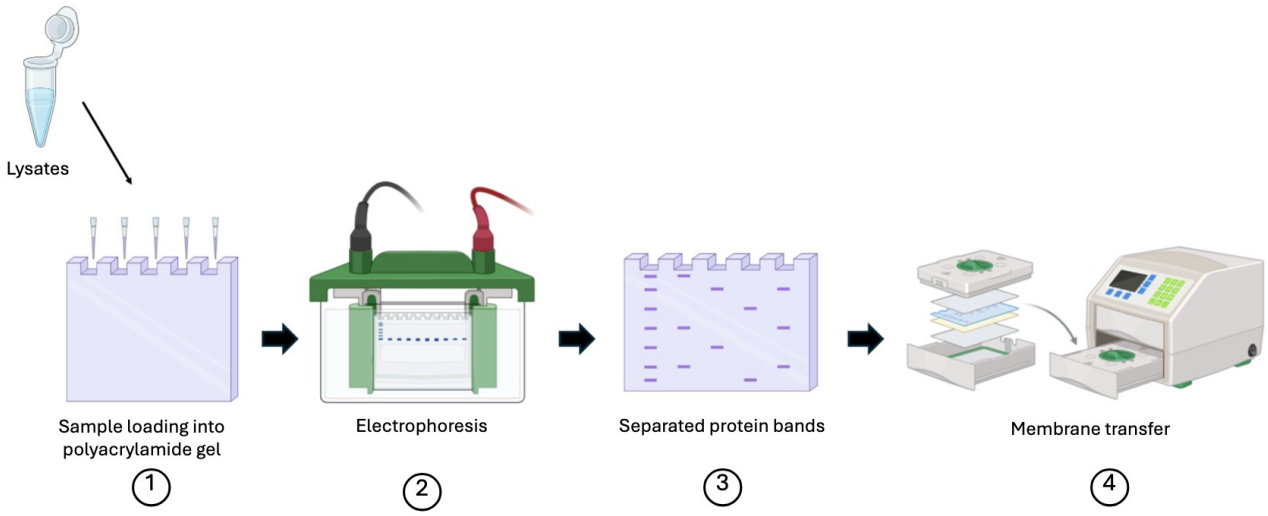


**Figure 10. Schematic representation of protein separation and transfer (created with BioRender).** 1) Loading of the prepared samples into polyacrylamide gel; 2) Electrophoresis; 3) Separated protein bands at the end of the electrophoresis; 4) Transfer from the gel to the PVDF membrane

**Immunodetection**

After protein transfer, the PVDF membrane was activated by incubation in methanol for 30 seconds, then washed with $dH_2O$ for 5 minutes and with 1X PBS for 5 minutes. To visualize the transferred proteins and facilitate membrane sectioning according to the molecular weight ranges of interest, the membrane was stained with Ponceau S and subsequently rinsed with $dH_2O$.

The target proteins were then detected by immunoblotting as described below:

| | |
|---|---|
| **Blocking** | Membranes were blocked for 1 hour at room temperature on an orbital shaker in 5% BSA and 0.05% Tween-20 in 1× PBS. |

| | |
|---|---|
| **Primary antibody incubation** | Membranes were incubated overnight at 4°C on an orbital shaker in 8 mL of 1% BSA and 0.05% Tween-20 in 1X PBS, containing the appropriate primary antibody. The following antibody dilutions were used: anti-Nrf2 antibody 1:1000 (ab62352, Abcam), anti- SOD1 antibody 1:2000 (10269-1-AP, Proteintech), anti-HO1 antibody 1:2000 (ab68477, Abcam), and anti-β-actin antibody 1:10000 (STJ11103683, St John's Laboratory), a housekeeping protein used as the loading control. |
| **Wash** | Membranes were washed 3 times for 10 minutes each with 1X PBS and 0.05% Tween-20 on an orbital shaker at room temperature. |
| **Secondary antibody incubation** | Membranes were incubated for 1h on an orbital shaker the oscillator at room temperature in 1% BSA and 0.05% Tween-20 in 1X PBS, containing HRP-conjugated secondary antibody (1:5000 anti-rabbit) (Bio-Rad). |
| **Wash** | Membranes were washed 3 times for 10 minutes each with 1X PBS and 0.05% Tween-20 on an orbital shaker at room temperature, followed by a final 5-minute wash with 1X PBS. |

Protein detection was performed using enhanced chemiluminescence (ECL), which is based on the HRP-catalyzed oxidation of luminol in the presence of hydrogen peroxide ($H_2O_2$).
This reaction generates an excited 3-aminophthalate intermediate, which emits light, producing a chemiluminescent signal proportional to the amount of target protein.
In this project, two ECL substrates with different sensitivities were employed: WESTAR ANTARES (Cyanagen) and the higher-sensitivity WESTAR HYPERNOVA (Cyanagen).

Membranes were incubated with the appropriate ECL substrate for 1 minute before image acquisition using Azure 600 Imaging System (Azure Biosystems, Dublin, CA, USA) under the parameters listed below:

| Protein | Reagent | Exposure time |
|---|---|---|
| Nrf2 | WESTAR HYPERNOVA | 10 seconds |
| SOD1 | WESTAR ANTARES | 5 seconds |
| HO-1 | WESTAR ANTARES | 10 seconds |
| β-Actin | WESTAR ANTARES | 5 seconds |

**Relative quantification**

Western blot images were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Band intensity was quantified by densitometric analysis using the Gel Analyzer tool. For each lane, the integrated density of the target protein band was measured from the corresponding intensity profile and normalized to the β-actin loading control. Statistical analyses and graph generation were performed using GraphPad Prism (GraphPad Software, Boston, MA, USA).

## 7. NULISA™

NULISA (NUcleic acid-Linked Immuno-Sandwich Assay) is an ultrasensitive protein detection platform that combines the specificity of antibody-based immunoassays with next-generation sequencing readout, enabling highly multiplexed protein profiling with attomolar sensitivity.

The NULISAseq™ platform employs a proprietary dual-selection proximity ligation approach to achieve high analytical sensitivity, high specificity, and an improved signal-to-noise ratio.

The assay is based on pairs of target-specific antibodies conjugated to unique DNA barcodes that recognize different epitopes on the same protein. When both antibodies bind simultaneously to their target, complementary bridging oligonucleotides promote the ligation of the DNA barcodes, generating a sequenceable DNA molecule whose abundance is proportional to the amount of the target protein (Fig. 11).

