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KNOWN BIOMARKER CANDIDATES
REDUCED ALPHA-SYNUCLEIN LEVELS IN CEREBROSPINAL FLUID IN PARKINSON’S DISEASE ARE UNRELATED TO CLINICAL AND IMAGING MEASURES OF DISEASE SEVERITY
ABSTRACT

**Background:** The cerebrospinal fluid (CSF) concentration of α-synuclein may reflect the aggregation of α-synuclein in brain tissue that neuropathologically characterizes PD. Although most studies in large cohorts report reduced CSF α-synuclein levels in PD, the available data to date are not consistent due to variation in group sizes, pre-analytical confounding factors and assay characteristics. Furthermore, it remains unclear whether CSF α-synuclein concentrations correlate with measures of disease severity. Acknowledging the methodological issues that emerged from previous studies, we evaluated whether CSF α-synuclein levels differ between PD patients and controls, and relate to disease duration or severity.

**Methods:** α-synuclein levels were measured in CSF samples of 53 well-characterized PD patients and 50 healthy controls employing a recently developed time-resolved Förster’s resonance energy transfer (TR-FRET) assay. In addition, we studied the relationship of CSF α-synuclein levels with disease duration, clinical measures of disease severity and the striatal dopaminergic deficit as measured by dopamine transporter (DAT) binding and Single Photon Emission Computed Tomography (SPECT).

**Results:** In PD patients, we observed a decrease in mean CSF α-synuclein levels that was unrelated to disease duration or measures of disease severity. Using total protein normalized α-synuclein, a sensitivity and specificity of 70 and 74% could be reached for distinguishing between PD patients and controls.

**Conclusion:** CSF α-synuclein levels are reduced in PD patients compared to healthy controls. However, sensitivity and specificity indicate that α-synuclein will not suffice as a single biomarker. CSF α-synuclein levels do not correlate with measures of disease severity, including striatal dopaminergic deficit.

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INTRODUCTION

The cerebrospinal fluid (CSF) is in direct contact with the extracellular fluid surrounding brain cells and may therefore reflect specific pathological brain processes occurring in Parkinson’s disease (PD) [1]. As such, the CSF can be an excellent source for biomarker discovery. Proteins in CSF that are associated with PD pathogenesis may not only help to increase the accuracy of an early diagnosis of PD, but may also serve as a measure of disease progression and/or response to therapy.

Alpha-synuclein in the CSF is a promising biomarker candidate for PD as its concentration may reflect the accumulation and aggregation of misfolded α-synuclein in Lewy bodies and Lewy neurites that neuropathologically characterize PD [2]. Its origin is likely to be brain- rather than blood-derived [3]. Furthermore, research in PD genetics has linked point mutations, duplications and triplications in the α-synuclein gene (SNCA) to familial forms of PD, while α-synuclein positive inclusions have also be observed in symptomatic patients carrying other mutations, for example in some but not all patients with leucine-rich repeat kinase 2 (LRRK2) mutations [4]. CSF α-synuclein may therefore serve both as a diagnostic biomarker and as a marker of disease progression as it may be sensitive to the increase of α-synuclein aggregates during the progression of PD [5].

To date, the results of biomarker studies evaluating total α-synuclein levels in lumbar CSF of PD patients have been inconsistent. Several studies reported reduced CSF α-synuclein in PD patients compared to controls [6-10] whereas other studies found no difference between groups [11-13]. This inconsistency in the literature may to a large extent be explained by differences in group size, since most of the studies that failed to find differences between patients and controls were performed in small groups. In addition, differences in patient characteristics, choice of the control group, assay characteristics, sample collection and processing protocols, and variations in the degree of blood contamination may have contributed to discrepancies.

