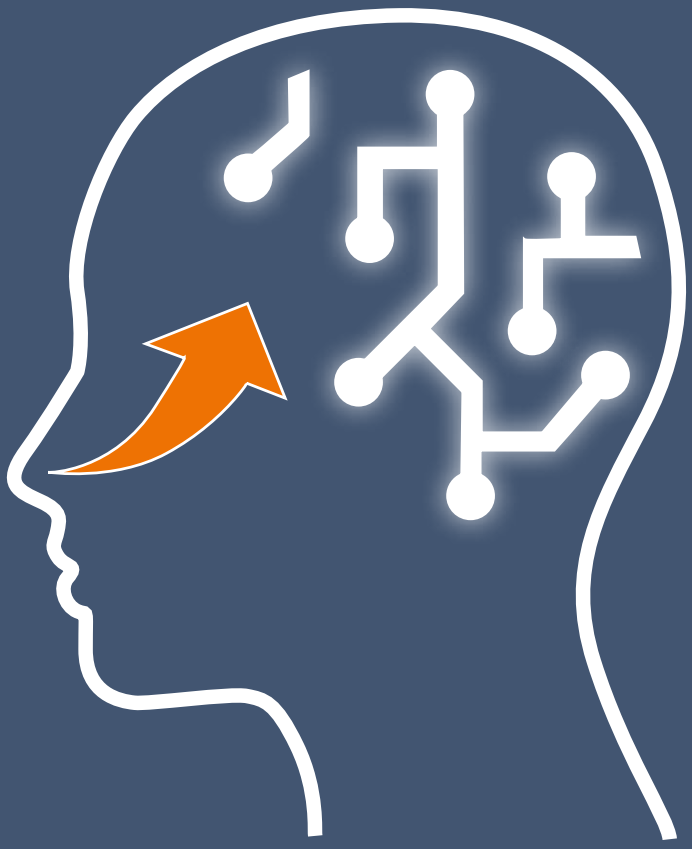
UNIVERSITY OF COPENHAGEN
FACULTY OF HEALTH AND MEDICAL SCIENCES

Early Signs, Symptoms, and Biomarkers of Lewy Body Disease



PhD Thesis

Oskar Hoffmann McWilliam

DANISH DEMENTIA RESEARCH CENTRE

REGION H Rigshospitalet

This thesis has been submitted to the Graduate School of Health and Medical Sciences, University of Copenhagen 14 November, 2025

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Danish Dementia Research Centre

Department of Neurology

Copenhagen University Hospital - Rigshospitalet

Memory can restore to life everything except smells, although nothing revives the past so completely as a smell that was once associated with it.
- Mary by Vladimir Nabokov

PhD thesis

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Author Oskar Hoffmann McWilliam, MD
Danish Dementia Research Centre, Department of Neurology,
Copenhagen University Hospital – Rigshospitalet,
Copenhagen, Denmark

Supervisors

Principal supervisor Associate Professor Kristian Steen Frederiksen, MD, PhD
Danish Dementia Research Centre, Department of Neurology,
Copenhagen University Hospital – Rigshospitalet, and
Department of Clinical Medicine, University of Copenhagen,
Copenhagen, Denmark

Primary co-supervisor Professor Gunhild Waldemar, MD, DMSc
Danish Dementia Research Centre, Department of Neurology,
Copenhagen University Hospital – Rigshospitalet, and
Department of Clinical Medicine, University of Copenhagen,
Copenhagen, Denmark

Co-supervisors Senior Researcher Aušrinė Areškevičiūtė, MSc, PhD
Danish Reference Center for Prion Diseases, Department of Pathology,
Copenhagen University Hospital, Copenhagen, Denmark

Professor Steen Gregers Hasselbalch, MD, DMSc
Danish Dementia Research Centre, Department of Neurology,
Copenhagen University Hospital – Rigshospitalet, and
Department of Clinical Medicine, University of Copenhagen,
Copenhagen, Denmark

This thesis was submitted to the Graduate School of Health and Medical Sciences, University of Copenhagen, on the 14th of November 2025

Assessment committee

Chairperson	Professor Lars Hageman Pinborg, MD, DMSc Epilepsy Clinic, Department of Neurology, Copenhagen University Hospital – Rigshospitalet, and Department of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark
Assessor	Professor Per Borghammer, MD, PhD, DMSc Department of Nuclear Medicine and PET, Aarhus University Hospital, Denmark, and Department of Clinical Medicine, Aarhus University, Denmark
Assessor	Professor Sonia Gandhi, MD, PhD The Francis Crick Institute, London, UK, and Department of Clinical and Movement Neurosciences, UCL Queen Square Institute of Neurology

Preface and acknowledgments

This PhD project was carried out at the Danish Dementia Research Centre, Department of Neurology, Copenhagen University Hospital, Rigshospitalet, between 2022 and 2025. Since the initial conception of the project to the present moment of submitting this thesis, the field has advanced significantly, with numerous new publications and emerging ideas to consider. At the same time, many new questions have arisen. Keeping pace with these accelerating developments has not always been easy, but as the saying goes, "No man is an island", and neither is work within this thesis. The work was carried out as part of a close collaboration between the Danish Dementia Research Centre, the Danish Reference Center for Prion Diseases from the Department of Pathology, and Head & Neck Surgery and Audiology from the Department of Otolaryngology. The combination of expertise in neurology, pathology, and otolaryngology has been invaluable in advancing this research and illustrates the strength of working across disciplines.

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Finally, and most importantly, I want to express my deepest gratitude to the study participants and their caregivers. Without your time and willingness to contribute, this research would not have been possible. I hope that this work will, in time, add to the effort of unraveling the mysteries within Lewy body disease and ultimately help improve the lives of coming patients.

List of Studies

This thesis is based on the following four original studies:

Study 1 Bsoul R*, **McWilliam OH***, Waldemar G, Hasselbalch SG, Simonsen AH, von Buchwald C, Bech M, Pinborg CH, Pedersen CK, Baungaard SO, Lombardía J, Ejlerskov P, Bongianni M, Bronzato E, Zanusso G, Frederiksen KS, Lund EL, and Areškevičiūtė A. *Contributed equally.
Accurate detection of pathologic α-synuclein in CSF, skin, olfactory mucosa, and urine with a uniform seeding amplification assay.
Acta Neuropathologica Communications. 2025 May 24[1]

Study 2 **McWilliam OH**, Bsoul R, Lund EL, Waldemar G, Hasselbalch SG, Simonsen AH, Bruun M, von Buchwald C, Aanæs K, Pedersen CK, Andersen ISB, Bech M, Areškevičiūtė A, and Frederiksen KS.
α-Synuclein seed amplification assay in Lewy body dementia versus Alzheimer's disease.
Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring.
2025 November 7[2]

Study 3 **McWilliam OH**, Bsoul R, Waldemar G, Hasselbalch SG, Simonsen AH, Bruun M, Andersen LSN, von Buchwald C, Aanæs K, Pedersen CK, Andersen ISB, Bech M, Pinborg CH, Areškevičiūtė A, Lund EL, and Frederiksen KS. Prodromal Lewy Body Symptoms and α-Synuclein Seeding in Idiopathic Olfactory Dysfunction
Movement Disorders Clinical Practice. 2025 Sepember 8[3]

Study 4 **McWilliam OH**, Bsoul R, Areškevičiūtė A, Andersen ISB, Bruun M, von Buchwald C, Hasselbalch SG, Lund EL, Pedersen CK, Simonsen AH, Waldemar G, Aanæs K, and Frederiksen KS.
Symptoms & α-Synuclein Seeding Parameters in Olfactory Dysfunction & Lewy Body Dementia
Submitted[4]

Abbreviations

AD = Alzheimer's disease; **AROMA** = Alpha-synuclein Rt-QuIC and neurologic symptoms in persons with idiopathic anosmia; **αSyn** = α-Synuclein (native or recombinant); **αSynD** = Misfolded self-aggregating α-synuclein depositions; **ANOVA** = Analysis of Variance; **AUC** = Area under the curve; **CBD** = Corticobasal degeneration; **CI** = 95% Confidence interval; **COVID-19** = coronavirus disease 2019, **CSF** = Cerebrospinal fluid; **CT** = Computed tomography; **DAT** = Dopamine transporter; **DISPLAY** = Diagnostic biomarkers and Symptoms in Patients with Alzheimer's disease and Lewy body dementia; **DLB** = Dementia with Lewy bodies; **EEG** = Electroencephalography; **ENS** = Enteric nervous system; **Fmax** = Maximum fluorescence intensity; **FM-100** = Farnsworth–Munsell 100 hue test; **GBA** = Glucocerebrosidase gene; **GI** = Gastrointestinal tract; **HC** = Healthy controls; **iLBD** = Incidental Lewy body disease; **iOD** = Idiopathic olfactory dysfunction; **iRBD** = isolated RBD; **IQR** = Interquartile range; **LB** = Lewy bodies; **LBD** = Lewy body disease; **LR** = Likelihood ratio; **LRRK2** = Leucine-rich repeat kinase 2; **MCI** = Mild cognitive impairment; **MDS** = Movement Disorder Society; **MDS-UPDRS** = Movement Disorder Society Unified Parkinson's Disease Rating Scale; **MIBG** = Metaiodobenzylguanidine; **MMSE** = Mini-Mental State Examination; **MoCA** = Montreal Cognitive Assessment; **MRI** = Magnetic resonance imaging; **MSA** = Multiple system atrophy; **N** = Number; **NSD-ISS** = Neuronal α-synuclein disease integrated staging system; **OD** = Olfactory dysfunction; **oHC** = Older healthy controls; **OM** = Olfactory mucosa; **OR** = Odds ratio; **PAF** = Pure autonomic failure; **PARS** = Parkinson Associated Risk Study; **PD** = Parkinson's disease; **PDD** = PD dementia; **PET** = Positron emission tomography; **PNS** = Peripheral nervous system; **PP** = Polypropylene; **PPMI** = Parkinson's Progression Markers Initiative; **PSG** = Video polysomnography; **PSP** = Progressive supranuclear palsy; **pPD** = Prodromal Parkinson's disease; **RBD** = REM sleep behavior disorder; **RBD1Q** = REM Sleep Behavior Disorder Single-Question Screen; **RBDSQ** = REM Sleep Behavior Disorder Screening Questionnaire; **RFU** = Relative fluorescence units; **ROC** = Receiver Operating Characteristic; **RT-QuIC** = Real-time quaking-induced conversion; **SAA** = Seed Amplification Assay; **SD** = Standard deviation; **SDMT** = Symbol Digit Modalities Test; **SIT-16** = 16-item Sniffin' Sticks Identification Test; **SOC** = α-Synuclein Origin site and Connectome; **SPECT** = Single-photon emission computed tomography; **SuStaIn** = Subtype and Stage Inference; **TDI** = Sniffin' Sticks Threshold–Discrimination–Identification test; **TH50** = Time-to-50% of maximal fluorescence; **UPSIT** = University of Pennsylvania Smell Identification Test; **USSLB** = Unified Staging System for Lewy Body disorders; **Vmax** = Maximum slope of fluorescence increase; **yHC** = Younger healthy controls.

Summary

Lewy body diseases (LBD), including Parkinson's disease (PD) and dementia with Lewy bodies (DLB), are caused by pathological accumulation of misfolded, self-aggregating α-synuclein. This process likely begins years before clinical onset, during a premanifest phase characterized by non-motor symptoms such as olfactory dysfunction (OD). Although the early pathophysiology remains unclear, α-synuclein pathology is hypothesized to originate in the olfactory mucosa and/or the gut before spreading to the brain, leading to motor and cognitive symptoms. The novel α-synuclein seed amplification assay (SAA) enables sensitive detection of self-aggregating α-synuclein. Improved understanding and early diagnosis are essential as the prevalence of LBD increases and disease-modifying treatments emerge for LBD and other dementias, such as Alzheimer's disease (AD).

This project investigated self-aggregating α-synuclein detected by SAA as a biomarker for early DLB across multiple biospecimens and explored idiopathic OD (iOD) as a potential prodromal LBD model. Participants were recruited from two cohorts: DISPLAY, including patients with DLB or AD, and AROMA, including individuals with iOD and normosmic controls. Clinical assessments and biospecimen sampling (cerebrospinal fluid (CSF), skin, olfactory mucosa, saliva, and urine) were performed to detect self-aggregating α-synuclein and assess clinical correlates. The risk of prodromal PD was calculated in the AROMA cohort.

Self-aggregating α-synuclein was detected in most DLB patients, nearly half of those with iOD, a few with AD, and rarely in controls. The assay showed high accuracy in CSF and skin. Detection in the olfactory mucosa was technically challenging but improved with methodological refinements. While self-aggregating α-synuclein was undetectable in saliva, it was identified for the first time with SAA in urine. LBD symptoms and skin-based SAA kinetics parameters increased stepwise from controls to iOD to DLB. Just under half of individuals with iOD met criteria for prodromal PD.

These findings demonstrate that α-synuclein detected by SAA is a promising biomarker that may support early clinical diagnosis of DLB, enabling more targeted patient care. CSF SAA will likely be implemented first, but the less invasive skin biopsies may become clinically relevant as they perform comparably. SAA may also aid in identifying patients for disease-modifying trials. Finally, iOD represents a valuable prodromal model, linking early LBD-related symptoms with α-synuclein pathology, and may aid future research in enhancing the understanding and hopefully also the prevention of manifest LBD.

Dansk Resumé

Lewy body sygdomme (LBD), herunder Parkinsons sygdom (PD) og demens med Lewy bodies (DLB), skyldes en patologisk ophobning af fejlfoldet, selv-aggregerende α-synuklein. Denne proces begynder sandsynligvis mange år før den kliniske sygdomsdebut i en præmanifest fase karakteriseret ved symptomer såsom nedsat lugtesans kendt som "olfaktorisk dysfunktion" (OD). Selvom de tidlige sygdomsmekanismer fortsat er uklare, antages det, at α-synuklein-patologien opstår i lugteslimhinden og/eller i tarmen, før den spreder sig til hjernen, hvor den giver bevægeforstyrrelser og kognitive symptomer. Det nye α-synuklein seed amplification assay (SAA) muliggør sensitiv påvisning af selv-aggregerende α-synuklein. En forbedret forståelse og tidlig diagnostik er afgørende, i takt med at forekomsten af LBD stiger, og sygdomsmodificerende behandlinger udvikles for LBD og andre demensformer, fx Alzheimers sygdom (AD).

Projektet undersøgte selv-aggregerende α-synuklein dedekteret med SAA som biomarkør for tidlig påvisning af DLB på tværs af flere biologiske materialer og udforskede idiopatisk OD (iOD) som en mulig præmanifest model for LBD. Deltagere blev rekrutteret fra to kohorter: DISPLAY, som inkluderede patienter med DLB eller AD, og AROMA, som inkluderede personer med iOD og kontroller med normal lugtesans. Kliniske undersøgelser og prøvetagning (rygmarvsvæske, hud, lugteslimhinden, spyt og urin) blev udført for at påvise selv-aggregerende α-synuklein og dennes korrelation med kliniske symptomer. Risikoen for præmanifest PD blev udregnet i AROMA.

Selv-aggregerende α-synuklein blev påvist hos de fleste DLB-patienter, næsten halvdelen af personer med iOD, enkelte med AD og meget sjældent hos kontroller. SAA viste høj nøjagtighed i rygmarvsvæske og hud. Påvisning i lugteslimhinden var teknisk udfordrende, men blev forbedret med metodologiske justeringer. Fejlfoldet α-synuklein kunne ikke påvises i spyt, men blev for første gang detekteret med SAA i urin. Graden af LBD-symptomer og SAA-kinetiske mål steg gradvist fra kontroller til iOD og videre til DLB. Næsten halvdelen af iOD-gruppen opfyldte kriterierne for præmanifest PD.

Resultaterne viser, at α-synuklein målt med SAA er en lovende biomarkør, der formentlig snart kan understøtte tidlig diagnostik af klinisk DLB og dermed muliggøre mere målrettet behandling. SAA på rygmarvsvæske vil sandsynligvis blive implementeret først, men mindre invasive hudprøver kan få klinisk relevans, da de præsterer tilsvarende. SAA kan muligvis bidrage til udvælgelsen af patienter til sygdomsmodificerende kliniske forsøg. Endelig repræsenterer iOD en værdifuld præmanifest model, der kan forbinde tidlige LBD-symptomer med patologisk α-synuklein og kan med fremtidig forskning, forbedre viden og forhåbentlig med tiden også forebyggelsen af manifest LBD.

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0. Introduction

Fritz Heinrich Lewy worked in Alois Alzheimer's laboratory, where he examined brains from patients with Parkinson's disease (PD) and, in 1912, was the first to describe the eosinophilic protein inclusions within neurons that are now known as *Lewy bodies* (LB). The intricate relationship between Lewy body disease (LBD) and Alzheimer's disease (AD) persists to this day, clinically and in research. Clinically, where the pathologies co-exist and shared features can challenge the differentiation, and in research, where AD can serve as a flagship for the development of biomarkers, improving the diagnostic accuracy, and disease-modifying treatments that may improve the lives of patients and caregivers.

1. Background

This thesis will focus on a novel biomarker for LBD, misfolded α-synuclein detected by the seed amplification assay (SAA), examining its potential to differentiate DLB from AD and its application in the prodromal stage of LBD. The background chapter begins with an overview of LBD, followed by its pathogenesis and theories on the onset and propagation of LB pathology. The clinical features of LBD, with emphasis on dementia with Lewy bodies (DLB) and relevant co-pathologies such as AD, are then presented. The mechanisms behind olfactory dysfunction (OD), other prodromal symptoms, and current biomarkers are described, followed by an introduction to the SAA. The chapter concludes with the potential use of SAA in preventive strategies for LBD and a summary of the knowledge gaps.

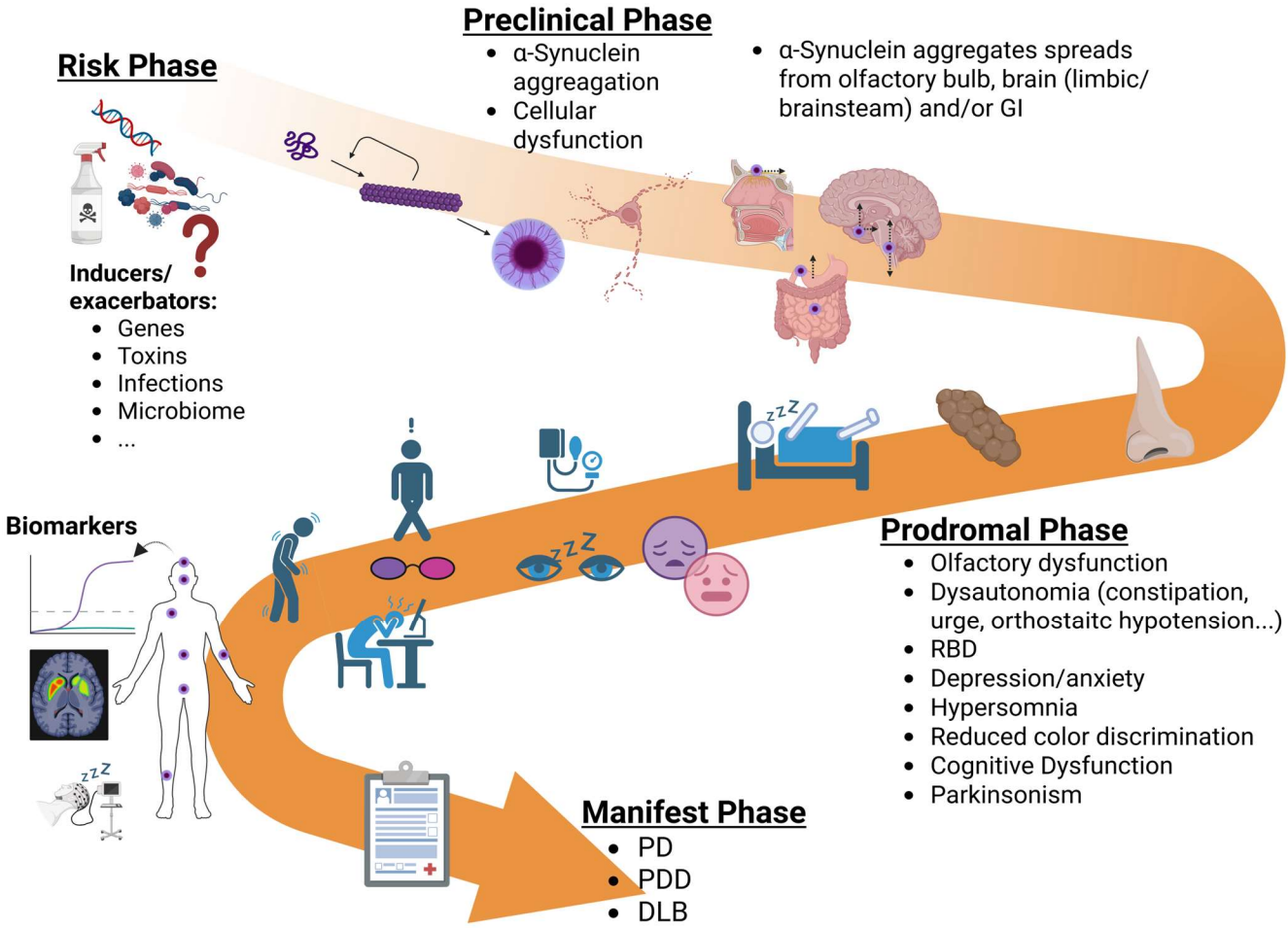
1.1 Lewy body disease

LBD encompasses a clinicopathological spectrum of neuronal α-synucleinopathies, including PD, PD dementia (PDD), and DLB, as well as a premanifest phase subdivided into a preclinical and a prodromal stage (Figure B1).

LBD is neuropathologically characterized by the presence of LB and Lewy neurites, consisting of misfolded self-aggregating α-synuclein depositions (αSynD)[5]. It is hypothesized that αSynD aggregation begins decades before the manifest disease stage, initially in the preclinical phase without symptoms, followed by a prodromal phase marked by further spreading of αSynD pathology and the appearance of early clinical features[6–8]. These prodromal symptoms include OD, REM sleep behavior disorder (RBD), autonomic dysfunction, decreased color discrimination, neuropsychiatric symptoms, subtle parkinsonism, and cognitive dysfunction. The presence of LB pathology in the absence of known LBD symptoms or a diagnosis of manifest LBD can neuropathologically be

referred to as incidental Lewy body disease (iLBD)[7–9]. Much of the knowledge of LBD is derived from research focused on PD. Even though the phenotypes of PD and DLB differ, a great part of the basic pathological mechanisms is believed to overlap[10].

Figure B1 Hypothesized Development of Lewy Body Disease



A schematic overview of the hypothesized development of LBD divided into the four stage: 1) The Risk Phase, in which genetic and environmental factors may influences the initial αSynD aggregation; 2) The Preclinical Phase, in which the initial αSynD aggregation accumulates, induces cellular dysfunction and spreads from the initial site(s); 3) The Prodromal Phase in which symptoms due to LB pathology emerge and biomarkers of neurodegeneration will begin to be able to detect the neurodegeneration. It is not known when αSynD can be detected by seed amplification assays; 4) The Manifest Phase, in which especially cognitive dysfunction and parkinsonism are at a level sufficient for a diagnosis of PD, PDD, or DLB. Created in Biorender.
Abbreviations: DLB = Dementia with Lewy bodies; GI = Gastrointestinal tract; PD = Parkinson's disease; PDD = Parkinson's disease dementia; RBD = REM sleep behavior disorder

1.1.1 Epidemiology of Lewy Body Disease

The global number of individuals living with LBD is rapidly increasing, driven by the aging population[11] and possibly environmental factors[12,13]. PD affects over 1% of individuals above the age of 60 years[14]. Between 2019 and 2050, the global number of people living with dementia is projected

to increase from 57 to over 150 million, while the prevalence of PD is expected to more than double, reaching approximately 25 million[11,13]. DLB is the second most common type of neurodegenerative dementia after Alzheimer's disease (AD)[15]. DLB and PDD account for approximately 8% of clinical dementia cases, whereas LB pathology is found in 8-17% of a cognitively unimpaired population and is involved in around 20% of diagnosed neurodegenerative dementia cases[7,15,16]. Beyond the significant impact on patients and caregivers, DLB imposes the greatest economic burden among dementias[17]. For reasons that remain unclear, RBD, autonomic dysfunction, PD, and particularly DLB are more frequently diagnosed in men than in women[18].

The increasing prevalence of neurodegenerative disease, together with the development of emerging disease-modifying therapies for LBD[19] and especially AD[20] underscores the need for precise and accessible biomarkers.

1.2 Pathogenesis

1.2.1 α-Synuclein

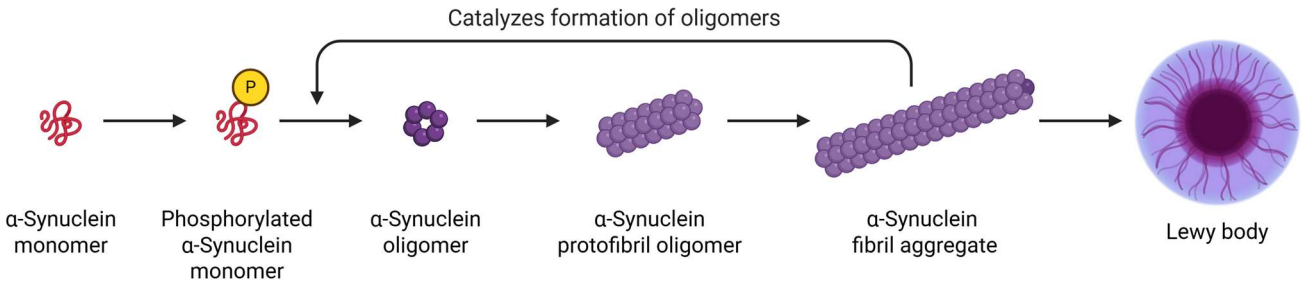
Native αSyn is a relatively simple, monomer protein predominantly localized to presynaptic terminals, where it is thought to regulate synaptic vesicle trafficking, neurotransmitter release, and may be involved in immune defense[19,21,22]. While neurons are the principal site of αSyn expression, it is also present in other CNS cells and peripheral cells, for instance, red blood cells, leukocytes, and cells of the enteric nervous system[21,23].

1.2.2 α-Synuclein Aggregation

In pathological contexts, αSyn can undergo post-translational modifications, the most studied being phosphorylation, which, with other modifications, changes the protein conformation and increases the αSyn aggregation propensity[21]. The misfolded αSyn monomers self-associate into soluble oligomers, which are considered the most neurotoxic species due to their ability to disrupt membranes, impair mitochondrial function, and alter synaptic transmission, ultimately leading to cell death. The αSyn oligomers can further assemble into protofibrils and ultimately mature fibrils, which are proteinase-resistant and insoluble[21] and together here termed as αSynD. Importantly, both oligomeric and fibrillar αSynD can act as "seeds" that template the misfolding of native αSyn, enabling a prion-like mechanism of aggregation and propagation (Figure B2)[24]. Fibrillar αSynD accumulates within neurons, forming characteristic intracytoplasmic inclusions known as LB, along with Lewy neurites in axons. Together, these structures represent the pathological hallmarks of LBD[25].

Although LBD has been perceived primarily as a proteinopathy, some studies focus on other pathological processes contributing to cell dysfunction parallel to or independent of αSynD accumulation[26–28], but this will not be treated in this thesis.

Figure B2 The Misfolding and Aggregation of α-Synuclein into Lewy Bodies



The proposed mechanism involves phosphorylation (P), misfolding, and aggregation of endogenous α-synuclein, which promotes further aggregation in a prion-like manner. In combination with other proteins, such as ubiquitin, α-synuclein fibrils aggregate to form Lewy bodies. Created in Biorender.

The phosphorylation and aggregation of αSyn are thought to result from a complex interplay of genetic, environmental, and systemic factors. Genetic mutations in SNCA (encoding αSyn) or in genes involved in lysosomal and mitochondrial function (e.g., LRRK2, GBA) increase the protein's aggregation propensity by altering its folding dynamics or impairing clearance mechanisms[21]. Environmental toxins, such as pesticides (e.g., rotenone, paraquat), air pollution, and heavy metals, induce oxidative stress and mitochondrial dysfunction, thereby promoting αSyn misfolding and phosphorylation[12,29]. Infections have also been proposed as triggers, as viral or bacterial pathogens upregulate neuronal αSyn expression as part of an innate immune response; sustained overexpression may favor pathological aggregation[22,30,31]. Emerging evidence also indicates a role for the gut microbiome in modulating αSyn pathology. Dysbiosis (imbalanced microbiome) and specific bacteria can increase intestinal permeability and local inflammation, potentially driving αSyn overexpression and aggregation in enteric neurons[32]. The risk phase, in which many of these processes occur, may precede the clinical diagnosis of PD and DLB by several decades and can thus be a challenge to study in vivo.

1.2.3 α-Synuclein Propagation

The misfolded αSynD aggregates propagate from cell to cell via synapses and/or extracellular vesicles. Another possible pathway is a direct transfer from cell to cell, where the αSynD is tunneled via nanotubules[19]. αSynD can spread antero- and retrogradely preferentially along structurally and functionally connected neural pathways, rather than randomly across regions[33]. The prion-like

propagation from the enteric nervous system (ENS) to the brain via the vagus nerve has been shown to be possible in a rodent model[34]. The rodents were injected with αSynD in the gastrointestinal (GI) tract, which spread to the dorsal motor nucleus of the vagus, locus coeruleus, amygdala, substantia nigra, and even the olfactory bulb. This propagation was inhibited by vagotomy and by the removal of endogen αSyn. Likewise, in a rodent model[35], the αSynD injected in the olfactory bulb spread to other structures, e.g., amygdala, hippocampus, olfactory tubercle, entorhinal cortex, and other brain structures, but not to brainstem structures like the locus coeruleus or substantia nigra, although the latter has been observed in other rodent models[29]. The seeding pattern and especially the disposition to contralateral propagation depended on the strain of αSynD fibrils.

1.2.4 Lewy Body Disease Propagation Models and Staging Systems

The initial site of αSynD aggregation and its subsequent propagation are critical determinants of where pathological changes can be detected. Numerous neuropathological staging systems have been proposed for LBD, reflecting the marked heterogeneity in the presentations, resulting in difficulties in classification and with inter-pathologist disagreement[36]. Among the previously most widely used are the neuropathological consensus criteria[37], which divide LB pathology into three ascending stages: 1) "brainstem-predominant", 2) "limbic/transitional", and 3) "diffuse neocortical". Most PD/PDD and nearly all DLB cases correspond to the diffuse neocortical stage by the time of death. These criteria have subsequently been revised into the LB pathology consensus system[36], which incorporates the two additional stages, "olfactory-only" and "amygdala-predominant", preceding or independent of the brainstem stage. Unlike other semiquantitative systems, the LB pathology consensus system employs a dichotomous classification (present/absent), where a single LB or Lewy neurite suffices for a classification as LB pathology positive. Beyond these, four principal staging frameworks or hypotheses have been proposed to describe the origin and progression of αSynD pathology and its relation to symptom development (Table B1):

Table B1 Overview of the Hypotheses on the Origin and Progression of Lewy Body Pathology

Hypothesis (Author)	**Subtype**	**Pathological Origin and Progression**	**Likely Diagnosis at Death**	**Hypothesized Clinical Profile**
Dual-Hit Hypothesis[23,30] (Braak & Hawkes)	Single trajectory	Olfactory mucosa → amygdala + ENS → brainstem	PD, DLB	Early: OD, dysautonomia Early/Mid: Depression/Anxiety, RBD Mid: Parkinsonism Late: CD
USSLB[38] (Adler & Beach)	A: Brainstem-predominant	Olfactory bulb → A) brainstem or B) limbic	PD	Early: OD Mid: RBD, parkinsonism Late: Dysautonomia
	B: Limbic-predominant		DLB, AD	Early: OD Mid: Psychiatric, parkinsonism, RBD Late: Dysautonomia
SOC model[39–41] (Borghammer)	Brain-First	Olfactory bulb, Amygdala. Slow Progression Speed	PD	Early: Parkinsonism (asymmetrical and tremor dominant) Mid: OD (mild) Late: RBD (rare), dysautonomia (mild), CD (mild)
	Body-First	ENS Faster progression	DLB, PD	Early: Dysautonomia, OD Mid: RBD, psychiatric Late: CD, Parkinsonism (symmetrical)
The α-Synuclein SuStaIn model[9] (Mastenbroek/ Algorithm)	S1: Olfactory bulb-early / Limbic-early	Olfactory bulb + Amygdala	AD, DLB, PD	Early and pronounced: CD Mid and moderate: OD and Parkinsonism
	S2: Olfactory bulb-early / Brainstem-early	Olfactory bulb + Brainstem Slow progression	iLBD, PD, AD	Early: Parkinsonism (tremor dominant) Mid: OD (mild) Late: CD (mild)
	S3: Brainstem-early / Olfactory bulb-later	Brainstem Faster progression	PD, DLB	Early: Parkinsonism (postural/gait dominant) Mid: OD, CD

Abbreviations; AD = Alzheimer's disease; CD = Cognitive dysfunction; DLB = Dementia with Lewy bodies; ENS = Enteric nervous system; ILBD = Incidental Lewy body disease; LBD = Lewy body disease; OD = Olfactory dysfunction; PD = Parkinson's disease; RBD = REM sleep behavior disorder; SOC = α-Synuclein Origin site and Connectome; SuStaIn = Subtype and Stage Inference; USSLB = Unified Staging System for Lewy Body disorders

The Dual-Hit Hypothesis[23,30,42] proposes that the αSynD aggregates enter the nervous system through two peripheral routes: (1) the olfactory mucosa (OM) and (2) the enteric mucosa. Although termed the Dual-Hit Hypothesis, the authors suggested that a single initiating event, such as exposure to a neurotropic pathogen, could simultaneously affect both sites. In this model, an olfactory viral infection could directly invade the OM, while swallowing virus-laden saliva might introduce

the pathogen to the gastrointestinal (GI) tract, including the ENS, at the same time. From these peripheral entry points, αSynD pathology is proposed to spread anterogradely from the olfactory mucosa (OM) via the olfactory bulb and tract to the temporal lobe and associated olfactory cortical regions, and retrogradely from the GI tract to the enteric nervous system (ENS) and via the vagus nerve to the dorsal motor nucleus of the vagus in the brainstem. From here, αSynD may subsequently seed other central structures, including the locus coeruleus and substantia nigra, before eventually reaching limbic and cortical regions.

This Dual-Hit hypothesis provides a mechanistic explanation for the early non-motor manifestations observed in prodromal LBD, such as OD, autonomic dysfunction, and RBD. The hypothesis was originally proposed to describe the pathogenesis of sporadic Parkinson's disease[42], but has since also been extended to DLB[7].

The Unified Staging System for Lewy Body Disorders (USSLB)[38] provides a pathology-based staging framework that classifies LBD into five stages with two distinct subtypes: Stage I (olfactory bulb only), IIa (brainstem predominant), IIb (limbic predominant), III (brainstem + limbic), and IV (neocortical involvement)[38]. This system, derived from 280 autopsy-confirmed cases, demonstrated stronger clinico-pathological correlations than the traditional Braak staging. Importantly, it accounts for αSynD pathology restricted to the olfactory bulb as well as limbic-predominant patterns that bypass the brainstem.

The USSLB posits that LBD pathology develops as an olfactory/CNS-first process, with later spread to the peripheral nervous system (PNS) and GI tract. This notion is supported by only a few human postmortem cases demonstrating ENS/PNS αSynD pathology preceding CNS involvement[43]. In contrast to the Dual-Hit Hypothesis, the USSLB delineates two distinct clinico-pathological subtypes (IIa and IIb), which may explain some of the clinical heterogeneity observed across LBDs.

The α-Synuclein Origin site and Connectome (SOC) model[40,44–46] is based on a variety of studies, including especially neuropathological and imaging data. In short, it describes two distinct clinico-pathological phenotypes, Brain-First and Body-First. In the Brain-First phenotype, αSynD aggregation begins in the amygdala, sometimes preceded by olfactory bulb onset, before the pathology spreads to the substantia nigra, locus coeruleus, other brainstem structures, and cortical areas. It is only in later stages that the PNS may be affected.

In the Brain-First phenotype, αSynD pathology develops and spreads relatively slowly from the amygdala or olfactory bulb, while the brainstem and PNS are initially relatively spared, resulting in

a short prodromal period and less prominent RBD and dysautonomia symptoms. The pathology begins in one hemisphere, which explains the primary asymmetrical motor symptoms found in PD. Despite early affection of the olfactory-associated system (for description see 1.4), OD is a late and less prominent feature of the Brain-First phenotype. This may be explained by the lateralized αSynD pathology, whereas the olfactory-associated system functions bilaterally[39].

In contrast, the Body-First phenotype αSynD aggregation begins in PNS/ENS and propagates retrogradely via the vagal nerve and sympathetic fibers to the dorsal motor nucleus of the vagus bilaterally due to the bilateral innervation of the vagus nerve. The pathology spreads much like the ENS pathway described in the Dual-Hit hypothesis. This PNS to CNS phenotype has a long prodromal phase with autonomic dysfunction, but is also characterized by prodromal RBD due to brainstem involvement. Due to the bilateral pathological affection, the parkinsonism in the Body-First phenotype is more symmetrical. Counterintuitively, OD is a prodromal and prominent symptom in Body-First. This is explained by the more widespread and bilaterally αSynD pathology in Body-First[39]. Whereas the Brain-First phenotype will primarily be diagnosed with PD, the Body-First phenotype is a combination of PD and DLB[10].

The α-Synuclein Subtype and Stage Inference (SuStaIn) model[9] is a data-driven algorithm that grouped cross-sectional neuropathological data into subtypes to infer a progression model and then combined the subtypes with clinical data[47]. For LBD, the model phenotypes are clustered into three distinct subtypes (S): S1 “Olfactory bulb-early / Limbic-early”, S2 “Olfactory bulb-early / Brainstem-early”, and S3 “Brainstem-early / Olfactory bulb-later”. The model is based on the same but larger clinical and neuropathological sample as the USSLB.

S1 is the most frequent subtype, in which αSynD pathology begins in the OB and limbic system, especially the amygdala, before it spreads to the brainstem and cortex. S1 exhibits more αSynD pathology in the OB than the other subtypes, but less peripheral pathology. The αSynD pathology in S1 often coexists with AD pathology, and the subtype is therefore associated with early and pronounced cognitive decline, most frequently diagnosed as AD, followed by DLB and PD, but to a lesser extent than S2 and S3.

Like S1, the αSynD pathology in S2 begins in the olfactory bulb but spreads to the brainstem instead of the limbic regions. Subsequently, it propagates to the limbic and cortical areas. S2 has earlier and more peripheral αSynD than S1. Subjects with S2 are most often not clinically diagnosed with any neurodegenerative disorder during life (iLBD); however, when a diagnosis is made, it is typically PD or AD.

In S3 αSynD pathology begins in the brainstem but spreads to the olfactory bulb and limbic structures at a relatively early stage before continuing to cortical structures. S3 has less dense pathology in more regions and is thus thought to have a faster regional spread compared to S1 and S2, which were associated with a slower progression due to more regional build-up of pathology before propagating to other regions. Although pathology in the olfactory bulb appears slightly later in S3, it eventually surpasses that observed in the other phenotypes. S3 is like the Body-First associated with more cognitive impairment, postural parkinsonism, and OD than the other subtypes, and is often diagnosed with PD or DLB.

The inherent challenges of neuropathological studies are that pathology has progressed substantially since symptom onset and that cross-sectional findings must be inferred to represent temporal disease progression. Nevertheless, such studies have provided an important framework for accurate diagnosis and the correlation between symptoms and pathology.

1.3 Manifest Lewy Body Disease and Co-Pathologies

Although neuropathological confirmed diagnosis still is the gold standard for DLB, PD, and other neurodegenerative diseases, a probable/established or possible antemortem diagnosis is useful in the clinic and research settings.

