Lysosomal and synaptic dysfunction markers in longitudinal cerebrospinal fluid spanning from healthy subjects to prodromal and manifest Parkinson’s disease

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Abstract

Lysosomal and synaptic dysfunctions are hallmarks in multiple neurodegenerative diseases including Alzheimer's disease and Parkinson's disease (PD) and could be relevant from a biomarker perspective. Biomarker data on prodromal and early PD are not yet available. We performed targeted mass spectrometry measurements cross-sectionally and longitudinally over 10 years with an established biomarker panel, assessing autophagy and synaptic function in cerebrospinal fluid (CSF) in prodromal subjects with isolated REM sleep behavior disorder (iRBD), drug-naïve de novo PD subjects at baseline, and sex- and age-matched healthy controls. Multiple markers of autophagy, synaptic plasticity, and secretory pathways showed reduced expression in PD and iRBD compared to controls. Machine learning identified neuronal pentraxin receptor and neurosecretory protein VGF as the most relevant for discriminating between groups. CSF levels of LAMP2, neuronal pentraxins, and syntaxins correlated with clinical progression and showed predictive potential for motor- and non-motor symptoms as a valid basis for future drug trials.

Introduction

Parkinson's disease (PD) is an increasingly prevalent, progressive, and complex neurodegenerative disease that lacks a conclusive panel of biomarkers that assess the rate, trait, fate, and state of the disease. PD diagnosis is still clinically based and usually made when the majority of affected neurons have already degenerated. In this context, non-motor symptoms like hyposmia and isolated REM (rapid eye movement) sleep behavior disorder (iRBD) are very valuable in identifying those at risk of developing the disease. iRBD is very specific, and therefore, the most powerful prodromal entity of PD. It is characterized by dream-enacting behaviors during REM sleep and a high conversion rate to an α-synucleinopathy, including PD and dementia with Lewy bodies and multiple system atrophy (MSA).

PD, mostly but not exclusively, affects dopaminergic neurons, especially in the substantia nigra pars compacta. α-synuclein (αSyn) plays a key role in its pathology and is physiologically localized mainly in presynaptic terminals of neurons. In certain synucleinopathies, including PD and iRBD, it accumulates within the neuronal soma, described as Lewy body inclusions. Nevertheless, the utility of quantifying total levels of αSyn as a biomarker is limited. It is lower in the cerebrospinal fluid (CSF) and plasma of PD patients compared to healthy controls, but no longitudinal changes or predictive potential have been detected. αSyn seeding aggregation assays (SAAs) were recently established that diagnose α-synucleinopathy in PD with high sensitivity and specificity, therefore enabling the identification of molecular heterogeneity and increased PD risk among patients. However, SAAs have not yet been qualified as progression markers as they have not been sufficiently linked to the specific pathophysiology. Therefore, many questions remain to be answered. Research for effective diagnostic and prognostic biomarkers in early and prodromal disease stages needs to be widened.

Synaptic dysfunction is a hallmark of many neurodegenerative diseases, including PD. Multiple established risk factors and causative PD genes are known to influence synaptic functioning. Lysosomal dysfunction is also implicated in PD: multiple variants in lysosomal storage disorder genes are associated with an increased risk of PD and previous studies suggest that there are alterations of the autophagic and
endolysosomal system in PD. Furthermore, accumulation of autophagic vacuoles is evident in PD patients’ brain cells, accompanied by a decrease in endolysosomal markers like LAMP2.

Assessing lysosomal and synaptic functioning biomarkers in PD could potentially improve diagnostic and prognostic accuracy by focusing on the early disease phase and enabling treatment response monitoring in future drug trials. Previous attempts to assess synaptic functioning are based on research in synaptic proteins in CSF of patients with Alzheimer’s disease. Some earlier studies, mainly focused on PD, either found no differences between PD and controls or had inconsistent results.

Data on de novo PD and longitudinal analysis are generally lacking and no studies on prodromal PD stages are available to date.

We sought to fill this gap by investigating an established panel of lysosomal and synaptic markers (Table 2) validated in different cohorts. Over a 10-year follow-up, we used this approach to longitudinally assess differently expressed proteins in CSF between subjects with iRBD, de novo PD, and healthy controls from our ongoing, prospective, single-center de novo Parkinson’s disease (DeNoPa) cohort. We integrated our data into one of the two newly proposed neuronal αSyn-disease integrated staging systems and correlated the results with the available longitudinal clinical data. Linear mixed modelling was performed to analyze progression over time, and we evaluated the predictive and discriminative potential of these markers with machine learning algorithms.

**Results**

**The DeNoPa cohort**

We analyzed CSF samples from subjects of our longitudinal de novo PD (DeNoPa) cohort and included, from baseline (BL), 88 PD patients (62 men, 70.5%) and 46 healthy controls (HC) (34 men, 73.9%) for whom CSF samples were available at all time points. The mean age was 65 years (± 9.8) for PD and 66 years (± 7.2) for HC. The mean Hoehn and Yahr Stage in the PD group was 2 (± 0.4), and the mean MDS-UPDRS part III score was 23 (± 11.4). MMSE (mini-mental state examination) did not significantly differ between PD and HC (28 in both groups), but the MoCA (Montreal cognitive assessment) score was lower in PD (PD: 24, healthy controls: 26). Further, we included nine subjects at BL with iRBD (2 men, 2.2%) mean age 65 (± 9). The demographics and clinical characteristics of the cohort are shown in Table 1.

We added our available αSyn–seed amplification assay (αS-SAA) data, based on high-throughput CSF αS-SAA, showing 65 of 82 PD subjects were positive, as were 8 of the 9 iRBD subjects.

