Alpha-synuclein as a Potential Biomarker for Parkinson's Disease and Other Synucleinopathies: Gaps, Challenges, and Opportunities

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Abstract

Parkinson's disease (PD), the second most common progressive neurodegenerative disease, develops and progresses for 10-15 years before the clinical diagnostic symptoms of the disease are manifested. Furthermore, several aspects of PD pathology overlap with other neurodegenerative diseases (NDDs) linked to alpha-synuclein aggregation, also called synucleinopathies. Therefore, there is an urgent need to discover and validate early diagnostic and prognostic markers that reflect disease pathophysiology, progression, severity, and potential differences in disease mechanisms between PD and other NDDs. The close association between alpha-synuclein (aSyn) and the development of pathology in synucleinopathies, along with the identification of aSyn species in biological fluids, has led to increasing interest in aSyn species as potential biomarkers to detect PD and differentiate it from other synucleinopathies. In this review, we 1) provide an overview of the progress toward mapping the distribution of aSyn species in the brain, peripheral tissues, and biological fluids; 2) present comparative and critical analysis of previous studies to measure total aSyn as well as specific forms of monomeric, modified and aggregated forms of aSyn in different biological fluids; and 3) highlight conceptual and technical gaps and challenges that could hinder the development and validation of reliable aSyn biomarkers and outline a series of recommendations to address these challenges. Finally, we propose a combined biomarker approach based on integrating total aSyn levels with specific biomarkers based on the biochemical features (posttranslational modifications), aggregation and structure of aSyn, in addition to other biomarkers of neurodegeneration. We believe that capturing the diversity of aSyn species is essential to developing robust assays and diagnostics for early detection, patient stratification, monitoring of disease progression, and differentiation between synucleinopathy subtypes. This could transform clinical trial design and implementation, accelerate the development of new therapies, and improve clinical decisions and treatment strategies.

Introduction

Parkinson's disease (PD) is the second most common progressive neurodegenerative disease (NDD), with a worldwide prevalence rate of approximately 1-4% in people aged over 60 years [1]. The incidence of PD is expected to increase as a result of higher life expectancy [2]. PD is characterized by the progressive loss of dopaminergic neurons and the deposition of aggregated alpha-synuclein (aSyn) into intracellular inclusions that accumulate in the form of Lewy bodies (LBs) in cell bodies and Lewy neurites (LNs) in axons and dendrites [3]. To date, clinical PD diagnosis has been based on motor features, along with nonmotor symptoms such as psychiatric and autonomic features and sleep disturbance [4-7]. Detection of aSyn pathology in the postmortem brain remains the primary means of reaching a conclusive diagnosis, often revealing that significant cases of PD have been misdiagnosed [8]. Because the diagnosis of PD relies on clinical symptoms that are manifested only after a substantial and irreversible loss of dopaminergic neurons in the substantia nigra (SN), there is an urgent need to identify PD-specific biomarkers that allow diagnosis at the onset and/or early stages of the disease[9]. Furthermore, given the clinical and neuropathological overlap between PD and other synucleinopathies (e.g., dementia with Lewy bodies (DLB) and multiple system atrophy (MSA)), there is also a need for biomarkers that would allow differentiation between synucleinopathies. The discovery of early diagnostic and prognostic markers that reflect disease pathophysiology, progression and severity and reflect potential differences in disease mechanisms are of paramount importance and hold great promise for improving the design of clinical trials and the development of novel disease-specific diagnostic tools and therapies for PD and other synucleinopathies.

aSyn as a potential biomarker

Several experimental observations have led to the emergence of aSyn as a leading therapeutic target and biomarker for PD. First, aggregated forms of aSyn are major components of LBs and LNs, the pathological hallmarks of PD [3,10-14]. aSyn aggregates are also found in the brains of patients with other synucleinopathies, including dementia with Lewy bodies (DLB), multiple system atrophy (MSA) and Alzheimer's disease (AD) [15-17]. Second, mutations or duplications of the SNCA gene appear to be sufficient to cause PD or Lewy body dementia (LBD) [18-27]. Several familial forms of PD have been linked to increased expression of aSyn due to SNCA gene duplication or missense point mutations (single amino acid substitutions), such as A30P,

E46K, H50Q, A53E and A53T [23,25,26,28-30]. Moreover, other aSyn point mutations, such as the G51D mutation, were reported to phenotypically display common neuropathological features of PD and MSA [31,32]. More recently, an E83Q mutation in aSyn was identified in a patient suffering from DLB and atypical frontotemporal lobar degeneration [27,33]. Third, the level of aSyn aggregates in the CSF and skin biopsies distinguishes PD patients from controls with high accuracy [34-36]. Fourth, several animal models show that overexpression of aSyn (the wild-type form or disease-associated mutant forms) or inoculation of aSyn into the central nervous system (CNS) and peripheral tissues induces aSyn pathology formation and/or pathology spreading into brain regions that are affected in PD and other synucleinopathies [37-39]. These observations, combined with the findings that aSyn aggregation and pathology spreading in rodent models increases with cumulative aSyn, point to aSyn as a central player in PD pathogenesis [40-43]. However, whether aSyn aggregation and neurodegeneration are the primary initiators of the pathological process in PD remains a subject of active debate. Although several aSyn-targeting therapies are being tested in the clinic, reliable tools and assays to assess aSyn target engagement are still lacking. This has led to increasing efforts to develop and validate assays to identify, quantify and validate different aSyn species as potential biomarkers for synucleinopathies.

aSyn in body fluids and peripheral tissues

The discovery that aSyn is readily secreted into extracellular spaces and can be found in different forms (monomeric and seeding-competent aggregated forms) in body fluids such as CSF [44-52], blood components [53-62], saliva [63-67] and tears [68,69] as well as in peripheral tissues (e.g., skin, esophagus, colon) [36,70-72] sparked even greater excitement about aSyn biomarkers. The diversity of peripheral sources of aSyn presents unique opportunities to develop noninvasive diagnostic and prognostic tools based on measuring the levels of individual or multiple aSyn species (reviewed in [73-75]) (Figure 1).

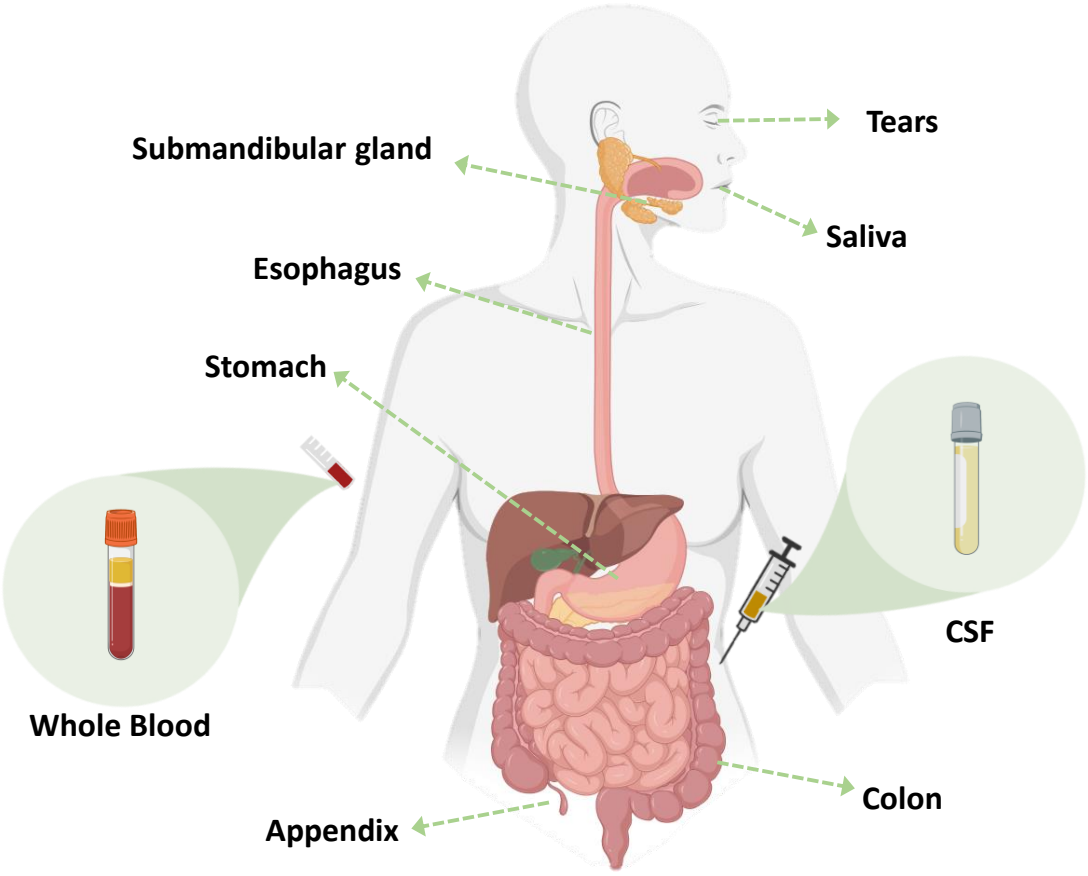

Figure 1. Detection and quantification of total aSyn in biological fluids and peripheral tissues.

Although various biomarker-based studies (using peripheral tissues and body fluids) have been performed to detect and quantify different aSyn species levels (i.e., total, oligomeric/aggregated or modified forms (those with posttranslational modifications, or PTMs) [73-75], there is not yet a consensus on which form(s) represent reliable biomarker(s) for early diagnosis, patient stratification or monitoring of disease progression.

Initial studies focusing on quantifying total aSyn levels in CSF reported a general trend of decreased aSyn levels in patients with PD compared to healthy controls [44-47]. However, several factors have called into question the utility and effectiveness of total aSyn in CSF as a biomarker in clinical practice; these factors include 1) the broad range of aSyn levels reported [44-46,52], 2) studies reporting no correlation with PD progression/severity as well as a considerable overlap of aSyn levels with controls and other NDDs [45,59,75,76] and 3) poor interlaboratory reproducibility. Because of these limitations, several groups have pursued modified aSyn species (i.e., aSyn phosphorylated at S129 (pS129) [49,50,53,77-80] or aggregated

forms of aSyn [79,81,82]) as potential biomarkers. Unfortunately, independent replication and validation of many of these studies remain challenging. This has hampered efforts to systematically evaluate and validate the diagnostic value of measuring aSyn species, emphasizing the need for more robust assays that capture the diverse range of aSyn species (total aSyn) or specific modified or aggregated proteins.

The use of specific assays designed to amplify and detect minute amounts of aggregated aSyn in biological samples, such as real-time quaking-induced conversion (RT-QuIC) and protein-misfolding cyclic amplification (PMCA), have consistently shown the presence of seeding-competent aSyn species in CSF and peripheral tissue biopsies (e.g., from the skin and colon), which could serve as a reliable diagnostic marker for PD with higher accuracy, sensitivity and specificity [35,72,83-86]. However, these assays do not yet allow reliable discrimination between different synucleinopathies or monitoring of disease progression and severity. However, recent applications of these assays suggest that they could be further optimized and developed to differentiate between PD and other synucleinopathies (MSA from PD and LBD) [36,87] and potentially identify presymptomatic cases years before they develop PD [34,88]. Considering that CSF and skin biopsies are relatively invasive and place considerable demands on the patient and clinician, expanding the application of these assays to other biological fluids or their derivatives, such as different blood components (e.g., whole blood, erythrocytes or plasma), saliva and tears, could pave the way for the development of noninvasive point-of-care diagnostics.

Several aSyn PTMs are closely associated with the progression of pathology in synucleinopathies

PTMs play a key role not only in modulating protein structure and function but also in regulating clearance, localization and secretion. Hence, PTMs could act as molecular switches for regulating biological processes in health and disease. In the context of NDDs such as PD, AD, and amyotrophic lateral sclerosis (ALS), PTMs have emerged as key markers of extra- and intracellular inclusions that represent the pathological hallmarks of NDDs. Biochemical studies of LBs, neurofibrillary tangles and other pathological aggregates linked to NDDs have consistently demonstrated that the aggregate-forming proteins (e.g., aSyn, Tau, TDP-43 and amyloid-beta) accumulating in these pathological inclusions or deposits are subjected to a wide range of PTMs (e.g., phosphorylation, glycosylation, acetylation, nitration, SUMOylation,

and ubiquitination) [89]. Moreover, many of these PTMs cluster in neighboring sites and compete for the same residue. The close association between specific PTMs (e.g., aSyn pS129 and phosphorylation of Tau at different residues such as Thr181, Ser262 and Ser404) and pathological aggregates [16,90,91] has led to the emergence of antibodies against such PTMs as the primary tools to detect, monitor and quantify pathology formation in the human brain and animal models of NDDs. However, the precise role of PTMs in regulating protein misfolding, aggregation, and the development and spread of pathology in NDDs remains incomplete. Interestingly, recent findings suggest that the great majority of PTMs in pathological aggregates may occur after aggregation (see recent reviews [92-94]). This suggests that they could play important roles in regulating the secretion of aSyn aggregates and influence their seeding activity in the CSF. Therefore, their detection in biological fluids may indeed provide a window to pathological aSyn in the brain.

Several aSyn PTMs have been identified in the postmortem brain tissues of patients with PD and other synucleinopathies using different approaches such as mass spectrometry (MS) and antibody-based assays (e.g., immunohistochemistry) [16,17,95-98]. Among the most frequently reported aSyn PTMs are acetylation (at the N-terminus and lysine residues), ubiquitination, phosphorylation (at S129 and, to a lesser extent, at Y39, S87, Y125), and nitration, as well as several N-terminal and C-terminal truncations [16,17,95-98]. In addition, pS129 aSyn species have been detected in several peripheral tissues, including the skin, esophagus and colon, of patients with PD and synucleinopathies [36,70-72], although the correlation between pS129 levels and peripheral aSyn pathology remains unclear, mainly because most studies describe the detection of pS129 immunoreactivity without assessing the aggregation state of aSyn.

Among the many aSyn PTMs found in the brain, pS129, truncations, and ubiquitination are the most commonly detected PTMs and correlate with pathology formation [89] (Figure 2A). However, most studies have focused mainly on exploring the role of aSyn pS129 in the pathogenesis of synucleinopathies and its potential as a PD-related biomarker. This is primarily because it is one the most abundant PTMs but also because several antibodies against this PTM are available, whereas few antibodies are available for other modified aSyn species, such as ubiquitinated and truncated forms.

Despite the increasing evidence pointing to N- and C-terminal truncations as the second most common type of aSyn PTM in the brains of patients with PD and other synucleinopathies [16,99-101], truncated aSyn species have also been reported to be present in the soluble fraction of healthy human brains, suggesting that they may be involved in regulating some physiological functions of the protein [16,95-98]. Nevertheless, analysis of human brain tissues has revealed that C-terminal truncations of aSyn are highly enriched in the pathological inclusions of different synucleinopathies, such as PD [16,98-100,102-111], DLB [16,98,101-103,106,109,112], and MSA [16,99,108,113], and in AD patient brains without LB pathology [105] compared to the insoluble fraction from control subjects. Although the majority of these studies relied primarily on antibodies and western blotting analyses, the presence of C-terminal truncations has been further verified by unbiased tandem mass spectrometry (MS/MS) approaches. These studies revealed the presence of C-terminal truncations in PD [95,96,98], DLB [16], MSA [17] and PD with dementia [98] (Figure 2A). Among the most representative truncated species were aSyn 1-119 and 1-122 [16,17,96,98]. Recently, N-terminal truncations (5-140, 39-140, 65-140, 66-140, 68-140 and 71-140) (Figure 2A, 2B) have also been detected in human LBD brains [101] and PD brains [95,96]; however, the pathological relevance of these N-terminally truncated forms of aSyn remains unclear. Surprisingly, aSyn truncations have also been reported to be present in the human appendix [104], with the great majority of truncated aSyn species being cleaved in both the N- and C-terminal regions of the protein. However, a comparison of the various aSyn species detected in the brain and the appendix (Figure 2B) reveals major differences in the cleavage sites and distribution of truncated aSyn species [16,95,96,98,104]. Figure 2C summarizes the different PTMs identified in the brain, biological fluids and peripheral tissues.

