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Short communication

Compartment-specific correlation of pathological α-synuclein in prodromal Parkinson's disease

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ABSTRACT

Introduction: Neuronal α-synuclein dyshomeostasis and aggregation are essential features of early Parkinson's disease, as seen in isolated REM sleep behavior disorder (iRBD). The link between these pathologies across body compartments remains unclear. Our aim was to assess correlations between α-synuclein aggregates in stool and urine with α-synuclein content in neuronally derived L1CAM extracellular vesicles (L1EVs) from serum in iRBD.
Methods: We conducted a cross-sectional study, analyzing concurrent biobank samples from 46 individuals with iRBD to quantify α-synuclein aggregates in stool and urine with surface-based fluorescence intensity distribution analysis (sFIDA) and α-synuclein content in L1EVs using electrochemiluminescence.
Results: Corrected for age and sex, α-synuclein concentrations in L1EVs significantly correlated with stool aggregate concentration. No significant correlation was observed between α-synuclein concentration in L1EVs and urine, nor between urine and stool aggregates.
Conclusion: The correlation of serum and stool α-synuclein suggests shared or linked pathology across these compartments, whereas the urinary compartment may be pathophysiologically distinct in prodromal Parkinson's Disease.

1. Introduction

Isolated REM sleep behavior disorder (iRBD) is considered an early-stage α-synucleinopathy, with pathological α-synuclein detectable in the vast majority of cases. Affected individuals are at a specific high risk of phenoconversion to Parkinson's disease (PD) or dementia with Lewy bodies (DLB) within the next decade. Growing evidence suggests that individuals with iRBD have an ascending pathology spread, likely evolving from the vagal nerve to more rostral brainstem nuclei, as suggested within the Braak staging scheme. In this sense, a "body-first" subtype of PD was proposed for individuals exhibiting iRBD before PD, as caudal brainstem nuclei seem earlier affected than the substantia nigra [1].

Following this framework, α-synuclein pathology might origin in the gastrointestinal system in individuals with iRBD and spread along neuronal connections from the peripheral to the central nervous system, invade the substantia nigra, and eventually lead to dopaminergic deficiency and pathognomonic PD motor symptoms. In recent studies, α-synuclein aggregates have been detected in the gastrointestinal autonomic nervous system preceding the diagnosis of PD and in individuals

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with iRBD [2]. Likewise, rodent models challenged with preformed α-synuclein fibrils via the gastrointestinal tract allow examining the proposed α-synuclein spread from the gastrointestinal system to the peripheral and eventually to the central nervous system [e.g. 3].

In support of a gastrointestinal α-synuclein dyshomeostasis in iRBD, we recently showed that α-synuclein aggregates were elevated in stool samples from individuals with iRBD compared to those from healthy control individuals [4]. Additionally, α-synuclein content in neuronally derived L1CAM-positive extracellular vesicles (L1EVs) isolated from blood was elevated in individuals with iRBD compared to healthy control individuals and further increased when more prodromal PD markers were present [5]. We have also demonstrated that α-synuclein aggregates are elevated in the urine of individuals with iRBD and PD compared to healthy control individuals [6]. However, the relationship between α-synuclein changes across different body compartments has been sparsely studied.

Here, we analyzed the correlation of α-synuclein aggregate concentrations in stool and urine, measured using surface-based fluorescence intensity distribution analysis (sFIDA), with those of α-synuclein content in L1EVs, measured using electrochemiluminescence, in concurrently collected samples from individuals with iRBD.

2. Materials and methods

2.1. Subjects and clinical assessments

We identified 46 individuals with polysomnography-confirmed iRBD from our local cohort [7], who concurrently provided stool, urine and blood samples to our biobank. For the establishment of our cohort, recruitment has been conducted through newspaper advertisements, and relevant inclusion and exclusion criteria are as follows: i) inclusion: age >35 years and <80 years, and ii) exclusion: known neurological disorder (e.g., PD, epilepsy, narcolepsy), early age of RBD symptom onset (<35 years), alcohol or drug abuse [7]. All individuals underwent overnight video-polysomnography to prove the presence of RBD according to the criteria of the American Academy of Sleep Medicine (AASM). We assessed cognition using the Montréal Cognitive Assessment (MoCA), motor symptoms with the Movement Disorder Society - Unified Parkinson's Disease Rating Scale (MDS-UPDRS) Part III and olfactory function with Sniffin' Sticks. The local ethics committee approved the study, and all subjects provided written informed consent following the Declaration of Helsinki.

