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Diagnostic biomarkers for Parkinson's disease: focus on α-synuclein in cerebrospinal fluid

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ABSTRACT

Diagnostic biomarkers are measures that detect or confirm the presence of a disease or identify individuals with a subtype of the disease. For Parkinson's disease, unlike other neurodegenerative diseases such as Alzheimer's disease and Creutzfeldt-Jakob disease, diagnostic biomarkers remain elusive as none are yet available or approved for clinical use. A biomarker to diagnose early or prodromal Parkinson's disease with high accuracy would significantly enhance clinical practice as well as advance clinical therapeutic trials. Multiple lines of evidence support a role of α-synuclein in the pathophysiology of Parkinson's disease and hence major ongoing efforts to identify biomarkers for Parkinson's disease are aimed at measuring α-synuclein in peripheral tissues and biofluids, including cerebrospinal fluid. This work is still in the early stages of biomarker development and has been accompanied by both losses and victories. Here, α-synuclein in cerebrospinal fluid as a diagnostic marker for Parkinson's disease is reviewed, including measures of total α-synuclein, oligomeric and phosphorylated α-synuclein, and seeding activity of α-synuclein.

1. Introduction to diagnostic biomarkers

A biological marker, or *biomarker*, is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [1] or "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" [2]. Biomarkers can be classified into various types according to their specific function (Table 1) [3]. For example, *diagnostic biomarkers* are measures that detect or confirm the presence of a disease or identify individuals with a subtype of the disease. While research into biomarkers for Parkinson's disease (PD) spans the range of biomarker types, a major focus has been on diagnostic biomarkers. This focus is justly warranted since a diagnostic biomarker for PD has the potential to confirm a pre-mortem diagnosis of PD in the clinical setting thereby enhancing clinical practice and having meaningful implications for patients and their families. Moreover, a biomarker that can diagnose with high accuracy PD at motor symptom onset, or ideally pre-motor or prodromal PD, will greatly facilitate clinical trials testing disease-modifying therapies intended to target early stages of neurodegeneration.

Similar to therapeutic development, developing a diagnostic biomarker involves a process with multiple phases. The initial *discovery phase* involves pilot or exploratory studies to identify candidate biomarkers as well as early assay development. This phase is enhanced by use of patient and control samples obtained from standardized sources. For this reason, initiatives such as the Parkinson Disease Biomarkers Program (PDBP) which provides access to biospecimens for biomarker research that have been collected and stored according to standard operating procedures together with clinical and laboratory data obtained using harmonized protocols [4,5] are critical for biomarker discovery. Next is a *validation phase* which includes establishing the performance characteristics of the diagnostic biomarker. The key performance measures are *sensitivity* (i.e., the proportion of true positives correctly identified by the biomarker among people with disease) and *specificity* (i.e., the proportion of true negatives correctly identified by the biomarker among people without disease) [6]. From these measures, biomarker performance can be further analyzed by generating receiver-operator curves (ROC) and calculating area under the curve (AUC), as well as by determining likelihood ratios [7,8]. An important component of the validation phase is determining reproducibility through replication studies. Prior to clinical use, potential diagnostic biomarkers are further assessed in a *clinical utility phase* and possibly a *qualification phase*. The clinical utility phase involves testing the

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Table 1
Types of Biomarkers [3].

Type of Biomarker	Function
Susceptibility/Risk	To indicate the potential for developing a disease in an individual who does not currently have clinically apparent disease
Diagnostic	To detect or confirm the presence of a disease or to identify individuals with a subtype of the disease
Prognostic	To identify the likelihood of a clinical event or progression in patients who have the disease
Predictive	To identify individuals who are more likely to experience a favorable or unfavorable effect from a treatment
Monitoring	Measured serially to assess status of a disease or medical condition or for evidence of exposure to (or effect of) a medical product or an environmental agent
Surrogate Endpoint	An endpoint supported by mechanistic and/or epidemiologic rationale and/or clinical data providing evidence that an effect on the surrogate endpoint predicts a specific clinical benefit
Pharmacodynamic/Response	To show that a biological response has occurred in an individual who has been exposed to a medical product or an environmental agent
Safety	Measured before or after an exposure to a medical product or an environmental agent to indicate the likelihood, presence, or extent of toxicity as an adverse effect

biomarker in large prospective clinical studies and are crucial since pilot studies have the tendency to significantly overestimate findings [9].

