Assessment of cerebrospinal fluid α-synuclein as a potential biomarker in Parkinson’s disease and synucleinopathies

Ioanna Chalatsa1, Katerina Melachroinou1, Evangelia Emmanouilidou1,2, Kostas Vekrellis1

1Center for Basic Science, Biomedical Research Foundation Academy of Athens, Athens 11527, Greece.
2Laboratory of Biochemistry, Department of Chemistry, National and Kapodistrian University of Athens, Athens 11527, Greece.

Correspondence to: Dr. Kostas Vekrellis, Center for Basic Science, Biomedical Research Foundation Academy of Athens, 4 Soranou Ephessiou St., Athens 11527, Greece. E-mail: vekrellis@bioacademy.gr

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Abstract
The discovery of diagnostic and prognostic biomarkers for neurodegenerative diseases represents an unmet clinical challenge. For example, the diagnosis of Parkinson’s disease (PD) relies mainly on the presence of clinical symptoms. Therefore, the identification and use of novel PD biomarkers would allow the application of disease-modifying treatments at the very early stages of neurodegeneration. The presynaptic protein, α-synuclein, has been genetically and biochemically linked with PD pathogenesis and has been considered as a potential biomarker for the diagnosis of PD and the related synucleinopathies. The vast majority of studies have assessed the measurement of α-synuclein, alone or in combination with other biomarkers in the cerebrospinal fluid (CSF), since it is the biofluid that most closely reflects the pathophysiology of the brain. The diagnostic value of the monomeric α-synuclein but also the oligomeric, the phosphorylated and the aggregated forms of the protein has been evaluated using a variety of immunoassays. The results have so far been reproducible but the assays used are still lacking the required diagnostic accuracy. Recent reports have shown that Protein misfolding cyclic amplification is a technique that has the potential to detect α-synuclein seeds in samples of CSF with high sensitivity and across different synucleinopathies. In an effort to increase the source of biomarker for PD and related synucleinopathies, α-synuclein has also been measured in neuronal exosomes, small vesicles of endosomal origin that are secreted from neurons into the CSF or the periphery. The potential diagnostic value of exosomes stems from the notion that exosomes carry a disease-specific repertoire of marker proteins. Therefore, the assessment of exosome-associated α-synuclein species may also open up new avenues for disease diagnosis in different synucleinopathies.
INTRODUCTION

The formation of large inclusions mostly containing protein aggregates is a common pathological hallmark in a wide spectrum of neurodegenerative disorders such as Alzheimer’s Disease (AD) and Huntington Disease\(^1\). Particularly three distinct neurological conditions, Parkinson’s Disease (PD) including Parkinson’s disease dementia, Dementia with Lewy Bodies (DLB) and Multiple System Atrophy (MSA), are characterized by the aberrant accumulation of the presynaptic protein \(\alpha\)-synuclein. In PD and DLB, \(\alpha\)-synuclein deposits are found either in the cytoplasm of neurons, where they are called Lewy bodies (LB), or in the neuronal terminals, where they are called Lewy neurites\(^2\) whereas in MSA, \(\alpha\)-synuclein deposition occurs in glial cells. \(\alpha\)-Synuclein is also genetically linked with the development of PD since specific point mutations or multiplications (duplications, triplications) of the \(SNCA\) gene encoding for \(\alpha\)-synuclein result in the familial forms of PD\(^3-8\). The genetic association of \(\alpha\)-synuclein with PD is further strengthened by all the genome-wide association studies performed so far which indicate a strong correlation of PD with variations in the \(SNCA\) gene\(^9,10\). These biochemical and genetic linkages of \(\alpha\)-synuclein with pathology as well as the observation that the protein is present in biological fluids or peripheral tissues led to the assumption that \(\alpha\)-synuclein could serve as a potential candidate biomarker for PD diagnosis and also aid the differential diagnosis between the synucleinopathies\(^11\).

