Aus der Klinik und Poliklinik für Neurologie
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Dermal α-synuclein oligomers and aggregates in Parkinson's disease

Inaugural - Dissertation
zur Erlangung der Doktorwürde der
Medizinischen Fakultät
der
Julius-Maximilians-Universität Würzburg

vorgelegt von
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Würzburg, April 2018

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Tag der mündlichen Prüfung: 28. April 2020

Die Promovendin ist Ärztin

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LIST OF ABBREVIATIONS

AD	Alzheimer disease
CBD	Corticobasal degeneration
CJD	Creutzfeldt–Jakob disease
DLB	Dementia with Lewy bodies
GIT	Gastrointestinal tract
H&Y	Hoehn and Yahr
HIER	Heat-Induced Epitope Retrieval
IHC	Immunohistochemistry
LB	Lewy bodies
MSA	Multiple system atrophy
p-α-syn	α-synuclein phosphorylated at position S129
PD	Parkinson's disease
PK	Proteinase K
PLA	Proximity ligation assay
PSP	Progressive supranuclear palsy
RT	Room temperature
SN	Substantia nigra
α-syn	α-synuclein

1. INTRODUCTION: PARKINSON'S DISEASE AND ITS DIAGNOSTIC WORK-UP - STATE OF THE ART

DEFINITION AND ETIOLOGY

Parkinson's disease (PD) is a neurodegenerative disease affecting approximately 1% of individuals older than 60 years (Tysnes and Storstein, 2017). It is the most common cause of the parkinsonian syndrome marked by tremor, rigidity, bradykinesia and postural instability. These characteristic motor features are accompanied and often preceded by a number of non-motor symptoms (Fig. 1). In this long prodromal period of 10-20 years a variety of symptoms have been described, most importantly, rapid eye movement (REM) sleep disorder, hyposmia, constipation, urinary urgency and depression (Schapira et al., 2017). Clinical diagnosis of PD is challenging, especially at early stages of disease. The insidious nature of PD is probably still best reflected by the words of James Parkinson, who first described the disease in 1817 referring to it as "paralysis agitans" (Parkinson, 2002): "So slight and nearly imperceptible are the first inroads of this malady, and so extremely slow its progress, that it rarely happens, that the patient can form any recollection of the precise period of its commencement."

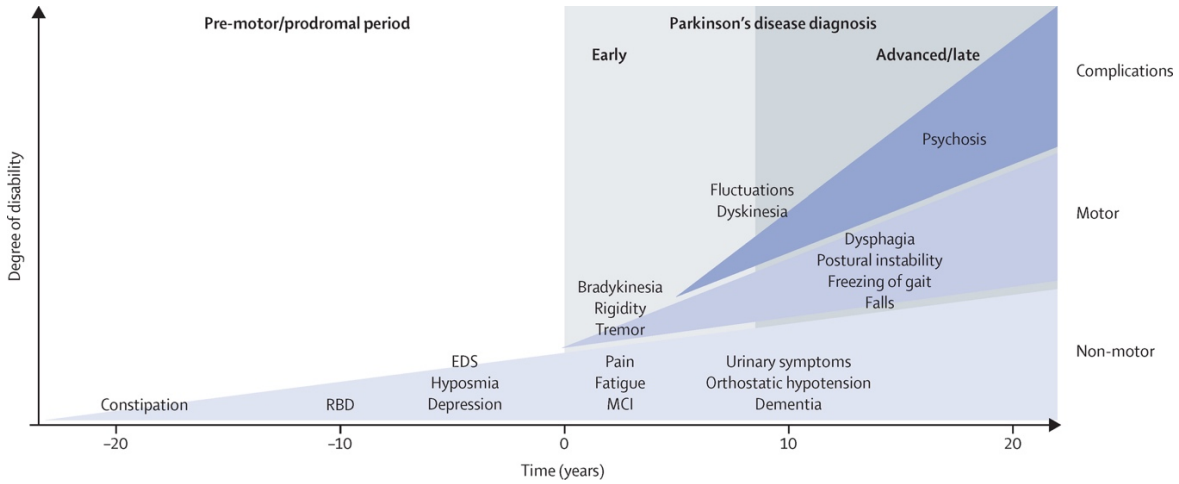


Figure 1 Schematic time course of PD (Kalia and Lang, 2015, License # 4324690564952)

The etiology of PD remains unknown, but is thought to involve both genetic and environmental factors. PD is more common in men with the male-to-female ratio being approximately 3:2 (Kalia and Lang, 2015) and its incidence increases 5-10 fold from the sixth to the ninth decade of life (Poewe et al., 2017). Several environmental factors were found to increase the risk of developing PD, most consistent of which is exposure to pesticides (Priyadarshi et al., 2000).

Epidemiological data are variable on whether history of traumatic brain injury (Gardner et al., 2015), farming work (Rocca et al., 1996), exposure to metals (Powers et al., 2003) increase the risk of PD. Other commonly mentioned associated factors such as depression (Shen et al., 2013) or constipation (Adams-Carr et al., 2016) might as well represent a prodromal stage of the disease and not a risk factor per se. Curiously, one epidemiological study postulated that subjects with higher education and in particular physicians have an increased risk of PD, though the data could be biased through a number of confounding factors, the most likely explanation being the so called surveillance bias - increased recognition and earlier detection of PD among educated subjects and physicians (Frigerio et al., 2005). Several protective environmental factors have been described as well, most strong of which appears to be history of smoking as shown by several large population studies. For example, a 2012 meta-analysis reported that the risk of PD was significantly lower for current smokers compared with never smokers (Noyce et al., 2012). A neuroprotective action of nicotine has been proposed as one possible explanation (Quik, 2004). An alternative theory that could elegantly explain these observations postulates that since dopamine is an integral component of the brain's reward system and dopamine is significantly depleted in the basal ganglia years before symptoms of PD appear, people who will later develop PD simply do not engage in reward-seeking behaviors, such as smoking (Ritz et al., 2014). Other factors associated with a reduced risk of PD according to at least some studies are intake of coffee (Hernán et al., 2002), statins (Bai et al., 2016), and ibuprofen, but curiously not of other non-steroid anti-inflammatory drugs (Samii et al., 2009). Although the majority of cases of PD are sporadic, there are genetic forms of parkinsonism due to mutations in genes involved in dopamine metabolism, mitochondrial function, detoxification or synaptic signal transduction, i.e. α-synuclein (α-syn), parkin, UCHL1, DJ1, PINK1, and LRRK2 (Klein and Westenberger, 2012).

PATHOGENESIS

The first insight into PD pathogenesis was gained in the early 20th century, when the German neurologist and neuropathologist Friedrich Lewy reported neuronal cytoplasmic inclusions in the brain of a patient with “paralysis agitans” that were later named after him (Holdorff, 2002). In 1919, a Russian neuropathologist, Konstantin Tretiakoff, observed that the most critical abnormality in PD was the loss of neurons in the substantia nigra (SN) pars compacta of the midbrain (Lees et al., 2008). In the 1950s Oleh Hornykiewicz and Herbert Ehringer at the University of Vienna discovered that dopamine

levels in the striatum in PD patients are lower than in unaffected brains and later it was clarified that the striatum's dopamine comes from neurons projecting from the SN (Hornykiewicz, 2010). These findings paved the way for the pharmacological dopamine substitution with L-DOPA first attempted in the early sixties in PD patients as well as in patients with postencephalitic parkinsonism leading to prompt and dramatic functional improvement that was even reflected in literature and film (Penny Marshall, 1990; Sacks, 1973). L-DOPA was approved for treatment in 1970 and since then remained the mainstay of therapy in PD. In 1997, a team led by geneticist Mihael Polymeropoulus in Bethesda, USA, identified a mutation in the α-syn gene that causes an inherited form of PD (Polymeropoulos et al., 1997). In the same year neurobiologists Michel Goedert and Maria Grazia Spillantini who were studying α-syn at the University of Cambridge, UK, when Polymeropoulus's work was published, discovered that the elusive primary constituent of Lewy bodies is α-syn (Spillantini et al., 1997). The original theory that the PD pathology starts with the degeneration of dopaminergic neurons in the SN was challenged by the German neuropathologist Heiko Braak, who proposed that the pathologic changes of PD start in the vagal nuclei in the lower brainstem and in the olfactory bulb, progressing rostrally over many years to the cerebral cortex in a predictable six-stage process (Braak et al., 2003).

The precise mechanisms of neurodegeneration in PD still remain not well understood, but dysfunction of α-syn is thought to play a central role in PD pathogenesis. Distinguishing the ever more important role attributed to α-syn in PD pathology, the term synucleinopathy was first coined in 2002 by Trojanowski and Lee referring to PD and related diseases (Trojanowski and Lee, 2002). The most widely accepted pathophysiological pathway to date is the so called α-syn cascade hypothesis that postulates that physiologic α-syn due to unknown reasons forms misfolded prefibrillar oligomeric species that in their turn assemble into fibrils which constitute Lewy bodies (Ingelsson, 2016). Once initiated, α-syn pathology is supposed to propagate in a prion-like fashion in which pathologically altered/misfolded proteins induce the templated misfolding of other protein molecules (Brundin and Melki, 2017) (Fig.2).

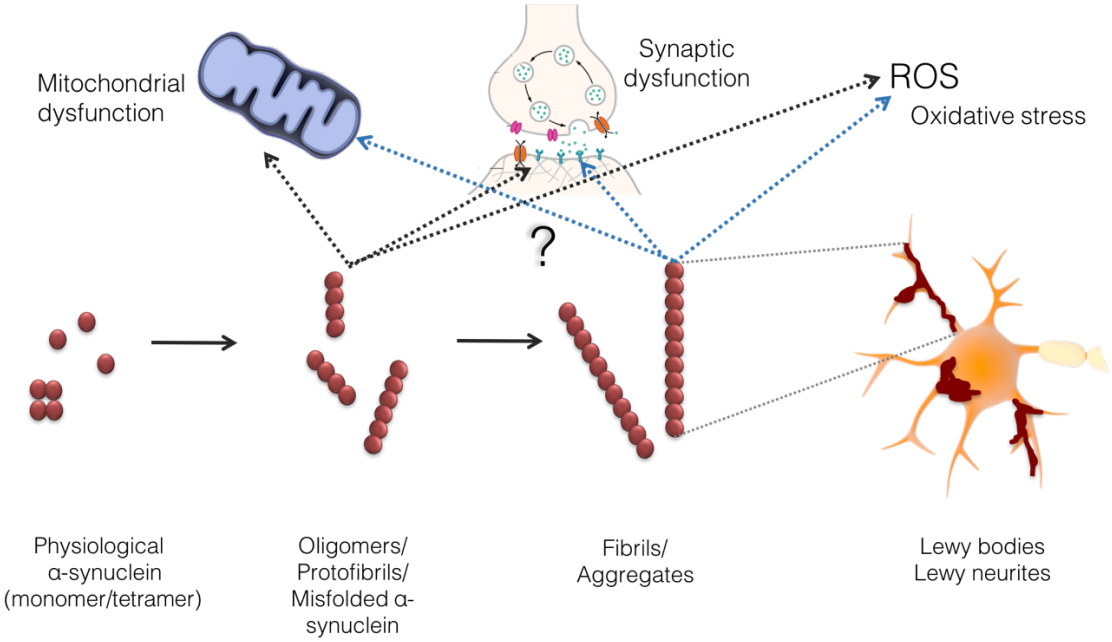


Figure 2 α-syn cascade: physiologic α-syn monomers/tetramers due to not precisely defined but most likely multifactorial pathologic stimuli assemble into oligomers or protofibrils that through further aggregation turn into fibrils that get sequestered in Lewy bodies/neurites. Intermediate species (oligomers) are thought to be neurotoxic through different proposed mechanisms, including synaptic dysfunction, mitochondrial disruption and induction of oxidative stress (image of synapse from Wikipedia, License for use CC BY-SA 4.0)

Aggregate rich Lewy bodies that were initially thought to be toxic and to contribute to neurodegeneration have more recently been postulated to represent a form of aggresome that develops in response to increased levels of oligomers to segregate and facilitate their clearance (Olanow et al., 2004). A lot of evidence suggests that it is the intermediate oligomers that are toxic to neurons through destabilization of lipid membranes, protein clearance pathways and mitochondrial function and not the fibrils (Andreasen et al., 2015; Roberts and Brown, 2015).

There is some inconsistency in terminology used in literature to describe α-syn conformations. In the strict sense of the word the term “aggregate” can refer to any abnormal association of misfolded proteins starting from a few molecules (Ross and Poirier, 2005). However, in the PD field the term “aggregate” usually refers to the end-stage of the aggregation process: large amyloid filamentous aggregates of α-syn, that are rich in β-sheet secondary structure and are the principal constituent of the Lewy bodies. One essential property of this conformation is proteinase K (PK) resistance (Neumann et al., 2004). The intermediate species in the process of aggregation that are commonly referred to as oligomers or protofibrils and are not that well defined. A variety of oligomers has been described and they probably represent a

continuum of species ranging in size from a couple of monomers to 15-30 monomer large constructs. (Cremades et al., 2012; Danzer et al., 2007; Ehrnhoefer et al., 2008; Lashuel et al., 2002; Pieri et al., 2016). Only so-called "on-pathway" oligomers possess seeding capacity and can form fibrils (Pieri et al., 2016), while some oligomers were shown to be harmless.

Notably, there is also accumulating evidence that native α-syn to at least some extent exists as an oligomer (Bartels et al., 2011; Wang et al., 2011). The exact nature of oligomers is not characterized and our knowledge is mostly based on indirect evidence using denaturing methods and in vitro studies that do not adequately reflect the protein conformation in vivo. Oligomers are believed to be practically not resistant to PK (Roberts et al., 2015). However, a type of "compact oligomers" directly preceding fibrils that are PK resistant has also been described (Cremades et al., 2012). β-sheet content that is believed to be responsible for PK resistance (Swietnicki et al., 2000) increases with the oligomer size (Chen et al., 2015). So the line separating oligomers and aggregates appears to be vague.

A-SYNUCLEIN CONFORMATIONAL STATES AND POST-TRANSLATIONAL MODIFICATIONS (PTM)

α-syn is a 140 amino acid long protein composed of three domains: N-terminal α-helix, non-Aß component of Alzheimer's disease amyloid central (NAC) domain and C-terminal acidic tail (Fig. 3). The primary function of the N-terminal domain is interaction with lipids suggested by it containing four amino acid repeats with a highly conserved hexamer motif (KTKEGV) similar to those found in lipid-binding domains of apolipoproteins (Emamzadeh, 2016). Moreover, upon interaction with lipid vesicles, the N-terminal region of α-syn adopts an α-helical conformation (secondary structure) (Davidson et al., 1998). All three clinical PD mutations are found in this region, which adds to the importance of this domain for the normal function of α-syn (Siddiqui et al., 2016). The central NAC domain comprises the highly amyloidogenic part of the molecule essential for the aggregating abilities of the protein. Mutation of only one single amino acid in this central domain of α-syn can alter the aggregation properties of the protein (Deleersnijder et al., 2013). The C terminus is thought to be responsible for protein-protein interactions, metal binding and to play a regulatory role in the aggregation and fibril formation of the protein. Deletion of C terminus increases the aggregation of α-syn (Hoyer et al., 2004).

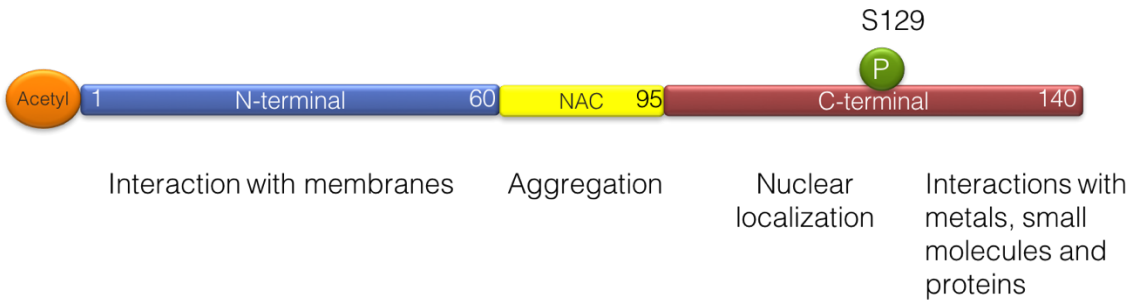


Figure 3 α-syn domains and their suggested functions. Also marked is the most important phosphorylation site - serine129.

α-syn is abundantly expressed in the central and peripheral nervous system, making up around 1% of total cytosolic protein in the brain (Stefanis, 2012). It is also very abundant, for unclear reasons, in erythrocytes and platelets (Barbour et al., 2008) as well as in melanocytes (Matsuo and Kamitani, 2010). The fact that α-syn normally localizes to the nerve terminal and is induced during establishment of synaptic connections in neuronal development suggested a role in neurotransmitter release (Bendor et al., 2013; Stefanis, 2012). The latest study could elucidate an exact mechanism through which α-syn affects neurotransmission: Using adrenal chromaffin cells and neuron culture authors could show that α-syn accelerates exocytotic release through dilation of the exocytotic fusion pore (Logan et al., 2017). Interestingly, mutations that cause Parkinson's disease abrogated this property of α-syn (Logan et al., 2017).

The native physiological conformation of α-syn remains controversial. α-syn has long been thought to exist as a natively unfolded monomer that would acquire α-helical secondary structure only upon binding to lipid vesicles. However, recent work suggests that it also occurs normally as α-helix-rich tetramers (Bartels et al., 2011; Wang et al., 2011). The concept of α-syn being an “intrinsically disordered” monomer shaped the research field for more than 20 years until in 2011 Bartels and Selkoe and independently a group led by Gregory Petsko (Wang et al., 2011) reported that endogenous α-syn isolated and analyzed under non-denaturing conditions from neuronal as well as non-neuronal cell lines and brain tissue occurs in large part as a folded tetramer in addition to monomers. α-syn tetramers were also shown to be resistant to aggregation while PD-causing mutations decreased the amount of tetramers in mouse brain and in cultured neurons (Dettmer et al., 2015). These new findings suggest that the shift of the supposed equilibrium between α-helical tetramers and unfolded monomers towards the latter plays a crucial role in PD pathogenesis and stabilizing the tetrameric form could be a promising

therapeutic approach similarly to already existing therapy for transthyretin-related hereditary amyloidosis (Said et al., 2012).

A number of post-translational modifications (PTM) of α-syn are known, many of which play a role in PD. α-syn within Lewy bodies has been shown to be phosphorylated (at S87, S129, or Y125), ubiquitinated (K12, K21, or K23), truncated (at 96, 115, 120) and oxidized by tyrosine nitration (Schmid et al., 2013). Interestingly, the majority of disease-associated PTMs (phosphorylation, nitration, and truncations) cluster within the C terminal.

The most abundant PTM in Lewy bodies is phosphorylation at S129 first described by Fujiwara et al. in 2002, who found that about 90% of brain α-syn is phosphorylated in dementia with Lewy bodies while less than 4% is phosphorylated in the normal brain. However, since then it was found that accumulation of phosphorylated α-syn (p-α-syn) occurred in about one fourth of the aged population who did not have record of synucleinopathy. Muntané et al., 2012 provided evidence that Ser129 α-syn phosphorylation is an intrinsically normal event and it is increased in human substantia nigra and nucleus basalis of Meynert compared to other brain regions both in patients with synucleinopathies and in control subjects. Total α-syn expression levels in the same regions were lower compared with other brain regions and were as well independent of age and pathology (Muntané et al., 2012).

So far, the studies that tried to elucidate the link between phosphorylation of α-syn and its aggregation and neuronal toxicity led to conflicting results (Tenreiro et al., 2014). It is clear that phosphorylation plays an important role in synucleinopathy, but it remains a matter of hot debate, whether phosphorylation is a cause or a consequence of aggregation and whether it contributes to neurotoxicity or is a compensatory mechanism. Phosphorylation is a very common PTM for proteins in general and can lead to a variety of effects. In particular, in the nervous system it is believed to be crucial for regulating synaptic plasticity by changing the functions of the synaptic proteins (Lee, 2006; Woolfrey and Dell'Acqua, 2015), and this is probably directly applicable to α-syn as it plays an important role in synaptic transmission (Bendor et al., 2013; Logan et al., 2017). Phosphorylation can allow certain interaction partners to bind by providing a docking site, trigger conformational changes, determine the subcellular localization of a protein or target a protein for degradation (Salazar and Höfer, 2009). Indeed, there has been accumulated a bulk of evidence for the role of α-syn phosphorylation in a variety of physiologic processes. S129 phosphorylation modulates α-syn membrane binding (Nübling et al., 2014), enhances interaction with metal ions (Liu and Franz, 2005; Lu et al., 2011), α-syn degradation (Machiya et al., 2010), protein-protein interactions (McFarland

et al., 2008) and subcellular localization (Gonçalves and Outeiro, 2013). S129 is the PTM that has been most commonly exploited as a potential diagnostic marker and this will be in detail discussed in the next chapter.

DIAGNOSIS

Although PD is the most common condition that can cause a parkinsonian syndrome, a number of other less common neurodegenerative diseases, including synucleinopathies such as dementia with Lewy bodies (DLB) and multiple system atrophy (MSA), as well neuropathologically distinct taupathies (corticobasal degeneration (CBD) and progressive supranuclear palsy (PSP)) can lead to parkinsonism (Litvan et al., 2003). Distinguishing PD from other parkinsonian syndromes can be difficult, particularly in the early stages of disease. Furthermore, secondary parkinsonism can develop due to drug exposure, most commonly due to neuroleptics and antiemetics with dopamine receptor blocking action (Shin and Chung, 2012), rarely (only a few cases described) but noteworthy - due to illicit drug contaminant MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) - as it led to rapid onset and permanent parkinsonism and introduced use of MPTP for PD modeling in animals (Langston, 2017), due to structural lesions of SN caused by a lacunar stroke (Robles, 2016) or a tumor (Gherardi et al., 1985; Gouider-Khouja et al., 2000). Interestingly, in one of the earliest case reports on parkinsonism published in 1893 by Blocq and Marinesco, destruction of the substantia nigra by a tuberculoma was revealed upon autopsy (Hostiuc et al., 2016).