1.3.1 Clinical Features and Diagnosis of DLB

The essential criterion for DLB is dementia, which is a syndrome of persistent cognitive decline to an extent that it affects activities of daily living[15,48]. Deficits in attention, executive function, and visuoperceptual ability are especially prominent in DLB, whereas memory dysfunction is milder. Mild cognitive impairment (MCI) is defined by objective evidence of cognitive decline in at least one cognitive domain or on global cognitive abilities, but with functional independence and a preserved or minimal effect on activities of daily living[49,50]. In addition to being common in PD, MCI due to LB pathology (MCI-LB) represents a late prodromal stage of DLB, reflected by the substantial overlap in diagnostic criteria[6,15,49]. The MCI-LB is like DLB, often diagnosed and managed in memory clinics. Furthermore, MCI-LB is useful as a model of early symptoms of DLB, as few studies report symptoms in newly diagnosed DLB.

Table B2 Consensus Criteria for DLB and MCI-LB

Core clinical features	1 Fluctuating cognition 2 Recurrent visual hallucinations 3 RBD 4 Parkinsonism (minimum one cardinal feature)
Indicative biomarkers	1 Reduced DAT in the basal ganglia on DAT imaging 2 Reduce cardiac uptake on ^{123}iodine-MIBG myocardial scintigraphy 3 PSG with REM sleep without atonia
Supportive clinical features	Neuroleptic sensitivity; postural instability/falls; syncopes; severe* autonomic dysfunction (e.g. constipation, orthostatic hypotension, urinary incontinence); hypersomnia; OD; other hallucinations; delusions; psychiatric symptoms of apathy, anxiety, and depression; sense of presence#, delirium#
Supportive biomarkers	MRI/CT with relative preservation of medial temporal lobes; SPECT/PET with reduced occipital activity +/- cingulate island sign; EEG with posterior slow-wave with periodic fluctuations in the pre-alpha/theta range; MRI with insular thinning#
Probable DLB/MCI-LB	a. ≥2 core features b. 1 core feature and ≥1 indicative biomarker
Possible DLB/MCI-LB	a. 1 core symptom, no indicative biomarkers b. ≥1 indicative biomarker but no core symptoms

** In MCI-LB only autonomic dysfunction (not severe). # MCI-LB but not DLB. The summary of antemortem criteria for DLB[15] and MCI-LB[49], besides the essential criterion of dementia and MCI. In the research criteria for MCI-LB indicative biomarkers are called "proposed" and supportive biomarkers are called "suggested".*

Abbreviations: CT = Computed tomography; DAT = Dopamine transporter; DLB = Dementia with Lewy bodies; EEG = Electroencephalography; MIBG = Metaiodobenzylguanidine; MRI = Magnetic resonance imaging; OD = Olfactory dysfunction; PD = Parkinson's disease; PET = Positron emission tomography; PSG = Polysomnography; RBD = REM sleep behavior disorder; SPECT = Single-photon emission computed tomography.

The DLB diagnosis has undergone several revisions, and the latest is the fourth consensus criteria[15], see Table B2. The primary updates from the third[37] to the fourth consensus report were: 1) the promotion of RBD from a supportive feature to a core symptom, as it is a very specific symptom related to DLB/LBD, 2) stronger emphasis on biomarkers (see also section 1.6), and 3) the addition of OD as a supportive clinical feature.

Noteworthy is that the clinical core criteria for parkinsonism in DLB only include at least one out of the three cardinal parkinsonian symptoms, in contrast to PD, which demands bradykinesia and either tremor or rigidity[51]. Besides core clinical features and indicative biomarkers, the diagnostic criteria also include supportive clinical features and biomarkers, which themselves were not judged to have enough specificity for DLB, but in conjunction with the core criteria increase the likelihood of a correct diagnosis. The supportive clinical features give a comprehensive, but not complete, description of symptoms in subjects with DLB and include several of the prodromal symptoms, which will be treated in sections 1.4 and 1.5.

Subjects with MCI instead of dementia and co-occurring LBD symptoms can be diagnosed with the research consensus criteria for MCI-LB[49], which generally parallel the DLB criteria. Two other phenotypes parallel to MCI-LB have been proposed: 1) the recurrent or prolonged delirium and 2) the psychiatric onset. None of these have been thoroughly studied and will thus not be treated in this thesis[49].

The clinical distinction between DLB/MCI-LB and PD/PDD is somewhat arbitrarily set as the 1-year rule, which states that in subjects with onset of dementia within one year of parkinsonism is defined as DLB, whereas later cognitive decline to the level of dementia is defined as PDD[15,52,53]. The challenge is that around 40% of de novo PD patients have cognitive dysfunction at the level of MCI[54].

1.3.2 Parkinson's Disease Criteria

PD is classified as a movement disorder diagnosed according to the UK PD Brain Bank diagnostic criteria[55] and the MDS diagnostic criteria[51]. The cardinal motor manifestations are bradykinesia in combination with either rigidity or rest tremor, which are often initially asymmetrical. The UK PD Brain Bank criteria additionally include postural instability as a core feature. Both diagnostic frameworks are primarily based on clinical evaluation, emphasizing the presence of cardinal motor symptoms and supportive features, while also considering red flags and exclusion criteria. The MDS-criteria include the utility of ^{123}iodine-MIBG myocardial scintigraphy and dopamine transporter (DAT) imaging and recognize that non-motor features such as sleep disturbances, dysautonomia, OD, and psychiatric symptoms are common in PD, although early and severe autonomic dysfunction represents a red flag suggestive of an alternative diagnosis such as multiple system atrophy (MSA).

PDD refers to patients who first develop PD and subsequently, after a minimum of one year, develop cognitive impairment severe enough to meet criteria for dementia. They often have a clinical profile similar to DLB[52,53].

1.3.4 Diagnostic Challenges

The diagnostic challenge of an early and precise diagnosis of DLB has been reported in several studies, and DLB is likely underrecognized and initially misdiagnosed. Galvin et al.[56] surveyed caregivers of individuals with DLB and PDD and found that 78% were initially given another diagnosis. Unsurprisingly, most (39%) were diagnosed with PD or related disorders, but a substantial number of patients (36%) were diagnosed with other dementias, especially AD, and 24% with psychiatric disorders. A meta-analysis on clinical versus neuropathological DLB diagnosis by Rizzo et

al.[57] found a pooled sensitivity, specificity, and accuracy of 60%, 94%, and 80%, respectively. AD was again the most frequent misdiagnosis. Unfortunately, the meta-analysis has some drawbacks as it is based on older, less accurate consensus criteria from 1996 and 2005[37,58], and it primarily includes late-stage cases, where clinical symptoms have developed and thus increased the accuracy of the clinical diagnosis.

The diagnosis of PD can also be a challenge. A recent study of cases from 2009–2019 reported an overall diagnostic accuracy of 84%[59]. PD diagnoses made by general neurologists had a lower accuracy of approximately 75%, primarily due to PD being diagnosed in individuals with non-LBD pathologies such as AD and vascular[60].

1.3.5 Co-pathology

The presence of co-pathology is more the rule than the exception in neurodegenerative diseases driven by protein aggregates such as tau species, β-amyloid, and TDP-43, as well as in the frequent coexistence of non-degenerative conditions such as vascular pathology and normal pressure hydrocephalus[61,62]. Several neurodegenerative and non-degenerative disorders may mimic the clinical presentation of LBD, particularly in the early stages. MSA is a non-LBD α-synucleinopathy that can resemble PD, due to overlapping features such as autonomic dysfunction, parkinsonism, and RBD. The tauopathies, progressive supranuclear palsy (PSP) and Corticobasal degeneration (CBD), may present with parkinsonism and cognitive impairment that can mimic both PD and DLB[61].

1.3.6 Alzheimer's Disease

AD pathology is the leading cause of dementia and frequently co-occurs with LBD pathology[63]. AD dementia is clinically characterized by progressive memory impairment, and later or alternatively executive dysfunction, behavioral changes, language, and visuospatial deficits[48]. AD is typically preceded by a prodromal stage defined as MCI-AD[64] and likely by an even earlier phase marked by more diffuse symptoms that may influence health-related behavior[63,65]. Neuropathologically, AD is defined by extracellular amyloid-β plaques and intraneuronal tau neurofibrillary tangles, which often progress in a stereotypical topographic pattern. The McKhann et al. and Albert et al. consensus criteria primarily focused on clinically differentiating AD dementia and MCI from other dementias, particularly DLB, frontotemporal dementia, and vascular dementia. In contrast, the 2018 and updated 2024 National Institute on Aging-Alzheimer's Association (NIA-AA) criteria define AD as a biological construct based on the presence of amyloid, tau, and neurodegeneration biomarkers. NIA-AA criteria also incorporate αSyn-SAA as a relevant test for co-pathology in AD.

LB pathology is detected in 33–66% of sporadic AD cases at autopsy, which include cases with sparse to widespread pathology. In AD, LB pathology is most often localized to the olfactory bulb and amygdala, with less involvement of the brainstem or neocortex[36,66,67]. Some AD cases have more widespread diffuse and/or cortical LB pathology, often resulting in a faster cognitive decline and potentially accompanied by a clinical profile overlapping with LBD, sometimes leading to a mixed AD/DLB diagnosis[15,66]. Co-pathology with αSynD detected by αSyn-SAA has been associated with accelerated cognitive decline in both unimpaired individuals and cognitively impaired across diagnostic groups, including AD[68,69]. While CSF αSyn-SAAs have shown a high sensitivity for cortical LB pathology, several studies reported lower sensitivity for detecting pathology restricted to the olfactory bulb and amygdala[66]. Whether amygdala-predominant LB pathology in AD represents an incidental finding or a distinct subtype of AD remains uncertain. Nonetheless, the frequent colocalization of pathological tau and αSynD in the amygdala and other brain structures (Table B4) suggests possible cross-seeding mechanisms that may accelerate aggregation and disease progression[66,70].

In DLB, AD pathology and biomarkers are also common, with approximately 50% of patients showing an amyloid+/tau+ profile, which is associated with worse cognition and increased mortality[71,72]. Differentiating AD from DLB in the early stages can be challenging, yet it is clinically important, as symptoms, prognosis, and disease trajectory may differ. Most crucially, with the recent approval of disease-modifying anti-amyloid therapies for AD[20], establishing an accurate etiological diagnosis has become clinically actionable. Whether patients with AD and concurrent LB pathology benefit from anti-amyloid and anti-tau therapy to the same extent as those with pure AD remains unknown. This adds to the need for biomarker-based approaches to disentangle AD, DLB, and their overlap to guide patient management and precision treatment strategies.

1.4 Olfactory System and Dysfunction

This section will give a background for the olfactory system and etiologies of OD, along with the proposed mechanistic link between OD and neurodegenerative diseases. Further background on OD as a prodromal symptom will be elaborated on in the next section, *Prodromal Lewy body disease*.

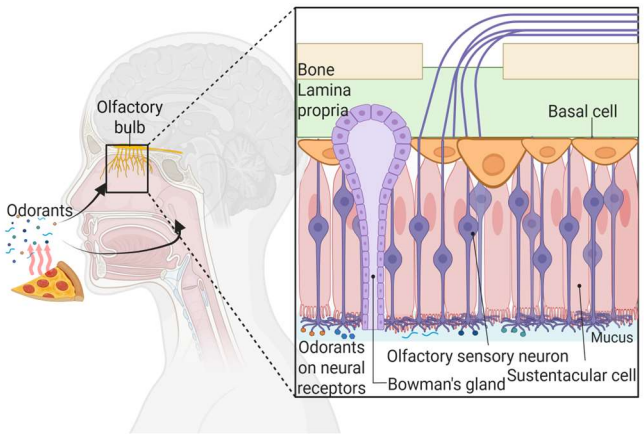
1.4.1 Olfactory System

The sense of smell is of great importance in our everyday life. It helps identify and enjoy food, detects dangers, and guides us in our social life[73,74]. The olfactory system has, in recent years, drawn attention as a marker for diseases like COVID-19 and neurodegenerative disorders[74].

Aromas called odorants enter the nasal cavity from the air via the nostrils and from the mouth via the pharynx. It is estimated that 70% of the sensory experience of food can be attributed to olfactory

stimulation. The OM is located at the roof of the nasal cavity over the cribriform plate of the ethmoid bone and the superior part of the middle turbinate (Figure B3). Around 400 different olfactory receptor neurons embedded in the OM detect the different odorants and convey the signal to interneurons in the olfactory bulb[74,75]. The olfactory receptor neurons are the only neurons in our body with direct contact with the outside world. They regenerate every few months and, along with olfactory bulb interneurons, are among the few neuron types capable of regeneration[76]. Besides the sensory neurons, the olfactory mucosa consists mainly of supporting columnar epithelium with microvilli and basal cells, which are progenitor cells for the regeneration of neurons and epithelial cells. The different OM cells can be recognized by morphology and immunostaining markers[77,78].

Figure B3 and Table B3 Odorant Pathway and Cell Types in the Olfactory Mucosa



Cell type	Key features
Olfactory sensory neurons	Bipolar sensory neurons with dendritic cilia that bind odorants.
Sustentacular/ Supporting cells	Pseudostratified columnar cells with microvilli provide metabolic and physical support, detoxify substances, and maintain ionic balance in the mucus.
Bowman's gland cells	Serous secretory cells that produce mucus to dissolve odorants
Basal cells	Progenitor cells for olfactory neurons and supporting cells

Schematic overview of the pathway of odorants to the olfactory mucosa. Created in Biorender. Table of the main cells of the olfactory mucosa.

These bipolar olfactory sensory neurons project unmyelinated axons through the cribriform bone plate that synapse in the olfactory bulb with neurons called mitral and tufted cells. The output of the olfactory bulb is transmitted via the olfactory tract to olfactory structures, including the piriform cortex, amygdala, entorhinal cortex, and olfactory tubercle, bypassing the thalamus initially. The neocortical structures generally provide the conscious olfactory perceptions, like identification, while limbic structures assign the emotional components[79,80]. Besides the processing structures, brainstem structures like locus coeruleus and substantia nigra, have a strong noradrenergic and dopaminergic innervation to the olfactory bulb, which is thought to modulate the olfactory signals and may be part of the development of OD[81]. AD and LBD pathology is found early in many structures associated with the olfactory system, as shown in Table B4.

Table B4 Olfactory Structures and the Association with LBD and AD Pathologies

Structure	Role in Olfactory Processing	LB Pathology	AD Pathology
Olfactory Bulb	Primary relay, odor feature extraction, projects to the amygdala, piriform, and entorhinal cortex	Early, common	Early, common
Piriform Cortex	Primary olfactory cortex. Initial cortical odor representation, pattern recognition	Late, common	Late, common
Anterior Olfactory Nucleus	Bilateral integration, feedback to the olfactory bulb, odor localization	Early, common	Early, common
Amygdala	Emotional valence, motivation, odor-driven behavior	Early, common	Early, common
Entorhinal Cortex	Pathways to hippocampus, contextual odor memory	Late, common	Early, common
Hippocampus	Episodic and contextual odor memory	Late, common	Early, common
Anterior Cingulate Cortex	Intensity and affective processing of odors.	Mid, common	Late, common
Substantia Nigra	Dopaminergic modulation affecting odor-guided motor/behavior.	Mid, common	Late, occasional
Locus Coeruleus	Noradrenergic modulation of olfaction, attention, and learning.	Early, common	Early, common

Main brain regions involved in olfactory processing and the connection to Lewy body (LB) and Alzheimer's disease (AD) pathologies [25,30,36,38,42,74–76,79,81–87].

1.4.2 Olfactory Dysfunction

OD affects around 15% of the population and around 30% of the population over the age of 60 years. OD can be subdivided into a reduced sense of smell, labeled hyposmia, and a complete loss of smell called anosmia, affecting more than 1% of the population[73,88]. In some articles, *hyposmia* is used synonymously for OD, including both hyposmia and anosmia. OD can be subdivided into conductive, sensorineural, and cognitive impairment (Table B5). The most common known causes are infection, inflammatory (rhinitis), and traumatic head injuries[73,74,89]. The olfactory function declines with progressive age, which is presumed to be caused by a multitude of different factors, such as inflammatory processes in the OM, calcification of the cribriform foramina, decreased density of sensory receptor neurons, but also neurodegenerative factors[75]. Idiopathic OD (iOD) can be defined as OD not associated with any known causes and may be a prodromal symptom of neurodegenerative disease, especially LBD, manifesting before patients fulfill diagnostic criteria, e.g., DLB, PD, or AD[75]. Practically, iOD can be defined as a non-congenital OD with no pathology on a nasal endoscopy, no response to corticosteroids (to exclude inflammatory causes), and no timely association with head trauma or infection[89]. Some definitions also include a gradual onset of OD and structural scans excluding tumors[90]. In a tertiary olfactory clinic, 24% of OD cases were classified as iOD[89].

Subjective OD refers to a self-reported decreased sense of smell, which may not always correlate with objective testing results, as around 72% of individuals with OD are unaware that they have OD [84,91]. Olfactory testing commonly assesses three dimensions of smell function: odor identification (the ability to correctly recognize odors, somewhat also dependent on memory and semantic knowledge), odor threshold (the lowest concentration of an odorant that can be detected), and odor discrimination (the ability to distinguish between different odors). The University of Pennsylvania Smell Identification Test (UPSIT) is a standardized 40-item scratch-and-sniff test that evaluates odor identification. The UPSIT can be self-administered, but includes odors designed for an American population[92]. In contrast, the Sniffin' Sticks test battery, developed in Europe, uses felt-tip pens filled with odorants to assess identification, along with the possibility of also assessing threshold and discrimination, allowing for calculation of a composite Threshold, Discrimination, and Identification (TDI) score. The full TDI is comprehensive but time-consuming and thus not often used outside otolaryngological research and clinical practice in designated smell units. The Sniffin' Stick identification test alone is more commonly used and can be found in sets with 8, 12, or 16 different odors. It is a suprathreshold test assessing the olfactory function with very intense odors, which may not detect more subtle OD within the threshold domain. OD can be defined at an age- and sex dependent or independent level for both UPSIT and Sniffin Sticks[93]. The 16-item Sniffin' Sticks Identification test (SIT16) has less variance than UPSIT with 40 odors, which, especially in older cohorts, can pose a challenge in differentiating OD from norsmomic[92].

Table B5 Categories and Causes of Olfactory Dysfunction

Category	Definition & Mechanism	Causes
Conductive	Odorants fail to reach the olfactory neuroepithelium due to obstruction or altered airflow.	Nasal polyps, nasal tumors, allergic rhinitis (also sensorineural component), and congenital malformations
Sensorineural	Damage to olfactory sensory neurons, supporting cells, or the olfactory nerve with reduced signal transduction.	Infection/post-infectious (e.g., COVID-19), traumatic*, toxins (e.g., heavy metals and pesticides), iatrogenic (medicine, surgery, and radiation), tumors, and congenital
Cognitive	Damage to central higher-order olfactory brain structures	Neurodegenerative, frontal or temporal lobe lesions (tumors, trauma, stroke), epilepsy, and psychiatric disorders (schizophrenia, depression)

** Most commonly, shearing of olfactory filaments. Other mechanisms can be intranasal lesions and brain injury, resulting in a conductive and cognitive OD, respectively.*

1.4.3 Olfactory Dysfunction in Neurodegenerative Disease

Both LBD and AD have been associated with prodromal OD, but LBD to a more severe degree, likely due to a lower olfactory detection threshold[94,95].

The mechanisms underlying OD in LBD and AD remain incompletely understood, including whether dysfunction originates peripherally or centrally. The high cellular turnover in the OM and, to some extent, in the olfactory bulb may further complicate this understanding. However, evidence suggests that OD is closely associated with dopaminergic, serotonergic, and cholinergic dysfunction in olfactory-related brain regions[96] and correlates with cognitive performance[82]. This has been substantiated by a neuropathological study of non-demented individuals that found tau, β-amyloid, and LB pathologies in the olfactory bulb and CNS, but only central tau and LB pathology correlated with olfactory performance. Table B4 gives an overview of LB and AD pathology in the olfactory-associated brain areas. Recent findings in AD suggest a possible retrograde propagation of pathology from the locus coeruleus and temporal lobe to the olfactory bulb[97,98]. Furthermore, studies showing that olfactory training can improve performance in other cognitive domains strengthen the link between olfactory dysfunction and cognitive impairment[82].

The correlation between OD and especially LBD has been substantiated by a large cohort study of cognitively unimpaired individuals[69], which reported an association between αSyn-SAA positivity and degree of OD, whereas no such relationship was observed for β-amyloid or tau. The olfactory test performed with an accuracy of 85% in predicting aSyn-SAA positivity in CSF. Other parkinsonian syndromes like PSP, CBD, and MSA are not affected to the same degree in olfactory areas as LBD and are not substantially affected by OD compared to healthy controls (HC)[84,99] (Figure B4).

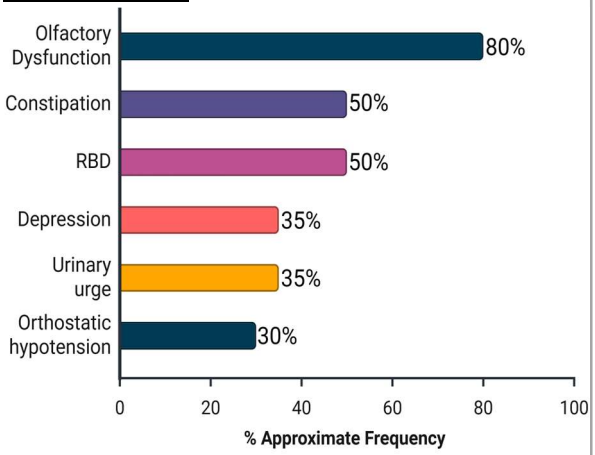
Figure B4 Olfactory Function in Neurodegenerative Disease

Created in Biorender.
Abbreviations: AD = Alzheimer's disease; CBD = Corticobasal degeneration; DLB = Dementia with Lewy bodies; MSA = Multiple system atrophy; PD = Parkinson's disease; PSP = Progressive supranuclear palsy

1.5 Prodromal Lewy Body Disease

After the preclinical phase with initial accumulation of αSynD and cell dysfunction, but often years before the diagnosis of PD or DLB, there is a prodromal phase with progressive pathological changes and development of LBD symptoms (Figure B1).
This section will go through the general knowledge on prodromal LBD symptoms, the two most studied models of prodromal LBD, along other prodromal cohorts.

Figure B5 Approximate Prevalence of Symptoms in Newly Diagnosed Manifest LBD/MCI-LB



The approximate frequency of symptoms based on newly diagnosed PD, DLB, MCI-LB. References in text of section 1.5. Created in Biorender
Abbreviations: RBD = REM-sleep behavior disorder

The frequency and chronology of prodromal symptom onset across the LBD spectrum remain uncertain, but findings from newly diagnosed PD and DLB, as well as MCI-LB, provide some approximate frequencies (Figure B5). Besides parkinsonism in PD and cognitive impairment in DLB, OD is the most prevalent prodromal symptom, affecting approximately 80% of newly diagnosed PD and MCI-LB cases[90,100]. Reported prevalence varies between 62% and 98%, depending on testing methods and definitions[101]. RBD is challenging to assess on a large scale but has been reported to occur in 19–38% of PD[102,103] and around 60% of MCI-LB[104]. Constipation is the most frequent autonomic symptom, present in roughly 60% of MCI-LB and DLB[104,105], but just under 40% in de novo PD[106]. Depression affects about 35% of PD and MCI-LB[104,107]. In a neuropathological study, probable RBD predicted LB pathology with 38% sensitivity and 89% specificity, whereas a smell test (UPSIT) achieved 74% sensitivity and specificity[108]. These prodromal symptoms are generally more prevalent in prodromal DLB, which is predominantly associated with the body-first phenotype, whereas prodromal PD theoretically encompasses both body-first and brain-first subtypes[41].

Currently, besides genetic PD and the late prodromal MCI-LB, there have been two prodromal models most investigated for LBD, which are isolated RBD (iRBD) and pure autonomic failure (PAF)[109,110]. However, recent studies, especially from the Parkinson's Associated Risk Study (PARS) cohort[111–113], have highlighted OD as an additional and promising prodromal model that may encompass a broader spectrum of prodromal LBD. The coming sections will focus on the current knowledge from prodromal cohorts.

1.5.1 REM Sleep Behavior Disorder

Much of the current understanding of prodromal LBD originates from longitudinal cohorts of individuals with iRBD. RBD has a prevalence of 0.7–6% and is a parasomnia with vivid dreaming, dream enactment, and loss of the normal atonia under REM sleep[114]. It can be caused by neurodegeneration in the brainstem as in LBD, but it is also seen in other conditions such as narcolepsy, alcohol withdrawal, encephalitis, and as a side effect of antidepressants[115,116]. Despite other potential etiologies, RBD is classified as an early and very specific prodromal symptom of LBD. Approximately 60% of participants with iRBD will, within 10 years, have phenoconverted to PD or DLB, and a few to MSA[115,117,118]. The conversion rate is higher in cohorts with longer follow-up time, participants with OD, and when including MCI in the phenoconversion[119].

A comprehensive study of iRBD participants found that OD typically was the first symptom to initiate, emerging more than 20 years before the diagnosis of manifest LBD[118]. Especially in the iRBD participants converting to DLB, OD was an early and pronounced symptom, while in the PD converters, it developed later with a more rapid increase, 13 years before the diagnosis. RBD symptoms followed OD, also preceding PD or DLB by approximately two decades. Autonomic dysfunction, including erectile dysfunction, constipation, and later urinary dysfunction, appeared 7–16 years before diagnosis, while color vision impairment emerged around a decade prior. Subtle parkinsonian signs and cognitive dysfunction developed within the final 10 years preceding clinical diagnosis. The cognitive dysfunction may begin with a decline in executive function, followed by attention, memory, and then visuospatial dysfunction[120].

1.5.2 Pure Autonomic Failure

PAF is a rare condition with neurogenic orthostatic hypotension, either in isolation or in combination with other features of autonomic dysfunction such as urge incontinence, constipation, impaired thermoregulation, and sexual dysfunctions[121]. Pathophysiologically, PAF is caused by neuronal or glial αSynD within the autonomic nervous system[122], but autonomic failure can also be caused by neuropathy and autonomic diseases[123]. PAF is associated with a phenoconversion rate of around 33% within six years to PD (42%), DLB (35%), and MSA (23%)[124]. In the longitudinal PAF cohort, the presence of possible RBD was 56% whereas OD was 86%. In a small cohort of 28 PAF participants, 93% were positive for CSF αSyn-SAA ten years after clinical onset[125]. PAF has also been associated with phosphorylated αSyn in the skin[126]. RBD and PAF have, particularly within the framework of the SOC model, been linked to the Body-First phenotype, characterized by αSynD pathology ascending from the peripheral autonomic nervous system to the brainstem[46], which is also the phenotype associated with early and severe OD.

1.5.3 Community, mixed, and OD cohorts

Although prodromal LBD symptoms have been extensively studied in iRBD and to some degree in PAF cohorts, LBD symptoms have also been the focus in large-scale prospective longitudinal community-based cohorts, e.g., the Honolulu-Asian Aging Study[127] and mixed cohorts with healthy controls, relatives of PD patients, other at-risk groups (RBD and OD), and manifest PD patients, e.g., PARS[111–113] and Parkinson's Progression Markers Initiative (PPMI)[128].

In the Honolulu-Asian Aging Study, an association was observed between the prodromal LBD symptoms: OD, constipation, excessive daytime sleepiness, and impaired executive function, and the later development of PD. Among these, OD showed the highest predictive accuracy with a sensitivity of 79% but a specificity of 53%. The relatively short interval between OD onset and phenoconversion of approximately four years may reflect the use of an olfactory test with low variance and the advanced age of the study cohort (71–95 years). Increasing severity of OD correlated with greater neuropathological iLBD burden in the locus coeruleus and substantia nigra, with an iLBD frequency of 17% in the group with the lowest olfactory performance compared to 1.8% in the best-performing tertile. The presence of multiple LBD-related symptoms further increased the risk of phenoconversion[127].

The PARS also found an association between participants with persistent OD and the phenoconversion to PD of 20% versus none of the normosmic. This was backed by abnormal DAT imaging in 29% of the persistent OD versus none of the normosmic[111].

1.5.4 Prodromal Lewy Body Disease Research Criteria and Emerging Biological Definitions

The MDS Research Criteria for Prodromal Parkinson's Disease from 2015[51], revised in 2019[129] is a research tool to estimate the likelihood that an individual has prodromal PD (pPD). The criteria have also been shown to predict the phenoconversion of PD alongside DLB with a sensitivity of 81% and a specificity of 68% in an iRBD cohort[130]. A 10-year follow-up community cohort study also found that the criteria had a high sensitivity of 98% but a lower specificity ranging from 67% (3-year follow-up) to 35% (10-year follow-up)[131]. The research criteria integrate demographical data (e.g. sex, age, smoking status, and pesticide/solvent exposure)), co-morbidities (e.g. diabetes mellitus type II), genetic disposition to PD, clinical symptoms (RBD, OD, constipation, excess daytime sleepiness, orthostatic hypotension, erectile dysfunction, urinary dysfunction, depression, cognitive dysfunction, and subthreshold parkinsonism), and biomarkers (DAT imaging and PSG) into a likelihood ratio (LR) and a percentual probability for pPD. Within the clinical symptoms, the strongest weight is given to neurogenic orthostatic hypotension, subthreshold parkinsonism, and OD. The absence of OD carries the strongest negative predictive weight among the prodromal markers. The criteria define the probability for pPD over 50% as possible pPD and over 80% as probable pPD.

Due to the development of biomarkers, particularly genetic, imaging, and αSyn-SAA in combination with the wish to investigate early risk factors, track and stage the development of manifest and prodromal LBD for drug development, two new biological classification systems SynNeurGe[132] and Neuronal α-Synuclein Disease Integrated Staging System (NSD-ISS)[133], have been proposed. Both classification systems incorporate clinical symptoms, αSyn-SAA, DAT imaging/neurodegeneration, and genetic testing. NSD-ISS is also a staging system from early prodromal to late manifest stages.

1.6 Selected Current Biomarkers in Lewy Body Disease

Imaging and neurophysiological biomarkers play an important role in the clinical diagnosis of DLB and PD, as well as in prodromal research. This section will describe some of the biomarkers for LBD.

1.6.1 DAT Imaging

DAT imaging, such as ^{123I}FP-CIT SPECT or ^{18}F-FE-PE2I PET, can establish a marked reduction of striatal uptake reflecting underlying nigrostriatal denervation in PD and in approximately 80-90% of DLB cases[134]. This modality can assist with high specificity in differentiating DLB from AD, although it does not distinguish LBD from other Parkinsonian syndromes such as MSA or PSP[135]. Clinical parkinsonism generally emerges once DAT binding is reduced by ~45-80%[112,132,136]). For this reason, DAT imaging has been employed in large prospective cohorts such as PPMI and PARS to identify individuals at risk of developing parkinsonism before symptom onset, although the sensitivity is markedly lower even in the late prodromal stages such as MCI-LB[137]. Other limitations to DAT imaging are that some types can be influenced by antidepressant drugs, and specific neurovascular insults, along with normal pressure hydrocephalus, can challenge the interpretation[138–140].

1.6.2 Cardiac ^{123}I-MIBG Scintigraphy

Cardiac ^{123}I-MIBG scintigraphy can demonstrate reduced sympathetic innervation in DLB (in contrast to AD), PD, and in prodromal LBD, particularly in patients with autonomic dysfunction. The limitations are that interpretation may be influenced by several comorbidities and medications, and a lower sensitivity in PD or patients less affected by autonomic dysfunction[134,141].

1.6.3 PSG

Video polysomnography (PSG)-confirmed RBD, defined by REM sleep without atonia, is also considered an indicative biomarker and is highly specific for manifest and prodromal α-synucleinopathies,

including MSA[115,117,118]. Nevertheless, the method is resource-intensive, may be impractical in patients with dementia, and can be influenced by comorbid sleep disorders and medication[142].

1.6.4 FDG-PET and Other Biomarkers

Among supportive biomarkers, occipital hypometabolism with the characteristic "cingulate island" sign on FDG-PET is useful in the differential diagnosis of dementia, though less applicable in prodromal disease stages with less cognitive dysfunction or DLB cases with substantial AD pathology[134,143]. Imaging for cholinergic deficits and αSyn PET are emerging as promising biomarkers, but they will not be further addressed in this thesis, as they are not in clinical use, or as α-Synuclein PET still lacks precision for LBD. Structural scans, especially cerebral MRI, are used diagnostically to exclude other pathologies and assess the medial temporal lobe atrophy, while other novel modalities may in the future support the antemortem diagnosis[134].

Although ^{123}I-MIBG cardiac scintigraphy, DAT imaging, and PSG may detect a pathological presymptomatic orthostatic hypotension, parkinsonism, or RBD, these biomarkers share the limitation that they primarily reflect neurodegenerative changes. Such changes include sympathetic cardiac denervation, degeneration of the substantia nigra, or other brainstem structures. These neurodegenerative processes are likely downstream consequences of the accumulation of αSynD. A biomarker capable of predicting neurodegeneration before substantial and irreversible damage has occurred and propagated would enable earlier therapeutic intervention. Particularly valuable would be detecting pathology before its spread into the CNS, where targeted interventions are more challenging.

1.7 Seed Amplification Assay

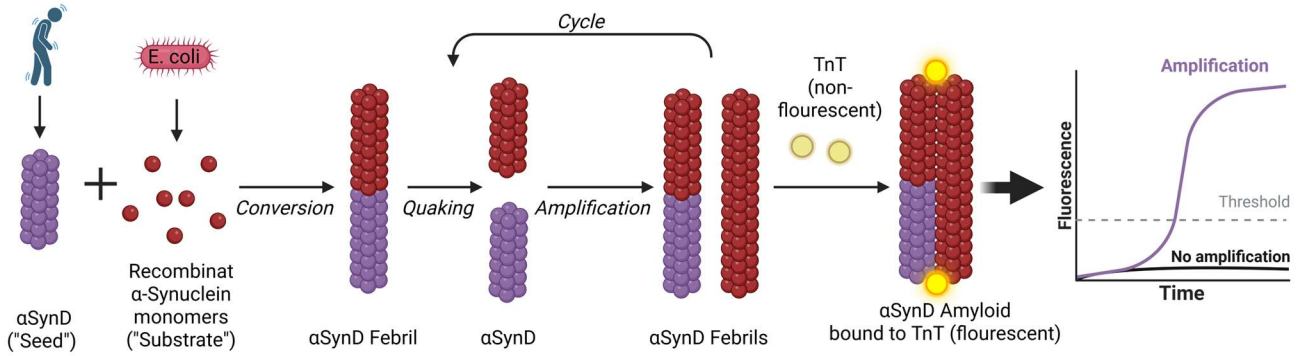
In this section, I will outline the development of SAA, followed by studies with αSyn-SAA in different biospecimens (CSF, skin, OM, and others), then the technical challenges, and finally cover kinetic parameters and quantitative αSyn-SAA.

1.7.1 SAA Development

SAAs were first developed in the early 2000s as protein misfolding cyclic amplification to detect the minute amounts of misfolded prion protein associated with Creutzfeldt–Jakob disease[144]. Protein misfolding cyclic amplification exploits the ability of misfolded prion-like proteins to template the conversion of normal proteins into protein aggregates. Repeated cycles of incubation and sonication promote protein aggregation and fragmentation, enabling exponential amplification from minute seed amounts. In 2011, a novel SAA called real-time quaking-induced conversion (RT-QuIC) was

developed as a refinement and less resource-demanding SAA, employing shaking ("quaking") for fragmentation and using real-time fluorescence detection instead of end-point immunoblotting[24]. The SAA principle was subsequently extended to other prion-like proteinopathies, including α-synucleinopathy with high sensitivity for PD and DLB but low for MSA, first reported in 2016[125,145] (Figure B6). The core methodology remains the same; however, the choice of substrate differs across proteinopathies, e.g., recombinant α-synuclein in αSyn-SAA compared to recombinant prion protein or tau.

Figure B6 Principals of α-Synuclein RT-QuIC



Created in Biorender.
Abbreviations: αSynD = Pathological self-aggregating α-Synuclein; TnT = Thioflavin T

1.7.2 CSF αSyn-SAA

Over the years, αSyn-SAA has emerged as a tool for detecting α-synuclein pathology in CSF and peripheral biospecimens. CSF αSyn-SAA has been widely studied in PD and, to a lesser extent, in DLB[24,146], and is recommended by the American Food and Drug Administration for clinical trials[147] as well as incorporated into the novel biological classifications of LBD[132,133]. Table B6 provides an overview of the diagnostic accuracy of αSyn-SAA in manifest LBD. Importantly, CSF αSyn-SAA studies indicate that assay sensitivity is influenced by the localization and stage of pathology. Lower sensitivities are found in early disease, when LB pathology is confined to the olfactory bulb (35-50%), limbic regions (60-70%), or brainstem (~70%), compared to near 100% sensitivity in cases with cortical involvement[148,149]. Furthermore, AD with amygdala-dominant LB pathology has seemed only to produce positive CSF αSyn-SAA in 16-50% of cases[66]. The sensitivity is also lower in clinically diagnosed DLB, with values as low as 64% but typically 72–90%[150–152], in contrast to neuropathologically confirmed cases that typically approach 100%[146]. The cause of these variations is not yet understood and could potentially be explained by the load of αSynD pathology, variations in the release of αSynD into the CSF, different strains, and co-seeding.

Besides AD, αSyn-SAA has also yielded some positive results in other parkinsonian syndromes such as MSA, as well as in a few cases of the tauopathies PSP and CBD. These positive findings may reflect

cross-seeding, e.g., from MSA-related αSynD or, given the correlation with LBD-related symptoms such as OD and RBD, may also indicate co-existing LB pathology[61,153]. In prodromal LBD, Iranzo et al.[154] demonstrated a strong correlation between CSF αSyn-SAA positivity and phenoconversion to manifest LBD in individuals with iRBD. Most phenoconverts were already positive at baseline, whereas others converted to positivity only upon subsequent CSF sampling, indicating that αSyn-SAA may detect pathology at different stages of disease progression.

Technically, CSF is relatively uncomplicated to use for αSyn-SAA as it can be used directly, although there are some potential inhibitory effects by other substances, e.g., proteins, lipids, and in some cases minute amounts of blood[24,155]. Even though assay parameters vary, there is generally a good correspondence between laboratories in dichotomous outcomes but not kinetic parameters[156]. CSF αSyn-SAA is currently the assay closest to clinical implementation for diagnostic and trial use, as indicated by the Food and Drug Administration recommendation. However, due to variability in reported accuracies across studies, further validation and standardization remain necessary, particularly in DLB, AD, and prodromal LBD.

Table B6 αSyn-SAA in different Biospecimens for manifest LBD

Biospecimen	Sensitivity	Specificity vs HC	Specificity vs AD	Notes
Brain[157–159]	~100%	100%	95–100%	Postmortem tissue. Mostly used for assay validation.
CSF[24,146,153]	72–100%	~95%	~85–95%	Lumbar puncture with some contraindications and few serious adverse events. May require prior structural scan. Sensitivity varies depending on the pathological stage.
Skin[24,146,153,160]	75-96%	90–96%	70%*	Minimally invasive punch biopsy with no contraindications and no serious adverse events. Sparse data on AD and early prodromal LBD.
OM[77,152,161,162]	46–86%	~94%	~75–85%	Minimally invasive but requires endoscopy. Sampling variability can affect results. Theoretically positive in early prodromal LBD.
Saliva[163,164]	~75%	78–98%	NA	Non-invasive. Variable results and sparse reproducibility.
GI[165–167]	20–100%	96–100%	NA	Invasive, requires endoscopic biopsy. Affected by the segment sampled. Sparse reproducibility.
Blood[168–172]	80-100%	92–100%	84%	Minimally invasive and very accessible biospecimen. Substantial reproducibility problems.

** Only based on one study with 17 clinical AD patients[160]*
AD = Alzheimer's disease; CSF = Cerebrospinal fluid; GI = Gastrointestinal tract; HC = Healthy control; LBD = Lewy body disease; NA = not applicable; OB = Olfactory bulb; OM = Olfactory mucosa.