A plethora of studies have assessed the absolute quantification of \(\alpha\)-synuclein levels as a marker of synucleinopathy with the ultimate aim to discriminate PD patients from healthy subjects or other unrelated neurological controls. In this regard, understanding the structural biology of \(\alpha\)-synuclein is critical; the protein is highly modified at the post-translational level and has the ability to adopt different conformations depending on the surrounding milieu. From all the modifications that have been reported so far, phosphorylation is considered most closely related to PD pathology since almost 90\% of \(\alpha\)-synuclein in LB appears to be hyper-phosphorylated\(^12\). In addition, the assembly into multiple-sized oligomers has been considered an early event in the pathological process of aggregate formation\(^13\). As such, different forms of \(\alpha\)-synuclein, i.e., monomeric, oligomeric and phosphorylated, have been targeted in order to increase diagnostic accuracy\(^14\). The measurements have been performed in bodily fluids [cerebrospinal fluid (CSF), blood plasma or serum, saliva], isolated secreted vesicles (exosomes) and peripheral tissues (skin, olfactory or gut mucosa, salivary gland) using a variety of analytical approaches depending on the nature of the biological sample and the form of \(\alpha\)-synuclein detected with each assay\(^15\).

In comparison with the other biological fluids, the assessment of \(\alpha\)-synuclein in the CSF has provided the most consistent results in terms of analytical validation by different laboratories\(^16\). CSF \(\alpha\)-synuclein is mostly detected by means of immunoassays that use specific antibodies to target the different \(\alpha\)-synuclein forms. Even though the absolute concentrations can vary from study to study, the results obtained so far are supported by several meta-analysis studies suggesting that CSF \(\alpha\)-synuclein could serve as a potential marker of synucleinopathy\(^17\). In this review, we aim to discuss the results from the assessment of \(\alpha\)-synuclein in CSF and exosomes and explain the factors responsible for the variability among the different studies.

MEASUREMENT OF CSF \(\alpha\)-SYNUCLEIN

Being primarily produced by the choroid plexus within the ventricles of the central nervous system, CSF is an established biological fluid to study neurodegenerative disorders since it is expected to mirror brain microenvironment. The quantification of total \(\alpha\)-synuclein, as well as its oligomeric and phosphorylated forms, can be measured in CSF using different techniques, such as ELISA\(^18\), xMAP technology\(^19\),
mass spectrometry\textsuperscript{[20]}, time-resolved fluorescence energy transfer\textsuperscript{[21]}, electrochemiluminescence immunoassay\textsuperscript{[22]} and western blot\textsuperscript{[23]}. In addition, new biochemical assays that can detect α-synuclein aggregates have emerged, such as Protein-misfolding cyclic amplification and Real-time Quaking-induced conversion, by taking advantage the ability of α-synuclein to nucleate further aggregation\textsuperscript{[24-26]} . Using all these different methods, it is important to note that, even though there are variations in the absolute concentrations measured, the results produced for total (or monomeric) CSF α-synuclein agree on a reduction in α-synuclein levels in PD patients when compared with control subjects. When oligomeric or phosphorylated α-synuclein was assessed in the CSF, both forms were found to be increased in PD patients compared with the controls. However, it is important to note that the of ligands such as ThT may affect the actual structure of the α-synuclein species.

Even though the above findings are consistent, the diagnostic accuracy (sensitivity and specificity) remains unsatisfactory either for the detection of the monomeric or the modified forms of α-synuclein. Additionally, some studies report contradictory results; some have found similar CSF α-synuclein levels between PD patients and control subjects\textsuperscript{[27-29]} , whereas others have reported increased CSF α-synuclein levels in samples from AD\textsuperscript{[30,31]}, progressive supranuclear palsy or Creutzfeldt-Jacob patients compared with the control group\textsuperscript{[32]}.

A number of factors could explain the observed variability in the results, as well as the differences reported in the absolute concentrations. First, the immunoassays used are based on divergent antibodies that recognize different fragments of the protein and with variable affinity. Second, the patient cohorts show great variability in terms of number, disease stage and clinical symptoms (affected mobility or dementia) present at the time of CSF collection. Third, the implementation of strict standardized guidelines concerning collection and storage protocols and allowed blood contamination have only recently started to be followed. Furthermore, common reference materials are still missing making the interpretation of results from assay to assay extremely difficult.

