Investigation of the presence of a-synuclein deposits in the skin of patients with familial Parkinson Disease mutations.

ΕΛΛΗΝΙΚΗ ΔΗΜΟΚΡΑΤΙΑ
Εθνικόν και Καποδιστριακόν Πανεπιστήμιον Αθηνών

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Abstract

Introduction: Parkinson's disease (PD) is a neurodegenerative disease that affects a considerable proportion of the population worldwide. The pathognomonic feature of PD is the misfolding of the protein named a-synuclein. A-synuclein misfolding also characterises a few other diseases, such as Multiple System Atrophy (MSA) and Dementia with Lewy Bodies (DLB), termed collectively, with PD, as synucleinopathies. Pathologic forms of a-synuclein are characterised by the presence of phosphorylation of the protein at Serine 129. This phosphorylated a-synuclein is found both in the CNS and the peripheral tissues of patients, including the skin.

Research question and methodology: In this study, we wanted to elucidate whether phosphorylated a-synuclein is present in the skin of patients carrying mutations causing genetic PD. We also attempted to clarify the neuronal populations that might differentially contain phosphorylated a-synuclein, depending on the genetic form. These genetic forms included cases with the A53T mutation in the SNCA gene, along with cases with GBA or PARKIN mutations. As an extra group, we also included patients with Multiple System Atrophy, in which skin a-synuclein deposition is controversial. We analysed skin tissues from patients and controls to assess for colocalization of pathologic a-synuclein in cervical and foot skin samples, by using markers against phosphorylated a-synuclein and neuron subtypes/compartments, including PGP 9.5, VIP and Synaptophysin. Finally analysis of epidermal fiber neurodegeneration was performed.

Results: We noted a trend of increased colocalization of phosphorylated a-synuclein with neuronal markers in the skin of A53T mutation carriers compared to controls. Patients with the A53T mutation also showed a trend of higher degeneration of their cutaneous autonomic

fibers when comparing to controls. Results from the other genetic groups and MSA were limited due to the very small number of subjects.

Conclusion: This research opens the pathway to further characterise the a-synuclein deposits in patients with genetic mutations, specifically in the SNCA gene. This will hopefully assist in better understanding the disease process and the evolution of treatment for genetic PD.

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Introduction

Neurodegenerative diseases are conditions that might affect the Central Nervous System (CNS) or the Peripheral Nervous System (PNS). The main outcome is the death of selected populations of neurons, that cause symptoms in the affected individuals (Wilson et al., 2023).

A-synuclein: physiology and pathology

Synucleinopathies constitute a major group of neurodegenerative diseases. This group is consists of 4 conditions that bear close resemblance to one another: Parkinson's Disease (PD), Multiple System Atrophy (MSA), Dementia with Lewy Bodies (DLB) and Pure Autonomic Failure (Galvin et al., 2001). The term synucleinopathy is derived from the main pathognomonic feature of those conditions, the aggregation of a protein called a-synuclein (Koga et al., 2021).

A-synuclein is a small protein composed of 140 aminoacids; it belongs to a family of proteins that are called 'intrinsically disordered proteins' (Lashuel et al., 2012). In the cellular environment, a-synuclein is thought to exist in the form of monomers, but there are advocates of the theory that a-synuclein can also form stable tetramers (Bartels et al., 2011). The protein is thought to assume an a-helical conformation when it is present as a monomer, in a well-

regulated cellular environment, especially upon binding to membranes (Stephens et al., 2019).

A-synuclein is subject to a range of post-translational modifications, which give different properties to the protein. A significant modification is the phosphorylation of a-synuclein at Serine 129 (Okochi et al., 2000). The phosphorylation at this amino-acid residue is found in the aggregates that are formed in synucleinopathies, and minimally in the non-parkinsonian brain (Fujiwara et al., 2002, Kawahata et al., 2022). The biological role of this phosphorylation remains unclear, as numerous studies have opposing findings regarding the possible neurodegenerative or neuroprotective role of this modification (Lee et al., 2011, Xu et al., 2015, Oueslati et al., 2013). Interestingly, phosphorylation as Serine 129 might have a role at regulating synaptic currents in the normal brain (Ramalingam et al., 2023). Other modifications of a-synuclein include phosphorylation in different residues, acetylation, ubiquitination, nitration and truncation (Manzanza et al., 2021).

A-synuclein is present in abundance in the nervous system, where it is mostly expressed by neurons; astrocytes might also produce endogenous a-synuclein but at lower levels (Mori et al., 2002). The protein is localised in neuronal synapses and the neuronal nucleus and is believed to play a key role in regulating many cell functions, including the neurotransmitter release at the synaptic level (Sharma et al., 2023) and the exocytosis of vesicles (Huang et al., 2019).

One of a-synuclein's main characteristics is its ability to self-aggregate. The aggregation is a complex, multi-stage event. The a-synuclein monomers interact through the central hydrophobic domain and gradually form oligomers and insoluble fibrils (Lashuel et al., 2012). The aggregates are also shown to propagate between cells (Woerman et al., 2015, Steiner et al., 2011) and organisms (Kim et al., 2019, Vermilyea & Emborg, 2015), including

humans (Li et al., 2008). The cellular prion-like transmission of a-synuclein is considered to play an important role in the pathogenesis of the synucleinopathies.

A-synuclein fibrils (Luk et al., 2009) and oligomers (Bengoa-Vergniory et al., 2017, Alam et al., 2019) are both toxic to cells. The general consensus is that the early forming oligomers are those that are significantly neurotoxic (Volpicelli-Daley et al., 2011), although this debate is long-running, with differing experimental data (Bengoa-Vergniory et al., 2017). A recent study has implicated fibrils as propagation vectors that release toxic oligomers while breaking down (Cascella et al., 2021). Distinct aggregate strains cause differing syndromes across the synucleinopathy spectrum (Peelaerts et al., 2015), and conversely, the formation of a-synuclein aggregates is impacted by the cellular environment, with varying strains being created in distinct cellular types (Peng et al., 2018).

The levels of intracellular a-synuclein are one of the main contributing factors leading to its aggregation. Such levels are tightly controlled via the regulation of its production and degradation. The autophagic-lysosomal pathway has a well-defined role in a-synuclein clearance (Fellner et al., 2021) and a-synuclein is degraded both by macro autophagy (Vogiatzi et al., 2008) and Chaperone Mediated Autophagy (CMA) (Xilouri et al., 2013).

Parkinson's disease and genetics

As it has already been underlined, a multitude of clinical syndromes involve cellular death due to the accumulation of a-synuclein and are termed synucleinopathies. The most well-known of those syndromes is Parkinson' Disease (PD), a neurodegenerative disease that mostly affects people of older age. The main pathognomonic feature of PD is the degeneration of the basal ganglia and more specifically, the dopaminergic neurons of the

substantia nigra pars compacta (SNpc) (Feraco et al., 2021). The SNpc owns an important role in the direct and indirect pathways of the basal ganglia regulating the striatum and providing input for fine control (Fuxe et al., 2006). The degeneration of these types of neurons results in the motor signs that characterise the disease: tremor, bradykinesia, rigidity and impaired posture (NICE guidelines, 2017).

The CNS of the patients with Parkinson's disease is characterised by the presence of inclusion bodies containing a-synuclein, the Lewy Bodies (LB). They exist in abundance in the Substantia Nigra (Goedert et al., 2012), although they can also be encountered in different areas of the brain, such as the locus coeruleus and the cortex. It should be underlined that a-synuclein is not their only component, as LBs are rich in lipid membranes (Shahmoradian et al., 2019). A-synuclein in LBs is specifically phosphorylated at Serine 129. Distinct LB conformations exist, with brainstem LBs containing a-synuclein in the periphery and cortical LBs displaying a diffuse immunohistochemical pattern (Kon et al., 2019).

Apart from the motor dysfunction, various other signs are also present in PD, due to the degeneration of other CNS areas. Spread of a-synuclein aggregates to the olfactory bulb distorts the sense of smell and is one of the first clinical signs of the disease, appearing over 10 years prior to the overt presentation of the motor signs (Jellinger, 2008). LBs in the cortex are associated with the disruption of higher brain functions (Jellinger, 2015).

PD is considered a multifactorial disease, with both environmental and genetic factors predisposing to it. The exposure to various pesticides, such as rotenone and paraquat, is thought to predispose individuals to PD, an epidemiological finding (Spivey, 2011) that is supported by experiments in cell culture and animal models (Van Laar et al., 2023). On the other hand, genetic studies have unveiled multiple loci that are associated with PD.

A prominent gene involved in PD is the *SNCA* gene, located on chromosome 4, encoding for the a-synuclein protein, which as mentioned above has a central role in the pathogenesis of PD (Siddiqui et al., 2016). Mutations in the gene that affect the protein's fibrillization properties have been discovered in kindreds with familial PD (Li et al., 2001). The mutations follow an autosomal dominant pattern of inheritance and cause an early onset form of PD (Klein & Westenberger, 2007). Examples of these mutant forms include A53T, E46K and A30P. Duplications or triplications in the *SNCA* gene, affecting the level of the wild type protein, have also been discovered (Konno et al., 2016, Singleton et al., 2003)

Of particular importance is the A53T mutation which is frequently encountered in patients of Greek descent (Polymeropoulos et al., 1997). This SNCA gene mutation leads to the substitution of alanine to threonine at the 53 amino acid position. This mutated protein forms protofibrils at a higher rate compared to the wild-type protein (Lashuel et al., 2002). The penetrance of this mutation is thought to be high, but not 100% (Papadimitriou et al., 2016). The age of onset is lower compared to idiopathic PD, but late onset PD has also been described (Trinh et al., 2018). The disease can have an atypical presentation, although this varies in the literature. Moreover, neuropsychiatric and cognitive symptoms are generally more severe compared to iPD (Koros et al., 2018). Treatment is via Levodopa or other dopaminergic drug administration, leading to a good response, although there are instances where this response is poor, in the context of very extensive deposition of Lewy Bodies (Spira et al., 2001).

Genetic Wide Association Studies (GWAS) have unveiled other polymorphisms around this gene that modify the risk of developing PD. Those polymorphisms mostly affect the stability of a-synuclein or the amount of the protein that is produced by the neuronal cells (Edwards et al., 2010).

The genetic studies have also uncovered multiple genes, apart from *SNCA*, that are linked to the emergence of PD. Mutations in the gene GBA, encoding for the protein beta-glucocerebrosidase, are the most common cause of genetic PD and are inherited in an autosomal dominant fashion, although this does not often appear in families as such, due to reduced penetrance; it may affect the lysosomal degradation of a-synuclein. The phenotype ranges from an isolated motor disorder to a picture similar to DLB (Smith & Schapira, 2022).

Other genes related to PD include LRKK2, which plays a role in the regulation of the immune system, and genes that affect mitochondrial function and dynamics such as PARKIN, PARK2 and VPS35 (Klein & Westenberger, 2007).

