University of Basilicata

PhD Program in Applied Biology

Sex-specific differences in Parkinson's disease: an integrated study of circulating biomarkers, metabolism and cellular response to α-synuclein toxicity

SSD

BIOS-07/A

Coordinator
Prof. Patrizia Falabella

PhD Student
Alessandro Pistone

Tutor
Prof. Angela Ostuni

Co-Tutor
Prof. Maria Antonietta Castiglione Morelli

XXXVIII Cycle

Table of Contents

List of abbreviations

CNS – Central Nervous System

SNpc – Substantia Nigra pars compacta

DA – Dopamine Agonist/ Dopamine

ENS – Enteric Nervous System

PGC-1α - Peroxisome Gamma Proliferator Coactivator 1

CSF – Cerebrospinal Fluid

ETC - Electron Transport Chain

mtDNA - mitochondrial DNA

ATP- Adenosine Triphosphate

ADP- Adenosine Diphosphate

UPR – Unfolded Protein Response

PD – Parkinson's Disease

HC – Healthy Control

SOD 2 - Superoxide Dismutase isoform 2

NF-kB - nuclear factor kappa-light-chain-enhancer of activated B cells

UCP2 - Mitochondrial uncoupling protein 2

Nrf2 - Nuclear Factor Erythroid 2-related factor 2

NQO1 - NAD(P)H dehydrogenase (quinone) 1

LC3 - Microtubule-associated proteins 1A/1B light chain 3B

BCL 2 - B-cell lymphoma 2

BAX - bcl-2-like protein 4

BiP - Binding immunoglobulin protein

CHOP - C/EBP Homologous Protein

P21 - CDK-interacting protein 1

P53 – Tumor protein P53

MMP 3/9 - Matrix Metalloproteinase 3/9

MoCA - Montreal Cognitive Assessment

MMSE - Mini Mental State Examination

Parkin - Parkin-RBR E3 ubiquitin protein ligase.

Abstract in Italian

Il morbo di Parkinson rappresenta la seconda malattia neurodegenerativa più diffusa dopo l'Alzheimer, emergendo come una crescente sfida sanitaria e sociale a livello globale. Si tratta di una patologia cronica e progressiva che colpisce selettivamente i neuroni dopaminergici della Substantia Nigra pars compacta, ed è caratterizzata non solo da sintomi motori, ma anche da un ampio spettro di manifestazioni non motorie che hanno un profondo impatto sulla qualità della vita dei pazienti. Sebbene l'eziologia della malattia rimanga in parte sconosciuta, è ormai evidente che il Parkinson è una condizione multifattoriale, in cui componenti genetiche, ambientali, immunologiche e metaboliche interagiscono tra loro. Tra i meccanismi molecolari implicati nella patogenesi, un ruolo centrale è svolto dallo stress ossidativo, dall'accumulo di α-sinucleina, dalla disfunzione dei sistemi di degradazione cellulare e dall'infiammazione cronica del sistema nervoso centrale. La presenza di aggregati citotossici di α-sinucleina rappresenta il principale marcatore distintivo del Parkinson. Le forme oligomeriche e fibrillari della proteina, presenti in equilibrio dinamico tra loro, alterano numerosi processi cellulari, tra cui l'omeostasi mitocondriale, il traffico vescicolare, l'autofagia, lo stress del reticolo endoplasmatico e la risposta infiammatoria.

Ad oggi, la diagnosi del morbo di Parkinson si basa principalmente su criteri clinici, fondati sui sintomi motori e non motori del paziente, nonché sull'uso di tecniche di neuroimaging e sulla somministrazione di test volti a valutare i deficit cognitivi e motori. La ricerca si è concentrata sull'identificazione di biomarcatori affidabili che possano supportare la diagnosi precoce, la stratificazione dei pazienti, il monitoraggio della progressione e la valutazione della risposta terapeutica. Sebbene siano in studio numerosi marcatori, particolare attenzione è rivolta all'α-sinucleina e alle sue forme aggregate o modificate, presenti nel liquido cerebrospinale, nelle cellule del sangue e negli esosomi plasmatici, oltre che a marcatori di danno neuronale.

Negli ultimi anni, la ricerca sulle malattie croniche ha iniziato a includere il sesso, e in parte anche il genere, nell'identificazione e caratterizzazione di nuovi biomarcatori. Nel Parkinson, uomini e donne presentano una diversa suscettibilità alla malattia, non solo in termini di sintomi, ma anche in termini di incidenza,

progressione della patologia, risposta ai farmaci e impatto sulla qualità della vita. Nonostante ciò, l'integrazione sistematica di sesso e genere nella ricerca sul Parkinson è ancora agli inizi, e molti aspetti restano poco esplorati.

In questo studio abbiamo adottato un approccio integrato basato sull'analisi di campioni di siero di pazienti affetti da Parkinson e modelli cellulari neuronali, con l'obiettivo di identificare e caratterizzare potenziali biomarcatori circolanti e di chiarire i percorsi molecolari influenzati in modo differenziale dalla α-sinucleina patologica, con particolare attenzione al contributo del sesso biologico nei meccanismi della malattia.

Nella prima parte dello studio, sono state effettuate analisi su campioni di siero di pazienti con Parkinson (PD) e controlli sani (HC) di entrambi i sessi per valutare alterazioni legate al sesso nello stress ossidativo, nell'infiammazione e nel metabolismo. Sebbene non siano state rilevate variazioni significative nell'equilibrio redox, sono stati osservati livelli ridotti di Nrf2 e SOD2 nei pazienti PD, in particolare nei maschi, suggerendo un indebolimento del sistema di difesa antiossidante. I livelli di interleuchina-6 (IL-6) sono risultati significativamente aumentati nei pazienti maschi con PD, e inversamente correlati con Nrf2, indicando un'interazione disfunzionale tra i percorsi ossidativi e infiammatori. La metalloproteinasi-3 della matrice (MMP3) è risultata elevata nelle pazienti femmine con PD rispetto ai maschi, e positivamente correlata con IL-6, supportando una modulazione sesso-specifica dell'infiammazione sistemica. L'analisi sierica dell'α-sinucleina ha rivelato quantità maggiori di specie oligomeriche resistenti alla proteasi (52 kDa) nei pazienti maschi con PD, mentre le pazienti femmine hanno mostrato una risposta anticorpale più marcata contro le forme monomeriche e aggregate della proteina.

Il profilo metabolomico ha evidenziato 17 metaboliti significativamente alterati, con tendenze dipendenti dal sesso: le pazienti PD femmine hanno mostrato livelli elevati di β-idrossibutirrato (BHB), coerenti con una disfunzione mitocondriale, mentre i pazienti PD maschi hanno evidenziato un aumento di acetato, acetone, citrato, creatina, glucosio, lattato e fenilalanina, riflettendo alterazioni nel metabolismo energetico e degli amminoacidi.

Nella seconda parte dello studio, modelli cellulari di origine maschile (BE(2)-M17) e femminile (SH-SY5Y) sono stati esposti a diverse specie di α-sinucleina (monomeri,

aggregati e fibrille) per indagare le risposte specifiche in base al tipo cellulare e al sesso. L'esposizione all'α-sinucleina ha influenzato la vitalità cellulare in modo dose- e conformazione-dipendente, con le fibrille che hanno mostrato la maggiore citotossicità. Le cellule SH-SY5Y di origine femminile hanno mostrato una maggiore resistenza allo stress indotto dall'α-sinucleina, attivando in modo robusto i percorsi antiossidanti (Nrf2, SOD2, NQO1), mitofagici (PARKIN, DJ-1, LC3, BECLIN) e della risposta alle proteine mal ripiegate (BIP, ATF6). Al contrario, le cellule BE(2)-M17 di origine maschile hanno mostrato un aumento del danno ossidativo, depolarizzazione mitocondriale, stress del reticolo endoplasmatico (attivazione di CHOP) e segnali pro-apoptotici (upregolazione di BAX, ridotto rapporto BCL2/BAX).

Complessivamente, questi risultati dimostrano che il sesso biologico influenza significativamente l'equilibrio redox, la regolazione infiammatoria e la vulnerabilità cellulare alla tossicità dell'α-sinucleina. L'integrazione di analisi biochimiche, immunologiche e metabolomiche con modelli cellulari differenziati per sesso fornisce nuove prospettive sui meccanismi molecolari sesso-dipendenti alla base del morbo di Parkinson e rappresenta una strategia promettente per l'identificazione di biomarcatori sesso-specifici e per approcci terapeutici personalizzati.

Abstract in English

PD represents the second most widespread neurodegenerative disease after Alzheimer's, emerging as a growing health and social challenge at a global level. It is a chronic and progressive pathology that selectively affects the dopaminergic neurons of Substantia Nigra pars compacta, and is characterized not only by motor symptoms, but also by a broad spectrum of non-motor manifestations that have a profound impact on the quality of life of patients. Although the etiology of the disease remains partly unknown, it is now evident that PD is a multifactorial condition, in which genetic, environmental, immunological and metabolic components interact with each other. Among the molecular mechanisms implicated in pathogenesis, a central role is played by oxidative stress, α-synuclein accumulation, dysfunction of cellular degradation systems and chronic inflammation of the central nervous system. The presence of cytotoxic aggregates of

α-synuclein is the main hallmark of PD. Oligomeric and fibrillary forms of the protein, present in dynamic equilibrium with each other, alter numerous cellular processes, including mitochondrial homeostasis, vesicular trafficking, autophagy, endoplasmic reticulum stress, and inflammatory response.

To date, the diagnosis of PD is mainly based on clinical criteria, based on the patient's motor and non-motor symptoms, as well as on the use of neuro-imaging techniques and the administration of tests aimed at evaluating cognitive and motor deficits. Research has focused on identifying reliable biomarkers that can support early diagnosis, patient stratification, monitoring of progression and evaluation of therapeutic response. Although several markers are being studied, particular attention is paid to the α-synuclein and its aggregated or modified forms, within the cerebrospinal fluid, blood cells and plasma exosomes, as well as markers of neuronal damage.

In recent years, research on chronic diseases has begun to include sex, and partly also gender, in the identification and characterization of new biomarkers. in PD, man and woman present a different susceptibility to the disease, not only in terms of symptoms, but also in terms of incidence, progression of the disease, response to drugs and impact on quality of life. Despite this, the systematic integration of sex and gender in PD research is still at the beginning, and many aspects remain little explored.

In this study we adopted an integrated approach based on the analysis of serum samples from PD patients and neuronal cellular models, with the aim to identify and characterize potential circulating biomarkers and to elucidate molecular pathways differentially affected by pathological α-synuclein, with a particular focus on the contribution of biological sex to disease mechanisms.

In the first part of the study, analyses were performed on sera from PD and HC of both sexes to evaluate sex-related alterations in oxidative stress, inflammation, and metabolism. Although no changes were detected in the redox balance, reduced levels of Nrf2 and SOD2 were detected in PD patients, particularly in males, suggesting an impairment of the antioxidant defense system. Interleukin-6 (IL-6) levels were significantly increased in male PD, and inversely correlated with Nrf2, indicating a dysfunctional interplay between oxidative and inflammatory pathways.

Matrix metalloproteinase-3 (MMP3) was elevated in female PD patients, compared to male counterpart, and positively correlated with IL-6, supporting a sex-specific modulation of systemic inflammation. Serum analysis of α-synuclein revealed higher amounts of protease-resistant oligomeric species (52 kDa) in male PD patients, while female PD patients exhibited a stronger antibody response against monomeric and aggregated forms of the protein.

Metabolomic profiling highlighted 17 significantly altered metabolites, with sex-dependent trends: PD females displayed elevated β-hydroxybutyrate (BHB) levels, consistent with mitochondrial dysfunction, whereas PD males showed increased acetate, acetone, citrate, creatine, glucose, lactate, and phenylalanine, reflecting alterations in energy and amino acid metabolism.

In the second part of this study, cellular models of male (BE(2)-M17) and female (SH-SY5Y) origin were exposed to different α-synuclein species (monomer, aggregates, and fibrils) to investigate cell-type- and sex-specific responses. α-Synuclein exposure affected cell viability in a dose- and conformation-dependent manner, with fibrils showing the highest cytotoxicity. Female-derived SH-SY5Y cells exhibited greater resistance to α-synuclein-induced stress, activating robust antioxidant (Nrf2, SOD2, NQO1), mitophagic (PARKIN, DJ-1, LC3, BECLIN), and unfolded protein response (BIP, ATF6) pathways. In contrast, male-derived BE(2)-M17 cells displayed increased oxidative damage, mitochondrial depolarization, ER stress (CHOP activation), and pro-apoptotic signaling (BAX upregulation, reduced BCL2/BAX ratio).

Overall, these findings demonstrate that biological sex significantly influences redox balance, inflammatory regulation, and cellular vulnerability to α-synuclein toxicity. The integration of biochemical, immunological, and metabolomic analyses with sex-differentiated cellular models provides novel insights into sex-dependent molecular mechanisms underlying PD and represents a promising strategy for the identification of sex-specific biomarkers and personalized therapeutic approaches.

Chapter 1: Introduction

Neurological Disorders: an overview

Neurodegenerative disorders, differently to metabolic and toxic disorders that affect selective neuronal death, are characterized by progressive loss of selectively vulnerable populations of neurons. These diseases can be classified according to primary clinical features (Dementia and Parkinsonism), anatomic distribution of neurodegeneration (frontotemporal degenerations) or molecular alteration. Although each neurodegenerative disease shows different pathophysiological characteristics, they share many fundamental processes associated with progressive neuronal dysfunction and death; such as proteotoxic stress and its attendant abnormalities in ubiquitin–proteasomal and autophagosomal/lysosomal systems, oxidative stress, programmed cell death, and neuroinflammation (Dugger B.N., 2017).

Unlike many somatic cells, mature neurons in the adult human brain are resilient to various stresses and pro-apoptotic stimuli, such as the deprivation of neurotrophic factors. This means that the majority of mature neurons in the Central Nervous System (CNS) are capable of enduring and perform their functions correctly (Kole A.J., 2013). However, it must be considered that in physiological conditions neurogenesis in an adult CNS is often accompanied by neuronal cell death by microglia, as a mechanism for maintaining functional homeostasis, as happens during aging or when the cell is unable to perform its function. As previously mentioned, in pathological conditions there is an increase in neuronal cell dysfunction resulting in death. A common hallmark that transpires in many neurodegenerative pathologies is aberrant protein aggregates. For example, in Alzheimer's disease there is an accumulation of tau and β-amyloid proteins involved in the formation of neurofibrillary plaques which hinder the transmission of nerve impulses. In the same way α-synuclein is involved in PD (Chi H., 2018).

1 Parkinson's Disease

1.1 Epidemiology of Parkinson

Due to various factors including difficulty in diagnosis and unfair access to healthcare services, it is extremely difficult to provide data on the incidence of PD; however, there are large epidemiological studies which, based on various risk factors, contribute to understanding the expansion of the disease. Although much research has focused on the incidence of the disease only in a few countries and on high-income countries, leaving a gap in our understanding of its impact on low- to upper-middle-income countries (Su D., 2025). For this reason, studying the prevalence and distribution of PD is crucial to acknowledge its burden and aid in planning healthcare services and public policies. Additionally, investigating specific ethnic and environmental risk factors influencing PD prevalence in different contexts can bring novel insights into understanding disease pathophysiology and epidemiology. Meta-analysis studies showed that prevalence of PD varied greatly among different ethnicities and that factors such as life expectancy at birth and disparities in access to health benefits were significantly associated with this prevalence (Pereira G.M., 2024). The Global Burden of Disease, Injury and Risk Factors Study (GBD) estimated that 6.1 million individuals worldwide had PD in 2016, up from just 2.5 million in 1990, and that the age-standardized prevalence rate (ASPR) increased by 21.7% over the same period. Notably, the number of patients was approximately 8.5 million in 2019, with an increase of 39.34% compared to 2016. Furthermore, according to data from the World Health Organization, from 2000 to 2023 there was an increase in disability of 81% and mortality of 100%. This data indicates a substantial increase in the global prevalence and incidence of PD over the past two decades (Figure 1) (Luo Y., 2025).

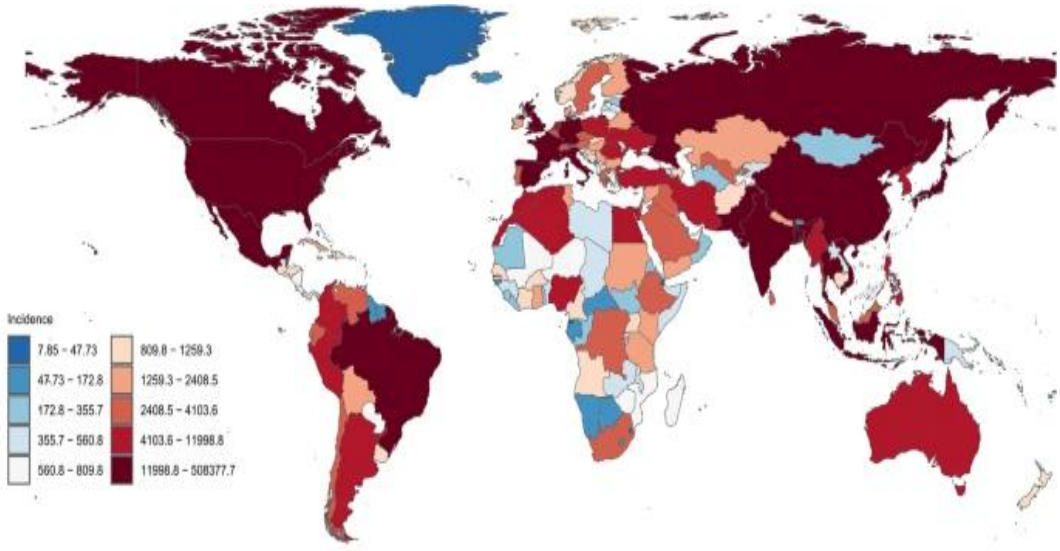


Figure 1: Incidence rate (per 100,000 population) of PD worldwide . (Luo Y., 2025)

1.2 Clinical feature of Parkinson's disease

PD, first described by James Parkinson in 1817, is the second most common age-related neurodegenerative disorder after Alzheimer's disease, and is an important societal issue and global priority. It is a chronic progressive multi-system neurodegenerative disease affecting about 3% of the population by the age of 65 and up to 5% of the people over 85 years people (Dexter D.T., 2013).

The disease is characterized by distinctive neuropathological alterations. The main pathological feature of PD is the progressive loss of midbrain dopaminergic (DA) neurons in the Substantia Nigra pars compacta (SNpc) and the formation of abnormal proteinaceous spherical bodies called Lewy bodies and Lewy's neurites, abnormal extensions of neurons, similar to filaments characterized by the presence of cytotoxic aggregates of alpha-synuclein (Figure 2) (Mandel S.A., 2010).

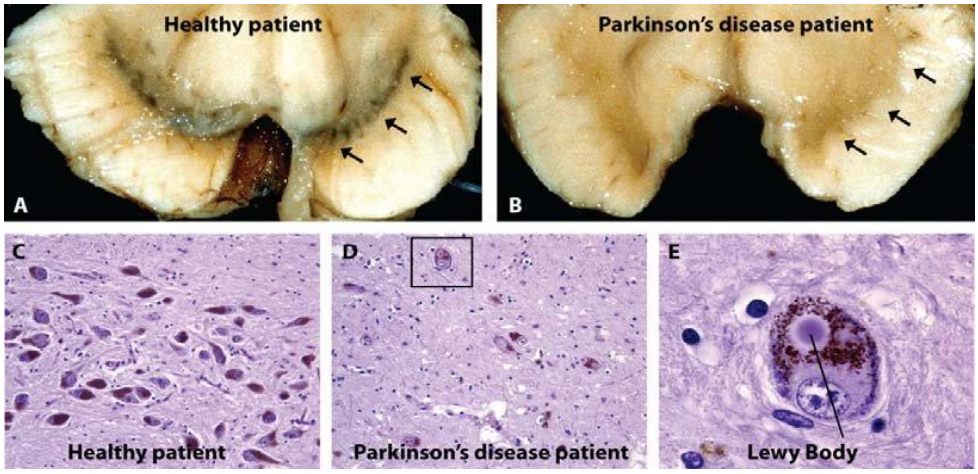


Figure 2: Pathological examination of a healthy patient (A) reveals typical pigmented DA neurons in the SN. Loss of SN neurons leads to pigment disappearance in the PD brain (B). Magnification of the SN area reveals a dense network of melanin-pigmented SN neurons in the healthy brain (C) while most of SN neurons are lost in PD (D). Some of the remaining neurons in PD contain insoluble cytoplasmic protein aggregates (Lewy Bodies) (E) that are made of aggregated alpha-synuclein and other proteins. (Mandel S.A., 2010)

In the pre-symptomatic stages of the disease the inclusion bodies are confined to the medulla oblongata and olfactory bulb or anterior olfactory nucleus. With progression of the disease, Substantia Nigra pars compacta (SNpc) and other nuclei of the midbrain and forebrain become affected. Specifically, Braak and others have proposed a pattern of spread of Lewy pathology, starting in the caudal brainstem and progressing rostrally through the upper brainstem, limbic regions, and finally the neocortex, which can lead to death; but such spread probably does not occur in all cases. It is believed that at this stage patients experience clinical symptoms (Braak H., 2002) (Sveinbjornsdottir S., 2016).

Although the pathogenesis of PD is not yet fully understood, many of the fundamental principles that have classically defined the disorder have recently been called into question. For example, although first described for their motor manifestations, non-motor characteristics are now also widely recognized and are an important factor in disease-related disability (Titova N., 2017). Similarly, while the discovery of Lewy body pathology has identified an important trigger for the disease, in some cases Lewy bodies are absent and other brain pathologies also play an important role. The emerging picture is more suitable for a syndrome, described as a set of different conditions with variable clinical and/or pathological overlap (Ye H., 2023).

Clinically, PD is characterized by motor and non-motor symptoms. Motor symptoms manifest themselves through bradykinesia, resting tremor, rigidity, and postural instability. The motor symptoms in PD are caused by dysfunction of the basal ganglia cortical motor circuit due to the neurodegeneration. Bradykinesia is a key motor symptom of PD, which presents as difficulties in planning, initiating, and executing movement, and performing sequential tasks. Other manifestations of bradykinesia include loss of spontaneous movements and swallowing difficulty, monotonic and hypophonic speech but also decreased eye blinking, mobility disabilities and postural instability, due to the loss of postural reflexes, and which manifests itself generally during the later stage of the disease. Rigidity manifests as increased resistance to passive limb movements and it can be associated with pain; this can lead to an incorrect diagnosis. The most recognizable symptom in PD is resting tremor. It is initially unilateral and prominent in the distal part of the extremities, but increases during walking; however, tremors may also occur at rest especially at the level of the facial plexus (Shin H.W., 2022).

Non-motor symptoms are universal features of idiopathic PD and involve a multitude of functions including sleep–wake cycle regulation, cognitive function, regulation of mood and hedonistic tone, autonomic nervous system function as well as sensory function. Studies have suggested that many patients, especially in the early stages of the disease, experience cognitive difficulties which worsen with the progression of the disease and in 30–40% of cases, generally over 65 years old, will develop clinically defined dementia. The development of dementia has a significant impact on the natural history of the disease and has been shown to be associated with more rapid progression of disability, increased risk for nursing home placement and increased mortality (Goldman J.G., 2025).

Sleep disorders are amongst the most frequent non-motor problems of PD. They include difficulties falling asleep, frequent awakenings, painful dystonia and REM phase disorders (Poewe W., 2008). It is easy to understand that the management of patients with multiple clinical manifestations represents an important challenge not only for patients and their caregivers, but also for the healthcare system.

1.3 Role of aging in Parkinson's pathogenesis

Ageing is the main risk factor for multiple pathological conditions, such as cardiovascular disease, cancer, and neurodegenerative disorders, including PD. Consequence of aging is immunosenescence, characterized by a progressive decline in the function of the immune system resulting in a weakened response to novel antigens. Consequently, immunosenescence contributes to increased susceptibility to infections as well as increased prevalence of autoimmunity and inflammatory conditions in the elderly (Kouli A., 2022).

Alterations in the peripheral immune system are well-described in PD, with changes in innate and adaptive immunophenotypes as well as proinflammatory cytokines being linked to a more aggressive disease phenotype. The role of aging on the molecular mechanisms of the immune system are not yet entirely clear, but justify the increase in incidence of the disease in patients over 85 years old (Williams-Gray C.H., 2016). Studies have shown that in CD8 T lymphocytes of patients affected by PD instead of healthy patients, there is a significant decrease in the mRNA levels of cyclin-dependent kinase inhibitor p16 (p16INK4a), a protein that plays a crucial role in inhibitions of cyclin-dependent kinases (CDKs), specifically CDK4 and CDK6, which are involved in cell cycle progression. This shows that the CD8 population is less senescent in patients affected by the disease. Furthermore, low levels of CD8 T lymphocytes have been found in PD patients. This finding might seem paradoxical, considering that aging is a risk factor for PD; however, one possible interpretation of these findings is that the typical shift towards more senescent CD8 T cells with aging, confers protection against PD, while impaired immunosenescence predisposes to an overactive response to new disease-related antigens (Vandenberk B., 2011) (Kouli A., 2022).

Interestingly, no correlation was observed between markers of T cell senescence and measures of disease progression, but low levels of terminally differentiated lymphocytes were observed in PD cohorts at various stages of the disease, including newly diagnosed cases. This shows that age-related immune dysregulation may be involved in disease pathogenesis rather than progression (Williams-Gray C.H., 2016).

The T cell response may also be influenced by changes in dendritic cells that play a critical role in triggering T lymphocytes. There is evidence that aging is associated with a decrease in the number of dendritic cells resulting in a reduction in the release of pro-inflammatory cytokines, suggesting that in PD dendritic cell immunosenescence occurs with aging and this could lead to a more pronounced immune response to disease-associated antigens such as oligomeric alpha-synuclein in the early stage of the disease, but autoantibodies may be present years before symptoms develop, suggesting a protective effect in the context of PD (Kouli A., 2022).

1.4 Environmental factors as a cause of onset of Parkinson's disease

Environmental factors have long been thought as contributing to neurodegenerative disorders and several neurotoxicants found in the environment cause behavioural and clinical symptoms in people with PD, although none of them have been linked to the development of such neurological conditions. Environmental pollution is believed to be the cause of diseases of the Central Nervous System (CNS) by causing oxidative stress, microglial cells activation, neuronal inflammation, and changes in blood-brain membrane permeability; as well as heavy metal toxicity has severe and long-term consequences on the brain, resulting in cognitive impairment (Ramirez Ortega D., 2020).

Pesticides, on the other hand, have a significant impact on the etiology of neurodegenerative illnesses. Chronic exposure can, therefore, contribute to the onset of the disease as well as aggravate the clinical picture (Mostafalou S., 2018) (Figure 3).

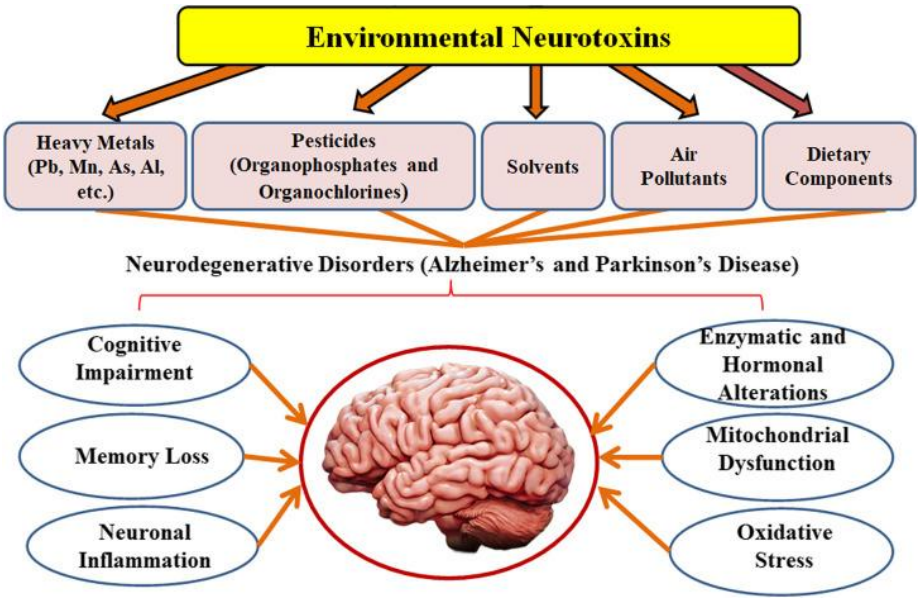


Figure 3: impact of environmental factors on the pathogenesis of neurodegenerative disorders. (Nabi M., 2022)

Many studies conducted mainly on animal models and brain tissue of post-mortem patients have shown that most pesticides elevate the incidence of neurodegeneration. The most well-defined pathway for this link is mitochondrial toxicity, due to an increase in Reactive Oxygen Species (ROS) and an accumulation of cytotoxic aggregate proteins. Most used pesticides such as Organophosphate and Organochlorines have a wide range of molecular targets, including hormones, neurotransmitters, neurotrophic variables, enzymes involved in the breakdown of β-amyloid protein, and proinflammatory alterations, in addition to their main activity of inhibiting the acetylcholinesterase enzyme (Sánchez-Santed F., 2016).

It was observed that several air pollutants, including nanoparticles, can rapidly translocate to the CNS and may stimulate innate immune responses; these induce cerebrovascular impairment, and changes in the blood-brain barrier which contribute to neurological disorders. Specifically, the vascular endothelium in the brain is a vital component of the blood-brain-barrier, due to its highly compact structure, which helps to maintain a functional and molecular barrier between the brain and the rest of the body, as well as to protect neurons from infections and toxins. Simultaneously, the blood-brain-barrier coordinates molecular transportation in and out of the Central Nervous System (CNS). Alterations in the

blood-brain-barrier cause the leakage of components into the CNS (Sweeney M.D., 2019). This results in the activation of glial cells including astrocytes, involved in the maintenance of neuronal homeostasis, with a consequent increase in the release of pro-inflammatory cytokines causing neuroinflammation and consequently neurodegeneration (Nabi M., 2022).

1.5 Involvement of the gut microbiome in Parkinson's disease

Diet, the gut microbiome and its metabolites have received huge scientific attention. Evidence of a dysfunctional gut-brain axis in PD emerged in the 1980's, when the first reports of the occurrence of Lewy pathology in the enteric nervous system (ENS) were made (Salim S., 2023).

Studies have shown that there is a relationship between the brain and the gut, known as gut-brain axis which they are able to communicate bidirectionally. This axis represents a complex network through which the gut and brain can influence each other’s functions and activities (Appleton J., 2018).

Imbalanced gut microbiota can impact the brain function through various mechanisms, including: activation of pro-inflammatory responses, production of neuroactive compounds, such as short-chain fatty acids (SCFAs) like acetate, propionate, and butyrate, initiation of immune responses triggered by microbial metabolites, such as lipopolysaccharides (LPS), disruption of neurotransmitter production in the gut, in particular serotonin and dopamine, alteration of gastrointestinal integrity, leading to microbial translocation into the bloodstream and brain, due to an increase in intestinal permeability, as well as dysfunction of blood–brain barrier integrity.

SCFAs regulate the integrity of the blood-brain barrier, are involved in modulating the synthesis or release of neurotransmitters and possess antioxidant and anti-inflammatory properties. Decreased levels of SCFAs as a result of a lesser abundance of a beneficial microbial population have been linked to intestinal barrier malfunction and neuroinflammation and to an increase in the susceptibility of neurons to injury in patients affected by PD (Silva Y.P., 2020).

Lipopolysaccharide (LPS) is a pro-inflammatory endotoxin released by Gram-negative bacteria. Increased levels of LPS have been linked to the disruption of the integrity of the blood brain barrier (BBB), causing the translocation of microbial substances into the brain and consequent neuroinflammation (He Q., 2013).

In itself, aging leads a change in the balance of bacterial communities in the gut, with a consequent decrease in beneficial bacteria and an increase in harmful ones. Such dysbiosis can lead to inadequate production of essential nutrients and a rise in toxins that cause inflammation, including neuroinflammation and neurodegeneration (Salim S., 2023).

It is known that diet, as well as drugs, influence the homeostasis of the gut microbiome. Considering what has been said, it is clear that an unbalanced diet can contribute to the risk of the onset of various pathologies including neurodegenerative diseases. To date, the impact of diet on gut dysbiosis, associated with PD is not yet well understood, however it is believed that a diet high in saturated fat and low in antioxidants could increase the risk, while a balanced diet rich in nutrients, like omega-3 fatty acids and fiber, such as the Mediterranean one, can promote the growth of beneficial bacteria in the gut, reducing inflammation and could potentially enhance the quality of life of patients affected by PD, reducing the risk of neurodegenerative diseases and have a protective effect, potentially slowing the progression of the disease (Vauzour D., 2010) (Heravi F.S., 2023).

To date, it is not yet known whether PD originates from the intestine or the brain, and new evidence suggests that gut-brain propagation of PD may be a subtype of the disease.

1.6 Genetics as a risk factor for Parkinson's

Cases of PD are mostly sporadic, not hereditary, called idiopathic PD. Only in a small percentage of cases, approximately 5–10% of cases, mostly in early-onset forms, the disease is linked to dysfunction of a single gene. We talk about monogenic PD. For the monogenic forms, there are several well-established genes, with autosomal dominant, for example SNCA and LRRK2, and autosomal recessive,

such as PRKN, PINK1 and DJ-1, modes of inheritance. In addition, there is X-linked inheritance (X-linked dystonia-parkinsonism) and atypical or complex parkinsonian phenotypes due to mutations in different genes. Furthermore, mutations in genes such as glucocerebrosidase (*GBA*) fall between a monogenic cause and a genetic susceptibility factor (Kumar K.R., 2012).

When discussing genetic risk in PD, one should differentiate risk variants from causal monogenic ones. Risk variants are relatively common, each with a small individual effect size, but collectively they significantly increase the risk of disease. A recent large meta-analysis of genomic association studies (GWAS) has identified 90 of these genomic risk alleles that collectively account for 16–36% of PD inheritance. A causal monogenic variant, on the other hand, is a rare variant with a large effect, considered the cause of the disease. Identifying these differences is difficult since autosomal dominant forms of monogenic PD have incomplete age-dependent penetrance to varying degrees, which can be influenced by the causal gene and specific pathogenic variant, as well as the patient's ethnicity. Advances in genomic technology offer people with PD greater access to genetic testing through clinic and research. The most important challenge is to combine the huge amount of data taking into account the age, gender and ethnicity of patients, in order to progress in genetic discoveries (Jia F., 2022).

Mutations in the SNCA gene, encoding for alpha-synuclein, were the first genetic cause of PD to be identified. Several point mutations, as well as structural alterations such as duplication and triplication, have been described. Generally, patients carrying SNCA mutations present early onset of PD with severe and early non-motor symptoms, including cognitive decline. In addition, pathogenic point mutations account for slightly different clinical phenotypes. Particularly, PD patients carrying the A53T and the E46K mutations have an early-onset disease with severe parkinsonism and dementia, while those carrying the A30P mutation have a less severe phenotype (Kasten M. K. C., 2013).

Tambasco et al. compared clinical presentations of patients with gene multiplications: triplications implied a more severe burden of non-motor symptoms, including higher prevalence of depression, psychosis, gastrointestinal and urinary dysfunctions and postural hypotension, compared to duplications which causes

rapid and severe cognitive decline to the point of determining a phenotype of dementia with disabling Lewy bodies (Tambasco N., 2016).

Studies have also confirmed the ability of mutations in the SNCA gene to modulate the immune response triggering pro-inflammatory pathways. In fact, carriers of the A53T mutation show greater production of the pro-inflammatory cytokine IL1-α and early activation of microglia (Alvarez-Erviti L., 2011). The discovery of dominant mutations in SNCA as a cause of PD is consistent with the critical role the alpha-synuclein protein plays in the pathogenesis. A53T, and E46K mutations affect the N-terminal domain of the alpha-synuclein protein triggering protofibril formation and smaller to larger aggregates. Furthermore, they increase N-terminal positive charge and improve N-terminal and C-terminal contacts and can cause alterations in mitochondrial function (Serratos I.N., 2022) (Figure 4).

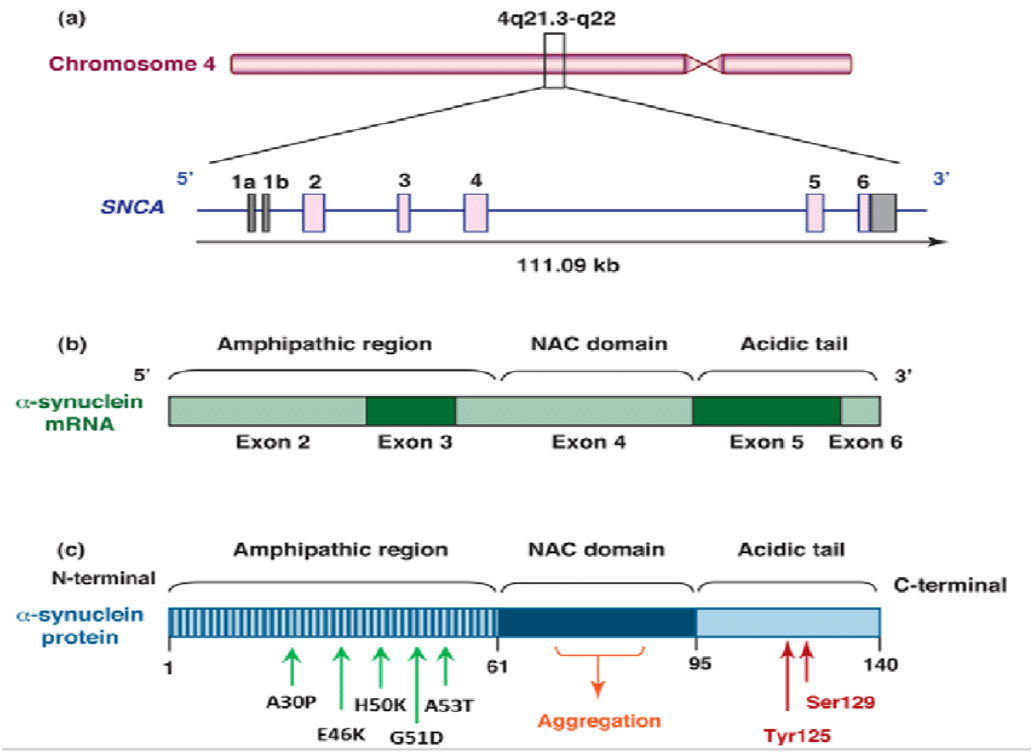


Figure 4: α-Synuclein is a major player in Parkinson's disease pathogenesis. (**A**) α-syn is encoded by SNCA gene, located on chromosome 4. (**B**) From the 6 exons comprising SNCA, only the last 5 encode for α-synuclein protein, which is composed by 140 amino acids. (**C**) Schematic representation of the different domains of α-syn and the localization of the familial mutations A30P, E46K, H50Q, G51D and A53T in the N-terminal domain. (Magistrelli L., 2021)

The LRRK2 gene encodes the Leucine-rich repeat kinase 2 protein located in the brain and other tissues of the body. This protein has kinase activity but its function is not yet well known. However, recent studies suggest that the protein is part of a complex signaling pathway that modulates neuronal activity by acting on the mechanism that regulates nerve signal transmission (Berwick D.C., 2019). At least seven missense variants in LRRK2 have been described as causing PD. However, unlike what was described above, these mutations lead to a milder phenotype. For example, LRRK2 mutation carriers are less likely to have non-motor symptoms such as olfactory impairment, cognitive features, and REM-behavior sleep disorder (Kestenbaum M., 2017).

The PRKN gene, also known as PARKIN, encodes the Parkin protein. This protein belongs to a family of proteins with a conserved ubiquitin-like domain (UBL) and RING motifs. Parkin functions as a multifunctional cytosolic E3 ubiquitin ligase and catalyzes the transfer of ubiquitinated molecules to multiple substrates. Through mono - and poly-ubiquitination chains bound by lysine-48 or lysine-63, the protein is involved in protein degradation signaling and non-degradation processes. Parkin, along with PINK1, encoding PTEN-induced kinase 1, a mitochondrial serine/threonine-protein kinase, is involved in a quality control system that "controls" the autophagy of depolarized, malfunctioning, or damaged mitochondria, a process known as mitophagy. Parkin is self-inhibited under normal conditions and is activated, by ubiquitination by PINK1 at the UBL domain, to induce mitophagy (Salles P.A., 2024). It is clear, therefore, that mutations in these two genes cause alterations in mitophagy with consequent accumulation of damaged or malfunctioning mitochondria, which contribute to neurodegeneration. Furthermore, alteration of PINK1, the second most common cause of autosomal recessive PD, is characterized by typical PD characteristics such as tremor, bradykinesia and rigidity, with a mean age of onset of 32 years. Additional phenotypic characteristics include dyskinesias, dystonia, and motor fluctuations, with cognitive impairment and psychosis occurring rarely; the disease is slowly progressive, with a sustained response to levodopa therapy, although with a greater tendency to levodopa-induced dyskinesias (Kasten M. H. C., 2018).

The DJ-1 gene, also known as PARK7, encodes a small, highly conserved protein of 189 amino acids, expressed ubiquitously and dimerically under physiological conditions in cells with high energy demands; In particular, it is highly expressed in astrocytes in the frontal cortex and substantia nigra of brains with idiopathic PD and in healthy patients. The protein plays an important role in protecting cells from oxidative stress, a major cause of cell damage in PD. DJ-1 is thought to perform this function by protecting cells from various forms of damage, including oxidative stress, reactive oxygen species, and DNA damage. Mutations in DJ-1 can cause early-onset autosomal recessive parkinsonism and can affect the three-dimensional structure of the protein causing a complete loss of its function or simply a functional alteration, by modulating the toxicity/misfolding of aggregation-prone proteins such as alpha-synuclein (Repici M., 2019).

Some studies conducted on brain tissue have shown that mutated DJ-1 was associated with the presence of Lewy bodies, hypothesizing a correlation with alpha-synuclein. Clinically, PD patients with DJ-1 mutations exhibit an early onset of dyskinesia, rigidity, and tremors, followed by later manifestation of psychiatric symptoms, such as psychotic disturbance, anxiety, and cognitive decline, and generally respond well to levodopa treatment. Although there are several studies conducted on DJ-1, to date the knowledge regarding the involvement of the protein in the pathogenesis of the disease is not yet entirely clear (Kasten M. H. C., 2018).

Due to its ability to protect from oxidative stress, DJ-1 is an interesting target for therapeutic interventions. One approach, which is the most utilized thus far in different pathological models, is to increase DJ-1 levels to obtain neuroprotection when oxidative stress arises. The efficacy of recombinant wild type (WT) DJ-1 for protection of dopaminergic neurons has been demonstrated in several studies employing rat PD models (Repici M., 2019).

1.7 Molecular mechanisms involved in Parkinson's disease

Although PD was first described over two centuries ago, understanding the molecular mechanisms underlying its pathogenesis has only made significant progress in recent decades. Traditionally considered a sporadic and idiopathic

disease, a complex interaction between genetic, environmental and cellular factors is now known to contribute to the onset and progression of the disease. Neuropathological studies, conducted initially on post-mortem brain tissue and later on biological fluids, cellular and animal models, and studies of the functions of genes implicated in inherited forms of PD, suggest that neurodegeneration is linked to numerous molecular and cellular changes, including mitochondrial dysfunctions and oxidative stress, alpha-synuclein aggregation, the aberrant management of proteins resulting in apoptosis and dysfunction of cellular homeostasis maintenance processes. The pathogenic factors cited above are not mutually exclusive and one of the main objectives of current research is to clarify the sequence in which they act and whether the points of interaction between these pathways are fundamental for the disappearance of dopaminergic neurons in SNpc (Dong-Chen X., 2023).