1.7.3 Skin αSyn-SAA

Skin αSyn-SAA has mostly been studied in PD and iRBD, with a few studies of DLB. The sensitivity and specificity parallel that of CSF in meta-studies[146,153], but few studies have been performed on prodromal stages of LBD besides RBD, where the sensitivity ranges from 90-98%, with higher sensitivities for participants with hyposmia, abnormal DAT-imaging, dysautonomia[173]. The anatomical site of the skin biopsy has been investigated with some indications of a slightly higher positivity in cervical sites compared to more distal sites like the leg or arm[126,174,175], although this may depend on the phenotype[176]. The reliability of skin αSyn-SAA has not yet been systematically investigated in prodromal models other than iRBD, where it has shown a 99% agreement with CSF[173]. Furthermore, an immunohistological staining study of patients with PAF demonstrated cutaneous nerves with intraneuronal phosphorylated α-synuclein in 100% of cases compared to none in HC [177], while another study found phosphorylated α-synuclein in only around 50% of PD patients[178]. Both immunohistochemical staining and αSyn-SAA are accepted methods for the biological anchors in SynNeurGe[132]. Immunohistochemistry allows confirmation of the localization of αSynD and phosphorylated α-synuclein within autonomic nerve fibers, but has a variable sensitivity, requires multiple biopsies, and labor-intensive staining procedures compared to αSyn-SAA, which is the reason that RT-QuIC represents a more feasible and scalable biomarker approach[176,179].

1.7.4 Olfactory Mucosa αSyn-SAA

OM αSyn-SAA is interesting for the detection of αSynD in the early phase due to the early accumulation of LB pathology in the olfactory bulb[36,38,42], while CSF αSyn-SAA shows low sensitivity when αSynD is confined to this region[148]. SAA for prion disease has shown high accuracy, close to 100% in OM [180]. However, several studies of OM αSyn-SAA in manifest LBD and iRBD have found a varying sensitivity of 44–83%[77,152,161,181,182]. Potential reasons for the varying sensitivities specific to OM sampling may be: 1) sampling site – Bongianni et al found a higher sensitivity in the agger nasi area than the middle turbinate[77]; 2) inhibiting factors – Kuzkina et al.[161] spiked the extracellular matrix/mucus from the nasal swabs with LBD brain homogenate and found that the mucus inhibited the seed amplification; and 3) nasal swabs type and processing. There is sparse data on OM αSyn-SAA in AD[152], and no studies on prodromal LBD besides a single study on iRBD with a sensitivity of 44%.

1.7.5 αSyn-SAA in other Biospecimens

Other biospecimens of interest for αSyn-SAA include easily accessible, minimally/non-invasive samples such as saliva, urine, and blood, which hold clinical potential. In addition, biospecimens that may shed light on the anatomical propagation of αSynD, such as tissue from the GI tract, are likely to be more of academic and mechanistic interest.

Saliva αSyn-SAA represents a non-invasive biospecimen of interest, as αSyn-SAA has shown positive results in submandibular salivary glands[183] and oral mucosa[184]. However, only a few groups have so far had success with saliva αSyn-SAA[163,164,185].

Blood-based αSyn-SAA is particularly attractive because blood sampling is routinely performed in memory and movement disorder clinics, and many laboratories maintain blood biobanks. However, a major challenge is that the complex composition of blood, containing abundant αSyn, lipids, and other interacting molecules, may interfere with seeding reactions[24]. To date, a few groups have successfully applied αSyn-SAA in blood using different strategies: (1) Kluge et al. isolated extracellular vesicles, including neuron-derived vesicles, from serum and performed SAA on these fractions, achieving 100% accuracy in a non-blinded PD cohort[168]; (2) Okuzumi et al. used immunoprecipitation to isolate αSynD seeds prior to RT-QuIC, yielding an overall accuracy of 86% in a partly blinded LBD cohort compared with HCs and tauopathies[171]; and (3) Kuang et al. removed lipid components before αSyn-SAA, achieving 89% accuracy in a blinded PD versus healthy control cohort [172]. Despite considerable interest, blood-based αSyn-SAA struggles with reproduction[186].

Urine, representing the filtrate of blood, has not previously been evaluated using αSyn-SAA. However, SAA applied to prion diseases has demonstrated very high diagnostic accuracy[187].

GI αSyn-SAA is particularly interesting, given the hypothesis that αSynD pathology may originate in the ENS and the fact that some LBD experience early GI symptoms. Research in this area remains limited, and sampling has been performed from different segments, including the stomach, duodenum, colon, and rectum. This heterogeneity in sampling sites likely contributes to the wide range of reported sensitivities[165–167].

1.7.6 Other Technical Challenges in αSyn-SAA

αSyn-SAA is an ultrasensitive technique, but it is also sensitive to a variety of preanalytical and analytical factors, which may explain some of the differences between studies[27,70]. Listed below are primary sources of potential variation:

1) The α-Syn substrate used is probably one of the most substantial factors. Some groups employ different recombinant α-synuclein produced in-house, while others rely on costly commercial recombinant protein. The substrate can differ in purity, folding state, amino acid composition, and aggregation propensity[125,158,188].
2) The sampling method and anatomical source. Most studies sample from live participants, while some studies have used cadavers. CSF sampling does not vary, except for potential circadian diversities, but other biospecimens like skin, OM, and GI may vary in anatomical site and sample size.

3) Storage duration, freeze-thaw cycles, and the need for lysis of solid tissues (unlike CSF) can influence assay[155,157,172].
4) Inhibitory compounds in the biospecimen affecting the seeding process, e.g., in CSF, saliva, and OM, higher volumes of material decrease fluorescent activity. This is especially challenging in blood-based SAA.
5) Assay reaction mixture, where solvent conditions (ionic strength, pH, presence of detergents) affect aSyn substrate aggregation kinetics, and assay readout[155,157,172].
6) Pre-analytical isolation of αSynD seeds, e.g., by immunoprecipitation or by isolation of extracellular vesicles.
7) SAA method (Protein misfolding cyclic amplification versus RT-QuIC) and in the number of replicates (wells) per sample, with most set-ups run in triplicate or quadruplicate. The definition of positivity is not standardized: Thresholds can be set at several standard deviations above negative or healthy controls, and criteria for positivity vary (e.g., 2/3 wells, 3/3 wells with rerun of 2/3, 2/4, 3/4 positive wells or algorithm-based)[156]. Furthermore, temperature, assay duration, shaking/rest cycles, and the type of beads used to promote mechanical shearing differ across protocols and can affect both sensitivity and specificity.

Together, these technical variations complicate direct comparisons between studies and underscore the need for methodological harmonization to ensure reproducibility and comparability of SAA results across centers. Conversely, the sensitivity of the αSyn-SAA to a wide range of compounds can be leveraged to investigate factors influencing aggregation as well as a method to screen potential aggregation-inhibiting drugs[189,190].

1.7.7 Kinetic Seeding Parameters and Quantitative αSyn-SAA

Besides testing inhibitory agents, quantitative or kinetic parameters αSyn-SAAs have been explored as a tool for monitoring prodromal disease and proposed as a potential for evaluating disease-modifying treatments, although the field is still very new, and replication of quantitative results is sparse[153,156]. There are generally two main approaches to continuous measures in αSyn-SAA: 1) An approach correlating kinetic αSyn-SAA parameters to disease, stage, symptoms, or longitudinal, and 2) a pseudoquantitative approach with endpoint dilution of the seed until only 50% of the wells cross the threshold. The former provides continuous variables that can be compared within or across groups or time, whereas the latter yields a measure of seeding dose or "seeding capacity", analogous to a titration method, but this is also more resource demanding.

Kinetic αSyn-SAA parameters have primarily been investigated in CSF and PD focusing on: 1) number of positive wells; 2) the aggregation speed (measured by time-to-threshold, time-to-50% of maximal

fluorescent (TH50), and slope of fluorescent increase (Vmax); 3) maximal fluorescent intensity (Fmax); and 4) the fluorescent AUC (see section 3.4.1 Figure M3 for definitions). Especially, time-to-threshold has been found to correlate with clinical parameters like MDS-UPDRS-III, MoCA, and Hoehn and Yahr stage[153]. A new study based on the PPMI cohort also found a correlation between time-to-threshold in CSF αSyn-SAA and phenoconversion in prodromal cohorts of OD, iRBD, and genetic prodromal PD[128]. In skin, a few studies have investigated the kinetic parameters, with some not finding any correlations[191], while another study[174] found the number of positive wells, time-to-threshold, TH50, and Fmax correlated with disease stage in PD participants. A study comparing skin αSyn-SAA in iRBD and PD showed faster seeding in iRBD, likely reflecting a higher peripheral αSynD burden in RBD-associated phenotypes. This was supported by faster seeding in PD patients with suspected RBD than in those without[175]. A challenge to quantitative and kinetic parameters αSyn-SAA in CSF may be that the seeding kinetics do not develop progressively at later stages of the disease[175,182].

1.8 Preventing LBD and Gaps of Knowledge

LBD is a complex, progressive disorder characterized by a wide range of clinical symptoms that often develop years before the diagnosis of PD or DLB. Beyond RBD, many prodromal symptoms are non-specific and may arise from other conditions, complicating early diagnosis. A biomarker capable of detecting the pathological hallmark of LBD in the early or even preclinical stages would substantially improve our understanding of the underlying pathophysiology and support the development of preventive and disease-modifying strategies.

Earlier intervention is likely to be more effective due to limited pathology and less irreversible neurodegeneration, although early detection of LB pathology also presents substantial ethical considerations[24,192]. Prevention can be considered at three levels. Primary prevention targets the general population by reducing exposure to modifiable risk factors such as pesticides, pollution, and microorganisms. If αSyn-SAA can detect early pathological changes, it may help link such exposures to αSynD initiation. Secondary prevention focuses on individuals at increased risk, including those with prodromal symptoms (iRBD, iOD, PAF) or genetic predisposition. αSyn-SAA could aid with the identification of participants with αSynD for clinical trials and potentially for post-treatment evaluation. Tertiary prevention targets manifest LBD to slow progression, where αSyn-SAA could serve both for patient selection and potentially as a surrogate marker of treatment response, like amyloid PET in AD. In order

to apply αSynD as a biomarker, its limitations and accuracies in different phenotypes and disease stages must be investigated.

Despite increased focus on disease-modifying therapies, most research has centered on PD, while DLB studies largely explore repurposed drugs[193]. Current approaches include αSyn-targeted immunotherapies, reduction of αSyn production or enhancement of clearance, inhibition of misfolding, anti-inflammatory treatments, and neuroprotective strategies[19,194,195]. Although antibody-based therapies have yet to show consistent benefits in primary outcomes, post hoc analyses suggest possible effects in subgroups with RBD, cognitive impairment, and autonomic dysfunction – features consistent with the proposed Body-First or S3 phenotype[196]. This highlights the importance of considering phenotype, including clinical presentation and αSynD propagation pattern, when selecting participants who may benefit from αSyn-targeted therapies. αSyn-SAA across different biospecimens may further aid in differentiating these phenotypes.

Taken together, substantial gaps remain in our understanding of the risk factors driving the development of αSynD, along with early pathophysiological processes of LBD, the sequence of prodromal symptom development, and how prodromal models such as iOD in combination with αSyn-SAA can bridge the translational gap between early pathology and therapeutic intervention. Addressing these gaps will be essential for improving early diagnosis, developing disease-modifying treatments, and ultimately preventing manifest LBD.

2. Aims

2.1 Overall aim

The overarching objective of this project is to investigate αSynD as a biomarker for the early diagnosis of DLB and investigate iOD as a prodromal model of LBD.

2.2 Study-specific aims

Study 1: To optimize sampling, processing, and uniform αSyn-SAA protocols for detecting LBD across multiple biospecimens (CSF, skin, OM, saliva, and urine).
Hypothesis: αSyn-SAA can detect αSynD in CSF, skin, OM, saliva, and urine.

Study 2: To investigate the sensitivity, specificity, and diagnostic accuracy of αSynD-SAA for differentiating DLB from AD in CSF, skin, olfactory mucosa, and urine.
To examine associations between αSyn-SAA positivity and clinical features of DLB.
Hypothesis: αSyn-SAA is positive in patients with a DLB phenotype but not in patients with an AD phenotype.

Study 3: To investigate the prevalence of αSynD pathology, detected by αSyn-SAA, and frequency and severity of LBD symptoms in individuals with iOD and normosmic HC.
Hypothesis: Individuals with iOD have more pronounced LBD symptoms and a higher prevalence of αSynD detected by αSyn-SAA than normosmic HC.

Study 4: To investigate the frequency and severity of LBD symptoms in αSynD-negative HC, αSynD-positive iOD, and αSynD-positive DLB individuals.
To investigate quantitative differences of αSynD-SAA kinetics between skin from iOD and DLB and explore the correlations with clinical parameters.
Hypothesis: There is a stepwise increase in the frequency and severity of LBD symptoms, as well as more rapid kinetic αSyn-SAA parameters, from HC to iOD, and most pronounced in DLB.

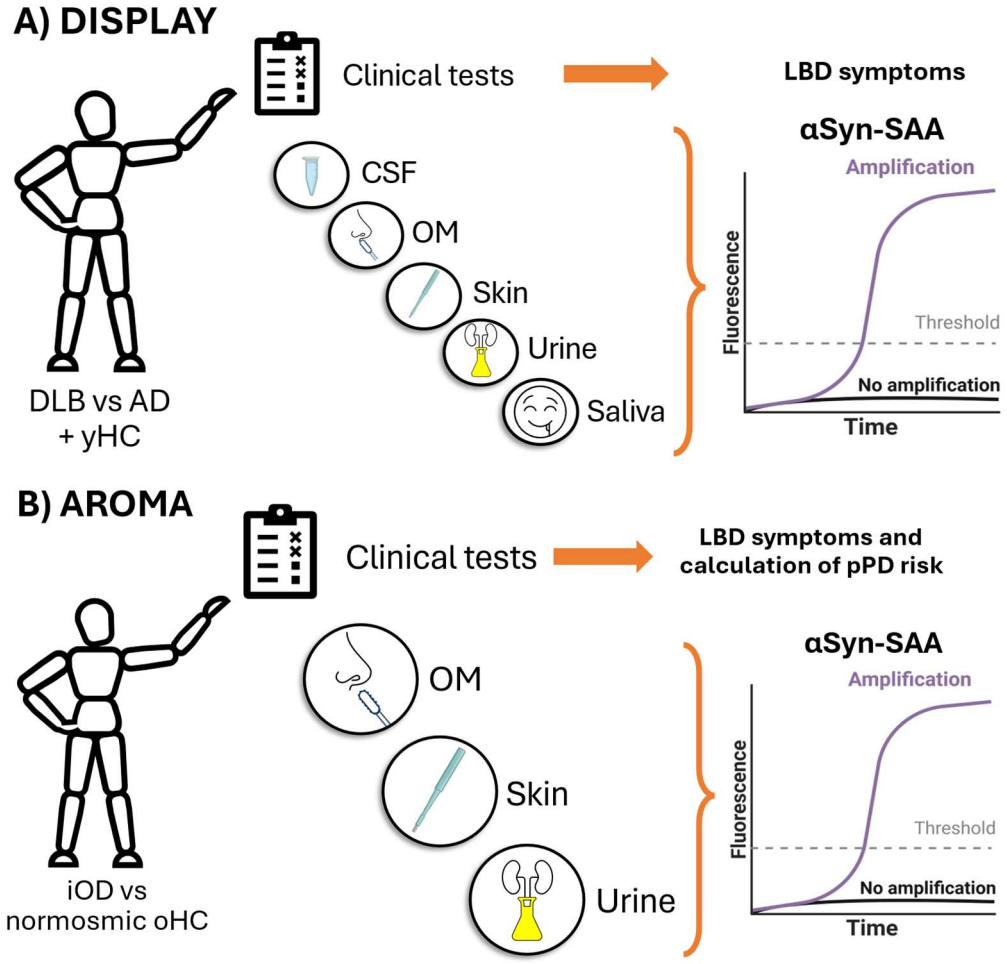
3. Methods

To answer the aims, we conducted four studies with participants from two clinical cohorts (illustrated in Figure M1):

A) DIagnostic biomarkers and Symptoms in Patients with Alzheimer's disease and Lewy bodY dementia (*DISPLAY*). This cohort focused on cognitively impaired patients with DLB or AD, who were investigated clinically and with αSyn-SAA. We also included young HC (yHC) to serve as negative controls for the development of the αSyn-SAA technique across the range of biospecimens.

B) Alpha-synuclein Rt-quic and neurologic symptoms in persons with idiOpathic anosMiA (*AROMA*). This cohort included older HC (oHC) and iOD investigated for clinical LBD features and αSyn-SAA in skin, OM, and urine.

Figure M1 The DISPLAY and AROMA Cohorts Overview



Abbreviations: AD = Alzheimer's disease; CSF = cerebrospinal fluid; DLB = Dementia with Lewy bodies; iOD = idiopathic olfactory dysfunction; LBD = Lewy body disease; oHC = older healthy controls; OM = olfactory mucosa; pPD = prodromal Parkinson's disease probability score; yHC = younger healthy controls.

3.1 Study Designs

Both DISPLAY and AROMA are cohort studies; the present analysis was cross-sectional and comparative. All participants in DISPLAY were recruited and seen by the same investigator, OHM, and most participants in AROMA were seen by OHM and some by LSNA, supervised by OHM.

Study 1 was designed to optimize the pre- and analytic factors of αSyn-SAA in DLB versus HC participants. The following parameters were evaluated and optimized: 1) Sampling method; 2) amount of biospecimen; 3) additives; 4) treatment before SAA, e.g., sonication and filtration; 5) assay parameters, especially running time. In the absence of neuropathological validation of the clinical DLB diagnosis, CSF αSyn-SAA was used as a reference/gold standard for DLB when comparing the αSyn-SAA in other biospecimens.

Study 2 was designed to investigate the accuracy of αSyn-SAA across biospecimens for the diagnosis of DLB in a clinically relevant setting.

Study 3 was designed to investigate the frequency of LBD symptoms measured by clinical scales and αSynD detected by αSyn-SAA in iOD compared with normosmic HC. The pPD criteria were us as a reference for the likelihood of having prodromal LBD.

Study 4 was designed to compare LBD symptoms between non-LBD (αSyn-SAA- HC), prodromal LBD (αSyn-SAA+ iOD), and manifest LBD (αSyn-SAA+ DLB). Furthermore, we explored the correlation between kinetic αSyn-SAA parameters and prodromal LBD symptoms.

For an overview of study participants, clinical data, and biospecimens used for αSyn-SAA in each study, see Table M1. For details on study participants, see Studies 1-4.

Table M1 Overview of studies with participants, clinical data, and biospecimens

Study	Participants	Clinical Data	Biospecimen for αSyn-SAA
1	DLB (DISPLAY) yHC (DISPLAY) oHC (AROMA)	Diagnosis, demographics, MoCA	CSF (DLB), skin, OM, urine, saliva
2	DLB (DISPLAY) AD (DISPLAY)	Diagnosis/work-up, demographics, MMSE, RBD1Q, SIT-16, MDS-UPDRS I–III, orthostatic hypotension	CSF, skin, OM, urine
3	iOD (AROMA) oHC (AROMA)	Demographics, cognitive tests (MoCA, SDMT, recall, fluency), RBDSQ, SIT-16/TDI, FM-100, MDS-UPDRS I–III, orthostatic hypotension, pPD calculation	Skin, OM
4	αSyn-SAA+* DLB (DISPLAY) αSyn-SAA+ iOD (AROMA) αSyn-SAA- oHC (AROMA)	Diagnosis, demographics, MoCA, RBD1Q, SIT-16, MDS-UPDRS I–III, orthostatic hypotension	Skin (also kinetic parameters), OM

** αSynD-SAA+ is defined as positive in skin and/or OM.*

Abbreviations: AD = Alzheimer's disease; CSF = cerebrospinal fluid; DLB = Dementia with Lewy bodies; FM-100 = Farnsworth–Munsell 100 hue test; iOD = idiopathic olfactory dysfunction; MDS-UPDRS = Movement Disorder Society Unified Parkinson's Disease Rating Scale; MoCA = Montreal Cognitive

Assessment; MMSE = Mini-Mental State Examination; oHC = older healthy controls; OM = olfactory mucosa; pPD = prodromal Parkinson's disease probability score; RBD1Q = REM Sleep Behavior Disorder Single-Question Screen; RBDSQ = REM Sleep Behavior Disorder Screening Questionnaire; SDMT = Symbol Digit Modalities Test; SIT-16 = 16-item Sniffin' Sticks odor identification test; TDI = Sniffin' Sticks Threshold-Discrimination-Identification test; yHC = younger healthy controls.

3.2 Study Participants

3.2.1 Study Participants DISPLAY

Patients diagnosed with probable AD dementia/MCI-AD[48,64] (referred to as AD) or DLB/MCI-LB[15,49] (referred to as DLB) were recruited from the Memory Clinic at Copenhagen University Hospital, Rigshospitalet, from February to October 2023.

Inclusion criteria were: 1) Mini-Mental State Examination (MMSE) score >18; 2) age >50 years; and 3) a caregiver willing and able to participate in the study. Exclusion criteria were: 1) other major neurological or psychiatric diseases; 2) ongoing alcohol or drug abuse; or 3) deemed unfit for completing the study.

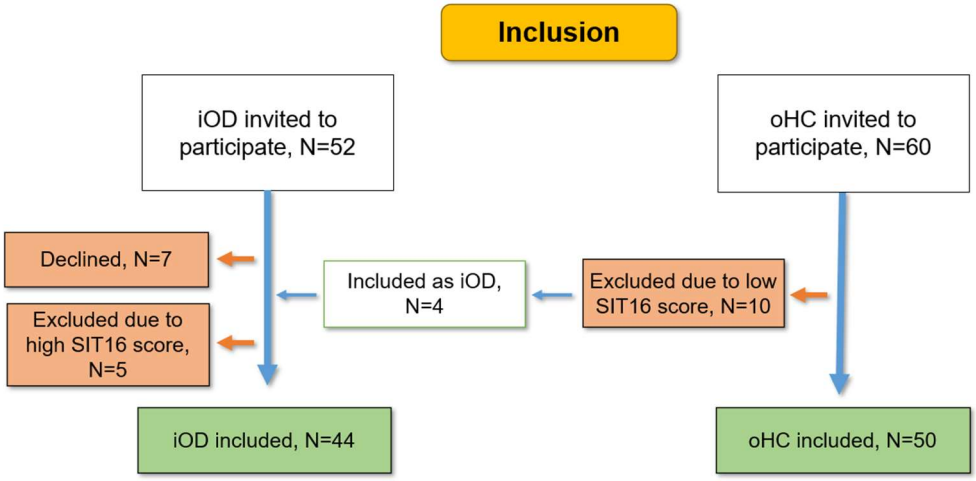
The diagnostic work-up for all patients included anamnesis, physical and neurological examination, blood screening tests, cerebral MRI/CT, FDG-PET, and, for a substantial subgroup, also neuropsychological testing and lumbar puncture (for detection of β-amyloid, p-tau-181, and total-tau). Some patients with DLB underwent polysomnography and DAT-imaging (see Study 2, Supplementary Materials Table S1). A multi-disciplinary team conference with medical doctors (within neurology, psychiatry, and geriatrics), nurses, and neuropsychologists reached a consensus on the diagnosis based on international clinical criteria[15,48,49,64]. The αSyn-SAA results were not available at the team conference.

The yHC were recruited to act as negative controls with a presumed low probability of being αSynD positive. The inclusion criteria were: 1) age 18–40 years; 2) MMSE score indicative of no cognitive impairment; and 3) no first-degree relatives with PD/DLB.

3.2.2 Study Participants AROMA

Participants with iOD were recruited from the Department of Ear, Nose, and Throat, Unit for Sense of Taste and Smell, at Rigshospitalet, private otolaryngologist practitioners, or from the assumed normosmic oHC from September 2023 to November 2024 (Figure M2). The oHC was recruited from previous studies at the Danish Dementia Research Centre, by media posts, and via a family medicine doctor.

Figure M2 AROMA Inclusion



Flow chart of participants in the study. Ten participants screened to be included as oHC were excluded due to low olfactory score (SIT16), four were referred to the Unit for Sense of Taste and Smell and diagnosed with iOD and included as such. The remaining excluded participants with OD did not wish to be referred. The figure was reproduced from Study 3.
Abbreviations: iOD = idiopathic olfactory dysfunction; N = number; oHC = older healthy controls; and SIT16 = 16-item Sniffin' Stick Identification Test.

We recruited iOD and oHC participants aged 55–75 years who did not have any significant neurological or psychiatric comorbidities. The age range was chosen to maximize the likelihood of including individuals in a prodromal stage of LBD, while at the same time minimizing the influence of age-related olfactory decline on the assessment of olfactory function. The criteria: absence of significant neurological or psychiatric comorbidities, were used to exclude participants with manifest LBD and other diseases affecting central clinical scales like MDS-UPDRS. Minor psychiatric/neurological co-morbidity, e.g., depression and minor stroke, was accepted.

OD was defined as SIT16 $\leq$11 for participants aged 55–59 years, which is equal to the cut-off at the 10th percentile and just under the 25th percentile of SIT16 in the age group 51-60 years. For participants aged over 60 years, we defined OD as a SIT16 $\leq$10, which is equal to the cut-off at the 10th percentile and just under the 25th percentile of SIT16 in the age group 61-70 years, but the 25th percentile for age 71-80[197].

We applied the following criteria for iOD: 1) noncongenital OD; 2) no temporal link to head trauma or iatrogenic treatment (surgery, medication, or radiotherapy); 3) MRI or CT excluding pathology relating to OD or progressive OD >2 years; 4) no temporal link to infection, defined by no infection within two weeks of OD onset; 5) no significant sino-nasal disease on endoscopy; and 6) no response to short-term systemic/topical corticosteroid treatment (if tried). As noted in the Background section 1.4, iOD are defined differently in studies. The criteria we used were based on previous

studies[89,90], except that MRI/CT, for practical reasons, was unnecessary if OD had been present for more than 2 years, making a tumor compressing the OT less likely.

We included oHCs with the same criteria as iOD except for OD. Participants presumed to be included as oHC, but with OD on the initial SIT16 (N=10), were asked if they wanted a diagnostic work-up for OD, and if they fulfilled iOD criteria, they were included as such (N=4) or otherwise excluded from the study. Participants with presumed iOD but with a normal SIT16 were excluded from the study. The SIT16 test only examines olfactory identification, but not discrimination and threshold, in which an individual could have a functional OD with a normal SIT16.

3.3 Clinical Assessments

We used a variety of different clinical scales and tests in the DISPLAY and AROMA cohorts (Table M2). In DISPLAY, we limited the visit to around 2.5 hours, including biospecimen collection, to avoid overloading cognitively vulnerable participants. Likewise, in AROMA, we limited the visits to 3 hours to avoid overloading vulnerable participants and discouraging participants with a busy schedule due to work and other activities. Because of the limited time, some features were assessed by screening rather than extensive scales. The descriptions of assessments are provided in Studies 1-4, but are provided here along with considerations. Disease duration in AD and DLB is difficult to assess due to memory issues combined with the heterogeneity and subtle onset of symptoms, so we chose to use the more clinically relevant time from the first visit instead.

Table M2 Overview of Clinical Assessments

Lewy body symptom	Assessment Methods
OD	SIT16, TDI* and subjective degree# and onset
Cognition	MoCA, MMSE#, SDMT*, Logical Memory subtest*, Animal Fluency*
RBD	RBD1Q, RBDSQ*
Parkinsonism	MDS-UPDRS-II, III
Fluctuations	Clinical assessment combined with medical records#
Visual hallucinations	Clinical assessment combined with medical records#
Hypersomnia	MDS-UPDRS item 1.8
Autonomic dysfunction	MDS-UPDRS item 1.10, 1.11, 1.12, 2.2
Orthostatic hypotension	Orthostatic blood pressure test
Urge incontinence	MDS-UPDRS item 1.10 and medication
Constipation	MDS-UPDRS item 1.11 and medication
Erectile dysfunction	Male sex and relevant medication*
Neuropsychiatric symptoms	MDS-UPDRS item 1.2, 1.3, 1.4
Depression	MDS-UPDRS item 1.3, medication, diagnosis
Anxiety	MDS-UPDRS item 1.4, medication, diagnosis
Color discrimination	FM-100*

** Only AROMA cohort. # Only DISPLAY cohort.*

Abbreviations: FM-100 = Farnsworth–Munsell 100 Hue Test; MDS-UPDRS = Movement Disorder Society – Unified Parkinson's Disease Rating Scale; MMSE = Mini-Mental State Examination; MoCA = Montreal

Cognitive Assessment; RBD1Q = REM Sleep Behavior Disorder Single-Question Screen; RBDSQ = REM Sleep Behavior Disorder Screening Questionnaire; SDMT = Symbol Digit Modalities Test; SIT16 = 16-Item Sniffin' Sticks Identification Test 16; TDI = Threshold, Discrimination, and Identification test.

3.3.1 Assessment of Olfactory Function

In both the DISPLAY and AROMA, the olfactory function was assessed using the Danish adaptation of SIT16[198]. It is a forced-choice test where participants identify 16 suprathreshold odors from four options. The maximum score is 16, and a score ≤8 indicates no functional sense of smell/anosmia. Each odor pen was presented for ca. 4 seconds at approximately 2 cm from both nostrils. For the DISPLAY cohort objective OD was evaluated by the 25th lower percentile of age-adjusted scores of SIT16. Additionally, in the AROMA cohort, the iOD group was also assessed with the Sniffin' Sticks Threshold and Discrimination tests[197] providing a more comprehensive olfactory score, abbreviated TDI. The TDI is the sum of scores for Threshold, Discrimination, and Identification, with a range between 1–48 points. The test was conducted according to validated protocols[197].

Subjective OD was assessed on a 3-point scale (normal, reduced, or absent), and participants with subjective OD were asked when they first noticed the reduction.

3.3.2 Assessment of Cognition Function

In the DISPLAY cohort, MMSE was used to include patients. MMSE assesses global cognitive function[199]. The MMSE is a widely used tool to measure cognitive decline in AD and DLB, but it is limited by a ceiling effect, especially in MCI[200] and LBD[201,202], compared to the Montreal cognitive assessment (MoCA), which also assesses global cognition on a scale from 0 to 30 points. The MoCA appoints an additional point to participants with ≤12 years of education. The MoCA was used in both DISPLAY and AROMA. The MDS criteria for pPD include a cognitive dimension, which warrants global cognitive deficits to the level of MCI or under 1.5 SD below the age and sex adjusted norm. Unfortunately, sufficient normative data for especially the younger participants in the AROMA cohort, corrected for educational level, was not available through a literature search. In AROMA, cognitive impairment was defined by a MoCA score ≤25, which is an abnormal test score. This threshold has shown a high sensitivity for MCI[203].

The Symbol Digit Modalities Test (SDMT) was used to assess processing speed and attention[204]. It is a reliable tool for both the detection of cognitive impairment and especially the longitudinal follow-up of PD and MCI[204,205].

To test other cognitive domains Logical Memory subtest of the Wechsler Memory Scale, Third Edition, section A+B (Recall test)[206] was used to evaluate immediate and delayed memory recall. Lastly, Animal Fluency testing was conducted to measure semantic fluency[207].

3.3.3 Assessment of Sleep Problems

In both DISPLAY and AROMA, screening for probable/possible REM Sleep Behavior Disorder was performed using the REM Sleep Behavior Disorder Single-Question Screen (RBD1Q), a single-question test, which has been reported to have a high sensitivity of 94% and specificity for RBD of 87%[208] in participants with iRBD versus controls (healthy and other sleep disorders). The RBD1Q was created as a self-administered test, but it is not validated for participants with dementia, so in the DISPLAY cohort, the question was read aloud and explained if necessary.

In the AROMA cohort, we also used the RBD screening questionnaire (RBDSQ), which includes questions that participants may be aware of themselves. The score ranges from 0 to 13, and a score above 5 is considered positive, indicating suggestive of RBD with a specificity of 84%[209]. Because PSG-confirmed RBD was unavailable for most participants, we applied the term *probable RBD* to RBD1Q-positive patients with dementia or MCI, and *possible RBD* to RBD1Q/RBDSQ-positive participants without a diagnosed neurodegenerative disorder.

Excess daytime sleepiness/hypersomnia can be evaluated with different tools. The Epworth Sleepiness Scale is generally recommended as the tool for clinical evaluation of LBD [210]. In DISPLAY and AROMA, hypersomnia was screened for with the MDS-UPDRS question 1.8 about daytime sleepiness and regarded as present if $\geq$2 (mild or higher), which means participants have spontaneously fallen asleep in monotone situations within the last week. The MDS-UPDRS question 1.8 about daytime sleepiness has shown an acceptable correlation with the Epworth Sleepiness Scale[210].

3.3.4 Assessment of Color Discrimination

Decreased color discrimination has previously been reported to develop in the prodromal phase of LBD[118]. To test this, we applied a comprehensive, sensitive, and validated test called the Farnsworth–Munsell 100 hue test (FM-100). The test consists of 85 colored squares (arranged into four rows), each representing a gradual step along the visible color spectrum. The participants were asked to arrange the squares in order of hue between two fixed anchor colors. Errors in the arrangement were plotted to generate an error score from 0 (perfect) to 1000 (completely random/no color discrimination). We used an online version available on a tablet with the maximum lighting setting. The participants had maximally 15 minutes to complete the test. Due to the difficulty of the test and the strong correlation with cognitive impairment within executive and visuospatial domains[211], the test was only used in the AROMA cohort due to the difficulty level. Decreased color discrimination is not part of the MDS pPD criteria.

3.3.5 Assessment of Other Nonmotor Symptoms

Movement Disorders Society-Unified Parkinson's Disease Rating Scale (MDS-UPDRS) Part I, along with medication, was applied to screen nonmotor LBD symptoms. A participant was rated as having urinary dysfunction if he/she reported taking drugs for urinary urge or any points on MDS-UPDRS question 1.10. Likewise, a participant was rated as having constipation if he/she reported taking drugs for constipation (fiber supplements or laxatives) or any points on MDS-UPDRS question 1.11. Severe erectile dysfunction was rated as present if male participants reported taking drugs for erectile dysfunction. If there were no drugs for erectile dysfunction, the item was rated as NA in the pPD calculation.

Orthostatic hypotension was tested with an automated blood pressure monitor. A test was deemed positive if there was a drop in systolic blood pressure of ≥20 mmHg, a drop in diastolic blood pressure of ≥10 mmHg, or a decrease in heart rate of ≥10 beats per minute within 3 minutes from the supine to the standing position[121].

For the MDS pPD calculation in Study 3, depression was classified as present if individuals exhibited a ≥3 points (moderate or higher) on the MDS-UPDRS question 1.3 depressive symptoms, or if there was a current or previous diagnosis of depression. If the patient had anxiety, it was rated as NA in the pPD calculation, as instructed in the criteria. In the other assessments, a diagnosis or any points on depressed mood or anxiety (question 1.4) or a diagnosis of depression/anxiety was taken as a sign of depressed or anxious mood.

Visual hallucinations and cognitive fluctuations were evaluated clinically and supported by medical records in DISPLAY.

For Study 4, the neuropsychiatric score was calculated as the sum of values from the questions on depression, anxiety, and apathy on the MDS-UPDRS. The dysautonomia score was calculated as the sum of values from the questions on urinary problems, constipation, light-headedness on standing, and drooling.

3.3.6 Assessment of Motor Symptoms

MDS-UPDRS II and III were used to evaluate subjective and objective motor symptoms, respectively. For Study 2 (DISPLAY cohort), MDS-UPDRS III was used to evaluate the presence of parkinsonism along with a clinical evaluation according to the diagnostic criteria for DLB[15,49]. In the AROMA cohort and for Study 4, subthreshold parkinsonism was defined as a MDS-UPDRS III score without action tremor above 6 points[129].

3.3.7 Prodromal PD Calculation

In Study 3, we used the MDS online tool "Prodromal PD Calculator" to estimate the likelihood of pPD according to the MDS criteria[129]. The tool calculates both the LR and the estimated probability of pPD. In previous studies, possible pPD has been defined as an estimated probability between 50–79%, whereas a probability of ≥80% has been used to define probable pPD[129]. See background section 1.5.4 for further details on the criteria.

3.4 Test for Pathological α-Synuclein

The same sampling procedures and αSyn-SAA set-up were used in both DISPLAY and AROMA, except for minor changes. All the biospecimens were run with the uniform αSyn-SAA set-up, but the preparation, e.g., lysis method and seeding amount, varied. The descriptions of sampling procedures and development of the uniform αSyn-SAA are described in detail in Study 1, but an overview and considerations are provided below.

3.4.1 Sampling and Processing of Biospecimens

CSF samples were collected from patients with DLB and AD (not iOD or HC) by lumbar puncture. If the participant had undergone a lumbar puncture stored at the Danish Dementia Biobank with 1.5 years of inclusion, this was used. If the participant had any contraindications for lumbar puncture, e.g., blood thinners, or did not consent, CSF was not collected. After the lumbar puncture, the CSF was centrifuged at 2000g, 4ºC, for 10 min. The centrifugated samples were aliquoted into 250 µL in polypropylene (PP) tubes and subsequently stored at -80°C until analysis. CSF samples were discarded if the total protein count was >1g/L, the erythrocyte count was >300, or the white blood cell count was >5, which could inhibit the seeding process. The CSF samples were used immediately after thawing and without any processing. In Study 1, both 7 µL and 15 µL of CSF were used for αSyn-SAA, whereas in Study 2, only samples with a volume of 15 µL were used for analysis, as this volume demonstrated higher sensitivity in Study 1 and a previous study from the same lab[212].

OM samples were collected via nasal endoscopy, collected bilaterally from the middle turbinate and the roof of the nasal cavity (agger nasi area) using either up to two fiber-based swabs (Nasopharyngeal Specimen Collection Swab, NEST®) in DISPLAY or up to four nylon-based swabs (FLOQswabs™ Copan® Diagnostics) in AROMA. The change of swab type from DISPLAY to AROMA was due to initial low sensitivity and based on experience from our collaborators in Verona, who have presented studies with higher sensitivity in DLB[152]. To reduce discomfort and improve sampling, topical anesthesia and detumescence were applied using cotton gauze placed

towards the middle turbinate and remained for at least 2 minutes prior to sampling. The nasal swabbing was performed by final-year medical students trained in the procedure by a rhinologist. The rhinologist was consulted for supervision in case of uncertainty. The technical difficulty of the sampling procedure was evaluated using a five-point scale reflecting anatomical recognizability, patient tolerance, and confidence in correct swab placement on each side. A score of 0 indicated that the procedure could not be performed, whereas a score of 5 represented flawless execution without any difficulties or uncertainties. Collected swabs were submerged in 15 mL PP tubes containing 3 mL saline solution at pH 7.4 and vortexed for one minute. All non-bloodied swabs were revortexed together in saline to produce an extra tube with OM. Blood contamination samples were discarded. To test for correct sampling of the OM and not regular respiratory epithelium without neurons, the technical difficulty of the sampling procedure was scored on a five-point scale, and we performed a cytological spot check of 19 random samples. From each participant, two αSyn-SAA analyses were conducted, with material obtained from each side when possible. The OM samples were prepared using two slightly different extraction methods. In method 1, all the material in the sample was used, and a mechanical lysis with silica beads was applied, combined with chemical lysing using sodium dodecyl sulphate. This method was applied to the first swab analyzed in DISPLAY. Method 2 was based on the method used by Bongianni et. al., with a few modifications[77]. The main differences were 1) a 10 µg inoculating loop to quantify the amount of material, and 2) instead of mechanical lysis, the cell dissociation was performed in an ultrasonic water bath. Method 2 was applied to the second swab in DISPLAY if this was available and in AROMA.

Two 3 mm punch skin biopsy samples were collected from the neck region (C7), 5 cm paramedially, after administration of local anesthetic and adrenaline. The neck area was chosen because of easy accessibility and indications of an increased sensitivity of αSynD detection in some studies[175]. The size of the skin biopsy may influence the sensitivity as the chance of sampling αSynD increases, but we chose, like most studies, to use small 3 mm samples[179], as it causes less bleeding and scarring. The biopsies were dabbed for blood on a cotton cloth, thoroughly washed with cold PBS, and stored dry at -80°C until the chemical and mechanical lysis. For participants in DISPLAY, one skin biopsy was used for αSyn-SAA, whereas for AROMA, both biopsies were homogenized into a single solution to increase the chance of detecting αSynD. The same weight-to-volume ratio was used for αSyn-SAA in DISPLAY and AROMA.