The clinical diagnosis of PD according to the Movement Disorder Society (MDS) criteria requires the essential presence of motor parkinsonism defined as bradykinesia plus resting tremor or rigidity. A diagnosis of clinically established PD additionally requires the presence of at least 2 supportive criteria and the absence of "red flags" or absolute exclusion criteria; red flags if present can be counterbalanced by supportive criteria to allow a diagnosis of clinically probable PD (Postuma et al., 2015). The pooled data from the studies using the pathologic examination as gold standard provided an accuracy of the clinical diagnosis made by experts using all available clinical data and after an adequate follow-up of 83.9%, with a sensitivity of 81.3% and a specificity of 83.5% (Rizzo et al., 2016). Another analysis (Adler et al., 2014a) showed a similar sensitivity of 88% and specificity of 68%. Remarkably, the accuracy of clinical diagnosis early in the course of the disease (<5 years) decreased dramatically to 53% (Adler et al., 2014a).

These findings stress the need for the development of an objective diagnostic biomarker. A biomarker that can help diagnose PD early in the course of disease would become of even more important once disease modifying therapies are available. And this might not be far from now as currently there are at least five therapies targeting α-syn in clinical trials ("Michael J. Fox Foundation Website," n.d.). An ideal diagnostic biomarker should be acquirable through minimally invasive means, allow to be studied with widely available methods, correlate with disease severity, and be able to detect the disease at early or even premotor stages.

BIOMARKER DEVELOPMENT

The search for a reliable biomarker can be divided into two main areas: neuroimaging and immunohistochemistry (IHC)/biochemical methods based on detection of α-syn pathology.

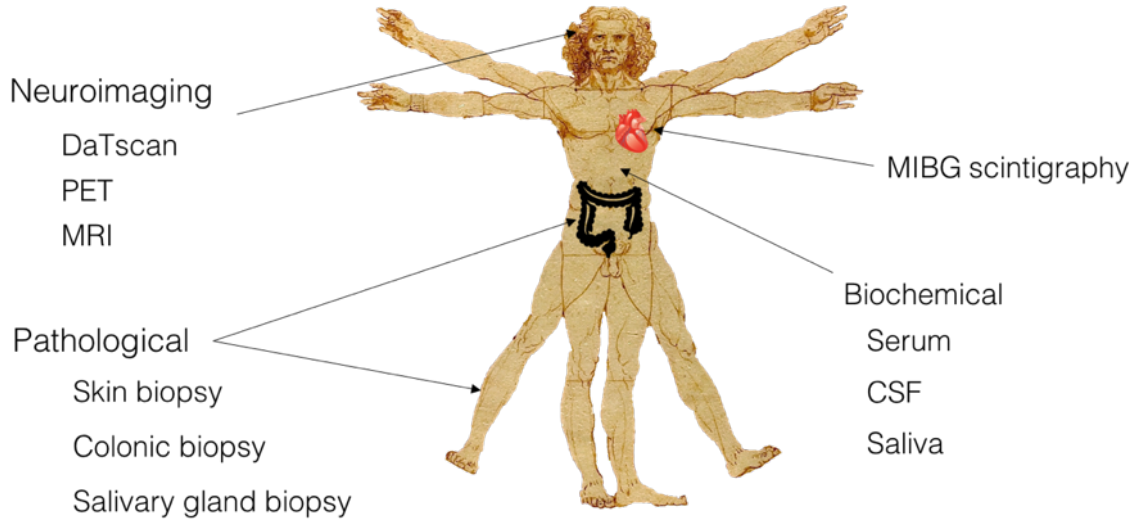


Figure 4 General methodological approaches to biomarker development

1.1.1. IMAGING

Neuroimaging techniques such as magnetic resonance imaging (MRI), positron-emission tomography (PET) and single-photon emission CT (SPECT) rely on different principles that could be useful depending on the question asked or the clinical setting. Most of the nuclear medicine imaging techniques focus on dopaminergic metabolism, although in the last years a great variety of new methodologies that could be potentially useful in PD have been developed: more than a dozen MRI-based techniques and over 100 PET and SPECT radioligands are available (Politis, 2014). Until now, however, very few of these new modalities have been successfully translated into clinical practice.

The imaging modality most widely used in PD diagnostics is single photon emission computed tomography (SPECT) with ioflupane (I123-FP-CIT), also called DaTSCAN. DaT-SPECT typically reveals normal dopamine transporter (DAT) levels in the caudate and putamen of healthy controls and patients with essential tremor or with drug-induced parkinsonism, but reduced DAT levels are seen in patients with PD, MSA or the neuropathologically distinct PSP (Djang et al., 2012). The accuracy of DaTSCAN for PD does not exceed that of the clinical diagnosis (de la Fuente-Fernández, 2012), but it can be used to support the diagnosis in ambiguous clinical presentations.

PET shows decreased [18F]-fluorodopa tracer uptake in the caudate and putamen in patients with early PD compared with controls. This imaging modality has a diagnostic accuracy similar to DaTSCAN (Eshuis et al., 2009). PET imaging using F-FDG shows an alteration in glucose metabolism in PD (75% sensitivity, 100% specificity), MSA (100% sensitivity, 87% specificity) and PSP (86% sensitivity, 94% specificity) compared to normal control subjects (Juh et al., 2004). One study could show that by FDG-PET scans, PD patients could be identified out of a pool of scans including patients with MSA, PSP and CBD (86% sensitivity, 91% specificity) (Hellwig et al., 2012).

Myocardial sympathetic denervation, assessed with PET, SPECT or scintigraphy using noradrenergic tracers such as MIBG (iodine-123-meta-iodobenzylguanidine) is common in PD, but is not seen in patients with atypical parkinsonism or other PD mimics (Braune et al., 1999; Shin et al., 2006).

Brain parenchyma sonography (transcranial ultrasound) is a safe and widely accessible imaging modality that shows loss of SN hyperintensity in PD. A sensitivity of 82-91% and a specificity of 82-100% have been reported (Gaenslen et al., 2008; Hellwig et al., 2014). The limitations of transcranial ultrasound are that it may not be possible in some subjects due to an insufficient bone-window and is highly dependent on the expertise of the examiner.

MRI is primarily used to exclude structural lesions causing secondary parkinsonism. Structural changes in the SN, in particular the loss of the normal “swallow tail” appearance, can also be detected using MRI with a variable sensitivity of 76-100% and a high specificity of about 95% (Cosottini et al., 2014; Oustwani et al., 2017; Schwarz et al., 2014). With advance of MRI technology, it might become possible to more accurately assess the structural changes in PD. The yet not widely available in clinical practice 7 Tesla MRI technique used in several studies revealed an abnormal architecture of the substantia nigra that allowed a discrimination between PD patients and healthy subjects with a sensitivity of 100% and a specificity of 92.3-100% (Cosottini et al., 2014; Kim et

al., 2016; Kwon et al., 2012). However, further trials will show the usefulness of the technique in clinical practice as only a small number of patients and controls were studied in either trial. In one of the studies (Kim et al., 2016) only patients with advanced stage disease (Hoehn und Yahr, H&Y >3) who can be easily diagnosed clinically were imaged.

There is no imaging method that allows to visualize α-syn load in the brain in vivo, as it has already been made possible for amyloid in Alzheimer's disease (Rice and Bisdas, 2017). The Michael J. Fox Foundation has announced a $2 million award for the first team to create a selective α-syn ligand to be used in PET ("Alpha–Synuclein Imaging Prize," n.d.), because α-syn is more likely to directly reflect the disease process unlike the currently available tracers that are surrogate markers of dopaminergic neurodegeneration.

1.1.2. A-SYNUCLEIN AS A "WET" BIOMARKER

Numerous studies have been conducted in attempt to specifically detect α-syn pathology in peripheral nerves of practically every existing tissue as well as in different biofluids.

1.1.2.1. SERUM

Serum represents the ideal substrate for diagnostics as it can be easily and safely acquired and α-syn was shown to be present in the serum (El-Agnaf et al., 2003). But so far the studies that examined total α-syn serum levels in PD patients gave controversial results, with some showing no difference while others – decreased level of α-syn (Besong-Agbo et al., 2013; Smith et al., 2012).

Another approach was to measure oligomeric α-syn performing ELISA with the same capture and detection antibody. Although an initial study observed significantly higher α-syn oligomer levels in plasma of PD patients (El-Agnaf, 2006), this could not be replicated in other laboratories and studies with larger patient populations (Gorostidi et al., 2012; Simonsen et al., 2016).

Foulds et al. looked at the level of S129 p-α-syn in the plasma of PD patients and controls and found that the mean level of p-α-syn was higher in PD patients (Foulds et al., 2011). The same group went on to conduct a first of a kind longitudinal study of α-syn and p-α-syn blood plasma levels in PD patients and controls (Foulds et al., 2013). They found that total α-syn levels increased with time for up to 20 years after the first symptoms appeared whereas p-α-syn levels remained constant. The authors suggested that the plasma levels of p-α-syn could have potential value as a diagnostic tool, whereas the level of total α-syn could act as a surrogate marker for PD progression.

The presence of autoantibodies to α-syn in PD was first shown in 2006 (Papachroni et al., 2006). The frequency of antibody presence in the sporadic PD was not different from controls, but they were found in a significantly higher proportion of patients with familial disease. Studies that followed have shown conflicting results. An international group led by L.A. Morozova-Roche found autoantibodies to α-syn to be significantly higher in the blood sera of PD patients compared to controls, their levels decreasing with disease progression (Yanamandra et al., 2011). One more group reported α-syn autoantibodies to be significantly higher in PD cohort than in controls (Shalash et al., 2017). These findings, however, could not be replicated by other groups (Besong-Agbo et al., 2013; Smith et al., 2012)

1.1.2.2. CEREBROSPINAL FLUID (CSF)

Unlike relatively easily detectible changes of tau and Aβ42 CSF levels in Alzheimer disease, α-syn is predominantly an intracellular protein with only minute quantities excreted in CSF. Its normal amount in CSF ranges according to one study (Mollenhauer et al., 2011) from 0,95 to 6,07 pg/µl being 1-2 orders of magnitude lower than those of tau (75–2497 pg/µl) and beta-amyloid (136–914 pg/µl). Most studies that measured CSF α-syn levels showed a decreased concentration in PD, however, the results were inconsistent (Mollenhauer, 2014). Difference in CSF α-syn level between PD and normal controls showed a range of diagnostic accuracies among the studies: 61 to 94% sensitivity and 25 to 64% specificity for distinguishing PD from controls (Atik et al., 2016). Two independent trials measured p-α-syn levels in CSF and found that these to be higher in PD patients and to correlate with disease progression (Stewart et al., 2015; Wang et al., 2012).

So far the most promising results in terms of high diagnostic specificity (88.5-92%) and sensitivity (96.9-100%) were reached using protein misfolding assays (Fairfoul et al., 2016; Shahnawaz et al., 2017). The obvious drawback of using CSF is a relative invasivity of the test making it unfeasible for screening or monitoring disease progression/therapy response. Although it might seem intuitive at first, CSF might not be the ideal substrate for α-syn detection.

1.1.2.3. SALIVA

Several studies have failed to show a significant difference in α-syn concentration in saliva of PD patients and normal controls (Devic et al., 2011; Goldman et al., 2018). Kang et al. showed that salivary samples from patients with PD contain high levels of α-syn oligomers (Kang et al., 2016). Another group

in Italy observed a similar tendency: total salivary α-syn was found to be lower, whereas oligomeric α-syn was higher in PD patients than healthy subjects as measured by sandwich ELISA with identical capture and detection antibody. The ratio of oligomeric to total α-syn was on average significantly higher in patients than in healthy subjects (Vivacqua et al., 2016), however there was overlap of the α-syn levels in some healthy subjects and PD patients and further trials are needed to confirm its utility for clinical diagnosis. With advance of methods sensitivity saliva is one very promising biomarker source as it can be acquired completely non-invasively.

1.1.2.4. SALIVARY GLAND BIOPSY

In 2010 two retrospective autopsy-based studies showed PD specific α-syn depositions in the submandibular gland in the majority of PD patients (Beach et al., 2010; Del Tredici et al., 2010). The first in vivo studies could detect α-syn pathology in labial minor salivary gland biopsy in 2 out 3 PD examined patients (Cersósimo et al., 2011). A follow-up study with a larger patient cohort could only find α-syn pathology in 3 out of 16 PD patients (Folgoas et al., 2013) designating minor salivary gland biopsy not useful. Submandibular gland biopsy showed promising results in a simulation of needle biopsy on autopsy material showing Lewy-type pathology in 17 of 19 PD patients (Beach et al., 2013). The in vivo studies showed phosphorylated α-syn in the nerve fibers of the submandibular gland in 74%–75% of PD subjects while 22% of controls were also positive (Adler et al., 2016, 2014b). Recently, p-α-syn deposits in the submandibular gland could also be shown in REM-sleep behavior disorder (Vilas et al., 2016). The major methodological problem of this method was the success rate of the biopsy - glandular parenchyma was missed in 50-57% of patients despite ultrasound guidance (Vilas et al., 2016). Side events including mild-to-moderate local pain and post-procedure subcutaneous hematoma were quite common (17-70%), but did not require treatment (Adler et al., 2016; Vilas et al., 2016).

1.1.2.1. GASTROINTESTINAL BIOPSIES

In the autopsy studies comparing the prevalence of α-syn pathology among different tissues, gastrointestinal tract (GIT) looked as one of the most promising peripheral tissues due to a relatively high prevalence of α-syn deposits (Beach et al., 2010; Gelpi et al., 2014). Since then a number of studies were conducted using biopsies of the gastrointestinal tissue that can be acquired through a colonoscopy. The results have, however, been mixed. Although some studies showed a virtually 100% specificity (Hilton et al., 2014;

Lebouvier et al., 2008; Pouclet et al., 2012; Sánchez-Ferro et al., 2015), others found no clear difference in α-syn protein expression pattern between PD and normal controls, even when staining for p-α-syn was performed (Aldecoa et al., 2015; Böttner et al., 2012; Visanji et al., 2015a). In attempt of determining the optimal protocol for the detection of α-syn pathology in colonic biopsies a multi-center blinded trial was conducted using a common set of slides and 4 different methods, 3 of which stained for p-α-syn (Corbillé et al., 2016). Disappointingly, also in this study no significant difference to normal controls could be found. Until now α-syn pathology in the GIT could not be conclusively characterized so as to make its use in diagnostics possible. Moreover, the required endoscopy presents another important limitation making this test too invasive for routine clinical use. These studies, however, gave an interesting insight into the multi-organ distribution of α-syn pathology, supporting Braak´s hypothesis that PD might originate in the gut (Lionnet et al., 2018).

1.1.2.2. SKIN

Use of skin biopsies is particularly attractive due to a relatively low invasiveness of the procedure which can be performed on outpatient basis and can be easily repeated. The first study to show a widespread p-α-syn accumulation in the nerve fibers from autopsy material in synucleinopathies was published in 2008 (Ikemura et al., 2008). In 2010 the possibility of skin biopsy use for the PD diagnosis was further explored by another Japanese group, who found abnormal accumulation of p-α-syn in the chest skin of two (10%) of 20 autopsied patients, but in none of the leg samples (Miki et al., 2010). In 2013 Roy Freeman's group performed a first in vivo skin biopsy trial and could show a higher total-α-syn deposition in dermal nerve fibers in PD patients compared to controls (Wang et al., 2013). Most groups since then have focused on p-α-syn deposits in the skin due to a higher specificity compared with total-α-syn. The sensitivity, however, differed greatly between the studies. Donadio and others (Donadio et al., 2016, 2014; Giannoccaro et al., 2015) have reported α-syn pathology in the cervical region in all PD subjects and in none of the controls. The initial study conducted in our group assessing distal and proximal leg, back, and finger, found that 16/31 PD subjects were positive for p-α-syn, and Haga et al. reported only 2/38 PD patients were positive in chest and leg biopsies (Doppler et al., 2014; Haga et al., 2015). It was recently proven, that p-α-syn is present in patients with REM-sleep disorder, a high-risk factor and suspected prodromal stage of PD (Antelmi et al., 2017; Doppler et al., 2017).

Neuropathologically, MSA and DLB are closely related to PD and are also characterized by α-syn aggregation, therefore it is logical to assume that α-syn deposits might also be found in peripheral tissues in these disorders. Interestingly, one group could find no p-α-syn deposits in MSA proposing to use anti-p-α-syn immunohistochemistry as a method to distinguish MSA from PD (Zange et al., 2015). However, different results were observed in our group: in MSA p-α-syn deposits were found in the majority of patients similarly to PD, but most of the deposits were found in somatosensory nerve fibers in contrast to PD where autonomic nerve fibers were predominantly affected (Doppler et al., 2015). As expected, pathological α-syn deposits were found in none of the patients with taupathies (CBD, PSP), indicating a potential utility for differential diagnosis (Doppler et al., 2015). Recently, Donadio et al. could also show that p-α-syn is deposited in DLB (Donadio et al., 2017).

In none of the studies conducted so far could any p-α-syn be detected in the skin nerves of normal controls, giving a promise of a 100% specificity. The sensitivity of the method in our lab could be increased to 80% by using serial sections, a laborious and time consuming procedure (Doppler et al., 2017).

In summary, irrespective of tissue/biofluid or methodological approach, the use of p-α-syn usually allowed to definitively distinguish between patient and control samples, unlike in case of measuring total α-syn where results have been contradicting and often no difference could be found (Atik et al., 2016; Foulds et al., 2013). The new biomarker findings should be cautiously tested in larger population of patients, as according to some reports p-α-syn that was thought to be very specific for synucleinopathies was shown to be present in a substantial quantity in colonic biopsies (Barrenschee et al., 2017), salivary gland biopsies (Adler et al., 2016), blood and CSF of control subjects (Eusebi et al., 2017).

THEORY BEHIND THE METHODS

The aim of this doctoral thesis was to examine whether dermal p-α-syn accumulation represent aggregates and/or oligomers of α-syn. For this a set of immunohistochemical methods was adopted:

1.1.3. PROXIMITY LIGATION ASSAY

Proximity ligation assay (PLA) is a technique for protein detection that was first introduced in 2002 (Fredriksson et al., 2002). It is based on labeling of

antigens with antibodies bound to one of two complementary oligonucleotides (Fig. 5).

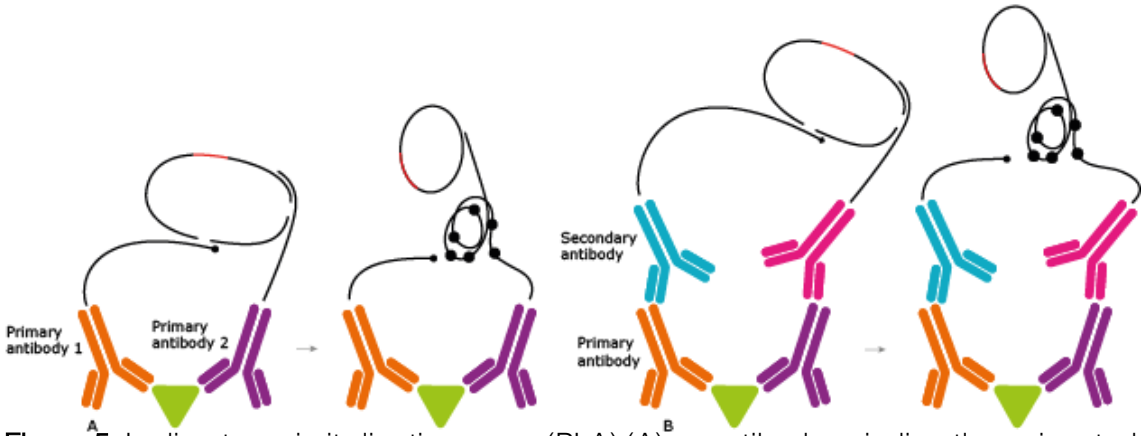


Figure 5. In direct proximity ligation assay (PLA) (A) an antibody pair directly conjugated to PLA probes is used, in indirect PLA (B) secondary antibody conjugates are used (https://www.proteinatlas.org, Creative Commons license CC BY-SA 3.0, https://creativecommons.org/licenses/by-sa/3.0/)

When antigens are lying in close proximity (as it is the case in oligomers), oligonucleotides can be hybridized to a connector oligonucleotide creating a circular template that can be amplified and quantified by methods such as real-time polymerase chain reaction (PCR) or in case of IHC by microscopy, made possible by the addition of oligonucleotides labeled either with fluorophores or with horseradish peroxidase (HRP). PLA has since then become an established complementary technique in IHC for detection of protein-protein interactions, posttranslational modifications (such as methylation or phosphorylation) and low expression proteins (Leuchowius et al., 2011). The first adaption of the technique for detection of oligomeric forms of protein was published in 2010 (Kamali-Moghaddam et al., 2010). In this study detection of Aβ protofibrils (aggregates associated with Alzheimer disease, AD) was based on one protofibril being simultaneously bound by 3 antibodies directed against the same epitope: a capture antibody immobilized on a microplate, an antibody bound to a minus nucleotide and an antibody bound to a plus nucleotide. Authors could specifically detect Aβ protofibrils in normal human CSF spiked with either in vitro prepared protofibrils or brain homogenates from genetically modified mice previously shown to express a high amount of aggregated Aβ. However, an attempt to detect of Aβ aggregates in human CSF failed to reveal signals above background for either AD patients or controls (Kamali-Moghaddam et al., 2010).