To increase the power of our longitudinal model, we also included additional samples for which there were no CSF samples available at baseline. This resulted in a total of 12 iRBD (11 of 12 αS-SAA positive), 104 PD (86 of 100 αS-SAA positive), and 58 HC. Applying the neuronal αSyn-disease integrated staging system (NSD-ISS) led to four subjects categorized as stage NSD-2A (iRBD, S + D-), six in stage NSD-2B (iRBD, S + D+) and 78 who met the criteria for stage 4 (PD, S + D+, moderate clinical impairment). See the methods and supplementary Table S4 for details.
Baseline demographics and some CSF biomarkers of the included subjects, SAA data displays positive and negative results of the available samples. PD subjects displayed a higher CSF albumin quotient, indicative of a blood-brain barrier dysfunction. Typical CSF Alzheimer’s disease markers (Aβ, t- and p-tau) showed no differences between the groups. As reported before, neurofilament light (NFL) was increased in PD samples. The P-value is adjusted for age and sex. Abbreviations: PD = Parkinson’s Disease, HC = healthy control, iRBD = isolated REM sleep behavior disorder, n = number, sd = standard deviation, CSF: cerebrospinal fluid, MDS-UPDRS = Movement Disorder Society – United Parkinson’s Disease Rating Scale, MMSE: Mini-Mental State Examination, MoCa: Montreal Cognitive Assessment Score

<table>
<thead>
<tr>
<th>Baseline</th>
<th>HC (N = 46)</th>
<th>PD (N = 88)</th>
<th>RBD (N = 9)</th>
<th>Adj. p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td>0.008*</td>
</tr>
<tr>
<td>- female</td>
<td>12 (26.1%)</td>
<td>26 (29.5%)</td>
<td>7 (77.8%)</td>
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</tr>
<tr>
<td>- male</td>
<td>34 (73.9%)</td>
<td>62 (70.5%)</td>
<td>2 (22.2%)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td>0.989</td>
</tr>
<tr>
<td>- Mean (SD)</td>
<td>65.6 (7.23)</td>
<td>65.136 (9.81)</td>
<td>64.78 (8.97)</td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td></td>
<td></td>
<td></td>
<td>0.012*</td>
</tr>
<tr>
<td>- Mean (SD)</td>
<td>26.97 (4.63)</td>
<td>28.447 (4.71)</td>
<td>24.99 (3.68)</td>
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</tr>
<tr>
<td>MDS-UPDRS-III</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>- Mean (SD)</td>
<td>0.63 (1.48)</td>
<td>22.966 (11.44)</td>
<td>3.11 (2.71)</td>
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<tr>
<td>MDS-UPDRS total score</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>- Mean (SD)</td>
<td>3.39 (3.63)</td>
<td>37.278 (16.76)</td>
<td>18.11 (10.17)</td>
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<tr>
<td>Hoehn &amp; Yahr</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001**</td>
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<tr>
<td>- Mean (SD)</td>
<td>0.00 (0.00)</td>
<td>2.045 (0.74)</td>
<td>0.11 (0.33)</td>
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<tr>
<td>MMSE total score</td>
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<td>0.278</td>
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<tr>
<td>- Mean (SD)</td>
<td>28.64 (1.18)</td>
<td>28.207 (1.48)</td>
<td>28.56 (0.88)</td>
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<td>MoCA total score</td>
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<td>- Mean (SD)</td>
<td>26.05 (2.33)</td>
<td>24.257 (3.265)</td>
<td>25.44 (2.60)</td>
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<td>CSF albumin quotient</td>
<td></td>
<td></td>
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<td>0.019*</td>
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<tr>
<td>- Mean (SD)</td>
<td>7.65 (3.74)</td>
<td>8.916 (4.507)</td>
<td>6.03 (1.88)</td>
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<td>CSF β-amyloid (Aβ)</td>
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<td>0.154</td>
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<tr>
<td>- Mean (SD)</td>
<td>870.65 (211.07)</td>
<td>870.401 (210.363)</td>
<td>673.67 (65.68)</td>
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<tr>
<td>CSF phospho-tau protein</td>
<td></td>
<td></td>
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<td>0.066</td>
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<td>- Mean (SD)</td>
<td>44.97 (16.72)</td>
<td>42.192 (16.326)</td>
<td>27.77 (3.36)</td>
<td></td>
</tr>
</tbody>
</table>
Baseline | HC (N = 46) | PD (N = 88) | RBD (N = 9) | Adj. p-values
--- | --- | --- | --- | ---
CSF Total tau protein | 256.84 (122.39) | 235.63 (115.46) | 159.00 (16.82) | 0.099

CSF neurofilament light chains (NfL) | < 0.001**
- Mean (SD) | 536.28 (203.38) | 702.10 (405.19) | 361.83 (85.92)

α-synuclein seeding aggregation assay (SAA) | 1/40 (+/-) out of 41 | 67/13 (+/-) out of 80 | 8/1 (+/-) out of 9 | < 0.001**

## Synaptic and lysosomal biomarker levels

The application of a targeted mass spectrometry biomarker panel (Table 2) showed nine markers that were differentially expressed between PD and healthy controls: neurosecretory protein VGF, amyloid-beta precursor protein (APP), the neuronal pentraxins and its corresponding receptor (NPTX1, NPTX2, NPTXR), secretogranin-2, neurogranin, syntaxin-7 and AP-2 complex subunit beta (AP2B1). The markers displayed significantly lower CSF levels in PD compared to healthy controls. All these markers also showed lower CSF levels in iRBD, with significant results for secretogranin-2 (p = 0.01). The analysis showed no changes in protein levels between PD and iRBD.

