Title	Alpha-Synuclein in bio fluids and tissues as a potential biomarker for Parkinson's disease
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Citation	Journal of Alzheimer's Parkinsonism and Dementia, 2017, v. 2 n. 1, p. 013:1-10
Issued Date	2017
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Journal of Alzheimer's Parkinsonism & Dementia

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Mini Review

Alpha-synuclein in Bio Fluids and Tissues as a Potential Biomarker for Parkinson's Disease

This article was published in the following Scient Open Access Journal:
Journal of Alzheimer's Parkinsonism & Dementia
Received July 05, 2017; **Accepted** July 17, 2017; **Published** August 02, 2017

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Abstract

Background: Parkinson's disease (PD) is a chronic neurological disorder that impairs normal motor function and has no cure at present. Diagnosis of PD is clinical only; biopsies confirming the presence of the disease can only be done *post-mortem*. Furthermore, similarities in the manifestation of PD symptoms to other diseases such as Multiple System Atrophy (MSA), make early diagnosis difficult and ambiguous. As a result, there is a high demand for research investigating biomarkers for timely diagnosis of PD.

Alpha-synuclein (α-SYN) is a protein found misfolded in the brain and other body tissues of PD patients. Its relevance and association to PD make it a prime biomarker candidate. However, reports in the literature suggest that the structural form and location of α-SYN are key to yield a reliable diagnosis. The aim of this Minireview is to highlight efforts made in studying α-SYN as a biomarker over the past decade.

Key Findings: Based on the literature surveyed, α-SYN was indeed the most widely studied candidate biomarker for PD. Cerebrospinal fluid (CSF) and skin were promising sites for assessing α-SYN effectiveness in differentiating PD from MSA. Furthermore, gastro-intestinal α-SYN was suitable for early diagnosis of PD. A combination of total α-SYN and other forms including but not limited to phosphorylated α-SYN were the best predictors of the disease.

Conclusion: Misdiagnosis of patients enrolled in clinical trials is a confounding factor for PD drug development. A robust biomarker for PD will help eradicate this problem. Identifying an accurate biomarker for PD will also ensure timely therapeutic intervention to manage symptoms better and improve the quality of life of patients. The promise α-SYN and its phosphorylated form show in different tissues is a step forward in this direction.

Keywords: Minireview, Parkinson's disease, α-SYN, Biomarker, Phosphorylation

Introduction

Background of Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder, with a prevalence of 100 to 180 per 100,000 people [1] and over 10 million patients worldwide [2]. In the UK, 1 in 500 people suffer from PD and at least one person is diagnosed with this disease every hour. PD mostly affects people aged above 50 years but about 1 in 20 people with this disease is aged under 40 [3]. Genetic predisposition and environmental toxins are both putative risk factors for the disease. Pathological changes in PD may be present as early as 30 years before patients showing any signs that could allow diagnosis [4,5]. The primary lesion of PD involves degeneration of dopaminergic (DA) neurons in the *substantia nigra pars compacta*, causing dopamine deficiency within the basal ganglia. This leads to impaired motor function such as tremor at rest, dysphagia and abnormal gait [5]. However, there is a significant body of evidence suggesting that PD is more than just nigrostriatal dopamine deficiency [6,7]. Alongside dopamine depletion, Lewy body pathology is another hallmark of PD [5,8]. Aggregates of alpha-synuclein (α-SYN) are deposited within the cell body as Lewy bodies (LB) and within neurites as Lewy neurites (LN) [5]. Current therapeutic strategies for PD are pharmacological or surgical (deep brain stimulation). Traditional Pharmaco-therapy of PD is based on replenishing or sustaining dopamine levels. Levodopa (L-DOPA), a precursor of dopamine, still remains a frontier in this category [9]. However, with an increasing knowledge of the pathological

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mechanisms of PD, a gamut of therapies are under trial. Antioxidants such as coenzyme Q10 have shown neuroprotection in this regard [10]. Agents reducing inflammation such as minocycline are also under trial [11].

Currently, the diagnosis of PD depends on the identification of motor symptoms, which include bradykinesia, resting tremor and muscle rigidity [12]. However, PD patients suffer not only from motor symptoms, but also non-motor symptoms. Non-motor symptoms, such as rapid eye movement (REM) sleeping disorder, anosmia, constipation, mood disorders, and cognitive impairment are likewise well-recognised key features of PD [3,13,14]. Some of these non-motor symptoms may be present before the motor symptoms and often predominate as the disease progresses [5,7,15,16].

The need for biomarkers for PD and current strategies

The accuracy of the clinical diagnosis of PD ranges from 46% to 90%, being particularly poor in early stages of the disease [17-20]. Clinical diagnosis depends upon symptomatic features, which increases the risk of misdiagnosis in PD due to the fact that these symptoms present similarly in other diseases. In addition, there are only around 30% of neurons left when the symptoms become noticeable [3]. As a result, therapeutic intervention is delayed. Ideally a biomarker based diagnosis could help discover new targets for treatment and manage symptoms better, improve quality of life and reduce societal burden of the disease [21].

A biomarker is defined as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or after disease"[22]. This suggests that a biomarker for PD can either be based on imaging techniques or biochemical measurements. A robust biomarker should therefore be: non-invasive to acquire, convenient to measure, sensitive and specific. In addition, it is crucial to produce consistent and reliable data. Ideally, its level should change with disease progression and be useful even for patients in preclinical phases to allow progress monitoring and early diagnosis.