Roberts et al. adapted the PLA technique for detection of oligomeric forms of α-syn (Roberts et al., 2015). Apart from staining in the Lewy lesions in PD, there was a diffuse staining virtually in all brain areas both in PD and control

samples, where the staining of reticular formation and intermediate reticular zone of the medulla was significantly more intense in PD than in controls. Surprisingly, in the midbrain there was no difference in the amount of staining in patients and controls.

1.1.4. PARAFFIN-EMBEDDED TISSUE (PET) BLOT

Paraffin-embedded tissue (PET) blot is a technique that was introduced by Schulz-Schaeffer for detection of pathological prion (Schulz-Schaeffer et al., 2000). It emerged from the so called “histoblot” (Hecker et al., 1992; Taraboulos, 1992) – a hybrid between western blot and immunohistochemistry. In this method freshly cut frozen tissue was placed on nitrocellulose membranes instead of glass slides and therefore could be subjected to a harsh proteolysis in a way that only pathological prion resistant to digestion remained attached to the membrane and then could be detected with antibody staining. The advantage of the method over western blot was that it not only could specifically detect prion but allowed to see its anatomical distribution in a way similar to IHC. The enzyme used in the proteolysis was PK. The only substantial difference of the PET blot technique from histoblot is the use of archived formalin fixed paraffin-embedded tissue. In a direct comparison of PET blot and histoblot techniques for the detection of a scrapie strain in C57/Bl6 mice authors showed that PET blot was superior to histoblot in terms of sensitivity and anatomical detail (Walter J. Schulz-Schaeffer et al., 2000). They also showed as a proof of principle that it could be used to detect prion in the brains of patients with CJD. PET blot has become an important ancillary method in prion research (Budka, 2003). IHC, however, remains in practice a very sensitive method for neuropathological diagnosis, as shown by the *European neuropathological study of human prion diseases* among almost 1000 patients fulfilling criteria for a human prion disease only 2 specimens were negative for PrP in immunohistochemistry (Budka, 1997).

The PET blot method was adopted to show α-syn aggregates in the main synucleinopathies PD, DLB and MSA (Neumann et al., 2004, 2002). Separating the PK resistant aggregates based on their size (sucrose gradient analysis) and analyzing the fractions for synaptic markers syntaxin and synaptophysin Schulz-Schaeffer lab could show the presence of numerous small α-syn aggregates that were located at presynaptic terminals in DLB (Kramer and Schulz-Schaeffer, 2007). The presence of presynaptic aggregates was linked to a loss of dendritic spines leading the authors to the conclusion that the presynaptic aggregates and not Lewy bodies are responsible for neurodegeneration.

If we think of a possible usefulness of this method for an α-syn biomarker, its application for detection of pathological protein in peripheral tissue is of particular interest. Peripheral tissue is free of prion pathology in sporadic Creutzfeldt-Jakob disease (sCJD), however in a distinct subtype of prion disease, namely variant CJD, pathologic prion has been shown in the lymphoreticular system. In a study of spleen, tonsil, lymph node and dorsal root ganglion the PET blot method showed a more intense labeling pattern for disease associated PrP compared to IHC, however, IHC (with PK antigen retrieval) still allowed to distinguish between CJD and normal controls in all cases and provided more anatomical detail (Ritchie et al., 2004).

Application of PET blot for the study of peripheral tissue in PD is as of now limited to two published studies: Wrede and others could not find any PET blot signal in paravertebral muscle tissue from 14 patients with PD with camptocormia (Wrede et al., 2012). α-syn staining within the muscle tissue (presumably in nerve fibers) disappeared after a very short incubation with PK similarly to a normal control (Wrede et al., 2012). Opposite to these findings but not less disappointing were the results of a recent study of colonic mucosal biopsies: PET blot–resistant aggregated α-syn was detected in 19 of 22 individuals with PD but also in 11 of 11 control subjects (Visanji et al., 2015a).

1.1.5. THE SYN211 STAIN AFTER PK DIGESTION

The same principle (making use of resistance of α-syn aggregates to PK digestion) can be applied to IHC (see Fig. 6). In order to preserve tissue, the incubation time and concentration of PK used in IHC is substantially lower than in PET blot. However, even after a 10-30 min incubation time with a relatively low concentration of PK (50 µg/ml) no staining can be seen in the brain of the normal control (Tanji et al., 2010). Stating that PK digestion only leaves aggregated or beta-sheet rich protein would be an oversimplification as this effect is dependent on many variables of the reaction (temperature, concentration, incubation time, pH). Proteolytic enzymes (trypsin, pepsin and PK) have been used for antigen retrieval in formalin-fixed, paraffin-embedded tissues even before heat induced antigen retrieval (HIER) was introduced (Shi and Taylor, 2010). As fixation is based on forming cross links that can mask an epitope, antigen retrieval through protein digestion is supposedly working by nonspecifically cleaving bonds formed by proteins and fixative. Apart from being used for detection of pathological prion or α-syn (Takeda et al., 2000), PK is still sometimes used for antigen retrieval to stain for such high molecular weight extracellular matrix proteins as laminin, collagen, fibronectin, but has also been used for antigen

retrieval to stain for smaller proteins like insulin, glucagon and calcitonin (Ramos-Vara and Beissenherz, 2000) or p53 (Cornelissen et al., 2004). PK concentration in protocols varies from 4 µg/ml to 400 µg/ml (Mcnicol and Richmond, 1998). For a specific and sensitive stain, antigen retrieval with PK digestion has to be finely adjusted. When applied to α-syn this means that digestion should be strong enough to destroy physiological protein while leaving aggregates intact. Other factors (duration of tissue fixation, frozen tissue vs paraffin embedded) should also be taken into account as it will dramatically change the susceptibility in a similar way as to HIER (Jiao et al., 1999). Among a variety of methods used for α-syn IHC by different laboratories, antigen retrieval with PK was superior in sensitivity and most importantly specificity, resulting in no staining in the normal control (Beach et al., 2008).

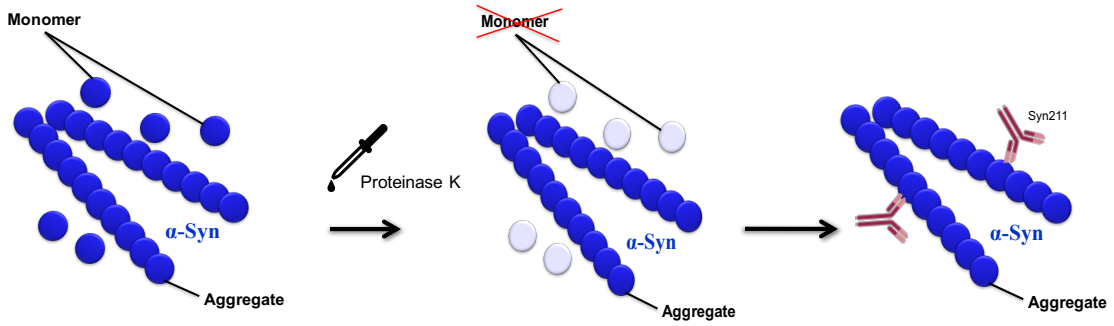


Figure 6 Principle of the proteinase K assay: cryosections are incubated with PK prior to staining. Monomers of α-syn being less resistant to PK than the aggregated form are digested. The stain with an antibody to total α-syn (syn211) reveals only aggregates.

1.1.6. THE STAIN WITH AGGREGATE SPECIFIC ANTIBODY 5G4

Kovacs et al. recently synthesized an antibody with high reactivity for disease-associated forms of α-syn. The monoclonal antibody created by immunizing mice with a peptide containing amino acid 44-57 of α-syn was specific for disease-associated α-syn while it did not show any background staining and immunoreactivity of normal presynaptic structures thus enabling to very specifically distinguish physiologic and disease-associated α-syn (Kovacs et al., 2012). 5G4 was shown to have virtually no immunogenicity towards monomeric α-syn. The authors developed an ELISA assay using this antibody and could detect aggregated α-syn in the CSF in a subset of patients synucleinopathies although the case number (N=7) was very low (Unterberger et al., 2014). The assay has been commercialized and used by another group to show a higher level of oligomers in the blood of PD patients carrying GBA mutation (Pchelina et al., 2017).

1.1.7. THE STAIN WITH OLIGOMER SPECIFIC ANTIBODY ASYO5

Brännström et al. used a novel approach in attempt to create antibodies that could selectively bind to oligomers or fibrils/aggregates using a two-step procedure in which the choice of an epitope, exposed within the monomeric and oligomeric forms but buried within the fibrillar/aggregated state, was combined with the effect of avidity to select for oligomer-specific binding properties (Brännström et al., 2014b). First, the authors found that interaction with antigen that engages both of the epitopes (i.e. with an oligomer/aggregate) increased the antibody affinity 1500 times compared to a monovalent interaction. This effect was dependent on concentration, i.e. the antibody was binding to divalent antigens while "ignoring" the monovalent antigens only within a certain concentration range. With a higher concentration this discrimination was lost (Fig. 7). This effect could be exploited to detect oligomeric α-syn by using a very high antibody dilution.

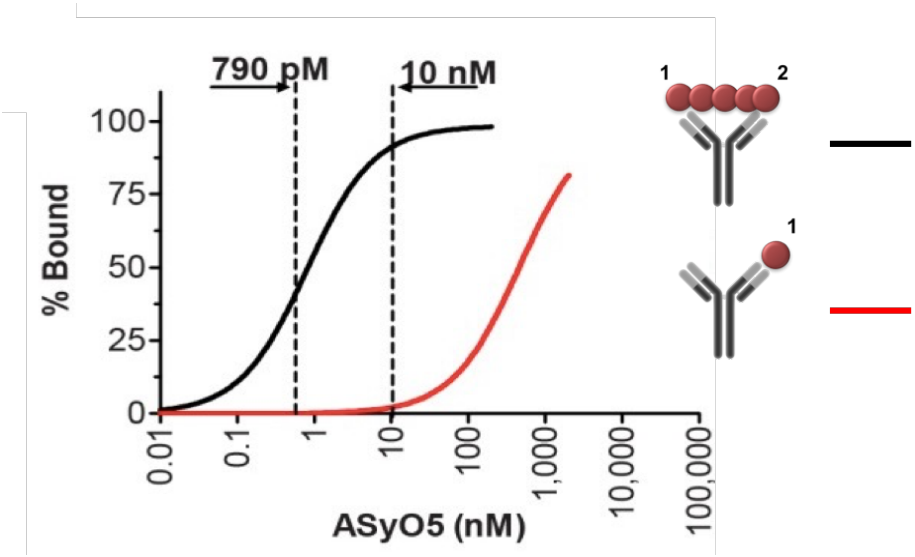


Figure 7 The level of ASyO5 saturation upon binding to oligomers (black line) and to monomers (red line). In the ASyO5 concentration range of 790pM-10nM the saturation level of binding to the oligomer is above 50% while the monovalent saturation level remains below 2%, adapted from Brännström et al., 2014a, License CC BY 4.0 https://creativecommons.org/licenses/by/4.0/).

STUDY OBJECTIVE

The objective of this study was to further characterize the dermal p-α-syn accumulations, in particular to assess whether the aggregation state of α-syn in the dermal nerve fibers in patients with PD is similar to that found in the Lewy bodies in the brain, as this is a prerequisite for the potential use of skin biopsy as a tool to study α-syn pathology in pre-mortem tissue. For this midbrain samples of a PD patient and a normal control were used to optimize the IHC with

antibodies specific for oligomeric/aggregated forms of α-syn (ASyO5 and 5G4), syn211 stain after PK digestion, PET-blot and PLA. Then skin biopsies from normal controls and two cohorts of patients were immunoreacted with the established methods to check for the following hypotheses:

1. Dermal phosphorylated α-syn in PD represents at least to an extent aggregated α-syn.
2. α-syn aggregates are not found in the dermal nerve fibers in normal controls.
3. Amount of α-syn aggregates increases with disease stage.

2. MATERIALS AND METHODS

PATIENTS AND CONTROLS

Autopsy brain samples

Frozen and paraffin embedded midbrain autopsy samples from a patient with neuropathologically confirmed PD (Braak stage VI) and a normal control were acquired from PD Dr. Monoranu (Abteilung für Neuropathologie, Pathologisches Institut, Universität Würzburg).

Skin biopsies

Skin punch biopsies from two cohorts of patients and controls recruited for earlier studies at our department (Doppler et al., 2017, 2015, 2014) were used in this study. Immediately after acquisition, samples were fixed in 4% paraformaldehyde for a minimum of 30 minutes, washed thrice with phosphate buffer, incubated in 10% sucrose overnight for cryoprotection, embedded in Tissue-Tek and stored at 80°C till sectioning.

Cohort #1

Archived biopsies from patients/controls recruited between 2011 and 2014 were used. 41 patients with the diagnosis of PD based on the UK brain bank criteria (Hughes et al., 1992) ranging from H&Y stage 1 to 5 (average 3) (Hoehn and Yahr, 1967), three patients with MSA and 22 normal controls. Parkinsonian motor symptoms were quantified using the Unified Parkinson's disease scale part III (UPDRS III) (Goetz et al., 2008). A minimum of two 20-µm sections per biopsy were analyzed. Choice of patients was biased towards those who had already been shown to have dermal p-α-syn deposits in previous studies conducted by our group (Doppler et al., 2017, 2015, 2014). Demographic data of all participants are summarized in Table 1, for detailed data for each participant see Table 3.

Cohort #2

27 patients with PD diagnosis based on the UK brain bank criteria in early disease stages (H&Y 1-2) and five normal controls were recruited between 2014 and 2016 (Hoehn and Yahr, 1967; Hughes et al., 1992). Skin punch biopsies were taken on one side of the body from four sites: distal leg, proximal thigh,

lower back (Th10), upper back (C7). For summarized data see Table 2, detailed information for each patient – Table 4.

Table 1: Summary of demographic data of patients and controls from the first cohort

	average age	female	male	average scores	
				H&Y	UPDRS3 (SD)
PD, N=41	66,6	13	28	3	41 (26)
NC, N=22	47	13	9	-	-

Table 2: Summary of demographic data of patients and controls from the second cohort

	average age	female	male	H&Y 1	H&Y 2	average UPDRS3 (SD)
PD, N=27	63	12	15	13	14	15.3 (7.8)
NC, N=5	65	1	4	-	-	-

Table 3: Demographic data of patients and controls in the cohort #1

Patients with Parkinson's disease				
#	age	sex	H&Y	UPDRS3
1	79	m	3,0	41
2	55	m	3,0	48
3	78	m	2,0	10
4	68	m	1,0	14
5	71	m	2,5	43
6	65	m	5,0	92
7	66	f	4,0	24
8	71	f	3,0	54
9	73	f	4,5	70
10	59	f	2,0	36
11	70	f	3,0	14
12	80	m	3,0	12
13	80	m	3,5	n/a
14	78	m	2,5	n/a
15	70	m	3,5	84
16	53	f	3,0	45
17	59	m	4,0	84
18	66	m	5,0	23
19	54	m	2,5	78
20	56	m	3,0	71
21	71	m	2,0	66
22	46	m	3,0	65
23	65	m	4,5	23
24	74	m	3,0	49
25	61	m	2,0	20
26	65	f	4,0	57
27	49	m	3,0	48
28	88	f	3,0	68
29	63	m	2,0	18
30	72	f	4,0	53
31	65	m	3,0	30
32	63	m	3,0	7
33	64	f	3,0	24
34	62	f	1,0	2
35	69	f	1,0	11
36	50	m	2,5	15
37	71	m	3,0	19

38	80	m	2,5	98
39	60	m	2,0	45
40	72	f	4,5	32
41	68	m	1,0	14
Patients with multiple system atrophy				
42	71	m	4,0	37
43	74	m	5,0	44
44	65	f	4,0	n/a
Controls				
1	50	m		
2	22	m		
3	20	f		
4	20	f		
5	28	f		
6	48	f		
7	49	m		
8	47	f		
9	49	m		
10	33	m		
11	30	f		
12	69	f		
13	44	f		
14	46	f		
15	44	f		
16	49	f		
17	66	f		
18	69	f		
19	75	m		
20	67	m		
21	68	m		
22	50	m		

Table 4: Demographic data of patients and controls in the cohort #2

#	age	sex	H&Y	UPDRS3
Patients with Parkinson's disease				
1	63	f	1	6
2	69	m	1	21
3	70	f	2	9
4	62	f	1	20
5	55	m	2	11
6	65	f	2	19
7	62	m	1	19
8	71	f	2	27
9	50	f	2	0
10	44	m	1	14
11	67	m	2	19
12	67	f	2	12
13	50	m	2	23
14	61	f	1	4
15	73	m	1	16
16	73	m	1	20
17	54	f	1	22
18	76	m	2	19
19	61	m	2	30
20	55	m	2	21
21	63	f	2	21
22	59	m	1	4
23	63	m	1	12
24	67	f	1	7
25	66	m	1	3
26	77	f	2	11
27	63	m	2	22
Controls				
1	69	m		
2	51	m		
3	80	m		
4	75	m		
5	48	f		

Primary antibodies[1]

Antibody against	Short clone name	Epitope (also see Fig. 8)	Dilution	Species	Cat. #	Manufacturer
α-synuclein	SNCA purified	n/a	1:1000	mouse	AM05094PU-N	Acris
α-synuclein	Syn211	121-125	1:1000	mouse	ab80627	abcam
α-synuclein	LB509	115-122	1:1000	mouse	180215	Invitrogen
aggregated α-synuclein	5G4	44-57	1:1000	mouse	MABN389	Merck, Millipore
α-synuclein, oligomer specific	ASyO5	111-125	1:10.000	mouse	AS132718	Agrisera
phosphorylated α-synuclein	P-Syn/81A	124-134, phosphorylated at Serine 129	1:500	mouse	MMS-5091	Biolegend
phosphorylated α-synuclein	Ser129, D1R1R	phosphorylated at Serine 129	1:2000	rabbit	23706	Cell Signaling
Protein Gene Product 9.5	Anti-PGP 9.5	n/a	1:1000	rabbit	516-3342	Zytomed Systems

[1] all primary antibodies except for anti-PGP9.5 are monoclonal

Secondary antibodies[2]

Antibody	Dilution	Catalogue #	Manufacturer
Cy™3 Donkey Anti-Mouse IgG	1:100	715-165-150	Jackson ImmunoResearch
Alexa Fluor® 488 Donkey Anti-Rabbit IgG (H+L)	1:400	711-545-152	Jackson ImmunoResearch

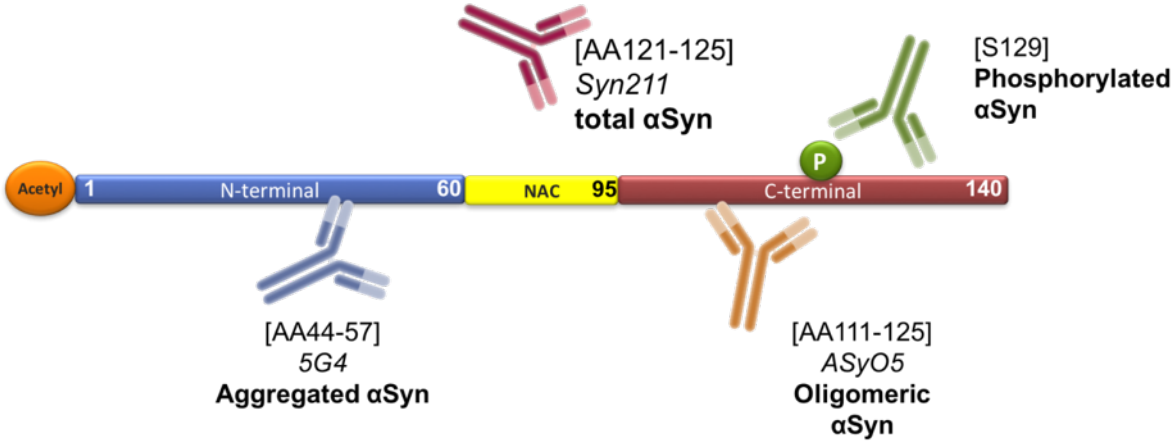


Figure 8 Epitopes that the anti-α-syn antibodies used in this study are directed at.

