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Alpha-synuclein as a biomarker in Parkinson's disease: focus on neural derived extracelluar vesicles

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The prevalence of Parkinson's disease (PD) is rapidly increasing, and more than 12 million people are expected to suffer from PD by 2040. PD is a highly invalidating neurodegenerative condition that arises from the progressive degeneration of dopaminergic neurons in the *substantia nigra pars compacta*. The main cause for such degeneration is the formation of cytoplasmic inclusions known as Lewy bodies, which include both misfolded α-synuclein (α-syn) protein and a multitude of fragmented membranes, organelles, and vesicles (Shahmoradian et al., 2019; Lashuel, 2020). No reliable biomarkers that can predict the onset of PD in its prodromal phase or could assess disease progression are currently available. α-Syn plays a pivotal role in the pathogenesis of PD and is present in peripheral tissues and biofluids; this led to the investigation of α-syn as a possible PD biomarker.

α-Synuclein in physiology and pathology: α-Syn was identified for the first time in amyloid plaques of patients suffering from Alzheimer's disease in the early '90s; the coding gene was subsequently identified and called SNCA. The whole α-syn transcript of 140 amino acids represents the major variant, but different alternatively spliced shorter isoforms of 98, 112, and 126 amino acids, characterized by different aggregating potentials, are known (Beyer and Ariza, 2013). The involvement of α-syn in the pathogenesis of PD is also strongly supported by the observation that both different missense mutations in *SNCA* gene (A30P, E46K, H50Q, G51D, A53E, A53T) and gene duplications/triplications were found to associate with PD (Burrè et al., 2018).

α-Syn is a soluble, highly acidic, and heat-resistant protein which includes three main domains: (1) the N-terminal domain, an 11-residues sequence repeated seven times that can form an amphipathic α-helix in the presence of lipids and membranes; (2) the central non-amyloid-beta component hydrophobic region, which is involved in fibril formation and aggregation; and (3) the C-terminal acidic and glutamate-rich sequence, which plays a role in the interaction with multiple proteins and in the modulation of binding with membranes (Burrè et al., 2018).

Under physiological circumstances, α-syn exists in a fine equilibrium between soluble and membrane-bound conditions, but in pathological situations, this protein changes into a β-sheet amyloid conformation that results in aggregation, oligomers/fibril formation, and deposition within Lewy bodies (Uversky, 2007). α-Syn is universally expressed throughout the brain, and, at the neuronal level, is predominantly localized at presynaptic terminals where it modulates the stability of the neuronal membrane, influences membrane trafficking via vesicular transport, and acts as a chaperone to promote the assembly of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor complex (SNARE) through direct binding to VAMP-2 (Braak et al., 2003).

We have recently described that oligomeric α-syn species are increased, whereas the SNARE components STX-1A and VAMP-2 are diminished in neural-derived extracellular vesicles (NDEVs) of PD patients (Agliardi et al., 2021), corroborating the hypothesis of a direct role for α-syn in the exocytosis of neurotransmitters. Under still unknown conditions, α-syn physiological conformations (monomeric species, dimers, and probably tetramers) undergo pathological aggregation (oligomers, fibrils) that subsequently degenerate into Lewy neuritis and Lewy bodies cytoplasmic inclusions, the hallmark of PD (Uversky, 2007) (**Figure 1A**). Oligomers, in particular, are aggregated α-syn structures that have not acquired a fibrillar conformation and include a wide spectrum of molecular weights. Notably, the oligomeric forms of α-syn are believed to be the most toxic species as they inhibit proteasomal activity; alter the endoplasmatic reticulum and impair endoplasmatic reticulum protein quality control; stimulate an inflammatory response in glial cells; induce membrane damage and synaptic alterations; and impair the autophagic-lysosomal pathway (Burrè et al., 2018). This latter dysfunction may also contribute to the further spreading, via exocytosis, of aggregated α-syn species, leading to a prion-like propagation (Bellomo et al., 2019).

In summary, α-syn oligomers clearly negatively impact many cellular processes and very likely play a central role in PD-associated neurodegeneration.