The relationship between CSF total α-synuclein levels and disease severity in PD is at present unclear. An inverse correlation between CSF α-synuclein and disease stage was reported in a single study [9] whereas in all other studies no such association could be found [6-8,10,12,13]. Of note, disease severity in these studies was measured using clinical scales that either have a low sensitivity for detecting small differences (i.e. Hoehn and Yahr staging) or can be influenced by the use of dopamine replacement therapy (i.e. motor section of the Unified Parkinson’s Disease Rating Scale; UPDRS-III). An alternative and more objective way to assess disease progression is through the measurement of the striatal dopaminergic deficit by means of dopamine transporter (DAT) single photon emission computed tomography (SPECT) tracers [14]. So far, striatal DAT binding has not been assessed in relation to CSF α-synuclein levels.
The aim of the present study was to overcome some of the methodological pitfalls of previous studies and analyze total α-synuclein levels in CSF of PD patients and healthy controls using a recently developed robust, reproducible and sensitive homogeneous time-resolved Förster's resonance energy transfer (TR-FRET) immunoassay [15]. Importantly, CSF with no or only minimal blood contamination was collected using standardized sample collection protocols [16]. Furthermore, we studied the relationship between CSF α-synuclein levels and both clinical and imaging measures of disease severity, including UPDRS-III, Hoehn and Yahr (H&Y) stage and striatal DAT binding.

METHODS

Study population
In this study, we included 53 PD patients that attended the outpatient clinic for movement disorders of the VU University Medical Center between September 2008 and February 2011, as well as 50 self-declared healthy controls, recruited through an advertisement in the periodical of the Dutch Parkinson Foundation. All PD patients fulfilled the United Kingdom Parkinson’s Disease Society Brain Bank (UK-PDSBB) clinical diagnostic criteria [17]. Patients were included only if they were able to understand the study aim and procedures. Mini-Mental State Examination (MMSE) and/or neuropsychological assessment in the patients did not indicate dementia. In the controls, dementia was excluded using the Cambridge Cognitive Examination (CAMCOG) scale [18]. Patients and controls underwent a standardized clinical assessment that included their medical history and a neurological examination. Disease duration was defined as the time period starting from the first motor symptom until the time of lumbar puncture. Severity of parkinsonism and disease stage in the “on” state were rated using the UPDRS-III [19] and the modified H&Y classification [20], respectively. In a subgroup of 24 PD patients DAT-SPECT scans were available to evaluate the relationship between CSF α-synuclein levels and the striatal dopaminergic deficit. The study was approved by the local ethics committee of the VU University Medical Center and all participants gave written informed consent.

CSF collection procedures
CSF was obtained by lumbar puncture and collected in polypropylene collection tubes. CSF was routinely assayed for cell counts, centrifuged at 1800 x g at 4°C for 10 minutes, aliquoted and stored at -80°C within 2 hours, in line with published guidelines [16].

Assays
Total protein amount was measured with a bicinchoninic acid assay (BCA) in triplicate according to the manufacturer’s instructions (Pierce BCA Protein Assay Kit #23225, Thermo Scientific, Rockford IL, USA). Total α-synuclein concentrations were determined using a time-resolved Förster's resonance energy transfer (TR-FRET) immunoassay. For an
extensive assay description, we refer to Bidinosti et al [15]. Only samples containing less than 500 erythrocytes per microliter were included, since traces of blood may influence CSF α-synuclein levels [7] (α-synuclein is known to be highly expressed in erythrocytes [21]). In short, complete protease inhibitor cocktail (Roche) and 1% TritonX-100 were added to the CSF after which 384-well low volume polypropylene (Greiner Bio-One) microtiter plates were loaded with 12 µl per well of analyte sample in triplicate that was subsequently mixed with 2.4 µl of antibody solution (50 mM NaHPO₄, 400 mM NaF, 0.1% BSA and 0.05% Tween-20) which contained 0.3 ng/µL of SynBa2–Tb antibody and 3 ng/µL of SynBa3–d2 antibody. Plates were incubated at 4°C for 20 h prior to measurement of time-resolved fluorescence at 620 nm and 665 nm on an Envision Multilabel reader (Perkin Elmer). For quantification, the TR-FRET signal was normalized against the background signal and compared to a standard curve of recombinant α-synuclein in immunodepleted human CSF. The median of the triplicate values was used for further analysis.