1.8 Role of Oxidative Stress in Parkinson

A feature that has been noticed to be common to neurodegenerative diseases is oxidative stress, a condition produced by the imbalance between oxidants and antioxidants in a biological system. The imbalance occurs as a result of the excess level of reactive species or improper functioning of the antioxidant system (Chiurchiù V., 2016).

In biological systems oxygen plays a crucial role in proper cellular functioning. Although oxygen is crucial for life and is involved in signal transduction, gene transcription and other cellular activities, it also has a deleterious effect on biomolecules when converted into free radicals and highly unstable reactive species, known as reactive oxygen species (ROS). There are various chemical species comprising free radicals, containing oxygen, such as the hydroxyl radical ($HO^{\bullet}$), generated from hydrogen peroxide (H_2O_2), superoxide radical anion ($^{\bullet}O_2^-$), hydroperoxyl radical ($HO_2^{\bullet}$), and peroxyl radicals ($ROO^{\bullet}$). Besides reactive oxygen species, other unstable molecules are produced in biological systems, among these important are the reactive nitrogen species (RNS). The most important is nitric oxide (NO), which performs several biological functions including relaxation and

proliferation of vascular smooth muscle cells, leukocyte adhesion, angiogenesis and others. Radical forms like nitric monoxide (NO•) and peroxynitrite ($ONOO^-$) are harmful for biomolecules. Furthermore, it is also appropriate to mention Lipid peroxides (LPO), highly reactive molecules that include malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), acrolein, isoprostanes (IsoPs), and neuroprostanes (NeuroPs). They can disrupt proteins and DNA structure and functions (Singh A., 2019).

An important aspect to consider is that free radicals perform important physiological functions necessary for cellular homeostasis. They are necessary to synthesize some cellular structures and are used as a defense against pathogens. In fact, phagocytes synthesize and store free radicals, in order to be able to release them following exposure of the pathogen. Free radicals play a key regulatory role in intracellular signaling cascades, in different cell types. For example, nitric oxide is an important cell-cell messenger necessary for correct modulation of blood flow and for normal neural activity (Pizzino G., 2017).

The redox balance is guaranteed by the presence of a complex antioxidant system. Antioxidants are generally divided into antioxidant enzymes and low molecular weight antioxidants. The most important antioxidant enzymes involve Superoxide dismutases (SODs), Catalase (CAT), Glutathione peroxidase (GPx), and others. The most important low molecular weight antioxidants involve vitamin C, vitamin E, carotenoids, flavonoids, glutathione, and other antioxidants. Antioxidant enzymes are a class of proteins that catalyze, by a multiphase process, the transformation of ROS and/or their byproducts into more stable and usually less harmful species, such as hydrogen peroxide and water. Low molecular weight antioxidants disrupt radical chain reactions. Some low molecular weight antioxidants are water soluble and act in the cytosol or cytoplasmic matrix. Another group of antioxidants, soluble in lipids, operates in membranes (Jomova K., 2023).

Reactive oxygen species are usually produced from exogenous and endogenous sources. Sources of exogenous ROS production includes ionizing radiation, xenobiotics, alcohol and smoking, but also poor nutrition and viral and bacterial infections. In this regard it is necessary to remember that the gut microbiome plays an important role in maintaining redox balance. As discussed above, dysbiosis can

cause damage to the intestinal mucosa. This causes the passage of endotoxins, such as lipopolysaccharides (LPS), into the bloodstream and consequent overactivation of the immune system. This ultimately leads to an increase in inflammation and consequent increase in ROS (He Q., 2013) (Hyży A., 2025). Finally, ROS can also be produced as by-products due to the metabolism of environmental chemicals. On the other hand, free radicals are produced in different compartments of our body as products of normal cellular metabolism. Production occurs by the cells of the immune system in response to pathogens, in the endoplasmic reticulum following some oxidation reactions during the biosynthesis of proteins and lipids, and in peroxisomes responsible for the beta-oxidation of long-chain fatty acids. However, the main source of production of reactive oxygen species is mitochondria. Most reactive oxygen species are the result of cellular respiration, a process in which an electron breaks away from the electron transport chain and attaches to oxygen, resulting in superoxide anions (O_2^-). The mitochondria take part in the formation of ROS through the electron transport chain (ETC) (Figure 5) (Singh A., 2019).

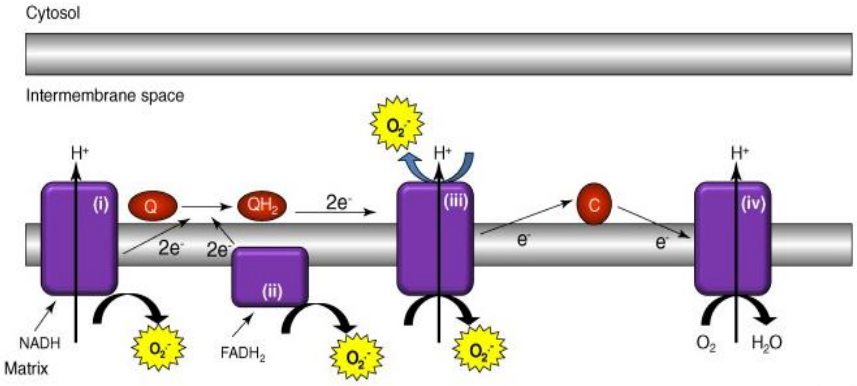


Figure 5: Schematic representation of the endogenous production of ROS by mitochondria. (Hamanaka R.B., 2010)

However, under pathological conditions or within different organs, these enzymes have different capacities for ROS generation. For example, Complex I is considered the main source for ROS production in the brain. Moreover, in the mitochondria, ETC Complex I and Complex III are considered to be the primary generators of ROS. During normal conditions, Complex III generates two times more ROS than Complex I, whereas under disease conditions, Complex I is the main source (Kim G.H., 2015).

Moreover, it must be considered that there are two main defense mechanisms that can act against ROS: dopamine transporter (DAT) and vesicular monoamine transporter 2 (VMAT2). These neurotransmitter transporters can remove free dopamine from the synapse and pack it into synaptic vesicles to be protected from oxidation. However, with age, nigral expression of DAT gradually declines, indicating an impaired synaptic dopamine clearance. In addition, alpha-synuclein interacts with VMAT2 during vesicle filling and inhibits DAT-mediated synaptic dopamine reuptake (Trist B.G., 2019).

Oxidative stress is thought to play a critical role in PD pathophysiology, as it is considered, along with energy stress and altered proteostasis, to be the most relevant pathogenetic process involved in the progression of PD. Excessive ROS production is due to neuroinflammation, mitochondrial dysfunction, age, caspase activation-inducing intracellular calcium influx, dopamine degradation, altered alpha-synuclein proteostasis and altered ubiquitin-proteasome system. Furthermore, it can be aggravated in case of environmental exposure to pesticides and neurotoxins. Specifically, alpha-synuclein accumulation in mitochondria is supposed to cause mitochondrial complex I deficiency. An additional link between ROS and disease is emerging from the study of monogenic forms associated with loss of function mutations of the PINK1 and PARKIN genes. Alterations in these genes cause incorrect mitophagy. This implies that damaged or malfunctioning mitochondria are not removed and contribute to the increase in oxidative stress. A malfunction of monoamine oxidase B (MAO-B), the enzyme that degrades dopamine, can occur in patients with PD; this causes the release of free radicals, causing excitotoxicity. ROS also affect the stability of nucleic acids, causing RNA oxidation and mitochondrial DNA mutations. Astrocytes and microglia represent an additional target of oxidative stress implicated in neurodegenerative processes. Glial cells help counteract the increase in ROS due to excessive dopamine activation in a specific nigral subpopulation. Interestingly, both astrocytes and microglia possess dopamine receptors, which become more active in the context of neuroinflammation (Kim G.H., 2015) (Caproni S., 2025).

1.9 Mitochondrial dysfunction

Mitochondria are independent organelles, typically ellipsoidal in shape and perform crucial functions for the cell. Eukaryotic cells contain many mitochondria, which occupy up to a quarter of the cytoplasmic volume. Mitochondria contain their own genome that encodes only 13 proteins in the respiratory chain. Each mammalian mitochondrion contains 2 to 10 copies of mtDNA, resulting in 1000 to 100,000 copies in every human cell. Individual mtDNA molecules replicate randomly, and if there are two or more different types of mtDNA in a cell, any one of them can replicate more frequently than the other. This is advantageous because in case of deleterious mutations, a compensatory mechanism would be created to continue expressing a protein. However, the copy number of mitochondrial DNA is not the same for everyone, with a lower copy number indicating reduced efficiency in the body and brain. Every aspect of the mitochondria's form is linked to a highly specialized function, with dynamic events allowing their appearance to range from their more typically described rod shape to more complex reticular networks (Klemmensen M.M., 2024).

From a structural point of view, the outer membrane, characterized by the presence of membrane receptors and proteins, including voltage-dependent anion channels that allow the transport of both hydrophilic and small proteins, has a porous structure and acts as a boundary between the inside of the mitochondria and the cytoplasm. Its composition is the same as that of the cellular lipid membrane, allowing the diffusion of fat-soluble molecules into the intermembrane space. Its porous nature makes it permeable to small molecules and ions. More internally, motochondria feature a second cardiolipin-enriched membrane and contain more proteins than the outer membrane, including enzymes involved in several biochemical pathways, including oxidative phosphorylation, this membrane is impermeable to most small ions and molecules. The two membranes separate the intermembrane space which is similar to the cytoplasm, but with greater specificity for larger mitochondrial proteins. Because of its strong impact on mitochondrial function, it serves as a limiting factor when targeting mitochondria with drug therapies. The double membrane is characterized by the presence of lamellar structures called ridges. They have a large surface area for oxidative

phosphorylation and maintenance of the proton gradient, demonstrating the high adaptability of mitochondria. The complexity of the ridges can vary depending on the tissue, cell type, stage of development, or physiological conditions (Frey T.G., 2000).

Approximately 90% of the ATP necessary for the correct functioning of the cell is produced through oxidative phosphorylation, the final phase of cellular respiration. This biological process consists of two parts: the electron transport chain (ETC), which is composed of four multi-subunit protein complexes, combined with the final complex, ATP synthase, the enzyme that generates ATP from ADP and phosphate. The complexes that make up the respiratory chain are located in the internal mitochondrial membrane. Specifically, ETC includes 4 lipoproteic multienzyme complexes such as Complex I (NADH dehydrogenase) and II (succinate dehydrogenase) which transfer electrons to ubiquinone Q; Complex III (cytochrome bc1) which transfers electrons from ubiquinol to cytochrome c; and Complex IV (cytochrome c oxidase, COX) involved in the transfer of electrons to molecular oxygen to form water. The movement of electrons is associated with the transport of protons (H+) in the intermembrane space, generating an electrochemical gradient called proton-motor force, which consists of the mitochondrial membrane potential (ΔΨ m), the main factor contributing to the proton-motor force, and in a pH gradient (Δ pH). The proton gradient is exploited by ATP synthase to produce energy. It is important to note that during glycolysis with 1 molecule of glucose only 2 molecules of ATP are produced, while in cellular respiration 15 times more ATP molecules are produced (Pham L., 2024) (Figure 6).

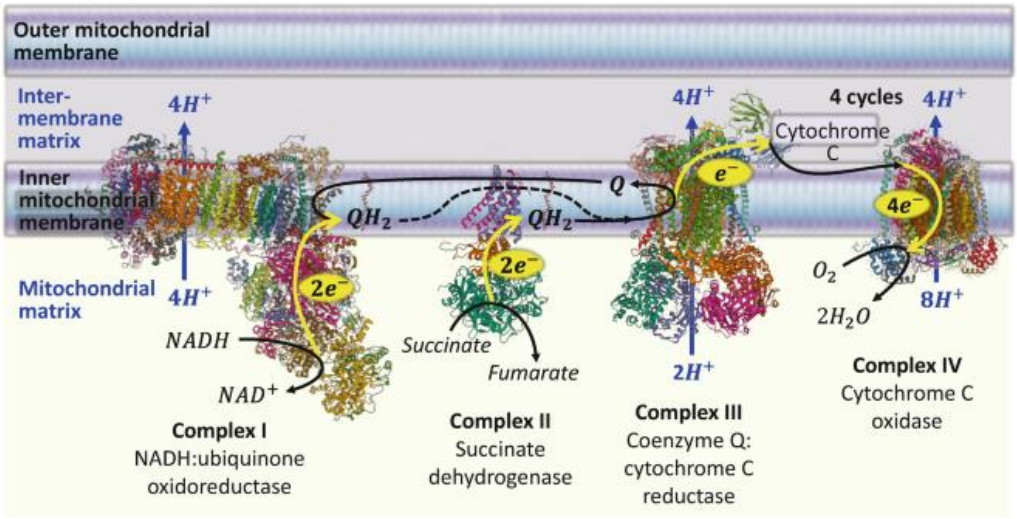


Figure 6: Complexes involved in cellular respiration. (Volodyaev I., 2023)

As discussed above, neurons, unlike other cell types, consume a greater amount of oxygen and therefore require a greater amount of ATP to ensure proper transmission of nerve impulses. However, it is interesting to note that a smaller amount of mitochondria are present in neurons, located mainly in synapses. Mitochondrial diseases result from dysfunction of the mitochondrial respiratory chain that occurs due to mutations in the mitochondrial or nuclear genome, such as mutations in the PINK-1, PARKIN, DJ-1 genes. In addition to this, there are other factors of mitochondrial damage including excessive production of ROS, due to several causes including defects in respiratory chain complexes, as already discussed, and reduction of antioxidant defenses. Redox imbalance, also favored by exposure to xenobiotics and neurotoxic substances, as well as by the accumulation of damaged or misfolded proteins that are not removed due to the compromise of the ubiquitin-proteasome system, in turn induces an increase in the production of pro-inflammatory cytokines, triggering a harmful cyclical mechanism which, especially in chronic diseases, it is also not counterbalanced due to dysfunctions in autophagy processes (mitophagy). All this, coupled with mtDNA defects and respiratory chain abnormalities, is linked to the pathogenesis and progression of many neurodegenerative disorders, including PD, as shown in Figure 7 (Caproni S., 2025).

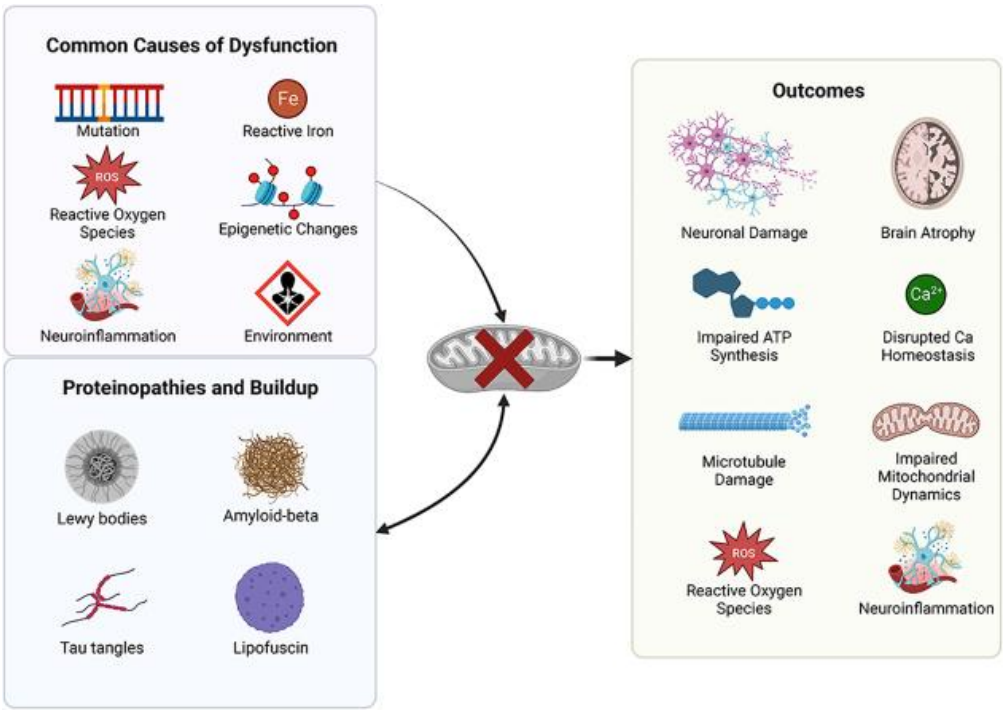


Figure 7: Common causes of Mitochondrial dysfunction and what they cause. (Klemmensen M.M., 2024)

More specifically, mitochondrial dysfunction can favor cell death processes such as apoptosis and necrosis. Apoptosis is a process of programmed cell death that is tightly regulated by the mitochondria. Apoptosis is often the result of intracellular signaling characterized by morphological changes such as cell shrinkage, chromatin condensation and nucleus fragmentation which ultimately result in the removal of the cell without damage to surrounding tissues. The two main apoptotic pathways that occur in mammalian cells are the intrinsic and extrinsic pathways, in which mitochondria play an essential role. The intrinsic pathway is controlled by the Bcl-2 family, which directs death signaling to the mitochondria, facilitating the release of pro-apoptotic proteins from the intermembrane space. The extrinsic pathway is characterized by a cascade of caspases, the most important of which are caspase 9, which acts as an activator, and caspase 3, which acts as an effector; a cascade of protein interactions is generated which lead to the permeabilization of the mitochondrial outer membrane and the release of cytochrome c from the intermembrane space. The main effectors of apoptosis are the antiapoptotic proteins Bcl-2 and the proapoptotic proteins BAX and BAK, which interact with mitochondria. In contrast, necrosis occurs in response to extracellular stimuli,

ischemia or trauma, leading to cell swelling and rupture of cell membranes. Cellular debris released into the extracellular space causes damages surrounding tissues, triggering an inflammatory process. (Orrenius S., 2015) (Klemmensen M.M., 2024).

Mitochondria play a critical role in maintaining calcium homeostasis, an important ion that serves as a secondary messenger in a variety of signaling pathways, is involved in the release of neurotransmitters, and helps regulate gene expression. Calcium transport across the outer mitochondrial membrane occurs through VDAC, the most abundant protein in the outer membrane and whose expression is directly proportional to the calcium concentration. Both calcium signaling and calcium uptake in mitochondria are linked to apoptosis and necrosis through the mitochondrial permeability transition pore (mPTP). An increase in intracellular calcium concentration, combined with oxidative stress, induces significant variation in inner mitochondrial membrane permeability, resulting in reduced membrane potential, disruption of oxidative phosphorylation, and matrix swelling. Subsequently, the rupture of the beautiful mitochondrial membrane occurs with the consequent release of cytochrome and activation of apoptosis or necrosis processes (Vakifahmetoglu-Norberg H., 2017).

The oxidative stress induced by ROS directly influences epigenetic modifications. This occurs both through the oxidation and damage of DNA and through inhibiting the activity of DNA methyltransferases, the enzymes responsible for adding methyl groups to DNA. In both cases hypomethylation occurs with consequent alteration of gene expression (Rackham O.J., 2017).

Another important factor closely related to mitochondrial dysfunction in neurodegenerative diseases, especially PD, is hypoxia. It is not a direct cause of the disease, but can be a consequence or an aggravating factor and can be caused by disease processes, toxins, chronic hypoxia, or a severe decrease in environmental oxygen levels, leading to cell injury and exacerbation of PD symptoms. This process is regulated by hypoxia-inducible factors (HIF) and factor-2-related nuclear erythroid factor 2 (Nrf2). In short, HIFs are transcription factors that respond to low oxygen levels by regulating molecular adaptations to maintain oxygen supply and energy metabolism. Among these, the most important factors that act at the brain level in PD are HIF-1, HIF-2. They are heterodimers made up of an α (oxygen-sensitive) and a

β (constitutive) subunit. Under physiological conditions HIF-a subunits are continuously degraded through hydroxylation, catalyzed by prolyl hydroxylase (PHD) and HIF inhibiting factor. As a result, under conditions of severe hypoxia, reduced oxygen levels inhibit PHD activity, preventing degradation of HIF-α subunits. Consequently, HIF is not degraded by the ubiquitin-proteasome system and induces HIF-1β transcription at the nuclear level, improves oxygen delivery to tissues and facilitates metabolic adaptation to hypoxia. HIF-1α is activated rapidly during acute and severe hypoxia and appears to have neuroprotective effects as it regulates the expression of genes such as VEGF, involved in angiogenesis, and tyrosine hydroxylase (TH), important for dopamine synthesis, and for survival and differentiation of dopaminergic neurons. However, in case of severe hypoxia, the factor can act by suppressing mitochondrial function, facilitating the formation of inflammasomes and triggering cell death by increasing the expression of pro-apoptotic genes (Chen H., 2022) (Ivy C.M., 2022).

Studies conducted on PD patients have shown that the function of HIF-1α is reduced and at the same time there is greater degradation of this factor. All this translates into less protection of the CNS neurons. In contrast, HIF-2α gradually accumulates during prolonged and moderate hypoxia and shares the same target genes as HIF-1α. Studies conducted on post-mortem brain tissue demonstrate the accumulation of HIF-2α can hyperphosphorylate alpha-synuclein at the serine 129 site contributing to abnormal aggregation. In addition, Nrf2 plays a crucial role in protecting cells from oxidative stress by activating the transcription of a wide range of antioxidant and anti-inflammatory genes. Under oxidative stress conditions, also induced by a condition of acute or severe hypoxia, Nrf2 translocates to the nucleus, where it forms a heterodimer with small MAF proteins and binds to the antioxidant response element (ARE) to activate a series of detoxification genes phase II. (Li G., 2022).

Dopaminergic neurons in SNpc are highly susceptible to hypoxia. During chronic hypoxia, hyperphosphorylation of alpha-synuclein occurs, a mechanism involved in the formation of oligomeric aggregates. The oligomers promote the accumulation of HIF-1α in primary normoxic microglia through the mediation of toll-like receptors 7 and 8, thus improving their migratory capacity. Furthermore, cytotoxic alpha-

synuclein can alter the structure and permeability of the mitochondrial membrane. This results in blockage of oxidative phosphorylation, impairment of autophagy, including mitophagy, preventing the removal of dysfunctional mitochondria. Hypoxia, in this context, can hinder the Nrf2/HO-1 pathway by reducing the expression of scavenger enzymes. This leads to an increase in oxidative stress which is associated with neuroinflammation and the blockage of the ubiquitin-proteasome system. All this damages brain tissue and accelerates neurodegeneration (Gao Y., 2024).

Evidence demonstrating the involvement of mitochondria in PD comes from studies conducted on MPP^+. MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is a molecule structurally similar to the herbicide Paraquat and was the first neurotoxicant shown to induce in humans a profound parkinsonian syndrome. MPTP injures, in a selective manner, the dopaminergic neurons in the nigrostriatal system, and when tested in various animal species, including non-human primates, it showed the ability to recreate most of PD typical symptoms. For example, the long exposure to low MPTP doses promotes the increase of oxidative stress, α-syn fibrillization, and loss of mitochondrial functionality. Since MPTP is a highly lipophilic compound, it rapidly crosses the blood–brain barrier and after systemic exposure, the toxin levels are already detectable in the brain within minutes. By itself, MPTP is not a toxic substance, however, once in the brain, it is metabolized to 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP) by the enzyme monoamine oxidase B (MAO-B) in non-dopaminergic cells such as astrocytes. Next, MPDP is oxidized to the active 1-methyl-4-phenylpyridinium (MPP^+) which is then released into the extracellular space, where it is taken up by the dopamine transporter (DAT) and is concentrated within the dopaminergic neurons, causing the specific loss of nigrostriatal neurons. MPP^+ exerts its toxicity by concentrating in the mitochondria, where it blocks the activity of NADH-ubiquinone oxidoreductase (complex I) of the mitochondrial electron transport system (ETS), leading to ATP depletion and the production of reactive oxygen species (ROS). Furthermore, prolonged exposure to the toxin causes a drastic inhibition of the synthesis of respiratory subunits encoded by mtDNA (Risiglione P., 2020).

1.10 Neuroinflammation

Neuroinflammation is an important contributing factor to PD pathogenesis. Epidemiological studies have found that various chronic inflammatory autoimmune diseases, such as rheumatic diseases, Crohn's disease, and ulcerative colitis, are associated with an increased risk of PD development. In contrast, anti-inflammatory drugs, such as nonsteroidal anti-inflammatory drugs or corticosteroids, can delay or prevent onset. Moreover, in genetic studies, more than 90 loci of human leukocyte antigen (HLA) genes encoded by major histocompatibility complex class II (MHC-II) have been identified. These genes participate in antigen presentation during the immune response and are associated with sporadic PD (Tan EK., 2020).

Evidence of inflammation in PD emerges from post-mortem brain tissue analyses and in vivo studies. The immune response in the central nervous system (CNS) is distinct from that in the peripheral system in terms of the blood-brain barrier, glial cells such as microglia and astrocytes, and the absence of an adaptive immune response itself. Specifically, preliminary studies have demonstrated the presence of innate immune cells in the brains of post-mortem patients. Both microglial and astrocytic activation was observed, with consistent microglial activation (Lai T.T., 2022).

Microglia constitute approximately 5%–12% of CNS cells and are capable of self-renewal independent of hematopoietic stem cells. In addition to microglia, astrocytes are the most abundant CNS cell type. They demonstrate diverse morphological and functional characteristics dependent on specific brain areas. Astrocytes are known to contribute to various physiological functions, including the maintenance of neurons, the formation of the blood-brain barrier, and the regulation of synaptic functions. The innate immune responses are considered the first line of defense against invading pathogens and malfunctioning proteins. Therefore, the activation of microglia is protective for the brain. However, sustained or chronic activation of microglia can lead to irreversible CNS damage. Recent observations add further levels of complexity to understanding the microglia-mediated mechanisms affecting the brain. In fact, it is believed that there are different subtypes of activated microglia, which can be defined by common cell surface markers, and which express heterogeneous cytokines that could contribute to

tissue damage. Microglia operate as safeguards of the CNS and they are activated following the presence of pathogens; this occurs because microglia are equipped with toll-like receptors (TLRs), which are transmembrane receptors characterized by a leucine-rich extracellular repeat domain that detects pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). The activation of downstream TLRs pathways leads to the production of pro-inflammatory cytokines or to the production of type I interferons, which induces the release of IFN-β and chemokines, such as the C-X-C motif chemokine ligand 10 (CXCL10) (Hickman S., 2018) (Siracusa R., 2019).

Aging, one of the main risk factors of PD, is associated with senescence, an involutional biological process characterized by structural modifications and the decay of various physiological activities and functions. Like neurons, senescence also involves microglia and astrocytes, therefore these cells are subject to age-dependent selective alterations. In PD, senescence, along with other factors including ROS, trigger a process of hyperactivation of microglia and astrocytes, known as gliosis, which results in alterations in these immune cells. In particular, senescent microglia express higher levels of IL-1β, TNF-α, IL-6 and IFN-γ than physiological microglia. The chronicity of the inflammatory process creates a vicious cycle that exacerbates the inflammatory insult, and promotes the incorrect folding and aggregation of endogenous alpha-synuclein, ultimately leading to neurodegeneration. (Muzio L., 2021) (Figure 8).

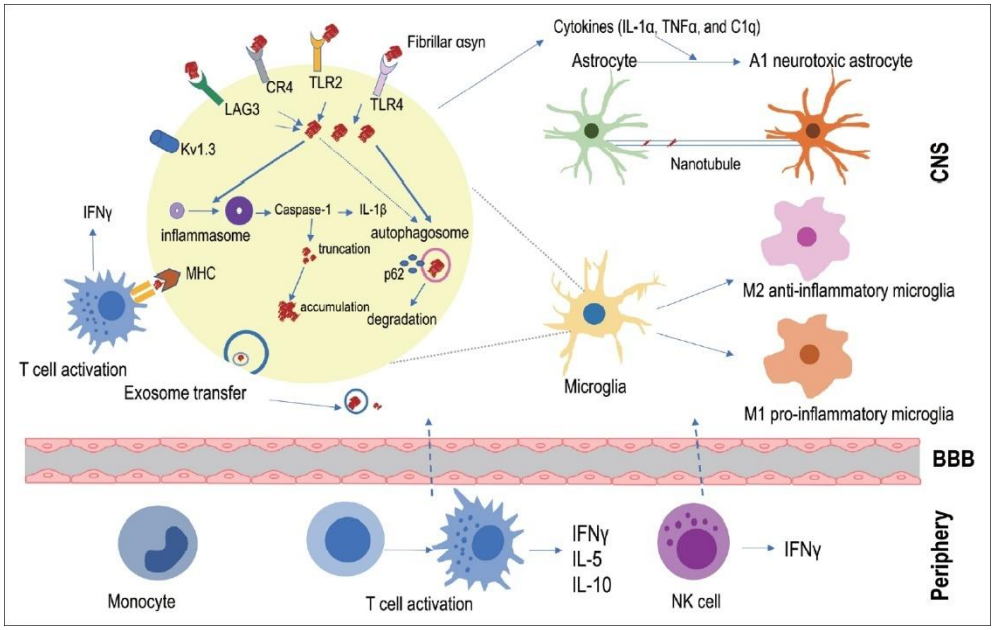


Figure 8: Mechanism of neuroinflammation involving glial cells, the accumulation of misfolded synuclein and the excessive production of pro-inflammatory molecules. (Lai T.T., 2022)

In addition to the activation of innate CNS immune cells, peripheral lymphocyte infiltration has also been observed in the PD brain and cerebrospinal fluid, which further demonstrates the immune association in PD. In particular, an increase in CD4 and CD8+ T cells was observed, but not in B cells or natural killer (NK) cells. It has been suggested that T cell infiltration and activation is due to increased expression of specific factors by astrocytes and microglia, during inflammatory response. Increased activation of monocytes or NK cells has also been observed in peripheral blood of PD patients. In addition to blood, CSF from PD patients has been shown to have greater activation of immune cells, such as T lymphocytes and monocytes, than healthy patients (Sorrentino Z.A., 2019).

Based on what has been said, it is clear that there is a correlation between inflammation and alpha-synuclein. Galiano-Landeira et al. observed robust infiltration of CD8+ T cells, especially in the early stage of Lewy body disease, observing increased T cell reactivity against the protein. These results indicate the possibility that inflammation could start from an early disease state and precede alpha-synuclein pathology (Galiano-Landeira J., 2020). Although synuclein is able to trigger an inflammatory process, it must be considered that, according to studies conducted in vitro and on animal models, microglia and astrocytes have a function

similar to that of macrophages which, through lilosomal processes, are able to incorporate and degrade the protein. They therefore exert a neuroprotective effect. In studies of the cellular mechanism underlying alpha-synuclein clearance, in vitro research has identified that the cytotoxic protein, when present in large quantities, alters microglial function through interactions with TLR. TLR4 deficiency results in over-expression of synuclein, which does not occur when Toll-like receptor functionality is restored. In conclusion, inflammation resulting in hyperactivation of microglia and astrocytes contributes to the propagation of alpha-synuclein and the progression of the disease through exosomal transfer from cell to cell with the involvement of Toll-like receptors (Lai T.T., 2022).

1.11 Impaired autophagy in Parkinson's disease

Autophagy is a fundamental cellular process that allows the cell to degrade and recycle its components, such as damaged proteins or unnecessary organelles. This process is essential for maintaining cellular homeostasis and responding to environmental stress. In eukaryotic cells, there are three major types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. Autophagy is activated in response to several signals, such as lack of nutrients, oxidative stress, hypoxia, cellular damages or cellular infection. It is characterized by several sequential steps: induction and nucleation, load sequestration, release and fusion of the autophagosome with the lysosome, degradation and recycling of the degraded material and reformation of the autophagic lysosome, regulated by several independent mechanisms including the unc-51-like autophagy-activating kinase 1 (ULK1) complex, the class III PI3K complex, and two ubiquitin-like conjugation systems (Ravikumar B., 2010). The induction step involves the activity of autophagy-related proteins (Atgs) and is regulated by upstream pathways. These include mammalian target of rapamycin complex 1 (mTORC1) pathway, which reduces ULK1 complex activity and is an important repressor of autophagy induction. Furthermore, the Bcl-2 pathway inhibits the class III PI3K complex by binding to Beclin 1, one of its major components. mTORC1 is negatively regulated by energy-sensitive kinases, such as AMP-activated protein kinase (AMPK) and positively regulated by Akt. The elongation step is the process in which the material to be degraded is surrounded by

a double membrane structure called autophagosome. This phase is regulated by two ubiquitin-like conjugation systems. First, the ATG5-ATG12 complex non-covalently connected to ATG16L1 is formed which is associated with the phagophore membrane and is responsible for membrane elongation and autophagosome formation. The second complex involved in phagophore elongation and load recognition is Atg8/LC3, also known as MAP1LC3B (protein associated with 3B light chain microtubules), which undergoes conjugation with phosphatidylethanolamine (lipidation), by the first complex (Nechushtai L., 2023). In the last phase the autophagosome recognizes the material to be incorporated through the intervention of adaptor proteins such as p62 which recognize ubiquitinated proteins. The autophagosome then moves to the perinuclear regions of the cell where the lysosomes are located by the microtubules and fuses with a lysosome, forming the autolysosome. This allows lysosomal enzymes to degrade internal components and recycle products. This process is regulated by the SNARE complex, which directly mediates fusion between the autophagosome and the lysosome, and involves regulatory proteins such as HOPS complex (Homotypic fusion and protein sorting) and Rab 7 (Figure 9) (Zhu Y., 2024).

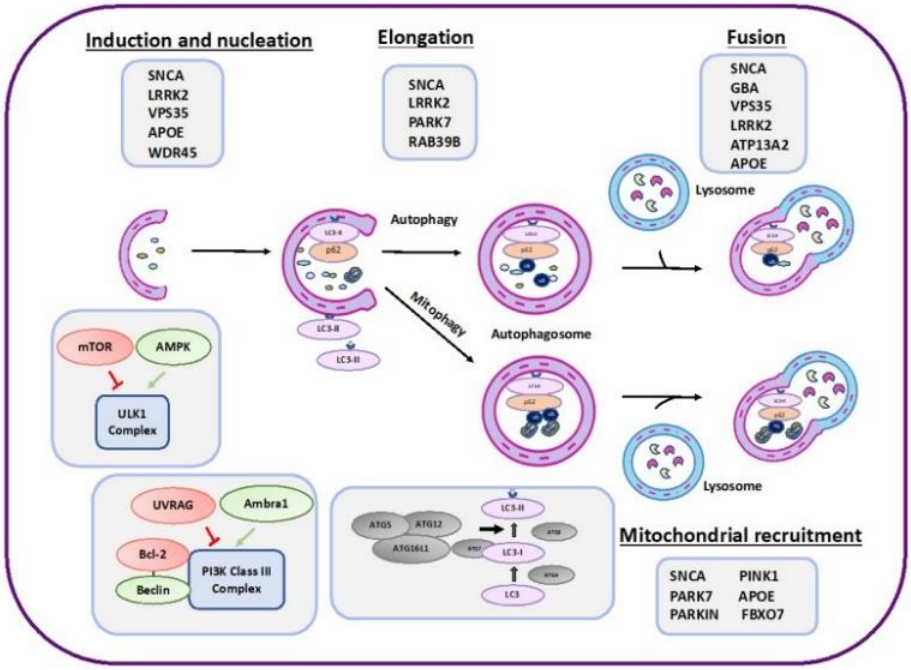


Figure 9: A schematic presentation of the various autophagy steps and proteins involved in autophagy regulation**.** (Nechushtai L., 2023)

Increasing evidence indicates that unregulated autophagy can contribute to the development of various neurodegenerative diseases, mainly those related to protein conformational disorders, by enhancing the accumulation of proteins and inducing cellular toxicity. Defects early in the process can lead to toxicity due to the accumulation of proteins or cellular components in the cytosol. Studies have shown that in the brain tissue of PD patients there were elevated levels of LC3 protein and impaired autophagy. In addition, there was a reduction in the levels and activities of lysosomal enzymes, such as glucocerebrosidase or cathepsin D protease (Moors T.E., 2019).

Autophagic process malfunction in PD can be due to several factors. First, genetic mutations related to PD directly or indirectly alter the mechanism. Mutations in LRRK2 (leucine-rich repeat kinase 2) can negatively affect autophagosome maturation, alter vesicular trafficking, and inhibit fusion with lysosomes. Several studies suggest that LRRK2 regulates lysosomal function through its kinase activity on Rab GTPases. PINK1, PARKIN and DJ-1 are involved in mitochondrial quality control pathways, activating mitophagy in response to mitochondrial damage, as already mentioned. Mutations in these genes inhibit mitophagy, thus allowing the accumulation of damaged mitochondria that ultimately lead to apoptosis (Rüb C., 2017).

Several studies show that α-synuclein can affect autophagy. Protein overexpression has been reported to increase the interaction between Bcl-2 and Beclin 1 by inhibiting autophagy. It was also demonstrated that synuclein leads to the accumulation of Parkin and that it compromises autophagy via inhibition of Rab1, resulting in ATG9 mislocalization or hindering the maturation of the autophagosome or by reducing the expression of SNARE complex proteins, preventing fusion with lysosomes. The degradation of α-synuclein aggregates depends on lysosomal degradation mediated by autophagy. Generally, the monomeric forms are degraded by chaperone-mediated autophagy, while the aggregated forms are degraded by macroautophagy. However, cytosolic aggregates characterized by mutations and dopamine-induced post-translational modifications have been found in the brain tissue of PD patients. These forms have been shown to inhibit autophagy (Nechushtai L., 2023).

1.12 Alpha-synuclein in Parkinson's disease

In 1985, a 140-amino acid protein that is present in both the cytoplasmic and nuclear regions of neurons, called α-synuclein, was identified in the brains of rats, although subsequent studies have never confirmed nuclear localization. Further studies showed that this protein is present in a wide variety of tissues but is more abundant in the brain and has homologies with proteins involved in the formation of amyloid plaques associated with Alzheimer's disease. A smaller protein of 134 amino acids was also identified, which showed a homology of 61% with the 140 amino acid protein, called β-synuclein. These proteins are encoded by the SNCA and SNCB genes respectively (Emamzadeh FN., 2016).

1.13 Structure and physiological functions

α-synuclein is characterized by three domains, namely the amphipathic N-terminal end, the central amyloid binding domain (NAC), also known as the non-Aβ component of amyloid plaques, and the C-terminal acid tail. The protein may be present as an α-helical structure in association with phospholipids or an unfolded conformation in the cytosol, suggesting that it plays specific roles at different cellular locations based on its dynamic structure. The N-terminal domain (residues 1–60) is a positively charged region, which includes seven sets of 11 amino acid repeats. Each repeat contains a highly conserved KTEGV hexameric motif, also present in the α-helical domain of apolipoproteins. These repeats, through their ability to induce the helical structure of synuclein and subsequently reduce the protein's tendency to form β structures, are important in interactions with lipids. Therefore, this region therefore allows α-synuclein to bind to membranes. The central region (residues 61–95), also known as NAC, is involved in the formation and aggregation of fibrils, as it can form crossed β structures. The C-terminal domain (residues 96-140) is an acidic tail of 43 amino acid residues, containing 10 residues of Glutamate and 5 residues of Aspartate. Structurally, the C-terminal domain is present in a spiral structure due to its low hydrophobicity and high net negative charge. In vitro studies revealed that aggregation of α-synuclein can be induced by pH reduction neutralizing these negative charges. An interaction between the C-

terminal domain and the NAC region is thought to be responsible for inhibiting aggregation of the protein (Figure 10) (Rodriguez J.A., 2015) (Sode K., 2006).

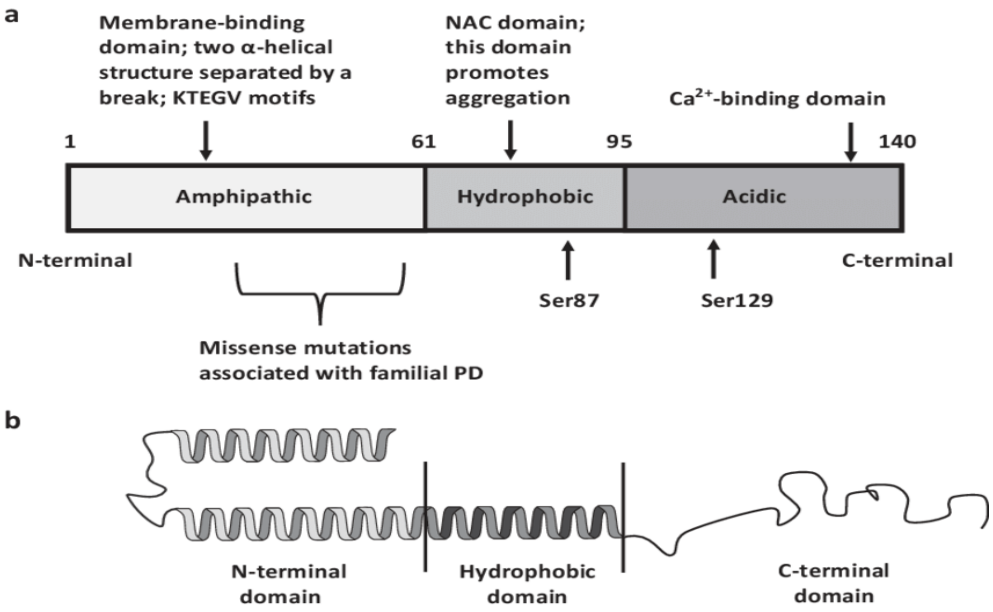


Figure 10: A schematic figure about the structure of α-synuclein. The protein can be divided into three distinct domains. The N-terminal amphipathic domain contains the evolutionary conserved KTEGV motifs. The hydrophobic NAC region is responsible for promoting aggregation. The C-terminal domain is negatively charged. It contains a Ca^{2+}-binding site. (Torok N., 2016)

As mentioned, the N-terminal end of α-synuclein, being rich in lysine residues, in the repeated domains, binds electrostatically to anionic lipids. In particular, the binding occurs more with phosphatidylethanolamine, phosphatidic acid, phosphatidylinositol and ganglioside rather than with phosphatidylserine or phosphatidylglycerol. In addition, polyunsaturated acyl chains can increase the ability of the protein to interact with the membrane. This may be due to the greater space between poorly packaged unsaturated lipids compared to compact saturated forms. The membrane-bound alpha-synuclein forms a helical structure that spans eleven residues in three distinct regions. A break at the center of this structure divide the synuclein into two helical zones, increasing its ability to bind to highly curved membranes. it has been observed that the A30P mutation in the SNCA gene causes a shift in two of these structures, reducing the interaction force; However, the A53T mutation does not appear to affect the conformation and affinity of interaction, while E46K can increase the tendency of synuclein to interact with the

membrane (Nuscher B., 2004). Although the first 100 amino acid residues are important for lipid interactions, different N-terminal regions bind to membranes based on lipid to protein ratio. Typically, α-synuclein binds to membranes with its first 25 residues when its ratio is high enough. However, reducing the lipid to protein ratio causes the protein to interact with the membrane by binding of the first 97 residues. Depending on this ratio and the curvature of the membrane, a structural transition of the protein from a disordered state to an a-helix, bifurcated or extended, occurs (Pfefferkorn C.M., 2012).

To date, the functions of α-synuclein are not entirely clear, however it seems that the protein is implicated in various physiological mechanisms. Protein kinase C (PKC) is a serine-threonine kinase that phosphorylates several target proteins and thus controls many mechanisms, such as apoptosis. This kinase is very sensitive to oxidative stress and triggers an apoptotic cascade in dopaminergic cells. It appears that alpha-synuclein is able to downregulate kinase, by inhibition of nuclear factor κB (NF-kB) protecting dopaminergic cells from apoptosis (Jin H., 2011). In addition to neurons, synuclein has also been identified in many other cell types, particularly secretion cells. Proteins are thought to be able to reduce insulin secretion by interacting with ATP-dependent potassium channels. These findings suggest a role for alpha-synuclein in diabetes (Geng X., 2011). Another function attributed to alpha synuclein includes the ability to modulate lipid synthesis by acting on the enzyme Acyl-CoA synthetase (Emamzadeh FN., 2016).