2.2. L1EV immunocapture and α-synuclein measurement

In brief, the serum was isolated, aliquoted, frozen at −80 °C, and sent on dry ice for L1EV analysis [5]. All samples were sent on dry ice and processed in a blinded fashion by SY. A three-step sequential spin (300 g for 10 min, 2000 g for 20 min and 10000 g for 30 min) was used to remove cellular debris, protein aggregates, and fatty material in the serum. We used a direct immunocapture assay using anti-L1CAM conjugated to poly(carboxybetaine methacrylate) (pCBMA) coated beads to isolate L1EVs from 250 μL of precleared serum as previously described and validated. Electrochemiluminescence was performed in 96-well Meso Scale Discovery (MSD) U-Plex plates using antibody pairs for α-synuclein: MJFR1 rabbit monoclonal antibody (Abcam) for capture and an MSD proprietary mouse monoclonal antibody for detection.

2.3. Stool collection and processing

In brief, samples were collected by the individual at home, stored in a plastic bag in a home freezer, transported to the clinic enveloped in an ice pack within a thermal bag, frozen at −80 °C, and transported on dry ice [4]. Typically, the sample was obtained at the same day of the visit or the day before. Stool samples were homogenized in cold extraction buffer using Simplix extraction tubes, followed by centrifugation at 3000×*g* for 5 min at 4 °C to pellet insoluble components, and the supernatant was stored at −80 °C until analysis. The supernatant after centrifugation was used for further analysis with sFIDA, which detects and quantifies protein aggregates by using antibodies of the same epitope for capture and detection. We used glass bottom microtiter plates, incubated with the Syn211 antibody for capture. After blocking and washing, 20 μL of the samples were incubated at RT for 1 h. After washing, the wells were incubated with the fluorescent detection antibodies Syn211-CF633 and Syn211-CF488A, after which the wells were rewashed for fluorescence imaging with an IRIS confocal fluorescence microscope. Images were analyzed using the in-house developed sFIDAta software tool. The sFIDA readout refers to the number of colocalized pixels of both channels. Additionally, we included a calibration in each measurement and converted the sFIDA readout to a calibration-based concentration [fM].

2.4. Urine processing

Samples were aliquoted, frozen at −80 °C and transported on dry ice for analysis [6]. Bovine serum albumin and protease inhibitors were added before 10-fold concentration by centrifugation. Samples were analyzed using the sFIDA assay, using 384-well glass-bottom plates that were coated with Syn211 antibody, blocked, and incubated with 20 μL of concentrated urine samples. After washing, fluorescently labeled Syn211 antibodies were used for detection. Imaging was performed using confocal fluorescence microscopy, and aggregate concentrations were quantified with calibration-based conversion to [fM].

2.5. Statistical analysis

Statistical analyses were performed using IBM SPSS version 28. The assumption of data normality was inspected with Shapiro-Wilk tests. Data is presented as mean ± standard deviation unless stated otherwise. Correlation analyses were calculated with Spearman rho tests. Multiple linear regression analyses included α-synuclein content in L1EVs as a dependent variable, α-synuclein aggregates in stool as a predictor and age and sex as covariates. Statistical significance was set at an alpha-level of $p \leq 0.05$.

3. Results

Table 1 provides demographic and clinical characteristics of 46 included individuals with iRBD (89 % male), with an average age of 66.45 years and an average self-reported duration of RBD symptoms of 7.91 ± 5.68 years. All subjects provided stool and urine samples obtained the same day or the day before blood collection.

Table 1
Clinical and demographic characteristics of 46 included individuals with iRBD.