**DAT SPECT imaging**

DAT-SPECT scans were performed and analyzed as described previously [22]. In short, ratios of specific to non-specific binding of the tracer [¹²³I]N-ω-fluoropropyl-2β-carbomethoxy-3β-(4-iodophenyl)nortropane ([¹²³I]FP-CIT) for the left and right putamen and caudate nucleus (Str) were calculated, using the occipital cortex (Occ) as a reference area ([Str–Occ]/Occ). To minimize the effect of age, DAT-SPECT binding was subsequently normalized for age by dividing the individual calculated ratios by the age-expected specific-to-nonspecific striatal binding ratios [14]. Age-expected values were obtained from a linear regression analysis of values from 19 healthy subjects (unpublished data) of which the age range was similar to the PD patients. The subsequent values represented the percentage of DAT-SPECT signal of a normal individual of similar age. Then, mean age-normalized values of the right and left putamen and caudate nucleus were calculated ([left+right]/2). These values were used to assess the relationship between CSF α-synuclein levels and mean DAT binding in the putamen and caudate nucleus.

**Statistical analysis**

Statistical analysis was performed using Statistical Package of the Social Sciences software version 15.0 (SPSS, Chicago, IL, USA). We analyzed both α-synuclein concentrations and the ratio of α-synuclein concentrations to total protein in CSF. As a consequence of the non-parametric distribution of most variables, we used Mann-Whitney U-tests for all group comparisons. Chi-squared tests were used for comparisons of categorical data. Correlations were assessed using bivariate Spearman’s rank correlation coefficients. Statistical significance was set at p<0.05. A receiver operating characteristic (ROC) curve was used to evaluate the ability of α-synuclein and the α-synuclein to total protein ratio to discriminate PD patients from controls. For sensitivity and specificity values, the cut-off value from the ROC curve was used at which the sum of sensitivity and specificity was maximal.
RESULTS

The PD patients and controls were matched for age but not for gender (p=0.002; table 1). We decided not to correct for gender, because CSF α-synuclein levels did not differ between male and female patients (p=0.85), or male and female controls (p=0.07; data not shown), in accordance with previous data [7]. Mean CSF total protein concentrations were higher in PD patients compared to controls (table 1; p=0.002). In the controls but not in the PD patients, CSF α-synuclein levels positively correlated with CSF total protein concentrations (r=0.31, p=0.03). Therefore, we also analyzed the relative levels of α-synuclein, that is, the ratio between α-synuclein and total protein concentrations.

Both mean α-synuclein levels and α-synuclein to total protein ratios in CSF were lower in PD patients (α-synuclein: mean ± SD 1.48 ± 0.41 ng/ml; α-synuclein ratio 1.58 ± 0.48 ng/mg) compared to controls (α-synuclein: 1.70 ± 0.47 ng/ml, p=0.02; α-synuclein ratio 1.99 ± 0.57 ng/mg, p=0.00005; figure 1), although a large overlap was observed between both groups. ROC curve analysis (figure 2) indicated that the sensitivity and specificity was maximal at cut-off values of 1.67 ng/ml and 1.79 ng/mg for α-synuclein and the α-synuclein to total protein ratio, respectively. Sensitivity and specificity values for discriminating PD patients from controls were 56% and 74% for α-synuclein and 70% and 74% for the α-synuclein to total protein ratio.