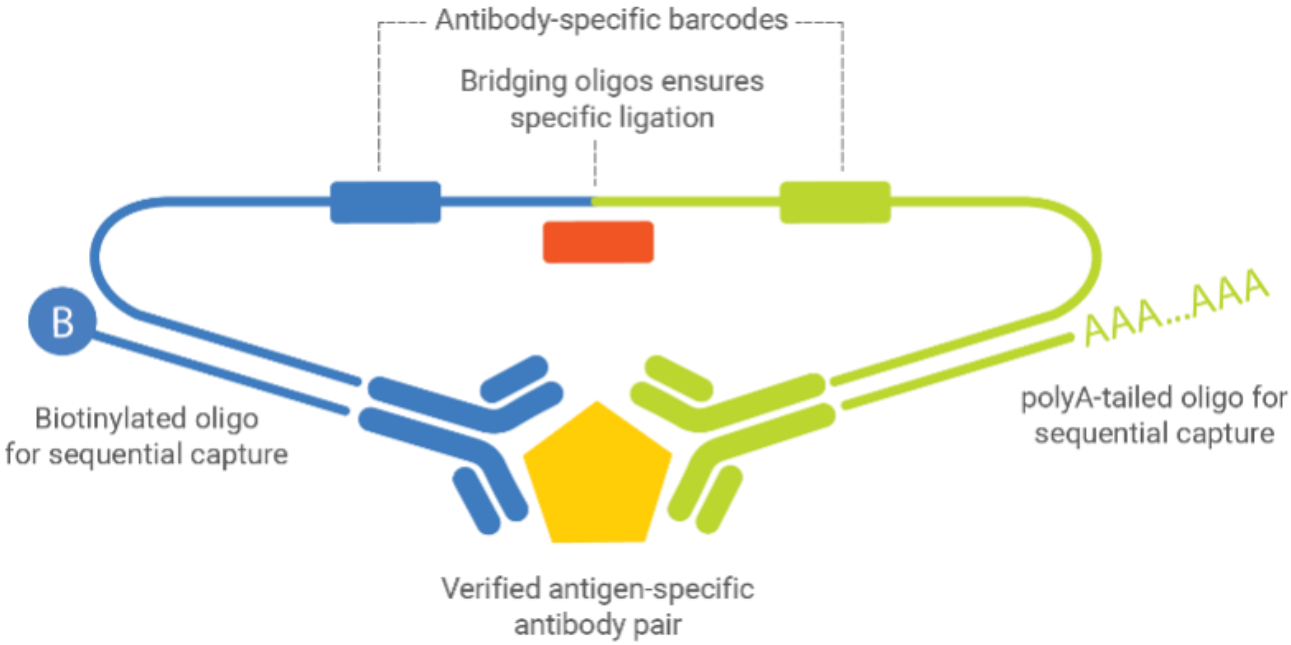


**Figure 11. Schematic representation of the key components of NULISA™ technology (Alamar Biosciences, Inc, CA, USA).**

Plasma samples were centrifuged at 10000xg for 10 minutes at 4°C. A volume of 25 ul µL of each sample was then loaded into the NULISAseq™ sample plate. Samples were subsequently analyzed using the NULISAseq™ platform (Alamar Biosciences, Inc, CA, USA), according to the manufacturer's instructions.

In particular NULISAseq™ CNS Disease Panel 120 and NULISAseq™ Neuro 220 Panel were used. The NULISAseq™ CNS Disease Panel 120 allows the simultaneous detection of more than 120 CNS-related biomarkers, including proteins associated with neurodegeneration and neuroinflammation. The NULISAseq Neuro 220 Panel provides a broader coverage of neurological biomarkers, with a particular focus on molecules relevant to Parkinson's disease and related neurodegenerative disorders.

## 8. Statistical Analysis

The normality of data distribution was assessed using the Shapiro–Wilk test. Based on the results of this assessment, statistical analyses were performed using an unpaired Student's t-test or Mann–Whitney U test for comparisons between two groups, and one-way ANOVA or Kruskal–Wallis test for comparisons among multiple groups, as appropriate. Statistical outliers were identified and removed. All statistical analyses were performed using Prism 9 software (GraphPad Software, Boston, MA, USA).

# Results

## 1. Evaluation of the total and aggregated α-synuclein levels in PBMCs

Total α-synuclein levels in PBMCs were evaluated using the LEGEND MAX™ Human α-Synuclein (Colorimetric) ELISA Kit (BioLegend), while aggregated α-synuclein levels in PBMCs were, measured using the LEGEND MAX™ α-Synuclein Aggregate ELISA Kit (BioLegend), allowing the evaluation of the aggregated α-syn/total α-syn ratio.

No statistically significant differences in aggregated α-syn levels or in the aggregated α-syn/total α-syn ratio were observed between PD patients and healthy controls (Fig. 12C-E).

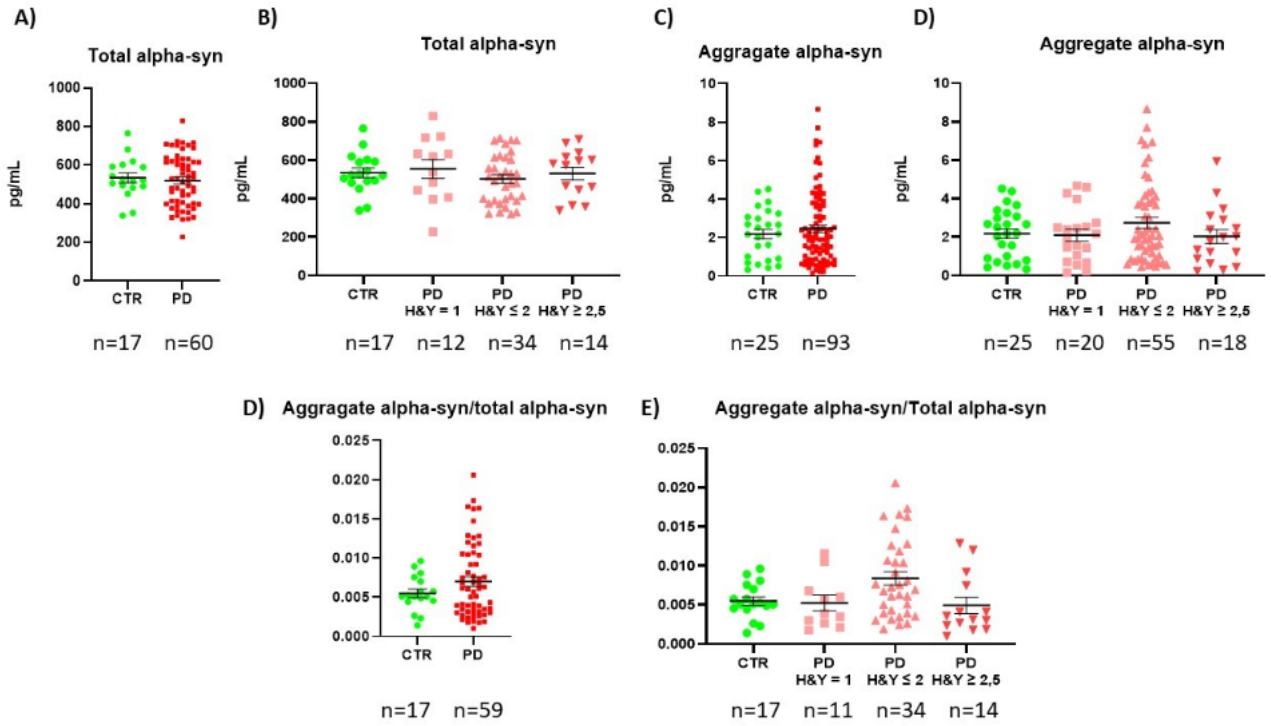


**Figure 12. Total and aggregated α-synuclein in PBMCs of PD patients and healthy controls** A-B) Total α-syn levels in PBMCs, shown as overall comparison and stratified by disease stage; C-D) aggregated α-syn levels in PBMCs, shown as overall comparison and stratified by disease stage; E-F) aggregated/total α-syn ratio, shown as overall comparison and stratified by disease stage. Mean ±SEM. Normality was assessed using the Shapiro-Wilk test. Statistical analysis was performed using an unpaired Student's t-test or Mann-Whitney U test (two group comparisons), and one-way ANOVA or Kruskal-Wallis test (multiple-group comparisons), as appropriate.

## 2. Evaluation of Nrf2 and Nrf2-downstream proteins in PBMCs

To investigate the systemic involvement of the Nrf2 pathway in Parkinson's disease, Nrf2 levels were assessed in PBMCs obtained from PD patients and healthy controls. Nrf2 protein expression levels did not show statistically significant differences between PD patients and controls (Fig. 13A), even after stratifying patients by disease stage according to the Hoehn and Yahr clinical scale (Fig. 13B).