Urine samples were collected by urination into a PP container. There were no requirements for the sample material, e.g., midstream collection or restrictions from the last urination. The content was

transferred to 50 ml PP tubes and stored at -80°C. After thawing, 15 mL of urine was ultracentrifuged, resulting in a 30x concentrated sample. The freeze/thawing process caused heavy precipitation in most of the urine samples and was avoided downstream.

Saliva samples were collected after participants had fasted for a period of minimum 1 hour, abstaining from food, beverages (water excepted), and smoking according to previous protocols[213]. The participants were asked to thoroughly rinse their mouths before providing the saliva sample. Samples were collected directly into PP tubes and kept on ice until centrifuged at 2000 g for a minimum of 10 min, at 4°C. The supernatant was aliquoted and stored at -80°C until use. The saliva samples were processed and tested in multiple ways as described in study 1 (see Study 1 Additional file 1)[1].

3.4.1 α-Synuclein Seed Amplification Assay

We used a locally produced recombinant full-length human αSyn N-terminal 6x-histidine-tag substrate for the αSyn-SAA. The production was performed similarly to previous studies[125,158] and described in detail in Bsoul et al.[212]. Different batches of recombinant αSyn were used within the DISPLAY cohort, while the same batch was used for the whole of the AROMA samples. The seeding properties varied substantially as described in Bsoul et al.[212] and Study 4, but they performed equally regarding the quality of the threshold.

The assay was performed on a FLUOStar™ Omega instrument, using a black 96-well plate with a clear flat bottom, at 42ºC. Intervals of 60 seconds double orbital shaking at 400 rpm, and 60 seconds resting time were performed throughout the duration of the assay. The fluorescence intensity expressed in Relative Fluorescence Units (RFU) was measured every 45 min. The coloring in urine interfered with the fluorescence of thioflavin, and thus the RFU for urine samples was corrected for baseline RFU.

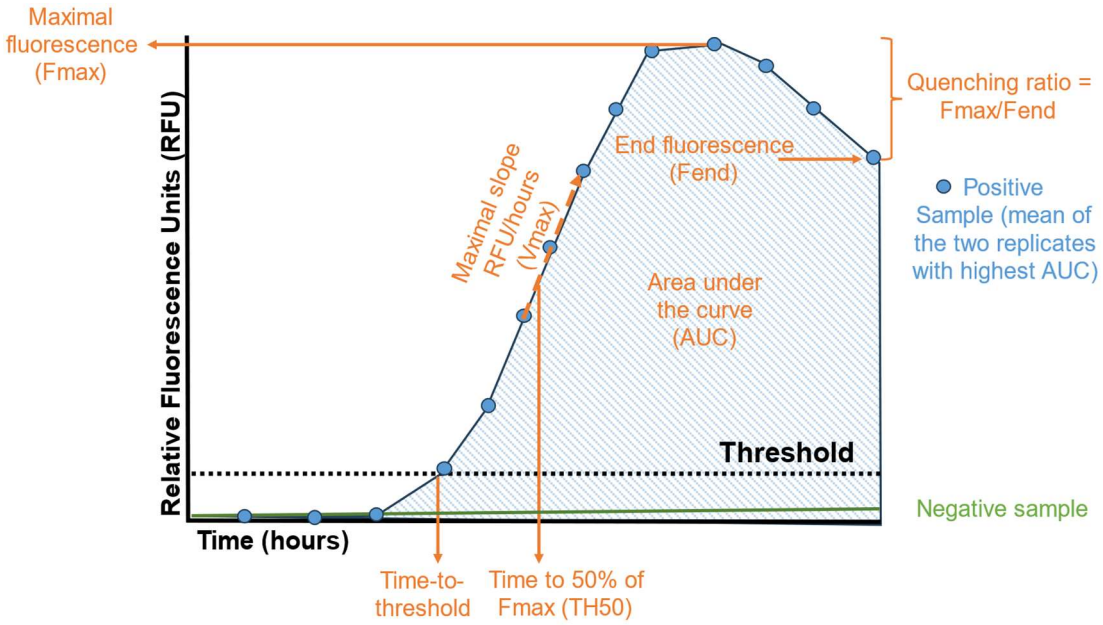
The total volume per well was 100 µL, including the sample and reaction mixture comprised of 40 mM phosphate buffer at pH 8.0, 170 mM NaCl, 10 µM Thioflavin T, 0.0015% SDS in PBS x 1, and 0.1 µg/µL of freshly thawed recombinant αSyn.

The RFU baseline was determined by averaging the fluorescence intensity of 120 wells of negative controls (60 from normal brain homogenate and 60 from unseeded reaction mixture). The positive controls used were made from DLB brain homogenate, described in detail in another study[212].

The assay was performed unblinded in the DISPLAY cohort but blinded in AROMA.

As described in Study 4, the kinetic αSyn-SAA analyses were performed on skin samples from all participants with positive results for skin αSyn-SAA. In line with previous studies of αSyn-SAA in CSF[153,214,215] and skin[175] the following kinetic parameters were analyzed, see Figure M3.

Figure M3 SAA Kinetic Parameters Overview



Visual definitions of kinetic αSyn-SAA parameters: (1) Maximum fluorescence (Fmax), representing the peak intensity of the relative fluorescence units (RFU) signal; (2) area under the curve (AUC), reflecting cumulative fluorescence over time; (3) time-to-threshold (sometimes referenced to as lag time), defined as the time in hours to the fluorescence reached the threshold; (4) TH50, the time in hours to reach 50% of Fmax; (5) maximum slope (Vmax), calculated as the highest rate of fluorescence increase across two time points (1.5 hours), to adjust for fluctuations in the fluorescence measurements; and 6) the quenching ratio was defined as Fmax divided by the last fluorescent value. Reproduced from Study 4.

3.5 Statistical Analysis

All analyses were performed using R, versions 4.3.0–4.3.3 (Studies 1–4) and GraphPad Prism 10 (Study 1). Continuous variables were compared using parametric tests (independent-samples t-test, ANOVA) when normally distributed, and non-parametric tests (Mann-Whitney-Wilcoxon rank-sum, or Kruskal-Wallis) when assumptions were not met. Categorical variables were analyzed using Fisher's exact test or chi-squared test as appropriate. Sensitivity, specificity, accuracy, PPV, and NPV (reference DLB and αSyn-SAA-positivity) were calculated with 95% confidence intervals using the Wilson method (binom package) as appropriate for the sample size. Agreement between biospecimens was assessed with Cohen's κ, interpreted according to Cohen's guidelines[216]. Odds ratios were calculated with Haldane–Anscombe correction where applicable. In Study 4, associations were assessed with Spearman's rank correlation, and regression models were used to examine relationships between SAA kinetic parameters and clinical measures, with and without adjusting for age and sex. The

accuracy AUC was calculated using ROC analysis and the Youden Index. In Study 4, the robustness of the AUC was evaluated with Leave-One-Out Cross-Validation and bootstrapping. In Study 3, both uncorrected p-values and p-values corrected for multiple comparisons using the false discovery rate method (annotated as *q-value*) were calculated. In Study 4, p-values for symptoms were corrected for multiple comparisons using Holm's post hoc correction. The level of significance was defined as a two-sided p- or q-value below 0.05. In Studies 1 and 2, p-value correction for multiple testing was not applied, as these analyses primarily concerned demographic data.

3.6 Ethics

The projects were registered on ClinicalTrials.gov (NCT05740683 and NCT05768425) prior to participant recruitment, except for the pPD calculation, which was added during recruitment for the AROMA study. Ethical approval was granted by the Capital Region Ethical Committee (H-22053428 and H-22046053), and we adhered to the Declaration of Helsinki. Personal participant information is protected by law, and the cohorts were approved by Videnscenter for Dataanmeldelse, Capital Region of Denmark (P-2022-668 and P-2022-669). All participants provided written informed consent.

Participants were not informed of their αSyn-SAA results as the use of αSynD as a biomarker is still on a research level, and the implications in participants with AD, iOD, and HC are not yet fully understood. Especially in the AROMA cohort, we acknowledge that disclosing prodromal LBD with a biological anchor such as αSynD could negatively affect the lives of participants. The participants who requested their clinical test results were provided with the information and offered a referral for further evaluation if clinically indicated.

Participants did not receive any economic compensation besides travel expenses according to the official compensation scheme in Denmark.

Lumbar puncture was optional and only performed in a limited number of cases, as most were performed for diagnostic investigation. The risk of adverse events in lumbar puncture is few and very seldom serious[217]. Skin and nasal swabbing are temporarily unpleasant but do not pose any significant serious adverse risk[182,218].

4. Summary of results

The main findings from the studies included in this thesis are summarized under each subsequent sub-heading. For more comprehensive descriptions, the full study papers and supplementary materials are available in the Appendices. Recruitment and key demographic characteristics for the DISPLAY and AROMA cohorts are presented in Table R1.

Table R1 Demographics of DISPLAY and AROMA cohorts

	DISPLAY			AROMA	
	DLB	**AD**	**yHC**	**iOD**	**oHC**
N	31 (3 MCI)	25 (3 MCI)	5	44	50
Age, mean (SD)	75 (5.9)	73 (5.2)	31 (4.8)	65 (5.5)	67 (5.0)
Sex, N female (%)	4 (12%)	11 (44%)	2 (40%)	20 (45%)	28 (56%)
Never smoker, N (%)	13 (42%)	10 (40%)	NA	21 (48%)	21 (42%)
Years of education, median (IRQ)	15 (12–17)	14 (12–16)	17 (17–17)	16 (13–17)	16 (15–17)
Depression (%)	7 (23%)	8 (32%)	NA	10 (23%)	5 (10%)
MoCA, mean (SD)	21 (4.3)	20 (4.4)	30 (1.2)*	25 (2.9)	27 (2.0)
SIT16, median (IRQ)	6 (4–8)	8 (7–10)	NA	6 (5–9)	14 (13–15)
OD onset, N (%)					
≤1 year	2 (8.3%)	2 (50%)	NA	3 (7.0%)	NA
>1–2 years	2 (8.3%)	0	NA	4 (9.3%)	NA
>2–5 years	5 (21%)	1 (25%)	NA	22 (51%)	NA
>5–10 years	3 (13%)	1 (25%)	NA	11 (26%)	NA
≥10 years	12 (50%)	0	NA	3 (7.0%)	NA

**MMSE instead of MoCA. The table was in part reproduced from Studies 1, 2, 3, and 4. In Study 1, only data on 48 oHCs were available at the time for analysis.*
Abbreviations: AD = Alzheimer's disease; DLB = Dementia with Lewy bodies; iOD = idiopathic olfactory dysfunction; MCI = mild cognitive impairment; MoCA = Montreal Cognitive Assessment; N = number; OD = olfactory dysfunction; oHC = older healthy controls; IQR = interquartile range; SD = standard deviation; SIT-16 = 16-item Sniffin' Sticks odor identification test; yHC = younger healthy controls.

4.1 Study 1

To optimize sampling, processing, and the uniform αSyn-SAA protocols, we compared data from the included participants at the time, which were 31 participants with DLB (DISPLAY) and 53 HCs (5 yHCs (DISPLAY) and 48 oHCs (AROMA)).

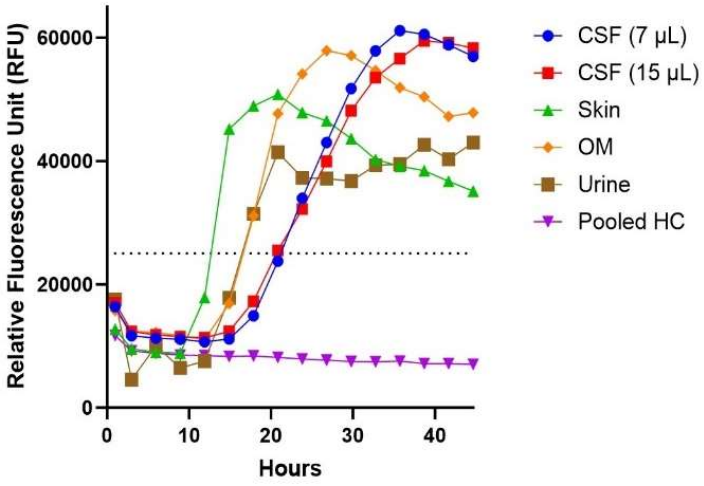
We found that CSF testing at 15 µL was positive for αSynD in 18 out of 22, 82% (CI: 61–93%) of patients with DLB, compared to 17, 77% (CI: 57-90%) DLB patients, when 7 µL CSF was used.

To benchmark the performance of different biospecimens against the most robust reference, we compared patients with DLB, who were CSF αSyn-SAA positive (N = 18/22), with HCs.

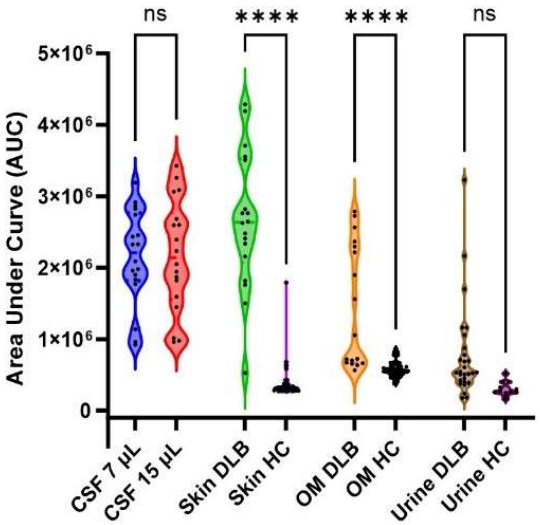
Both the αSyn-SAA in skin and OM produced fluorescence amplification signals resembling those observed in CSF, with significant differences in fluorescent AUC values compared to controls ($p<0.0001$; Figure R1). In contrast, only five urine samples from DLB patients tested positive, and the fluorescent AUC did not differ significantly from HC. The resulting sensitivities, specificities, and accuracies are summarized in Table R2. Saliva, despite variations in sample processing, additives, and αSyn-SAA protocols (see Study 1, Supplementary Materials S3), did not yield any reliable amplification signal. Due to the low sensitivity of OM αSyn-SAA, sampling, processing, and assay parameters were evaluated and optimized (see Table R3). In the optimization process, we also found that the assays were most stable within the first 48 hours. After this, replicate wells began to show spontaneous activity (data not shown).

Figure R1 αSyn-SAA Kinetics parameters for Biospecimens

A) Kinetics between biospecimens



B) AUC for the biospecimens



A) Aggregation plot across the different biospecimens from αSyn-SAA-positive DLB and from pooled HC, including varying CSF concentrations (no HC). The dotted line represents the threshold. B) Comparison of the kinetic parameter AUC across the biospecimen groups. The figure was partly reproduced from Study 1.
*Abbreviations: ns = non-significant; ****p<0.0001. AUC = Area under the curve; CSF = cerebrospinal fluid; DLB = Dementia with Lewy bodies, HC = healthy controls (young HC+ older HC); OM = olfactory mucosa*

Table R2 Performance of αSyn-SAA in Biospecimens

Biospecimens	N Samples	Sensitivity % (CI)	Specificity % (CI)	Accuracy % (CI)
Skin	18 DLB 52 HC	94 (74-99)	98 (90-100)	96 (90-100)
OM Swabs combined	17 DLB 53 HC	47 (26-69)	100 (93-100)	74 (63-84)
- OM Swab 1	17 DLB 52 HC	24 (10-47)	100 (93-100)	62 (52-71)
- OM Swab 2	8 DLB 39 HC	62 (31-86)	100 (91-100)	81 (67-95)
Urine	18 DLB 12 HC	22 (9-45)	100 (76-100)	61 (50-72)

Performance of αSyn-SAA in biospecimens for CSF αSyn-SAA positive DLB versus HC. Generally, swab 1 was treated with method 1 and swab 2 with method 2, except all oHC swabs were treated with method 2. The table was reproduced from Study 1.
Abbreviations: CI = 95% confidence interval; DLB = dementia with Lewy bodies; HC = healthy controls (young HC + older HC); OM = olfactory mucosa.

Notably, the sensitivity of OM αSyn-SAA was increased substantially between processing method 1 and method 2, rising from 24% (CI: 10-47%) to 62% (CI: 31-86%). Cytological spot checks were performed to test for the correct sampling site on 19 OM samples, of which 3 swabs (16%) contained either no cells or cells without recognizable morphology relating to OM. The remaining 16 swabs (84%) showed neuronal markers characteristic of the OM. The scores of technical difficulty were high on the five-point scale, with a median (IQR) of 4 (3-4), and did not differ between DLB and HC.
A total of 11 DLB and five yHC skin samples were repeated with 100% concordance, while the rerun of the five positive DLB urine samples was only positive in three samples, likely due to precipitation of proteins in the freeze/thaw cycle. We found that all CSF and skin samples were positive in all four wells tested (100%). This was the case for 95% of OM samples but only 40% of urine samples.

Table R3 Optimization of Olfactory Mucosa Methods

	Optimization	Evaluation/result
Sampling	Increase the number of swabs from two to four	Increased the possibility of material for dual αSyn-SAA testing
	Change of swab type to increase material retrieval and release from the swabs	Not evaluated
Processing	Measurement of the amount of material used for αSyn-SAA	Increased sensitivity in combination with other changes
	Homogenization from mechanical lysing to ultrasonic dissociation	Increased sensitivity in combination with other changes
	Additives tried to overcome the inhibition from the extracellular matrix	Did not change αSyn-SAA results
Assay parameters	Reduced running time to 48 hours (also for other biospecimens)	Reduced false positives wells.
	Dual testing of swabs	Increased sensitivity

Overview of methodological optimizations, especially applied to olfactory mucosa sampling, processing, and αSyn-SAA assay parameters.

4.2 Study 2

We included 31 patients with DLB and 25 patients with AD. Overall, the groups were comparable (Table R1) and had been referred relatively recently to the memory clinic, with a median time since first visit of 10 months (IQR 4.0–22) for DLB and 7.5 months (IQR 2.8–22) for AD. Age-adjusted OD was observed in 81% of DLB cases and 64% of AD cases (p=0.16). However, both the severity of OD (p=0.01) and the frequency of subjective OD (p<0.0001) were significantly greater in DLB (Table R1). In the DLB group, CSF was available for 24 patients, of whom 13 (54%) had an abnormal p-tau181/β-amyloid-42 ratio.

The frequency of αSyn-SAA positivity varied considerably across biospecimens, with CSF and skin showing the highest diagnostic accuracies (Table R4). Notably, one participant with DLB and five with AD were positive only in OM, but not in skin or CSF. The concordance between skin and CSF in AD and DLB was almost perfect, with a $\kappa = 0.87$ (CI 0.72–1.0). Combining CSF and skin results, or combining all biospecimens, did not improve overall diagnostic accuracy, primarily due to the CSF-skin concordance and the six (24%) AD patients testing positive in OM.

Table R4 Diagnostic accuracy of αSyn-SAA for the diagnosis of DLB versus AD

Biospecimen	Sensitivity % (CI)	Specificity % (CI)	Accuracy % (CI)
CSF	82 (61–93)	92 (75–98)	87 (77–97)
Skin	77 (60–89)	92 (75–98)	85 (76–94)
OM	40 (25–58)	76 (57–89)	58 (47–69)
Urine	17 (8–35)	100 (86–100)	59 (51–66)
CSF and skin combined	81 (64–91)	88 (70–96)	84 (75–94)
All biospecimens combined	84 (67–93)	68 (48–83)	76 (65–87)

The table was reproduced from Study 2.
Abbreviations: AD = Alzheimer's disease; CSF = cerebrospinal fluid; CI = 95% confidence intervals; DLB = dementia with Lewy bodies; OM = olfactory mucosa

When assessing the relationship between αSyn-SAA positivity and clinical features of DLB, we found that among the core diagnostic criteria, parkinsonism, cognitive fluctuations, and probable RBD showed the highest diagnostic accuracies (>79%), while visual hallucinations had a lower accuracy (Table R5). Objective OD demonstrated a very high sensitivity but poor specificity, whereas subjective OD achieved a more balanced performance with a higher overall accuracy but lower sensitivity. Among the LBD features, orthostatic hypotension, fluctuations, and visual hallucinations exhibited the highest specificities.

When analyzing the total number of core diagnostic criteria fulfilled, the presence of ≥2 criteria yielded the highest point estimate for diagnostic accuracy, although the confidence intervals substantially overlapped with those for ≥1 and ≥3 criteria.

Thirteen participants with DLB/MCI-LB received DAT imaging, of which 7/7 with abnormally low DAT signal and 3/6 with a normal/non-neurodegenerative DAT-imaging result were SAA positive in skin or CSF. In the DLB cohort, SIT16 scores did not differ significantly between participants with OM αSyn-SAApositive and negative.

Table R5 Predictive values of DLB Features for CSF αSyn-SAA positivity

CSF αSynD SAA	Sensitivity % (CI)	Specificity % (CI)	Accuracy % (CI)	OR (CI)
Fluctuations	75 (53–89)	89 (72–96)	83 (70–91)	9.8 (2.9–40)
Visual Hallucinations	50 (30–70)	89 (72–96)	72 (58–83)	2.9 (0.9–9.7)
Probable RBD	75 (53–89)	81 (63–92)	79 (65–88)	6.5 (2.0–25)
Parkinsonism	80 (58–92)	85 (68–94)	83 (70–91)	6.7 (2.0–28)
Objective OD	95 (76–99)	41 (25–59)	64 (50–76)	10 (1.8–271)
Subjective OD	80 (58–92)	78 (59–89)	79 (65–88)	5.9 (1.7–25)
Hypersomnia	70 (48–85)	67 (48–81)	68 (54–80)	3.16 (1.0–11)
Depression, anxiety, and/or apathy	60 (39–78)	33 (19–52)	45 (31–59)	0.85 (0.27–2.7)
Urge incontinence	80 (58–92)	59 (41–75)	68 (54–80)	3.8 (1.1–16)
Constipation	55 (34–74)	78 (59–89)	68 (54–80)	1.9 (0.6–6.0)
Orthostatic Hypotension	40 (22–61)	89 (72–96)	68 (54–80)	2.7 (0.8–9.6)
Core criteria ≥ 1	90 (70–97)	70 (52–84)	79 (65–88)	9.2 (2.2–70)
Core criteria ≥ 2	90 (70–97)	85 (68–94)	87 (75–94)	16 (3.8–124)
Core criteria ≥ 3	75 (53–89)	93 (77–98)	85 (72–93)	9.8 (2.9–40)
Core criteria = 4	25 (11–47)	96 (82–99)	66 (52–78)	2.6 (0.6–12)

Sensitivity, specificity, and accuracy of DLB features for αSyn-SAA positivity across both diagnostic groups (DLB, N=22, and AD, N=25). Colors indicate the relative values: Intense red <50%, Faded red = 50–64%, No color = 65–74%, Faded green = 75–84%, and Intense green >85%. The table was in part reproduced from Study 2.

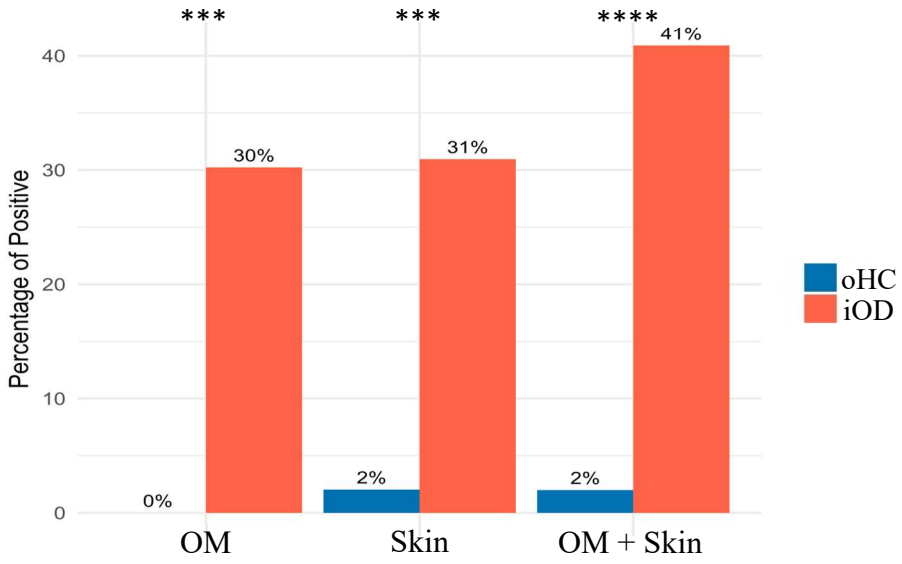
Abbreviations: AD = Alzheimer's disease; CSF = cerebrospinal fluid; CI = 95% confidence intervals; DLB = dementia with Lewy bodies; N = number; OD = olfactory dysfunction; OR = Odds Ratio; RBD = REM-sleep behavior disorder.

4.3 Study 3

To assess the prevalence of prodromal LBD symptoms and αSynD pathology in iOD, we included 44 participants with iOD and 50 with oHC. The groups were comparable in demographics (age, sex, education, smoking, and family history of PD/DLB) and medical history (e.g., DM2 and hypertension), except for a higher prevalence of anxiety in iOD (9% vs. 0%, p=0.045). The iOD αSyn-SAA positive vs negative was also comparable, but with more depression in the positive group (39% vs 12%, p=0.033) (Table R1 and Study 3, Table 1).

αSynD was detected by αSyn-SAA in 13 of iOD participants in OM and 13 in skin, compared with none and one of healthy controls, respectively (OR = 46 (CI 2.7–806) and 22 (CI 2.7–173)). Overall, 18 iOD participants were positive in at least one biospecimen, and eight (44%) of these were positive in both (Figure R2).

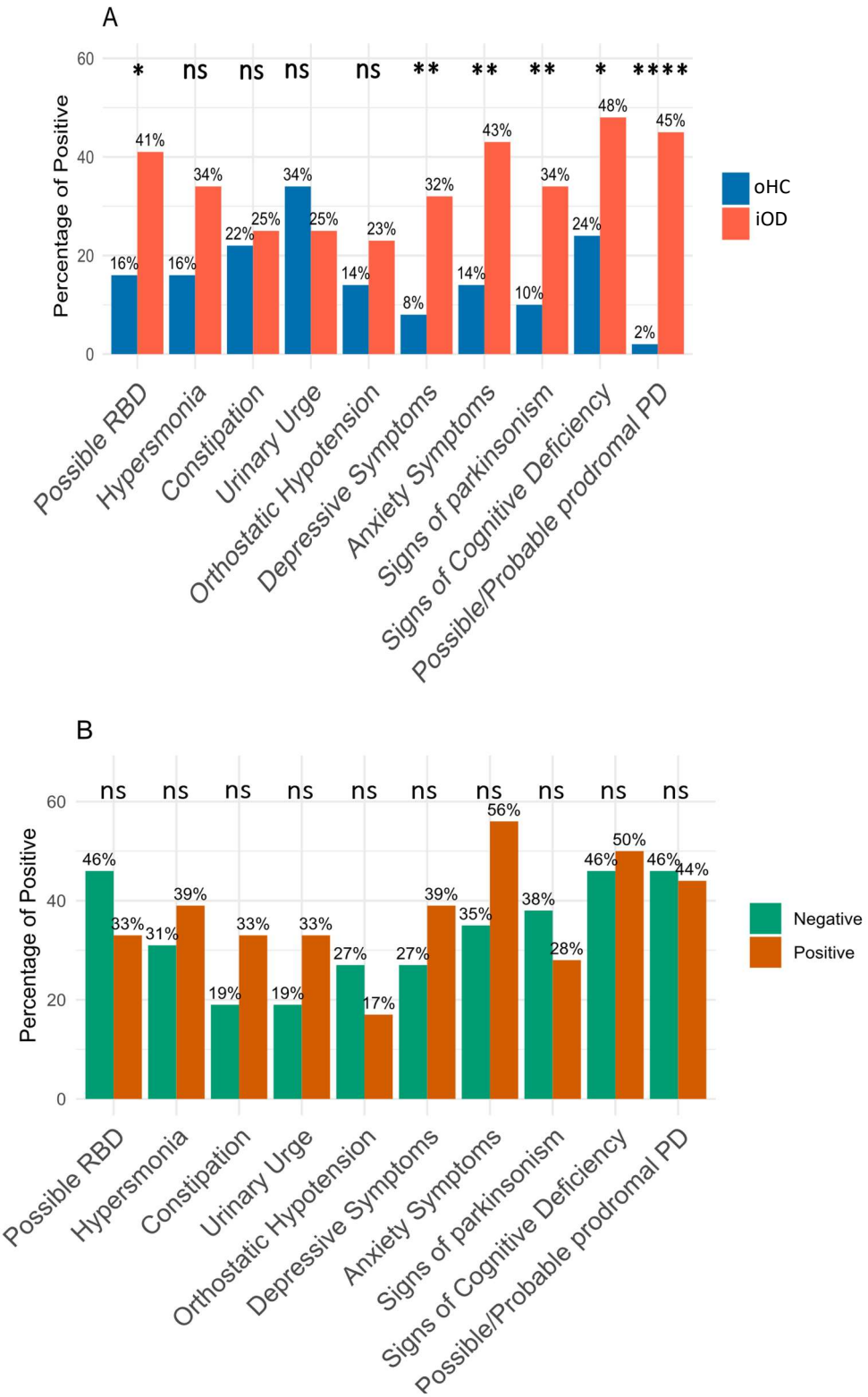
Figure R2 αSyn-SAA positivity



For seven oHCs and four iOD participants, only one swab was analyzed; in one iOD participant, no swabs were available due to blood contamination. Skin samples from one HC and two iOD participants were unavailable for analysis.
*Abbreviations: ***p<0.001; ****p<0.0001. iOD = idiopathic olfactory dysfunction; oHC = older healthy controls; OM = olfactory mucosa.*

When assessing the frequency of LBD-related symptoms in iOD and oHC, participants with iOD showed higher rates of possible RBD, neuropsychiatric symptoms, but not autonomic symptoms, compared with controls (Figure R3.A). No differences were found between the frequency of LBD-related symptoms in iOD αSyn-SAA positive vs negative (Figure R3.B, Table R6).

Figure R3 Frequency of Lewy body symptoms



A) iOD vs oHC; B) iOD αSyn-SAA positive vs negative. FDR correction did not change any significant results to non-significant. The figure was reproduced from Study 3.
*Abbreviations: ns = non-significant; *p<0.05; **p<0.01; ****p<0.0001; iOD = idiopathic olfactory dysfunction; oHC = older healthy control; PD = Parkinson's disease; possible RBD = possible rapid eye movement sleep behaviour disorder.*

When assessing the severity of LBD-related symptoms in iOD and oHC, participants with iOD performed worse on all cognitive tests (except for animal fluency) and exhibited more Parkinsonian symptoms on the MDS-UPDRS. This was primarily driven by higher scores on non-motor symptoms (Part I) and objective motor assessments (Part III), with smaller differences in subjective motor complaints (Part II). There were no significant differences between iOD αSyn-SAA-positive vs. negative, assessed by clinical scales, but the αSyn-SAA-positive had a numerically worse SDMT-score (Table R6).

Table R6 Clinical assessments associated with LBD symptoms

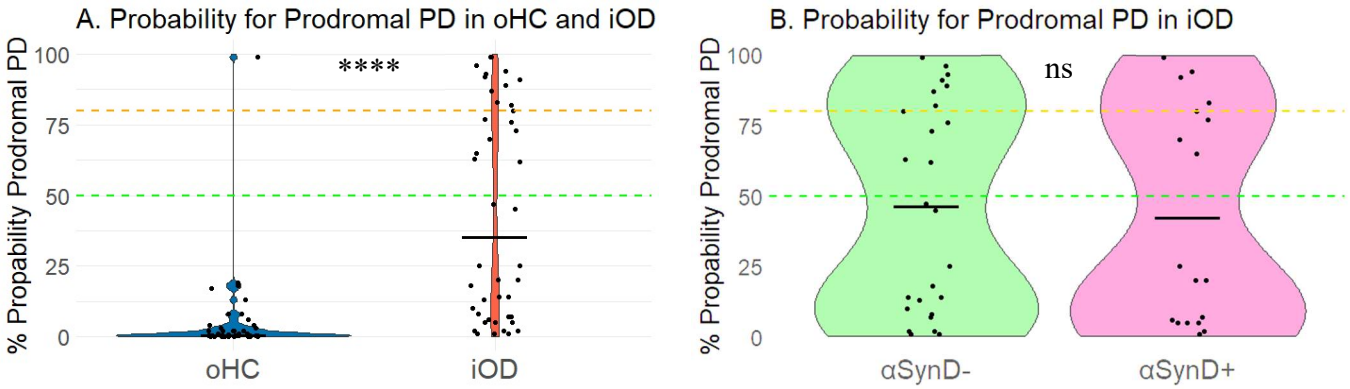
	oHC (N=50)	iOD (N=44)	q-value	αSyn-SAA- (N=26)	αSyn-SAA+ (N=18)	p-value
Age, mean years (SD)	67 (5.0)	65 (5.5)	0.11	64 (6.0)	67 (4.2)	0.057
Sex, N female (%)	28 (56%)	20 (45%)	0.31	9 (35%)	11 (61%)	0.083
SIT16, mean (SD)	14 (1.3)	6.5 (2.4)	**<0.001**	6.6 (2.5)	6.5 (2.4)	0.92
MDS-UPDRS I, mean (SD)	3.6 (2.6)	7.6 (5.3)	**<0.001**	7.2 (5.1)	8.1 (5.6)	0.57
MDS-UPDRS II, median (IRQ)	0 (0–0)	0 (0–2)	**0.005**	0 (0–2)	0 (0–3)	0.61
MDS-UPDRS III, median (IRQ)	2 (0–4)	5.5 (4–9)	**<0.001**	6 (3–9)	5 (4–7)	0.37
MDS-UPDRS I–III, median (IRQ)	5.5 (3–11)	15 (7–22)	**<0.001**	16 (7–21)	14 (7.5–24)	0.92
RBDSQ, median (IRQ)	2.0 (1.0–4.8)	3.0 (1.0–5.0)	**0.001**	4.0 (1.5–5.0)	2.0 (1.0–4.8)	0.30
MoCA, mean (SD)	27 (2)	25 (2.9)	**0.001**	26 (2.1)	25 (3.8)	0.53
SDMT, mean (SD)	47 (7.8)	43 (9.9)	**0.015**	45 (10)	39 (9.1)	0.068
Recall, mean (SD)	32 (6.7)	25 (8.7)	**<0.001**	25 (8.5)	25 (9.2)	0.92

For oHC vs iOD, q-values are presented except for age and sex, which are uncorrected p-values. For αSynD- vs αSynD+ p-values are displayed, as there were no significant differences. MDS-UPDRS I assesses subjective nonmotor symptoms, II subjective motor symptoms, and III objective motor symptoms. The table was in part reproduced from Study 3.
Abbreviations: αSyn-SAA = α-synuclein seed amplification assay; iOD = idiopathic olfactory dysfunction; IQR = interquartile range; MDS-UPDRS = Movement Disorder Society - Unified Parkinson's Disease Rating Scale; MoCA = Montreal Cognitive Assessment; N = number; oHC = older healthy controls; PD = Parkinson's disease; q-value = false discovery rate-adjusted p-value; RBDSQ = REM Sleep Behavior Disorder Screening Questionnaire; SD = standard deviation; SDMT = Symbol Digit Modalities Test; SIT16 = 16-item Sniffin' Sticks Identification Test.

The probability of pPD was higher in the iOD group compared with oHC (Figure R4), even when excluding OD in the calculation ($p<0.01$). In total, 12 (27%) iOD participants met criteria for probable pPD and 8 (18%) for possible prodromal PD, compared to 1 (2%) HC meeting criteria for probable pPD. Within iOD participants, αSyn-SAA status was not associated with significant differences in

probability of pPD (median: negative = 46% vs positive = 23%, p=0.73). Among the αSynD+ iOD participants, 8 (44%) met criteria for possible or probable prodromal PD.

Figure R4 Probability of Prodromal Parkinson's disease

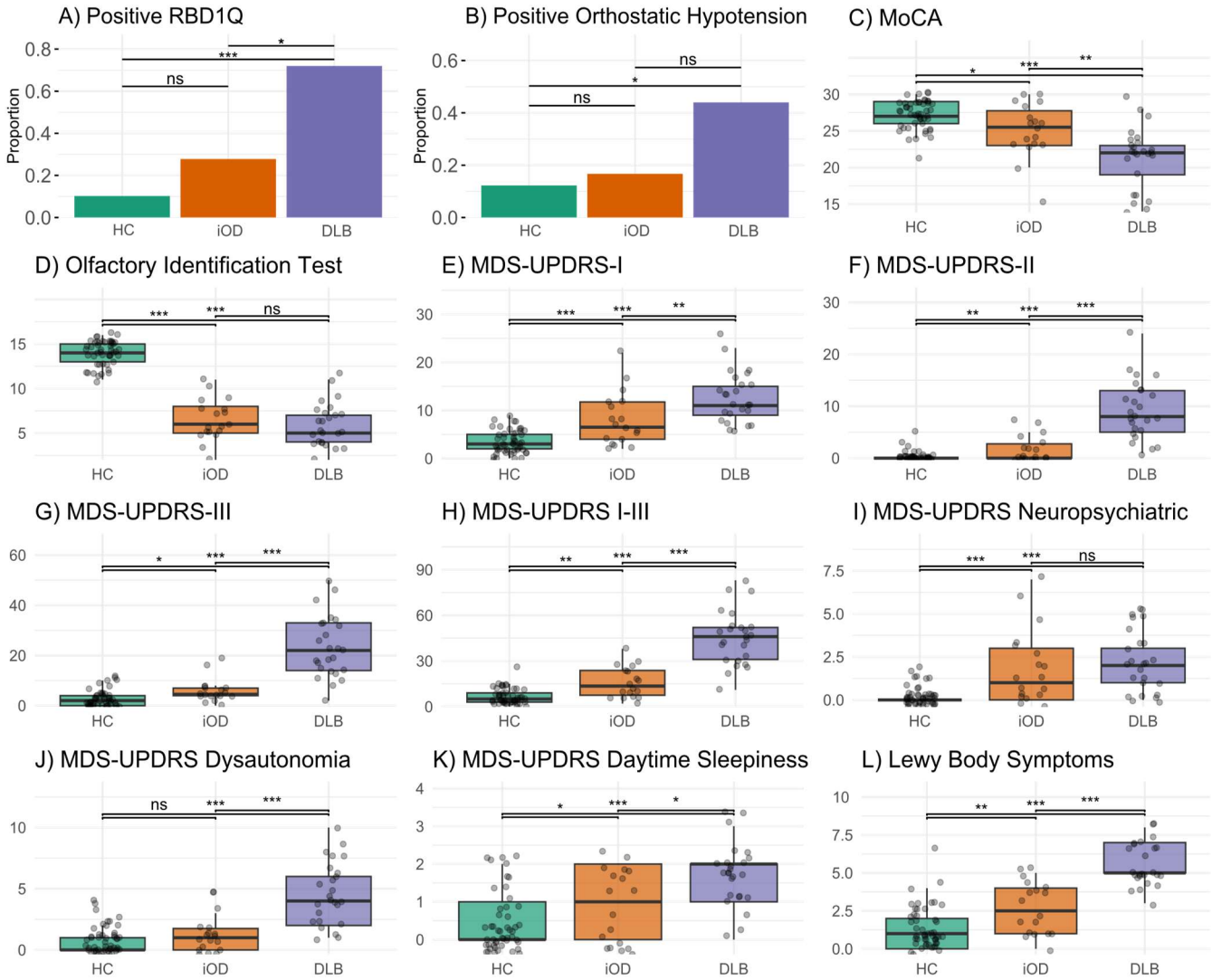


A Probability of pPD across groups calculated with the MDS pPD criteria. B: Probability of pPD comparing iOD αSyn-SAA-negative vs. positive. The dotted lines indicate possible (green) and probable (orange) prodromal PD. The figure was in part reproduced from Study 3.
*Abbreviations: ns p>0.05, **** p≤0.0001. αSyn-SAA = α-synuclein seed amplification assay; oHC = older healthy controls; iOD = idiopathic olfactory dysfunction; and pPD = prodromal Parkinson's disease*

4.4 Study 4

To examine the frequency and severity of LBD symptoms across non-LBD, assumed prodromal LBD, and manifest LBD, we included the 49 αSyn-SAA-negative oHC, 18 αSyn-SAA-positive iOD, and 25 αSyn-SAA-positive DLB participants. Symptom burden showed a stepwise increase from oHC to iOD to DLB, particularly in hypersomnia, cognitive impairment, parkinsonism, and the total number of LBD symptoms (Figure R5). In contrast, no significant differences were observed between iOD and DLB for olfactory dysfunction and neuropsychiatric symptoms, or between oHC and iOD for autonomic dysfunction.

