

VIEWPOINT

α -Synuclein Seed Amplification Assays from Blood-Based Extracellular Vesicles in Parkinson's Disease: An Evaluation of the Evidence

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We wish to express our thoughts on recent articles about α -synuclein seed amplification assays (SAA) from blood-derived neuronal extracellular vesicles (EV) as a potential biomarker for Parkinson's disease (PD). The initial pilot study in 2022 by Kluge et al¹ was followed by three publications in 2024,^{2–4} claiming that this test not only distinguishes PD from healthy controls, but can also detect α -synuclein pathology in *PRKN*-linked PD², identify prodromal and presymptomatic PD,³ and monitor disease progression.⁴ Undoubtedly, these studies address an unmet medical need, as underscored by the recently proposed SynNeurGe criteria for a biological definition of PD⁵.

Because this approach relies on a combination of two state-of-the-art, but also very sensitive and fault-prone techniques (EV purification and SAA), it is of highest importance to use standardized protocols making this

assay robust and comparable across laboratories. Despite our expertise in purifying EVs,^{6–8} performing α -synuclein SAA,^{9,10} and in α -synuclein protein biochemistry,¹¹ we struggle to reliably reproduce these results. Our efforts identified methodological aspects that warrant further scrutiny within the scientific community.

When comparing the conditions of neuronal EV purification, crucial steps within the protocols vary between the studies.^{1–4} In the original study,¹ plasma was used, whereas the following studies use plasma and serum samples interchangeably (plasma and serum,² serum,³ and plasma⁴). Second, the NCAM-L1 capture antibody to isolate neuronal EVs from a total EV fraction was changed between the studies. The two used antibodies recognize different epitopes, either N³- or C-terminal^{1,2,4} of the single-pass type I transmembrane protein, binding to the extra- or intracellular domain of the protein respectively, therefore, raising questions about their ability to purify intact EVs.

In all four studies,^{1–4} total EVs were first incubated with the NCAM-L1 capture antibody, an immunoglobulin G (IgG), followed by protein A/G-coated agarose beads for immunoprecipitation. Protein A/G is a fusion protein that binds indiscriminately to a wide variety of mammalian IgGs.¹² In samples rich in soluble IgGs, like EV extracts derived from blood, these beads mostly bind to endogenous IgGs rather than the NCAM-L1 capture antibody. This means that the beads do not specifically bind neuronal exosomes, but rather any existing IgGs, leading to uncertainty as to which components are actually immunoprecipitated. To avoid this nonspecific extraction, NCAM-L1 must first be bound to the A/G-coated agarose beads before being added to the solution for specific extraction of neuronal EVs.

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Hence, we suggest that future studies include additional EV quality controls and comparisons between the different antibodies used for EV isolation. Because the EV field is fast-developing and rapidly growing, much needed quality control standards, which are regularly updated and released by the International Society for Extracellular Vesicles (Minimal Information for Studies of Extracellular Vesicles),¹³ should have been additionally considered in the three most recent studies from 2024.²⁻⁴ Although the original study¹ provided CD63, CD9, CD81, and neuronal markers for the purified EVs, as well as size measurements by dynamic light scattering, following studies did not provide any quality control measures of the purified EVs, although crucial parts of the neuronal EV purification protocol had been changed.²⁻⁴ Additionally, analysis of EV size was only performed in the original publication,¹ but is lacking in the three following publications,²⁻⁴ raising questions regarding the purity, integrity, and identity of the enriched particles as bona fide EVs.

Concerning the SAA conditions applied in the studies,¹⁻⁴ essential components and procedures of the assay did not remain constant. Specifically, the source of the monomeric α -synuclein as substrate for the SAA plays a critical role and needs to be chosen carefully. In the initial pilot study, in-house *E. coli*-derived α -synuclein was used.¹ Although, strict quality control measures were applied,¹⁴ the purity of this recombinant protein is not comparable to any commercial source. It is noteworthy that an interchanging use of α -synuclein from different sources (self-made^{1,3} and commercial)^{2,4} still resulted in similar end results (SAA curves) across the studies. We believe that self-produced α -synuclein may be used for experimental studies, however, for any clinical read-outs and interpretations, we recommend using commercially available and well-characterized monomeric α -synuclein, which undergoes rigorous quality control checks to enhance the comparability between studies.

