CHAPTER 3

Clusterin and ApoE as biomarkers for neurodegenerative disease, and their role in the etiology of Alzheimer’s disease
3.2

Cerebrospinal fluid and plasma clusterin levels in Parkinson’s disease

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ABSTRACT

Clusterin is a multifunctional chaperone protein that has repeatedly been linked to Alzheimer’s disease (AD) pathogenesis and, more recently, also to Parkinson’s disease (PD) by both genetic and proteomic analyses. Although clusterin is detectable in cerebrospinal fluid (CSF) and plasma, studies comparing clusterin levels in PD patients and controls have been scarce and yielded conflicting data. The aim of the present study was to determine whether CSF and/or plasma clusterin levels differ between PD patients and controls and are related to disease severity. We measured CSF and plasma clusterin levels in a group of 52 PD patients and in 50 age-matched neurologically healthy controls and found that clusterin levels in CSF and plasma were not different between the two groups. Furthermore, clusterin levels in CSF and plasma were not associated with disease duration, stage or severity. CSF clusterin levels did, however, correlate with CSF levels of total tau, phospho-tau and amyloid-β-42. We elaborate on the identified correlations between levels of clusterin and AD related proteins and on possible explanations for the discrepant findings in clusterin studies in PD so far.
INTRODUCTION

Clusterin is a multifunctional glycoprotein that has been related to cholesterol and lipid metabolism, regulation of complement mediated cell lysis, as well as to inhibition of neuronal apoptosis. Furthermore, clusterin acts as an extracellular chaperone that maintains stressed proteins in a soluble state, thereby preventing their precipitation.

A role for clusterin in Alzheimer’s disease (AD) pathogenesis has become apparent from genome-wide association studies in which certain polymorphisms in CLU, the clusterin encoding gene, were found to confer susceptibility for AD. In addition, expression studies showed up-regulated clusterin expression in AD-affected brain areas and levels of clusterin were reported to be increased in cerebrospinal fluid (CSF) and plasma of AD patients. Moreover, higher plasma clusterin levels were associated with more severe cognitive dysfunction. Furthermore, clusterin was found to co-localize with amyloid plaques and neurofibrillary tangles in immunohistochemical studies.

Recently, a specific CLU single-nucleotide polymorphism (SNP; rs11136000) was reported to be associated with Parkinson’s disease (PD). This association was most pronounced in PD patients with dementia and independent of APOE genotype and known risk factors for PD, suggesting that cortical Lewy Body pathology or concomitant AD pathology in the PD patients might play a role.

Using unbiased quantitative proteomic techniques, increased levels of clusterin were observed in both plasma and CSF of PD patients. These studies had a relatively small sample size. In two studies lumbar CSF clusterin levels were evaluated in larger numbers (n = 11-32) of PD patients and controls. The results of these studies were not in accord, clusterin levels being unchanged in one study and increased in the other. The increased clusterin levels reported were mainly found in patients with a short (<2 years) disease duration. Consequently, it remains unclear whether CSF or plasma clusterin levels differ between PD patients and controls, and whether clusterin could potentially serve as a diagnostic marker for PD.

The aim of the present study was to evaluate whether CSF and/or plasma clusterin levels differ between PD patients and age-matched neurologically healthy controls. We compared plasma clusterin and lumbar CSF clusterin levels between 52 well-characterized PD patients and 50 controls, and analyzed the relationship between clusterin levels and disease duration, stage and severity of motor symptoms. In addition, we investigated whether clusterin levels were related to those of the AD-related proteins amyloid-β-42 (Aβ42), total tau (T-tau) and phospho-tau (P-tau).

METHODS

Study population
For this study we included 52 PD patients from our outpatient clinic for movement disorders, as well as 50 self-declared healthy controls recruited through an advertisement in the periodical of the Dutch Parkinson Foundation. PD was diagnosed according to...
the United Kingdom Parkinson’s Disease Society Brain Bank (UK-PDSBB) clinical
diagnostic criteria\textsuperscript{15}. Patients were included only if they were able to understand the study
aim and procedures, and if no signs of dementia were detectable upon Mini-Mental State
Examination (MMSE) and/or neuropsychological assessment. In the controls, dementia
was excluded using the Cambridge Cognitive Examination (CAMCOG) scale\textsuperscript{16}. Patients
and controls underwent a standardized clinical assessment that included their medical
history and a neurological examination. Severity of parkinsonism and disease stage in
the “on” state were rated using the motor subscale of the Unified Parkinson’s Disease
Rating Scale (UPDRS-III) and the Hoehn and Yahr classification\textsuperscript{17}, respectively. In addition,
PD patients were divided into clinical subgroups according to Lewis et al.\textsuperscript{18}: (1) younger
onset, (2) tremor-dominant or (3) non-tremor dominant. None of the PD patients fulfilled
the criteria for the rapid disease progression subtype. The study was approved by the
local ethics committee of the VU University Medical Center and all subjects gave written
informed consent.