Many of the ideal features of a diagnostic biomarker for PD are the same as for other diseases [10]. From a practical point of view, the ideal biomarker would be readily quantifiable in accessible clinical samples, such as blood or cerebrospinal fluid (CSF). The assay developed to perform the measure should be reliable, quick, and inexpensive to allow for its use in most clinical settings. For optimal performance characteristics, the biomarker should demonstrate an appreciable increase or decrease specifically in the relevant disease without being affected by unrelated conditions or comorbid factors. Furthermore, the values measured should not show a wide variation in the general population. Ideally, there would be no overlap in these values between patients and healthy control subjects to allow for a clear cut-off for diagnosis. A logical expectation is that a measure which reflects the underlying pathophysiology of the disease would be most likely to meet these criteria of an ideal diagnostic biomarker for PD.

2. Rationale for α-synuclein-based biomarkers

The classical strategy for discovery of a biomarker is the hypothesis-driven *targeted approach* in which a candidate biomarker is selected for investigation based on existing knowledge about the pathophysiology of the disease. This strategy contrasts the hypothesis-generating *unbiased approach* in which many possible biomarkers are screened simultaneously (i.e., "-omics") without *a priori* assumptions regarding their potential for reflecting important pathophysiological information [11]. From the targeted approach, several candidate biomarkers for PD have been discovered including *clinical markers* (e.g., olfactory impairment measured by University of Pennsylvania's Smell Identification Test (UPSIT) and REM sleep behaviour disorder (RBD) diagnosed by polysomnography), *imaging markers* (e.g., ligand-based imaging methods, such as positron emission tomography (PET) or single photon emission tomography (SPECT), to measure reduction of dopaminergic nerve terminals within the striatum), and *genetic markers* (e.g., *GBA* mutation carrier status) [12].

Major ongoing efforts using targeted approaches to identify PD biomarkers are aimed at measuring aspects of α-synuclein. The rationale for these efforts is based on multiple lines of evidence supporting a key role for α-synuclein in the neurodegenerative process in PD including its aggregation into fibrillar structures leading to Lewy pathology, the neuropathological hallmark of PD that frequently accompanies neurodegeneration. Unequivocal evidence for pathogenicity of α-synuclein in PD came from early genetic studies which demonstrated that missense mutations or multiplications of *SNCA*, the gene encoding for the protein α-synuclein, cause autosomal dominant forms of PD [13]. Missense mutations cause α-synuclein misfolding and multiplications cause α-synuclein overexpression, both promoting α-synuclein aggregation with fibril and oligomer formation. Additional genetic evidence came from genome-wide association studies (GWAS) which have consistently identified variations at the *SNCA* locus as one of the strongest genetic risk factors for developing sporadic PD [14]. In addition, experimental findings from *in vitro* and *in vivo* studies strongly support a cytotoxic role for α-synuclein in PD through various mechanisms including impairment of protein folding and degradation, endoplasmic reticulum stress, mitochondrial dysfunction and oxidative stress, and neuroinflammation [15].

Based on earlier demonstrations of α-synuclein within the peripheral nervous system [16], several investigators are exploring whether α-synuclein in peripheral tissues, detected using methods such as immunohistochemistry, could be used as *pathological markers*. The impetus for this area of exploration arises partly from the hypothesis that abnormal aggregation of α-synuclein begins in the periphery and spreads to the central nervous system (CNS). Thus, a biomarker that measures abnormal peripheral α-synuclein could have the potential to detect premotor disease (i.e., before the neurodegenerative process causes motor symptoms by involving the substantia nigra pars compacta). Peripheral tissues being examined for the development of an α-synuclein-based pathological marker include gastrointestinal tract (e.g., stomach, colon), salivary glands (e.g., submandibular, minor), skin, and olfactory epithelium [17,18].