However, α-synuclein acts mainly at the neuronal level, where it participates in vesicular trafficking and in the modulation of neurotransmitter exocytosis by promoting vesicle fusion with the presynaptic membrane and reuptake. This causes that α-synuclein promotes interaction between proteins of the SNARE complex (Scott D., 2012). α-synuclein, as a downregulator of tyrosine hydroxylase (TH) activity, can modulate dopamine production and control cell levels. Thus, its reduced expression or aggregation leads to increased dopamine synthesis, resulting in oxidative stress caused by dopamine metabolism. The inhibitory effect on TH activity is not direct and depends on the interaction between synuclein and protein phosphatase 2A (PP2A). Following this interaction, dephosphorylation of the Ser 40 residue occurs resulting in TH inhibition (Yang W., 2013). Finally, some studies

suggest that the protein associates with the external mitrochondrial membrane, thanks to binding with cardiolipin and could participate in mitochondrial transport, mitochondrial biogenesis or the response to oxidative stress. However, this role is still being studied (Emamzadeh FN., 2016).

1.14 Mechanism of aggregation and formation of cytotoxic oligomers of alpha synuclein

Synucleinopathies, such as PD, dementia with Lewy Bodies (DLB), and multiple system atrophy (MSA), are a group of neurodegenerative disorders of unknown aetiology, which share some typical characteristics. The most significant being the aggregation of α-synuclein, present in cytoplasmic inclusions bodies in the cells of the affected brain regions. These cytoplasmic inclusion bodies are different in nature and cellular localization across these synucleinopathies. Lewy bodies, eosinophilic inclusions consisting of filamentous structures in a dense core surrounded by a peripheral structure containing numerous membranous components and dysmorphic organelles, are found in neurons of PD and DLB patients' brains, while glial cytoplasmic inclusions, filamentous structures in the shape of a sickle, are detected in oligodendrocytes of MSA patients' brains. The neuronal pathology, known as Lewy pathology, is the most studied in PD (Estaun-Panzano J., 2023).

α-synuclein exists in various conformational forms. In fact, it is present in at least two structural isoforms that comprise a soluble, natively unfolded monomeric form and a membrane-bound form, rich in helixes. Some authors have suggested the existence of an alternative, tetrameric, aggregation-resistant organization of the protein (Bartels T., 2011). Isoforms can undergo radical structural changes, resulting in increasingly assembled structures that are the precursors of more complex amyloid forms of the protein. Specifically, α-helix structures tend to organize themselves into more compact and stable oligomeric structures made up mainly of β-sheets. Continuous polymerization leads to the formation of insoluble, higher molecular weight protofibrils that can polymerize into amyloidogenic fibrils following the reassembly of multiple protofibrils contained in Lewy bodies (Figure

11.A). This mechanism has been proposed by numerous in vivo studies; however, it still remains under investigation.

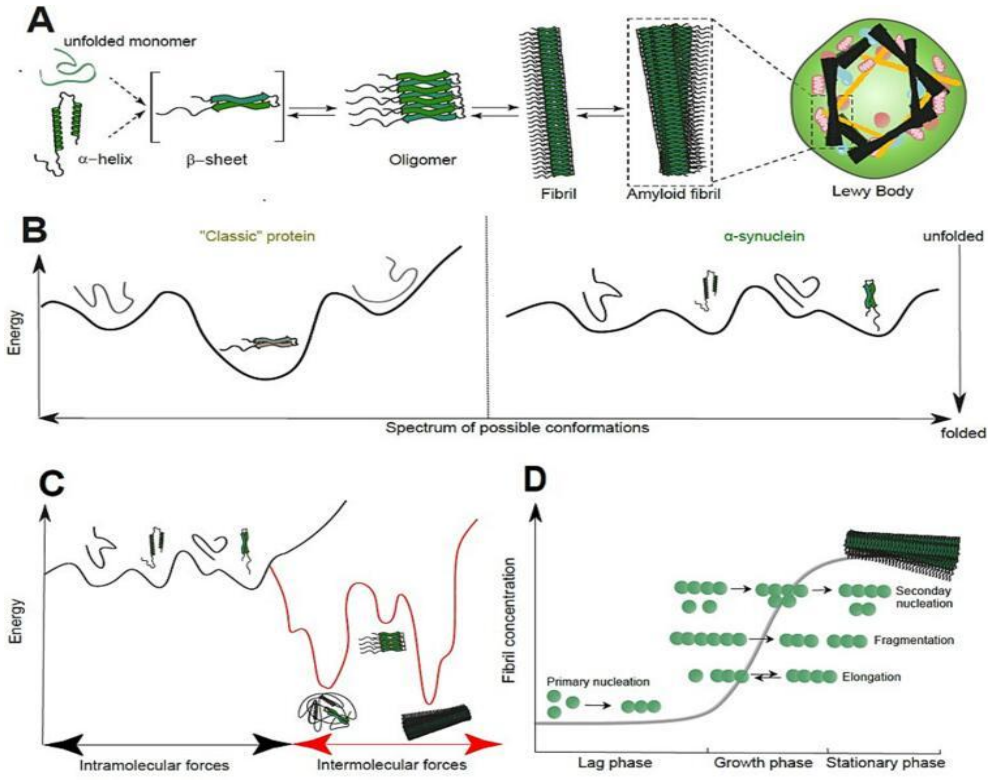


Figure 11: A Schematic summary of the aggregation pathway of alpha-synuclein. Different monomeric species exists in a continuous equilibrium. **B; C** Energy diagrams illustrating the structural transition of the protein and the formation of highly stable structures called amyloid structures. **C** Diagram representing the different aggregation phases of alpha-synuclein. (Estaun-Panzano J., 2023)

α-synuclein is one of the most intrinsically disordered proteins. Its structure oscillates continuously between various conformations, ranging from a fully deployed to a fully folded protein. As can be seen from the diagram in Figure 11.B, a well-structured protein takes the form in which the energy is maintained the lowest. In the case of α-synuclein, all the forms that it assumes during aggregation have about the same energy. This explains why the protein continually oscillates between different energy states (Robustelli P., 2018). However, in some cases the protein can form highly stable structures, called amyloid structures, which constitute the pathological form of the protein, so this conformational balance is lost and the protein is unable to oscillate between various energy states (Figure 11.C). Many environmental factors influence protein misfolding and aggregation. Among these,

there are physical factors, such as molecular crowding and temperature, chemical factors, such as changes in pH or the presence of other proteins or biomolecules, including nucleic acids, phospholipids and proteoglycans, and finally post-translational modifications (Naiki H., 2009).

Further factors that influence the misfolding and aggregation characteristics of alpha-synuclein are missense point mutations in the SNCA gene, coding for the protein in question. In particular, the A53T and A53E mutations, as previously discussed, as well as the potential increase of synuclein expression levels, induced by duplications or triplications of SNCA. A53T causes conformational changes with structural alterations of β-sheet structures, which are thermodynamically more stable in aqueous solutions, exhibiting a significant influence on the aggregation process (Coskuner O., 2013).

A low pH as well as a higher concentration of metal ions, in particular small polyvalent cations, can increase the net surface charge, and therefore improving side chain repulsion, allow exposure of the hydrophobic core prone to aggregation. It has been shown that the growth of synuclein fibrils is impaired at neutral pH while it is amplified at slightly acidic pH values (Buell A.K., 2014).

Polyamines are a group of physiological polycations involved in different biological functions, ranging from DNA replication to protein synthesis. They exhibit multivalent cations separated by aliphatic hydrocarbon chains, through which they can interact with natively unfolded proteins via both electrostatic and hydrophobic interactions. High-order polyamines, such as putrescine, spermine, and spermidine, are more involved in cellular toxicity during PD development Polyamines have been shown to enhance aggregation in a manner proportional to the cationic charge and length of the aliphatic chains separating the amino groups (Krasnoslobodtsev A.V., 2012).

Proteoglycans are glycoproteins containing sulfated glycosaminoglycan chains. It has been observed that due to the presence of both a negatively charged moiety due to the presence of sulfated groups and positively charged amino acids, their binding to amyloids is both harmful and protective (Candelise N., 2020).

As already mentioned, unsaturated fatty acids, in particular arachidonic acid or docosahexaenoic acid, facilitate the binding of alpha synuclein through the N-terminal domain, inducing a conformational change that favors fibrillation (Gorbenko G.P., 2006).

Some studies have highlighted the presence of nucleic acids in association with amyloid plaques and tangles in protein misfolding diseases. Since they possess a high binding affinity for amyloids, due to electrostatic interaction between the positive charges of amyloids and the negatively charged phosphate groups. This interaction can increase the local concentration of amyloid proteins and is supposed to act as a scaffold to promote protein aggregation (Liu C., 2011).

Finally, the interaction of α-synuclein with the microenvironment is influenced by the presence of point mutations, which can modify the degree and type of the interaction, resulting in an improvement or reduction in aggregation kinetics. Briefly, it has been observed that phosphorylation of Ser129 residue of the protein may promote aggregation as well as glycation at the amino terminal end. This supports the hypothesis that hyperglycemia may contribute to the onset of synucleinopathies, supporting the role of diabetes as a risk factor for PD (Sato H., 2013).

In contrast, in its physiological state, alpha-synuclein appears to be constitutively acetylated at the N-terminal end in several lysine residues. The presence of this point mutation appears to affect binding to vesicles with low negative charge, while binding to highly negatively charged structures appears to be unaffected. Overall, acetylation helps maintain the balance between soluble monomeric forms and membrane-bound tetrameric assemblies (Dikiy I., 2014).

Finally, physical factors such as temperature, concentration and the application of an external force can increase the entropy of the system, thus increasing the chances of triggering aggregation (Candelise N., 2020).

In conclusion, amyloid fibril formation is a complex process that relies on several steps that include primary nucleation, elongation, and secondary nucleation (Figure 11.D). Primary nucleation is the initial and determining stage of the alpha-synuclein aggregation process and is critical to understanding how the first toxic aggregates

are formed in PD and other synucleinopathies. In this phase monomers of α-synuclein, initially in a disordered state, spontaneously self-associate into small stable aggregates called nuclei. These nuclei are thermally and kinetically unstable until they reach a critical stability threshold. Once this threshold is exceeded, the nucleus acts as a seed and triggers the exponential growth of the fibrils. The process is energetically disfavored, it occurs slowly at the beginning, and is considered the limiting step in fibril formation. The process can only occur if optimal conditions of pH, and intracellular or extracellular environment occur. It has been observed that the C-terminal region of the protein can have an inhibitory role on nucleation following interaction with the NAC region (Buell A.K., 2014). The elongation step is the process by which soluble synuclein monomer units add to the end of the preformed seeds and stretch them to form the mature fibrils. This occurs because the monomer units interact with the exposed ends of the fibrils, especially those rich in β-sheets, in the NAC region, between residues 61–95, through hydrophobic interactions, hydrogen bonds and electrostatic forces. Once bound to the end of the fibril, the protein adopts a well-organized β-structured conformation and aligns with the β-sheet motif already present by incorporating it into the existing structure, which turns out to be well-structured in overlapping β-sheets. Fibrils typically grow at both ends, but one of them may be more kinetically active. Unlike primary nucleation, elongation is rapid, energetically favored and autocatalytic (Mao H., 2022). Secondary nucleation is the last aggregation step. This step differs profoundly from primary nucleation both in the mechanism, and in the amplifier impact it has on fibril formation. It is an autocatalytic process in which soluble synuclein monomers interact with the surface of existing fibrils, giving rise to new aggregative nuclei; in order to happen, the presence of preformed fibrils is necessary and occurs on their lateral surface and not at the end. The monomers transiently bind to the lateral surface of the fibrils by hydrophobic interactions and hydrogen bonds that form between the NAC region and the N-terminal domain. On the surface of the fibril, the monomer undergoes partial folding, adopting β-sheet conformations. The final result is the formation of an insoluble, cytotoxic aggregate. Once this has occurred, the detachment of structured oligomers can occur which will act as new nuclei, triggering elongation or secondary nucleation, in order to form a growing network of aggregates, or the fission of the mother fibril, with the

generation of new ends (Bezard E., 2022). Although these insoluble aggregates represent the most stable forms of the protein, growing experimental evidence points to smaller soluble species, usually called oligomers, as the main widespread toxic species. These smaller aggregates, precisely because of their instability and small size, would be responsible for the cytotoxicity and expansion of the disease (Emin D., 2022).

1.15 Alpha-synuclein propagation: The prion-like hypothesis

We discussed the mechanisms of formation of cytotoxic aggregates and the factors that influence these processes. At this point it is necessary to ask ourselves how the propagation of cytotoxic α-synuclein occurs in the context of a complex neurodegenerative disease such as PD. In recent years, the identification of the gut-brain axis and its bidirectional communication have opened new perspectives in understanding the progression of PD. In particular, there has emerged a growing interest in the mechanisms by which alpha-synuclein pathology spreads between the gut and the Central Nervous System (CNS), both in a bottom-up and top-down direction, has emerged (Figure 12) (Salim S., 2023).

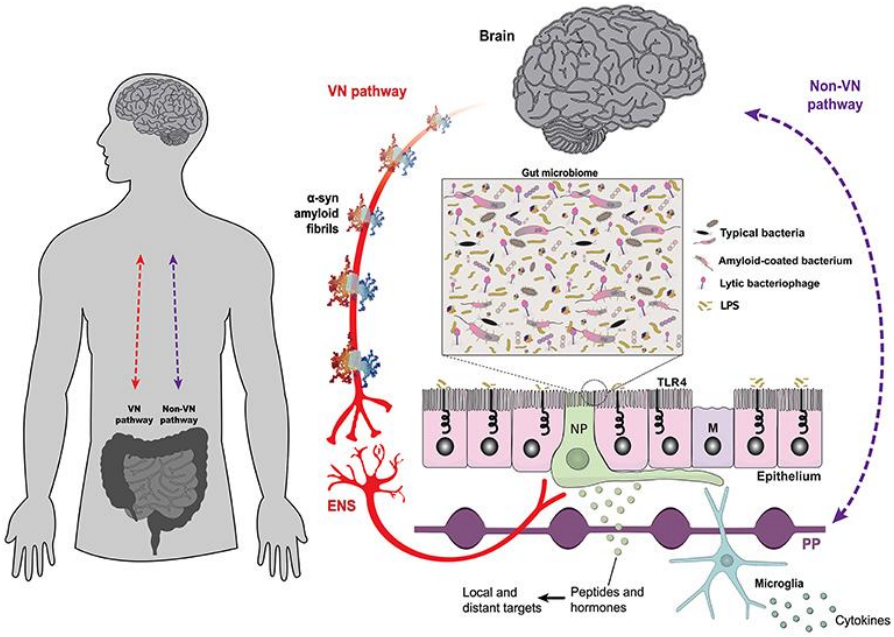


Figure 12: Grafical Abstract of alpha-synuclein pathology in the gut-brain axis. (Salim S., 2023)

The use of anti α-synuclein antibodies has made possible to identify Lewy pathology not only in Substantia Nigra pars compacta, but also in numerous other tissues; in fact, Lewy bodies and Lewy neurites are found in several other brain regions and also in the peripheral nervous system (Volpicelli-Daley L., 2018). According to Braak hypothesis and based on systematic analyses conducted on numerous autopsies, it has been suggested that Lewy pathology progressively involves multiple regions of the nervous system as the disease progresses. In this regard, two types of staging have been proposed, which explain the presence of neurites and Lewy bodies in the various districts of nervous system (Braak H., 2002). Specifically, sporadic PD could originate from an invasive pathogen that penetrates the body via the nasal and intestinal pathways, triggering α-synuclein aggregation when intestinal microbial products interact with synuclein present in olfactory or enteric neurons. Once aggregated, alpha-synuclein would propagate to the CNS through the olfactory bulb and vagus nerve, until arriving at the Substantia Nigra pars compacta and, subsequently, to the hippocampus and primary and higher-order sensory and motor cortical areas, causing neurodegeneration. The appearance of inclusions in CNS coincides with the onset of motor symptoms, while the appearance of inclusions in the temporal cortex is associated with cognitive decline (Rietdijk C.D., 2017).

A clinical support to this hypothesis is represented by the fact that in 60–80% of PD patients, enteric nervous system (ENS) dysfunctions are observed with symptoms such as constipation that can precede the onset of motor symptoms by years. In addition, postmortem studies have suggested that the vagus nerve may serve as a communication pathway between the SNE and the CNS, conveying α-synuclein pathology from the gut to the brain (Braak H., 2002) (Adams-Carr K.L., 2016).

In parallel, a solid body of evidence is emerging to support the possibility that α-synuclein may propagate in the opposite direction, that is, from the brain towards the gut, according to a top-down model. The myenteric plexus, which represents the main motor innervation of the gastrointestinal tract, plays a key role in intestinal motility, regulated by excitatory neurotransmitters (e.g. acetylcholine) and inhibitors (e.g. nitric oxide). It is believed that such a plexus may also act as a transmission channel for aggregated α-synuclein to the gastrointestinal tract (Harsanyiova J., 2020). This hypothesis was supported by animal models treated with 6-

hydroxydopamine (6-OHDA), a neurotoxin selective for dopaminergic neurons. Injection of 6-OHDA into Substantia Nigra induced enteric inflammation, associated with increased oxidative stress and production of pro-inflammatory cytokines (Salim S., 2023). Other studies have further elucidated the possibility of bidirectional vagal transmission. In particular, it was observed that overexpression of human α-synuclein in the vagal system of rats caused its significant spread to the gastric nerve endings, confirming the vagus nerve as a privileged route also for transfer from the brain to the intestine (Ulusoy A., 2017).

The concept of prion-like propagation of synuclein fits into this context. Similarly to what is observed in prion diseases, small quantities of fibrillar α-synuclein can act as pathogenic seeds, capable of converting soluble endogenous protein into an aggregate and insoluble form such as that present in Lewy bodies and neurites. These aggregates can be released by neurons into extracellular fluid or within extracellular vesicles (exosomes), and subsequently taken up by nearby cells, triggering a knock-on effect (Kuo G., 2025). The possibility that these pathological forms cross cellular compartments is supported by data that α-synuclein fibrils interact with heparan sulfate proteoglycans (HSPGs) and are internalized by macropinocytosis, including through receptors such as LAG3 (Abounit S., 2016). After endocytosis, these pathological forms can rupture endosomal membranes and enter the cytoplasm, initiating new cycles of aggregation, with a mechanism similar to that used by some intracellular viruses. Furthermore, the recently described MAPS secretion pathway represents an alternative mechanism of extracellular release of aberrant forms of α-synuclein (Brás I.C., 2021) (Volpicelli-Daley L., 2018).

Finally, it has been demonstrated that the propagation of α-synuclein can also occur via tunneling nanotubes, cytoplasmic structures that allow the direct transfer of aggregates between neurons (Abounit S., 2016).

1.16 Involvement of alpha-synuclein in molecular mechanisms

Cytotoxic α-synuclein plays a fundamental role in PD. Although the way in which altered α-synuclein proteostasis is not yet fully understood, it is evident that

oligomeric and fibrillary forms can alter different physiological mechanisms leading to neurodegeneration.

There is increasing evidence that the interplay between α-synuclein misfolding and impaired mitochondrial biology may be at the center of PD pathology. As previously mentioned, mitochondria are dynamic organelles that play a critical role in many physiological processes within the cell, including energy metabolism, programmed cell death, cell signaling and calcium homeostasis. As a result, mitochondrial dysfunction is severely harmful to cellular health, negatively impacting key biosynthetic processes and compromising overall homeostasis (Shen L., 2024). The main factor contributing to mitochondrial dysfunction is the accumulation of ROS, and the reduction of antioxidant defenses, which results in a redox imbalance with a consequent increase in oxidative stress (Jomova K., 2023).

Mitochondrial biogenesis plays a critical role in mitochondrial quality control, as it is responsible for producing new, healthy mitochondria to replace damaged ones. Mitochondrial biogenesis can be activated by a number of factors, including environmental toxins, temperature and oxygen variation, nutrient availability, growth factors and hormones (Uittenbogaard M., 2014) (Jornayvaz F.R., 2010). Mitochondrial biogenesis is therefore a complex process involving the synthesis of proteins encoded by mtDNA, the synthesis of mitochondrial proteins encoded by nuclear DNA, the synthesis of internal and external mitochondrial membranes and the replication of mtDNA, and it is regulated by the PGC-1 a-NRF-TfamA pathway (Cardanho-Ramos C., 2021). Adenosine monophosphate protein kinase (AMPK) and silent information regulator 1 (Sirt1) serve as upstream regulators of the peroxisome proliferator-activated gamma-1 alpha co-activator (PGC-1α). Upon activation, PGC-1α stimulates nuclear respiratory factors 1 and 2 (NRF1 and NRF2), which in turn activate mitochondrial transcription factor A (Tfam). This last factor is involved in mtDNA transcription and replication, as well as transcription of mitochondrial proteins encoded by the nucleus (Popov L., 2020). Several studies, both in vitro and in vivo, have shown that oligomeric α-synuclein can bind to the promoter sequence by inhibiting the synthesis of PGC-1α and its target genes in dopaminergic neurons. This determines an alteration of mitochondrial biogenesis (Piccinin E., 2021).

Synuclein can also lead to abnormal mitochondrial biogenesis through interactions with the PINK1/Parkin signaling pathway. These proteins regulate PGC-1α through the degradation of PARIS, a transcriptional repressor of PGC-1α, and interact with TfamA to improve mitochondrial transcription. It has been observed that exposure to exogenous alpha-synuclein oligomers and fibrils reduces Parkin expression, inducing a decrease in PGC-1α levels (Wilkaniec A., 2021).

Furthermore, the cytotoxic aggregates of α-synuclein can interact with mitochondrial dynamics, a process of fundamental importance for the correct functioning and maintenance of organelle homeostasis and consists of continuous cycles of fusion and fission. Fusion refers to the combination of two mitochondria into a single mitochondrion, while fission refers to the separation of the mitochondria into smaller parts (Rambold A.S., 2018). The mechanism underlying the alteration of mitochondrial dynamics is not yet well known, however it is believed that oligomeric α-synuclein can cause mitochondrial fragmentation by interacting directly with mitochondrial membranes and interfere with the PINK1/Parkin pathway, involved in fission. It has also been observed that α-synuclein with A53T and A30P mutations induce mitochondrial fragmentation, preventing its fusion (Guardia-Laguarta C., 2014).

The downregulation of Parkin by α-synuclein can influence various aspects of mitophagy, a fundamental process for the degradation and recycling of damaged or malfunctioning mitochondria. Treatment of a neuronal cell model with exogenous α-synuclein aggregates caused a reduction in Parkin levels, resulting in alterations in mitophagy as evidenced by decreased mitochondrial protein ubiquitination and accumulation of abnormal mitochondria (Shen L., 2024).

A crucial mechanism involved in the recycling and degradation of accumulated material and in the maintenance of cellular homeostasis is the autophagy-lysosome pathway, which has been shown to be involved in the clearance of pathological α-synuclein by cathepsins, combined with the ubiquitin-proteasome system (Xilouri M., 2013). It is clear, therefore, that lysosomal dysfunction is involved in the pathogenesis of neurodegenerative diseases, including PD. Studies conducted in vitro and in animal models have shown that dopaminergic neurons derived from PD patients that present α-synuclein aggregation show impaired lysosomal trafficking

of cathepsins, probably due to the impairment of the SNARE complex involved in vesicular fusion, resulting in reduced proteolytic activity of cathepsins in the lysosome. In particular, pathological α-synuclein aggregation impairs the maturation and proper targeting of these enzymes to lysosomes, reducing their proteolytic activity. This triggers a vicious cycle as the more synuclein accumulates, the more the functioning of the lysosomes is altered (Mazzulli J.R., 2016). Furthermore, the activity of GCase, an important lysosomal enzyme that hydrolyzes glucosylceramide into glucose and ceramide has been found reduced in the brain tissues of PD patients. The reduced functionality of this enzyme is associated with the accumulation of cytotoxic forms of α-synuclein (Drobny A., 2023).

The folding of secreted and transmembrane proteins is one of the main functions of the Endoplasmic Reticulum (ER). Membrane and extracellularly targeted proteins are translated onto ribosomes located on the cytosolic surface of the rough endoplasmic reticulum and promptly inserted into the membrane or lumen. Subsequently the proteins reach a specific folded conformation, acquire post-translational modifications such as glycosylation and the formation of disulfide bridges and are selectively destined for secretion or to the plasma membrane or other cellular compartments. ER is also responsible for the biosynthesis of lipids and steroid hormones and it is a primary site for Ca^{2+} accumulation (Colla E., 2019). Misfolded proteins that are potentially harmful to cell biology are brought back into the cytosol by the Endoplasmic Reticulum-Associated Degradation (ERAD) system where they are degraded by the ubiquitin-proteasome system. If this pathway is disturbed by the accumulation of misfolded proteins, a condition known as ER stress is generated, which can have deleterious consequences and lead to the collapse of the entire secretory pathway and cellular homeostasis (Smith M.H., 2011). However, one mechanism that intervenes to ensure homeostasis is the unfolded protein response (UPR), which works by increasing the cellular folding capacity and transient reduction of the flow of proteins entering the ER. UPR is highly conserved and activated by ER stress sensors. These include inositol-requiring enzyme 1 (IRE1), double-stranded RNA (PKR)-activated protein kinase (PERK)-like ER kinase, and activating transcription factor 6 (ATF6); they act by activating signaling cascades resulting in the activation of downstream proteins (Figure 13). The exacerbation of ER stress and the inability of UPR to restore

homeostasis mean that this mechanism activates the apoptosis process (Colla E., 2019).

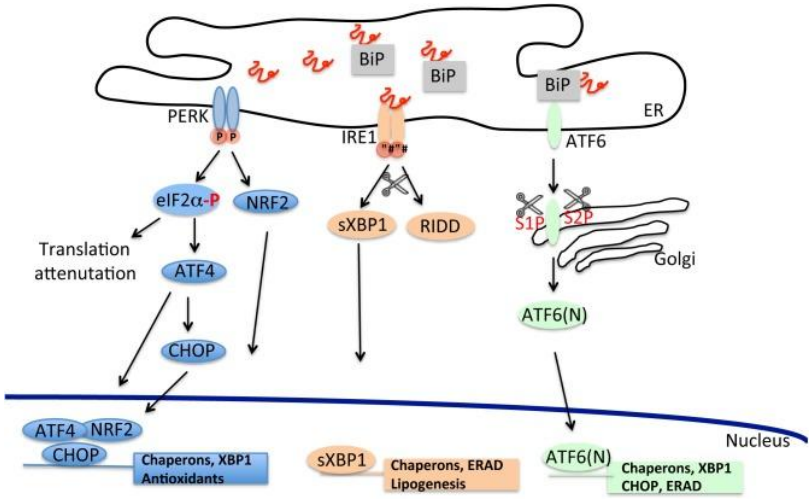


Figure 13: The Unfolded protein response cascade. Stressful conditions due to starvation, infections, oxidative damage, and changes in ER Ca^{2+} concentration can lead to accumulation of misfolded proteins in the ER. Induction of the unfolded proteins response through the activation of its three independent arms (PERK, IRE1, and ATF6) counteracts the build-up of misfolded proteins and improves the ER folding capacity. (Colla E., 2019)

Several studies, based on the use of MPP^+, 6-hydroxydopamine (6-OHDA) or rotenone on neuronal cultures, support the link between ER stress and PD pathogenesis, as the activation of UPR genes has been observed. Activation of the ER stress response has also been reported in the human brain of PD patients The accumulation of ER chaperones has been found in Lewy bodies, while increased PERK/p-eIF2 a signaling has been demonstrated in dopaminergic neurons of the Substantia Nigra in post-mortem tissue of PD cases (Valdés P., 2014) (Hoozemans J.J., 2007).

In addition, genes such as Parkin and LRRK2 have been observed to be associated with endoplasmic reticulum stress. Parkin, was increased after inducing ER stress and this increase was mediated directly by binding of activating transcription factor 4 (ATF4) to the Parkin promoter. Parkin overexpression protected cells from ER stress by promoting XBP-1 splicing and induction of UPR pro-survival response. Mutations causing loss of function of LRRK2 have also been shown to modulate upregulation of BiP expression after 6-OHDA treatment or α-synuclein overexpression, increasing neuronal death in vitro and in vivo (Duplan E., 2013) (Colla E., 2019).

Cytotoxic α-synuclein oligomers not only interfere with the correct folding of proteins in the ER, causing an accumulation of misfolded or unfolded proteins, thus activating ER stress and generating a loop in which stress induces further aggregation of α-synuclein with further accumulation of misfolding proteins; but even by blocking the SNARE complex, they hinder vesicular traffic. ERAD and UPR responses ultimately fail to restore homeostasis, triggering apoptosis and therefore neuronal loss (Zeng H., 2025).

Microglia have a crucial role in the transmission of harmful protein aggregates between cells, which are thought to contribute to the spread of α-synuclein neuropathology and underlie the pathogenesis of Parkinson's disease. Microglia have a strong ability to absorb surrounding synuclein-containing exosomes, leading to their activation and subsequent intracellular processing (Guo M., 2020). Internalization of the protein by resting and activated microglia is a critical step driving neuroinflammatory responses, and multiple processes have been proposed underlying this function. Endocytosis is thought to be the primary process by which pathological proteins are internalized. Another mechanism is phagocytosis by which microglia can recognize and absorb extracellular targets via specific receptors. Monomeric α-synuclein has been observed to increase microglial phagocytosis in a dose- and time-dependent manner through the involvement of TLR4 receptors. TLR-4 mediates microglial phagocytosis of soluble full-length synuclein, fibrillar form, and C-terminal truncated protein, but not the oligomeric form, which conversely can inhibit this process. This suggests that microglia show different responses when it incorporates distinct forms of synuclein (Muzio L., 2021) (Bido S., 2021). It has been proposed that several cell-surface receptors, including TLR2 and TLR4, are involved in α-synuclein internalization by clathrin-dependent endocytosis (Filippini A., 2019). The activation of the microglial surface receptors TLR2 and TLR4, by pathological α-synuclein, cause the activation of the intracellular NF-κB pathway and the release of inflammatory factors such as TNF-α and IL-6. The various forms of synuclein have distinct preferences for the two receptors. The oligomeric forms act predominantly on TLR2 while the fibrillary forms act on TLR4. Furthermore, the NF-kB pathway promotes NLRP3 inflammasome assembly, mitochondrial dysfunction, reactive oxygen species (ROS) production, and oxidative stress (Wu A.G., 2021). In addition to microglia, cytotoxic forms of α-synuclein can also involve peripheral immune

cells, such as macrophages and CD4+ and CD8 T lymphocytes, through the release of chemokines, mostly including CXCL10. Therefore, it is clear that inflammation is confined not only to brain tissue, but can also affect peripheral areas. This can be seen from the presence of the gut-brain axis by which activated microglia release oligomeric and fibrillar synuclein through vesicles, leading to intestinal dysbiosis, and from the recruitment of peripheral immune cells. An important aspect to consider is that inflammation, up to the appearance of motor symptoms, becomes chronic. In a context of chronic inflammation there is increasingly formation of cytotoxic aggregates which exert harmful effects not only through neurodegeneration but also through damage in other tissues (Miao Y., 2024).

1.17 Parkinson's diagnosis

Although PD is defined as a movement disorder and predominantly associated with motor symptoms that include bradykinesia, resting tremor and stiffness, as well as alterations in posture and gait, which cause progressive disability with impairment of daily activities and a reduction in quality of life, the disease is associated with a wide variety of non-motor symptoms, which occur in all patients and include hyposmia, constipation, urinary dysfunction, orthostatic hypotension, memory loss, depression, pain and sleep disorders, as previously discussed. While classic motor signs of PD are linked to nigral degeneration and striatal dopamine depletion, non-motor symptoms are likely related to neurodegeneration of other structures, including the peripheral autonomic nervous system. They are frequent in the initial stages in which they manifest themselves with a mild entity and then worsen as the disease progresses (Poewe W., 2008) (Shin H.W., 2022). Several non-motor symptoms associated with PD, such as smell loss or constipation, are commonly experienced by patients prior to the onset of classic motor symptoms, sometimes preceding the occurrence of motor features by years or even decades. This is called the prodromal phase of the disease, in which neurodegenerative changes involve extranigral sites, such as the lower brainstem, the olfactory bulb and tracts, and the peripheral autonomic nervous system. Similarly to Alzheimer's disease, it has also been hypothesized that PD has an even earlier period in which patients are still

symptom-free, defined as a preclinical phase potentially evident through biomarkers (Siderowf A., 2012).

1.18 Clinical aspects in the diagnosis of the disease

The diagnosis of idiopathic PD is essentially clinical in cases with a classic clinical history, typical asymmetric motor signs, absence of atypical characteristics and exclusion of alternative etiologies. However, a recent meta-analysis found an overall diagnostic accuracy for the clinical diagnosis of PD of only 80.6% in eleven clinical-pathological studies, with error rates ranging from 15% to 24%. Common errors in clinical practice include non-PD disease tremor disorders, such as essential tremor, as well as different types of secondary parkinsonism. However, the biggest challenge is the differentiated early detection of PD from atypical parkinsonian disorders. Atypical parkinsonism encompasses a variety of neurodegenerative disorders, including multisystem atrophy (MSA), progressive supranuclear palsy (PSP), and corticobasal degeneration (CBD), in which parkinsonian syndrome is a prominent clinical feature, but the entire clinical spectrum, underlying pathology, progression, and prognosis differ fundamentally from those of PD. In the early stages of the disease, all three conditions can be very difficult to distinguish from PD and each other (Adler C.H., 2014). To enhance the diagnostic accuracy of a clinical diagnosis of PD, the International Parkinson and Movement Disorder Society (MDS) has proposed a set of criteria that essentially represent a revised version of the Queens Square Brain Bank (QSBB) Criteria that have been the most commonly used over the past decades. These criteria rest on the expert clinical neurological examination showing of a parkinsonian syndrome defined by the presence of bradykinesia and at least one additional cardinal motor feature, plus the application of supportive and exclusionary features. Based on the presence of supportive characteristics and the absence of exclusion characteristics, as well as on the presence or absence of "red flags", clinical characteristics that rarely occur in PD, the MDS criteria propose two levels of diagnostic certainty, namely "clinically ascertained" and "clinically probable". The first category establishes a set of criteria aimed at maximizing specificity at the expense of sensitivity, while the criteria for the

second level aim at greater sensitivity. The use of these criteria has significantly increased the sensitivity and specificity of the diagnosis (Tolosa E., 2021).

Furthermore, the International Parkinson and Movement Disorder Society (MDS) has introduced the use of tests in clinical practice, which are essential for diagnosis. These include the Montreal Cognitive Assessment (MoCA) test and the Mini-Mental State Examination (MMSE) test. Of the two, the most widely used test is MoCA, as it has been shown to be more sensitive than MMSE (Fiorenzato E., 2024). MoCA evaluates short-term memory, visuospatial function, executive function, attention, concentration and working memory, language and orientation, and is based on a maximum score of 30 points which is corrected in function of the patient's level of education. The original study indicated a normal range for MoCA of 26 to 30 points. People with more advanced disease often score below 26 compared to those in the early stages. MoCA can help identify people who need specialist assessment and treatment for dementia. Diagnosis of dementia can reduce uncertainty for individuals, their families and potential caregivers, facilitate access to appropriate services and encourage planning for the future (Davis D.H., 2021). To date, these tests are joined by the UPDRS scale (Unified Parkinson's Disease Rating Scale), a tool used to evaluate and monitor the severity and progression of PD. It consists of several parts that examine aspects such as cognitive and behavioral functions, mood, activities of daily living, motor functions and complications related to treatment. In particular, part I evaluates cognitive functions, behavioral disorders and mood changes; part II evaluates the patient's autonomy and ability to carry out daily activities; part III examines motor skills, such as tremor, stiffness, bradykinesia and postural instability, part IV analyzes complications that can arise from drug therapy; part V allows to evaluate the general severity of the disease, through the use of the Hoehn and Yahr scales; part VI evaluates the degree of autonomy and disability of the patient, as reported by the patient himself, on the basis of the Schwab and England scale (AlMahadin G., 2020).

In addition to cognitive tests, the clinical diagnosis of PD is based on the use of neuroimaging techniques and olfactory function. Olfactory function tests have been extensively studied in PD and other parkinsonian syndromes. Hyposmia or anosmia have been found in approximately 90% of patients with PD, while normosmia is the

norm in the early stages of atypical or secondary degenerative parkinsonisms. The MDS criteria for PD list hyposmia as one of the four supporting criteria for the diagnosis of PD, and although in their validation study the olfactory test achieved only 63.4% specificity, this characteristic showed a high diagnostic accuracy in distinguishing PD from other forms of dementia. Magnetic resonance imaging (MRI) is usually of little significance in PD. However, it should be part of the routine diagnostic process to distinguish PD from secondary or atypical parkinsonian syndromes, as several features of MRI are highly specific to atypical parkinsonisms, although sensitivity is low. New imaging techniques including neuromelanin (NMI) imaging, quantitative susceptibility mapping (QSM), or visual assessment of dorsal nigra hyperintensity have been the focus of recent research. NMI exploits the paramagnetic properties of neuromelanin, while QSM allows quantification of iron deposition in Substantia Nigra pars compacta. These new MRI techniques are typically unable to distinguish between PD and other types of degenerative parkinsonism, as nigral pathology is common to all of these pathologies. Several radionuclide tracers are available to examine presynaptic and postsynaptic striatal dopaminergic function by positron emission tomography (PET) and Single Photon Emission Computed Tomography (SPECT). Among them, only DAT-SPECT has an established role in the clinical routine due to its availability and low cost. The presynaptic monoamine transporter ligands used in DAT-SPECT are sensitive in detecting dysfunction or loss of striatal dopaminergic terminals and allow the identification of parkinsonian syndromes with nigral neurodegeneration such as PD (Tolosa E., 2021).

1.19 Blood-based biomarkers for the diagnosis and prognosis of Parkinson's

A biomarker is a measurable biological indicator, such as a protein, gene, or cell, that signals the presence or evolution of a biological process, pathology, or response to medical therapy or treatment. These indicators can be measured in biological samples such as tissues and biological fluids, for example blood, saliva and urine. Biomarkers can be distinguished based on their function. We have diagnostic markers that are used to identify a pathology; prognostics that allow

predicting the course of the disease, regardless of treatment; predictors that serve to evaluate how an individual might respond to a treatment; monitoring or effect biomarkers, used to follow the progression of a disease, evaluate the response to a treatment, or detect the effects of exposure on a system or organ. Similarly, they can be classified on the basis of the type of indicator that is taken into account. We have, therefore, molecular biomarkers that include DNA, proteins, enzymes, biochemical metabolites, and cellular or physiological ones that refer to the evaluation of specific physiological responses or biological states. Biomarkers therefore represent a resource of fundamental importance in the diagnosis of a large number of pathologies, including neurodegenerative diseases such as PD. There are several reasons why PD biomarkers are clinically beneficial (Tönges L., 2022).

First, a PD diagnosis is made clinically and can only be made definitively with post-mortem brain examination. Potentially, a specific biomarker may be able to narrow a differential diagnosis. Finally, although there are currently no disease-modifying therapies for PD, treatment development could be aided with the use of biomarkers since biomarkers could help identify patients before they become symptomatic. It is necessary to consider that to date there are no clinically validated markers for PD. This is essentially due to the complexity of the disease, caused by both phenotypic, genotypic or epigenetic factors, the presence of different subtypes of PD, the molecular characteristics shared between various neurodegenerative diseases and the fact that many altered molecular pathways have not yet been fully understood. Cerebrospinal fluid (CSF) analysis is widely used to diagnose a variety of neurological diseases. The pathophysiological changes in the Central Nervous System can be detected through CSF analysis because it is in direct contact with the extracellular space of the nervous tissue. The CSF offers a real overview of brain pathology and can be used to identify people at risk of developing various neurological diseases and distinguish between infectious, autoimmune and degenerative diseases (Rizzo G., 2016).

Since α-synuclein, as already discussed, is one of the main factors associated with PD, several studies have focused on the detection of this protein in CSF. However, it was observed that total synuclein levels in PD were significantly lower than in controls. This is probably due to the fact that aggregation occurs within neurons

resulting in low levels in the cerebropinal fluid. Reduced sensitivity and specificity for total α-synuclein in CSF as a biomarker have been shown to be insufficient to differentiate between patients with PD and controls (Parnetti L., 2019) (Shim K.H., 2020). Recently, the CSF α-synuclein seed amplification assay has been investigated as a possible alternative to traditional CSF analysis. The assay relies on the seeding ability of proteins to show small amounts of disease-specific protein aggregates. In a study of 172 patients, this methodology showed a sensitivity of 95.3% and a specificity of 98% for the detection of alpha-synuclein in various alpha-synucleinopathies related to Lewy bodies allowing to distinguish healthy controls from PD patients (Rossi M., 2020).

Astrocytes contain an intermediate strand called glial fibrillar acid protein (GFAP). When astrocytes suffer damage, they release GFAP and its degradation products into cerebrospinal fluid and serum. Thus, higher levels of GFAP and its degradation products are indicative of astrocytic damage, suggesting neurodegenerative disease. Although an increase in GFAP in the cerebrospinal fluid indicates an abnormal pathological process, GFAP does not allow to distinguish between various neurodegenerative diseases leading to false positives. Nonetheless, this marker could prove useful in tracking the progression of PD and the extent of neurological degeneration (Oeckl P., 2019).

To date, research is mainly focused on the study of biomarkers detectable in peripheral blood and serum. This is because unlike CSF, peripheral blood sampling is not invasive and being able to identify and characterize blood-based biomarkers allows to reduce costs and speed up diagnosis. There are numerous potential serum biomarkers that could facilitate the diagnosis of PD in general and to make a differential diagnosis, while others could predict the severity of symptoms or cognitive decline in patients already diagnosed (Yamashita K.Y., 2023). α-synuclein has been studied not only as a biomarker of cerebrospinal fluid, as discussed above, but also as a serum biomarker. However, studies have revealed inconsistent results. Compared to controls, total serum or plasma synuclein levels of PD patients were increased, in other cases reduced or even not significant higher plasma and serum protein levels showed a significant correlation with motor severity and cognitive decline. Additionally, exosomes are part of extracellular vesicles that can

transfer alpha-synuclein through cells and from the brain to peripheral blood. Significantly higher concentrations of exosomal synuclein were observed in PD patients compared to controls. Due to conflicting data, attention has been paid to different forms of the protein. Oligomers are considered toxic forms that precede neuronal aggregation and death. Increased levels of oligomeric forms of alpha-synuclein have been found in plasma, serum and red blood cells with moderate ability to differentiate PD patients from controls. PD patients showed elevated levels of phosphorylated synuclein on serine 129 in plasma and red blood cells (Tönges L., 2022).

The neurofilament light chain (NfL), like GFAP, is a central nervous system ubiquitous structural protein that is released when neurons are damaged. NfL concentrations in blood and CSF are strongly correlated, therefore blood NfL is a promising biomarker for neurodegeneration, including PD. NfL in the blood is higher in atypical parkinsonian syndromes such as multisystem atrophy (MSA), progressive supranuclear palsy (PSP) and corticobasal syndrome (CBS) than in PD and therefore could serve for a differential diagnosis, with a level of accuracy up to 90%. Furthermore, blood NfL appears to be higher in patients with more advanced PD than in controls, while the situation is controversially described for the early stages of the disease. Cross-sectional studies have shown heterogeneous results regarding the association between blood concentrations of NfL and motor impairment in PD. In particular, higher levels of NfL are associated with more severe motor impairment in the long-term course of the disease. Consistent inverse associations between blood NfL levels and cognitive scores have been reported. Most studies have described a positive association between baseline blood NfL levels and cognitive impairment. This association was found for all stages of severity of PD (Lin C.H., 2019) (Ygland Rödström E., 2022).