There are also unexplained variations in the concentrations of recombinant α -synuclein used across experiments, ranging from 0.005 mg/mL^{2,3,4} to 0.001 mg/mL¹, which are 100-fold lower than the 0.1 to 0.5 mg/mL used in validated cerebrospinal fluid (CSF) SAAs.¹⁵⁻²⁰ The use of such monomer concentrations may further interfere with the detection of α -synuclein aggregates, especially in blood, where such α -synuclein species are potentially less abundant than in CSF. Further, the multiwell plates in which the SAA was performed were changed. Whereas non-coated plates were used initially,¹ immunoassay-compatible (MaxiSorp) 96-well plates exhibiting hydrophilic binding properties were used in two subsequent studies^{3,4} (incorrectly described as uncoated in the material and method section). We believe that choosing appropriate and comparable plasticware for performing an assay working with “sticky”

amyloid protein is crucial for a standardized outcome of this assay.

Altogether, all above-mentioned modifications in the protocols of EV purification as well as SAA conditions across the different studies¹⁻⁴ did not result in any changes in the outcome values of the SAA, as the thioflavin T (ThT) fluorescence curves remained similar across all studies, (eg, the time to threshold was ~20 hours). This is quite notable and warrants further investigation to understand the underlying reasons.

In addition, the manual addition of ThT before each measurement is unconventional, given that ThT exhibits stable fluorescence on binding to amyloid fibrils without significant quenching and could introduce variability and potential contamination. Adding 1/100 volume of 1 mM ThT before each measurement over 10 time points increases the ThT concentration from 0.01 to 0.1 mM in the reaction, potentially causing ThT self-fluorescence.²¹ Self-fluorescence occurs when ThT molecules emit fluorescence independently, without the presence of α -synuclein aggregates. Furthermore, repeated ThT addition also introduces the challenge of managing sample concentration because of increased evaporation, because the plate has to be opened before each measurement. Further concerns regarding the reproducibility of the assay arise from the use of detergents and other buffer components, of which the final amount in the reaction is not deducible from the given description of the assay conditions.¹⁻⁴ Recent studies have shown that these components can significantly affect SAA.²² Therefore, for standardization and comparability, we recommend specifying not only protein amounts, but also the volumes and detailed component compositions of the samples used in the SAA.

The absence of technical and biological replicates in independent cohorts to assess experimental stability and reproducibility is another limitation. Standard SAA protocols require analyses in triplicates or quadruplicates (technical replicates) to mitigate risks such as auto-aggregation, which can lead to false-positive results. Additionally, test-retest-reliability analyses and interlaboratory ring-tests are essential to ascertain stability of results over time and across laboratories.

The omission of a recognized diagnostic gold standard, such as immunohistochemistry on autopsy or CSF SAA, crucial for validating diagnostic claims, introduces an additional level of uncertainty. Given that CSF SAA is an established marker for pathological α -synuclein aggregates, it is essential that studies involving blood SAA also include comparisons with matched CSF samples.

Importantly, different maximum ThT signals in SAAs are believed to indicate distinct seed strains rather than quantitative differences in the amount of seeds. This principle challenges the interpretation of the increase in

maximum ThT signals over time in the preclinical disease course, as reported by Kluge et al,³ since no currently available data suggest any change in the Lewy-fold α-synuclein strains during the course of PD.

Although the concept of a blood-based EV SAA test is innovative and of significant interest, our experience suggests that its application in research and clinical practice requires a high degree of standardization, validation against diagnostic gold standards, replication in independent cohorts and laboratories, and cautious interpretation. Therefore, we encourage further discussion and investigation of this methodology, which may constitute a game-changing turning point for the field, if confirmed.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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