Other synucleinopathies

Synucleinopathies also include 3 more syndromes: MSA, DLB and PAF. They have various differences compared to PD in the systems that are affected, the symptoms and the management. As far as MSA is concerned, a-synuclein aggregation-induced cell death is considered the main culprit, as in PD. Nevertheless, in this disorder, the aggregated synuclein is mostly localised in oligodendrocytes, the myelinating cells of the CNS (Lee et al., 2019). Three distinct forms of MSA are thought to exist: MSA-P, with primary parkinsonian symptoms, MSA-C, where the degeneration is mostly localised in the cerebellum and mixed MSA, where both systems are affected. MSA has a more sinister disease course compared to PD with prominent autonomic dysfunction (Lipp et al., 2009).

DLB is a clinical entity that is distinguished by the early presence of dementia and parkinsonian features. There is overlap between this syndrome and Parkinson disease with Dementia, and are mostly distinguished by the time of onset of dementia, that appears very early in the course of DLB, compared to PDD (Outeiro et al., 2019).

In PD, it is thought that the accumulation of a-synuclein in the brain starts occurring many years prior to the onset of motor symptoms (Goldman & Postuma, 2014). An early diagnosis of synucleinopathy may be helpful in order to advise patients regarding their life choices or alterations of their life style towards a more energetic one, including exercise, but does not currently have a major impact regarding some form of preventive drug treatment; in the future, as neuroprotective therapies advance, such early diagnosis may have a more profound impact. As our knowledge in the field is expanding, new ways of tackling the disease might be discovered, either through lifestyle changes or with pharmaceutical means. An earlier intervention in this setting could be more effective. Moreover, there are multitude of clinical syndromes that mimic PD and the differential diagnosis can prove a challenge to even experienced clinicians. Focus has been given in researching objective methods in differentiating between those conditions and most importantly diagnosing PD from an early stage.

Efforts have been made to uncover distinguishing features of the syndrome. DATscan is a diagnostic method that involves the injection of Ioflupane together with the use of single photon emission computed tomography (SPECT) in order to uncover the loss of dopaminergic striatal neurons (Bega et al., 2021). Although this method is useful for differentiating from some disorders, more specifically from essential tremor, it has some limitations in differentiating between causes of dopaminergic cell death in the basal ganglia, such as PD compared to MSA. Furthermore, it is an expensive technique that can only be carried out by specific tertiary centres, limiting its usefulness in the general population.

Given the central role of a-synuclein in the pathogenesis of PD, the detection of pathogenic forms of a-synuclein in an individual would assist in the early diagnosis of the syndrome. Total levels of a-synuclein in the CSF are slightly lower overall in PD vs. controls (Gao et al.,

2014), however such an assay does not provide a useful biomarker. In contrast, the recently developed and established CSF Seeding Amplification Assay (SAA) of a-synuclein is highly sensitive and specific for the detection of prodromal PD and its differentiation from other (Siderowf et al., 2023). The spinal tap required to obtain CSF is however a rather invasive procedure.

In following the principle of using a-synuclein as a PD biomarker, the researchers have made efforts to uncover other tissues that can be studied as sources of a-synuclein. As has already been mentioned, a-synuclein is present in abundance mostly in neurons, including the neurons of the peripheral nervous system.

Autonomic and Peripheral Nervous System

The peripheral nervous system includes all the nerves and the ganglia that are located outside of the spinal cord and the brain. Its main components are the autonomic nervous system and the somatic nervous system. The main purpose of the somatic nervous system is to control voluntary movement. It is comprised of neurons that transfer information towards the periphery (efferent pathway) and neurons that do the opposite, transferring information towards the CNS (afferent pathway). For instance, primary neurons that are located in the primary motor area of the human cortex project to the bodies of spinal motor neurons, which have their neuronal bodies in the ventral horn of the spinal cord. Signals of these pathways are used for the execution of voluntary movement. Neuronal fibers that innervate the muscles form the neuromuscular junction, which uses the neurotransmitter acetylcholine. (Catala & Kubis, 2013).

The autonomic nervous system (ANS) is involved in the precise regulation of involuntary processes and reflexes. The main three components of the ANS are the sympathetic, the parasympathetic and the enteric nervous system.

The sympathetic nervous system (SNS) is comprised of neurons the cell bodies of which are located in the lateral horns of the spinal cord. Presynaptic fibers exit the spinal cord and reach either the paravertebral or the prevertebral ganglia. SNS contains both afferent and efferent fibers, in order to gather sensory information and guide motor signals from the CNS to the periphery. Colloquially, the SNS guides a 'fight or flight' response to environmental stimuli, through the innervation of various organs, such as the heart, skin, and gastrointestinal system. For example, activation of the SNS provokes an increase in the heart rate, raise of blood pressure, reduced gastrointestinal motility and various other functions. The main presynaptic neurotransmitter of the SNS is acetylcholine. Postsynaptic fibers typically have norepinephrine as their main neurotransmitter, although various other substances can also be encountered.

On the other hand, the parasympathetic nervous system (PNS) innervates the head, the viscera and the genitalia. The PNS fibers exit the CNS through cranial nerves and the sacrum. The vagus cranial nerve plays a significant role in the PNS, as it innervates most of the viscera, such as the heart, intestine and diaphragm. Similar to the SNS, the PNS also contains both efferent and afferent fibers. The PNS plays a significant role in various functions, including controlling the heart rate, respiratory secretions, gastrointestinal motility and others. The effects of the PNS activation on body functions can be summarised as preparing to 'rest and digest', in opposition to the SNS. Regarding neurotransmitters, both the preganglionic and the postganglionic fibers use acetylcholine, although, in a similar manner to the ANS, postganglionic fieres might also use various other neurotransmitters (Robertson & Biaggioni, 2012).

The final subdivision of the ANS is the enteric nervous system (ENS). The ganglia that comprise the ENS are organised in two distinct organisations. The Auerbach plexus, which is also known as the myenteric plexus contains neurons and neuronal fibers in the muscularis externa. It receives parasympathetic and sympathetic input. The second organisation of the ENS is the Meissner's plexus, located in the submucosal layer of the gastrointestinal tract, and is also called the submucosal plexus (Fleming II et al., 2020). Various neurotransmitters are used by the ENS, including vasoactive intestinal peptide, nitric oxide, pituitary adenylate cyclase-activating peptide, purine and serotonin (Mittal et al., 2017).

Given the knowledge about the presence of pathogenic forms of a-synuclein that cause the various forms of synucleinopathies in the brain, and the fact that a-synuclein is present in the neurons of the peripheral and autonomic nervous system, a hypothesis was made regarding the possible discovery of pathogenic a-synuclein forms in the PNS and ANS.

Synuclein in the PNS: the GI tract

As previously mentioned, PD patients can experience gastrointestinal symptoms. LBs were discovered first in the oesophagus and colon of patients with PD that displayed GI symptoms (Qualman et al., 1984). This finding was corroborated by the discovery of LB in the Meissner and Auerbach's plexuses in patients with PD (Wakabayashi et al., 1988). Further studies have analysed samples derived from colonic biopsies and have supported that people with prodromal PD have increased a-synuclein pathological deposits compared to controls (Hilton et al., 2013). Beach et al showed a rostro-caudal gradient of phosphorylated synuclein in PD patients, with lower amounts being in the colon and higher in the Upper

Gastrointestinal Track (Beach et al., 2010). Nevertheless, valid criticisms, including regarding the specificity of those findings, raise concern whether the gut can be a valid site to accurately diagnose PD (Visanji et al., 2013).

Anatomy of the skin

Similar efforts have shown that patients with PD develop skin conditions, such as dyshidrosis and dermatitis to a different frequency compared to the unaffected population, and levodopa treatment affects those symptoms (Skorvanek & Bhatia, 2016). The main components of the skin are three layers: the epidermis, the dermis and the hypodermis.

The epidermis is the outermost layer of the human skin. It is comprised of a stratified epithelium and contains mostly keratinocytes. It has the capacity to self-renew. Other cells in the epithelium include Langerhans cells, Merkel Cells, melanocytes and lymphocytes. The epidermis is comprised of multiple continuous layers: the basal membrane, the stratum spinosum, the granular layer, the striatum disjunctum, with the presence of a fifth layer evident in some body parts, the striatum lucidum, between the granular layer and the striatum disjunctum (Agarwal & Krishnamurthy, 2023).

Significant components of the epidermis are the sweat glands, which are either apocrine or eccrine. The eccrine glands are present throughout the whole body, while the apocrine glands are present in the perineal and axillary areas. Both glands offer thermoregulatory regulation, but they differ in their structure and their development over the human's life. The sweat glands also receive different innervation: eccrine sweat glands send signals to the CNS via cholinergic fibers, while apocrine glands respond via norepinephrine. It should be noted that no significant vasculature is contained in the epidermis (Kreyden & Scheidegger, 2004).

The dermis is located below the epidermis and is comprised mostly of connective tissue. The dermis contains hair follicles, which together with the hair shaft, the sebaceous gland and the arrector pili muscle form the pilosebaceous unit. The arrector pili muscle innervation comes from the ANS, and more specifically by fibers of the sympathetic nervous system, which use the neurotransmitter norepinephrine (Brown & Krishnamurthy, 2023).

The skin is a significant organ, and among its function is to receive sensory input. As a result, it is not extraordinary that the skin has significant and complex innervation, through multiple pathways. The skin is innervated by fibers from the ANS. The main component of the cutaneous innervate is the sympathetic system, although some fibers also stem from the parasympathetic system and the somatic system.

The fibers originating from the sympathetic system contain neurotransmitters from different subtypes. A comprehensive study by Donado et al. (Donadio et al., 2019) analyses thoroughly the sympathetic skin innervation. According to this study, the main subsets of sympathetic fibers are the adrenergic and the cholinergic. The sympathetic fibers can be distinguished by specific antibody markers, in order to understand in what part of the ANS they belong to. The cholinergic fibers are characteristically differentiated through their staining with markers such as VIP (vasoactive intestinal peptide) CGRP (calcitonin gene-related peptide) and Substance-P. On the other hand, Tyrosine Hydroxylase (TH) and Neuropeptide-Y staining characterise the adrenergic fibers. A significant number of cutaneous fibers are parasympathetic; they lack obvious TH staining, and contain neuropeptides, such as VIP.

The main cutaneous structures that are innervated are the arrector pili muscles of the pilosebaceous unit, the arterioles and the sweat glands. The autonomic component of each structure is different. For example, it is well known that the sweat glands are innervated by

cholinergic fibers, although they also contain some amount of TH. On the other hand, the arrector pili muscles and the arterioles contained mostly adrenergic innervation.

The skin is the largest organ by size, and it is involved in a number of pathologies. Diseases involving the skin include infections, autoimmune disorders and neoplasms. From a neurological perspective, small fiber neuropathy of the skin has been identified as a condition with various causes, or as part of greater syndromes, such as diabetes mellitus. The epidermal neurodegeneration can be studied via assessing the Intra Epidermal Nerve Fiber Density (IENFD), which is significantly reduced in most types of peripheral neuropathies compared to healthy individuals (Devigili et al., 2019).