<table>
<thead>
<tr>
<th>Table 1: Demographics and CSF values of total protein and blood cell count</th>
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<tr>
<td>Controls</td>
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<tr>
<td>Number of subjects</td>
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<td>Sex (M/F)</td>
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<td>Age (years)</td>
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<td>Disease duration (years)</td>
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<td>Hoehn and Yahr stage (number per stage 1 / 1.5 / 2 / 2.5 / 3 / 4 / 5)</td>
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<td>UPDRS-III</td>
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<td>CSF total protein (mg/ml)</td>
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<td>CSF red blood cell count per µl</td>
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Data are mean ± SD and range unless specified otherwise.

No significant correlations were observed between α-synuclein levels or α-synuclein to total protein ratios and disease duration (α-synuclein: r=0.10, p=0.47; α-synuclein ratio: r=0.07, p=0.63), Hoehn and Yahr stage (α-synuclein: r=0.07, p=0.62; α-synuclein ratio: r=0.04, p=0.75) or UPDRS-III (α-synuclein: r=0.12, p=0.39; α-synuclein ratio: r=0.005, p=0.97). In addition, α-synuclein levels or α-synuclein ratios did not correlate with average DAT
binding in putamen (α-synuclein: \( r=-0.14, \ p=0.52; \) α-synuclein ratio: \( r=-0.07, \ p=0.75 \)) or caudate nucleus (α-synuclein: \( r=-0.32, \ p=0.13; \) α-synuclein ratio: \( r=-0.21, \ p=0.33 \); figure 3).

**Figure 1:** Total CSF α-synuclein levels and α-synuclein to total protein ratios in PD patients and controls. Whiskers indicate minimal and maximal values.

**Figure 2:** Combined receiver operating characteristic (ROC) curve for α-synuclein and the α-synuclein to total protein ratio of Parkinson's disease patients versus controls. The area under the curve (AUC) was 0.63 for α-synuclein and 0.73 for the α-synuclein to total protein ratio.

**DISCUSSION**

Acknowledging some of the methodological issues that emerged from previous studies, we analyzed CSF α-synuclein levels in PD patients using the following methodology: (1) a novel, robust, reproducible and sensitive immunoassay, (2) a standardized approach to CSF sample collection and processing, (3) strict limits on the degree of blood contamination, and (4) a control group consisting of well-defined neurologically healthy controls. Using this approach, we found a 13% reduction in CSF α-synuclein levels in PD patients compared to healthy controls that was unrelated to disease duration or clinical and imaging measures of disease severity.
Our finding of reduced CSF α-synuclein levels in PD is consistent with the results of several large cohort studies [6-10], though at odds with the findings of others in which CSF α-synuclein levels were unchanged in PD patients compared to controls [11-13].

A reduction of CSF α-synuclein levels in a disease that is typically characterized by the accumulation of α-synuclein may seem unexpected. A plausible explanation for this reduction is 'pathological protein trapping', a phenomenon whereby low 'free' α-synuclein in CSF results from the sequestration of pathological α-synuclein in the brain [23]. Increased uptake of α-synuclein in neurons [8] has been proposed as an alternative explanation for decreased α-synuclein levels in CSF of PD patients, while yet others have suggested that extensive synaptic and/or neuronal loss could result in lower α-synuclein secretion in CSF [11]. Lastly, previously reported decreases in CSF α-synuclein levels in PD could, hypothetically, result from biased oligomerization of α-synuclein monomers and loss of detection antibody epitopes during the assay [11]. However, for several reasons it is highly unlikely that this would have occurred with the current detection assay. First, owing to the TR-FRET assay's design, CSF α-synuclein molecules were quantified in-solution and not concentrated on a surface (as in standard ELISAs), thereby reducing the possibility of artifactual oligomerization. Secondly, the antibody pair used here was previously demonstrated to yield similar detection signals for either monomeric or oligomeric alpha-synuclein [15].
Using the ratio of CSF α-synuclein to total protein, a sensitivity and specificity of 70% and 74% for the discrimination of PD patients from controls could be reached in the current study. These values suggest that α-synuclein levels in CSF could be helpful as a diagnostic marker. However, α-synuclein will not suffice when used as a single biomarker. A combination of α-synuclein with other markers could improve the discrimination of PD patients from controls. This has been observed, for example, for the combination of α-synuclein and α-synuclein oligomers levels in CSF [24]. Of note, disease specificity is an issue for CSF α-synuclein, because similar reductions have also been observed in other synucleinopathies, including dementia with Lewy bodies (DLB) and multiple system atrophy (MSA) [6,8,10]. Previous studies indicate that CSF markers other than α-synuclein (i.e. neurofilament light chain and β-amyloid 1-42 [6]) could help in the differentiation between PD and atypical parkinsonian syndromes.