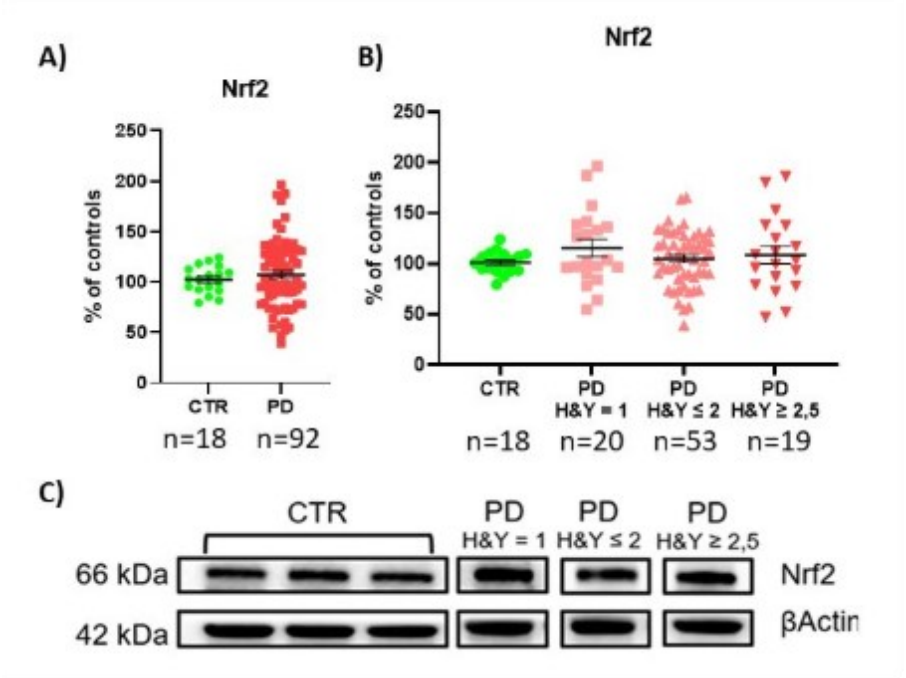


**Figure 13. Nrf2 protein expression in PBMCs of PD patients and healthy controls.** A) Levels of Nrf2 protein expression in PBMCs; B) expression levels after stratification of PD patients based on disease stage; C) representative Western blot membrane. Mean ±SEM. Normality was assessed using the Shapiro-Wilk test. Statistical comparisons were performed using an unpaired Student's t-test (A) and ordinary one-way ANOVA (B) for normally distributed data.

Similarly, there are no differences in the expression levels of SOD 1 between the study groups (Fig. 14).

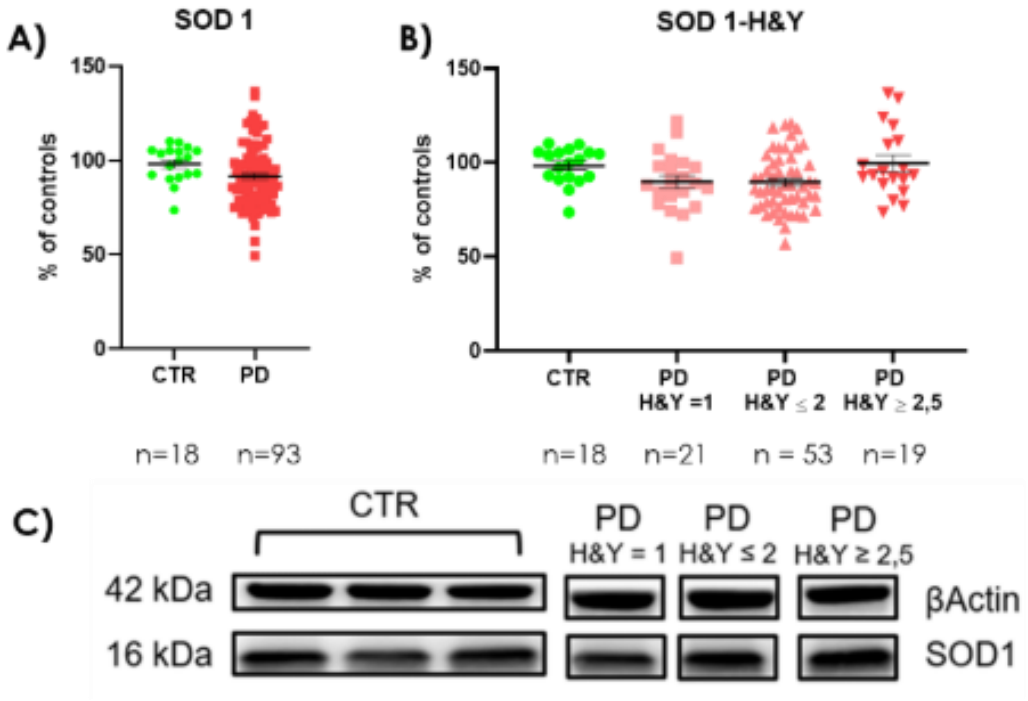


**Figure 14. SOD1 protein expression in PBMCs of PD patients and healthy controls.** A) Levels of SOD1 protein expression in PBMCs; B) expression levels after stratification of PD patients based on disease stage; C) representative Western blot membrane. Mean ±SEM. Normality was assessed using the Shapiro-Wilk test. Statistical comparisons were performed using an unpaired Student's t-test (A) and ordinary one-way ANOVA (B) for normally distributed data.

Data on HO-1 protein expression show a statistically significant impairment in PD patients compared with controls (Fig. 15A; Mann-Whitney test: CTR vs. PD, p = 0, 0008). In particular, reduced expression of this antioxidant enzyme was observed in de novo and intermediate-stage patients, but not in advanced-stage patients (Fig. 15B; Kruskal-Wallis test followed by Dunn's multiple comparisons test: CTR vs. PD H&Y = 1, p = 0, 0073; CTR vs. PD H&Y ≤ 2, p = 0, 0102).

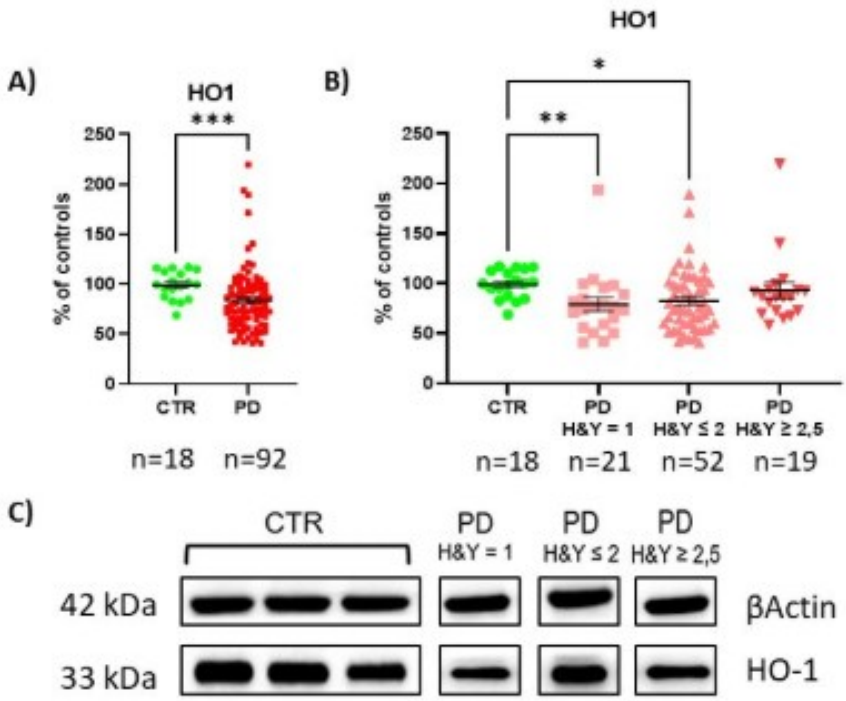


**Figure 15. HO-1 protein expression in PBMCs of PD patients and healthy controls.** A) Levels of HO-1 protein expression in PBMCs; B) expression levels after stratification of PD patients based on disease stage; C) representative Western blot membrane. Mean ±SEM. Normality was assessed using the Shapiro-Wilk test. Statistical comparisons were performed using a Mann-Whitney test (A) and Kruskal-Wallis test (B) for non-normally distributed data. *p<0.05; **p<0.01; ***p<0.001.