Dying neurons, glial and immune cells can release inflammatory mediators. Numerous studies have previously found correlations between PD and various cytokines, interleukins and other inflammatory markers such as serum C-reactive protein (PCR). However, many results are heterogeneous and conflicting, and, when significant, the effects tend to be small. Although individually these findings are sensitive and non-specific, as they are general markers of inflammation, the hope is

that they can be used to build a fairly useful model. A recent systemic review and meta-analysis examining 152 studies found significant increases in levels of inflammatory cytokines including IL-6, IL-1β, and TNF-α and chemokines in the blood of PD patients compared to healthy controls. IL-4 and IFN-gamma were instead reduced. Cytokine levels correlated positively with disease severity. As with many other biomarkers, inflammatory biomarkers within neuron-derived extracellular vesicles in plasma were specifically examined. The results were broadly in line with what has just been said (Qu Y., 2023).

In recent years, metabolomics has established itself as a promising tool for the study of PD, offering new perspectives for the identification of diagnostic and prognostic biomarkers, as there is a growing awareness that metabolic alterations could contribute not only to the onset, but also to the progression of the disease. The data collected so far highlight profound alterations in numerous metabolic pathways in patients with PD compared to healthy subjects. These alterations seem to correlate not only with the presence of the disease, but also with its clinical severity, response to therapy and the presence of motor complications, such as L-DOPA-induced dyskinesias. Although this, critical issues persist relating to the variability of each individual, the size of the cohort under study and the fact that in most cases the studies do not take into account the evolution of the disease over time (Shao Y., 2019).

1.20 Parkinson's therapy: current approaches and future prospects

To date, there are no definitive therapies for PD, but current therapeutic approaches aim to alleviate the symptoms in the advanced stages of the disease. The main therapeutic approach is based on the use of Levodopa, direct-acting dopamine (DA) agonists and monoamine oxidase inhibitors (IMAO). Dopamine, levodopa formulations, monoamine oxidase B inhibitors, and dopamine agonists are valid initial therapies for the management of motor symptoms, especially in younger patients with marked tremor. Levodopa can effectively cross the blood–brain barrier to convert into dopamine, which is then stored in the presynaptic terminals of striatal neurons. This medication is highly efficient in the initial years of use, as there are adequate dopamine neurons capable of storing dopamine and regulating its release in the striatum. However, as the disease advances, long-term use of

levodopa can lead to dyskinesias that significantly impact patients' quality of life (Kwon D.K., 2022). Monoamine oxidase inhibitors work by inhibiting the degradation of levodopa, thus aiming to prolong and improve its impact on dopaminergic neurotransmission. Although these drugs improve motor symptoms, their effectiveness is slightly lower than levodopa; however, they carry a reduced risk of inducing dyskinesia. As a result, PD patients often undergo treatments involving multiple classes of drugs to maximize benefits and minimize adverse effects (Muleiro Alvarez M., 2024).

There are also other classes of drugs including catechol-O-methyltransferase inhibitors, which act by directly increasing levodopa levels in the CNS by reducing catechol-O-methyltransferase levels, involved in the conversion of levodopa into a secondary product. Anticholinergic agents were among the first pharmacological treatments developed for PD and are generally considered for initial therapy in patients under the age of 65 who primarily have tremor, and work by blocking dopamine reuptake at the synapse level. However, to date the most used drug is levodopa. (Pirker W., 2023).

Recent advancements in genetics and regenerative medicine have spurred the quest for innovative and personalized therapies aimed at halting or slowing the advancement of specific neurodegenerative disorders through the utilization of pluripotent stem cells, which represent a promising alternative for cellular therapy in localized neurodegeneration, such as PD. The primary challenge of this therapeutic approach lies in precisely directing the differentiation of pluripotent stem cells into specific cell lineages. In the context of PD, this involves generating dopaminergic cells specific to the mesencephalon through directed differentiation technologies, crucial for producing these essential cell types (Church F.C., 2021).

Gene therapy has emerged as a therapeutic option to correct defective genes and introduce therapeutic genes into several diseases. In PD, various methods of gene therapy have been explored in both animal models and humans, showing promising results (Axelsen T.M, 2018).

Non-pharmacological treatments, such as physical activity, are recommended to improve functional capacity, slow disease progression, and improve motor skills in patients with PD. Rehabilitation and physiotherapy are critical to address both non-

motor and motor symptoms, potentially also slowing disease progression. A balanced diet, characterized by the intake of calcium and vitamin D and essential for PD patients, as they have a greater risk of developing sarcopenia and osteoporosis. There are several studies demonstrating the role of the ketogenic diet in PD, indicating that this dietary approach has an anti-inflammatory effect, thus reducing the inflammatory process associated with neurodegenerative diseases (Muleiro Alvarez M., 2024).

1.21 Gender medicine

The concept of gender refers to the ever-changing social construction of what is considered "feminine" and "masculine", based on power and sociocultural norms about women and men. The first distinction to be made is between "sex" and "gender". Sex corresponds to the set of phenotypic characteristics that express the inherited genotype. On the other hand, gender refers to the economic, social and cultural conditions of man in general. Initially, the diagnosis, prevention and treatment of many pathologies derive from studies conducted mainly on male subjects. Historically, for multiple reasons, including the alleged safety of women and their offspring, women of childbearing age have been excluded from clinical trials. As a result, medical research focused on male physiology believing that male and female individuals were biologically identical. Preclinical research and drug development studies have also predominantly used male animal models and cells. However, many studies show that women and men have different abilities to metabolize the active ingredients contained in drugs. Failure to recognize physiological differences related to sex increases vulnerability to adverse effects due to inappropriate administration of the drug. An emblematic example concerns the intake of antidepressant drugs which, particularly in women of childbearing age, can lead to the onset of life-threatening cardiac arrhythmias. It is necessary, therefore, to consider that sex and gender are aspects of fundamental importance and can influence the risk factors, the age of onset, the clinical phenotype of a pathology and the effectiveness of pharmacological treatments as well as the adverse effects connected to the active ingredient (Clayton J.A., 2016). Gender medicine is not a medical branch in itself, but is the study of how diseases differ

between men and women in terms of prevention, clinical signs, therapeutic approach, prognosis, psychological and social impact. In recent years, research has increasingly focused on the study of diseases, considering sex and gender as two important variables to take into consideration. Thanks to new knowledge on the molecular, genetic and epigenetic basis of complex diseases and thanks to the pharmacogenetic approach for drug design/prescription, several diseases are now addressed in a personalized way. However, while sex inclusion is an ongoing process, with clear results from both preclinical and clinical studies, the impact of gender in the medical and scientific fields is still at an early stage, with difficulties and delays due to its intrinsic complexity (Franconi F., 2017).

1.22 Sex differences in Parkinson's: an overview

A fundamental aspect for the development of future research studies, diagnoses and treatments for PD is the understanding of the differences in its manifestation in men and women and to what extent these differences are influenced by sex and/or gender. Although sex and gender are considered very important factors in PD, studies including these two factors are only just beginning. Furthermore, in case-control studies, sex is often treated inconsistently and not considered as an interacting or contributing factor to altered molecular pathways, leading to incorrect conclusions. Studies conducted on a large cohort of disease patients and healthy controls, the growing availability of public data for secondary analysis and rapidly evolving technologies constitute key opportunities for analyzing the sex-related molecular basis of PD and their interactions with gender (Schaffner S.L., 2025). Based on what it has been said, it is clear that neurological disorders can be influenced by sex differences in the organization, structure and function of the brain and genetic and hormonal factors determined by sexual characteristics are important in the development and functioning of brain structures. In addition to these intrinsic biological factors, other external socioeconomic and environmental factors, including lifestyle, can contribute to sex differences in the risk of developing the disease, influencing its course and prognosis (Cattaneo C., 2025). Gender differences in both prevalence and incidence of PD are widely reported. The prevalence of the disease is 1.5 times higher in men than in women and the

incidence, adjusted for age, is on average twice as high in men as in women; data report about 19 new diagnoses per 100,000 patients for men and 9.9 per 100,000 patients for women. However, at a very advanced age, the prevalence in women may become higher, mainly for reasons related to longevity (Ben-Shlomo Y., 2024). The onset of symptoms in women occurs late compared to men. This is due to the fact that sex differences begin during the embryonic development of the brain and continue during its growth, influencing brain morphology and neuronal connectivity. It was reported that at the onset of the disease, women showed higher levels of striatal dopamine binding and a higher baseline number of dopaminergic neurons than men. Furthermore, a significant reduction in the content of gangliosides and phospholipids in the Substantia Nigra pars compacta of men but not of women was highlighted. These neurochemical changes provide evidence of selective neuronal loss that correlates with disease duration and severity. However, women have a higher mortality rate and faster progression of the disease; in addition, women show distinctive symptoms and differences in response to drug therapies and the deep brain stimulation procedure and in personal assessment of quality of life compared to men. Other risk factors may differ between the sexes. Some studies have shown an increased risk of PD in men due to head trauma, immunological disease, or exposure to pesticides, solvents, and metals (Dahodwala N., 2018) (Georgiev D., 2017).

Although women diagnosed with PD represent a considerable part of the population, it must be considered that today they are less likely to receive specialist care such as pharmacological and non-pharmacological treatments such as physiotherapy, occupational therapy and speech therapy; similarly, they are less likely to receive caregiver support than their male counterparts. Consequently, more women than men use paid care services. The reasons for this discrepancy may be related to women's higher average life expectancy and their natural inclination to be carers rather than care recipients (Cerri S., 2019).

1.23 Sex-related predictive and risk factors

In order to have an accurate diagnosis of PD, it is necessary to highlight differences in predictive markers in both sexes. Urate, an endogenous purine metabolite with antioxidant and neuroprotective properties, is a genetically and environmentally

determined modifiable factor and a potential biomarker of PD, as elevated urate levels are associated with reduced risk and slower progression of idiopathic PD. However, the contribution of urate levels to PD risk based on biological sex is still controversial. Higher urate levels were associated with reduced prevalence and slower progression of PD in men, while the opposite trend was observed in women, a situation that changes when considering women of childbearing age when urate levels are comparable to those of men (Cortese M., 2018).

Gene expression patterns in dopaminergic neurons differ between men and women. The genes overexpressed in females are mainly involved in signal transduction, neuronal maturation and protection from oxidative stress, while the genes overexpressed in males are implicated in PD pathogenesis. These sex differences may create a more disadvantageous condition for neurodegeneration in men. Some studies have found a higher prevalence of LRRK2 mutations among women than men, with increased risk of neurodegeneration through its kinase activity and downstream effects on alpha-synuclein and neuroinflammation. A genetic risk factor in PD is the mutation in the gene encoding the lysosomal enzyme glucocerebrosidase which degrades the glycosphingolipid glucocerebroside. This mutation has a higher prevalence in women than men (Balestrino R., 2020). Much attention has been focused on the X and Y sex chromosomes, in particular the SRY region of the Y chromosome which encodes transcription factors responsible for gonadal differentiation and consequently the production of sex hormones. In conclusion, it is hypothesized that alterations in chromosomal, hormonal and other yet unknown factors would lead to a dimorphism of the basal ganglia, with consequent distinct pathogenetic mechanisms for the two sexes highlighted at a clinical level (Cattaneo C., 2025).

Sex differences relating to the risk of PD due to exposure to neurotoxic agents have been highlighted. Numerous in vivo and animal model studies suggest greater male susceptibility to dopaminergic neurodegeneration caused by exposure to environmental toxicants compared to women. While there are several potential mechanisms underlying the observed sex differences, there is evidence that estrogen plays an important protective role in protecting females from exposure to neurotoxic substances. Furthermore, emerging data suggest that genetic sex

characteristics, vesicular glutamate transporter 2 (VGLUT2) expression and microbiome influences are also potential mechanisms that provide female resilience to toxic substances (Adamson A., 2022).

Sex hormones are among the main factors in structural differences and brain function, as well as critical factors for sex differences in disease susceptibility. The most important hormone is estrogen, more precisely 17β-estradiol, which is recognized for its neuroprotective activity. It has been observed that the development of symptomatic PD can be delayed by higher physiological levels of striatal dopamine, probably due to the increased exposure to estrogen during a woman's lifetime, furthermore, these hormones could also prevent the deposition of Lewy bodies through specific anti-aggregating properties of α-synuclein and fibril destabilization (Arabia G., 2022).

Several studies have also shown that estrogen increases antioxidant responses, stimulating the synthesis of scavenger enzymes, and the activity of the mitochondrial respiratory chain; this is partly due to the restoration of mitophagy. Likewise, they are able to modulate the inflammatory response of the glia, in response to oxidative damage and inflammation, resulting in the protection of dopaminergic neurons (Shiraishi T., 2024).

Another important female gonadal hormone is progesterone, which has demonstrated neuroprotective activity in animal models of PD. In mice injected with MPP^+, progesterone prevented striatal depletion of dopamine and its metabolites and prevented downregulation of the dopamine transporter (DAT) in the striatum and SNpc (Bassani T.B., 2023). On the other hand, the role of androgens related to PD is not yet clear, as both neuroprotective and harmful effects have been highlighted. The higher androgen level compared to estrogen in postmenopausal women and its role in the increased incidence of PD after menopause have yet to be studied. However, in female animal models, treatment with dihydrotestosterone had no effect on 6-Hydroxydopamine-induced toxicity, while estradiol showed a protective effect, suggesting that increased androgen levels in females have no harmful effects on the dopaminergic system (Bourque M., 2021).

1.24 Influence of sex and gender in the clinical aspects of Parkinson's

Sex-related differences are also evident in the clinical phenotype of PD (Figure 14).

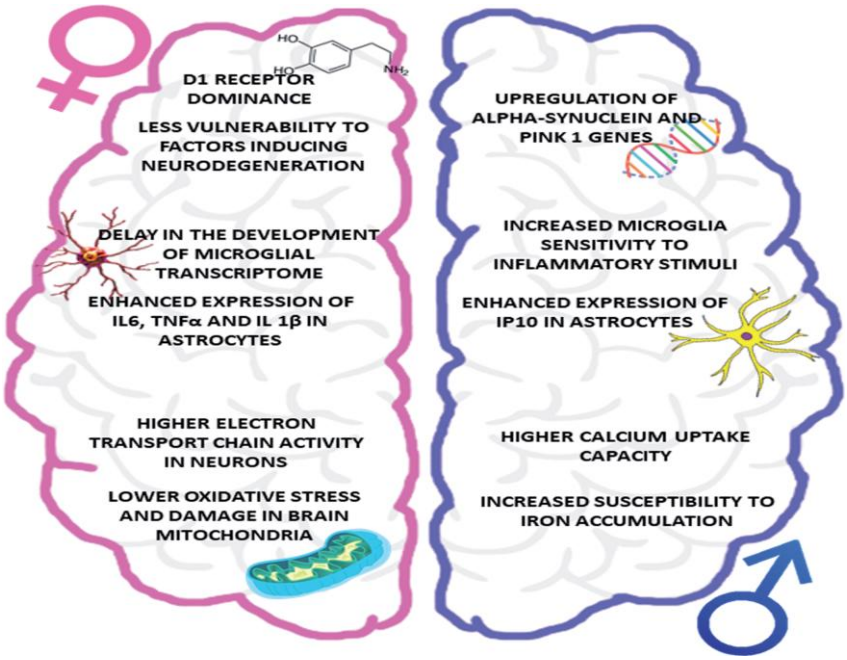


Figure 14: Main sex-related clinical evidence in Parkinson's. (Boccalini C., 2022)

The differences mainly concern motor and non-motor symptoms. The characterization of possible sex-related differences in motor symptom patterns can play a crucial role in terms of diagnostic accuracy and therapeutic strategies. Motor symptoms emerge late in women, with specific characteristics such as reduced stiffness, tremor as the most common first presenting symptom and a greater propensity to develop postural instability; while in men, stiffness and reduced walking ability are more frequent. Another motor disorder specific to PD is camptocormia, which refers to abnormal, severe forward flexion of the trunk that occurs in a standing position or during walking and subsides or disappears in a supine position. It has recently been reported that affected male patients are at increased risk of developing this symptom during disease progression (Nicoletti A., 2023). A 5-year longitudinal analysis of 423 patients with PD reported that men scored significantly higher than women on the Movement Disorder Society Unified Parkinson's Disease Rating Scale (MDS-UPDRS), used clinically to assess disease severity and progression. These findings suggest that disease progression is faster in men than in women. In contrast to this more benign phenotype of PD parkinsonian

motor symptoms, women have a higher risk of complications due to pharmacological treatment with levodopa. In particular, women have a three times higher risk of developing dyskinesias following treatment with levodopa(Cattaneo C., 2025).

In recent years, many studies have avoided sex-related differences in non-motor symptoms. Anxiety, depression, fatigue, dysphagia, constipation and pain are more common in women, while men suffer more from sialorrhea, urinary dysfunction, hypotension, REM sleep disorders (Bovenzi R., 2024). Mild cognitive impairment is present in 30-40% of patients from the early stages of the disease and may evolve in the late stages towards dementia, with a significant impact on the quality of life of patients and caregivers. Women progress to cognitive impairment more slowly than men and male sex is considered an important predictor of cognitive impairment in PD. Finally, among the autonomic disorders that occur in PD, gastrointestinal dysfunctions play an important role, also due to their profound impact on the quality of life. Based on the evidence that intestinal inflammation can act as a trigger for the disease, several studies have found an increase in numerous immune mediators and angiogenesis in female patients compared to controls, while no differences have emerged in male patients (Iwaki H., 2021).

Gender influences how clinical symptoms affect the quality of life of PD patients and their ability to carry out daily activities. Health-related quality of life (HrQoL) is a multidimensional scale used to evaluate the impact of disease and treatments on patients' lives. A recent study exploring the relationship between three HrQoL domains (physical functioning, cognition, socioemotional) and sociodemographic variables did not reveal a global gender effect. However, female gender proved to be a negative predictor for physical functioning and socioemotional HrQoL, while male gender mainly influenced the cognitive domain (Ophey A., 2018).

1.25 Sex and gender differences in major Parkinson's-related molecular pathways

As widely discussed, neuroinflammation is closely involved in the pathogenesis and progression of PD. It is therefore very likely that the sex differences observed in the

development and clinical course of this disease can be explained, at least in part, by sex differences in the inflammatory and/or anti-inflammatory functions and responses of the various cell types present in the innate and adaptive immune systems (Figure 15) (Bourque M. S. D., 2023).

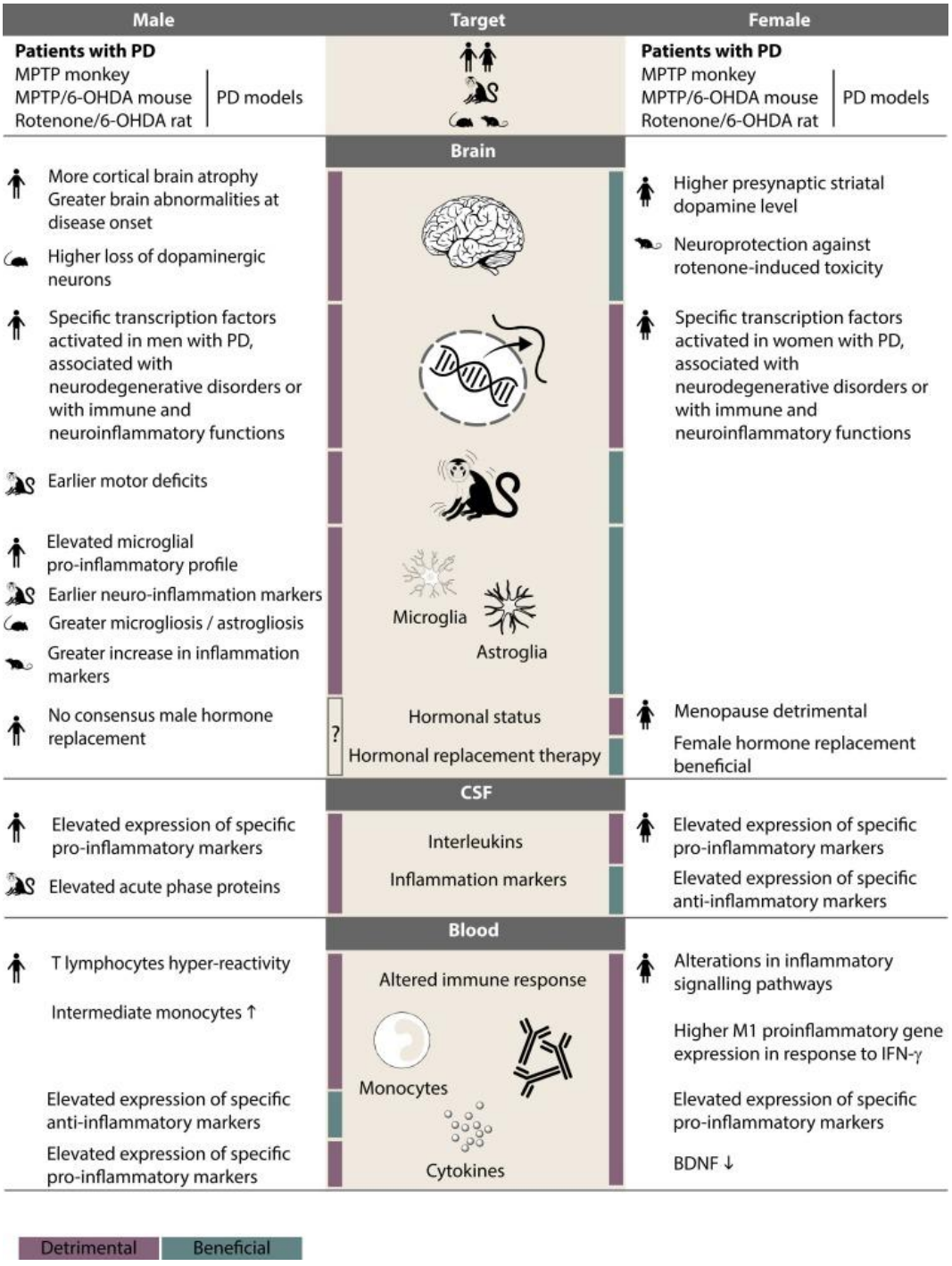


Figure 15: Overview of gender differences in patients and animal models of Parkinson's disease. (Bourque M. S. D., 2023)

There are few reports of sex-associated morphological and functional changes in the microglia of the nigrostriatal pathway. A study demonstrated that in young mice the density and morphology of microglia in the striatum are similar in male and female mice; just as no difference in striatal microglia morphology has been reported between male and female mice with intact gonads and gonadectomized (Isenbrandt A., 2023). One study showed that the transcriptional profile of microglia in the midbrain is different from that of the prefrontal cortex and striatum. In the first case there is a greater expression of genes involved in the immune response, while in the second case genes involved in synaptic remodeling. Transcriptional analysis showed sex differences; specifically, male microglia show a more inflammatory transcriptional profile than female microglia. In rodents, there is compelling evidence of sex differences in microglial physiology regarding number and morphology, and these differences are heterogeneous and more evident in some brain regions such as the hippocampus and amygdala. In addition, gender differences in gene expression of different cytokines, as well as chemokines and their respective receptors in the parietal cortex and hippocampus of adult rats and particularly high levels of IL-1β in females have been reported (Barko K., 2022).

In humans, analysis of the serum profile of immune markers in female and male controls, idiopathic PD, and PD associated with gene mutations shows that a large percentage of these markers differ between males and females. In addition, the relationship between CSF inflammatory markers and PD clinical characteristics was studied. The analysis of CSF inflammatory markers in female and male PD patients treated with dopaminergic drugs showed that, in women, higher levels of IL-8 and lower levels of IL-18 were associated with higher motor scores at the Unified Parkinson's Disease Rating Scale (UPDRS) and Hoehn and Yahr staging. In both men and women, high levels of inflammatory markers associated with higher levels of alpha-synuclein in the cerebrospinal fluid were found. In patients with untreated early PD, a difference in gene expression in blood monocytes between males and females has been reported. In females the pathway analysis showed changes in inflammatory signaling and enrichment of gene sets associated with IFN-γ activation, while males showed a more heterogeneous pattern and mainly related to groupings of disease states rather than specific mechanisms. The reactivity of T lymphocytes towards α-synuclein was also observed and it was observed that,

although a clear gender difference is not observed between sex and T cell reactivity, male patients with PD showed greater reactivity and response immune to the antigen (Bourque M. S. D., 2023) (Brockmann K., 2016).

It has been discussed that mitochondria are the main source of oxidative stress associated with neurodegeneration. Due to their exclusive maternal transmission, mitochondria exhibit strong sex-specific behavior and exert differential effects in males and females. Studies in animals and postmortem human samples have shown that female neurons have greater electron transport chain activity and greater functional capabilities than male neurons. In general, less oxidative stress and damage were observed in the brain mitochondria of female rats compared to males, regardless of age and estrous cycle (Harish G., 2013).

Contrary to the positive influence of female gender on cellular respiration and oxygen redox species generation, calcium absorption capacity is lower in female than male brain mitochondria, which can negatively affect mitochondrial calcium buffering and consequently cellular homeostasis (Kim H.J., 2012).

Mitochondria are both the site of steroidogenesis and the target of sex steroids in the stimulation of mitochondrial functions, particularly biogenesis. Estradiol counteracted the loss of mitochondria in the elderly female hippocampus, restoring mitochondrial number. Therefore, it is believed that it can reduce oxidative stress, apoptosis, depolarization of the mitochondrial membrane and the influx of Ca^{2+} through the inhibition of transient thermosensitive receptor potential (TRP) channels. It is interesting to note that the effects of estrogen are not limited to neuronal cells but are also carried out at the level of astrocitary mitochondria (Yazğan Y., 2017) (González-Giraldo Y., 2019). Few studies have addressed sex-related differences in oxidative stress in the context of PD. Gender differences in prooxidant-antioxidant balance and levels of malondialdehyde, a product of lipid peroxidation, have been observed in PD patients. Neuromelanin acts as a scavenger by removing potentially toxic substances through autooxidation of catecholamines and/or by binding redox-active metal ions such as iron. The relationship between estrogen and iron in PD has been known for decades. Experimental and epidemiological evidence suggests that estrogen plays a regulatory role in iron metabolism. The striatum of male mice has been shown to be more susceptible to

iron accumulation than females. Similarly, a study conducted in humans showed that at the same plasma iron concentrations, women were less likely to develop the disease (Mariani S., 2016).

In conclusion, a growing body of experimental and clinical evidence supports the idea that PD differs between women and men. Not only do men and women experience the disease differently, but different mechanisms or the same mechanisms but regulated differently seem to be involved in the pathogenesis of the disease. However, we are still far from understanding what underlies those differences. Studies in this area are underrepresented, both from a clinical and research point of view, especially those regarding women. The overall goal is to develop tailor-made interventions and design innovative programs that meet the specific needs of men and women with PD (Bourque M. S. D., 2023).

Aim of the thesis

The results of this research are divided into two sections.

In the first section (chapter 2), studies conducted on the serum of healthy controls and patients suffering from PD will be covered, with the aim of identifying and characterizing sex-related biomarkers through an integrated approach of biochemical and metabolomics studies.

In the second section (chapter 3), will be considered studies conducted on cellular models of PD by using cell cultures of both sexes: SH-SY5Y for females, BE(2)-M17 for males. The aim is to investigate molecular pathways that could be differentially altered by cytotoxic forms of α-synuclein, in order to evaluate the possible role of biological sex in cellular vulnerability associated with PD.

Chapter 2: Results and Discussions

Blood-based biomarkers in healthy controls and Parkinson's disease patients

2 Results

2.1 Oxidative stress and Inflammatory biomarker in serum

Alterations in redox balance due to an increase in the production of unstable reactive species, in particular reactive oxygen species (ROS), and to reduced detoxifying activity, are the basis of the process that leads to chronic inflammation and neurodegeneration (Jomova K., 2023) (Chiurchiù V., 2016).

To assess whether there were any sex differences in PD and HC serum related to redox balance, preliminary investigations were conducted by two colorimetric assays. The D-ROMs test was used to evaluate the level of oxidative stress by measuring the reactive oxygen metabolites (ROMs) responsible for cell damage and aging. The experiments conducted did not reveals significant differences between HC and PD sera regardless of sex; not even segregating by sex (Figure 16).

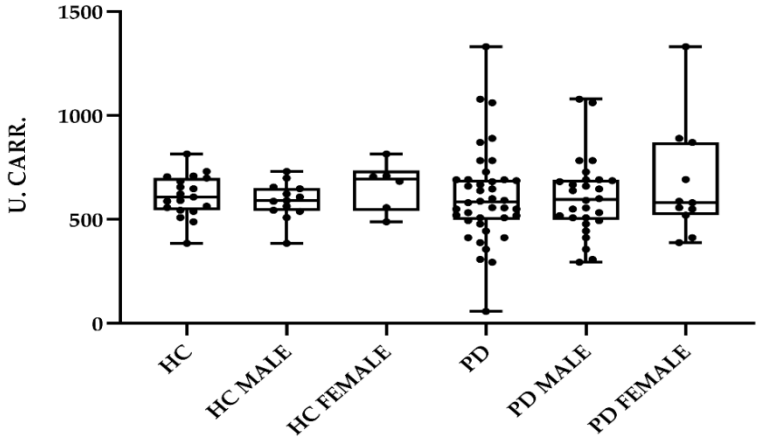


Figure 16: Analysis of Reactive Oxygen Metabolites (ROMs). The graphs show the levels of ROMs expressed in CARR units compared to patients. The box plots show medians and whiskers of the values. Statistical analysis was performed by Wilcoxon-Mann-Whitney test, comparing HC with PD regardless of sex, HC and PD of opposite sex, and HC with PD of the same sex. No significant differences were detected between groups ($p = 0.12$)

The BAP test was used to evaluate the overall ability of antioxidant molecules (exogenous and endogenous) present in plasma, such as vitamins, glutathione, uric acid and proteins, to neutralize free radicals. As shown in Figure 17, there were no significant differences between patients and healthy controls.

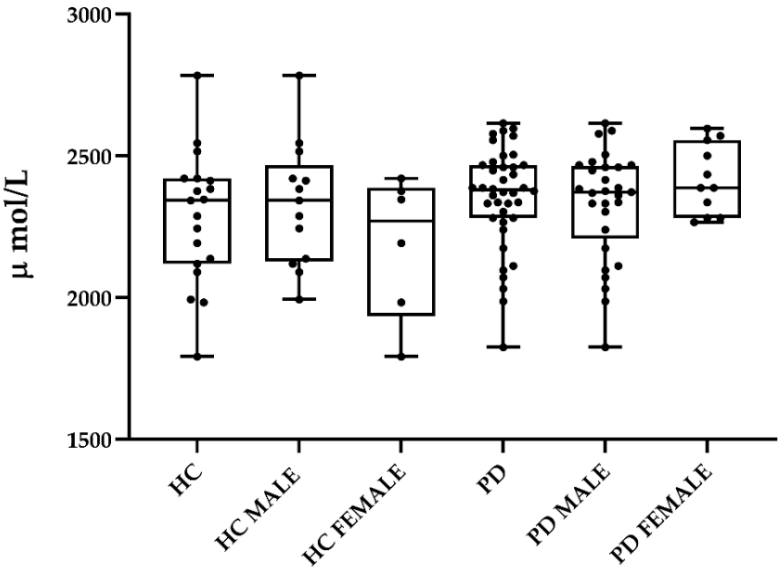


Figure 17: BAP test analysis of HC and PD sera. The graphs show the levels of antioxidant activity expressed in µmol/L. The box plots show medians and whiskers of the values. Statistical analysis was performed by Wilcoxon-Mann-Whitney test, comparing HC with PD regardless of sex, HC and PD of opposite sex, and HC with PD of the same sex. No significant differences were detected between groups ($p = 0.12$).

In order to investigate whether different molecular pathways are involved in controlling the redox balance in the sera of PD patients compared to HC, we investigated by Western blotting analysis, the serum levels of Nrf2, a transcriptional factor that under conditions of cell damage can be released into the bloodstream (Figure 18).

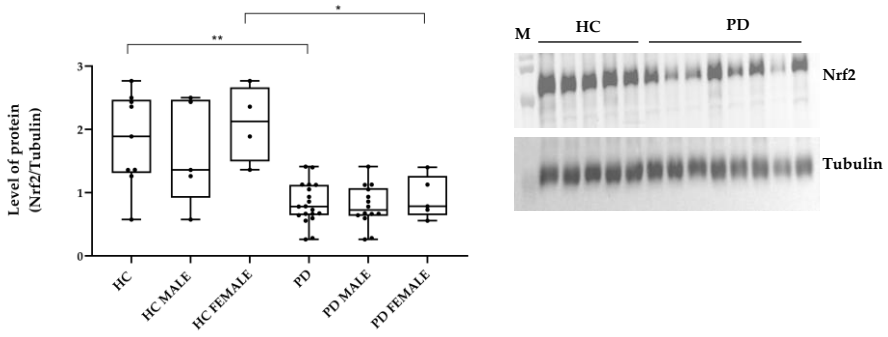


Figure 18: Expression level of Nrf2 in serum samples from healthy subjects (HC) and patients with Parkinson's disease (PD), assessed by Western blot. Densitometric analysis of immunoreactive bands was performed in three independent experiments. After densitometric analysis, western blot signals of target proteins are normalized for Tubulin. Densitometric quantification and representative blot are reported. The box plots show medians and whiskers of the values. Statistical analysis was performed by Wilcoxon-Mann-Whitney test, comparing HC with PD regardless of sex, HC and PD of opposite sex, and HC with PD of the same sex. $p < 0.03$ *, $p < 0.002$ **.

In the serum of HC, Nrf2 expression levels were higher than in PD, regardless of sex. The segregation of data by sex, showed that the observed difference is due to higher protein expression in the serum of HC females.

NRf2 is a transcription factor that controls the expression of numerous other genes encoding proteins that regulate redox balance, such as NQO1 and SOD 2. NAD(P)H Quinone Oxidoreductase 1 (NQO1) catalyzes the reduction of potentially toxic quinone compounds to less toxic hydroquinones. Superoxide Dismutase 2 (SOD 2) belongs to the oxidoreductase family and is essential for neutralizing reactive oxygen species (ROS). In particular, SOD 2 catalyzes the reduction of superoxide radicals ($O_2^{\bullet -}$) in more stable compounds such as water and hydrogen peroxide (H_2O_2), which will then be further reduced. The expression level of these biomarkers was evaluated by Western blot (Figure 19).

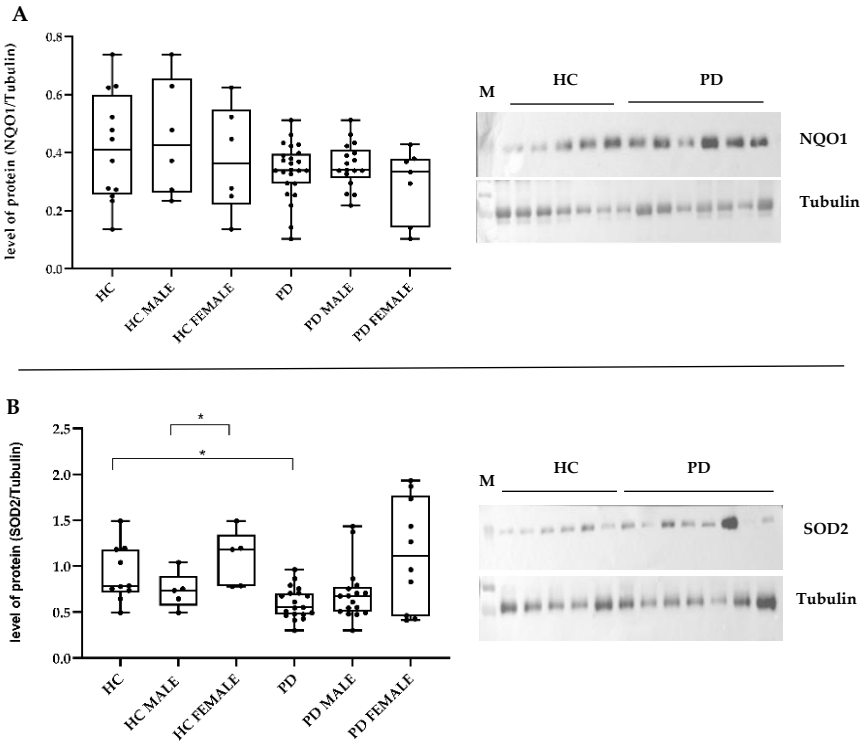


Figure 19: Expression level of NQO1 **(A)** and SOD 2 **(B)** in sera of HC and PD, assessed by Western blot. Densitometric analysis of immunoreactive bands was performed in three independent experiments. After densitometric analysis, western blot signals of target proteins are normalized for Tubulin. Densitometric quantification and representative blot are reported. The box plots show medians and whiskers of the values. Statistical analysis was performed by Wilcoxon-Mann-Whitney test, comparing HC with PD regardless of sex, HC and PD of opposite sex, and HC with PD of the same sex. $p < 0.03$ *. No significant differences were detected between groups for NQO1 ($p = 0.12$).

No statistically significant difference was observed between HC and PD for NQO1, not even segregating by sex. Serum SOD2 levels were significantly lower in PD patients compared with HC, and in male HC relative to female HC.

Another important protein involved in resolving oxidative stress is UCP2, that can be released into the bloodstream during cell lysis due to neurodegeneration (Jomova K., 2023). Western blot analysis of UCP2 showed no difference in the expression levels of this protein both in HC and PD patients' sera (Figure 20).

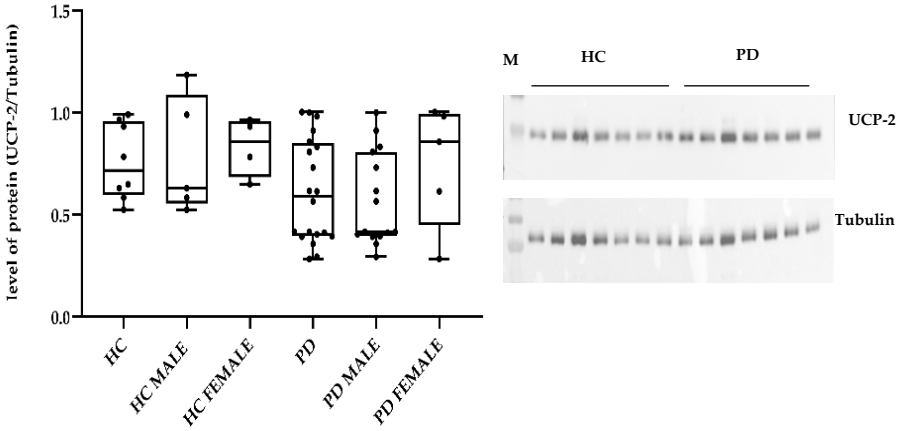


Figure 20: Expression level of UCP-2 in serum from healthy controls (HC) and Parkinson's disease patients (PD)**,** assessed by Western blot. Densitometric analysis of immunoreactive bands was performed in three independent experiments. After densitometric analysis, western blot signals of target proteins are normalized for Tubulin. Densitometric quantification and representative blot are reported. The box plots show medians and whiskers of the values. Statistical analysis was performed by Wilcoxon-Mann-Whitney test, comparing HC with PD regardless of sex, HC and PD of opposite sex, and HC with PD of the same sex. No significant differences were detected between groups ($p = 0.12$).

Inflammation is closely involved in neurodegeneration. IL-6 is a pleiotropic pro-inflammatory cytokine involved in immune response, inflammation, and metabolic regulation (Muzio L., 2021). The ELISA sandwich assay showed a higher amount of IL-6 in PD compared to HC, regardless of sex, precisely in males affected by PD compared to HC of the same sex (Figure 21).

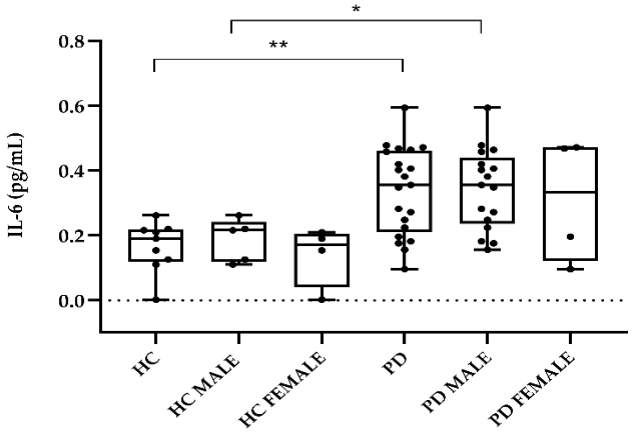


Figure 21: ELISA test measurement of IL-6 amount in the serum of HC and PD patients. The test was performed as reported by the manufacturer. The box plots show the median and whiskers. Statistical analysis was performed by Wilcoxon-Mann-Whitney test, comparing HC with PD regardless of sex, HC and PD of opposite sex, and HC with PD of the same sex. $p < 0.03$ *, $p < 0.002$ **.

Inflammation and oxidative stress are two closely related mechanisms; in this regard we conducted Spearman correlation analyses between the amount of IL-6 and the expression of Nrf2 in HC serum, regardless of sex (Figure 22).

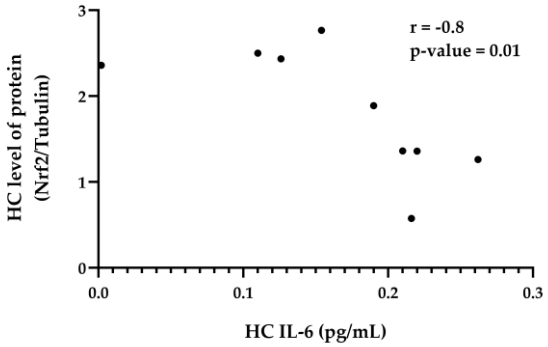


Figure 22: Scatter plot graphs of Spearman correlation. Correlation analysis conducted on marker of inflammation and oxidative stress in serum from healthy patients (HC). Each symbol represents the measure of a case. $p < 0.03$ *.

In HC, regardless of sex, there is a strong negative correlation between the amount of IL-6 and the level of Nrf2 expression.

2.2 Evaluation of matrix metalloprotease in serum of healthy controls and Parkinson's patients

Matrix metalloproteinases (MMPs) are enzymes crucial for tissue remodeling. Their dysregulation in neurodegenerative diseases contributes to neuronal damage because they can exacerbate brain damage by disrupting the blood-brain barrier and promoting inflammatory cell migration (Kim E.M, 2011) . We focused our attention on MMP3 and MMP9. The activity of MMP9 in the patients' serum was measured by zymography on gelatin, a functional assay that allows us to quantify the active forms, potentially involved in PD, and the latent forms of the enzyme (Figure 23).

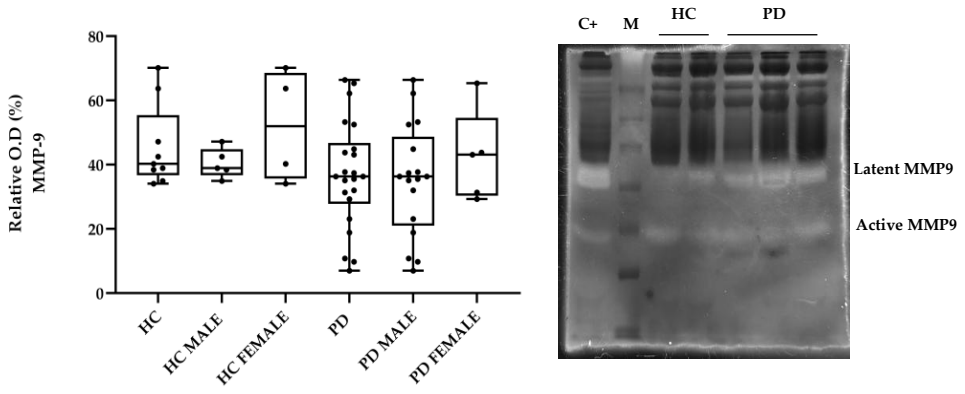


Figure 23: Activity of MMP9 in HC and PD patients' serum. Gelatin gel zymography comparing MMP 9 activity in serum of sex-independent and sex-segregated HC and PD. Densitometric band analysis was performed in three independent experiments. The graph shows the percentage of the relative optical density normalized for the total content. Statistical analysis was performed by Wilcoxon-Mann-Whitney test, comparing HC with PD regardless of sex, HC and PD of opposite sex, and HC with PD of the same sex. No significant differences were detected between groups (p = 0.12).