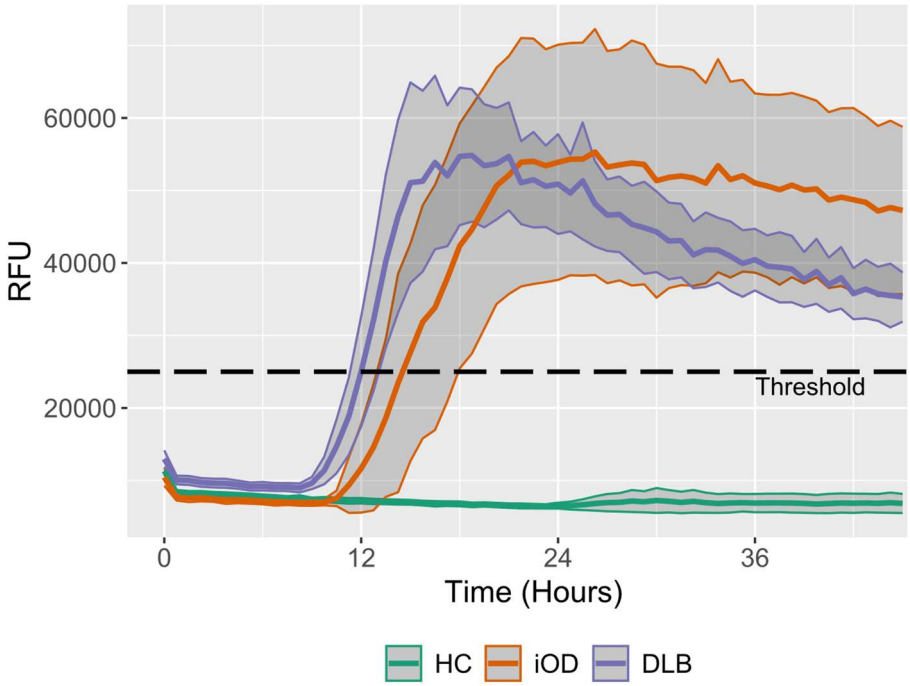
Figure R5 Clinical Assessments



Clinical assessments across groups (oHC, iOD, and DLB). The p-values are corrected pairwise with Holm's test. Reproduced from Study 4.
*Abbreviations: ns = non-significant; *p ≤ 0.05; ** p<0.01; ***p≤0.001; **** p≤0.0001. DLB = dementia with Lewy bodies; HC = (older) healthy controls; iOD = idiopathic olfactory dysfunction;, MDS-UPDRS = Movement Disorders Society unified; MoCA = Montreal Cognitive Assessment;, and RBD1Q = REM sleep behavior disorder single question.*

Kinetic αSyn-SAA parameters in skin samples from oHC, iOD, and DLB participants revealed faster seeding in DLB, as indicated by shorter time-to-threshold and TH50 values (Figure R6 and Table R7). Among the analysed parameters, TH50 provided the highest classification accuracy for distinguishing DLB from iOD, at 72% (CI: 55–89).

Figure R6 α-Synuclein Aggregation in Skin



Time plot of the αSyn-SAA from skin sample analysis with mean and CI for each group. The plot is based on the mean values from the two best-performing wells out of four. Reproduced from Study 4.
Abbreviations: DLB = Dementia with Lewy bodies;, HC = (older) healthy controls; iOD = idiopathic olfactory dysfunction; RFU = relative fluorescence unit.

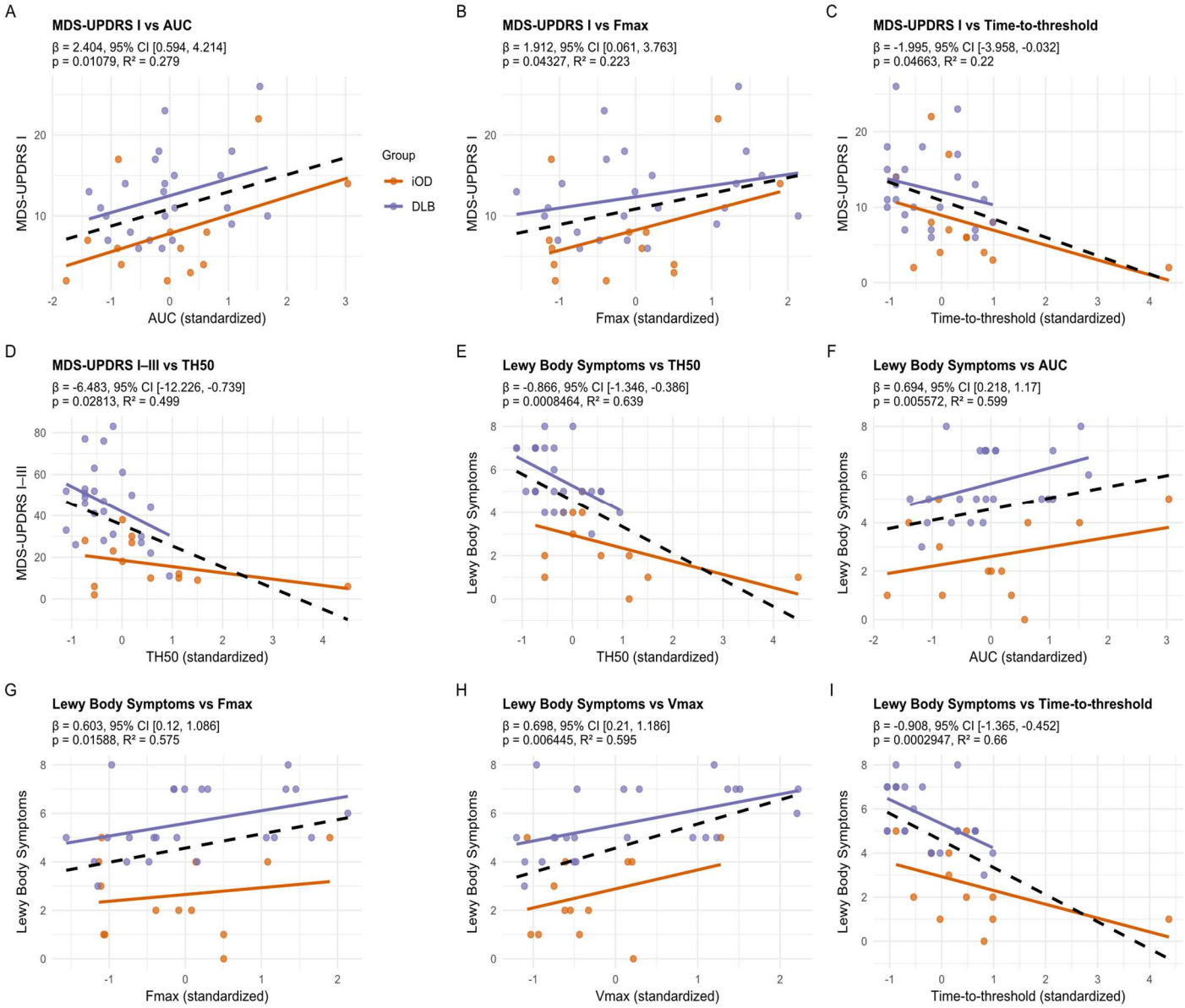
Table R7 α-Synuclein SAA Kinetic Parameters in Skin

	iOD (N=13)	DLB (N=24)	p-value
Time-to-threshold, median, (IRQ) hours	16 (14–19)	13 (11–17)	**0.029**
TH50, median (IRQ) hours	17 (15–20)	14 (13–16)	**0.017**
Vmax, median (IRQ)×10³ RFU	11 (8.8–17)	14 (8.9–26)	0.31
Fmax, median (IRQ)×10³ RFU	66 (41–81)	65 (50–96)	0.63
AUC, median (IRQ)×10⁶	1.6 (1.1–1.9)	1.6 (1.3–1.7)	0.91
Quenching Ratio, median (IRQ)	1.4 (1.3–1.4)	2.1 (1.5–2.2)	**0.0015**

Kinetic parameters for skin αSyn-SAA-positive iOD and DLB. See also Supplementary Materials Fig S1 for illustrated definitions of kinetic parameters. Reproduced from Study 4.
Abbreviations: AUC = area under the curve; DLB = Dementia with Lewy bodies; Fmax = maximal fluorescence measured; iOD = idiopathic olfactory dysfunction; IRQ = interquartile range; RFU = relative fluorescence unit; TH50 = time to 50 of Fmax; Vmax = maximal velocity of RFU change over 1.5 hours

The total number of Lewy body symptoms and MDS-UPDRS (I + I–III) correlated with the kinetic parameter (Figure R7), whereas cognitive and olfactory dysfunction were not significantly related to kinetic parameters, see Study 4 Supplementary Tables S1–S2 for full data, including non-significant associations.

Figure R7 Association of Symptoms and Kinetic Seeding Parameters



Linear regression models between clinical assessment scale scores (outcome) and αSyn-SAA kinetic parameters (predictors) in skin samples corrected for age and sex. Orange line = iOD, purple line = DLB, and dashed line represents the combined group (iOD and DLB). Only significant associations are displayed. Reproduced from Study 4.

*Abbreviations: ns non-significant; *p ≤ 0.05; ** p<0.01; ***p≤0.001; **** p≤0.0001. AUC = area under the curve; DLB = Dementia with Lewy bodies; Fmax = maximal fluorescence measured; iOD = idiopathic olfactory dysfunction; TH50 = time to 50% of Fmax; Vmax = maximal velocity of RFU change over 1.5 hours*

5. Discussion

The key findings of the studies are presented in Table D1 and can be summarized as follows:
1) αSynD can be detected by αSyn-SAA across different biospecimens with CSF and skin performing with the highest accuracy in DLB and may thus be a good clinical biomarker for DLB; and 2) iOD participants have increased frequency of LBD symptoms and αSynD. In the following, I will discuss the key findings divided into three sections: 1) αSynD detected by αSyn-SAA as a biomarker for LBD; 2) The correlation between LBD symptoms and αSyn-SAA positivity; and 3) iOD as a prodromal model for LBD. For further discussions, please refer to the study papers in the Appendix.

Table D1 Key Findings

Study	N Participants	Key Findings
1	31 DLB 5 yHC 48 oHC*	1. CSF: High sensitivity and reproducibility. 2. Skin: Slightly lower sensitivity, but high accuracy and reproducibility. 3. OM: Low sensitivity, but improved methods increased sensitivity. 4. Urine: Very low sensitivity and poor reproducibility. Saliva: Not successful.
2	31 DLB 25 AD	1. CSF and skin: High diagnostic accuracies. 2. OM: Low-medium accuracy. 3. High diagnostic accuracies of the LBD symptoms (parkinsonism, RBD, and fluctuations) for αSyn-SAA+. 4. OD: Very high sensitivity for αSyn-SAA+.
3	44 iOD 50 oHC	1. αSyn-SAA+: 41% of iOD vs 2% of oHC. 2. iOD displayed more prodromal LBD symptoms (cognitive, neuropsychiatric, motor, and RBD) than oHC. 3. 45% of iOD fulfilled pPD criteria vs. 2% of oHC. 4. Few significant clinical differences between iOD αSyn-SAA+ vs -.
4	25 αSyn-SAA+ DLB 18 αSyn-SAA+ iOD 49 αSyn-SAA- oHC	1. Stepwise increase in sleep disturbances, cognitive impairment, parkinsonism, and the number of prodromal LBD symptoms across groups (oHC → iOD → DLB). 2. No significant differences between iOD and DLB in OD and neuropsychiatric symptoms. 3. DLB showed faster seeding than iOD with the accuracy of 74% (CI: 57–91%) for TH50. 4. Some kinetic parameters correlated with MDS-UPDRS scores and LBD symptom burden.

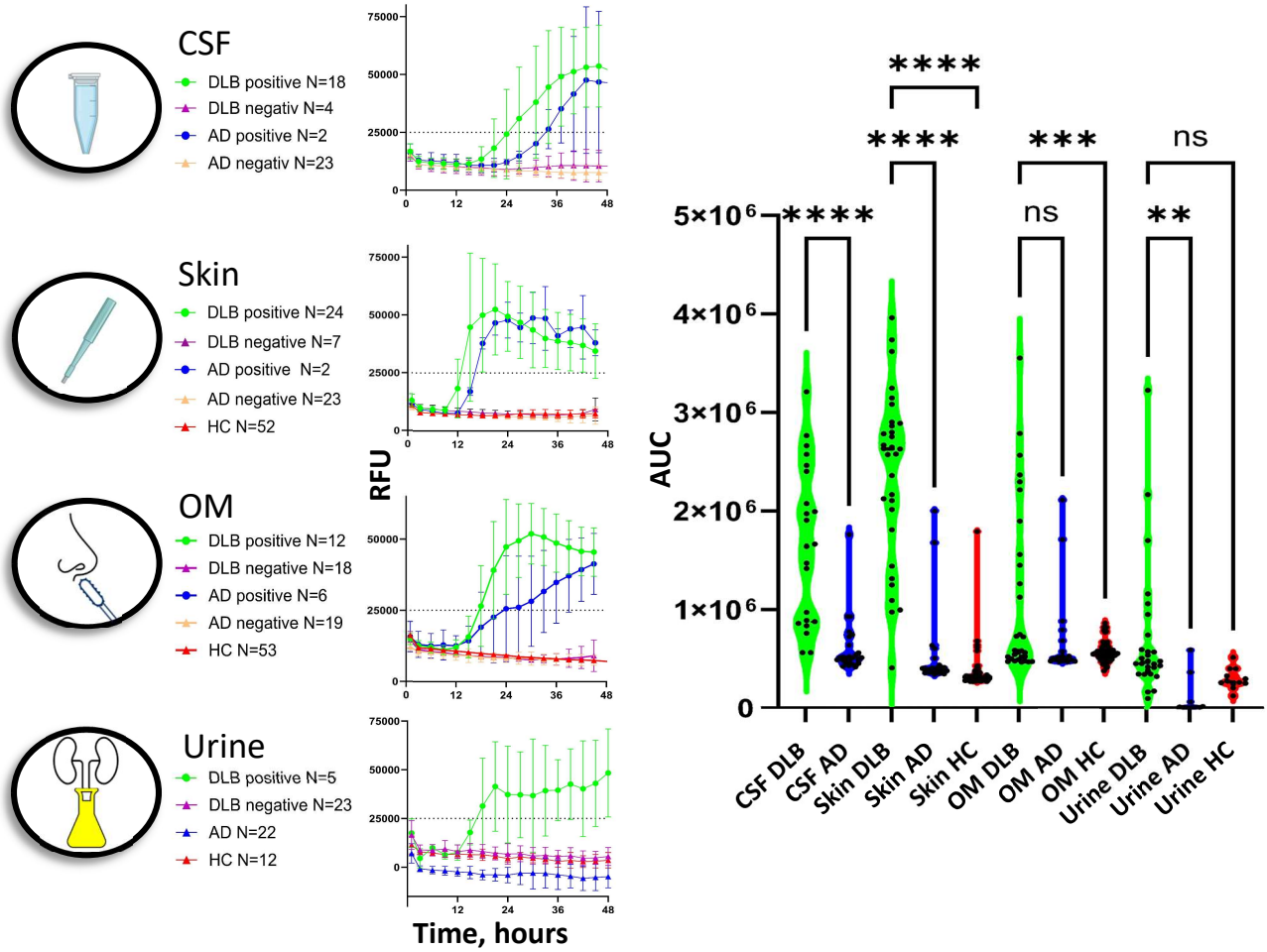
** At the time of analysis, αSyn-SAA data were only available for 48 out of 50 oHC.*
Abbreviations: AD = Alzheimer's disease; DLB = Dementia with Lewy bodies; iOD = idiopathic olfactory dysfunction; LBD = Lewy body symptoms; CI = 95% confidence interval; MDS-UPDRS = Movement Disorder Society Unified Parkinson's Disease Rating Scale; N = number; OD = olfactory dysfunction; oHC = older healthy controls; pPD = prodromal Parkinson's disease; TH50 = time to 50% of Fmax; yHC = younger healthy controls.

5.1 αSynD as a Biomarker

There is an inherent challenge in identifying accurate diagnostic biomarkers for pathologies that are themselves diagnostically difficult, such as DLB[56,57], or in cohorts like AROMA, where the true αSynD status is even more unknown. This uncertainty complicates the interpretation of αSyn-SAA results, as the assay may detect underlying pathology not clinically meaningful, and diagnostic misclassifications can affect the accuracy.

The optimization, processing, and αSynD-SAA protocols were investigated in Study 1, whereas Study 2 examined a more clinically relevant comparison between DLB and AD. The analyses of individual biospecimens are presented in the following subsections. The αSynD-SAA kinetics in Studies 1 and 2 are summarized in Figure D1, in which seeding over time and AUC can be compared.

Figure D1 αSynD-SAA Kinetics in Studies 1 and 2



Aggregation plots and comparisons of AUC across biospecimens between DLB, AD, and HC.
*Abbreviations: ns non-significant; *p≤0.05; **p<0.01; ***p≤0.001; **** p≤0.0001. AD = Alzheimer's disease; AUC = area under the curve; DLB = Dementia with Lewy bodies; HC = Healthy controls; iOD = Idiopathic olfactory dysfunction; N = Number; OM = Olfactory mucosa; RFU = Relative fluorescence unit.*

5.1.1 αSyn-SAA in CSF

αSyn-SAA has been mostly tested in CSF, especially in PD, AD, and iRBD cohorts, and to a lesser extent in OD and DLB. In Studies 1 and 2, we found that CSF αSyn-SAA had a sensitivity of 82% for the clinical diagnosis of probable DLB/MCI-LB. This is consistent with other studies reporting sensitivities of approximately 72–95%[68,150,152], although our results lie toward the lower end of this range. Neuropathologically confirmed studies generally report near-perfect sensitivities in neocortical LB cases[157,158] encompassing around 55–90% of DLB depending on the staging system[36]. The discrepancy between clinical and neuropathological studies may reflect the inherent challenges of the clinical diagnosis, the αSyn-SAA setup, insufficient sensitivity for lower disease burden, or the selection of participants for studies. The effect of the selection of stereotypical DLB patients can be illustrated when the results of Studies 1 and 2 are compared with a recent paper from the same laboratory with an identical αSyn-SAA set-up, which found 100% sensitivity on 12 selected DLB patients[212]. Whether the CSF contained substances that inhibited the αSyn-SAA, or whether the assay exhibited a reduced sensitivity to specific αSynD strains, was not investigated in Study 1.

The specificity of CSF αSyn-SAA against AD is particularly interesting due to the reportedly high αSynD co-pathology in AD, which is present in approximately 20–50% of neuropathologically confirmed AD cases[219,220]. Comparing DLB with AD, we found a significant difference in the fluorescent AUC ($p<0.0001$) and a specificity of 92%, which is close to that found in a meta-analysis reporting a pooled specificity in CSF of 88% for DLB versus AD[146] and a recent study by Plastini et al.[221] reporting a specificity of 86% of DLB against AD. The apparent discrepancy between the rate of αSynD co-pathology in AD and the high specificity of αSyn-SAA may be explained by the accumulation of αSynD between diagnosis and death, and/or by that clinical DLB phenotypes are characterized by more widespread αSynD pathology sufficient to be detected in the assay[149,222]. Conversely, in AD, the LB pathology is frequently confined to the olfactory bulb, amygdala, and to a lesser degree brainstem, with only limited involvement of the neocortex[38]. Recent large studies have demonstrated that the sensitivity of αSyn-SAA varies across brain regions, being highest in the neocortex and substantially lower when αSynD pathology is confined to the amygdala, brainstem, and limbic regions[148,222]. This could explain the lower αSynD detection rate in AD, but may also correlate with more relevant LB pathology.

In summary, CSF αSynD detected by αSyn-SAA proved to be a useful biomarker for distinguishing DLB from AD with high accuracy. However, the lower sensitivity observed in this clinical study compared to neuropathological studies may reflect diagnostic misclassification of DLB or that a substantial burden of αSynD is required for detection by αSyn-SAA in CSF.

5.1.2 αSyn-SAA in Skin

Skin αSyn-SAA represents a minimally invasive alternative to CSF and a marker of peripheral αSynD. We detected αSynD in 77% of clinically diagnosed DLB cases and in 94% of CSF αSyn-SAA–positive DLB cases, with specificities of 98% versus HC and 92% versus AD. These findings are consistent with previous studies in manifest LBD and iRBD, which have reported comparable sensitivity and accuracy between skin and CSF assays[146,173,223,224]. However, our results showed sensitivity at the lower end of the reported range but high specificity. Few studies have compared skin αSyn-SAA results on DLB and AD, but Wang et al.[160] reported 29% skin αSyn-SAA positivity in AD, which is substantially higher than the 8% observed in Study 2.

Among iOD participants, 31% were αSyn-SAA positive in skin. Depending on αSynD origin and propagation (olfactory, central, or peripheral), skin involvement may occur at different stages of LBD. The 31% positivity was lower than the 48% CSF positivity in the PARS OD cohort[113], but close to the combined positivity of 41% across skin and OM. Differences between the PARS and Study 3 could reflect assay variation, tissue distribution of αSynD, and sampling heterogeneity. Notably, the PARS study excluded inconclusive αSyn-SAA cases (4%) and performed CSF analyses only in a subset of participants enriched for higher LBD risk (familial PD history and male sex). This selective sampling complicates direct comparisons and limits the estimation of the true αSynD frequency in iOD.

The very low positivity rate in normosmic controls in Study 3 matched rates in other cohorts[113,148]. Some other skin αSyn-SAA studies not selecting on olfactory function have higher positive rates in the HC, around 10%[175,225].

In summary, skin αSyn-SAA parallels CSF αSyn-SAA with a capable sensitivity in manifest LBD and high specificity against AD and HC (particularly normosmic individuals). However, the disease stage at which skin becomes positive remains uncertain and warrants longitudinal investigation.

5.1.3 Kinetic αSyn-SAA parameters in Skin

Traditional αSyn-SAAs have primarily been interpreted as a dichotomous readout, limiting the utility for early detection, disease monitoring, and assessment of therapeutic efficacy. In Study 4, we explored kinetic αSyn-SAA parameters in skin from iOD and DLB participants and identified significant differences between these disease stages, along with correlations between seeding kinetics and clinical severity (Figure R6–R7).

These findings are in line with the work of Kuzkina et al.[174], who reported associations between skin αSyn-SAA kinetic parameters (Fmax and TH50) and both the disease stage (Hoehn & Yahr) and disease duration in PD. However, in another paper, Kuzkina et al. compared participants with iRBD with PD and interestingly found a faster seeding in the prodromal iRBD group. This may be

explained by that RBD is a phenotype associated with the Body-First/S3 that has more peripheral αSynD than non-RBD phenotypes. We found a nominal but non-significant faster seeding in iOD and DLB with possible/probable RBD.

However, we did not find a correlation with cognitive decline, which is in contrast with the findings in CSF correlation faster seeding activity and cognitive decline in large cohorts of especially PD patients[226–228]. Although all studies also used MoCA to assess cognition, we only had cross-sectional data and not longitudinal follow-up, as was assessed in most of the other cohorts.

A technical limitation in the kinetic comparison between iOD and DLB was the significant difference in seeding activity of the recombinant αSyn substrates used (Study 4, Supplementary Materials Figure S2). The substrate used in the iOD group showed stronger and faster seeding activity in the positive control wells than the substrate used for the DLB group. As DLB samples nonetheless exhibited higher seeding activity, the substrate properties are unlikely to account for the observed differences but may have attenuated the magnitude of group differences and correlations.

To our knowledge, this Study 4 is the first to explore kinetic differences in skin αSyn-SAA between a broad prodromal LBD model, such as iOD, and manifest LBD. Overall, these findings support the feasibility of kinetic αSyn-SAA analysis in minimally invasive biospecimens such as skin and highlight its potential to provide monitoring beyond conventional binary assays. Further optimization, standardization, and cross-laboratory validation will be essential to establish robustness and reproducibility across studies, along with an understanding of the potential influence of phenotypes.

5.1.4 αSyn-SAA in Olfactory Mucosa

OM αSyn-SAA is especially interesting for the detection of αSynD in the early prodromal phase or as a minimally invasive alternative to CSF. We found that the sensitivity for clinically probable DLB increased from 24% to 62% by optimizing swabbing and processing. Cytological analyses showed that about 16% of swabs lacked neuronal material, prompting us to analyze two swabs per participant and consider a single positive swab sufficient for αSynD detection in OM. The variability in sensitivity across other studies may reflect this obstacle, but may also be due partly to the inhibitory effect of the nasal extracellular matrix that interferes with αSyn-SAA[161]. We tried to overcome this inhibitory effect in the optimization process but failed in finding an addictive that improved the detection rate.

Interestingly, OM αSyn-SAA detected αSynD in a quarter of participants with AD. While this limits its clinical biomarker utility for differentiating DLB from AD, it may provide insight into the accumulation of αSynD in olfactory-related regions, including the amygdala in AD.

Among iOD participants, 30% were αSyn-SAA positive in OM, and 40% of these were also positive in skin. This may represent antemortem evidence of early αSynD spread or a "Dual-Hit" onset. Unfortunately, the cross-sectional design precludes conclusions about directionality or spread.

No OM αSyn-SAA-positive cases were found among HCs, contrasting with previous reports of about 10% positivity[174,182]. This difference likely reflects our inclusion of normosmic HCs, underscoring the importance of excluding individuals with OD from control cohorts to minimize the risk of including cases with iLBD, as also indicated by our findings with skin αSyn-SAA.

Although nasal swabbing is minimally invasive, it requires endoscopic equipment and carries risks of a transient mild olfactory impairment and insufficient sampling. Overall, OM αSyn-SAA currently lacks clinical utility for distinguishing DLB from AD, but with improved sensitivity and sampling reliability, it may become valuable for detecting early αSynD or as a complementary biomarker in prodromal LBD.

5.1.4 αSyn-SAA in Urine and Saliva

Urine αSyn-SAA represents an appealing biomarker due to its accessibility and non-invasive nature and may offer an alternative to blood-based assays, which remain technically challenging. In our study, minimally processed urine samples showed low sensitivity for DLB (17%), increasing modestly to 22% in CSF αSyn-SAA–positive cases. While SAA has successfully detected prions in urine using Protein misfolding cyclic amplification in prion diseases[187], αSyn-SAA has not previously been applied to urine. Recent work using fluorescence-based assays reported increased urinary αSynD in iRBD versus controls [229]. Several factors, including protein precipitation, low levels of αSynD, and inhibitory agents, have likely affected the amplification. Currently, urine αSyn-SAA requires substantial methodological optimization before clinical or research use.

Contrary to other groups[163,164,185], we were unable to achieve reliable amplification in saliva despite several methodological attempts, likely due to inhibitory agents in saliva.

5.1.5 αSyn-SAA versus other LBD biomarkers

Several validated biomarkers increase diagnostic certainty in DLB and MCI-LB by detecting neurodegeneration, most notably dopamine imaging[134]. Although Study 2 was not designed for direct comparison between biomarkers, αSyn-SAA detected αSynD even when DAT imaging did not support the DLB diagnosis. Likewise, Marek et al.[113] reported a higher rate of αSyn-SAA positivity (48%) than DAT imaging (17%) among individuals with OD, indicating that αSyn-SAA may have a greater sensitivity also for prodromal LBD.

5.2 The correlation between Lewy Body Symptoms and αSyn-SAA

5.2.1 Lewy Body Symptoms in DLB and the Association with αSyn-SAA

Understanding the relationship between LBD symptoms and αSynD detected by αSyn-SAA is essential for assessing its diagnostic and clinical relevance. In Study 2, the core DLB features, including probable RBD, parkinsonism, and cognitive fluctuations, showed high accuracy (>79%) for predicting CSF αSyn-SAA positivity. Visual hallucinations were less predictive, likely due to their lower prevalence (55%) in this recently diagnosed cohort. OD was frequent in both DLB and AD but more severe in DLB, and objective OD was the strongest predictor of αSyn-SAA positivity (Table R5). These findings are consistent with Coughlin et al.[128], who reported a strong correspondence between αSyn-SAA positivity and core DLB symptoms, with few αSyn-SAA–positive participants lacking OD. Their UPSIT smell test achieved a higher AUC (0.87) than our SIT-16 (0.75), possibly reflecting the greater test variance in UPSIT. Similarly, a study including both neurodegenerative and cognitively unimpaired participants found that only a small proportion of neuropathological LB cases did not exhibit OD and showed that combining olfactory testing with CSF αSyn-SAA markedly improved diagnostic accuracy while reducing the need for lumbar puncture by a quarter. These observations collectively support OD as an important clinical marker for LBD.

Autonomic symptoms showed weaker associations with αSynD in CSF. Urinary urgency had relatively high sensitivity but low specificity, while constipation and orthostatic hypotension were more specific but less sensitive, consistent with previous reports[104,105]. These results support the use of constipation and orthostatic hypotension as clinical indicators that can help rule in αSyn-SAA positivity.

Psychiatric symptoms did not differ between DLB and AD and were not associated with αSyn-SAA positivity. This is despite the psychiatric features being part of the criteria for DLB and a significantly higher prevalence found in MCI-LB versus AD[104].

Applying a threshold of more than two core clinical criteria yielded the highest predictive value, consistent with the McKeith criteria[15,49]. Although the inclusion of probable DLB cases may have influenced these outcomes, the strong association between core symptoms and αSyn-SAA positivity supports the biological validity of αSynD detection for the diagnosis of DLB. OD may merit a more central role in future diagnostic frameworks, as preserved olfaction can serve as a strong indicator against the presence of LB pathology.

Although it would have been interesting to explore the clinical profiles of the αSyn-SAA-positive AD patients or the αSyn-SAA-negative DLB, these analyses were not feasible due to the sample size and

the small number of discordant cases. Future studies with larger and more heterogeneous cohorts are needed to clarify these relationships and to better delineate the clinical boundaries of αSyn-SAA positivity across the LBD spectrum.

5.2.2 Lewy Body Symptoms and αSyn-SAA in iOD

In Study 3, iOD participants showed a higher prevalence and severity of LBD-related symptoms, including parkinsonism, cognitive dysfunction, probable RBD, and psychiatric symptoms, compared with normosmic oHC, while autonomic dysfunction did not differ significantly. Nearly half of iOD participants met criteria for possible or probable pPD compared to just 2% of HCs. Noteably, the elevated risk in iOD was still present even when OD as a parameter in the calculation was excluded, indicating a broad spectrum of prodromal LBD features in the iOD. Few studies have directly compared individuals with OD and normosmic HC. However, our findings of increased LBD symptoms in iOD are generally consistent with, though possibly exceed, previous reports. In the PARS cohort, Siderowf et al.[230] observed that participants with OD more frequently exhibited depressive and anxiety symptoms, and a modestly higher prevalence of signs of parkinsonism, constipation, and RBD symptoms. A more recent study from the PARS cohort found that 29% of participants with persistent OD demonstrated or developed an abnormal DAT imaging result, compared with none in the normosmic HC group[111]. The substantial differences we found between iOD and oHC do not likely stem from a super healthy HC cohort, as the oHC group in the AROMA cohort did not appear to have a particularly reduced risk of pPD compared with other large HC cohorts[231]. The more prominent symptoms in iOD participants from the AROMA cohort may reflect the more clinically ascertained iOD population recruited mainly through otolaryngologist clinics, compared with community-based recruitment.

Contrary to expectations, αSyn-SAA–positive and –negative iOD participants showed no major clinical differences (Figure R3–R4, Table R6). This finding aligns with PARS results[113], who reported no group differences between OD αSyn-SAA–positive and –negative in terms of parkinsonism, cognition, RBD symptoms, or constipation, although αSyn-SAA-positive participants were more likely to develop DAT deficits and later convert to PD. Similarly, in the PPMI cohort, no significant differences in MDS-UPDRS I–III scores were observed between αSyn-SAA–positive and -negative PD cases, except for OD[232]. Relatedly, Iranzo et al.[173] reported that αSyn-SAA–positive iRBD participants exhibited more frequent OD, cognitive impairment, and abnormal DAT imaging compared with αSyn-SAA–negative iRBD, but the motor scores did not differ. The absence of clear clinical differences between αSyn-SAA–positive and –negative iOD participants may reflect: 1) A reduced αSyn-SAA sensitivity in the prodromal phase; 2) alternatively, a detection of very early LBD before overt symptom emergence; or

3) contributions from coexisting pathologies such as AD, which can produce OD and have even been reported to mimic some parkinsonian features[55,60].

5.3 iOD as a Prodromal Model for LBD

To further elucidate the role of iOD as a potential prodromal model for LBD, Study 4 compared αSyn-SAA–negative oHC, αSyn-SAA–positive iOD, and DLB participants. The findings revealed a stepwise increase in hypersomnia, cognitive impairment, and parkinsonism (MDS-UPDRS II+III), suggesting that these symptoms emerge during the prodromal phase in individuals with αSyn-SAA-positive iOD. Due to the absence of longitudinal data, it remains uncertain whether these symptoms will progress and whether the iOD participants will progress to PD or DLB or continue with a submanifest symptomatology. In a longitudinal cohort of individuals with OD, approximately one quarter of αSyn-SAA-positive OD cases progressed to PD within a median follow-up of four years, whereas none of the αSyn-SAA–negative cases converted[113]. However, the conversion rate in OD is lower than the 66% of αSyn-SAA-positive iRBD participants that progressed to PD or DLB within a median of 3.5 years[154]. Whether this lower conversion rate reflects a distinct phenotype with slower progression, a symptom profile less closely linked to clinical syndromes such as PD and DLB, or simply that OD represents an earlier manifestation of disease, remains unclear. Nonetheless, these findings support that combining LBD-related symptoms, such as RBD or OD, with biomarkers of αSynD may improve the identification of individuals likely to phenoconvert.

Interestingly, the degree of OD and neuropsychiatric symptoms did not differ significantly or substantially between iOD and DLB, which may indicate that these symptoms emerge early and subsequently plateau. A potential selection bias for the degree of OD in the AROMA study must be considered, as inclusion required iOD, which in most cases was to a substantial degree. However, findings from iRBD cohorts also suggest that OD is prominent many years preceding and plateauing before phenoconversion to DLB[118].

The same study reported that neuropsychiatric symptoms tend to emerge later in the prodromal course of iRBD. Whether our observation reflects a feature specific to the iOD phenotype or a cohort-related bias cannot be determined from our data alone. Previous research[107] has shown a higher risk of depression among women, and the relatively low proportion of female participants in the DLB group may partly explain the absence of significant group differences in neuropsychiatric symptoms. Larger longitudinal cohorts with more detailed neuropsychiatric assessments are needed to determine whether such symptoms also arise early in the prodromal phase of iOD.

Autonomic dysfunction assessed through medication use, test of orthostatic hypotension, and by relevant items on the MDS-UPDRS was not increased in the αSyn-SAA-positive iOD compared to oHC. Furthermore, the degree of OD was inversely correlated with the degree of collective severity of dysautonomia in both iOD and DLB. These findings do not align with the Body-First phenotype, with both greater OD and more pronounced autonomic dysfunction. A possible explanation is that OD is a more common LBD symptom than autonomic dysfunction and RBD. The iOD phenotype model may thus span across different subtypes of LBD, sustained by the substantial OD in all subtypes in the SuStaIn model[9]. Consequently, the OD phenotype may encompass both LBD individuals with autonomic dysfunction and those without, thereby attenuating the differences in autonomic dysfunction observed between oHC and iOD. However, we found substantial differences in almost all the other LBD features investigated. The findings on autonomic dysfunction should be interpreted cautiously, given the small sample size.

Collectively, these findings suggest that OD represents an early symptom and that αSyn-SAA-positive iOD constitutes a clinically and research-relevant prodromal model within the LBD spectrum. The symptom profile, besides OD, is characterized by early neuropsychiatric symptoms, a range of cognitive changes, mild motor impairment, minimal subjective parkinsonism, and hypersomnia, but not prominent autonomic dysfunction. This pattern suggests early αSynD involvement in olfactory and limbic regions as well as brainstem and possibly cortical involvement.

Together with the slower αSyn-SAA kinetic parameters observed in iOD, the substantial overlap in symptomatology with DLB supports the concept of a continuum from a prodromal phase with limited αSynD pathology to clinically manifest LBD with more extensive αSynD accumulation. The presence of αSynD in skin from most of the iOD also indicates a substantial propagation of αSynD between the PNS and CNS at an early stage. Further longitudinal follow-up of αSyn-SAA-positive iOD cohorts would clarify the phenoconversion rate and delineate distinct trajectories of LBD symptoms. Such studies could provide critical insights into early disease mechanisms and inform the design of stratified prevention trials targeting prodromal LBD.

5.4 Strengths and limitations

The main limitation of our studies is the absence of a gold standard, such as a neuropathological validated diagnosis, since evaluating a diagnostic test against a potentially less accurate reference standard may lead to biased conclusions. To mitigate this limitation, we included only participants with probable DLB/MCI-LB or AD dementia/MCI-AD diagnosed at multidisciplinary conferences after a comprehensive work-up. CSF αSyn-SAA was used as the reference/gold standard for αSyn-

SAA in other biospecimens, as CSF αSyn-SAA corresponds to neuropathological LB in advanced disease.

The absence of neuropathological validation also constrains the interpretation of the frequency of αSynD in iOD. Although we applied the MDS criteria for pPD as a reference, αSyn-SAA positivity in iOD was not associated with a higher risk of prodromal LBD. More detailed phenotyping, including DAT imaging, PSG, cardiac scintigraphy, or AD biomarkers, might have improved the understanding of this discrepancy. Another major limitation, especially in Studies 3 and 4, is the absence of longitudinal data. This challenges the interpretation of symptom progression and the clinical meaningfulness of αSynD.

αSyn-SAA for the detection of αSynD remains a novel biomarker with several technical challenges, as outlined in section 1.7.6. We observed considerable differences in sensitivity across OM processing methods, and although we have improved the sensitivity, the detection rates were still lower than expected. The use of a fixed preset threshold across all biospecimens may have reduced sensitivity, whereas a dynamic threshold could potentially have improved detection, maybe at the cost of a lower specificity or a dual threshold with an intermediate zone. The preset threshold was chosen based on CSF because of the similarities between the amplification curves in CSF, skin, and OM, and to make the positive findings more credible, especially in the unblinded DISPLAY cohort. Nevertheless, almost all results were based on four out of four positive wells, and CSF and skin results were reproducible across independent runs, but validation in an external laboratory would have further strengthened these findings.

Analyses of discordant cases in Studies 1 and 2 were limited by small sample sizes. In Studies 3 and 4, the modest number of participants may have reduced the sensitivity to subtle differences (type II error), particularly within symptoms of autonomic dysfunction.

It should be noted that patients with DLB were recruited from a memory clinic, which likely resulted in a cohort with a more pronounced cognitive profile and less prominent motor profile compared to DLB patients typically seen in movement disorder clinics. This may have affected the distribution of αSynD, clinical diagnostic accuracy, and thus potentially the sensitivity of αSyn-SAAs in DISPLAY.

