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PhD Course in Life Sciences and Biotechnologies

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Parkinson's disease: Immune System, Infections and Alpha-synuclein protein

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Foreword

This thesis is based on several manuscripts that were published during my PhD.

The work of this PhD thesis has been completed during my enrolment as PhD student at the Department of Biomedical Sciences, Section of Microbiology and Virology, University of Sassari, Italy, in the period from November 2015 to October 2018 under the supervision of Professor Leonardo Sechi. The studies of the thesis were also conducted for a period of five months at the University of Edinburgh, from September 2017 to January 2018, under the supervision of Professors Jürgen Haas and Richard Lathe. All the subjects enrolled in the study were kindly enrolled thanks to the collaboration of Dr Kai Paulus, Dr Giannina Arru and Professor GianPietro Sechi belonging to the Neurology Clinic of the University Hospital, Department of clinical and experimental medicine, University of Sassari, Italy.

Abbreviations

AD: Alzheimer's disease

AMPs: Antimicrobial peptides

Aβ: Amyloid beta

BBB: Blood-Brain Barrier

BDNF: Brain-derived neurotrophic factor

CFS: Cerebrospinal Fluid

CNS: Central Nervous System

COMT: Catecol-O-methyltransferase

DBS: Deep Brain Stimulation

ELISA: Enzyme-Linked Immunosorbent Assay

FCS: Fetal bovine serum

GFP: Green Fluorescent Protein

GSH: Glutathione

HCs: Healthy Controls

HSV1: Herpes simplex virus type 1

IgG: Immunoglobulin G

IL: Interleukin

Ldopa: Levodopa

MAO-B: monoaminoxidase B

MHC I-II: Major histocompability complex I-II

MPTP: 1-metil 4-fenil 1,2,3,6-tetraidro-piridina

MSA: Atrophy multisystemic

OND: Other neurologic disease

PBS-T: PBS-Tween 20

PD: Parkinson's disease

PET: Positron Emission Tomography

PMBCs: Peripheral Blood Mononuclear Cells

ROC: Receiver operator characteristic

SD: Standard deviation

SNpc: Substantia Nigra Pars Compacta

SNPs: Single nucleotide polymorphism

SSRIs: Reuptake inhibitor Serotoninergic

TNF: Tumor necrosis factor

UPS: Ubiquitin-proteasome system

α-syn: Alpha-synuclein

Abstract

Parkinson's disease (PD) is a neurodegenerative disorder and its etiology is unknown, but environmental factors are implicated in the development of this disease. In this project we wanted to analyze different roles played by α-syn, HSV1 and Immune System in PD. We have investigated autoimmunity in PD by ELISA test and a specific immune-stimulation using homologous peptides of HSV-1 and α-syn in PD patients versus HCs. Moreover, we have investigated the potential role of α-syn as an antimicrobial peptide and how this may contribute to α-syn aggregation, neuroinflammation, and widespread dopaminergic neuron death. Lastly, we have analyzed selected circulating miRNAs as noninvasive diagnostic candidate biomarkers of PD patients and neuroinflammation. The results obtained are in line with the hypothesis of a possible involvement of the immune system, in particular autoimmunity, in the pathogenesis of PD, and that HSV1 infections may lead to a progression of the disease. Concerning the role of α-syn as a potential antimicrobial peptides further studies are needed in order to clarify the complexity of the functions of this protein. Regarding identification of specific miRNA in PD, we have highlighted different levels of expression of some miRNA, 155 and 146a, between PD patients and HCs.

Riassunto

La malattia di Parkinson (MP) è una patologia neurodegenerativa e la sua esatta eziologia è ad oggi ancora sconosciuta, ma è noto che essendo una patologia multifattoriale i fattori ambientali possano avere un ruolo importante nella patogenesi di questa malattia. Visti questi presupposti lo scopo di questo progetto è stato quello di analizzare il ruolo svolto da diversi fattori come: la proteina α-syn, HSV1 ed il sistema immunitario nella patogenesi della MP. Abbiamo studiato il ruolo della risposta umorale, analizzando l'omologia molecolare tra HSV1 e α-syn umana, col presupposto che questa interazione possa condurre ad una più rapida progressione della MP, ed inoltre abbiamo analizzato il ruolo dell'immunità cellulo-mediata attraverso una specifica immuno-stimolazione, *in vitro,* con peptidi del HSV1 e gli omologhi umani dell' α-syn attraverso la metodica ICC, analisi delle citochine intracellulari, sui pazienti con MP e controlli sani. Inoltre, attraverso degli studi in vitro, abbiamo analizzato il potenziale ruolo antimicrobico dell'α-syn, che potrebbe contribuire sia all'aggregazione di se stessa che portare a fenomeni di neuroinfiammazione e alla morte diffusa dei neuroni dopaminergici. Infine abbiamo analizzato il potenziale ruolo dei miRNA circolanti come dei possibili biomarcatori non invasivi sia di diagnosi che di neuroinfiammazione nei pazienti con MP. I risultati ottenuti indicano il mimetismo molecolare come meccanismo molecolare di autoimmunità, in particolare correlato alla cross-reattività tra peptidi HSV1 e suoi omologhi dell α-syn, nelle membrane dei neuroni dopaminergici della SNpc. Inoltre, i risultati hanno mostrato, per la prima volta, una risposta cellulare specifica per le popolazioni di CD8, CD4 e NK secernenti TNF-α dopo stimolazione nei pazienti con MP. Quindi i nostri dati sono in linea con l'ipotesi di un possibile coinvolgimento del sistema immunitario, in particolare dell'autoimmunità, nella patogenesi della MP, e che le infezioni da HSV1 possano portare a una progressione della malattia. Per quanto riguarda α-syn come un potenziale peptide antimicrobico sono necessari ulteriori studi per chiarire la complessità delle funzioni di questa proteina. Rispetto all'identificazione del miRNA come potenziali biomarcatori dell'infiammazione nella PD abbiamo evidenziato diversi livelli di espressione di alcuni miRNA, 155 e 146a, tra pazienti con MP e controlli sani. Il miRNA 155 potrebbe non solo essere un obiettivo interessante per la terapia anti-infiammatoria nella MP, ma anche la sua valutazione putrebbe aiutare la diagnosi sugli stadi della malattia.

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Chapter 1: Introduction

1.1 Parkinson's disease: General hallmarks

Parkinson's disease (PD) is a complex and progressive neurodegenerative disease whose crucial physiopathogenic moment is represented by the loss of dopaminergic neurons localized in the Substantia Nigra Pars Compacta (SNpc), in particular in its ventrolateral portion. This leads to a deficit in the dopaminergic transmission involved in complex subcortical neuronal circuits exercise the Gangli of the base, which is a group of grey substance nuclei located at the base of both the cerebral hemispheres and densely interconnected with the cerebral cortex, the thalamus and the brain stem. The basal ganglia become part of the extra-pyramidal movement control and the correct functioning of these and their connections is of crucial importance to ensure the normal execution of the motor act. In fact one of the alteration is responsible for extra-pyramidal diseases that can extrinsecate influencing speed, fluidity and quality of movements in the hypercinetic sense, such as in Huntington's disease, both in the hypokinetic sense, such as in PD. In fact, the neuronal deficit found in SNpc, leads to a reduction of neurons that send dopaminergic projections to putamen. This, in turn, determines a hyperactivity of inhibiting signals that from the Gangli Base lead to the thalamus and, in consequence, a reduction of facilitator impulses from thalamus lead pre-motor cortex (areas 6-8 Brodmann); the result is an inhibition of movement. It should be recalled that in PD, in addition to the Nigro-striatal circuit just described, other dopaminergic pathways are also involved: the mesolymbic pathway, the mesocortical pathway and the Via Tubero-Infundibolare.

Dopaminergic depletion in SNpc neurons and the consequent alteration of the subcortical circuits that preside the execution of the movement, are responsible for the onset of the pivotal motor symptoms of PD:

1. muscular rigidity of the plastic type, initially unilateral, frequently associated with trochlea phenomenon;
2. resting tremor at medium-low frequency (4-6 Hz) which reduces with movement and sleep and is accentuated during emotional states. Initially unilateral, with onset in the distal part of the upper limb, it occurs characteristically in the act of "making pills" or "counting coins";
3. bradykinesia with alteration of the velocity, amplitude and rhythm of the movement which is considerably slowed down, frequently accompanied by akinesia and therefore difficulties in initiation the motor act. Manifestations related to akinesia are: facial hypomimia, fixity of the gaze with reduction of blinking, the monotony of speech, the sialorrhea caused by the reduced frequency of swallowing, the loss of the pendular movement of the upper limbs during walking, poverty of gestural language, walking with small steps and bending attitude of the bust, micrography and so on;
4. Postural instability with late appearance and generally crippling. It is the result of akinesia, stiffness, loss of postural reflexes and straightening [1]. These are generally associated with a variety of non-motor symptoms that can sometimes also precede the above motor manifestations [2]. In fact, they would seem to be also involved in the pathology other dopaminergic pathways placed at the plexus myoenteric, intestinal level and the olfactory system.

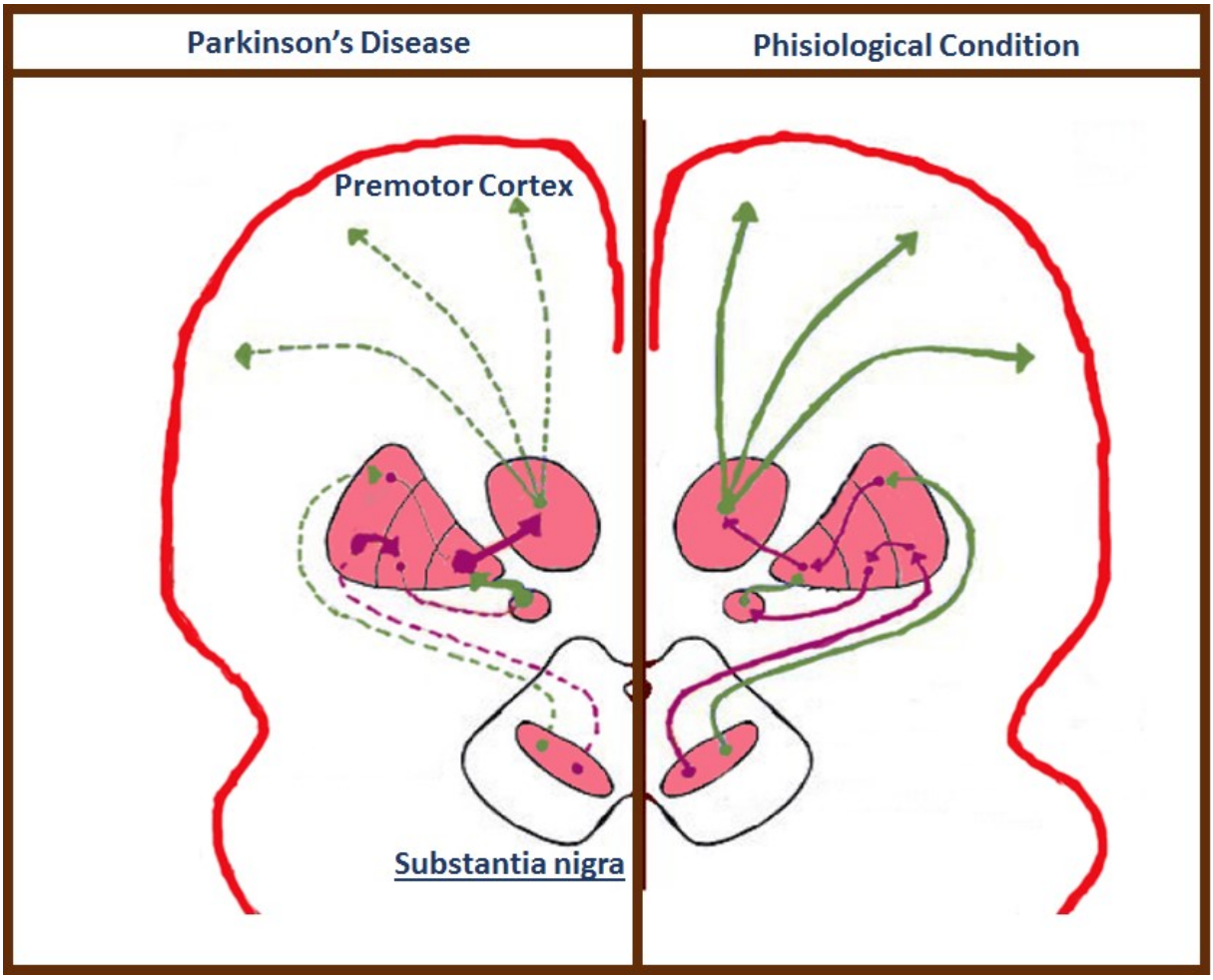

Figure 1. Shows the alteration, consequent of dopaminerg neurons dead, tipical of Parkinson's disease respect to the normal subcortical circuits that govern the execution of movement.

Givenits heterogeneity regarding the modalities of onset, the clinical

appearance, the symptoms and the speed of progression, we can distinguish mainly two forms: i) A form tremorigenic essentially characterized by tremor at rest, poor bradykinesia, generally deambulation well controlled, speed of progression of slow and disabled functional mild disease; ii) a form rigid-akinetic, in which the tremor does not represent the characterizing symptom and that is frequently associated with pictures more complex phenotypics such as rigidity-akinesia, early postural instability and disorders debilitating cognitive [3].

As far as non-motor symptoms are concerned, let us remember: hyposmia or olfactory dysfunctions, cognitive decay (from a dysexecutive syndrome to forms of real dementia), psychiatric symptoms such as depression (for alteration of the serotoninergic pathways, dopaminergic and noradrenergic), REM sleep disorders, constipation and dysfunction autonomic, pain, indefatigability, apathy (for degeneration of the corticosubcortical circuits that mediate the reward system). As already mentioned, some of these symptoms may precedes motor manifestations, thus representing podromic symptoms or of alarm for the development of PD and could become elements of consideration for an early diagnosis of the disease. Also, since symptoms as hyposmia or REM sleep disorders may precedes even 13-15 years the appearance of the motor symptoms, it would be identified a useful therapeutic window for prevent further damage and further loss of dopaminergic neurons, which causes of the onset of motor symptoms [4]. It is noted, in fact, that motor dysfunctions appear with the loss of at least 60% of the dopaminergic neurons of the SNpc [5]. The disease is progressive and to date, unstoppable. The only available therapeutic options are based on a symptomatic treatment of the disease which, so, can it be adequately monitored in its initial clinical manifestations, but not completely resolved. The progression of the disease implies the appearance of drug-resistant symptoms and complications determined by the long-term

symptomatic therapy: fluctuations motor and non-motor, dyskinesias and psychosis (visual hallucinations and less commonly tactile, auditory or olfactory) [6]. In the last stages of the disease the clinical picture gets complicated with the appearance of freezing, instability postural and frequent falls, cognitive alterations, dysphagia and dysarthria. Moreover, autonomic dysfunctions such as: urinary incontinence, constipation and orthostatic hypotension are frequent [6;7]. PD has a very important social and economic-health impact, both for its great prevalence and for the functional disable to which it inevitably leads. This disease represents the second neurodegenerative disease by frequency, second only to Alzheimer's disease (AD) [8], with a prevalence of 1-3% in the population over 50 [9] and a male ratio: females of 3:2 [10]. Although the first detailed description of PD was made more than two centuries ago (James Parkinson, "An essay on the Shaking Palsy", 1817), the pathogenesis of this disease has not been yet completely clarified [11]. The first piece of its understanding is been placed in 1912, when Lewy described of intraneuronal cytoplasmic inclusions which, subsequently, were denominated in his honor "Lewy Bodies" by Tretiakoff [12]. Subsequently the alpha-synuclein (α-syn) was discovered, described as a precursor to the non-amyloid component of the highlighted senile plaques in AD [13]. The possible pathogenetic role of this protein in PD was hypothesized in 1997, following the discovery of the SNCA gene that it is mutated in some very rare forms of familial PD with autosomal dominant transmission. Later, genetic studies have shown that polymorphisms and SNCA mutations are also related to sporadic Parkinson's forms [14], which represent the vast majority of cases. This gene, located in the chromosome 4q21-Q23, coding for the α-syn which would therefore play a fundamental role of in the pathogenesis of PD [15]. To confirm this, it was demonstrated how α-syn is the main component of Lewy bodies and Lewy neuritis, both characteristic neuropathologic elements of PD, but not only that as a result, this

disease must be considered as a full-fledged synucleinopathy, meaning by this term a heterogeneous group of neurodegenerative diseases that share common neuropathological lesions: intracellular proteinaceus inclusions consisting primarily of α-syn [16]. Although genetic, biochemical, and neuropathological studies suggest a clear pathogenic role of α-syn, how this or its derivatives actually trigger the neurodegenerative process is not still totally defined [17]. Furthermore, although several risk factors have been identified, the primum movens of the disease has not yet been identified. In this regard, efforts and studies are consistent to better understand the complex pathogenetic framework of PD.

The main risk factor to be considered is, of course, the age: the prevalence of PD increases with aging and has a spike after 80 years [18]. Among the environmental risk factors includes nutritional factors [19,20], exposure to some metals [21] and pesticides such as Paraquat [22] and Diquat [23]. As stated above, PD definitely has a certain genetic component. A story family of PD or tremor is therefore an additional risk factor for the development of the disease [24]. In addition to the SNCA gene coding for α-syn, numerous other genes related to PD have been identified: LRRK2, Parkin, PINK1 and others [25].Among the environmental factors it is also suggested the role of several infectious agents such as viruses, including Herpes Simplex virus, influenza viruses, HIV and several hepatotrophic viruses. In fact, there are many infectious agents able to overcome the blood-brain barrier (BBB) and induces inflammatory processes of cerebral parenchyma such as encephalitis. Furthermore, it should be emphasized that in the symptomatology of patients with encephalitis, we can to detect also characteristic clinical elements of Parkinson's [26], as proof of a possible correlation between the two pathologies.

The diagnosis is purely clinical and is based on the search for the pivotal symptoms of disease: tremor, bradykinesia and rigidity, while the postural instability is generally a late

symptom. In this regard, the criteria set out by the UK Parkinson's Disease Society Brain Bank are used [27]. The gold standard is, in fact, represented by the anatomopathological identification of the typical alterations of the disease, which the diagnosis is certainly post-mortem. At the present time there are no known biomarkers that to arrive at an early diagnosis, even if numerous studies are underway. A considerable help is given by imaging diagnostics. Techniques such as the DaTSCAN ™ (Ioflupane I 123 injection) allow you to make differential diagnoses with movement disorders not distinguished by the loss of dopaminergic neurons, such as the essential tremor. However these techniques are able to put into evidence of neuronal loss in the SNpc only when this has already significant [28], thus not being useful for an early diagnosis.

The main differential diagnosis of PD is represented by Parkinsonian syndromes of other nature such as: multisystemic atrophy, supranuclear palsy progressive, corticobasal degeneration, disease from diffuse Lewy bodies. In establishing diagnosis helps the combination of Parkinsonian signs, rapidly evolving and poorly suited to replacement therapy, and other neurological signs specific to the disease. Other alternative diagnoses are: parkinsonism post-encephalitis lethargicor other forms of viral encephalitis; vascular Parkinsonism (generally characterized by focal neurological signs caused by ischemia, worsening steps for the succession of ischemic events and a neuroradiological picture of encephalopathy multinophartual, together with a poor dopamine response); rare forms of toxic parkinsonism from manganese, mercury, carbon monoxide or drugs (phenothiazines, CA-antagonists, butiferrones). In the forms of tremorigenic Parkinson's, the main diagnoses differentials are represented by: the essential tremor, a condition almost always familial trait with autosomal dominant transmission; the tremor associated with hyperthyroidism, high frequency and associated with other signs of thyroid dysfunction; the tremor associated with

multiple sclerosis or chronic alcoholism. As already mentioned, the therapy of PD is exclusively symptomatic and focuses on the enhancement of dopaminergic transmission at the subcortical circuits level through drugs such as: levodopa, a natural precursor of dopamine, in the form of able to cross the BBB. Generally it is associated with drugs DOPA-decarboxylase peripheral inhibitors (Carbidopa or benserazide) in such a way as to reduce the daily intake dose. Other commonly used drugs are the MAO-B (monoaminoxidase B) and COMT (Catecol-O-methyltransferase) inhibitors that allow to reduce the dopamine metabolism at the Central Nervous System (CNS) level and increase its availability. Let us also remember the dopamino-agonists, ergolinic (Bromocriptine, Pergolide, Cabergoline) or non-ergolinic (Ropinorol and Pramipexole), which act through activation of dopaminergic postsynaptic receptors. In this class of drugs should be mentioned Apomorphine which has a pharmacological profile comparable to dopamine, and it is used in cases of PD characterized by severe motor fluctuations.

All these drugs just mentioned, act by improving the symptomatology of the patient and improving the quality of life sometimes with really amazing results. The tremor, unlike the other symptoms, responds inconsistently to replacement therapy and therefore its requires the association of other drugs such as anticholinergic, although these have a limited efficacy and numerous central and peripheral side effects that allow use only in patients more young people with PD predominately tremorigenic.

The medical challenge is the management of adverse effects (nausea, daily drowsiness and impulse control disorders such as chronic gambling, hypersexuality, hyperfagia, compulsive buying syndrome) and complications of long-term therapy associated, in particular, to the use of levodopa (Ldopa). In fact, although Ldopa, represent the most drug effective for the symptomatological management of the pathology, prolonged use is accompanied by a gradual

reduction of its effectiveness and to the onset of motor complications (dyinesias and motor fluctuations). These can be, at least in part, controlled with a better synergy between different classes of drugs, in order to optimize the concentration of dopamine at central level. The symptoms non-engines instead turn out to be more difficult to control. Talking about effects collateral it is must also mention a study that exposed a case of hyperpyrexia malignant with delirium consequent the sudden suspension of therapy with Ldopa. In this case, a rapid reduction in the concentration of dopamine, may have had an important role in the determination of the syndrome [29].