Synuclein in the PNS: the skin

Nevertheless, a moderately new finding is the role of the skin in neurodegenerative disorders, and more specifically in PD and the other synucleinopathies. A-synuclein is normally expressed by various cells of the human skin, and its physiological role is not clear with our present knowledge. A-synuclein pathological forms have been detected in the skin of Parkinson's disease patients. The first study that showed the presence of pathologic a-synuclein in the skin was conducted by Ikemura et al and was published in 2008 (Ikemura et al., 2008). The researchers used paraffin embedded skin tissue derived from patients with Lewy Body disease disorders and stained with antibodies for phosphorylated a-synuclein. They noted the presence of phosphorylated a-synuclein fibers in various structures, such as small blood vessels, via simple immunocytochemistry. Moreover, using immunofluorescence, they showed that a-synuclein colocalises with fibers expressing TH.

Following this landmark study, multiple efforts have been made in order to further elucidate the presence of a-synuclein in the skin of synucleinopathy patients, which could be

considered as a biomarker for early diagnosis of the disease. In the literature, skin biopsies are from the C7 level of the cervical spine or from the distal leg, sites that are characterised by their rich innervation. The proximal and distal site to the CNS is being studied to assess whether any gradient of deposits is visible. Both simple immunochemistry and immunofluorescence have been used. The targets are the phosphorylated a-synuclein or the total a-synuclein . Very recently, the a-synuclein SAA has also been applied to skin (Siderowf et al., 2023).

When immunofluorescence is used, double label confocal microscopy is utilised to assess the colocalization of the PD protein with a neuronal marker. This is most usually PGP 9.5, a pan-neuronal marker that also labels skin fibers. Some studies have utilised specific antibodies, such as Synaptophysin, VIP, TH to target selected neuronal fibers. Usually, the biopsy is collected using a 3mm punch, which is then frozen and subsequently sliced. The thickness of each slide differs between each study and can range between 10mm and 50mm, depending on the technique used (Tsukita et al., 2019).

Doppler et al showed colocalization of PGP 9.5 and phosphorylated a-synuclein in PD patients but not in controls (Doppler et al., 2014). This association was greater in proximal compared to distal biopsies. Similar findings were reported by Donadio ‘s Team (Donadio et al., 2014, Donadio et al., 2017), who further supported the presence of a proximal-to-distal gradient of phosphorylated synuclein in iPD. Phosphorylated a-synuclein is mostly located in autonomic nerve fibers (Donadio et al., 2018). The specificity of the identification of colocalization of phosphorylated a-synuclein with PGP 9.5 is quite high, with almost no control subjects or patients with atypical parkinsonism exhibiting any positive samples.

PD is a progressive disease and thus, it has been suggested that the amount of phosphorylated a-synuclein deposits in iPD patients may be proportional to the disease

duration and severity, when adjusted for sex (Melli et al., 2018). Moreover, phosphorylated a-synuclein in the skin might spread from autonomic ganglia to other innervated structures, as evident by a longitudinal study of a patient with PD and orthostatic hypotension, but the available data is limited (Infante et al., 2020). The early deposition of cutaneous phosphorylated a-synuclein in the disease process is supported by a landmark study showing phosphorylated a-synuclein deposits colocalizing with PGP in patients who suffer from REM sleep disorder (Doppler et al., 2017).

Phosphorylated synuclein is also located in the neuronal fibers of patients with genetic forms of PD. All patients with the SNCA mutations that have been studied have a significant amount of deposits in the cutaneous tissue - this includes gene copy number alterations, the point mutation E46K and one patient carrying the A53T mutation (Isonaka et al., 2021). PARK2, GBA, LRRK2 and DJ1 gene mutations may also lead to the cutaneous deposits of phosphorylated a-synuclein, while this likely does not apply to PARKIN (Carmona-Abellan et al., 2019, (Isonaka et al., 2021). Nevertheless, all of the studies performed in genetic PD contain a small number of patients, thus further research needs to be undertaken to solidify these findings.

The higher presence of cutaneous phosphorylated a-synuclein compared to controls characterises all synucleinopathies. Analysis of samples from PAF patients shows a sensitivity of 100% for phosphorylated a-synuclein reactivity and colocalization with neuronal fibers (Donadio et al., 2013). The team managed to replicate the findings in another study (Donadio et al., 2015), also showing that PAF might have a significantly higher amount of colocalized deposits compared to PD. Finally, phosphorylated a-synuclein in DLB is mostly located in autonomic skin fibers (Khan, 2018).

Research regarding cutaneous a-synuclein in the skin of patients with MSA is conflicting. Initial reports differentiated between MSA and PD by supporting that there is virtually no positive colocalization in the autonomic skin fibers of MSA subjects while there is there is some deposition in unmyelinated somatic fibers (Doppler et al., 2015, Zange et al., 2015, Donadio et al., 2020). Moreover, samples from MSA patients show a distal-to-proximal gradient, with the sites away from the CNS having a greater possibility of being positive for deposits, which is in stark contrast with samples from PD patients (Donadio et al., 2020). Nevertheless, Gibbons' s team found significant involvement of autonomic fibers in MSA, while also supporting the presence of this caudorostral gradient which is specific for MSA (Gibbons et al., 2023). This could possibly be explained by the elusive nature of the disease and the high rate of misdiagnosis (Kim et al., 2015).

The presence of phosphorylated a-synuclein in the skin is only considered significant when it colocalizes with a neuronal marker. PGP 9.5 is the most widely used one, as mentioned but various other cell specific markers have also been utilized. Significant colocalization has been shown for TH, VIP, DbH, Substance P and CGRP (Doppler. *et* al., 2014, Donadio et al., 2014). A paper published in 'Brain' (Mazzetti et al., 2020) promoted the colocalization of a-synuclein oligomers with Synaptophysin, a specific neuronal marker that targets non-somatic fibers. The researchers used quite a different methodology compared to the mainstay literature, without evaluating the presence of phosphorylated a-synuclein. Finally, similar studies have been conducted in MSA (Doppler et al., 2015, Donadio et al., 2020). When critically appraising those findings, it should be underlined that the findings are limited, and further research needs to be conducted to assess any differences between the various synucleinopathies and the cutaneous neuronal populations affected.

All in all, while we can see the evidence about the presence of phosphorylated deposits in the skin of patients affected with synucleinopathies, nevertheless, we should

underline that the process is not standardised. The main difference is observed in the preparation of the tissues, where some teams use paraffin embedded samples and others frozen in 4% paraformaldehyde. Moreover, the thickness of the samples ranges from 10mm to 50mm depending on the publication. Transfer of this knowledge to clinical practise requires the creation of specific protocols.

Finally, epidermal neurodegeneration characterises iPD. Significantly reduced IENFD values have been shown in patients compared to controls (Melli et al., 2018), with a negative correlation between IENFD and disease duration and severity (Jeziorska et al., 2019). PD patients might also show reduced IENFD compared to MSA (Haga et al., 2015).

Research question and aim

Based on the literature analysed above, the presence of phosphorylated a-synuclein in the skin of PD patients is a widely researched topic. Nevertheless, the distinct fibers affected in the disease have been poorly studied, while the data on genetic PD is limited. Thus, we embarked on our study to further investigate the cutaneous involvement in PD. We had the opportunity to obtain samples from a cohort of patient harboring the A53T mutation, which has only been minimally previously evaluated in the literature (Isonaka et al., 2019). All in all, we were in the position to investigate the involvement of distinct cutaneous neuronal fibers in a rare cohort of patients, together with patient carrying other mutations for genetic PD as well as MSA patients. The degeneration of epidermal skin fibers was also analysed thoroughly for the first time in the A53T PD cohort.

Materials and Methods

Sample collection and processing

In order to analyse the presence of a-synuclein deposits in human tissue, skin samples were obtained from volunteers. The recruitment of patients took place in Aiginiteion Hospital, Athens, Greece. The population of the study composed of people with Idiopathic Parkinson's Disease, Genetic Parkinson's Disease, and Multiple System Atrophy. The diagnosis of the synucleinopathies was clinical, while in the case of the genetic forms, the patients had known mutations in specific genes. In the case of controls, samples were obtained from three healthy controls with no family history of movement disorders or any clinical features suggestive of synucleinopathy. Both groups comprised of ethnically Greek individuals of both genders.

At least 2 tissue samples were obtained from each individual. One sample was obtained at the approximate level of cervical spondylus C6, which was selected due to the high density of neuronal fibers that could be used for immunocytochemistry. A further site of sampling was selected just above the lateral malleolus. The side of the sampling was ipsilateral to the side with the highest severity of clinical symptoms. Punch biopsies were collected as previously described: aseptic technique and local anaesthetic injection were used and 3mm tissue sample was collected from each site.

Following this, the tissues were fixed in 4% PFA for 3 hours in 4^{o}C. The samples were theN incubated in 0.1 Phosphate Buffer for 10 minutes, and this step was repeated three times. Finally the samples were left to settle for at least 24 hours in 4^{o}C. The samples were cut into slices of 10mm (confocal analysis), 40mm (fiber density analysis) and 20mm (any further experiments) via a cryotome.

10mm slices were incubated with a blocking buffer for 30 minutes (10% BSA, 0.1% Triton X10% and PBS). Double staining was performed with a mouse derived antibody against phosphorylated a-synuclein (G81A,dilution 1/500) and

- A rabbit derived antibody against PGP 9.5 (PGP 9.5 516-3344, Zytomed, dilution 1/500) or
- A rabbit derived antibody against Synaptophysin (Synaptophysin D8F6H, Cell Signalling, dilution 1/500) or
- A rabbit derived antibody against VIP (VIP PA185958, Thermo Scientific dilution 1/500)

in a solution of 1% BSA, 0.3% Triton X 10% and PBS. The primary antibodies were incubated for a period of 48 hours and subsequently the samples were washed and incubated for 1 hour with anti-mouse rabbit CF533 and anti-rabbit CF488A (dilutions 1/2000), DAPI (dilution 1/2000) and TOPRO (dilution 1/1000) in a solution of 1% BSA, 0.3% Triton X and PBS. The slides were stored in 4^{o}C.

For IENFD analysis, rabbit PGP (dilution 1/500, PGP 9.5 516-3344, Zytomed, dilution 1/500) was used instead of double immunostaining, and the rest of the buffers were similar to the ones described above.