Results are shown in Table 3 and box plots appear in Fig. 1.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Abbreviation</th>
<th>Accession</th>
<th>Sequence</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurosecretory protein</td>
<td>VGF</td>
<td>O15240</td>
<td>AYQGVAAPFPK</td>
<td>Neurotransmitter secretion</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>NSEPQDEGFQGVDPR</td>
</tr>
<tr>
<td>Neuronal pentraxin receptor</td>
<td>NPTXR</td>
<td>O95502</td>
<td>NNYMYAR</td>
<td>Glutamate receptor recruitment; synaptic plasticity</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LVEAFGGATK</td>
</tr>
<tr>
<td>Neuronal pentraxin-1</td>
<td>NPTX1</td>
<td>Q15818</td>
<td>ETVLQQK</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CESQSTLDPGAGEAR</td>
</tr>
<tr>
<td>Neuronal pentraxin-2</td>
<td>NPTX2</td>
<td>P47972</td>
<td>VAELEDEK</td>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
<td>ETVVVQQK</td>
</tr>
<tr>
<td>Amyloid-beta precursor protein</td>
<td>APP</td>
<td>P05067</td>
<td>VESLEQEAANER</td>
<td>Neuronal surface receptor</td>
</tr>
<tr>
<td>Secretogranin-2</td>
<td>SCG2</td>
<td>P13521</td>
<td>ALEYIENLR</td>
<td>Neuroendocrine protein, biogenesis of secretory granules</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>VLEYLNQEK</td>
</tr>
<tr>
<td>Neurogranin</td>
<td>NEUG</td>
<td>Q92686</td>
<td>KGPGPPGPGGAGVAR</td>
<td>Binds calmodulin, enhances synaptic transmission</td>
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<tr>
<td>Syntaxin-7</td>
<td>STX7</td>
<td>O15400</td>
<td>EFGSLPTTPSEQR</td>
<td>Vesicle trafficking</td>
</tr>
<tr>
<td>Syntaxin-1B</td>
<td>STX1B</td>
<td>P61266</td>
<td>QHSAILAAPNPDEK</td>
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<tr>
<td>AP-2 complex subunit beta</td>
<td>AP2B1</td>
<td>P63010</td>
<td>AVWLPAVK</td>
<td>Mediating endocytosis</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>IQPGNPNYTLSLK</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>QVFLATWK</td>
</tr>
<tr>
<td>Chromogranin-A</td>
<td>CMGA</td>
<td>P10645</td>
<td>GLSAEPGWQAK</td>
<td>Aggregation and processing of secretory granules</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>EDSLEAGLPLQVR</td>
</tr>
<tr>
<td>Ganglioside</td>
<td>SAP3</td>
<td>P17900</td>
<td>EVAGLWIK</td>
<td>Binding gangliosides, stimulating GM2 degradation</td>
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<tr>
<td>GM2 activator</td>
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<td></td>
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<td>IESVLSSSGK</td>
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<tr>
<td>β-synuclein</td>
<td>SYUB</td>
<td>Q16143</td>
<td>EGVVQGVASVAEK</td>
<td>Presynaptic functioning</td>
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<td>Protein</td>
<td>Abbreviation</td>
<td>Accession</td>
<td>Sequence</td>
<td>Function</td>
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<tr>
<td>γ-synuclein</td>
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<td>O76070</td>
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<td>Complexin-2</td>
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<td>Q6PUV4</td>
<td>AALEQPCEGS-LTRPK</td>
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<td>Rab GDP dissociation inhibitor alpha</td>
<td>GDIA</td>
<td>P31150</td>
<td>QLICDPSYIPDR</td>
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</tr>
<tr>
<td>Phosphatidylethanolamine-binding protein 1</td>
<td>PEBP1</td>
<td>P30086</td>
<td>NRPTSISWDGLDSGK</td>
<td>Regulatory protein, presynaptic functioning</td>
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<td>LYEQLSGK</td>
<td></td>
</tr>
<tr>
<td>Lysosome-associated membrane glycoprotein 2</td>
<td>LAMP2</td>
<td>P13473</td>
<td>IPLNDLFR</td>
<td>Chaperone-mediated autophagy</td>
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<td>Cathepsin-F</td>
<td>CATF</td>
<td>Q9UBX1</td>
<td>TLLCSFQVLDLGR</td>
<td>Protein turnover, intracellular degradation</td>
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<td>1433E</td>
<td>1433E</td>
<td>P62258</td>
<td>IISSIEQK</td>
<td>Adapter proteins, modulating general and specific</td>
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<td>pathways by activity regulation of the binding</td>
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<td>P27348</td>
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<td>1433Z</td>
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<td>P63104</td>
<td>VVSSIEQK</td>
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Table 3
Results of the assessed markers in CSF samples, significantly differently expressed values are marked with *, P-value is adjusted for age and sex Abbreviations: PD = Parkinson’s Disease, HC = healthy control, iRBD = isolated REM sleep behavior disorder