α-SYN, DJ-1, β-amyloid and tau present in CSF, and urate in blood are considered as optimal diagnostic biomarkers of PD [20]. DJ-1, β-amyloid and tau have shown association with PD. Mutations in the *DJ-1* gene contribute to the incidence of early-onset familial PD. The primary function of the cellular protein DJ-1 is attributed to its defense against reactive oxygen species [23]. Presence of neurofibrillary tangles and β-amyloid plaques are features closely associated with Alzheimer's disease. However studies have also shown the prevalence of these in PD, with a greater link to Parkinson's disease dementia (PDD) [24].

Other compounds, such as heart-type fatty acid-binding protein (H-FABP), hypocretin, homocysteine and osteopontin are also candidate biomarkers of PD. There are also studies on tissue transglutaminase and serpins, which are suggested to have an association with α-SYN aggregation. MicroRNA 19b has been shown to correlate with patients having rapid eye movement (REM) sleeping disorder before synucleopathy diagnosis [25]. Neurotransmitter metabolites [26], such as homovanillic acid (HVA) and methoxy-hydroxy-phenyl-ethylene glycol (MHPG), growth hormones and amino acids which are associated with nitrogen monoxide and metals, e.g. iron and zinc, are all under research to assess their potential links with PD [27]. However, the studies involving these candidate markers are very preliminary and the results are inconsistent [27]. Among all of these potential biomarkers, α-SYN is considered to be the most promising biomarker. As a hallmark of PD, α-SYN attracts great interest to identify its potential usefulness in diagnosis and/or treatment of PD [28].

The importance of α-SYN

α-SYN is a 140-amino-acid protein, which is one of the three-protein family comprising β-SYN, γ-SYN and α-SYN in humans [29]. While its normal physiological role still remains ambiguous, docking of synaptic vesicles, serving as a molecular chaperone, and maintenance of polyunsaturated fatty acid levels in the brain are among some of its reported functions [30]. Recent evidence also proposes a protective role of α-SYN against infectious agents in the central nervous system (CNS), wherein neurons were more susceptible to certain species of RNA viruses in α-SYN knockout mice [31]. Another study proposed the role of α-SYN in normal functioning of the nigrostriatal system. A deficiency in striatal dopamine and loss of locomotor function was seen in α-SYN knockout mice [32]. Perez et al. also demonstrated the ability of overexpressed α-SYN to reduce tyrosine hydroxylase activity *in- vitro*, consequently interfering with dopamine synthesis [33]. Overexpression of mutant α-SYN also reduced the dopamine storage pool, leading to impaired motor function in transgenic mice [34] and subsequently increasing cytoplasmic dopamine, leading to its oxidation *in-vitro* [35]. α-SYN also plays a role in modulating the human dopamine transporter. Lee and colleagues show that this results in intracellular uptake of dopamine, leading to apoptosis [36].

LBs are widespread in nature, present throughout the brain, spinal cord, peripheral nervous system, enteric nervous system, salivary glands, adrenal medulla, cutaneous nerves and sciatic nerve. Braak et al. have proposed the prevailing theory that in early stages Lewy pathology is seen in regions of the brain stem, ultimately spreading to the midbrain and cortex [37]. Highlighting the involvement of the gastric system further is the "dual-hit hypothesis", which accounts for the early presence of Lewy pathology seen in this region. This hypothesis proposes initiation of PD by a pathogen, possibly entering the body via nasal and gastric routes [38]. This theory might be further enhanced by Braak and colleagues who successfully demonstrated that inclusions of α-SYN are present in innervations to the gastro-intestinal (GI) tract [39]. Recent evidence has furthermore shown the gut micro biome to play a role in PD pathology [40]. Therefore, we see that there is an active involvement of the GI tract in Lewy body pathology.

Oligomeric and fibrillar α-SYN are formed from the aggregation of α-SYN via different pathways and due to different factors, such as phosphorylation on specific sites [41], addition of metal ions and small charged molecules [42], and mutations [43]. Conformations and aggregation states (soluble oligomers, amorphous aggregates or amyloid-like fibrils) of α-SYN vary depending on the conditions and co-factors [44]. Although several aggregation states of α-SYN may be neurotoxic, the oligomeric form is suggested to be highly correlated with neurotoxicity [45]. While the native structure of α-SYN remains unclear, there are studies hinting at either the monomeric [46,47] or alpha-helical tetrameric [48] as the physiological form. Since α-SYN is found

in various regions of the body and in different forms, several discrepancies about its potential as a biomarker for PD remain.

The aim of this review is to examine the literature focusing on α-SYN as a biomarker for PD, compared to other molecules. The biomarker potential of α-SYN for PD is then evaluated with an emphasis on the sites where it is found.