PLA Kit components (Sigma-Aldrich)

1. Duolink® In Situ Probemaker Minus/Plus: Duolink In Situ oligonucleotide Minus/Plus, Conjugation Buffer, Stop Reagent, Storage Solution, 20x Assay Reagent, Blocking Solution and PLA probe Diluent.
2. Duolink® In Situ Wash Buffers A and B
3. Duolink® In Situ Detection Reagents
   - Ligation (5x): oligonucleotides that hybridize to the PLA probes
   - Ligase (1 unit/µL)
   - Amplification (5x) Orange: components needed for Rolling Circle Amplification (RCA) as well as oligonucleotide probes labeled with a fluorophore (λex 554 nm; λem 576 nm) that hybridize to the RCA product.
   - Polymerase (10 units/µL)
4. Duolink® In Situ Mounting Medium with DAPI

[2] all secondary antibodies are polyclonal

2.1.1. OTHER REAGENTS

Name	Purpose	Vendor	Cat. #
Proteinase K	Protein digestion, epitope retrieval	Sigma-Aldrich	P6556
PBS 0.1M	Buffer for IHC	Self-made with Sodium dihydrogen Phosphate Dihydrate and di-Sodium hydrogen phosphate dehydrate (Millipore)	1.06345.1000, 1.06580.1000
Hoechst 33342	Nuclear stain	Thermo Fisher	62249
Albumin bovine serum (BSA)	Blocking	Sigma-Aldrich	A2153
Normal donkey serum	Blocking	Millipore	S30
Brij-35	Detergent Solution	ThermoFisher	85117
Tween 20	Detergent Solution	Roth	9127
Triton X-100	Detergent Solution	Sigma-Aldrich	T8787
VECTASTAIN® Elite® ABC-HRP Kit	avidin/biotin-based peroxidase system	Vectastain	PK-6100
DAB3,3'-diaminobenzidine Peroxidase (HRP) Substrate Kit	Immunohistochemical staining	Vectastain	SK-4100

2.1.2. EQUIPMENT

- Cryostat: Leica CM3050 S (Leica Biosystems Nussloch, Germany)
- Microscopy: Fluorescence microscope (Ax10, Zeiss, Oberkochen, Germany) with CARVII-system and Visiview software (Visitron GmbH, Puchheim, Germany)

METHODS

2.1.3. THE STAIN FOR ANTI-AGGREGATED A-SYNUCLEIN WITH THE 5G4 ANTIBODY

Slides with 20-µm sections were retrieved from -20°C and air dried for 30-60 min. After this, an additional post-fixation with 4% PFA for 10 min was done to help preserve the tissue morphology during antigen retrieval. Then, slides were washed thrice in PBS. The sections were then subjected to antigen retrieval with 80%[3] formic acid for 5 min. The slides were washed thrice in PBS. Blocking and permeabilization was done with PBS/5% Donkey Serum/1%BSA/0.1% TritonX100 for 30 min. After this, the slides were washed once in PBS to remove TritonX100. The sections were incubated with primary antibodies (5G4, mouse, 1:1000 and anti-PGP 9.5, rabbit, 1:200) diluted in 1%BSA/PBS buffer overnight at 4°C. After this the slides were washed thrice in PBS. Incubation with secondary antibodies (Cy3 Donkey Anti-Mouse 1:200, Alexa Fluor 488 Donkey Anti-Rabbit 1:400) was done for 2 hours at RT. After this the slides were washed thrice in PBS. For nuclear stain, sections were incubated with 1:2000 Hoechst (in PBS) for 2 min, after that the slides were washed 2-3 times in PBS, embedded in Vectashield media and allowed to dry before microscopy.

2.1.4. THE STAIN WITH ASYO5 – OLIGOMER-SPECIFIC ANTIBODY

Slides with 20-µm sections were retrieved from -20°C and air dried for 30-60min. Blocking and permeabilization was done with PBS/5% Donkey Serum/1%BSA/0.1% TritonX100 for 30 min. After this the slides were washed once in PBS to remove TritonX. The sections were incubated with primary antibodies (ASyO5, mouse, 1:10,000 and anti-PGP 9.5, rabbit 1:200) diluted in

[3] In case of serial sections that had been stored at -20°C for longer than 6 months the formic acid concentration had to be reduced to 50%.

1%BSA/PBS buffer overnight at 4°C. After this the slides were washed thrice in PBS. Incubation with secondary antibodies (Cy3 Donkey Anti-Mouse 1:200, Alexa Fluor 488 Donkey Anti-Rabbit 1:400) was done for 2 hours at RT. The slides were then washed thrice in PBS. For nuclear staining, sections were incubated with 1:2000 Hoechst (in PBS) for 2 min, after that the slides were washed 2-3 times in PBS, embedded in Vectashield media and allowed to dry before microscopy.

2.1.5. THE STAIN FOR PHOSPHORYLATED A-SYNUCLEIN

The stain with anti-p-α-syn was done identically to the ASyO5 stain described in detail in 2.3.2., but instead of ASyO5 antibody the anti-p-α-syn (Biolegend, mouse, 1:500) was used in a double stain with anti-PGP 9.5.

2.1.6. THE A-SYNUCLEIN STAIN AFTER DIGESTION/ANTIGEN RETRIEVAL WITH PK

Slides with 20-µm sections were retrieved from -20°C and air dried for 30-60 min. After this an additional post-fixation with 4%PFA for 10 min was done to help preserve the tissue morphology during antigen retrieval. Then slides were washed thrice in PBS and the sections subjected to digestion with 10µg/ml proteinase K in TBS-B (10 mM Tris–HCl, pH 7.8, 100 mM NaCl, 0.1% Brij 35) for 20 min at 37°C (10min for serial sections in cohort #2). The slides were washed thrice in PBS. Blocking and permeabilization was done with PBS/5% Donkey Serum/1%BSA/0.1% TritonX for 30 min. After this the slides were washed once in PBS to remove the excess of TritonX. The sections were incubated with a primary antibody against total (either syn211, Abcam or anti-SNCA, Acris, mouse, both 1:1000 and anti-PGP 9.5, rabbit 1:200) diluted in 1%BSA/PBS buffer overnight at 4°C. The slides were then washed thrice in PBS. Incubation with secondary antibodies (Cy™3 Donkey Anti-Mouse IgG 1:100 and Alexa Fluor® 488 Donkey Anti-Rabbit IgG 1:400) was done for 2 hours at RT. After this the slides were washed thrice in PBS. For nuclear stain sections were incubated with 1:2000 Hoechst (in PBS) for 2 min, after that the slides were washed 2-3 times in PBS, embedded in Vectashield media and allowed to dry before microscopy.

- To control for specific labeling additionally stainings with primary antibodies omitted were performed (applicable to 2.1.3-2.1.6 IHC stains and PLA).

2.1.7. DETECTION OF A-SYNUCLEIN OLIGOMERS USING PROXIMITY LIGATION ASSAY (PLA)

Reagents from Duolink® In Situ, Sigma-Aldrich

I. Conjugation: 4 µl of Conjugation Buffer was added to 40 µg of Syn211 (1mg/ml in PBS) and mixed (this was done in duplicate). Half of the antibody solution was transferred to the vial with Plus and the other half with Minus lyophilized oligonucleotide, mixed gently and incubated at RT overnight. After this the reaction was stopped by adding 2µl of Stop Reagent and incubating at RT for 30 min. 24 µl of storage solution was added and probes stored at +4°C until use.

II. PLA staining

1) In case of cryosections skin biopsy samples on glass slides were air dried at RT for 30 min. In case of paraffin embedded tissue samples were deparaffinized in xylene for 30 min and subsequently rehydrated in increasing ethanol concentrations (100%, 96%, 90%, 80%, 70%, 50%), then washed in PBS twice. HIER was done by heating slides submerged in citrate buffer pH6.0 for 30min in the microwave. Slides were then cooled down, washed in distilled water thrice and then in PBS.

2) Duolink® blocking solution or 1%BSA in PBS was added at 37°C for 1 h. Optionally a permeabilization agent (0.1-0.2% Tween 20 or 0.1-0.3% Triton X) was added to the blocking solution.

3) Conjugated antibodies (Plus and Minus PLA probes) diluted to a 1:100, 1:200, 1:400, 1:500 or 1:1000 concentration in the PLA probe diluent were added to the samples, slides were incubated in a humidity chamber at +4°C overnight.

4) Detection: after washing slides in Wash Buffer A for 2 x 5 min an appropriate amount of freshly made Ligation-Ligase solution (ligase taken from -20°C was added to the Ligation stock prediluted 1:5 in high purity water) was added to each sample. Slides were incubated in a pre-heated humidity chamber for 30 min at +37°C.

5) Amplification: Slides were washed in 1x Wash Buffer A for 2 x 2 min under gentle agitation. Amplification-Polymerase solution was freshly prepared by diluting Amplification stock 1:5 in high purity water and adding Polymerase (-20°C) at a 1:80 dilution. Samples were incubated with Amplification-Polymerase solution in a pre-heated humidity chamber for 90-120 min at +37°C.

6) Slides were subsequently washed in 1x Wash Buffer B for 2 x 10 min and 0.01x Wash Buffer B for 1 min.

7) Slides were coverslipped using Duolink In Situ Mounting Medium with DAPI and dried at RT in the dark. Slides were analyzed using the Ax10 Zeiss fluorescence microscope.

Bright field PLA was done in a similar manner with the following alterations:

- Peroxidase quenching was performed with ready-to-use hydrogen peroxide supplied with the kit for 5 min before blocking
- Detection was performed using HRP-labeled probes diluted 1:5 in water that were added to the samples for 60min at 37 RT followed by addition of substrate – a mix of reagents A (1:70), B (1:100), C (1:100) and D (1:50) - for 5-10 min at RT.

2.1.8. PARAFFIN-EMBEDDED TISSUE (PET) BLOT

III. Mounting of sections onto the membranes:

PVDF/nitrocellulose membranes were cut to the size of glass coverslips (20x50mm). PVDF membranes were pre-wetted in methanol. 5µm sections of paraffin embedded tissue were cut using a microtome and placed into a water bath heated to 40°C. Tissue sections were collected onto membranes in a similar fashion as with a glass slide. The PET blots were then placed onto a glass plate and labeled in a corner. Samples were dehydrated by placing the glass plate and membranes into a 60°C oven for 30 min/ 37°C incubator overnight

IV. Deparaffinization:

Membranes carrying samples were dipped into xylene for 5 minutes (x2) and air dried. After this the samples were rehydrated through subsequent incubation in 100%/95%/70% ethanol and air dried.

V. Proteinase K (PK) digestion:

Membranes with samples were incubated in 50 (as described by Neumann et al., 2002), 100 or 250 µg/ml (as described by Walter J. Schulz-Schaeffer et al., 2000) PK (Sigma) in TBS-T (10 mmol/L Tris-HCl, pH 7.8; 100 mmol/L NaCl; 0.1% TritonX100) or TBS-B (10 mM Tris–HCl, pH 7.8, 100 mM NaCl, 0.1% Brij 35) or a following lysis buffer (0.5% Nonidet P-40, 0.5% sodium deoxycholate, 100mM NaCl, 10mM EDTA, 10mM Tris-HCl pH 7.8) for 8, 15 or 18 hours at 37 or 55°C. Membranes were washed with TBS/T (x3). Optionally samples were additionally treated with 3 mol/L guanidine isothiocyanate (Sigma, G6639-25G) in 10 mmol/L Tris-HCl (pH 7.8) for 10 min and washed in TBST (x3)

VI. Staining:

Blocking was done with 1%BSA + 10% goat serum for 1 hour at RT. A primary antibody solution (syn211, Abcam, 1:100, LB509 1:1000, anti-p-α-syn,

Biolegend, 1:500) was applied and incubated at 4°C overnight. Membranes were washed in TBS (x3). To block the intrinsic peroxidase activity an incubation in 0.5% H_2O_2 in methanol was done for 20 min. Membranes were washed in TBS thrice. Biotinylated goat-anti-mouse (Vector) was preincubated 1:1 with human serum at 37°C for 1 hour and after that diluted 1:200 in 1%BSA/TBS. Samples were incubated in the secondary antibody solution for 45min at RT. Membranes were washed in TBS thrice. Freshly prepared streptavidin-biotin complex (Vectastain) was added to the membranes for 30min at RT. 3,3'-diaminobenzidine (DAB) solution was prepared according to manufacturer's instruction (Vectastain). After washing slides in TBS thrice, DAB solution was added for 10 minutes. After development with DAB, membranes were washed in distilled water. If PVDF was used the developed PET-blots were placed into a small petri dish containing a 3:2 mix of dimethyl sulfoxide (DMSO): ethanol for 5 min until they become transparent. PET blots were mounted onto a glass slide using the 3:2 DMSO:ethanol mix as a mounting solution.

2.1.9. MICROSCOPY

All sections were analyzed using a fluorescence microscope (Ax10, Zeiss, Oberkochen, Germany) with CARVII system, Visiview software (Visitron GmbH, Puchheim, Germany). All biopsies were carefully scanned for immunoreactive nerve fibers and were considered positive if at least one dermal nerve fiber was immunoreactive both for α-syn stain and the axonal marker PGP9.5. In case of a double stain with 5G4 and anti-p-α-syn, nerve fibers were identified only by morphology. In attempt to quantify the extent of α-syn deposits the number of distinct dermal structures supplied (arrector pili muscle, sweat gland, vessel) or containing (dermal nerve bundle) at least one nerve fiber positive for a chosen α-syn marker was counted. The number of single nerve fibers was not counted as thin sectioning through the biopsy or dissociation during sample preparation could make a single nerve fiber appear as multiple due to its winding course.

To assess colocalization in the double stains of 5G4 with p-α-syn in the midbrain, the average Mander's coefficient of five scans of the representative region of substantia nigra was calculated in ImageJ Colocalization Analysis plugin (Manders et al., 1993). To quantify the extent of lesions five representative scans of SN were analyzed using Object finder 3D (Bolte and Cordelières, 2006) in ImageJ. The scans were taken with the same magnification (10x) and exposure settings. After manually setting the threshold (that was kept the same across images for the same channel) so as to include the maximum number of

lesions while excluding the background the number of objects and their volume were automatically calculated by the plugin. The average “lesion burden” was calculated as volume times number of objects for each of the scans, then the average lesion burden for 5 scans was calculated.

2.1.10. STATISTICAL ANALYSIS

R Version 3.2.2 was used to perform the statistical tests. A quantile-quantile (Q-Q) plot was constructed to check whether the microscopy data on lesion burden among the five scans is normally distributed. Subsequently a two-sided Student t-test was used to determine statistical significance.

A Q-Q plot was used to check whether the data on average number of deposits per patient is normally distributed. Subsequently to compare the average number of deposits per patient between stage I and II H&Y two sided unpaired Wilcoxon test was applied.

3. RESULTS

ESTABLISHING THE STAINS: WHAT IT LOOKS LIKE IN THE BRAIN.

Stains used in this study were optimized using cryosections and paraffin embedded sections (in case of PET blot) of the midbrain (including SN) from a normal control and a patient with neuropathologically confirmed Parkinson's disease in Braak stage VI.

3.1.1. PK DIGESTION IN IHC

Using sections of frozen brain tissue from a patient and a normal control the optimal conditions (incubation time of 20 min at 37°C with 10 µg/ml PK) that led to complete disappearing of α-syn stain in the normal brain while enhancing the staining of Lewy bodies and neurites in the diseased brain and preserving the tissue morphology were determined (see Fig. 9). Higher concentration of PK or longer incubation times led to dramatic damaging of tissue morphology and detachment of sections from the slides.

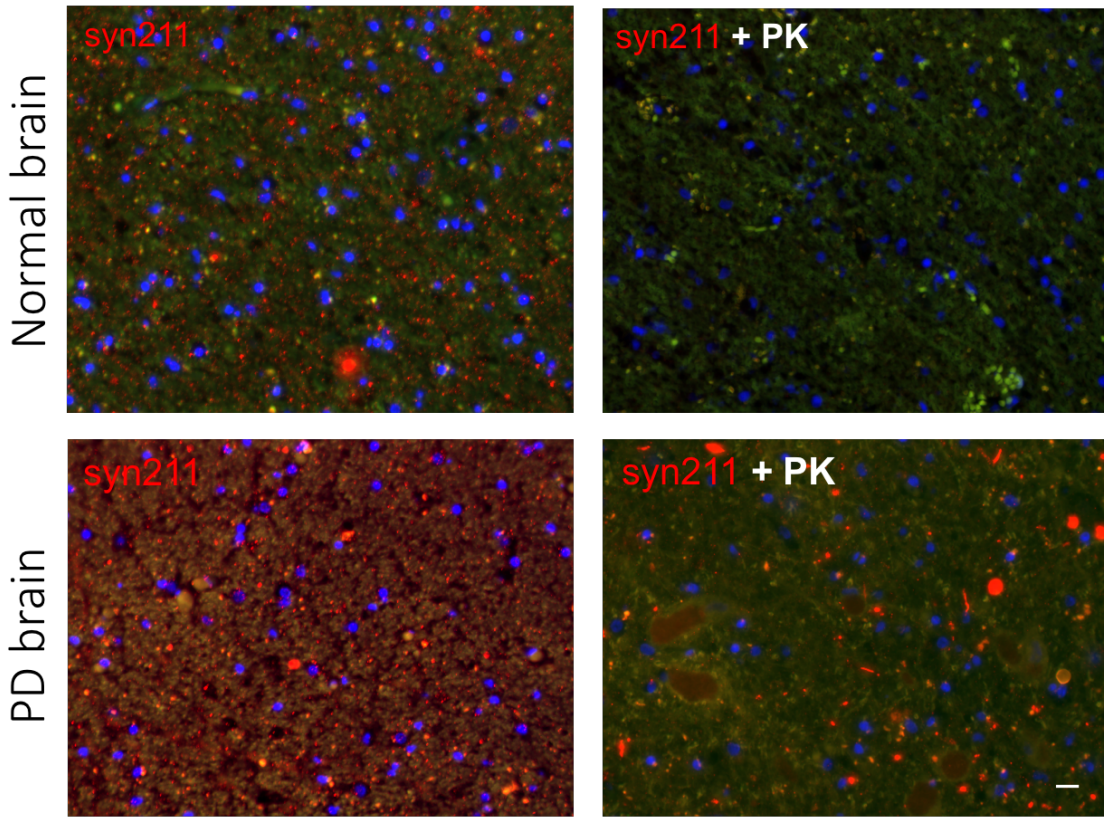


Figure 9 The stain with syn211 (red) after PK digestion of a midbrain section of a patient with neuropathologically confirmed PD and a normal control. Pretreatment with 10 µg/ml PK for 20 min eliminates α-syn stain in the normal control, while in the PD brain it increases the stain intensity of the characteristic Lewy bodies and neurites, scale bar = 10µm.

3.1.2. ASYO5 (ANTI-OLIGOMERIC ALPHA-SYNUCLEIN)

The anti-oligomeric α-syn antibody ASyO5 diluted 1:100 resulted in a diffuse stain in the normal control and revealed characteristic lesions in Parkinson's disease (Fig. 10A). A higher dilution (1:10,000) resulted in no stain in the normal control while the Lewy pathology in the PD brain was still visible, albeit the staining was weaker (Fig. 10B). To check whether ASyO5 is able to distinguish between oligomeric and aggregated forms the brain tissue was subjected to digestion with 10 µg/ml (Fig. 10C) or 50µg/ml (Fig. 10D) PK for 20 min. Some staining remained even after digesting with 50µg/ml PK for 20 min (Fig. 10D) suggesting that the protein being stained is aggregated. Of note, the faded Lewy body and neurites revealed with ASyO5 stain became even more distinguished after antigen retrieval with PK (Fig. 10C, D).

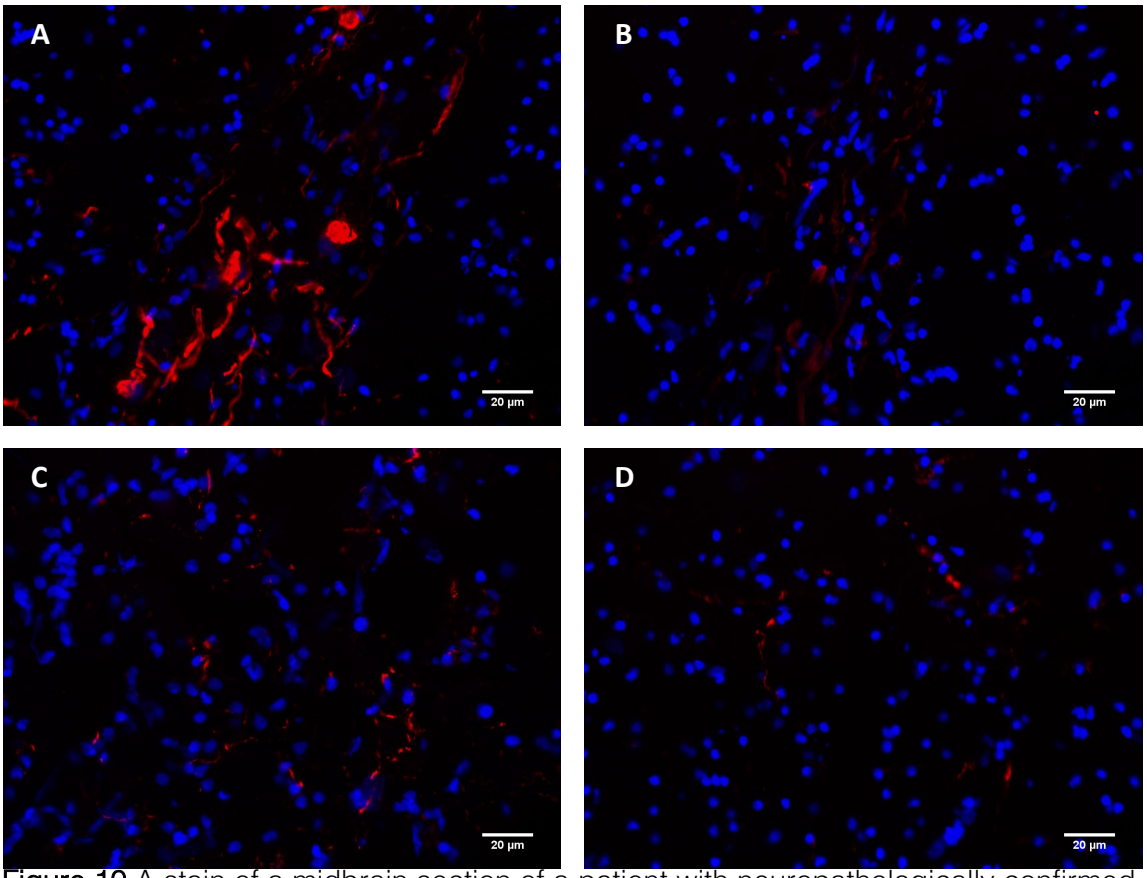


Figure 10 A stain of a midbrain section of a patient with neuropathologically confirmed PD with ASyO5 at 1:100 (A) and 1:10,000 (B) dilution. The stain with ASyO5 (1:10,000) persists after 20 min digestion with 10µg/ml (C) and 50 µg/ml (D) PK, scale bar = 20um.