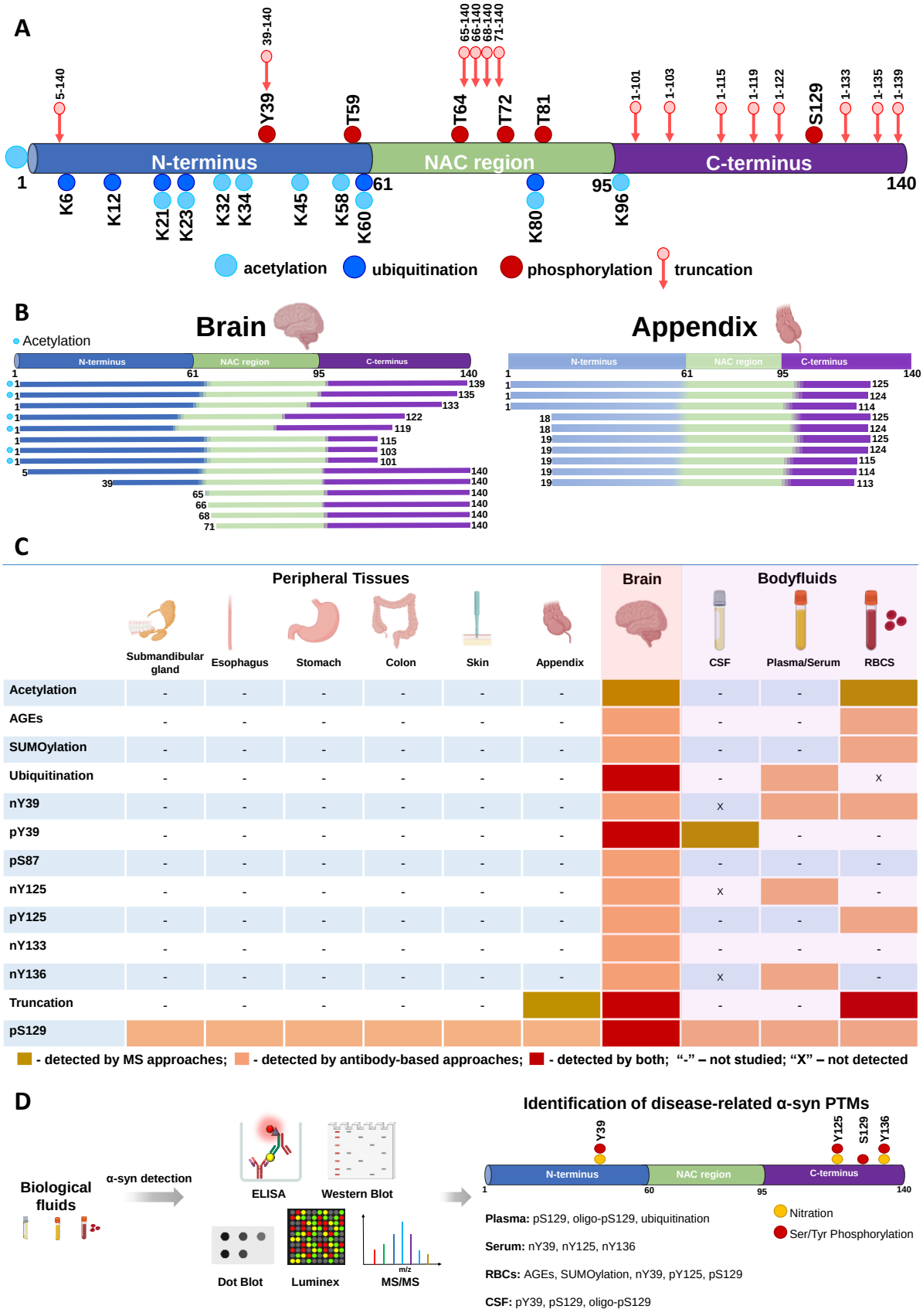

Figure 2. Schematic illustration of aSyn PTMs identified in biological specimens from human synucleinopathy patients. A) aSyn PTMs detected in the brains of patients with

synucleinopathies by MS studies and immunohistochemistry. B) Comparison of the truncated aSyn species identified in the brain and appendix identified by MS and antibody-based approaches. C) Overview of aSyn PTMs identified in the peripheral tissues, brain and body fluids. Various aSyn PTMs have been reported to be present in the human brain using different approaches, i.e., MS or antibody-based assays. Remarkably, some of these PTMs have also been identified in different peripheral tissues and body fluids. aSyn pS129 is the most extensively studied disease-related PTM and is reported to be present in various biological specimens. D) PTMs of human aSyn in body fluids. Several assays have been developed and used to profile, identify and quantify unmodified aSyn along with its modified species in different biological fluids. Various PTMs related to aSyn diseases have been identified in different constituents of the blood [plasma, serum and red blood cells (RBCs)] and CSF.

The close association of different modified and aggregated forms of aSyn with pathological aSyn inclusions in the brain in the context of PD and other synucleinopathies has led to increasing interest in potentially quantifying the levels of these species in biological fluids as potential disease biomarkers. This section presents a comparative and critical analysis of previous studies to measure total aSyn and specific forms of monomeric, posttranslationally modified, and aggregated (oligomer and fibril) forms of aSyn in different biological fluids.

aSyn PTMs in biological fluids and their potential as biomarkers

The close association between aSyn pathology in the brain and several aSyn PTMs, combined with converging findings demonstrating that neurons secrete different forms of aSyn, has led to the pursuit of modified aSyn species in different peripheral tissues (e.g., appendix, skin, colon and esophagus tissue) [36,70-72] and biological fluids [73,75] (Figure 2C) as potential biomarkers of peripheral pathology or PD. The assumption here is that the distribution of aSyn species in biological fluids, such as the cerebrospinal fluid (CSF) or blood, may provide a window to the dynamics of aSyn proteoforms in the PD brain and reveal changes that reflect the extent of pathology and/or disease progression. Over the past decades, various methods and assay platforms have been employed to profile, detect and quantify modified aSyn isoforms in body fluids (Figure 2D), including western blots; dot blots; ELISA; biotin ELISA; phospholipid ELISA; electrochemiluminescence; immunomagnetic reduction (IMR)-based, Luminex, and Singulex assays; MS; and modified paired surface plasma wave biosensors coupled to immunoassays.

In this review, we will 1) provide an overview of the progress made toward mapping aSyn PTMs in different biological fluids (CSF, plasma, serum, and RBCs); 2) present critical analyses of previous studies that have sought to explore the potential of total aSyn and aSyn PTMs as biomarkers to monitor disease progression or differentiate between PD patients and healthy controls or between PD and other synucleinopathies; 3) present a gap analysis to help guide future aSyn biomarker studies; 4) highlight some of the current challenges in targeting modified and aggregated aSyn species as potential biomarkers for PD; and 5) provide an overview of how recent advances in protein synthesis and more sensitive approaches (MS) to detect PTMs may help address these challenges. Finally, we outline a series of specific recommendations for the design of future biomarker studies, sample handling, and research tool development and validation that we believe will pave the way to develop sensitive and accurate assays that capture and more accurately measure the diversity of aSyn forms in biological fluids and samples. The need for a more integrative approach that combines multiple biomarkers linked to different disease mechanisms implicated in PD and other synucleinopathies is also discussed. Table 1 summarizes the different studies regarding aSyn PTM biomarkers of PD and other synucleinopathies using biological fluids; these studies will be described in detail in the next sections.

aSyn in the CSF

CSF remains one of the main accessible body fluids and provides a window to biochemical and neuropathological changes in the brain. Therefore, it is not surprising that the search for aSyn biomarkers has focused primarily on CSF, especially in the absence of validated aSyn-pathology-specific brain positron emission tomography (PET) tracers and biomarkers. Initial studies focused on measuring total aSyn levels, but interest in aggregated and phosphorylated forms (namely, pS129) increased over time, with converging evidence suggesting links between these species and aSyn pathology in the brains of patients with PD and other synucleinopathies.

Total aSyn levels in the CSF: Although total aSyn is consistently detected in the CSF, mainly using ELISA and other immunoassays, the reported levels of CSF aSyn in control individuals and patients suffering from PD or other synucleinopathies vary significantly from one study to another [44-52]. For example, levels of total aSyn were reported to be in the range of 67-68900

pg/mL and 94.2 to 55000 pg/mL in controls and PD patients, respectively [44-47,52,114-116] (Figure 3A, Table 2). The majority of these studies had small sample sizes, and, remarkably, the numbers of samples derived from PD patients and controls used did not increase across the years (Figure 3B). In other synucleinopathies, the reported total aSyn levels are as follows: 1) DLB: 58 to 59000 pg/mL; 1420±1260 pg/mL, 2) MSA: 108 to 56000 pg/mL and 3) progressive supranuclear palsy (PSP): 428 to 63000 pg/mL [44,45,50,114-116] (Figure 3C). A single study by Foulds and colleagues reported much higher levels of total aSyn, specifically in the range of µg/mL instead of the observed pg/mL, for patients suffering from different synucleinopathies as well as control subjects (PD: 1.85 ±2.40 µg/mL (average); DLB: 2.31±2.51 µg/mL; MSA: 3.80±2.40 µg/mL; PSP: 1.45±1.97 µg/mL and healthy controls (HC): 1.87±2.29 µg/mL) [49]. These large variations have been attributed to several preanalytical and analytical confounding factors as well as to clinical and demographic data heterogeneity, comorbidities and potential medical treatments that have been extensively discussed in recent reviews (see [75,76,117]). This has led to inconclusive findings regarding differences in aSyn levels between PD patients and other parkinsonism patients and whether changes in total aSyn levels could be used to monitor disease progression. For example, several cross-sectional studies showed a decrease in total aSyn levels in PD patients [44-47] compared to HC individuals and controls with other neurodegenerative diseases, which was corroborated to some extent by a number of meta-analysis studies [51,118-120]. Nonetheless, several studies did not show that there was any correlation between total aSyn levels and disease progression/severity or that changes in aSyn levels provided a reliable marker that distinguishes between PD and other synucleinopathies, such as MSA or PSP [45,59,76]. However, two studies comparing PD patients with controls reported an association with PD severity/progression [78,121]. Furthermore, in a recent meta-analysis study, Eusebi and colleagues reported that aSyn levels did not differentiate PD from other types of parkinsonism [51]. One consistent finding in the majority of these studies is that aSyn can be reliably detected in the CSF (Figure 3A).

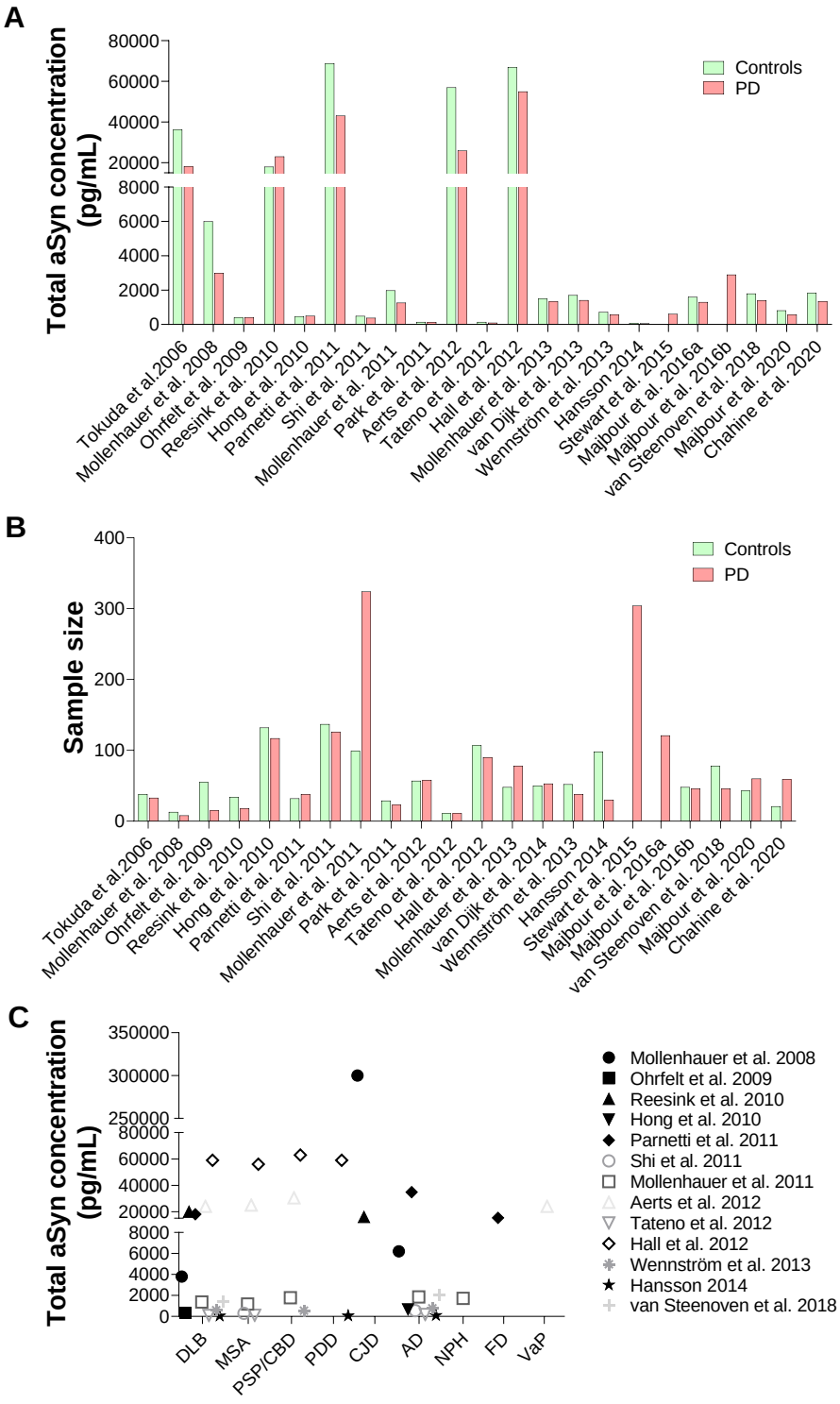

Figure 3. Graphs depict the CSF levels of total aSyn in PD, controls and patients suffering from other synucleinopathies (e.g., DLB, MSA, PSP) and tauopathies. A) Comparison of CSF levels of total aSyn between PD and controls in different studies. B) Sample size of PD and controls used in the studies that aimed to measure the levels of total aSyn. C) Variability of CSF levels of total aSyn across other disease-group patients. Each dot in the graph displays the average values of aSyn pS129 reported in the respective study.

Key – CBD: corticobasal degeneration; MCI: mild cognitive impairment; VaP: vascular parkinsonism; CJD: Creutzfeldt–Jakob disease; NPH: normal-pressure hydrocephalus; FD: frontotemporal dementia

In addition to the confounding factors highlighted above, differences in the distribution of modified aSyn forms could also contribute to the large variations in aSyn levels, especially since the great majority of the capture and/or detection antibodies used in most aSyn immunoassays target the C-terminal domain of aSyn (residues 110-130), which harbors the most abundant pathology-associated aSyn modifications (e.g., phosphorylation and C-terminal truncations; Figure 2A).

pS129: The search for posttranslationally modified forms of aSyn in CSF initially focused on aSyn pS129 because of the converging evidence demonstrating that pS129 is the predominant modified form of aSyn in LBs (reviewed in [122]) and a reliable marker of pathology that correlates with the increase in pathology formation in the brain [49,50,53,77-80]. Several studies suggested that CSF pS129 levels might enable the diagnosis of PD [50,51,77,79] or differentiate not only between PD patients and control subjects but also between different synucleinopathies [49,123,124] (Figure 4A). Nevertheless, the results from these studies revealed high variability in terms of the detected pS129 levels in the CSF, ranging from no detection to 7.14±9.19 µg/mL [49,53] (Figure 4B).