Age, years	66.45 ± 6.74
Symptom duration, years	7.91 ± 5.68
Sex	41m/5f
MDS-UPDRS III	3.87 ± 2.54
Sniffin' Sticks, correct	6.54 ± 2.93
SCOPA-AUT	7.51 ± 3.20
MoCA	27.52 ± 1.74
α-syn aggregate concentration in stool [fM], median [IQR]	10.95 [21.93]
α-syn aggregate concentration in 10x concentrated urine [fM], median [IQR]	13.01 [9.62]
α-syn content in L1EVs [pg/mL], median [IQR]	19.36 [13.21]

Displayed is the mean ± standard deviation if not stated otherwise. Abbreviations: α-syn, α-synuclein; MDS-UPDRS, Movement Disorder Society - Unified Parkinson's Disease Rating Scale; MoCA, Montreal Cognitive Assessment; SCOPA-AUT, Scales for Outcomes in Parkinson's Disease - Autonomic Dysfunction; IQR, Interquartile Range.

α-synuclein aggregate concentrations in stool and α-synuclein content in L1EVs correlated significantly (rho(46) = 0.398, p = 0.008, Fig. 1A). In the multiple linear regression analysis including age and sex as co-variates, α-synuclein aggregates in stool remained a significant predictor of α-synuclein content in L1EVs ($F(3,42) = 4.720$, $p = 0.006$, $R^2 = 0.252$, $R^2adj = 0.199$, $\beta = 0.281$, $p = 0.050$).

There was no significant correlation between α-synuclein content in L1EVs and α-synuclein aggregate concentrations in urine (rho(46) = 0.012, p = 0.937, Fig. 1B). Similarly, correlation analysis revealed no significant association between α-synuclein aggregate concentrations in stool and urine (rho(46) = 0.066, p = 0.664, Fig. 1C).

4. Discussion

We quantitatively analyzed α-synuclein concentrations in multiple body compartments in individuals with iRBD, namely aggregate concentrations with sFIDA in stool and urine as well as α-synuclein content in L1EVs isolated from serum. Correlation analysis revealed a link between α-synuclein levels in L1EVs and stool aggregates. In contrast, no correlation was found between L1EV α-synuclein and urine aggregates, nor between stool and urine aggregates. Taken together, the data suggest that the gastrointestinal and neuronal compartments are interconnected in iRBD, but remain distinct from the urinary compartment.

Growing evidence suggests that gastroenteric dysbiosis can initiate α-synuclein aggregation within the gastrointestinal tract, prompting α-synuclein pathology in the autonomic nervous system of the gut, which may spread along neuronal structures to the central nervous system [8]. However, such processes are bidirectional and it remains elusive whether detected α-synuclein aggregates in stool stem from primarily intraluminar processes or if they are shed into the lumen from the host, potentially originating even from non-neuronal structures [9]. It is known that neuronally derived EVs can cross the blood-brain barrier and, likewise, the intestinal barrier [5]. Hence, circulating EVs in the blood might also be a source of α-synuclein aggregates detectable in stool [9]. Furthermore, specific gut microbial profiles may influence the aggregation and propagation of α-synuclein in the gut, potentially mediating the correlation observed between serum L1EVs and stool aggregates.

In contrast, EVs circulating in the blood are unlikely to pass into the renal tubular system, as they are retained by the glomerular filtration barrier [10]. However, renal epithelial cells express α-synuclein, which may contribute to its presence in tubular fluid and urine [11]. Moreover, recent data show that seeding activity in urine samples is low and only weakly concordant with CSF-based assays, potentially resembling technical challenges in urine sample preparation; however, it also supports the hypothesis of compartmentalized α-synuclein pathology in iRBD [12]. In that sense, α-synuclein may originate from cellular sources, depending on the biological compartment. Although we could find elevated urinary α-synuclein aggregate concentrations in individuals with iRBD compared to controls [6], we found no significant correlation between urinary aggregates and either stool aggregates or α-synuclein content in L1EVs. This suggests that urine-based α-synuclein analyses have diagnostic value in group comparisons but might not reflect pathophysiological connectivity with the stool or blood compartment.

