Exploring the potential role of the protein, Alpha-Synuclein, as diagnostic target for Parkinson's disease

Majbour, N.K.

2019

document version
Publisher's PDF, also known as Version of record

Link to publication in VU Research Portal

citation for published version (APA)
Majbour, N. K. (2019). *Exploring the potential role of the protein, Alpha-Synuclein, as diagnostic target for Parkinson's disease.*

CHAPTER 7 | SUMMARY & GENERAL DISCUSSION

Biomarkers for Parkinson's disease (PD) are of paramount importance, since the clinical symptoms are often nonspecific and show overlap with other neurodegenerative diseases, the clinical progression is highly variable, and multiple clinical phenotypes exist for the same disease [1]. Primary endpoints of most clinical trials in PD depend on measures of motor and cognitive impairment, which are critically hindered by a lack of disease specificity or sensitivity [2]. Biomarkers reflecting the underlying disease mechanisms at the molecular level might prove useful in improving diagnostic accuracy, monitoring disease progression, and serving as surrogate endpoints in clinical trials. However, at this point we are still far beyond having solid biomarkers for PD.

In this thesis, we address the challenges for the identification of informative body fluid biomarkers for PD, and we highlight the effort made to develop robust immunoassays targeting different species of alpha-synuclein (α-syn), aiming to provide a panel of disease-reflecting biomarkers for the early diagnosis of PD, and for the monitoring of disease progression. The recognition that α-syn protein is abundant in Lewy bodies (LBs) [3], the finding that mutations in the gene encoding α-syn protein (*SNCA*) [4], as well as that duplication and triplication of this gene lead to early-onset, familial PD [5], and the reports of transgenic flies and animals with human wild-type or mutant α-synuclein genes developing neuronal dysfunction and PD-like pathology [6] were behind the growing interest in α-syn as a critical player in PD pathogenesis. After a 20-year journey of α-syn research [7], we have come to understand that α-syn can adopt several different pathological conformations. In addition, α-syn can be detected in extracellular fluids, such as CSF, blood and saliva [8, 9]. These findings prompted us to explore the role of α-syn as a diagnostic and progression biomarker for PD.

In this chapter, we recount and discuss our discoveries from the previous chapters of this thesis. Thereafter, we summarize our conclusions concerning the potential role of α-syn species as diagnostic and progression biomarkers for PD. We then describe how our findings can facilitate reaching the goal of an early diagnosis of PD and pave the way towards the development of more effective therapeutic strategies for the disease. At the end of the chapter, we comment on future directions for biomarker discovery in PD and related disorders.

In **Chapter 1**, we reviewed the progress made to date towards the development and validation of body fluid biomarkers to improve the diagnosis and treatment of PD. Despite the progress made in the past decades in understanding the etiology of PD [10], the diagnosis still depends on clinical criteria, hindering the early detection of a disease that has a long prodromal or premotor phase. The existing literature was reviewed looking for the answer to the most important question; What might be the ideal biomarker for PD? While the answer is yet to be found, there are reasons to be believe that we are getting closer to that goal. Perhaps,

the most insightful conclusions made in **Chapter 1**, are: 1) a panel of biomarkers rather than a single marker is the way forward, 2) some biomarkers better differentiate PD from other forms of parkinsonism (NF-L levels), while others better predict PD progression (CSF t-a-syn, CSF o-a-syn), 3) it is important to adhere to strictly and thoroughly standardized protocols for body fluid sample collection and processing to reach consistent results.