3.1.3. 5G4 (ANTI-AGGREGATED ALPHA-SYNUCLEIN)

The 5G4 antibody required antigen retrieval with 70-80% formic acid. Staining of the midbrain from a PD patient revealed extensive Lewy pathology in the form of cytoplasmatic inclusions and neurites (Fig. 11). Staining of control brain showed no signal.

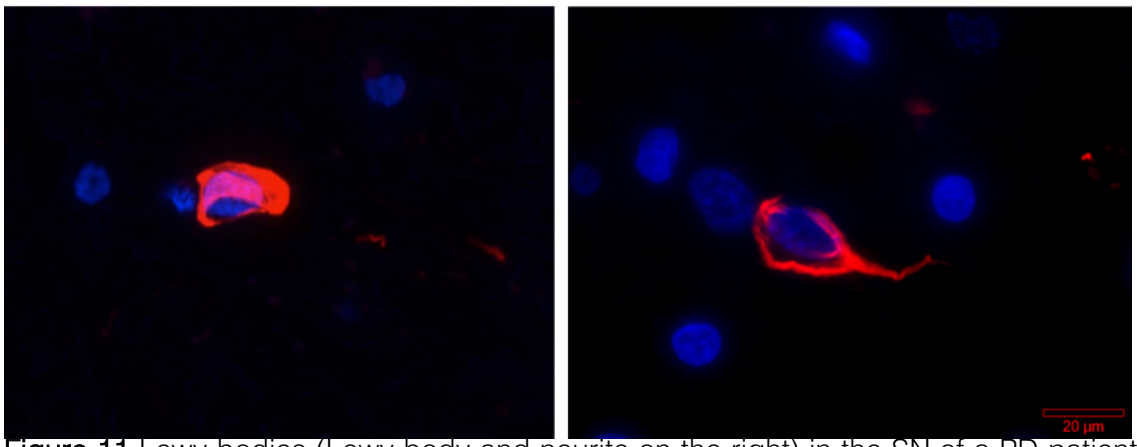


Figure 11 Lewy bodies (Lewy body and neurite on the right) in the SN of a PD patient stained with 5G4.

3.1.4. A DOUBLE STAIN OF P-SYN AND AGGREGATED SYN (5G4)

A double stain with an anti-p-α-syn (rabbit, Cell signaling) and 5G4 (mouse, Millipore) revealed Lewy bodies and Lewy neurites that were mainly positive for both markers (Fig. 12, arrows); some of the neurites were exclusively positive for p-α-syn (Fig. 12, arrowheads), few small lesions positive only for 5G4 (Fig. 12, marked *).

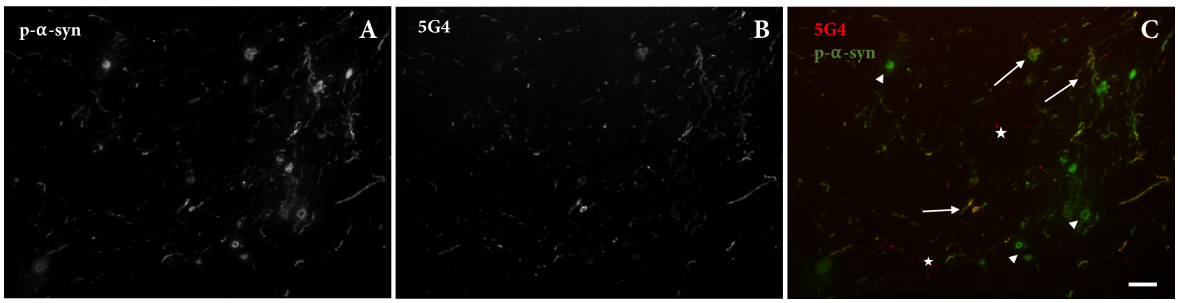


Figure 12 Double-immunofluorescence stain of a midbrain section of a PD patient showing colocalization of p-α-syn (green, A) and 5G4 (red, B) in the Lewy bodies and neurites. Many lesions colocalize completely (arrows), some are positive only for p-α-syn (arrowheads), and very few small lesions are only positive for 5G4 (*). Scale bar=20 µm

Image analysis showed a high degree of colocalization with Mander's coefficient=0,89 (average for five representative regions within SN),

colocalization can be best visually appreciated in Fig.14. To quantify the amount of p-α-syn+ and 5G4+ lesions five representative scans of SN were analyzed using Object finder 3D (Bolte and Cordelières, 2006), ImageJ. The median lesion burden for 5G4 scans was 60% of that in anti-p-α-syn scans (647.5 vs 1010.5), n=5, Fig. 13B. As the data was normally distributed (following a straight line on a q-q plot, Fig.13A), a paired two-sided Student t-test was applied, p=0.0002101.

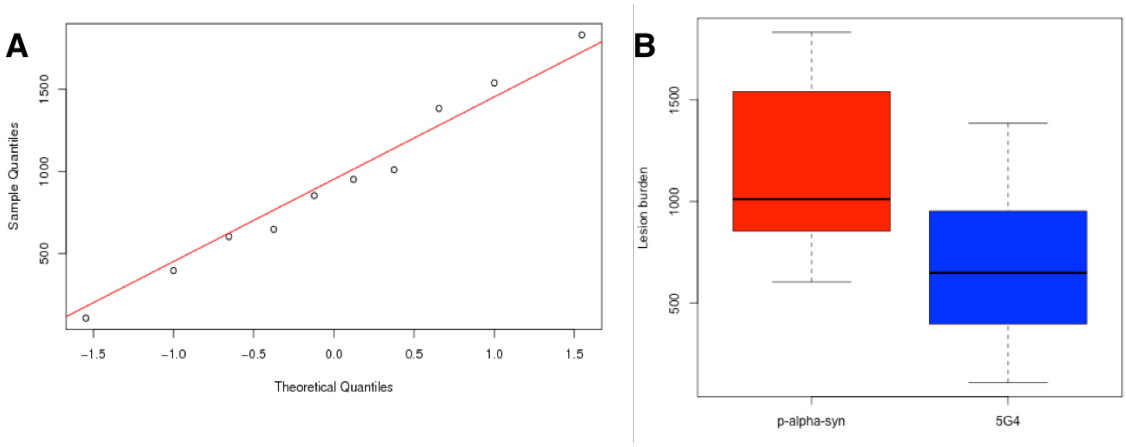


Figure 13 A q-q plot of calculated "lesion burden" for each of the five scans for both stains, the data is likely normally distributed as the data points are following a straight line (A). A box plot showing lesion burden for p-α-syn and 5G4 over 5 representative scans of SN. Median lesion burden for 5G4=647.5 and for p-α-syn=1010.5 (B).

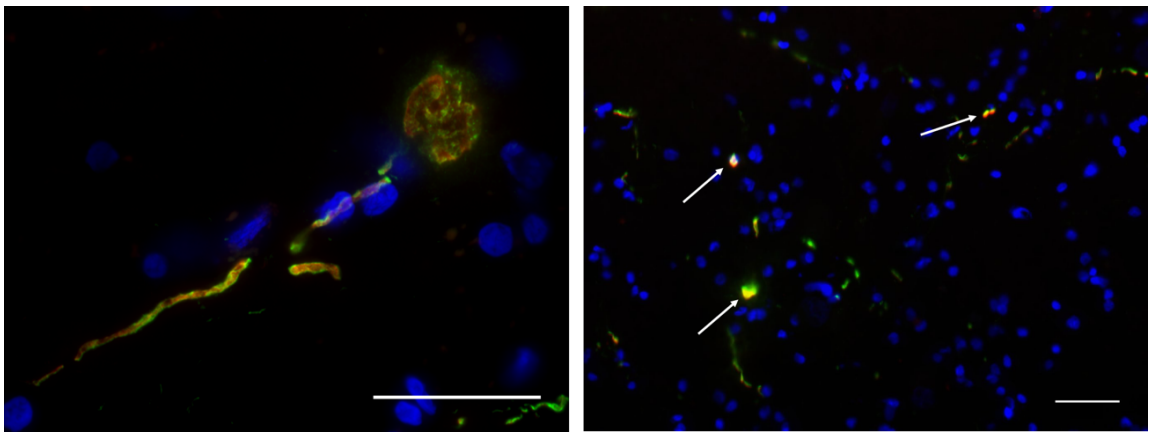


Figure 14 Double immunofluorescence stain for phosphorylated (anti-p-α-syn , green) and aggregated (5G4, red) α-syn. The majority of Lewy-bodies and –neurites are positive for both (arrow, yellow overlay), also neurites positive solely for p-α-syn are visible (scale bar = 50µm).

3.1.5. TECHNICAL CONTROLS

When omitting either of the primary antibodies used (anti-p-α-syn, anti-total-α-syn, ASyO5, 5G4 or PGP9.5) no staining was detected either in the brain or skin of normal controls or diseased samples.

3.1.6. PROXIMITY LIGATION ASSAY (PLA)

PLA was reproduced based on the publication by Roberts et al. to stain for oligomeric α-syn in the brain and, for the first time, in the skin samples (Roberts et al., 2015). In order to be able to use a double stain with an axonal marker the proximity ligation assay (PLA) was first attempted using fluorescence detection. However, the extensive accumulation of lipofuscin (pigment normally accumulating in the brain with aging (Goyal, 1982)) interfered significantly with the analysis. Lipofuscin has an autofluorescence maximum in the range of 540 nm to 570 nm (Mochizuki et al., 1995) making it impossible to distinguish from the signals emitted by the chosen PLA fluorophore (λem 576 nm). The detection system was changed to horseradish peroxidase (HRP) labeled probes that can be evaluated in bright field.

PLA performed on paraffin-embedded brain samples revealed an extensive diffuse labeling of the midbrain that was even more extensive in the normal control than in the patient with Parkinson's disease (see Fig. 15). Changes in protocol (primary antibody concentration, permeabilization, in situ reaction times) failed to make any difference.

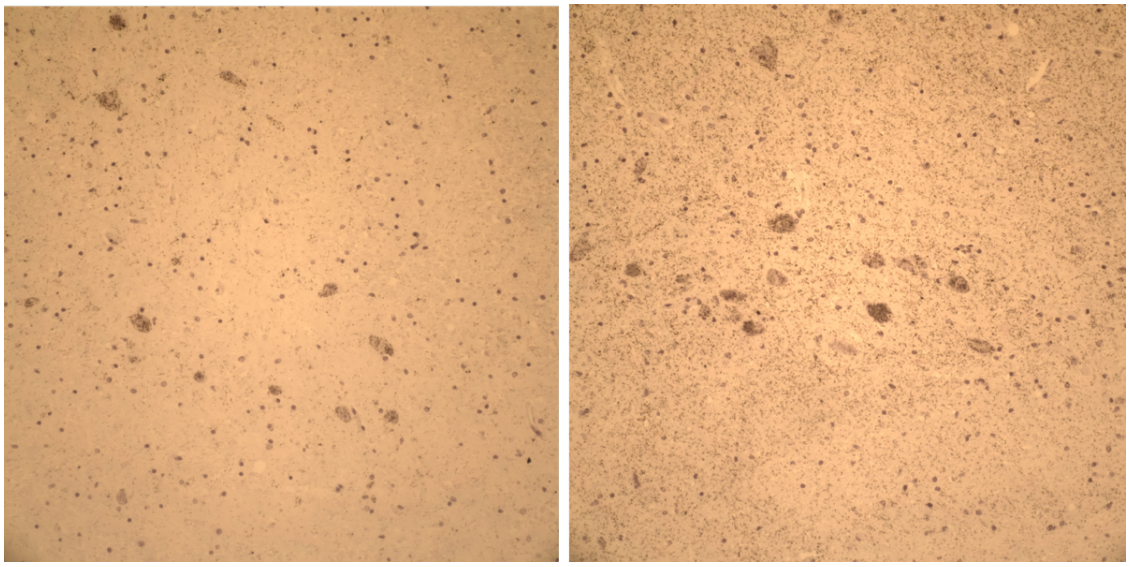


Figure 15 Bright field PLA of a PD (on the left) and normal (on the right) midbrain. Unlike expected there are more lesions in the sample from a normal control.

PLA stain of paraffin embedded and cryopreserved skin samples revealed an occasional signal in the dermis, in particular surrounding vessels and sweat glands in a plausible location for autonomic nerve fibers (Fig. 16). However, these stains could be observed in some PD patients as well as in normal controls.

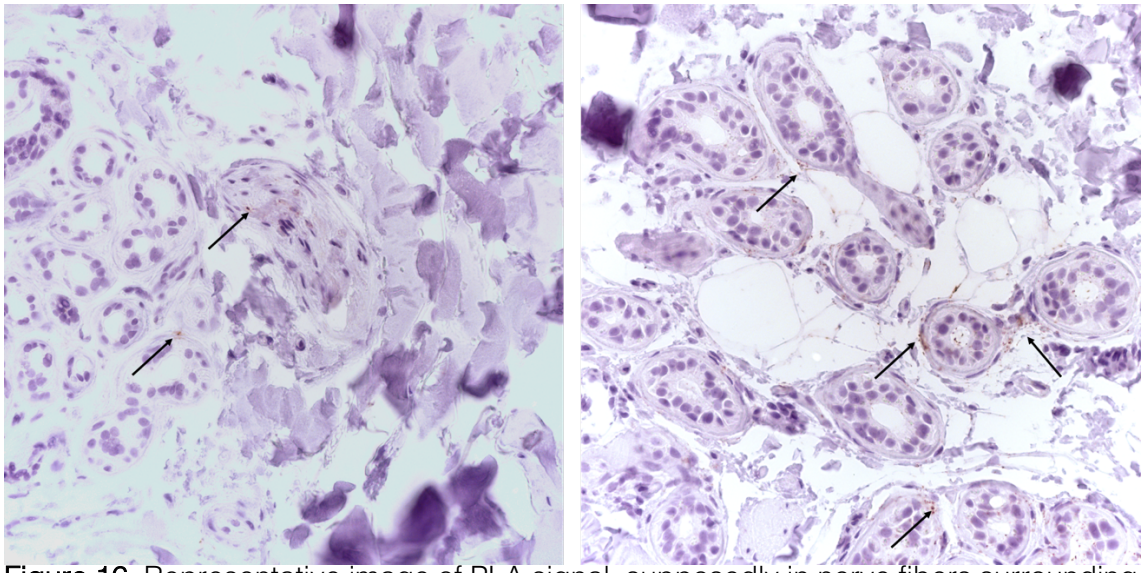


Figure 16. Representative image of PLA signal, supposedly in nerve fibers surrounding sweat glands (right image, lower arrow left image) as well as presumably a vessel (upper arrow in the left image).

PLA also resulted in a diffuse intracellular stain in some of the cells of the basal epidermis layer, most likely melanocytes (Fig. 17).

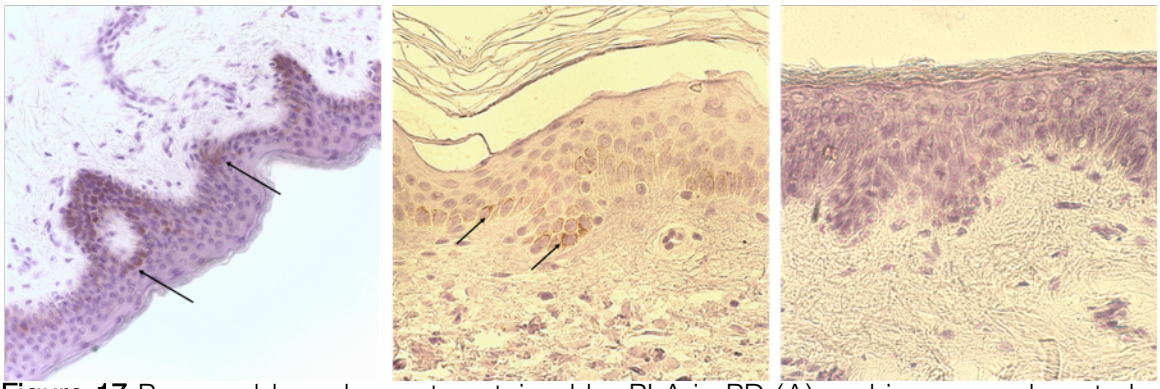


Figure 17 Presumably melanocytes stained by PLA in PD (A) and in a normal control (B). Negative control with no primary antibody is shown in (C).

As no apparent difference in the PLA staining pattern of either the midbrain or the skin was seen between patients and normal controls, this method was not systematically tested in a larger cohort.

3.1.7. PET BLOT

Despite different protocol optimizations, in particular adjusting incubation time, PK concentration, antibody or detergent, no specific stain could be achieved in the PD brain tissue. Additional treatment with guanidine thiocyanate also had no difference on the stain result.

Example of one such stain is shown in Fig. 18. As the substantia nigra is showing virtually no α-syn, the signal in other midrain regions is probably representing non-specific staining due to an incomplete blocking of internal peroxidases.

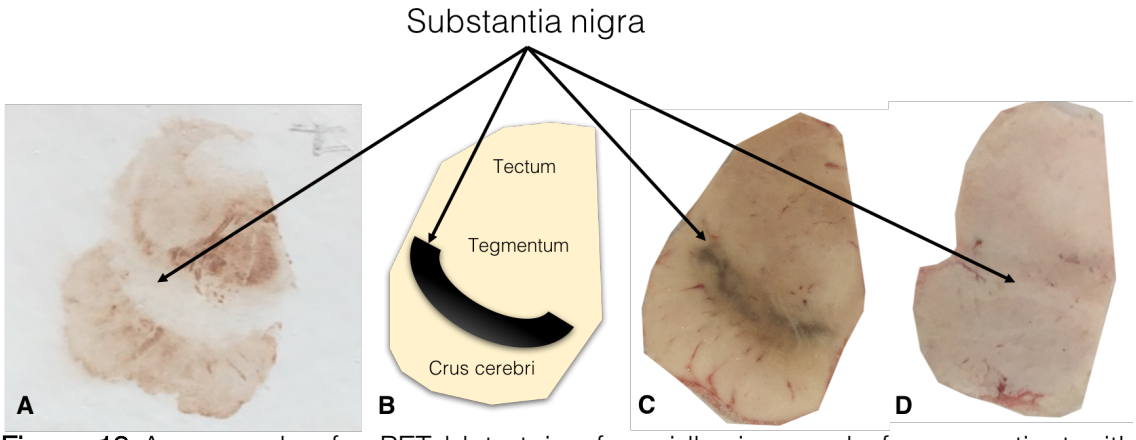


Figure 18 An example of a PET blot stain of a midbrain sample from a patient with neuropathologically diagnosed PD in Braak stage VI (A). A schematic representation of SN within the midbrain (B). Midbrain specimen from a control (C) and a PD patient (D) easily distinguishable by loss of SN pigmentation in PD.

THE STAIN FOR PHOSPHORYLATED AND AGGREGATED FORMS OF A-SYNUCLEIN IN SKIN BIOPSY SAMPLES OBTAINED FROM PD PATIENTS AND NORMAL CONTROLS.

3.1.8. COHORT 1 (OPTIMIZATION, PROOF OF PRINCIPLE)

Stains established using the brain samples were then performed on a set of skin biopsy samples from patients and normal controls. 26 out of 46 patients were found to be positive for at least one stain. Number and location of biopsy sites per patients in this cohort differed as predominantly those biopsies that showed the maximum amount of p-α-syn in previous studies were taken. If expressed in the percentage of all sections stained, 69% of sections showed p-α-syn accumulation, 62% showed positive α-syn signal after digestion with PK, 39% were positive for 5G4 and 28% for ASyO5 stains.

Among biopsies from the p-α-syn positive patients 75% were also positive in the syn211 stain after PK digestion, 91% for ASyO5 and all stained positive for 5G4, the overlap between the stains is schematically shown in Fig. 19. Detailed results are summarized in the Table 5.

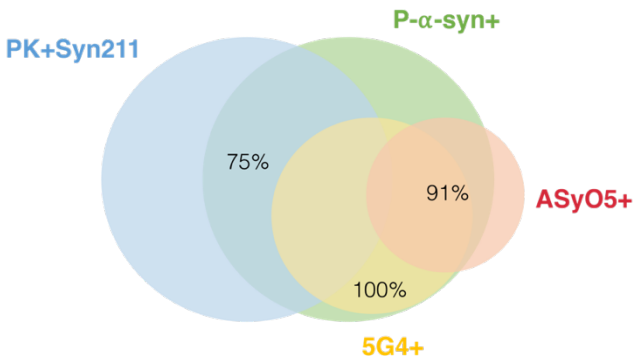


Figure 19 Overlap between biopsies in the cohort #1 positive in different stains expressed as percentage of p-α-syn biopsies positive for other markers.

Only deposits within the dermal nerve fibers identified through colocalization with PGP 9.5 were considered as a positive stain (e.g. see Fig. 20).

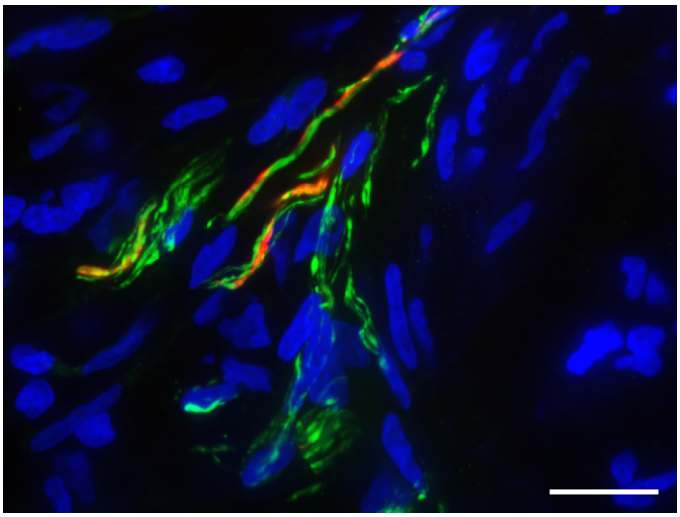


Figure 20 Dermal nerve fibers supplying a blood vessel (identified by anti-PGP 9.5 stain, green), some of which stained positive for aggregated α-syn as detected by 5G4 stain (red). Colocalization can be seen in yellow. Scale bar = 10μm.