Methods

Identification of studies for the minireview

For the purpose of this Minireview near 100 publications were examined after following strict criteria. Studies were identified by searching electronic databases, i.e., Embase and PubMed. The free text and MeSH terms used in this search were "PD", "alpha-SYN" and "biomarkers". The last search was run on 2nd December 2016. Other searches in PubMed included the following combinations, in which the free text and MeSH terms of "PD", "biomarkers" and (a) "tau" or "tau protein"; (b) "DJ-1" or "DJ protein"; (c) "amyloid-b" or "amyloid beta"; (d) "uric acid" or "urate" were used to retrieve the number of papers which are studying these biomarkers. Another "PD", "biomarkers" and (a) "plasma"; (b) "saliva" or "salivary gland" or "submandibular gland"; (c) "gastrointestinal tract" or "stomach" or "colon"; (d) "skin"; (e) "cerebrospinal fluid" to identify the number of publications per biological matrix. The last search was "PD", "alpha-SYN", "biomarkers" and (a) "oligomer" or "oligomeric"; (b) "phosphorylated"; (c) "monomer" or "monomeric"; (d) "native" to identify the number of papers which were published per forms of α-SYN.

Only studies involving human participants were included; any animal studies were excluded. Studies published in English language in the top 50 journals on the subject area of "Neuroscience" and top 200 journals on the subject area of "Biochemistry, Genetics and Molecular Biology" ranked by SC imago Journal & Country Rank [49] were selected. Also a restriction on year of publication was applied to the last ten years (2006-2016) and any review articles identified from the search were excluded.

Results and Discussion

α-SYN and other potential biomarkers for PD

A considerable difference in the literature published between α-SYN and other main potential PD biomarkers, including tau, β-amyloid, DJ-1 and uric acid was observed (Figure 1). Till date, the number of publications studying α-SYN has reached 324, which is at least twice as much as the other candidates according to the database search (see Methods). In addition, it is apparent that there has been a rapid rise in number of publications on α-SYN (a two-fold increase) over the last five years (Figure 1). This is closely followed by tau and β-amyloid, for which the number of papers published in the past five years were almost double in comparison with the amount of papers available from 2006 to 2010 (Figure 1). Considering these trends, a growing interest in the roles of α-SYN for the diagnosis of PD was clearly observed.

Locations and conformations of α-SYN as a potential PD biomarker

α-SYN was detected in various places of the body. Based on the data retrieved from the PubMed search, cerebrospinal fluid (CSF), plasma, GI tract, saliva and related glands, and skin were studied in recent investigations. There is a vast amount of literature focusing on CSF α-SYN, having 83 papers published in the last ten years with a threefold increase over the last five years (Figure 2). Publications discussing the GI tract increased greatly from 2 to 22 papers over a five-year period (Figure 2). Remarkable growth on this area leads us to believe it is another considerable site for future tests. In contrast, the numbers of publications concerning plasma in the past five years (8 papers) were the least, greatly overtaken by the GI tract (Figure 2). It also seems that researchers have started exploring the significance of salivary and cutaneous α-SYN with a gradual increase in the number of studies. However, reports are still rare with only 14 and 12 publications respectively, over these ten years (Figure 2). Overall, it is evident that experts are intensively studying CSF α-SYN in comparison with other biological matrices. Meanwhile, the potential of other sites are yet to be fully explored.

Trends in the forms of α-SYN as biomarkers was also looked

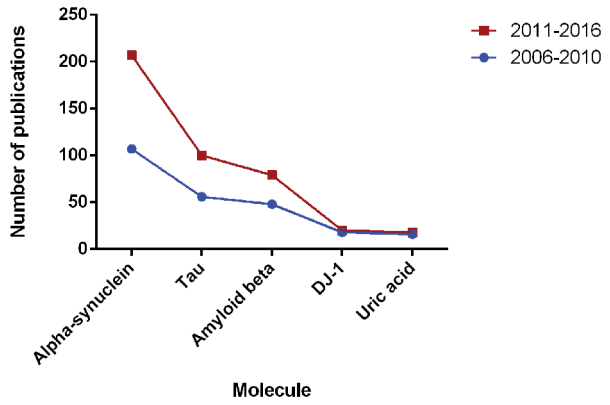

Figure 1. The number of papers studying the potential of α-SYN, tau, amyloid beta, DJ-1 and uric acid as PD biomarkers, published in the last ten years (2006 – 2016).

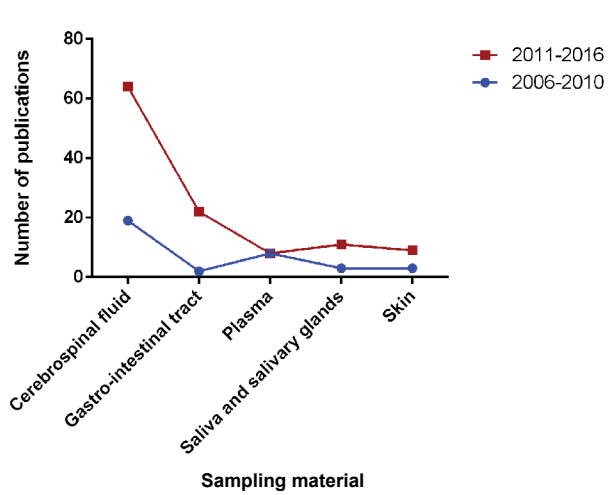

Figure 2. The number of papers studying α-SYN in different locations, including cerebrospinal fluid, gastrointestinal tract, plasma, saliva, and salivary glands and skin, published in the last ten years (2006 – 2016).