Considering the minute levels of the different species of α-syn in body fluids, studying their presence in different biological compartments of the human body in association with the pathophysiology of PD demands robust tools with high specificity and affinity. The optimal way to address these challenges was through the generation of conformation-specific monoclonal antibodies (mAbs) that can specifically target PD pathology. This aim was successfully achieved in **Chapter 2**, where we described the generation and characterization of conformation-specific mAbs against α-syn aggregates. Our mAbs were generated following the hybridoma technology, where recombinant α-syn aggregates were used to immunize female balb/C mice. Only IgG isotype clones that showed specificity towards α-syn aggregates were selected for further characterization. The specificity of the mAbs towards α-syn aggregates was assessed by means of inhibition ELISA, dot blotting, and preadsorption experiments. Our mAbs did not cross-react with monomeric or fibrillar forms of β- or γ-syn, or any other amyloidogenic proteins or peptides such as Aβ, Tau40, IAPP or ABri. This clearly established that although our mAbs were raised against a conformational epitope, they are still highly selective, unlike many other conformation-specific mAbs that recognize the conformation of multiple amyloid proteins [11]. To ensure that the affinity of our mAbs towards α-syn aggregates would outreach their peer antibodies, surface plasmon resonance analysis was deployed where most of our antibodies showed affinity towards α-syn fibrils in the picomolar range. In an attempt to acquire more details about the epitope of our mAbs, pepscan using a peptide library spanning the entire α-syn sequence was performed. MAbs exhibited very weak reactivity for the C-terminal peptide spanning the α-syn sequence between amino acids 127 and 140, confirming that our mAbs do not recognize linear peptides and that the C-terminus of α-syn may be a part of the complex conformational epitope. The potential of the mAbs was additionally explored by staining human brain sections of patients with PD, dementia with Lewy bodies (DLB), Alzheimer's disease (AD), and multiple system atrophy (MSA) along with healthy control cases. Immunohistochemical analysis showed that our mAbs were able to recognize α-syn pathology in synucleinopathies, as well as identify pathological structures that were not detected by other commercially available antibodies. In summary, **Chapter 2** detailed the generation and characterization of six novel conformation-specific mAbs against PD pathology. These mAbs can either be exploited to develop immunoassays that may serve as diagnostic tools for PD, or be engineered to serve as therapeutic agents in the form of vaccines in the field of passive immunotherapy [12].

Our quest for developing robust tools that can improve our understanding of PD pathology did not stop there. In **Chapter 3**, we went on to use the mAbs described in **Chapter 2** for the development of quantitative analytical methods, which resulted in highly sensitive and specific enzyme-linked immunosorbent assays (ELISA). In this chapter, we additionally reported the characterization of more mAbs (11D12, and PS129), as well as our polyclonal antibody Syn-140. We also elaborated on the sensitivity and specificity of the three ELISA assays that were developed to capture total- (t-), oligomeric- (o-), or phosphorylated S129 (p-S129-) a-syn. **Chapter 3** highlights the value of adding a biochemical test to the diagnostic procedures in PD to overcome the limitations of current clinical criteria and reduce the high rates of misdiagnosis in the early stages of the disease. We explored the potential of our novel assays for the discovery of CSF biomarkers in a Dutch cohort of PD patients and age matched healthy controls. Analyses of this cross-sectional cohort revealed reduced CSF t-α-syn, and elevated CSF o- and p-S129-α-syn levels in PD compared to healthy controls. Highlighting the diagnostic power of multiple CSF biomarkers, combining both o-/t-α-syn and p-S129-/t- α-syn ratios improved the ability to discriminate between PD and healthy controls, which was further enhanced by including p-tau. When investigating the correlations of biomarker levels with disease severity in the PD group, CSF t-α-syn levels inversely correlated with MMSE scores, whereas CSF o-α-syn levels inversely correlated with H&Y scores. In conclusion, the results described in **Chapter 3**, 1) highlight the potential of our biomarker assays in the field of PD and other synucleinopathies, 2) address the shortcomings of previous ELISA platforms [13](that used a generic antibody for capture, and the biotinylated form for detection) by using a novel conformation-specific mAb (Syn-O2), and 3) validate the usefulness of combining multiple CSF biomarkers in improving diagnostic accuracy in PD.