When comparing the CSF pS129 levels between healthy controls and PD patients, several studies reported a significant elevation in the latter [50,51,77,79]. One study suggested that the observed change in pS129 levels allows differentiation between diagnostic cohorts [50]. However, other studies observed no significant change in the mean pS129 values between healthy controls and PD patients [49,80,123,125] or samples were compared between patients with PD and those with other synucleinopathies [49,123,124] (such as DLB [49,123], MSA or PSP [49,124]) or those with tauopathy [123,124]. One study reported the following pS129 concentrations in different subgroups: MSA (7.14±9.19 µg/mL); PSP (5.14±9.73 µg/mL); DLB (1.63±1.42) and controls (3.58±3.85 µg/mL). Despite the lack of significant differences, MSA and PSP patients showed increased CSF pS129 values, while DLB patients exhibited reduced levels in comparison to healthy individuals [49]. Furthermore, pS129 levels were slightly higher in MSA patients than in PSP patients and higher than those in DLB patients [49]. However, in another report, Wang and collaborators evaluated pS129 levels in different subgroups, comprising

samples derived from PD, MSA and PSP patients along with controls, and reported values on the scale of pg/mL [50]. This study revealed reduced pS129 levels in patients suffering from MSA and PSP in comparison with PD patients and controls. Interestingly, they observed similar concentration levels between the MSA and PSP disease groups, while the PSP cohort displayed significantly lower pS129 levels than controls [50].

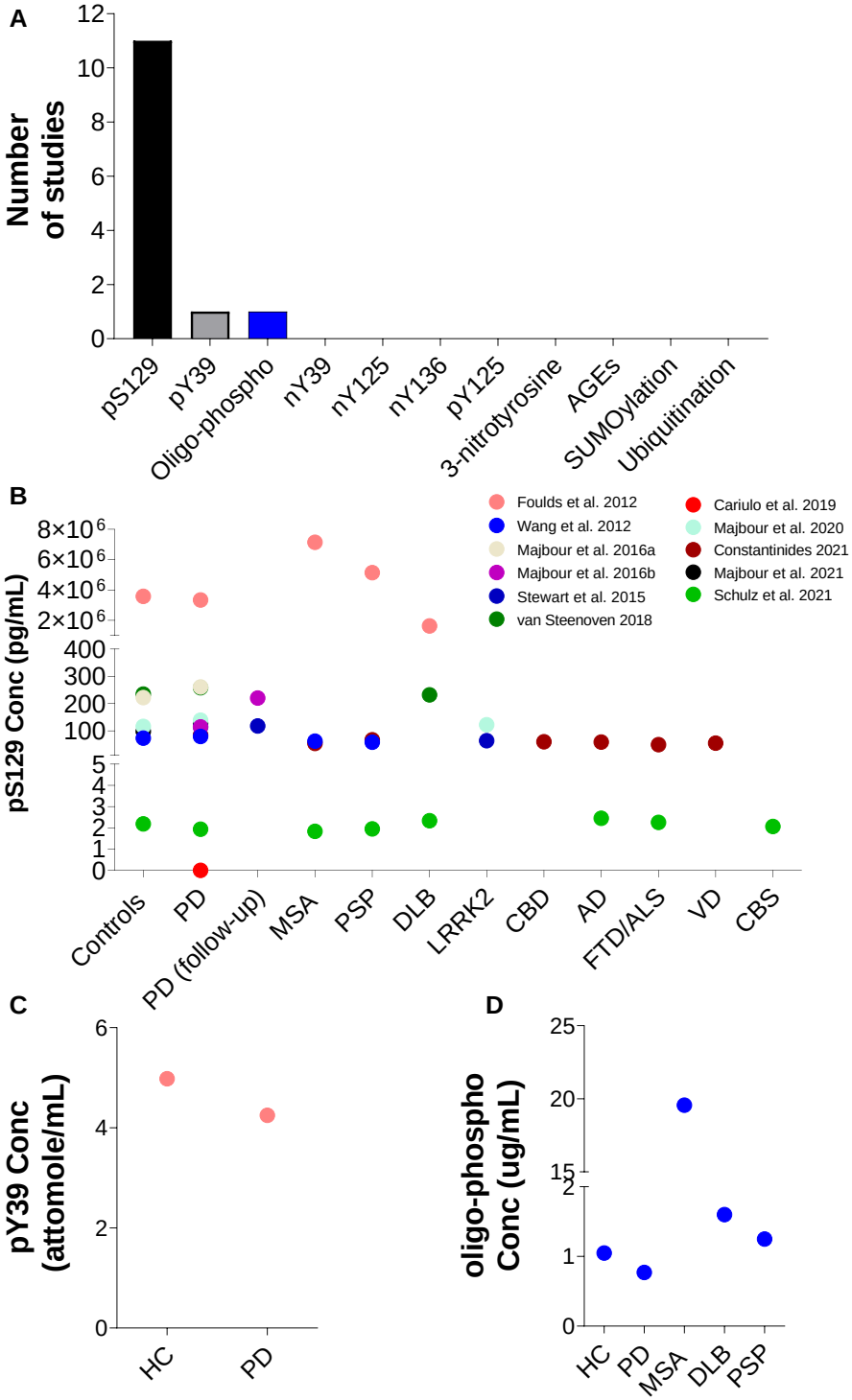

Figure 4. Landscape of aSyn PTMs detected in CSF. A. Number of studies that identified CSF aSyn PTMs. B. Variability of pS129 levels across the different disease groups and controls. Each dot in the graph corresponds to the average values of aSyn pS129 reported in the respective study. Other aSyn PTMs, i.e., C. pY39 and D. oligo-phosphorylation, were reported in single studies.

The correlation between pS129 aSyn levels and disease severity was also assessed in several different studies, but inconsistent results were reported. Wang et al. [50] reported a correlation of decreased pS129 levels with PD severity, which was further corroborated by others [77,78], suggesting that pS129 in the CSF may serve as a progression biomarker for PD and is associated with different PD clinical phenotypes. However, a study by Majbour et al. [79] did not show any association between pS129 levels and disease severity and progression. Moreover, in a recent study, Majbour and colleagues reported that the aSyn pS129 levels of PD patients were lower at the two-year follow-up than at baseline; however, this observed reduction did not display statistical significance [126].

It has been suggested that the ratios of pS129 to total aSyn and/or oligomeric aSyn to total aSyn, as opposed to pS129 levels only, may provide a superior diagnostic marker and could differentiate between PD and other synucleinopathies [50,77,80,81,127,128]. Two studies reported an elevated ratio of pS129 aSyn to total aSyn in PD and symptomatic and asymptomatic LRRK2 mutation carrier patients compared with controls [50,80]. The level of pS129 aSyn/total aSyn was also shown to increase with disease progression in PD patients in two different follow-up studies [77,78], enabling the differentiation between MSA and PSP patients [50]. To the best of our knowledge, only one study has sought to assess the level of phosphorylated aggregated forms of aSyn in the CSF. Remarkably, in this study, the authors reported that the phosphorylated oligomeric form of aSyn varied among the PD, DLB, PSP, MSA and control groups, with highly significant differences reported in MSA (19.56±1.66 µg/mL) in comparison with all the other groups (PD: 0.75±1.15 µg/mL (average); DLB: 1.60±3.02; PSP: 1.25±3.32 µg/mL; and controls: 1.05±2.23 µg/mL) (Table 1) [49]. Interestingly, the level of the phosphorylated oligomeric form of aSyn was marginally decreased in PD patients in comparison to controls (Figure 4A and C) as well as DLB and PSP patients. Furthermore, DLB and PSP patients displayed slightly higher levels than controls, while the DLB group displayed marginally higher levels than PSP patients. However, in MSA patients, this aSyn species was estimated to be at an approximately 20-fold

higher concentration than in other diseased patients (Figure 4C) [49]. Surprisingly, the levels of pS129 detected in CSF have displayed great variability [49,50,77-80,123,124] (see Table 1).

Recently, Cariulo and colleagues [53] developed the Singulex Erenna immunoassay, an ultrasensitive immunoassay based on a quantitative fluorescent sandwich immunoassay coupled to single-molecule counting technology, for quantifying total aSyn and pS129 species in the range of pg/mL. They reported that pS129 could be readily detected in human plasma (at approximately 878.5±317.4 pg/mLa0 but not in CSF. This sensitive assay could detect recombinant and homogeneous pS129 aSyn with a detection limit of 0.15 pg/mL. It is noteworthy that using an IP-MS/MS method with a detection limit of 78 pg/mL, we failed to detect pS129 in the CSF from PD patients or healthy controls (unpublished data). One major difference between these two studies and previous reports is that these studies relied on the use of a highly pure pS129 protein calibrant, which is site-specifically and homogeneously phosphorylated at S129, whereas many of the previous studies used impure calibrants that were generated by in vitro phosphorylation of recombinant aSyn using kinases that were later shown to only partially phosphorylate aSyn (Table 1). Although recent reports have used more efficient kinases, such as PLK2 or PLK3, to generate pS129 calibrants [129], the extent of phosphorylation was not assessed or reported, and it was not clear if additional steps were implemented to purify pS129 aSyn (Table 1). The great variability of pS129 levels identified in the CSF of patients suffering from different synucleinopathies ranging from no detection [53] to values ranging from pg/mL to µg/mL scale [(1.84±0.71 to 261(206.8–296.3) pg/mL; (7.14±9.19) µg/mL] [49,50,77-80,123-125], combined with the failure of several groups to replicate some of these studies and lack of validation by antibody-independent methods, has precluded the use of pS129 levels as a reliable biomarker. Given that many of the early studies relied on the use of poorly characterized pS129 calibrants (Table 1) and pS129 antibodies that show high cross-reactivity to other proteins [130-132], we recommend repeating some of these studies using homogeneously modified pS129 aSyn monomers and oligomers and thoroughly characterized and validated pS129 antibodies.

pY39: In addition to pS129, recent studies have also explored the potential of assessing pY39 aSyn levels as a potential CSF biomarker. These studies were motivated by prior studies demonstrating that the levels of the activated form of c-Abl kinase, which phosphorylates aSyn efficiently at Y39 [133], are increased in the striatum and substantia nigra in PD brains [134]

and that an inhibitor of c-Abl increases aSyn clearance and is neuroprotective in preclinical models of PD [135-138]. Furthermore, in a small nonrandomized study of twelve PD patients, treatment with the c-Abl inhibitor nilotinib was reported to lead to an improvement in motor and cognitive symptoms [139]. However, a recent Phase 2 study reported that nilotinib showed no effect on symptoms or disease progression in either moderate or advanced PD.

To determine whether pY39 could serve as a biomarker differentiating PD patients from healthy controls or patients with other synucleinopathies, Na et al. [140] developed a targeted MS approach for the quantification of pY39 in the CSF. The assay was used to assess pY39 in CSF from a small cohort of PD patients (n=4) and healthy controls (n=4) and showed no significant differences in pY39 aSyn levels between the two groups. However, the ratio of pY39 to Y39 was significantly increased in PD patients [140]. It is noteworthy that pY39 levels in the CSF are very low, in the range of 0.00002-0.00067 pg/mL (1.5–5 attomoles/mL) [140] (Figure 4A and C). Therefore, the presence of minute amounts of unlabeled pY39 peptide standards could complicate the accurate estimation of its levels. Therefore, for targeted proteomics, it is of critical importance that the heavy standard peptide is spiked in at low quantities for reliable retention time and MS identification of the heavy peptide standard. The heavy/light ratio should be well above 1% to rule out any analytical bias and discard light isotope contamination derived from the standard and ultimately allow accurate quantification of the peptide/PTM of interest. Therefore, the reported levels of pY39 should be interpreted with caution [140], and further studies in larger cohorts are needed to confirm the recent findings and to validate the assays used to detect and quantify aSyn pY39 as a potential PD biomarker.

aSyn in the blood (plasma and serum)

Human blood represents an alternative biological fluid that can be easily obtained using minimally invasive methods. A few studies have reported the identification and quantification of total plasma aSyn, which has been reported in varying concentrations on the scale of thousands of pg/mL (i.e., 3600 to 1777100± 3609600 pg/mL) [53-59] (Table 2). The presence of posttranslationally modified forms of aSyn in plasma, particularly pS129, has also been investigated (Figure 5A) and was reported to vary significantly across different studies (0.8 ± 0.6 fg/mL to 12.9±8.7 fg/mL [141] and 878.5±317.4 pg/mL to 756.8±2419.9 ng/mL [53,55]) (Figure 5B and Table 1).

The levels of total, pS129 and oligomeric aSyn levels in different blood-derived components, including plasma and serum, have also been assessed as potential diagnostic and prognostic biomarkers of PD (reviewed in [73,75]). Using PTM-specific antibodies, Foulds et al. [54] reported that aSyn pS129 levels were slightly increased in the plasma of PD patients compared to healthy controls, but no significant differences were reported at the levels of oligomeric pS129 aSyn between the groups (Figure 5C). Remarkably, using the rabbit polyclonal anti-ubiquitin antibody FL-76 (Santa Cruz Biotechnology), they described the detection of mono- and polyubiquitinated aSyn species in plasma; however, the ubiquitination sites and chain lengths were not defined. In a follow-up study, the authors showed that pS129 levels remain unchanged over a span of 20 years after the initial manifestations of PD symptoms [55]. However, subsequent studies showed an increase in pS129 levels in the plasma of PD patients [53,55,141], suggesting that plasma pS129 may be a valuable PD biomarker. Interestingly, in a recent study, the plasma pS129 levels did not correlate with cognitive decline [141] but were associated with motor severity and disease progression (i.e., increasing levels of pS129 over time) in a follow-up study of 3.5±2.1 years [141].

Recently, Chen and colleagues [142] developed a novel approach using a modified paired surface plasma wave biosensor (PSPWB) coupled to an immunoassay and a label-free technique for quantifying aSyn pS129 in diluted human serum and assessing its suitability as a diagnostic biomarker for PD. They reported that pS129 could be detected in diluted human serum human plasma in the range of 0.5 to 5 ng/mL in healthy controls, whereas in PD, its levels ranged from 4 to 12 ng/mL. Interestingly, when comparing the levels of aSyn pS129 levels between PD patients and healthy controls, they observed a substantial area under the curve (AUC) value (0.92), indicating that aSyn pS129 in diluted serum could be a potential PD biomarker. However, this study relied on a small sample size (10 PD patients and 11 HCs); thus, further studies are required to validate these findings.

In a brief report published in 2013, Fernandez et al. [143] described that nitration of aSyn could be detected in serum but not in CSF samples and suggested higher levels of nitration at Tyr125/136 residues and lower levels of nitration at Y39 in PD patients than in controls (Figure 5C). Moreover, the ratio of Tyr125/136 to Tyr39 aSyn was higher in early PD patients than in controls or advanced PD patients. However, no subsequent studies have confirmed or validated these findings or the antibodies used by the authors.