<table>
<thead>
<tr>
<th>Marker</th>
<th>HC</th>
<th>iRBD</th>
<th>PD</th>
<th>HC-iRBD</th>
<th>HC-PD</th>
<th>iRBD-PD</th>
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<tr>
<td>Secretogranin-2</td>
<td>12.42</td>
<td>11.79</td>
<td>12.12</td>
<td>0.01*</td>
<td>0.03*</td>
<td>0.43</td>
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<tr>
<td>VGF</td>
<td>13.42</td>
<td>13.12</td>
<td>13.09</td>
<td>0.12</td>
<td>0.03*</td>
<td>0.92</td>
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<tr>
<td>Neuronal Pentraxin receptor</td>
<td>10.99</td>
<td>10.91</td>
<td>10.67</td>
<td>0.26</td>
<td>0.03*</td>
<td>0.96</td>
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<td>Amyloid-beta precursor protein</td>
<td>12.79</td>
<td>12.71</td>
<td>12.49</td>
<td>0.24</td>
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<td>0.96</td>
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<td>Neuronal Pentraxin-2</td>
<td>6.16</td>
<td>5.99</td>
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<td>7.77</td>
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<td>0.24</td>
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<tr>
<td>Neurogranin</td>
<td>4.36</td>
<td>4.36</td>
<td>4.08</td>
<td>0.39</td>
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<td>Syntaxin-7</td>
<td>3.53</td>
<td>3.37</td>
<td>3.34</td>
<td>0.26</td>
<td>0.05*</td>
<td>0.96</td>
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<td>AP-2 complex subunit beta</td>
<td>7.62</td>
<td>7.57</td>
<td>7.41</td>
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<td>β-synuclein</td>
<td>3.29</td>
<td>2.99</td>
<td>3.10</td>
<td>0.11</td>
<td>0.12</td>
<td>0.47</td>
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<td>Syntaxin-1B</td>
<td>3.69</td>
<td>3.51</td>
<td>3.48</td>
<td>0.26</td>
<td>0.07</td>
<td>0.96</td>
</tr>
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<td>Chromogranin-A</td>
<td>12.12</td>
<td>12.14</td>
<td>11.86</td>
<td>0.48</td>
<td>0.07</td>
<td>0.96</td>
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<tr>
<td>Ganglioside GM2 activator</td>
<td>12.70</td>
<td>12.43</td>
<td>12.59</td>
<td>0.22</td>
<td>0.27</td>
<td>0.82</td>
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<td>γ-synuclein</td>
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<td>4.13</td>
<td>4.40</td>
<td>0.12</td>
<td>0.43</td>
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<td>Complexin-2</td>
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<td>5.71</td>
<td>0.37</td>
<td>0.16</td>
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<td>Rab GDP dissociation inhibitor alpha</td>
<td>6.96</td>
<td>6.86</td>
<td>6.85</td>
<td>0.42</td>
<td>0.16</td>
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<td>Lysosome-associated membrane glycoprotein 2</td>
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<td>6.72</td>
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<tr>
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<td>0.45</td>
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<td>Cathepsin-F</td>
<td>9.60</td>
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<td>9.59</td>
<td>0.39</td>
<td>0.90</td>
<td>0.84</td>
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Linear mixed models and longitudinal changes
The established linear mixed models, adjusted for age and sex, revealed no significant changes in the assessed peptides over time in the healthy controls, PD, or iRBD groups. As the PD subjects were de novo at baseline and started drug treatment during the follow-up, we wanted to monitor possible treatment effects on the marker levels and, therefore, analyzed the influence of the LEDD (levodopa equivalent daily dosage) on the marker panel by including it as a fixed effect term in the models. This showed no significant relationship to the medication. Only age and sex contributed significantly to the model. Results can be found in supplementary Table S2.

**Correlation analysis**

Multiple markers correlated with the available clinical and diagnostic data at baseline. This was especially the case for MDS-UPDRS part I: the rating of non-motor symptoms was strongly positively correlated with 20 markers, including VGF, NPTX1, NPTXR, neurogranin, γ-synuclein, β-synuclein, and AP2B1. Nine markers correlated positively with the MoCA domain “language sentence repetition”: SAP3, secretogranin-2, GDIA, PEBP1, AP2B1, syntaxin-7, syntaxin-1B, complexin-2, and NPTX1. Syntaxin-1B and 7 correlated positively with MDS-UPDRS parts I, II, III, and the total score, as well as with the MoCA domain “language sentence repetition”. This suggests a strong connection between the syntaxins and motor and non-motor impairment. The results from the dopamine-transporter–single-photon emission computed tomography (DAT-SPECT), namely the specific binding ratios of caudate nucleus and striatum on both sides, correlated negatively with Cathepsin-F. All correlations can be found in the correlation matrix (Fig. 2).

**Predictive potential**

The correlation of the baseline biomarker levels and the last available MDS-UPDRS scores after 10 years of follow-up revealed a predictive potential for several markers. Higher CSF levels at baseline predicted worse outcomes in MDS-UPDRS I for LAMP2, γ-synuclein, and Neurogranin. The markers Cathepsin-F, SAP3, LAMP2, PEBP1, Syntaxin-7, Syntaxin-1B, CPLX2, 1433Z, and γ-synuclein were predictive for MDS-UPDRS part II, Cathepsin-F, LAMP2, Syntaxin-7, and γ-synuclein for MDS-UPDRS part III, and cathepsin-F, SAP3, LAMP2, PEBP1, Neurogranin, Syntaxin-7, CPLX2, 1433Z and γ-synuclein for the MDS-UPDRS total score. LAMP2 and γ-synuclein CSF baseline levels were predictive of the outcome in all MDS-UPDRS subscores. Cathepsin-F was predictive for all subscores of the MDS-UPDRS except for part I.

**Machine learning approach**

The Boruta analysis performed 100,000 iterations and the Boruta algorithm detected two proteins of high importance for the discrimination of the PD subjects from healthy controls, namely VGF and Neuronal pentraxin receptor (Fig. 4).

**DISCUSSION**

In prodromal and early PD, we need effective biomarkers that reflect specific pathophysiological processes and are directly connected to disease progression and clinical phenotypes. This led us to apply a well-established biomarker panel in the CSF of prodromal and manifest PD patients as well as healthy subjects.
Synaptic and lysosomal dysfunction has been shown to play an important role in neurodegenerative diseases and the proteins included in the analyzed panel have been implicated in Alzheimer’s disease, PD, and other Parkinson syndromes\textsuperscript{26–28}. Nevertheless, longitudinal data and studies in early and prodromal PD disease stages are lacking. To fill this gap, we (i) analyzed the potential of lysosomal and synaptic function markers to detect or monitor endolysosomal dysfunction and synaptic degeneration in an established cohort of \textit{de novo}, unmedicated PD and matched healthy controls at baseline and longitudinally. We extended our study by including subjects with polysomnography (PSG)-verified iRBD. We further analyzed (ii) correlations with clinical data to evaluate the potential of these evaluated markers in routine clinical diagnostics and for monitoring therapeutic effects. The data were further tested for their potential to (iii) predict progression of motor and non-motor symptoms. Linear mixed modeling was performed to assess longitudinal changes and progression. Finally, we applied machine-learning algorithms to evaluate how the different markers discriminate between groups.