Cerebrospinal fluid (CSF) and plasma samples
CSF was obtained by lumbar puncture, collected in polypropylene collection tubes,
routinely assayed for cell counts, centrifuged at 1800xg at 4°C for 10 min, aliquoted and
stored at -80°C within 2 h, in line with published guidelines\textsuperscript{19}. Only samples containing
less than 500 erythrocytes per microliter were included in the study, because traces of
blood, in which clusterin expression is around 15 times higher than in CSF\textsuperscript{20}, may influence
CSF clusterin levels. EDTA plasma was collected directly before or after lumbar puncture,
centrifuged at 1800xg at 4°C for 10 min, aliquoted and stored at -80°C within 2 h.

Assays
Clusterin was quantified by an in-house sandwich ELISA. In short, disposable microtiter
plates (Costar 9018, Corning) were coated with a clusterin-specific mouse monoclonal
antibody (clone G7;\textsuperscript{21}) and blocked with PBS containing 2% milk, where after 100 µl of
either CSF samples (1:200 diluted) or EDTA plasma samples (1:5000 diluted) were added
to the wells in duplicate and incubated for 1 h at room temperature. Bound clusterin was
detected with biotinylated rabbit anti-human clusterin polyclonal antibody (Alexis
Biochemicals, Enzo Life Sciences, Zandhoven, Belgium) and visualized after subsequent
incubations with streptavidin poly-HRP (1:10,000 diluted; Sanquin, Amsterdam, The
Netherlands), and 3,5,30,50-tetramethylbenzidine (TMB; Sigma, Germany). The reaction
was stopped using sulfuric acid after 20 min and the absorbance was measured at 450
nm in a spectrophotometer (Bio-Tek Synergy HT, Winooski VT, USA). Clusterin purified
from human plasma by affinity chromatography with Sepharose 4B coupled G7
monoclonal antibodies as described previously\textsuperscript{21}, was serially diluted to prepare a
 calibration curve. The linear range of the assay was from 0.5 to 38.9 ng/mL, the inter-
assay and intra-assay coefficients of variation were 8.8% and 1.4%, respectively. CSF levels
of Aβ\textsubscript{42}, T-tau and P-tau were determined using commercially available ELISAs (Innotest;
Innogenetics, Gent, Belgium) as described previously\textsuperscript{22}. The technicians performing the
ELISAs were not aware of the clinical diagnoses.
Statistical analysis

Statistical analysis was performed using Statistical Package of the Social Sciences software version 15.0 (SPSS, Chicago, IL, USA). T-tau and P-tau data were square root transformed, while plasma clusterin data required inverse transformation to obtain a normal distribution. Group comparisons between PD patients and controls were performed using univariate analyses of variance (ANOVA) for continuous data, corrected for age and gender, while group comparisons within the PD patients were corrected for age alone. Mann-Whitney U tests were used for ordinal data and chi-squared tests for categorical data. Correlations were assessed using bivariate Pearson and Spearman’s rank correlation coefficients when appropriate. A Kruskal-Wallis test was used to compare CSF and plasma clusterin data between clinical subgroups of PD patients. For CSF clusterin, we excluded 1 outlier (PD patient) with a clusterin concentration higher than 10 ng/µl (>3 standard deviations from the mean). For plasma clusterin, we excluded 2 PD patients with a clusterin concentration >3 standard deviations from the mean. These exclusions did not alter the outcome of this study (data not shown).

Standard in-house diagnostic cut-offs were used for Aβ42 (<550 pg/mL), T-tau (>375 pg/mL) and P-tau (>52 pg/mL) that were based on the optimal separation of AD patients from patients with subjective memory complaints. Statistical significance was set at p < 0.05.