A potential limitation in the comparison between αSyn-SAA-positive iOD and DLB is the substantial sex imbalance in the DLB group, with only 12% female participants, which is a pattern consistent with most DLB cohorts[18]. To mitigate this bias, we adjusted for both age and sex in the correlation analyses between clinical symptom severity and kinetic SAA parameters. In contrast, the iOD (61% female) and HC groups were well balanced regarding sex and age. However, this may have affected the severity of some clinical features when comparing the iOD and DLB groups.

A key strength of our studies is the comprehensive collection of multiple biospecimens from the same participants, which has not been performed to the same extent in previous studies. We used an RT-QuIC setup that has been validated in several previous studies[125,158,212] and applied it to additional biospecimens, demonstrating that the protocol is feasible for implementation in other laboratories. The assay was performed in a blinded manner within the AROMA cohort, thereby increasing the validity of the findings.

We applied a comprehensive battery of clinical assessments covering a broad spectrum of LBD symptoms, enabling clinical phenotyping and comparisons across disease stages. The oHC and iOD groups were comparable in age, sex, and educational level, which strengthens the validity of the observed differences between them. All DISPLAY participants and most AROMA participants were assessed by the same investigator (OHM), who also closely supervised the remaining assessments, ensuring homogeneity across evaluations.

6. Conclusions and perspectives

6.1 Conclusions

In Studies 1 and 2, we established uniform multi-biospecimen αSyn-SAA procedures demonstrating high accuracy in CSF and skin for differentiating DLB from AD. These results confirm CSF αSyn-SAA for the detection of αSynD as a robust DLB biomarker and highlight skin as a less invasive alternative. OM αSyn-SAA performance improved from low to moderate accuracy, supporting further exploration in prodromal LBD and AD, where it may detect regionally restricted LB pathology. αSynD was not reliably detected in saliva, but we provided the first proof-of-concept of prion-like αSynD in urine as a potential non-invasive biomarker.
Study 2 also showed strong correspondence between αSyn-SAA positivity and core DLB symptoms, reinforcing its clinical relevance. OD was associated with αSyn-SAA positivity, while preserved olfaction indicated non-LB pathology. Together, CSF and skin αSyn-SAA, combined with OD, may play key roles in future DLB diagnostic criteria.

Study 3 showed that nearly half of iOD participants were αSynD-positive and half met pPD criteria, though concordance was moderate. Combining skin and OM αSyn-SAA improved detection, and prevalence aligned with prior OD studies, supporting iOD as a prodromal LBD phenotype. The discordance in iOD between LBD symptoms and αSyn-SAA results may reflect mixed pathology to further investigate. Notably, αSyn-SAA was negative in almost all normosmic HC, which, together with the higher prevalence of αSynD and LBD symptoms in iOD, indicates that olfactory testing is a simple way of enriching cohorts with and without LBD symptoms and pathology in future studies.

Study 4 demonstrated a stepwise increase in LBD symptoms from αSynD-negative HC to αSynD-positive iOD and DLB, reinforcing iOD as a prodromal LBD model characterized by early OD and neuropsychiatric symptoms, along with motor and sleep symptoms, while autonomic dysfunction remained limited. Kinetic analyses of skin αSyn-SAA revealed faster seeding in DLB than in iOD, correlating with clinical severity. These results could suggest a progressive increase in αSynD seeding activity and support kinetic αSyn-SAA parameters as potential markers of prodromal LBD and disease progression, warranting validation in larger, longitudinal cohorts.

This project aimed to evaluate αSynD detected by αSyn-SAA as an early diagnostic biomarker for DLB and to assess iOD as a prodromal LBD model. Analogous to Fritz Lewy's microscopic

identification of LBs postmortem, αSyn-SAA may now enable antemortem detection of αSynD pathology across biospecimens. Given its high prevalence of LBD symptoms and αSynD, iOD emerges as a valuable model for early-stage LBD, which may encompass a broader spectrum of LBD than the previous models, iRBD and PAF. Together, these findings may advance early diagnosis, improve clinical trial stratification, and enhance understanding of LBD pathophysiology.

6.2 Clinical implications

αSynD detected by αSyn-SAA provides a potentially robust biological marker for antemortem LBD diagnosis, complementing current clinical and neurodegenerative biomarkers. It may enable earlier, more accurate diagnosis, improve understanding of LBD heterogeneity, and aid in identifying LB copathology, supporting tailored use of emerging proteinopathy-specific therapies. These findings further reinforce the strong link between OD and LBD, highlighting olfactory testing as a simple, cost-effective tool to screen for possible LB pathology, while preserved olfaction should raise suspicion of non-LBD causes. The high frequency of LBD-related symptoms in iOD underscores the need for greater clinical awareness, particularly in primary care, geriatrics, and ENT, to promote earlier recognition and specific care.

6.3 Future Research

Antemortem biological anchors such as αSynD may enhance staging and longitudinal research in LBD by linking pathology with symptomatology, biomarkers, and treatment response. Future studies should clarify the relationship between αSynD detected by αSyn-SAA and underlying pathology in the CNS and periphery, including quantitative correlations with αSynD load, strain, and seeding kinetics. As αSyn-SAA is highly sensitive, standardization, technical improvements, and cross-laboratory validation will be essential to ensure reproducibility. The interaction between tau and αSyn seeding, and the impact of anti-amyloid and anti-tau therapies in AD with substantial LB pathology, also warrants investigation. Using iOD as a prodromal model for LBD instead of iRBD and PAF cohorts may extend the understanding of the clinical heterogeneity of LBD, which might bridge current knowledge gaps between early risk factors, such as male sex and microbial disturbances, and the development of manifest disease. Studies of alternative biospecimens, for instance, feces, may aid earlier detection and refine hypotheses on αSynD onset, while the development of blood-based αSyn-SAA could enable large-scale clinical implementation.

7. References

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8. Appendix (Study 1 – 4)

Study 1 Accurate detection of pathologic α-synuclein in CSF, skin, olfactory mucosa, and urine with a uniform seeding amplification assay.

Bsoul R*, McWilliam OH*, Waldemar G, Hasselbalch SG, Simonsen AH, von Buchwald C, Bech M, Pinborg CH, Pedersen CK, Baungaard SO, Lombardía J, Ejlerskov P, Bongianni M, Bronzato E, Zanusso G, Frederiksen KS, Lund EL, and Areškevičiūtė A. *Shared first authorship.

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Study 2 α-Synuclein seed amplification assay in Lewy body dementia versus Alzheimer's disease.

McWilliam OH, Bsoul R, Lund EL, Waldemar G, Hasselbalch SG, Simonsen AH, Bruun M, von Buchwald C, Aanæs K, Pedersen CK, Andersen ISB, Bech M, Areškevičiūtė A, and Frederiksen KS.

Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring. 2025 November 7. doi.org/10.1002/dad2.7020[2]

Study 3 Prodromal Lewy Body Symptoms and α-Synuclein Seeding in Idiopathic Olfactory Dysfunction

McWilliam OH, Bsoul R, Waldemar G, Hasselbalch SG, Simonsen AH, Bruun M, Andersen LSN, von Buchwald C, Aanæs K, Pedersen CK, Andersen ISB, Bech M, Pinborg CH, Areškevičiūtė A, Lund EL, and Frederiksen KS.

Movement Disorders Clinical Practice. 2025 Sep 8. doi: 10.1002/mdc3.70333[3]

Study 4 Symptoms & α-Synuclein Seeding Parameters in Olfactory Dysfunction & Lewy Body Dementia

McWilliam OH, Bsoul R, Areškevičiūtė A, Andersen ISB, Bruun M, von Buchwald C, Hasselbalch SG, Lund EL, Pedersen CK, Simonsen AH, Waldemar G, Aanæs K, and Frederiksen KS.

Submitted[4]

8.1 Study 1: Accurate detection of pathologic α-synuclein in CSF, skin, olfactory mucosa, and urine with a uniform seeding amplification assay

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Accurate detection of pathologic α-synuclein in CSF, skin, olfactory mucosa, and urine with a uniform seeding amplification assay

Remarh Bsoul[1†], Oskar H. McWilliam[2†], Gunhild Waldemar[2,3], Steen G. Hasselbalch[2,3], Anja H. Simonsen[2], Christian von Buchwald[3,4], Magne Bech[4], Clara H. Pinborg[4], Christian K. Pedersen[4], Sara O. Baungaard[2], José Lombardía[2], Patrick Ejlerskov[2], Matilde Bongianni[5], Erika Bronzato[5], Gianluigi Zanusso[5], Kristian S. Frederiksen[2,3], Eva L. Lund[1,3] and Aušrinė Areškevičiūtė[1*]

Abstract

Currently, early diagnosis of dementia with Lewy bodies (DLB) is based on clinical criteria, which is challenging due to overlapping symptoms with other neurodegenerative diseases. Seeding amplification assays, detecting minute amounts of disease causing α-synuclein (αSyn^D), are emerging as a promising diagnostic tool for α-synucleinopathies including DLB and Parkinson's disease. This study aimed to test whether the same seeding amplification assay established for αSyn^D detection in cerebrospinal fluid (CSF) could be applied to other biospecimens, including skin, olfactory mucosa, saliva, and urine, obtained from the same patients. A total of 31 patients with probable DLB and 53 healthy controls were recruited. When evaluating the assays' applicability to different biospecimens, only those collected from participants with a positive CSF αSyn^D result were considered. Seeding amplification assay results were evaluated based on the αSyn^D amplification rate over 48 h and the value of the area under the curve. The sensitivity and specificity were 94% and 98% for skin, 47% and 100% for olfactory mucosa, and 22% and 100% for urine, respectively for the CSF positive DLB and healthy controls. αSyn^D was undetectable in saliva. Cohen's Kappa analysis (κ) showed almost perfect agreement between CSF and skin assays ($\kappa = 0.86$) but slight to no agreement for CSF versus olfactory mucosa ($\kappa = 0.12$) and urine ($\kappa = 0.094$). In summary, the seeding amplification assay established for αSyn^D detection in CSF demonstrated comparable diagnostic performance in minimally invasive skin biopsies. Olfactory mucosa, saliva, and urine sample preparation pose technical challenges resulting in the established assays' low diagnostic accuracy, for now, limiting their use in diagnostics. Nevertheless, the proof-of-concept for αSyn^D detection in urine expands the potential for non-invasive diagnostics of α-synucleinopathies in the future.

Keywords Real-time quaking-induced conversion, RT-QuIC, Amplification assay, Neurodegenerative, Lewy body, Skin, Cerebrospinal fluid, Olfactory mucosa, Urine

†Remarh Bsoul and Oskar H. McWilliam contributed equally to this work.

*Correspondence:
Aušrinė Areškevičiūtė
ausrine.areskeviciute@regionh.dk

Full list of author information is available at the end of the article

Introduction

Driven by demographic changes and increased life expectancy, dementia prevalence is rising. Dementia with Lewy bodies (DLB) is the second most common neurodegenerative dementia after Alzheimer's disease (AD). It accounts for 4–8% of diagnosed cases but is assumed to be underdiagnosed [39]. DLB is clinically defined by the McKeith criteria [28], yet misdiagnosis can happen especially in the early disease stages [13, 42] making accurate diagnostic tests necessary.

Neuronal alpha-synucleinopathies such as DLB and Parkinson´s disease (PD) are characterized by the disease causing aggregation of alpha-synuclein (αSyn^D), called Lewy bodies, in the central nervous system (CNS) and peripheral tissues [26].

Besides dementia and parkinsonism, hallmark features of DLB and PD include REM sleep behavior disorder (RBD) and visual hallucinations, which are often preceded by a prodromal phase with hyposmia [11, 37, 46]. Pathologically, Lewy bodies are detected early in the olfactory bulb [26], and the gut, both of which are hypothesized to serve as entry sites for the prion-like propagation of αSyn^D from the periphery to CNS as alternative to CNS-first theory [9, 10, 41].

Seeding amplification assays (SAA) such as Real-Time Quaking-Induced Conversion (RT-QuIC) and Protein Misfolding Cyclic Amplification (PMCA) have emerged as highly sensitive and specific assays for detecting αSyn^D aggregates. αSyn^D SAA exploits the prion-like properties of αSyn^D to amplify minute amounts of misfolded protein, enabling its detection through a fluorescence dye [18]. αSyn^D RT-QuIC is highly specific on both PD, DLB, and RBD but is less sensitive for αSyn^D seeds in multiple system atrophy [7, 38, 40]. In a meta-analysis, PD and DLB could not be discriminated by measurement of αSyn^D using RT-QuIC [36], but there are some reports of DLB having a slightly higher amplification rate [5, 15].

Cerebrospinal fluid (CSF) is a well-established source for biomarker discovery in neurodegenerative diseases and several research groups have demonstrated RT-QuIC to be highly capable of identifying neuropathologically confirmed DLB cases with sensitivity and specificity close to 100% [36]. CSF can thus be considered a golden standard for RT-QuIC in detecting αSyn^D. Recently, the US Food & Drug Administration encouraged the use of αSyn^D SAA for patient screening in clinical trials [17].

However, CSF sampling is invasive and may not be feasible for all patients or for screening protocols, and therefore there is a need for reliable and minimally invasive biomarkers that can enhance diagnostic accuracy in the early stages of DLB and PD, as well as in their prodromal phases. Such biomarkers could play a critical role in identifying candidates for potential disease-modifying therapies. Therefore, other biospecimens like skin [18, 19, 21, 24, 25, 49], olfactory mucosa (OM) [11, 20, 37], and saliva [22, 48, 50] have been tested as alternatives to CSF.

Skin biopsies offer a convenient and minimally invasive method for obtaining peripheral tissue samples. Multiple studies [18, 19, 21, 24, 25, 49] have demonstrated the presence of αSyn^D aggregates in skin biopsies from DLB and PD patients with RT-QuIC, with sensitivity and specificity typically of > 90% and > 85%. In a recen meta-anaysis, skin αSyn^D RT-QuIC has also shown promising results, which almost parallel the sensitivity and specificity reported with CSF samples [52].

The olfactory bulb is one of the first regions affected by Lewy body pathology [2, 10, 26], making OM a promising target for confirming prodromal α-synucleinopathies. Studies have shown that RT-QuIC can detect αSyn^D by nasal swab brushings in DLB/PD/RBD but the sensitivity varies substantially from 48 to 81% [20, 37, 46].

RT-QuIC has also been adapted successfully to detect αSyn^D in saliva with a sensitivity in PD of 84–90% and a specificity versus healthy controls (HC) of 83–92%, potentially offering an entirely non-invasive and easily accessible diagnostic biomarker [22, 48].

Urine has not yet been tested for αSyn^D detection using SAA in alpha-synucleinopathies. However, a study has shown that urine is a suitable material for the detection of variant Creutzfeldt-Jakob's disease, using the PMCA approach with a sensitivity of 93% and 100% specificity [30].

In this study, we aimed to expand the established αSyn^D CSF RT-QuIC assay protocol to multiple biospecimens which include skin, OM, saliva, and urine - all collected from a single cohort. The cohort consisted of patients with DLB, who were positive in RT-QuIC for αSyn^D in CSF, and HC. We describe the optimized preparation of these different biospecimens, report the sensitivity and specificity achieved for each biospecimen using a uniform αSyn^D RT-QuIC protocol with a rapid result, and discuss the opportunities and challenges these biospecimens present for improving clinical diagnostics.

Materials and methods

Study design and ethics

Patients fulfilling the consensus criteria for probable DLB [28] or probable mild cognitive impairment with Lewy bodies (MCI-LB) [29] with Mini-Mental State Examination (MMSE) > 18 were included (from now on both annotated as DLB).

The HC cohort consisted of samples collected from individuals in two groups: (1) Young HCs (yHC); age < 40 years, MMSE score indicative of no cognitive impairment with no first-degree relatives with PD/DLB, and (2) Older HCs (oHC); age 55–75 years with a normal sense of smell defined by Sniffin´ Stick Identification test 16 (a test using 16 different smells, that quantitatively assesses

olfaction adopted to a Danish population [32]) over the 25th age-adjusted percentile [33].

This study is an unblinded single-center cross-sectional case-control study with patients with DLB versus HC. In total, the study cohort consisted of 84 individuals, including 31 clinically diagnosed with probable DLB, 5 yHC, and 48 oHC. CSF sample collection was possible for 22 patients with DLB. Using the established RT-QuIC, all CSF samples were screened for αSyn^D. Only biospecimens from patients with a positive CSF αSyn^D were used for RT-QuIC sensitivity and specificity calculations in different biospecimens. See Table 1 for an overview of patients' demographics, clinical tests, and sample collection. The αSyn^D RT-QuIC data for the whole cohort is available in Additional file 1.

All patients with DLB were recruited within three years of diagnosis. The participants with DLB were older than oHC and included significantly fewer females, see Table 1. Around half of the DLB patients had significant co-pathology of both amyloid-β42 and/or p-tau in CSF. In the CSF SAA negative group, 3 (75%) were p-tau positive compared to the CSF SAA positive group where only 5 (28%) were positive ($p = 0.076$).

Reagents

All reagents used for the collection and preparation of samples, and for αSyn^D RT-QuIC protocol were from Sigma Aldrich® unless otherwise specified.

Collection and preparation of samples

Brain

Brain samples acquired from the biobank of the Danish Reference Center for Prion diseases, with neuropathologically confirmed Lewy body deposits, were used as a standard αSyn^D RT-QuIC positive control. These samples were prepared using fresh frozen frontal cortex tissue collected during cranial autopsy and stored at -80 °C. The presence of Lewy body deposits was confirmed by immunohistochemistry on adjacent formalin-fixed brain tissue samples.

The brain DLB controls were prepared as a 10% *w/v* (10^{-1}) brain homogenate (BH) solution through chemical lysis using tris-buffered saline x 1 at pH 7.4, containing 0.5% sodium deoxycholate and 0.5% tergitol. The BH solution was then transferred to M-tubes™ compatible with the Dispomix™ cell dissociator (Xiril®). Mechanical lysis was conducted at 4000 rpm for 15 s. The resulting BH supernatant was cleared by centrifugation at 800 g for 5 min at 4 °C and diluted a millionfold (10^{-6}) in 1:10 serial dilution in DEPC-treated water.

Cerebrospinal fluid

CSF samples were collected from patients with DLB by lumbar puncture and cleared by centrifugation at 2000 g, 4 °C, 10 min. The cleared CSF samples were aliquoted into 250 µL in polypropylene (PP) tubes and subsequently stored at -80 °C until use. CSF samples were discarded if the total protein count was > 1 g/L, erythrocyte count > 300, and white blood cell count > 5. CSF samples were less than two years old when tested in RT-QuIC.

Table 1 Demographics and biospecimen collection of the study cohort

	Total DLB cohort	DLB CSF SAA+	DLB CSF SAA-	yHC	oHC	All HC
N	31 (3 MCI)	18 (3 MCI)	4	5	48	53
Age, mean (SD)	75.0 (5.9)	74.5 (4.2)	74.5 (9.5)	31.2 (4.8)	67.5 (5.1)	64.1 (11.9)
Sex, N female (%)	4 (12%)	1 (6%)	0	2 (40%)	28 (58%)	30 (57%)
MoCA, mean (SD)	27 (2.0)	21 (4.4)	18.2 (4.3)	29.6 (1.2)*	27 (2.0)	NA
β-amyloid-42, N Positive (%)	16 (67%)	11 (61%)	3 (75%)	NA	NA	NA
P-tau, N Positive (%)	10 (42%)	5 (28%)	3 (75%)[a]	NA	NA	NA
CSF, N	22	18	4	0	0	0
Skin, N	31	18	4	5	47	52
Olfactory Mucosa Swab 1, N	30#	17¤	4	4	48	52
Olfactory Mucosa Swab 2; N	17	8	3	1	37	39
Saliva, N	31	11	4	5	NA	5
Urine, N	29	18	4	4	8	12

Table showing the demographics, cognitive status and markers for co-pathology in the total cohort of DLB, the DLB with CSF available (positive and negative), and healthy controls (young and old). Note that not all DLB (31) had CSF available for SAA testing. Generally, swab 1 was treated with method 1 and swab 2 with method 2, except all oHC swabs were treated with method 2

Abbreviations: CSF = cerebrospinal fluid, CI = 95 % Confidence intervals, DLB = Dementia with Lewy body, MoCA = Montreal Cognitive Assessment, N = number of samples, NA = not applicable, OM = olfactory mucosa, SAA = seeding amplification assay, SD = standard deviation, oHC = older healthy controls, yHC = young healthy controls

* MMSE score

[a] Difference between SAA+/- DLB $p = 0.076$

3 DLB swabs were discarded due to no pellet

¤ 2 DLB swabs discarded due to no pellet

The CSF samples were used immediately after thawing and without any processing. Freeze/thaw cycles were noted and did not exceed more than three times [1].

Olfactory mucosa

OM samples were collected via nasal endoscopy in both nostrils via swabbing from the agger nasi area and anterosuperior part of the middle turbinate bilaterally using either nylon (FLOQswabs™ Copan® Diagnostics) (N of oHC = 48) or fiber-based swabs (Nasopharyngeal Specimen Collection Swab, NEST®) (N of DLB = 30, and yHC = 5). Topical anesthesia with Lidocaine and Epinephrine (20 mg + 6,7 ug/mL) was administered using cotton gauze placed towards the middle turbinate. The gauze remained in place for at least two minutes.

Two final-year medical students were trained in the procedure by a rhinologist. The rhinologist was consulted for supervision in case of uncertainty. For patients with DLB and yHC, a maximum of two samples were collected, one from each side. For oHC, a maximum of four swabs were collected, two from each side. In certain cases, fewer samples were obtained if not technically feasible. Collected swabs were submerged into 15 mL PP tubes containing 3 mL saline solution at pH 7.4. Blood contamination was noted.

The technical difficulty of the sampling procedure was scored on a five-point scale based on the following variables: Anatomical recognizability, patient tolerance, and certainty of correct placement of swabs on each side. A score of zero indicated that the procedure could not be performed, while a score of five points meant flawless execution without any issues or uncertainties.

Tubes with swabs were vortexed separately at a speed of 18,0000 rounds per minute for 1 min., followed by a second vortex with a combination of all swabs in a new tube. Sample solutions were pelleted at 2000 g (DLB and yHC) and 550 g (oHC), 4 °C, 20 min to yield a visible cell pellet (off-white to custard yellow). Cell pellets in solution were stored at -80 °C until the lysis protocol initiation. Bloody pellet samples were discarded.

To confirm the presence of olfactory mucosa in the nasal swabs, we performed a cytological spot check of 19 random samples. For the description of this method see Additional file 1.

The OM samples were prepared using two extraction methods. In method 1, a thawed cell pellet from a single swab was suspended in 0.075% sodium dodecyl laurate (SDS) in phosphate-buffered saline (PBS) x 1 at pH 7.4. To the suspension were added 800-micron silica beads (OPSDiagnostics®) and vortexed at maximum speed for 30 s. All 30 DLB OM samples were first processed using method (1) Method 2 was inspired and instructed by M. Bongianni et al. with few modifications [8]. OM cell pellets were thawed, and a sterile 10 µg inoculating loop (Sarstedt®) was used to transfer one loop of cells to 100 µL 40 mM sodium phosphate buffer (ThermoFisher®), pH 8. The cell suspension was subjected to cell dissociation in an ultrasonic water bath (Emag® EMMI, 120 W) at 100% ntensity until total dissociation (~ 30 s) of the pellet. Overall, 17 out of 30 DLB OM samples were processed with both methods 1 and 2, while 13 DLB OM were only processed with method 1 due to lack of material (Additional file 1). All oHC OM were processed with method (2) The same RT-QuIC script was applied for both methods.

Skin biopsy

Skin biopsy samples were collected from the neck region (C7) five cm paramedially after administration of local anesthetic and adrenaline. Two biopsies were taken from each participant, each measuring 3 mm. The biopsies were dabbed for blood on a cotton cloth, thoroughly washed with cold PBS x 1 at pH 7.4, and stored dry at -80 °C until lysis protocol. For participants with DLB and yHC, one skin biopsy was used for RT-QuIC, whereas for oHC, both biopsies were used.

The skin biopsies were prepared as a 5% *w/v* stock solution by chemical lysis using PBS x 1 at pH 7.4, 1% triton x-100, 1 x total protease cocktail inhibitor (Promega®), and 150 mM NaCl. The solution containing the biopsy was transferred to M-tubes™ compatible with the Dispomix™ cell dissociator (Xiril®). Mechanical lysis was conducted by a 30-second interval program increasing from 1100 rpm to 4000 rpm. The resulting supernatant was cleared by centrifugation at 800 g for 5 min at 4 °C. The 5% *w/v* stock solution was further diluted to a 1:10 (10^{-2}) solution in DEPC-treated water.

Saliva

Saliva samples were collected after participants adhered to a minimum fasting period of 1 h, abstaining from food, beverages, and smoking (water excepted). Samples were collected directly into PP tubes and kept on ice until centrifuged at 2000 g for a minimum of 10 min, at 4 °C. The supernatant was aliquoted and stored at -80 °C until use.

The saliva samples were processed in multiple ways which are listed in Additional file 1.

Urine

Urine samples were collected by urination into a 100 ml PP beaker. There were no requirements for the sample material and no restrictions from the last urination. The content was transferred to 50 ml PP tubes and stored at -80 °C.

After thawing 15 ml urine was transferred to a suitable-sized spin filter (sartorius®) with a molecular weight cut-off of 100 kilo Daltons. Filters were centrifuged at 3200 g, 30 min at 4 °C until filter dead volume (< 100 µL). The

dead volume was dissolved into a total of 500 µL 40 mM phosphate buffer which resulted in a 30x concentrated sample. The resulting solution was used to flush the filter before transferring it to a microcentrifuge tube.

αSyn^{D} RT-QuIC

Production of recombinant αSyn

The production of recombinant αSyn (rec-αSyn) substrate with an N-terminal 6x-histidine-tag was optimized from a previously reported protocol [14]. A 5 mL liquid culture of Lysogeny Broth media was inoculated from a glycerol stock of transformed E. coli until it reached an OD600 of 0.3–0.5. The culture was then diluted 1:1000 in Overnight Express Terrific Broth (OETB™, Novagen®) media and cultivated in 2 L baffled flasks at 30 °C, 180 rpm, overnight. Cells were pelleted at 4000 g for 15 min and carefully resuspended in 25 mL osmotic shock buffer (40% sucrose, 2 mM EDTA, 30 mM Tris, pH 7.2) per 250 mL of culture media. The suspension was stirred in rotation for 10 min at room temperature and pelleted at > 7000 g for 20 min at 20 °C. The supernatant was discarded, and each wet pellet was resuspended in 10 mL ice-cold 20 mM Tris-Hydrochloric acid (HCl) buffer supplemented with 20 mM imidazole, pH 7.5. Each pellet was sonicated on ice (Sonifier SFX150, microtip) at 70% intensity for 30 s over two rounds with a 10-second pause in between. The resulting suspension was stirred in rotation for 10 min at 4 °C and pelleted at > 7000 g for 30 min at 4 °C. Pellets were discarded, and the supernatant was acidified to ~ pH 3.5 with 1 M HCl. The solution, along with the resulting precipitate, was centrifuged at 7000 g for 30 min at 4 °C, and the pH was elevated to ~ 7.5 with 1 M NaOH. The solution was sterile filtered (0.45 µm) and loaded (20 mM imidazole, 20 mM Tris, pH 7.5) onto a 5 mL prepacked HisTrap™ HP column (Cytiva®). The column was washed with 50 mM imidazole, pH 7.5. The protein was eluted in a linear gradient with 15 column volumes of 20 mM Tris, 500 mM imidazole, pH 7.5. The eluate was sterile filtered (0.22 µm) and dialyzed overnight in 40 mM sodium phosphate buffer, pH 8 (ThermoFisher®). The dialysis buffer was exchanged, and dialysis continued for a further 4 h. Protein concentration was estimated with the Qubit® protein assay kit. Each production batch was labeled with a batch number and validated by an RT-QuIC test run. Best yield was 60 mg rec-αSyn pr. 1 L culture.

RT-QuIC set-up

The αSyn^{D} RT-QuIC assay was conducted using a FLUOStar™ Omega instrument from BMG LABTECH®, with a black 96-well plate featuring a clear flat bottom (Greiner®). Each well contained six 800-micron silica beads (OPSDiagnostics®). The total reaction volume per well was 100 µL, including the sample and master mix comprised of 40 mM phosphate buffer (ThermoFisher®) at pH 8.0, 170 mM NaCl, 10 µM Thioflavin T, 0.0015% SDS in PBS x 1, and 0.1 µg/µL of freshly thawed rec-αSyn. CSF samples were added in volumes of 7 and 15 µL. Processed BH, skin, and OM samples were added in 2 µL volumes. Urine samples were added in 15 µL volumes, while saliva samples were added in volumes ranging from 1 to 10 µL, Additional file 1. The plate was sealed with adhesive film (MicroAmp®) and subjected to alternating intervals of 60 s of double orbital shaking at 400 rpm and 60 s of resting, continuously throughout the assay duration. Fluorescence measurements were taken every 45 min for a total of 48 h, expressed in Relative Fluorescence Units (RFU), at 450 ± 10 nm excitation and 480 ± 10 nm emission.

RT-QuIC interpretation

αSyn^{D} RT-QuIC assay experiments were performed in quadruple wells (referred to as replicates) for each volume. The threshold for a positive replicate was set at 25.000 RFU (4 standard deviations from the Thioflavin T background RFU), and any given sample and control were considered positive if ≥ 2 of 4 replicates crossed the threshold within 48 h. For samples with > 2 positive replicates, the two best-performing replicates were used for further analysis to ensure an optimal comparison of seeding activity between samples. For samples with < 2 positive replicates (deemed negative), the mean of all four was used for further analysis, to ensure that the variance of the single positive replicates was transparent in the data visualization (Fig. 1, f). We included the single positive replicates in the plot (Fig. 1, f) to demonstrate the robustness of the assay towards the healthy control cohort when pooled together.

The kinetic performance of RT-QuIC is interpreted based on the increase of RFU over time. The performance progress can be divided into a lagging phase (lag-phase), a logarithmic phase (log-phase), and a stationary phase (plateau in RFU) which are all quantified by the Area Under Curve (AUC). Additionally, maximum RFU, lag-phase time, final RFU and time to half the maximum RFU (TH50) are also calculated. DLB sample performance is evaluated by the shortest lag-phase, broadest log-phase, and earliest stationary phase, while HC is expected to have a constant lag-phase under the set RFU threshold.

Statistics

The visualization of results was performed using GraphPad Prism 10. RFU over time displaying the kinetic development is presented as the mean RFU with accompanying 95% confidence intervals (CI). The AUC was computed with the software's default parameters and comparisons of AUC values across groups were performed using a one-way analysis of variance (ANOVA).

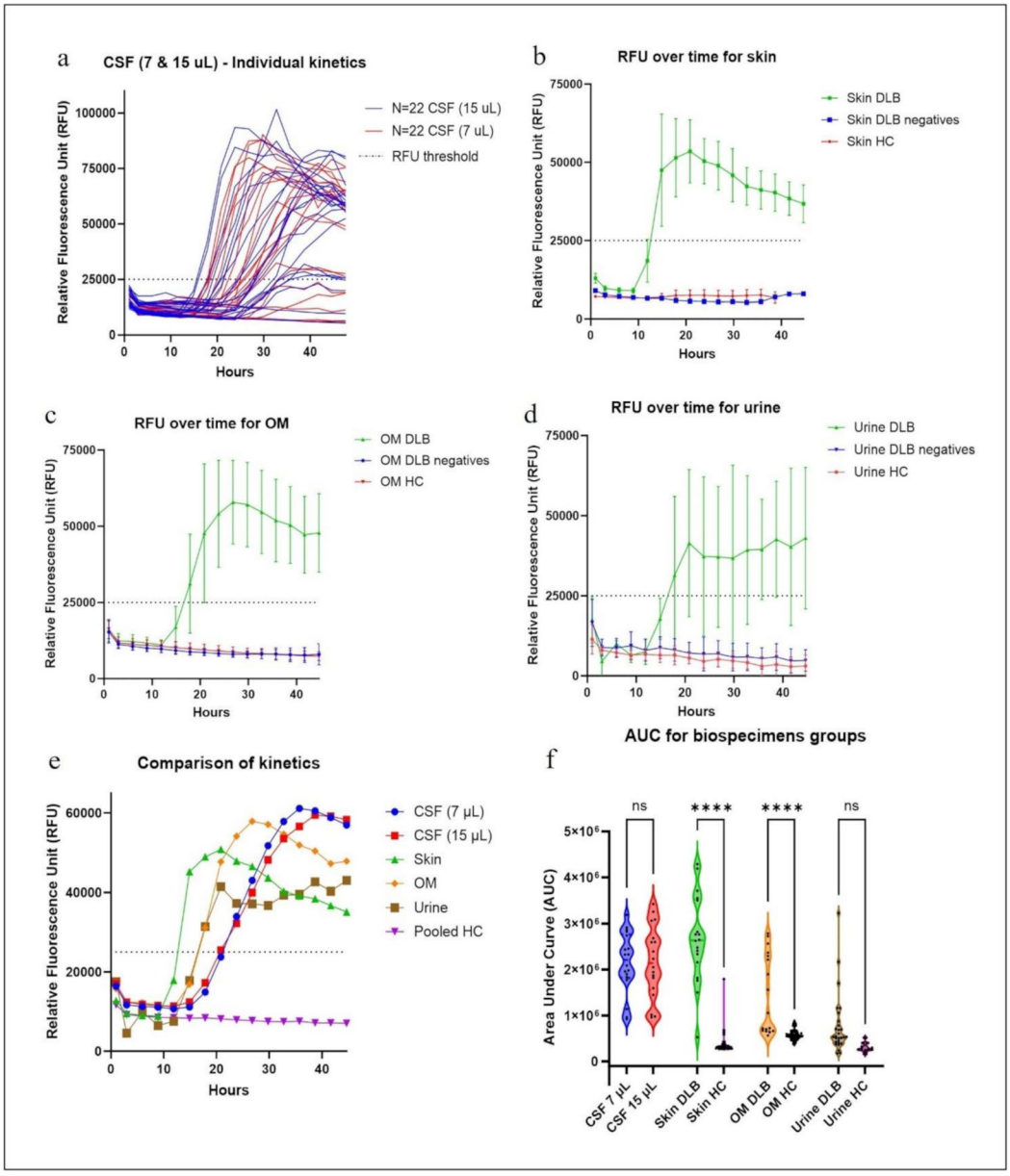

Fig. 1 Kinetics and AUC for each biospecimen group. (**a**) Individual curves for all CSF samples at CSF volumes 7 µL and 15 µL (**b**) RFU over time for skin groups (**c**) RFU over time for OM groups (**d**) RFU over time for urine groups (**e**) Comparison in kinetics between biospecimens (**f**) Area Under Curve (AUC) for the biospecimen groups. Each black dot is a patient sample, and the violin plot displays the distribution

Sensitivity, specificity, and diagnostic accuracy were calculated in R (version 4.3.3) and reported with CI using Wilson's method in the *binom* package. The agreements between biospecimens were calculated using the *psych* package for Cohen´s Kappa (κ) analysis, with Cohen´s interpretation: below 0 = No agreement, 0.01–0.2 = None to slight agreement, 0.21–0.39 = Fair agreement, 0.40–0.59 = Moderate agreement, 0.60–0.79 = Substantial agreement, above 0.80 = Almost perfect agreement [27].

Results

αSyn^D RT-QuIC sensitivity and specificity for individual groups of biospecimens

Twenty-two CSF samples were analyzed for the presence of αSyn^D at 7 µL and 15 µL CSF volumes per reaction and the kinetic parameters were compared (Fig. 1, a). The CSF sensitivity was calculated based on agreement with clinical diagnosis. Out of the 22 samples; 18 samples were positive at 15 µL and 17 samples were positive at 7 µL. The sensitivity at 15 µL and 7 µL CSF volumes were 82% (CI = 61–93%) and 77% (CI = 57–90%), respectively.

Table 2 The sensitivity, specificity, accuracy and kinetic parameters of biospecimens from CSF SAA-positive DLB versus HC

	Sensitivity % (CI)	Specificity % (CI)	Accuracy % (CI)	MaxRFU (CI)	AUC (CI)	Lag-phase (h)	Final RFU (48 h)	T50 (h)
Skin	94 (74–99)	98 (90–100)	96 (90–100)	54,894 (31176–39556)	1.56×10^6 ($1.33–1.79\times10^6$)	12	35,547	13
Olfactory Mucosa combined	47 (26–69)	100 (93–100)	74 (63–84)	52,200 (30894–38933)	1.65×10^6 ($1.38–1.92\times10^6$)	15	46,603	17
Olfactory Mucosa Swab 1	24 (10–47)	100 (93–100)	62 (52–71)	-	-	-	-	
Olfactory Mucosa Swab 2	62 (31–86)	100 (91–100)	81 (67–95)	-	-	-	-	
Urine	22 (9–45)	100 (76–100)	61 (50–72)	48,375 (25866–33385)	1.39×10^6 ($1.08\times1.79\times10^6$)	15	48,375	17

The sensitivity, specificity, accuracy, Maximum RFU, AUC, hours of lag-phase, final RFU and TH50 of the biospecimens calculated only for the SAA CSF positive DLB samples. Abbreviations: CI = 95% Confidence Interval, MaxRFU: Maximum RFU, h: hours, T50: Time to reach 50% of maximum RFU

The sensitivity, specificity, diagnostic accuracy and kinetic parameters were calculated for skin, OM, and urine to evaluate the efficiency of the αSyn^D RT-QuIC in discriminating between the DLB CSF positive and HC groups (Table 2). The RT-QuIC performed excellently for the skin group (Fig. 1, b), with a diagnostic accuracy of 96% (CI. = 90–100%) for CSF SAA positive DLB versus HC.

The OM samples preparation (but not the SAA parameters) was optimized from method 1 to method 2, increasing the sensitivity from 24 to 62% without changing the specificity. The combined swabs resulted in a final diagnostic accuracy of 74% (CI = 63–84%) (Table 2; Fig. 1, c). There was a broad gap between the diagnostic accuracies when comparing OM samples processed solely by either method 1 or method 2. Increasing the assay time for OM up to 80 h did not change the outcome of the result.

Lastly, the urine group demonstrates a low αSyn^D detection, with a sensitivity of 22% and with a lack of reproducibility in 3 out of 5 samples (Fig. 1, d). It was noted that the urine samples had significant precipitations, and this was seen for every freeze and thaw cycle. The urine HC group was negative, resulting in a 100% specificity, indicating that there is not a common tendency for false positivity across urine samples using this method.

When comparing the Cohen's agreement of DLB CSF RT-QuIC versus skin RT-QuIC results the agreement was almost perfect, $\kappa = 0.86$, CI 0.6–1), whereas CSF versus OM (combined method) had a none to slight agreement, $\kappa = 0.12$, CI -0.17–0.42), and CSF versus urine had a none to slight agreement, $\kappa = 0.094$ (CI -0.027–0.22). The agreement was higher when comparing skin (DLB and HC) to OM (combined), $\kappa = 0.52$ (CI 0.31–0.72, moderate agreement), and skin to urine, $\kappa = 0.19$ (CI 0.028–0.35, none to slight agreement). The agreement between OM methods 1 and 2 was only fair ($\kappa = 0.34$, CI -0.062–0.75).

When regarding the entire DLB cohort (which includes DLB patients that were negative in CSF RT-QuIC), the sensitivities were 77% (CI = 60–89%) for skin, 40% (CI = 25–58%) for OM, and 17% (CI = 8–35%) for urine (Additional Table S1).

Multiple approaches were tried on saliva, both in sample preparation and to adjustments of the RT-QuIC set-up, but no detection of αSyn^D was achieved. We suspected that the lack of amplification of potentially present αSyn^D is due to strong suppressive effects from the saliva matrix. Therefor we tested the effect of the saliva matrix by spiking HC saliva with DLB BH (10^{-2} to 10^{-5}), which demonstrated a strong suppressive effect, with only the 10^{-2} dilution (10^4-fold concentrated compared to the standard DLB BH control) being significantly amplified.