Other manifestations of the disease may benefit from targeted therapy: symptoms of depression can be controlled by the use of SSRIs (reuptake inhibitors Serotoninergic), the sialorrhea with the use of anticholinergic and the injection of botulinum toxin, sleep disorders improving their hygiene and controlling the nocturia, and so on. We also remember how the motor symptomatology of PD can also benefit from surgical treatments such as Deep Brain Stimulation (DBS), which is the stimulation of nuclei involved in subcortical circuits altered by the pathology, such as Sub-thalamic nucleus of Luys or on the Globus pallidus Internal [30]. In particular, DBS is an option for patients responding to levodopa, but with a quality of life deteriorated by complications of long-term therapy.

1.2 Alpha-synuclein

1.2.1 Structure and function of alpha-synuclein

α-syn is a small neuronal protein that is closely associated with the etiology of PD. The α-syn is a protein of 140 amino acid residues, being part of a family of small proteins that also includes the beta-synuclein and the gamma-synuclein. Originally described as a non-amyloid

component of the senile plaques in AD, α-syn certainly plays a pathogenic role in the PD. It is the major component of intraneuronal inclusions called Lewy bodies and Lewy's neurites, observable in SNpc but also in other districts such as spinal cord and peripheral nervous system (vague nerve, sympathetic ganglia, enteric plexus nervous system, salivary glands, adrenal medulla, cutaneous nerves and sciatic nerve) [31,32,33,34]. The same neuropathologic elements are also found in a group of diseases therefore called synucleinopathies. These diseases are represented by: dementia with Lewy bodies and MSA (atrophy multisystemic) and other lesser known forms [35]. The physiological role of α-syn does not completely known: it would act modulating the release of synaptic vesicles and would therefore play a role in the regulation of synaptic transmission [36]. In physiological conditions, α-syn, actually, could be found in numerous presynaptic terminals, in proximity of vesicles containing neurotransmitters. How this modulation happen is another element still to be clarified; however it is thought that its action may be carried out by stabilizing the proteins of the complex SNARE, whose assembly and disassembly is essential for the release of neurotransmitters [37]. It has been shown how that protein is naturally "unfolded", i.e in its pure form at neutral PH it is completely deployed, without structure secondary or tertiary. However, as a result of binding with acid phospholipids of membranes of synaptic vesicles, it assumes a structure folded to alpha-helical [38]. In its structure can be identified three domains: one N-terminal, one part called NAC (non-amyloid component of the senile plaques) and a domain C-terminal. Each of these gives of the protein a particular characteristic. The N-terminal domain would determine the tendency of the protein to take on a conformation to alpha-helical when linked to vesicular membranes; the central part hydrophobic NAC, it would be responsible for the conformation Beta-leaflet and the protein's

tendency to form fibrillary aggregates. The C-terminal portion, full of negative charges, which would depend on the characteristic of protein "unfolded" [39].

Considering the different studies, we can therefore affirm that this protein, physiologically, exist in various unstructured and oligomeric states in equilibrium between them thanks to factors that promote and inhibit fibrillar aggregation [40], i.e. aggregation in structures in Beta-leaflet.

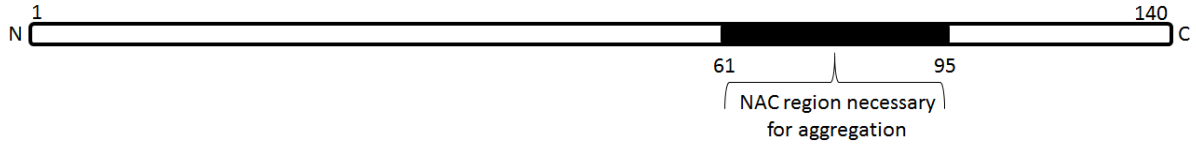

Figure 2: Structure of alpha-synuclein.

The toxic subtype of α-syn is not still identified with certainty. Some studies indicate how toxic the insoluble amyloid-like fibrils identifiable in Lewy bodies. Other studies indicated as toxic the soluble prefibrillary intermediates such as oligomers. Recently, a growing number of studies have shown how species oligomeric, rich in structures to Beta-Leaflet, are neurotoxic [41,40,42]. Then these oligomers can aggregate to form protofibrils of higher molecular weight, insoluble, whose further polymerization brings to the formation of amyloid fibrils, resembling those found in the Lewy bodies.

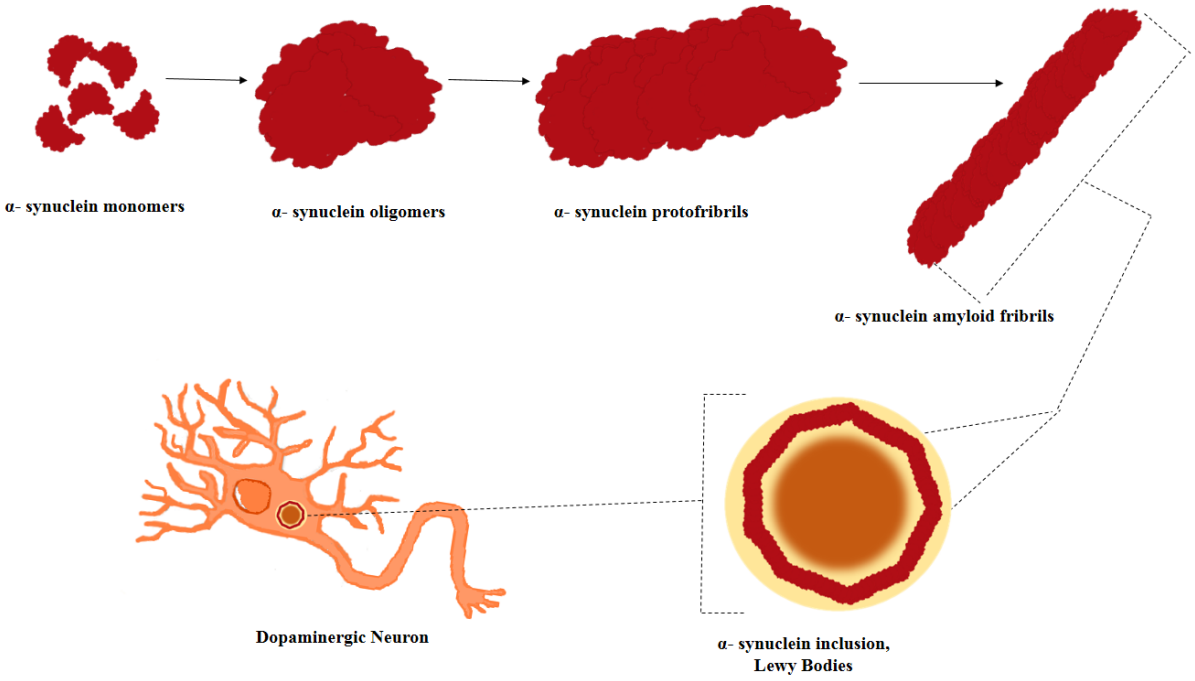

Figure 3: The image shows the different unstructured forms assumed by α-syn. In physiological conditions it is found in an inherently unfolded native form and a alpha-Helix form when it is linked to membrane phospholipids. Studies show how monomeric forms of α-syn can be aggregated into oligomeric species rich in structures in beta-leaflet and later in higher molecular weight protofibrils, insoluble, which can then further undergo polymerizing and therefore to the formation of amyloid fibrils similar to those found in Lewy bodies.

The latter could even be the result of a protective mechanism activated by neurons [43]. It would seem that dopamine and its metabolites succeed in inhibit, in vivo, the conversion of protofibrils into fibrils, thus determining accumulation of neurotoxic protofibrillar intermediates. That would explain the high sensitivity of the of dopaminergic neurons to the toxic effects of α-syn, which instead are less significant if we consider the other neuronal systems. [44]

1.2.2 Degradation of alpha-synuclein and pathologic correlations

α-syn is encoded by the SNCA gene. Six mutations are currently known that affect this gene and that as a result cause an alteration of α-syn. These mutations are associated with a parkinsonian phenotype characterized by early onset, rapid disease progression and rapid onset of non-motor symptoms such as hallucinations, autonomic dysfunctions, dementia [15]. It should be remembered that is thanks to the study of these very rare forms of familial Parkinson's that the interest int o α-syn research was born. However, studies have shown that, if overexpressed, may cause onset of PD (as well as expression of α-syn wild type). In addition it has been confirmed that the severity of disease is directly related to the degree of overexpression of the protein. To confirm this, patients who have triplication of the SNCA gene have a more severe clinical picture and rapidly progressive than patients that present a duplication of the gene [45-46]. This indicates that the levels of the protein should be maintained into a certain physiological range, thanks to a balance between production, degradation and secretion.

In the pathogenesis of PD, alterations of the normal mechanisms to maintain proper proteostasis could be implicated, in particular: the ubiquitin-proteasome system (UPS) and the lysosome mediated autophagy system (including microautophagy, macroautophagy and autophagy chaperone-mediated according to the modalities with which the protein that it to be degraded is transported within the lysosome) [47]. Several studies have shown how the alteration of these physiological pathways can actually lead to an accumulation of α-syn, through a reduction in its clearance. For example: it is demonstrated how, total protein concentration, increases after the inhibition of lysosomal-mediated autophagy [48], while the results about the actual recognition and destruction of α-syn by the proteasome are discordant. In particular, it would seem that the wild a α-syn is selectively degraded from a chaperone-

mediated process, whereas all forms of α-syn can be degraded by macroautophagy processes [49, 50].

The inhibition of chaperone-mediated protein degradation leads to an accumulation and to the aggregation of high molecular weight intermediates and insoluble α-syn species in neurons [49], and therefore, physiologically, it would have a fundamental role in prevention of accumulation and aggregation of α-syn. So, it can be said that an alteration of the chaperone-mediated autophagy plays a pathogenetic role in the PD. Moreover, it would appear that α-syn itself determines the alteration of the lysosomal function, thus going to create a vicious circle: accumulation of α-syn→ lysosomal damage → further accumulation of protein. In this regard some studies show how the main proteins involved in chaperone-mediated autophagy (Hsc70 and Lamp2a) are reduced in the brain tissue of patients with PD [51].

The alteration of the chaperone-mediated function could occur through a compensatory activation of macroautophagy processes. In fact, several animal models of synucleinopathies have shown indicative elements of an excessive activation of macroautophagy processes (e.g. certain specific markers) or indicative, however, of an alteration of the fusion of the autophagic vacuoles with lysosomes [52].The consequences of this compensatory activation are not clear: some studies indicate how this mechanism can be protective against α-syn neurotoxicity; other studies, on the contrary, indicate how the activation of macroautophagy is deleterious. These different effects may not exclude each other and also depend on the stage of the disease.

In the early stages, the compensatory activation of macroautophagia could represent a defense mechanism that proves to be counterproductive and harmful in the advanced stages of the disease [17]. Further evidence of the involvement of the lysosomal function in PD, comes from the study of Gaucher disease. This is a storage disease, hereditary, determined by

mutations “Loss of function”of the GBA gene coding for the glucocerebrosidase, a lysosomal enzyme. It is characterized by the accumulation of glucosilceramide in the reticulo-endothelial cells of the spleen, bone marrow and liver. The presence of this mutation also leads to an increase in the risk of developing PD or other types of diseases with Lewy bodies [53]. In fact, the mutation determines a whole series of consequences:

1. A lesser affinity of the enzyme Glucocerebrosidase for α-syn compared to the enzyme Wild-Type; [54]
2. A reduction in the activity of Glucocerebrosidase and consequently an α-syn accumulation and neurotoxicity; [55]
3. Accumulation of glucosylceramide that would seem to “stabilize” species oligomeric α-syn, and increase its toxic potential. [55]

It was also demonstrated that overexpression of the Glucocerebrosidase enzyme wild-type in animal models of Gaucher disease, is able to invert the process of accumulation of α-syn and its histopathological alterations [56]. So, most likely, the accumulation of α-syn plays a role in the pathogenesis of PD.

1.2.3 Interaction between alpha-synuclein and biological membranes

The mechanisms through which α-syn or its different isoforms exert their toxic potential are different and not completely known. Some studies suggest that normal and neuropathogenic functions of α-syn may be different from the molecular point of view, contributing to neurodegeneration. The role of α-syn oligomers in binding and permeabilization of cell membranes has been also reported [57], the α-syn oligomers have the ability to bind themselves to the lipids of different biological membranes, going to increase its permeability

at the mitochondrial level, lysosomal and of synaptic vesicles. Through this link, it could be explained both the physiological effects of proteins, as well as pathological ones. In fact it has been shown as this link can lead to an increase in the influx of calcium ions within the organelles, alterations in ionic equilibrium and finally cellular death through activation of the pathway activated by the Caspase 3 [58].

It has also been shown how the α-syn molecules be subject to a series of post-translational modifications that might somehow affect its toxic potential and the ability to bind to membranes. Among these modifications we remember: phosphorylation in sites Ser87 and Ser129 of α-syn isolated from tissue from encephalic patients with PD [59]; processes of nitration and oxidation, which they should reduce the propensity of α-syn to form insoluble fibrils and they would "stabilize" the oligomers, there by increasing the toxicity [60] and many other modifications. So this protein could play a role physiopathological also through the connection with the biological membranes, going to alter the functionality of different intracellular organelles. The interactions between α-syn and mitochondria have been widely studied: in different works, the protein has been found in association with outer membranes of these organelles, closely tied to Cardiolipin [61]; its bondto the inner membrane of the same, turns out to be less certain [62]. Furthermore α-syn is able to determine a down-regulation of complex I, i.e the succinate-coenzyme Q reductase involved in the mitochondrial respiratory chain.

It has also demonstrated as both the mutated protein and the wild-type can determine alterations of mitochondrial morphology [63]. These alterations consist in: swelling, membrane distortion or crystal formation even to the fragmentation of mitochondria. These effects would seem to be mediated by the bond of the protein with the mitochondrial membrane, towards which it presents marked affinity due to its wealth in Cardiolipin [61].

What kind of α-syn can to determine these effects, is not yet been clarified. The fragmentation of mitochondria could trigger a cascade of events that culminates with the loss of potential of mitochondrial transmembrane, respiratory chain alteration and neuronal death [61]. The same interactions could also occur with the biological membranes of other organelles like the already mentioned lysosomes, presynaptic vesicles, endoplasmic reticulum or the Golgi apparatus. Also, the effects exercised by the α-syn on the mitochondria, could determine the release of reactive oxygen species, thus triggering a vicious cycle that leads to the further accumulation and aggregation of the protein [64]. As already affirmed the presence of α-syn causes excessive activation of the macroautophagy processes. In parallel, a specific pathway appears to activate the mitophagia who, together to the already mentioned macroautophagy, leads to a depletion of mitochondria. Also the mitophagia, therefore, could represent a mode through which the process of neurodegeneration is determined.

However, it is necessary to clarify how the aforementioned subcellular alterations may represent also a consequence of normal ageing, which always represents the main risk factor for the development of PD [20].

The involvement of mitochondria in the pathogenesis of PD has been widely described by several studies using mitochondrial toxins such as MPTP (1methyl-4 Phenyl-1, 2, 3.6 tetrahydropyridine) or 6-Ohda. These molecules are able to induce onset of Parkinson's symptoms without determining the appearance of Lewy bodies. For example the MPTP, found for the first time as a contaminant of a drug in a cluster of young students who have developed PD, is metabolized by the enzyme MAO-B to form the MPP + ion, which is able to inhibit complex I of the mitochondrial respiratory chain by inducing cell apoptosis.

Furthermore, mutations that alter mitochondrial function have been identified and are responsible for some cases of autosomal recessive PD. These mutations involve the genes:

1 Parkin coding for an E3 ligase involved in mitophagy processes.

2 PINK1 is a mitochondrial kinase that is always involved in the process of mitophagy.

3 Others like LRRK2, DJ-1.

1.2.4 Interactions between alpha-synuclein and cytoskeleton proteins

α-syn could exert its pathological role, as well as on organelles cytoplasmic, also on other cellular constituents. The interactions between this protein and the elements of the cytoskeleton were therefore studied, in order to highlight a possible physiopathogenetic correlation with PD. The studies in this regard are manifold and sometimes contrasting. The extracellular application of α-syn would seem to lead to a reduction in polymerization of tubulin [65]. Vice versa, studies conducted on yeast cultures, have shown how the inhibition of polymerization of tubulin may trigger the aggregation of α-syn [66]. This last statement has been questioned by other studies which, on the contrary, have shown as the oligomerization of tubulin promotes the aggregation of α-syn [67]. It has also been shown that α-syn is able to determine the hyperphosphorylation of Tau free protein, this protein stabilizes the structure of microtubules and regulates their spatial organization [68]. The effects of this phosphorylation remains to be clarified. We remember that Tau protein, like α-syn, is involved in the development of a whole series of neurodegenerative disease characterized by an alteration of its metabolism, such as the AD.

A confirmation of a possible interaction between cytoskeleton and PD comes, once again, from genetic studies. In fact, there are very rare cases of autosomal recessive hereditary Parkinson related to mutations in the gene LRRK2. This gene coding for a kinase whose activity is directly related to the functions of the cytoskeleton. In fact, similarly to α-syn,

LRRK can determine cytoskeletal instability through the hyperphosphorylation of tau or directly through the phosphorylation of beta-tubulin.

1.2.5 Alpha-synuclein and synaptic transmission

Given the physiological role of α-syn in the release of synaptic vesicles, it follows that its alteration will inevitably reflect on the synaptic transmission. It has been shown that an alteration of the α-syn leads to:

1. Loss of pre-synaptic proteins essential for the release of neurotransmitter-rich vesicles; [69]
2. Reduction of the pool of synaptic vesicles and consequently the release of neurotransmitters in synaptic space; [70]
3. Redistribution of SNARE proteins; [71]
4. Morphological alterations of synaptic vesicles;
5. Reduction of synaptic vesicle recycling.

As Calcium plays a key role in synaptic transmission, probably α-syn acts by altering the homeostasis of this ion. In fact, it would seem that α-syn determines the formation of pores at the cellular membrane level [72], significantly increasing the intracellular flow of calcium ions. This leads to an alteration of the membrane potential of the synaptic terminal and the loss of physiological pace-maker activity in dopaminergic neurons that normally is guaranteed by an optimum calcium concentration and the voltage-dependent calcium channels L [29]. Through the same mechanism, it is thought that α-syn can also bind to synaptic vesicles, forming pores with consequent loss of molecules of neurotransmitter within the cytosol. In particular, in dopaminergic neurons, an excess cytosolic dopamine can be detrimental to the cell with induction of oxidative stress and cellular death [73]. Finally, we

remember that the name of α-syn is due to the fact that this protein has been initially highlighted both at the synaptic level but also at the nuclear level. However the levels of protein found at the nuclear level are generally inconsistent [74].

1.2.6 Secretion and propagation of alpha-synuclein

Although the studies are countless and the results are sometimes promising, the triggers that lead to the accumulation of α-syn are not yet identified, and it is not known what it is exactly the way that towards which it presents marked affinity due to its wealth in Cardiolipin leads to neurodegeneration.

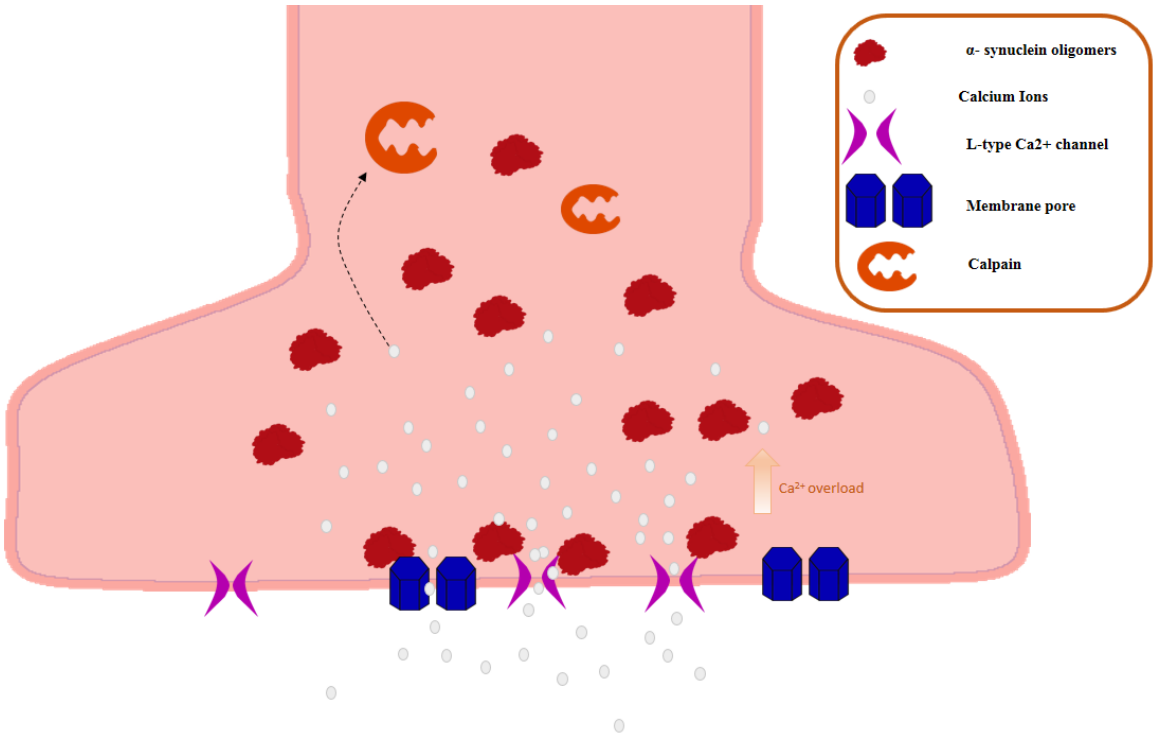

Figure 4: α-syn and synapses. Notes the accumulation of Ca^{++} in the cytoplasm of synaptic terminal.

The understanding of these mechanisms would lead to the resolution of numerous question marks that characterize the different aspects of the PD, but it would also open the way to the understanding of other related neurodegenerative diseases and especially to new horizons and therapeutic possibilities to reduce the neurotoxicity of α-syn [75]. The new knowledge and information about it are constantly increasing.

For example: α-syn has been considered, for a long time, a protein exclusively intracellular. In fact, new evidence underline how it can be found also in different extracellular fluids, such as plasma and Cerebrospinal Fluid (CFS) [76,77]. This new data also opens the way to new diagnostic opportunities. Studies have been conducted on saliva samples of PD patients, to seek a correlation between protein levels and the onset of the disease, in the hope of identifying a cut-off that may lead to an early diagnose. To that regard we quote one study where the total amount of α-syn in the saliva of PD patients was reported considerably lower than that of HCs. Also, the same study, highlighted a greater salivary levels of α-syn oligomers in PD patients versus HCs [78]. This data could be used in the future in order to develop a test for the early diagnosis of PD.