RESULTS

PD patients and controls were matched for age, but not for gender (Table 1). CSF clusterin levels did not differ between male and female subjects in patients or controls (PD p = 0.82; controls: p = 0.88). In PD patients, plasma clusterin levels were higher in females compared to males (female PD 79.3 ± 10.7 ng/µl; male PD 69.1 ± 9.4 ng/µl; p = 0.003). No difference was observed for controls (p = 0.22). In both PD patients and healthy controls, plasma and CSF clusterin levels did not correlate significantly with age, although in controls a trend towards higher CSF clusterin levels with increasing age was observed (r = 0.25, p = 0.08).

No significant differences in mean CSF or plasma clusterin levels were observed between PD patients (CSF clusterin mean ±SD: 4.9±1.39 ng/µl; plasma clusterin: 73.2±11.1 ng/µl) and controls (CSF clusterin: 4.6±1.1 ng/µl; p = 0.19; plasma clusterin 76.1±12.6 ng/µl; Figure 1). Furthermore, we did not find correlations between CSF or plasma clusterin levels and disease duration, Hoehn and Yahr stage, or disease severity as measured by the UPDRS-III. Also, no significant differences in CSF or plasma clusterin levels were found between patients with a disease duration shorter than 3 years and patients with a longer disease duration (CSF clusterin: p = 0.31; plasma clusterin: p = 0.45). CSF and plasma clusterin levels did not differ between distinct clinical PD subgroups (CSF clusterin: younger onset 5.4±1.3 ng/µl; tremor-dominant 4.9±2.0 ng/µl; non-tremor dominant 4.9±1.2 ng/µl, p = 0.46; plasma clusterin: younger onset 71.9±11.1 ng/µl; tremor-dominant 72.0±8.8 ng/µl; non-tremor dominant 74.5±11.8 ng/µl; p = 0.77). Neither CSF
nor plasma clusterin levels correlated with MMSE values (CSF clusterin in controls: $r = 0.15$, $p = 0.29$ and in PD: $r = 0.20$, $p = 0.15$; plasma clusterin in controls: $r = 0.06$, $p = 0.70$ and in PD: $r = 0.05$, $p = 0.72$).

In controls but not in PD patients, CSF clusterin levels positively correlated with plasma clusterin levels (controls: $r = 0.39$, $p = 0.005$; PD: $r = 0.03$, $p = 0.82$). In both PD patients and controls, CSF clusterin levels were positively correlated with CSF levels of $\mathrm{A_\beta}_{42}$ (controls: $r = 0.45$, $p = 0.001$; PD: $r = 0.41$, $p = 0.002$), T-tau (controls: $r = 0.40$, $p = 0.004$; PD: $r = 0.46$, $p = 0.001$) and P-tau (controls: $r = 0.41$, $p = 0.003$; PD: $r = 0.32$, $p = 0.02$). CSF levels of $\mathrm{A_\beta}_{42}$, T-tau and P-tau in PD patients were however not different from the levels in controls ($p > 0.05$). None of the PD patients or controls showed a classical AD pattern of decreased CSF levels of $\mathrm{A_\beta}_{42}$ in combination with increased CSF levels of both T-tau and P-tau.

**DISCUSSION**

In this study, we found no significant difference in CSF or plasma clusterin levels between PD patients and neurologically healthy controls, illustrating that CSF nor plasma clusterin...
are potential diagnostic biomarker candidates. Furthermore, clusterin levels were not associated with disease duration, stage or severity of motor symptoms. Interestingly though, CSF levels of clusterin were positively correlated with levels of A\textsubscript{42}, T-tau and P-tau in both PD patients and controls.

The absolute concentration of CSF clusterin in controls (4.6±1.1 ng/µl) in the current study, is lower than reported previously (Lidstrom et al.\textsuperscript{13}: 5.6±1.1 ng/µl). Also, plasma clusterin levels in controls (76.1±12.6ng/µl) were lower than expected from previous studies (for example Schrijvers et al.\textsuperscript{7}: 114±25 ng/µl). These differences between studies are likely influenced by different approaches to detect clusterin (i.e. pre-analytical variables, platform of detection, antibodies used).

Our finding of unchanged clusterin levels in PD patients compared to controls is in accordance with the study by Lidstrom et al.\textsuperscript{13}, but is at odds with the increased CSF clusterin levels, in particular in patients suffering from PD for less than 3 years, reported by Přikrylová Vranová et al.\textsuperscript{14}. The discrepancies between the three studies, including our own, may be due to differences in patient or sample characteristics or to technical variations of the assay used (Table 2).