The quantification of CSF α-synuclein could aid the differential diagnosis in clinically overlapping neurodegenerative diseases, as suggested for PD, DLB and AD\textsuperscript{[33-35]}. However, it is unclear whether the levels of CSF α-synuclein could be correlated with the severity of disease, indicating for example a more rapid decline in motor performance or the appearance of dementia. Interestingly, recent reports have shown that simultaneous measurement of α-synuclein levels along with other proteins, such as tau, Aβ\textsubscript{42} and Glucocerebrosidase 1 could be more effective in discriminating PD patients with synucleinopathies from healthy individuals or those with other neurodegenerative diseases\textsuperscript{[30,36-38]}.

Other biological fluids could also serve as promising candidates for α-synuclein detection and subsequent PD diagnosis. The majority of reports studying plasma α-synuclein have exhibited increased levels in PD patients\textsuperscript{[39-46]} relative to control subjects, whereas other studies have reported similar\textsuperscript{[27,47,48]} or decreased plasma α-synuclein levels\textsuperscript{[49]} between PD patients and healthy participants. Interestingly, it was found that plasma levels of phosphorylated α-synuclein were higher in the PD samples than the controls\textsuperscript{[44,49,51]}. The results obtained from plasma have been controversial, mainly due to the fact that red blood cells are a major source of α-synuclein and the rest erythrocytes that remain in plasma\textsuperscript{[52]} can be subjected to hemolysis markedly affecting α-synuclein values\textsuperscript{[47]}.

As erythrocytes are the major source of peripheral α-synuclein, a recent report has proposed erythrocytic α-synuclein as a potential PD biomarker, as it was found that the total and aggregated α-synuclein levels were significantly higher in the membrane fraction of PD patients compared to healthy controls\textsuperscript{[53]}. Saliva α-synuclein has also been considered as a prospective biomarker, as α-synuclein pathology has been found in submandibular salivary glands\textsuperscript{[54,55]} and saliva α-synuclein could be easily accessible and poorly affected
by blood contamination\textsuperscript{[56-59]}. Some studies have reported that total $\alpha$-synuclein levels were reduced in the saliva of PD patients compared with control subjects, whereas oligomeric $\alpha$-synuclein appeared to be elevated in the saliva of PD patients\textsuperscript{[56,57]}.

MEASUREMENT OF $\alpha$-SYNUCLEIN FROM NEURONAL EXOSOMES AS A POTENTIAL BIOMARKER

$\alpha$-synuclein was considered to be localized mostly in the cytoplasm of neuronal cells, until several studies demonstrated its presence in human CSF, human plasma and in the conditioned medium of various cell lines\textsuperscript{[23,60]}. Many studies have shown that $\alpha$-synuclein is physiologically secreted in the extracellular space, but the mechanism of $\alpha$-synuclein release is still unclear. Evidence from recent studies has also suggested that extracellular $\alpha$-synuclein can confer to the progression of PD\textsuperscript{[1-3]} and it has been proposed that $\alpha$-synuclein secretion, either in a monomeric or oligomeric state, induces $\alpha$-synuclein propagation via cell-to-cell transfer\textsuperscript{[61]}. Thus, elucidating the mechanism by which $\alpha$-synuclein is secreted in the extracellular space is of great importance in understanding cellular pathways that may cause PD.