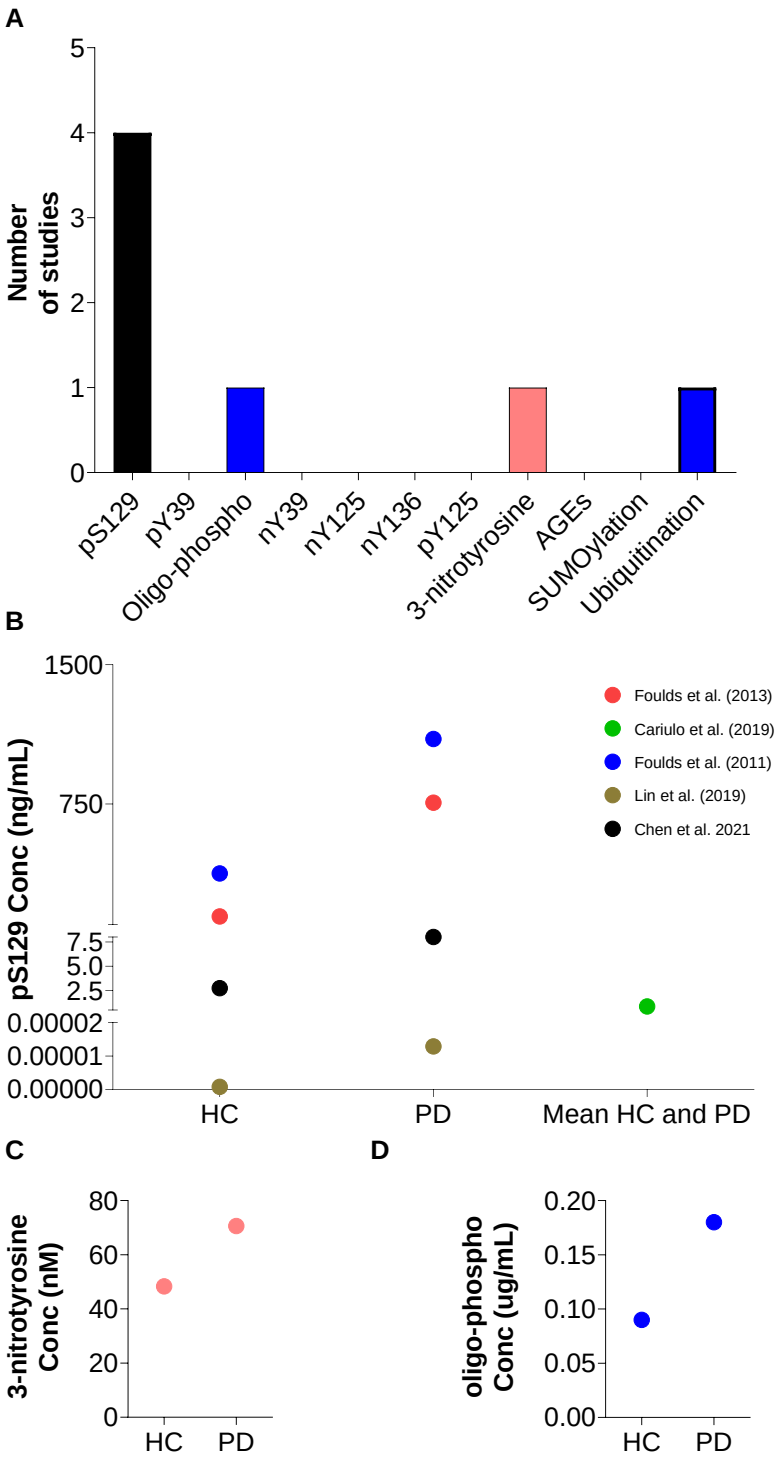

Figure 5. Plasma/serum aSyn PTM overview. A. Numbers of studies that reported the detection of aSyn PTMs in plasma/serum. B. Variability of pS129 concentration between HCs and PD in the different studies. Each dot represents the mean concentration of aSyn pS129 reported in each study. The mean of HC and PD is displayed for Cariulo et al. [53] because only one pS129 level was provided in that study. Other aSyn PTMs, namely, C. serum 3-nitrotyrosine [143] and D. plasma oligo-phosphorylation [54], were also reported, but only in a single study each. Ubiquitinated aSyn levels are not shown because this modification was assessed and quantified only by WB.

aSyn in red blood cells

RBCs, or erythrocytes, are an additional blood-derived component that has been extensively explored for potential biomarkers. This compartment is the most abundant cellular fraction of human blood and is recognized as the primary source of aSyn in the blood [60]. Initial studies by Barbour et al. reported that approximately 99% of blood aSyn species are derived from RBCs, where its concentration is 26200±3000 ng/mL [60]. Recently, this was confirmed, to some extent, by two independent studies based on quantitative MS/MS approaches [61,62], where aSyn was reported to be among the 20 most abundant proteins in RBCs [61], and its concentration in RBCs was later reported to be in the range of 40 µg/mL [62] (Table 2).

Vincent-Miranda *et al.* reported the detection of several posttranslationally modified forms of aSyn in RBCs, including phosphorylation (Y125), nitration (Y39), glycation and SUMOylation [144]. They reported increased levels of pY125, nY39, and glycated aSyn in the RBCs of PD patients compared to controls. In contrast, SUMOylated aSyn levels were decreased in PD patients compared to healthy controls. Furthermore, the authors failed to detect ubiquitinated aSyn in these samples [144]. This study was based on the detection and quantification of modified aSyn forms through immunoblotting analysis (dot blots) using a selected set of antibodies against aSyn PTMs, mainly a single antibody against each selected PTM. However, in this study, it was not clear whether several antibodies were screened before the selection of antibodies against PTMs, and the main findings were not validated by independent techniques such as MS/MS. In addition, the method used in this study (comparison of dot blots) is not considered a robust, precise and sensitive quantitative assay for the assessment of PTM concentrations in RBCs and could lead to inconclusive evidence regarding their potential value as biomarkers of PD.

Because RBCs are considered the major source of aSyn in the blood [60], different studies were conducted with the main aim of purifying aSyn from RBCs through extensive protocols based on chromatography steps [145,146]. Using immunoblotting analysis and MS-based approaches, it has been shown that full-length aSyn is the predominant species in RBCs. In addition, a truncated form of aSyn was consistently detected by WB in samples from healthy donors. However, to the best of our knowledge, the precise sequence of this truncated form has not been mapped, and there have been no studies to determine whether its levels change in synucleinopathies or during disease progression.

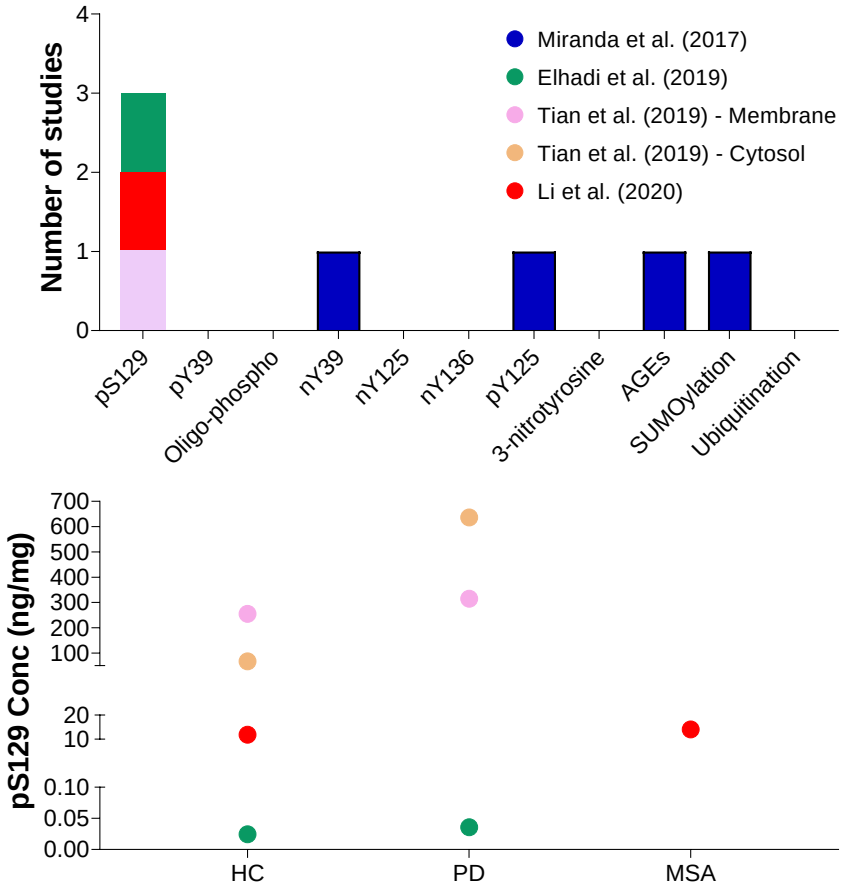

Figure 6. Summary of aSyn PTMs in RBCs. A. Number of studies that reported the identification of different aSyn PTMs in RBCs. B. Variability of pS129 levels across the different studies comparing PD to HCs or to patients with other synucleinopathies such as MSA. In this graph, it is also depicted that RBC pS129 levels differ between the membrane and cytosol fractions [147]. Each dot represents the mean levels of aSyn pS129 reported in each study.
The levels of aSyn PTMs such as nY39, pY125, AGEs or SUMOylation are not depicted in the figure because they were assessed only by dot and western blotting analysis [144]; no assay for obtaining an "absolute" quantification was carried out.

In addition to the abovementioned aSyn PTMs in RBCs, pS129 levels have also been identified and detected in the range of 24.48±7.6 to 636050±6030 pg/mg [147-149] (Figure 6A-B, Table 1). The levels of pS129 in RBC subcellular fractions were reported to be increased significantly in the cytosolic fraction and to a lesser degree in the membrane fraction of samples from PD patients compared to controls [147]. Elevated pS129 levels in RBCs were also reported in patients with purely motor PD compared to healthy controls, whereas lower levels of pS129 were observed in PD with cognitive impairment than in purely motor PD [148]. In another study using WB and ELISAs, Li et al. [149] reported significantly higher pS129 aSyn levels in MSA patients than in healthy controls. The authors also described significant differences between

two subgroups of MSA patients [parkinsonian (MSA-P) and cerebellar (MSA-C)]. MSA-P patients displayed higher pS129 values than MSA-C patients [149].

The increased levels of aSyn in RBCs compared to the other blood components and biological fluids could explain why it is easier to detect higher levels of modified forms of aSyn and suggest that RBCs may be a valuable source of aSyn biomarkers for the diagnosis and prognosis of PD. However, more systematic studies are needed to profile and quantify the levels of the different aSyn species in RBCs from large cohorts using a combination of robust unbiased and targeted proteomic approaches [62,150].

aSyn PTMs in saliva and tears

In addition to CSF and blood-derived fluids, saliva and tears have also been investigated as potential sources of biomarkers for PD because both types of fluids can be collected via a noninvasive method and are free from blood contamination. To the best of our knowledge, no studies have reported the identification and quantification of posttranslationally modified aSyn in saliva or tears, which could be attributable to the low levels of aSyn in these fluids compared to CSF, plasma and RBCs [concentrations in saliva are 7.104±5.122 to 314.03±435.90 pg/mL [63-66] or even on a ng/mL scale (i.e., 159.4 ± 61.6 to 229.9 ± 64 ng/mL) [67]; concentrations in tears range from 32.02 to 361.16 pg/mg [68,69]].

Oligomeric aSyn

The lack of correlation between LB pathology and PD development or severity, combined with accumulating evidence pointing toward the increased toxicity of oligomeric forms of aSyn, inspired efforts to search for oligomeric aSyn species in biological fluids as well as assess their potential as biomarkers for PD and other synucleinopathies. Over the past two decades, several groups have reported the detection of aSyn oligomers in CSF [52,81,82,128], blood constituents (plasma and RBCs) [82,151-155] and other biofluids such as saliva [65,66,156], relying on immunoassays such as ELISA.

In CSF, oligomeric aSyn levels were mainly reported to be increased in PD compared to controls [51,52,81,82,120,128]. The diagnostic potential of aSyn oligomeric species was assessed in a meta-analysis report considering the levels of this aSyn species from different studies. In this report, the authors demonstrated that the sensitivity and specificity of assays for oligomeric aSyn forms remain unsatisfactory and not adequate to support clinical decision-making [51].

Nevertheless, the ratio of oligomeric aSyn to total aSyn levels in CSF [81,123,127,128] displayed an improved diagnostic accuracy over utilizing the levels of the former alone, implying that this ratio is potentially a reliable PD biomarker.

The association between oligomeric aSyn levels and disease progression was also evaluated in different follow-up studies. Majbour et al. [78] reported the association of oligomeric aSyn levels with PD severity and progression. This finding was further confirmed in subsequent studies by the same group [79,80,126], suggesting that oligomeric aSyn species could be utilized as prognostic PD biomarkers. On the other hand, Murakami and colleagues [157] observed no association between oligomeric aSyn levels and disease progression.

Additionally, aSyn oligomer levels have been explored in blood constituents. In 2006, El-Agnaf and colleagues reported that plasma aSyn oligomeric levels were significantly higher in PD patients than in control subjects [151]. These findings were further confirmed in consequent studies from different groups reporting an increase in aSyn oligomeric levels in plasma [152] and red blood cells [154]. However, in other reports comparing the levels of blood aSyn oligomeric levels between PD and control individuals, no significant difference was identified [82,153,155]. Interestingly, in a recent report, Tian and colleagues, using an electrochemiluminescence (ECL) immunoassay, reported that oligomeric aSyn levels were significantly increased in the membrane fraction of RBCs in PD patients compared to control subjects, while no significant changes were identified in the cytosolic fraction of RBCs [147]. Furthermore, the levels of aSyn of oligomeric species were assessed in studies of salivary biomarkers, showing an increase in oligomeric aSyn levels in addition to a higher ratio of aSyn (oligo) to aSyn (total) in PD patients than in controls [65,66,156]. Nonetheless, these findings need to be confirmed in further studies using larger cohorts and by independent laboratories.

Posttranslationally modified oligomers. A review of the literature revealed a lack of studies evaluating and determining the levels of modified aSyn oligomeric and/or aggregated species. Indeed, to the best of our knowledge, only two studies, published by the same group, attempted to quantify the levels of aggregated phosphorylated species of aSyn in CSF and plasma (see sections: pS129 in CSF and aSyn PTMs in plasma/serum; Table 1). In both studies, the quantification of oligomeric phosphorylated aSyn species was performed using an in-house ELISA that relied on a commercially available antibody (anti-pS129 from Epitomics) as the capture and an in-house biotinylated p129 antibody as the detector. Outside this work,

the presence of oligomeric phosphorylated species and their value as a biomarker have not been further explored. Moreover, the in-house ELISAs used for the quantification of oligomeric-phosphorylated species could not differentiate between the different forms of aSyn, i.e., oligomers and fibrils [49,54]. Therefore, it remains unknown whether the aggregated phosphorylated species reported in CSF and plasma represent oligomers, fibrils or other aggregated forms of the aSyn protein.

One of the major challenges associated with measuring oligomeric forms of aSyn is that we have no insight into the biochemical and structural properties of native oligomeric aSyn. This is because of their dynamic and unstable nature compared to the highly stable and protease-resistant fibril forms of the protein. Therefore, artificial oligomers, usually one type, are used as calibrants, and in most cases, a chemically modified oligomeric form of the protein is used. The extent to which these chemical modifications affect the binding of the calibrants to antibodies or the ability to measure their concentration accurately has not been assessed. This, combined with the diversity of oligomer calibrants used and differences in the purity of the calibrants (i.e., the presence of other aSyn species (monomers or fibrils)), could also contribute to the considerable variation in absolute levels of aSyn oligomers measured by the different assays across different laboratories. Finally, it is important to stress that no current assay used to measure oligomers distinguishes between oligomers and fibrils [49,54]; thus, labeling these assays as oligomer assays is misleading. Until we have tools that differentiate between oligomers and other aggregated forms of aSyn, we should use the terms *aSyn aggregates* instead of *aSyn oligomers* and *aSyn aggregate assays* instead of *aSyn oligomeric assays*. Another major limitation in the measurement of aSyn oligomeric species as a diagnostic and prognostic marker for PD and other synucleinopathies is the scarcity of commercially available kits. This drawback drove the PD research community to develop different in-house assays. Despite the efforts in developing assays to monitor and quantify oligomers, comprehensive characterization of the employed calibrants and antibodies was not always carried out or made available, including independent validation of the biotinylated and in-house antibodies and the aggregate-species specificity of the antibodies (i.e., specificity for aSyn oligomers vs. other aggregated species) in addition to the purity of the calibrants (i.e., characterization of oligomers or aggregated forms by EM) [79,80,126].

Therefore, it is of paramount importance to 1) conduct further studies using a well-characterized and validated set of antibodies against the aggregation states of the aSyn protein [158] utilizing not only recombinantly generated standards but also cellular models to mimic physiological conditions; 2) extensively share data regarding the new antibodies and include the validation data in the reports; 3) openly communicate the characterization and validation data of the newly generated antibodies in the published reports; and 4) use properly characterized calibrants, ensuring that the reported observations are against aSyn oligomers and no other aggregated species.

Seeding-competent aSyn species

Sensitive and specific assays, termed RT-QuIC and/or PMCA, have been developed and optimized to amplify and detect minute amounts of aggregated aSyn not only in CSF [35,85-87] but also in peripheral tissues (e.g., skin or colon biopsies) [35,36,72,83-85].