The present study was performed in the largest sample of PD patients and controls so far. Contrary to the two previous studies of CSF clusterin levels in PD, we used only samples with less than 500 erythrocytes per microliter, since clusterin is highly expressed in blood\textsuperscript{20}. Therefore, blood contamination of CSF samples might influence CSF clusterin levels and could be an explanation for different findings between studies.

In the study by Přikrylová Vranová et al.\textsuperscript{14}, PD patients with high clusterin levels also had high T-tau levels. While T-tau levels are mostly reported to be unchanged in PD\textsuperscript{23}, increased T-tau levels have recently been observed in non-tremor dominant PD phenotypes\textsuperscript{24,25}. Likewise, clusterin could be phenotype dependent, in which case

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Table 1. Demographics and CSF values

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Parkinson's disease</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cases</td>
<td>50</td>
<td>52</td>
<td>n.a.</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>16/34</td>
<td>32/20</td>
<td>0.003</td>
</tr>
<tr>
<td>Age (years)</td>
<td>63 ± 7</td>
<td>63 ± 10</td>
<td>0.75</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>n.a.</td>
<td>4; 1-22</td>
<td>n.a.</td>
</tr>
<tr>
<td>Hoehn and Yahr stage (number per stage 1 / 1.5 / 2 / 2.5 / 3 / 4 / 5)</td>
<td>n.a.</td>
<td>3 / 4 / 23 / 16 / 6 / 0 / 0</td>
<td>n.a.</td>
</tr>
<tr>
<td>UPDRS motor subscale</td>
<td>n.a.</td>
<td>22 ± 8</td>
<td>n.a.</td>
</tr>
<tr>
<td>CSF red blood cell count per µl</td>
<td>1.5; 0-490</td>
<td>1; 0-499</td>
<td>0.25</td>
</tr>
<tr>
<td>CSF clusterin (ng/µl)</td>
<td>4.6 ± 1.1</td>
<td>4.9 ± 1.39</td>
<td>0.19</td>
</tr>
<tr>
<td>CSF A\textsubscript{42} (ng/l)</td>
<td>989 ± 214</td>
<td>926 ± 202</td>
<td>0.15</td>
</tr>
<tr>
<td>CSF total tau (ng/l)</td>
<td>218 ± 72</td>
<td>209 ± 87</td>
<td>0.35</td>
</tr>
<tr>
<td>CSF phospho-tau (ng/l)</td>
<td>41 ± 13</td>
<td>41 ± 16</td>
<td>0.86</td>
</tr>
</tbody>
</table>

Data are expressed as either mean ± SD or median and range unless specified otherwise. UPDRS = Unified Parkinson's Disease Rating Scale
differences in phenotypic patient characteristics between studies could therefore explain the different findings in clusterin levels. We could not confirm this hypothesis in the current study, as clusterin levels did not differ between distinct clinical PD subgroups. However, we cannot draw firm conclusions from these data, because our cohort of PD patients consisted of only 7 patients with a tremor-dominant phenotype. Furthermore, an alternative explanation for the dissimilar findings could be a difference in the assays used to determine clusterin levels, for example the use of different antibodies that bind to different clusterin epitopes.

The use of neurological controls by Přikrylová Vranová et al. (e.g. vertebrogenic disease, psychogenic disease, migraine, tension headache, diabetic neuropathy) in contrast to neurologically healthy controls in the two other studies is not likely responsible for the different outcome of this study. Specifically, clusterin levels can be increased (and not decreased) in other neurological diseases, such as demyelination and spinal cord compression.

As far as we know, this is the first study to describe plasma clusterin levels in a large sample of PD patients compared to controls. We could not confirm the observation in an unbiased quantitative proteomics study of higher plasma levels of clusterin in pooled PD samples compared to healthy controls. Studies in AD patients have shown associations of plasma clusterin levels with entorhinal cortex atrophy, baseline disease severity and rapid clinical progression in AD. Additional neuropathological data are necessary to determine whether plasma clusterin levels also reflect neuropathological disease progression in PD.

