Evolution of cerebrospinal fluid total \( \alpha \)-synuclein in Parkinson’s disease

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Abstract
**Introduction:** Cerebrospinal fluid (CSF) total α-synuclein is considered a potential biomarker for Parkinson’s disease (PD), but little is known about the evolution of this marker during the course of the disease. Our objective was to investigate whether CSF total α-synuclein concentrations change over time and are associated with motor and cognitive function in PD.

**Methods:** CSF total α-synuclein concentrations were quantified in 56 longitudinally followed PD patients, 27 of whom provided CSF repeatedly 2 and/or 4 years later. Another 18 subjects were included as controls. The samples were analyzed using two independent, validated ELISA methods: our recently developed and validated in-house ELISA and a commercial kit from BioLegend.

**Results:** CSF total α-synuclein levels did not distinguish PD patients from controls, displayed no substantial changes during a period of up to 4 years, and did not predict subsequent motor or cognitive decline. These findings were consistent for both analytical methods.

**Conclusion:** Our findings do not support the clinical utility of total α-synuclein as a single diagnostic or prognostic biomarker in PD.
Introduction

Parkinson’s disease (PD) is characterized by a remarkable clinical heterogeneity, with substantial variation in disease progression and considerable overlap with other neurodegenerative diseases. Therefore, reliable biomarkers for diagnosis and prognosis of PD are urgently needed. Given the central role of α-synuclein aggregation in PD pathogenesis, and because α-synuclein levels are a major determinant of its neurotoxicity [1], it is of great interest to investigate the potential of α-synuclein as a diagnostic and prognostic biomarker. In this respect, cerebrospinal fluid (CSF) is considered the most promising source. Most, but not all previous studies report a trend towards lower total CSF α-synuclein levels in PD compared to controls, though with substantial overlap between diagnostic groups [2]. Only a few studies have reported longitudinal measurements of α-synuclein, also with conflicting results [3-6]. Hence, there is a need for additional longitudinal studies to track CSF α-synuclein changes during the disease course. We also examined whether total α-synuclein concentrations were associated with motor symptoms and neuropsychological performance, and their changes over a period of up to 4 years. In addition, we compared total α-synuclein levels of PD patients with those of the controls.
Materials and methods

Patients

All PD patients were enrolled in the Norwegian ParkWest study, an ongoing, prospective, population-based, longitudinal cohort study of PD in Southwestern Norway [7]. Participants included a subset of 56 PD patients (median disease duration 4.8 years), of whom 27 also provided CSF at one or two follow-up visits 2 and 4 years later (Figure 1 and Table 1). The patients were followed prospectively by movement disorders neurologists and fulfilled the National Institute of Neurological Disorders and Stroke [8] and UK Brain Bank [9] diagnostic criteria of PD from study entry to latest visit. Exclusion criteria were secondary parkinsonism and dementia during the first year of motor onset to eliminate patients with dementia with Lewy bodies and other dementias, including Alzheimer’s disease. All patients underwent a uniform and standardized examination program at each visit. Disease severity was determined using the Unified Parkinson’s Disease Rating Scale (UPDRS) [10] part II and III, and disease stage was evaluated using the Hoehn and Yahr scale (H&Y) [11]. Cognitive assessments included the Mini-Mental State Examination (MMSE) [12] as a measure of global cognition, and a neuropsychological test battery assessing the following domains: verbal memory (California Verbal Learning Test II [CVLT-II] [13], which included total immediate recall [sum of trials 1–5], short-delay, and long-delay free recall scores), attention (Stroop word reading and color naming) [14], executive functioning (Semantic Verbal Fluency Test [15], and Stroop interference condition) and visuospatial skills (Visual Object and Space Perception battery [VOSP] Silhouettes and Cube subtests) [16]. Severity of depressive symptoms was determined using the Montgomery and Aasberg Depression Rating Scale (MADRS) [17].

Controls

The control group consisted of 18 subjects without known brain disease, who underwent elective neurological examination or orthopedic surgery at Stavanger University Hospital.
**CSF sample treatment**

CSF collection and sample treatment was conducted according to standardized procedures, as described elsewhere [18]. The CSF samples were centrifuged at 2000g for 10 minutes and frozen in polypropylene tubes at -80°C. The samples were subjected to one freeze-thaw event for aliquotation purposes.