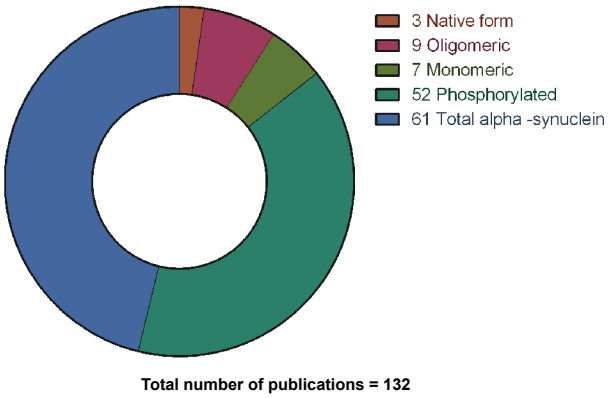

Figure 3. The number of papers studying different forms of α-SYN i.e., native, oligomeric, monomeric phosphorylated and total α-SYN, published within the last ten years (2006 – 2016).

into. Most researchers focus on total α-SYN in their studies (61 papers), closely followed by phosphorylated forms with just 9 papers less (Figure 3). The phosphorylated form, predominantly at serine 129, seems to be the most popular focus in comparison with all the other forms (Figure 3). Phosphorylated α-SYN (p-α-SYN) is widely recognised as the pathological form of PD [50]. This might have directed the interest of experts towards phosphorylated species.

Potential of α-SYN in Cerebrospinal Fluid (CSF) as a biomarker

PD vs. other neurological conditions: In a large study, Mollenhauer *et al*. detected the total concentration of α-SYN by using ELISA and Western blotting [51]. Their results could distinguish PD from Alzheimer's disease (AD) and non-synucleopathies, including Progressive Supranuclear Palsy (PSP) and Normal-pressure Hydrophalus (NPH), with a sensitivity and specificity of 70.72% and 52.83% respectively. Their results match the findings of Hall and colleagues who also observed a decrease in CSF α-SYN in PD and other parkinsonian disorders when compared to AD [52]. Furthermore, a combination of α-SYN and other AD biomarkers, including total tau, phosphorylated tau (p-tau), and $A\beta_{1-42}$ has been proposed to differentiate PD and AD [52]. A sensitivity of 93% and specificity of 84% was achieved when using the combination of α-SYN and $A\beta_{1-42}$, the fragment of β-amyloid abundant in AD patients, as an additional indicator [53]. Concentration of oligomers and the ratio of oligomeric α-SYN to total α-SYN (o/t-α-SYN) were higher in Parkinson's disease with dementia (PDD) patients when compared with subjects with AD, but with lower diagnostic accuracy than total α-SYN [54]. This is in accordance with another study, proving the possible use of oligomers to differentiate PD from AD and PSP [55]. Wang and colleagues recommended the use of a combination of p-α-SYN and total α-SYN to distinguish PD from MSA and PSP [56]. In contrast, the study by Foulds *et al*. did not show appreciable differences in total α-SYN concentrations between PD and PSP in a post-mortem study [57].

A high degree of concordance in total α-SYN concentration between PD and MSA was reported in a small number of studies [51-53,57]. Levels of oligomeric and phosphorylated forms did not show significant difference among these two groups of subjects in the *post mortem* study [57]. Oligomeric phosphorylated α-SYN (o-p-α-SYN) was found useful in this study, being able to distinguish PD from MSA and other neurological patients, including PSP and DLB. Also, Shi and colleagues noticed a more significant reduction of oligomeric phosphorylated species in MSA when compared to PD cases [53].

PD vs. Controls: The results of these studies [52,53,56,58-63] showed a significant decrease in total concentrations of α-SYN in PD compared to healthy controls with a specificity of 25% -75.7% [53,59,63,64] and generally high sensitivity of 92%-100% [53,59,63], except one report that showed a relatively low sensitivity (63%) [64]. Unlike total α-SYN, levels of p-α-SYN [57,58] and o-α-SYN [54,55,58,64] were found to be higher in PD. By studying o/t- α-SYN instead of oligomers alone, Tokuda et al. claimed that sensitivity and specificity were improved from 75% to 89.3% and 87.5% to 90.6% respectively [55]. A study carried out by Parnetti *et al*., however, demonstrated increased specificity from 41.6% to 85.7% but a poorer sensitivity of 56.2% by analysing o/t-α-SYN instead of o-α-SYN [64]. Besides, combining p-α-SYN and total α-SYN together also gave better performance than using the phosphorylated form only [56]. However, the study undertaken by Foulds and colleagues again found no differences in the levels of these forms between PD and controls, but was suggestive of the ability of o-p-α-SYN in differentiating PD patients from healthy controls [57]. It must be noted that the conflicting results showing no noteworthy difference in levels of α-SYN in all individuals, except o-p-α-SYN, were obtained from the only *post mortem* study [57].

Combination of α-SYN and other biomarkers: There are few CSF studies supporting the theory that a combination of α-SYN with other biomarkers could improve the diagnostic accuracy of PD. For example, a study conducted by Shi *et al*., revealed the possibility of distinguishing PD from AD, MSA and also healthy controls by using seven biomarkers including α-SYN [53]. This finding is vital as the similarities in clinical manifestations between MSA and PD are a major problem for differential diagnosis, while α-SYN *per se* is inadequate to work as a biomarker. In addition, higher sensitivity (90%) and specificity (71%) could be achieved by using a combination of α-SYN and p-tau to distinguish PD from MSA [53]. Besides, distinctions between synucleopathies and tauopathies could be made by using the ratios of total tau to α-SYN (t-tau/α-SYN) or p-tau/α-SYN [59]. t-tau/-SYN could also determine PD from other neurological conditions, i.e., primary headache, post-infective myelopathy, and polyneuropathy with high sensitivity of 89% and specificity of 61% [59]. The combination of p-tau with o/t-α-SYN or p-α-SYN also allows better discrimination between PD and healthy controls [58]. Thus there are strong evidences in literature which highlight combinations of different CSF biomarkers as a promising strategy to improve diagnostic accuracy for PD.