OM sampling and cytological spot check

There were no significant differences in the technical grading of the sampling procedures between DLB and oHC (Median (IQR): DLB = 4 (3–5) vs. oHC = 4 (3–5), $p = 0.8$). The cytological spot check of 19 samples revealed that three swabs (16%) either had no cells or cells with morphology that could not be studied, while the 16 swabs (84%) were positive for neuronal markers, which are specific for the olfactory mucosa but absent in the additional nasal mucosa, Additional file 1, Fig. S1).

Comparison of αSyn^D RT-QuIC AUC and kinetics across biospecimen groups

The best-performing biospecimens, ranked by highest mean AUC values, are CSF 7 µL, CSF 15 µL, OM, skin, and urine, respectively. However, the difference in mean AUC of the positive samples (Fig. 1, f) between biospecimen groups is minimal. There was no significant difference in AUC values (Fig. 1, f) when comparing 7 µL and 15 µL, and no correlation in a linear model between maximum RFU or AUC with MoCA (data no shown). When taking the best performing volume (CSF

7 µL) as a reference, the mean AUC difference in percentage is 2.3%, 2.7%, and 5.5% for CSF 15 µL, OM, and skin, respectively. This demonstrates an identical AUC pattern between biospecimens in the assay set-up. When comparing the lag-phase duration for all biospecimens (Fig. 1, e), we observe an early stationary phase within 16 to 36 h, regardless of sensitivity. The skin samples are the first to reach the stationary phase, followed by OM, and lastly CSF. When evaluating best-performing biospecimens on the shortest lag-phase duration, we observe the opposite trend than observed for AUC with the best being skin, OM, and CSF, respectively.

Lastly, we evaluated the number of positive replicates (quadruple set-up) for each positive sample. We found that every replicate was positive (100%) for each positive CSF and skin sample, and for OM and urine, the positive replicate rates were 95% and 40%, respectively.

Discussion

We aimed to test an established CSF αSyn^D RT-QuIC assay for its application to screen multiple biospecimens for the presence of αSyn^D without altering the assay protocol, and to compare their efficiency to CSF. We demonstrate that the described αSyn^D RT-QuIC can detect αSyn^D from multiple minimally invasive biospecimens. Our results on skin biopsy samples for αSyn^D RT-QuIC demonstrate a diagnostic accuracy of 96%, similar to other studies [19, 24] and a κ of 0.86, confirming the validity of using skin biopsies for the detection of αSyn^D instead of the more invasive CSF. The sensitivities for detecting αSyn^D in OM and urine were moderate to low in comparison to CSF and skin, which we primarily attribute to obstacles in sampling and processing techniques.

The OM varied in viscosity and amount of cell material, and these variations can certainly influence the outcome of the result. Less variation such as biopsy weight were noticeable in skin processing and total homogenization, and thereby lowering the failure risk. We compared the AUC of each biospecimen group and observed that the early stationary phase is not correlated to a higher AUC, as the stationary phase tends to drop over time (Table 2). Skin had the most rapid amplification with a lag phase of 12 h, and this can be attributed to skin samples being lysed with a buffer containing 1% Triton X-100 (with a final concentration of 0.002% Triton X-100 in the RT-QuIC assay well), which has been proven to accelerate αSyn^D amplification [24]. The prolonged lag phase of CSF (18 h) compared to the other biospecimens is likely due to minor inhibitory effects exhibited by CSF [45]. This inhibitory effect may also explain why CSF at 7 µL (Fig. 1, a, blue lines) performs slightly better than at 15 µL (Fig. 1, a, red lines), even though the αSyn^D seeding should theoretically be higher. Despite the observed differences in kinetics between biospecimens, arising from the nature of the samples or processing technique, we still observe an overall similarity. This similarity demonstrates that the SAA methodology plays a crucial role in the kinetic development and altering the SAA methodology would likely alter the kinetics as well. This is significant regarding what these kinetics can actually tell us about the patient. The reported performance of αSyn^D RT-QuIC on OM samples has varied in previous studies, with sensitivities ranging from 46% [46], 48% [20] to 56% [11] for PD and 83% for DLB [8]. The variance could partly be explained by different groups (PD, RBD, and DLB) but the sampling and further processing could also be important aspects. When we changed the OM extraction method to the described method by Bongianni M. et. al. [8] we achieved a doubling in sensitivity without any other alterations, e.g. the RT-QuIC script. Unfortunately, we did not have sufficient swabs to perform method 2 on all the swabs in the DLB group. The increased sensitivity is likely due to method 2 being more efficient in releasing intracellular material and assessing the pellet for aliquoting, and is therefore our recommended method. To assess the quality of the OM sampling material we randomly selected 19 nasal swabs for cytology spot check and 16% of these were not positve for neurons specific for olfactory mucosa. Even though the cytology spot checks were performed on a small number of samples it demonstrates the challenge of successful sampling, which could be a partial explanation for the lower sensitivity. Perhaps, a cytology spot check would be a standard test along with the SAA result to validate proper sampling. We sought to overcome the risk of incorrect sample material by using two swabs.

Previous studies on OM [11, 20, 37] with varied sensitivities were all performed on RT-QuIC assay parameters that differed (primarily a longer rest cycle time of 14 and 29 min). This results in a prolonged lag-phase of + 30 h and with an assay time of + 70 h, when compared to assays using short resting cycles (~ 1 min) requiring only 48 h at maximum [5, 24]. Our assay on OM is therefore, to our knowledge, the most rapid with a lag-phase of only 16 h and a stationary phase reached at 24 h.

We have investigated the use of saliva samples for αSyn^D detection but did not achieve a successful result, despite numerous efforts. The strategies were aimed at overcoming a strong suppressive effect from the biological material. This suppressive effect was clearly observed when we seeded HC saliva samples with our DLB BH control at dilutions ranging from 10^{-3} to 10^{-6}, inspired by Kuzkina A. et al., who investigated a suppressive effect in OM samples, collected from nasal brushings [20]. Luan M. et al. have reported the successful detection of αSyn^D in saliva samples from PD patients [22, 23]. Of notice, the reported assay parameters are different from ours, especially in the amount of added rec-αSyn pr well, which is

ten-fold higher. We have tried multiple assay modifications but have not substantially increased the rec-αSyn. Another published successful approach is to combine SAA with immuno-precipitation (IP) strategies [50] to isolate present αSyn species.

We report the first proof-of-concept αSyn^D SAA detection in minimally processed urine samples. With thorough optimization, urine may have the potential to become a new biospecimen suitable for detecting αSyn^D in patients with α-synucleinopathies. The lack of reproducibility for 3 out of the initial 5 positive DLB samples is primarily attributed to the possible impact of freezing and thawing urine samples. Saetun et al. demonstrated that the freezing and thawing of urine samples reduced the presence of urinary proteins [43]. The specificity for urine was 100% and we therefore do not attribute the initial positive results to be false. Furthermore, requirements such as urination restriction prior to sampling could be considered. In this study, such requirements were not applied due to a high presence of urge incontinence in participants with DLB. The freezing of urine samples could also be avoided totally by diluting urine samples in a preservation medium (i.e. phosphate buffer supplemented with protease inhibitor cocktail), which could keep samples stable at 4 °C, prior to sample concentration. To our best knowledge, the depositions of αSyn^D in urine have not yet been explored for αSyn^D detection with SAA, but Nam D. et al. [31] reported utilizing urine samples for detecting abnormal levels of αSyn^D in PD patients using an Enzyme-Linked Immunosorbent Assay based method.

Although SAA exhibited a high combined sensitivity (81%), six participants diagnosed with probable DLB remained negative in all biospecimens (Table S1). Given the absence of neuropathological confirmation and the limited sample size, it remains uncertain whether these DLB cases represent phenocopies of other pathologies or if they are false negatives attributed to SAA testing. It is worth noting that there was a trend for higher p-tau pathology in the SAA negative CSF versus SAA positive (Table 1). A study of αSyn^D copathology in AD found a negative correlation between tau and αSyn^D SAA positivity [44]. Weather the increased p-tau represent a different pathology e.g. AD or tau have an inhibitory effect on the SAA cannot be determined from this study. Furthermore, recent studies have reported reduced sensitivity of SAA for Lewy body pathology confined to the olfactory bulb/brainstem [6, 16, 44], amygdala-predominant [3, 16, 44, 47], and limbic system [47]. Consequently, future research involving larger, clinically more divers cohorts with available neuropathological analyses may clarify the issue of SAA-negative DLB cases.

The RT-QuIC sensitivity of the total DLB cohort (Additional Table S1) is lower compared to the subgroup identified as positive for CSF SAA (Table 2). This distinction is important to consider when implementing the assay in a typical clinical setting where the cohort has not been prescreened for CSF SAA positivity.

Despite the promising results, there are few limitations in our study design. The study lacked complete sample blinding. However, the RFU threshold applied in this study for positive and negative RT-QuIC results was predefined in advance based on our own preliminary data and published recommendations [10]. The CSF samples collected from patients with clinical DLB were used to screen for the presence of αSyn^D. It is a limitation that we do not have CSF samples from the HC group. However this decision was made to increase the number of voluntiers in the HC group, who were more accepting of other biospecimen collection, contrary to lumbar puncture. Additional limitation regarding the HC group is that this group was younger than the DLB group by 10 years. The lower mean age and higher cognitive score in the HC group has likely contributed to increased specificity in this study. However, it also provides valuable context regarding the technical performance in HC participants, whom we consider true negative controls.

αSyn^D RT-QuIC has advanced since its first report by Fairful G. et al. [12], with over a hundred studies on various biospecimens, RT-QuIC set-ups, and recombinant αSyn substrate types. Initially, RT-QuIC was adopted to diagnose Creutzfeldt-Jakob Disease [4, 35, 51] and a comprehensive ring-trial with definitive samples was later conducted among established laboratories [34], demonstrating that different in-house assay protocols can yield the same diagnostic conclusions. Similar ring-trials are needed on reported αSyn^D RT-QuIC protocols to evaluate their diagnostic potential, and would also help to understand why some biospecimens, like OM and saliva, perform well only in certain laboratories.

Conclusion

We have established a multi-biospecimen αSyn^D RT-QuIC with rapid performance and feasible sample processing techniques. The diagnostic performance of skin is as powerful as CSF with a diagnostic accuracy of 96% (CI = 90–100%), while OM demonstrates moderate accuracy at 74% (CI = 63–84%), and with opportunities for further improvement in sampling and processing. Detection of αSyn^D in saliva samples failed, likely due to the sample material inhibiting the assay performance. Lastly, we have demonstrated the first proof-of-concept detection of αSyn^D in urine with minimal sample processing, potentially bringing another non-invasive strategy into the diagnostic toolbox for α-synucleinopathies.

Abbreviations

AD	Alzheimer's disease
AUC	Area under curve

BH	Brain homogenate
CSF	Cerebrospinal fluid
CNS	Central nervous system
CI	Confidence intervals
DLB	Dementia with lewy bodies
HC	Healthy controls
HCl	Hydrochloric acid
lag-phase	Lagging phase
log-phase	Logarithmic phase
MCI-LB	Mild cognitive impairment with Lewy bodies
MMSE	Mini-mental state examination
MoCA	Montreal cognitive assessment
OM	Olfactory mucosa
oHC	Older HCs
ANOVA	One-way analysis of variance
PD	Parkinson´s disease
PBS	Phosphate-buffered saline
αSyn^{D}	Disease causing α-synuclein
PP	Polypropylene
PMCA	Protein misfolding cyclic amplification
RT-QuIC	Real-time quaking-induced conversion
RFU	Relative fluorescence units
RBD	REM sleep behavior disorder
RBD	Seeding amplification assays
SDS	Sodium dodecyl laurate
yHC	Young HCs

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40478-025-02034-8.

Supplementary Material 1

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Author contributions

Conceptualization was the effort of RB, OHM, GW, SGH, CvB, CKP, KSF, ELL and AA.Writing-original draft was the effort of RB, OHM and AA. AHS, MB, CHP, SOB, JL, PE, MB, EB, GZ, RB, OHM, GW, SGH, CvB, CKP, KSF, ELL and AA contributed to the Methodology and Writing – review & editing. Supervision and funding were the effort of GW, SGH, KSF. ELL and AA.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

The study was approved by The Danish Research Ethics Committee (H-22046053 and H-22053428) and The Danish Data Protection Agency (P-2022-668 and P-2022-669). All patients gave written informed consent to participate in the study.

Consent for publication

All authors have read and approved this manuscript for publication. All patients gave consent for publication of study results.

Competing interests

The authors declare no competing interests.

Author details

[1]Danish Reference Center for Prion Diseases, Department of Pathology, Copenhagen University Hospital, Copenhagen, Denmark
[2]Danish Dementia Research Centre, Dept. of Neurology, Copenhagen University Hospital - Rigshospitalet, Copenhagen, Denmark
[3]Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark
[4]Dept. of Otolaryngology, Head & Neck Surgery and Audiology, Copenhagen University Hospital - Rigshospitalet, Copenhagen, Denmark
[5]Department of Neurosciences, Biomedicine, and Movement Sciences, University of Verona, Policlinico G. B. Rossi, Verona, Italy

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Publisher's note

Accurate Detection of Pathologic α-Synuclein in CSF, Skin, Olfactory Mucosa, and Urine with a Uniform Seeding Amplification Assay

Remarh Bsoul[1*], Oskar H. McWilliam[2*], Gunhild Waldemar[2,3], Steen G. Hasselbalch[2,3], Anja H. Simonsen[2], Christian von Buchwald[3,4], Magne Bech[4], Clara H. Pinborg[4], Christian K. Pedersen[4], Sara O. Baungaard, José Lombardía[2], Patrick Ejlerskov[2], Matilde Bongianni[5], Erika Bronzato[5], Gianluigi Zanusso[5], Kristian S. Frederiksen[2,3], Eva. L. Lund[1,3], Aušrinė Areškevičiūtė[1]

Affiliations

[1]Danish Reference Center for Prion Diseases, Department of Pathology, Copenhagen University Hospital, Denmark

[2] Danish Dementia Research Centre, Dept. of Neurology, Copenhagen University Hospital - Rigshospitalet, Copenhagen, Denmark

[3] Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

[4] Dept. of Otolaryngology, Head & Neck Surgery and Audiology, Copenhagen University Hospital - Rigshospitalet, Copenhagen, Denmark

[5]Department of Neurosciences, Biomedicine, and Movement Sciences, University of Verona, Policlinico G. B. Rossi, Verona, Italy

[*]Contributed equally.

Corresponding author: Aušrinė Areškevičiūtė, ausrine.areskeviciute@regionh.dk, +45 21535902

Additional Materials

S1 SAA sensitivity and specificity for DLB versus clinical DLB diagnosis and HC

Table S1 Sensitivity, Specificity and Accuracy for all clinically diagnosed with probable DLB

aSyn SAA	Samples, N DLB/HC	Sensitivity % (CI)	Specificity % (CI)	Accuracy % (CI)
CSF (15 µL)	22/0	82 (61-93)	-	-
Skin	31/52	77 (60-89)	98 (90-100)	88 (80-95)
Olfactory Mucosa combined	30/53	40 (25-58)	100 (93-100)	70 (62-78)
Olfactory Mucosa Swab 1	30/52	27 (14-44)	100 (93-100)	63 (56-71)
Olfactory Mucosa Swab 2	17#/39	35 (17-59)	100 (91-100)	68 (57-78)
Saliva	22/5	NA	NA	NA
Urine	29/12	17 (8-35)	100 (76-100)	59 (50-68)
All biospecimens combined (ex. CSF)	31/53	81 (64-91)	98 (90-100)	89 (82-97)
All biospecimens combined (incl. CSF)	22/0	84 (67-93)	-	-

Table showing the number of samples in the total cohort of DLB and HC. Sensitivity is shown for the clinical diagnosis of probable DLB. The specificity and accuracy are measured against HC. Generally, swab 1 was treated with method 1 and swab 2 with method 2, except all oHC swabs was treated with method 2. # 3 DLB swabs discarded due to no pellet. Abbreviations: CSF = cerebrospinal fluid, CI = 95 % Confidence intervals, DLB = Dementia with Lewy body, N = number, NA = not applicable, OM = olfactory mucosa, SAA = seeding amplification assay, SD = standard deviation, oHC = older healthy controls, yHC = young healthy controls.

Table S2 Overview of Olfactory Mucosa results in the DLB cohort

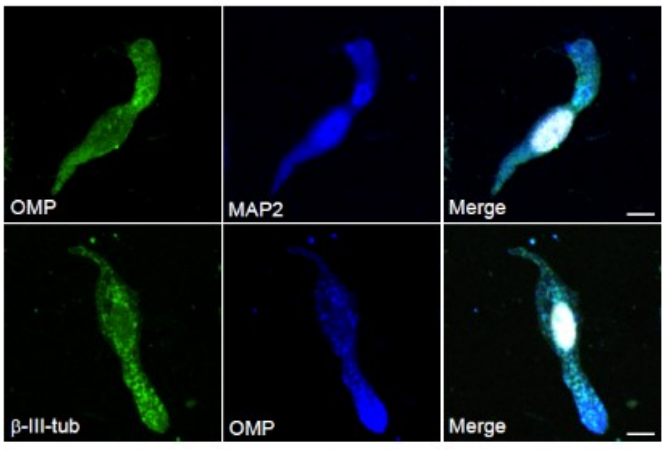
	Method 1	Positive wells	Method 2	Positive wells
CSF SAA positive				
DLB1	Positive	4	No pellet	
DLB2	Negative	0	No swab	
DLB3	Negative	0	No swab	
DLB4	Negative	0	No swab	
DLB5	Negative	0	No pellet	
DLB6	Negative	1	No swab	
DLB7	Positive	2	No swab	
DLB8	Negative	0	No swab	
DLB9	Negative	1	Negative	0
DLB10	Positive	4	No swab	
DLB11	Negative	0	Positive	4
DLB12	Negative	0	Negative	0
DLB13	Positive	4	Positive	4
DLB14	No swab		No swab	
DLB15	Negative	0	Positive	4
DLB16	Negative	0	No swab	
DLB17	Negative	0	Positive	4
DLB18	Negative	0	Positive	4
No SAA CSF available				
DLB19	Negative	0	No pellet	
DLB20	Negative	1	Negative	0
DLB21	Negative	0	Negative	1
DLB22	Negative	1	Negative	0
DLB23	Negative	1	Negative	0
DLB24	Positive	2	Negative	0
DLB25	Positive	4	No swab	
DLB26	Negative	1	Negative	1
DLB27	Positive	4	Positive	4
CSF SAA negative				
DLB28	Positive	2	Negative	0
DLB29	Negative	0	Negative	0
DLB30	Negative	0	Negative	0
DLB31	Negative	0	No swab	

Table of olfactory mucosa analysis with method 1 and method 2. In both methods 4 wells were run with 2 positive needed for a positive test. All HCs were negative with none having any positive wells. Abbreviations: CSF = cerebrospinal fluid, DLB = Dementia with Lewy body, SAA = seeding amplification assay

S2 Cytological spot check protocol

To verify the anatomical location was OM and, we assessed the presence of neurons in the cells collected from the nasal swabs (N = 19). The cells were gently removed from the swab with a cell scraper in ice-cold PBS-/- (without calcium/magnesium) and transferred to a 15 ml PP tube. The cells were centrifuged at 500 x g, for 10 minutes, at 4°C, and following the cell pellet was resuspended in 250 ice cold PBS and the cells were counted. Then 250 µl of paraformaldehyde (PFA) 8% was added (final concentration 4%) and 20.000 cells were transferred to each well in an Ibidi 12-well chamber. To attach the fixed cells to the bottom of the Ibidi chamber slide, the cells were cyto-spun in a custom-made 3D printed holder at 1000 x g, for 5 minutes, at room temperature. The adherent cells were incubated in the PFA for additional 5 minutes in a flow bench before a gentle wash in PBS-/-. For immunofluorescence staining the cells were blocked and permeabilized in a TX-100 blocking buffer (5% normal goat serum, 2% bovine serum albumin, and 0.25% TX-100 in PBS-/- for 45 minutes before adding primary antibodies diluted in blocking buffer overnight, at 4°C. The following day the cells were washed 3 times in PBS-/- and then incubated with secondary antibodies diluted in blocking buffer for 1 hour at room temperature. Subsequently, the cells were stained with DAPI for 5 minutes, washed 3 times in PBS-/-, and mounted with ProLong™ Gold Antifade Mountant (Molecular Probes). Images were acquired in a Nikon Ti-E inverted Crest confocal spinning disk microscope with a 60x oil objective. The following antibodies were used; primary antibodies: □-III-tubulin (Merck cat. no. MAB1637), Olfactory Marker Protein (Novus cat. no. NB110-74751); and secondary antibodies: Alexa fluor 488, 568, and 633 (Invitrogen).

Fig S1 Olfactory neurons from the nasal cavity.



Cells were gently removed from the nasal swab and fixed in 4 % paraformaldehyde solution (PFA). The cells were cytospun and immunostained for neuronal markers olfactory marker protein (OMP), MAP2, and β-lll-tubulin. Scale bar, 10 µM.

S3 Strategies for saliva RT-QuIC

Below are listed different options tried for saliva samples, individually and in multiple combinations with each other. Codes in parentheses () indicate that the combinations were used with and without these. Totally were 22 saliva samples tried in various combinations.

Additives:

A1 Protease inhibitor cocktail

A2 Triton x-100 (0.1 %)

A3 Reducing agent (1x DTT – 20 min incubation at 30 °C)

Treatments before RT-QuIC:

T1 Sonication in water bath (1 min, 37 C, 100 % intensity at 120 V)

T2 Sterile filtration (0.2 um) of saliva samples

Concentrating (**C1**=2x, **C2**=5x, **C3**=10x) in 100 kDa spin filter and redissolve in 40 mM Phosphate buffer.

Diluting (**D1**=1:10, **D2**=1:2) in phosphate buffer

Volumes (**VX** µl) of saliva added to RT-QuIC:

Script parameters adjusted:

S 800 rpm instead of 400 rpm

Tries with Concentration:

C1 + V1 + (V2) + (A2) + (A3)

C2 + V4 (+ A1) + V4 (+A2)

C3 + T1 + V4 + (A1)

Tries with Diluting:

D1 + V5

D2 + V1 (+ V2) + (V3) + (V4) + (A2) + (A2)

Tries with different volumes of saliva added to RT-QuIC:

V2 + (+ A2) + D2 (+ A3)

V4 + (+ A2) + C3 (+T1) + D2 (+ A3) +S

V5 + (+ A1) + (A3) + D2

V7 + T1 + A1 (+ C2) + (+ A2) + D2

V10 + D1 + (C3)

8.2 Study 2: α-Synuclein seed amplification assay in Lewy body dementia versus Alzheimer's disease.

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RESEARCH ARTICLE

α-Synuclein seed amplification assay in Lewy body dementia versus Alzheimer's disease

Oskar H. McWilliam[1] | **Remarh Bsoul**[2] | **Eva L. Lund**[2,3] | **Gunhild Waldemar**[1,3] | **Steen G. Hasselbalch**[1,3] | **Anja H. Simonsen**[1] | **Marie Bruun**[1,3] | **Christian von Buchwald**[3,4] | **Kasper Aanæs**[3,4] | **Christian K. Pedersen**[4] | **Ida S. B. Andersen**[4] | **Magne Bech**[4] | **Aušrinė Areškevičiūtė**[2] | **Kristian S. Frederiksen**[1,3]

[1]Danish Dementia Research Centre, Department of Neurology, Copenhagen University Hospital - Rigshospitalet, Copenhagen, Denmark

[2]Department of Pathology, Danish Reference Center for Prion Diseases, Copenhagen University Hospital - Rigshospitalet, Copenhagen, Denmark

[3]Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

[4]Department of Otolaryngology, Head & Neck Surgery and Audiology, Copenhagen University Hospital - Rigshospitalet, Copenhagen, Denmark

Correspondence
Oskar Hoffmann McWilliam, Inge Lehmanns Vej 6, 2100 Copenhagen, Denmark.
Email: oskar.mcwilliam@regionh.dk

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Abstract

INTRODUCTION: Differentiating dementia with Lewy bodies (DLB) from Alzheimer's disease (AD) is challenging. Seed amplification assay (SAA) is sensitive for the detection of misfolded α-synuclein.

METHODS: Patients with DLB ($N = 31$) and AD ($N = 25$) were recruited and evaluated. Misfolded α-synuclein was assessed in cerebrospinal fluid (CSF), skin, urine, and olfactory mucosa using SAA.

RESULTS: The accuracy of α-synuclein-SAA for DLB was 87% (95% confidence interval [CI]: 77% to 98%) in CSF, 85% (95% CI: 75% to 98%) in skin, 58% (95% CI: 47% to 69%) in olfactory mucosa, and 59% (95% CI: 51% to 66%) in urine. The core symptoms – fluctuations, REM sleep behavior disorder, and parkinsonism – had accuracies for SAA positivity of ≥79%. Notably, 95% of SAA-positive patients also had hyposmia.

DISCUSSION: These findings support the use of CSF and skin α-synuclein-SAAs as diagnostic tools for DLB, with strong associations between SAA and clinical phenotype. In particular, intact olfactory function is associated with a lower risk of SAA positivity.

KEYWORDS

Alzheimer's disease, anosmia, atypical parkinsonism, autonomic dysfunction, biomarker, cerebrospinal fluid, core criteria, dementia, dementia with Lewy bodies, hyposmia, mild cognitive impairment, olfactory dysfunction, olfactory mucosa, Parkinson's disease, real-time quaking-induced conversion, REM sleep behavior disorder, seeding amplification assay, skin, urine, visual hallucinations

Highlights

- CSF and skin biopsies show high diagnostic accuracy for α-synuclein, demonstrating good concordance.

Aušrinė Areškevičiūtė and Kristian S. Frederiksen contributed equally to the study and share last authorship.

- Strong correlations exist between core symptoms of DLB and pathological α-synuclein.
- A very high sensitivity of hyposmia for pathological α-synuclein is observed.
- A novel proof-of-concept is offered for the potential detection of pathological α-synuclein in urine, marking the first such comparative analysis between patients with DLB and AD.

1 | BACKGROUND

Dementia with Lewy bodies (DLB) and Parkinson's disease (PD) are characterized by pathologically misfolded neuronal α-synuclein deposition (αSyn^D). The protein aggregates into Lewy bodies (LBs) and may be implicated in the neurodegeneration observed in both diseases. In most but not all LB disease cases, the olfactory bulb is the first place LB pathology is observed. A proposed mechanism underlying DLB and PD pathology is the prion-like propagation of αSyn^D, with the olfactory bulb serving as the earliest site of pathological changes in most phenotypes.[1–5]

The discrimination between DLB and Alzheimer's disease (AD) in clinical practice can be a challenge, especially early in the disease course.[6] In a population of patients with DLB and PD dementia, 36% were initially misdiagnosed with AD or related dementias and 24% with a primary psychiatric disorder before receiving a correct diagnosis.[7] An early correct diagnosis of DLB is important to ensure proper treatment, potentially minimizing complications such as falls, psychiatric problems, and dysautonomia and improving quality of life. Recently, focus on a biological definition of α-synucleinopathies has been described in several papers.[8,9] Moreover, high-precision diagnostic tests could be critical in selecting patients for drug trials involving disease-modifying therapies.[10]

In this context, seed amplification assays (SAAs) for detecting αSyn^D have demonstrated promising diagnostic performance in the discrimination of DLB from AD, with pooled sensitivity and specificity of 95% and 88%, respectively, across multiple biospecimens (brain, CSF, skin, and olfactory mucosa).[11] In studies with neuropathologically confirmed αSyn^D pathology, the sensitivity and specificity of the CSF αSyn^D SAA approach 100%.[12,13] In contrast, when a clinical diagnosis of DLB is used, the reported sensitivities are lower, around 72% to 90%.[14–16] Therefore, in the absence of neuropathological confirmation, CSF represents a gold standard for the detection of αSyn^D by SAA in peripheral biospecimens.

SAA performed on peripheral biospecimens like skin, saliva, blood, olfactory mucosa, and urine, which may accumulate αSyn^D earlier than CSF,[1,2] could provide an opportunity for early detection, along with the possibility to map the *ante mortem* spread of αSyn^D. Less invasive procedures can also be more feasible than lumbar puncture for diagnosis in the prodromal stages of DLB and PD. The studies assessing biospecimens other than CSF for DLB versus AD have generally been few and with small sample sizes (<17 AD patients[17,18]). Peripheral biospecimens have been studied more thoroughly in PD than in DLB. The sensitivity for different SAA in olfactory mucosa has varied considerably from 46% to 75%,[17,19–21] while skin showed a pooled sensitivity reaching around 90%.[11,22,23] Few studies have investigated multiple biospecimens in the same patient cohort,[12,24] enabling more precise diagnostic accuracy comparisons.

In this study, we aimed to determine the sensitivity, specificity, and diagnostic accuracy of αSyn^D -SAA for DLB versus AD in CSF, skin, olfactory mucosa, and urine. Second, we aimed to assess the association of DLB symptoms with SAA positivity.

2 | METHODS

2.1 | Participants

Patients diagnosed with AD or DLB were recruited from the Memory Clinic at Copenhagen University Hospital, Rigshospitalet, from February to October 2023. The DLB patients were investigated in a technical paper on SAA kinetics and development by Bsoul et al.[25]

Inclusion criteria were (1) a diagnosis of probable dementia or mild cognitive impairment (MCI)[26,27] due to DLB or AD,[28,29] (2) Mini-Mental State Examination (MMSE) score > 18, (3) age > 50 years, and (4) a caregiver willing and able to participate in the study. Exclusion criteria were (1) other major neurological or psychiatric diseases or (2) ongoing alcohol or drug abuse.

The diagnostic work-up for all patients included anamnesis, physical and neurological examination, blood screening tests, cerebral magnetic resonance imaging/computed tomography scan, fluorine-18 fluorodeoxyglucose positron emission tomography scan, and for a substantial subgroup also neuropsychological testing, and lumbar puncture (for detection of β-amyloid, p-tau, and tau). Some patients with DLB underwent polysomnography and dopamine transporter scans (Table S1). A consensus diagnosis was reached at a multidisciplinary team conference and based on international clinical criteria.[26–29] Results from the analysis of αSyn^D were not available at the team conference. The study was registered at www.clinicaltrials.gov (ID: NCT05768425) before recruitment.

2.2 | Study design and procedures

This was a single-center, cross-sectional, unblinded case-control study. Following informed consent, participants were seen for a single study

visit at which several scales/tests were administered (see below). Further, patients underwent lumbar puncture if no contraindications (e.g., treatment with anticoagulants/thrombotics) were present. If patients had undergone lumbar puncture with CSF stored at the Danish Dementia BioBank within 1.5 years of inclusion, a new lumbar puncture was not performed, and the CSF from the biobank was used for SAA. Lastly, skin biopsies and olfactory mucosa sampling were performed, and urine was collected.

2.3 | Assessments and definitions of outcomes

All participants were seen by a single investigator (OHM). MMSE was applied to assess global cognitive function.[30]

Movement Disorders Society-Unified Parkinson's Disease Rating Scale (MDS-UPDRS) I–III was used to evaluate parkinsonian symptoms. Parkinsonism was assessed by MDS-UPDRS III elements specific to parkinsonism in DLB and MCI-DLB, as outlined by McKeith et al.[26,27]

The olfactory function was assessed using the multiple-choice Sniffin' Sticks Smell Identification Test 16 (SIT16).[31] The maximum score is 16, and a score around 4 indicates anosmia. We defined hyposmia as scoring below the 25th percentile age-adjusted score (< 11 for age 60-70; < 10 for age 70-80; < 6 for age 81+), based on a study with normative data.[32]

The presence of probable REM sleep behavior disorder (RBD) was assessed using the RBD1Q, a single-question test validated for RBD[33,34] combined patient history. Cognitive fluctuations and visual hallucinations were clinically assessed based on patient history. For further definitions of clinical evaluations (Supplementary Materials S1).

2.4 | Collection and handling of biospecimens

CSF was collected by lumbar puncture in polypropylene vials. After collection, the CSF was kept at 4°C and centrifuged at 2000 × g for 10 min. The resulting supernatant was stored at −80°C within 2 h of sampling.

One-punch skin biopsy (3 mm diameter) was obtained paramedially 5 cm from the C7 vertebra following subcutaneous administration of local anesthetic and adrenaline. The skin samples were dabbed for blood, rinsed in 5 mL phosphate-buffered saline, and kept on ice in polypropylene tubes until stored dry at −80°C within 2 h of sampling.

Olfactory mucosa sampling was performed under visual guidance by nasal endoscopy by ear-nose-throat specialists or a medical student specially trained in the procedure. Samples were collected with a fiber-based swab (Nasopharyngeal Specimen Collection Swab, NEST) from the roof of the nasal cavity (agger nasi area) and upper portion of the middle turbinate in both nostrils following administration of local anesthetics and detumescent agents. Two samples (one on each side with different swabs) were taken when possible. The method is described in Bsoul et al.[25]

Urine samples (50 mL) were collected in polypropylene containers without imposing restrictions on the interval since the last urination. Samples were stored at −80°C.

RESEARCH IN CONTEXT

1. **Systematic review**: We conducted a review to identify studies evaluating SAAs for detecting misfolded α-synuclein in DLB and AD. While most studies focused on CSF, limited evidence exists for peripheral tissues such as skin, olfactory mucosa, and urine. No prior study assessed these biospecimens in the same patients.
2. **Interpretation**: CSF and skin α-synuclein-SAA are highly accurate diagnostic biomarkers for DLB versus AD, which could improve the early identification of patients with DLB in clinical settings. The strong association between SAA positivity and clinical features, parkinsonism, REM sleep behavior disorder, fluctuations, and hyposmia supports α-synuclein-SAA's clinical relevance.
3. **Future directions**: Future research should validate these findings, particularly those related to skin, in larger, independent cohorts. Key priorities include investigating α-synuclein in prodromal Lewy body disease and identifying minimally invasive biomarkers to improve early diagnosis and refine clinical trial inclusion criteria.

2.5 | Seed amplification assay

For αSyn^{D}-SAA, we used a uniform real-time quaking-induced conversion assay for all biospecimens performed on a FLUOStarTM Omega instrument with recombinant α-synuclein with an N-terminal 6x-histidine-tag, as described previously.[25] The assay was not performed blinded, but the threshold was preset to 4 standard deviations from a negative control group in a pilot study of SAA CSF. All samples were run in quadruplicate wells and deemed positive if at least two replicates crossed the threshold within 48 h. Buffers are described in Supplementary Materials S2. No blood-contaminated CSF or nasal swabs were used for SAA. Two different olfactory mucosa swabs were analyzed, when possible, but in 16 cases, only one swab was analyzed due to insufficient material. The SAA kinetics are presented in Figure S1.

2.6 | Statistical analysis

All analyses were performed using R (version 4.3.3). For comparisons of numerical variables between DLB and AD, the unpaired *t* test was applied for normally distributed data, while the exact Wilcoxon–Mann–Whitney test was used for non-normally distributed data. Fisher's exact test was employed for categorical data comparisons. Sensitivity, specificity, accuracy, positive predictive value (PPV), and negative predictive value (NPV) were calculated using the binom package. Accuracy is defined as the overall proportion of correct classifications (both true positives and true negatives) out of all individuals tested. We used 95% confidence intervals (CIs) computed using the Wilson method.[35] Cohen's kappa (κ) was calculated using the psych package to assess concordance between CSF and other

TABLE 1 Demographics and features of DLB and AD.

Characteristic	AD	DLB	*p* value
N	25	31	
Age, mean (SD)	73.4 (5.2)	75.0 (5.9)	0.079
Sex, *N* female (%)	11 (44%)	4 (12%)	0.015
MMSE, median (IQR)	25 (23 to 26)	26 (24-29)	0.040
Months from first visit, median (IQR)	7.5 (2.8 to 22)	10 (4.0-22)	0.23
MDS-UPDRS III, median (IQR)	7.0 (3.0 to 9.0)	19 (14-33)	<0.0001

Abbreviations: AD, Alzheimer's disease; DLB, Dementia with Lewy bodies; IQR, interquartile range; MDS-UPDRS, Movement Disorders Society-Unified Parkinson's Disease Rating Scale; MMSE, Mini-Mental State Examination; *N*, number; SD, standard deviation.

biospecimens. Cohen's interpretation: below 0 = no agreement, 0.01 to 0.2 = none to slight agreement, 0.21 to 0.39 = fair agreement, 0.40 to 0.59 = moderate agreement, 0.60 to 0.79 = substantial agreement, above 0.80 = almost perfect agreement.[36] *P* values were not corrected in this study and were considered significant if less than 0.05.

3 | RESULTS

3.1 | Demographics and clinical characteristics

We included 31 patients with DLB and 25 patients with AD, of which three participants in each group were in the MCI stage. In general, the groups were well matched. As expected, there were more females in the AD group (Table 1). FAQ-IADL was higher in DLB, and scores on assessments measuring symptoms and features related to α-synucleinopathies, for example, MDS-UPDRS III (Table 1) and SIT16 (Figure S2), indicated more impairment in the DLB group.

All but one patient with DLB fulfilled two or more core diagnostic criteria for DLB,[27] and none of the AD patients fulfilled more than one. All AD patients were positive for increased phosphorylated tau at threonine 181 (p-tau181)/amyloid beta (Aβ)-42 ratio in CSF. In the DLB group, 54% (13 out of 24 DLB patients for whom CSF had been analyzed) were positive for an increased p-tau181/Aβ-42 ratio.

3.2 | Sensitivity, specificity, and accuracy of αSyn^D SAA

The frequency of αSyn^D SAA positivity varied substantially across the different biospecimens. CSF and skin showed the highest diagnostic accuracy of 87% (95% CI: 77% to 97%) and 85% (95% CI: 76% to 94%) for the clinical diagnosis of DLB (Table 2). The concordance between skin and CSF in AD and DLB was almost perfect, with $\kappa = 0.87$ (95% CI: 0.72 to 1.0). Taking CSF αSyn^D SAA positivity as the reference instead of clinical DLB diagnosis, the sensitivity of skin increased to 94% (95% CI: 74% to 99%), the specificity to 96% (CI: 82% to 99%) with an accuracy of 95% (95% CI: 83% to 100%) (Table S2). Both CSF and skin SAA had high PPV, ≥90%, with a slightly lower NPV of 85% and 77%, respectively for DLB. The PPV and NPV in relation to DLB prevalence are displayed in Figure S3. The olfactory mucosa showed a lower sensitivity at 40% (95% CI: 23% to 59%) and the lowest specificity of all the specimens at 76% (95% CI: 57% to 89%). The concordance between CSF and olfactory mucosa was fair, with $\kappa = 0.26$ (95% CI: −0.02 to 0.54). Urine had a very low sensitivity of 17% (95% CI: 8.0% to 35%) but exhibited a high specificity of 100% (95% CI: 86% to 100%). The concordance with CSF was fair, with $\kappa = 0.22$ (95% CI: 0.03 to 0.41). One participant with DLB and five with AD were only positive in the olfactory mucosa and not skin or CSF (Table S3). A total of 84% of DLB cases and 32% of AD cases tested positive for SAA in at least one biospecimen, resulting in an overall accuracy of 76% (95% CI: 65% to 87%).

3.3 | Sensitivity, specificity, and accuracy of DLB features predicting SAA positivity

To identify which clinical features of DLB were predictive of SAA positivity in CSF, we evaluated the diagnostic accuracy of different clinical features (Table 3). Among the core diagnostic criteria, parkinsonism and fluctuations demonstrated the highest accuracy of 83% (95% CI: 70% to 91%), probable RBD had an accuracy of 79% (95% CI: 65% to 88%), while visual hallucinations had the lowest at 72% (95% CI: 58% to 83%) of the four core criteria. Furthermore, objective age-adjusted hyposmia had the highest sensitivity at 95% (95% CI: 76% to 99%) but a low specificity of 41% (95% CI: 25% to 59%). Applying a fixed cut-off of SIT16 < 8 dropped the sensitivity to 85% (95% CI: 64% to 95%) but raised the specificity to 52% (95% CI: 34% to 69%). Orthostatic hypotension, fluctuations, and visual hallucinations had the highest specificity at 89% (95% CI: 72% to 96%). When considering the number of core diagnostic criteria fulfilled, the presence of 2+ yielded the highest point estimate of accuracy of 87% (95% CI: 58% to 83%), although confidence intervals overlapped considerably with 1+ and 3+ core criteria.