PD is a progressive neurodegenerative disease with synaptic loss\textsuperscript{28}. Markers of synaptic dysfunction are increased in the CSF\textsuperscript{28} of patients with Alzheimer’s disease, while the levels of many markers (e.g. AP2B1, LAMP2, Secretogranin-2) were decreased in a cross-sectional PD cohort\textsuperscript{33}.

In our approach, longitudinal samples of prodromal and \textit{de novo} PD (NSD-ISS Stage 4 at baseline) subjects were assessed, identifying nine markers that were significantly decreased in CSF samples of PD subjects compared to healthy controls (VGF, APP, NPTX1/2 and NPTXR, neurogranin, secretogranin-2, syntaxin-7 and AP2B1). Interestingly, all these nine markers were lower in iRBD, but only secretogranin-2 was already significantly decreased in prodromal iRBD (NSD-ISS stage 2A/B). As we discuss below, the role of secretogranin-2 in vesicle-mediated transport could be important in early pathophysiological steps when αSyn aggregation takes place.

The correlation analysis of all measured markers showed a strong relationship between 20 markers and the baseline MDS-UPDRS part I score (that is, a mix of non-motor symptoms, like dementia, but also other features like sleep and depression, etc.). These included NPTX1 and the corresponding receptor, neurogranin, secretogranin-2, β- and γ-synuclein, and syntaxin-1B and 7.

From the MoCA score (cognition only), the domain “language sentence repetition” correlated the most with nine markers, including neuronal pentraxins, secretogranin-2, and the syntaxins. Syntaxin-1B and 7 also correlated positively with MDS-UPDRS parts I-III and total score. Cathepsin-F was the only marker correlating with several DAT-SPECT parameters.

As the longitudinal DeNoPa cohort collects extensive data every two years we analyzed longitudinal CSF samples over 10 years. The CSF levels of proteins, that were significantly differentially expressed at BL, remained at a stable expression level over time and showed no further significant change. The linear mixed model estimated the influence of dopaminergic drug intake, but found no significant correlation between the LEDD and the marker levels. As previously shown in many different studies, age and sex significantly influenced the model\textsuperscript{34}. LAMP2 and γ-synuclein were the markers with the highest predictive potential regarding the progression of the MDS-UPDRS total score as well as its subscores (I, II, and III). The additional
machine-learning approaches via the Boruta algorithm showed that the proteins VGF and NPTXR were most powerful in discriminating between PD and healthy controls.

Below we discuss the markers according to their biological function:

**Synaptic function and plasticity**

The neuronal pentraxins (NPTX1, NPTX2, NPTXR) are widely expressed at excitatory synapses and are very important in synaptic plasticity and in the clustering of glutamate receptors\(^{35}\).

Lower levels of the secreted glycoproteins NPTX1 and NPTX2 are related to more severe non-motor PD symptoms and cognitive deficits, measured by MDS-UPDRS part I, supporting previous findings in PD\(^{33,36,37}\).

NPTX1/2 is upregulated in the substantia nigra of PD subjects while NPTXR is downregulated. NPTX2, similar to αSyn, is present in Lewy bodies in the substantia nigra\(^{38}\). Lower NPTX2 CSF levels in PD could be connected to the accumulation in Lewy bodies, similarly to αSyn. NPTXR contributed significantly to our machine-learning model.

Neurogranin is a post-synaptic protein, a neuronal injury marker connected to calmodulin regulation. Higher CSF levels of neurogranin have been reported in Alzheimer's disease\(^{19,21}\). However, in contrast to Alzheimer's disease, CSF levels in PD were lower in our study compared to healthy controls and positively correlated with MDS-UPDRS part I.

In this study, CSF levels of markers associated with synaptic plasticity and presynaptic functioning showed lower values in PD compared to healthy controls, and correlated mostly with non-motor symptoms. Synaptic dysfunction seems to be already present in prodromal and early disease stages. It reflects motor as well as non-motor symptoms, including cognitive decline, which is in line with previous studies in neurodegenerative disorders\(^{28,39}\).

**Secretory pathways and vesicle trafficking**

Neurosecretory protein VGF is a member of the chromogranin/secretoygranin family. It regulates secretory pathways.

VGF levels were lower in PD in our study and the protein was marked as important by the machine-learning model, revealing a discriminatory potential between early PD and healthy controls. Available data for VGF reports decreased levels in dementia with Lewy bodies, amyotrophic lateral sclerosis, Alzheimer's disease, and brain tissue of PD subjects\(^{33,40}\). VGF is also decreased in patient plasma samples. In a 6-hydroxydopamine-(6OHD)-induced lesions rat model (a common experimental PD animal model with lesions in the medial forebrain bundle), brain tissue and plasma samples were reduced compared with controls. Interestingly, these were restored by levodopa treatment\(^{41}\).

Secretogranin-2 regulates the packing and sorting of peptide hormones and neuropeptides into secretory vesicles and is generally considered a marker for secretory granules, dense-core vesicles in neurons and
Secretogranin-2 CSF levels were not only significantly lower in PD but also in iRBD compared with healthy controls, with the PD levels lying in between. They were also positively correlated with MDS-UPDRS part I and MoCA domain “language sentence repetition”.

Recently, the SCG2 gene was reported as a signal integrator of glutamate and dopamine inputs\textsuperscript{43}. Lower CSF levels have been shown in PD as well as PD with \textit{GBA}-lysosomal enzyme glucocerebrosidase) mutation, the most common genetic risk factor for PD\textsuperscript{33}. In a 6OHD rat model, increased SCG2 mRNA levels were found in brain tissue samples and validated via immunohistochemical staining. In addition, these levels even increased during chronic levodopa treatment\textsuperscript{43}. Nevertheless, in our linear mixed-model analysis LEDD did not significantly influence Secretogranin-2 or VGF CSF levels.