A potential limitation of our comparative study is that matrix effects across biofluids may affect the detectability of α-synuclein aggregates. Moreover, possible differences in aggregate conformation between compartments may further influence assay performance. Another limitation is the cross-sectional design, which precludes analysis of longitudinal changes in α-synuclein levels and their temporal relationship to clinical phenoconversion. The marked predominance of male participants represents a limitation, as sex-related biological factors may

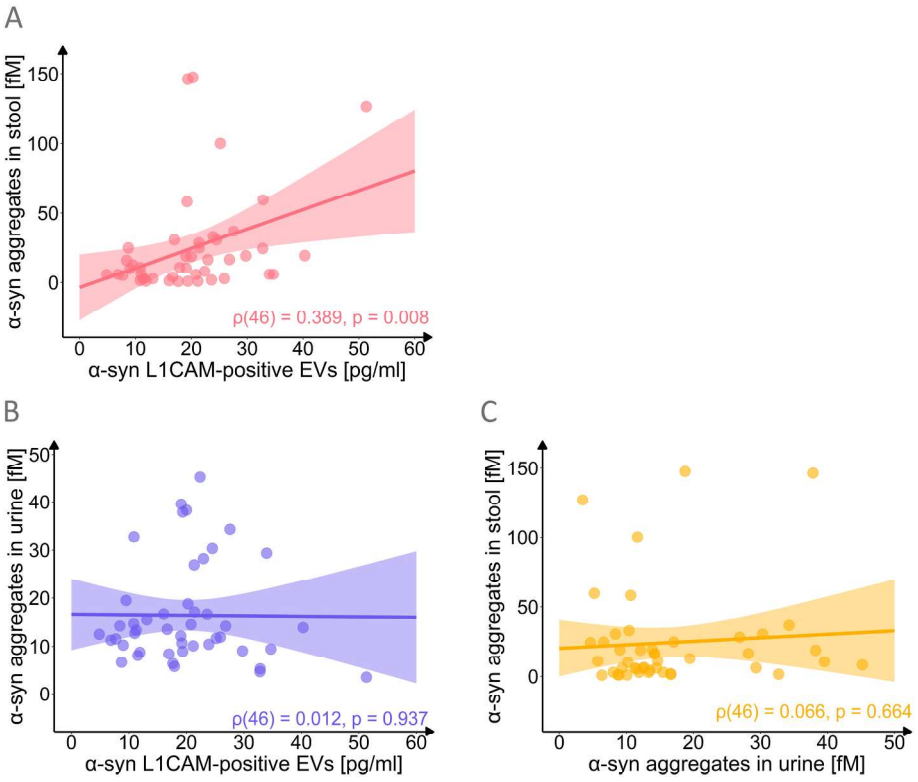

Fig. 1. Spearman rho correlation plots of α-synuclein aggregate concentrations in stool and 10x concentrated urine measured with surface-based fluorescence intensity distribution analysis (sFIDA), and α-synuclein content in L1CAM-positive extracellular vesicles (L1EVs) measured by electrochemiluminescence in N = 46 concurrently collected samples from individuals with iRBD. Panel A shows the significant correlation between α-synuclein levels in blood and stool (rho(46) = 0.398, p = 0.008). Panels B and C show no correlation between α-synuclein levels in blood and urine (rho(46) = 0.012, p = 0.937), and stool and urine (rho(46) = 0.066, p = 0.664), respectively.

influence α-synuclein metabolism; thus, replication in cohorts with more balanced gender distribution is warranted. Future studies should also include qualitative analyses of α-synuclein aggregates to further elucidate compartment-specific differences in pathology.

In conclusion, our data indicate that serum L1EV α-synuclein and stool α-synuclein aggregates are linked and might represent the same pathological process in the prodromal phase of PD, while both compartments did not correlate with urine α-synuclein aggregates. Future studies are needed to elucidate the mechanisms of this interaction.

CRediT authorship contribution statement

Sinah Röttgen: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Michael Sommerauer:** Writing – original draft, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Shijun Yan:** Writing – review & editing, Investigation. **Carolin Hungerland:** Writing – review & editing, Investigation. **Gereon R. Fink:** Writing – review & editing, Supervision, Resources. **Anja Ophey:** Writing – review & editing, Software. **Michael T. Barbe:** Writing – review & editing, Supervision, Resources. **Laura Müller:** Writing – review & editing, Investigation. **Gültekin Tamgüney:** Writing – review & editing, Supervision, Resources. **George K. Tofaris:** Writing – review & editing, Supervision, Resources.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data are available on request to the corresponding author because of privacy/ethical restrictions.

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