The modality of secretion of this protein has not yet been completely clarified, although we think of a mechanism of non-classical exocytosis through exosomes, of the endocytic vesicles derived from multivesicular bodies and released with a Ca-mediated mechanism.

This process could represent a real language of communication between one cell and the other, but it could also represent a way of propagation of α-syn. This hypothesis has been processed as a result of the following discovery: fetal dopaminergic transplants in human striatum develop Lewy bodies disease years after the transplant, suggesting a possible transmission "Host-Graft" of α-syn. However it has not been possible to ascertain that the α-syn included in the transplant were derived from the host and were not primarily formed in

the tissue transplanted [79]. This ability to propagate α-syn, would depend on the capacity of the cells to capture the protein. Actually, studies, show how α-syn oligomers are particularly prone to being captured by the cells and therefore they would have a role in the dissemination of pathology. [80] The transmission cell-to-cell of α-syn has been demonstrated in vitro [81], but also in vivo in transgenic mice [82] [83].

These notions suggest the hypothesis of a possible prion-like transmission of α-syn, which could also explain the advancement of the pathology in PD and explained in the Braak staging. This in fact divide the pathology into different stages based on the location of the neuropathological alterations. Initially there is an exclusive involvement of the ganglia autonomic, the anterior olfactory nucleus and of the dorsal motor nucleus of the vagus. After the disease progresses in an upward way, involving extensive areas of the CNS and in particular the SNpc, determining the appearance of the motor manifestations of PD: tremor, bradykinesia and rigidity. As the disease progresses, the anatomopathological alterations also spread at the cortical level, associated to cognitive decline and psychiatric disorders [84].

According to prion-like theory, α-syn misfolded could have a pathogenetic behavior similar to PRPSC prion protein and, like this, it would propagate from cell to cell inducing formation of further α-syn misfolded, thus contributing to the progression of the disease.

We recall that prion diseases includes different forms of transmissible spongiforms encephalopathies, where the pathogen agent is not a common microorganism, but it is represented by an abnormal protein (PRPSC) which, once formed, is able not only to spread in the individual, but is also endowed with infectious properties.

These diseases, such as Creutzfeldt-Jacob disease and its variants, Gerstmann-Straussler-Scheinker disease, Kuru and fatal familial insomnia, can be transmitted from individual to individual.

We talk about prion-like transmission of α-syn, because this protein could have the same transmission capacity cell and the ability to induces the formation of a further protein misfolded, namely the ability to induce alteration of endogenous α-syn normally conformed. However, the infectious capacity of the protein has not been demonstrated, and in fact no reports of cases of inter-human transmission of synucleinopathies are so far reported.

According to the Braak's theory of and his collaborators and called "dual-hit" hypothesis, a pathogen agent with prion-like properties, such as α-syn, would initially be localized at the olfactory epithelium level, probably in response to the inhalation of a neurotropic agent not better specified. Then it could be also localized at the level of the intestinal epithelium following the ingestion of nasal secretions together with saliva. Again according to Braak's theory, this hypothetical agent, it would be propagated from the olfactory epithelium to the temporal lobe and in via retrograde from the intestinal epithelium to the CNS, through the fibers of the vagus nerve [85,86]. Actually Lewy bodies were found both along the structures of the olfactory via (anterior olfactory nucleus, olfactory tubercle and olfactory cortex)[87] which in nerve cells of the enteric plexus of patients with PD [88].

It is unlikely that α-syn may penetrate into the organism directly through the olfactory via. It's more likely to be present in these locations, as a result of contact with substances present in the environment, such as viral agents or pesticides, which actually represent a risk factor for the development of the disease. This theory was very successful, as the filaments of the olfactory nerve are the only nerves that are directly in contact with elements from the environment. To confirm a possible propagation of the α-syn, a study has put in evidence also the correlation between the activity of this protein and mitochondrial activity. Indeed the intragastric administration of Rotenone, an inhibitor of the mitochondrial complex I, leads to the appearance of α-syn included in the intestinal epithelium, which gradually also affect the

CNS, including dopaminergic neurons. This study, therefore, indicates how α-syn can propagate independently from the point of origin of its accumulation [89].

Although this theory is appealing, there are still numerous unresolved questions and arguments against it. For example: there are no studies that confirm how the dopaminergic neurons, primarily affected by PD, have the ability to capture α-syn from other cells or that the transfer of the protein could induces the formation of Lewy bodies. Furthermore, it should be stressed out that the central role of α-syn in triggering neurodegenerative events is not accepted unanimously. In fact, there are numerous alternative theories that explain the etiopathogenesis of PD. Some studies, for example, have shown a decrease in the concentration of reduced Glutathione (GSH) in the SNpc of PD patients. This could uncover other mechanism involved in neurodegeneration and also possible new therapeutics target. A study has highlighted the therapeutic efficacy of the GSH in patients with PD untreated with a substitute therapy [90]. Moreover, other studies have shown that GSH deficiency is directly proportional to the severity clinical pathology, further emphasizing the potential role of this substance in the pathogenesis of PD [91]. Other studies suggest that alteration of the α-syn and its aggregation, could be an epiphenomenon caused by other processes such as, for example, neuroinflammation, which actually is detected in postmortem histologic preparations of patients with PD [92]. It's also true though that the same extracellular α-syn, once secreted, is able to induces neuroinflammation by activating glial cells. To support this theory, it has been shown how the glial cells are able to capture and degrade the α-syn aggregates in a more effective way than neurons [93]. Activation of microglial cells would cause the release of protective molecules as brain-derived neurotrophic factor (BDNF) but also pro-inflammatory cytokines, reactive oxygen and nitrogen species [94], there by definitely playing a role in the progression of neurodegenerative disease. Also, it has been

shown as the α-syn nitrated is not recognized as a self and, therefore, causes the formation of harmful species of helper T lymphocytes that could be another cause of neuronal damage [95]. This indicates the importance of the Immune System in the pathogenesis of PD. The maintenance of a correct extracellular homeostasis of α-syn, would be another key piece to keep a correct brain function and could represent a possible therapeutic target in the near future.

1.2.7 Alpha-synuclein as an antimicrobial peptide

Currently, the exact physiological function of α-syn is still not fully known, and also the exact mechanisms that lead to toxicity with subsequent neuronal death are still unclear, but the deposition of α-syn fibrils in Lewy Bodies in dopaminergic neurons is one of the main features of PD, Lewy Bodies are composed largely of beta-sheet rich α-syn amyloid fibrils [96]. Oligomerization in particular is viewed as a pathogenic pathway and α-syn oligomers are assumed to be intrinsically abnormal. Antimicrobial peptides (AMPs), an evolutionarily very old family of proteins, have the characteristic of generating oligomers and fibrils, as well as α-syn. These properties play a key role in mediating the processes of defense of innate immunity, AMPs are the first-line of defense against pathogens and act as potent broad-spectrum antibiotics and immunomodulators that target bacteria, mycobacteria, enveloped viruses, fungal, and protozoans, and in some cases, transformed or cancerous host cells [97]. AMPs are expressed in many tissues, but it has been reported that there is a notable expression in the brain [98], as well as in other tissues where the intervention of the adaptive immune system is limited. Normally the action of this class of peptides turns out to be protective, but their dysregulation can lead to toxic effects in the host cells [99,100], such as chronic inflammation [101,102,100,103] and degenerative diseases [104]. Recent studies

reveal a possible antimicrobial action of amyloid-beta (Aβ), suggesting that Aβ deposits may be a consequence of the protective action of this peptide against infections, for example Bourgade et al showed Aβ as an antiviral activity *in vitro* study of HSV-1 infection of human fibroblasts, epithelial and neuronal cells [105,106,107,108, 109].

Regarding the α-syn Park et al demonstrate that α-syn exhibits antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. In addition, the authors demonstrate a role for α-syn in inhibiting various pathogenic fungal strains such as *Aspergillus flavus*, *Aspergillus fumigatus* and *Rhizoctonia solani*. They also analyzed localizations of recombinant α-syn protein in *E. coli* and *Candida albicans*. These results suggest that in addition to α-syn's role in neurotransmitter release, it appears to be a natural AMP [110]. Beatman et al reported that the neuronal expression of α-syn is able to inhibit viral infection by West Nile Virus (WNV), in fact the authors observed an increase of α-syn expression in neurons infected with WNV. At the same time they observed that knockout mice for α-syn develop a higher viral titre respect to wild-type animals. this data suggest a possible action of α-syn as AMPs [111].

1.3 Inflammation in Parkinson's disease

Inflammation has been increasingly studied as part of the pathophysiology of neurodegenerative diseases, corroborating the hypothesis that the immune system may be the nexus between environmental and genetic factors, and the abnormal immune function can lead to disease. Since 1988, McGeer's research team suggested that inflammation could be the first mechanism for PD [112]. At the same time the role of inflammation in PD is indirectly proven by the use of non-steroidal anti-inflammatory drugs that decrease the risk for PD. Several scientific evidences documented inflammatory processes in PD patients, such as microglia activation, cytokine production and the presence of autoantibodies [113, 114, 112].

In vitro studies showed the presence in serum of PD patients of antibodies that recognize some of the membrane proteins in a model of dopaminergic neurons. McGeer through immoistological assay reported the presence of microglia in the striatum of PD patients with a consequent production of pro-inflammatory cytokines[115]. Nagatsu et al documented in striatum autopsy findings and in CSF of PD patients high levels of cytokines and also high levels of proapototic proteins, a clear sign that inflammation is a constant element in the disease [116]. However, it remains to be explained whether inflammation represents the primary cause of neurodegeneration or it is the results of damage processes and cell degeneration. Inflammation appears to be a constant feature in this disease, and at the same time neurons death would seem to support the inflammatory process in the CNS [117]. It is known that the neurotoxin MPTP is able to cause neuronal damage and parkinsonian syndromes and a study conducted by Langston et al showed that people who had been exposed to this toxin presented an activation of microglia that could be found post-death up to 16 years after death in the autopsy findings [118]. This is a further evidence that neuronal damage is associated to a neuro-inflammation process. Moreover all these data are supported by the numerous studies conducted on Parkinson's animal models.

Several studies conducted in animal models showed that the MPTP [119] rotenone (Gao et al., 2002a; Sherer et al., 2002) and 6-hydroxydopamine (6-OHDA) are able to activate microglia. In the same way it has been observed that microglial activation LPS-inducted cause dopaminergic neurodegeneration in vitro and in vivo studies [122, 121, 122, 123, 124, 125].

1.3.1 Activation of Microglia

The microglia cells protect and repair neurons in the CNS [126]. Different kind of insults can activate the microglia, and they can be external or internal signals such as neuronal dysfunction, trauma or some toxin. Furthermore a wide range of substances such as viral or bacterial proteins, α-syn, cytokines and antibodies can induce the activation of microglia [127]. Usually after that microglia produces different molecular mediators that have a chemotactic and immunomodulatory function, such as reactive oxygen species, prostanoids and cytokines. In PD Tumor necrosis Factor (TNF) has an important role: it would seem to be able to modulate synaptic plasticity [128, 129, 130]. In the state of chronic neuroinflammation it has been observed the expression of MHC-II molecules in microglial cells while it has not been observed in the CNS of healthy people [131]. Different authors reported in PD brains the presence of HLA-DR+ microglia, and observed that the microglia and macrophage activation marker CD68 had a positive correlation with disease duration and with the deposit of α-syn. Moreover studies conducted by PET confirmed the activation of microglia in PD [112, 132, 133]. People with single nucleotide polymorphism (SNPs) in MCH-II locus showed an increased risk to develope PD, it is an indirect proof of the importance of adaptive immunity in PD [134]. Furthermore the expression of MHC-II in neurons can modulate the immunoresponse and the neuroinflammation in the CNS. There are numerous factors that can induce microglia activation leading to neuroinflammation and destruction of dopaminergic neurons. Activation factors can be proteins such as: α-syn aggregates, Neuromelanin, MMP-3, Fibrinogen; or environmental toxins such as: LPS, MPTP, Rotenone, Paraquat, Pesticides, Proteasome, Heavy metals[135].

1.3.2 The role of T cells

Naïve and memory T cells perform homeostatic surveillance in the CNS [136, 137], for this reason they may be involved in initiating and propagating PD pathogenesis. T-cell infiltration has been observed in postmortem brain sections of PD patients [138]. Several studies have analyzed the composition of T-cell subsets in the peripheral blood of PD patients showing that immune response is altered in these patients. The overall numbers of lymphocytes was decreased but not the frequency [139, 140]. Besides an increased number of memory T cells but a decreased number of naïve T cells was observed in PD in comparisonto other neurologic diseases (OND) [141]. Memory T cells responded faster and with greater magnitude than activated naïve T cells [142]. In PD patients a decreased CD4+:CD8+ ratios and a shift to more IFNγ− versus IL-4-producing T cells was observed suggesting a the cytotoxic T-cell response [143, 140, 144]. To date, nothing is known about the identity of CNS antigens to which activated and memory T cells are responding in PD patients.

CD8+ T cells in these kind of patients have a lower frequency of Vβ8 expressing cells [145]. Some candidate proteins such as β-fibrinogen and transaldolase have been identified within T cells and investigated as possible biomarkers [146]. PD patients presented some pathogenic changes in PBL. Lymphocytes displayed an increased incidence of micronuclei, single-strand DNA breaks, and oxidized purine bases [147]. Interestingly, DNA damage seemed to shrink after levodopa treatment [148]. Moreover in lymphocytes of PD patients were observed increased level of apoptosis, caspase-3 activation, and Cu/ Zn superoxide dismutase activity [149]. T cells contribution to PD-like pathology has been assessed mainly in animal models using neurotoxins. In mouse model of PD the overexpression of intranigral AAV-human-α-synuclein, caused a B- and T-lymphocyte infiltration that persisted in the CNS after peak of

microglial activation, suggesting that microglia in the CNS involved adaptive immune cells to propagate inflammation [150].

1.3.3 Humoral immunity

Humoral immunity plays an important role in many neurodegenerative diseases, and B lymphocytes with the production of antibodies are the main protagonists. Different authors report in the peripheral blood of PD patients a decrease of B cells number [151].Some studies suggest that the proliferation of lymphocytes can be influenced by Ldopa treatment, while other authors suggest that levodopa treatment is not correlated with the decrease of lymphocyte [144, 140].Some authors reported the presence of antibodies against DA neurons in PD patients respect to healthy controls [152, 153]. Furthermore the presence of immunoglobulins near of DA neurons in neurohistological reperts from PD brains has been reported [154]. It can be hypothesized an interaction between microglia and B lymphocytes. Some studies with a mouse model transfected with AVV-α-syn vector showed a significant IgG deposition in the midbrain, suggesting that humoral immunity can play an important role in neurodegeneration of PD [155]. All of these data suggest that humoral immunity can induced a progression of PD.

1.3.4 **Proinflammatory cytokines**

Chronic neuroinflammation with production of cytokines has been observed during PD. Probably neuroinflammation doesn't represent an initiating factor but it's clear that sustained inflammatory responses involving microglia and astrocytes may contribute to progression of PD. It has been observed that some cytokines such as TNF and IFN-γ have a high affinity for dopaminergic neurons [156, 157]. Microglia is the biggest producer of these cytokines in the

CNS, and is able to induce a great sensitivity of dopaminergic neurons [158]. Furthermore, some cytokines such as IL-1 IL-1β, TGF-β, IFN-γ, and IL-6 appear to be present at high concentrations in CSF and in striatum of patients with Parkinson's compared to healthy controls [159,113, 160]. In addition other studies have reported that high levels of cytokines have also been found in people with PD at the peripheral level and it would seem to have a positive correlation with the degree of disability [161]. Elevated level of some pro-inflammatory cytokines including IL-1α, IL-1β, IL-6, and iNOS are a risk factor for PD as demostred from genetic analyses of DNA polymorphism for these cytokines [162, 163]

1.4 miRNA, neuroinflammation and Parkinson's disease

Neuroinflammation has been increasingly studied as a chief mediator in the pathogenesis and progression of PD [112]. miRNAs, small non coding RNA, are involved in several pathologies since their activity consists in controlling the genetic expression and their dysregulation contribute to different pathologies, including PD [164]. miRNA could be perfect candidates as biomarkers for diseases in which they are altered. Furthermore, they could be potentially used in order to monitor the progression of the disease. Peripheral blood mononuclear cells (PBMCs) share more than the 80% of the transcriptome with other tissues, including the CNS, so peripheral blood could be considered a great source of biomarkers being also widely available [165]. Several studies show how a dysregulation of miRNA is involved in the pathogenesis of different neurodegenerative diseases like: Alzheimer's disease, Parkinson's disease, Huntington's disease and Amyotrophic lateral sclerosis. The molecular mechanisms underlying the pathological implications of misregulated miRNA expression and the regulation of the key genes involved in neurodegenerative disorders remain largely unknown [166, 167, 168, 169].

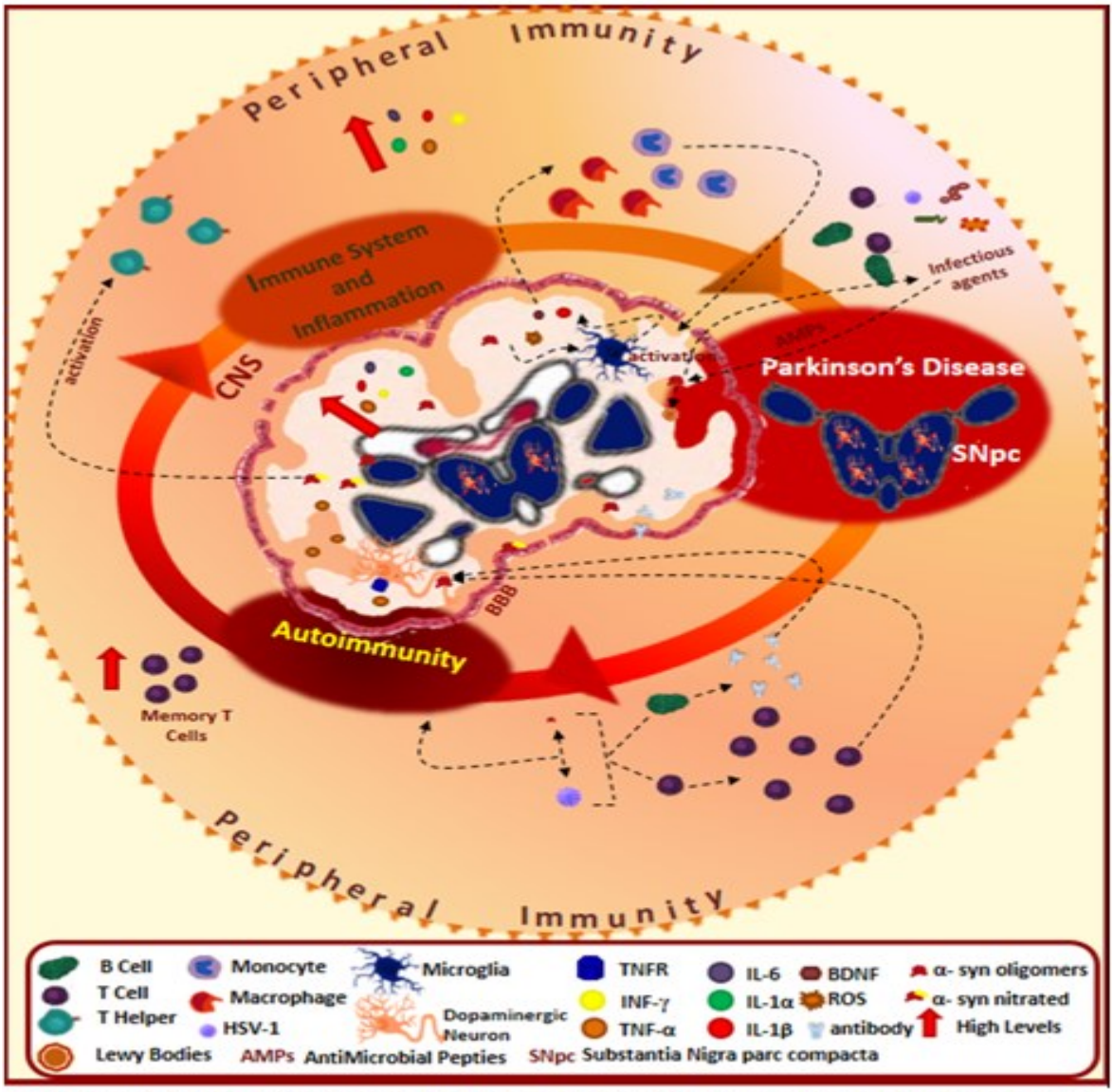

Figure 5. The immage represents the possible role of inflammatory and immune processes in the etiology and progression of PD, underling the possible role of autoimmunity, which could be induced by a molecular mimicry between α-syn and HSV1. it is shown how several factors could activate microglia, monocytes, macrophages and lymphocytes in PD. Furthermore, the possible role of α-syn as AMPs is represented.

1.5 Herpes simplex virus type 1: outline

The Herpes simplex virus type 1 (HSV1) is a ubiquitous virus in the Herpesviridae family, which is transmitted by direct inter-human contagion. HSV1 is a double-stranded DNA virus of considerable size, the virus is coveredby a large enveloped and its genome encodes for 84 proteins [170, 171]. Generally, in the adult, this virus determines the appearance of benign, transient clinical manifestations, represented by small febrile vesicles involving the facial region, especially the perioral, perinasal area and the periocular region. These manifestations are resolved spontaneously in a matter of days, without leaving any relic. However, the virus has the characteristic of remain in the latent state at the level of the ganglion cells, in particular at the level of the *ganglio of Ganges*, ganglio of the cranial nerve V, the trigeminal. This explains why the manifestations of HSV1 can easily reappearance following the reactivation of the virus, coinciding with episodes of deficit, even transient, of the immune response: situations of high stress, menstrual cycle, excessive exposure to sunlight and so on. This means that HSV1 infection remains dormant and its genome can be found at the level of ganglion cells. HSV1 is a markedly neurotropo virus and is able to determine the onset of specific inflammatory processes of the cerebral parenchyma, i.e. Herpes simplex virus 1 encephalitis. These herpetic encephalites occur in the form of an acute temporal necrotizing encephalitis, as a result of either a primitive infection or a recurrent infection. Clinical onset is acute (less than 48 hours) with 40 °C fever, headache, behavioural disturbances, movement disorders, alterations in language and mnemonic abilities. These initial signs are followed by a confusion and coma, which may be associated with convulsions or paralysis. The emergency treatment involves intravenous administration of antiviral drugs as soon as the diagnosis is suspected. The course of the disease is very variable and potentially can be severe with a mortality rate of 20% and after effects for the surviving patients.