## 3. Evaluation of inflammatory and neurodegeneration markers in plasma

Inflammatory and neurodegeneration markers were further evaluated using ELLA (Bio-Techne). Specifically, the following markers were assessed: NfL, IFN-γ, TNF-α, fractalkine, IL-6, TREM2, and S100B.

NfL levels, a well-established marker of neurodegeneration, were significantly increased in PD patients compared with controls (Fig. 16A; Mann Whitney test, $p < 0{,}0001$), with a further stratification showing significant differences in both intermediate (H&Y ≤ 2) and advanced disease stages (H&Y >2) (Fig. 16B); Kruskal-Wallis test followed by Dunn's multiple comparisons test: CTR vs. PD H&Y ≤ 2, $p = 0{,}0002$; CTR vs. PD H&Y ≥ 2,5, $p = 0{,}0010$).

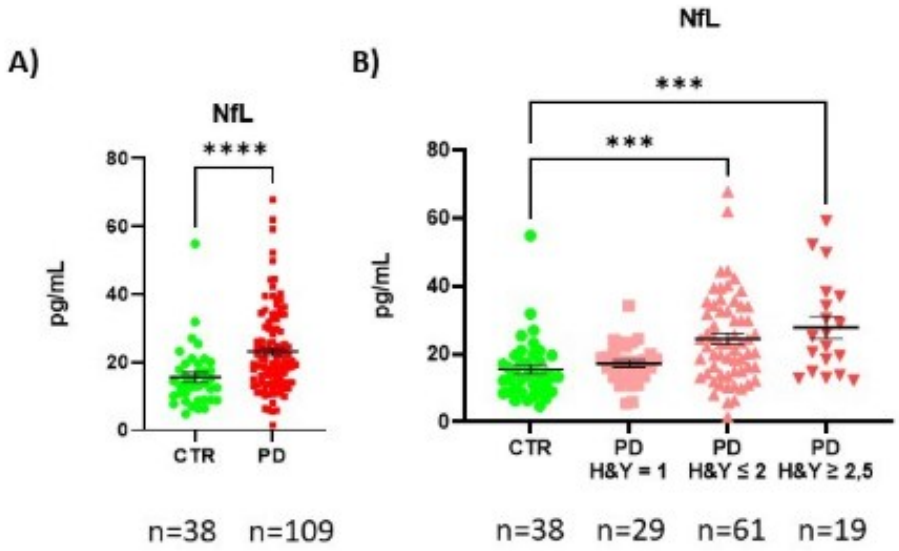


**Figure 16. Levels of NfL in plasma of PD patients and healthy controls.** Mean ±SEM. Normality was assessed using the Shapiro-Wilk test. Statistical comparisons were performed using a Mann-Whitney test (A) and Kruskal-Wallis test (B) for non-normally distributed data. ***p<0.001; ****p<0.0001.

While in the levels of Fractalkine, IFN-$\gamma$ , IL-6, TREM2, and S100B no differences were observed between Parkinson's patients and healthy controls (Fig. 17).

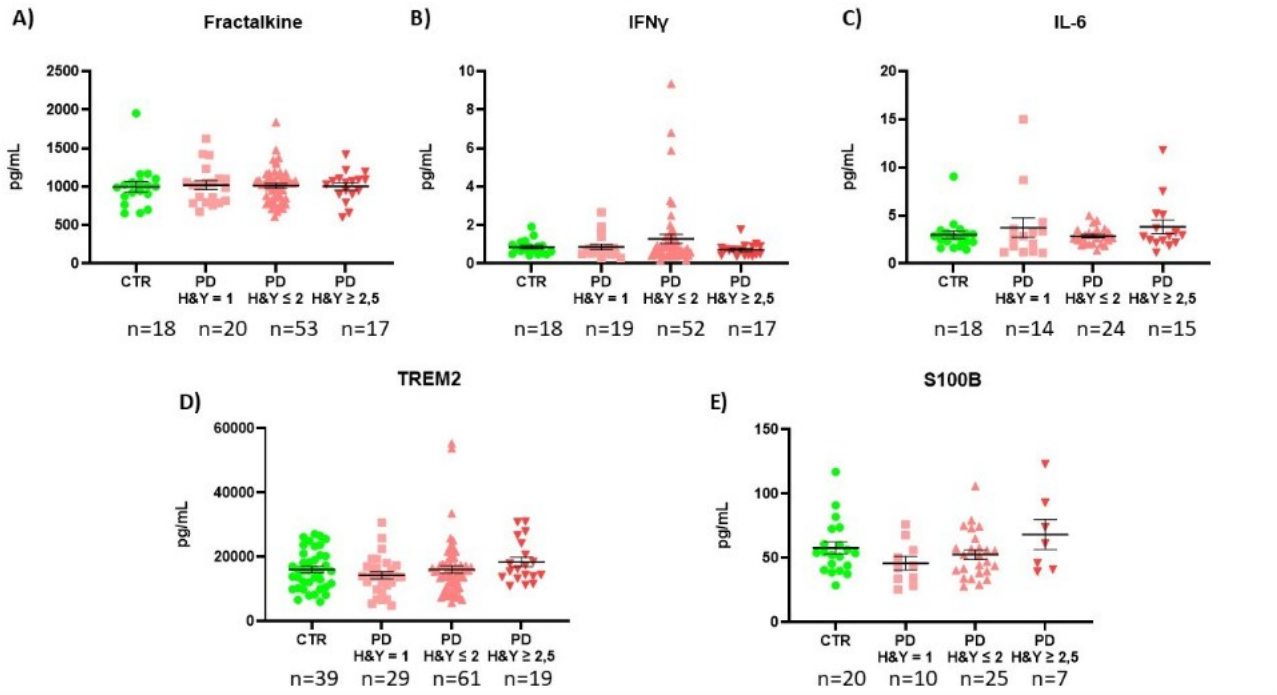


**Figure 17. Levels of Fractalkine (A), IFN-$\gamma$ (B), IL-6 (C), TREM2 (D), and S100B (E) in plasma of PD patients and healthy controls**. Mean ±SEM. Normality was assessed using the Shapiro-Wilk test. Statistical analysis was performed using a Mann-Whitney U test (two-group comparisons), and Kruskal-Wallis test (multiple-group comparisons) for non-normally distributed data.

Notably, TNFα levels were significantly reduced in PD patients compared with controls (Fig. 7 A, Mann-Whitney test, CTR vs. PD, p = 0,0372); however, this difference was no longer statistically significant after stratification by disease stage (Fig. 18B), likely due to the limited sample size of the individual subgroups.

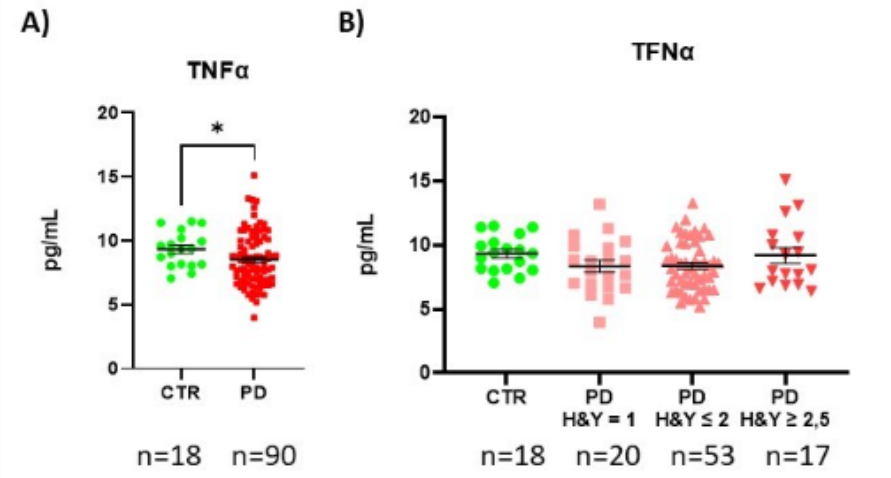


**Figure 18. Levels of TNFα in plasma of PD patients and healthy controls**. Mean ±SEM. Normality was assessed using the Shapiro-Wilk test. Statistical comparisons were performed using a Mann-Whitney test (A) and ordinary one-way ANOVA (B). *p<0.05.