Another area of active investigation is the discovery and development of α-synuclein-based *biochemical markers*. Assays for these biomarkers attempt to measure α-synuclein within biofluids, such as saliva, blood (e.g., serum, plasma, red blood cells), and CSF, which tend to be more easily accessible than tissue. Similar to pathological markers in peripheral tissues, α-synuclein in saliva is being explored as diagnostic biomarkers for PD because of its potential to detect a pathological process that may first occur outside the CNS. Measures of α-synuclein in blood are of interest because α-synuclein might be transported from the CNS to blood. Indeed, rodent studies have demonstrated that labelled α-synuclein injected into the brain can be detected in plasma, including within a fraction containing exosomes likely derived from the CNS [19]. When α-synuclein levels were measured from this exosome fraction in human plasma, they were found to be higher in PD subjects versus healthy controls whereas total plasma α-synuclein levels were not different [19]. Whether measures of α-synuclein in blood directly reflect abnormalities of α-synuclein occurring within neurons of the CNS remains to be determined. As of yet, CSF is the biofluid more likely to reflect CNS abnormalities [20].

3. α-Synuclein levels in cerebrospinal fluid

CSF is a relatively accessible biofluid that is commonly used for diagnostic assays in neurology (e.g., albuminocytologic dissociation for Guillain-Barre syndrome, oligoclonal bands for multiple sclerosis, autoantibodies for autoimmune encephalitis). To date, the most extensively studied biochemical biomarker for PD is CSF α-synuclein. Optimism for this line of investigation came, in part, from the successes achieved in CSF biomarker development for Alzheimer's disease (AD). Neuropathological hallmarks of AD include amyloid plaques and

neurofibrillary tangles which are primarily composed of amyloid-β and tau, respectively. Assays to measure CSF levels of amyloid-β (specifically amyloid-β1-42) and tau (including total tau and phosphorylated tau) have been developed. In combination, reduced levels of amyloid-β1-42 and elevated levels of total tau and tau phosphorylated on threonine 181 (pThr181) in CSF are 85%–95% sensitive and specific for AD at the stage of dementia or earlier at the mild cognitive impairment (MCI) stage [21]. Thus, these CSF biomarkers are now included in research diagnostic criteria for both prodromal and dementia stages of AD and there are recommendations to move them toward clinical implementation [22]. Unfortunately, similar successes have not yet been achieved in the development of CSF α-synuclein as a diagnostic biomarker for PD.

3.1. Total α-synuclein

Multiple studies have investigated levels of total α-synuclein in CSF as a potential diagnostic biomarker for PD. These studies demonstrated that α-synuclein is readily quantifiable in CSF using traditional antibody-based assays, such as enzyme-linked immunosorbent assays (ELISA). The most recent meta-analysis which assessed 17 of these studies (in total n = 1482 PD subjects and n = 1012 healthy controls) demonstrated that total α-synuclein levels in CSF are lower in PD compared to healthy controls [23]. However, this finding was inconsistent across studies. For those studies that did demonstrate a difference between PD subjects and healthy controls, this difference tended to be modest (only 10%–20%). Moreover, total α-synuclein levels demonstrated wide variation in both PD and healthy populations which was accompanied by significant overlap in values between the two populations. In this meta-analysis, diagnostic data were available for 14 studies and, using these data, pooled sensitivity and pooled specificity were calculated as 72% and 65%, respectively, which are suboptimal. At least 2 meta-analyses aimed at examining whether levels of total α-synuclein in CSF are decreased specifically in PD found no difference between PD and other neurodegenerative diseases associated with α-synuclein pathology, including dementia with Lewy bodies (DLB), multiple system atrophy (MSA), and AD [23,24].