Since 1978 there have been scientific evidence of a possible association between HSV1 infections and PD, in fact a high antibody titre against HSV1 was observed in PD patients but not in healthy controls [172]. Other studies conducted with the microindirect hemagglutination (IHA) technique have found the same trend [173]. Outbreak of viruses have been linked to specific case clusters of early onset Parkinson's. Viral encephalitis, causing by Herpes simplex virus, have all been followed by unusual spike in Parkinon's diagnosis. Moreover, HSV1, as well as other neurotropic viruses, has been shown to induce long-term neuroiimune activation, and this could be one of the mechanisms underlying neurodegeneration [174].

* Part of this chapter is based on the following article:

Inflammation, infectious triggers and Parkinson's Disease.

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Chapter 2: Aims of the project

PD is a complex and progressive neurodegenerative disease, where the main physiopathological features are represented by the degeneration of dopaminergic neuronspresent in the SNpc. The consequent depletion of dopamine at the level of the Nigro-Striatal via, leads to the onset of symptoms that are typical of the symptomatic picture: tremor at rest, rigidity and bradykinesia. Although the disease has been known for several centuries, its exact etiology still represents a discussion topic, that it has many controversial aspects. During the last decades some discoveries have proved to be milestones for its understanding.

- First, the discovery of the Lewy bodies and neuritis of Lewy, neuropathologic elements, features of PD, although not pathognomonic.
- The discovery of very rare forms of hereditary Parkinson with autosomal transmission dominant as, for example, the one determined by mutations in the SNCA gene coding for α-syn that shifted the focus of research into the study of this protein.

Numerous researches have been conducted to explain the physiologic functions of α-syn and its possible pathological role in the PD and other sinucleinopathies. It has been shown how the neurotoxic forms of α-syn is represented by oligomeric prefibrillary intermediates, which act through different modalities: by increasing the permeability of biological membranes, altering the homeostasis of many intracellular organelles such as mitochondria, altering proteostasis, inducing a neuroinflammation process and so on. Despite the research effort, however, the primum movens that determines the alteration or accumulation of this protein is not yet known. Several risk factors related to the development of the disease have been identified: age, familiarity, nutritional factors, exposure to toxic substances such as pesticides or metals.

Among the environmental risk factors we could also hypothesize the role of different infectious agentsas viruses, including the HSV1. In fact there are numerous infectious agents able to overcome the BBB and induce inflammatory processes of the brain parenchyma such as encephalitis. Furthermore, it should be emphasized that in the symptomatology of patients with encephalitis, it is also possible to identify clinical features characteristic of PD [26], proof of a possible correlation between the two pathologies. Other evidences that would support this theory are:

1 Presence of a neuroinflammatory component in animal models of PD [175];

2 Activation of microglia in the SNpc of PD patients which would cause the release of protective molecules to be released BDNF (Brain-derived neurotrophic factor), but also pro-inflammatory cytokines, reactive species of oxygen and nitrogen [94];

3 High number of studies that underlines the role of the Immune System (IS) in the pathogenesis of PD [177];

4 The onset of forms of viral post-encephalitis parkinsonism, such as those documented in association with encephalitis developed after 1918 influenza pandemic (*encephalitis lethargic of Von Economo*) [178].

These and other evidence led to hypothesi a viral cause as a possible trigger of PD associated to α-syn expression and accumulation. In particular, this project focused on the role played by α-syn, HSV1 and the Immune System. HSV1 is frequently found in a latent form whitin the CNS, in particular the genome of this virus is found with greater frequency in the brain tissues of older people rather than that of young people; this could be explained by the aging that inevitably leads to a reduction in the efficiency of the IS and consequently to a greater probability that the virus may cross the BBB [178].

Some genetic studies in PD patients showed that mutations of APOE gene seem to be associated with a more rapid decline of the disease, showing a decrease in memory performance, attention, speech and execution of movements. APOE gene mutations in AD seem to be associated with an increased accumulation of amyloid-beta (Aβ) protein, in a similar way in PD patients, Apo-ε4 mutation seems to stimulate the aggregation of α-syn, in fact patients carrying Apo-ε4 have an early onset. It has also been reported that mutations of the APOE gene are a predisposing factor for HSV1 infections [179,180, 181]

Aims of this project was to analyze the role of α-syn and HSV1 in inducing an autoimmune response in PD in patiens included those genetically susceptible. Therefore, through *in vitro* studies, we analyzed the potential role of α-syn as an AntiMicrobial Peptide (AMP), initially on HSV1 and then against the most common pathogens of the CNS. Furthermore, we want to analyze different roles of α-syn having a potential protective/damaging duality in human health and in PD.

- Firstly we tried to understand if α-syn can lead to an autoimmune response in PD patients. The hypothesis start from the assumption that a common pathogen of the CNS, such as HSV1, could play a role in triggering autoimmunity in PD. Destruction of dopaminergic neurons of the SNpc may occur through a mechanism of molecular mimicry or immunological cross-reactivity. In genetically predisposed individuals antibodies produced against HSV-1, due to a previous infection, may cross-react with α-syn peptides homologues linked to the membrane of neurons resulting in their impairment by the host immune system. We blasted the α-syn protein against the HSV1 proteome and identified different regions with high homology: Ul42 bound to C-terminus of HSV 1 pol and the Transcriptional Regulatory Protein VP16. Based on

these findings, we tested the humoral response against these peptides in PD patients and HCs by an enzyme liked immunoassay (ELISA), moreover we investigated the role of cell-mediated immunity upon a specific immune-stimulation with HSV1 and human α-syn homologues peptides by using the intracellular cytokine (ICC) method.

- The second step answered the question whether α-syn and its post-transcriptional modification may have an antiviral action, and if its over-expression following infection, may lead to an excessive accumulation of α-syn in the cytoplasm resulting in the progression of the disease. α-syn might play a protective role in CNS innate immunity, and its alteration due to a neurological infection, in particular HSV1 infection, may directly promote α-syn aggregation and damage. Ongoing α-syn deposition can drive neuroinflammation, leading to neuropathology and widespread dopaminergic neuron death. We hypothesize that α-syn can have antimicrobial activities, such as pathogen agglutination and entrapment, neutralization of endotoxin and interacting with the membranes. However, dysregulated α-syn oligomerization can also lead to serious pathologies, including inflammation, tissue degeneration and deposition in Lewy bodies. Thus, α-syn oligomerization carries a potential protective/damaging duality in human health. Therefore, through in vitro studies, we would like to analyze the potential role of α-syn as AMP, initially on HSV-1 and then with the most common pathogens of the CNS.
- Lastly we investigated the potential of circulating miRNAs as noninvasive diagnostic candidate biomarkers of PD patients and neuroinflammation.

The confirmation of the hypothesis that HSV-1 infection, in genetically predisposed individuals, may induce the development of an autoimmune reaction towards α-syn with

consequent PD progression will open the way to new lines of research for the development of new diagnostic and therapeutic systems. The findings raise the possibility that the death of neurons in PD could be prevented by a targeted immunotherapy, as well as considering the hypothesis that we may be able to treat UL42 positive patients with antiviral drugs. Furthermore the results could revolutionize the diagnostic test for PD and could help us to identify individuals at risk or in the early stages of the disease with an analysis of specific T/B-cells in blood samples. Moreover, if the role of α-syn as an antimicrobial peptide will be confirmed, the role of this protein could be revolutionized. It would open the way to new studies aimed at assessing whether, following recurrent HSV1 with its cycles of latency and reactivation, may lead to an increase in the expression of α-syn aimed at neutralizing the virus with consequent accumulation of the protein resulting in neuronal damage.

Chapter 3: Materials and Methods

3.1 Analysis of the humoral response

3.1.1 Subjects for immunoenzymatic assay

The peripheral venous blood of Parkinson's patients and that of healthy control donors were collected. The diagnosis of PD was based on the established criteria [182]. Parkinson's patients were enrolled at the Neurology Clinic of the University Hospital of Sassari, the healthy controls (blood donors) at the transfusion center of Sassari. The cohort included 40 PD patients(M/F = 2.33, mean age 69.83 ± 7.95, mean of years disease =8.42 ± 4.29, mean of Hoehn–Yahr scale = 3.01 ± 0.88) and 40 ageand-sex-matched healthy controls (M/F = 2.33, mean age 65.76 ±9.60). The local ethic committee approved the study (Prot. N 2159/CE2015, Azienda Sanitaria Locale 1, Sassari, Italy) and all participating subjects gave informed consent.

3.1.2 Antigens for immunoenzymatic assay

The following peptides were included in the study: $Ul42_{22–36}$ [LGQPEEGAPCQVVLQ], $\alpha\text{-synuclein}_{100–114}$ [LGKNEEGAPQEGILE],$Vp16_{324–340}$ [KNNYGSTIEGLL-DLPDD] and $\alpha\text{-synuclein}_{102–119}$ [KNEEGAPQEGILEDMPVD] (Table 1). All peptides were synthesized commercially at 90% purity (LifeTein, South Plainfield, NJ 07080, USA).

Table 1 characteristics of selected peptides

Peptide name	Sequence	Protein name	Organism
Ul42 $_{22-36}$	LGQPEEGAPCQVVLQ	UL42	Herpes simplex virus 1
α–synuclein$_{100-114}$	LGKNEEGAPQEGILE	Alfa-synuclein	Human
Vp16 $_{324-340}$	KNNYGSTIEGLL-DLPDD	Vp16	Herpes simplex virus 1
α–synuclein$_{102-119}$	KNEEGAPQEGILEDMPVD	Alfa-synuclein	Human

3.1.3 Enzyme-Linked Immunosorbent Assay (ELISA)

The selected peptides were dissolved in carbonate/bicarbonate togive the concentration of 10 mM, 96 wells were coated with this solutionand incubated o/n at 4 °C. The next day the wells were saturated with 200 ml of blocking solution (PBS-Tween containing 5% milk) for1 h at room temperature. After the incubation the plates were washed with PBS-T, diluted sera (1:100) were added, in duplicate, and incubated for 2h. The plates were subjected to 5 washes with PBS-T after which we proceeded with the addition of the alkaline phosphatase-conjugated goat antihuman IgG polyclonal Ab at a dilution of 1:1000, and left to incubatefor 1 h at room temperature environment. Finally, after another five washes with PBS-T, para-nitrophenyl phosphate substrate solution was added to each well and the plates were incubated at room temperature in the dark for 5, 10 and 15 min. The optical density (OD) was read at a wave length of 405 nm using VersaTunableMAX microplate reader. Data was normalized to a positive control serum included in all experiments, the reactivity of which was fixed to 10,000 arbitrary units (AU)/ml.

3.1.4 Competitive assay

The competitive assay were performed by pre-incubating the serum (1:100 on PBS-T) of three patients for 2h at room temperature with α-syn$_{100–114}$ and Ul42$_{22–36}$ at saturating concentrations of 50 μM. Three PD patient sera were subjected to ELISA on plates coated with Ul42$_{22–36}$ or α-syn$_{100–114}$, respectively. Ul42$_{22–36}$ or α-syn$_{100–114}$ [50 μM] was added as positive control. In the same assay serum without the peptide was used as a negative control.

3.1.5 Statistical analysis

Statistic studies were carried out using GraphPad Prism 6.0 software (San Diego, CA, USA). Continuous variables are presented as mean ±standard deviation (SD), and categorical variables as numbers and percentages. PD patients and HCs were compared by Student's t-test. Correlations among continuous variables were calculated as well. Optimal cut-off values were determined by setting the specificity at 95% and the corresponding sensitivity was calculated using ROC analysis. A p value of 0.05 was regarded as significant.

3.2 Analysis of the cell-mediated respons

3.2.1 Samples for flow cytometry analysis

The peripheral blood of Parkinson's patients and Healthy Controls (HCs) were collected in BD Vacutainer NH (Sodium Heparin). The diagnosisof Parkinson's disease was based on the established criteria[182]. The Parkinson's patients were enrolled at the Neurology Clinic of the University Hospital of Sassari, Italy, while the Healthy Controls were provided by a family physician of Li Punti district,Sassari. The experiment included 9 PD patients (M/F = 0.8, meanage 71.3 ± 9.6, mean disease duration 8.3 ± 4.8 years, mean Hoehn-Yahr scale 3.3 ± 1.2) and 7 HCs (M/F =1.3, mean age 65 ± 5.9) (Table 2). The samples were processed,

immediately after harvest, for flow cytometry analysis. The local ethics Committee approved the study (Prot. N 2159/CE, Azienda Sanitaria Locale 1, Sassari, Italy) and all participating subjects gave informed consent.

Table 2 Demographic and clinical characteristics of study participants.

Group	Age, y, Mean ± SD	Sex, females/ Males, n	Disease duration, Mean ± SD	H&Y score, Median ± SD	Levodopa mg Mean ± SD
PD (# 9)	71,3 ± 9,6	4/5	8,3 ± 4,8	3 ± 1,2	516,7 ± 259,8
HC (#7)	64.9 ± 5,8	4/3			

3.2.2 Antigens

Synthetic peptides derived from HSV1 and human α-syn were used in this study for the antigenic stimulation. Peptides have been described in detail in a previous paragraph. All peptides were synthesized commercially at 90% purity (LifeTein, South Plainfield, NJ 07080, USA).

3.2.3 Cytokines and phenotyping antibodies

Monoclonal antibodies (mAbs) CD3 FITC, CD8 PreCP-Cy5.5, TNFPE-Cy7, INF-γ APC, CD14 PE were used for the analysis of T lymphocytes,while NK were detected using: CD3 FITC, CD56 PE, IL6 APC, TNFPE Cy 7, CD16 PerCP-Cy5.5. The antibodies are used at the manufacturer's recommended concentrations (BD Biosciences, San Diego CA).

3.2.4 Cell preparation and antigenic stimulation

Sodium heparinized venous blood was aliquoted into 15 ml falcon tubes at 500 µl for tube, including positive and negative controls. Positive control included: 500 µl of sample, 500 µl of RPMI and PMA (phorbol 12-myristate 13-acetate) 25 µg/ml cells suspension and calcium ionophore A23187 1 µg/ml (Sigma Aldrich). Negative control included: 500 µl of sample, 500 µl of RPMI. Antigenic stimulation sample were performed with: 500 µl of sample, 500 µl of RPMI, the costimulatory mAB CD28/49d 5 µl/ml and two different peptides($UL42_{22–36}$ and $\alpha\text{-syn}_{100–114}$, 25 µM). The culture tubes were incubatedin a diagonal position at 37 °C and 7% of 5% CO2 for 8 h, the first 2 h of incubation were done without Brefeldin A (BFA) to enable antigen processing by antigen presenting cells (APC). The BFA (10 µg/ml) was included for the final 6 h of activation in order to inhibit the secretion and paralysis of the Golgi apparatus. After wards, the samples were centrifuged for 10 min at 1200 rpm, then the supernatant was aspired and discerned. The pellet was vortexed and the samples were incubated for 10 min with 2 ml of 1× FACS Lysing Solution at room temperature (RT). Cells were washed in 2 ml of PBS and centrifuged for 10 min at 12000 rpm and then the supernatant was discarded by inversion. The samples were gently resuspended in 2 ml of 1× FACS Lysing Solutionand incubated at 4 °C overnight (o/n).

3.2.5 Intracellular cytokine staining and phenotyping analysis

The following day the cells were washed by adding 2 ml of PBS 1X and centrifuged for 10 min at 1200 rpm, the supernatant was discarded by inversion, then 100 µl of permeabilizating solution (0.05% saponinin PBS solution) were added to each sample and incubated for 10 min. Subsequently, a mix of antibodies was added for marking and samples were further incubated for 30 min in the dark. After washing with 2 ml of permeabilizing solution,

centrifuging for 10 min at 1200 rpm and discarding the supernatant by inversion, cells were resuspended in 300 µl of FACS Flow solution. The labeled cells were analyzed by flowcytometry on FACSCanto™ using FACSDiva 2.2 (Becton & Dickinson).

3.2.6 Statistical analysis

The comparison of each lymphocyte population and the ratio of INF-γ, TNF-α, IL-6 producing by T cell ad Natural Killer between PD patients and healthy controls were analyzed by GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA) using the Mann-Whitney U test. The data are presented as means with standard deviation, a p value lower than 0.05 was considered statistically significant. We have also investigated the correlation of cell responses stimulated by homologous peptides; the analysis was performed using Pearson's correlation coefficients with a confidence interval of 95%.

3.3 Alpha-synuclein as a antimicrobial peptide

3.3.1 Effect of alpha-synuclein on HSV-1 replication (first protocol)

The first day Hela cells were seeded in black 96-well plates at $1X10^4$ well in 100 µl normal growth media, the cells were incubated at 37° C in a humidifield incubator with 5% CO_2 overnight for cells to adhere. The day after HSV1 strain C12 GFP was diluited to MOI 0.5 in 25ul red-free media, then media was removed from wells to be infected and it has been replaced with 25 µl diluited HSV1, media alone was added to 'unifected' control cells. Cells were returned to the incubator and left for 1hr at 37° C in ahumidified incubator with 5% CO_2. Whilst cells were incubating, serial dilutions of α-syn were prepared (Sigma Aldrich) as calculated (10ug/ml to 39ng/ml). After 1 hour, virus was removed and the medium was

changed with 100ul of serial dilutions of α-syn. Cells were returned to the incubatorand left for ~ 20 hrs post- infection before measuring virus replication. The next day virus replication was monitored as a function of eGFP fluorescence from 24 h to 72 h post-infection using the Clariostar plate reader. Day 5→ test cell viability with CellTiter-Blue assay. Replication growth curves were monitored, and endpoint replication (as determined by fluorescence) was normalized to unifected cells.

3.3.2 Effect of alpha-synuclein on HSV-1 replication (second protocol)

The first day Hela cells were seeded in black 96-well plates at $1X10^4$ well in 100 µl normal growth media, cells were incubated at 37° C in a humidifield incubator with 5% CO_2 overnight for cells to adhere. The next day HSV1 strain C12 GFP was diluited to MOI 0.5 in 25ul red-free media, then media was removed from wells to be infected and it has been replace with 25 µl diluited HSV1, media alone was added to 'unifected' control cells. Cells were returned to the incubator and leave for 1hr at 37° C in ahumidifield incubator with 5% CO_2. Whilst cells are incubating: **i)** serial dilutions of α-syn were prepared (Sigma Aldrich) from (500ug/ml to 125ng/ml) as a physiological concentrations; **ii)** amyloid-beta Aβ (1-2 ng/*ml), Aβ (1-2ng/ml) were used as positive controls; **iii)** only medium was used as negative controls. After 1 hour, virus was removed and the medium was changed with 100ul of: **1)** serial dilutions of α-syn; **2)** different concentration of Aβ; **3)** negative controls. Cells were returned to the incubatorand left for ~ 20 hrs post- infection before measuring virus replication. The next day virus replication was monitored as a function of eGFP fluorescence from 24 h to 72 h post-infection using the Clariostar plate reader. Day 5 →test cell viability with CellTiter-Blue assay. Replication growth curves were monitored, and endpoint replication (as determined by fluorescence) was normalized to unifected cells.

3.3.3 Effect of alpha-synuclein on HSV-1 replication (third protocol)

The first day Hela cells were seeded in black 96-well plates at $1X10^4$ well in 100µl normal growth media, cells were incubated at 37° C in a humidifield incubator with 5% CO_2 overnight for cells to adhere.The next day:

- HSV1 strain C12-GFPto MOI 0.5 was preincubated for 1h at 37°C (in medium without FCS) with serial dilutionsof α-syn (Sigma Aldric) physiological range→**1)** α-syn 500 µM ; **2)** α-syn 250 µM; **3)** α-syn 125µM.
- HSV1 strain C12-GFPto MOI 0.5 was preincubated for 1h at 37°C (in medium without FCS) with Aβ, produced by cells line CHO-CAB (1-2 ng/ml) as a positive controls (provided by Harvard University);
- HSV1 strain C12-GFP to MOI 0.5 was preincubated for 1h at 37°C (in medium without FCS) with Aβ, produced by cells line H4 (4-2 ng/ml) as a positive controls (provided by Harvard University);
- HSV1 strain C12-GFP to MOI 0.5 was preincubated for 1h at 37°C (in medium without FCS) with Negative control, medium produced by cells line CHO-N and H4-N;
- HSV1 strain C12-GFP to MOI 0.5 was preincubated for 1h at 37°C in medium without FCS as negative controls.

After Hela cells were infected with different HSV1 conditions, media alone was added to 'unifected' control cells. After 1 hour, when HSV1 has been absorbed by the cells, virus was removed and the medium was changed with 100ul of fresh medium. Cells were returned to the incubatorand leave for ~ 20 hrs post- infection before measuring virus replication. The

next day virus replication was monitored as a function of eGFP fluorescence from 24 h to 72 h post-infection using the Clariostar plate reader. Day 5→test cell viability with CellTiter-Blue assay. Replication growth curves were monitored, and endpoint replication (as determined by fluorescence) was normalized to unifected cells.