## 4. The role of gender on clinical and biochemical measurements

The role of gender on clinical and biochemical measurements was also investigated. Sexual hormones, including LH, FSH, estrogen, testosterone, and progesterone, were successfully examined within our study population (in progress). Additionally, all female patients underwent the FSFI questionnaire to assess the quality of sexual life, providing further insight into an aspect that has received limited attention in PD patients.

Total α-syn levels were significantly higher in female than in male across the entire study cohort (Fig. 19 A; unpaired Student's *t*-test, M vs. F, p = 0,0253). In addition, female showed significantly higher TREM2 levels (Fig. 19 B; Mann Whitney test, M vs. F, p = 0,0408) and significantly lower IL-6 levels (Fig. 19 C; Mann Whitney test, M vs. F, p = 0,0386) than male. These sex-related differences suggest a potential role of estrogens in the regulation of these biomarkers and support the influence of sex hormones on molecular pathways relevant to Parkinson's disease, particularly those involved in neuroinflammatory and immune responses.

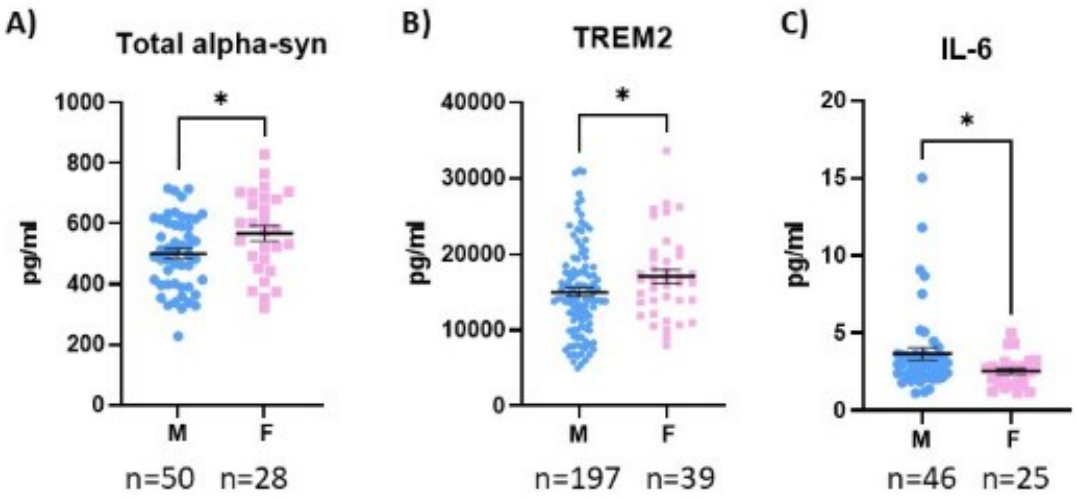


**Figure 19. Total α-syn (A), TREM2 (B), and IL-6 (C) levels in male (M) and female (F) subjects.** Mean ±SEM. Normality was assessed using the Shapiro-Wilk test. Statistical analysis was performed using an unpaired Student's t-test or Mann-Whitney U test, as appropriate.

## 5. Plasma proteomic profiling by NULISA

To further explore peripheral protein alterations associated with Parkinson's disease, plasma samples were analyzed using the NULISAseq™ CNS Disease Panel 120 (V2). Differential expression analysis was performed using the NULISAseqR R package (v1.5.1).

In the overall PD group, DOPA decarboxylase (DDC) showed increased levels compared with healthy controls (Fig. 20).

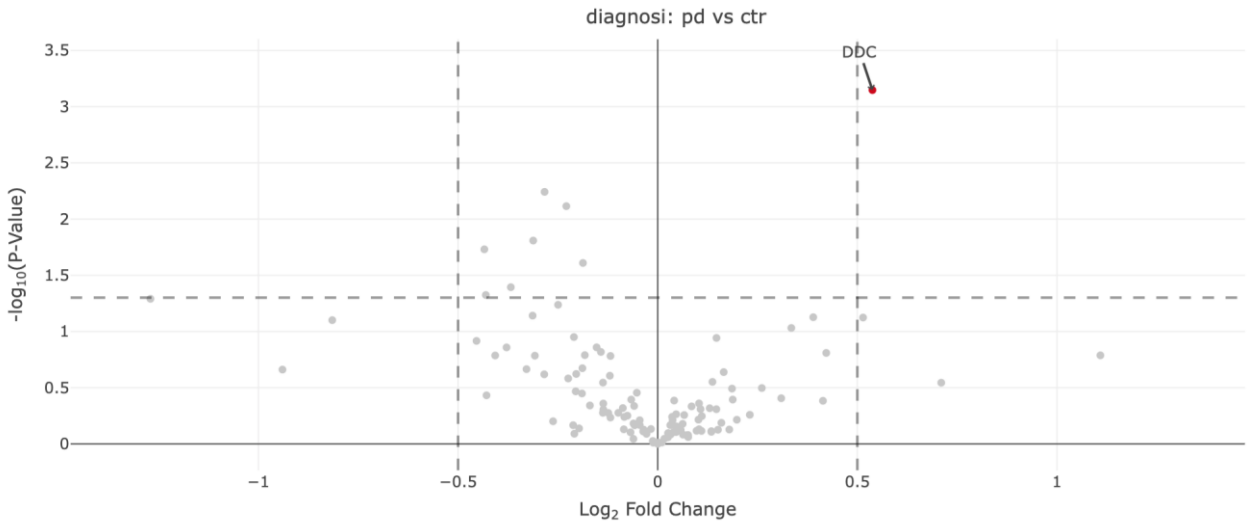


**Figure 20. Differential plasma protein abundance in PD patients compared with healthy controls assessed by NULISAseq™.** Volcano plot showing log fold change on the x-axis and −log10(p-value) on the y-axis. Differentially expressed targets were identified based on an adjusted p-value < 0.05 and a log fold change > 0.5 for up-regulation or < −0.5 for down-regulation. DDC showed significantly increased levels in PD patients compared with controls.

Stage-specific analysis identified additional differences in de novo and advanced PD patients.

In de novo PD patients. C-C motif chemokine ligand 13 (CCL13), amyloid-beta 38 (Aβ38), and NfL showed significantly lower levels compared with healthy controls, whereas GDNF levels were significantly increased (Fig. 21).