The majority of the positive fibers were found near blood vessels (66.7% of ASyO5-positive structures, 47.2% of anti-syn211 after PK, 55% of 5G4 and 48.7% of anti-p-α-syn), followed by dermal nerve bundles (respectively 25%, 33.4%, 25% and 33.3%), fibers supplying sweat glands (8.3%, 8.3%, 10%, 7.7%) and arrector pili muscles (0%, 11.1%, 10%, 10.3%), see Fig. 21. Structures were identified only by their morphology. There was virtually no difference in the distribution of deposits between the stains.

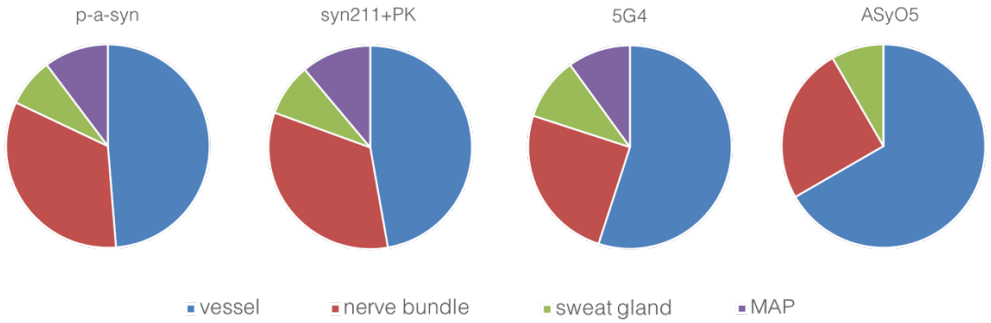


Figure 21 Distribution of p-α-syn, PK resistant, 5G4- and ASyO5-positive deposits in regards to dermal structures.

By analyzing consecutive sections, immunoreactive fibers could be identified in the same location within each section, suggesting colocalization. An

example of a nerve bundle with one of the nerves being positive for p-α-syn (Fig. 22A), PK resistant (Fig. 22B), oligomeric (ASyO5, Fig. 22C) and aggregated (5G4, Fig. 22D) α-syn.

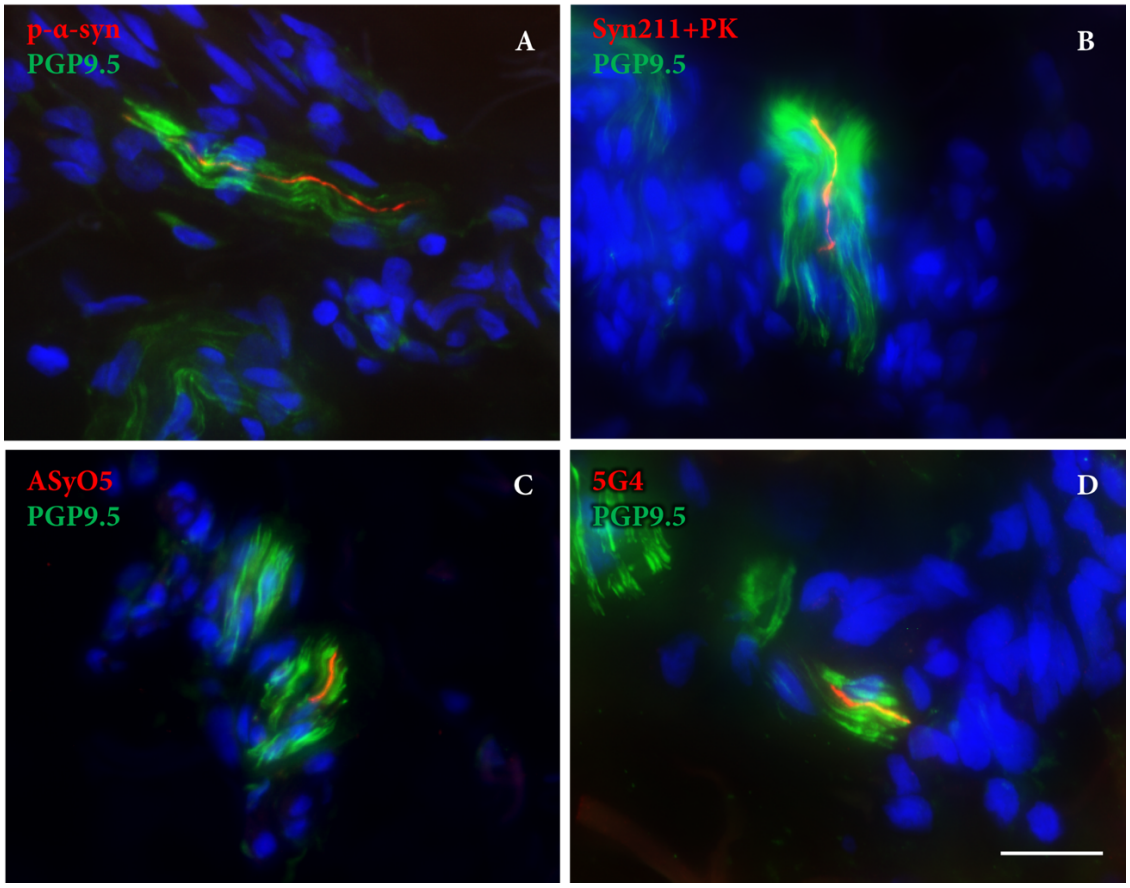


Figure 22. Double-immunofluorescence of a skin biopsy of a PD patient with anti-p-α-syn (red) and anti-PGP 9.5 (green) (A), syn211 (red) and anti-PGP 9.5 (green) after 10 min PK digestion (B), AsyO5 (red) and anti-PGP 9.5 (green) (C), and 5G4 (red) and anti-PGP 9.5 (green) (D). All images show the same dermal nerve bundle with one nerve fiber immunoreactive for p-α-syn, syn211 after PK digestion, ASyO5 and 5G4 in the subsequent sections of the biopsy, indicating colocalization of these markers. Scale bar=20 µm.

Another common location for Lewy-like pathology in the skin are blood vessels. In Fig. 23 a single nerve fiber supplying the same vessel stained positive for phosphorylated, aggregated α-syn (5G4, syn211 after PK digestion) and oligomeric α-syn (ASyO5).

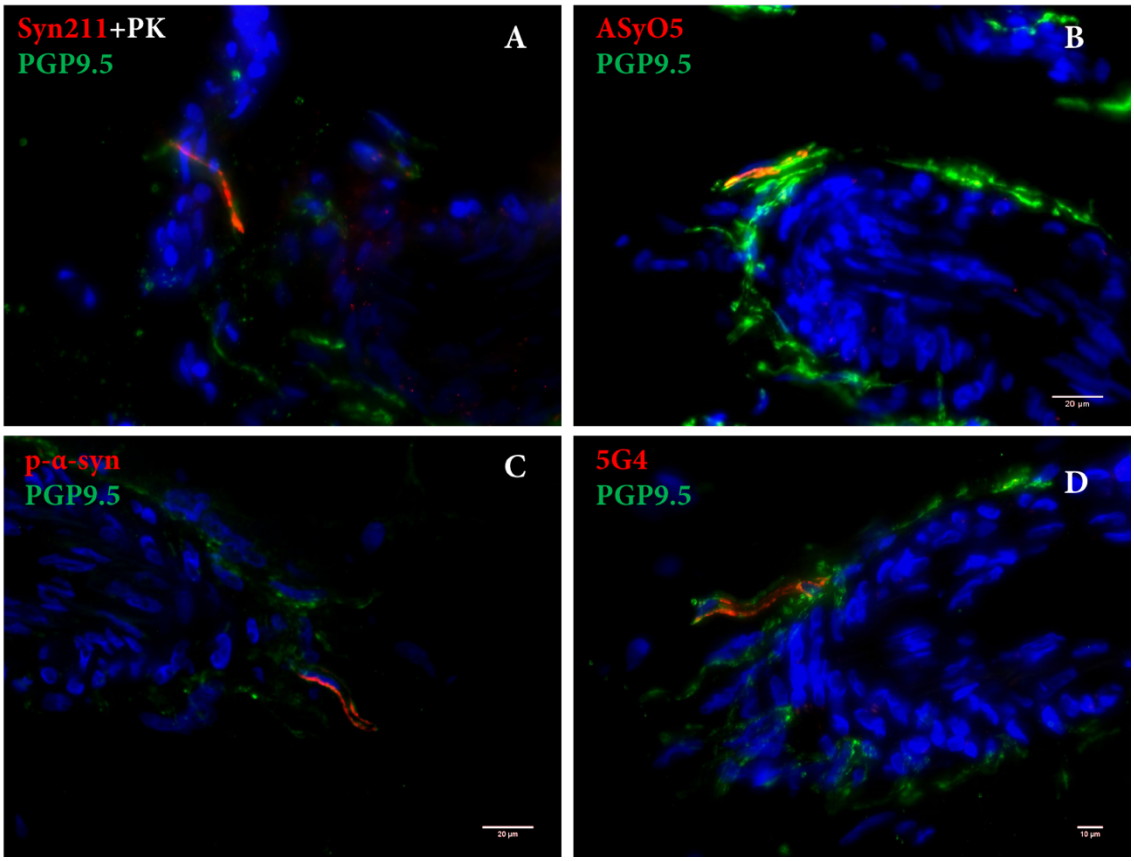


Figure 23. Double-immunofluorescence of a skin biopsy of a PD patient with syn211 (red) and anti-PGP 9.5 (green) after PK digestion (A), AsyO5 (red) and anti-PGP9.5 (green) (B), anti-p-α-syn (red) and anti-PGP 9.5 (green) (C), and 5G4 (red) and anti-PGP 9.5 (green) (D). All images show the same vessel (identified by nuclear stain with Hoechst) in subsequent sections of the biopsy. Nerve fibers that are immunoreactive for p-α-syn, syn211 after PK digestion, AsyO5 and 5G4 are found in all subsequent sections, indicating colocalization of these markers. PK digestion in (A) was done for 20 min, accordingly PGP 9.5 signal is comparatively weak. Scale bar=20 µm.

None of the stains in the normal controls were positive (a minimum of two sections from lower leg and paravertebrally at Th10 were stained against phosphorylated α-syn, aggregated α-syn (5G4), oligomeric α-syn (ASyO5) and total-α-syn after PK digestion). However, the ASyO5 stain showed a weak diffuse signal in many of the nerves in the normal controls as well as in PD (Fig. 24). This weak stain was not considered positive and is explained by staining of α-syn monomers as dilution dependent binding of the ASyO5 to the higher-molecular weight protein is preferential but not exclusive (Bränström et al., 2014a).

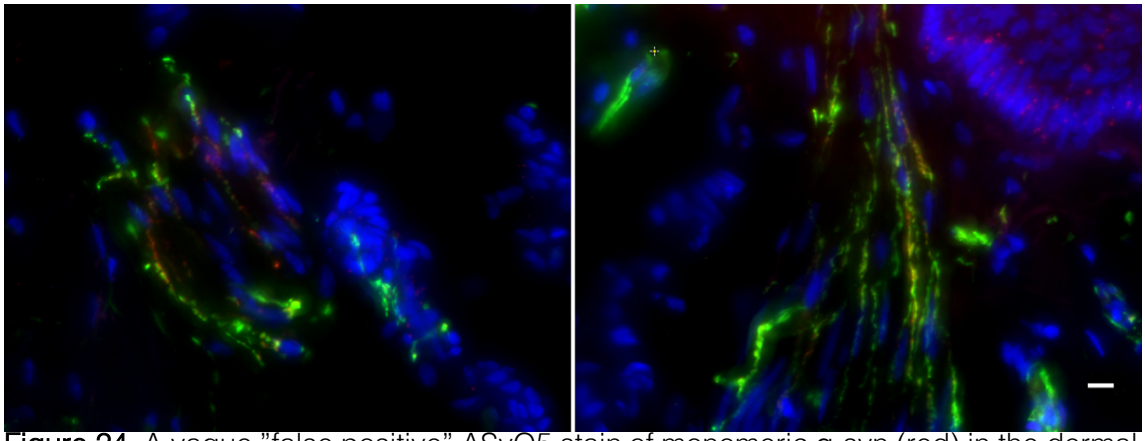


Figure 24. A vague "false positive" ASyO5 stain of monomeric α-syn (red) in the dermal nerve fibers (identified by PGP 9.5, green) of a normal control, scale=10μm.

It is worth mentioning that α-syn expression in the skin is not limited to the nerve fibers, e.g. melanocytes stain positive for α-syn (Fig. 25, also see https://www.proteinatlas.org), where it probably plays a role in melanin synthesis (Matsuo and Kamitani, 2010; Pan et al., 2012). In order to exclude false-positive results, only α-syn pathology within the nerve fibers, i.e. in case of colocalization with PGP 9.5, was considered a positive signal.

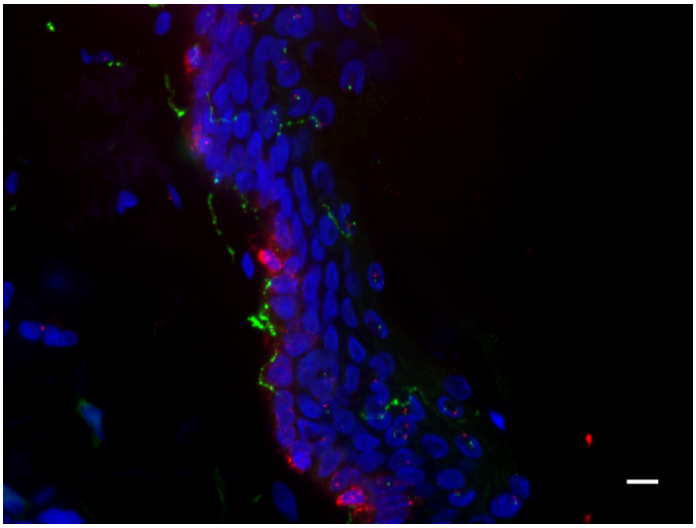


Figure 25 Melanocytes stain positive for α-syn (syn211, red), some intraepidermal nerve fibers are visible (PGP 9.5, green), anti-α-syn and anti-PGP 9.5 do not colocalize. Scale bar = 20μm.

Notably, within the studied cohort there were 3 patients with MSA, all of which showed positive results in at least one of the stains: 1 patient positive for anti-p-α-syn, 5G4 and syn211 after PK digestion, 1 only for syn211 after PK digestion, and 1 for anti-p-α-syn, 5G4 and AsyO5.

The mean H&Y score among positive patients (for any of the stains) was higher than in the patients whose skin samples stained negative in all the stains 3.29 (+/-1.0) vs 2.7 (+/-1), statistical significance was not determined as choice of patients was biased and number of stained sections was uneven.

Table 5: Positive patients in cohort #1 (including 3 MSA patients, marked *)

#	age	sex	H&J	UPDRS3	Biopsy localization	ASyO5	syn211 + PK	5G4	p-α-syn	sum
1*	71	m	4	37	back	0	1	1	1	3
2	79	m	3	41	back	0	0	0	1	1
3	55	m	3	48	prox. thigh	1	1	1	1	4
					back	0	0	0	0	0
4	78	m	2	10	back	0	0	0	1	1
5	68	m	1	14	dist. leg	0	1	1	1	3
					back	1	0	0	0	1
6	71	m	2,5	43	back	0	1	0	1	2
7	65	m	5	92	back	0	1	0	1	2
8	66	f	4	24	dist. leg	0	0	0	1	1
					back	0	0	0	1	1
9	71	f	3	54	back	0	1	0	0	1
10	73	f	4,5	70	dist. leg	1	1	1	1	4
					back	1	1	1	1	4
11	59	f	2	36	prox. thigh	0	1	1	1	3
12	70	f	3	14	dist. leg	0	1	0	0	1
					prox. thigh	0	0	0	0	0
13	80	m	3	12	prox. thigh	0	1	0	0	1
14	80	m	3,5	na	dist. leg	1	1	0	1	3
					prox. thigh	0	1	1	1	3
					back	0	1	0	0	1
15	78	m	2,5	n/a	prox. thigh	0	1	1	1	3
16	70	m	3,5	84	dist. leg	1	1	1	1	4
					back	0	0	0	0	0
17*	74	m	5	44	dist. leg	0	1	0	0	1
18	53	f	3	45	dist. leg	0	0	0	1	1
					prox. thigh	1	1	0	1	3
					back	0	1	1	1	3
19	59	m	4	84	prox. thigh	0	1	0	0	1
20*	65	f	4	n/a	back	1	0	1	1	3
21	66	m	5	23	dist. leg	0	1	0	0	1
					prox. thigh	0	0	0	0	0
					back	0	0	1	1	2
22	54	m	2,5	78	back	1	1	0	1	3
23	56	m	3	71	dist. leg	1	0	1	1	3

					prox. thigh	0	1	0	1	2
24	71	m	2	66	back	0	0	1	1	2
25	46	m	3	65	prox. thigh	1	1	1	1	4
26	65	m	4,5	23	back	0	0	0	1	1

3.1.9. COHORT 2: SERIAL SECTIONS (VALIDATION)

In this cohort, five sections for each of the four biopsy sites (distal leg, upper thigh, Th10 and C7 paravertebrally) from 27 patients and five normal controls were stained with syn211 after PK digestion, 5G4, ASyO5 and in parallel with anti-p-α-syn. In total 540 sections from PD patients were studied in each stain.

Deposits of p-α-syn were found in 48% (13 out of 27) of patients, which is significantly lower than the 80% positivity rate for p-α-syn in the same cohort performed using exactly same protocol several months earlier by cand. med. Lena Schulmeyer (most likely explanation being loss of antigenicity during storage, see Discussion). In total 47 p-α-syn deposits across all biopsies were found.

Aggregated α-syn identified by PK resistance was detected in 13 of 27 patients (48%), in 5 of these, positive fibers were found in the biopsies from the distal leg, in 6 from the proximal leg, in 4 from Th10 and in 6 from C7. In total 58 PK resistant α-syn deposits were found.

In the stain with 5G4, aggregates were found in 10 of 27 patients (37%) or 13 biopsy samples (2 cases in distal leg, 4 in proximal leg, 3 in Th10 and 4 in C7). In total 22 5G4-positive deposits were found.

ASyO5-positive fibers, supposedly representing oligomeric α-syn, were found in 9 patients (33%), among them 6 positive biopsies originated from the distal leg, 3 from the proximal leg, 2 paravertebrally from Th10 and C7 each. In total 21 ASyO5+ deposits were found. Detailed distribution of deposits can be seen in Table 8.

Within the biopsies that were found to have p-α-syn deposits (n=22) 59% were also positive for PK resistant α-syn (n=13), 41% for 5G4 (n=9) and 45% for the ASyO5 (n=10) stain. Most of the patients were positive for >2 stains, 4/18 patients were positive for all 4 markers (Fig. 26B), most positive patients were found in anti-p-α-syn and syn211 after PK digestion stains (Fig. 26A). An example of a single nerve fiber positive for all 4 markers can be seen in Fig. 27. Detailed results for each patient can be seen in Table 7 and 8.