Release of $\alpha$-synuclein via extracellular vesicles termed exosomes has been demonstrated by our group and others\textsuperscript{[62,63]}. Exosomes are extracellular vesicles of ~50 to 200 nm diameter and can mediate proximal and distal cellular communication through the transfer of biological molecules between cells. They originate from the inward budding of multi-vesicular bodies (MVBs) and are released to the extracellular space upon fusion of MVBs with the plasma membrane in an exocytic manner. Exosomes are released from numerous cell types including neurons and glia\textsuperscript{[64]} and in several studies have been observed to be associated with pathologic proteins including $\alpha$-synuclein\textsuperscript{[63,65]}. Based on the current knowledge, exosomes are functionally active entities, with a highly versatile role, ranging from intercellular communication by delivering specific protein, lipid or RNA cargo, and removal of obsolete or misfolded proteins, as a means of cell detoxification, to deleterious shuttles that impair cell homeostasis\textsuperscript{[66]}.

Some well-characterized functions of exosomes are protein secretion and intracellular uptake, immune response regulation and toxicity induction\textsuperscript{[67]}. Interestingly, Danzer et al.\textsuperscript{[65]} demonstrated that exosome associated $\alpha$-synuclein is more potent in transmitting aggregation pathology between neurons than free-secreted $\alpha$-synuclein. One study has shown that patients with PD have higher $\alpha$-synuclein levels in plasma exosomes compared to healthy controls\textsuperscript{[68]}, while Stuendl et al.\textsuperscript{[69]} found decreased neural exosome $\alpha$-synuclein levels in PD patients, consistent with the total $\alpha$-synuclein levels in CSF. In addition, the quantification of CSF exosomal $\alpha$-synuclein exhibited distinct differences between patients with PD and DLB. Moreover, exosomal $\alpha$-synuclein levels correlated with the severity of cognitive impairment in cross-sectional samples from patients with DLB. In the same study, Stuendl et al.\textsuperscript{[69]} showed that exosomes from PD and DLB patients contain pathogenic $\alpha$-synuclein species which serve as seeds to induce the oligomerization of soluble $\alpha$-synuclein in recipient cells. Shi et al.\textsuperscript{[70]} have shown that CNS-derived exosomes can efflux into blood. Importantly, they found a substantially augmented $\alpha$-synuclein concentration in the plasma-isolated exosomes from PD patients compared to healthy control subjects, despite the fact that no differences were detected in plasma total $\alpha$-synuclein levels. Additionally, they report a significant increase of plasma exosomal $\alpha$-synuclein/total $\alpha$-synuclein ratio in PD patients, negatively correlated with the disease severity, further supporting the importance of the disease-related exosomal cargos as PD biomarkers with high sensitivity and specificity. The authors concluded that plasma, CNS-derived exosomal $\alpha$-synuclein can serve as a PD biomarker with high sensitivity and specificity\textsuperscript{[70]}. The same group has also shown that CNS-derived exosomal tau in plasma is significantly higher in PD patients than in controls and is correlated with CSF total tau and phosphorylated tau\textsuperscript{[71]}. Furthermore, distinct circulating exosome entities have been identified in the serum of patients with PD\textsuperscript{[72]}. A recent study demonstrated that the levels of DJ-1 and $\alpha$-synuclein in plasma CSF-derived exosomes and the
ratio of plasma CSF-derived exosomal DJ-1 to total DJ-1 were significantly higher in patients with PD, compared with controls\[4\]. Several factors have been shown to affect the release of α-synuclein through exosomes such as the activity of Glucocerebrosidase enzyme (GCase), ion homeostasis, such as Zn\(^{2+}\), Ca\(^{2+}\) and Mn\(^{2+}\), as well as neuronal activity and neurotransmitter release. The heterozygous mutations in the GBA1 gene are considered as an important risk factor for PD. In this regard, it has been demonstrated that GCase overexpression leads to a decrease of exosome secretion while chronic pharmacological inhibition of GCase activity \textit{in vivo} profoundly increased exosomes levels, as well as exosome-associated α-synuclein oligomers \[73\]. In addition, decreased GCase activity has been demonstrated in brain samples with increased α-synuclein levels and in CSF from sporadic PD patients \[74\]. More recently, a study by Cerri \textit{et al.} \[75\], showed that exosomes from PD patients contain a greater amount of α-synuclein compared to healthy subjects whereas no differences were found in plasma total α-synuclein levels. Importantly, the authors showed a significant inverse correlation between GCase activity and this ratio in PD patients.