Quantification of Immunofluorescence co-localization

Images were obtained via the upright confocal microscope Leica SP5 II in DM6000 CFS. Image magnification was set to 40x. To quantify co-localization of phosphorylated α-synuclein with PGP 9.5, VIP or synaptophysin we used the 3D volume measurement features of Imaris software (Bitplane, version 7.4.2). Using the 3D co-localization module would not

be informative as the extensive presence of cellular debris, exhibiting autofluorescent signal in all channels (blue, green and red fluorescence collection channels), would interfere with the co-localization measurements **(Figure 1)**. Instead, we decided to use the masking feature of Imaris program to remove all signal co-localizing with the signal detected in the blue channel, before quantifying red and green signal volumes. In order to achieve that, automatic thresholding for each confocal image was applied on the blue channel for the whole Z-stack. A "surface" (visualization of thresholded blue voxels volume) was created and all green channel voxels within this volume were set to zero intensity value, thus excluding them from further analysis. As nerve fibers (for every combination of staining found in the green channel) are not co-localized with tissue nuclei and were never found co-localized with cellular debris, their analysis was not affected. Thresholding for each confocal image was applied on the rest of green channel signal (threshold value varying depending on the staining combination but not within each set of images examined), and red channel voxel intensity values were set to zero everywhere outside the thresholded green voxels. Thresholding (again stable for each staining combination) was applied on the remaining voxels of the red channel for each confocal image. Threshold values were chosen studying the images of each studied group in an attempt to expose differences of staining intensity and are found in **Table 1**. 3D volume quantifications of green and red channel surfaces were provided by the software, and the volume values for all images of each sample added up. Co-localization was calculated as the percentage of the red voxels volume upon the total volume of green voxels. Values represent mean ± Standard Deviation (SDEV). At least three and up to 15 images were evaluated per biopsy. Analysis sequence is shown in **Fig. 2**.

		Blue channel threshold	Green channel threshold	Red channel (phospho-a-synuclein) threshold
Cervical	**Synaptophysin**	automatic	10	38
	PGP 9.5	automatic	10	38
Foot	**Synaptophysin**	automatic	10	60
	PGP 9.5	automatic	15	30
	VIP	automatic	15	60

Table 1. Threshold values used for each nerve fiber marker/phospho-a-synuclein volume analysis.

Quantification of intraepidermal nerve fiber density (IENFD)

In order to measure IENFD we used PGP 9.5 immunofluorescence on foot skin punch biopsies. PGP 9.5-immunofluorescence-positive nerve fibers found on the basement membrane were counted on at least 33 and up to 56 fields from at least 8 different sections of each biopsy. IENFD was quantified as the count of PGP 9.5-immunoreactive nerve fibers found on the basal membrane and travelling from the dermis to the epidermis per mm of basal membrane. Z-stacks were obtained using a confocal microscope, and quantifications were performed on the maximum projections. Fiji image analysis software and its segmented line tool were used to delineate basal membrane and quantify its length in each image.

Statistical Analysis

All immunofluorescence data were analyzed by one way analysis of variance (ANOVA). The level of statistical significance was set at 0,05. Follow-up analysis with Student's t-test assuming equal variances was subsequently conducted where the data allowed this.

Results

Extensive presence of cellular debris in cervical and foot punch biopsy samples.

Extensive presence of cellular debris, exhibiting autofluorescent signal either in all channels (blue, green and red fluorescence collection channels) (**Fig. 1**, white arrows), or in two of the fluorescence collection channels (blue and red) (**Fig. 1**, white arrowheads) was observed in the vast majority of the skin biopsy samples. Analysis to measure co-localization of nerve fiber markers with phospho-a-synuclein in z-stacks of skin samples was appropriately chosen so that the volume of the cellular debris is excluded from the analysis **(Fig. 2)**.

Phosphorylated a-synuclein is present in distinct cutaneous neuronal fibers of patients that carry the A53T Mutation

We have observed the presence of phosphorylated a-synuclein in the neuronal fibers that are positive for Synaptophysin (**Fig. 3, 5**) or VIP (**Fig. 7**) in carriers of the A53T mutation. Synaptophysin is characteristic of the purely autonomic fibers while VIP is located on cholinergic fibers. Colocazization of phosphorylated a-synuclein with synaptophysin positive fibers was not both in the proximal (**Fig. 3**)and distal areas (**Fig. 5**). On the other hand, colocalization of VIP with phosphorylated a-synuclein was found in the distal sites (**Fig. 7**), since cervical samples were not examined with this immunocytochemical stain.

A53T, MSA, GBA and parkin patients do not show significantly elevated expression of phosphorylated α-synuclein in synaptophysin-positive cervical skin nerve fibers, but A53T-derived biopsies show a trend for higher values

In order to study whether A53T, MSA, GBA and parkin patients have a higher level of phospho-α-synuclein in their cervical skin nerve fibers in comparison to control subjects we performed immunofluorescence on skin punch samples staining against phospho-α-synuclein and synaptophysin to mark the fibers **(Fig. 3)**. Quantification of the co-localization of phosphorylated α-synuclein and synaptophysin, a marker of non-somatic neuronal fibers, shows that even though there is a trend showing A53T patient skin nerves containing higher levels of phospho-α-synuclein, there is no statistical significance between any of the groups examined (p=0,317). Specifically, while 14,46 ±17,05% of the control subject nerve fibers are co-localized with phospho-α-synuclein, co-localization in A53T patients skin nerves is approximately 90% higher 35,77 ±15,15% (n=3 for both groups) **(Fig. 3G)**. The lack of statistical significance observed is probably due to high variation within each group. For the one studied MSA patient, nerve fibers are co-localized with phosphorylated α-synuclein by 37,32%, for the GBA patient 2,06% and for the parkin patient 44,54%. Specifically, control vs. A53T p=0,073, control vs. MSA p=0,077, control vs. GBA p=0,625, control vs. parkin p=0,063, A53T vs. MSA p=0,938, A53T vs. GBA p=0,194, A53T vs. parkin p=0,666. 2-tailed t-test p= 0.072 between controls and A53T.

A53T, MSA, GBA and parkin patients do not show significantly elevated expression of phospho-α-synuclein in PGP 9.5-positive cervical skin nerve fibers, fibers, but A53T-derived biopsies show a trend for higher values

In order to study whether A53T as well as MSA, GBA, and parkin patients have a higher level of phospho-α-synuclein in PGP 9.5-positive cervical skin nerve fibers in comparison to control subjects we performed immunofluorescence on skin punch samples staining against phospho-α-synuclein and PGP 9.5 to visualize the fibers **(Fig. 4)**. Quantification of the co-localization of phosphorylated α-synuclein and PGP 9.5 shows that there is no statistical significance between the five groups (p= 0,312). More particularly, 0% of the control subject fibers are co-localized with phospho-α-synuclein (n=2), 5,74 ±5,1% of the A53T patient fibers are co-localized with phospho-α-synuclein (n=3), 0,45 ±0,64% of MSA patient fibers (n=2), 0,03 ±0,04% of parkin patient fibers (n=2), and 0% of GBA patients fibers are co-localized with phospho-α-synuclein (n=1) **(Fig. 4J)**. The previous result regarding the lack of significance between control subjects and patients using synaptophysin to mark the nerve fibers is corroborated by the equivalent analysis using PGP 9.5. Again we observe a high level of variation between the values within each studied group, and there is again a definite trend for the A53T group to show higher values compared to controls. Control vs. A53T p=0,433, control vs. MSA p=0,667, control vs. parkin p=0,676, A53T vs. MSA p=0,260, A53T vs. GBA p=0,433, A53T vs. parkin p=0,231, MSA vs. parkin p=0,450, MSA vs. GBA p=0,667. 2 Tailed t-test p=0.43 between the control and A53T cohorts.

<u>A53T, MSA, GBA and parkin patients do not show significantly elevated expression of phosphorylated α-synuclein in foot skin nerve fibers, fibers, but A53T derived biopsies show a trend for higher values.</u>

An equivalent analysis of whether A53T, MSA, GBA or parkin patients have a higher level of phospho-α-synuclein in their foot skin nerve fibers in comparison to control subjects was performed on skin punch samples staining against phospho-α-synuclein and

synaptophysin to mark the fibers **(Fig. 5)**. Quantification of the co-localization of phospho-α-synuclein and synaptophysin shows that even though there is a trend showing A53T, GBA and parkin patient foot skin nerves contain higher levels of phospho-α-synuclein, there is no statistical significance between any of the groups examined (p=0,612). Specifically, while 0,2544% of the control subject nerve fibers are co-localized with phosphorylated a-synuclein (n=1), co-localization in A53T patients foot skin nerves is approximately 100 times higher, at 20,46 ±30,57% (n=3), in parkin patients 7,80 ±3,17% (n=2), in the GBA patient it is found at 45,84% (n=1), and in the MSA patient it is found 0% (n=1) **(Fig. 5M)**. Specifically, control vs. A53T p=0,625, control vs. parkin p=0,302, A53T vs. GBA p=0,547, A53T vs. parkin p=0,618, A53T vs. MSA p=0,621, GBA vs. parkin p=0,065, parkin vs. MSA p=0,294.

A trend for patients showing higher co-localization of foot skin nerve fibers with phospho-a-synuclein without a statistically significant result is corroborated by the examination of co-localization using two more nerve fiber markers, the pan-neuronal PGP 9.5 **(Fig. 6)** and the cholinergic marker VIP **(Fig. 7)**. For PGP 9.5 (p=0,713), 0,23% of the control subject nerve fibers are co-localized with phospho-α-synuclein (n=1), 27,83 ±17,26% of the A53T patients (n=2), 29,37 ±23,46% of the MSA patients (n=2), 12,29% of the GBA patient (n=1) and 4,52% of the parkin patient (n=1) foot skin nerve fibers are co-localized with phospho-a-synuclein **(Fig. 6J)**. For VIP (p=0,306), 24,43% of the control subject nerve fibers are co-localized with phospho-α-synuclein (n=1), 31,91 ±32,59% of the A53T patients (n=3), 13,55% of the GBA patient (n=1) and 53,33 ±45% of the parkin patients (n=2) foot skin nerve fibers are co-localized with phospho-a-synuclein **(Fig. 7J)**. For PGP 9.5/phospho-a-synuclein: Control vs. A53T p=0,416, control vs. MSA p=0,495, A53T vs. MSA p=0,947, A53T vs. GBA p=0,596, A53T vs. parkin p=0,469, MSA vs. GBA p=0,659, MSA vs. parkin

p=0,546. For VIP/phospho-a-synuclein: Control vs. A53T p=0,861, control vs. parkin p=0,693, A53T vs. GBA p=0,674, A53T vs. parkin p=0,573, GBA vs. parkin p=0,602

Intraepidermal nerve fiber density (IENFD) in A53T and MSA patients.

We used PGP 9.5 immunofluorescence to study the amount of nerve fibers travelling through the basal membrane from the dermis to the epidermis per mm of basal membrane to quantify IENFD on foot biopsy samples. We observed that A53T patients and one MSA patient show a reduced IEFND in comparison to one control subject, while there is no statistical significance. In particular: in the control subject IENFD is 7,74 fibers/mm of basal membrane (n=1), for A53T 5,29 ±1,64 (n=3), 31,6% lower than in control, and MSA 2,91 nerve fibers/mm of basal membrane (n=1), 62,4% lower than in control, while p=0,220 **(Fig. 8)**. Control vs. A53T p=0,240, A53T vs. MSA p=0,249.