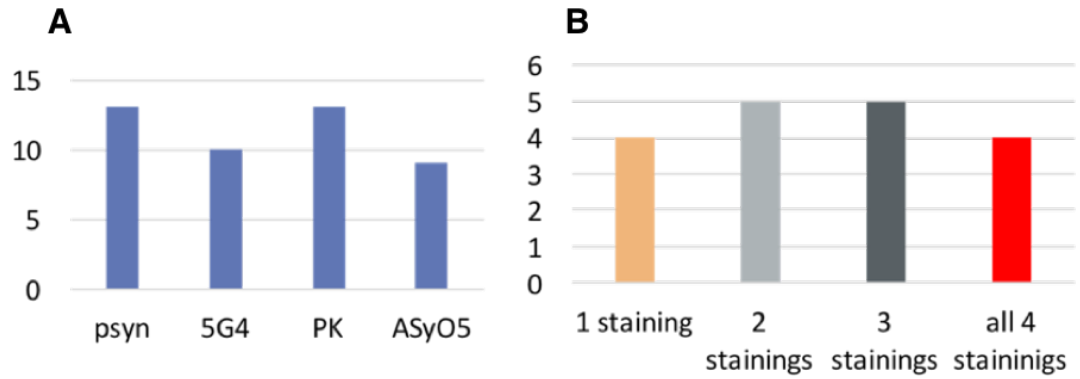


Figure 26 The number of positive patients (y axis) in anti-p-α-syn, 5G4, syn211 after PK digestion and ASyO5 stains (A) and the number of patients (y axis) positive for any 1, 2, 3 or all 4 stains (B)

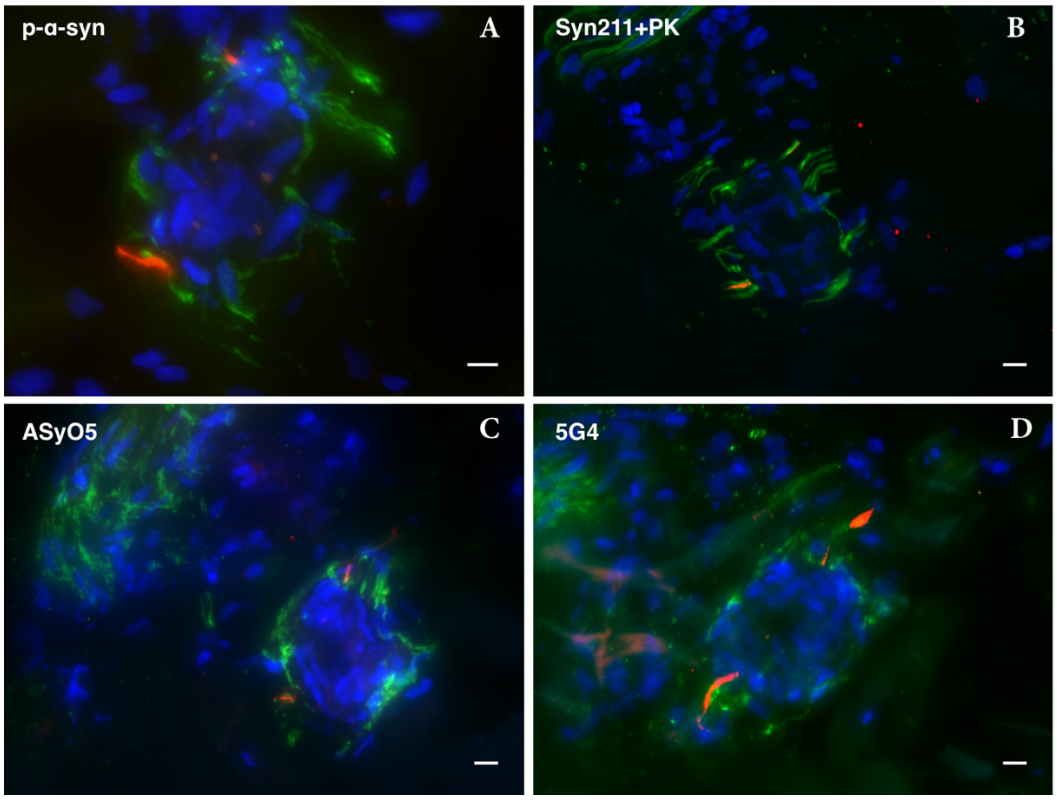


Figure 27 Double-immunofluorescence of a skin biopsy of a PD patient with anti-p-α-syn (red) and anti-PGP 9.5 (green) (A), syn211 (red) and anti-PGP 9.5 (green) after PK digestion (B), AsyO5 (red) and anti-PGP9.5 (green) (C) and 5G4 (red) and anti-PGP 9.5 (green) (D). All images show the same vessel (identified by nuclear stain with Hoechst) in subsequent sections of the biopsy. Nerve fibers that are immunoreactive for p-α-syn, syn211 after PK digestions, AsyO5, and 5G4 are found in all subsequent sections, indicating colocalization of these markers. PK digestion in (A) was done for 10 min, accordingly PGP 9.5 signal is still visible. Scale bar=20 µm.

Most pathological protein deposits were found within the nerve fibers surrounding blood vessels followed by isolated dermal nerve bundles, see Fig. 28 (structures were identified only by morphology). There was no significant difference in the distribution of positive structures between the stains.

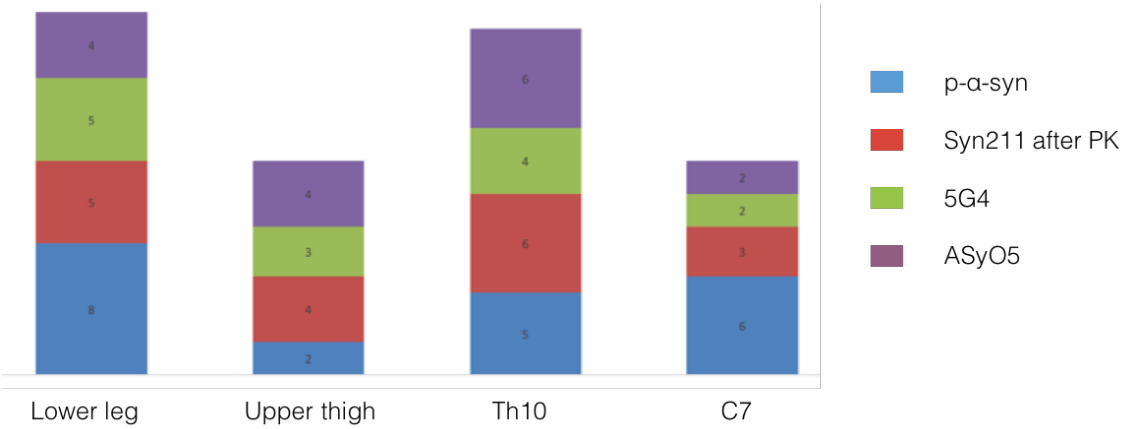
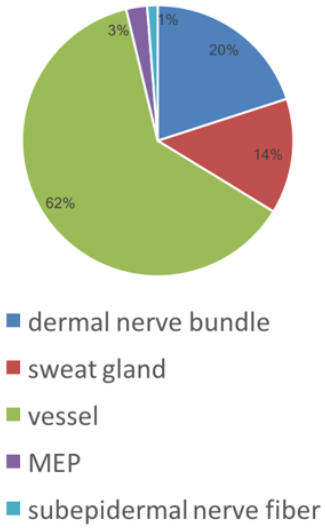


Figure 28 Distribution of pathological α-syn deposits (for all 4 stains) among different dermal structures.

The majority of positive sites were found in the lower leg, followed by Th10 (see Fig. 29), so there was no proximal gradient in the distribution of pathological deposits.

Figure 29 Distribution of positive samples among 4 anatomical biopsy sites and for different stains: anti-p-α-syn – blue, syn211 after PK digestion – red, 5G4 – green, ASyO5 – violet.

A double stain with anti-p-α-syn and 5G4 of 18 biopsies from 18 patients revealed 21 structures only positive for p-α-syn and 8 structures positive both for p-α-syn and 5G4 (no structures solely positive for 5G4 were found). In this staining, 39% of patients were positive for p-α-syn and 17% for p-α-syn and 5G4. An example of colocalization of 5G4 with p-α-syn is shown in Fig. 30.



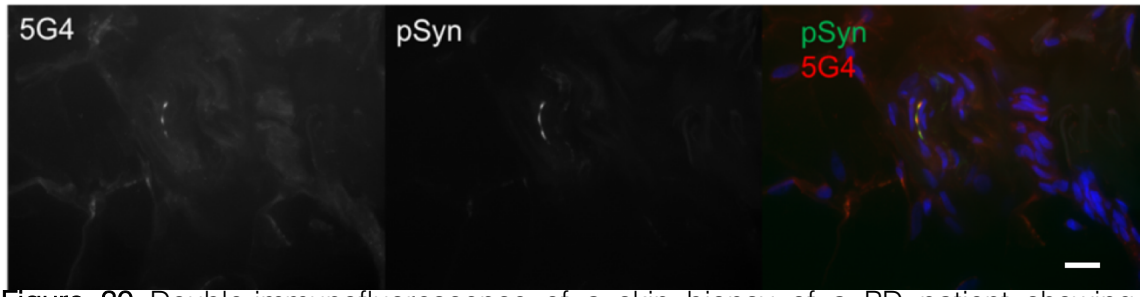


Figure 30 Double-immunofluorescence of a skin biopsy of a PD patient showing colocalization (right image) of 5G4 (red, left image) and p-α-syn (green, middle image) in a dermal nerve fiber. Scale bar=20 μm.

There was a trend for increased number of deposits in H&Y stage II (7.1 deposits per patient on average) compared to H&Y stage I (3.8 deposits per patient on average, see Table 6), that however did not reach statistical significance (the data was not normally distributed, see Fig. 31, so a two-sided unpaired Wilcoxon analysis was performed, $p>0.05$).

Table 6

	H&Y I	H&Y II
Average # pos. structures	3.8	7.1
Median # pos. structures	2.0	4.0
Standard deviation	4.5	8.6

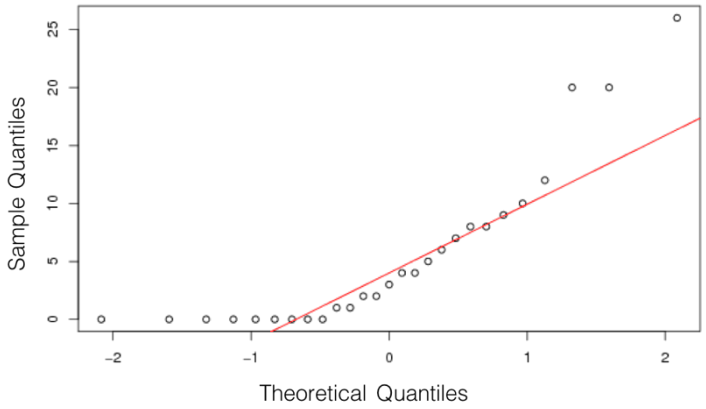


Figure 31 A Q–Q plot of the number of deposits found across the patient cohort #2, stages H&Y I and II. The data does not follow a normal distribution.

Overall, pathologic dermal α-syn deposits were not so commonly encountered in the stained sections. Among 2160 sections that were stained with all 4 studied markers in this study only 118 positive sections (with at least one positive nerve fiber) were found, that means that on average the chance of seeing a positive signal in any single analyzed section is only 5.5%. This explains why the use of serial sections and multiple biopsy sites is required to increase the sensitivity.

Table 7 Summary of staining results in cohort #2

Pat.#	H&Y	age/sex	p-α-syn	5G4	total α-syn after PK-digestion	ASyO5	# of positive stains
1	1	63,f	0	0	0	0	0
2	1	69,m	0	0	0	0	0
3	2	70,f	0	1	1	0	2
4	1	62,f	1	0	1	0	2
5	2	55,m	0	0	0	0	0
6	2	65,f	1	1	1	1	4
7	1	62,m	1	1	1	0	3
8	2	71,f	1	1	0	1	3
9	2	50,f	1	0	1	1	3
10	1	44,m	0	0	0	0	0
11	2	67,m	0	0	0	0	0
12	2	67,f	0	0	0	0	0
13	2	50,m	0	0	1	1	2
14	1	61,f	1	1	1	1	4
15	1	73,m	1	0	1	0	2
16	1	73,m	1	1	1	1	4
17	1	54,f	1	1	0	0	2
18	2	76,m	0	0	1	0	1
19	2	61,m	0	0	1	0	1
20	2	55,m	0	0	0	0	0
21	2	63,f	1	1	1	1	4
22	1	59,m	1	0	0	0	1
23	1	63,m	0	0	0	1	1
24	1	67,f	1	1	1	0	3
25	1	66,m	0	0	0	0	0
26	2	77,f	0	0	0	0	0
27	2	63,m	1	1	0	1	3

Table 8 Classification and distribution of α-syn deposits detected by anti-p-α-syn, 5G4, ASyO5 stains and staining with syn211 after PK digestion over different biopsy sites in the patient cohort #2

N	H&Y	p-α-syn				5G4+				PK resistant			
		lower leg	proximal leg	Th10	C7	lower leg	proximal leg	Th10	C7	lower leg	proximal leg	Th10	C7
1	1												
2	1												
3	1	4*nb		nb						3*nb			
4	1	2*sg			sg	sg			ves	2*sg	2*nb	MAP, nb	sg
5	1												
6	1	ves	ves			ves				2*ves			
7	1				sg, subepi						ves		ves
8	1	5*ves	nb	ves			4*ves		ves		2*ves	2*ves	ves
9	1		ves	ves			ves	ves					
10	1	ves											
11	1												
12	1				7*ves				3*ves				7*ves,3* sg
13	1												
14	2						ves				nb		
15	2												
16	2				3*ves				5*ves				ves
17	2	nb					MAP						
18	2	ves		2*ves						3*ves, 1*sg		ves	
19	2												
20	2												
21	2										ves, nb		
22	2												ves
23	2									2*nb			
24	2												
25	2		4*ves, 3*nb	ves				ves			6*ves, 4*nb	2*ves, 3*nb	
26	2												
27	2	ves, sg	sg					nb					
N of deposits/positive biopsies		17/8	11/5	6/5	13/4	2/2	7/4	3/3	10/4	13/5	18/6	10/4	17/6
Sum of deposits, all			47				22				58		
Dermal nerve bundles (nb)			5				1				8		
Sweat glands (sg)			5				1				4		
Blood vessel (ves)			14				10				14		
Muscle arrector pili (MAP)			0				1				1		
Subepidermal fibers (subepi)			1				0				0		

Table 8 Classification and distribution of α-syn deposits detected by anti-p-α-syn, 5G4, ASyO5 stains and staining with syn211 after PK digestion over different biopsy sites in the patient cohort #2

ASyO5+				Total N deposits
lower leg	proximal leg	Th10	C7	
				0
				0
				8
				12
				0
ves	ves			7
				6
ves		ves	ves	20
				4
				1
2ves, 2*sg				4
				20
				0
				2
				0
			ves	10
3*nb				5
ves				9
				0
				0
	ves			3
				1
				2
				0
	ves	ves		26
				0
2*ves,2* nb				8
14/6	3/3	2/2	2/2	148/69
	21			
	2			
	1			
	12			
	0			
	0			

4. DISCUSSION

In this study, a set of distinct immunohistochemical methods targeted at detecting oligomeric or aggregated forms of α-syn were adapted for use in skin biopsies. Subsequently, serial sections of a cohort of PD patients and controls were systematically tested to check the distribution of pathological α-syn forms in relation to p-α-syn and to evaluate the sensitivity and specificity of the methods. Using three different staining methods, it could be shown that α-syn in the dermal nerve fibers in PD is not only phosphorylated, but represents aggregated protein similarly to α-syn found in Lewy bodies in the brain.

THE STAINS WITH 5G4, ASYO5 AND SYN211 AFTER PK DIGESTION ARE HIGHLY SPECIFIC FOR PATHOLOGICAL A-SYN DEPOSITION

Neither in the midbrain of the normal control nor in any of the skin biopsies from 27 normal controls that were stained in parallel with a PD brain sample as a positive control was any positive signal seen in any of the new stains (5G4, ASyO5 and syn211 after PK digestion). Specificity for p-α-syn also remained at 100%, as previously shown (Doppler et al., 2017, 2014). Thus deposits of aggregated α-syn do not occur in healthy controls and represent a finding specific for PD. They might provide an additional benefit in terms of specificity as aggregation is the hallmark of α-syn pathology, while phosphorylation on the other hand does not represent an unambiguously pathologic event. As some studies have shown, s129 phosphorylation occurs in the normal brain, and is more commonly found in the elderly (Muntané et al., 2012; Saito et al., 2003). Although p-α-syn has shown a 100% specificity in the skin biopsies in the studies performed so far, in several IHC studies of colonic biopsies, p-α-syn positive fibers were found in normal controls as well, its incidence increasing with age (Barrenschee et al., 2017; Böttner et al., 2012). Thus, although staining for aggregated/oligomeric α-syn is somewhat more elaborate than p-α-syn staining, it probably reflects a genuinely pathological finding and might provide a benefit of higher specificity when applied to larger patient populations.

DETECTION OF AGGREGATED A-SYN BY PK DIGESTION

Among the newly established methods, the highest positivity rate was achieved by staining with an antibody against total α-syn after PK digestion (38.7% of patients in cohort #1, 48% in cohort #2), which was similar to that reached by an anti-p-α-syn stain performed in parallel (43.6% cohort #1, 48% cohort #2). PK is believed to completely break down native α-syn, sparing

aggregated α-syn (Neumann et al., 2004, 2002), analogously to the way PK breaks down normal prion protein, but spares aggregated prion protein (Schulz-Schaeffer et al., 2000). However, it would be an overstatement to conclude that PK digests all monomeric proteins only leaving aggregates as this effect depends on concentration, temperature, buffer and incubation time as well as on the properties of the sample (in particular fixation, storage time). Therefore, in a narrow range of conditions, other physiologic proteins may remain antigenic while monomeric α-syn is already digested, e.g. in our scans PGP 9.5 signal was mostly still visible after PK pretreatment. As the originally used 20 min incubation time led to almost complete disappearance of staining signal in stored tissue cryosections as well as destruction of tissue morphology, it was reduced to 10 min. The shorter incubation time led to better preservation of PGP 9.5 signal, but did not affect the specificity of the stain, i.e. did not lead to any false positive results among normal controls. Similarly, in the brain, physiologic α-syn was destroyed by PK while staining of the Lewy bodies was further intensified by PK pretreatment. This staining method reveals α-syn aggregates that are specific for PD pathology (and other synucleinopathies, e.g. MSA) and has a sensitivity comparable to anti-p-α-syn. The limitation of the method is the relative complexity of PK pretreatment itself, that as it is dependent on many parameters (see above), cannot be as easily translated between laboratories and makes routine processing of many samples difficult, as the brief incubation time must be adhered.

DETECTION OF A SUBSET OF AGGREGATES BY 5G4

The positivity rate of the anti-aggregated α-syn 5G4 stain (37%) was lower than that of anti-p-α-syn or staining after PK pretreatment. So 5G4 positive structures likely represent a part of PK resistant α-syn species. 5G4 has been shown to be highly specific for disease associated α-syn (Kovacs et al., 2012), accordingly no immunoreactivity in the normal control samples of the midbrain or dermal nerve fibers was seen in this study. In order to allow a better comparison to p-α-syn deposition, a double stain with 5G4 and anti-p-α-syn was performed that revealed dermal fibers positive for both p-α-syn and 5G4 as well as fibers containing only p-α-syn. In the substantia nigra of a PD patient, there was a high degree of colocalization between anti-p-α-syn and 5G4, while some lesions contained either only p-α-syn (more commonly) or 5G4 positive α-syn (fewer small lesions).

Although the antibody is marketed as “anti-aggregated α-syn” it might not be an accurate description of its specificity. While having virtually no

immunoreactivity against monomeric α-syn, 5G4 was shown to be immunoreactive against α-syn conformations of a wide range of molecular weight, e.g. on immunoblotting it also gave a distinct band at 35kDa, likely corresponding to a dimer (Kovacs et al., 2012), so 5G4 cannot differentiate between small oligomers (starting with dimers) and fibrils/aggregates. The same authors could further show 5G4 to be immunoreactive against nitrated α-syn oligomers, but not oxidized oligomers (Kovacs et al., 2014); oxidation has previously been shown to interfere with fibril formation (Binger et al., 2008; Breydo et al., 2005) – placing such oligomers into the "off-pathway" class, i.e. unable to form fibrils or likely non-pathogenic (Breydo and Uversky, 2015). The authors conclude that 5G4 is only staining the "on-pathway" high molecular weight fraction of β-sheet rich oligomers as well as fibrils. The epitope of 5G4 (44-57) lies within the fibril core that comprises residues 35–96 (Vilar et al., 2008) unlike the majority of α-syn antibodies that are directed towards the C-terminal (Croisier et al., 2006). The proposed characteristic quality of the epitope is that it is only available in the β-sheet, that can be acquired by oligomers as well as aggregates. Therefore, 5G4 antibody is specifically staining the higher molecular weight α-syn (larger oligomers and aggregates), though with a lower sensitivity than an anti-total-α-syn stain after PK pretreatment. In the dermal nerves the aggregates/oligomers detected by 5G4 were mostly PK resistant and the overall yield of 5G4 positive aggregates was lower than that of PK resistant deposits. This is probably explained by a lower sensitivity of 5G4 compared to syn211. Relation of 5G4 positive lesions to PK resistant lesions and α-syn phosphorylation will be discussed in more detail in section 4.5. It should also be kept in mind, that this staining requires antigen retrieval with formic acid, that would have to be adjusted when using a different set of samples (fixation and storage time) to maintain a similar sensitivity and might present an obstacle for routine use.

ASYO5 AS A MARKER FOR AGGREGATES AND LARGE OLIGOMERS RATHER THAN AN ANTI-OLIGOMER SPECIFIC ANTIBODY

ASyO5-positive deposits were found in a similar proportion of patients as in the 5G4 stain (33%) and in none of the normal controls, similarly to the midbrain, where ASyO5+ lesions resembling Lewy bodies and neurites were only present in the PD sample. As oligomers are believed to have a transient/low resistance to PK (Roberts et al., 2015), the fact that the ASyO5 stain was still visible even after an intensive digestion with PK (50µg/ml for 20min) strongly suggests that the structures being stained either represent fibrillary forms of α-

syn or very large oligomers that are also resistant to PK. ASyO5 specificity towards oligomers is based on two theoretical principles: the discrimination between monomers and higher molecular forms (both oligomers and aggregates) is achieved due to a higher antibody affinity to a ligand with more epitopes within a certain concentration range (Brännström et al., 2014b). The discrimination between oligomers and aggregates is then achieved by choosing an epitope that should not be available on the aggregated form – this in case of anti-ASyO5 was determined using in vitro preparations of fibrils and oligomers which can greatly vary depending on incubation conditions and might not exactly reflect the structures found in vivo. Interestingly, the same principle can probably explain results of an earlier study (Wang et al., 2013) where a higher α-syn deposition in PD compared to normal control was found using a high α-syn antibody dilution (1:13,000). This could not be reproduced by others who found no difference in total α-syn protein expression in the skin either using immunoblot (Michell et al., 2005) or conventional IHC (Donadio et al., 2016b) and in our own observations (unpublished). Accordingly, a very weak α-syn signal, that was not considered positive, could be seen in many dermal nerve fibers in PD patients as well as in controls, bringing some subjectivity into the evaluation and making this method not suitable for routine diagnostics.