Zymographic analysis shows no difference in MMP9 activity between HC and PD.

The amount of MMP3 measured by western blot was higher in the serum of female PD patients than in male PD patients (Figure 24).

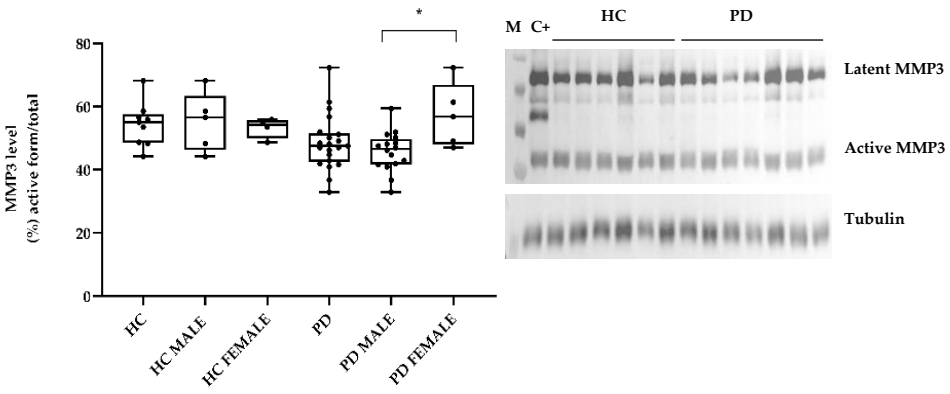


Figure 24: Levels of MMP3 in HC and PD sera, assessed by Western blot. Densitometric analysis of immunoreactive bands was performed in three independent experiments. After densitometric analysis, western blot signals of target proteins are normalized for Tubulin. The relative levels of the active form were normalized for the total MMP3 content, and reported as a percentage. Densitometric quantification and representative blot are reported. The box plots show medians and whiskers of the values. Statistical analysis was performed by Wilcoxon-Mann-Whitney test, comparing HC with PD regardless of sex, HC and PD of opposite sex, and HC with PD of the same sex. $p < 0.03$ *.

The correlation analyses between metalloprotease and IL-6 showed that in patients with PD, the amount of IL-6 is positively correlated with the total form of MMP3. This

correlation is more evident when considering PD, regardless of sex, and weaker, when considering only male patients. (Figure 25).

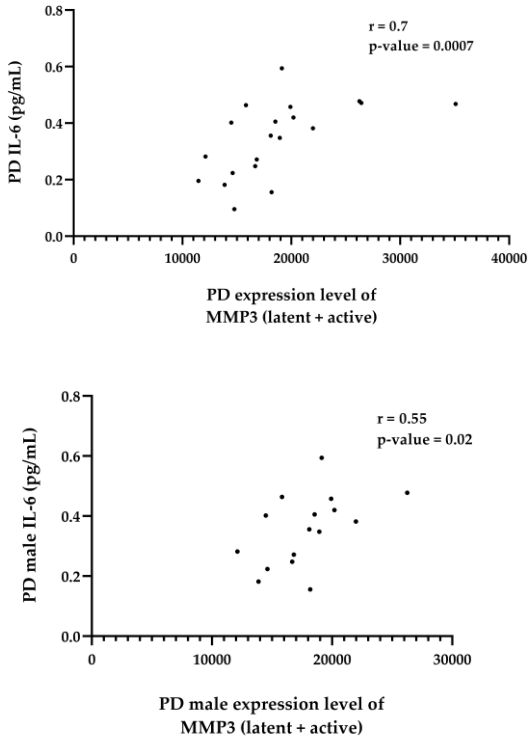


Figure 25: Scatter plot graphs of Spearman correlation. Correlation analysis between MMP3 and IL-6 in PD patient serum. Each symbol represents the measure of a case. p< 0.03 *, p-value: 0,002 **.

2.3 Alpha-synuclein as a potential biomarker in biological fluids

Although the molecular mechanisms dysregulated by α-synuclein have not yet been fully understood, to date this protein is considered an important biomarker for diagnosis and prognosis. Numerous studies focus on the detection of α-synuclein in the biological fluids of PD patients, with conflicting and variable results depending on the biological fluid analyzed (Shu H., 2024).

Western blot analysis of serum from healthy subjects (HC) and Parkinson's patients (PD), after controlled proteolysis with proteinase K, showed a different profile of immunoreactive bands (Figure 26A). Densitometric analysis of immunoreactive bands showed a higher amount of PK-resistant α-synuclein at 52 kDa in PD serum, compared to HC. In particular, this evidence emerges in males PD (Figure 26B).

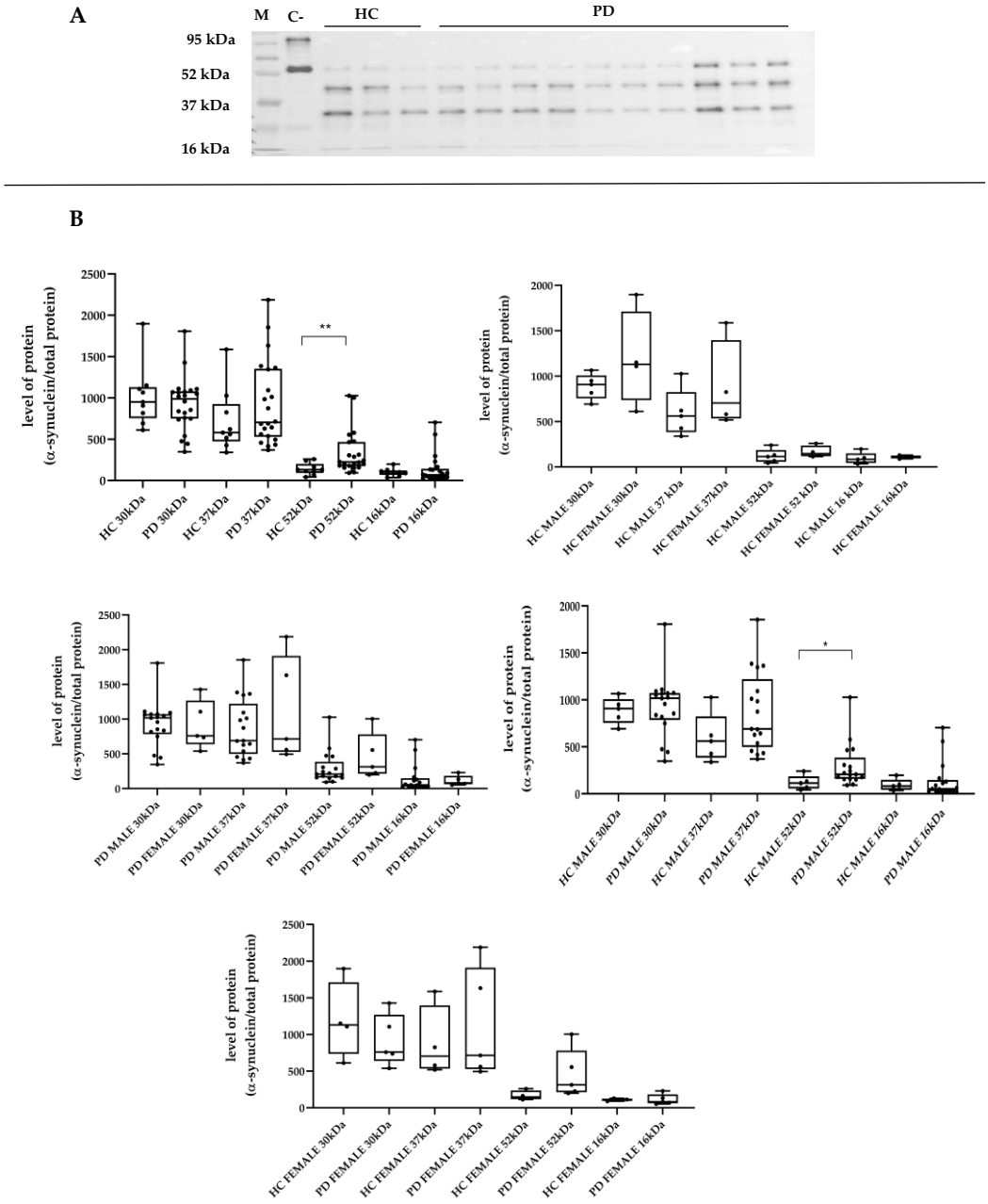


Figure 26: Western blot of α-synuclein profile on HC and PD sera. Representative blot of α-synuclein profile after controlled digestion with proteinase K is reported **(A).** Densitometric analysis of immunoreactive bands was performed in three independent experiments. After densitometric analysis, western blot signals of target proteins are normalized for total proteins. The box plots show medians and whiskers of the values **(B)**. Statistical analysis was performed by Wilcoxon-Mann-Whitney test, comparing HC with PD regardless of sex, HC and PD of opposite sex, and HC with PD of the same sex. $p < 0.03$ *, $p < 0.002$ **.

2.4 Assays for Structural Characterization of α-Synuclein Aggregation

In PD, there is a close correlation between oxidative stress, inflammation, and conformational changes in α-synuclein (Shen L., 2024).

It is known that in PD, α-synuclein is present in a dynamic equilibrium between various forms, namely monomers, aggregate oligomers, and early fibrils. These conformational states present different characteristics among themselves including the different composition of β-sheets. These, along with other factors, are responsible for the formation of insoluble and cytotoxic oligomers of the protein responsible of neurodegeneration (Bartels T., 2011) (Estaun-Panzano J., 2023).

In order to evaluate the presence of antibodies against different forms of α-synuclein, recombinant synuclein was expressed using recombinant DNA technology in E. coli as a monomer and subsequently aggregates as reported in (Dandurand J., 2020). Synuclein fibrils starting from the monomeric form were produced as reported in the Materials and Methods section.

α-synuclein aggregates were characterized by Thioflavin T assay; thioflavin fluorescence was observed only in the presence of an increase of β-structures of aggregated synuclein as well as in the oligomeric forms of the protein (Figure 27).

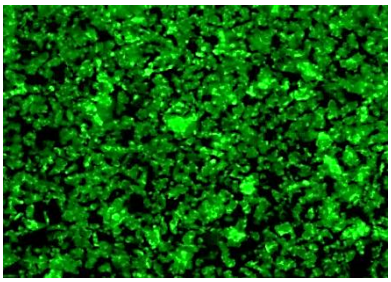


Figure 27: Fluorescence micrographs of aggregate α-synuclein. Thioflavin (50 μM) was added to α-synuclein (25 μM). The images were acquired with a fluorescence microscope using the green light filter (510–560 nm) immediately after the addition of Thioflavin.

Moreover, TEM analysis showed the presence of large aggregated structures in the α-synuclein sample subjected to continuous stirring for 7 days at 45 °C (Figure 28A), compared to the control sample (Figure 28B).

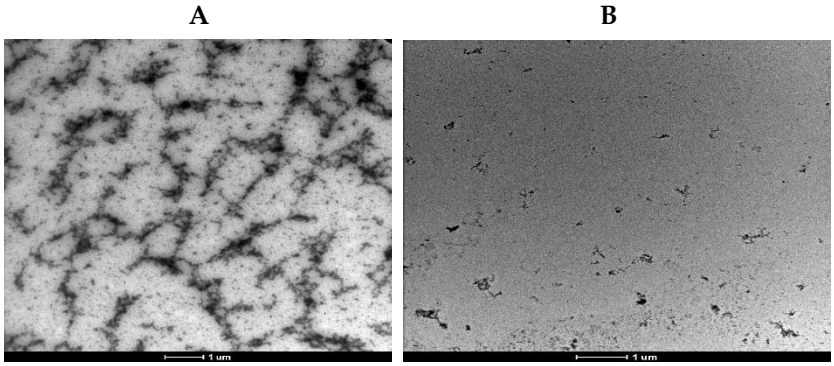


Figure 28: TEM micrographs of α-synuclein structures. **(A)** protein solubilized and subjected to aggregation process; **(B)** freshly solubilized protein, used as a negative control. Image **A** was captured at 9500 x, while image **B** at 11,500 x magnification.

Circular dichroism spectroscopy and deconvolution analysis of the spectra allowed to characterize the secondary structures of monomer, aggregate and fibrils forms of synuclein (Figure 29).

	% Helix	% Strand	% Turns	% Unordered
Fibrils	8	35	23	34
Aggregate	10	32	24	33
Monomer	16	23	27	34

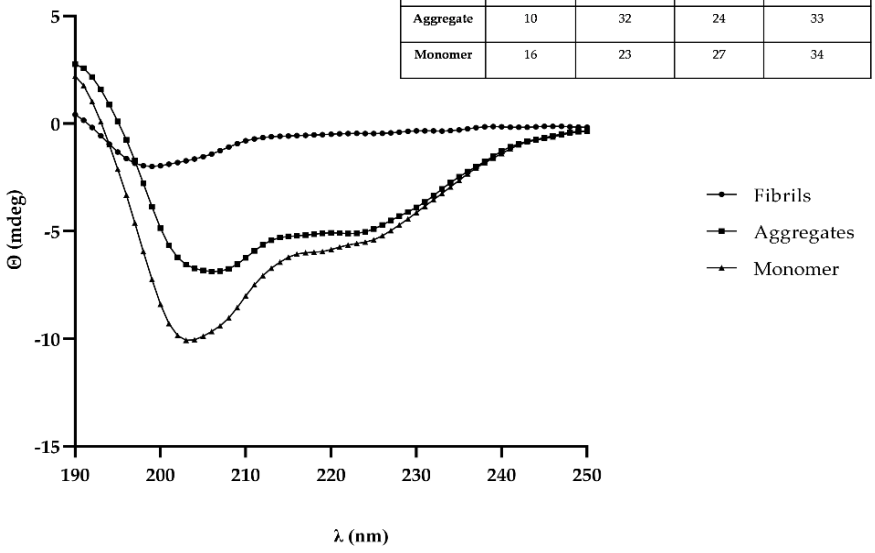


Figure 29: CD spectrum of the three forms of a-synuclein. The spectra were recorded at a wavelength between 190 and 250 nm. The deconvolution analysis was performed by the Contin algorithm.

Alpha-helix structures prevail in the monomer, while β-sheets prevail in the aggregate and fibrils.

Tertiary structure analysis of the protein was evaluated by fluorescence spectroscopy. Fluorescence spectra were acquired by exciting at wavelengths of 264 nm and measuring the emitted fluorescence in a range between 270 and 400 nm. α-synuclein monomer showed a fluorescence spectrum with an emission maximum around 320 nm, due to the presence of tyrosine and phenylalanine residues. The aggregates are characterized by an increase in fluorescence and a blue shift a 305 nm; the fibrils are characterized by a decrease in the intensity of the emitted fluorescence and a blue shift at 300 nm. Blue shifts suggest the presence of more folded structures in both aggregate and fibrillar forms (Figure 30).

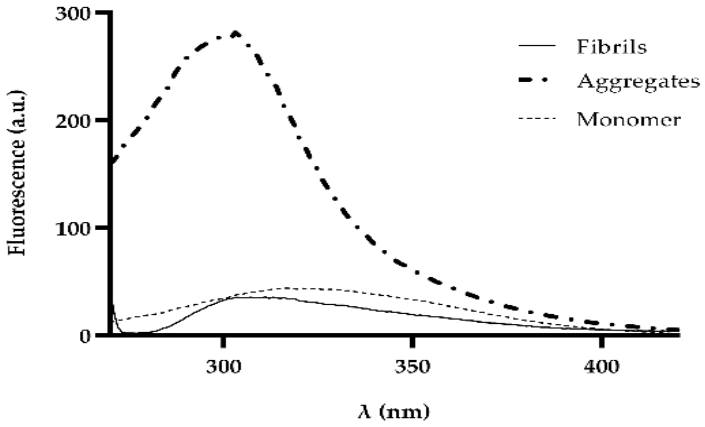


Figure 30: Fluorescence emission spectrum of α-synuclein. The three forms of α-synuclein were used at a concentration of 70 μM. Phosphate buffer was used as negative control. Fluorescence spectra were obtained by exciting the protein at a wavelength of 264 nm and measuring the emitted fluorescence in a wavelength range of 270 to 400 nm.

2.5 Evaluation of the presence of antibodies against different forms of synuclein in human serum

Based on the evidence emerging from the Western blot on the presence of specific α-synuclein resistant to proteolytic cleavage, we decided to investigate, with indirect ELISA assay home-made, if in the serum of HC and PD there were antibodies able to recognize the different forms of α-synuclein, specifically the monomeric form, aggregates, and fibrils. A significantly amount of antibodies against monomeric and aggregated forms is observed in PD females compared to PD males (Figure 31).

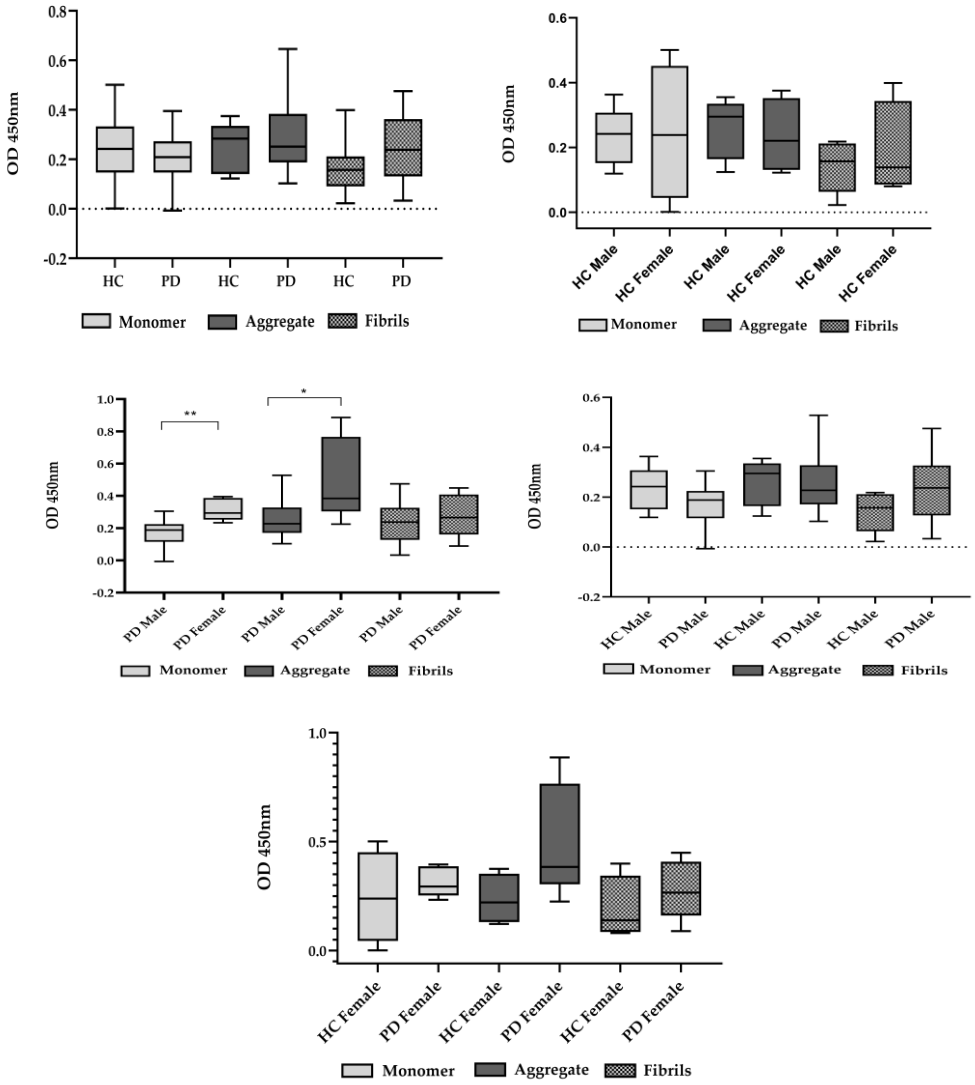


Figure 31: Indirect ELISA on HC and PD serum for the research of antibodies capable of recognizing and binding monomeric, aggregate, and fibrillary form of α-synuclein. The box plots show the optical density (O.D) measured at 450 nm obtained from the colorimetric reaction of the bond of antibodies to the immobilized antigen. Statistical analysis was performed by Wilcoxon-Mann-Whitney test, comparing HC with PD regardless of sex, HC and PD of opposite sex, and HC with PD of the same sex. $p < 0.03$ *, $p < 0.002$ **.

2.6 Metabolomic Analysis

To identify metabolites that may reflect a different physiological and pathological condition in healthy controls and PD patients, as well as in patients of different sexes, we conducted metabolomics studies using proton nuclear magnetic resonance (^{1}H NMR) spectroscopy.

A panel of 17 metabolites produced significantly different results in HC and PD (Figure 32).

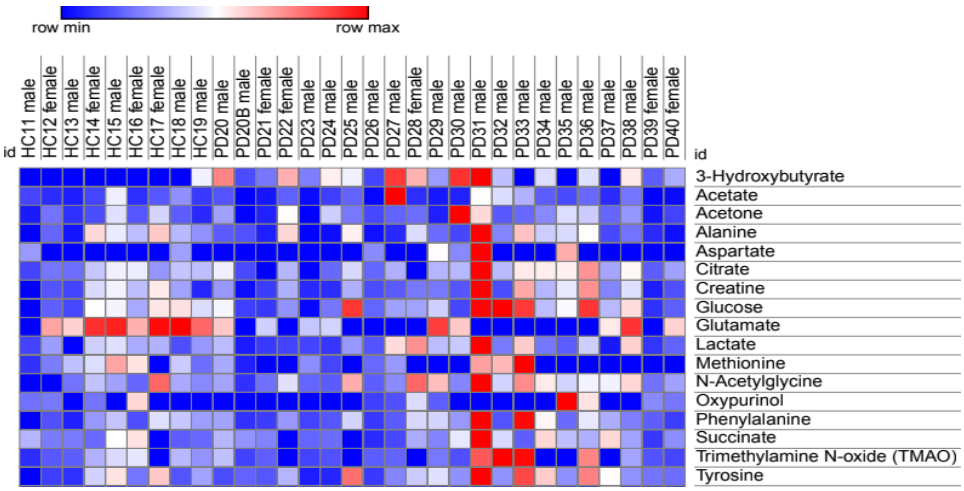
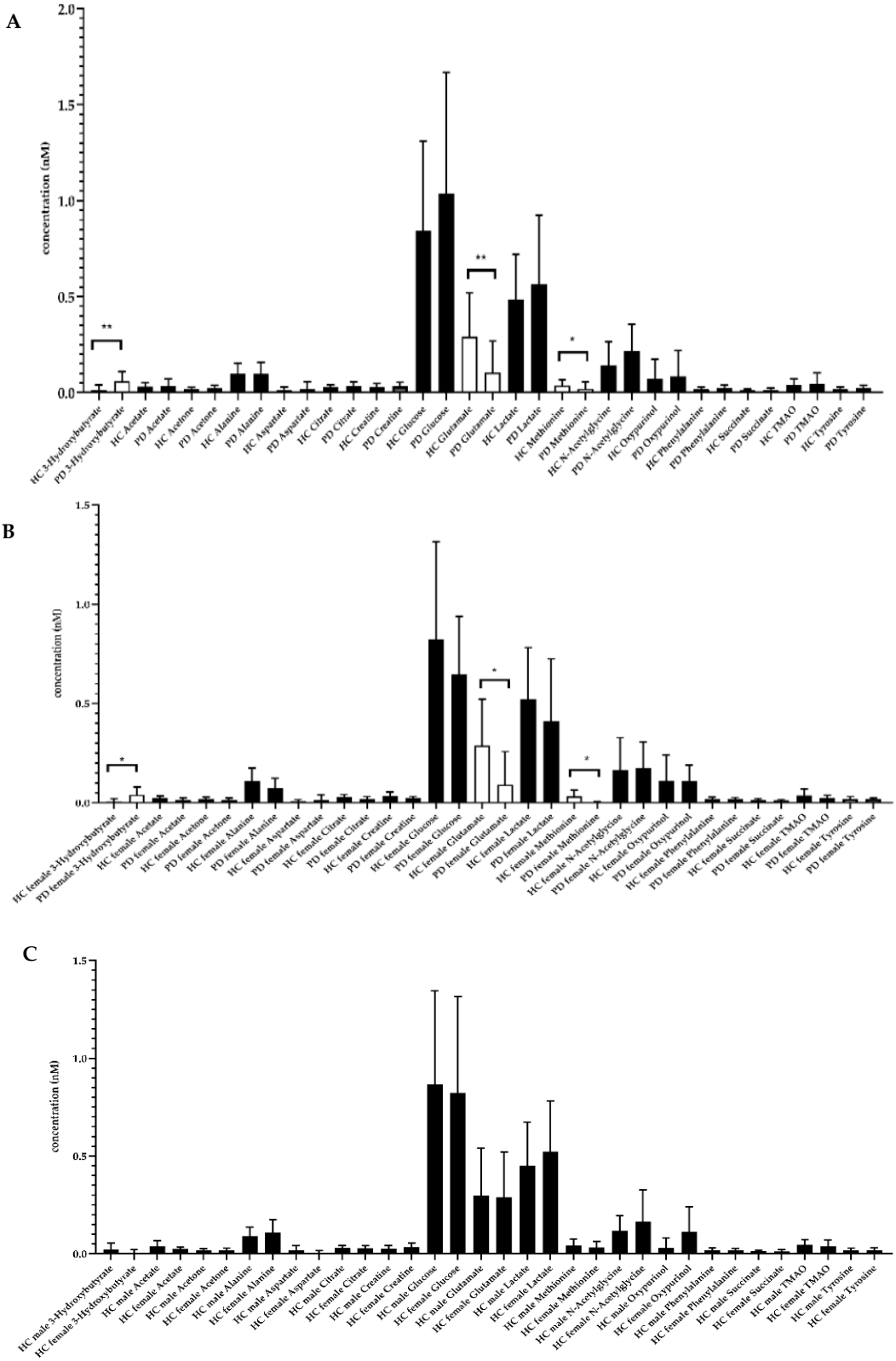


Figure 32: Heatmap of the most relevant metabolites between male and female HC and PD. The values in red (row max =1) indicate the most abundant metabolites; the values in blue (row min =1) indicate the less abundant metabolites. * $p < 0,05$

Regardless of sex, HC sera showed a greater quantity of Glutamate and Methionine, while PD sera, showed a greater quantity of beta-hydroxybutyrate (BHB) (Figure 33A). stratifying by sex, it emerges that these alterations are due to HC and PD, both female (Figure 33B). No sex-related differences were observed within HC (Figure 33C), and no significant differences were detected between PD and HC males (Figure 33D). Notably, PD males exhibited increased levels of acetate, acetone, citrate, creatine, total glucose, lactate, and phenylalanine compared to PD females (Figure 33E).


A
concentration (nM)
2.0
1.5
1.0
0.5
0.0
**
**
*
HC 3-Hydroxybutyrate
PD 3-Hydroxybutyrate
HC Acetate
PD Acetate
HC Acetone
PD Acetone
HC Alanine
PD Alanine
HC Aspartate
PD Aspartate
HC Citrate
PD Citrate
HC Creatine
PD Creatine
HC Glucose
PD Glucose
HC Glutamate
PD Glutamate
HC Lactate
PD Lactate
HC Methionine
PD Methionine
HC N-Acetylglycine
PD N-Acetylglycine
HC Oxypurinol
PD Oxypurinol
HC Phenylalanine
PD Phenylalanine
HC Succinate
PD Succinate
HC TMAO
PD TMAO
HC Tyrosine
PD Tyrosine
B
concentration (nM)
1.5
1.0
0.5
0.0
*
*
*
HC female 3-Hydroxybutyrate
PD female 3-Hydroxybutyrate
HC female Acetate
PD female Acetate
HC female Acetone
PD female Acetone
HC female Alanine
PD female Alanine
HC female Aspartate
PD female Aspartate
HC female Citrate
PD female Citrate
HC female Creatine
PD female Creatine
HC female Glucose
PD female Glucose
HC female Glutamate
PD female Glutamate
HC female Lactate
PD female Lactate
HC female Methionine
PD female Methionine
HC female N-Acetylglycine
PD female N-Acetylglycine
HC female Oxypurinol
PD female Oxypurinol
HC female Phenylalanine
PD female Phenylalanine
HC female Succinate
PD female Succinate
HC female TMAO
PD female TMAO
HC female Tyrosine
PD female Tyrosine
C
concentration (nM)
1.5
1.0
0.5
0.0
HC male 3-Hydroxybutyrate
HC female 3-Hydroxybutyrate
HC male Acetate
HC female Acetate
HC male Acetone
HC female Acetone
HC male Alanine
HC female Alanine
HC male Aspartate
HC female Aspartate
HC male Citrate
HC female Citrate
HC male Creatine
HC female Creatine
HC male Glucose
HC female Glucose
HC male Glutamate
HC female Glutamate
HC male Lactate
HC female Lactate
HC male Methionine
HC female Methionine
HC male N-Acetylglycine
HC female N-Acetylglycine
HC male Oxypurinol
HC female Oxypurinol
HC male Phenylalanine
HC female Phenylalanine
HC male Succinate
HC female Succinate
HC male TMAO
HC female TMAO
HC male Tyrosine
HC female Tyrosine

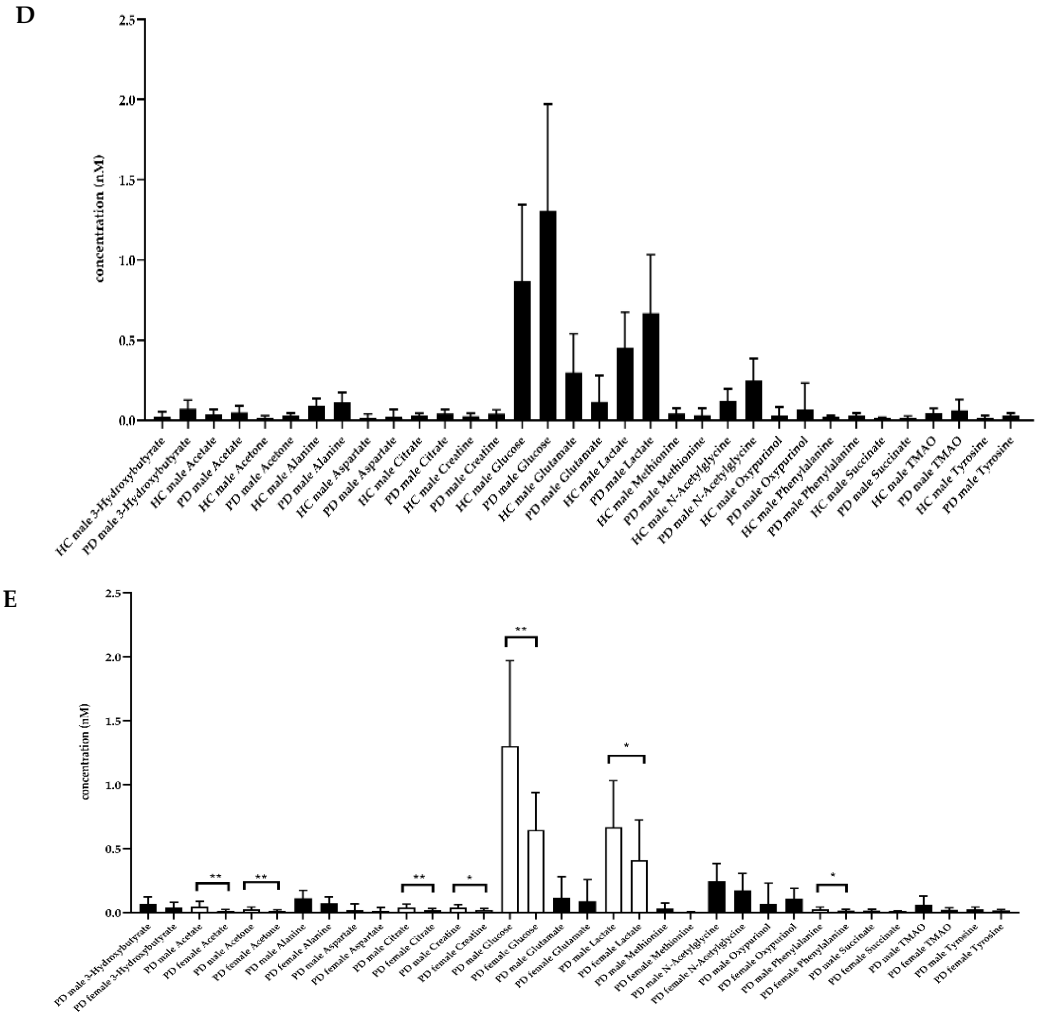


Figure 33: Metabolomic analysis. The graph shows the concentration of metabolites (nM) of HC and PD regardless of sex **(A)**, and HC and PD segregated by sex: female HC and PD **(B)**, male and female HC **(C)**, HC and PD male **(D)**, male and female PD **(E).** Statistical analysis was performed by Wilcoxon-Mann-Whitney test. $p < 0.03$ *, $p < 0.002$ **

2.7 Discussion

The diagnosis, prognosis, as well as the monitoring of disease progression and response to pharmacological treatments require the presence of validated biomarkers. Given the complexity of PD, different types of markers are being studied but, to date, there are still no validated biomarkers. In this context, the identification and characterization of potential markers for PD, in biological fluids, particularly in blood and serum, is fundamental not only because the diagnosis is essentially based on clinical evidence, but also to be able to distinguish the subtypes of the pathology, which could be characterized by distinct molecular mechanisms, with the aim of obtaining a precise and early diagnosis (Tönges L., 2022) (Rizzo G., 2016).

The search for new biomarkers faces several critical challenges. Comparative approaches, such as meta-analyses, are often hindered by the limited number of studies, particularly for novel or difficult to measure markers, due to small sample sizes, and substantial heterogeneity across studies. Moreover, biological and sex-related differences among patients must be taken into account, as findings from one cohort may not be reproducible in another. It should also be noted that sex and gender differences have only relatively recently begun to be systematically considered. In addition, many studies are cross-sectional rather than longitudinal. These factors, combined with the intrinsic complexity of biological matrices, such as body fluids, may result in outcomes that are inconclusive and not fully representative of the underlying biological reality (Huang A., 2025).

In the first phase of our research, we aimed to perform serum analyses in healthy controls and PD patients of both sexes to identify potential circulating biomarkers.

As reported in the Introduction section, oxidative stress, acting through mitochondrial dysfunction and the alteration of pathways involved in the removal of misfolded proteins, such as α-synuclein, is one of the mechanisms most involved in neurodegeneration (Caproni S., 2025). The oxidative stress is closely connected to inflammation. The increase in ROS determines an overactivation of glial cells, responsible for the release of large quantities of cytokines and pro-inflammatory mediators, ultimately responsible of neurodegeneration (Lai T.T., 2022) (Siracusa R., 2019).

We investigated the presence of any sex-related differences of the oxidative stress and antioxidant capacity in serum levels of patients PD compared to HC, by the combined employment of colorimetric assays and protein analyses. The absence of significant differences in oxidative stress levels or antioxidant capacity between groups, including stratifying by sex, suggest that probably, in the patients included in our study, there is no petrurbation of redox homeostasis, or that the dynamic redox modifications, that could occur at the cellular level, are not completely reflected in the circulating markers.

To explore these aspects, some key proteins involved in the response to oxidative stress were analysed. In particular, Western blot analysis showed a reduction in serum Nrf2 levels in PD compared to HC, a difference mainly determined by the high expression of Nrf2 in female HC. This result is particularly relevant because it suggests a possible systemic adaptive response to conditions of oxidative stress and could suggest a greater basal antioxidant capacity in healthy female controls, probably due to the role of estrogen (Arabia G., 2022). Downstream of Nrf2, two of its main targets, NQO1 and SOD2, were investigated. The expression level of NQO1 is not different between groups. This could be due to alterations in the structure or stability of the protein, due to post-translational modifications, hindering its correct detection. Serum SOD2 expression was increased in healthy controls compared to patients, particularly in females, in line with what was observed by others (Wang Z., 2021). This finding, partly consistent with Nrf2 data, reinforces the hypothesis of greater efficacy of the antioxidant response in healthy subjects, potentially compromised in PD patients, probably due to the dysfunction of the Nrf2/Keap1-ARE pathway.

The mitochondrial protein UCP2, also involved in the reduction of oxidative stress by regulation of mitochondrial membrane potential, showed no significant variation between the groups analyzed. Although the increase in UCP2 has been shown to have a protective effect against oxidative stress and neuronal loss, studies are predominantly based on animal and cellular models. Therefore, our results may be due to the fact that in patients the regulation of UCP2 is more relevant at the tissue level and the protein is probably not systematically released into the serum (Conti

B., 2005). Overall, these observations highlight the complexity of redox mechanisms in PD.

IL-6 levels were significantly increased in patients compared to controls, namely in sera of male PD. This data could reflect a chronic systemic inflammatory state associated with neurodegeneration. The negative correlation between IL-6 and Nrf2 levels further reinforces the link between oxidative stress and inflammation, suggesting that greater activation of the antioxidant response is associated with lower inflammatory activation. These results, highlight a possible dysfunctional interaction between antioxidant and inflammatory pathways in PD patients, modulated in part by biological sex. Failure to effectively activate the Nrf2 pathway with a consequent increase of SOD2 and concomitant increase of serum IL-6 levels suggest a systemic imbalance that could contribute to neurodegenerative progression.

Matrix metalloproteinases are essential for tissue formation, neuronal network remodeling, and blood-brain barrier integrity. Several hypotheses have been put forward that support the involvement of these enzymes in the onset of PD. It is believed that MMPs, released by neurons subject to strong oxidative stress, induce neuroinflammation by activating glial cells and consequently also destabilize the structure of the blood-brain barrier, causing the passage of harmful substances from the blood to brain tissue (Behl T., 2021). It is known that MMP3 is involved in the incorrect cleavage of α-synuclein, favoring the formation of insoluble and cytotoxic aggregates (Kim E.M, 2011), and that is detected at low levels in serum of PD compared to controls, especially at an early stage of the disease (Chuan Z.L., 2022); on the contrary, MMP9 levels were higher in PD at both an early and advanced stage.

We find no difference in MMP9 expression levels in our HC and PD serum groups, also considering the sex variable. In contrast, we observed a greater amount of the active form of MMP-3 in female patients with PD than in males. However, serum MMP-3 show a positive correlation with IL-6. These results suggesting a possible sex-specific involvement of this metalloprotease in the systemic inflammatory response.

The main hallmark of PD is the presence of cytotoxic aggregate forms of α-synuclein in the Substantia Nigra pars compacta. It must be considered that, to date, the

structural properties of mature oligomers are known well, but much less is known about those of the transient species that form during aggregation. It is believed that these transient species (early fibrils) are more harmful than mature forms. These structures can dysregulate numerous physiological processes leading to the protein accumulation which causes loss of dopaminergic neurons (Chen S.W., 2024) (Emamzadeh FN., 2016).

The higher abundance of 52 kDa oligomeric forms resistant to PK-controlled proteolysis observed in male PD patients indicates the accumulation of degradation-resistant protein aggregates, which are likely involved in the pathogenetic mechanisms of the disease.

Although α-synuclein represents a promising biomarker, and many studies focus on the dosage of this protein or anti-synuclein antibodies in serum, to date, the results are inconsistent and often conflicting between healthy controls and disease patients. In addition, as already discussed, there are different forms of α-synuclein in dynamic equilibrium with each other, some of which have a greater toxic effect (Tönges L., 2022).

An in-house indirect ELISA using different α-synuclein conformers as antigens showed that female PD patients exhibited a stronger antibody response to both monomeric and aggregated forms than males, suggesting sex-specific modulation of the immune recognition of toxic protein species, and, probably, a greater efficiency of the women's immune system to recognize different forms of the pathological protein.

Since PD is a multifactorial disease, the search for new circulating biomarkers should also focus on altered metabolites in PD patients. In this context, a promising approach is metabolomics, as it provides a comprehensive overview of metabolic pathways that could be altered in both sexes and contribute to the onset of the disease. Although initially conducted on cerebrospinal fluid, today studies focus on plasma and serum, as matrix that can be obtained with non-invasive approaches. Metabolic changes are the direct results of alterations in protein and enzyme activities. Therefore, metabolomics may offer valuable information on PD-related physiological process, molecular interactions and metabolic pathways. PD exhibits high heterogeneity, having multiple pathways and molecular mechanisms mediating

its molecular pathogenesis. Based on metabolomic findings in clinical and experimental models, the metabolic pathways that are majorly perturbed in PD are related to the metabolism of lipids, energy such as glycolysis, fatty acids and amino acids. In addition, treatment with dopaminergic drugs, such as L-DOPA, can contribute to the dysregulation of metabolic pathways (Shao Y., 2019).

Our metabolomics studies identified 17 significantly altered metabolites in serum PD compared to HC, while highlighting sex-specific differences.

In particular, in in PD females compared to HC females there is an increase in serum levels of ketone bodies, in particular β-hydroxybutyrate (BHB), probably due to mitochondrial dysfunction or alterations in glycolysis. This result is consistent with what was previously reported (Toczylowska B., 2020). In female HC, higher levels of glutamate and methionine were detected, suggesting differences in amino acid metabolism and oxidative stress regulation compared with PD. This finding was unexpected, as previous evidence indicates elevated levels of these metabolites in PD; glutamate, acting as a neurotransmitter, may accumulate at the synaptic level due to excitotoxicity caused by impaired post-synaptic receptor function, thereby contributing to neurodegeneration (Iovino L., 2020). The presence of higher concentrations of acetate, acetone, citrate, total glucose, lactate and phenylalanine found in male PD compared to the female counterpart could indicate alterations in the main energetic cycles, including glycolysis and the tricarboxylic acid cycle. Presumably, the increase in acetone and citrate could indicate an increase in beta-oxidation and oxidative stress, respectively (Luo X., 2024). In addition, there are studies that have highlighted high levels of phenylalanine in early-onset PDs. Since this aromatic amino acid is involved in the formation of dopamine, there could probably be an alteration in the biogenesis of neurotransmitters which could contribute to neurodegeneration (Li S., 2024).

Ultimately, these results support the hypothesis that in PD there are differences linked to the metabolome in the two sexes due to a complex dysregulation between various metabolic pathways.

Chapter 3: Results and Discussions.

Molecular mechanisms altered by pathological α-synuclein in cell cultures deriving from males and females.

3 Results

It is known that extracellular α-synuclein is capable of modifying cell metabolism (Colla E., 2019) (Shen L., 2024). In order to assess whether synuclein may influence some pathways of neuronal cells, we treated with α-synuclein two neuroblastoma cell lines, used as models for the study of PD: SH-SY5Y deriving from a female patient (SH) and BE(2)-M17 deriving from a male patient (M17).

3.1 α-synuclein affects viability of SH-SY5Y and BE(2)-M17 cell lines

To evaluate the effect of the three forms of α-synuclein on the viability of cells, we conducted MTT viability assays. Cells were treated at increasing concentrations of monomers, aggregate and fibrils (0.03 - 0.3 - 3 μM) for 24, 48 and 72 hours (Figure 34).