3.2. Oligomeric or phosphorylated α-synuclein

α-Synuclein is an endogenously expressed protein that is present in high amounts in the healthy CNS. The physiologic state of α-synuclein is likely in a monomeric and possibly tetrameric form (Fig. 1). The physiologic functions of α-synuclein have yet to be completely elucidated but appear to include a role in exocytosis and synaptic vesicle-mediated release of neurotransmitter. The underwhelming performance of total CSF α-synuclein as a diagnostic biomarker for PD may be due to assays that measure both physiologic and pathogenic forms of α-synuclein and cannot discriminate between the two. Putative pathogenic forms of α-synuclein include those with aberrant tertiary or quaternary protein conformations (e.g., oligomers, fibrils) or with specific post-translational modifications (e.g., phosphorylated, ubiquitinated, nitrated). In addition, truncated or cleaved α-synuclein (e.g., at the C-terminus) as well as α-synuclein compartmentalized within exosomes are proposed to be pathogenic forms. Specifically measuring these putative pathogenic forms of α-synuclein in CSF would be expected to produce a more promising diagnostic biomarker for PD than measuring total α-synuclein. Assays measuring oligomeric α-synuclein or α-synuclein phosphorylated on serine 129 (pSer129) have been the most studied and it appears that these pathogenic forms of α-synuclein are increased in CSF in PD compared to healthy or neurological disease controls [23,24]. Early studies have shown that CSF α-synuclein oligomer levels are also increased in PD subjects who report RBD features [25] but not in subjects with idiopathic RBD diagnosed by polysomnography [26], suggesting that CSF oligomeric α-synuclein may not be a useful susceptibility/risk biomarker or diagnostic biomarker for prodromal PD. In contrast, elevated CSF oligomeric α-synuclein may be of value as a diagnostic biomarker for DLB or PD dementia [26,27]. Initial studies investigating phosphorylated α-synuclein in CSF suggest that pSer129 levels may have diagnostic value; for example, in differentiating PD from atypical parkinsonism such as progressive supranuclear palsy (PSP) [28]. CSF pSer129 α-synuclein levels have also been found to change over the course of PD [28,29], suggesting the possibility of a monitoring biomarker. However, only a limited number of studies investigating oligomeric α-synuclein or pSer129 α-synuclein in CSF in PD have included both discovery and validation cohorts (e.g., [28,30]). Also, findings have been inconsistent, and assays have not been widely replicable, possibly due to preanalytical differences (e.g., CSF sampling, processing, storage, and handling), contamination of CSF samples with blood (which contains high levels of α-synuclein), variations in the assays used to detect α-synuclein (e.g., differences in the anti-α-synuclein antibodies used in ELISAs), and confounding variables (e.g., medications and co-morbidities) [31,32]. Furthermore, since the levels of these putative pathogenic forms of α-synuclein are very low, it may be that using current methods results in a floor-effect that precludes reliable detection of differences between PD and controls.

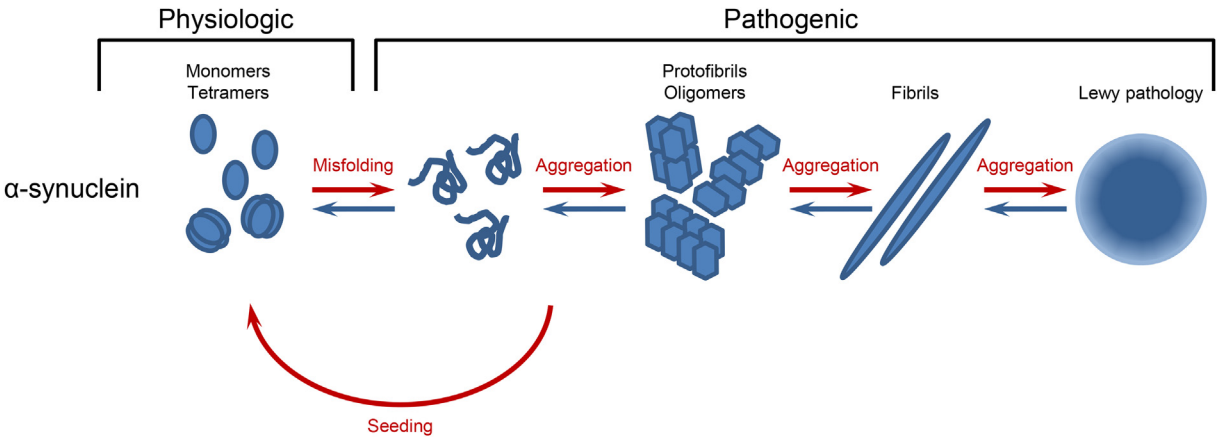

Figure 1. Pathogenic aspects of α-synuclein. The physiologic state of α-synuclein is likely a monomeric and possibly tetrameric form. Emerging evidence suggests that α-synuclein shares similar pathogenic features to prion protein. In particular, misfolded α-synuclein has a propensity to aggregate to form pathogenic oligomers and fibrils, as well as large inclusions termed Lewy pathology. Pathogenic α-synuclein can seed the misfolding and aggregation of non-pathogenic α-synuclein.