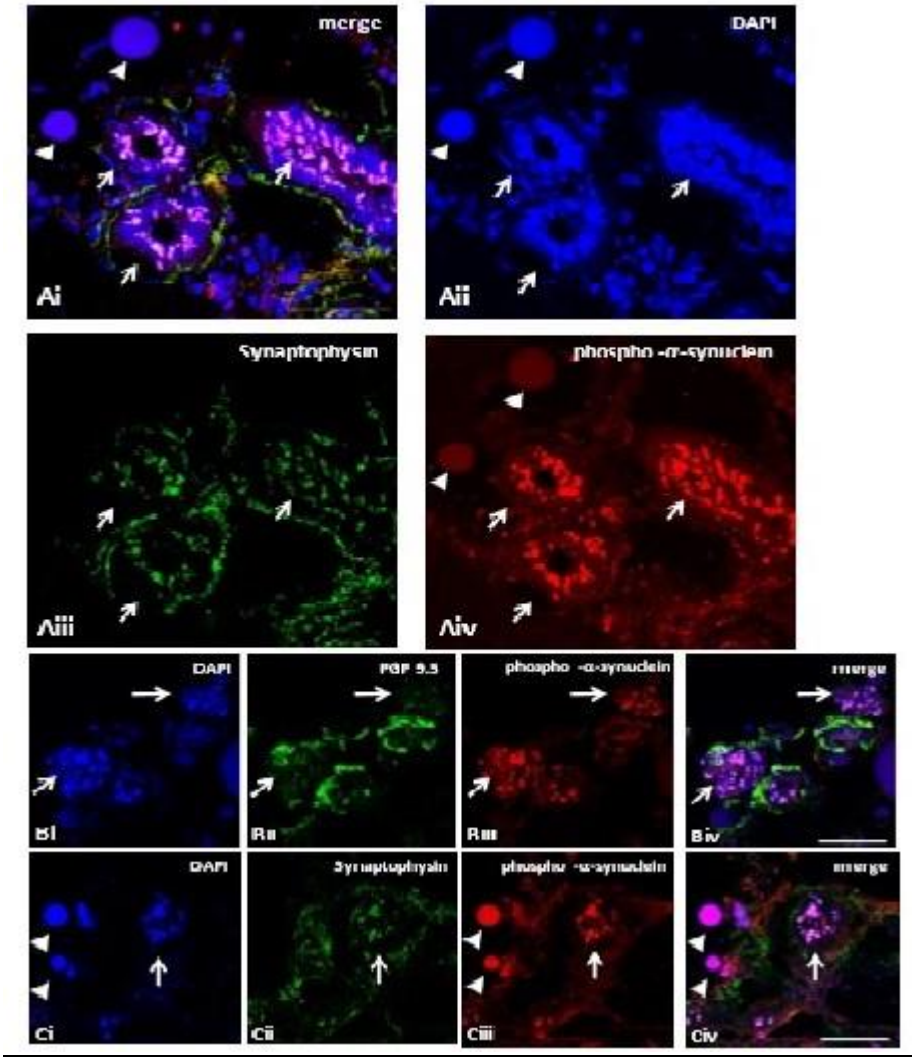

Figure 1. Cellular debris: A. An A53T patient cervical skin biopsy immunofluorescence staining for Synaptophysin and phospho-a-synuclein. Autofluorescent cellular debris that can be detected in all three fluorescent signal collection channels is indicated by white arrows. Autofluorescent cellular debris that can be detected in blue and red fluorescent signal collection channels is indicated by white arrowheads. **Ai.** merge, **Aii.** blue, (DAPI signal collection channel, 430-470nm), **Aiii.** green, (Alexa Fluor 488 signal collection channel, 510-540nm), **Aiv.** red, (Alexa Fluor 555 signal collection channel, 560-620nm). **Bi-Biv.** Control subject cervical skin biopsy immunofluorescence staining for PGP 9.5 and phospho-a-synuclein. **Ci-Civ.** Parkin patient cervical skin biopsy immunofluorescence staining for PGP 9.5 and phospho-a-synuclein. Scale bars 50μm.

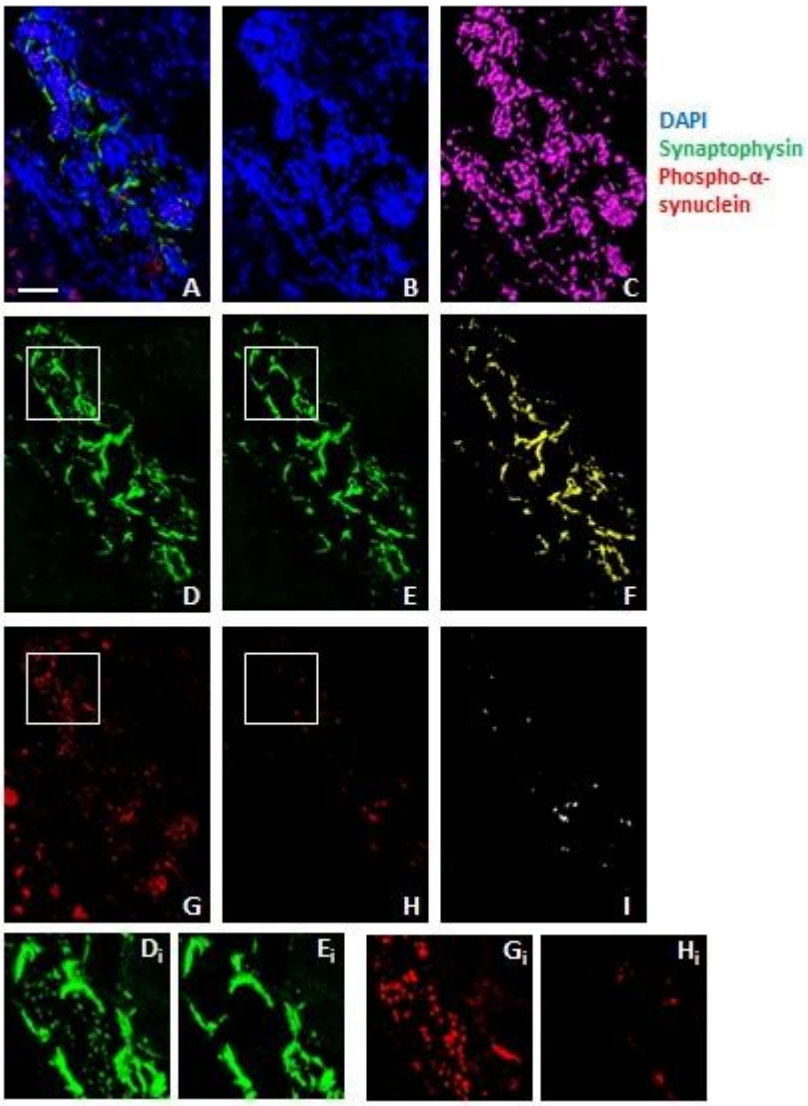

Figure 2. Analysis workflow example from a cervical skin biopsy of an A53T patient: A. Image with three channels (Blue: DAPI, green: Synaptophysin, red: phospho-α-synuclein). **B.** Blue channel shown. **C.** Blue channel volume created using automated threshold, shown in magenta. **D.** Green channel shown. **E.** Green channel after masking based to blue channel volume (all voxels inside blue channel volume set to zero). **F.** Masked green channel volume created using threshold value 10 (for cervical samples, synaptophysin as a nerve fiber marker), shown in yellow. **G.** Red channel shown. **H.** Red channel after masking based to

green channel volume (all voxels outside green channel volume set to zero). **I.** Masked green channel volume created using threshold value 38 (for cervical samples, synaptophysin as a nerve fiber marker), shown in gray. **Di, Ei.** Insets shown in **D** and **E** in higher magnification. Green channel before and after masking. Note how cellular debris accumulations are not found (and, thus, not calculated in synaptophysin volume) after masking, in **Ei** . **Gi, Hi.** Insets shown in **G** and **H** in higher magnification. Red channel before and after masking. Only red voxels co-localizing with synaptophysin-stained nerves are shown after masking of the red channel. Scale bar 50μm.

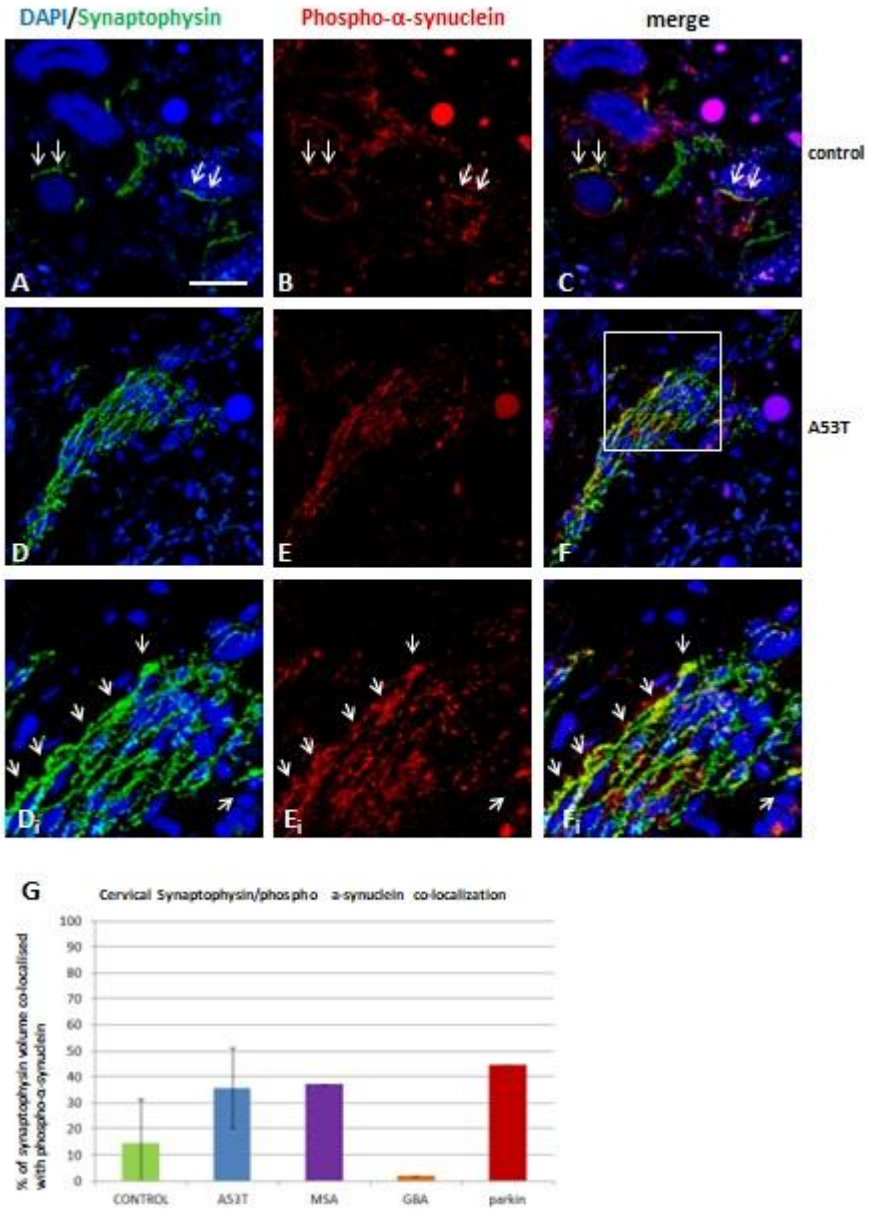

Figure 3. Co-localization of phospho a-synuclein with synaptophysin on nerve fibers of patient cervical skin samples. A-C. Image of immunofluorescence on a healthy subject skin sample (Blue: DAPI, green: Synaptophysin, red: phospho-α-synuclein). **D-F.** Image of immunofluorescence on a A53T patient skin sample. **D_i-F_i.** Field shown in orange frame in **F** in higher magnification, showcasing co-localization of synaptophysin and phospho-α-synuclein in the A53T patient skin sample in **D-F.** White arrows indicate positions of co-

localization. Scale bar 50µm. **G.** Graph of quantification of percentage of synaptophysin volume co-localized with phospho-α-synuclein. p=0,317. For control and A53T n=3. MSA, GBA and parkin n=1.