Correlation of α-SYN with Age, Treatment, Disease Severity and Disease Duration: Overall, different forms of CSF α-SYN have no correlation with age [52,59,65]. Treatments likewise showed no effects on α-SYN levels [51,61,63]. These results may suggest that changes in CSF α-SYN levels are solely due to the pathology of PD.

A small number of studies were performed to identify the possible link of α-SYN with disease progression. The increase in pattern of total and o-α-SYN found in longitudinal studies supports the potential of CSF α-SYN serving as a biomarker for disease progression [61,66]. Interestingly, Hall and colleagues noticed that the correlation pattern was not observed in the cases of PD for less than or equal to five years [66]. In contrast with the patterns observed in total and o-α-SYN, Majbour et al. noticed an obvious reduction in p-α-SYN concentration when compared to baseline level [61]. However, Stewart et al. detected an opposite longitudinal trend in p-α-SYN levels in PD subjects [65]. This could be due to disease stages of PD subjects studied. Levels of p-α-SYN were suggested to decrease during the early stages but increase at the later stages [65]. This explains the converse findings of both studies as all PD subjects in Majbour *et al*. research were at initial disease stages.

A study conducted over two years suggested that higher levels of α-SYN within PD may indicate faster progression of motor symptoms and cognitive impairment [60]. Two cross-sectional studies, however, found converse correlation of α-SYN [62] or those in oligomeric form [58] with motor symptoms [65].

On the other hand, Hong et al. and Stewart et al. both showed a lack of correlation between α-SYN levels and PD progression [65]. It is worthy to mention that the post-mortem study conducted by Fould *et al*. reported similar findings, suggesting the lack of correlation between total α-SYN, o-α-SYN, p-α-SYN and o-p-α-SYN with disease severity [57]. Moreover, no correlation was found between α-SYN and disease duration [51,54,57,59].

Potential of α-SYN in GI tract as a biomarker

PD vs. Other Neurological Conditions: The patterns of α-SYN in gastric and colonic mucosa detected in PD and MSA were reported to be similar using immunohistochemical techniques [67]. However, this was not the case for AD patients with LBs. While the PD group showed more than a 60% frequency in cases showing positive staining for p-α-SYN, the AD group had a frequency of less than 20% [68].

PD vs. Controls: Besides PD patients, total [67,69-72] or p-α-SYN [70,72,73] immunostaining were likewise found positive in the study controls, whilst there are few studies showing positive immunostaining for total [73-76] and p-α-SYN [77,78] in PD subjects but not in controls. The study conducted by Sánchez-Ferro et al. [79] reported that only 2 out of 29 controls showed the presence of α-SYN [79]. Although α-SYN was expressed in both PD and controls in a study set up by Gold and colleagues, the frequency and intensity of α-SYN expression were much higher in PD, suggesting its usefulness in differentiating PD patients from controls [80]. α-SYN immunostaining was positive in 100% of the PD subjects in three of these studies [74,76,81]. Besides identifying α-SYN in all nine PD individuals of the study conducted by Shannon and colleagues [74], it was also present in normal control subjects and ulcerative colitis patients but with a distinct pattern and low percentage, 8% and 23% respectively. Studies with 100% of α-SYN immunostaining in PD involved only 3-9 patients in total [74,81]. Despite the fact that none of the 161 controls in a large cohort study was found with α-SYN immunoreactivity, it is surprising that only 7 out of 62 PD patients showed accumulation of α-SYN [75]. These results lead us to believe that GI α-SYN may not be sensitive enough to use as a robust PD biomarker. The sensitivity and specificity of α-SYN reported in studies range between 36.1 -85%and 53.2-95%, respectively. In addition, patients in early stages of PD [77], including individuals with biopsies taken before diagnosis of PD [75,81] were investigated in a few separate studies. Detection of total and p-α-SYN immunostaining proved their possible usefulness for early diagnosis. By showing α-SYN as specifically present in subjects with preclinical PD, the large scale study conducted by Hilton et al. provides clear evidence to propose GI α-SYN as a specific early diagnostic biomarker for PD [75]. However, more studies with larger cohorts are needed to further support this hypothesis.

Rostro-caudal gradient: α-SYN was found in different parts of the GI tract, including oesophagus [68], duodenum [68], jejunum [68], ileum [68], sigmoid colon [67-69,74,81], descending colon [78,81], transverse colon [67,68], ascending colon [67,77,78], rectum [68,69,78], rectosigmoid segment [72], upper digestive tract [76], appendix [71], fundus, antral and pyloric region of stomach [67,79]. By analysing immunoreactivity of α-SYN in the colon and rectum, α-SYN exhibited decreasing rostro-caudal gradient patterns [78]. This was in line with the findings of two other studies [67,68], however in contrast with a study having the most abundant and highest density of α-SYN in the appendix in comparison with stomach, ileum and colon [71]. The rostro-caudal gradient detected in various studies suggest that gastric biopsies may be a better choice for studying α-SYN accumulation for PD diagnosis. However, the potential of appendicular sites cannot be disregarded.