4. Seeding activity of α-synuclein from cerebrospinal fluid

4.1. Assays to measure seeding activity

An alternative to directly measuring amounts of pathogenic forms of α-synuclein in CSF is instead measuring pathogenic activity of α-synuclein. Emerging evidence suggests that α-synuclein shares many of the same pathogenic activities as prion protein (PrP) which is implicated in Creutzfeldt-Jakob disease (CJD), a rapidly progressive neurodegenerative disease [33]. These activities of PrP and α-synuclein include a propensity to aggregate as well as the ability of misfolded pathogenic forms of the protein to induce non-pathogenic forms of the protein to misfold and take on pathogenic conformations in a process termed self-templating (Fig. 1). Thus, pathogenic α-synuclein can act as a seed that initiates exponential increase of misfolded and aggregated α-synuclein.

Methods to measure seeding activity of PrP have been developed and applied to the development of diagnostic biomarkers for CJD. These methods are protein amplification techniques that exploit the ability of pathogenic PrP to induce a conformational change in non-pathogenic PrP so that small amounts of pathogenic PrP in biofluids can be amplified to a threshold of detection by conventional laboratory techniques. The two most developed protein aggregation assays are the protein misfolding cyclic amplification assay (PMCA) [34] and real-time quaking-induced conversion assay (RT-QuIC) [35,36]. The methods of both assays involve combining a test sample (e.g., CSF) containing small amounts of the pathogenic protein which will act as a *seed* with non-pathogenic protein (e.g., recombinant protein, protein from cell homogenates, protein from brain homogenates) which acts as the *substrate* [37,38]. This combination of pathogenic and non-pathogenic proteins undergoes multiple cycles of *mechanical disruption* (e.g., sonication, shaking) which promotes seeding, misfolding, and aggregation. A read-out of misfolded and aggregated proteins (e.g., immunoblot of proteinase K resistant protein, thioflavin T fluorescence) is then used as a measure of seeding activity.

Using protein aggregation assays to develop a diagnostic biomarker for CJD appears very promising. RT-QuIC has been developed with recombinant prion protein as the substrate, intermittent shaking as the method for mechanical disruption, and thioflavin T fluorescence as the read-out of misfolded and aggregated proteins [39]. This RT-QuIC allows for detection of ≥1 femtogram of pathogenic prion protein. Using CSF as the test sample, sensitivity and specificity of this RT-QuIC for diagnosis of sporadic CJD are 77%–97% and 99%–100%, respectively [39]. Thus, this assay is starting to be used to support a pre-mortem diagnosis of CJD. For example, CSF RT-QuIC is now used by the National Prion Disease Pathology Surveillance Center in the United States and the Prion Laboratory of the National Microbiology Laboratory in Canada to assist in making a CJD diagnosis. These assays are also being adapted to be used with other biospecimens. An initial study investigated an assay using RT-QuIC with nasal brushings and demonstrated that this assay may have better performance characteristics than CSF RT-QuIC for diagnosis of sporadic CJD (sensitivity 97%, specificity 100%) [40]. In addition, PMCA has been developed with brain homogenates from prion protein transgenic mice as the substrate, sonication as the method for mechanical disruption, and immunoblotting of proteinase K digested protein as the read-out of misfolded and aggregated proteins [41]. Using urine as the test sample, this PMCA may be a useful diagnostic biomarker for variant CJD (sensitivity 93%, specificity 100%) [41]. The RT-QuIC for CJD does appear to have some advantages over PMCA including the use of recombinant protein as the substrate (which is more reliable and reproducible than brain homogenate), the use of shaking for mechanical disruption (which tends to be easier to standardize and replicate between laboratories compared to sonication), and the use of thioflavin T fluorescence for misfolded protein detection (which is faster, less laborious, and more sensitive than the immunoblotting protocol) [42].