3.3.4 Transient over-expression of α-synuclein

For transient overexpression of α-syn in Hela cells line at first plasmid for the expression of α-syn has been prepared. pDONOR223 contaning SNCA gene in *E.coli* has been used, (kindly provided by the University of Edinburgh). After it proceeded amplify the DNA from the avaible clone and the next day the DNA of interest has been extracted. Quality and concentration of DNA has been evaluated with NanoDrop. The extracted DNA has been sent for sequence analysis to GATC Biotech company. At the same time through digestion with specific restriction enzymes the insert in the plamisde was inspected and it has been confirmed through analysis on agarose gel. After sequence confirmation, through Gateway ™ technology, plasmid pCR3 was produced with LR reaction. Then the selected clones have been used for large-scale DNA production and extraction with Promega Midi-Prep kit. For transient over-expression, $1.56*10^4$ Hela and HuH7.5 cells were seeded in black 96-well plates. The following day, cells were transfected with: **i)** 100ng pCR3-SNCA; **ii)** 100ngpCR3-Med23 as a positive controls [183]; **iii)** 100ng pCR3-GFP as controls of transfection, **iv)** 100ng pCR3-free as a negative controls. Transfection was done using LipofectamineTM LTX (Invitrogen) and incubated for 48 h before infection with the recombinant HSV1 reporter viruses C12 at MOI 0.5. Replication growth curves were monitored, and endpoint replication (as determined by fluorescence) was normalized to untransfected cells.

3.3.5 Cell viability assay

The cytotoxicity of α-syn was determined using the CellTiter Blue (CTB, Promega) reagent, which gives a fluorescent or absorbance signal relative to the number of live cells. Briefly, 5 µl CTB was added per well using the Multidrop 384. Plates were incubated at 37°C in a humidified incubator with 5% CO2 for 2h before measuring fluorescence (POLARstar OPTIMA plate reader). Readings were normalized to viability untreated cells, per plate, and mean cell viability over three replicates was calculated. Distribution analysis of cell viability values identified median viability as 60%, and values <60% were considered cytotoxic.

3.3.6 Statistical analysis

Statistical analysis of *in vitro* studies included means and SDs (e.g., slope of GFP fluorescence, replication slopes during linear growth were normalized to controls) for a minimum of five replicate experiments. Regarding Cell viability the readings were normalized to viability of untreated cells, per plate, and mean cell viability over three replicates was calculated. Distribution analysis of cell viability values identified median viability as 60%, and values <60% were considered cytotoxic.

3.4 Expression of circulating miRNA analysis

3.4.1 Samples for miRNA analysis

The peripheral blood of Sardinian PD patients, enrolled at the Neurology Clinic of the University Hospital of Sassari, Italy, and Healthy Controls (HCs) provided by a family physician of Li Punti district, Sassari, Italy, were collected. The diagnosis of PD was based on the established criteria [182]. The cohort included 37 PD patients (M/F = 0.8, mean age 71.3

± 9.6, mean disease duration 8.3 ± 4.8 years, mean Hoehn-Yahr scale 3.3 ± 1.2) and 43 HCs (M/F =1.7, mean age 60 ± 13.14). Immediately after collection, PBMCs were isolated from 10ml of blood by density gradient centrifugation on Ficoll-Paque Plus, (GE Healthcare Bioscience, Sweden), washed twice in phosphate-buffered saline (PBS), counted and stored at -80°C with RNA later (Sigma) until further use.The study was approved by ethics committee of the Azienda Sanitaria Locale 1, Sassari, Italy (Prot. N 22, 2015). The patients and the volunteers gave written informed consent.

3.4.2 miRNAs cDNA synthesis and real-time PCR

Purification of total RNA containing miRNA from PBMCs was performed using miRNeasy Mini kit (Qiagen, USA) according to the manufacturer's recommendations. Quality of extracted RNA was determined according to 260/280 absorbance ratio, measured by Nano Drop spectrometer (Thermo Scientific, USA). 500 ng/RNA were used in reverse-transcription reaction. cDNA synthesis for miR-155, miR-132, miR-146a and miR-26a was fulfilled using a miSCript II RT Kit (Qiagen) according to the manufacturer's leaflet. MiRNAs quantification was performed with Custom miScript miRNA PCR Array.

3.4.2 Heat Maps

We performed heat maps using GeneGlobe Data Analysis Center (Qiagen). The heat map provides a visualization of the fold changes in expression between the selected groups for every gene in the array in the context of the array layout. The table provides the fold regulation data used for the map as well as the comments associated with each one. The color of the square denotes the relative up- or down-regulation of the miRNA in that sample. In addition, it produces dendrograms for the rows and columns, which are computed using

hierarchical clustering. The ordering of the rows and columns is the most compatible with the dendrograms.

3.4.3 Statistical analysis

miRNAs data analysis was performed using the ΔΔCT method by Qiagen miRNA detection software and final data were normalized for small nuclear RNA, miRTC (median Ct = 24.86 ± 0.614) PPC (median Ct = 21.27± 0.302), RNU6-6P (median Ct = 23.34 ± 0.116), SNORD68 (median Ct = 22.54± 0.211) expression levels as endogenous controls.

Chapter 4: Analysis of the humoral response against alpha-synuclein peptides homologous to Herpes simplex virus 1 proteins in PD patients VS healthy controls.

Part of this chapter is based on the following article:

- 2016. Elisa Caggiu, Kai Paulus, Giannina Arru, Rosanna Piredda, Gian Pietro Sechi, Leonardo A. Sechi. Humoral cross reactivity between a α-synuclein and Herpes simplex -1 epitope in Parkinson's disease, a triggering role in the disease? Journal of Neuroimmunology 291 (2016) 110–114.

In this study we suggested that a common pathogen of the central nervous system, such as HSV1, could play a role in triggering autoimmunity in PD. Destruction of dopaminergic neurons of the *substantia nigra* may occur through a mechanism of molecular mimicry or immunological cross-rectivity. A few studies suggest that the HSV1 DNA is abundantly present in latent form in the brains of people observed, with a greater frequency in older people than in young adults and children. This could be due to the fact that, with increasing age, one can have a lower immune system efficiency and thus passage of the virus in the brain [184]. The assumption is that antibodies developed against HSV1, owing to a previous infection, may cross-react with α-syn linked to the membrane of neurons resulting in their impairment by the host immune system. We blasted the α-syn protein against the HSV1 proteome and identified different regions with high homology: Ul42 bound to C-terminus of HSV1 pol and Trascriptional Regolatory Protein VP16. Based on these findings, in this study we tested the humoral response against these peptides in PD patients and healthy controls by an enzyme immunoassay, in order to examine the association between α-synuclein and HSV1.

4.1 Results

4.1.1 ELISA

Humoral response against the selected peptides was evaluated both in patients and in controls. The peptides $Ul42_{22\text{-}36}$ homologous to $\alpha\text{-syn}_{100\text{-}114}$ and $Vp16_{324\text{-}336}$ homologous to $\alpha\text{-syn}_{102\text{-}119}$were obtained by a BLAST analysis between the α-syn and the proteome of HSV1. Regarding $Ul42_{22\text{-}36}$ analysis of the antibody response showed a statistically significant difference between patients and controls, with p value of 0.0022 analyzed by T test (Fig 6 A). Observed seropositivity was 58% in patients and 18% in healthy controls, determined by ROC analysis with a cut-off value of 0.26. In the same way, upon evaluation of antibodies reactivity against the homologous peptide $\alpha\text{-syn}_{100\text{-}114}$,we observed that the two populations reacted statistically different, evaluated by the t test with a p value of= 0.0025 (Fig 6 B).

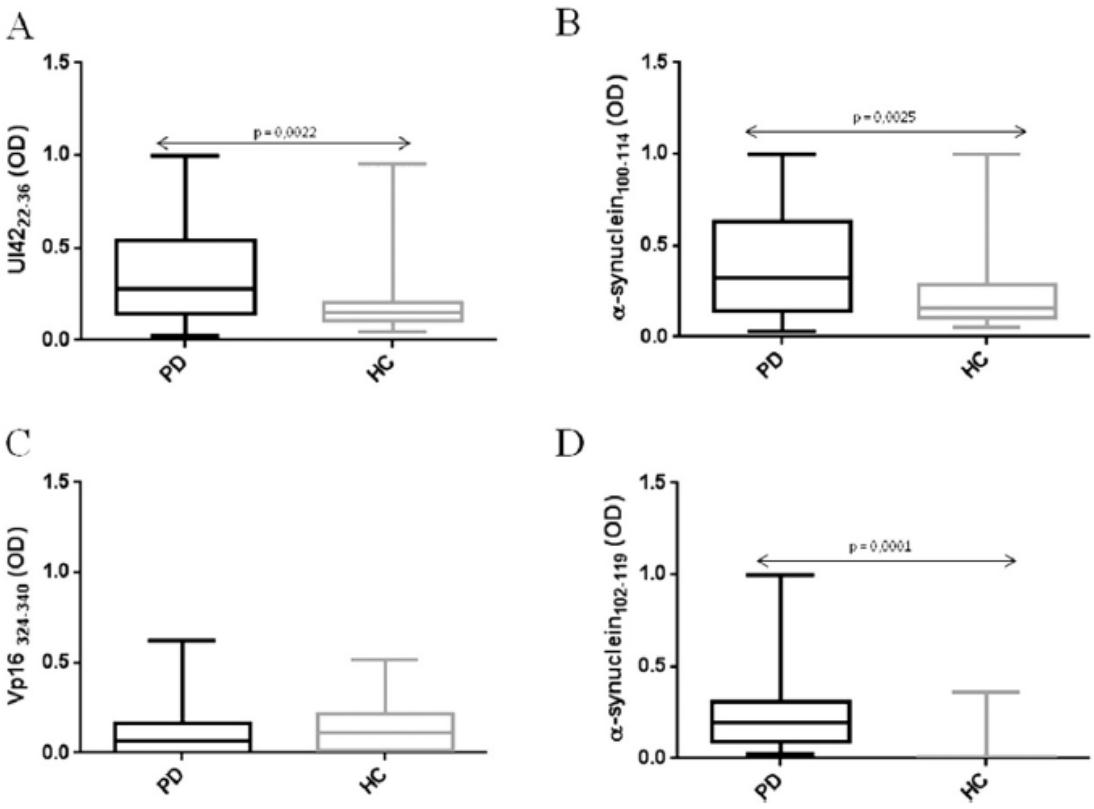

Figure 6. Box-whisker plot. ELISA-based analysis. 40 PD patients and 40 healthy controls were screened for humoral response (IgG) against four peptides: (A) Ul42$_{22–36}$, (B) α-syn$_{100–114}$, (C) Vp16$_{324–340}$, and (D) α-syn$_{102–119}$. Mean and standard deviation were represented. A, B, D showed a statistical difference between PD patients and healthy controls.

We observed 55 % seropositivity in patients and 20 % in healthy controls, calculated by ROC analysis with a cut-off value of 0.29. Thereafter, we evaluated the correlation of the humoral response in PD patients against the homologous α-syn$_{100-114}$ and Ul42 $_{22-36}$ peptides, observing a very strong correlation coefficient R^2 of 0.8294(Fig. 7 A). Similarly,we observed a correlation coefficient of comparable value in healthy controls with R^2= 0.7443 (Fig 7 B). Regarding the antibody response against the peptide Vp16 $_{324-336}$ no significant difference between PD patients and controls was registered, as indicated by the t test with a p value of 0.3903. The percentages of seropositivity were 15% in patients and 20% in control according

to the ROC analysis with a cut-off value 0.123 (Fig 6 C). On the other hand ahumoral reaction against the homologous peptide α-syn$_{102-119}$ was observed with a statistically significant response in PD patients compared to healthy controls, with a p value of 0.0001 (Fig 6 D). Eighty percent of PD patients and 18% controls were positive after ROC analysis with a cut-off value = 0.066. We also evaluated the correlation between α-syn$_{102-119}$ and its homologous Vp16$_{324-340}$ peptides, in the humoral response in the patients obtaining a correlation coefficient R^2 0.5154 (Fig 7 C). Similarly in the healthy controls the correlation coefficient equaled R^2 0.1614 (Fig 7 D).

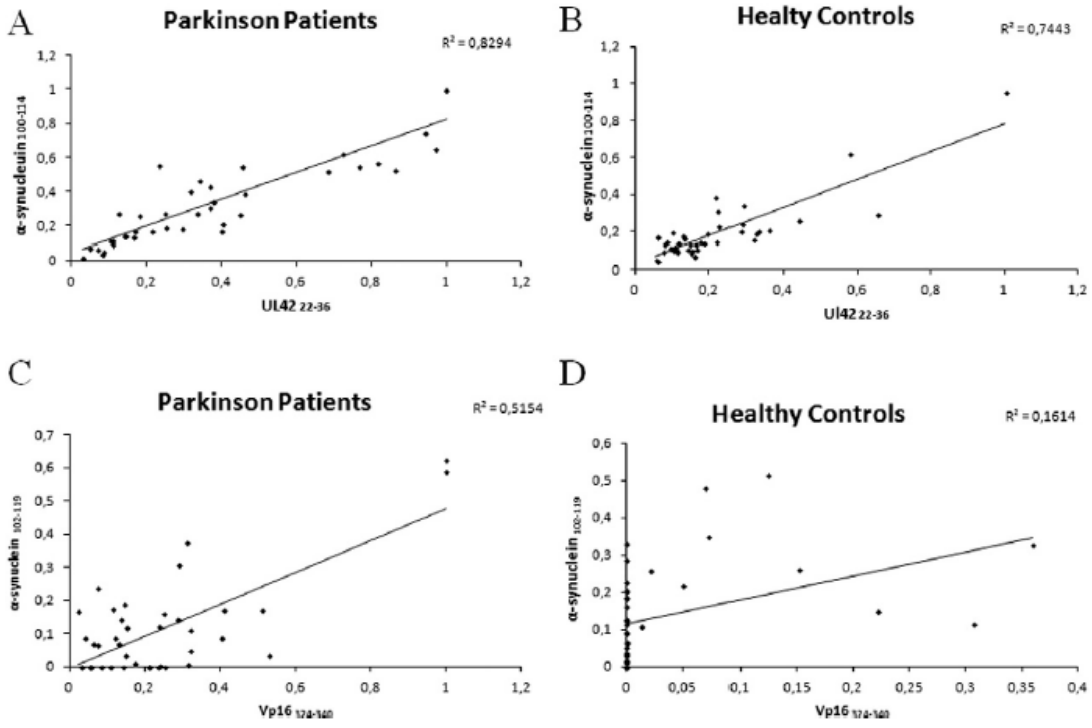

Figure 7. Correlation analysis. Correlation between titer antibodies recognizing (A) α-syn$_{100–114}$ and Ul42$_{22–36}$ on 40 PD patients, (B) α-syn$_{100–114}$ and Ul42$_{22–36}$ on 40 HCs, (C) α-syn$_{102–119}$ and Vp16$_{324–340}$ on 40 PD patients, and (D) α-syn$_{102–119}$ and Vp16$_{324–340}$on 40 HCs.

4.1.2 Competitive assay

In order to confirm the specificity of the antibodies against the Ul42 $_{22\text{-}36}$ and the homologous α-syn$_{100\text{-}114,}$ we performed two competition assay with sera of three selected patients. The PD patients were selected on the basis of the immune response against the peptides (one strong, one medium and one weak responder). In the first assay the sera of the three patients were incubated with the α-syn$_{100\text{-}114}$ peptide and there after incubated within the plate coated with homologous Ul42$_{22\text{-}36}$ peptide (Fig. 8A). In the second experiment the opposite was performed. Sera were incubated first with peptide Ul42$_{22\text{-}36}$ and successively they were incubated in a plate coated with the α-syn$_{100\text{-}114}$ homologous peptide (Fig. 8B). Patient 1 showed an excellent antibodies cross-reactivity with a marked reduction of the antibody reaction after the incubation with the first peptide in both experiments (Fig. 8A and B) Likewise, Patients 2 and 3 showed a good cross-reactivity of antibodies (Fig. 8A and B).

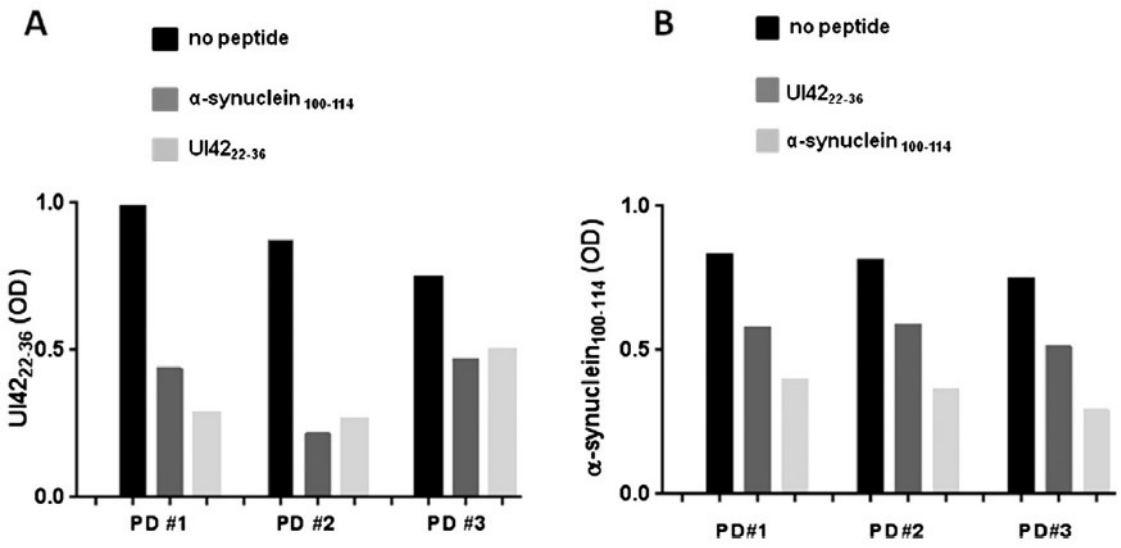

Figure 8. Competition assay with $Ul42_{22–36}$ (A) and $\alpha\text{-syn}_{100–114}$ (B) coated plates. (A) Sera from 3 PD patients were pre-incubated for 2 h at room temperature with saturating concentrations [50 μM] of $\alpha\text{-syn}_{100–114}$ and using $Ul42_{22–36}$ as positive control. The first bar represents a regularly performed ELISA (1:100 sera in PBS-T), the second bar represents the ELISA (1:100 sera in PBS-T) incubated with $\alpha\text{-syn}_{100–114}$, and the third bar represents the ELISA (1:100 sera in PBS-T) incubated with $Ul42_{22–36}$. (B) Sera from 3 PD patients were pre-incubated for 2 h at room temperature with saturating concentrations [50 μM] of $Ul42_{22–36}$ and using $\alpha\text{-syn}_{100–114}$ as positive control. The first bar represents a regularly performed ELISA (1:100 sera in PBS-T), the second bar represents the ELISA (1:100 sera in PBS-T) incubated with $Ul42_{22–36}$, and the third bar represents the ELISA (1:100 sera in PBS-T) incubated with $\alpha\text{-synu}_{100–114}$. Bars show means of triplicate wells and results are representative of two separately performed experiments.

4.2 Discussion

The α-syn protein is the main component of PD Lewy bodies and Lewy neuritis and at the molecular level, neuronal α-syn inclusions and pathological α-syn transmission play a key role in initiation of Parkinson-like neurodegeneration [185; 186]. Several studies suggest that from a molecular point of view the normal and neuropathogenic functions of α-syn may be different. The role of α-syn oligomers in binding and permeabilization of cell membranes has been also reported [57]. The aim of the study was to ascertain if HSV1 peptides and the homologous α-syn peptides were cross recognized in PD patients and consequently, it may be

hypothesized a possible role in the rate of progression of PD. We have supposed a molecular mimicry between HSV1 and human α-syn that could foster the progression of this pathology. Infection mediated by HSV1 that carry an exogenous molecular mimic, can enhance the development of autoimmunity; in particular, in predisposed subjects autoreactive antibodies could recognize α-syn linked to the membrane of dopaminerginc neurons leading to their destruction. We have observed a statistically significant difference of the Ab levels against $Ul42_{22\text{-}36}$ in PD patients in comparison to HC, observing the same response against the human homologous peptide $\alpha\text{-syn}_{100\text{-}114}$. However we haven't observed the same humoral response against the other couple of homologous peptides, reactivity against $Vp16_{324\text{-}340}$ was not different among PD patients and HC whereas a statistically significant difference was observed against the $\alpha\text{-syn}_{102\text{-}119}$ human peptide. This could indicate that only $Ul42_{22\text{-}36}$may have a triggering role in PD. Statistical correlation analysis confirmed the previous results, in fact a strong correlation was observed between $Ul42_{22\text{-}36}$ and $\alpha\text{-syn}_{100\text{-}114}$ peptides in PD patients and HC respectively. We have further investigated the hypothesis of molecular mimicry through competition assays between $Ul42_{22\text{-}36}$ and $\alpha\text{-syn}_{100\text{-}114}$ by the competition experiments. Our results highlighted that autoantibodies recognizing HSV1-$Ul42_{22\text{-}36}$ peptide are able to cross-react with the homologous human $\alpha\text{-syn}_{100\text{-}114}$ epitope. This was not confirmed for the other HSV1 peptide considered in this study $Vp16_{324\text{-}340}$ where as its human homologous $\alpha\text{-syn}_{102\text{-}119}$ was highly recognized in PD patients. The fact that only an HSV1 epitope was strongly recognized in PD patients may strength the value of the results since it demonstrate that the peptide homology alone is not sufficient to induce autoimmunity. Of note, the α-syn sequence can be divided in three regions: the N-terminal amphipathic region, which contains the three point mutations related to autosomal dominant early onset PD; the central region, which promotes aggregation, and the acid C-terminal portion, which tends to

decrease protein aggregation [185]. Interestingly, since the human homologous peptide α-syn$_{100-114}$ is part of the acidic C-terminal region, it is hypothesizable that the autoantibodies recognizing the HSV1-Ul42$_{22-36}$ peptide may also react with the α-syn$_{100-114}$ residues in vivo. These may consequently interfere with the physiological function of this region and, ultimately, foster α-syn aggregation. In conclusion the obtained results indicate the molecular mimicry as a molecular mechanism of autoimmunity, specifically related to the cross reactivity between Ul42$_{22-36}$ and α-syn$_{100-114}$ peptides, in membranes of dopaminergic neurons of the substantia nigra pars compacta. This mechanism may play a role in Parkinson-like neurodegeneration.

Chapter 5: Study of cell-mediated response, following stimulation with homologous alpha-synuclein and Herpes simplex virus 1 peptides, in PD patients VS healthy controls through intracellular cytokine method

Part of this chapter is based on the following article:

- 2017. Caggiu Elisa, Paulus Kai, Galleri Grazia, Arru Giannina, Manetti Roberto, Sechi Gian Pietro, Sechi A Leonardo. Homologous HSV1 and alpha-synuclein peptides stimulate a T cell response in Parkinson's disease. Juornal of neuroimmunology. 310; 26-31.