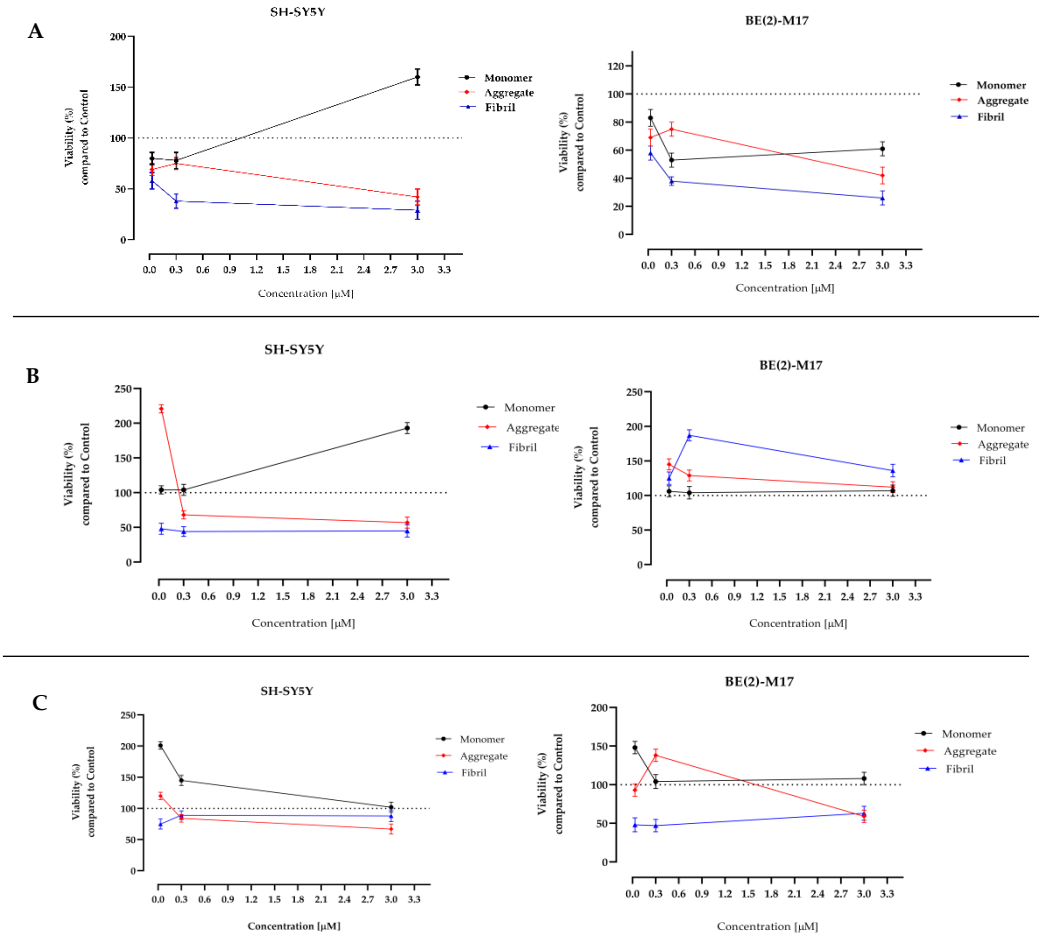


Figure 34: Dose-response plot of the three forms of α-synuclein on SH-SY5Y and BE(2)-M17 neuroblastoma cell viability. Cells were treated with monomer, aggregate and fibrils at three different concentrations (0.03, 0.3, 3 µM), for **(A)** 24 hours, **(B)** 48 hours, **(C)** 72 hours. Cell viability was assessed by MTT assay, of three independent experiments, and expressed as a percentage of control treated with vehicle alone (PB 0.5%), and set to 100%. Each data point represents the average of three independent experiments, with vertical bars indicating the standard deviation.

After 24 hours, the exposure to all three forms of synuclein showed the same trend in SH cell viability, which decreases as concentration increases, except for cells treated with monomer, in which viability increases by 50% compared to control. In cells treated with aggregates, viability decreases from 80% to 50%; while, with fibrils, cell viability decreases from 60% to 30%. A similar trend is observed in M17 cells, whose viability decreases from 70% to 40%, and from 60% to 30%, in cells treated with aggregates and fibrils, respectively; however, unlike the observed increase in viability for SH, after treatment with monomer (3 µM), M17 show a viability of only 60% compared to the control. Ultimately, in both cell types, fibrils impair cell viability more (compared to aggregates) over the entire range of concentrations used; surprisingly, after exposure to the monomer (3 µM), the two

cell types show an opposite trend (Figure 34A). After 48 hours of treatment with the monomer, SH show a similar trend observed at 24 hours, in fact, the viability increases by approximately 80% at 3 μM. After treatment with fibrils, cell viability remains (over the entire range of concentrations used) around 50%. Surprisingly, treatment with aggregates at the lowest concentration (0.03 μM) determines an increase in cell viability of 100% compared to control, which is reduced in a dose-dependent manner as the concentration increases. In contrast, in M17, the three forms of α-synuclein do not affect viability at any concentration considered, compared to control (Figure 34B). After 72 hours of treatment, in SH, at lower concentrations (0.03 μM), there is an increase in cell viability of approximately 100% and 30% with monomers and aggregates respectively; while, fibrils reduce viability by approximately 30%. At the highest concentration (3 μM), the viability of cells treated with monomer decreases but does not change compared to control, while the viability of cells treated with aggregate and fibril remains within 60%. A similar trend is observed in M17. At all fibril concentrations and the highest concentration of aggregates, viability is about 50%; while, after treatment with monomer (0.03 uM), cell viability increases by 50% compared to the control, and does not undergo variations at higher concentrations (Figure 34C).

In both cell types, α-synuclein exposure affected cell viability in a dose-dependent manner. In SH cells, fibrils showed higher cytotoxicity than aggregates and monomers, except at low concentrations of aggregates where a stimulatory effect was observed. In M17 cells, fibrils consistently showed the most cytotoxic effect at all concentrations, while aggregates were harmful even at higher concentration.

Based on these observations, and considering that aggregate and fibrils are considered the most harmful forms, subsequent experiments were conducted using concentrations of 3 μM for all three forms, and for 24 hours of exposure.

3.2 Evaluation of endogenous α-synuclein expression

To understand whether the effects observed on the two cell lines lead to an increase in endogenous expression of α-synuclein, we conducted Real-time and Western blot analysis (Figure 35).

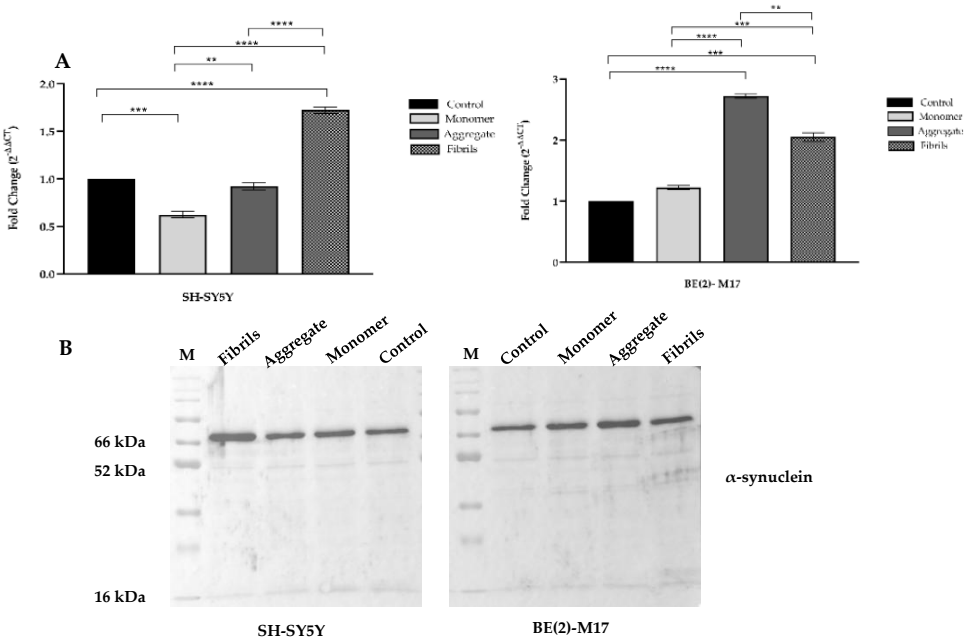


Figure 35: Effect of monomer, aggregate and fibrils on endogenous α-synuclein expression, in SH-SY5Y and BE(2)-M17 cells. Cells were treated with 3 μM of monomer, aggregate and fibrils, for 24 hours. **(A)** The expression level of SNCA gene was calculated as Fold Change ($2^{-\Delta\Delta ct}$), with β-Actin as reference gene, and compared to the control, treated with vehicle alone (PB 0.5%). Data are presented as mean ± SEM of at least three independent experiments. **(B)** Representative blot of α-synuclein profile. Statistical significance was assessed using one-way ANOVA followed by Holm-Sidak post hoc correction for multiple comparisons. p < 0.002 **, p< 0.0002 *** p< 0.0001 ****.

The qRT-PCR graphs showed that, after treatment with α-synuclein preparations for 24 hours, in both cell lines there is a significant increase in the expression of the SNCA gene, coding for α-synuclein. Alpha-synuclein expression increased when SH cells were treated with fibrils, while the monomer reduced gene expression, compared to control. Treatment of M17 with all three forms of synuclein resulted in an increased gene expression, most evident with aggregate and fibrils (Figure 35A). What emerged from the qRT-PCR is confirmed by the Western blot, which showed the presence of a greater quantity of synuclein at 66 kDa and weaker bands at 52 and 16 kDa, indicative of the presence of oligomeric and monomeric forms (Figure 35B).

3.3 Treatment with alpha-synuclein modifies intracellular ROS

To understand the reduced viability observed by MTT assay with aggregate and fibril, we measured the presence of intracellular reactive oxygen species (ROS) by the oxidation of a non-fluorescent probe (2',7'-dichlorodihydrofluorescein diacetate, DCFH-DA) to its fluorescent form (2',7'-dichlorofluorescein, DCF). As shown in Figure 36, in SH cells, all three form of α-synuclein increased ROS by approximately 30% compared to control; unlike what happens in M17, where ROS decreased.

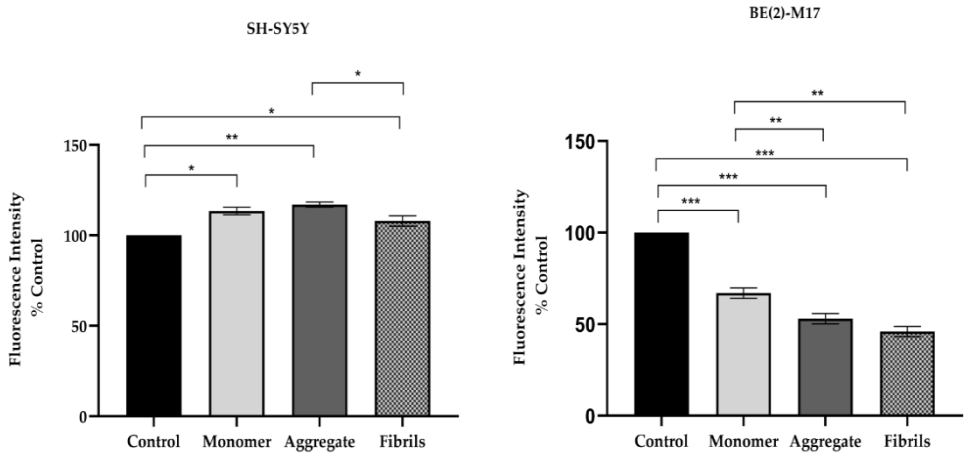


Figure 36: Effect of the three forms of α-synuclein on intracellular ROS accumulation, in SH-SY5Y and BE(2)-M17 cells. Cells were treated with 3 μM of monomer, aggregate and fibrils for 24 hours. It was determined the fluorescence intensity as a percentage of the control, treated with vehicle alone (PB 0.5%). Data are presented as mean fluorescence ± SEM of three independent experiments. Statistical significance was assessed using one-way ANOVA followed by Holm-Sidak post hoc correction for multiple comparisons. $p< 0.03$ *, $p < 0.002$ **, $p< 0.0002$ ***.

To investigate what emerged from the ROS analysis, we evaluated the ability of α-synuclein preparations to influence gene expression and the synthesis of proteins involved in oxidative stress and inflammation (Figure 37).

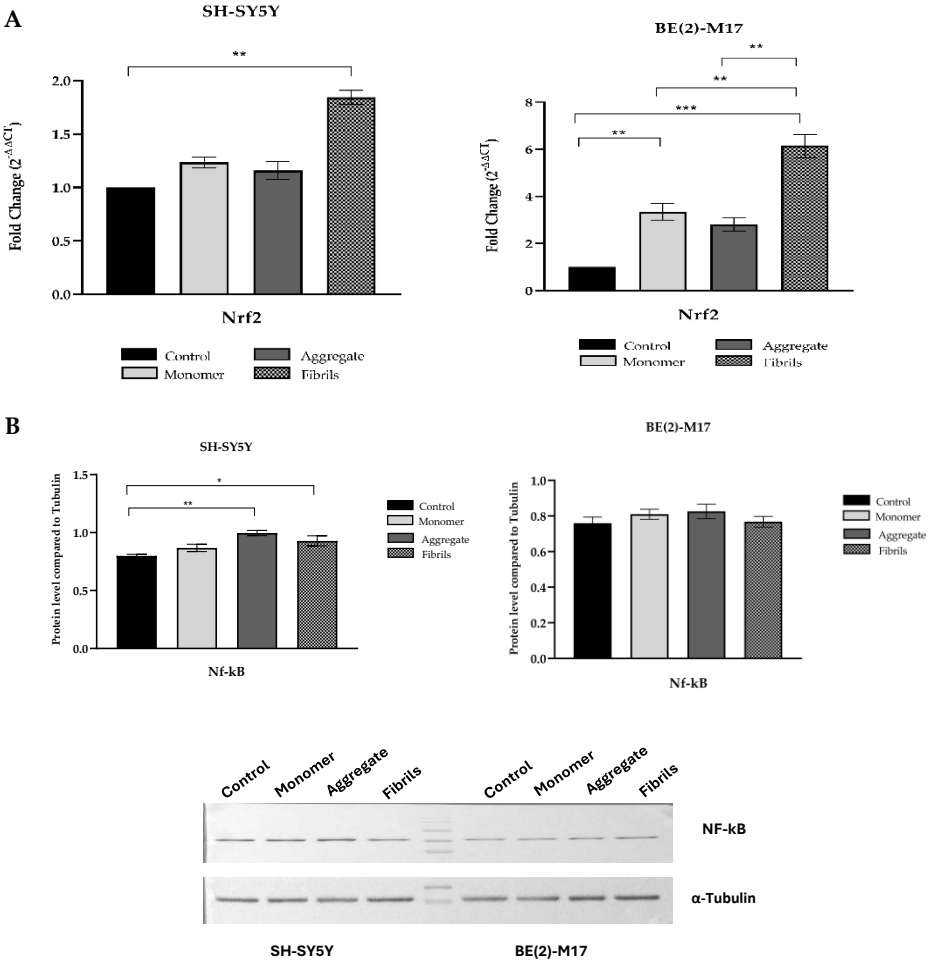


Figure 37: Effect of the three forms of α-synuclein on the expression of genes and proteins involved in Oxidative Stress and inflammation, in SH-SY5Y and BE(2)-M17 cells. Cells were treated with 3 μM of monomer, aggregate and fibrils for 24 hours. **(A)** The expression level of Nrf2 gene was calculated as Fold Change ($2^{-\Delta\Delta ct}$), with β-Actin as reference gene, and compared to the control, treated with vehicle alone (PB 0.5%). Data are presented as mean ± SEM of at least three independent experiments. **(B)** Western blot analysis of relative levels of the Nf-kB protein normalized for α-Tubulin. Data are presented as mean ± SEM of two independent experiments. Statistical significance was assessed using one-way ANOVA followed by Holm-Sidak post hoc correction for multiple comparisons. $p< 0.03$ *, $p < 0.002$ **, $p< 0.0002$ ***.

In both cell lines, after 24 hours of exposure, fibrils induced a significant increase in Nrf2 expression levels; in M17, a significant increase in Nrf2 expression was observed even with treatments with monomer and aggregate.

From Western blot analyses, an increase of Nf-kB levels in SH-SY5Y, due to both aggregates and fibrils, emerged. No change in NF-κB levels was observed in M17.

To explain the increase in Nrf2 expression, we evaluated, by Western blot, the level of proteins downstream of the transcription factor (SOD2), and involved in

resolution of oxidative stress and maintenance of redox homeostasis (UCP-2) (Figure 38).

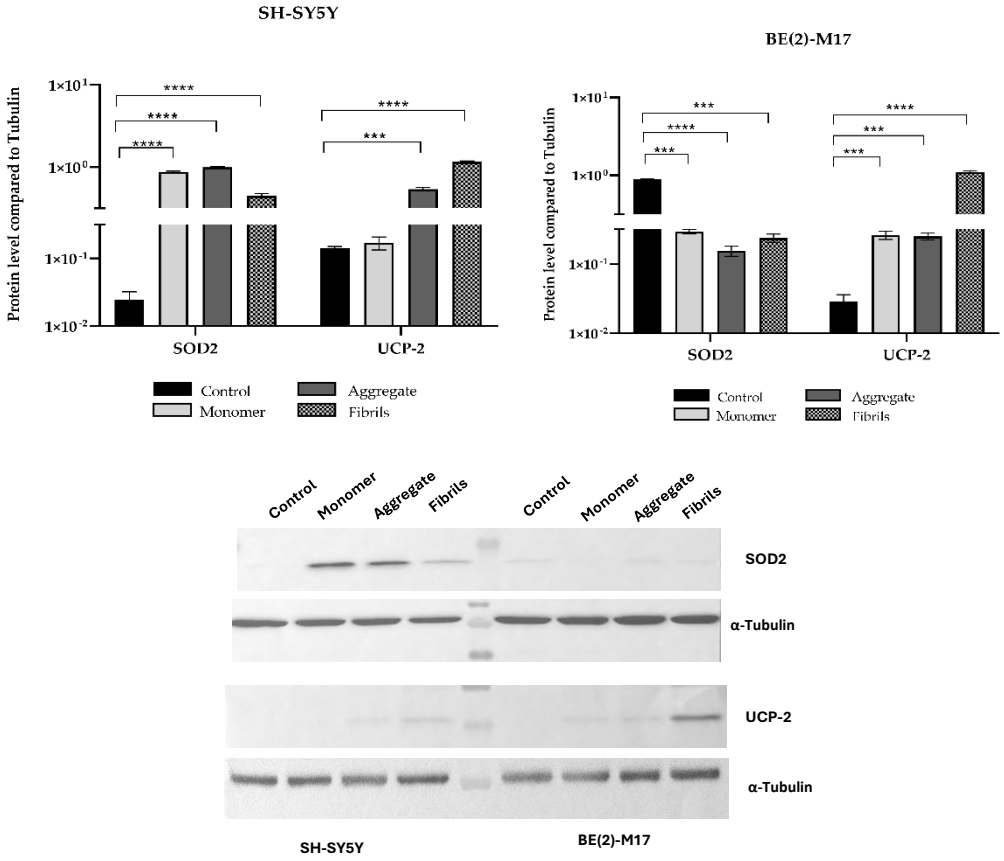


Figure 38: Effect of α-synuclein on oxidative stress biomarkers, in SH-SY5Y and BE(2)-M17 cells. Cells were treated with 3 μM of monomer, aggregate and fibrils for 24 hours. The relative level of SOD2 and UCP2 proteins was normalized for α-Tubulin. Data are presented as mean ± SEM of two independent experiments. Expression level of protein and representative blot are reported. Statistical significance was assessed using one-way ANOVA followed by Holm-Sidak post hoc correction for multiple comparisons. $p < 0.0002$ ***, $p < 0.0001$ ****.

In SH the three forms of α-synuclein induced a significant increase in SOD2 expression, compared to the control; while, in M17 an opposite trend was observed. Furthermore, in both cell lines, UCP-2 expression increased due to the effect of aggregate and fibrils, and due to the effect of the monomer only in M17.

The qRT-PCR analysis showed a significant overexpression of NAD(P)H Quinone Oxidoreductase 1 (NQO1), in SH-SY5Y treated with all three forms of α-synuclein; while, in M17, an under expression have emerged with aggregate compare to control (Figure 39).

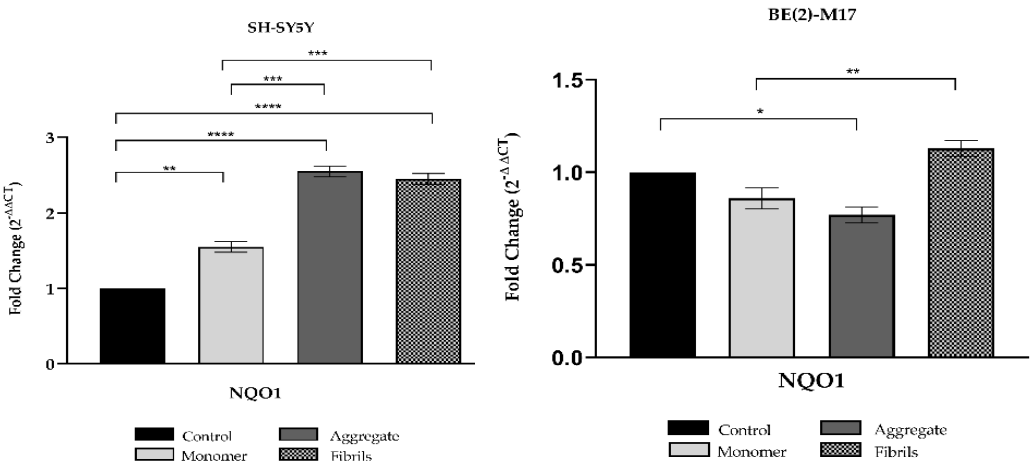


Figure 39: Effect of the three forms of α-synuclein on the expression level of NQO1 gene involved in reduction of oxidative stress, in SH-SY5Y and BE(2)-M17 cells. Cells were treated with 3 µM of monomer, aggregate and fibrils for 24 hours. The expression level was calculated as Fold Change ($2^{-\Delta\Delta ct}$), with β-Actin as reference gene, and compared to the control (PB 0.5%). Data are presented as mean ± SEM of three independent experiments. Statistical analysis was performed by one-way ANOVA followed by Holm-Sidak post hoc correction for multiple comparison. p< 0.03 *, p < 0.002 **, p< 0.0002 ***, p < 0.0001 ****.

3.4 Effect of α-synuclein preparations on mitochondrial homeostasis and mitophagy

To assess whether the α-synuclein preparations influence mitochondrial function, we investigated the mitochondrial membrane potential (Figure 40).

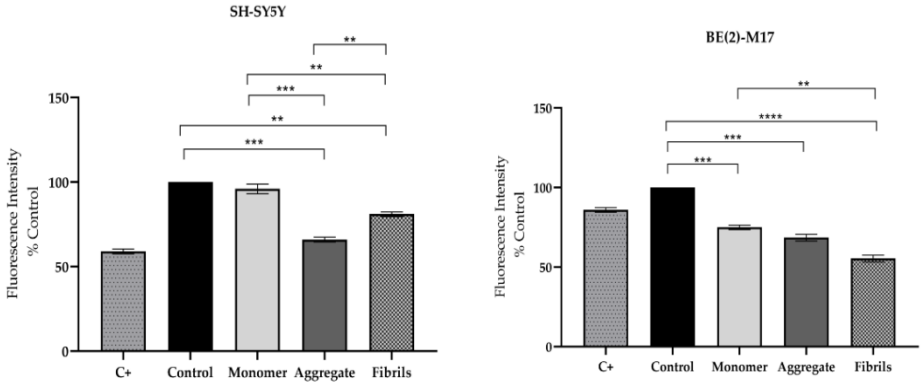


Figure 40: Effect of the three forms of α-synuclein on mitochondrial membrane potential (ΔΨm), in SH-SY5Y and BE(2)-M17 cells. Cells were treated with 3 μM monomer, aggregate and fibrils for 24 hours, and the fluorescence emitted by the JC-1 molecular probe was measured. Interference of the fluorescence signals emitted by the probe was resolved by calculating the ratio of red (510-570 nm) to green (580-640 nm) fluorescence. The fluorescence intensity obtained was reported as a percentage compared to the control, treated with 0.5% phosphate buffer alone. FCCP (10 uM) was used as a positive control. Data are presented as mean fluorescence ± SEM of three independent experiments. Statistical significance was assessed using one-way ANOVA followed by Holm-Sidak post hoc correction for multiple comparisons. $p < 0.002$ **, $p< 0.0002$ ***, $p < 0.0001$ ****.

In both cell lines, aggregate and fibrils reduced the mitochondrial membrane potential. In M17 cells, treatment with the monomer also led to a decrease in membrane potential.

Through qRT-PCR analysis we evaluated the level of expression of some genes involved in the maintenance of mitochondrial homeostasis (Figure 41).

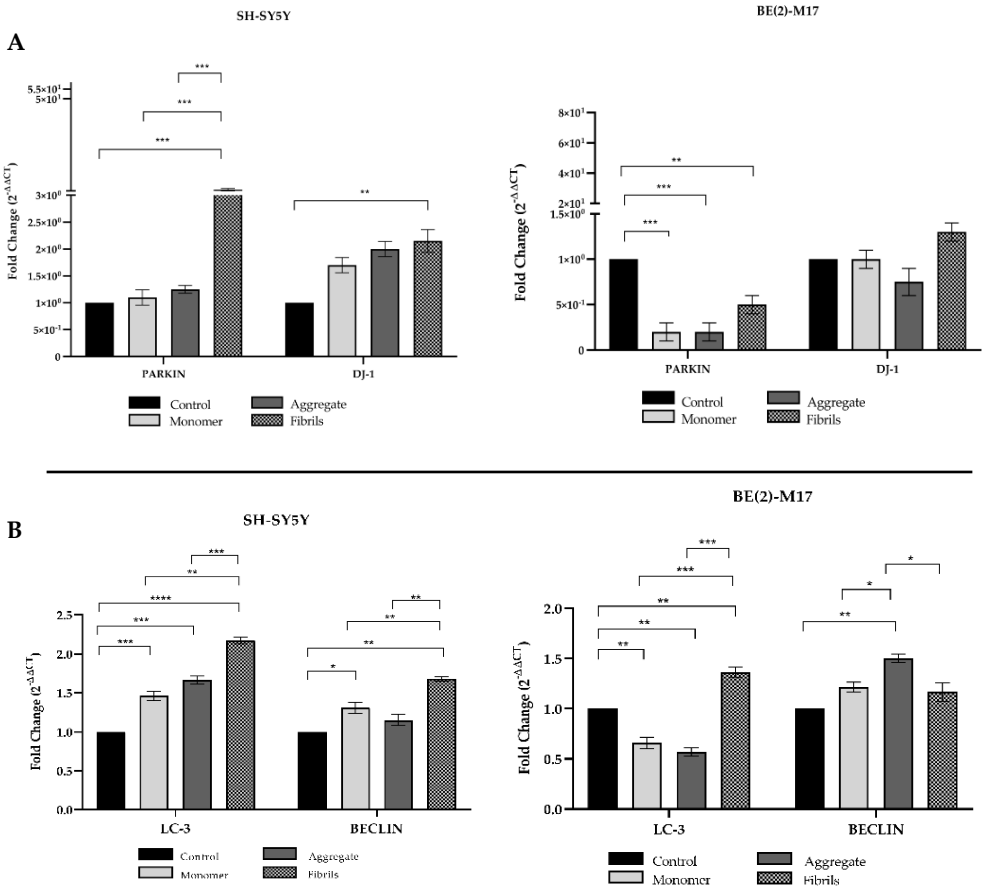


Figure 41: Effect the three forms of α-synuclein on mitochondrial homeostasis and mitophagy in SH-SY5Y and BE(2)-M17 cells. Cells were treated with 3 μM of monomer, aggregate and fibrils for 24 hours. The expression level of PARKIN and DJ-1 **(A)**; and LC-3 and BECLIN **(B)**, was calculated as Fold Change ($2^{-\Delta\Delta ct}$), with β-actin as reference gene, and compared to the control, treated with vehicle alone (PB 0.5%). Data are presented as mean ± SEM of at least three independent experiments. Statistical analysis was performed by one-way ANOVA followed by Holm-Sidak post hoc correction for multiple comparisons. p< 0.03 *, p < 0.002 **, p< 0.0002 ***, p < 0.0001 ****.

As shown (Figure 41A), fibrils induced overexpression of both PARKIN and DJ-1 in SH cells. In contrast, in M17 cells, all forms of α-synuclein decreased PARKIN expression and did not change the expression level of DJ-1.

In SH cells, the expression level of LC-3 and BECLIN was increased mainly due to the effect of fibrils. In M17, monomer and aggregate decreased the expression level of LC-3; on the contrary fibrils increased the transcript level of it. In addition, the expression level of BECLIN increased due to the aggregates (Figure 41B).

3.5 Effect of aggregate α-synuclein on ER stress and UPR pathway

In PD, the accumulation of cytotoxic α-synuclein oligomers causes a condition known as Endoplasmic Reticulum stress (E.R stress). Although cells have a defense mechanism known as Unfolded Protein Response (UPR), in many cases this mechanism fails. We investigated the effect of α-synuclein, on this mechanism, evaluating the expression of markers involved in the UPR pathway (Figure 42).

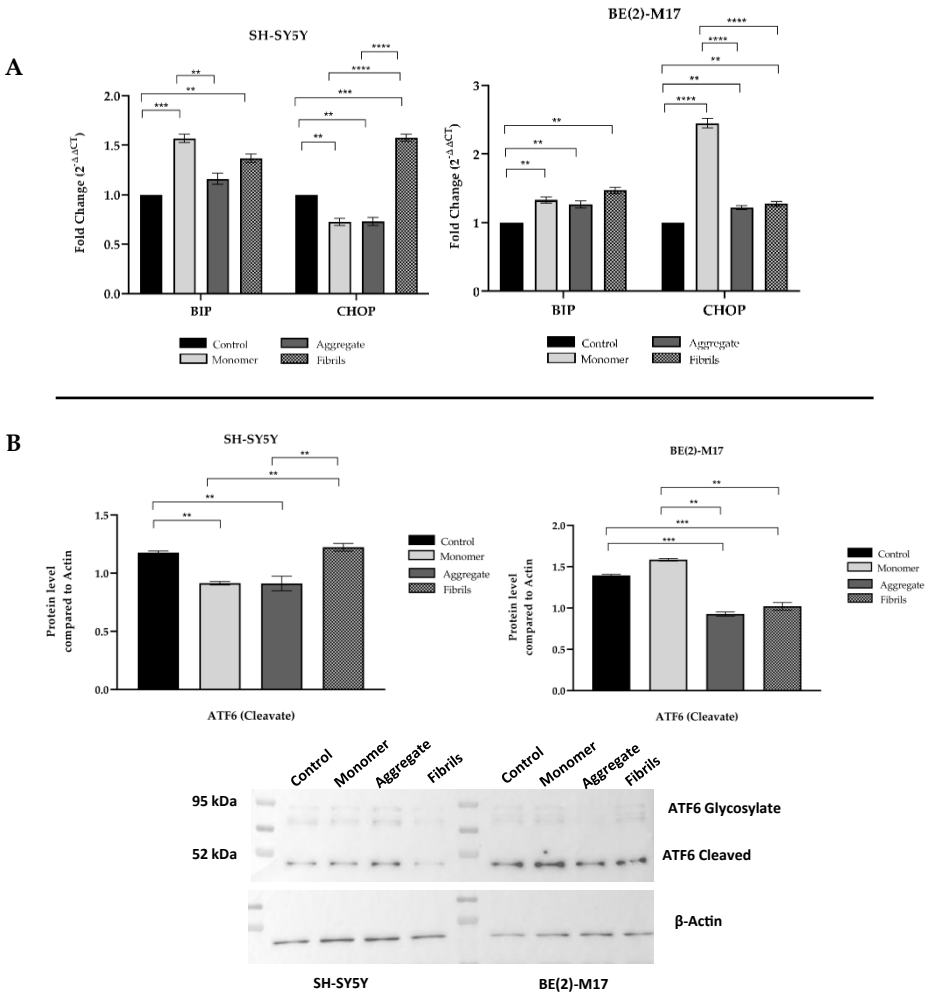


Figure 42: Effect of the three forms of α-synuclein on the expression of markers involved in ER stress and UPR pathway, in SH-SY5Y and BE(2)-M17 cells. Cells were treated with 3 μM of monomer, aggregate and fibrils for 24 hours. **(A)** The expression level of BIP and CHOP was calculated as Fold Change ($2^{-\Delta\Delta ct}$), with β-Actin as reference gene, and compared to the control (PB 0.5%). Data are presented as mean ± SEM of at least three independent experiments. **(B)** Expression level of ATF6 protein was normalized for β-Actin and reported as the ratio of the clivate form to the glycosylated form. Graphs and representative blot are shown. Data are presented as mean ± SEM of at least two independent experiments. Statistical analysis was performed by one-way ANOVA followed by Holm-Sidak post hoc correction for multiple comparisons. p< 0.03 *, p < 0.002 **, p< 0.0002 ***, p < 0.0001 ****.

α-synuclein induced a significant, yet variable, increase in BIP expression in both male and female cell lines. On the contrary, CHOP is over expressed in SH-SY5Y by fibrils, and in M17 by monomer. The expression level of CHOP decreased with treatment with monomer and aggregate in SH-SY5Y (Figure 42A).

Western blot analysis revealed the presence of both the glycosylated (~90 kDa) and cleaved (~50 kDa) forms of ATF6. Quantification was therefore based on the cleaved form, which represents the transcriptionally active species. These results indicate that α-synuclein modifies the activation of ATF6 rather than its total expression. In SH cells the active form of ATF6 is significantly decreased due to the effect of monomer and aggregate, while in M17 the same effect is due to aggregate and fibrils (Figure 42B).

3.6 Effect of α-synuclein on apoptosis balance

To further investigate whether synuclein influences the balance between adaptive and pro-apoptotic responses, we analyzed the expression levels of key regulators of the intrinsic apoptotic pathway, BCL-2 and BAX. qRT-PCR analysis revealed that synuclein modulated both proteins in a cell line–dependent manner. To quantify the overall effect on cell survival potential, we calculated the BCL-2/BAX ratio. Treatment with aggregate and fibrils significantly increased this ratio in SH cell line suggesting a shift toward an anti-apoptotic phenotype. In M17, aggregates determine an increase in survival capacity but, conversely, a reduced ratio is observed after treatment with fibrils, indicating limited activation of survival pathways (Figure 43).

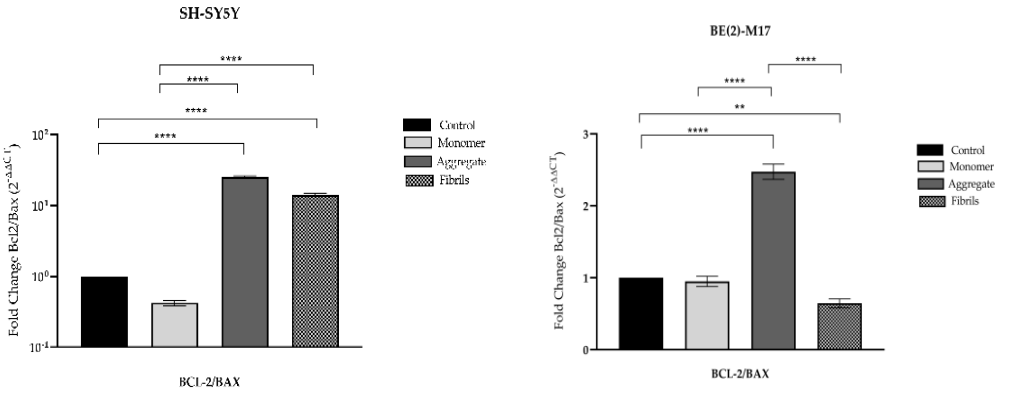


Figure 43: Effect of α-synuclein on apoptosis regulatory genes, in SH-SY5Y and BE(2)-M17 cells. Cells were treated with 3 μM of monomer, aggregate and fibrils for 24 hours. The expression level of BCL2 and BAX was calculated as Fold Change ($2^{-\Delta\Delta ct}$), with β-actin as reference gene, and compared to the control (PB 0.5%). After calculating the Fold Change, the BCL2/BAX ratio was determined. Data are presented as mean ± SEM of at least three independent experiments. Statistical analysis was performed by one-way ANOVA followed by Holm-Sidak post hoc correction for multiple comparisons. $p < 0.002$ **, $p < 0.0002$ ***, $p < 0.0001$ ****.

Caspase-3 transcripts and Procaspase-3 levels were reduced after treatment of SH cells with aggregate or aggregate and monomer, respectively. These data suggest modulation of caspase-3 processing, although direct evidence of caspase activation is lacking. On the contrary no modulation was observed in M17 cell line (Figure 44).

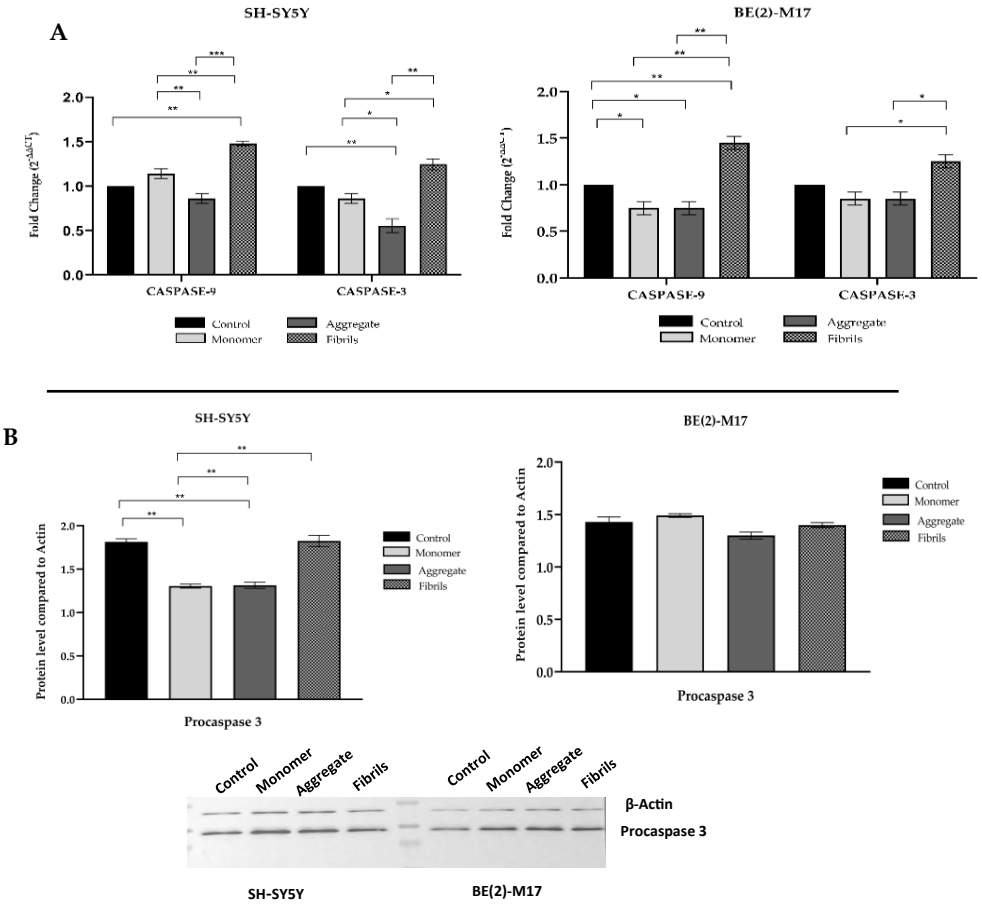


Figure 44: Effect of the three forms of α-synuclein on apoptosis pathway, in SH-SY5Y and BE(2)-M17 cells. Cells were treated with 3 μM monomer, aggregate and fibrils for 24 hours. **(A)**The expression level of CASPASE-9 and CASPASE-3 was calculated as Fold Change ($2^{-\Delta\Delta ct}$), with β-actin as reference gene, and compared to the control, treated with vehicle alone (PB 0.5%). Data are presented as mean ± SEM of at least three independent experiments. **(B)** Expression level of Procaspase 3 protein was normalized for β-Actin. Graphs and representative blot are shown. Data are presented as mean ± SEM of two independent experiments. Statistical analysis was performed by one-way ANOVA followed by Holm-Sidak post hoc correction for multiple comparisons. $p < 0.03$ *, $p < 0.002$ **, $p < 0.0002$ ***.

The qRT-PCR analysis showed that, in both cell lines, the fibrils induced a significant increase in the expression of caspase 9, which acts as an initiator of the apoptotic process, while monomer and aggregate reduced its expression, only in M17 (Figure 44A). Since only caspase-9 mRNA and procaspase-3 protein were measured (cleaved caspase forms or caspase activity were not evaluated), the increase observed in caspase-9 transcripts is reported as indicative of a potential trigger of the intrinsic apoptotic pathway rather than as definitive evidence of apoptotic execution.

Ultimately, we evaluated the expression of two genes encoding proteins involved in cell cycle regulation: P53 involved in monitoring DNA integrity, and P21 involved in cell cycle arrest by inhibiting the activity of cyclin-dependent kinases (CDK) (Figure 45).

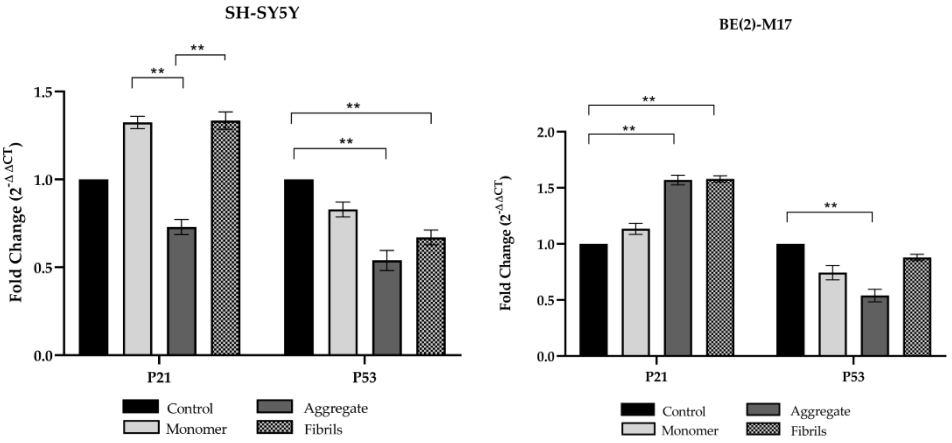


Figure 45: Effect of the three forms of α-synuclein on cell cycle markers, in SH-SY5Y and BE(2)-M17 cells. Cells were treated with 3 µM monomer, aggregate and fibrils for 24 hours. The expression level of P53 and P21 was calculated as Fold Change ($2^{-\Delta\Delta ct}$), with β-actin as reference gene, and compared to the control (PB 0.5%). Data are presented as mean ± SEM of at least three independent experiments. Statistical analysis was performed by one-way ANOVA followed by Holm-Sidak post hoc for multiple comparisons. $p < 0.002$ **.

The qRT-PCR analyses highlighted that P21 does not change significantly, while the expression of P53 decreases due to the effect of aggregate and fibrils in SH. In contrast, in M17 cells, the same trend is observed for P53 due to the aggregate effect, while P21 expression increases due to the aggregate and fibrils effect.

3.7 Discussion

It is known that in synucleinopathies, including PD, α-synuclein oligomers are able to alter numerous molecular mechanisms causing neurodegeneration (Yaribash S., 2025). Aggregate cytotoxicity is not a prerogative of oligomeric species, but it can also be induced by intermediate amyloid forms that possess specific structural properties, such as those present in early fibrils. These intermediate amyloid forms are more similar to toxic oligomers than to late aggregates in terms of secondary structure, flexibility of the N-terminal end and exposure of hydrophobic residues (Chen S.W., 2024).

Based on this, in this second part of our research, we analyzed the effects of three forms of α-synuclein: monomers, aggregates and fibrils, on viability, oxidative stress, mitochondrial function and cellular response in two lines of neuroblastoma of different origin: SH-SY5Y, of female origin and BE(2)-M17, of male origin. The aim was to assess possible differences in susceptibility and response mechanisms to protein-induced proteotoxic stress, with a particular focus on any variations related to the biological sex of the cells.