**α-synuclein measurement**

The in-house assay procedures and characteristics have been described in detail previously [19]. In brief, the ELISA assay fulfills the newest validation criteria [20], has a lower limit of quantification of 36.3pg/mL and requires less than 15µL CSF. The samples were analyzed in duplicates at the Neuroscience Research Laboratory at Stavanger University Hospital, Norway, with plate-to-plate variation below 6%. An identical set of CSF aliquots were sent to the Clinical Neurochemistry Laboratory at Sahlgrenska University Hospital, Sweden for analysis with the commercial ELISA kit from BioLegend (#844101; BioLegend, San Diego, USA), with plate-to-plate variation below 5%. There was a high correlation between the two assays (ρ=0.785, *P*<0.001), which were independent in terms of site, date of analysis and personnel.

**Hemoglobin measurement**

To assess possible blood contamination as source of α-synuclein, the samples were analyzed for hemoglobin content using a commercial kit (#E88-135, Bethyl Laboratories, Montgomery, USA). Samples with hemoglobin concentrations above 200µg/mL were excluded from the study.

**Statistical analysis**

Standard curve fitting (4-parameter logistic fit), plotting of calibrator curves and calculation of total α-synuclein concentrations were performed using the Meso Scale Discovery Workbench 4.0 software (Meso Scale Discovery, Gaithersburg, USA). Descriptive statistics for continuous
variables are presented as means for symmetrically distributed data, as geometric means for right skewed, small samples, or otherwise as medians, and with full ranges. Categorical variables are presented with counts and percentages. Between-group comparisons were performed by Mann-Whitney U for continuous variables. Within-patient comparisons were performed by paired samples t-tests for symmetrically distributed differences, or Wilcoxon paired signed rank tests for non-normally distributed data. Possible relationships between CSF total α-synuclein and clinical and neuropsychological performance at CSF sampling were assessed by linear regression analysis with adjustment for age and sex. To assess associations between CSF total α-synuclein and clinical and neuropsychological measures within 4 years after CSF sampling, we used linear random intercept models with adjustment for age and sex, and with time and CSF total α-synuclein included as main effects and with an interaction term, that measures the possible differential effect of levels of α-synuclein on progression slopes. Spearman’s rank correlation was used to assess strength of association between the two analytical methods. All analyses were conducted using SPSS 23 (IBM, Armonk, USA). Two-tailed P-values <0.05 were considered statistically significant. For multiple comparisons, Bonferroni correction was applied with two-tailed P-values <0.0036 (14 tests) considered significant.

Research Ethics

All procedures were approved by The Norwegian Regional Committee for Medical and Health Research Ethics, and signed written informed consent was obtained from all participants.
Results

There was no significant difference in CSF total α-synuclein concentrations between PD patients (N=56, 55% males, median age 69.6) and controls (N=18, 50% males, median age 67.7), as measured by the in-house assay (469 [178-1333] pg/ml vs. 424 [175-852] pg/ml; $P=0.392$), and the BioLegend kit (677 [259-1723] pg/ml vs. 747 [306-1623] pg/ml; $P=0.366$) (Figure 2A and B). In the PD patients, CSF levels were not significantly associated with clinical or cognitive performance cross-sectionally (Table s1), nor did they predict the rate of motor or cognitive decline longitudinally during up to 4 years of follow-up (Table s2), after correction for multiple testing. A separate analysis excluding patients with PD dementia or depression did not alter the overall result (data not shown). Finally, we found no consistent pattern nor any statistically significant changes in CSF total α-synuclein concentrations during 4 years of follow-up (N=27, all $P>0.1$; Table 1, Figure 2A and B), although some variation over time was observed in a few individuals (Figure 2C and D).
Discussion

In this study of PD patients recruited from a prospective, population-based longitudinal study, we found that total CSF α-synuclein levels, measured by two independent analytical methods, 1) did not differ from controls, adding to the list of studies reporting comparable levels between PD and controls [2]; 2) displayed no significant changes during a period of up to 4 years, and 3) did not predict the rate of motor, cognitive or neuropsychological decline, in agreement with previous studies [2]. Overall, these findings do not support the clinical utility of total α-synuclein as a single diagnostic or prognostic biomarker in PD.