The validation of decreased Secretogranin-2 levels in iRBD points towards its role early in the disease course during the loss of synapses and preceding neuronal degeneration. Its function is strongly connected to vesicle-mediated transport and lysosomal dysfunction, leading to a potential relation to αSyn and its aggregation.

The proteins syntaxin-7 and syntaxin-1B are relevant for vesicle trafficking and higher CSF levels of the syntaxins have been reported in Alzheimer’s disease\textsuperscript{44}. Here, PD subjects had lower CSF levels and the markers also correlated positively with MDS-UPDRS part I, II, III and total score and the MoCA domain “language sentence repetition” and were predictive of MDS-UPDRS II and STX-7, as well as MDS-UPDRS part III and total score. Therefore, secretion and vesicular trafficking seem to reflect parts of PD pathophysiology, especially regarding the correlation with the clinical picture\textsuperscript{33}.

**Autophagy and lysosomal function**

Endolysosomal function and chaperon-mediated autophagy are key components of PD pathology\textsuperscript{33,45}. Several markers are involved in these pathways according to our panel. Previous data reported increased LAMP2 CSF levels in Alzheimer’s disease and lower in PD compared to controls\textsuperscript{46}. Here, LAMP2 levels were not significantly different between the groups but seemed to be a strong predictor for the clinical outcome in PD. LAMP2 CSF levels at baseline were predictive of higher scores in MDS-UPDRS I-III and the total score over the ten years of follow-up.

SAP3 is a lysosomal protein that catalyzes the degradation of gangliosides and is involved in α-synuclein proteostasis\textsuperscript{46}. SAP3 levels were associated with worse clinical performance in PD, indicated by the positive correlations with MDS-UPDRS part I and total score and the MoCA domain “language sentence repetition”. Previous data reported higher CSF levels in Alzheimer’s disease and dementia with Lewy bodies and lower levels in PD\textsuperscript{33}.

Cathepsin-F was predictive for MDS-UPDRS parts II, III, and total score and correlated with the DAT-SPECT quotient of N. caudatus and striatum on both sides. It is a member of the papain-like cysteine protease family. It is involved in protein degradation after endocytosis and presentation of protein fragments via major histocompatibility complex (MHC) class II to T lymphocytes\textsuperscript{47}. Therefore, proteins of αSyn proteostasis are already altered in the early disease stages and are linked to immunological presentation, which is congruent
with the known activity of αSyn-specific T-cell populations both prior to and at the onset of motor symptoms\textsuperscript{48}.

**Predictive potential and clinical implications**

Besides the illustration of specific disease pathways, biomarkers have clinically relevant implications. We found several markers, whose baseline CSF levels were correlated with motor and non-motor clinical symptoms, that were associated with clinical outcomes. MDS-UPDRS part I correlated with 20 proteins. It rates non-motor symptoms in PD including cognitive impairment, hallucinations, affective symptoms like anxiety and depression, sleeping problems, and vegetative symptoms like urinary problems, which are known to significantly impair disease-related quality of life\textsuperscript{49}. Synaptic dysfunction seems to affect pronounced non-motor aspects of the disease, which is in line with findings showing a connection between synaptic dysfunction and cognitive decline in Alzheimer’s disease\textsuperscript{26}.

Regarding prediction, LAMP2 levels were strongly connected with the performance of all MDS-UPDRS subscores and the total score. Cathepsin-F was associated with the outcome of all but part I of the MDS-UPDRS and correlated with DAT-SPECT results. LAMP2 seems to be an ideal marker to predict disease progression over time, for motor and non-motor symptoms, independent of drug therapy. Previous data showed that αSyn preformed fibrils impair autophagy flux resulting in the degradation of LAMP2 in activated microglia, forming a direct connection between synaptic dysfunction, αSyn pathology and neuroinflammation\textsuperscript{50}.

As the described synaptic markers are correlated with cognitive functioning and are prominently altered in Alzheimer’s disease, it is interesting, that many correlated with the MoCA score, especially the part “language sentence repetition”. Studies show that sentence repetition and generation are altered in PD before\textsuperscript{51}.

A strength of our study is the validated and established protein panel as well as the established study cohort. We present for the first time, longitudinal, high quality and deeply phenotyped clinical data from prodromal and PD subjects staged with the new NSD-ISS. This has been previously recommended by researchers in the field\textsuperscript{33}.

Limitations include the small number of iRBD subjects, the lack of validation in corresponding brain or blood samples, and the panel itself. This may not be fully representative of all relevant autolysosome and synaptic processes in PD.

**Conclusion**

Autophagy, and lysosomal and synaptic dysfunction play a relevant role in PD pathology, but not all known synaptic markers showed the same importance in PD and prodromal subjects. LAMP2, Cathepsin-F and the Syntaxins were most predictive for the clinical outcome and progression over time. Neurosecretory protein VGF and the Neuronal pentraxin receptor were most able to discriminate the groups and correlated with clinical measures.
The validation of these markers in other cohorts with independent approaches would be a promising way to assess new biomarker candidates for clinical trials and possible translation into clinical practice in the future.

Methods

The DeNoPa cohort

Recently diagnosed patients with PD and matched healthy controls were enrolled at the Paracelsus-Elena-Klinik, Kassel, Germany between 2009 and 2012. Participants had to be aged between 40 and 85 years old with newly diagnosed PD with at least two of the following criteria: resting tremor, bradykinesia, and rigidity according to UK Brain Bank Criteria; UKBBC1). To be eligible for inclusion, participants had to meet the criteria for de novo PD: any exposure to L-dopa had to have been less than 2 weeks and not within the 4 weeks prior to study entry2. Reasons for exclusion were severe vascular encephalopathy, normal-pressure hydrocephalus (NPH) shown on magnetic resonance imaging (MRI) (when available at screening or when detected during imaging studies), evidence for multiple system atrophy (MSA) or progressive supranuclear palsy (PSP) as well as medication-induced PD.