The data obtained from the viability assays highlighted a differential effect between the two cell lines in response to the different forms of α-synuclein and at different exposure times. After 24 hours, SH-SY5Y showed greater tolerance to monomeric forms and, in part, to aggregates at low concentrations, with a significant increase in cell viability in the presence of monomers at 3 μM. This data suggests that the toxic protein apparently induces compensatory activation leading to an increase of cell proliferation, as reported for SH by (Rodríguez-Losada N., 2020). What has just been said, was confirmed by the increase in expression of α-synuclein observed in the two cell lines due to the effect of aggregate and fibrillary forms. On the contrary, fibrils have proven to be toxic already at low concentrations, showing a more marked dose-dependent effect. A similar response was also observed in the BE(2) - M17 line, but with an even more accentuated sensitivity to aggregate and fibrillar forms. As the exposure time increased, the differences between the two lines were maintained, but with an attenuation of the cytotoxic effects. In particular, in SH-SY5Y the toxic effect of the fibrils was reduced over time, while for the aggregate a biphasic trend was observed, with initial stimulation at low concentrations and a

marked reduction in viability at higher doses. This may be due to adaptive mechanisms of cells towards cytotoxic forms. In the male cell line, however, the effects are more stable and the cytotoxicity induced by fibrils remains constant or even more marked at prolonged times.

A key element that emerged from our experiments concerns the production of ROS. We observed an opposite trend in the two cell lines; in fact, while in SH-SY5Y there is a significant increase in ROS, due to all three forms of α-synuclein, with a greater effect for aggregates, in M17 the amount of ROS is significantly decreased, especially due to the effect of fibrils. What has emerged demonstrates not only that aggregates, under our experimental conditions, are responsible for the increase in oxidative stress, but also that the two cell lines probably respond differently to oxidative stress. In response to the increase in ROS, we observed the activation of the antioxidant pathway of Nrf2, due to fibrils, in both cell lines but this is more evident in cells of male origin. Nrf2 activation is accompanied by increased expression of SOD2, UCP2 and NQO1, albeit with differences between the two cell lines. In particular, in SH-SY5Y the expression is more evident, suggesting a more efficient response to oxidative stress. On the contrary, in the M17, the downstream response, as in the case of SOD2, appears to be compromised. This could be due to post-transcriptional modifications, or to factors that hinder the expression of downstream enzymes. What was observed is consistent with the role of Nrf2 as a central transcription factor in the antioxidant response. However, there is evidence that over-expression of α-synuclein, although it activates the Nrf2 pathway, is not always accompanied by increased expression of downstream scavenge proteins (Perfeito R., 2017).

It is widely demonstrated that oligomeric α-synuclein induces mitochondrial dysfunctions, particularly at the level of the electron transport chain, causing a reduction in the synthesis of ATP necessary for the transmission of nerve impulses. In addition, α-synuclein can block mitophagy by hindering the formation and assembly of the phagolysosome, and, ultimately, hindering the removal of damaged mitochondria (Caproni S., 2025)(Nechushtai L., 2023). Under our experimental conditions, monomers, aggregate and fibrils act differently at the mitochondrial level differently in the two cell lines. The effect of α-synuclein on mitochondrial

function was confirmed by the loss of mitochondrial membrane potential (ΔΨm), observed in both lines after exposure to aggregates and fibrils. However, again, BE(2)-M17 cells appears more sensitive to this effect, showing a greater degree of depolarization. The involvement of mitochondria is further supported by the activation, in SH-SY5Y, of mitophagy-related genes such as PARKIN, DJ-1, LC3 and BECLIN, involved in the recognition of mitochondrial damage and in the formation and assembly of the phagolysosome, mainly due to the effect of fibrils. Although this, however, the mechanism appears not to be activated in the male counterpart. These data suggest that female cells are able to activate mechanisms for removing damaged mitochondria more efficiently, and this difference may contribute to the greater resistance observed towards α-synuclein toxicity.

Closely linked to mitochondrial dysfunction is ER stress, caused by the accumulation of misfolding proteins. Although in many cases reticular stress is attenuated by the defense mechanism known as Unfolding protein response (UPR), when exacerbated, it creates a condition in which the formation of misfolded proteins is not counterbalanced by its removal, leading to neuronal death (Colla E., 2019). Most studies on cellular models have explored this pathway through the use of the neurotoxin MPP^+. Based on this and considering the effect of our heterogeneous preparations of α-synuclein, we evaluated some markers that regulate the UPR mechanism. In SH-SY5Y, the increase in BIP, mainly due to monomers, suggests the activation of the UPR pathway. This is confirmed by the presence of the active form of the transcriptional regulator ATF6. However, the stress is not high enough to involve the CHOP protein as an inducer of apoptosis. The increase in the expression of BIP and CHOP due to the fibrils suggests that these intermediate structures, at least at the concentrations tested, determine an exacerbation of ER stress, directing towards apoptosis. However, in the male line, an increase in CHOP is observed with all forms of α-synuclein, suggesting greater lattice vulnerability and a less efficient UPR response.

Finally, the analysis of apoptotic pathways and cell cycle confirms partial involvement of the intrinsic apoptosis pathway. The slight increase in caspase 9 observed in both cell lines as a result of fibrils, is not assisted by an activation of caspase 3; in fact, from Western blot analyses, we did not observe the presence of

cleaved caspase 3, but only of inactive Procaspase 3. This suggests that the cells feel a damage but there is a tendency towards survival rather than apoptosis. This is also confirmed by the prevalence of BCL2 compared to BAX in both cell lines, although in SH-SY5Y, BCL2 levels increased due to the effect of aggregate and fibrils, while in M17 only due to the effect of aggregate. Furthermore, in the latter, due to the effect of the fibrils, the relationship between the two regulatory proteins of apoptosis is in favor of BAX. This suggests that cells of female origin are more resistant and likely activate survival mechanisms than their male counterparts.

The picture is completed by the modulation of p53 and p21. In SH cells, the decrease in p53 without changes in p21 suggests that the cell is engaging alternative survival mechanisms (e.g., consistent with the increased BCL-2/BAX ratio and BIP expression). In M17 cells, p21 activation independently of p53 may indicate the induction of adaptive stress responses, potentially reflecting p53-independent senescence. Ultimately, the results obtained suggest that α-synuclein-induced toxicity is modulated not only by the form of aggregation and concentration, but also by the intrinsic characteristics of opposite-sex cell lines. SH-SY5Y cells appear more resistant to stress, activating more efficient antioxidant and mitophagic responses, while BE(2)-M17 cells appear more susceptible to oxidative, mitochondrial and stress damage, with a less effective response at the level of cellular protection. These observations, although coming from in vitro models, emphasize the importance of considering biological sex in the analysis of the pathogenetic mechanisms of PD.

Chapter 4: Materials and Methods

4 Expression of α-synuclein and preparation of aggregate and fibrillar forms

Expression and purification of α-synuclein was performed by recombinant DNA technology as described (Dandurand J., 2020). After purification, dialysis and lyophilization, aggregate and fibrils were prepared.

For the preparation of aggregate of α-synuclein:

Monomeric α-synuclein was solubilized in 1.2 mL of phosphate buffer (PB) containing 3.3 mM KH2PO4 and 3.8 mM Na2 HPO4, pH 6.8. The concentration was determined spectrophotometrically at 280 nm using extinction coefficients 5960 M^{-1} cm^{-1}. The 0.7 mM α-synuclein solution was subjected to stirring at 200 rpm, for 7 days at 45 °C.

For the preparation of fibrils of α-synuclein:

The lyophilized α-synuclein was solubilized, at the final concentration 100 μM, in sterile phosphate salt buffer (PBS 1X) containing 1.37 M NaCl, 27 mM KCl, 100mM Na2HPO4, 18mM KH2PO4, pH 7.4. To the PBS 1X were added 0.5 M (NH4)2SO4 and 2-methyl-2,4-pentanediol (MPD) (Sigma, Saint Louis, MO, USA) 10% (v/v).

The solution was subjected to stirring at 200 rpm, for 3 days at 37 °C. After incubation the solution was centrifuged at 16000 rpm for 15 minutes, and the pellet was resuspended in the same buffer used for the preparation.

4.1 Thioflavin-T (ThT) Aggregation Assay

The presence of aggregate and oligomeric forms of α-synuclein is evaluated by Thioflavin-T assay. Thioflavin (Sigma, Saint Louis, MO, USA) selectively interact with β-sheets present in the amyloid aggregates and oligomers, resulting in an increase in the fluorescence intensity and a red shift of the emission maximum. A concentration of 50 μM of ThT was added to 25 μM α-synuclein. Fluorescence was evaluated by fluorescence microscopy (Nikon Eclipse TS100, Nikon Europe B.V. Stroombaan 14, 1181 VX Amstelveen, The Netherlands) with the green light filter, using monomeric α-synuclein solubilized in phosphate buffer (PB) as a negative control.

4.2 Transmission Electron Microscopy (TEM)

Carbon coating on 400 mesh copper grids (Agar Scientific Ltd, Unit 7, M11 Business Link, Parsonage Lane, Stansted, Essex CM24 8GF United Kingdom) was used. One drop of the α-synuclein at 0.7 mM concentration was applied to the grid and was left to stand for 4 min. Then grids were dabbing with Whatman paper, stained with 5 µL microfiltered uranyl acetate solution 2% (w/v) for 2 min, and finally dabbed with Whatman paper. The samples were analyzed using a FEI Tecnai G2 20 Twin Transmission Electron Microscope (FEI Italia S.r.l.-Via Monte Nero 84, 20135 Milano, Italy) at 120 kV.

4.3 Circular Dichroism

The presence of the amyloidogenic forms of α-synuclein was also assessed by Circular dichroism. Circular dichroism (CD) spectra were recorded on a Jasco J-185 CD spectrophotometer (Jasco, 28600 Mary's Court Easton, MD 21601) using a 0.1 cm cylindrical quartz cell. The spectra were recorded at 25 °C across wavelengths 190 nm to 250 nm, with a scanning speed of 100 nm/min, 1 nm bandwidth, a time-constant of 0.5 s, 20 mdeg of sensitivity, and a total number of 16 accumulations for each spectrum. Then, the baseline spectrum of the PBS 1X and PB was subtracted, and spectra were smoothed using the Fourier transform. α-synuclein was used at the concentration of 7 µM. Data were obtained in terms of the molar ellipticity per residue in units of deg × cm2 × dmol−1. and in θ(mdeg). Analysis of spectra for the evaluation of secondary structure content was performed with DichroWeb using the CONTINLL algorithm.

4.4 Fluorescence Spectroscopy

Characterization of alpha synuclein was performed by fluorescence spectroscopy. Monomer, aggregate and fibrillar α-synuclein were used at the 70 µM concentration. Fluorescence spectra were recorded at the excitation wavelength of 264 nm and collecting the emitted fluorescence in a range between 270 and 400 nm. Spectra were recorded immediately (T0) and after 3 hours (T3) using a Cary Eclipse fluorescence spectrophotometer (Agilent Technology, 5301 Stevens Creek Blvd

Santa Clara, CA 95051 United States). The respective synuclein-free buffers were used as a negative control.

4.5 Serum samples collection

Sera from healthy patients and patients with Parkinson's disease were obtained from Padova BioBank, Department of Biomedical Sciences, University of Padova (Table 1).

Groups	Number	Age at sample collection. (average)	Age from symptoms onset (average)
Healthy (HC) male	13	68 ± 1.8	-
Healthy (HC) female	6	58 ± 2.7	-
Parkinson's (PD) male	29	64 ± 1.8	7 ± 1
Parkinson's (PD) female	12	65 ± 2	10 ± 1.8

Table 1: Clinical data from healthy controls (HC) and Parkinson's patients (PD), used in our studies (Padova BioBank).

The serum samples were aliquoted and frozen at -80 °C until analysis.

4.6 Evaluation of anti α-synuclein antibodies in human serum

The dosage of the antibodies against the α-synuclein forms, previously synthesized, was performed by indirect Enzyme-Linked Immunosorbent Assay (ELISA) as described.

Polystyrene microplates for ELISA (Costar) were pretreated for 1 hour at room temperature with 5% (v/v) glutaraldehyde (Sigma). After five washes with phosphate buffer (PBS) for 1 minute each, 2 μg of antigen (monomer, aggregate and fibrils of α-synuclein) was immobilized at the bottom of the wells and incubated at 4 °C overnight. After three washes of 1 minute, the non-specific sites were saturated using a saturation buffer (5% skimmed milk in PBS) for 2 hours at room temperature. After further three washes the plates were treated with PD and HC serum diluted

1:50 in the saturation buffer for 1 hour at room temperature. Subsequently, four washes were carried out with phosphate buffer with the addition of 0.05% Tween (PBST), and the plates were treated with conjugated anti-human IgG-peroxidase secondary antibody (A8792 Sigma) diluted 1:5000 in the saturation buffer for 1 hour at room temperature. Four washes were carried out with PBST and, finally, 3,3',5,5' tetramethyl benzidine (TMB) (Sigma) was added for 5 minutes, until blue coloration appeared. The reaction was stopped with sulfuric acid 1M and the reading was immediately carried out using a microplate reader at 450 nm (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific, Waltham, MA, USA). Absorbance values of wells with antigen were subtracted from those without antigen to remove background.

4.7 Measurement of pro-inflammatory cytokine IL-6

To evaluate the concentration of IL-6 in the human sera, the Human IL-6 ELISA kit (Proteintech, Am Klopferspitz, Planegg-Martinsried, Germany) was used, according to the manufacturers' instructions.

4.8 Evaluation of oxidative balance

Two tests were used to evaluate the oxidative balance: d-ROMs test and BAP test. In the first case, reactive oxygen metabolites (ROMs) are measured, while the second is based on the determination of biological antioxidant protection (BAP). The measures were conducted according to the manufacturer's instructions (Diacron Diagnostic International Srl, Grosseto, Italy).

4.9 Evaluation of matrix metalloprotease enzymatic activity

The enzyme activity of matrix metalloprotease 9 (MMP9) was assessed by zymography. PD and HC sera were dosed into microplates by Bradford's method at 595 nm using a microplate reader (Multiskan™ GO Microplate Spectrophotometer,

Thermo Scientific, Waltham, MA, USA). Approximately 10 ug of protein were added to non-reducing sample buffer (50 mM Tris–HCl pH 6.8, 10% glycerol, 4% SDS, 0.002% bromophenol blue) in a 1:1 ratio. The samples were resolved by electrophoresis on 8% SDS-polyacrylamide gel copolymerized with high purity gelatine from bovine skin (Sigma) to the final concentration of 0.2 %. The electrophoretic run occurred at a constant voltage (150 V) at 4 °C, to preserve the enzymatic activity. Subsequently the gels were washed twice for 30 minutes with 2.5% Triton-X 100 solution, to reactivate the enzyme and incubated for 18 hours at 37 °C in buffer containing 50 mM Tris–HCl pH 6.8, 10 mM CaCl2, and 1% Triton X-100, to promote the enzymatic degradation reaction. The gels were stained with 0.25% Coomassie Brillant Blue R-250 (Sigma) and 0.05% Coomassie Brillant Blue G-250 (Sigma), and MMP activities were detected as transparent bands on the blue background.

4.10 Nuclear Magnetic Resonance Spectroscopy

Serum samples were defrosted on ice and subsequently kept at room temperature before use. 20 µl of serum was mixed with 580 µl of deuterate water (D2O) and 5 µl of TSP (3-trimethylsilyl propionic acid-sodium salt d4), which was used as reference for chemical shift (δ = 0), and transferred to five mm NMR tubes (ST500, NORELL, Inc., Morganton, NC, USA). All 1H NMR spectra were acquired at 25 °C on a Varian Inova 500 MHz spectrometer (Varian, Palo Alto, CA, USA). A total of 100 scans and 16K points were acquired with a spectral width of 5,995 Hz and a recycling delay of 5 s. The spectra were Fourier transformed with an FT size of 32 K and a line broadening of 1 Hz, phased and a polynomial baseline correction was applied over the entire spectral range.

Identification of relevant metabolites was conducted from raw spectra using NMR Suite 8.6 software (Chenomx Inc., Edmonton, Canada), based on recent literature data. The TSP signal and the 4.7-5.1 ppm region, around the water signal, were excluded from the analysis.

4.11 Proteinase K controlled digestion

The digestion pattern of serum α-synuclein was evaluated by controlled digestion with Proteinase K. Proteinase K is a broad-spectrum serine protease, obtained from the fungus *Tritirachium album*, widely used for protein digestion. HC and PD patients sera were diluted 1: 2 in phosphate buffer (PBS) and dosed spectrophotometrically using Bradford's method. 20 µg of serum protein was reacted with 100 ng of Proteinase k (Sigma), at final concentration of 10 µg/ml. The digestion reaction was made to occur at 37 °C for 20 minutes in a heated bath, and subsequently inactivated at 95 °C for 5 minutes. To separate insoluble fragments, samples were centrifugated at 12,000 x g for 3 minutes. The supernatant was solubilized in Laemmly Sample buffer containing 60 mM Tris–HCl pH 6.8, 10% glycerol, 2% SDS, 1% dithiothreitol (DTT) and 0.002% bromophenol blue. Samples were separated by electrophoresis on 12% SDS-polyacrylamide gel and analyzed by Western blot.

Analysis of immunoreactive bands was conducted by ImageJ software (Bethesda, MD, USA) and normalized for total protein. A PD serum and an HC serum not subjected to digestion were used as control.

4.12 Western Blotting analysis on serum

Serum samples were subjected to albumin depletion, before proceeding to Western blot analyses, to reduce interference in the immunoreactive bands. Albumin depletion was achieved adding 20 µl of serum to 4 volumes of a cold solution of acetone and 10% TCA (trichloroacetic acid). After mixing gently, serum was incubated at -20 °C for 90 minutes, followed by centrifugation at 15,000 x g for 20 minutes in cold centrifuge. The supernatant, containing a large part of the albumin, was discarded and the pellet was subjected to a second depletion cycle. Subsequently the pellet was washed once exclusively with 1 ml of acetone, incubated in ice for 5 minutes, centrifuged at 15,000 x g for 20 minutes, and the acetone residues were evaporated. To completely remove acetone, which could interfere with subsequent analyses, some washes were done with PBS. The pellet was solubilized in PBS and total proteins were measured using the Bradford assay at

595 nm. 8 µg of serum protein were loaded onto SDS-polyacrylamide gels at different percentages of acrylamide, and separated electrophoretically. They were subsequently transferred to a 0.2 µm nitrocellulose membrane, using the Trans-Blot Turbo Transfer System (BioRad, Hercules, California, USA). The membranes were stained with Ponceau S solution (Sigma) for 15 minutes and rinsed with distilled water. Membranes were saturated in a saturation buffer (5% non-fat milk in PBS with 0.05% Tween 20, PBST) for 1 hour at room temperature with gentle shaking and incubated overnight at 4 °C with specific primary antibodies: 1:1000 anti-α-Tubulin monoclonal antibody (cat no. 11H10, Cell Signaling Technology, Inc., Danvers, MA, USA); 1:5000 anti-Nrf2 polyclonal antibody (cat no. 16396-1-AP, ProteinTech Group, Inc., Chicago, IL, USA); 1:800 anti-UCP2 polyclonal antibody (cat no. 11081-1-AP, ProteinTech Group, Inc., Chicago, IL, USA); 1:100 anti-NQO1 (cat no. SC-32773, Santa Cruz Biotechnology, Inc., Texas, CA, USA); 1:20000 anti-SOD2 polyclonal antibody (cat no. 66474-1-Ig, ProteinTech Group, Inc., Chicago, IL, USA); 1:1000 anti-α-Synuclein polyclonal antibody (ca no. PA1-18264, Invitrogen, California, USA); 1:1000 anti-MMP3 polyclonal antibody (cat no. 17873-1-AP, ProteinTech Group, Inc., Chicago, IL, USA). The membranes were washed twice for 10 minutes each with PBST and then incubated at room temperature for 1 hour with the appropriate horseradish peroxidase-conjugated secondary antibody. The signal was visualized using either ECL™ Western Blotting Detection Reagents (GE Healthcare, Chicago, IL, USA) or SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific, Waltham, MA, USA), with a Chemidoc™ XRS detection system and Image Lab 5.1 software for image acquisition (BioRad). Densitometric analysis was performed by using ImageJ software (Bethesda, MD, USA). The bands related to the proteins of interest were normalized for α-Tubulin.

4.13 Mammalian cell cultures and treatments

Two immortalized human neuroblastoma cell lines were used. The cell line SH-SY5Y, derived from a female patient, and BE(2)-M17, derived from a male patient. Cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (1:1) medium (DMEM F12) (Gibco, Thermo Fisher Scientific; Waltham, MA), containing 365 mg/L L-Glutamine and 2.384 g/L sodium bicarbonate, supplemented with 10%

fetal bovine serum (FBS) (EuroClone), penicillin (100 U/mL) and streptomycin (100 µg/mL) (EuroClone). Cultures were maintained at 37 °C in a 5% CO2 humidified incubator. Monomeric α-synuclein, aggregate and fibrils, prepared as described previously, were defrosted gradually, to preserve the structures, and were diluted in the respective phosphate buffer to the concentration of 100 uM and subsequently diluted in the culture medium to the concentrations used for our experiments. The culture medium with the addition of phosphate buffer (as a vehicle) was used as a control.

4.14 Viability Assay

Cell viability was assessed by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma). SH-SY5Y and BE(2)-M17 cells were seeded into 96-well plates at densities of 1 x 10^4 and 2 x 10^4 cells/well respectively. After 24 hours, cells were treated with the three forms of α-synuclein at three different concentrations (0.03 - 0.3 - 3 µM) for 24 hours, using phosphate buffer added to the culture medium as a control. After treatment, cells were incubated with medium containing 0.75 mg/mL MTT for 4 hours at 37 °C. The medium was then removed and the formazan crystals were solubilized with 1:1 DMSO: isopropanol solution containing 1% Triton X-100 for 1 hour. Absorbance was measured at 570 nm, with background subtraction at 630 nm, using a microplate reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific, Waltham, MA, USA). Data are represented as viability % compared to control.

4.15 Intracellular Reactive oxygen Species (ROS) Assay

Intracellular levels of reactive oxygen species (ROS) were measured using the 2',7'-dichlorodihydrofluorescein (DCFH-DA) diacetate assay (Sigma). Cells were seeded into 24-well plates at the density of 5 x 10^4 cells/well. After 24 hours, the cells were treated with α-synuclein monomers, aggregates and fibrils diluted in the culture medium to the concentration of 3 µM for 24 hours, using phosphate buffer as a control. Tert-butyl hydroperoxide (t-BOOH) 5 mM for 1 hour was used as a ROS

induction control. After treatment, medium was removed and cells were incubated with DCFH-DA (10 uM in sterile PBS) for 30 minutes at 37 °C protected from light. At the end of the incubation, the cells were removed from the wells, centrifuged at 1200 rpm for 5 minutes at 4 °C and resuspended in sterile PBS. Fluorescence intensity was measured by BD FACSCanto II flow cytometer (BD Pharmingen, San Jose, CA, USA), at excitation wavelength of 485 nm and emission wavelength 515-540 nm. The control was considered to be 100%. The data were relative to the control set to 100.

4.16 Evaluation of mitochondrial membrane potential (ΔΨm)

SH-SY5Y and BE(2)-M17 were seeded in a 96-well black polystyrene plate with a clear bottom at a density of 1.5 x 10^4 cells/well, and treated with the various forms of α-synuclein for 24 hours. Carbonyl cyanide-p-trifluoromethoxyphenyl hydrazone (FCCF) (Thermo Scientific, Waltham, MA, USA) was used as a positive control: 10 μM FCCP was added to the culture medium for 1 hour at 37 °C, to induce membrane decoupling. Then the medium was removed and the cells were incubated for 20 minutes at 37 °C with 5 μM of JC-1 (Thermo Scientific, Waltham, MA, USA), a cationic carbocyanine-based dye, solubilized in culture medium. Cells were washed in sterile PBS once and fluorescence reading was taken immediately. Fluorescence intensity was measured using a GloMax™ Multi-Detection System (Promega), using green filter (excitation: 525 nm; emission: 580-640 nm) for red fluorescence and blue filter (excitation: 490 nm; emission: 510-570 nm) for green fluorescence. To remove the interference of the probe signal and obtain a representative value of the mitochondrial membrane potential (ΔΨm), the ratio between red and green fluorescence was calculated. The data were relative to the control set to 100.

4.17 Total RNA extraction and cDNA synthesis

RNA extraction was performed using Quick-RNA™ MiniPrep kit (Zymo Research; Irvine, CA), according to manifacturer's protocol. RNA concentration and purity were measured spectrophotometrically using a MultiskanTM GO Microplate Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Absorbance ratio at

260/280 and 260/230 nm were used as indicators of contamination by proteins or phenols respectively.

cDNA synthesis was carried out on 1 µg of template RNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem; Waltham, MA), on a peqSTAR 96 Universal Thermo Cycler (EuroClone; Siziano, PV, Italy), using the program suggested by the producer of the kit.

4.18 Real-time PCR Analysis

Amplification and relative quantification of cDNA templates were performed via real-time PCR using QuantStudio™ Real-Time PCR Instrument (Applied Biosystem), using iTaq-Universal-SYBR® Green Supermix (Bio-Rad; Hercules, CA). Primers were designed to span exon-exon regions to avoid unwanted genomic DNA amplification and PCR products were subjected to a melting-curve analysis to confirm amplification specificity. ΔCT value of each gene of interest was calculated according to the following formula:

$$\Delta CT\ (gene\ of\ interest) = CT\ (gene\ of\ interest) - CT\ (housekeeping\ gene)$$

β-actin was used as housekeeper gene in order to calculate the relative expression.

Then ΔΔCT is determined by the difference between the single ΔCTs, treated against the internal experimental control. $2^{-\Delta\Delta CT}$ calculate the fold change of the gene of interest relative to its expression in the control condition.

Genes used in the analysis are reported (Table 2).

Gene	Accession Number	Primer Forward	Primer Reverse
B-ACTIN	NM_001101.3	5'-CCTGGCACCCAGCACAAT-3'	5'-GCCGATCCACACGGAGTACT-3'
NRF2	NM_001145412.3	5'-AACTACTCCCAGGTTGCCCA-3'	5'CATTGTCATCTACAAACGGGAA-3'
NQO1	NM_000903.3	5'-GGTGGTGGAGTCGGACCTCTA-3'	5'-AGGGTCCTTCAGTTTACCTGTGAT-3'
PARKIN	NM_004562.3	5'-ACTGTGCAGAATTGTGACCT-3'	5'-TTCTGGGGTCGTCGCCTCC-3'
DJ-1	NM_001123377.2	5'-GCCATCTGTGCAGGTCCTAC-3'	5'-TGTCTTTAGCAAGAGGGTGT-3'
LC-3	NM_032514.4	5'-GAGAGCAGCATCCAACCAAAA-3'	5'-CCGTTCACCAACAGGAAGAAGG-3'
BECN1	NM_001313998.2	5'-AGCTGCCGTTATACTGTTCTG-3'	5'-ACTGCCTCCTGTGTCTTCAATCTT-3'
CASP 3	NM_001354777.2	5'-AGAGGGGATCGTTGTAGAAGTC-3'	5'-ACAGTCCAGTTCTGTACCACG-3'
CASP 9	NM_001229.5	5'-CTGTCTACGGCACAGATGGAT-3'	5'-GGGACTCGTCTTCAGGGGAA-3'
BCL-2	NM_000633.3	5'-GGTGGGGTCATGTGTGTGG-3'	5'-CGGTTCAGGTACTCAGTCATCC-3'

BAX	NM_001291428.2	5'-CCCGAGAGGTCTTTTTCCGAG-3'	5'-CCAGCCCATGATGGTTCTGAT-3'
BIP	NM_005347.5	5'-GAATCGCCTGACACCTGAAGA-3'	5'-GTTTGCTGATAATTGGTTGAACA-3'
CHOP	NM_001195053.1	5'-GTACCTATGTTTCACCTCCTG-3'	5'-TCTCCTTCATGCGCTGCTTTC-3'
P53	NM_000546.6	5'-TGAATGAGGCCTTGGAACTC-3'	5'-ACTTCAGGTGGCTGGAGTG-3'
P21	NM_000389.5	5'-CTGTCTTGTACCCTTGTGCCT-3'	5'-CGTTTGGAGTGGTAGAAATCTGTC-3'
SNCA	NM_000345.4	5'-TGCATGGTGTGGCAACAGTG-3'	5'-TGGGCTACTGCTGTCACAC-3'

Table 2: List of primers used in Real-time PCR assay.

4.19 Western Blotting analysis on cells

Cells were suspended in radioimmunoprecipitation assay (RIPA) buffer (0.1% sodium dodecyl sulfate, 1% NP-40, and 0.5% sodium deoxycholate in PBS at pH 7.4) supplemented with a protease and phosphatase inhibitor cocktail (Roche, Penzberg, Germany) and lysed via sonication. Then, the lysates were centrifuged at 13000 rpm for 10 minutes at 4 °C, and dosed with Bradford reagent at 595 nm. An amount of 30 µg of proteins was used for our experiments, according to the protocol described previously. Below are reported the primary antibodies specific for the proteins of interest we used: 1:1000 anti-α-Tubulin monoclonal antibody (cat no. 11410, Cell Signaling Technology, Inc., Danvers, MA, USA); 1:1000 anti-α-Synuclein polyclonal antibody (ca no. PA1-18264, Invitrogen, California, USA); 1:1000 anti-NfkB polyclonal antibody (cat no. 14673181, Invitrogen, California, USA); 1:800 anti-UCP2 polyclonal antibody (cat no. 11081-1-AP, ProteinTech Group, Inc., Chicago, IL, USA); 1:20000 anti-SOD2 (cat no. 66474-1, ProteinTech Group, Inc., Chicago, IL, USA); 1:250 primary antibody, 1:100 secondary antibody Western Blot Apoptosis Cocktail (cat no. Ab136812, Abcam); 1 µg/ml anti-ATF6 monoclonal antibody (cat no. PA5-20215, Invitrogen, California, USA). The bands related to the proteins of interest were normalized for the respective endogen. Data are reported as level of protein/endogenous as a function of cell line.

4.20 Statistical Analysis

Statistical analysis on serum was conducted using GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA). Shapiro-Wilk test was used to evaluate data normality. Wilcoxon-Mann-Whitney test, considering a 95% confidence interval

($p < 0.03$ *, $p < 0.002$ **, $p < 0.0002$ ***, $p < 0.0001$ ****), was conducted based on the experimental design and nature of the samples.

Correlation analyses were performed between the various markers evaluated on serum. The correlation was performed using Spearman non-parametric tests for values that do not have a Gaussian distribution. A confidence interval of 95% was considered ($p < 0.03$ *, $p < 0.002$ **, $p < 0.0001$ ***). Experiments were conducted in triplicate except for ^{1}H NMR.

Statistical analysis on cells was conducted using one-way ANOVA in GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA), followed by Holm–Sidak's test post hoc for multiple comparisons. A significance level of $\alpha = 0.05$ was used to determine statistical significance ($p < 0.03$ *, $p < 0.002$ **, $p < 0.0002$ ***, $p < 0.0001$ ****). All assays were performed in triplicate, Westerns in duplicate. Data are expressed as mean ± standard error of mean (SEM).

5 Final conclusions

PD represents one of the main challenges of modern neurology, as a multifactorial neurodegenerative pathology, progressive and still without validated diagnostic and prognostic biomarkers. In this context, we investigated in an integrated way the molecular mechanisms associated with oxidative stress, inflammation and α-synuclein proteotoxicity, placing a special focus on differences related to biological sex, both in serum samples from PD patients and in neuronal cellular models of male and female origin.

From a serological point of view, the analysis of specific biomarkers circulating in a cohort of patients and healthy controls has highlighted that serum levels of key proteins of the Nrf2 antioxidant pathway, such as SOD2, are significantly reduced in subjects with PD, especially in women, suggesting a impairment of the ability to respond to oxidative stress. In parallel, the increase of pro-inflammatory cytokines, such as IL-6, and its correlation with MMP-3 and MMP-9 support the hypothesis of a chronic systemic inflammatory state, potentially modulated by sex, and interconnected with redox regulation. These findings highlight the potential role of metalloprotease as inflammatory biomarkers of disease and the complex interaction between inflammation, oxidative stress and mitochondrial dysfunction in the context of neurodegeneration.

Furthermore, the evaluation of the immune response against the different forms of α-synuclein highlighted a greater ability of women with PD to produce antibodies against monomeric and aggregate forms, suggesting a possible sex-specific modulation of the immune system in the recognition of toxic species of the protein. The metabolomic profile further strengthened the relevance of sex in PD pathogenesis, showing significant variations in the levels of energy metabolites, amino acids and chetone body, indicative of alterations in mitochondrial metabolism and neurotransmitter biogenesis.

The use of cellular models for study PD, treated with the different forms of α-synuclein, allowed us to investigate the main cellular mechanisms altered by toxic protein forms in both sexes. We have highlighted greater resistance of cells of female origin (SH-SY5Y), which more effectively activate the antioxidant response, mitophagy and anti-apoptotic mechanisms. On the contrary, the cellular model of

male origin, BE(2)-M17, was found to be more vulnerable to oxidative stress and mitochondrial dysfunction associated with endoplasmic reticulum stress. The differences observed in the two cell lines indicate a sex-specific response to α-synuclein-induced insults at different aggregation stages. However, it is necessary to consider that, as in vivo, even in vitro α-synuclein preparations are never homogeneous, but constitute a set of structures in balance with each other in which there is a prevalence of one structure rather than of the other.

In conclusion, biological sex represents a real modulatory element in the pathophysiology of PD, capable of influencing both neuronal vulnerability and the immune-metabolic and inflammatory response. It is clear, therefore, that research on chronic diseases must be conducted from a gender perspective, because the identification of sex-specific differences in circulating biomarkers, as well as in cellular responses to proteotoxic stimuli, open new perspectives for precision medicine, aimed at a more accurate stratification of patients and the development of targeted therapeutic strategies. In light of the results obtained, it is necessary to further explore sex-specific differential biomarkers, through longitudinal and larger cohort studies, integrating multi-omic approaches and in vitro and in vivo models.

6 References

Abounit S., Bousser. L., Loria F., et al. (2016). Tunneling nanotubes spread fibrillar α-synuclein by intercellular trafficking of lysosomes. *EMBO J., 35*(19), 2120-2138. doi:10.15252/embj.201593411.

Adams-Carr K.L., Bestwick J.P., Shiribman S., et al. (2016). Constipation preceding Parkinson's disease: a systematic review and meta-analysis. *J Neurol Neurosurg Psychiatry., 87*(7), 710-716. doi:10.1136/jnnp-2015-311680.

Adamson A., Buck S.A., Freiberg Z., et al. (2022). Sex Differences in Dopaminergic Vulnerability to Environmental Toxicants - Implications for Parkinson's Disease. *Curr Environ Health Rep., 9*(4), 563-573. doi:10.1007/s40572-022-00380-6.

Adler C.H., Beach T.G., Hentz J.G., et al. (2014). Low clinical diagnostic accuracy of early vs advanced Parkinson disease: clinicopathologic study. *Neurology., 83*(5), 406-412. doi:10.1212/WNL.0000000000000641.

AlMahadin G., Lotfi A., Zysk E., Siena F.L., Carthy M.M. (2020). Parkinson's disease: current assessment methods and wearable devices for evaluation of movement disorder motor symptoms - a patient and healthcare professional perspective. *BMC Neurol., 20*(1), 419. doi: 10.1186/s12883-020-01996-7.

Alvarez-Erviti L., Couch Y., Ritchardson J., et al. (2011). Alpha-synuclein release by neurons activates the inflammatory response in a microglial cell line. *Neurosci Res., 69*(4), 337-342. doi:10.1016/j.neures.2010.12.020.

Appleton J. (2018). The Gut-Brain Axis: Influence of Microbiota on Mood and Mental Health. *Integr Med., 17*(4), 28-32.

Arabia G., De Martino A., Moro E. (2022). Sex and gender differences in movement disorders: Parkinson's disease, essential tremor, dystonia and chorea. *Int Rev Neurobiol., 164*, 101-128. doi:10.1016/bs.irn.2022.06.010.

Axelsen T.M, Woldbye D.P.D. (2018). Gene Therapy for Parkinson's Disease, An Update. *J Parkinsons Dis., 8*(2), 195-215. doi:10.3233/JPD-181331.

Balestrino R., Tunesi S., Tesei S., et al. (2020). Penetrance of Glucocerebrosidase (GBA) Mutations in Parkinson's Disease: A Kin Cohort Study. *Mov Disord., 35*(11), 2111-2114. doi:10.1002/mds.28200.

Barko K., Shelton M., Xue X., et al. (2022). Brain region- and sex-specific transcriptional profiles of microglia. *Front Psychiatry., 13*. doi: 10.3389/fpsyt.2022.945548

Bartels T., Choi J.G., Selkoe D.J. (2011). α-Synuclein occurs physiologically as a helically folded tetramer that resists aggregation. *Nature., 477*(7362), 107-110. doi:10.1038/nature10324.

Bassani T.B., Bartolomeo C.S., Oliveira R.B., Ureshino R.P. (2023). Progestogen-Mediated Neuroprotection in Central Nervous System Disorders. *Neuroendocrinology., 113*(1), 14-35. doi:10.1159/000525677.

Behl T., Kaur G., Sehgal A., et al. (2021). Metalloproteinases in Neurodegenerative Diseases: Pathophysiological and Therapeutic Perspectives. Int J Mol Sci.,22(3), 1413. doi: 10.3390/ijms22031413.

Ben-Shlomo Y., Darweesh S., Llibre-Guerra J., et al. (2024). The epidemiology of Parkinson's disease. *Lancet., 403*(10423), 283-292. doi:10.1016/S0140-6736(23)01419-8.

Berwick D.C., Heaton G.R., Azeggagh S., Harvey K. (2019). LRRK2 Biology from structure to dysfunction: research progresses, but the themes remain the same. *Mol Neurodegener., 14*(1), 49. doi:10.1186/s13024-019-0344-2.

Bezard E., Dehay B. (2022). Maladie de Parkinson - Le rôle de la synucléine [Aggregation and spread of synuclein in Parkinson's disease]. *Med Sci (Paris)., 38*(1), 45-51. doi:10.1051/medsci/2021241.

Bido S., Muggeo S., Massimino L., et al. (2021). Microglia-specific overexpression of α-synuclein leads to severe dopaminergic neurodegeneration by phagocytic exhaustion and oxidative toxicity. *Nat Commun., 12*(1), 6237. doi:10.1038/s41467-021-26519-x.

Boccalini C., Carli G., Pilotto A., Padovani A., Perani D. (2022). Gender differences in dopaminergic system dysfunction in de novo Parkinson's disease clinical subtypes. *Neurobiol Dis., 167*.

Bourque M., Soulet D., Di Paolo T. (2021). Androgens and Parkinson's Disease: A Review of Human Studies and Animal Models. *Androg Clin Res Ther., 2*(1), 294-303. doi:10.1089/andro.2021.0011.

Bourque M., Soulet D., Morisette M., Di Paolo T. (2023). Impact of sex on neuroimmune contributions to Parkinson's disease. *Brain Research Bulletin., 199*. doi:https://doi.org/10.1016/j.brainresbull.2023.110668

Bovenzi R., Conti M., De Franco V., et al. (2024). Sex differences in Parkinson's disease-related non motor symptoms: a focus on sleep problems. *Acta Neurol Belg., 124*(5), 1525-1534. doi:10.1007/s13760-024-02535-8.

Braak H., Del Tredici K., Bratzke H., et al. (2002). Staging of the intracerebral inclusion body pathology associated with idiopathic Parkinson's disease (preclinical and clinical stages). *J Neurol.*, (249): 1-5. doi:10.1007/s00415-002-1301-4.

Brás I.C., Outeiro T.F. (2021). Alpha-Synuclein: Mechanisms of Release and Pathology Progression in Synucleinopathies. *Cells., 10*(2), 375. doi:10.3390/cells10020375.

Brockmann K., Apel A., Schulte C., et al. (2016). Inflammatory profile in LRRK2-associated prodromal and clinical PD. *J Neuroinflammation, 13*, 122. doi:https://doi.org/10.1186/s12974-016-0588-5

Buell A.K., Galvagnion C., Gaspar R., et al. (2014). Solution conditions determine the relative importance of nucleation and growth processes in α-synuclein aggregation. *Proc Natl Acad Sci U S A., 111*(21), 7671-7676. doi:10.1073/pnas.1315346111.

Candelise N., Schmitz M., Thüne K., Cramm M., Rabano A. (2020). Effect of the micro-environment on α-synuclein conversion and implication in seeded conversion assays. *Transl Neurodegener., 9*, 5. doi:10.1186/s40035-019-0181-9.

Caproni S., Di Fonzo A., Colosimo C. (2025). Oxidative Stress: A New Pathophysiological Pathway in Parkinson's Disease and a Potential Target of the Brain-Sport Crosstalk. *Parkinsons Dis.* doi:10.1155/padi/6691390.

Cardanho-Ramos C., Morais V.A. (2021). Mitochondrial Biogenesis in Neurons: How and Where. *Int J Mol Sci., 22*(23), 13059. doi:10.3390/ijms222313059.

Cattaneo C., Pagonabarraga J. (2025). Sex Differences in Parkinson's Disease: A Narrative Review. *Neurol Ther., 14*(1), 57-70. doi:10.1007/s40120-024-00687-6.

Cerri S., Mus L., Blandini F. (2019). Parkinson's Disease in Women and Men: What's the Difference? *J Parkinsons Dis., 9*(3), 501-515. doi:10.3233/JPD-191683.

Chen H., Ma D., Yue F., et al. (2022). The Potential Role of Hypoxia-Inducible Factor-1 in the Progression and Therapy of Central Nervous System Diseases. *Curr Neuropharmacol., 20*(9), 1651-1666. doi:10.2174/1570159X19666210729123137.

Chen S.W., Barritt J.D., Cascella R., et al. (2024). Structure-Toxicity Relationship in Intermediate Fibrils from α-Synuclein Condensates. *J Am Chem Soc., 146*(15), 10537-10549. doi:10.1021/jacs.3c14703.

Chi H., Chang H.Y., Sang T.K. (2018). Neuronal Cell Death Mechanisms in Major Neurodegenerative Diseases. *Int J Mol Sci.*, 19(10). doi:10.3390/ijms19103082.

Chiurchiù V., Orlacchio A., Maccarrone M. (2016). Is Modulation of Oxidative Stress an Answer? The State of the Art of Redox Therapeutic Actions in Neurodegenerative Diseases. *Oxid. Med. Cell. Longev.*, 2016. doi:10.1155/2016/7909380.

Chuan Z.L., Liu C.Z., Guo S., et al. (2022). Correlation of matrix metalloproteinase 3 and matrix metalloproteinase 9 levels with non-motor symptoms in patients with Parkinson's disease. *Frontiers in Aging Neuroscience., 14*. doi:https://doi.org/10.3389/fnagi.2022.889257

Church F.C. (2021). Treatment Options for Motor and Non-Motor Symptoms of Parkinson's Disease. *Biomolecules., 11*(4), 612. doi:10.3390/biom11040612.

Clayton J.A. (2016). Studying both sexes: a guiding principle for biomedicine. *FASEB J., 30*(2), 519-524. doi:10.1096/fj.15-279554.

Colla E. (2019). Linking the Endoplasmic Reticulum to Parkinson's Disease and Alpha-Synucleinopathy. *Front Neurosci., 13*, 560. doi:10.3389/fnins.2019.00560.

Conti B., Sugama S., Lucero J., et al. (2005). Uncoupling protein 2 protects dopaminergic neurons from acute 1,2,3,6-methyl-phenyl-tetrahydropyridine toxicity. *J Neurochem., 93*(2), 493-501. doi:10.1111/j.1471-4159.2005.03052.x.