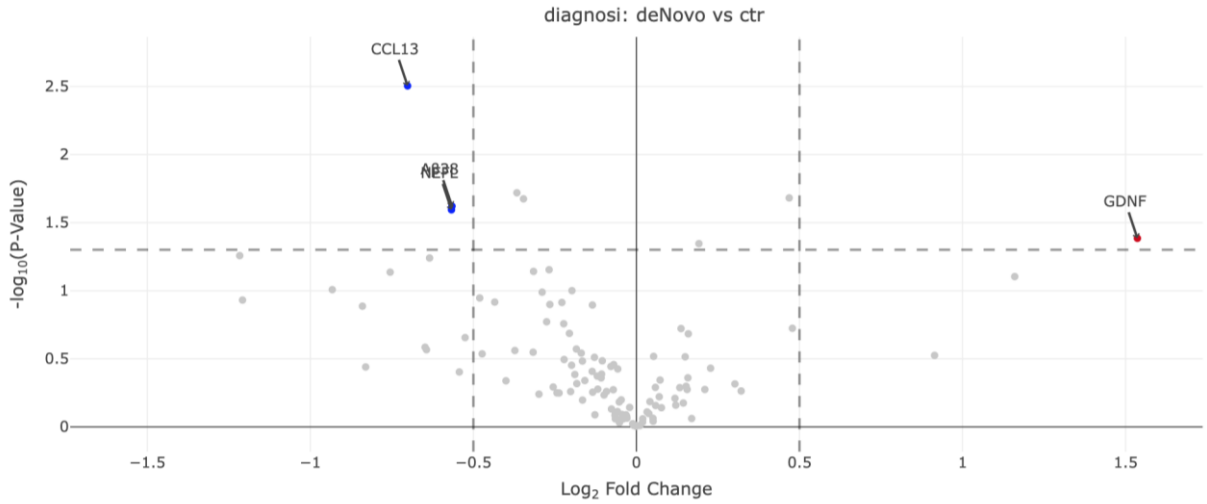


**Figure 21. Differential plasma protein abundance in de novo PD patients compared with healthy controls assessed by NULISAseq™.** Volcano plot showing log fold change on the x-axis and −log10(p-value) on the y-axis. Differentially expressed targets were identified based on an adjusted p-value < 0.05 and a log fold change > 0.5 for up-regulation or < −0.5 for down-regulation. CCL13, Aβ38 and NfL showed significantly lower levels in de novo PD patients, whereas GDNF showed significantly increased levels.

In advanced PD patients, contactin-2 (CNTN2) and CD40LG showed significantly lower levels compared with healthy controls, whereas glial cell line-derived neurotrophic factor (GDNF) showed significantly increased levels (Fig. 22).

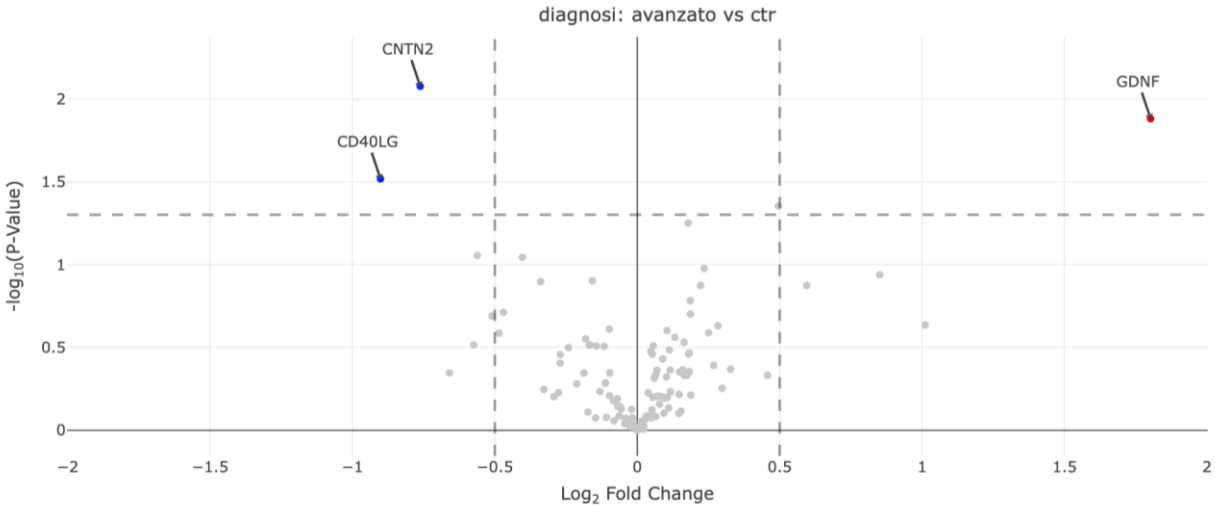


**Figure 22. Differential plasma protein abundance in advanced PD patients compared with healthy controls assessed by NULISAseq™.** Volcano plot showing log fold change on the x-axis and −log10(p-value) on the y-axis.

Differentially expressed targets were identified based on an adjusted p-value < 0.05 and a log fold change > 0.5 for up-regulation or < −0.5 for down-regulation. CNTN2 and CD40LG showed significantly lower levels in advanced PD patients, whereas GDNF showed significantly increased levels.

No significantly differentially abundant plasma proteins were identified between intermediate PD patients and healthy controls.

To investigate whether the protein alterations identified by differential expression analysis were associated with coordinated changes in biological processes, pathway enrichment analysis was performed using Gene Set Enrichment Analysis (GSEA) and the MSigDB Hallmark gene sets.
No significantly enriched pathways were identified in the overall PD versus healthy control comparison or in the analyses of de novo, intermediate, and advanced PD patients versus controls. Across all comparisons, none of the 23 analyzed hallmark pathways reached the predefined False Discovery Rate (FDR) threshold of 0.05.

# Discussion

Parkinson's disease (PD) is a complex and multifactorial neurodegenerative disorder, and its pathogenesis involves several interconnected mechanisms, including α-synuclein aggregation, neuroinflammation, and oxidative stress. Despite the biological complexity underlying PD pathogenesis, the clinical diagnosis of PD still largely relies on the identification of motor symptoms. However, the biological neurodegenerative processes may have begun decades before the onset of clinical symptoms. This discrepancy highlights the need for biomarkers reflecting disease-associated biological processes that could potentially facilitate earlier diagnosis, monitor disease progression, and improve patient stratification.

## Total and aggregated α-synuclein levels and the aggregated/total α-syn ratio do not show statistically differences between PD patients and healthy controls

Given its involvement in PD pathogenesis and its role as a hallmark of the disease, α-synuclein has been investigated as a potential biomarker. In this thesis, both total and aggregated α-synuclein levels were evaluated in PBMCs, along with the corresponding aggregated α-synuclein/total α-synuclein ratio.

No significant differences in total α-synuclein levels were observed between PD patients and healthy controls. This finding is consistent with previous evidence suggesting that total α-synuclein is not a reliable peripheral biomarker of PD (Zubelzu et al., 2022).

Similarly, aggregated α-synuclein levels did not show significant differences between groups.

To investigate whether α-synuclein levels were associated with disease severity, PD patients were also stratified according to Hoehn and Yahr motor scale. However, neither total nor aggregated α-synuclein levels showed significant differences across disease stages.

However, although preliminary, aggregated α-synuclein levels in intermediate PD patients showed a trend toward an increase compared with healthy controls. This trend is consistent with the central role of α-synuclein misfolding and aggregation in PD pathogenesis and with its close interaction with other disease-associated mechanisms, including oxidative stress and neuroinflammation. Oxidative conditions can promote α-synuclein misfolding and aggregation, while pathological α-synuclein species may further enhance cellular stress and inflammatory responses (Dias et al., 2013).

These results are also consistent with previous work by Veltri et al., 2026, who assessed total and oligomeric α-synuclein in PBMCs from venous blood samples. They observed no differences in total

α-synuclein levels in PD patients, compared with healthy controls, while oligomeric α-synuclein levels were significantly higher in PD patients compared with controls after adjustment for age.

In addition to the individual assessment of total and aggregated α-synuclein, the aggregated/total α-synuclein ratio was evaluated as an index of the relative abundance of aggregated species within the detectable α-synuclein pool. This approach may provide complementary information to absolute protein levels, such as changes in α-synuclein aggregation that may occur independently of variations in total protein abundance. In this study, the aggregated/total α-synuclein ratio did not differ between PD patients and healthy controls, suggesting that no detectable relative shift toward α-synuclein aggregation occurred in PBMCs under the experimental conditions examined.