Initial studies attempted to validate the seeding activity of aSyn by comparing brain and CSF samples. Interestingly, the great majority of the studies using these assays with CSF samples displayed good performance (high accuracy, sensitivity and specificity) as a potential diagnostic tool for PD [159]. Fairfoul et al. [85] compared a small cohort that comprised patients suffering from DLB, DLB with AD, AD with incidental LB, PD and HC. They reported that aSyn PMCA could identify controls and patients with tauopathy-related conditions with perfect specificity and that it had high sensitivity in discriminating the different synucleinopathy-related cohorts except for DLB with AD. Remarkably, several groups have corroborated and demonstrated similar results in discriminating PD from controls, highlighting the performance of aSyn PMCA as a potential diagnostic tool [35,72,83,84].

Interestingly, other studies have also compared the performance of aSyn PMCA in distinguishing PD from other synucleinopathies. Using these seeding assays, CSF aSyn strains could be discriminated [87,160], suggesting that pathogenic aSyn species might display different conformations depending on aSyn-related disorders. Moreover, these assays may play a key role in the preclinical identification of patients who may progress to PD. [34,88]. In greater detail, Shahnawaz and colleagues demonstrated that the aSyn PMCA could discriminate PD and controls with high sensitivity and specificity as well as correlate with the severity of the disease. Interestingly, they also identified that aSyn PMCA could open new avenues in

preclinical risk stratification, identifying patients who may develop PD [88]. This was further confirmed to some extent in a recent report of idiopathic rapid-eye-movement sleep behavior disorder (iRBD) [88]. Another exciting finding is related to a recent report by Kang and colleagues that reported the findings of two independent groups evaluating the same sample set by PMCA and RT-QuIC. They showed that both assays led to similar results [35], highlighting the reproducibility and putative diagnostic tool of these assays.

In addition to studies focusing on aSyn PMCA in CSF, several other studies have pursued to evaluate the performance of these assays using peripheral tissues (e.g., skin [36], olfactory mucosa and colon biopsies [35,72,83-85]), also displaying suitable results in terms of sensitivity and specificity. For more recent reviews on the development of aSyn amplification assays and their recent applications in biomarker discovery and characterization of different synucleinopathy cohorts, please refer to [159].

Despite the high sensitivity and specificity of CSF and peripheral tissues, their invasive collection methods may limit their clinical implementation and application. Therefore, we recommend the application of these assays to blood components (e.g., whole blood, erythrocytes or plasma), saliva and tears. This could pave the way for noninvasive novel diagnostic assays that could be used to support early diagnosis, patient recruitment and stratification for clinical trials; to assess target engagement in clinical trials of aSyn targeting therapies; or to monitor disease progression.

- **Summary of main findings**

1) Although total aSyn levels are consistently detected in CSF, displaying a trend of reduction in PD patients compared to controls, the reported levels vary significantly from one study to another.

2) aSyn PTMs are relatively scarce in biological fluids but are abundant in pathological aggregates in the brain and peripheral tissues.

2) The great majority of studies have focused primarily on the detection and quantification of pS129, which is readily detected in appreciable amounts in the blood components (plasma or red blood cells) but not in the CSF.

3) The detection of various modified forms of aSyn increases in biological fluids containing higher concentrations of aSyn. In particular, higher concentrations and a greater number of distinct modified forms of aSyn were detected in RBCs than in other blood components or CSF.

4) Most studies on aSyn levels or PTMs have relied on the use of selected antibodies targeting specific species rather than an unbiased approach aimed at profiling all aSyn species, and in most cases, the antibodies have not been validated for their ability to capture the diversity of modified aSyn species.

5) Oligomeric aSyn levels were mainly reported to be increased in PD patients in comparison with controls across the different biological fluids. However, the sensitivity and specificity of assays for oligomeric aSyn forms remain unsatisfactory or understudied.

6) Although several groups have investigated the levels of unmodified oligomeric forms of aSyn in biological fluids, the great majority of the antibodies and assays used do not allow differentiation among oligomeric, fibrillar and other aggregated forms of aSyn. Therefore, it remains unclear what form or forms of the protein are measured in these studies.

7) Only two studies have investigated the presence of levels of posttranslationally modified oligomeric forms of aSyn in biological fluids.

8) The lack of standardization in aSyn protein concentration determination methods and the reliance on poorly characterized protein standards are major contributors to the large variations in the quantification of total and unmodified aSyn measured in different studies.

Outlook

Clinical PD research: challenges and recommendations

Our review of the literature on the presence of aSyn species in biological fluids exposed several challenges that hinder progress in aSyn biomarker discovery and validation: 1) the lack of standardized guidelines for sample collection and handling (i.e., preanalytical and analytical confounding factors) and patient selection criteria; 2) the nature of the methods/assays that focus only on detecting a single aSyn proteoform rather than embracing the PTM complexity; 3) the use of nonspecific antibodies or antibodies that have not been well characterized using the appropriate protein standards; and 4) the usage of poorly characterized protein standards. Therefore, in the following section, we expand on discussing these challenges and provide recommendations to address them as an essential step to enable (in the near future) systematic assessment of the potential of aSyn (total, PTMs or aggregated forms or their combination) as biomarkers for 1) early detection and monitoring of disease progression; 2) patient stratification and 3) evaluating the efficacy of novel therapies.

Sample size and diversity. Our review of the literature also revealed that the vast majority of the studies on aSyn PTMs as synucleinopathy biomarkers relied on small sample sizes. The small sample size in PD research led to a poor assessment of variability between patients and experimental reproducibility across different laboratories (Figure 7A-C).

Particularly, in the CSF studies, aSyn PTMs have mainly been assessed in healthy controls and PD (Figure 7A) with patient cohorts ranging in size from 4 to 399 individuals, and the sample sizes decrease dramatically in studies on other synucleinopathies and neurodegenerative disorders (e.g., MSA, DLB, PDD, AD), between 8 to 76 patients (Figure 7A). Interestingly, in aSyn PTM studies, when considering the average size of the cohorts, an average of 60 controls and 90 PD patients were observed (Figure 7C), showing that future biomarker studies should use larger cohorts for accurate aSyn measurements. Among the studies investigating CSF aSyn PTMs as potential biomarkers, only a few studies evaluated their levels using samples derived from patients suffering from synucleinopathies other than PD (Table 1, Figure 4B and Figure 7A) [49,50,77,80,123,124]. Indeed, the average sample size decreases dramatically in studies on other synucleinopathies and neurodegenerative disorders (e.g., MSA: 20; DLB: 30; PSP: 30) (Figure 7C). Moreover, only a single study included patients suffering from CBD, FTD and VD. Thus, these numbers highlight the dearth of studies on other synucleinopathies

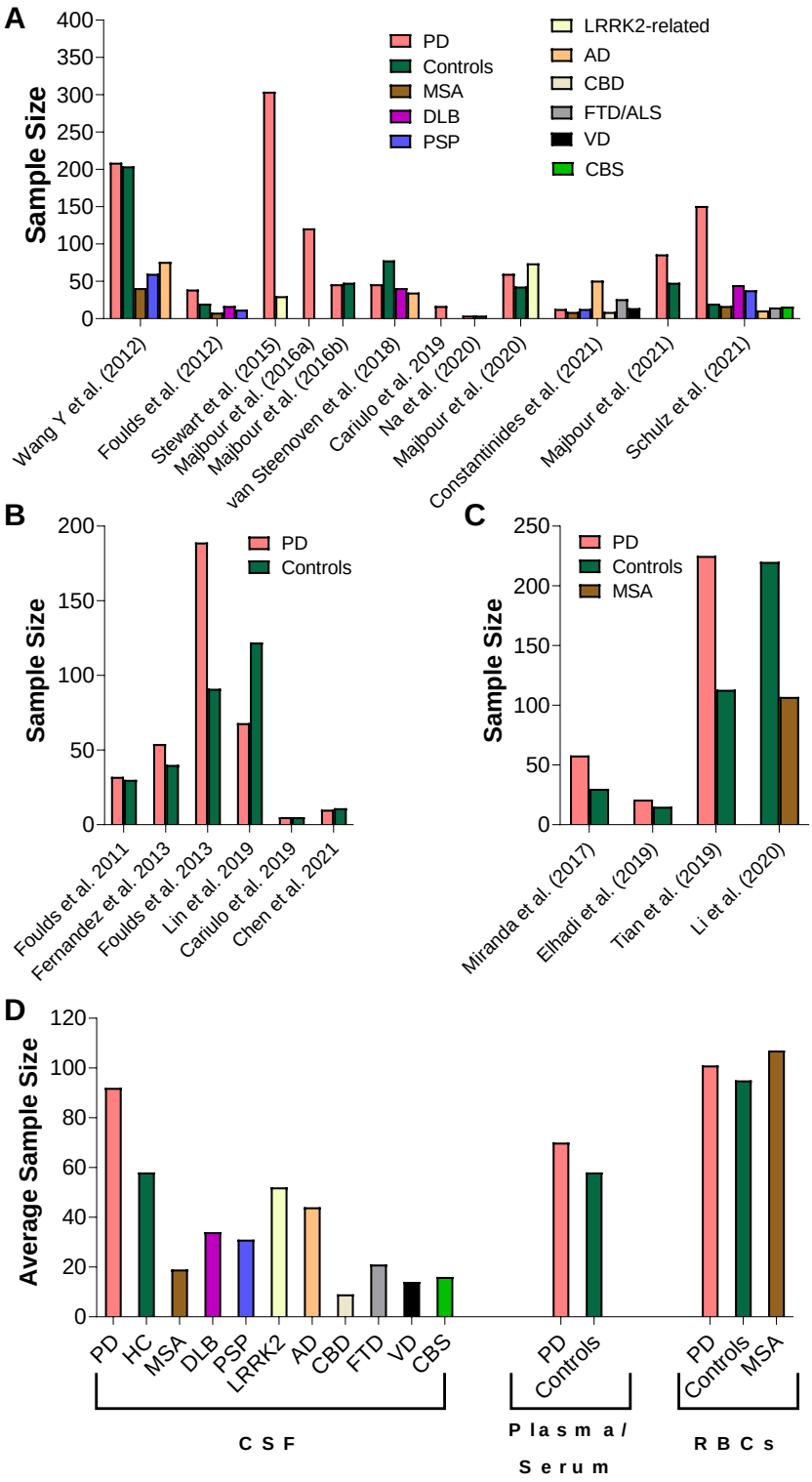

Figure 7. Sample sizes and averages of the different disease groups in studies aimed at quantifying aSyn PTMs in different body fluids. Sample sizes in A. CSF; B. plasma/serum; and C. RBCs. D. Average sample size of different diagnostic cohorts in different body fluids (i.e., CSF, plasma/serum and RBCs). The average sample sizes of the LRRK2-related, CBD, FTD, VD and CBS groups in CSF studies, as well as the MSA group in RBC studies, represent absolute values, since these disease groups were evaluated in only a single study each.

(MSA and DLB); further studies using larger cohorts are highly necessary. Remarkably, studies focusing on aSyn PTMs in blood constituents (i.e., plasma/serum and red blood cells) have mainly compared PD with controls [53-55,141,144,147,148,161] (Figure 7B, 7C) and relied mainly on less sensitive biochemical techniques such as ELISAs. To the best of our knowledge, aSyn PTMs in plasma/serum studies have been evaluated solely in controls and PD patients with sample sizes ranging from 5 to 189 individuals (Figure 7B). However, when all the studies are considered, the average sample size is 60 HCs and 70 PD patients (Figure 7E). Only in one study was the number of PD patients above the average sample size (n=189) [55]. As in the plasma/serum studies, the levels of aSyn PTMs in erythrocytes were assessed in relatively small cohorts ranging in size from 21 to 225 subjects (Figure 7C), with a mean cohort size of 90 HC and 100 PD individuals, while only a single study assessed the levels of aSyn pS129 in erythrocytes in other synucleinopathies, i.e., a comparison of 107 MSA patients with 220 controls [149] (Figure 7C, D). Additionally, regarding gender representation, most of the studies present gender bias, with male patients being overrepresented (Figure 8).

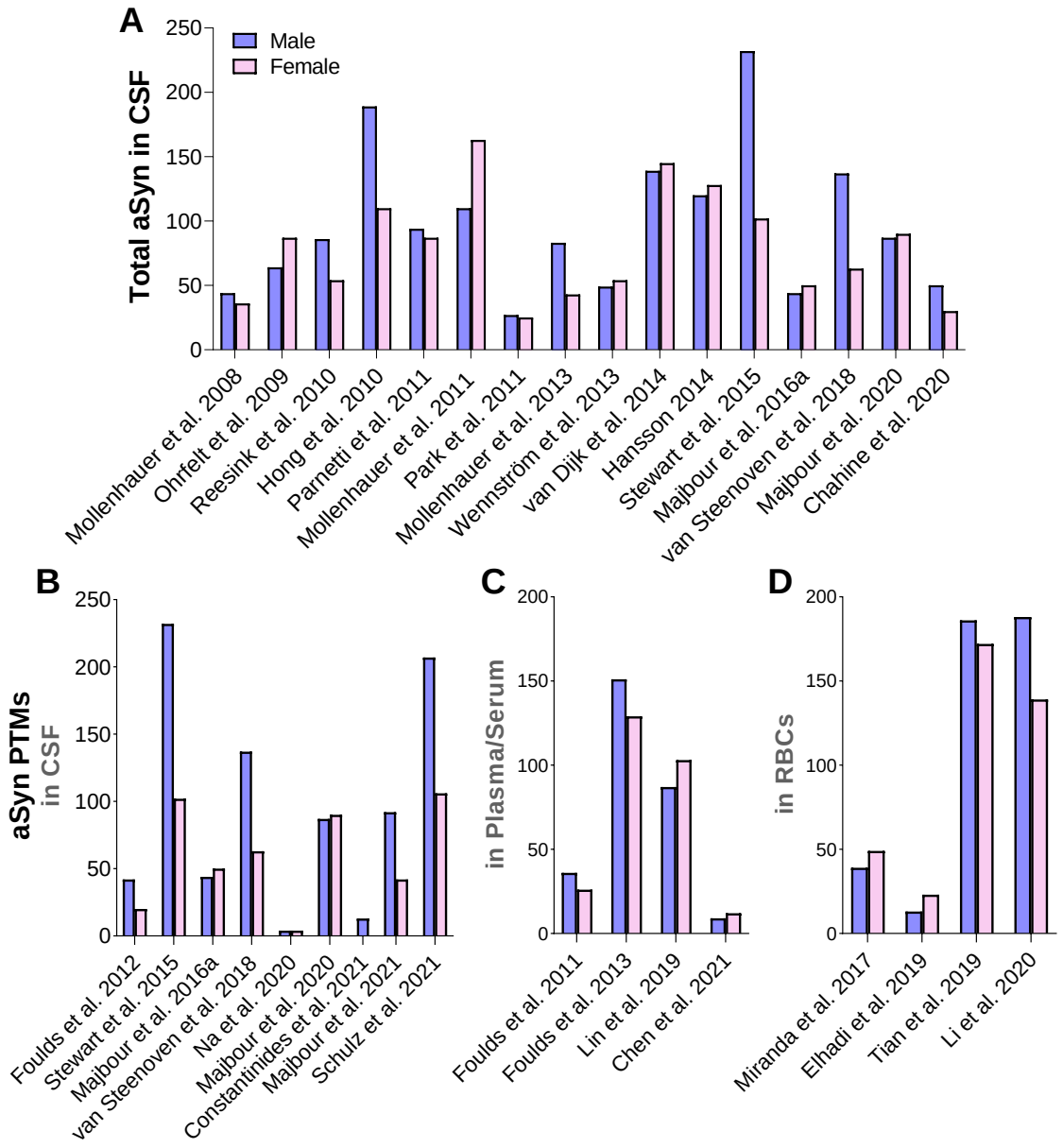

Figure 8. Gender representation across the different studies ((A) total aSyn levels and (B, C, D) aSyn PTMs). Male vs. female representation in studies that aimed to measure the levels of A. total aSyn in CSF and aSyn PTMs in B. CSF, C. plasma/serum and D. RBCs.