Cortese M., Riise T., Engeland A., Ascherio A., Bjørnevik K. (2018). Urate and the risk of Parkinson's disease in men and women. *Parkinsonism Relat Disord., 52*, 76-82. doi:10.1016/j.parkreldis.2018.03.026.

Coskuner O., Wise-Scira O. (2013). Structures and free energy landscapes of the A53T mutant-type α-synuclein protein and impact of A53T mutation on the structures of the wild-type α-synuclein protein with dynamics. *ACS Chem Neurosci., 4*(7), 1101-1113. doi:10.1021/cn400041j.

Dahodwala N., Shah K., He Y., Wu S.S., Schmidt P. (2018). Sex disparities in access to caregiving in Parkinson disease. *Neurology., 90*(1), 48-54. doi:10.1212/WNL.0000000000004764.

Dandurand J., Ostuni A., Armentano M.F., et al. (2020). Calorimetry and FTIR reveal the ability of URG7 protein to modify the aggregation state of both cell lysate and amylogenic α-synuclein. *AIMS Biophysics., 7*(3), 189-203. doi:10.3934/biophy.2020015

Davis D.H., Creavin S.T., Yip J.L., et al. (2021). Montreal Cognitive Assessment for the detection of dementia. *Cochrane Database Syst Rev., 7*(7). doi:10.1002/14651858.CD010775.pub3.

Dexter D.T., Jenner P. (2013). Parkinson disease: from pathology to molecular disease mechanisms. *Free Radic Biol Med.*, 62:132-144. doi:10.1016/j.freeradbiomed.2013.01.018.

Dikiy I., Eliezer D. (2014). N-terminal acetylation stabilizes N-terminal helicity in lipid- and micelle-bound α-synuclein and increases its affinity for physiological membranes. *J Biol Chem., 289*(6), 3652-3665. doi:10.1074/jbc.M113.512459.

Dong-Chen X., Yong C., Yang X., et al. (2023). Signaling pathways in Parkinson's disease: molecular mechanisms and therapeutic interventions. *Sig. Transduct. Target. Ther., 8*, 73. doi:https://doi.org/10.1038/s41392-023-01353-3

Drobny A., Boros F.A., Balta D., Prieto Huarcaya S., Caylioglu D. (2023). Reciprocal effects of alpha-synuclein aggregation and lysosomal homeostasis in synucleinopathy models. *Transl Neurodegener., 12*(1), 31. doi:10.1186/s40035-023-00363-z.

Dugger B.N., Dickson D.W. (2017). Pathology of Neurodegenerative Diseases. *Cold Spring Harb Perspect Biol*, 9(7). doi:10.1101/cshperspect.a028035

Duplan E., Giaime E., Viotti J., et al. (2013). ER-stress-associated functional link between Parkin and DJ-1 via a transcriptional cascade involving the tumor suppressor p53 and the spliced X-box binding protein XBP-1. *J Cell Sci., 126*(9), 2124-2133. doi:10.1242/jcs.127340.

Emamzadeh FN. (2016). Alpha-synuclein structure, functions, and interactions. *J Res Med Sci., 21*, 29. doi:10.4103/1735-1995.181989.

Emin D., Zhang Y.P., Lobanova E., et al. (2022). Small soluble α-synuclein aggregates are the toxic species in Parkinson's disease. *Nat Commun., 13*(1), 5512. doi:10.1038/s41467-022-33252-6.

Estaun-Panzano J., Arotcarena M.L., Bezard E. (2023). Monitoring α-synuclein aggregation. *Neurobiol Dis., 176*. doi:10.1016/j.nbd.2022.105966.

Filippini A., Gennarelli M., Russo I. (2019). α-Synuclein and Glia in Parkinson's Disease: A Beneficial or a Detrimental Duet for the Endo-Lysosomal System? *Cell Mol Neurobiol., 39*(2), 161-168. doi:10.1007/s10571-019-00649-9.

Fiorenzato E., Cauzzo S., Weis L., et al. (2024). Optimal MMSE and MoCA cutoffs for cognitive diagnoses in Parkinson's disease: A data-driven decision tree model. *J. Neurol. Sci., 466*, 123283. doi:https://doi.org/10.1016/j.jns.2024.123283

Franconi F., Raparelli V., Regitz-Zagrosek V. (2017). Sex and gender landscape in pharmacology. *Pharmacol Res., 123*, 93-94. doi:10.1016/j.phrs.2017.07.001.

Frey T.G., Mannella C.A. (2000). The internal structure of mitochondria. *Trends Biochem Sci., 25*(7), 319-324. doi:10.1016/s0968-0004(00)01609-1.

Galiano-Landeira J., Torra A., Vila M., Bové J. (2020). CD8 T cell nigral infiltration precedes synucleinopathy in early stages of Parkinson's disease. *Brain., 143*(12), 3717-3733. doi:10.1093/brain/awaa269.

Gao Y., Zhang J., Tang T., Liu Z. (2024). Hypoxia Pathways in Parkinson's Disease: From Pathogenesis to Therapeutic Targets. *Int. J. Mol. Sci., 25*(19), 10480. doi:https://doi.org/10.3390/ijms251910484

Geng X., Lou H., Wang J., et al. (2011). α-Synuclein binds the K(ATP) channel at insulin-secretory granules and inhibits insulin secretion. *Am J Physiol Endocrinol Metab., 300*(2), 276-286. doi:10.1152/ajpendo.00262.2010.

Georgiev D., Hamberg K., Hariz M., Forsgren L., Hariz G.M. (2017). Gender differences in Parkinson's disease: A clinical perspective. *Acta Neurol Scand., 136*(6), 570-584. doi:10.1111/ane.12796.

Goldman J.G. (2025). Non-motor Symptoms and Treatments in Parkinson's Disease. *Neurol Clin., 43*(2), 291-317. doi: 10.1016/j.ncl.2024.12.008.

González-Giraldo Y., Forero D.A., Echeverria V., Garcia-Segura L.M., Barreto G.E. (2019). Tibolone attenuates inflammatory response by palmitic acid and preserves mitochondrial membrane potential in astrocytic cells through estrogen receptor beta. *Mol Cell Endocrinol., 468*, 65-78. doi:10.1016/j.mce.2019.02.017.

Gorbenko G.P., Kinnunen P.K. (2006). The role of lipid-protein interactions in amyloid-type protein fibril formation. *Chem Phys Lipids., 141*(1-2), 72-82. doi:10.1016/j.chemphyslip.2006.02.006.

Guardia-Laguarta C., Area-Gomez E., Rüb C., et al. (2014). α-Synuclein is localized to mitochondria-associated ER membranes. *J Neurosci., 34*(1), 249-259. doi:10.1523/JNEUROSCI.2507-13.2014.

Guo M., Wang J., Zhao Y., et al. (2020). Microglial exosomes facilitate α-synuclein transmission in Parkinson's disease. *Brain, 143*(5), 1476-1497. doi:10.1093/brain/awaa090.

Hamanaka R.B., Chandel N.S. (2010). Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes. *Trends Biochem. Sci., 35*(9), 505-513. doi:https://doi.org/10.1016/j.tibs.2010.04.002

Harish G., Venkateshappa C., Mahadevan A., et al. (2013). Mitochondrial function in human brains is affected by pre- and post mortem factors. *Neuropathol Appl Neurobiol., 39*(3), 298-315. doi:10.1111/j.1365-2990.2012.01285.x.

Harsanyiova J., Buday T., Kralova Trancikova A. (2020). Parkinson's Disease and the Gut: Future Perspectives for Early Diagnosis. *Front Neurosci., 14*, 626. doi:10.3389/fnins.2020.00626.

He Q., Yu W., Wu J., et al. (2013). Intranasal LPS-mediated Parkinson's model challenges the pathogenesis of nasal cavity and environmental toxins. *PLoS One., 8*(11). doi:10.1371/journal.pone.0078418.

Heravi F.S., Naseri K., Hu H. (2023). Gut Microbiota Composition in Patients with Neurodegenerative Disorders (Parkinson's and Alzheimer's) and Healthy Controls: A Systematic Review. *Nutrients., 15*(20), 4365. doi:10.3390/nu15204365.

Hickman S., Izzy S., Sen P., Morsett L., El Khoury J. (2018). Microglia in neurodegeneration. *Nat Neurosci., 21*(10), 1359-1369. doi:10.1038/s41593-018-0242-x.

Hoozemans J.J., Van Haastert E.S., Eikelenboom P., et al. (2007). Activation of the unfolded protein response in Parkinson's disease. *Biochem Biophys Res Commun., 354*(3), 707-711. doi:10.1016/j.bbrc.2007.01.043.

Huang A., Foti R.S., Gunawardana C.G., Shen H., Xiao Y. (2025). Bioanalytical Considerations for Biomarkers of Parkinson's Disease. *J Neurochem., 169*(6). doi:10.1111/jnc.70131.

Hyży A., Rozenek H., Gondek E., Jaworski M. (2025). Effect of Antioxidants on the Gut Microbiome Profile and Brain Functions: A Review of Randomized Controlled Trial Studies. *Foods., 14*(2), 176. doi:10.3390/foods14020176.

Iovino L., Tremblay M.E., Civiero L. (2020). Glutamate-induced excitotoxicity in Parkinson's disease: The role of glial cells. *J Pharmacol Sci., 144*(3), 151-164. doi:10.1016/j.jphs.2020.07.011.

Isenbrandt A., Coulombe K., Morissette M., et al. (2023). Three-Dimensional Analysis of Sex- and Gonadal Status- Dependent Microglial Activation in a

Mouse Model of Parkinson's Disease. *Pharmaceuticals., 16*(2), 152. doi:https://doi.org/10.3390/ph16020152

Ivy C.M., Velotta J.P., Cheviron Z.A., Scott G.R. (2022). Genetic variation in HIF-2α attenuates ventilatory sensitivity and carotid body growth in chronic hypoxia in high-altitude deer mice. *J Physiol., 600*(18), 4207-4225. doi:10.1113/JP282798.

Iwaki H., Blauwendraat C., Leonard H.L., et al. (2021). Differences in the Presentation and Progression of Parkinson's Disease by Sex. *Mov Disord., 36*(1), 106-117. doi:10.1002/mds.28312.

Jia F., Fellner A., Kumar K.R. (2022). Monogenic Parkinson's Disease: Genotype, Phenotype, Pathophysiology, and Genetic Testing. *Genes, 13*(3), 471. doi:10.3390/genes13030471.

Jin H., Kanthasamy A., Ghosh A., et al. (2011). α-Synuclein negatively regulates protein kinase Cδ expression to suppress apoptosis in dopaminergic neurons by reducing p300 histone acetyltransferase activity. *J Neurosci., 31*(6), 2035-2051. doi:10.1523/JNEUROSCI.5634-10.2011.

Jomova K., Raptova R., Alomar S.Y., et al. (2023). Reactive oxygen species, toxicity, oxidative stress, and antioxidants: chronic diseases and aging. *Arch Toxicol., 97*(10), 2499-2574. doi:10.1007/s00204-023-03562-9.

Jornayvaz F.R., Shulman G.I. (2010). Regulation of mitochondrial biogenesis. *Essays Biochem., 47*, 69-84. doi:10.1042/bse0470069.

Kasten M., Hartmann C., Hampf J., et al. (2018). Genotype-Phenotype Relations for the Parkinson's Disease Genes Parkin, PINK1, DJ1: MDSGene Systematic Review. *Mov Disord., 33*(5), 730-741. doi:10.1002/mds.27352.

Kasten M., Klein C. (2013). The many faces of alpha-synuclein mutations. *Mov Disord., 28*(6), 697-701. doi:10.1002/mds.25499.

Kestenbaum M., Alcalay R.N. (2017). Clinical Features of LRRK2 Carriers with Parkinson's Disease. *Adv Neurobiol., 14*, 31-48. doi:10.1007/978-3-319-49969-7_2.

Kim E.M, Hwang O. (2011). Role of matrix metalloproteinase-3 in neurodegeneration. *J. of Neurochemistry, 116*(1), 22-32. doi: https://doi.org/10.1111/j.1471-4159.2010.07082.x

Kim G.H., Kim J.E., Rhie S.J., Yoon S. (2015). The Role of Oxidative Stress in Neurodegenerative Diseases. *Exp Neurobiol., 24*(4), 325-340. doi:10.5607/en.2015.24.4.325.

Kim H.J., Magranè J., Starkov A.A., Manfredi G. (2012). The mitochondrial calcium regulator cyclophilin D is an essential component of oestrogen-mediated neuroprotection in amyotrophic lateral sclerosis. *Brain., 139*(9), 2865-2874. doi:10.1093/brain/aws208.

Klemmensen M.M., Borrowman S.H., Pearce C., Pyles B., Chandra B. (2024). Mitochondrial dysfunction in neurodegenerative disorders. *Neurotherapeutics., 21*(1). doi:10.1016/j.neurot.2023.10.002.

Kole A.J., Annis R.P., Deshmukh M. (2013). Mature neurons: equipped for survival. *Cell Death Dis*, 4(6). doi:10.1038/cddis.2013.220.

Kouli A., Williams-Gray C.H. (2022). Age-Related Adaptive Immune Changes in Parkinson's Disease. *J Parkinsons Dis, 12*, 93-104. doi:10.3233/JPD-223228.

Krasnoslobodtsev A.V., Peng J., Asiago J.M., et al. (2012). Effect of spermidine on misfolding and interactions of alpha-synuclein. *PLoS One., 7*(5). doi:10.1371/journal.pone.0038099.

Kumar K.R., Lohmann K., Klein C. (2012). Genetics of Parkinson disease and other movement disorders. *Curr Opin Neurol., 25*(4), 466-474. doi:10.1097/WCO.0b013e3283547627.

Kuo G., Kumbhar R., Blair W., Dawson V.L., Dawson T.M., Mao X. (2025). Emerging targets of α-synuclein spreading in α-synucleinopathies: a review of mechanistic pathways and interventions. *Mol Neurodegeneration., 20*, 10. doi:https://doi.org/10.1186/s13024-025-00797-1

Kwon D.K., Kwatra M., Wang J., Ko H.S. (2022). Levodopa-Induced Dyskinesia in Parkinson's Disease: Pathogenesis and Emerging Treatment Strategies. *Cells., 11*(23), 3736. doi:10.3390/cells11233736.

Lai T.T., Kim Y.J., Ma H., Kim Y.E. (2022). Evidence of Inflammation in Parkinson's Disease and Its Contribution to Synucleinopathy. *J. Mov. Disord., 15*(1), 1-14. doi:https://doi.org/10.14802/jmd.21078

Li G., Liu J., Guo M., et al. (2022). Chronic hypoxia leads to cognitive impairment by promoting HIF-2α-mediated ceramide catabolism and alpha-synuclein hyperphosphorylation. *Cell Death Discov., 8*, 473. doi:https://doi.org/10.1038/s41420-022-01260-6

Li S., Song H., Yu C. (2024). Causal association between phenylalanine and Parkinson's disease: a two-sample bidirectional mendelian randomization study. *Front. Genet., 15*. doi:https://doi.org/10.3389/fgene.2024.1322551

Lin C.H., Li C.H., Yang K.C., et al. (2019). Blood NfL: A biomarker for disease severity and progression in Parkinson disease. *Neurology., 93*(11), 1104-1111. doi:10.1212/WNL.0000000000008088.

Liu C., Zhang Y. (2011). Nucleic acid-mediated protein aggregation and assembly. *Adv Protein Chem Struct Biol., 84*, 1-40. doi:10.1016/B978-0-12-386483-3.00005-7.

Luo X., Liu Y., Balck A., et al. (2024). Identification of metabolites reproducibly associated with Parkinson's Disease via meta-analysis and computational modelling. *NPJ Parkinsons Dis., 10*(1), 126. doi:10.1038/s41531-024-00732-z.

Luo Y., Qiao L., Li M., Wen X., Zhang W., Li X. (2025). Global, regional, national epidemiology and trends of Parkinson's disease from 1990 to 2021: findings from the Global Burden of Disease Study 2021. *Front. Aging Neurosci*, 16. doi: 10.3389/fnagi.2024.1498756

Magistrelli L., Contaldi E., Comi C. (2021). The Impact of SNCA Variations and Its ProductAlpha-Synuclein on Non-Motor Features of Parkinson's Disease. *Life., 11*(8), 804. doi:10.3390/life11080804

Mandel S.A., Morelli M., Halperin I., Korczyn A.D. (2010). Biomarkers for prediction and targeted prevention of Alzheimer's and Parkinson's diseases: evaluation of drug clinical efficacy. *EPMA J., 1*(2), 273-292. doi:10.1007/s13167-010-0036-z.

Mao H., Ye Y., Sun X., et al. (2022). Quiescent Elongation of α-Synuclein Pre-form Fibrils Under Different Solution Conditions. *Front Neurosci., 16*. doi:10.3389/fnins.2022.902077.

Mariani S., Ventriglia M., Simonelli I., et al. (2016). Association between sex, systemic iron variation and probability of Parkinson's disease. *Int J Neurosci., 126*(4), 354-360. doi:10.3109/00207454.2015.1020113.

Mazzulli J.R., Zunke F., Isacson O., Studer L., Krainc D. (2016). α-Synuclein-induced lysosomal dysfunction occurs through disruptions in protein trafficking in

human midbrain synucleinopathy models. *Proc Natl Acad Sci U S A., 113*(7), 1931-1936. doi:10.1073/pnas.1520335113.

Miao Y., Meng H. (2024). The involvement of α-synucleinopathy in the disruption of microglial homeostasis contributes to the pathogenesis of Parkinson's disease. *Cell Commun Signal., 22*(1), 31. doi:10.1186/s12964-023-01402-y.

Moors T.E., Paciotti S., Ingrassia A., et al. (2019). Characterization of Brain Lysosomal Activities in GBA-Related and Sporadic Parkinson's Disease and Dementia with Lewy Bodies. *Mol Neurobiol., 56*(2), 1344-1355. doi:10.1007/s12035-018-1090-0.

Mostafalou S., Abdollahi M. (2018). The link of organophosphorus pesticides with neurodegenerative and neurodevelopmental diseases based on evidence and mechanisms. *Toxicology., 409*, 44-52. doi:10.1016/j.tox.2018.07.014.

Muleiro Alvarez M., Cano-Herrera G., Osorio Martínez M.F., et al. (2024). A Comprehensive Approach to Parkinson's Disease: Addressing Its Molecular, Clinical, and Therapeutic Aspects. *Int J Mol Sci., 25*(13), 7183. doi:10.3390/ijms25137183.

Muzio L., Viotti A., Martino G. (2021). Microglia in Neuroinflammation and Neurodegeneration: From Understanding to Therapy. *Front. Neurosci., 15*. doi:https://doi.org/10.3389/fnins.2021.742065

Nabi M., Tabassum N. (2022). Role of Environmental Toxicants on Neurodegenerative Disorders. *Front Toxicol., 4*. doi: 10.3389/ftox.2022.837579

Naiki H., Nagai Y. (2009). Molecular pathogenesis of protein misfolding diseases: pathological molecular environments versus quality control systems against misfolded proteins. *J Biochem., 146*(6), 751-756. doi:10.1093/jb/mvp119.

Nechushtai L., Frenkel D., Pinkas-Kramarski R. (2023). Autophagy in Parkinson's Disease. *Biomolecules., 13*(10), 1435. doi:10.3390/biom13101435.

Nicoletti A., Baschi R., Cicero C.E., Iacono S., Re V.L. (2023). Sex and gender differences in Alzheimer's disease, Parkinson's disease, and Amyotrophic Lateral Sclerosis: A narrative review. *Mech Ageing Dev., 212*, 111821. doi:10.1016/j.mad.2023.111821.

Nuscher B., Kamp F., Mehnert T., et al. (2004). Alpha-synuclein has a high affinity for packing defects in a bilayer membrane: a thermodynamics study. *J Biol Chem., 279*(21), 21966-21975. doi:10.1074/jbc.M401076200.

Oeckl P., Halbgebauer S., Anderl-Straub S., et al. (2019). Glial Fibrillary Acidic Protein in Serum is Increased in Alzheimer's Disease and Correlates with Cognitive Impairment. *J. Alzheimer Disease., 67*(2), 481-488. doi:https://doi.org/10.3233/JAD-180325

Ophey A., Eggers C., Dano R., Timmermann L., Kalbe E. (2018). Health-Related Quality of Life Subdomains in Patients with Parkinson's Disease: The Role of Gender. *Parkinsons Dis., 2018*. doi:10.1155/2018/6532320.

Orrenius S., Gogvadze V., Zhivotovsky B. (2015). Calcium and mitochondria in the regulation of cell death. *Biophys. Res. Commun., 460*(1), 72-81. doi:10.1016/j.bbrc.2015.01.137.

Parnetti L., Gaetani L., Eusebi P., Paciotti S., Hansson O. (2019). CSF and blood biomarkers for Parkinson's disease. *The Lancet Neurology., 18*(6), 573-586. doi:https://doi.org/10.1016/S1474-4422(19)30024-9

Pellegrini C., Fornai M., Colucci R., et al. (2016). Alteration of colonic excitatory tachykininergic motility and enteric inflammation following dopaminergic

nigrostriatal neurodegeneration. *J Neuroinflammation., 13*(1), 146. doi:10.1186/s12974-016-0608-5.

Pereira G.M., Teixeira-dos-Santos D., Soares N.M., et al. (2024). A systematic review and meta-analysis of the prevalence of Parkinson's disease in lower to upper-middle-income countries. *npj Parkinsons Dis.*, 10(181). doi:https://doi.org/10.1038/s41531-024-00779-y

Perfeito R., Ribeiro M., Rego A.C. (2017). Alpha-synuclein-induced oxidative stress correlates with altered superoxide dismutase and glutathione synthesis in human neuroblastoma SH-SY5Y cells. *Arch Toxicol., 91*(3), 1245-1259. doi:10.1007/s00204-016-1788-6.

Pfefferkorn C.M., Jiang Z., Lee J.C. (2012). Biophysics of α-synuclein membrane interactions. *Biochim Biophys Acta., 18*(2), 162-171. doi:10.1016/j.bbamem.2011.07.032.

Pham L., Arroum T., Wan J., et al. (2024). Regulation of mitochondrial oxidative phosphorylation through tight control of cytochrome c oxidase in health and disease - Implications for ischemia/reperfusion injury, inflammatory diseases, diabetes, and cancer. *Redox Biol.*, 78. doi:10.1016/j.redox.2024.

Piccinin E., Sardanelli A.M., Seibel P., Moschetta A., Cocco T., Villani G. (2021). PGC-1s in the Spotlight with Parkinson's Disease. *Int J Mol Sci., 22*(7), 3487. doi:10.3390/ijms22073487.

Pirker W., Katzenschlager R., Hallett M., Poewe W. (2023). Pharmacological Treatment of Tremor in Parkinson's Disease Revisited. *J Parkinsons Dis., 13*(2), 127-144. doi:10.3233/JPD-225060.

Pizzino G., Irrera N., Cucinotta M., et al. (2017). Oxidative Stress: Harms and Benefits for Human Health. *Oxid Med Cell Longev.* doi:10.1155/2017/8416763.

Poewe W. (2008). Non-motor symptoms in Parkinson's disease. *European Journal of Neurology, 15*, 14-20. doi: https://doi.org/10.1111/j.1468-1331.2008.02056.x

Popov L. (2020). Mitochondrial biogenesis: An update. *J Cell Mol Med., 24*(9), 4892-4899. doi:10.1111/jcmm.15194.

Qu Y., Li J., Qin Q., et al. (2023). A systematic review and meta-analysis of inflammatory biomarkers in Parkinson's disease. *Npj Parkinson Dis., 9*(1). doi:10.1038/s41531-023-00449-5

Rackham O.J., Langley S.R., Oates T., Vradi E., Harmston N. (2017). A Bayesian Approach for Analysis of Whole-Genome Bisulfite Sequencing Data Identifies Disease-Associated Changes in DNA Methylation. *Genetics., 205*(4), 1443-1458. doi:10.1534/genetics.116.195008.

Rambold A.S., Pearce E.L. (2018). Mitochondrial Dynamics at the Interface of Immune Cell Metabolism and Function. *Trends Immunol., 39*(1), 6-18. doi:10.1016/j.it.2017.08.006.

Ramirez Ortega D., Ovalle Rodríguez P., Pineda B., et al. (2020). Kynurenine Pathway as a New Target of Cognitive Impairment Induced by Lead Toxicity During the Lactation. *Sci Rep., 10*(1), 3184. doi:10.1038/s41598-020-60159-3.

Ravikumar B., Sarkar S., Davies J.E., et al. (2010). Regulation of mammalian autophagy in physiology and pathophysiology. *Physiol Rev., 90*(4), 1383-1435. doi:10.1152/physrev.00030.2009.

Repici M., Giorgini F. (2019). DJ-1 in Parkinson's Disease: Clinical Insights and Therapeutic Perspectives. *J Clin Med., 8*(9), 1377. doi:10.3390/jcm8091377.

Rietdijk C.D., Perez-Pardo P., Garssen J., van Wezel R.J., Kraneveld A.D. (2017). Exploring Braak's Hypothesis of Parkinson's Disease. *Front Neurol.*(8), 37. doi:10.3389/fneur.2017.00037.

Risiglione P., Leggio L., Cubisino S., et al. (2020). High-Resolution Respirometry Reveals MPP^+ Mitochondrial Toxicity Mechanism in a Cellular Model of Parkinson's Disease. *Int J Mol Sci., 21*(21), 7809. doi:10.3390/ijms21217809.

Rizzo G., Copetti M., Arcuti S., Martino D, Fontana A., Logroscino G. (2016). Accuracy of clinical diagnosis of Parkinson disease. *Neurology., 86*(6), 566-576. doi:10.1212/wnl.0000000000002350

Robustelli P., Piana S., Shaw D.E. (2018). Developing a molecular dynamics force field for both folded and disordered protein states. *Proc Natl Acad Sci U S A., 115*(21), 4758-4766. doi:10.1073/pnas.1800690115.

Rodriguez J.A., Ivanova M.I., Sawaya M.R., et al. (2015). Structure of the toxic core of α-synuclein from invisible crystals. *Nature, 525*(7570), 486-490. doi:10.1038/nature15368.

Rodríguez-Losada N., de la Rosa J., Larriva M., et al. (2020). Overexpression of alpha-synuclein promotes both cell proliferation and cell toxicity in human SH-SY5Y neuroblastoma cells. *J Adv Res., 23*, 37-45. doi:10.1016/j.jare.2020.01.009.

Rossi M., Candelise N., Baiardi S., et al. (2020). Ultrasensitive RT-QuIC assay with high sensitivity and specificity for Lewy body-associated synucleinopathies. *Acta Neuropathol., 140*(1), 49-62. doi:10.1007/s00401-020-02160-8

Rüb C., Wilkening A., Voos W. (2017). Mitochondrial quality control by the Pink1/Parkin system. *Cell Tissue Res, 367*(11), 111-123. doi:10.1007/s00441-016-2485-8.

Salim S., Ahmad F., Banu A., Mohammad F. (2023). Gut microbiome and Parkinson's disease: Perspective on pathogenesis and treatment. *J Adv Res.*(50), 83-105. doi:10.1016/j.jare.2022.10.013.

Salles P.A., Pizarro-Correa X., Chanà-Cuevas P. (2024). Genetics of Parkinson´s disease: Recessive forms. *Neurol Perspectives., 4*(2). doi:https://doi.org/10.1016/j.neurop.2024.100147

Sánchez-Santed F., Colomina M.T., Herrero Hernández E. (2016). Organophosphate pesticide exposure and neurodegeneration. *Cortex, 74*, 417-426. doi:10.1016/j.cortex.2015.10.003

Sato H., Kato T., Arawaka S. (2013). The role of Ser129 phosphorylation of α-synuclein in neurodegeneration of Parkinson's disease: a review of in vivo models. *Rev Neurosci., 24*(2), 115-123. doi:10.1515/revneuro-2012-0071.

Schaffner S.L., Tosefsky K.N., Inskter A.M., et al. (2025). Sex and gender differences in the molecular etiology of Parkinson's disease: considerations for study design and data analysis. *Biol Sex Differ., 16*(1), 7. doi:10.1186/s13293-025-00692-w.

Scott D., Roy S. (2012). α-Synuclein inhibits intersynaptic vesicle mobility and maintains recycling-pool homeostasis. *J Neurosci., 32*(30), 10129-10135. doi:10.1523/JNEUROSCI.0535-12.2012.

Serratos I.N., Hernández-Pérez E., Campos C., Aschner M., Santamaría A. (2022). An Update on the Critical Role of α-Synuclein in Parkinson's Disease and Other Synucleinopathies: from Tissue to Cellular and Molecular Levels. *Mol Neurobiol., 59*(1), 620-642. doi:10.1007/s12035-021-02596-3.

Shao Y., Le W. (2019). Recent advances and perspectives of metabolomics-based investigations in Parkinson's disease. *Mol Neurodegeneration., 14*(3). doi:https://doi.org/10.1186/s13024-018-0304-2

Shen L., Dettmer U. (2024). Alpha-Synuclein Effects on Mitochondrial Quality Control in Parkinson's Disease. *Biomolecules., 14*(12), 1649. doi:10.3390/biom14121649.

Shim K.H., Kang M.J., Suh J.W., et al. (2020). CSF total tau/α-synuclein ratio improved the diagnostic performance for Alzheimer's disease as an indicator of tau phosphorylation. *Alz Res Therapy., 12*(83). doi:https://doi.org/10.1186/s13195-020-00648-9

Shin H.W., Hong S.W., Youn Y.C. (2022). Clinical Aspects of the Differential Diagnosis of Parkinson's Disease and Parkinsonism. *J Clin Neurol.*, 18(3):259-270. doi: 10.3988/jcn.2022.18.3.259.

Shiraishi T., Bono K., Hiraki H., et al. (2024). The impact of VPS35 D620N mutation on alternative autophagy and its reversal by estrogen in Parkinson's disease. *Cell Mol Life Sci., 81*(1), 103. doi:10.1007/s00018-024-05123-4.

Shu H., Zhang P., Gu L. (2024). Alpha-synuclein in peripheral body fluid as a biomarker for Parkinson's disease. *Acta Neurol Belg., 124*(3), 831-842. doi:10.1007/s13760-023-02452-2.

Siderowf A., Lang A.E. (2012). Premotor Parkinson's disease: concepts and definitions. *Mov Disord., 27*(5), 608-616. doi:10.1002/mds.24954.

Silva Y.P., Bernardi A., Frozza R.L. (2020). The Role of Short-Chain Fatty Acids From Gut Microbiota in Gut-Brain Communication. *Front Endocrinol (Lausanne)., 11*, 25. doi:10.3389/fendo.2020.00025.

Singh A., Kukreti R., Saso L., Kukreti S. (2019). Oxidative Stress: A Key Modulator in Neurodegenerative Diseases. *Molecules, 24*(8), 1583. doi:https://doi.org/10.3390/molecules24081583

Siracusa R., Fusco R., Cuzzocrea S. (2019). Astrocytes: Role and Functions in Brain Pathologies. *Front Pharmacol., 10*, 1114. doi:10.3389/fphar.2019.01114.

Smith M.H., Ploegh H.L., Weissman J.S. (2011). Road to ruin: targeting proteins for degradation in the endoplasmic reticulum. *Science., 334*(6059), 1086-1090. doi:10.1126/science.1209235.

Sode K., Ochiai S., Kobayashi N., Usuzaka E. (2006). Effect of reparation of repeat sequences in the human alpha-synuclein on fibrillation ability. *Int J Biol Sci., 3*(1), 1-7. doi:10.7150/ijbs.3.1.

Sorrentino Z.A., Giasson B.I., Chakrabarty P. (2019). α-Synuclein and astrocytes: tracing the pathways from homeostasis to neurodegeneration in Lewy body disease. *Acta Neuropathol., 138*(1), 1-21. doi:10.1007/s00401-019-01977-2.

Su D., Cui Y., He C., et al. (2025). Projections for prevalence of Parkinson's disease and its driving factors in 195 countries and territories to 2050: modelling study of Global Burden of Disease Study 2021. *BMJ*, 388. doi: https://doi.org/10.1136/bmj-2024-080952

Sveinbjornsdottir S. (2016). The clinical symptoms of Parkinson's disease. *Journal of Neurochemistry*, 318-324. doi: https://doi.org/10.1111/jnc.13691

Sweeney M.D., Zhao Z., Montagne A., Nelson A.R., Zlokovic B.V. (2019). Blood-Brain Barrier: From Physiology to Disease and Back. *Physiol Rev., 99*(1), 21-78. doi:10.1152/physrev.00050.2017.

Tambasco N., Nigro P., Romoli M., Prontera P., Simoni S., Calabresi P. (2016). A53T in a parkinsonian family: a clinical update of the SNCA phenotypes. *J Neural Transm (Vienna)., 123*(11), 1301-1307. doi:10.1007/s00702-016-1578-6.

Tan EK., Chao YX., West A., Chan LL., Poewe W., Jankovic J. (2020). Parkinson disease and the immune system - associations, mechanisms and therapeutics. *Nat Rev Neurol., 16*(6), 303-318. doi:10.1038/s41582-020-0344-4.

Titova N., Padmakumar C., Lewis S.J.G., Chaudhuri K.R. (2017). Parkinson's: a syndrome rather than a disease? *J Neural Transm (Vienna), 124*(8), 907-914. doi:10.1007/s00702-016-1667-6.

Toczylowska B., Zieminska E., Michałowska M., et al. (2020). Changes in the metabolic profiles of the serum and putamen in Parkinson's disease patients - In vitro and in vivo NMR spectroscopy studies. *Brain Res., 1748*. doi:10.1016/j.brainres.2020.147118.

Tolosa E., Garrido A., Scholz S.W., Poewe W. (2021). Challenges in the diagnosis of Parkinson's disease. *Lancet Neurol., 20*(5), 385-397. doi:10.1016/S1474-4422(21)00030-2.

Tönges L., Buhmann C., Klebe S., Klucken J., Kwon E.H. (2022). Blood-based biomarker in Parkinson's disease: potential for future applications in clinical research and practice. *J Neural Transm (Vienna)., 129*(9), 1201-1217. doi:10.1007/s00702-022-02498-1.

Torok N., Majlath Z., Szalardy L., Vecsei L. (2016). Investigational a-Synuclein Aggregation Inhibitors: Hope For Parkinson's Disease. *Expert Opin. Investig. Drugs, 25*, 1281-1294. doi:10.1080/13543784.2016.1237501

Trist B.G., Hare D.J., Double K.L. (2019). Oxidative stress in the aging substantia nigra and the etiology of Parkinson's disease. *Aging Cell., 18*(6). doi:10.1111/acel.13031.

Uittenbogaard M., Chiaramello A. (2014). Mitochondrial biogenesis: a therapeutic target for neurodevelopmental disorders and neurodegenerative diseases. *Curr Pharm Des., 20*(35), 5574-5593. doi:10.2174/1381612820666140305224906.

Ulusoy A., Phillips R.J., Helwig M., et al. (2017). Brain-to-stomach transfer of α-synuclein via vagal preganglionic projections. *Acta Neuropathol., 133*(3), 381-393. doi:10.1007/s00401-016-1661-y.

Vakifahmetoglu-Norberg H., Ouchida A.T., Norberg E. (2017). The role of mitochondria in metabolism and cell death. *Biochem Biophys Res Commun., 482*(3), 426-431. doi:10.1016/j.bbrc.2016.11.088.

Valdés P., Mercado G., Vidal R.L., et al. (2014). Control of dopaminergic neuron survival by the unfolded protein response transcription factor XBP1. *Proc Natl Acad Sci U S A., 111*(18), 6804-6809. doi:10.1073/pnas.1321845111.

Vandenberk B., Brouwers B., Hatse S., Wildiers H. (2011). p16INK4a: A central player in cellular senescence and a promising aging biomarker in elderly cancer patients. *J Geriatr Oncol, 2*(4), 259-269. doi:https://doi.org/10.1016/j.jgo.2011.08.004

Vauzour D., Rodriguez-Mateos A., Corona G., Oruna-Concha M.J., Spencer J.P. (2010). Polyphenols and human health: prevention of disease and mechanisms of action. *Nutrients., 2*(11), 1106-1131. doi:10.3390/nu2111106

Volodyaev I., Vladimirov Y.A. (2023). Enzymatic Sources of Free Radicals. *Springer. Nat.*, 219-261. doi: https://doi.org/10.1007/978-3-031-39078-4_14

Volpicelli-Daley L., Brundin P. (2018). Prion-like propagation of pathology in Parkinson disease. *Handb Clin Neurol., 153*, 321-335. doi:10.1016/B978-0-444-63945-5.00017-9.

Wang Z., Liu Y., Gao Z., Shen L. (2021). The Relationship between Levels of Serum Metal Ions and Parkinson's Disease. *Clin Lab., 67*(5). doi:10.7754/Clin.Lab.2020.200854.

Wilkaniec A., Lenkiewicz A.M., Babiec L., Murawska E., Jęśko H.M. (2021). Exogenous Alpha-Synuclein Evoked Parkin Downregulation Promotes Mitochondrial Dysfunction in Neuronal Cells. Implications for Parkinson's Disease Pathology. *Front Aging Neurosci., 13*. doi:10.3389/fnagi.2021.591475.

Williams-Gray C.H., Wijeyekoon R., Yarnall A.J., et al. (2016). Serum immune markers and disease progression in an incident Parkinson's disease cohort. *Mov Disord, 31*(7), 995-1003. doi:10.1002/mds.26563. Epub 2016 Mar 21

Wu A.G., Zhou X.G., Qiao G., Yu L., Tang Y. (2021). Targeting microglial autophagic degradation in NLRP3 inflammasome-mediated neurodegenerative diseases. *Ageing Res Rev., 65*. doi:10.1016/j.arr.2020.101202.

Xilouri M., Brekk O.R., Stefanis L. (2013). α-Synuclein and protein degradation systems: a reciprocal relationship. *Mol Neurobiol., 47*(2), 537-551. doi:10.1007/s12035-012-8341-2.

Yamashita K.Y., Bhoopatiraju S., Silverglate B.D., Grossgerg G.T. (2023). Biomarkers in Parkinson's disease: A state of the art review. *Biomarkers in Neuropsychiatry., 9*, 100074. doi:https://doi.org/10.1016/j.bionps.2023.100074

Yang W., Wang X., Duan C., Lu L., Yang H. (2013). Alpha-synuclein overexpression increases phospho-protein phosphatase 2A levels via formation of calmodulin/Src complex. *Neurochem Int., 63*(3), 180-194. doi:10.1016/j.neuint.2013.06.010.

Yaribash S., Mohammadi K., Sani M.A. (2025). Alpha-Synuclein Pathophysiology in Neurodegenerative Disorders: A Review Focusing on Molecular Mechanisms and Treatment Advances in Parkinson's Disease. *Cell Mol Neurobiol., 45*(1), 30. doi:10.1007/s10571-025-01544-2.

Yazğan Y., Nazıroğlu M. (2017). Ovariectomy-Induced Mitochondrial Oxidative Stress, Apoptosis, and Calcium Ion Influx Through TRPA1, TRPM2, and TRPV1 Are Prevented by 17β-Estradiol, Tamoxifen, and Raloxifene in the Hippocampus and Dorsal Root Ganglion of Rats. *Mol Neurobiol., 54*(10), 7620-7638. doi:10.1007/s12035-016-0232-5.

Ye H., Robak L.A., Yu M., Cykowski M., Shulman J.M. (2023). Genetics and Pathogenesis of Parkinson's Syndrome. *Annu Rev Pathol., 18*, 95-121. doi:10.1146/annurev-pathmechdis-031521-034145.

Ygland Rödström E., Mattsson-Carlgren N., Janelidze S., et al. (2022). Serum Neurofilament Light Chain as a Marker of Progression in Parkinson's Disease: Long-Term Observation and Implications of Clinical Subtypes. *J Parkinsons Dis., 12*(2), 571-584. doi:10.3233/JPD-212866.

Zeng H., Liu Y., Liu X., et al. (2025). Interplay of α-Synuclein Oligomers and Endoplasmic Reticulum Stress in Parkinson'S Disease: Insights into Cellular Dysfunctions. *Inflammation, 48*(8), 1590-1606. doi:10.1007/s10753-024-02156-6.

Zhu Y., Liu F., Jian F., Rong Y. (2024). Recent progresses in the late stages of autophagy. *Cell Insight., 3*(2). doi:https://doi.org/10.1016/j.cellin.2024.100152

Scientific publications

1. Dandurand J., Monné M., Samouillan V., Rosa M., Laurita A., **Pistone A.,** Bisaccia D., Matera I., Bisaccia, F., Ostuni A. (2024). "*The 75-99 C-Terminal Peptide of URG7 Protein Promotes α-Synuclein Disaggregation.*" *International journal of molecular science, 17;25(2):1135*.
2. Castiglione Morelli M. A., Iuliano A., Viggiani L., Matera I., **Pistone A.,** Schettini S. C. A., Colucci P., Ostuni A. (2024) "Redox Balance and Inflammatory Response in Follicular Fluids of Women Recovered by SARS-CoV-2 Infection or Anti-COVID-19 Vaccinated: A Combined Metabolomics and Biochemical Study." International journal of molecular sciences, 25(15), 8400.
3. **Pistone A.,** Matera I., Abruzzese V., Castiglione Morelli M.A., Rosa M., Ostuni A. "Enhancing Doxorubicin Efficacy in Hepatocellular Carcinoma: The Multi-Target Role of Muscari comosum Extract". Appl. Sci. 2025, 6509; 15(12). DOI: **https://doi.org/10.3390/app15126509**

Poster Presentations (Presenter)

1. **Pistone A.,** Matera I., Castiglione Morelli M.A., Sinisgalli C., Abruzzese V., Ostuni A. "*Muscari comosum bulbs extract modulates the doxorubicin effects in human hepatocarcinoma cells.*" FEBS Open Bio13 (Suppl. S2) (2023) 61-258 DOI: 10.1002/2211-5463.13646
2. **Pistone A.,** Dandurand J., Monnè M., Samouillan V., Rosa M., Laurita A., Bisaccia D., Matera I., Bisaccia F., Ostuni A. "*The 75-99 URG7 peptide and its analogs modulate the a-synuclein structures".* 48th FEBS Congress (2024)

Poster Presentations (Contributor)

1. Castiglione Morelli M.A., Iuliano A., Matera I., **Pistone A**., Viggiani L., Ostuni A. "*Protein and metabolic markers of oxidative and inflammatory stress are useful for delineating the biochemical profile of human follicular fluids in a pathophysiological context*". 62° SIB Congress (2023).
2. Matera I., Castiglione Morelli M.A., Zaccagnino R., **Pistone A.,** Bisaccia F., Abruzzese V., Ostuni A. "*Human ABCC6 protein comes into play in mechanisms which control the aggressiveness of hepatocarcinoma cells*". 62° SIB Congress (2023).
3. Matera I., Castiglione Morelli M.A., **Pistone A.,** Bisaccia F., Ostuni A. "*ABCC6 transporter: a player in cancer cell migration*". FEBS Open Bio13 (Suppl. S2) (2023) 61-258 DOI: 10.1002/2211-5463.13646

4. Matera I., Miglionico R., Abbruzzese V., Marchese G., Ventola G.M., Castiglione Morelli M.A., Zaccagnino R., **Pistone A.,** Rosa M., Bisaccia F., Ostuni A. *"The Abcc6 Knockdown alters the adhesion dynamics and aggressiveness of Human Hepatoma HepG2"*. 48th FEBS Congress (2024)
5. Ostuni A., Iuliano A., Viggiani L., Matera I., **Pistone A.**, Rosa M., Castiglione Morelli M. A.; "*A combined metabolomics analysis by 1H-NMR spectroscopy and biochemical studies as a useful strategy to evaluate the biochemical characteristics of follicular fluids of women approaching in vitro fertilization*" SIB (2024).
6. Matera I., Abruzzese V., Castiglione Morelli M.A., **Pistone A.**, Bisaccia F., Ostuni A. "*Old drugs for new molecular targets: the complex activity of Probenecid*." SIB (2024).