As these analyses are still ongoing, the current absence of significant differences should be interpreted considering the available sample size. Increasing the sample size will improve statistical power and allow a stronger assessment of possible differences in total or aggregated α-synuclein, or in their ratio, that may become detectable in larger cohorts.

Previous evidence supports the presence of peripheral alterations in aggregated or oligomeric α-synuclein in PD. Petrillo et al. (2020) reported increased α-synuclein oligomers in blood leukocytes from PD patients, together with systemic activation of the Nrf2 pathway and alterations in redox and mitochondrial parameters.

Furthermore, total α-synuclein levels were significantly higher in female patients than in male patients. This could suggest the presence of sex-related differences in α-synuclein homeostasis.

This finding is consistent with the hypothesis that sex hormones, particularly estrogens, may contribute to the regulation of α-synuclein metabolism and accumulation, although the mechanisms underlying these differences remain unclear.

Estrogens, particularly 17β-estradiol, have been proposed as neuroprotective agents in mesencephalic neurons through multiple mechanisms, including the regulation of apoptosis-related gene transcription, antioxidant activity, and interactions with neurotrophic factors (Sawada and Shimohama, 2000).

Beyond their effects on neuronal survival, estrogens may also influence α-synuclein homeostasis more directly. Hirohata et al. (2009) reported that estrogens, especially estriol, can inhibit α-synuclein aggregation and destabilize preformed α-synuclein fibrils, suggesting that sex hormones may influence α-synuclein homeostasis by modulating its aggregation.

Importantly, the literature suggests that the effects of estrogens on α-synuclein aggregation and fibril stability may alter its distribution among different molecular species without directly affecting its overall abundance. Therefore, the higher total α-synuclein levels observed in female patients in the present study may reflect sex-dependent differences in α-synuclein homeostasis, metabolism, or clearance rather than increased aggregation per se. Further studies assessing the α-synuclein

aggregation state, potentially using RT-QuIC, and examinanting its correlation with circulating sex hormone levels, would be necessary to clarify the mechanisms underlying this sex-related difference.

## Nrf2 and SOD1 levels do not show significant differences between PD patients and healthy controls, while HO-1 show a statistically significant reduction in PD patients compared with controls

The Nrf2 pathway was investigated as a key component of the cellular response to oxidative and inflammatory stress in PBMCs. Nrf2 regulates the transcriptional response to redox imbalance and other cellular stressors, promoting the expression of several genes involved in antioxidant mechanisms (Saha et al., 2020; Zhuang et al., 2019). Its assessment in peripheral cells may therefore provide information on the systemic activation of stress-response mechanisms associated with PD. Nrf2 protein expression levels did not show statistically significant differences between PD patients and controls, even after stratification according to Hoehn and Yahr scale.

However, the absence of differences in total Nrf2 protein expression does not necessarily exclude alterations in Nrf2 pathway activity, as Nrf2 activation also depends on its stabilization and translocation to the nucleus. In this regard, Ramsey et al. (2007) reported increased nuclear localization of Nrf2 in surviving nigral neurons from PD brains. Importantly, the authors noted that this response may nevertheless be insufficient to prevent neuronal degeneration.

Therefore, a further step will be to evaluate Nrf2 expression separately in the nuclear and cytoplasmic fractions, in order to investigate whether alterations in its subcellular localization may occur in the absence of changes in total protein expression.

To further investigate the Nrf2-related antioxidant response, SOD1 was subsequently evaluated as one of the antioxidant enzymes associated with Nrf2 signalling. SOD1 catalyzes the dismutation of superoxide into hydrogen peroxide and molecular oxygen, thereby limiting the accumulation of this reactive oxygen species (Fridovich et al., 1995). Alterations in SOD1 expression or function may therefore influence the ability of cells to counteract increased ROS production.

SOD1 expression levels showed no significant differences between the study groups, even after PD patients were stratified according to Hoehn and Yahr stages. This finding contrasts with previous evidence of peripheral alterations in SOD1 in PD. Smith et al. (2018), for example, observed reduced SOD1 transcript levels in PBMCs from recently diagnosed PD patients compared with controls.

The discrepancy between these findings and the present results may be related to differences in patient clinical characteristics, disease stage, inclusion and exclusion criteria, and experimental approaches. In particular, the present study evaluated SOD1 at the protein level by Western blot, whereas previous studies have also investigated its expression at the transcript level. Moreover, the semi-quantitative

nature of Western blot analysis may contribute to differences in the detection of relatively subtle changes in protein expression.

HO-1 is one of the major antioxidant and anti-inflammatory downstream proteins of the Nrf2 pathway. It catalyzes the degradation of heme, generating biliverdin, carbon monoxide and ferrous iron (Wu and Hsieh, 2022).

In this study, HO-1 expression showed a statistically significant impairment in PD patients compared with controls. Reduced expression of this antioxidant enzyme was observed in de novo and intermediate-stage patients, but not in advanced-stage patients.

The reduction in HO-1 expression observed in de novo and intermediate-stage patients may suggest an impaired or insufficient cytoprotective response to oxidative and inflammatory stress during the earlier stages of PD. This alteration was observed despite the absence of significant changes in total Nrf2 protein expression, potentially indicating a differential regulation of downstream antioxidant mechanisms rather than a generalized alteration in Nrf2 abundance.

The absence of a similar reduction in HO-1 expression in advanced-stage patients may reflect the activation of compensatory mechanisms that emerge during disease progression, potentially involving feedback regulation or the recruitment of alternative pathways contributing to the maintenance of antioxidant and cytoprotective responses. However, further investigation is required to determine whether such mechanisms contribute to the preservation of HO-1 expression at advanced disease stages.

A further step will be to evaluate not only protein expression but also the corresponding mRNA levels, in order to determine whether disease-stage-related differences may occur at the transcriptional level. For example, an increase in Nrf2 or SOD1 transcription during disease progression could represent a compensatory response to increasing cellular stress, without necessarily resulting in a corresponding increase in functional protein levels. Comparing transcript and protein expression may therefore help to clarify whether alterations in the Nrf2 pathway and its downstream antioxidant mechanisms occur primarily at the transcriptional or post-transcriptional level.

## NfL plasma levels were significantly increased in PD patients compared with controls

Neurofilament light chain (NfL) is a neuronal cytoskeletal protein released following axonal damage and is widely used as a biomarker of neuroaxonal injury (Gaetani et al., 2019).

In this study, NfL levels were evaluated in plasma, and showed a significant increase in PD patients compared with healthy controls. Significant differences were observed in both intermediate (H&Y $\leq$ 2) and advanced disease stages (H&Y > 2).

This increase is consistent with the neurodegenerative mechanisms underlying PD, which are characterized by the progressive loss of dopaminergic neurons.

The significant differences observed in both intermediate and advanced disease stages, compared with healthy controls, are consistent with the presence of neuroaxonal damage across clinically established phases of PD. However, the absence of a significant difference between intermediate and advanced stages does not support a clear stage-dependent increase in plasma NfL levels in this cohort. These findings suggest that NfL could provide information on the presence of neuroaxonal damage in PD, although its ability to reflect differences in disease severity or progression may be limited.

However, NfL is not specific to PD, as increased circulating levels can also be observed in other neurological and neurodegenerative conditions characterized by neuronal and axonal injury. Therefore, its use as a biomarker for PD may be more informative when combined with additional biomarkers that provide greater disease specificity.

## Fractalkine, TREM2, IL-6, IFN-$\gamma$ and S100B plasma levels did not show significant differences between PD patients and healthy controls

Given the contribution of neuroinflammatory processes to PD pathogenesis, the inflammatory profile was further investigated through the assessment of markers reflecting different components of immune and glial responses. Fractalkine and TREM2 were evaluated as molecules associated with microglial regulation, IL-6 and IFN-γ as inflammatory cytokines, and S100B as predominantly astrocyte-associated protein (Chu et al., 2025; Huang et al., 2024; Sathe et al., 2012).