Our findings imply that neither CSF nor plasma clusterin can serve as a reliable biomarker for the discrimination between PD patients and controls. However, in spite of the absence of a correlation between PD subtype and clusterin levels in the present study, clusterin

Table 2. Overview of CSF clusterin studies in PD

<table>
<thead>
<tr>
<th></th>
<th>Current study</th>
<th>Lidström et al.</th>
<th>Přikrylová Vranová et al.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects (PD/CTRL)</td>
<td>52 / 50</td>
<td>18 / 11</td>
<td>32 / 30</td>
</tr>
<tr>
<td>Age, mean ± SD (PD/CTRL)</td>
<td>63 ± 7 / 63 ± 10</td>
<td>65 ± 8 / 70 ± 6</td>
<td>60 ± 11 / 59 ± 10</td>
</tr>
<tr>
<td>Disease duration, mean ± SD</td>
<td>6 ± 5</td>
<td>NR</td>
<td>4 ± 4</td>
</tr>
<tr>
<td>Presence of dementia in PD patients</td>
<td>Non-demented</td>
<td>NR</td>
<td>Non-demented</td>
</tr>
<tr>
<td>Type of controls</td>
<td>Healthy controls</td>
<td>Healthy controls</td>
<td>Neurological controls*</td>
</tr>
<tr>
<td>CSF red blood cell count cut-off</td>
<td>500 / µl</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>CSF clusterin in PD vs CTRL</td>
<td>=</td>
<td>=</td>
<td>↑</td>
</tr>
<tr>
<td>CSF Aβ42 in PD vs CTRL</td>
<td>=</td>
<td>NR</td>
<td>=</td>
</tr>
<tr>
<td>CSF total tau in PD vs CTRL</td>
<td>=</td>
<td>NR</td>
<td>↑</td>
</tr>
<tr>
<td>CSF phospho-tau in PD vs CTRL</td>
<td>=</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Assay</td>
<td>Sandwich ELISA, in-house developed</td>
<td>Direct ELISA, in-house developed</td>
<td>Sandwich ELISA, Biovendor, Czech Republic</td>
</tr>
</tbody>
</table>

PD = Parkinson’s disease, CTRL = control, NR = not reported; *vertebrogenic disease, psychogenic disease, migraine, tension headache, diabetic neuropathy.
levels might still be associated with certain PD subtypes. Furthermore, a previous report of increased CSF clusterin levels in AD patients suggests that clusterin levels could potentially be useful to discriminate between different dementia syndromes, such as PD-related dementia, dementia with Lewy bodies and AD. Further studies that include these additional patient groups are needed to address this issue. We cannot draw firm conclusions from the correlation analysis between clusterin levels and PD stage and severity. More solid data to confirm the absence of a correlation between clusterin levels and disease stage and severity, will require testing in a larger number of patients with a more extensive range of PD stages and severity. We observed positive correlations between clusterin levels and the AD-related proteins \( \alpha_42 \), T-tau and P-tau in both PD patients and controls. Similar correlations were reported previously for T-tau and \( \alpha_42 \) in AD patients but not in control subjects. The correlations in the present study could point to an association between clusterin and AD pathology in PD patients and healthy elderly. Recent findings suggest that disposition of tau and \( \alpha_42 \) could play an important role in PD, even during early phases of the disease. The identified correlations between levels of clusterin and \( \alpha_42 \), T-tau and P-tau might, furthermore, reflect a direct interaction between these proteins. Interactions of clusterin with \( \alpha_42 \), such as chaperoning of amyloid peptides, fibrils and aggregates, have been widely recognized (reviewed in Ref.1). The finding of increased levels of tau and phosphorylated tau after injection of clusterin in rat hippocampus suggests that clusterin might also have a direct effect on tau metabolism. Recently, CSF T-tau levels were put forward as a potential marker of the severity of neuronal degeneration in PD. From this perspective, the positive correlation between CSF clusterin and T-tau levels might be explained by an association of both proteins with the intensity of neuronal degeneration. This assumption is in line with previous studies in which increased clusterin expression was observed in the human brain in response to ischemia, and in experimental animals after experimental lesioning. The absence of a relation between CSF clusterin levels and disease duration or disease severity in the present study, however, pleads against clusterin as a marker of the severity of neuronal degeneration, as well as the finding of a correlation between reduced CSF tau levels and lower striatal dopaminergic function as measured by PET scan in carriers of a G2019S LRRK2 mutation. In conclusion, we found no difference in CSF or plasma clusterin levels between PD patients and controls, nor a correlation of CSF or plasma clusterin levels with clinical measures of disease duration or severity. The present results do not exclude the possibility that high clusterin levels are an indicator of later cognitive decline or correlate with specific phenotypic variations in PD. The identified correlations between CSF levels of clusterin and T-tau, P-tau and \( \alpha_42 \) may reflect a direct interaction between these proteins.

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REFERENCES