The selective loss of specific neuronal populations while others are spared suggests a role for adaptive immunity in this process. In a previous study, we had found a possible association between HSV1 infection and PD with a statistically significant difference in antibody response between patients and controls. The aim of this study was to investigate the role of cell-mediated immunity upon a specific immune-stimulation with HSV1 and human α-syn peptides by using the intracellular cytokine (ICC) method. This assay permitted to quantify the peptide-specific memory CD4 and CD8 frequencies with incorporated antigens after a brief exposure able to induce production of cytokines, such as interferon gamma (INF-γ) or tumor necrosis factor alpha (TNF-α), and intracellular retention of cytokines by blocking with Brefeldin A. We also investigated the possible role of CD14 and CD56 using the same assay.

5.1 Results

5.1.1 Population of peripheral T lymphocytes

The analysis of the peripheral lymphocyte subset in HCs and PD patients are shown in Table 3. The percentage of $CD3^+$ was higher in HCs than in PD patients (p=0.00082), as was the percentage of $CD4^+$ $CD8^-$ (p= 0.00389). An increase of $CD4^-$ $CD8^+$ percentage was observed in HCs respect to PD patients (p=0.02). The calculated ratio of $CD4^+$ to $CD8^+$cells was similar between the two groups. Likewise, a decrease of NK percentage was observed in PD patients compared to HCs group (p=0.0052). The percentage of CD14 was not significantly different between the groups (Table 3).

Table 3. Comparison of peripheral lymphocytes subsets in control subjects and patients with Parkinson's disease

Cell Type	Controls (n=7)	PD patients (n=9)	P value
$CD3^+$, %	65,49 ±7,19 %	46.31 ± 11,42 %	0.00082
$CD4^+$ $CD8^-$, %	51,60 ± 7,75 %	37,93 ± 9,84 %	0.00389
$CD4^-$ $CD8^+$, %	19,89 ± 2.67 %	14,38 ± 6,14 %	0.02
$CD4^+$/ $CD8^+$, %	2.59 ± 0,65 %	2.64 ± 1,87 %	NS
CD14 , %	6,08 ± 2,33 %	7,85 ± 4,76 %	NS
CD56, %	42,06 ± 5.08 %	31,47 ± 3,13 %	0.0052

5.1.2 Activation of CD8 and CD4

T-cell activation against HSV1 and human α-syn peptides in 9 PD and 7 HCs blood samples was assessed by intracellular cytokine flow cytometry. Patients's responses were compared to healthy controls by analyzing the percentage of cells secreting TNF-α and INF-γ for $CD8^+$ and $CD4^+$. The mean percentage of TNF-α-secreting CD8 T cells specific for $\alpha\text{-syn}_{100\text{-}114}$ equaled 0.066% in PD patients versus 0.015% among HCs with statistically significant p value (p=0.02; Fig. 9A). Similarly, after stimulation with $UL42_{22\text{-}36}$, we observed an increase percentage of TNF-α-secreting CD8 T cells in PD patients (0.06%) compared to HCs (0.012%) with a p value of 0.03. A statistically significant difference in the response to peptide-specific activation was registered only for PD patients (Fig. 9B). Correlation analysis of PD responses after stimulation with homologous peptides have shown similar patterns characterized by a very good coefficient value (R^2=0.876; Fig. 11A).

Regarding the activation of CD8 T cells producing INF-γ, we detected a statistically significant difference only after stimulation with $\alpha\text{-syn}_{100\text{-}114}$(p= 0.032); the mean percentage of CD8 T cells accounted for 0.15% in PD patients and 0.02% in HCs (Fig. 9C), while no difference was observed after $UL42_{22\text{-}36}$ stimulation (Fig 9D).

CD4 T cells secreting TNF-α incubated with $\alpha\text{-syn}_{100\text{-}114}$ were present at a higher percentage in PD patients compared to HCs (0.055% vs. 0.006%, respectively, p= 0.0045; Fig. 9A). Similarly, we observed a statistically significant difference in responses following $UL42_{22\text{-}36}$ stimulation with respective mean values among PD patients and HCs (0.055% vs. 0.005%, p=0.03; Fig. 9B). Also in this case we registered a very good correlation between responses against $\alpha\text{-syn}_{100\text{-}114}$ and $UL42_{22\text{-}36}$ peptides in PD patients (R^2=0.979; Fig. 11B).

As regards CD4 T cell secreting INF-γ, we observed a statistical difference only when the cells were stimulated with $UL42_{22-36}$,with a mean of percentage of 0.06% in PD patients and 0.007% in HCs with a p value of 0.016.

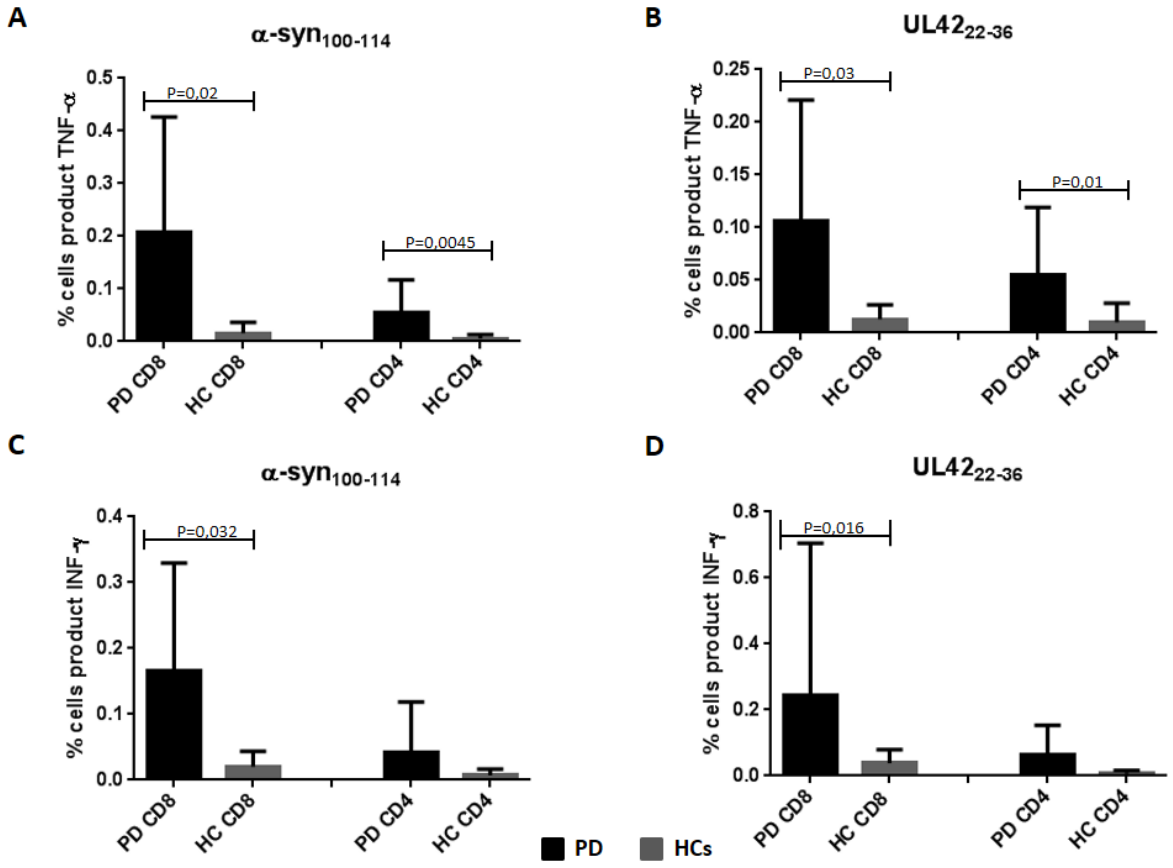

Figure 9. Frequencies of CD8 and CD4 T cells. (A) Percentage of CD8 and CD4 T cells secreting TNF-α after 8h incubation with $α\text{-syn}_{100-114}$.(B) Percentage of CD8 and CD4 T cells secreting TNF-α after 8h incubation with $UL42_{22-36}$. (C) Percentage of CD8 and CD4 T cells secreting INF-γ after 8h incubation with $α\text{-syn}_{100-114}$.(D) Percentage of CD8 and CD4 T cells secreting INF-γ after 8h incubation with $UL42_{22-36}$. Means and standard deviation are represented. A, B, C, D show statistical differences between PD patients and healthy controls.

5.1.3 Stimulation of CD14 and CD56

We also investigated the CD14 and CD56 responses after 8h of incubation with specific peptides, analyzing the percentages of CD14 cells secreting TNF-α, INF-γ and CD56 producing IL-6 and TNF-α.

Only CD14 cells secreting INF-γ displayed responses to a specific stimulation with $UL42_{22-36}$ peptide was and their percentages reached 0.06% in PD patients and 0.007% in HCs ($p=0.028$; Fig. 10A), while no specific stimulation was observed with $\alpha\text{-syn}_{100-114}$. However,a good correlation in this cell subset was obtained after stimulation with $UL42_{22-36}$ and $\alpha\text{-syn}_{100-114}$ ($R^2=0.976$; Fig. 11C). In the same way we didn't observe any statistically significant difference in the percentage of CD14 cells producing TNF-α (Fig. 10B).

Next we analyzed the frequency of CD56 cells producing IL-6 and TNF-α; only TNF-α-secreting cells responded to the specific stimulation with both $\alpha\text{-syn}_{100-114}$ and $UL42_{22-36}$ peptides. In the first case we observed a mean percentage of 0.04% in PD patients compared to 0.0006% among HCs ($p= 0.002$). Cells incubated with $UL42_{22-36}$ peptide accounted for 0.056% in PD patients versus 0.001% in HCs ($p=0.002$; Fig.10D). Correlation between percentages of CD56 cells secreting TNF-α upon stimulation with each peptidein PD patients reached $R^2= 0.944$ (Fig.11D).

In contrast, CD56 cells secreting IL-6 did not show differences in response to stimulation with both peptides in PD patients and HCs.

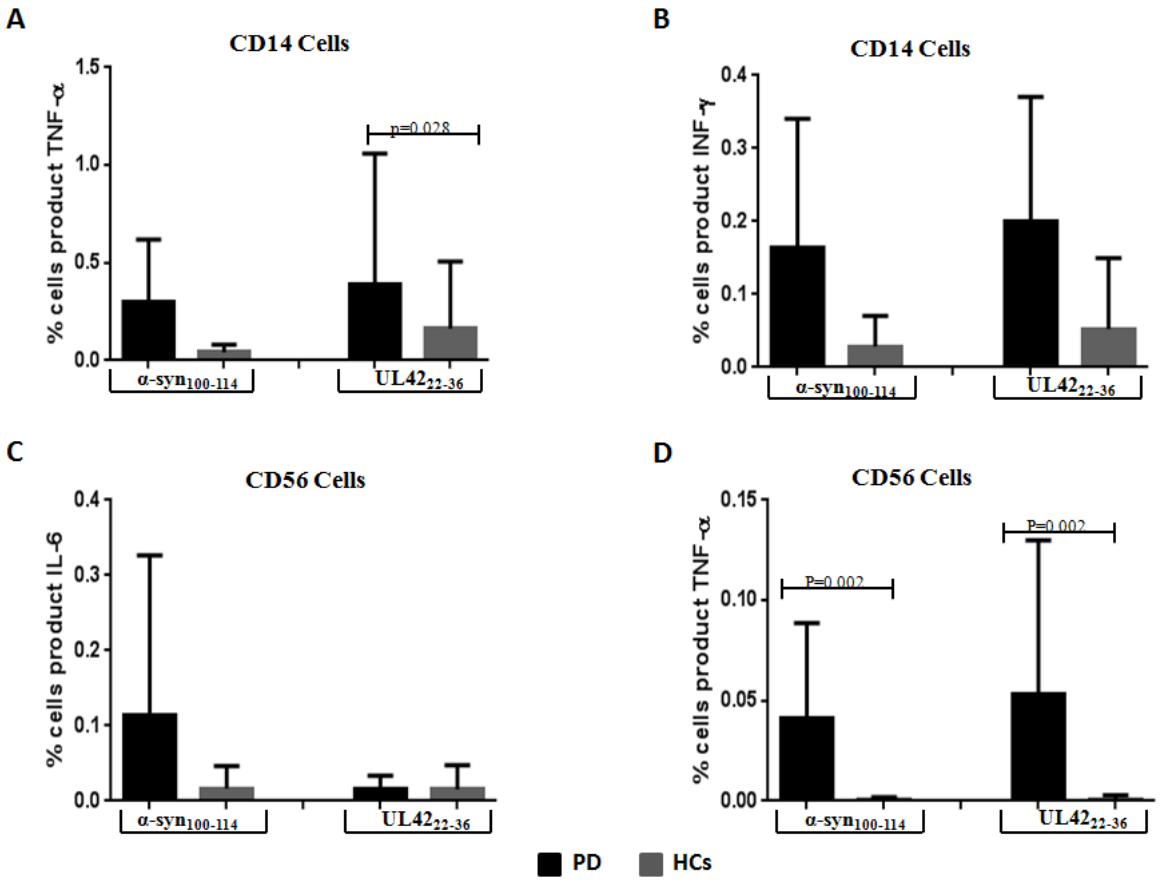

Figure 10. Frequencies of CD14 and CD56 cells. (A) Percentage of CD14 cells secreting TNF-α after 8h incubation with α-syn$_{100-114}$.and UL42$_{22-36}$ (B) Percentage of CD14 cells secreting INF-γ after 8h incubation with α-syn$_{100-114}$.and UL42$_{22-36}$. (C) Percentage of CD56 cells secreting IL-6 after 8h incubation with α-syn$_{100-114}$.and UL42$_{22-36}$. (D) Percentage of CD56 cells secreting TNF-α after 8h incubation with α-syn$_{100-114}$.and UL42$_{22-36}$. Means and standard deviation are indicated. A and D show statistical differences between PD patients and healthy controls.

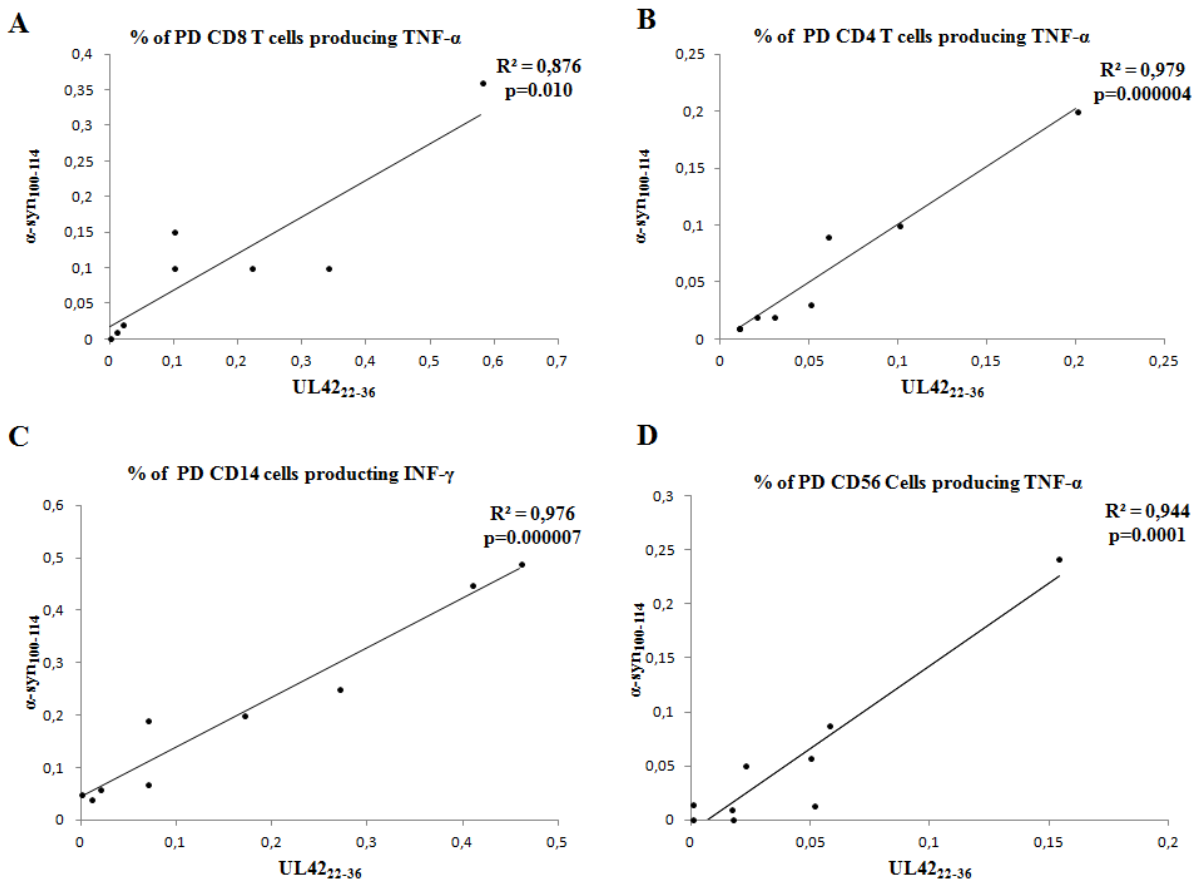

Figure 11 Correlation analysis. Correlation between specific immune responses after stimulation with homologous peptides. (A) Correlation of PD CD8 T cells secreting TNF-α after stimulation with α-syn$_{100-114}$ and UL42$_{22-36}$. (B) Correlation of PD CD4 T cells secreting TNF-α after stimulation with α-syn$_{100-114}$ and UL42$_{22-36}$. (C) Correlation of PD CD14 cells secreting INF-γ after stimulation with α-syn$_{100-114}$ and UL42$_{22-36}$. (D) Correlation of PD CD56 cells secreting TNF-α after stimulation with α-syn$_{100-114}$ and UL42$_{22-36}$.

5.2 Discussion

Parkinson's disease causes death of only dopamine-producing neurons in the brain suggesting a possible role of autoimmunity in this pathology. Although many evidences suggest a significant role of the immune system in PD pathogenesis, the exact nature of interaction with environmental factors is still unclear, though, at least in part, an autoimmune process is supposed to drive PD. Whether this apparent autoimmune response is in turn triggered by another process, such as infection or other source of inflammation,

remains to be discovered as well. For many years, the scientific world thought that neurons were unscathed by the immune system attacks, since it was believed that they were not subject to antigen presentation on their surface. Cebrian et al. demonstrated that human catecholaminergic substantia nigra and locus coerules neurons express MHC-I, then the neurons that have the MCH-I are able to present antigen and may be particularly susceptible to T-cell-mediated cytotoxic attack [187].

In a previous study, we suggested that a common pathogen of the central nervous system, such as HSV1, could play a role in triggering autoimmunity in PD. Destruction of dopaminergic neurons of the substantia nigra may occur through a mechanism of molecular mimicry or immunologic cross-reactivity; we have supposed a molecular mimicry between HSV1 and human α-syn that could foster the progression of this pathology. We have observed a statistically significant difference of antibody levels against $Ul42_{22–36}$ in PD patients in comparison to HCs; the same trend was mirrored by responses against the human homologous peptide $\alpha\text{-syn}_{100–114}$. Our results highlighted that auto-antibodies recognizing HSV1-$Ul42_{22–36}$ peptide are able to cross-react with the homologous human $\alpha\text{-syn}_{100–114}$ epitope. In this study, we investigated the involvement of the immune system, in particular cell-mediated immunity, using the intracellular cytokine (ICC) method.

In the first analysis, we detected lower percentages of different subsets of lymphocytes such as CD3, CD4, CD8 and CD56 in PD patients compared to HCs. Some authors suggest that levodopa treatment may have an inhibitory effect on the proliferation of lymphocytes [144] while other authors highlight that the decrease of lymphocytes is independent of levodopa treatment [140]. Our data have shown the presence of an immunological alteration among Parkinson's patients. Several reports indicate that individuals with PD have increased memory T cells but decreased naïve T cells compared to people with other neurologic diseases [141].

Flow cytometry analyses showed a significant increase of TNF-α CD8, CD4 and NK cells after stimulation with either α-syn$_{100-114}$ or UL42$_{22-36}$ peptides in PD patients respect to healthy controls. Interestingly, in PD patients the specific responses in PD patients display similar pattern after stimulation with both homologous peptides, indeed there is a strong correlation between the two kinds of responses registered for CD8, CD4 and NK.

We have showed an increase of CD8 percentage in PD patients and these cells may have an important role in Parkinson's pathogenesis. The action of CD8 cells in this pathology is well documented; indeed, their presence in CNS of patients may suggest an interaction between CD8 T cells and antigen-presenting functions of microglia. Moreover, the levels of B2 microglobulin are increased in the striatum of PD patients, and CD8 T cells activated by MCH-I can exemplify their cytotoxic activity leading to neuronal death [138,188, 112,113]. Thus, T-cell-mediated neurotoxicity may be driven by direct cell lysis, engagement of cell death receptors, and cytokine secretion through recognition of peptide: MHC molecules. We also showed increased levels of CD4 T cells subjected to stimulus of human α-syn protein and its homologous peptide derived from HSV1, suggesting a possible role of these immune cells in PD; furthermore, some authors report an increase in CD45RO^{+} T cells a subpopulation of CD4 T cells in PD patients [189].

We have observed a specific stimulation only in TNF-α secreting cells and this cytokine have an important role in the pathogenesis of PD. It is reported that dopaminergic neurons are very susceptible to TNF-α, which seems to have an effect on their plasticity. Furthermore, neuronal death can occur in response to TNF-α ligation with its receptors TNFRs [190, 130, 129].

In the same period of our studies another groups of Columbia University showed, through a flow cytometry study, a possible role of autoimmunity in PD. Sulzer et al analyzed the T cell

response in 67 PD patients and 36 healthy controls after stimulation with different α-syn peptides. The author observed a strong response toward two specific peptides of this protein Y39 and S129, while they didn't have the same response in control subjects. They analyzed the T-cell response in correlation with HLA risk alleles, showing that the expression of 4 specific alleles risk in patients determine a major responses against α-syn epitope Y39. This study supports the hypothesis that α-syn can activate T-cell response and the possible implication of cell-mediated immunity and in particular autoimmunity in PD [191].