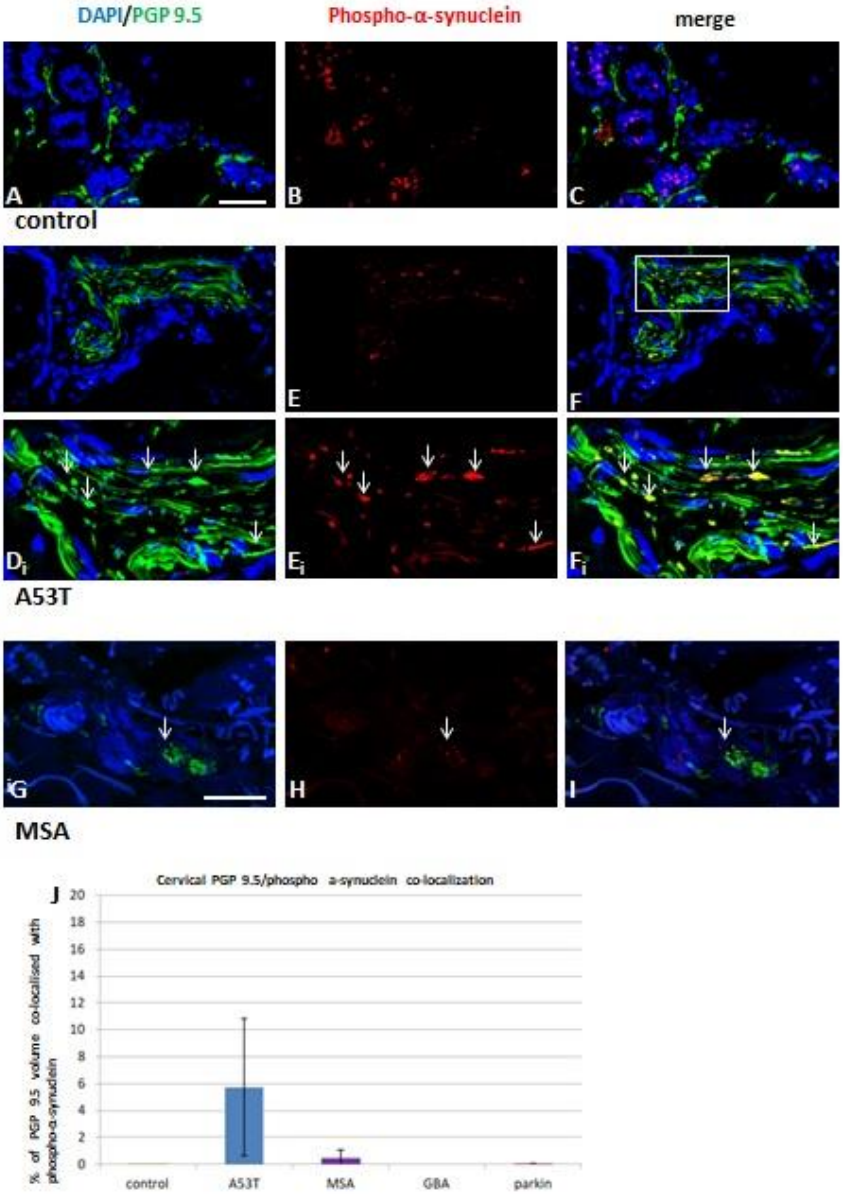

Figure 4. Co-localization of phospho a-synuclein with PGP 9.5 on nerve fibers of patient cervical skin samples. A-C. Image of immunofluorescence on a healthy subject skin sample

(Blue: DAPI, green: PGP 9.5, red: phospho-α-synuclein). **D-F.** Image of immunofluorescence on a A53T patient skin sample. **D_i-F_i**. Field shown in white frame in **F** in higher magnification, showcasing co-localization of PGP 9.5 and phospho-α-synuclein in the A53T patient skin sample in **D-F**. **G-I.** Image of immunofluorescence on a MSA patient skin sample. White arrows indicate positions of co-localization. Scale bars 50µm. **J.** Graph of quantification of percentage of PGP 9.5 volume co-localized with phospho-α-synuclein. p=0,312. For control, MSA and parkin n=2, for A53T n=3, and for GBA n=1

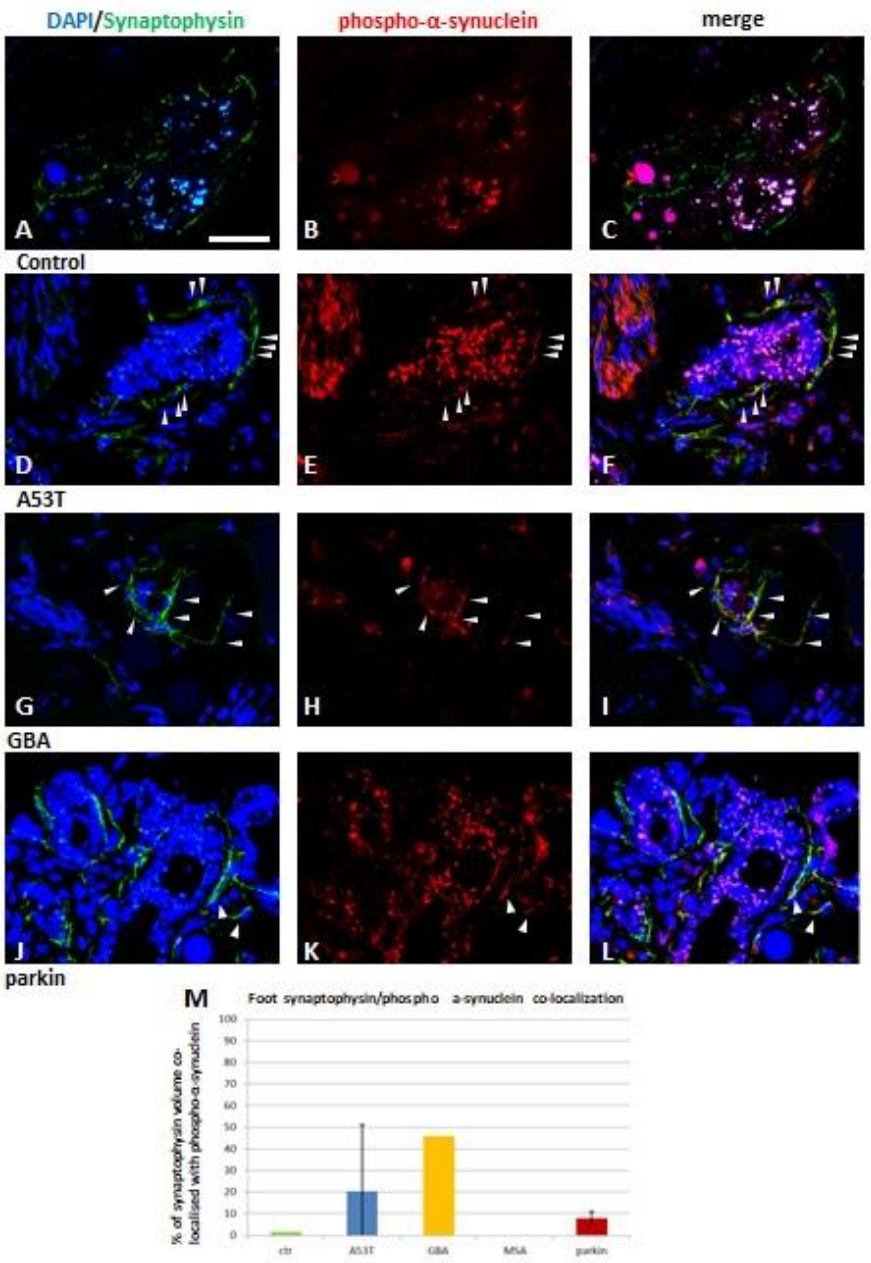

Figure 5. Co-localization of phospho a-synuclein with synaptophysin on nerve fibers of patient foot skin samples. A-C. Image of immunofluorescence on a healthy subject skin sample (Blue: DAPI, green: synaptophysin, red: phospho-α-synuclein). **D-F.** Image of immunofluorescence on an A53T patient skin sample. **G-I.** Image of immunofluorescence on a GBA patient skin sample. **J-L.** Image of immunofluorescence on a parkin patient skin sample. White arrowheads indicate positions of co-localization. Scale bar 50µm. **M.** Graph of

quantification of percentage of synaptophysin volume co-localized with phospho-α-synuclein. p=0,612. For control, GBA and MSA n=1, for parkin n=2, and for A53T n=3