We found no correlations between CSF α-synuclein levels and disease duration or clinical measures of disease severity, similar to most, but not all [9] previous studies. Notably, however, the clinical measures of disease severity used, i.e. H&Y stage and UPDRS-III, may have low sensitivity for small differences in disease severity or could be influenced by dopamine replacement therapy. Also disease duration may not be a very accurate measure for disease progression, as the rate of disease progression varies quite substantially between patients [20]. Therefore, we also studied the relationship between CSF α-synuclein levels and striatal DAT binding, which is an objective measure of the integrity of the nigrostriatal dopamine system. Furthermore, striatal DAT binding progressively declines with increasing disease duration, correlates with increasing disease severity in PD in cross-sectional studies (reviewed in [14]), and is not likely influenced by dopaminergic medication [25]. In spite of this, we found no correlation between CSF α-synuclein levels and striatal DAT binding in the PD patients.

Taken together, our results indicate that α-synuclein is not suitable as a single marker of disease severity in PD. This conclusion is in line with the results of a recent positron emission tomography (PET) scan study showing that CSF α-synuclein levels do not correlate with loss of striatal dopaminergic function in asymptomatic and symptomatic LRRK2 mutation carriers [26]. Of note, in some cases with LRRK2 mutations, α-synuclein positive inclusions are not observed [4]. As suggested previously [10], the lack of an association between CSF α-synuclein levels and measures of disease progression might indicate that there is a “floor” effect for CSF α-synuclein levels. CSF α-synuclein levels in PD may decrease only to a certain level that is reached before the appearance of clinical signs of disease, and remain stable thereafter. Alternatively, the variability of α-synuclein might be too high to identify correlations with measures of disease severity.

Furthermore, it is important to realize that both clinical and imaging measures of disease severity evaluated in the current study either reflect motor symptoms or the underlying...
progressive loss of nigrostriatal dopaminergic neurons. However, CSF α-synuclein levels may reflect α-synuclein aggregation throughout the brain, including non-dopaminergic systems. As such, CSF α-synuclein levels may be more representative of the overall progression of α-synuclein pathology, rather than the progressive loss of nigrostriatal dopaminergic function. To further address this issue, CSF α-synuclein levels should be analyzed in relationship to a composite measure of disease progression that includes non-motor symptoms such as olfactory dysfunction [22]. Additionally, longitudinal assessment of CSF α-synuclein levels in patients should be pursued. The development of techniques that visualize α-synuclein aggregates in vivo [27] may make it possible to directly relate CSF α-synuclein levels to the degree of α-synuclein aggregation.

In conclusion, the results of our study confirm that CSF α-synuclein levels are reduced in PD patients compared to healthy controls. Sensitivity and specificity values suggest that α-synuclein can not stand alone as a diagnostic biomarker, but may be useful in a panel of biomarkers. CSF α-synuclein levels do not correlate with measures of disease progression that reflect the striatal dopaminergic deficit. Further studies should focus on a longitudinal analysis of CSF α-synuclein levels within subjects and use non-dopaminergic parameters and/or in vivo imaging of α-synuclein aggregation in the brain to determine the relationship between CSF α-synuclein levels and the overall progression of α-synuclein pathology, rather than the progressive loss of nigrostriatal dopaminergic function.

REFERENCES