It remains to be clarified whether autoimmunity represent the primary causes or if it a consequence of the neurodegenerative process that bring to a progression of the disease.

All of these data support the hypothesis of autoimmunity in PD, and if it will be confirmed in future studies the diagnostic and therapeutic approaches could be improved, through the use of immunotherapies and using T-cells as biomarkers.

In conclusion, the results of our experiments support the assumption that α-syn$_{100\text{-}114}$ and UL42$_{22\text{-}36}$ peptides are able to induce cell-mediated responses in PD patients highlighting the relevant role of TNF-α in this process with statistically significant differences respect to healthy controls. Furthermore, our data are in line with the hypothesis of a possible involvement of the immune system, in particular autoimmunity, in the pathogenesis of PD, and that HSV1 infections may lead to a progression ofthe disease.

Chapter 6: Alpha-synuclein as a potential Antimicrobial peptide

Currently, the exact physiological function of α-syn is still not fully known, and also the exact mechanisms that lead to toxicity with subsequent neuronal death are still unclear, but the deposition of α-syn fibrils in Lewy Bodies in dopaminergic neurons is one of the main features of PD. Lewy Bodies are largely composed of beta-sheet rich α-syn amyloid fibrils [96]. Oligomerization in particular is viewed as a pathogenic pathway and α-syn oligomers are assumed to be intrinsically abnormal. Antimicrobial peptides (AMPs), an evolutionarily very old family of proteins, have the characteristic of generating oligomers and fibrils, as well as α-syn. These properties play a key role in mediating the processes of defense of innate immunity, AMPs are the first-line of defense against pathogens and act as potent broad-spectrum antibiotics and immunomodulators that target bacteria, mycobacteria, enveloped viruses, fungi, and protozoans, and in some cases, transformed or cancerous host cells [97]. AMPs are expressed in many tissues, but it has been reported that there is a notable expression in the brain [98], as well as in other tissues where the intervention of the adaptive immune system is limited. Normally the action of this class of peptides turns out to be protective, but their dysregulation can lead to toxic effects in the host cells [99,100], such as chronic inflammation [101,102,100,103] and degenerative diseases [104]. Recent studies reveal a possible antimicrobial action of Aβ, suggesting that Aβ deposits may be a consequence of the protective action of this peptide against infections [105, 106, 107]. Some genetic studies in PD patients showed that mutations of APOE gene seem to be associated with a more rapid decline of the disease, showing a decrease in memory performance, attention, speech and execution of movements. APOE gene mutations in AD seem to be associated with an increased accumulation of Aβ protein, whereas in PD patients, Apo-ε4

mutation seems to stimulate the aggregation of α-syn, patients carrying Apo-ε4 have an early debut. It has also been reported that mutations of the APOE gene are a predisposing factor for HSV-1 infections [179,180]

We hypothesized that α-syn may also perform functions such as AMPs, considering its ability to form oligomers, it is possible that infections can lead to a dramatic accumulation of a α-syn in the cytosol with subsequent formation of deposits in Lewy Bodies. An increasing number of studies show a possible role of the immune system in PD, in this study we want to analyze if α-syn can be a key protein of innate immunity through an action like AMPs, through *in vitro* studies we analyzed the antiviral action of α-syn against HSV1.

6.1 Results

α-syn mediated antiviral activity was tested in cell culture infection models, in different cell lines. Initially we have used for the first round of experiments, the epithelial Hela cells line due to their ease of transfection and susceptibility to HSV-1 infection and we tested produced in *E.coli* system, Aβ produced by CHO-CAB and H4-$A\beta_{42}$ cells lines as a positive controls and only medium as a negative controls. To generate a robust and reliable dataset the screening was carried out three times in triplicate, with one replicate used in a cell viability assay to determine any cytotoxic effects, and duplicates infected for the virus infection assay. After this series of experiments we test the possible antiviral activity in transient α-syn over-expression cell such as Hela and HuH7.5.

6.1.1 α-syn inhibition of HSV-1 infection in Hela cells

We analyzed the protective activities of α-syn against HSV1 in Hela monolayer infection model using Green Fluorescent Protein (GFP) labeled virus (HSV1strain C12) and we monitore virus growth kinetics as a measure of GFP-fluorescence. We used Aβ as a positive controls, and untreated cells as negative controls. We only reported some of the different experiments performed.

In the first experiment we did not observe any inhibition of viral replication after α-syn (produced in *E.coli*) addition to Hela cells infected with HSV1 (Fig. 12), neither the positive controls showed an inhibitory activity. Cell-viability assay was performed to determine the toxicity of α-syn protein, no toxicity was reported (Fig. 13).

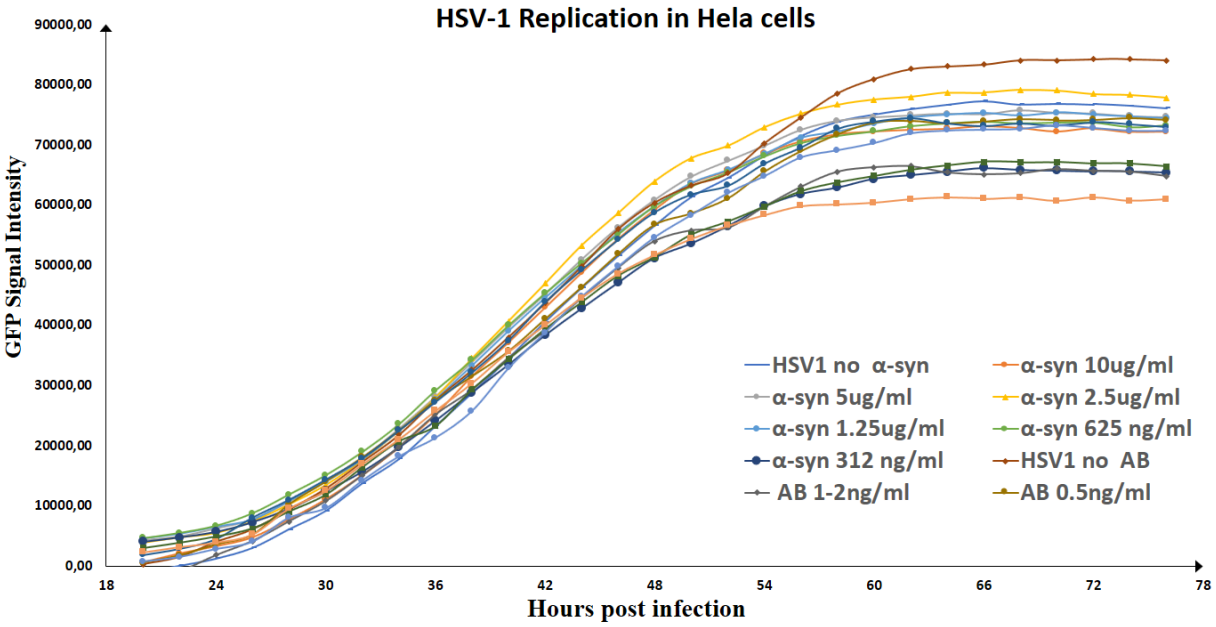

Figure 12. Hela cells infected with HSV1 recombinant strain C12 expressing GFP (MOI = 0.5). Virus growth has been monitored by GFP fluorescence.

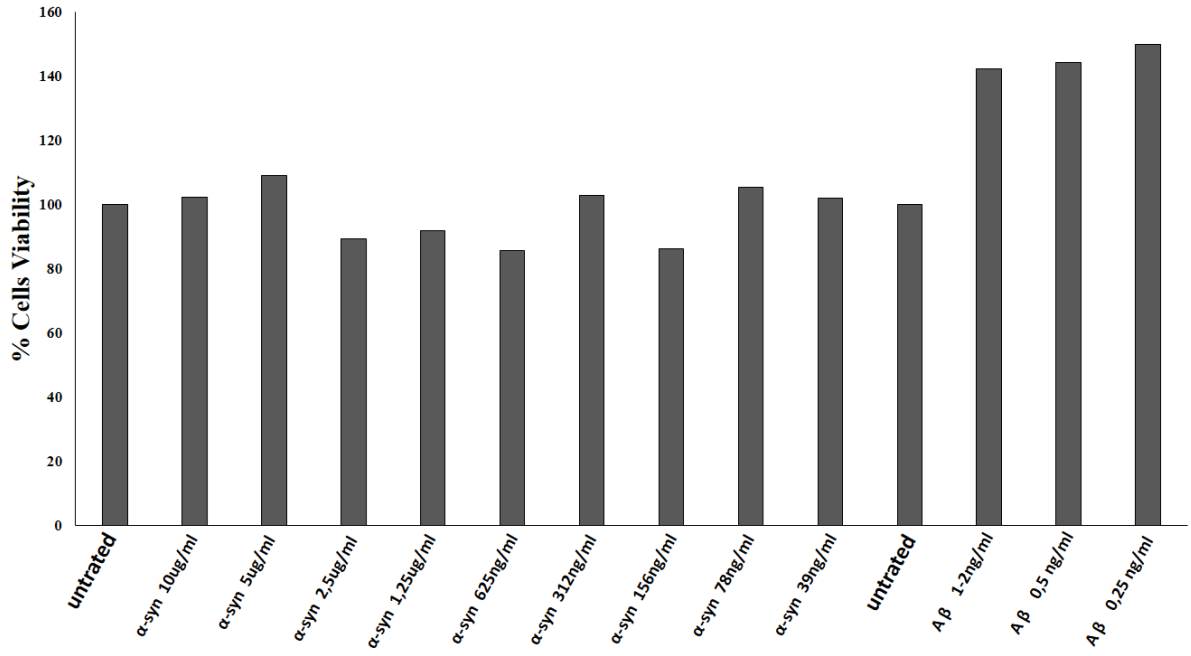

Figure 13 Relative viability of cells upon treatment with α-syn and Aβ

Then we have analyzed the possible α-syn antiviral activity by HSV1 pre-incubation with different condition: the α-syn protein, Aβ and medium as a negative control, for 1 hour before the infection of Hela cells. HSV1 was not inhibited by α-syn or Aβ, (Fig. 14) and the same result could be observed from the slope analysis that showed no inhibitory effect as showe on Fig. 15, at the same time we tried to show the α-syn activity increasing its concentration but without any positive results (Fig. 16).

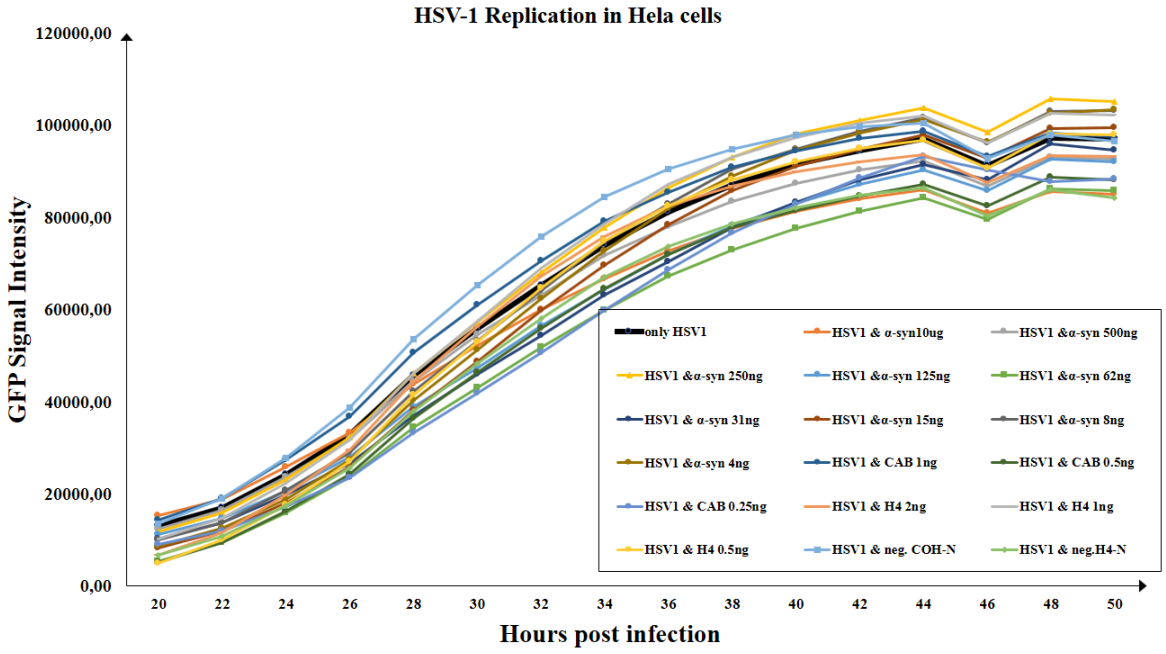

Figure 14 Hela cells infected with HSV-1 recombinant strain C12 expressing GFP (MOI = 0.5). Virus growth has been monitored by GFP fluorescence

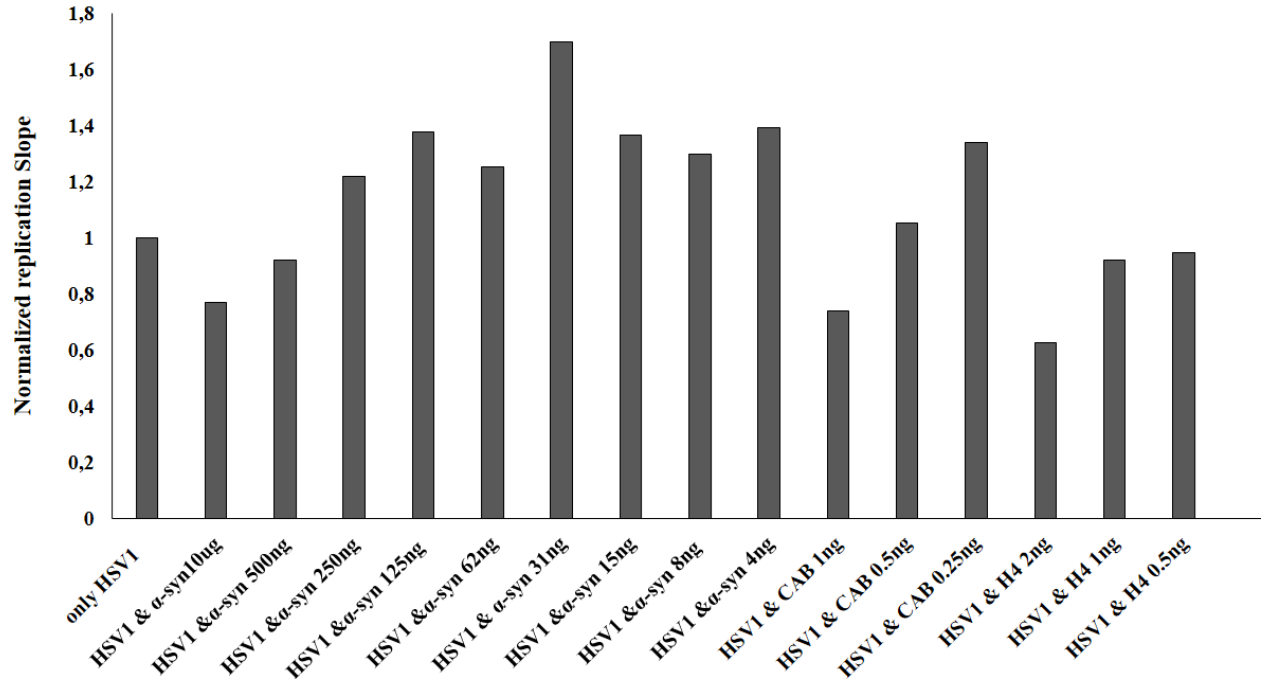

Figure 15 Histogram shows the results of the experiment where it has been analyzedα-syninhibits HSV-1 C12-GFP. Hela cells were infected with HSV-1 C12-eGFP at MOI 0.5. Replication slopes were monitored and normalized to control untreated virus. Error bars represent the mean of at least three independent experiments

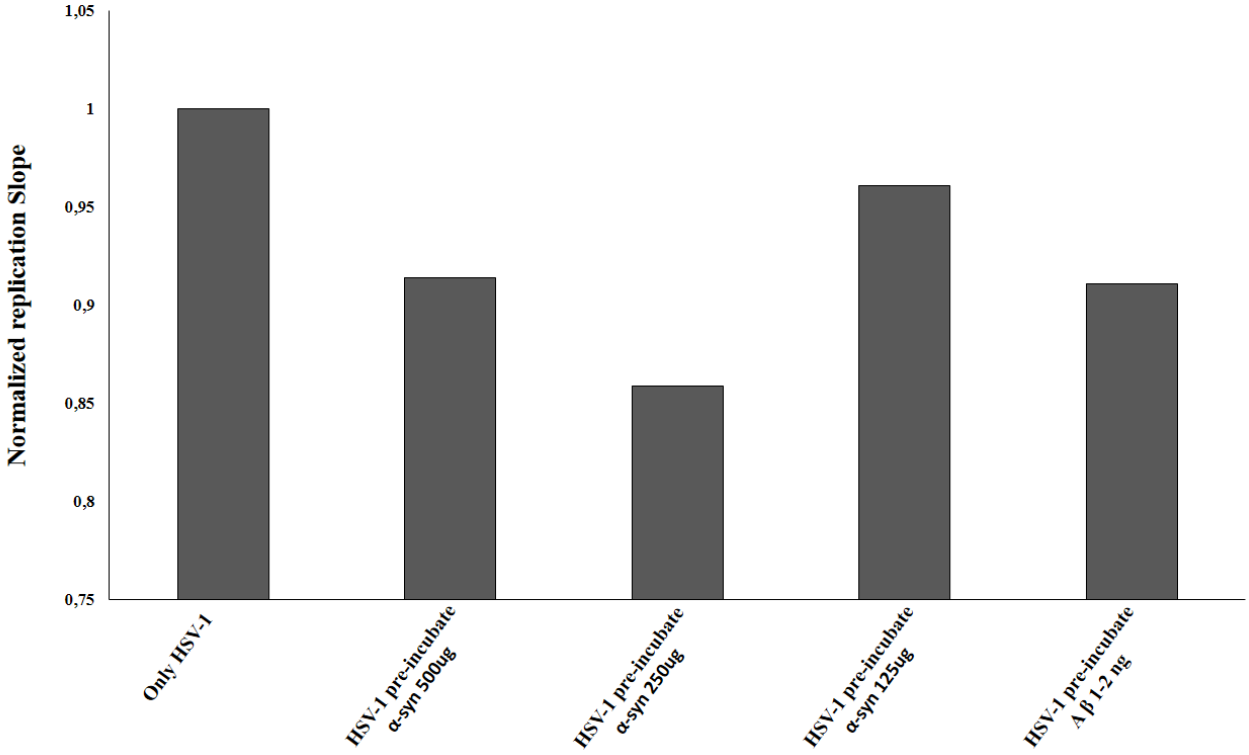

Figure 16 Histogram shows the results of the experiment where it has been analyzed α-syn inhibits HSV-1 C12-GFP infection in Hela cells. Cells were infected HSV-1 C12-eGFP at MOI 0.5. Replication slopes were monitored and normalized to control untreated virus. Error bars represent the mean of at least three independent experiments

6.1.2 HSV-1 infection on transient over-expression of α-syn

The effect of α-syn transient overexpression was investigated in Hela and HuH7.5 cells, to analyze the possible antiviral effect of α-syn expressed in a eukaryotic system. After the infection with HSV1 strain C12 the replication growth curves, and endpoint replication were monitored (as determined by fluorescence). In the Hela cells line a consistent inhibition of viral replication was observed (Fig. 17), the same result was showed in figure 18 from the slope analysis. Inhibition of viral replication is observed in the cells transfected with the plasmid containing SNCA and in those transfected with the positive control, but at the same

time a low viral replication is observed even in the cells transfected only with the negative control.

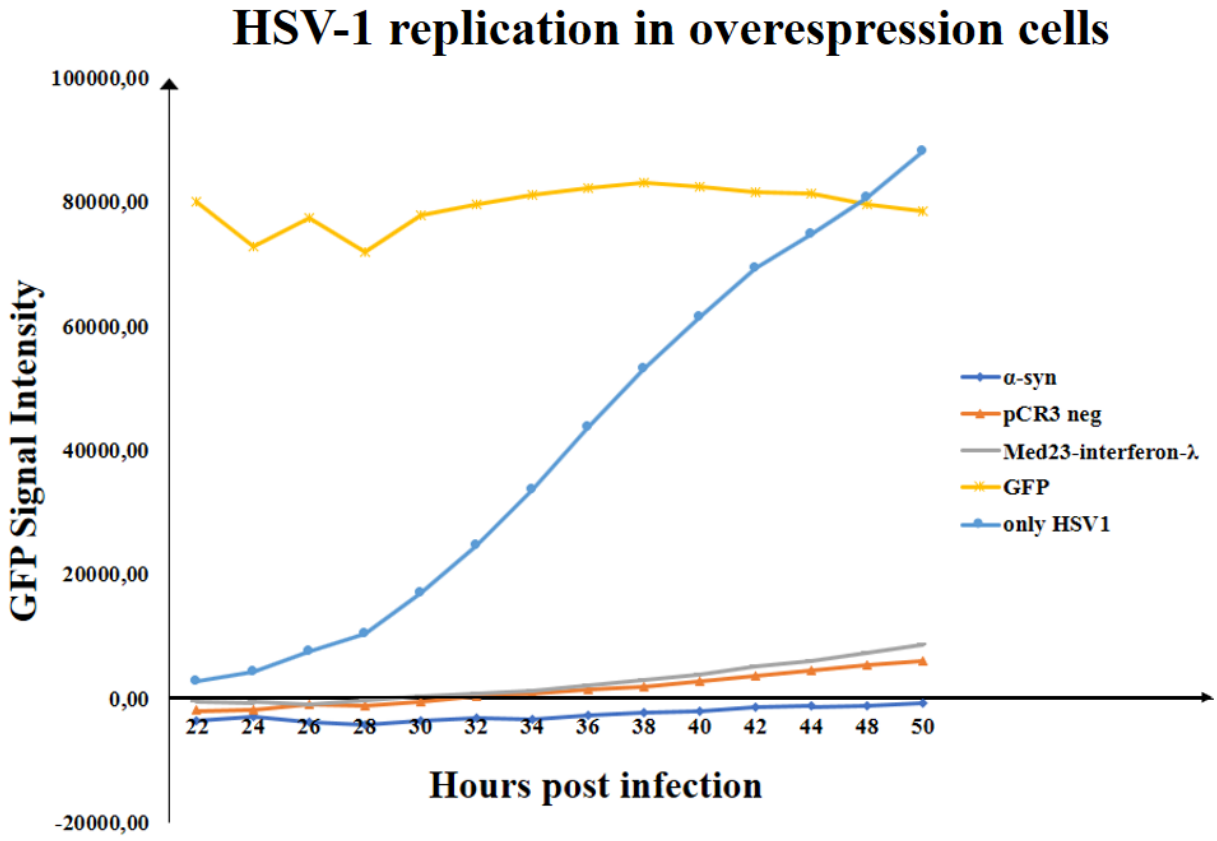

Figure 17 Hela over-expressing α-syn cells infected with HSV-1 recombinant strain C12 expressing GFP (MOI = 0.5). Virus growth has been monitored by GFP fluorescence.