Healthy individuals between 40 and 85 years, matched to the PD group by age, sex, and education level showing no pathological condition of the central nervous system and a negative family history of PD were included as controls. Biannual longitudinal clinical data were collected at baseline and at 2, 4, 6, 8, and 10 years of follow-up in 104 PD and 94 healthy controls (flow-chart see supplementary figure S1a/b). Clinical diagnosis was reassessed in the On-state for all patients at each follow-up by consensus of two teams of independent neurologists (CT/FS-D and BM/JE) as described5.

Motor function in DeNoPa was assessed with the Movement Disorder Society-Unified Parkinson’s Disease Rating Scale (MDS-UPDRS) parts I, II, III, and total score. Cognitive decline was assessed using the Mini-Mental-State Examination (MMSE) and MoCA (Montreal cognitive assessment) in all patients at baseline, and 2, 4, 6, 8, and 10 years of follow-up.

iRBD was diagnosed through video polysomnography by experienced raters (CT, FS-D, MLM) on two consecutive nights according to established criteria5,55. The LEDD (levodopa equivalent daily dosage) was calculated as previously described56.

Final diagnosis refers to the consensus diagnosis that was made up to 10 years of follow-up, including initial dopamine-transporter–single-photon emission computed tomography (DAT-SPECT), biannual clinical evaluations, levodopa challenge, lasting response to levodopa, and the emergence of advanced PD features such as motor fluctuations or levodopa–induced dyskinesias. Abnormal/pathological DAT-SPECT was determined by a specialist in nuclear medicine by visual inspection or quantification.

Sample collection
CSF was collected in polypropylene tubes (Sarstedt, Nümbrecht, Germany) directly after the plasma collection by lumbar puncture in the sitting position. Tubes were centrifuged at 2500 g at room temperature (20°C) for 10 minutes and aliquoted and frozen within 30 minutes after collection at −80°C until analysis. Before centrifugation, white and red blood cell counts in CSF were determined manually. CSF αSyn, β-amyloid 1–42, total tau protein (t-tau), and phosphorylated tau protein (p-tau181) concentrations were measured as previously described. The α-Syn Seed Amplification Assay has also been previously described. Consent to collect CSF samples was not successful for all the subjects, see Table 1 and Supplementary Table S3 for details.

**Neuronal α-synuclein-disease integrated staging system (NSD-ISS)**

Neuronal αSyn-disease is defined by the biological anchors S: presence of *in vivo* detected pathologic αSyn species, measured usually by αSyn seeding aggregation assays, (independent from clinical syndrome) and D: dopaminergic neuronal dysfunction, assessed by DAT-SPECT, leading to a proposed NSD Integrated Staging System (NSD-ISS) that includes clinical signs and symptoms. Stages 0–1 are defined by the presence of pathogenic variants in SNCA gene (Stage 0), S alone (Stage 1A), or S and D (Stage 1B) without clinical signs/symptoms. The occurrence of clinical manifestations defines the transition to Stage 2 and higher. Stage 2 includes subtle signs/symptoms without functional impairment. Stages 2B-6 require S and D to be positive and for there to be stage-specific increases in functional impairment. NSD-ISS was applied in all 88 participants where α-Syn-SAA and DAT-SPECT were available (supplementary Table S4).

**Statistical analysis**

All analyses were performed with the statistical software R (version 4.0.5). Baseline continuous variables were expressed as mean (standard deviation), median, and the range as given by the minimum and maximum values. Group comparisons were performed using the nonparametric Mann–Whitney Kruskal–Wallis test because some of the parameters had nonnormal distribution. For the binary variable “sex”, the count in each category is provided, and the Fisher exact Chi-square tests were used for comparison. Differential expression was assessed using the empirical Bayes approach as implemented in the Bioconductor limma package. Multiple hypothesis testing corrections were performed by using Benjamini and Hochberg’s (BH) false discovery rate at α = 5%. Linear mixed models were used for longitudinal data analysis that allowed fitting only for random intercept models. They were implemented using the function *lmer* from the cran package *lmerTest*. The correlation between the assessed proteins and the clinical parameters was assessed via a nonparametric Spearman coefficient using the base R function *cor.test* from the cran *psych* package. Here again, the BH procedure was used to correct for multiplicity.

For machine learning, the Boruta algorithm from the CRAN package *Boruta* was used, and algorithms were built around the random forest classification algorithm. It aims to capture all the relevant features in the
dataset concerning the outcome variables of PD vs. healthy controls. The algorithm adds randomness to the dataset by creating shuffled copies of all features (Shadow Features) and trains a random forest classifier on the extended dataset (original attributes plus shadow attributes), applying a feature importance measure (The Mean Decrease Accuracy), evaluating the importance of each feature. At every iteration, the Boruta algorithm checks whether a real feature is more important than others and removes features that are marked as highly unimportant. As a stopping rule, we used 100000 iterations with a maximum of 500 random forests as indicated by the parameter maxRuns in the Boruta function. With actual CRAN implementation of Boruta, warm-up rounds are removed, and the multiple testing corrections are introduced, marking all features that are either strongly or weakly relevant to PD diagnosis.

**Standard protocol approvals, registrations, and patient consent**

Approval was received from the local ethical standards committee on human experimentation for all human participants in all cohorts (FF 89/2008, FF 130/2012, MC 310/2010). Written informed consent for research was obtained from all study participants. DeNoPa is registered in the German Register for Clinical trials (DRKS00000540).