α-Synuclein in blood, cerebrospinal fluid, and saliva as a biomarker for Parkinson's disease: By definition, an ideal biomarker should be strictly related to the pathogenesis of a disease, non-invasive, easy to measure, detectable in a peripheral tissue or, even better, in a biofluid, and endowed with both high sensitivity and high specificity. Since α-syn is biologically, genetically and pathologically associated with PD and can be measured in biofluids, it is potentially a perfect biomarker for the disease, and numerous studies have investigated this possibility. The observation that α-syn can be present in different forms and in different matrices, though, complicates the issue. The most investigated species of this molecule are: (1) total α-syn, (2) oligomeric α-syn, and (3) phosphorylated α-syn. Phosphorylation at the S129 residue is characteristic of PD and is the most studied among post-translational modifications. There is no consensus on its role in PD pathogenesis as phosphorylation could have an active role in α-syn aggregation or on the contrary, could be a mechanism used by cells to label and eliminate toxic species (Oueslati, 2016).

The principal matrices in which α-syn has been analyzed are blood, cerebrospinal fluid (CSF), and saliva, but the presence and the quality of this protein have been investigated in urine and other tissues as well over the years. To further complicate things, α-syn is present physiologically in different populations of blood cells, including erythrocytes, platelets, leucocytes, as well as in body fluids like plasma, CSF, and saliva. Thus, in order to determine if α-syn could be considered a biomarker for PD, a cut-off value must be established that can discriminate between pathology and physiology. A number of studies have investigated this topic but, unfortunately, a consensus on the threshold defining normality is missing. To summarize: whereas the majority of these studies report that increased α-syn concentrations are seen in plasma and serum of PD patients compared to heal\thy controls, other results failed to demonstrate such differences, and, as a consequence, the potential usefulness of plasma or serum α-syn as a biomarker of PD has been questioned.

The measurement of oligomeric α-syn in blood, on the other hand, led to concordant results and showed that increased quantities of this protein are seen in PD patients compared to HC (Parnetti, 2019). However, the quantity of α-syn in the blood is strongly influenced by red blood cells contamination and/or hemolysis. Indeed, red blood cells are the major source (> 99%) of α-syn in blood and even a low degree of contamination could result in a substantially increased concentration in plasma and serum.

Results of a meta-analysis of data collected in the CSF showed that the concentration of total α-syn is reduced whereas that both oligomeric α-syn and phosphorylated α-syn increased in PD compared to patients suffering from other neurological conditions and non-PD controls. (Eusebi et al., 2017). The authors however concluded that the overall sensitivity and specificity results obtained in pooled data are far from being optimal and do not lend support to the use of total α-syn CSF concentration as a biomarker for PD. It has also to be underlined that, even if other results indicated that the oligomeric/total α-syn CSF ratio could be a more specific and sensitive biomarker for PD, CSF is clearly not an ideal specimen for routine monitoring due to the invasiveness of lumbar puncture.

Another repeatedly investigated source of α-syn as a biomarker of PD is saliva, an easily accessible fluid which is normally free of blood contamination. The use of saliva α-syn as a diagnostic factor is based on its presence in the nerve fibers that innervate salivary glands. Different studies reported that total α-syn is reduced whereas oligomeric α-syn and the oligomeric α-syn/total α-syn ratio are increased in the saliva of PD patients (Pawlik and Blochowiak, 2021). A challenge with using saliva as a source of biomarkers for PD nevertheless is that, even if easily accessible, these patients have clinical symptoms that can prevent its proper collection. Moreover, the stimulation of salivary secretion significantly changes its composition.

These discouraging outcomes led to the exploration of new techniques to measure different forms of α-syn in biological fluids in the attempt of identifying a solid and reproducible biomarker for PD.

Extracellular vesicles as a source of central nervous system α-Synuclein: A very interesting matrix to measure different α-syn species and verify their possible usefulness as biomarkers

for PD are extracellular vesicles (EVs). EVs are membrane particles of cellular origin that are endowed with the ability to regulate both physiological and pathological processes. EVs were recently suggested to play an important role in PD pathogenesis as well. Thus, EVs may have a key role in both the generation and the spreading of toxic α-syn species, promoting cell-to-cell transfer. In fact, it was hypothesized that the balance between the cellular protein degradative mechanisms and the secreted or EV-released α-syn may contribute to cellular integrity and homeostasis.