Submucosa vs. Mucosa: Among all studies, α-SYN was mostly found in submucosa [74,75,77,78,80,81] of PD patients, whereas some studies also detected its presence in mucosa [67,69,70,75,79]. For those studying the mucosa of subjects, α-SYN was not only detected in PD patients, but also in healthy controls [28,69-71,79]. This further suggests the lack of usefulness of mucosa for PD diagnosis. Although there is one study detecting α-SYN in the submucosa of neurologically intact patients [72], all the other studies reported high potential of submucosa α-SYN in differentiating PD from controls. Taken together, submucosal α-SYN, but not mucosal, was strongly believe to be a useful diagnostic tool for PD.

Correlation of α-SYN with age, disease severity and disease duration: Bottner et al. detected an increase in immuno-reactive signals of p-α-SYN with increasing age while the native form of α-SYN remained the same [72]. Ali and colleagues observed a correlation between density and intensity of α-SYN and severity of disease [76].

Contrary to this, most studies suggest that α-SYN does not reflect the disease status [67,70,78], duration [67] and is not related to age [72]. Chung and colleagues demonstrated that treatment has no effects on α-SYN [67].

Potential of α-SYN in blood as a biomarker

PD vs. Controls: A disparate pattern was observed regarding the presence of α-SYN in blood. A longitudinal study investigated the levels of p-α-SYN, total α-SYN, o-α-SYN and o-p-α-SYN in plasma over a three-month period, using ELISA [82]. In comparison to healthy controls, the level of p-α-SYN was observed as most differentiated in PD, suggesting it might be a

better biomarker. The outcome was different from their previous study [83], which showed a statistically significant rise in o-α-SYN levels in PD patients. This discordance might be explained by variations in control subjects recruited, detection systems and assay sensitivity. Another study detected elevated α-SYN in PD groups [84].

Interestingly, Papaiannakis and colleagues investigated monomeric and dimeric conformations of α-SYN in erythrocytes of PD patients [85]. Dimeric α-SYN was found to be significantly high in PD when compared to healthy controls. There was however, no difference in monomeric α-SYN levels in both PD and healthy subjects. This opens a new area of investigation for researchers to find out the potential of α-SYN dimers in PD diagnosis.

Correlation of α-SYN with Age, Treatment, Disease Severity and Disease Duration: In the study by Foulds and colleagues, p-α-SYN has no link with age, whereas other forms of α-SYN showed a weak correlation with age [82]. This is in agreement with other studies [84,86], reporting no association between PD patient age and total α-SYN and p-α-SYN. Duran *et al.* recruited PD subjects with and without treatments in a study, proving that the relatively high levels of α-SYN was not due to the medications used, supporting the usefulness of α-SYN as a biomarker for PD [84].

Although total α-SYN remains stable within the duration of the cohort studied by Foulds and colleagues, the short duration of this study (four months) may warrant further investigation [82]. In another longitudinal study, levels of total -SYN and p-α-SYN were determined for up to four years [86]. Over this period, substantial elevation in total -SYN levels was observed, whereas p-α-SYN remained high with no longitudinal change. Results found by Duran *et al.* contradicted this, finding no relationship between α-SYN concentration and disease duration [84].

Potential of α-SYN in saliva and salivary glands as a biomarker

PD vs. Other neurological conditions & controls: There are discrepancies in the findings regarding the submandibular gland [68,87-89]. A post-mortem study successfully demonstrated the presence of α-SYN in all PD and 2 out of 3 incidental LB disease, i.e. prodromal PD patients, but not any α-SYN immunostaining in controls and MSA patients, suggesting its usefulness for early diagnosis with the caveat of being an invasive technique [87]. In contrast, Stewart *et al*. suggested that α-SYN level in PD was indistinguishable from controls [89]. Two independent studies [68,88] observed the most p-α-SYN in submandibular gland. Also, p-α-SYN was present in many more sections of PD submandibular gland when compared to AD [68]. However, no controls were recruited in one of the studies [88] for specificity validation. In accordance, the post-mortem study conducted by Beach *et al.* demonstrated that p-α-SYN was mostly located in the submandibular gland and lower oesophagus in comparison with other parts of the GI system in PD [68]. However, it should be noted that most of these studies were explorative with less than 50 subjects [87-91].

The presence of α-SYN in minor salivary gland has also been shown [88,90]. Although it was detected in 2 out of 3 PD cases but none of the 3 controls [90], its usefulness could not be validated as the subjects involved are insufficient to provide reliable and unbiased results. Furthermore, in one study, only 1 in 15 PD patients was identified with p-α-SYN in the minor salivary gland, showing its poor sensitivity [88]. Thus, minor salivary gland may not be an ideal site for PD diagnosis.

In all 3 studies focusing on α-SYN in saliva [89,91,92], total α-SYN, showed no appreciable difference between PD and healthy controls. In fact, 2 out of 3 studies [91,92] noticed a slight reduction in α-SYN in PD when compared to controls but this was not significant enough to prove its usefulness. Instead, a high level of o-α-SYN was observed in the saliva of PD patients compared to controls, suggesting the potential of oligomeric form [92].