4.2. Pilot studies in Parkinson's disease

To date, three pilot studies have investigated protein amplification techniques like those developed for CJD to measure seeding activity of α-synuclein in CSF as a potential diagnostic biomarker for PD [43–45]. The first study included an exploratory phase to investigate the use of a RT-QuIC assay with CSF as well as a small validation phase [43]. The validation phase included 20 PD subjects of which 19 had a positive RT-QuIC response and 15 healthy controls of which none were positive. Thus, the sensitivity and specificity for PD was calculated as 95% and 100%, respectively. Interestingly, 3 subjects with idiopathic RBD, hence at high risk of developing PD, were also included in the validation phase and all had a positive RT-QuIC response, suggesting this measure has the potential to be a susceptibility/risk biomarker or a diagnostic biomarker for prodromal PD. For this RT-QuIC assay, 5 days were required to determine the test response. A second study examining RT-QuIC with CSF adapted the methodology (e.g., using of recombinant K23Q mutant α-synuclein instead of wild-type α-synuclein as substrate) to improve the assay including shortening its duration to only 1–2 days [44]. This assay was tested with samples from 12 PD subjects and 12 healthy controls as well as samples from 17 DLB, 16 AD, 2 PSP, and 1 corticobasal degeneration (CBD) subjects. Almost all of the PD (11 out of 12) and DLB (16 out of 17) CSF samples had positive RT-QuIC responses, whereas none of the AD subjects or non-synucleinopathy controls (i.e., healthy controls, PSP, CBD) met criteria to be considered positive RT-QuIC responses. Thus, this assay had a sensitivity and specificity of 93% and 100%, respectively, for either PD or DLB. The largest study so far to examine a protein amplification technique to measure α-synuclein seeding activity in CSF for PD used PMCA [45]. Unlike the PMCA developed for CJD, this PMCA used recombinant protein instead of brain homogenates as the substrate, cyclic agitation instead of sonication for mechanical disruption, and thioflavin T fluorescence instead of immunoblotting as the read-out of protein misfolding and aggregation. CSF from 2 different cohorts of PD samples were tested (n = 76) as well as CSF from healthy and other neurologic disease controls (n = 65) and subjects with DLB (n = 10), MSA (n = 10), AD (n = 14), and other chronic neurodegenerative diseases (n = 18). Duration of this assay was 13 days. Sensitivity was 88% for PD, 100% for DLB, and 80% for MSA. Specificity against controls and neurodegenerative diseases was 94%. Importantly, the investigators included an experiment in which they immune-depleted PD CSF samples using a cocktail of anti-α-synuclein antibodies. When immune-depleted samples were used for PMCA, they observed reduced kinetics of α-synuclein aggregation, demonstrating that PMCA does indeed detect α-synuclein seeds. Furthermore, the investigators examined whether α-synuclein seeding kinetics may correlate with disease severity. Interestingly, they found that time to reach 50% aggregation using their PMCA was negatively correlated with modified Hoehn and Yahr scores for both PD cohorts, suggesting the potential of CSF PMCA as a monitoring biomarker for PD.

5. Conclusions

Current assays that directly measure levels of total α-synuclein or putative pathogenic forms of α-synuclein in CSF are unlikely to provide viable biomarkers for clinical use to diagnose PD. Pilot studies on CSF α-synuclein seeding activity measured by protein amplification assays, such as RT-QuIC or PMCA, have demonstrated promising results but further validation, including replication studies and larger sample sizes, are required since pilot studies have the tendency to significantly overestimate findings. In addition to a possible diagnostic biomarker, CSF α-synuclein seeding activity may have the potential to serve as other biomarkers for PD, such as susceptibility/risk or monitoring biomarkers. However, in their present form, these seeding assays are unable to differentiate between the different synucleinopathies (PD *versus* DLB *versus* MSA) which is an important requirement of a

biomarker to diagnose early PD. Whether the use of other biospecimens (biofluids or tissues) can improve the performance of α-synuclein seeding activity assays as a PD biomarker remain to be explored.

Conflicts of interest

None. I certify that I have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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