Overall, these markers did not show significant differences between PD patients and healthy controls. The absence of detectable peripheral alterations does not necessarily argue against the involvement of neuroinflammation in PD, since the markers evaluated represent different aspects of the inflammatory response rather than a single uniform pathway.

Previous studies investigating peripheral inflammatory signatures in PD have reported inconsistent results, with differences in the levels of individual inflammatory and glial markers not being consistently observed across cohorts. This variability may reflect differences in patient characteristics, disease stage, treatment status, and experimental approaches. Therefore, the absence of significant differences observed in the present study is in line with the heterogeneous findings reported in the literature and may indicate that these specific peripheral markers are not uniformly altered in PD (Qu et al., 2023) (Hu et al., 2026).

Biological sex could be a potential confounding factor, playing a role in increasing the variability of peripheral cytokine levels.

In this study female patients showed significantly higher TREM2 levels and lower IL-6 levels, suggesting that sex-related differences could influence peripheral inflammatory biomarkers.

The results regarding IL-6 are consistent with previous work that focused on sex-dependent differences in immune and neuroinflammatory responses in PD. The work of Broque et al. (2023) reported differences between the serum immunoprofiles of male and female patients. In particular, the authors referred to the results obtained by Brockmann et al. (2016), in which male patients showed higher levels of various immune markers compared to females, including IL-6.

Differently, previous work by Wilson et al. (2020) showed significantly higher CSF soluble TREM2 levels in male patients, compared to females, but not in plasma TREM2 levels. A review by Filipello et al. (2023) reported that results regarding TREM2 levels are inconsistent across different studies and study populations.

Overall, these findings support biological sex as an important source of variability in inflammatory biomarkers in PD, although future studies are needed to clarify whether sex-specific mechanisms should be considered when evaluating TREM2 and IL-6 as potential biomarkers.

## TNFα plasma levels were significantly reduced in PD patients compared with controls

Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine known to be involved in inflammatory phenomena (Pasparakis et al., 1996).

In this study, TNFα levels were evaluated in plasma samples, and were significantly reduced in PD patients compared with controls. Although TNF-α is generally considered a pro-inflammatory cytokine and has been reported to be elevated in serum of PD patients, findings from peripheral blood studies remain inconsistent across clinical cohorts. This variability may reflect several confounding factors, including disease heterogeneity, treatment status, and temporal fluctuations in systemic inflammatory responses.

Moreover, after stratification by disease stage, this difference was no longer statistically significant, likely owing to the reduced sample size within individual subgroups.

Importantly, although increased TNF-α levels have been associated with motor symptom severity and disease progression in some studies, peripheral cytokine levels may not necessarily reflect the inflammatory processes occurring within the central nervous system (Wang et al. 2023).

Further studies in larger, preferably drug-naïve cohorts are therefore needed to clarify the biological significance of reduced peripheral TNF-α levels in PD. This is particularly relevant given the limited representation of drug-naïve patients in clinical PD cohorts, and expanding their recruitment represents an important objective of our ongoing work.

## NULISA proteomic analysis identifies preliminary protein alterations, but no significantly enriched pathways were identified

NULISA is a recent technology that enables highly multiplexed protein profiling with attomolar sensitivity. In the work of Durcan et al. (2025), the authors recently applied NULISA to different neurodegenerative disorders, demonstrating high sensitivity and reliability for the detection of a broad range of neurological and inflammatory proteins, and supporting the potential of this technology for biomarker discovery.

In the overall comparison between PD patients and healthy controls, DDC showed increased levels. Following stratification by disease stage, de novo PD patients showed lower levels of CCL13, Aβ38 and NfL, whereas GDNF levels were increased. These preliminary findings suggest that peripheral protein alterations may differ across disease stages, potentially reflecting changes in neurodegenerative, neurotrophic, and immune-related processes. However, the biological relevance of these alterations remains to be established.

The reduction in NfL levels observed in de novo PD patients was not consistent with the targeted ELLA analysis performed in this study, which showed increased plasma NfL levels in later disease stages. However, NULISA was performed in a subset of the cohort, and analysis of the remaining samples will be important to determine whether this finding is reproducible.

In advanced PD patients, CNTN2 and CD40LG showed lower levels, while GDNF was again increased. GDNF is a neurotrophic factor that promotes the survival and maintenance of dopaminergic neurons and has been investigated as a potential therapeutic strategy in PD (Barker et al., 2020). The increase in GDNF observed in both de novo and advanced patients may therefore be compatible with a compensatory neurotrophic response to neuronal stress, although this interpretation remains speculative (Węgrzynek-Gallina et al., 2026).

The reduction in CNTN2 observed in advanced PD patients may also be of interest in the context of synaptic dysfunction and Lewy body pathology. Chatterjee et al. (2020) suggest a potential association between contactin-2 and α-synuclein-related pathology.

The lower levels of CD40LG observed in advanced PD may reflect alterations in CD40-mediated inflammatory signaling. Indeed, CD40-mediated signaling in microglia and astrocytes has been associated with the induction of inflammatory mediators involved in dopaminergic neuronal loss (Ots et al., 2022). However, whether the reduction in circulating CD40LG observed in the present study reflects a modulation of neuroinflammatory processes in advanced PD remains unclear.

In the present analysis, 121 targets were evaluated in only 70 samples, and further stratification according to disease stage reduced the number of subjects within each subgroup. Consequently, statistical power to detect differences was limited. Expansion of the cohort will therefore be essential to determine whether the proteins identified could help clarify their potential relevance as peripheral biomarkers of PD. This need for validation in larger and more diverse cohorts is also emphasized by Durcan et al. (2025) in their assessment of NULISA-based proteomic signatures in neurodegenerative diseases.

To investigate whether the protein alterations identified by NULISA were associated with broader changes in biological pathways, Gene Set Enrichment Analysis (GSEA) was performed using the MSigDB Hallmark gene sets. However, no significantly enriched pathways were identified after FDR correction in the overall PD group or in the de novo, intermediate, and advanced PD subgroups. This suggests that, within the present cohort, the observed protein-level alterations did not converge into a coordinated pathway signature. This absence of significant enrichment may reflect the biological heterogeneity of PD, together with the small sample size and the limited number of proteins represented within each pathway by the targeted NULISA panel.

# Conclusions

This thesis investigated potential peripheral biomarkers associated with interconnected mechanisms involved in Parkinson's disease pathogenesis, including α-synuclein aggregation, oxidative stress and neuroinflammation. This study focused on patients with sporadic Parkinson's disease at different disease stages, with particular emphasis on the Nrf2 pathway.

Taken together, the results obtained so far do not support Nrf2 or SOD1 protein expression as reliable peripheral biomarkers of sporadic PD. Similarly, α-synuclein and most of the inflammatory markers investigated did not clearly distinguish PD patients from healthy controls, while the early reduction in HO-1 identifies this protein as a potential candidate for further investigation.

Notably, NfL emerged as the marker most clearly associated with neuroaxonal damage and with more advanced disease stages.

Sex-related differences were observed in peripheral markers, including higher total α-synuclein, higher TREM2 levels and lower IL-6 levels in females compared with male PD patients. These results suggest that sex may represent a relevant source of biological variability when evaluating possible peripheral biomarkers in PD.

The human data presented in this study remain preliminary, and an important limitation is the relatively small sample size. Expansion of the study cohort will therefore be necessary to validate these findings and to better define their relationship with disease progression and biological variability.

Future studies will include additional plasma analyses using the ELLA platform to investigate a broader panel of glial- and neurodegeneration-related biomarkers. Rather than focusing on individual markers, the integration of multiple complementary biomarkers may provide a more comprehensive representation of the biological processes underlying PD and facilitate the identification of molecular signatures associated with different stages of disease.

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