Correlation of α-SYN with Age, Treatment, Disease Severity and Disease Duration: Investigations by Kang and colleagues demonstrated that the level of total salivary α-SYN was not influenced by any kind of treatment, but negatively correlated with age in PD patients [92]. Another study does not agree with this finding, reporting no association between α-SYN and age [89].

Kang *et al.* also found no correlation between disease duration and severity of motor symptoms with salivary α-SYN [92], whereas another study found a minor inverse relationship [91]. The potential of o-α-SYN as a biomarker of PD progression was corroborated by showing great differences at each Hoehn & Yahr stage [92].

Potential of α-SYN in skin as a biomarker

PD vs. Other neurological conditions: Although immunostaining of p-α-SYN distinguished PD patients well from individuals with tauopathies and normal controls, sensitivity was the same in MSA cases [93]. Different locations of p-α-SYN staining in PD and MSA, however, allowed these two groups to be distinguishable. It was found present in autonomic fibres in PD, but in somatosensory fibres in MSA. This was further confirmed by Zange and colleagues [94].

PD vs. Controls: Good specificity was observed with cutaneous α-SYN as none of the healthy controls showed immunostaining for total or p-α-SYN [93-97]. However, p-α-SYN was only found in 51% of PD subjects in another study [95]. This could be justified by the immunohistochemical technique applied, which is less sensitive, and the difference in skin thickness analysed. This hypothesis was confirmed by Donadio and colleagues, proving that p-α-SYN staining could be affected by sample thickness in their studies [96]. A study by Wang et al. detected α-SYN in both PD and healthy subjects with higher deposition in PD patients [98].

Five of the six studies used skin biopsies of proximal and distal regions of the leg [93,95-98]. Their results were in high concordance with each other, suggesting that detection of α-SYN from skin biopsies of leg and thigh might be a robust PD biomarker. In addition, no positive p-α-SYN staining was found in the abdominal skin of any of the PD patients in a post-mortem study [90]. This may be due to the difference of biopsy sites taken, suggesting the relative limited usefulness of abdominal sites when compared to legs and thighs.

Interestingly, Donadio and colleagues studied the native form of α-SYN in skin nerve fibres [96]. A similar expression pattern of

α-SYN was observed in PD and control subjects. Thus, this form might be not a useful diagnostic tool.

A decreasing gradient in p-α-SYN deposition from proximal (sites on the back) to distal (distal portions of the leg) sites was found [93,95,96]. Both studies focused on the cervical sites showed 100% of α-SYN detection in PD patients [96,97].

Correlation of α-SYN with disease severity and disease duration: A correlation between α-SYN with PD severity was revealed [98], whereas, p-α-SYN was suggested to have no association with disease duration [94,96].

Comparison between potential of α-SYN on all sites

The biomarker potential of α-SYN in different biofluids and tissues has been summarised in table 1.

The importance of CSF for biomarker studies simply cannot be overstated. Its proximity to the CNS, and the functional role it plays in clearing the CNS of pathogenic material make it an important asset. However, the invasive techniques used to obtain CSF samples make it a less convenient method. In addition, slight contamination of blood in CSF can lead to a great elevation of CSF α-SYN levels, giving a false result. Although no correlation between α-SYN levels and haemoglobin levels was suggested by some authors, a lot of evidence demonstrates otherwise [62,63,99]. Hong and colleagues [63] found no statistical difference in α-SYN levels of PD and controls when high levels of haemoglobin were permitted [63]. Also, CSF α-SYN levels was modestly increased when haemoglobin contamination was more than 1000 ng/mL [62]. Although the authors claimed that levels of haemoglobin were similar among all subject groups, the assumption might be misleading as the levels had a wide range from 4400 - 6100ng/mL in their cohort. Lastly, CSF α-SYN might not be the most suitable candidate for a longitudinal biomarker when taking accessibility into account.

GI -SYN seems to be an ideal tool for early diagnosis, but the major limitation is its inconsistent sensitivity and specificity among different studies. GI α-SYN also seemed to show no association with disease duration or status. Therefore, it might not be an ideal biomarker for disease progression. It is noteworthy that sensitivity of sub mucosal α-SYN was better than mucosal α-SYN as shown by some studies [78,79,81].

Collection of blood is less invasive compared with GI or CSF. Moreover, consistent results in regards to negative correlation of blood α-SYN with age and treatment act as mounting evidence of its promise for diagnosis.

A high accessibility of saliva and low blood contamination risk make it an ideal candidate for disease progression monitoring. However, the salivary gland was more useful than saliva in differentiating PD from the healthy controls. Although the minor salivary gland proved to be inconclusive, the submandibular gland showed better promise in differentiating PD from AD and MSA. p-α-SYN was useful in differentiating PD subjects from MSA, based on its deposition in different nerve fibres. Furthermore, skin biopsies taken from the proximal portions of the legs and back were shown to be the most efficacious. However there remains a need to standardise the methods of investigation as sensitivity and specificity are believed to be influenced by the number of sections and thickness of samples taken.