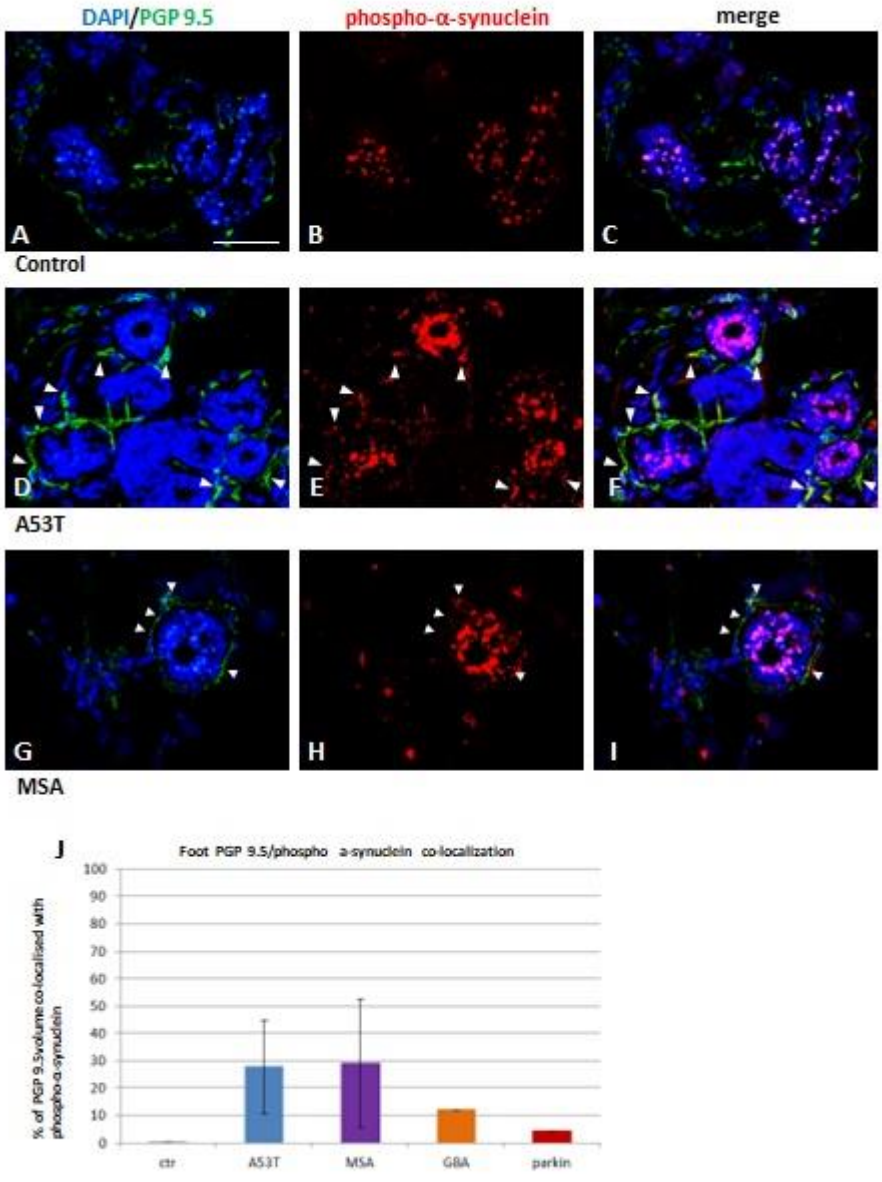

Figure 6. Co-localization of phospho a-synuclein with PGP 9.5 on nerve fibers of patient foot skin samples. A-C. Image of immunofluorescence on a healthy subject skin sample (Blue: DAPI, green: PGP 9.5, red: phospho-α-synuclein). **D-F.** Image of immunofluorescence on an A53T patient skin sample. **G-I.** Image of immunofluorescence on an MSA patient skin sample. White arrowheads indicate positions of co-localization. Scale bar 50µm. **J.** Graph of quantification of percentage of PGP 9.5 volume co-localized with phospho-α-synuclein. p=0,612. For control, GBA and parkin n=1, and for MSA and A53T n=2

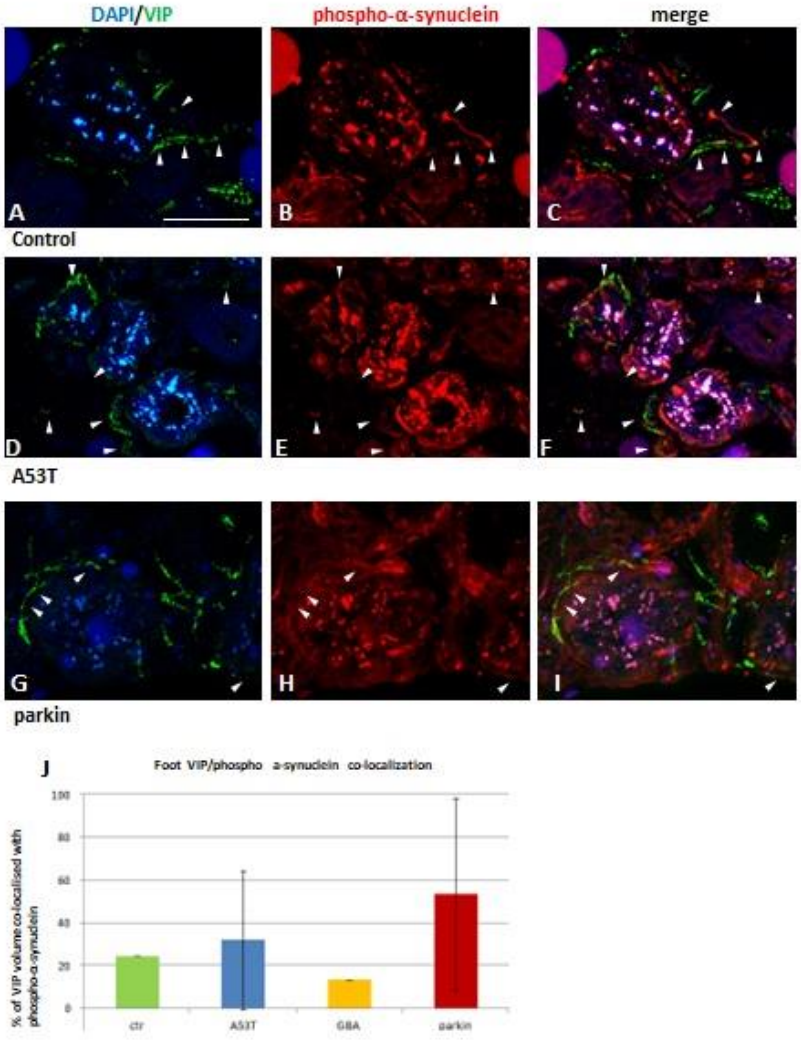

Figure 7. Co-localization of phospho a-synuclein with VIP on nerve fibers of patient foot skin samples. A-C. Image of immunofluorescence on a healthy subject skin sample (Blue: DAPI, green: VIP, red: phospho-α-synuclein). **D-F.** Image of immunofluorescence on an A53T patient skin sample. **G-I.** Image of immunofluorescence on a parkin patient skin sample. White arrowheads indicate positions of co-localization. Scale bar 50µm. **J.** Graph of quantification of percentage of VIP volume co-localized with phospho-α-synuclein. p=0,306. For control and GBA n=1, for parkin n=2, and for A53T n=3.

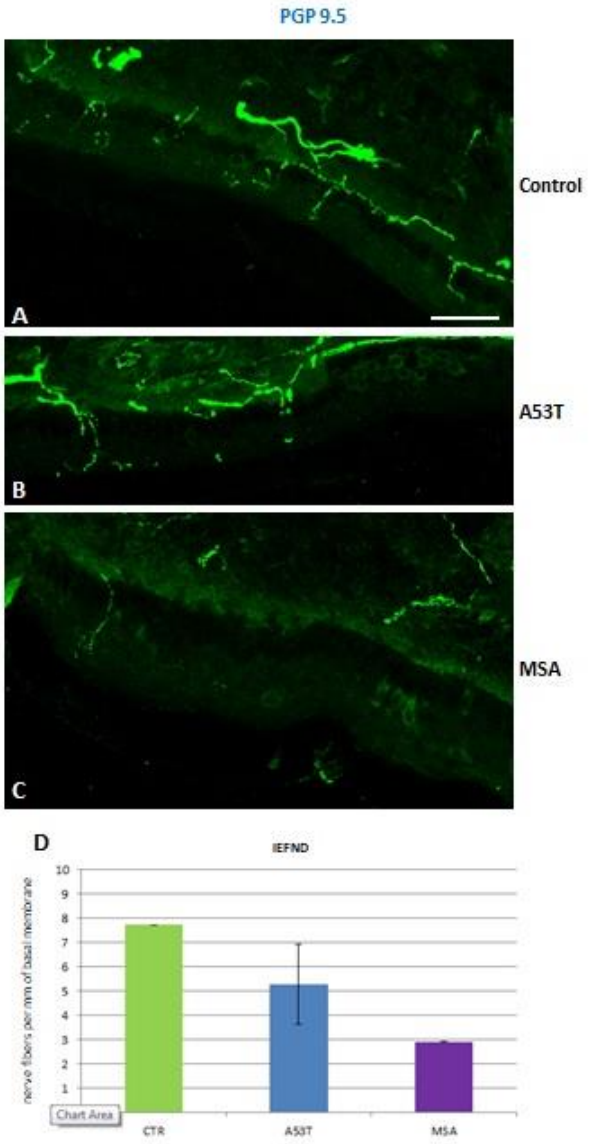

Figure 8. Intraepidermal nerve fiber density (IEFND) in patient foot skin samples. A-C. Representative images of PGP 9.5 immunofluorescence on a healthy subject, an A53T patient and an MSA patient skin sample respectively. Scale bar 50µm. **D.** Graph of quantification of IEFND, measured as the amount of nerve fibers travelling through the basal membrane from the dermis to the epidermis per mm of basal membrane. p=0,220. For control and MSA n=1, for A53T n=3

DISEASE	GENDER	AGE	DISEASE DURATION
Control	Male	58	N/A
Control	Female	57	N/A
Control	Female	60	N/A
SNCA – A53T	Male	57	2
SNCA – A53T	Female	56	10
SNCA – A53T	Male	55	15
SNCA – A53T	Male	38	5
MSA	Male	51	1
MSA	Male	42	7
GBA	Male	53	9
GBA	Female	52	7
PARKIN (heterozygous)	Female	54	11
PARKIN (heterozygous)	Male	34	4

Figure 9. Basic demographic data of the study participants.

Discussion

We will focus our discussion first on the results of the A53T mutation cohort, since this is the first time the presence of a-synuclein deposits in cutaneous tissues has been thoroughly studied in this group. Afterwards, we will also converse about the other categories. We will discuss the results of both the cervical and the foot skin biopsies and attempt to analyse any results that might differ from the currently available literature. Finally, mention of the limitations and the next steps proposed will be given.

In this dissertation, we attempted to assess the presence of phosphorylated a-synuclein in the skin of patients with forms of genetic PD. More specifically, as described by the literature, there is extensive colocalization of phosphorylated a-synuclein with skin fibers in PD individuals, which sets them apart from the non-affected population (Ikemura et al., 2008, Donadio et al., 2014, Doppler et al., 2014). Moreover, a study of the neuronal nerve fiber density was performed to assess whether peripheral nerve loss is evident in those individuals, which has been previously described (Jeziorska et al., 2019).