Altogether, the small sample sizes may be associated with different drawbacks of current approaches, such as 1) the invasive nature of CSF collection; 2) the nature of the patients' demographic data (e.g., elderly and nonautonomous); 3) difficulties in the accessibility to the specialist centers that are mainly in urban areas; 4) low incentives for participation in the studies; and 5) the requirement of multiple sample donations.

Nonetheless, because of the heterogeneity of PD and other synucleinopathies, multicenter biomarker studies with large sample sizes and appropriate patient selection (e.g., age, gender,

stages of the disease, patient clinical manifestations, lifestyle or comorbidities) encompassing PD and other synucleinopathy and neurodegenerative groups (e.g., MSA, DLB, PDD, PSP, AD) are needed. The large sample sets would allow the generation of a discovery cohort and independent validation cohorts with the appropriate sample sizes to enable rigorous statistical analysis and validation of reliable single and/or panels of synucleinopathy-related biomarkers. Thus, methodological mapping of aSyn species coupled with multicenter cohort studies of specific patient groups is necessary to allow systematic assessment of the potential of aSyn species as diagnostic biomarkers and to gain additional insights into the pathophysiology and PD and synucleinopathies. These studies will be facilitated by access to different types of biofluids and biopsies from the same patients and control cases and access to biofluids from cases with autopsy-confirmed diagnoses.

Sample collection: The great majority of the samples used in the published studies were collected under conditions that did not account for the reversibility of aSyn PTMs and were not optimized to ensure preservation of the diversity of aSyn species in biological fluids during sample collection and handling. Previous studies to standardize sample collection focused primarily on preanalytical confounding cofactors [117,162,163] such as 1) temperature at which the samples were collected; 2) type of collection tubes; 3) time between sample collection and sample storage; 4) multiple tube transfers (aliquots) and freeze-thaw cycles; 5) usage of nonionic detergents; and 6) collection of samples from various disease groups at different periods. When PTMs were focused on or otherwise considered in the study, sample collection guidelines and standardization focused primarily on trying to preserve phosphorylation. Therefore, standardization and validation of sample collection and handling techniques that both preserve the chemical integrity of the aSyn proteome (i.e., prevent aSyn degradation) and preserve the diversity of PTMs are needed. This is essential to achieve accurate measurements of total aSyn and specific aSyn species and to reduce inter- and intralaboratory aSyn measurement variations.

Modified aSyn protein standards: Initially, researchers in the field did not have access to pure protein standards of site-specifically modified aSyn proteins. Therefore, many preceding studies, aiming to quantify pS129, used aSyn protein standards that were generated by incubating recombinant aSyn with kinases that were later shown to phosphorylate aSyn only

partially and not to be efficient in phosphorylating aSyn at S129 (e.g., casein kinase II) [49,50,54,55,77,149]. In many of these studies, it is not shown whether the phosphorylated protein was purified or whether mixtures of unmodified and pS129 aSyn of unknown proportion were used as pS129 standards [49,54,55,77,149].

The discovery of the Polo-like kinases (PLKs) such as PLK2 and PLK3, as kinases that efficiently and quantitatively phosphorylate aSyn in vitro and in cells, made the generation of pure pS129 protein standards possible [53]. Unfortunately, in many cases with PLK2 or PLK3 being used, the extent of aSyn S129 phosphorylation was not reported. Indeed, it was not described whether the aSyn pS129 proteins used were purified from the in vitro phosphorylation reaction mixture (Table 1). Furthermore, in the majority of published biomarker studies, the analytical data supporting the biochemical and biophysical purity of the protein standards used are not presented. Such data should be included in future studies to demonstrate that the protein standards used are highly pure and free of aSyn oligomers or other aggregated forms of the protein.

Over the past 10 years, our group has pioneered the development of several semisynthetic strategies that allow for the site-specific introduction of single or multiple PTMs throughout the sequence of aSyn [129,164-169]. These advances have enabled us to generate highly pure and homogeneously modified forms of aSyn that encompass the great majority of aSyn species that have been detected in the brain, CSF and blood. The availability of these reagents and other semisynthetic proteins has already been instrumental in facilitating the development of novel and more robust tools and assays for the detection and quantification of aSyn through 1) the production of homogenously and site-specifically modified aSyn protein standards bearing single or multiple PTMs and 2) the generation of PTM-targeting antibodies [53,129,133,167,170]. Notably, the main pS129 aSyn protein standards used currently by researchers in the PD research community were produced on a large scale (hundreds of milligrams) using protein semisynthetic strategies developed by our group [129] and through close collaboration with a contract research organization funded by the Michael J. Fox Foundation, or MJFF (e.g., pS129 aSyn protein, which is now commercially available through MJFF and Proteos, Inc). Altogether, the methods for generating high-quality aSyn protein standards exist and should be used, and the description of the analytical data (the biochemical and biophysical purity of the standard) should be provided.

Antibodies: The vast majority of biomarker discovery studies relied on the usage of antibodies as the primary tool for detecting and quantifying the different aSyn PTMs in the brain, peripheral tissues and body fluids. Our review of this work revealed that 1) the available antibodies recognized only a limited number of aSyn PTMs (e.g., nitration, pS129). The two major reasons for targeting pS129 aSyn as a biomarker are as follows: 1) it is among the most common and abundant PTMs in the brain, as demonstrated by several studies, and 2) the availability of a large number of antibodies against pS129 enables the detection of this proteoform in the brain as well as in some biological fluids and peripheral tissues [16,49,54,55,72,78-80,91,171]. Several antibodies against aSyn pS129 have been developed and made commercially available (e.g., pSyn#64 from Fujifilm Wako; pS129 antibody from Epitomics, now acquired by Abcam (EP1536Y); anti-pS129 antibody (825701) from BioLegend) [49,53,141,148], although antibodies against many other PTMs are not yet available. Other antibodies against pS129 were developed in house [50,77-80,123,124,126] and are not directly accessible to the scientific community.

Interestingly, the majority of the antibodies against pS129 aSyn have not been validated against proper protein standards that account for confounding effects due to the presence of other proteins and co-occurring or multiple aSyn PTMs. This validation is of key importance given the clustering of multiple PTMs in different parts of the aSyn sequence. Our group has shown that the presence of multiple PTMs could, for example, interfere with the detection of pS129 by some pS129 antibodies [172]. In a more recent study, we demonstrated that the co-occurrence of pS129 with other disease-associated PTMs in the vicinity of S129, including phosphorylation at Y125 (pY125), nitration at Y125 (nY125) or C-terminal truncations at residues 133 and 135, dramatically decreases or abolishes pS129 detection by many pS129 antibodies. Moreover, using immunoblotting analysis, we have also shown that these antibodies cross-react with other proteins whose molecular weights are similar to those of aggregated aSyn species present in the brain (unpublished data). These findings underscore the critical importance of validating aSyn antibodies under physiologically relevant conditions using protein standards to account for the complexity and diversity of aSyn PTMs in the brain and biological fluids. It also highlights the reality that using these antibodies to detect and quantify pS129 in body fluids can lead to inaccurate measurements of aSyn levels and consequently high variability between the studies. Although a number of studies have investigated the specificity of pS129 aSyn antibodies, there are no published studies where

the specificity and cross-reactivity of antibodies against aSyn PTMs, such as nY39, pY39, and pS87, have been systematically investigated.

Regarding the detection of nitrated aSyn, despite the commercially available antibody selection (nY39, 36-012 (Millipore); nitrated aSyn (nY125/136): nSYn12 (Millipore); nitrosylated aSyn (nY39): nSYn14 (Millipore)), only a handful of studies have reported the quantification of nitrated aSyn species in body fluids using these antibodies [144,161].

Furthermore, despite the detection of other aSyn PTMs such as ubiquitination, acetylation, SUMOylation, glutathionylation, and glycosylation in the brain [89], reliable antibodies against these PTMs are still lacking.

In human brain tissues, the second most commonly observed modified aSyn species are the results of C- and N-terminal cleavage of aSyn, particularly C-terminally truncated species [95,96,98]. The truncation-directed antibodies recognize a limited number of truncated species, namely, at the C-terminus (predominantly truncations at 119 and 122). Indeed, only a handful of specific antibodies were previously used to characterize these truncated aSyn forms, such as Syn105 from Prothena and aSyn-131 and aSyn-134 from Roche [173]. Despite their existence, they are not readily accessible to the scientific community.

The mapping and profiling of truncated aSyn fragments in the brain are achieved primarily by WB analyses and more recently by MS approaches (Figure 2) [16]. However, the available antibodies that target cleaved aSyn fragments do not yield a precise mapping of all truncated species associated with synucleinopathy pathophysiology. Interestingly, none of the body-fluid-based reports described in this review attempted to specifically detect and quantify truncated aSyn or evaluate whether truncations could interfere with the detection of other aSyn PTMs such as pS129.

Overall, the majority of the immunoassays used relied on antibodies that were not thoroughly validated, or the validation data were not reported. In other words, the limitations of the assays included the following: 1) Some of the antibodies used may detect different forms of aSyn and not only the target aSyn species of interest, thus leading to inconclusive or irreproducible measurements. 2) Body-fluid-based biomarker studies have mainly focused on the quantification of the most prominent aSyn PTM, pS129, which does not fully address the multiplicity of PTM patterns [49,54,79,123,124,126]. For example, neither the possible presence of aSyn truncations nor the potential disruption of antibody binding to aSyn due to the co-occurrence of multiple modifications [95,96,98] was taken into consideration in the

measurements of aSyn species in body fluids. This could lead to underestimation of aSyn levels and contribute to the variability of aSyn levels across different studies. 3) A large number of the total aSyn biomarker studies relied on antibodies and assays that are designed to detect and quantify a single specific aSyn form rather than evaluating the diversity of aSyn PTMs. In other biomarker studies designed to detect and quantify single aSyn PTMs, the potential impact of the co-occurrence of multiple PTMs in close proximity to the target PTM was not considered during the selection of antibodies or optimization of the assays. Therefore, we recommend prioritizing 1) the development of more specific aSyn antibodies; 2) the development of antibodies that can recognize the different aSyn PTMs, preferably with residue specificity and selectivity; 3) the characterization and validation of antibodies using a library of aSyn standards bearing the desired PTM, but also including neighboring PTMs and other proteins comprising multiple physiological and pathologically relevant PTMs; and 4) thorough investigation of the specificity and cross-reactivity of all antibodies in different experimental settings. It is critical to validate all commercially available and in-house antibodies through independent third-party entities [174], which could catalyze the progress of novel and improved antibody-based tools for accurately assessing/mapping aSyn proteoforms and ultimately establish them in clinical practice as diagnostic and prognostic biomarkers.

Methods for measuring protein concentration: Immunoassays and antibody-based assays are among the most used for the identification and validation of protein-based markers, such as aSyn. Interestingly, despite using similar antibodies or antibodies that target the same region of aSyn, studies using these immunoassays have reported significant differences in the concentration of aSyn. Several factors have been proposed to contribute to the large deviations in aSyn levels measured with different kits [76,117]. One additional factor is the lack of well-characterized protein standards. The protein standards provided by different vendors have different concentrations and quality and are prepared using different sample preparation and handling procedures. This could also contribute to the large differences in the aSyn levels measured by researchers. Unfortunately, none of the commercial immunoassays provides information about the purity (chemical integrity and aggregation state) of the protein standards used in their kits or provides sufficient material to allow quality

control and independent validation of the concentration or purity of the standards by the users.

Protein concentration determination of the calibrant is of key importance for the quantification of biomarkers. Several methods have been used to assess protein amounts, such as Lowry's method, the Bradford method, the BCA approach and amino acid analysis (AAA) [175-178]. Spectrophotometric and colorimetric approaches are characterized as sensitive and reproducible, but their measurements can be affected by the conformation of the protein (aggregation state) [176-178]. On the other hand, AAA, which relies on protein hydrolysis, allows protein quantity and amino acid composition to be ascertained accurately. Thus, it is considered a gold standard method for determining an absolute and exact quantitative measure of the protein standard [175,179,180]. Moreover, this method is independent of any external protein standard curve, protein charge, protein state, or dye-binding percentage. Hence, we recommend using AAA for assessing the absolute quantification of protein standards that will be used as the reference material in the standard curve for different protein concentration assays, such as ELISA and IP-MS/MS.

Unbiased mapping of aSyn species. Although the assays used in aSyn biomarker research could support multiplexing, the majority of studies on aSyn PTMs in biological fluids have mainly focused on a single combination of antibodies (detector and capture antibodies), with a strong emphasis on the detection of one PTM at a time and a focus on pS129.

Given the lack of antibodies that cover the diversity of aSyn species and the scarcity of data on the role of different PTMs in the physiology and pathogenic properties of aSyn, we recommend revisiting mapping of the aSyn proteoforms in the brain and biological fluids using unbiased experimental approaches, such as MS. This would enable researchers to identify differences in the aSyn proteoform in individuals with synucleinopathies compared to healthy controls, thus enabling the field to prioritize the development of new antibodies targeting disease/pathology-associated aSyn and other abundant PTMs, tools, reagents and assays for biomarker discovery and validation. Altogether, this would lead to the development of novel antibodies and more sensitive approaches (e.g., targeted MS/MS, biosensors) for detecting and quantifying aSyn PTMs and pave the way for decoding aSyn PTMs in health and disease. Beyond their important use in biomarker discovery and validation, these tools will also help shed new light on the pathophysiology of this protein and

consequently offer a better understanding of the molecular mechanisms underpinning synucleinopathies.

Future directions

To address the aforementioned limitations and challenges, it is essential to 1) standardize protocols for sample collection and handling, aiming to maintain the properties and diversity of aSyn and its modified species; 2) access a large sample set from PD patients and controls as well as patients suffering from other synucleinopathies to conduct biomarker-based studies that reliably assess patient-to-patient variability; 3) standardize selected cohorts regarding disease diagnosis and progression and additional factors (e.g., medical treatment, lifestyle, and comorbidities); 4) harmonize guidelines for the development of novel assays, relying on a panel of well-characterized antibodies and pure and homogeneously modified standards; 5) optimize assays for mapping aSyn PTM patterns at the single-molecule level; and 6) improve the efficiency and sensitivity of MS-based approaches to profile aSyn species using unbiased methods and subsequently quantify these species by targeted methods. Many PTMs may exert their effects by acting on later stages of aSyn aggregation and pathological progression [89]. Hence, developing new approaches to investigate the role of post-aggregation PTMs in regulating aSyn aggregation, pathology spreading and toxicity is essential. In the context of biomarker discovery and validation, future studies should focus on measuring the levels of modified aggregated proteins. Collectively, this will facilitate the development of novel assays to simultaneously measure and quantify the ratios of various aSyn species (e.g., total, phosphorylated, nitrated, aggregated, or modified aggregated species).

The detection of monomeric and aggregated aSyn forms in body fluids (e.g., CSF (40-48), blood components (49-58), saliva (59-63) and tears (64, 65)) as well as in peripheral tissues (66-69) presents unique opportunities for the identification and validation of novel disease-relevant aSyn-based markers. However, increasing evidence suggests that it is unlikely that measuring a single aSyn species or total aSyn on its own will provide a sensitive diagnostic biomarker for early disease detection and monitoring of progression.

We propose that the use of a combination of biochemical and structural aSyn biomarkers and neurodegenerative disease biomarkers is likely to yield a better performance diagnostic for early detection, patient stratification and monitoring of disease progression, thus paving the

way for more personalized therapies for the management and treatment of PD and other synucleinopathies. Thanks to recent advances in the detection and amplification of aSyn aggregates in biological fluids and peripheral tissues as well as cryogenic electron microscopy (cryo-EM) in enabling near-atomic-level structural insight into aSyn brain pathology, we are closer to achieving this goal.

The development of PMCA and RT-QuIC has enabled the efficient amplifications of minute amounts of aggregated aSyn in CSF [35,86,87], skin biopsies [36], or colon biopsies [35,72,83-85], thus facilitating the development of assays that enable the differentiation of PD patients from controls with remarkable specificity and accuracy [36,87]. Most notable is the finding that assessments of the same sample set using the two most commonly used amplification assays (PMCA and RT-QuIC) by two independent groups yielded similar results [35]. Recent studies suggest that these assays can be further developed and refined to potentially make it possible to differentiate between PD and other synucleinopathies (MSA from PD and LBD) [36,87] and to predict disease development.