EVs are produced by almost all cell types and can be recovered in diverse body fluids, including plasma, serum, urine, and CSF (Manek et al., 2018). The three main categories of EVs are exosomes, microvesicles, and apoptotic bodies; they differ in size and are characterized by different mechanisms of release into the intercellular space. EVs-based detection of biomarkers is of particular relevance for diseases of the central nervous system (CNS), because the specific exosomes and microvesicles produced by neurons (NDEVs: neural derived extravesicles), have the ability to cross the blood-brain barrier and can be recovered in peripheral body fluids, and in particular in blood. Their cargoes of proteins, lipids, and nucleic acids species can be analyzed and measured, and are considered to be surrogate markers of CNS cells. The investigation of EVs cargoes in different CNS pathologies is a hot area of research, and, as expected, a number of researchers focused their attention on molecules that have a known involvement in the pathogenesis of CNS diseases, including α-syn.

Shi et al., in 2014, injected radiolabeled α-syn into mouse brain, demonstrated that CSF α-syn was transported to blood, and showed that a small proportion of CSF α-syn was contained in exosomes originating from neurons. They subsequently isolated exosomes from plasma samples of PD patients and HC using superparamagnetic microbeads coated with the neural cell adhesion molecule L1CAM and measured α-syn content. Results showed that plasma neural derived exosomes from PD patients contain higher levels of α-syn compared to HC and indicated that α-syn levels correlate with disease severity. Similar results were obtained by analyzing NDEVs isolated from plasma by precipitation with the polymer ExoQuick® (System Bioscience, CA, USA), subsequent incubation with a biotinylated L1CAM antibody, and selection using a streptavidin resin (Fiandaca et al., 2014) (**Figure 1B**). These two different methods have been used by many research groups to isolate NDEVs. Analyses performed in these NDEVs showed that: (1) α-syn is augmented in PD patients compared to HC (Johnson, 2020); (2) α-syn is reduced in PD compared to essential tremor patients (Si et al., 2019); and (3) oligomeric α-syn is augmented in PD patients compared to HC (Agliardi et al., 2021).

Conclusions and future challenges: The analysis of NDEVs is a rapidly evolving and extremely promising field of research; a number of hurdles need nevertheless to be overcome to allow these analyses to become a standard way to measure biomarkers in SNC diseases. Nowadays the major bottle-neck hindering the use of NDEVs as biomarkers is the lack of an optimal and reproducible methodology for sample collection, EVs isolation, and the evaluation of the molecules they carry. An essential challenge is the identification of novel and alternate neuron-specific markers that identify NDEVs: L1CAM is indeed highly expressed in neurons, but is also expressed, although at a much lower level, on the surface of other cells including lymphocytes. A standard and reproducible method to quantify α-syn in NDEVs is also needed: it is well known that all antibodies-based methods have limitations due to the type and the quality of the antibody used. In conclusion, efforts are still needed but with further investigation and standardization of the methods, as well as the use of larger sample groups, the measurement of α-syn in peripheral NDEVs has the potential to become the golden standard in the diagnosis of PD.

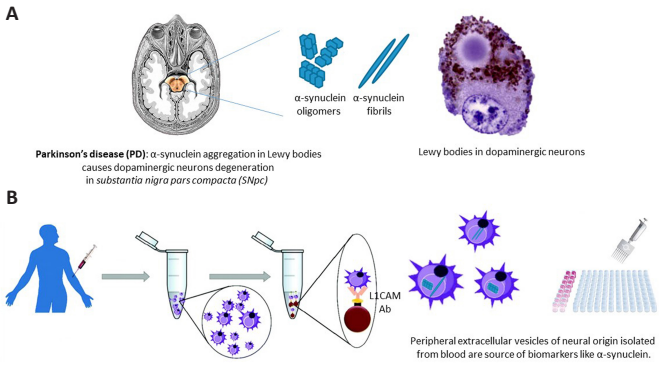

Figure 1 (A) The pathological hallmarks of Parkinson's disease (PD) are Lewy bodies, cytoplasmic inclusions leading to a degeneration of neurons. The main components of Lewy bodies are misfolded and aggregated α-synuclein protein, fragmented membranes, organelles, and vesicles. (B) Extracellular vesicles of neural origin can be isolated from peripheral blood and are a source of biomarkers for pathologies of the central nervous system like PD. Unpublished data.

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