**LC-MS/MS analysis**

For detailed sample preparation, refer to previously published work\textsuperscript{26,28}. In summary, a mix of heavy standard peptides serving as an internal standard (JPT Peptide Technologies (Berlin, Germany; SpikeTides L). 25 µL (for concentration see Suppl. Table. 1A) was added to 100 µL of CSF samples, which were then reduced, alkylated, digested, and desalted. The quantification was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) with a micro-high-performance LC-MS/MS system (6495 Triple Quadrupole LC/MS system, Agilent Technologies) equipped with a Hypersil Gold reversed-phase column (100 × 2.1 mm, 1.9 µm particle size, Thermo Fisher Scientific). The method involved the measurement of a panel of 38 synaptic and lysosomal proteins as indicated in Table 2. For further details, see Suppl. Table 1B, which describes the settings used. To monitor the assay's performance, two different quality control (QC) samples comprising CSF pools were periodically injected, where one of them was used to adjust for potential plate differences and the second to evaluate the final analytical performance. The analytical performance of the different proteins had a high precision within and between runs with a few exceptions (Suppl. Table. 1A). Skyline 20.1 (MacCoss Lab Software) was utilized to analyze the mass spectrometric data. One peptide, the one with the best analytic performance, per target protein was selected for statistical analyses.

**Declarations**

**Author contributions**

MB, BM: Conceptualization, Data acquisition, Data curation, Methodology, Writing – original draft.
JH, BG AB: Conduction of mass spectrometry, Conceptualization, Supervision, Writing – review & editing

MD: Data curation, Formal analysis, Methodology, – review & editing

ME, CT, HZ, FSD: Supervision, – review & editing

SW: Figure design

SS, SW, MX, MLM: Data acquisition – review & editing

All authors read and approved the final manuscript.

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Competing interests

MB has received funding from the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – 413,501,650.

CT has received honoraria for consultancy from Roche, and honoraria for educational lectures from UCB, and has received research funding for the PPMI study from Michael J. Fox Foundation and funding from the EU (Horizon 2020) and stipends from the (International Parkinson’s and Movement Disorder Society) IPMDS.

BM has received honoraria for consultancy from Roche, Biogen, AbbVie, UCB, and Sun Pharma Advanced Research Company.

BM is a member of the executive steering committee of the Parkinson Progression Marker Initiative and PI of the Systemic Synuclein Sampling Study of the Michael J. Fox Foundation for Parkinson’s Research and has received research funding from the Deutsche Forschungsgemeinschaft (DFG), EU (Horizon 2020), Parkinson Fonds Deutschland, Deutsche Parkinson Vereinigung, Parkinson’s Foundation and the Michael J. Fox Foundation for Parkinson’s Research.

MLM has received honoraria for speaking engagements from Deutsche Parkinson Gesellschaft e.V. and royalties from Gesellschaft für Medien + Kommunikation mbH + Co.
FSD has received honoraria for speaking engagements from AbbVie, Bial, Ever Pharma, Medtronic and royalties from Elsevier and Springer. She served on an advisory board for Zambon and Stada Pharma.

**FSD** participated in Ad Boards for consultation: Abbvie, UCB, Bial, Ono, Roche and got honorary for lecturing: Stada Pharm, Abbvie, Alexion, Bial.

**SS** received institutional salaries supported by the EU Horizon 2020 research and innovation program under grant agreement No. 863664 and by the Michael J. Fox Foundation for Parkinson's Research under grant agreement No. MJFF-021923. He is supported by a PPMI Early Stage Investigators Funding Program fellowship of the Michael J. Fox Foundation for Parkinson's Research under grant agreement No. MJFF-022656.

**HZ** has served at scientific advisory boards and/or as a consultant for Abbvie, Acumen, Alector, Alzinova, ALZPath, Annexion, Apellis, Artery Therapeutics, AZTherapies, Cognito Therapeutics, CogRx, Denali, Eisai, Merry Life, Nervgen, Novo Nordisk, Optoceutics, Passage Bio, Pinteon Therapeutics, Prothena, Red Abbey Labs, reMYND, Roche, Samumed, Siemens Healthineers, Triplet Therapeutics, and Wave, has given lectures in symposia sponsored by Alzecure, Biogen, Cellecricton, Fujirebio, Lilly, and Roche, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work).

**MD, MX, SW, ME, AB, JN, BG** have no competing interests to report.

**Data availability**

All data generated or analysed during this study are included in this published article and its supplementary information files.

**References**


**Figures**
Figure 1

Figure 2

Correlation matrix of the measured panel and the available clinical data at baseline. The colours of the circles represent positive (blue) and negative (red) correlations. The circle sizes represent the size of the absolute correlation coefficients, larger signifying stronger correlations. *p<0.05 **p<0.01 ***p<0.001, The p-values are Benjamini-Hochberg adjusted for multiple testing. Abbreviations: MDS-UPDRS = Movement
Figure 3

Predictive potential of the analyzed markers evaluated by the correlation of the baseline CSF levels with the available clinical data after 10 years of follow-up. The colours of the circles represent positive (blue) and negative (red) correlations. The circle sizes represent the size of the absolute correlation coefficients, larger...
signifying stronger correlations. *p<0.05 **p<0.01 ***p<0.001, The p-values are Benjamini-Hochberg adjusted for multiple testing. Abbreviations: MDS-UPDRS = Movement Disorder Society – United Parkinson’s Disease Rating Scale, MoCa: Montreal Cognitive Assessment Score,

![Graph showing results of the Boruta algorithm](image)

**Figure 4**

Results of the Boruta algorithm, revealing Neurosecretory protein VGF and Neuronal pentraxin receptor with significant relevance in discriminating between Parkinson’s Disease and Healthy controls out of 100,000 iterations,

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarymaterial.docx](file)