On the structural side of aSyn aggregates, our understanding of their structural properties was initially primarily based on solid-state nuclear magnetic resonance (NMR) studies performed on aSyn oligomers and fibrils [181,182]. Recently, thanks to advancements in cryo-EM, near-atomic models of aSyn fibrils were obtained from in vitro preparations [28,183-186] as well as postmortem brain patients with multiple system atrophy (MSA) [17]. The resulting cryo-EM structures revealed the folding landscape of the aSyn monomers into different polymorphs. To date, most of the structures have been derived from in vitro preparations due to the ease of sample preparation for cryo-EM imaging. The complexity and instability of native brain-derived aggregates during the isolation and purification procedures have hindered efforts to the structure of brain-derived aggregates in PD and all synucleinopathies.

Interestingly, cryo-EM structures of aSyn fibrils derived from the brains of patients with Parkinson's disease, MSA and dementia with Lewy bodies demonstrated that they have different structural and pathological properties, supporting the idea of disease-specific polymorphism [187]. Strikingly, the structure of MSA fibrils from the brain is dramatically different from the diverse structures of aSyn obtained from different preparations of recombinant aSyn fibrils. This underscores the critical importance of establishing that the tools and assays we use to detect or quantify aSyn aggregates are able to detect native (brain-derived) aggregates, in addition to other fibril structures.

Greater success has been achieved in solving amyloid fibrils from other neurodegenerative diseases, such as AD [188,189] and ALS [190]. We believe that it is only a matter of time before the structure of aSyn aggregates from different synucleinopathies will be solved. Although several groups have shown progress toward amplifying brain and peripheral-tissue-derived aSyn aggregates using the PMCA or RT-QuIC methods, it remains unclear whether the in vitro amplified structure faithfully reproduces the structural properties of the native aggregates. However, we predict that we will soon have access to methods that allow us to replicate and amplify their structure in vitro. These advances will pave the way for a new structure-based classification of synucleinopathies as was recently achieved for tauopathies. Achieving this goal could enable the differentiation of proteinopathies by targeting the different polymorphs and the development of disease-specific structure-based diagnostics and therapeutics.

To fully realize the potential of these new advances and develop more reliable biochemical and structural aSyn biomarkers, it is crucial to first map the biochemical and structural diversity of aSyn in biological fluids (CSF and plasma), peripheral tissues and postmortem brain tissues. Quantitative proteomics (MS methods) can be used to determine the distribution of aSyn species defined by different PTMs, as well as the aSyn proteome profile associated with each sample type. A combination of PMCA/RT-QuIC and cryo-EM approaches can also be used to map the polymorphism and structural diversity of aSyn aggregates (Figure 9A). For the first time, these methods will allow researchers to ascertain the relationship between the distribution of aSyn species in the brain and CSF and whether the CSF provides a window to aSyn pathology in the brain. Similarly, these studies will also enable the first comparative studies to identify the similarities or differences between aSyn pathology in the brain and peripheral tissues.

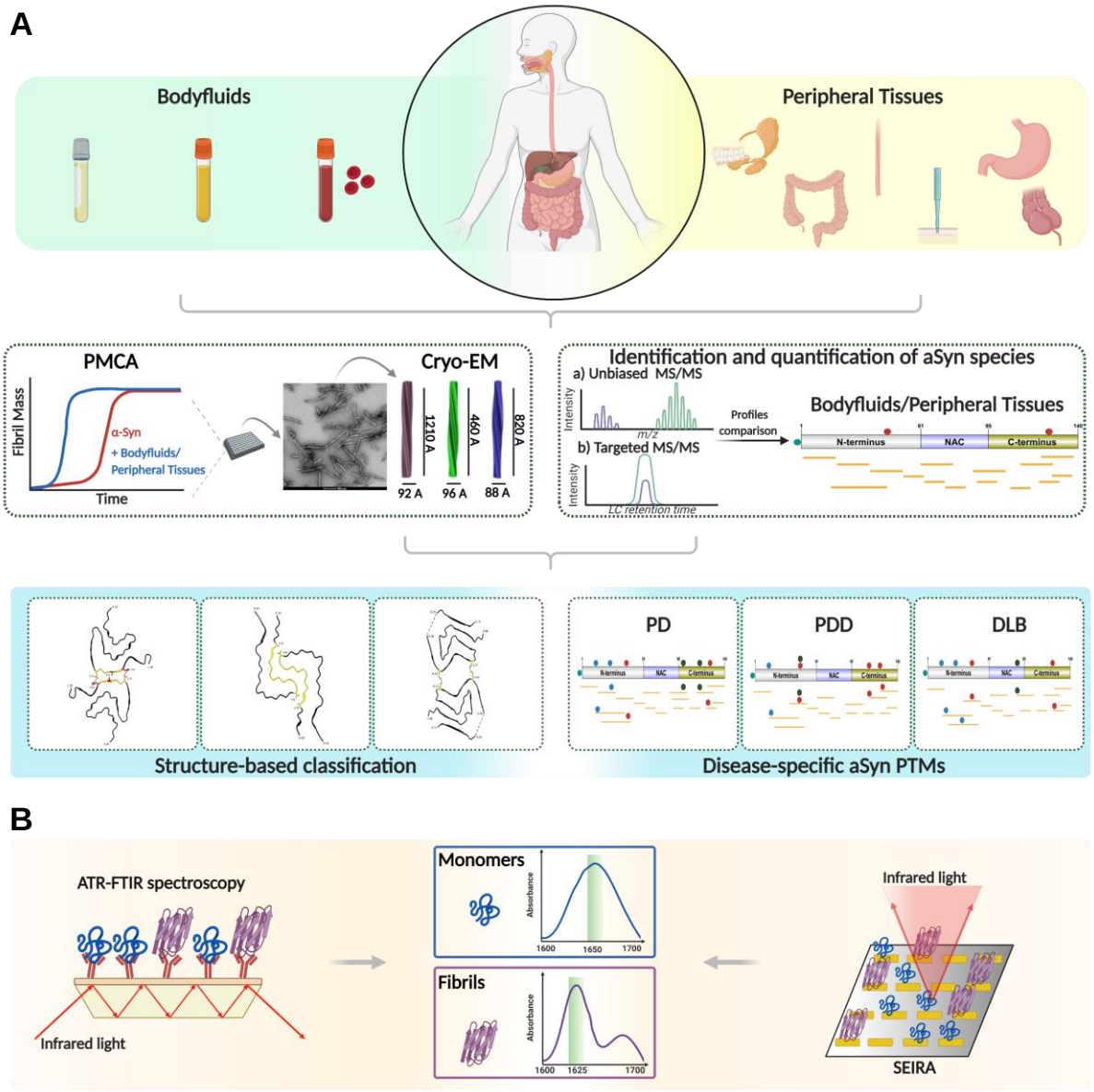

Figure 9. Molecular and structural biomarkers of synucleinopathies. A. The combination of 1) amplification and detection of minute amounts of aggregated aSyn in biological samples (e.g., PMCA) coupled with cryo-EM and 2) identification and quantification of aSyn species by MS/MS can lead to the discovery and validation of novel biomarkers, relying on structure-based classification and disease-specific aSyn PTMs. Together, these approaches can open new avenues and may be of key importance in differentiating PD patients from controls and from patients with other synucleinopathies. B. Attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) and surface-enhanced infrared absorption spectroscopy (SEIRA) as a complementary approach in this workflow for high-throughput analysis, allowing molecular-level differentiation of a monolayer of aSyn monomers and fibrils.
The cryo-EM structures of the aSyn fibrils depicted in the figure are derived from different aSyn recombinant proteins.

We envision a future in which samples from various peripheral tissues and body fluids will be collected at different time points over a follow-up period from patients with different

synucleinopathies. These samples will be simultaneously analyzed by 1) PMCA to quantify the levels of aSyn aggregates and perhaps obtain the signature aggregation kinetic profile of the sample; 2) detection of total aSyn forms using assays that capture the diversity of aSyn species; and 3) detection and quantification of a panel of 4-8 disease-associated posttranslationally modified forms of aSyn using immunoassays or MS-based approaches. The low throughput of cryo-EM precludes its inclusion as a diagnostic tool. However, we anticipate that the availability of a large number of structures from native aSyn fibrils and aggregates will pave the way for simplified high-throughput methods for profiling structural diversity in biological samples. This can be achieved by assembling panels of antibodies targeting different sequences and conformations, which would enable rapid indirect profiling of aSyn fibril structure and polymorphism. Furthermore, the mid-infrared spectroscopy technique could play a major complementary role in this workflow, as it is a fast, chemically specific, label-free and nondestructive method capable of identifying secondary structures by leveraging the infrared absorption fingerprints of proteins [191] (Figure 9B). To overcome the inherent limitations of classical bulk infrared sensing (low sensitivity and the overlap in absorbance between water and proteins), its derivatives, such as attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) and surface-enhanced infrared absorption spectroscopy (SEIRA), have been developed to serve as potential structural biomarker sensors by differentiating pathological beta-sheet-enriched neurodegenerative markers from intrinsically disordered/α-helix-enriched healthy monomers. For example, an immunological IR sensor based on the ATR-FTIR technique was developed for the early detection of AD through the overall secondary structure distribution of Abeta in body fluids such as blood and CSF [192,193]. SEIRA is still in its infancy but has already demonstrated its potential to perform conformational differentiation at the molecular level in a monolayer of aSyn fibrils and monomers [194] and identify structural changes in the protein monolayer at high resolution in real time [195]. The identification of the aSyn proteoforms that are specific to each synucleinopathy (e.g., PD, PDD, DLB) could also enable the development of complementary assays that target specific species or PTM signatures, which would further improve the fingerprinting of biological samples.

In summary, this workflow could enable comprehensive quantitative biochemical and structural profiling of disease-specific aSyn species. When integrated into a point-of-care

platform, it could enable early diagnosis and prognosis, patient stratification and guide clinical decisions.

Other neurodegeneration-related biomarkers for synucleinopathies

Increasing evidence support the hypothesis that PD and most likely other synucleinopathies do not represent a single entity diseases, but are rather heterogeneous and should be categorized into different subtypes based on the main underlying molecular and mechanisms associated with each subtype. In addition, there is consensus today that PD is characterized by the presence of multiple pathological aggregates, including Tau, amyloid-b and TDP-43 aggregates [196,197], which may contribute to the clinical heterogeneity of PD and other neurodegenerative diseases [198,199]. Although some studies have shown a correlation between the presence of multiple pathology and diseases progression or disease symptomology [200], the extent to which the levels of the different copathologies varies during disease progression or between the different synucleinopathies remains a subject of active investigation. The relative contributions of aSyn loss and gain of toxic mechanisms and other pathological aggregates to the development and progression of the different disease subtypes remain unknown. Therefore, it is likely that relying solely on aSyn biomarkers may not be sufficient for differentiating between the different subtypes or monitoring the disease progression for some of PD subtypes. Therefore, we agree with previous recommendations calling on expanding the range of biomarkers to include clinical markers, neurodegenerative disease biomarkers and biomarkers of biological pathways that have emerged as key drivers of disease development and progression [201].

This includes biomarkers linked to neuronal injury; axonal integrity and glia (e.g., Tau and phosphorylated Tau, neurofilament light chain (NFL), glial fibrillary acidic protein (GFAP), vilip-1, YKL-40, TREM2); synaptic integrity/function (e.g., granins, neurotransmitter metabolites, SCG2, PDYN, synaptobrevin); LRRK2-related variables (total and phospho-LRRK2, phospho-RAB); GBA-related variables (Lamp-1, Lamp-2); amyloid-beta; or proteins related to oxidative stress, inflammation, energy failure and the extracellular matrix, such as DJ-1, neurosin, neprilysin, and complement [9,125,202]. For example, CSF levels of the axonal degeneration biomarker NFL may help to differentiate MSA, PSP, and CBD from PD, since its levels were found to be increased in the CSF in the rare synucleinopathy groups compared to PD [125]. Other

promising biomarkers are linked to synapse neurodegeneration, i.e., VGF, SCG2, and PDYN were found to be reduced in PD and DLB but also correlated with cognitive measurements [202]. For more information on the assessment of the performance of these neurodegeneration biomarkers and their characterization as biomarkers for different synucleinopathy cohorts, please refer to [9,202]. Although some of these biomarkers have been linked to different NDDs [202], we predict that the panel of biomarkers encompassing the quantification of different aSyn species with some of the abovementioned neurodegenerative markers could pave the way for more reliable methods for early diagnosis, patient stratification, monitoring disease progression and evaluating target engagement in clinical trials (Figure 10). These advances would facilitate the development of targeted and potentially personalized therapeutic interventions or disease-modifying therapies for PD and other synucleinopathies.

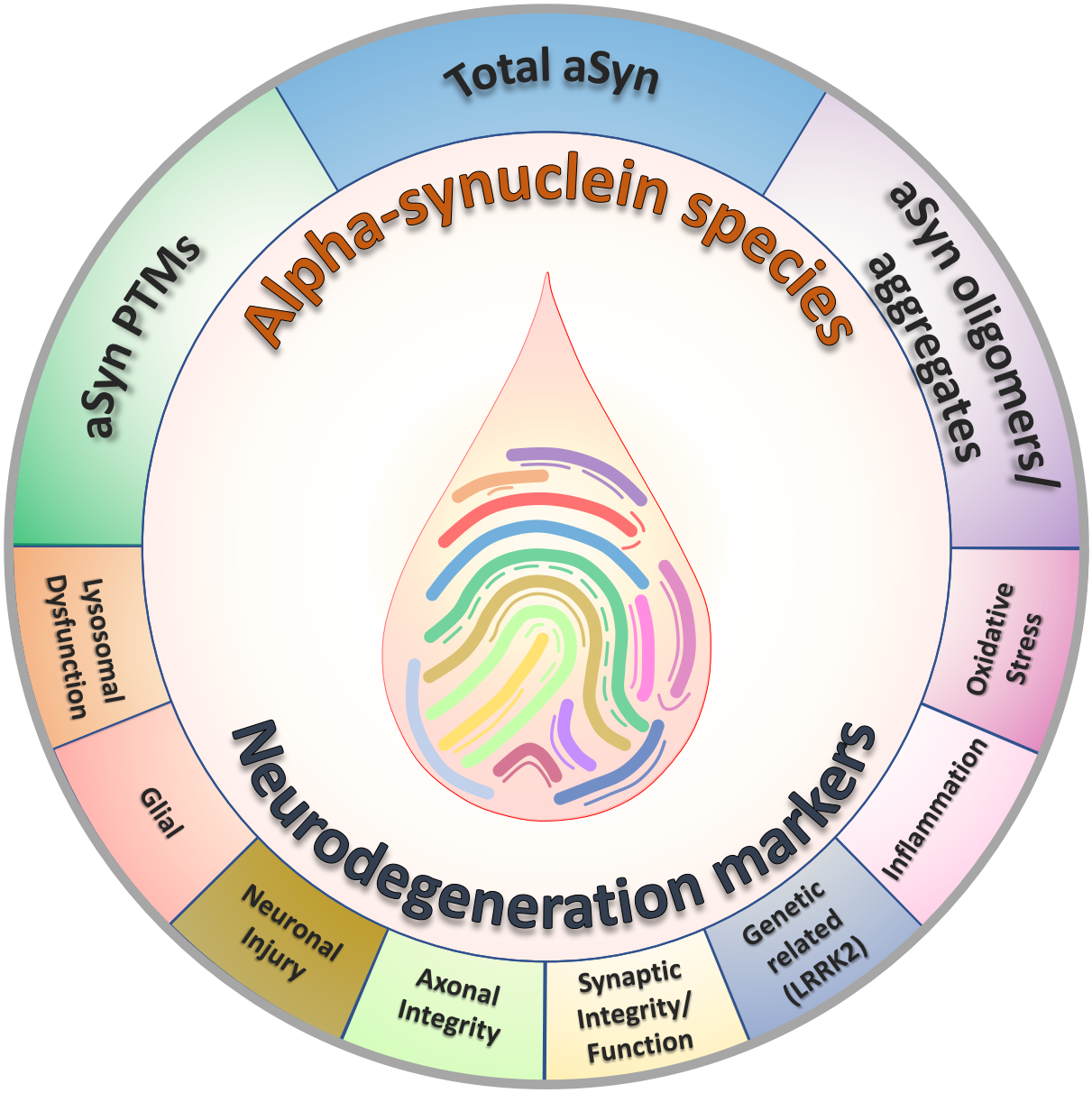

Figure 10. Schematic illustration of biomarker fingerprinting: a multimarker approach including the diversity of aSyn species with other neurodegenerative biomarkers.