To take full advantage of the merits of the assays described in Chapters 2 and 3, and to expand our understanding of the role that α-syn species may have as progression biomarkers in PD, we measured the different forms of α-syn (t, o- and p-S129-α-syn) in CSF from 121 participants in the longitudinal Deprenyl and Tocopherol Antioxidative Therapy for Parkinsonism (DATATOP) study cohort, who were clinically diagnosed with early-stage PD (probability between 90%-100%), which is described in **Chapter 4**. DATATOP is a multicenter, placebo-controlled clinical trial involving patients with early-stage PD, who underwent repeated CSF sampling during a 2-year period, prior to starting dopamine replacement therapy [14]. We examined the changes in CSF t-, o-, p-S129-α-syn, t-tau, p-tau, Aβ 1-40, and Aβ 1-42 during the 2-year follow-up period. Compared to baseline levels, follow-up CSF levels of t- and o-α-syn levels were significantly higher, whereas p-S129-α-syn levels were significantly lower. In addition, the changes in t- and o-α-syn levels, but not p-S129-α-syn levels positively correlated with duration of follow-up, confirming the increase in CSF t- and o-α-syn levels with disease progression. Although changes in CSF t- or o-α-syn levels alone did not correlate with clinical measures of motor progression, the change in the ratio of o-/t-α-

syn was associated with worsening UPDRS motor scores. Interestingly, patients with predominant postural instability and gait difficulty revealed a stronger correlation between the change in the o-/t-α-syn ratio and the change in UPDRS motor scores compared to other clinical phenotypes, defined as tremor dominant or intermediate subtypes, thus coupling the CSF biochemical profile of α-syn species with the clinical phenotype of PD. The data in **Chapter 4** shed light on the dynamic pattern of change in CSF α-syn species over the course of the disease, supporting their potential role as disease progression biomarkers and their association with PD clinical phenotypes. The findings in **Chapters 3 and 4** together demonstrate the potential role of α-syn species as surrogate biomarkers for PD diagnosis and disease progression monitoring. Despite the limitations surrounding the DATATOP cohort, including long storage of CSF samples (> 25 years), no tandardized protocols for sample collection across participating centers, and the lack of a control group, our findings are of paramount importance especially in the context of PD progression. Clearly, to fully appreciate the role CSF α-syn species can play in monitoring PD progression, our findings should be extended to larger cohorts where PD patients are followed longitudinally until autopsy, providing a definitive neuropathological confirmation of the clinical diagnosis.

The co-existence of α-syn and tau pathology observed in the brains of patients with AD, PD and DLB[15, 16], prompted us to study the association between CSF α-syn and tau in an AD cohort. The results of this study, described in **Chapter 5,** demonstrated an improvement of diagnostic sensitivity/specificity of classical AD CSF biomarkers when adding CSF α-syn species. Using the same immunoassays described in **Chapters 3** and **4**, we measured CSF levels of different α-syn species in an Italian cohort of AD patients, who showed a CSF profile typical of AD at baseline, as well as in cognitively intact controls. The AD patients in this cohort were selected based on both clinical evaluation and a CSF profile of Aβ-42, t-tau and p-tau supporting the clinical diagnosis of AD. CSF t-α-syn levels were significantly higher in the AD group compared to the control group, whereas CSF o- and p-S129-α-syn levels were not significantly different between the groups. A positive correlation between t-α-syn and tau species was found only in the AD group. The role of t-α-syn in AD pathology taken together with the lack of o- and p-S129-α-syn contribution to AD diagnosis, further support the hypothesis that o- and p-S129-α-syn species are intimately connected to the pathogenesis of synucleinopathies rather than tauopathies. To broaden our understanding of the potential role of α-syn as a cognitive biomarker [17], cohorts of subjects with α-syn related dementias, including DLB and PD dementia, should be investigated [18]. Furthermore, combining α-syn and AD core biomarkers with brain imaging data might contribute to the identification of individuals at higher risk of MCI and conversion to AD. In conclusion, the results in **Chapter 5** obtained in a well-characterized cohort of AD patients and neurological controls further support the notion that t-α-syn most likely represents a marker of neuronal loss and/or synaptic failure in AD [19].