Our study is pioneering in the fact that we analyse the presence of a-synuclein deposits in specific cutaneous neuronal fibers of patients suffering from genetic PD. Most genetic studies on SNCA patients focus only on the presence of the deposits without analysing the affected fibers' architecture (Carmona-Abellan, et al., 2019, Isonaka et al., 2019). In our study, we have used antibodies targeting PGP 9.5, Synaptophysin and VIP. PGP 9.5 is the most widely used marker, while studying synaptophysin is quite robust, since it is a specific pre-synaptic marker that allows us to associate the involvement of the cutaneous deposits with the eautonomic dysfunction previously described (Goldman & Postuma, 2014). On the other hand, VIP is a widely accepted marker of the cholinergic system. Based on our

findings, we support that the peripheral cholinergic system is directly affected in the patients carrying the p.A53T mutation.

While analysing the results, it became evident that there is a trend of increased colocalization of a-synuclein with cervical neuronal fibers stained with the marker PGP 9.5 in PD patients carrying the A53T mutation (figure 4). This is similar to the discovery of phosphorylated a-synuclein in the skin of individuals carrying the E46K mutation (Carmona-Abellan, et al., 2019, Isonaka et al., 2019). Our results in this regard were not however statistically significant, due to the variation observed and the small number of subjects in both the control and the A53T group.

The perceived finding of the presence of phosphorylated a-synuclein in the skin of A53T patients could be of various origins. More specifically, the most straightforward explanation could be that a-synuclein that is endogenously produced in neuronal skin fibers, and, because of the presence of the p.A53T mutation, becomes misfolded and phosphorylated. The effects of Serine 129 phosphorylation on the aggregation state of the protein have been debated (Chen et al., 2009, Paleologou et al., 2008). Nevertheless, in our present study we have not analysed the presence of a-synuclein fibrillar conformations in the subjects' skin neuronal fibers; previous studies have shown reduced specificity of markers against fibrillar species (Donadio et al., 2018).

Another proposed mechanism for the presence of phosphorylated a-synuclein in the neuronal fibers of patients with genetic PD is the possible prion-like propagation of the pathologic protein (Li et al. 2008). It is possible that phosphorylated a-synuclein originating from a different part of the nervous system, such as the alimentary tract, eventually invades the adjacent skin tissue and self-propagates.

Pathologic a-synuclein deposits are located in the CNS of individuals carrying the A53T mutation (Huang et al., 2012), thus anterograde propagation from the CNS is also a highly probable scenario. On the other hand, given the current knowledge, we cannot exclude that a-synuclein is actually propagated from the peripheral tissues to the CNS in those patients. Further studies analysing both the peripheral – and particularly skin tissue – and the CNS deposits could help further elucidate this issue. It will be remarkably interesting in particular to examine such subjects longitudinally, from the asymptomatic to the symptomatic stage, assessing both peripheral (skin) and CNS a-synuclein deposition, possibly with the newly developed SAA in skin and CSF.

In PD patients carrying the A53T mutation, we can observe a trend of increased colocalization between synaptophysin positive fibers and phosphorylated a-synuclein, indicating the involvement of autonomically innervated structures. Dysautonomia has been prescribed in patients with both sporadic and genetic forms of PD. The above finding is added to the previous study of a-synuclein oligomers in synaptophysin positive fibers (Mazzetti et al., 2020) but correlation with the degree of autonomic dysfunction in those specific individuals studied is required in order to establish a causal relationship.

Moving on the foot skin samples, colocalization analysis showed similar results to the cervical skin fibers, as far as the A53T cohort is concerned. More specifically, a trend of higher colocalization between the antibody staining for phosphorylated a-synuclein and PGP was noted. This can be explained in similar ways to the results of the cervical PGP positive fibers and a-synuclein. Quite interestingly though, we can observe an even higher degree of colocalization when comparing to the cervical spine skin fibers. This could be explained by the lower amount of PGP 9.5 positive fibers in the control subject's lower limb skin tissue, due to the perceived degeneration of such fibers, rather than a true increase in the

phosphorylated a-synuclein deposits. There is a perception of a positive proximal-to-distal gradient in idiopathic PD (Donadio et al., 2017), which has not been verified in our study.

We have also studied the a-synuclein deposits that are present in VIP immunoreactive fibers which are characterising cholinergic neurons. Here the results are more inconclusive, and this could be explained by the limitations of the study that will be analysed below. It is certain that further investigations of the neuronal fibers expressing this marker are required to understand their involvement in the pathology of genetic PD.

We had the opportunity to study other synucleinopathy cohorts, including patients with GBA and MSA, as well as presumed non-synucleinopathy Parkinsonism, such as that due to Parkin mutations. Again, the results show no statistical significance. Perhaps the most outstanding one is the particularly low amount of colocalization of phosphorylated a-synuclein and the cellular markers (PGP 9.5, synaptophysin, VIP) in the cervical skin of GBA. Throughout the literature, the GBA mutation is one of the most studied and phosphorylated a-synuclein, similar to idiopathic PD, has been shown to exist in the skin of those subjects (Doppler et al., 2018). Nevertheless, it is possible that the only studied subject did not have deposits in those fibers. Moreover, limitations regarding the technique might have played a role, and those will be discussed below.

Regarding PARKIN mutation carries, we again note an extremely limited amount of colocalization in the cervical skin fibers. There is the possibility of lack of phosphorylation of a-synuclein in the periphery in those individuals, but the data is conflicting (Isonaka et al., 2021, Fadda et al., 2019) Nevertheless, in this case, we also note some positive results when analysing the foot skin sections. This could possibly indicate that there is a gradient of a-synuclein phosphorylation, with distal sites being affected greater than the proximal. Finally,

the MSA cohort had minimal colocalization in PGP 9.5 positive cervical and foot skin fibers, which could be secondary to the nature of disease progression of this individual.

When we studied the colocalization of phosphorylated a-synuclein and various neuronal markers, we noted a large amount of particles during the taking of the confocal pictures. These particles were seen in all the available channels thus do not correspond to any of the relevant structures or proteins we are studying. They are mostly found within the lumen of the sweat glands, and have been previously described mostly when using widefield microscopy (Minota et al., 2019). The nature of those particles is unclear, and they likely represent debris, either cellular or gland secretions. The automated method we used excluded this debris from the analysis to a large extent.

An interesting finding was that of minor amounts of phosphorylated a-synuclein deposits in healthy controls. This was evident when analysing sections stained for synaptophysin or VIP and phosphorylated a-synuclein. This could be the consequence of the methodology of our experiment or of the analysis. Small amounts of a-synuclein oligomers have been shown in the skin of healthy controls, and 'false-positive' colocalization of phosphorylated a-synuclein and PGP 9.5 has been discovered in a non-parkinsonian subject (Donadio et al., 2019). This particular finding is quite interesting because almost all publications up to this point exclude the presence of pathologic a-synuclein in the skin of healthy people, and Donadio's and Doppler's team claim that this is a false positive signal because it is only found in one sample of the particular patient.

Finally, another part of our study was focused on the peripheral neurodegeneration. This was assessed via the measurement of the IENFD values of the control group and the A53T cohort. Remarkably, there was significant loss of intraepidermal fibers in the genetic A53T PD patients compared to the control, similar to idiopathic PD (Jeziorska, M. *et al.*

2019), nevertheless due to the limitations of the small number of samples, this needs to be further studied to ensure the results are valid. Interestingly, the loss of epidermal fibers was greater in the MSA patient compared to the A53T group, which appears to be similar to the findings of Haga et al, although they compared MSA with iPD patients.

Limitations

As with every study in the relevant field, there are a number of limitations that have affected our findings.

A quite important issue in the study is the collection of the samples. To be more precise, the human volunteers provided our lab with samples at completely different timeframes, with a range of more than a year. Given the samples were stored at a temperature of -20C, it is probable that they were not of the exact same quality at the time of immunofluorescence, thus the results might have been affected by this. Ideally, the people of the various groups should be recruited in a set timeframe and / or the relevant samples be stored at a lower temperature of -80C.

Our results are also limited by the small number of the patients and samples available. Only the A53T cohort had a group size equal or greater to two for most analyses. Further experiments with larger numbers of subjects are needed to corroborate our current findings.

Moreover, secondary to the random timeframe of each subject's recruitment, not all samples were available at the same time for the experiments. This is evident by the fact that, in the presented results, the cohorts' numbers differ depending on the particular analysis performed. It should be noted that in the circumstances when the patient's sample had already been stored for a prolonged period of time, the necessary slides may not have been available, as they might have been used beforehand, thus limiting the available material.

Regarding the technique, the antibodies and the dilutions were standardised. Nevertheless, due to initial issues with the confocal machine availability, the nuclei staining might differ. Both TOPRO-3 and DAPI were used, but in some cases TOPRO-3 was removed

from the antibody mix due to poor staining. Moreover, during imaging analysis, we removed most of the debris, but small quantities might be present in the final analysis.

Regarding the image-taking, images were initially collected via the inverted confocal microscope available at the BRFAA, while the latter images were obtained via the upright confocal microscope. The initial images (inverted) have not been analysed, as there was a significant difference in the laser strength that would affect the comparison of the images. As a result, some of the older tissue samples could not be analysed and are not presented in this dissertation. This fact, together with the large discrepancy between each tissue sample collection, could further aggravate any possible tissue deterioration overtime.

Significant time constraints also appeared in image taking. Consulting with the relevant staff who are experts in confocal imaging, each section was imaged in slices of a very small diameter. Thus, a significant amount of time was required to obtain high quality images of each section, which meant that sometimes the days of image-taking differed for sections of the same experiment, although thorough attention was given to avoid this happening for sections of the same sample tissue.

Last, but not least, a significant obstacle that affected multiple parts of this dissertation was the COVID19 pandemic. Due to the restraints imposed, the recruitment of volunteers was significantly limited. Moreover, the lockdowns imposed reduced access the researcher had to the laboratory and the various facilities, including imaging. This unfortunately led to some parts of the dissertation requiring further refinement, which has been impossible to do due to the limitations above and the researcher’s work schedule.

Conclusion

To our knowledge this is the first study that attempted to analyse the cutaneous changes of PD patients harboring the A53T mutation. Moreover, this is one of the limited genetic studies that tried to elucidate the involvement of specific neuronal types in the skin.

Our study provides initial evidence that cutaneous nerve fibers, and in particular autonomic fibers, of manifesting A53T SNCA mutation carriers indeed harbour aberrant deposits of alpha-synuclein. While not entirely surprising, and still needing to be corroborated through further experimentation, this finding adds to the body of knowledge regarding the aberrant deposition of a-synuclein in peripheral and central tissues of patients with genetic parkinsonism.

Longitudinal prospective studies in such individuals with genetic synucleinopathies, with multiple sampling sites, may provide essential information regarding the spread of a-synuclein pathology. At the same time, we have attempted to further analyse the differences between PD and MSA regarding aberrant cutaneous a-synuclein deposition, but inferences are limited due to the experimental constraints mentioned above.

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Acknowledgements

First of all, I would like to thank Professor Leonidas Stefanis for her encouragement, and professional advice.

I also would like to thank the Stefanis Lab members for their assistance.

.I would also like to thank our project participants who were willing to sacrifice their time, despite their difficult situation.

Lastly, I would like to thank my wife and my family for believing in me and for their

endless support.