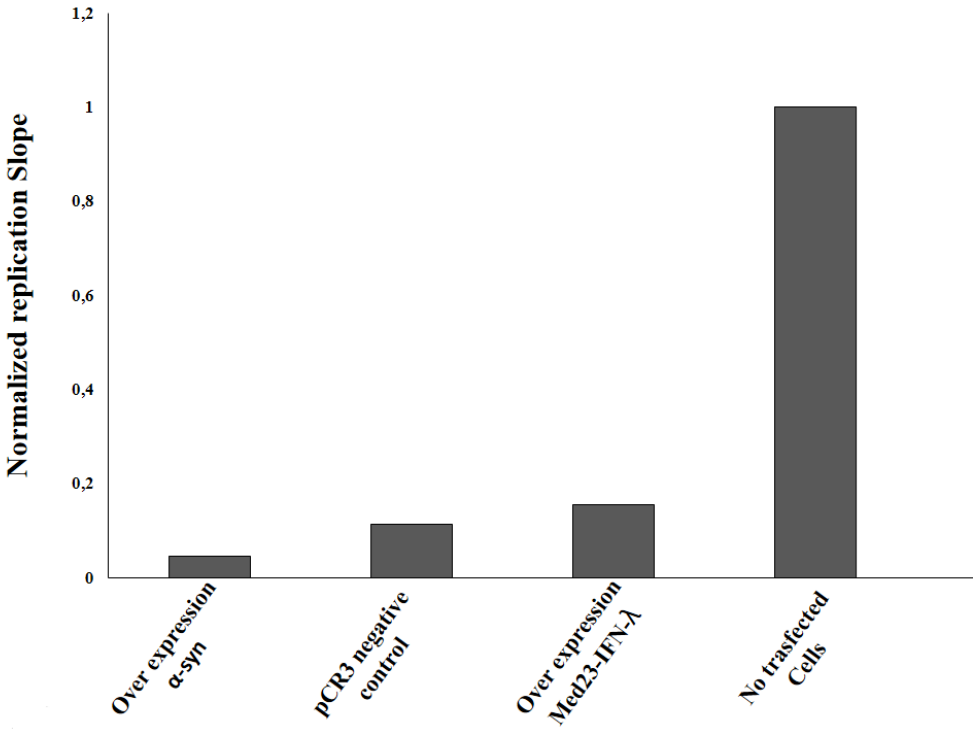

Figure 18 Histogram showing the results of the experiment where over-expression of α-syn inhibits HSV-1 C12-GFP. Hela cells were infected with HSV-1 C12-eGFP at MOI 0.5. Replication slopes were monitored and normalized to control untreated virus. Error bars represent the mean of at least three independent experiments

We tried to conduct the same type of experiment using another cell line HuH7.5, as it is shown Figure 19, we have not found any viral inhibition either for the line transfected with SNCA or for the line expressing the positive control.

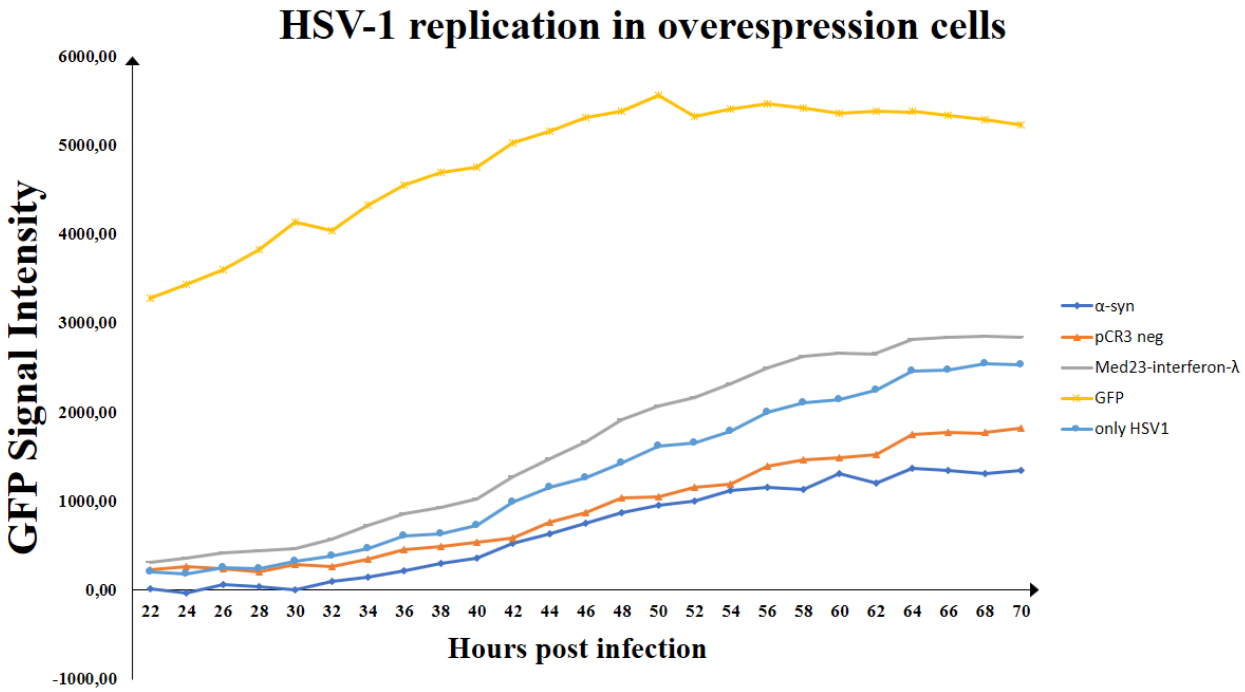

Figure 19 HuH7.5 over-expressing α-syn cells infected with HSV-1 recombinant strain C12 expressing GFP (MOI = 0.5). Virus growth has been monitored by GFP fluorescence.

6.2 Discussion

In previous studies we have highlighted a possible association between HSV1 infections and PD, in this work the aim was to analyze whether the α-syn could have an antiviral activity. The assumption was that recurrent HSV1 infections, with its latency-activation cycles, could lead to an over-expression of the α-syn, in first time to counteract the viral replication and then lead to the formation of oligomers with consequent accumulation inside the cells.

Currently in literature there are only two studies documenting a possible role of α-syn as a possible AMPs. In the first Park et al demonstrate that α-syn exhibits antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. In addition, the authors demonstrate a role for α-syn in inhibiting various pathogenic fungal strains such as *Aspergillusflavus*,

Aspergillus fumigatus and Rhizoctoniasolani [110]. In the second study Beatman et al reported that the neuronal expression of α-syn is able to inhibit viral infection by West Nile Virus, in fact the authors observed an increase of α-syn expression in neurons infected with WNV. At the same time they observed that knockout mice for α-syn develop a higher viral titre respect to wild-type animals [111]. All these data suggest a possible role of the α-syn as a AMPs.

In this study we wanted to analyze α-syn as potential AMPs by following different experimental approaches. In the first we infected cells with HSV1 and once the virus was adsorbed we incubated the cells with the medium containing different concentrations of α-syn, but we did not observe any inhibition. Several authors reported that usually Aβ and LL-37 show an antiviral property when the virus was pre-incubated with the peptide before the infection, because usually these peptides exert their action by binding to membrane receptors of the virus preventing their binding with the cell membrane[192,193; 194]. So, we conducted another series of experiments trying to pre-incubate the virus with α-syn before infection but we have not had any positive result, probably because as reported by authors natural peptides with antimicrobial activity have to be used at the physiological contractions to higher concentrations [195]. Following a careful analysis of our protocols we have highlighted that α-syn previoulsly used a concentration of 0.5 μM which is 100 times lower than the physiological concentration range (5-50μM) [196]. In the light of these observations, another series of experiments were carried out by pre-incubating HSV1 with physiological concentrations of the protein, but also in this case we did not obtain any positive results. Following a careful bibliographic study we have found that the α-syn produced in *E.coli* is not able to give rise to oligomers, several studies conducted using different methodologies, such

as NMR, light scattering and circular dichroism, have all put in evidence that α-syn produced in *E.coli* is predominantly in the monomers state and is not subject to the oligomerization phenomenon [197;198], being produced in a prokaryotic system the protein can not undergo all the post-transcriptional modifications typical of a eukaryotic system. As we have pointed out above, the formation of oligomers is a key and necessary feature of the AMPs to be able to perform their function. So to overcome this type of problem we have adopted another experimental approach transfecting, with lipofectamine, the Hela cells line with a plasmid containing the SNCA gene under the control of the Cytomegalovirus promoter, so that the cells line over-expressing the α-syn. As showing the result (Fig. 18) there is an important inhibition of viral replication in the cells transfected with SNCA, but the same result was also observed in the negative control. An explanation could be given by the fact that the chemical treatment for the transfection could, in some way, have altered the plasma membrane of the cells with consequent inhibition of the virus binding the membrane.

In conclusion, based on our results,we are not able to state that α-syn has an antiviral action against HSV1, but further studies are needed to clarify the possible antiviral function of it. In the future eukaryotic stable cell lines expressing α-syn through the use of lentiviral vectors may be generated and α-syn fibrils may be directly tested.

Chapter 7:Analysis of the expression of different miRNAs in PD patients compared to healthy controls

Part of this chapter is based on the following article:

- 2018. Elisa Caggiu, Kai Paulus, Giuseppe Mameli, Giannina Arru, Gian Pietro Sechi, Leonardo A. Sechi. Differential expression of miRNA 155 and miRNA 146a in Parkinson's disease patients. eNeurologicalSci. 13 : 1–4

Neuroinflammation has been increasingly studied as a chief mediator in the pathogenesis and progression of PD [112]. miRNAs, small non coding RNA, are involved in several pathologies since their activity consists in controlling the genetic expression and their dysregulation contribute to different pathologies, including PD [164]. miRNA could be perfect candidates as biomarkers for diseases in which they are altered. Furthermore, they could be potentially used in order to monitor the progression of the disease. Peripheral blood mononuclear cells (PBMCs) share more than the 80% of the transcriptome with other tissues, including the CNS, so peripheral blood could be considered a great source of biomarkers being also widely available [165]. Several studies show how a dysregulation of miRNA is involved in the pathogenesis of different neurodegenerative diseases like: Alzheimer's disease, Parkinson's disease, Huntington's disease and Amyotrophic lateral sclerosis. The molecular mechanisms underlying the pathological implications of misregulated miRNA expression and the regulation of the key genes involved in neurodegenerative disorders remain largely unknown [166, 167, 168, 169]. Since most PD symptoms are caused by a lack of dopamine in the striatum, many Parkinson's drugs are aimed at either temporarily replenishing or mimicking the action of dopamine, Ldopa is most commonly used drug [199].

In this study, we profiled the expression of different candidate PD miRNAs in PBMCs of L-dopa-treated PD patients and unaffected controls. We tested different miRNA such as miR-155, miR-26a, miR-146a and miR-132. We have selected these miRNAs because they are commonly studied in neurodegenerative diseases, but to date only few studies have been conducted in PD patients except for miR-155 that has only been studied in a mouse model of Parkinson disease [200, 201, 202, 203, 204, 205]. Our primary aim is to investigate the potential of circulating miRNAs as non invasive diagnostic candidate biomarkers of PD patients.

7.1 Results

7.1.1 miRNA expression in patients with PD and their matched controls

microRNAs derived from PBMC samples of PD patients and HCs were extracted and the total miRNA isolation was analyzed for the expression of miRNA 155-5p, 146a-5p, 132-3p and 26a-5p. The analysis of different miRNA expression showed that miRNA-155-5p (fold change = 27.18; $p > 0.000001$) were generally up-regulated in PD patients compared to HCs where as miRNA-146a-5p (fold change = -1.76; $p = 0.0015$) were down-regulated in PD patients in comparison to HCs. Other miRNA (miRNA-132-5p and miRNA-26a-5p) did not show a different expression between PD patients and HCs (Fig. 20).

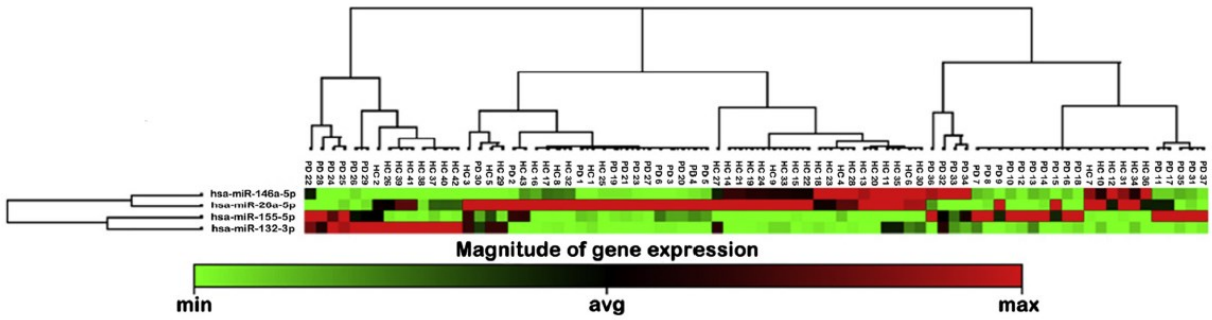

Figure 20 Heat map of microRNA (miRNA) microarray expression date from plasma samples of PD patients (PD 37) and Healthy controls (HCs 43). The miRNA species are shown on the left. Cluster analysis classified the samples in groups based on the miRNA expression levels in each samples. The dendogram shows different expression levels of miRNA among samples. Red indicates high expression of miRNA, and green indicates relatively low expression of miRNA.

7.1.2 miRNA expression in PD patients with different Levodopa dosages

Patients were classified into two groups based on the median daily dosage of L-dopa: lower of 458 mg per day in the first group and greater than 458 mg per day in the second group. The statistical analysis with Mann Whitney Test showed no difference in the distribution of the two groups regarding to age and sex, with the respective p values of p>0,7569 for the age, and p>0,7569 for the sex. We analyzed the expression of different miRNA to verify if it can be modified by levodopa treatment. Upon quantification of the miRNAs 155 -5p, 146a-5p, 132-3p and 26a-5p expression in the two groups we observed a down-regulation of miR-155-5p in PD patients with the highest dosage (fold change = -1.67; p= 0.029). The other miRNA did not show a different expression pattern in the different groups (Fig 21.).

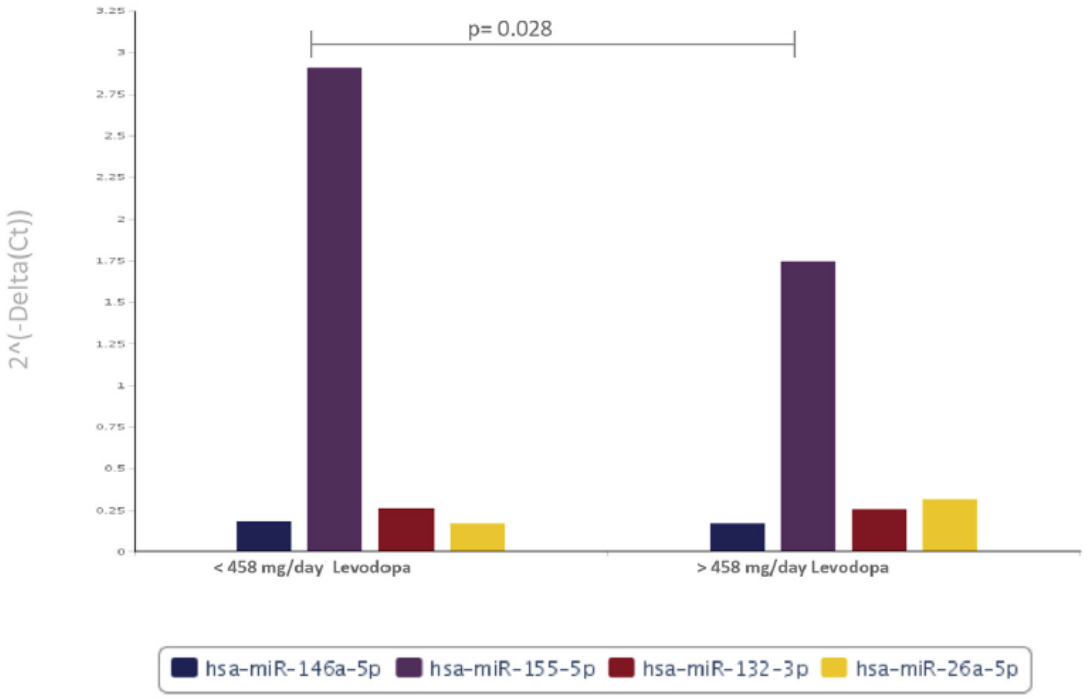

Figure 21 The Column Chart showing the average miRNA expression in different grouped, the PD patients are divided according to different dosage of Levodopa, the average daily dosage of Levodopa is indicated.

7.2 Discussion

miRNAs are more important in post-transcriptional regulation of target gene expression and each of them acts on the expression of a specific target. The maturation of miRNA is an highly controlled process and they undergo further post-transcriptional control. Abnormal miRNA expression may play a role in the pathogenesis of different diseases [206]. miRNAs appear to be suitable tools for the diagnosis, prognosis, and therapy of several disorders on the basis of their functional roles in diverse biological pathways.

Dysregulation of miRNA has been implicated in several neurological disorders, such as neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, Amylotrophic lateral sclerosis and Huntington's disease. Numerous reports demonstrate the role of miRNAs

in Parkinson's disease. Studies conducted to PD patients compared with healthy controls on the expression of different miRNA revealed that miR-34c-5p and miR-637 were significantly down-regulated in the amygdala of PD patients [207]. Other authors reported that miR-133b was down-regulated in the midbrain of PD patients compared with HCs [208]. Several studies indicated miR-335, -374a/b, -199, -126, -151-5p, 29b/c, -147, -28-5p, -30b/c,-301a, and -26a to be decreased in PBMCs of PD patients compared with controls [209].

In the present study, we investigated the expression of four miRNA (155 -5p, 146a-5p, 132-3p and 26a-5p) in PD patients compared to HCs and analyzed their possible association to inflammation and neurodegeneration. Our data showed an up-regulation of miR-155-5p in PD patients respect to HCs whereas miRNA 146a was under regulated. miR-155 in fact, it is up-regulated in the inflammatory processes with a particular ability to suppress the expression of anti-inflammatory molecules such as SOCS-1 and SOCS-3 [210]. Numerous studies indicate miR-155 to be over-expressed in inflammatory disorders of the central nervous system such as Multiple Sclerosis and Amyotrophic Lateral Sclerosis [211]. In addition, studies on animal models of the two diseases document how the inhibition of this miRNA through the use of complementary oligonucleotides can reduce disease-related dysfunctions [212]. For the first time we observed an overexpression of miRNA 155 in PBMCs of PD patients. Indeed we confirmed what has been previously reported in an in vivo mouse model of PD produced by adeno-associated-virus-mediated expression of α-syn. suggesting that miR-155 has a central role in the inflammatory response to α-syn in the brain [213].

In this context, our findings suggest that miR-155 is altered also in PD and, indirectly, could be a further evidence that inflammation may play an important role in the pathogenesis of this disease. It is also reported in the literature that, besides being miRNA a key regulator of

neuroinflammation, it may be crucial in regulating neuroinfiammation following the production of α-syn oligomers. Indeed, in miR-155 $^{(-/-)}$ mice there is a marked reduction in inflammation induced by α-syn fibrils [213].

Regarding miRNA 146a, a downregulation was observed. The same data has been reported by Ma et al. [214]. A previous study conducted by Jayadev et al. in 2013 reported that miR-146a, a negative regulator of the monocyte pro-inflammatory response, is constitutively downregulated in mice microglia with dysfunctional presenilin 2 (PS2) [215] whose mutations were shown to condition autosomal dominant AD [216]. MiRNA 146a acts regulating negatively the powerful pro-inflammatory pathway mediated by nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB), a transcription factor able to regulate inflammation, immunity and cell survival [217] and attenuating the proinflammatory response. Our results on miRNA 146a are in according with the above mentioned studies, where a downregulation is observed of this miRNA.

We also report that the expression of miR-155 is reduced in patients receiving a higher dosage of Levodopa. It remains to be seen whether the drug can somehow reduce the levels of this miRNA or indirectly it can reduced the inflammation. In contrast to many studies reporting that levodopa treatment can increase the levels of different miRNA [218], we observed a consistent decrease in miRNA 155 in patients with higher doses of Ldopa. The alterations of miR-155 levels in untreated PD patients needs to be verified before and after therapy in order to assess a modulatory potential of this drug. We did not find any difference in expression levels of miRNA 155 when compared to the degree of disability.

In conclusion, miRNA 155 could not only be a promising target for the anti-inflammatory therapy in PD but also its evaluation can help the disease progression. The role of levodopa in modulating the levels of miRNA 155 requires further studies.

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List of papers done during the PhD (01-11-2015 / 31-10-2018

1. 2016. Elisa Caggiu, Kai Paulus, Giannina Arru, Rosanna Piredda, Gian Pietro Sechi, Leonardo A. Sechi. Humoral cross reactivity between a α-synuclein and Herpes simplex -1 epitope in Parkinson's disease, a triggering role in the disease? Journal of Neuroimmunology 291 (2016) 110–114.

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