While most cases of PD occur in a sporadic manner, a subset of cases are attributable to genetic causes. Mutations in the leucine-rich repeat kinase 2 (*LRRK2*) gene are the most common causes of inherited PD. In **Chapter 6**, we aimed to determine whether any of the CSF α-syn species have the potential to serve as preclinical biomarkers for PD. Using our in-house developed ELISA assays, we measured t-, o- and pS129-α-syn species in a well-characterized Norwegian cohort of 72 subjects with *LRRK2* mutations: 21 symptomatic cases already diagnosed with PD and 51 asymptomatic *LRRK2* mutation carriers. In parallel, we also included 60 patients with sporadic PD (sPD) and 31 healthy control subjects. The observations in this cohort suggested that CSF o-α-syn holds potential as a surrogate biomarker for the early detection of PD. In addition, we observed a correlation between body fluid biomarker levels and PET scan images in asymptomatic *LRRK2* mutation carriers. Larger cohorts, where asymptomatic LRRK2 subjects are followed up longitudinally, would further validate these findings.

Interpreting the findings from **Chapters 3-5** together, the reduction of CSF t-α-syn in PD is most likely due to the protein folding tendency and its subsequent sequestration in LBs, while the increased CSF o-α-syn levels are most likely related to clearance failure of the oligomerized forms of α-syn. The scarce longitudinal data challenge our speculations about the reasons behind the longitudinal changes in CSF t- and o-α-syn levels over the course of PD. Elevated levels of α-syn have been linked to degree of neurodegeneration and a worse prognosis of PD [20]. Having said that, only a weak inverse correlation between CSF o-α-syn and motor impairment, and a moderate inverse correlation between t-α-syn and cognitive function were noted in **Chapter 3,** while the levels of neither of these CSF α-syn species correlated with disease duration. Longitudinal follow-up of cases, as described in **Chapter 4**, did show a correlation between the changes in t- and o-α-syn levels and disease duration, a correlation that may grow stronger with a longer follow-up period. In interpreting our findings, we have to keep in mind that PD is a disease that develops slowly over many years. The average follow-up duration of two years in most longitudinal studies may be too short to capture the true strength of the relationship between body fluid biomarkers and disease progression in PD. As discussed in the previous chapters of this thesis, α-syn is subject to several post-translational modifications (PTMs) [21], where phosphorylation at Serine 129 is among the most frequent ones [22]. So far, we do not know exactly in what way α-syn phosphorylation alters the folding propensity or neurotoxicity of the protein [23]. The fact that α-syn in LBs is predominantly phosphorylated at Ser129 [24] encouraged us to think that developing immunoassays measuring CSF p-S129-α-syn would best reflect LB pathology. This hypothesis was supported by findings in cross-sectional studies by our group and others, where higher levels of CSF p-S129-α-syn were found in PD [25, 26]. However, longitudinal studies have come up with conflicting results. Stewart *et al.* reported a longitudinal increase in CSF p-S129-α-syn levels, while we found a longitudinal decrease in cases from the DATATOP study [26]. Although differences in

selection criteria of the patients included in each study may explain these differences, this sparked a controversy about the potential role of p-S129-α-syn as a PD biomarker.

In AD, the increase in CSF t-α-syn can be attributed to protein over-expression and/or excessive neuronal damage in AD patients [27, 28], leading in turn to α-syn leakage from damaged cells into the brain's interstitial fluid and then into the CSF. This leakage, however, was not reflected in the levels of other forms of α-syn, such as o- or p-S129-α-syn.

Among the different species of α-syn explored in this thesis, and the original findings these cohorts yielded, the data suggest that the link between CSF α-syn biomarkers and PD severity can be summarized as follows: 1) CSF o-α-syn rather than t- or p-S129-α-syn is more connected to PD motor severity, 2) CSF t-α-syn is associated with cognitive decline in PD, 3) CSF o-α-syn holds the biggest potential for early detection of PD. Large-scale, prospective studies in well-controlled cohorts, where CSF samples are collected and processed using rigorously standardized procedures are needed to further validate these findings [29].