Main recommendations

1. Develop optimized protocols for sample collection and handling that take into account the stability of the different modified forms of aSyn.
2. Use multicenter cohorts and a large number of biological fluid samples from PD and other synucleinopathies.
3. Use highly pure and homogeneously modified protein standards.
4. Use amino acid analysis as the gold standard method for determination of aSyn protein concentration.
5. Use well-characterized and validated antibodies that detect the specific forms of the protein (PTM specific) or capture the diversity of aSyn species (for total aSyn concentration determination).
6. Generate new antibodies against different aSyn PTMs, as the current antibody toolsets cover only a limited number of aSyn PTMs.
7. Assess the cross-reactivity of the available and newly generated antibodies (particularly pS129) to other aSyn PTMs and the proteome of biological fluids.
8. Use a combination of biochemical and mass spectrometry approaches, to conduct systematic and unbiased analysis of aSyn species and PTMs in biological fluids, peripheral tissues and postmortem brain tissues from healthy controls and patients with PD and other synucleinopathies and NDDs (e.g., PDD, MSA, DLB, AD).
9. Develop a diagnostic workflow that integrates biochemical and structural aSyn biomarkers and other biomarkers of disease-relevant mechanisms (neurodegeneration, synaptic dysfunction, inflammation, etc.).

Acknowledgements:

We are grateful to Ahmed Sadek, Deepthy Kavungal, Enzo Morro, Galina Limorenko, Rajasekhar Kolla and Senthil Kumar for their critical review and valuable feedback, as well as all members of the Lashuel laboratory for their help during the preparation of the review article.

Ethics declarations

***Competing interests*:** Prof. Hilal A. Lashuel is the founder and chief scientific officer of ND BioSciences, Epalinges, Switzerland, a company that develops diagnostics and treatments for neurodegenerative diseases (NDs) based on platforms that reproduce the complexity and diversity of proteins implicated in NDs and their pathologies.

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Table 1 - Analysis of aSyn PTMs species in the different biological fluid specimens: Overview of the different techniques, antibodies employed and aSyn PTMs concentration range across control and patient groups.

PTM	Biological fluid	Techniques employed	Antibody or Enrichment strategy used (Vendor)	Mean of modified aSyn in controls	Mean of modified aSyn in cases	Preparation of calibrants	Methods used for calibrant characterization	Calibrant purity and characterization – Data Shown	References
nY39	Red blood cells	Immunoblotting analysis (Western blot and dot blots)	Anti-nitro-α/β-Synuclein, nY39, 36-012 (Upstate/Millipore)	NA	NA	NA	NA	NA	Vicente Miranda et al. [145]
Nitrotyrosine	Serum	ELISA, Western Blot and Mass spectrometry	Monoclonal antibody to nitrotyrosine (Hycult Biotech); Nitrosylated aSyn (nY125/136): anti-nitro-a/b-synuclein antibody - nSYn12 (Millipore); Nitrosylated aSyn (nY39): anti-nitro-a/b-synuclein antibody Tyr39 - nSYn14 (Millipore)	3-nitrotyrosine protein: 48.3 ± 6.8 nM	70.6 ± 4.7 nM (Serum)	NA	NA	NA	Fernandez et al. [162]
pY39	CSF	Targeted Mass spectrometry	PTMScan Phospho-Tyrosine antibody; P-Tyr-1000 (Cell Signaling Technology); TiO_2 beads (ThermoFisher)	1.67 – 4.98 attomole/mL	PD: 1.53 – 4.25 attomole/mL	Synthetic	Synthesis	NA	Na et al. [141]
pY125	Red blood cells	Immunoblotting analysis (Western blot and dot plots)	ab10789 (Abcam)	NA	NA	NA	NA	NA	Vicente Miranda et al. [145]
pS129	CSF	IP-MS, Luminex	IP-MS: pS129 (Abcam) and ExactaCruz IP kit; Luminex: Capture: ASY-1[#]; Detection: biotinylated anti-human pS129[#]	68.61±17.25 to 73.03±17.20 pg/ml	PD: 77.73 ± 20.45 to 79.23±23.22 pg/ml MSA:58.12±20.24 to 61.97 ± 14.19 pg/ml PSP: 55.54±16.87 to 58.24 ± 24.93 pg/ml AD: 67.50±15.68 to 72.64±19.57	Recombinant aSyn was incubated with casein kinase II (New England Biolabs)	Immunoblotting with a phosphorylation-dependent anti-aSyn antibody, pS129 (Epitomics) and mass spectrometry	X (MALDI-TOF/MS)	Wang Y et al. [50]
	CSF	ELISA	Capture: anti-α-synuclein N-19 (Santa Cruz Biotechnology); Detection: anti-pS129 (Epitomics)	3.58±3.85 µg/ml	PD: 3.43±6.18 µg/ml PD (nonD): 4.41±8.68 µg/ml PD (Cog): 1.76±1.02 µg/ml PD (Dem): 3.67±5.73 µg/ml DLB: 1.63±1.42 µg/ml PSP: 5.14±9.73 µg/ml MSA: 7.14±9.19 µg/ml	Recombinant aSyn was incubated with casein kinase II (New England Biolabs)	Immunoblotting with a phosphorylation-dependent anti-aSyn antibody, pS129 (Epitomics) and mass spectrometry	NA	Foulds et al. [49]
	CSF	Luminex	Biotinylated anti-human pS129 antibody [#] Streptavidin-R-PE (Prozyme)	NA	Baseline: 114.66 ± 17.14 (pg/ml) Follow-up: 117.89 ± 17.92 (pg/ml)	Recombinant aSyn was incubated with casein kinase II (New England Biolabs)	Immunoblotting with a phosphorylation-dependent anti-aSyn antibody, pS129 (Epitomics) and mass spectrometry	NA	Stewart et al. [77]
	CSF	ELISA, dot plot	Mouse anti-pS129-α-syn monoclonal antibody [#]	222 (180.5–275) pg/mL	261 (206.8–296.3) pg/ml	Methodology is not described	Not described in the method section	NA	Majbour et al. [79]
	CSF	ELISA	Mouse anti-pS129-α-syn monoclonal antibody [#]	NA	Baseline: 220.2 (145.0-316.4) pg/ml Follow-up: 180.8 (125.0-252.2) pg/ml	Methodology is not described	Not described in the method section	NA	Majbour et al. [78]

	CSF	ELISA	Mouse anti-pS129-α-syn monoclonal antibody #	116 (103–145) pg/mL	Sporadic PD: 139 (114.25–163) pg/mL Asymptomatic LRRK2 mutation carriers: 121 (94–150) pg/mL Symptomatic LRRK2 mutation carriers: 122 (106–145) pg/mL Follow-up: 117.89 ± 17.92 (pg/ml)	Methodology is not described	Not described in the method section	NA	Majbour et al. [80]
	CSF	ELISA	Mouse anti-pS129-α-syn monoclonal antibody #	235 ± 54 pg/mL	PD: 258 ± 52 pg/mL DLB: 232 ± 79 pg/mL AD: 220 ± 61 pg/mL	Methodology is not described	Not described in the method section	NA	van Steenoven et al. [123]
	CSF	ELISA	Mouse anti-pS129-α-syn monoclonal antibody #	NA	PD: 85 (55–110) pg/mL MSA: 54 (46–64) pg/mL PSP: 67 (56–78) pg/mL CBD: 60 (53–109) pg/mL AD: 59 (47–79) pg/mL FTD: 49 (34–72) pg/mL VD: 55 (46–93) pg/mL	Methodology is not described	Not described in the method section	NA	Constantinides et al. [124]
	CSF	ELISA using the Erenna Immunoassay System	Capture: PRTA-11A5 Detection: PRTA-23E8	2.19 0.83 pg/mL	PD: 1.94 0.90 pg/mL MSA: 1.84 0.71 pg/mL DLB: 2.34 0.97 pg/mL FTD/ALS: 2.28 1.04 pg/mL AD: 2.45 1.08 pg/mL CBS: 2.07 0.83 pg/mL PSP: 1.95 0.90 pg/mL	Recombinant aSyn was incubated with PLK2	mass spectrometry	NA	Schulz et al. [125]
	CSF	ELISA	Mouse anti-pS129-α-syn monoclonal antibody #	- Baseline: 112 (89–129) pg/mL - 24months follow-up: 101 (75–131) pg/mL - 48 months follow-up: 98 (89–140) pg/mL	- Baseline: 116 (89–160) pg/mL - 24months follow-up: 105 (78–126) pg/mL - 48 months follow-up: 128 (92–174) pg/mL	Methodology is not described	Not described in the method section	NA	Majbour et al. [126]
	Plasma and CSF	IP, Western Blot and Singulex Assays	MJF-R13 (8-8) (ab168381; Abcam)	Plasma: 878.5 ± 317.4 pg/ml CSF: NA (below detection limit)	NA	Recombinant aSyn was incubated with PLK3	UPLC, mass spectrometry, SDS-PAGE and WB analysis using pS129 antibody (ab168381)	NA	Cariulo et al. [53]
	Plasma	Western blot and biotin ELISA	ELISA- Capture: anti-α-synuclein N-19 (Santa Cruz Biotechnology); Detection: anti-pS129 (Epitomics); Western blot: pS129 (Epitomics)	HC: 0.15 to 0.6 ug/mL	PD:0.2 to 2 ug/mL	Recombinant aSyn was incubated with casein kinase II (New England Biolabs)	Immunoblotting with a phosphorylation-dependent anti-aSyn antibody, pS129 (Epitomics) and mass spectrometry	NA	Foulds et al. [54]
	Plasma	ELISA	Capture: anti-α-synuclein N-19 (Santa Cruz Biotechnology); Detection: anti-pS129 (Epitomics)	143.4 ± 531.8 ng/mL	756.8 ± 2419.9 ng/mL	Recombinant aSyn was incubated with casein kinase II (New England Biolabs)	Immunoblotting with a phosphorylation-dependent anti-aSyn antibody, pS129 (Epitomics) and mass spectrometry	NA	Foulds et al. [55]
	Plasma	Immunomagnetic reduction (IMR)-based immunoassay	Dextran-coating magnetic Fe3O4 nanoparticles (MF-DEX-0060, MagQu) bio-functionalized with monoclonal antibody - 825701, (Biolegend)	0.8 ± 0.6 fg/mL	12.9 ± 8.7 fg/mL	Synthetic: Alpha-synuclein pS129 peptide (ab188826)	Synthesis*	NA	Lin et al. [141]

	Serum	Modified paired surface plasma wave biosensor coupled to an immunoassay and non-labelled technique	Rabbit monoclonal anti-α-syn (phosphor S129) antibody (anti-p-S129-α-syn; Abcam, Cambridge, MA, USA)	0.5 to 5 ng/mL	4 to 12 ng/mL	Human phosphorylated aSyn ELISA kit (MyBioSource Co., Vancouver, Canada)	NA	NA	Chen et al. [142]
	Red blood cells	Phospholipid-ELISA assay	pSyn#64 (WAKO)	24.48 ± 7.6 pg a-Syn/mg protein	- PD-M: 35.820 ± 15.19 pg a-Syn/mg protein - PD-D: 27.370 ± 9.76 pg a-Syn/mg protein	Human Alpha-synuclein pS129 (RP-004; Proteos)	Semi-synthetic*	NA	Elhadi et al. [148]
	Red blood cells	ELISA, Western blot and immunodepletion	Nonbio sc-135638 (Santa Cruz Biotechnology)	11.89 ± 3.57 ng/mg	MSA: 14.02 ± 4.02 ng/mg MSA-P:13.27 ± 1.91 ng/mg MSA-C: 12.19 ± 3.04 ng/mg	Recombinant aSyn was incubated with casein kinase II (New England Biolabs)	SDS-PAGE and Western blotting analysis with a phosphorylation-dependent anti-aSyn antibody	NA	Li et al. [149]
	Red blood cells	Electrochemiluminescence assay	Anti-pS129 (BioLegend)	- Cytosol: 67.36 ± 0.48 pg/µg - Membranes: 255.05 ± 1.98 pg/µg	- Cytosol: 636.05 ± 6.03 pg/µg - Membranes: 315.35 ± 0.95 pg/µg	Human Alpha-synuclein pS129 (RP-004; Proteos)	Semi-synthetic*	NA	Tian et al. [147]
Oligo-phosphorylation	CSF	ELISA	Capture: anti-pS129 (Epitomics); Detection: biotinylated pS129 #	1.05±2.23 µg/ml	PD: 0.77±1.51 µg/ml PD (nonD): 0.26±0.03 µg/ml PD (Cog): 0.68±0.78 µg/ml PD (Dem): 1.28±2.27 µg/ml DLB: 1.60±3.02 µg/ml PSP: 1.25±3.32 µg/ml MSA: 19.56±1.66 µg/ml	Recombinant aSyn was incubated with casein kinase II (New England Biolabs)	Immunoblotting with a phosphorylation-dependent anti-aSyn antibody, pS129 (Epitomics) and mass spectrometry	NA	Foulds et al. [49]
	Plasma	Biotin ELISA	Capture: anti-pS129 (Epitomics); Detection: biotinylated pS129 #	HC: 0.04 - 0.09 ug/mL	PD: 0.04 to 0.18 ug/mL	Recombinant aSyn was incubated with casein kinase II (New England Biolabs)	Immunoblotting with a phosphorylation-dependent anti-aSyn antibody, pS129 (Epitomics) and mass spectrometry	NA	Foulds et al. [54]
Advanced glycation end-products (AGEs)	Red blood cells	Immunoblotting analysis (Western blot and dot blots)	AGEs KAL-KH001 (Cosmo-Bio)	NA	NA	NA	NA	NA	Vicente Miranda et al. [145]
SUMOylation	Red blood cells	Immunoblotting analysis (Western blot and dot blots)	Sc-9060 (Santa Cruz Biotechnology)	NA	NA	NA	NA	NA	Vicente Miranda et al. [145]
Ubiquitination	Plasma	Western blot and biotin ELISA	Anti-ubiquitin antibody FL-76 (Santa Cruz Biotechnology)	NA	NA	NA	NA	NA	Foulds et al. [54]
	Red blood cells	Immunoblotting analysis (Western blot and dot blots)	ab24686 (Abcam)	NA	NA	NA	NA	NA	Vicente Miranda et al. [145]

NA- Not applicable; # - antibodies generated in-house; * Commercial available

Table 2 - Overview of the concentration of unmodified aSyn and pS129 aSyn in the different biological fluid specimens, along with the other PTMs detected

Biological fluid	aSyn levels range in controls and PD	pS129 levels range in controls and PD	Other PTMs detected
CSF	67 to 68900 pg/mL [44-47,52,114-116]; 1.45±1.97 to 3.80±2.40 µg/mL [49]	no detection to 7.14±9.19 µg/mL [49,50,53, 77-80,123-125]	pY39; oligo-pS129
Plasma	3600 to 1777100± 3609600 pg/mL [53-59]	0.8 ± 0.6 fg/mL to 12.9±8.7 fg/mL [141]; 878.5±317.4 to 756800±2419900 pg/mL [53,55]	oligo-pS129; ubiquitination
Red blood cells	76.44±0.08 to 307±100 ng/mg [147-149; 26200±3000 to 40000 ng/mL [60,62]	24.48 ± 7.6 to 636050 ± 6030 pg/mg [147-149]	AGEs; SUMOylation; nY39; pY125
Saliva	7.104±5.122 to 314.03±435.9 pg/mL[63-66]; 159.4 ± 61.6 to 229.9 ± 64 ng/mL [67]	NA	NA
Tears	32.02 to 361.16 pg/mg [68,69]	NA	NA

*NA- Not applicable;