IMPACT OF DIFFERENT STAINS OF AGGREGATED A-SYN AND ITS RELATION TO P-A-SYN

In this study the highest number of aggregated α-syn deposits could be found in the stain with syn211 after PK digestion with a positivity rate similar to the anti-p-α-syn stain. The yield of other aggregate-specific methods (ASyO5 and 5G4) was lower. One explanation could be that S129 phosphorylation is more widespread than aggregation. However, others have previously found that aggregates of α-syn are only partially phosphorylated, in particular Lewy bodies were found to contain p-α-syn as well as aggregates while α-syn at the presynapse was not phosphorylated (Schulz-Schaeffer, 2010; Tanji et al., 2010). On the other hand, >90% of brain α-syn in dementia with Lewy bodies was shown to be phosphorylated by quantitative immunoblotting (Fujiwara et al., 2002). This apparent contradiction can be explained by the difference in methods used. If not separated into soluble and particulate fractions, brain lysates did not differ in α-syn content, including p-α-syn, between LBD and normal controls (Anderson et al., 2006). However, when measured separately in the particulate fraction, there was significantly more p-α-syn. As soluble p-α-syn is most likely not picked up by IHC, what is being detected is the insoluble

fraction that logically and as supported by this study represents aggregated protein. Higher positivity rate in the syn211 stain after PK digestion is most likely explained by a higher sensitivity of syn211 compared to 5G4 or ASyO5 that picks up total α-syn, while due to preceding PK digestion physiologic α-syn is removed resulting in a high specificity and simultaneously more epitopes are exposed on the remaining aggregates. To exclude the possibility that a harsher antigen retrieval with formic acid could lead to a lower detection of aggregated α-syn with 5G4 as compared to p-α-syn, a double stain with 5G4 and p-α-syn was performed, showing a substantial part of deposits that were p-α-syn positive but did not stain with 5G4, while 31% of deposits were positive for both, thus antigen retrieval is unlikely responsible for the lower detection rate in the 5G4 stain.

IHC is a semiquantitave method, since the immunoreaction is a complex product of the availability of a particular epitope within the permeabilized tissue, antibody affinity, the type and timing of fixation, antigen retrieval etc. Antigenicity is always a result of interplay of numerous factors in the IHC procedure that can potentially influence the end result so any quantitative conclusions for positivity rates gained across different staining methods and across tissues that were treated differently prior to staining can only be made cautiously. An alternative possible explanation for lower positivity yield with one or the other staining method directed against different epitopes or PTMs of the same protein can always be that the chosen antibodies have different affinities or the epitopes detected by these antibodies are less resistant to prolonged storage or are affected differently by fixation (O'Hurley et al., 2014).

The question whether all phosphorylated α-syn in the dermal nerve fibers represents aggregates cannot be definitively answered with all available data, although the positivity rate of PK resistant deposits that was similar to p-α-syn is highly suggestive. The proposed characteristic quality of the epitope detected by 5G4 is that it is only available on the β-sheet, that can be acquired by oligomers as well as aggregates (Kovacs et al., 2014). Deposits stained with supposedly oligomer specific ASyO5 were shown to be PK-resistant; PK resistance, however, was previously shown not only a quality of aggregates but also of higher molecular weight oligomers. This means that essentially all three methods were aimed at the higher molecular weight α-syn without definite further subclassification into oligomers or aggregates, which probably also reflects the complexity of the disease pathogenesis as both species represent a continuum in the α-syn aggregation process. IHC can only indirectly tell the supposed molecular size of the stained protein. In order to specify this further, other methods such as e.g. Western blotting, size exclusion chromatography or analytical ultracentrifugation, need to be applied. But although an exact

distinction between oligomeric and fibrillary aggregates could not be achieved using currently available IHC methods, all 3 methods essentially revealed depositions of higher molecular forms of α-syn that were exclusively present in PD patients.

A-SYNUCLEIN AGGREGATES IN DIFFERENT STAGES OF PD AND IN MSA

The mean H&Y score in positive patients (for any of the stains) in the first cohort was higher than in the patients whose skin samples stained negative in all the immunoreactions: 3.29 (+/-1,0) vs 2.7 (+/-1), however, the uneven distribution of stains due to biased biopsy selection in the first cohort does not allow to evaluate the statistical significance. All patients in the second cohort had earlier disease stages: H&Y I and II. Here, as well there was a trend towards increased number of deposits in the H&Y II that, however, did not reach a statistical significance due to a low number of subjects.

Within cohort 1 there were 3 patients diagnosed with another disease belonging to the spectrum of synucleinopathies, namely MSA, that is neuropathologically characterized by deposition of α-syn in glial and neuronal cytoplasm and nuclei. Skin was positive for aggregated α-syn in all 3 of the patients with MSA studied, along with p-α-syn that has already been shown to occur in dermal nerves in MSA in an earlier study done by our group (Doppler et al., 2015). In all samples but one, pathological α-syn deposits were found in the nerve fibers supplying blood vessels (in 1 – nerve fibers supplying a sweat gland). Based on these results, MSA cannot be clearly histopathologically distinguished from PD using skin biopsy, unlike suggested by an earlier study (Zange et al., 2015). Like in PD, dermal nerve fibers in MSA reflect the pathologic α-syn aggregation in the brain, where α-syn aggregates that are differently distributed but biochemically similar to those in PD can be found (Burn and Jaros, 2001).

COMPARISON WITH OTHER PERIPHERAL NERVOUS SYSTEM TISSUES

There has been only one study that aimed to detect aggregated α-syn in peripheral nervous system, namely in colonic mucosal biopsies (Visanji et al., 2015a). In that study, the PET blot method was used (Schulz-Schaeffer, 2010; Schulz-Schaeffer et al., 2000), i.e. tissue was subjected to a much harsher digestion with PK than used in this study. Surprisingly, PK resistant aggregates were found in the enteric nervous system of the normal controls as well as patients. In the IHC of SN, antigen retrieval with PK led to complete disappearance of signal in the normal controls both in PET blot in the study by

Visanji et al (Visanji et al., 2015a) as well as using IHC in this work, and in accordance with earlier reports (Beach et al., 2008; Schulz-Schaeffer, 2010), meaning that PK resistance is a pathologic finding specific for PD brain. Further studies will be needed to check whether α-syn aggregation is indeed a physiologic phenomenon found in the enteric nervous system (ENS) as it represents an obviously pathologic finding in CNS and, according to our results, in dermal nerve fibers in PD.

A-SYN AGGREGATES AT DIFFERENT BIOPSY SITES

In this study, no proximal gradient in the distribution of pathological α-syn was seen. The majority of positive sections for the sum of all stains came from lower leg (41) followed by thigh (30) and proximal back C7 (29), Th10 had the lowest (18) positivity. There is inconsistency in the studies in regard to whether there is a proximal gradient in the prevalence of pathological α-syn in the skin. While highly suggested by the presence of p-α-syn in the cervical biopsy site in all patients in some reports (Donadio et al., 2014), several other studies did not find a significant difference between distal and proximal biopsy sites (Doppler et al., 2017, 2014; Gibbons et al., 2016). As pathological α-syn accumulations are very rare there will be a big variation in detection/not detection merely by chance. The size and in particular depth of biopsy (more vessel and dermal nerve bundles are present in deeper biopsy levels) have crucial effects on the amount of deposits found. Based on the experience with skin biopsies gained in our lab, often punch biopsies from the back cannot be that easily disattached and have to be cut off with a scalpel what limits the size of the material taken.

LIMITATIONS AND TECHNICAL CONSIDERATIONS

The main methodological problem that interfered with the study was the loss of antigenicity during biopsy storage. The antigenicity of tissue substantially deteriorated during storage of cryosections at -20°C, falling from initial 80% to 48% (gradual decline could be seen by comparing the anti-p-α-syn stains done in an interval of several months by cand. med. L. Schulmeyer). This stresses that the timing of biopsy staining and analysis should be taken into account. The deterioration of tissue could be slowed e.g. by optimization of fixation method or use of paraffin tissue. An appropriate fixation time should be determined extra for the studied antigen; as an inadequate fixation can lead to artifacts and loss of antigens due to premature deterioration as well as to loss of epitopes due to overfixation. The type, concentration, incubation time and ratio of tissue volume to that of fixative all can have dramatic effects on the staining results. In some

other studies that reported higher sensitivity a solution with reduced aldehyde (Zamboni: 2%PFA, 12.5% picric acid) was used for a substantially longer (12-14h) fixation (Donadio et al., 2014). Exposure to humidity is another factor that was shown to lead to faster antigen degradation (Xie et al., 2011), a higher concentration of sucrose (up to 30% as compared to 10% routinely used) could be used to displace water and achieve a better preservation.

Detection of post-translationally phosphorylated proteins represents another challenge, as it is known to be highly dependent on the fixative (Burns et al., 2009) and the phosphorylation state can even change during fixation due to still active phosphatases/kinases (Espina et al., 2008).

In an attempt to set up a procedure that could be replicable in laboratories across the world the planned S4 study is aiming to use formalin fixed paraffin embedded tissue (Visanji et al., 2017). The likely obstacle will be a limited section thickness that will inevitably limit the sensitivity unless compensated by analysis of a higher number of sections.

PLA AND PET BLOT ARE NOT SUITABLE FOR DIAGNOSTICS USING SKIN BIOPSY TISSUE

PLA was adopted from the pioneer and so far only study that used this method to assess α-syn oligomerization (Roberts et al., 2015). As in the sections of SN positive signal was even more widespread in the normal control than in the PD and the singular trials in the skin samples showed staining in the normal controls as well, it wasn't considered specific. These results, however, do not diverge from Roberts et al, as in the study no significant difference in signal intensity between normal controls and PD subjects for the most brain regions (including SN) was found. The two studies that adapted PLA to study protein oligomerization in neurodegenerative diseases though perfectly specific in cell culture failed to give specific results when applied to patient material (Kamali-Moghaddam et al., 2010; Roberts et al., 2015). It is also worth mentioning that the native physiological state of α-syn as well as relation of α-syn conformation change to pathology is controversial and there is accumulating evidence that α-syn physiologically at least to an extent exists as a tetramer (Bartels et al., 2011; Wang et al., 2011). Detection of tetramers with PLA could explain the widespread signal in the normal control.

The PET blot method for detection of aggregated α-syn could not be optimized to give a specific result in the midbrain sections. Depending on the protocol alterations either no signal was seen both in the samples from the normal control and PD or there was a universal non-specific signal. PET blot is

an elaborate multi-step method that is practically not feasible to adapt for routine laboratory diagnostics. The only published study conducted in PD patients that stained peripheral tissue (colonic biopsy) found a positive signal in PET blot in normal controls as well as in PD (Visanji et al., 2015b). In the studies of brain tissue in sporadic and lymphoid tissue in variant CJD, PET blot showed a marginally better sensitivity than IHC (Bergström et al., 2009; Budka, 1997). Protease resistant PrP could be detected in the tonsillar tissue using more conventional Western blot as well (Hill et al., 1997). Therefore, PET blot did not find a utility in the diagnostics of prion disease due to its complexity and only marginally higher sensitivity to IHC. Since its introduction in 2000 (Schulz-Schaeffer et al., 2000) only 21 research papers that used the PET blot method for both prion and neurodegenerative diseases have been published (according to pubmed.gov search parameter: "pet blot"[Title/Abstract]).

5. CONCLUSION

Here for the first time it could be demonstrated that α-syn deposits in the dermal nerve fibers are similar in regards to their aggregation state to the α-syn lesions in the brain.

HYPOTHESIS #1: DERMAL P-A-SYN IN PD REPRESENTS AT LEAST TO AN EXTENT AGGREGATED A-SYN

The nerve fibers containing p-α-syn, which has already been to shown to be highly-specific peripheral marker for PD, mostly correspond to higher-molecular weight α-syn. This hypothesis could be supported by evidence gained through several independent methods. Firstly, PK resistance that is a marker of pathologic α-syn and is attributed to higher molecular forms of α-syn, namely aggregates or preceding them beta-sheet rich oligomers, could be shown in a similar proportion of patients as p-α-syn lesions and in none of the controls. Then by using a higher antibody dilution (ASyO5) deposits of higher molecular weight α-syn could be exposed. Oligomeric α-syn forms stained by this method were highly PK-resistant hinting that these lesions may also represent aggregates. And lastly, the stain with 5G4, an antibody that is directed towards beta-sheet of oligomers/aggregates revealed dermal nerve deposits that also often colocalized with PK-resistant, ASyO5 positive and phosphorylated lesions. All three methods directed towards higher molecular weight α-syn revealed dermal nerve and brain lesions in PD and mostly colocalized with p-α-syn and with each other.

HYPOTHESIS #2: A-SYN AGGREGATES ARE NOT FOUND IN THE DERMAL NERVE FIBERS IN NORMAL CONTROLS

These lesions were only present in PD patients and in none of the controls, what taken together with previous studies in which p-α-syn was stained, further supports the hypothesis that phosphorylated and aggregated α-syn in peripheral nervous tissue is a pathologic finding specific for PD.

HYPOTHESIS #3: AMOUNT OF A-SYN AGGREGATES INCREASES WITH DISEASE STAGE

There was a trend towards increase in the number of pathological α-syn deposits with disease progression (in higher H&Y stages), however, this hypothesis could not be supported by sufficient statistical data.

Altogether, this proves again the utility of peripheral α-syn post-translational modifications as a biomarker for PD. The methods used in the current study are somewhat more elaborate than the established anti-p-α-syn stain, and in terms of sensitivity are not advantageous for the diagnostic purpose, however, they might provide a benefit of higher specificity. The main limiting factor is the need for additional antigen retrieval, intensity of which (incubation time, reagent concentration) has to be adjusted based on the way tissue was processed or even the time it has been stored.

Methods (in particular biopsy spots, tissue fixation and antibody choice) used to stain for α-syn deposits differ considerably among laboratories, which leads to a wide range in sensitivity of skin α-syn IHC assays varying from 0 to 100% (Beach et al., 2010; Donadio et al., 2014). A standardization of protocols would be required to allow its widespread use in clinical practice. A multicenter blinded study similar to one conducted for colonic biopsies (Corbillé et al., 2016) could help to determine the best conditions to achieve the highest diagnostic accuracy.

Apart from the optimization of the existing methods, new technologies could be used to increase the sensitivity of α-syn detection. One attractive approach is light sheet fluorescent microscopy (LSFM); its potential role in clinical diagnostics is only starting to be realized (Glaser et al., 2017). This technique allows processing of biopsy samples en-bloc and scanning of the whole depth of a biopsy at once, potentially further increasing sensitivity and reducing processing time compared to staining of serial sections.

The major inherent limitation of IHC as a method is that it is hard to quantify, and changes in protein expression due to e.g. therapy targeting α-syn, would be hard to monitor. Here, a different more sensitive and quantifiable than IHC methodological approach might be advantageous. Now that it has been shown that skin contains α-syn aggregates it is feasible to use it in a protein misfolding assay – an emerging biochemical diagnostic method for synucleinopathies. This method, that has been already established for routine prion disease diagnostics, makes use of the ability of pathological α-syn to seed new aggregates, amplifying the amount of pathological protein and thus greatly increasing the sensitivity (Atarashi et al., 2011; Orrú et al., 2015). The pioneer trials have already shown to allow detection of pathological α-syn with very high diagnostic accuracy (sensitivity 88,5-95%; specificity 96,9-100%) in the CSF in synucleinopathies (Fairfoul et al., 2016; Groveman et al., 2018; Shahnawaz et al., 2017).

ABSTRACT (EN)

Lewy bodies and Lewy neurites are neuropathological hallmarks of Parkinson's disease (PD). These depositions in the brain mostly consist of aggregated α-synuclein (α-syn) phosphorylated at Ser129. A number of studies reported detection of phosphorylated α-syn (p-α-syn) in the dermal nerve fibers in Parkinson's disease. The objective of this study was to investigate whether pathological α-syn accumulations detected in the skin represent aggregated protein. A number of methods aimed at detecting α-syn oligomers and aggregates were first tested and optimized on the brain samples in PD and normal control. These methods included proximity ligation assay (PLA), PET-blot, immunohistochemical (IHC) stains with α-syn aggregate (5G4) or oligomer specific (ASyO5) antibodies and a stain against native α-syn (syn211) after proteinase K (PK) digestion. Subsequently, the most specific methods (stains with 5G4, ASyO5 and syn211 after PK digestion) were studied in two separate patient and control cohorts. Anti-p-α-syn stain was performed in parallel.

Single sections from at least 2 biopsy sites from 44 patients and 22 controls (cohort 1) as well as serial sections of 4 biopsy sites from 27 patients and 5 controls (cohort 2) were systematically studied for presence of aggregated and oligomeric α-syn. In total, 5G4 positive deposits were found in 24% (cohort 1) and 37% (cohort 2), ASyO5 positive lesions in 17,7% (cohort 1) and 33% (cohort 2), syn211 positive lesions after PK digestion in 38,7% (cohort 1) and 48% (cohort 2) of cases. There was a major overlap among positivity for a particular staining on the patient level and in most cases, the same nerve fiber was found to be positive for all 4 markers in neighboring sections. Among the skin biopsies which contained p-α-syn accumulation, 59% were also PK resistant, 41% were 5G4 positive and 45% were ASyO5 positive. The samples belonging to normal controls did not show any positive signal in either of the newly established stainings or in the anti-p-α-syn staining.

Using 3 distinct IHC methods, α-syn oligomers and aggregates were detectable in the majority of p-α-syn positive skin biopsies. This finding supports the hypothesis that α-syn aggregation occurs in the peripheral (i.e. dermal) nerves and can be specifically detected using skin biopsy.

ABSTRACT (DE)

Die neuropathologischen Kennzeichen des Morbus Parkinson sind Lewy-Körperchen und Lewy-Neuriten. Diese Ablagerungen im Gehirn bestehen hauptsächlich aus aggregiertem α-Synuclein (α-Syn), das am Ser129 phosphoryliert ist. Mehrere Studien konnten zeigen, dass phosphoryliertes α-Syn (p-α-Syn) auch in Nervenfasern der Haut von Parkinsonpatienten nachweisbar ist. Das Ziel dieser Arbeit war, zu untersuchen, ob es sich bei den pathologischen Ablagerungen von p-α-Syn in der Haut wie im Gehirn um Aggregate handelt. Mehrere Methoden, die dem Nachweis von α-Syn-Oligomere und Aggregate dienen, wurden zuerst an Gehirnen von einem Parkinsonpatienten und Normalkontrolle getestet und optimiert, darunter: Proximity Ligation Assay (PLA), PET-Blot, immunhistochemische Färbungen mit α-Syn-Aggregat- (5G4) oder Oligomer-spezifischen Antikörper (ASyO5) und eine Färbung mit einem Antikörper gegen natives α-Syn (syn211) nach Verdau mit Proteinase K (PK). Danach wurden die spezifischsten Methoden (Färbung mit 5G4, ASyO5 und syn211 nach PK-Verdau) an den Hautstanzbiopsien von zwei Patienten- und Normalkontrollkohorten untersucht. Parallel wurde in den Biopsien das p-α-Syn angefärbt.

Einzelschnitte von je mind. 2 Biopsiestellen von 44 Patienten und 22 Kontrollen (Kohorte 1) sowie Serienschnitte von je 4 Biopsiestellen von 27 Patienten und 5 Kontrollen (Kohorte 2) wurden systematisch nach Vorliegen von aggregierten und oligomerischen α-Syn untersucht. Zusammenfassend, wurden 5G4-positive Ablagerungen in 24% (Kohorte 1) und 37% (2. Kohorte), ASyO5-positive Läsionen in 17,7% (Kohorte 1) und 33% (Kohorte 2), syn211-positive Läsionen nach PK-Verdau in 38,7% (Kohorte 1) und 48% (Kohorte 2) der Fälle gefunden. Das p-α-Syn wurde entsprechend in 43,6% und 48% der Fälle detektiert. Es zeigte sich die Tendenz, dass Patienten, bei denen p-α-Syn nachweisbar war, auch für mehrere der neuen Marker positiv waren; auch häufig waren für alle 4 Marker positive Nervenfasern in naheliegenden Schnitte zu sehen, was für eine Kolokalisation spricht. Unter den Hautbiopsien, in den p-α-Syn-Ablagerungen zu sehen waren, hatten 59% gleichzeitig PK-resistente, 41% 5G4- und 45% ASyO5-positive Ablagerungen. Bei Kontrollen waren Ablagerungen weder mit den neu eingeführten Methoden noch mit anti-p-α-Syn-Färbung detektierbar.

Mit Hilfe von drei unterschiedlichen immunhistochemischen Methoden waren Oligomere und Aggregate vom α-Syn im Großteil der p-α-Syn-positiven Hautbiopsien nachweisbar. Dieser Befund unterstützt die Hypothese, dass die

Ablagerung von α-Syn-Aggregaten auch in peripheren (v.a. dermalen) Nerven vorkommt und spezifisch nachgewiesen werden kann.

6. LITERATURE

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ACKNOWLEDGEMENTS

First and foremost, I would like to thank Prof. Dr. Claudia Sommer and PD Dr. Kathrin Doppler for the opportunity to work on a fascinating research question. The immense support and guidance on the project, clinical practice and career in general is something I'll be indebted to forever. I am also grateful to Prof. Dr. Nurcan Üçeyler for most valuable critical remarks and indispensable clinical mentorship. I am deeply grateful to all of you for inspiring me through your own example to pursue both research and clinical career and, in particular, rekindling my interest in neurology, what has made my life thrilling and fulfilling.

A special gratitude goes to Barbara Dekant who introduced me to IHC and immensely helped by sectioning and staining a bulk of samples, and Hiltrud Klüpfel who was always helpful with any technical issues and ready to share her expertise. I am also thankful to Kathleen Stahl, Sonja Mildner, Barbara Reuter, Lydia Biko, Barbara Broll and other technicians for their friendliness and indispensable help with day-to-day technical issues.

I would like to thank my fellow students Thomas Klein, Helena Stengel, Anja Hock, Dimitar Evdokimov, Lena Schulmeyer and others for fruitful discussions, helpful suggestions, and a friendly and supportive atmosphere that made working in the lab fun.

A special thanks to Apoorva Baluapuri for being the best partner and closest friend and always patiently listening to me jabbering about my challenges and giving constructive advice.

Last but not least, I am forever grateful to my family who supported me in all my academic endeavors even when it meant that I would stay away from homeland.