Current limitations and on-going efforts

The major limitations of current studies are the lack of standardisation on methodologies and outcome measures. This is commonly attributed to inter-laboratory discrepancies and

Site	Strengths	Limitations
CSF	(1) Site with the most reproducible and consistent findings (2) High sensitivity (3) Direct reflection on disease states (4) Relatively cheap in comparison with imaging biomarkers (5) Combination of CSF biomarkers able to distinguish PD from AD and MSA	(1) Inadequate specificity (2) Unable to access in most clinical settings (3) Risk of false positive results due to blood contamination (4) Invasive method
GI tract	(1) Consistent findings in submucosa (2) Relatively easy sampling method by using endoscopy (3) Potential for early diagnosis	(1) Inconsistency of diagnostic accuracy due to rostro-caudal gradient (2) Lack of usefulness on mucosa (3) Relatively invasive (4) Require a large amount of preparation before sampling process
Blood	(1) Accessible (2) Cost-effective (3) Effective in differentiating PD patients from controls	(1) Inconsistent findings (2) Risk of false results due to lysis of blood cells
Saliva and salivary glands	Saliva (1) Accessible (2) No risk of blood contamination (3) Easy and direct collection methods (4) Cost-effective (5) Painless (6) Ideal for longitudinal monitoring Glands (1) Able to be performed in out-patient (2) Less invasive (3) Submandibular gland is useful in detection of prodromal stages of PD	Saliva (1) Lack of usefulness in differentiating PD from controls Glands (1) Lack of usefulness of minor salivary gland (2) Side effects, e.g., swollen cheek and sore throat
Skin	(1) Accessible (2) No risk of blood contamination (3) Less invasive (4) Consistent findings on the proximal sites of the leg (5) Ideal for longitudinal monitoring	(1) Difficulty with keeping sample thickness consistent. (2) Abdominal sites not useful

Table 1. Strengths and limitations of α-SYN as a PD biomarker in different sites of the body.

leads to a lot of consequential problems on the validation of a biomarker for PD. The differences in assay methods, subjects recruited, α-SYN species and duration of studies, make it difficult to compare data all together.

The absence of a standardised assay for α-SYN makes it difficult to identify well-defined sensitivity and specificity of α-SYN for diagnosis of PD. Furthermore, a clear cut-off point of the diagnostic range of α-SYN level is still absent due to the varying results across laboratories. This is achievable if standardised protocols are set up and used across different studies. It is crucial to identify the upper or lower limits of normal α-SYN levels in different sites because this would allow α-SYN to provide true positive results accurately on the diagnosis of PD.

To address this limitation, well-characterized methodologies, including number and types of subject recruited, clinical data acquisition, affinity of antibody, sampling methods, assay design and controlled variables need to be established. For sampling methods, it is recommended to collect more than one sample from each subject for assaying. For studies on skin and colon, thickness of samples should likewise be outlined. Methods of handling, storage and processing the samples would all affect study outcomes. Several standardized protocols for the collection and storage of CSF have been established, such as those during the BioMS-eu (2007) annual meeting [100]. Factors such as volume of collection and temperature of storage greatly affect the concentration and stability of biomarkers [100]. The type of needle used (atraumatic Sprotte or Whitacre) greatly affects patient comfort levels, in turn influencing compliance. Storage of the CSF should ideally be done in polypropylene tubes, which have the least protein binding affinity, and will thus influence α-SYN levels the least [100].

A small degree of contamination with red blood cells could easily affect the levels of α-SYN present in CSF and plasma. Thus, an acceptable range of blood contamination should be determined to allow consistent exclusion criteria in all future studies. Another example of important variables is the disease state and number of subjects recruited.

A few programmes have been set up to resolve the challenges and speed up PD biomarker validation, such as Bio FIND [101] and The PD Biomarker Program (PDBP) [102]. A project supported by PDBP tries to evaluate all ranges of data and identify the patterns across different investigating sources. The Michael J. Fox Foundation (MJFF) has also funded grants to evaluate different assays and performance consistencies of α-SYN [103]. This can be done by sharing samples, reagents, methods and findings. After identifying the most powerful assay for α-SYN detection and quantification, it can then be used across different investigations and inter-laboratory data generated can be comparable.

Conclusions & Future Perspectives

CSF α-SYN produced highly consistent results in different studies, and seems to be the most promising diagnostic biomarker for PD. However, the potential blood contamination and relatively invasive methods for obtaining CSF samples are its major drawbacks. Based on the very limited data currently available on this aspect, plasma might be a better option due to its great accessibility. The GI tract shows the most usefulness during pre-symptomatic stages. This is extremely important for PD, allowing intervention as soon as possible to minimize decline. Also, it is very encouraging to identify the potential of cutaneous p-α-SYN on differential diagnosis between PD and MSA. This is a big step forward in this field as it could barely be achieved by using traditional clinical diagnosis. Furthermore, a combination between α-SYN and other biomarkers such as $A\beta_{1-42}$ are believed to be a better option to achieve optimum sensitivity and specificity.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Koo Wee Hiew, Anuri Shah, Cristina Legido-Quigley designed and drafted the manuscript; all authors read and revised the final manuscript.

Acknowledgements

Anuri Shah is funded by the Postgraduate Scholarship from The University of Hong Kong. Pei Han is sponsored by the China Scholarship Council.

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101. Parkinson's Disease | The Fox Investigation for New Discovery of Biomarkers (BioFIND)

102. PDBP: Parkinson's Disease Biomarkers Program | PDBP : Parkinson's Disease Biomarkers Program.

103. Parkinson's Disease | Alpha-Synuclein Assay Standardization LEAPS.