In recent years, CSF total α-synuclein and other α-synuclein species have emerged as potential diagnostic and prognostic biomarkers in PD [2]. However, while numerous cross-sectional studies have investigated the diagnostic value of total α-synuclein, very few longitudinal studies have been conducted to explore changes of CSF total α-synuclein over time, and with inconsistent findings. Two studies utilizing data from the DATATOP cohort found either decreasing [3] or increasing [4] α-synuclein levels over a 2-year period. These apparently contradicting findings might be explained by differences in pre-analytical factors or detection methods [4]. Another study, using the Swedish BioFINDER cohort, found a minor increase at the group level in CSF α-synuclein levels over a period of 2 years in PD patients with long (>5 years) but not shorter (≤5 years) disease duration [5]. Finally, a recent report from the PPMI study revealed no change over a period of ½ and 1 year in early PD [6], in line with results from our 4-year longitudinal study.

In Alzheimer’s disease, it is well established that the deposition of amyloid-beta into extracellular plaques translates into decreased CSF concentrations of this protein [21]. In PD however, α-synuclein aggregates into Lewy bodies intracellularly, making it less clear how these pathological events, and their spread in the brain, influence the dynamics of α-synuclein in CSF. Different hypotheses explaining CSF changes of total α-synuclein levels in either
direction have been proposed. A reduction over time could be explained by the sequestration and aggregation of α-synuclein in soluble fibrils and Lewy bodies [2], while an increase in levels could result from progressive neuronal damage [22], possibly due to release of α-synuclein from degenerating cells. Indeed, some PD studies have shown strong correlations between CSF levels of α-synuclein and tau, an acknowledged marker of neuronal degeneration [23, 24]. Furthermore, a marked increase in levels of α-synuclein has been found in disorders with widespread and progressive neurodegeneration, such as Creutzfeldt-Jakob disease and AD [25-29]. A co-occurrence of such contrary events may result in no overall change in total α-synuclein levels, as found in our study. Furthermore, Hall et al. [5] hypothesized that the evolution of α-synuclein levels in PD might be bimodal with levels decreasing in early disease due to intracellular accumulation, followed by an increase with clinical disease progression reflecting progressive neuronal damage. In our study, CSF was obtained from patients at least 3 years after motor onset, and applying the bimodal hypothesis, patients may already have reached a plateau in the second stage of the disease development. This might explain why there was no further increase in α-synuclein levels with continued disease progression. However, more longitudinal studies with multiple sampling covering longer periods are needed to fully clarify this issue.

Another implication of the bimodal hypothesis is that the potential diagnostic value of α-synuclein may be offset by individual differences in the neuropathological effects accountable for changes in total α-synuclein levels in CSF. Therefore, an approach with α-synuclein as part of a biomarker panel, including other species of α-synuclein, such as oligomeric- and phosphorylated α-synuclein, could be a promising alternative [30].

A limitation of this study is the relatively small sample size, particularly of those with repeated CSF measurements, contributing to wide confidence intervals. In addition, the limited sample size did not allow for subgroup analysis (e.g. after stratification into different endophenotypes).
Therefore, we cannot exclude the possibility of associations of CSF α-synuclein with clinical features in certain PD subgroups. Another limitation, shared by similar studies, is that patients with milder symptoms are possibly more likely to consent to repetitive lumbar puncture, which could result in a potential selection bias. Finally, the lack of other CSF markers than α-synuclein (e.g. tau) and the inhomogeneous time intervals between lumbar punctures in those with repeated measures can be considered further limitations of this study. Strengths include the unselected PD cohort, comprehensive, prospective clinical and neuropsychological assessments, and study design with repeated CSF samples over a longer time interval than in previous studies. Finally, we followed well-established and standardized routines for collection and handling of CSF samples, and applied two independent, validated methods to quantify total α-synuclein levels with consistent findings.
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References