As highlighted in **Chapter 1**, several studies have reported inconsistent findings on α-syn levels among the different labs suggesting there are gaps in our knowledge that should be filled. This has led research groups to collaborate in order to identify the best strategies to move forward with body fluid biomarker studies. This includes an increasing number of recent studies directed towards understanding the leading cause for the lack of reproducibility between laboratories [30]. Many factors contributing to this inconsistency have already been identified, such as: 1) protocols for biofluid collection and processing (storage duration, types of tubes, centrifugation, etc.), 2) CSF contamination with blood traces, and hemoglobin levels in serum/plasma, 3) immunoassays platforms, and 4) patient selection criteria.

Future Directions

There is an increasing body of knowledge suggesting that multiple forms of α-syn can improve the diagnostic accuracy of PD, especially when used as a panel of biomarkers and supported by neuroimaging and clinical data. However, further studies using validated quantitative platforms and including patients with different synucleinopathies that are followed longitudinally until autopsy are highly needed. When further deployed in larger cohorts with longer follow-up durations, the assays we developed can facilitate the search for reliable CSF biomarkers that correlate with disease severity. We will make our assays widely available for the research community to help provide us with objective data from different biomarkers discovery studies to assist in making informed decisions about the diagnosis in individual patients in a timely manner. An accurate early diagnosis is desperately needed in the development of PD-specific therapies to be able to distinguish PD cases from patients suffering from other synucleinopathies or forms of parkinsonism that require different therapies.

Although considerable research is needed to translate α-syn biomarkers from the research setting to diagnostic strategies, it is worth acknowledging the rapid advances that have been made in the field of PD biomarkers, and the work towards accelerating the pace of developing disease modifying therapies for PD and related disorders. Given the association of multiple proteins and markers other than α-syn with PD, such as tau, Aβ42, neurofilament light chain, or inflammatory markers, it is very likely that the search for an ideal panel of biomarkers will continue. Furthermore, multiple large-scale, longitudinal studies in well-controlled cohorts, where standardized protocols are followed, like the Parkinson's Progression Markers Initiative, are now underway and will yield important new data within the next decade. In addition, an increasing number of studies have extended their search for biomarkers far beyond CSF and blood to also include peripheral tissues such as submandibular salivary glands [31] and skin biopsies [32]. Although this approach has not yet culminated in complete success, it might facilitate reaching the ultimate goal of an ideal (panel of) biomarker(s) for PD in the near future for early and differential diagnosis, and monitoring disease progression in PD.

In summary, while our understanding of PD over the past two centuries has vastly progressed and many potential biomarkers have come to the fore, the ideal biomarker or panel of biomarkers for PD has yet to be validated. On the bright side, multiple large-scale, longitudinal studies in well-controlled cohorts, where standardized protocols are followed, like the Parkinson's Progression Markers Initiative, are now underway and will yield important new data within the next decade.

Key findings of the thesis

1. Conformation-specific mAbs for α-syn aggregates were generated and employed to identify α-syn pathology compared to pan antibodies;

2. 11D12 and Syn-140 antibodies were generated and served as good candidates for t-α-syn detection, whereas PS129 mAb was specific for p-S129-α-syn;

3. Robust, sensitive and specific ELISA assays for t-, o-, or p-Ser129-α-syn forms were developed and characterized;

4. In PD patients, CSF t-α-syn levels were significantly decreased, whereas CSF o-α-syn and p-s129-α-syn levels were significantly increased compared to healthy controls;

5. The combination of CSF o-/t-α-syn, p-S129-α-syn and p-tau provided the best fitting predictive model for discriminating PD patients from healthy controls;

6. CSF o-α-syn levels correlated with the severity of PD motor symptoms;

7. CSF t- and o- α-syn levels significantly increased over a two-year follow-up period in early-stage PD, and the CSF o-/t-α-syn ratio was associated with the change in UPDRS motor scores;

8. CSF t-α-syn levels were significantly higher in AD patients compared to non-demented controls, and corresponded to worsening of cognitive functions in the AD patients;

9. Levels of CSF t-α-syn were comparable among sporadic PD, symptomatic and asymptomatic *LRRK2* mutation carriers, but significantly higher than in healthy subjects;

10. CSF o-α-syn levels were significantly higher in asymptomatic *LRRK2* subjects than in healthy controls or symptomatic *LRRK2* patients;