Notably, exosomes being a snapshot of the intracellular milieu, comprise a great source of bioactive molecules, including various RNA species. In a study conducted by Gui and co-workers, where exosomal miRNAs were isolated from the CSF of PD patients, 16 and 11 exosomal miRNAs were found upregulated and down-regulated, respectively, in PD patients compared to controls \[76\]. Validated hits were found to be miR-1 and miR-19b-3p, significantly reduced in PD CSF exosomes, in contrast to miR-153, miR-409-3p, miR-10a-5p, and let-7g-3p which were found to be increased \[76\]. This evidence highlights the potential diagnostic value of CSF exosomal RNA in the assessment of PD.

Although the role of exosome-associated α-synuclein as a potential biomarker remains relatively controversial, there are specific parameters that should be taken into account. Firstly, with regards to the source of exosomes, including plasma, saliva, CSF, there are certain protocols for their acquisition, followed by even more meticulous procedures for exosome isolation and purification. Differences during the aforementioned protocols may account for deviations between groups. In the same context, total exosome isolation may mask differences that could be found in exosomes of specific cellular origin. For example, Tomlinson and colleagues \[72\], following an unbiased proteomic approach, did not find any significant increase of α-synuclein in total exosomes isolated by ultracentrifugation from the serum of PD patients. On the contrary, Shi \textit{et al.} \[76\] showed a significant α-synuclein enrichment in neuron-specific exosomes, isolated from PD patients' plasma by immunoprecipitation of the neuronal adhesion molecule L1CAM. More importantly, the notion that α-synuclein exists and exerts its detrimental effects in different strains, leading to different aggregates that cause as many distinct synucleinopathies, i.e., PD, DLB, MSA has been cemented \[77,78\]. Given the fact that exosomal cargo mirrors the state of the cell from which it originates, exosome-associated α-synuclein may reflect the dynamic nature of α-synuclein species. To this end, it is of a pivotal importance to develop techniques that could allow detection and quantification of all the different α-synuclein conformers. Overall, although in its infancy, the study of exosome-associated α-synuclein as a potential biomarker is quite promising, yet, it requires more combinatorial approaches.

\textbf{CONCLUSION}

Over the last 10 years there has been considerable amount of research effort placed in the evaluation of neuronal α-synuclein as a diagnostic or, even, a prognostic biomarker for PD and related synucleinopathies. The majority of studies have indicated that CSF α-synuclein could be useful for the diagnosis of synucleinopathy that could also aid the distinguishing of PD patients from patients with other neurodegenerative conditions. However, its utility as a biomarker is hampered by the lack of a universally validated assay of high diagnostic accuracy. To this end, following the strict recently established standard operating protocols for CSF collection and storage and correlating the measurement of monomeric α-synuclein with the oligomeric or phosphorylated forms would greatly improve the diagnostic value of the assessment of α-synuclein in CSF. To further ameliorate the specificity of α-synuclein measurement, recent...
 experimental approaches involve the assessment of α-synuclein in neural exosomes. The discoveries of pathogenic misfolded proteins such as α-synuclein in them has generated intensive research into their use as biomarkers considering that they carry proteins with disease-specific fingerprints reflecting the presence and staging of the disease. However, in order to further verify this potential we need to have a very good understanding of the actual mechanisms behind their biogenesis and release. Importantly, we need to have those tools in place that will assist us in the identification of the α-synuclein species responsible for disease generation and pathology progression. The fact that exosomal cargo mirrors the state of the cell from which it originates\(^7\) unravels the promising role of the plasma/CSF -derived exosomes as potential biomarkers. Proteomic profiling of exosomal proteins in PD patients with different disease stages and healthy subjects may also aid the identification of specific protein changes that occur in response to pathology progression. Finally, modulating exosome biogenesis and release may have a promising prospect in PD therapy.

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Contributed to the writing of the manuscript and in its revision: Chalatsa I, Melachroinou K, Emmanouilidou E, Vekrellis K

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