Regarding the scaled variables, a ROC curve analysis was performed. MDS–UPDRS I-III, MDS–UPDRS III, and core DLB criteria including all indicative biomarkers had an area under the curve (AUC) ≥ 85%, while SIT16 had AUC = 75% (Figure S4).

Regarding participants with αSyn^D SAA-positive and negative olfactory mucosa, we did not find any significant differences in SIT16 (median [IRQ] for positive 5.5 [5.5 to 8.8] versus negative 7.0 [5.0 to 10], $p = 0.38$) in the DLB cohort.

4 | DISCUSSION

This study investigated the diagnostic accuracy of αSyn^D detection by SAA for the differential diagnosis of DLB versus AD in various biospecimens. We found that SAA in CSF performed best in identifying patients with a clinical diagnosis of DLB. The detection of αSyn^D in the skin

TABLE 2 Diagnostic accuracy of αSyn^D SAA for diagnosis of DLB versus AD in different biospecimens.

Biospecimen	DLB: SAA+/total AD: SAA+/total	Sensitivity, % (95% CI)	Specificity, % (95% CI)	Accuracy, % (95% CI)	PPV, % (95% CI)	NPV, % (95% CI)
CSF	18/22 2/25	82 (61 to 93)	92 (75 to 98)	87 (77 to 97)	90 (70 to 97)	85 (68 to 94)
Skin	24/31 2/25	77 (60 to 89)	92 (75 to 98)	85 (76 to 94)	92 (76 to 98)	77 (59 to 88)
Olfactory mucosa	12/30 6/25	40 (25 to 58)	76 (57 to 89)	58 (47 to 69)	67 (44 to 84)	51 (36 to 67)
Urine	5/29 0/24	17 (8 to 35)	100 (86 to 100)	59 (51 to 66)	100 (57 to 100)	50 (36 to 64)
CSF + Skin combination	25/31 3/25	81 (64 to 91)	88 (70 to 96)	84 (75 to 94)	89 (73 to 96)	79 (60 to 90)
Any	26/31 8/25	84 (67 to 93)	68 (48 to 83)	76 (65 to 87)	76 (60 to 88)	77 (57 to 90)

Abbreviations: AD, Alzheimer's disease; Any, a combination of all bio-specimens analyzed; CI, confidence interval; CSF, cerebrospinal fluid; DLB, Dementia with Lewy bodies; *N*, number; NPV, negative predictive value; PPV, positive predictive value.

TABLE 3 Sensitivity, specificity, and accuracy of DLB features for CSF αSyn^D SAA positivity.

CSF αSyn^D SAA	Sensitivity, % (95% CI)	Specificity, % (95% CI)	Accuracy, % (95% CI)
Fluctuations	75 (53 to 89)	89 (72 to 96)	83 (70 to 91)
Visual hallucinations	50 (30 to 70)	89 (72 to 96)	72 (58 to 83)
Probable RBD	75 (53 to 89)	81 (63 to 92)	79 (65 to 88)
Parkinsonism	80 (58 to 92)	85 (68 to 94)	83 (70 to 91)
Objective hyposmia	95 (76 to 99)	41 (25 to 59)	64 (50 to 76)
Subjective hyposmia	80 (58 to 92)	78 (59 to 89)	79 (65 to 88)
Hypersomnia	70 (48 to 85)	67 (48 to 81)	68 (54 to 80)
Urge incontinence	80 (58 to 92)	59 (41 to 75)	68 (54 to 80)
Constipation	55 (34 to 74)	78 (59 to 89)	68 (54 to 80)
Orthostatic hypotension	40 (22 to 61)	89 (72 to 96)	68 (54 to 80)
Core criteria ≥ 1	90 (70 to 97)	70 (52 to 84)	79 (65 to 88)
Core criteria ≥ 2	90 (70 to 97)	85 (68 to 94)	87 (75 to 94)
Core criteria ≥ 3	75 (53 to 89)	93 (77 to 98)	85 (72 to 93)
Core criteria = 4	25 (11 to 47)	96 (82 to 99)	66 (52 to 78)

Note: The total number of DLB and AD with αSyn^D SAA analysis in CSF = 22 and 25, respectively. Sensitivity, specificity, and accuracy of DLB features for αSyn^D SAA positivity across both diagnostic groups (DLB and AD). Colors indicate relative values: intense red <50%, faded red = 50% to 64%, no color = 65% to 74%, faded green = 75% to 84%, and intense green >85%. Hyposmia was evaluated by the 25th lower percentile of age-adjusted scores of SIT16, applying a non-age-adjusted cut-off at SIT16 ≤ 9 gave a similar result with a sensitivity, specificity, and accuracy of 95% (95% CI: 76% to 99%), 44% (CI: 28% to 63%), and 66% (95% CI: 52% to 78%), respectively. A cut-off at SIT16 ≤ 8 resulted in sensitivity, specificity, and accuracy of 85% (95% CI: 64% to 95%), 52% (95% CI: 34% to 69%), and 66% (95% CI: 52% to 78%), respectively. The correlation between objective and subjective hyposmia is shown in Figure S4.
Abbreviations: AD, Alzheimer's disease; CI, 95% confidence intervals; CSF, cerebrospinal fluid; DLB, Dementia with Lewy bodies; *N*, number; RBD, REM sleep behavior disorder; SAA, seed amplification assay; SIT16, Smell Identification Test 16.

demonstrated diagnostic performance nearly identical to that of CSF. In contrast, SAA in olfactory mucosa and urine showed substantially lower diagnostic accuracies. Clinically, almost all participants with a positive CSF SAA test exhibited the supportive DLB feature of hyposmia, suggesting that normosmia may effectively exclude SAA positivity in CSF. Additionally, the core features of fluctuations, parkinsonism, and probable RBD demonstrated notably high accuracy in identifying CSF SAA positivity.

4.1 | CSF and skin SAA

The high diagnostic accuracy (>80%) of CSF SAA for DLB versus AD observed in this study reinforces the potential of SAA as an integrational biomarker for distinguishing DLB from AD. We observed a sensitivity of 82%, in line with prior clinical studies reporting sensitivities ranging from 72% to 90%.[14,15,17] Neuropathologically confirmed studies generally report near-perfect sensitivities.[12,13] The

discrepancy between clinical and neuropathological studies may be due to the inherent challenge of clinical diagnosis[6,7] or the selection of participants.

The specificity of CSF SAA in this study at 92% aligns with previous studies, which are summarized in a meta-analysis reporting a pooled specificity in CSF of 88% (95% CI: 84% to 92%) for DLB versus AD.[11] The high specificity of SAA is particularly notable given the high co-occurrence of αSyn^D pathology in AD, which is present in approximately 20% to 50% of neuropathologically confirmed AD cases.[37,38] The discrepancy between the high rate of αSyn^D co-pathology in AD and the high specificity may be partly explained by the accumulation of incidental αSyn^D co-pathology from the time of diagnosis to death and/or that the clinical DLB phenotypes have widespread and sufficient αSyn^D pathology to trigger the SAA.[39,40] The variable sensitivity of SAA was investigated in a recent large study, which reported the sensitivity for detecting LB pathology to be highest in cortical regions, with lower sensitivities observed in the amygdala and limbic regions.[40] Further studies investigating factors like αSyn^D localization or co-pathology that influence SAA may, in the future, clarify these discrepancies.

In this study, skin SAA demonstrated performance comparable to CSF, with a near-perfect concordance between the two. While skin-based assays have been studied in PD, investigations in DLB have so far been limited to small sample sizes[18,41] with an accuracy against AD of 79%. The high accuracies of CSF and skin SAA reinforce the involvement of both central and peripheral pathophysiological processes in DLB, consistent with findings from previous immunohistochemical studies[22,42] and the hypotheses on the spread of αSyn^D.[1–3,5] Although CSF and skin in terms of assessment of αSyn^D may be interchangeable, there are other notable differences between the two in the diagnostic process. CSF analyses provide additional information about other biomarkers, but the lumbar puncture also has several limitations, including medical contraindications[41] and association with adverse events.[43] Skin, on the other hand, does not provide any additional information in the routine diagnostic process for dementia, but skin biopsies have no contraindications or serious adverse events.[44]

4.2 | Olfactory mucosa and urine SAA

Olfactory mucosa is especially interesting for the detection of αSyn^D. This is in part due to the early accumulation of LB pathology in the olfactory bulb[1,3–5] and the strong association between hyposmia and DLB/PD in this and other studies.[14,45] However, we found a low sensitivity in olfactory mucosa for DLB and a slightly higher specificity. In other studies of PD, DLB, and RBD, the sensitivity varied around 46% to 83%, and a specificity of ~90% was achieved versus healthy controls with very sparse data on AD.[17,19,21,46] The variability across studies in sensitivity and the lower specificity in our study may be due to the inhibitory effect of the nasal swab matrix that interferes with SAA as found by Kuzkina et al.[21] Moreover, technical challenges in terms of sampling from the olfactory mucosa may also play a role, specifically the relative difficulty of identifying the olfactory mucosa via endoscopy and obtaining sufficient non-bloody material from nasal swabs. Due to the technical challenges and low accuracy, the biospecimen may not be ideal for diagnostic discrimination between DLB and AD, but it has potential in the future to be a tool for early detection.

Urine has not previously been investigated for the detection of αSyn^D using SAA in DLB versus AD, and as such our finding represents a proof of concept. Urine may face some of the same technical challenges as SAA in blood, including low analyte concentrations and the presence of interfering substances.[47]

4.3 | Predictive value of DLB symptoms for αSyn^D SAA positivity

The three core features with the highest accuracy for predicting CSF SAA positivity were fluctuations, RBD, and parkinsonism, all with accuracies of ≥79%, which underlines the association of clinical DLB symptoms with SAA positivity. A similar pattern was also found in another SAA-studied DLB cohort.[14] Visual hallucinations, on the other hand, had a lower sensitivity of 50% but a high specificity of 89% for prediction of SAA positivity. The low sensitivity may be linked to the low prevalence (55%) of visual hallucinations in the studied participants with DLB compared to other studies showing up to 80% of DLB with visual hallucinations,[27] but it is comparable to other studies.[14] Additionally, our findings support the existing threshold of two or more core symptoms, reinforcing the utility of the consensus criteria for identifying αSyn^D. The study design may have influenced the predictive findings, particularly regarding the core criteria, as the DLB cohort was selected based on their fulfillment of the criteria for probable DLB.

The high association between hyposmia and SAA positivity has also been reported by other researchers.[14,48] However, the specificity of hyposmia is limited, given its relatively high prevalence (64%) within AD. While hyposmia is not a distinguishing feature between DLB and AD, its absence in a patient suspected of having DLB should prompt the consideration of alternative, non-LB pathologies.

4.4 | Limitations

Our study has limitations, particularly in connection with the lack of neuropathological confirmation, which necessitates relying on clinical diagnosis as the reference against which αSyn^D is detected. To address this constraint, we ensured that only patients who met the criteria for probable DLB, MCI-LB, AD, and MCI due to AD were included, based on a comprehensive clinical evaluation. To prevent circularity, we also did not base the clinical diagnosis on results from the SAA analysis.

The SAA was not carried out blinded regarding the clinical diagnosis, but samples were deemed positive or negative based on the prior set parameter. All the CSF, skin, and 77% of the DLB olfactory mucosa samples were positive in four out of four wells, underscoring the robustness of the results.

The sample size was sufficient to ensure reliable diagnostic accuracies between DLB and AD. However, the study was underpowered

to robustly assess the subtle difference in accuracy of individual symptoms or co-pathology relative to SAA status. Larger cohorts or meta-analysis would be needed to gain deeper insights into co-pathologies and clinical phenotypes of SAA-positive and SAA-negative patients.

4.5 | Strengths

This is the first study in DLB versus AD participants to investigate SAA in several biospecimens, all taken from the same participant with a uniform SAA. This allowed for strong comparisons of diagnostic accuracies and may lay the foundation for detecting the spread of αSyn^D.

4.6 | Conclusion

In conclusion, CSF and skin αSyn^D detected by SAA are promising diagnostic biomarkers for DLB and potentially other α-synucleinopathies. Separating DLB from AD in clinical practice can be difficult, and biomarkers of AD are less valuable due to the frequent occurrence of co-pathology. Our finding of high diagnostic accuracy for DLB versus AD is thus encouraging in terms of improving the diagnosis of DLB. The assay exhibited particularly high specificity, making it a reliable tool for confirming LB pathology. Larger prospective studies could further the validation of SAA use in memory and movement disorder clinics.

αSyn^D in olfactory mucosa and urine detected by SAA has the potential to serve as valuable minimally invasive biomarkers. However, technical optimization is required before they can be reliably implemented in clinical practice.

The current core criteria for DLB demonstrate high accuracy in predicting αSyn^D SAA positivity. Notably, hyposmia shows a sensitivity of 95%, consistent with previous studies. These findings from SAA research suggest that hyposmia, as a supportive clinical feature, could play a more prominent role in the diagnostic criteria for DLB.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest relevant to this work. Author disclosures are available in the Supporting Information.

CONSENT STATEMENT

The Ethical Committee of the Capital Region of Denmark (H-22046053) and the Data Protection Agency (P-2022-668) approved the project. All patients gave written informed consent to participate.

ORCID

Oskar H. McWilliam https://orcid.org/0000-0001-9728-8773

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Supplementary Materials

α-Synuclein Seed Amplification Assay in Lewy Body Dementia vs Alzheimer's Disease

Oskar H. McWilliam, MD;[1] Remarh Bsoul, MSc;[2] Eva L. Lund, MD, PhD;[2,3] Gunhild Waldemar, MD, DMSc, FEAN;[1,3] Steen G. Hasselbalch, MD, DMSc;[1,3] Anja H Simonsen, MSc, PhD;[1] Marie Bruun, MD, PhD;[1,3] Christian von Buchwald, MD, DMSc;[3,4] Kasper Aanæs MD, PhD;[3,4] Christian K Pedersen MD;[4] Ida SB Andersen MD;[4] Magne Bech, MD;[4] Aušrinė Areškevičiūtė, MSc, PhD;[2]*, Kristian S. Frederiksen, MD, PhD;[1,3]*

1) Danish Dementia Research Centre, Dept. of Neurology, Copenhagen University Hospital - Rigshospitalet, Copenhagen, Denmark
2) Danish Reference Center for Prion Diseases, Dept. of Pathology, Copenhagen University Hospital - Rigshospitalet, Copenhagen, Denmark
3) Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark
4) Dept. of Otolaryngology, Head & Neck Surgery and Audiology, Copenhagen University Hospital - Rigshospitalet, Copenhagen, Denmark
*Contributed equally to the study and share last authorship

Table S1 Diagnostic work-up

	AD	*DLB*	*p-value*
MRI-C/CT-C, N (%)	25 (100%)	31 (100%)*	1
FDG-PET, N (%)	25 (100%)	31 (100%)	1
Dopamine transporter imaging, N (%)	0 (0%)	13 (42%)	0.0002
Neuropsychological assessment, N (%)	21 (84%)	23 (74%)	0.52
Polysomnography, N (%)	0 (0%)	7 (23%)	0.01
CSF analysis, N (%)	25 (100%)	24 (77%)	0.013

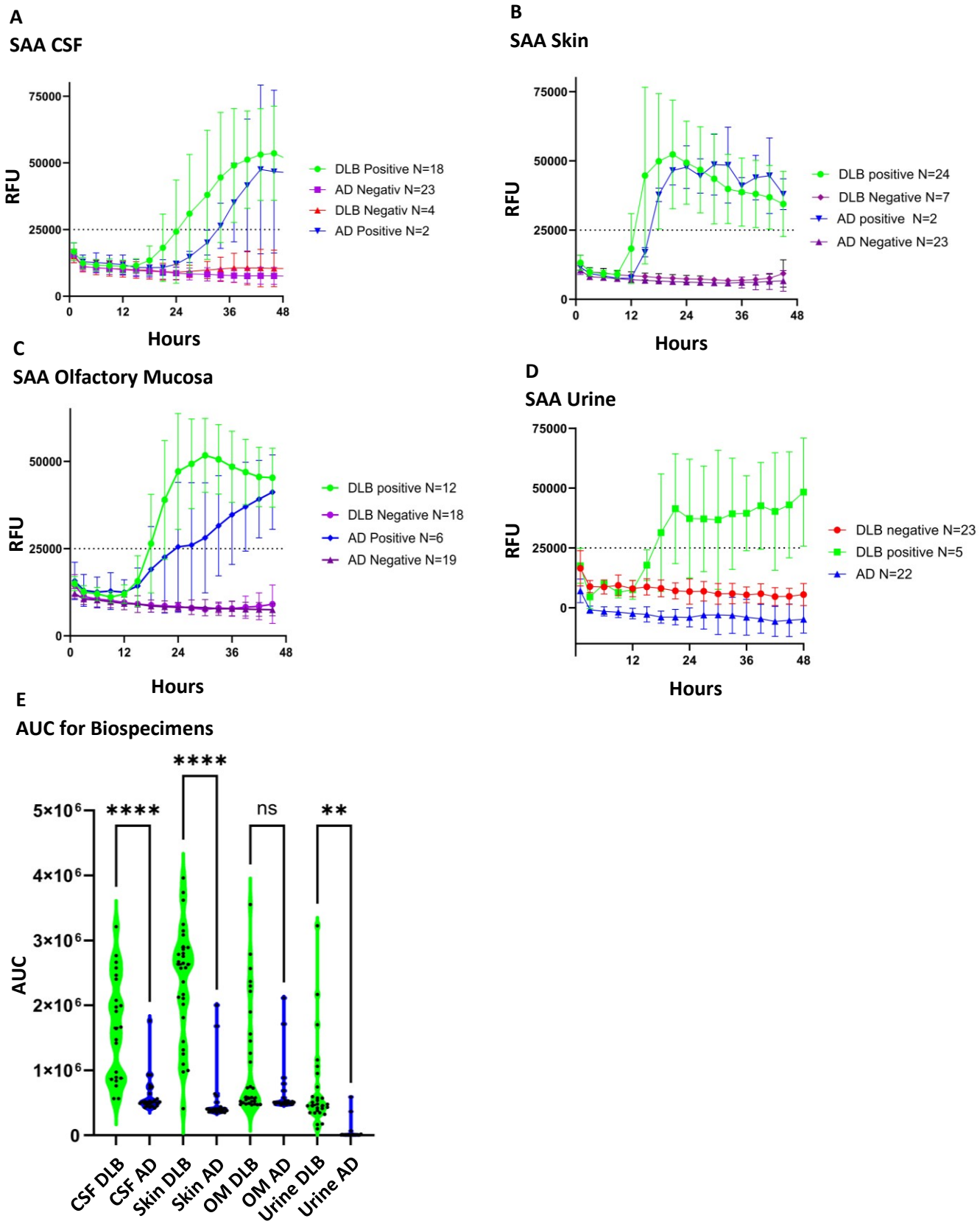
** Two received CT-C instead of an MRI-C.*

Abbreviations: AD = Alzheimer's Disease, CSF = cerebrospinal fluid, CT-C = Computed Tomography of the cerebrum, DLB = Dementia with Lewy bodies, FDG-PET = fluorodeoxyglucose positron emission tomography, MRI-C = Magnetic resonance imaging of the cerebrum, N = number

S1 Evaluation of clinical criteria

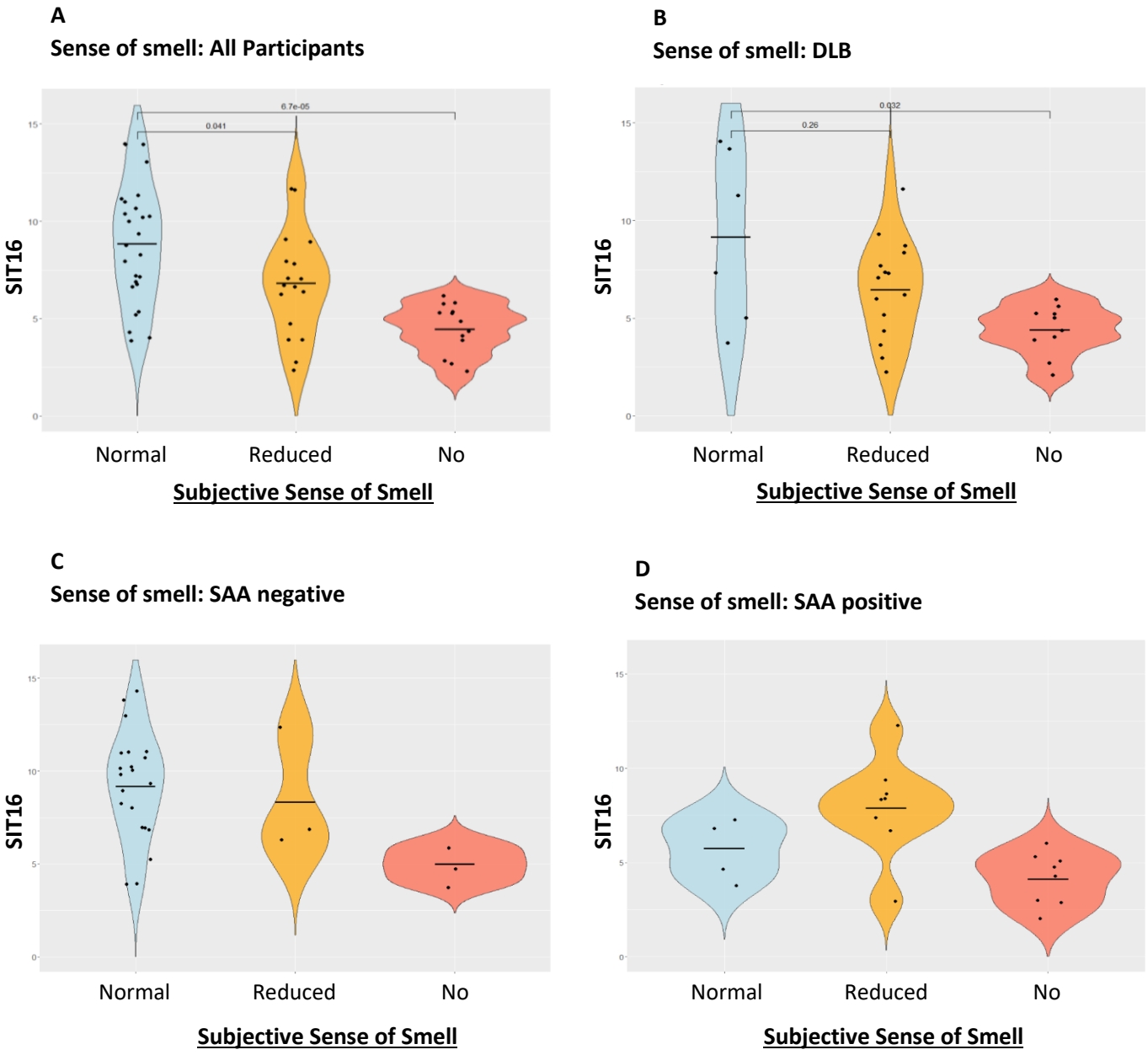
Impairment in activities of daily living was evaluated using the Functional Activities Questionnaire Instrumental Activities of Daily Living (FAQ-IADL caregiver interview).
Subjective hyposmia was assessed with a single question as to whether the patient experienced normal, reduced, or no sense of smell, with both reduced and no sense of smell labeled as subjective hyposmia. Hypersomnia was defined as a score $\geq$ 2 on MDS-UPDRS question 1.8 about daytime sleepiness. The evaluation of urge incontinence was based on medication for urge incontinence or a score $\geq$ 1 on MDS-UPDRS question 1.10 about urinary problems. The presence of constipation was based on medication for constipation or a score $\geq$ 1 on MDS-UPDRS question 1.11 about constipation. Orthostatic hypotension was assessed with blood pressure measurement after 5 minutes in the supine position and up to 3 minutes standing. A positive result was defined as a decrease in systolic blood pressure $\geq$20 mmHg, diastolic pressure $\geq$10 mmHg, or a decrease in heart rate of $\geq$10 beats per minute.

Figure S1 SAA Kinetics



AD = Alzheimer's Dementia, AUC = area under the curve, CSF = cerebrospinal fluid, N = number, DLB = dementia with Lewy bodies, OM = olfactory mucosa, RFU = relative fluorescent unit, SAA = seed amplification assay.

Figure S2 Subjective smell association with objective identification of odors by Sniffin' Stick 16



Comparison between subjective sense of smell and SIT16 in A) the whole cohort, B) DLB, C) AD, and D) the αSyn^{D} SAA positive. The concordance between objective and subjective hyposmia is κ=0.30 (CI 0.066 –0.53) for the whole cohort, κ=0.30 (CI -0.024–0.78) for DLB, κ=0.11 (CI -0.13–0.35) for AD and κ = -0.087 (CI -0.23 – 0.057) for αSyn^{D} SAA CSF positive.
Abbreviations: AD = Alzheimer's Disease, DLB = Dementia with Lewy bodies, SIT16 = Sniffin' Sticks identification test 16.

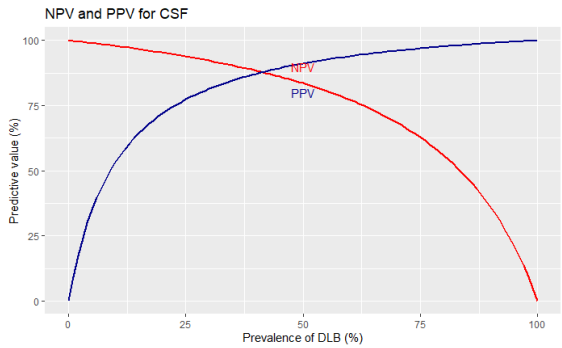
Table S2 Diagnostic accuracy of SAA for CSF SAA+ DLB versus CSF SAA- AD in different biospecimens

Biospecimen	*Sensitivity, % (CI)*	*Specificity, % (CI)*	*Accuracy, % (CI)*
Skin	94 (74–99)	96 (82–99)	95 (83–100)
Olfactory mucosa	47 (26–69)	78 (59–89)	63 (48–78)
Urine	22 (9–45)	100 (85–100)	61 (51–71)

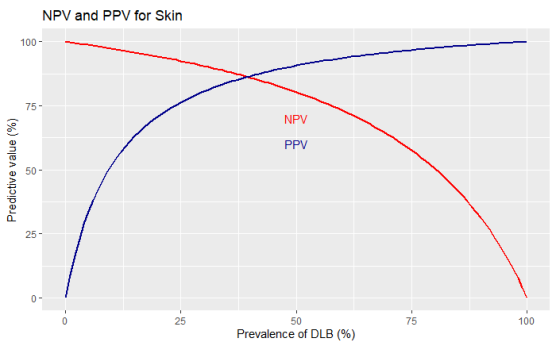
Diagnostic accuracy in patients with DLB and a positive SAA test in CSF versus patients with AD and a negative SAA test in CSF. Abbreviations: αSyn^{D} = α-synuclein, CSF = cerebrospinal fluid, CI = 95% confidence intervals, N = number, SAA = seed amplification assay.

Figure S3 A-E PPV and NVP depending on the prevalence of DLB

A



B



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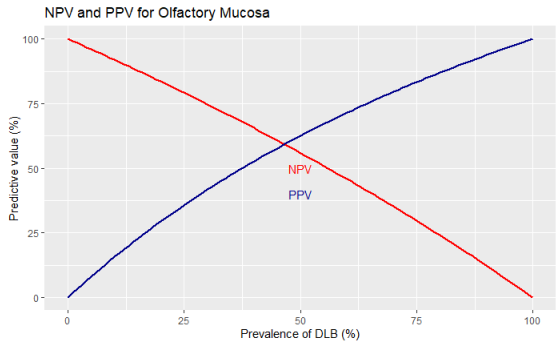

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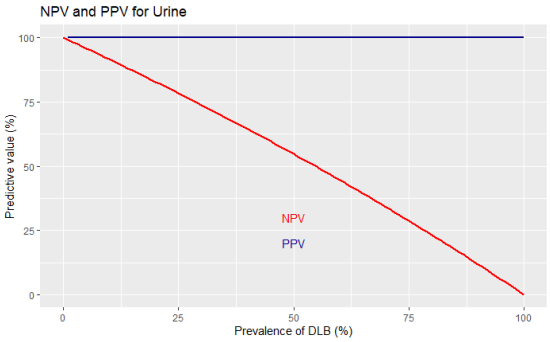

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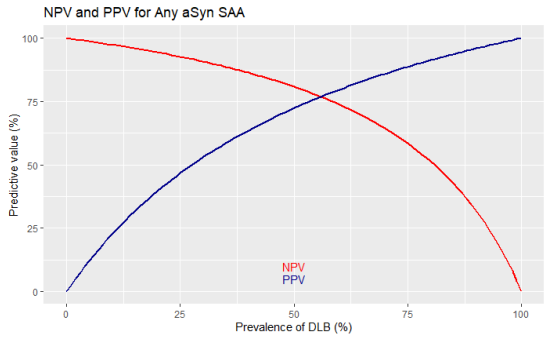


AD = Alzheimer's Disease, DLB = Dementia with Lewy bodies, NPV = negative predictive value, and PPV = positive predictive value

Table S3 Overview of positive samples in participants

Phenotype	Number	CSF	Skin	OM	Urine	Positive	Samples	Phenotype	Number	CSF	Skin	OM	Urine	Positive	Samples
DLB	D1	1	1	1	1	4	4	AD	A4	0	0	1		1	3
DLB	D2	1	1	1	1	4	4	DLB	D26	0	0	1	0	1	4
DLB	D3		1	1	1	3	3	AD	A5	0	0	1	0	1	4
DLB	D4	1	1	1	0	3	4	AD	A6	0	0	1	0	1	4
DLB	D5	1	1	1	0	3	4	AD	A7	0	0	1	0	1	4
DLB	D6	1	1	1	0	3	4	AD	A8	0	0	61	0	1	4
DLB	D7	1	1	1	0	3	4	DLB	D27 §		0	0		0	2
DLB	D8	1	1	1	0	3	4	DLB	D28 ¶		0	0	0	0	3
DLB	D9	1	1	1	0	3	4	AD	A9	0	0	0		0	3
DLB	D10	1	1	0	1	3	4	AD	A10	0	0	0	0	0	4
DLB	D11	1	1	0	1	3	4	AD	A11	0	0	0	0	0	4
DLB	D12	1	1		0	2	3	DLB	D29 ¶	0	0	0	0	0	4
DLB	D13	1	1	0	0	2	4	DLB	D30 #	0	0	0	0	0	4
DLB	D14	1	1	0	0	2	4	DLB	D31 **	0	0	0	0	0	4
DLB	D15	1	1	0	0	2	4	AD	A12	0	0	0	0	0	4
DLB	D16	1	1	0	0	2	4	AD	A13	0	0	0	0	0	4
DLB	D17	1	1	0	0	2	4	AD	A14	0	0	0	0	0	4
DLB	D18	1	1	0	0	2	4	AD	A15	0	0	0	0	0	4
AD	A1 *	1	1	0	0	2	4	AD	A16	0	0	0	0	0	4
DLB	D19		1	1		2	2	AD	A17	0	0	0	0	0	4
DLB	D20		1	1	0	2	3	AD	A18	0	0	0	0	0	4
AD	A2 †	1	0	1	0	2	4	AD	A19	0	0	0	0	0	4
DLB	D21	1	0	0	0	1	4	AD	A20	0	0	0	0	0	4
DLB	D22		1	0	0	1	3	AD	A21	0	0	0	0	0	4
DLB	D23		1	0	0	1	3	AD	A22	0	0	0	0	0	4
DLB	D24		1	0	0	1	3	AD	A23	0	0	0	0	0	4
DLB	D25		1	0	0	1	3	AD	A24	0	0	0	0	0	4
AD	A3 ‡	0	1	0	0	1	4	AD	A25	0	0	0	0	0	4

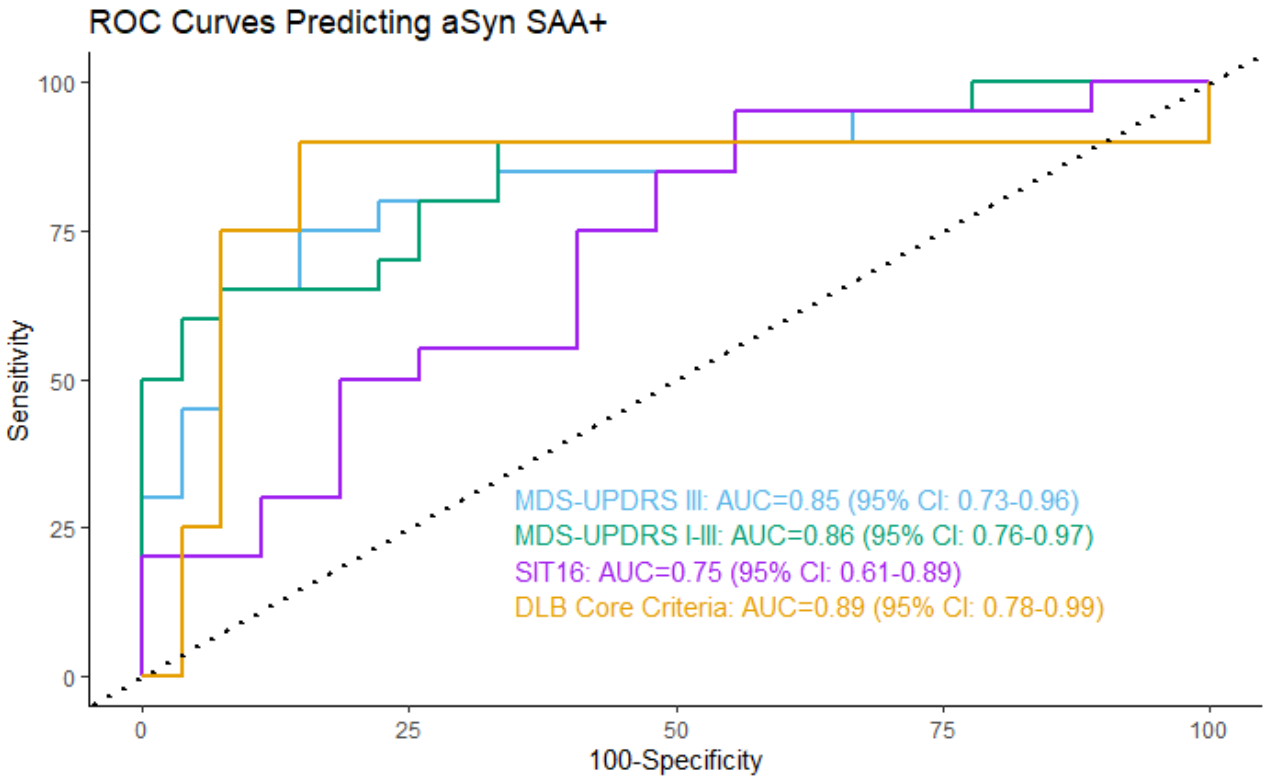
SAA+ AD
** Hyposmia, neurogenic orthostatic hypotension, and relative preservation of the medial temporal lobe.*
† Hyposmia, depression, hypersomnia, visuospatial affected, and occipital hypometabolism on PET-FDG.
‡ Hyposmia, parkinsonism, hypersomnia, and dysautonomia.

SAA- DLB
§ RBD, visual hallucinations, dysautonomia, cingulate island sign on FDG-PET but normosmia.
¶ DLB phenotypes with mixed AD clinical phenotype.
 # RBD, parkinsonism, fluctuations, dysautonomia, PD, cingulate island sign on FDG-PET, hyposmia, but normal DAT scan.
*** RBD, discrete parkinsonism, visual hallucinations, dysautonomia, depression, preserved medial temporal lope, but normosmia.*

Abbreviations: AD = Alzheimer's Disease, CSF = cerebrospinal fluid, DLB = dementia with Lewy bodies, and OM = olfactory mucosa

F

Figure S4 Predicting CSF αSyn^{D} SAA+ with quantitative variables



MDS-UPDRS III, MDS-UPDRS I-III, SIT16 and core diagnostic criteria were used in calculating AUC ROC curves. α-Syn SAA = α-synuclein seed amplification assay; MDS-UPDRS = Movement Disorder Society Unified Parkinson's Disease Rating Scale part III; SIT16 = Sniffin' Stick Smell Identification Test 16.

8.3 Study 3: Prodromal Lewy Body Symptoms and α-Synuclein Seeding in Idiopathic Olfactory Dysfunction

Movement Disorders
CLINICAL PRACTICE

RESEARCH ARTICLE

Prodromal Lewy Body Symptoms and α-Synuclein Seeding in Idiopathic Olfactory Dysfunction

Oskar Hoffmann McWilliam, MD,[1,*] Remarh Bsoul, MSc,[2] Gunhild Waldemar, MD, DMSc, FEAN,[1,3] Steen Gregers Hasselbalch, MD, DMSc,[1,3] Anja Hvid Simonsen, MSc, PhD,[1] Marie Bruun, MD, PhD,[1,3] Laura Storm Næsborg Andersen,[1] Christian von Buchwald, MD, DMSc,[3,4] Kasper Aanæs, MD, PhD,[3,4] Christian K. Pedersen, MD,[4] Ida S.B. Andersen, MD,[4] Magne Bech, MD,[4] Clara Hageman Pinborg,[4] Aušrinė Areškevičiūtė, MSc, PhD,[2] Eva Løbner Lund, MD, PhD,[2,3] and Kristian Steen Frederiksen, MD, PhD[1,3]

Abstract: Background: Early identification of pathological α-synuclein deposition (αSynD) may improve understanding of Lewy body disorder (LBD) progression and enable timely disease-modifying treatments.
Objectives: We investigated αSynD using a seed amplification assay and assessed prodromal LBD symptoms in individuals with idiopathic olfactory dysfunction (iOD).
Methods: In this cross-sectional, case–control study, we included iOD participants and normosmic healthy controls (HC) aged 55 to 75 years without diagnoses of dementia with Lewy bodies, Parkinson's disease (PD), or other major neurological disorders. iOD was defined as hyposmia without any known causes. The primary outcome was αSynD detection in skin and olfactory mucosa. Secondary outcomes included prodromal LBD symptoms: cognitive dysfunction, rapid eye movement sleep behavior disorder (RBD), motor and nonmotor symptoms (Movement Disorders Society-Unified Parkinson's Disease Rating Scale [MDS-UPDRS] Parts I–III), and prodromal PD risk.
Results: We recruited 44 iOD participants (mean age 65) and 50 HCs (mean age 67). Eighteen iOD participants (41%) were αSynD positive in skin and/or olfactory mucosa compared to 1 HC (2%, $P < 0.0001$). iOD participants had higher rates of cognitive dysfunction (48% vs. 24%, $P = 0.02$), possible RBD (41% vs. 16%, $P = 0.01$), and elevated MDS-UPDRS I–III (median [interquartile range]: 15 [7–22] vs. 5.5 [3–11], $P < 0.0001$). Dysautonomia symptoms did not differ significantly. In the iOD group, 45% met probable/possible prodromal PD criteria versus 1 control ($P < 0.0001$). However, αSynD-αSynD-negative iOD participants had a nonsignificantly higher prodromal PD risk compared to αSynD-positive individuals.
Conclusions: iOD exhibits high αSynD prevalence and prodromal LBD symptoms, supporting its role as an early LBD marker and potential model for early intervention and mechanistic studies.

Parkinson's disease (PD) and dementia with Lewy bodies (DLB) may be considered late-stage manifestations of Lewy body disorders (LBD) associated with pathological accumulation of α-synuclein deposition (αSynD) and neuronal dysfunction.[1,2] The olfactory mucosa is innervated by neurons projecting from the olfactory bulb and is believed to be the earliest site of αSynD pathology in the central nervous system (CNS).[3–6] Olfactory dysfunction (OD) (ie, hyposmia) is present in about 90% of patients with clinically verified LBD.[7–9] Other prodromal symptoms such as dysautonomia, neuropsychiatric symptoms, reduced color vision, and especially rapid eye movement sleep behavior disorder (RBD) may also precede the cognitive and motor

[1]Department of Neurology, Danish Dementia Research Centre, Copenhagen University Hospital—Rigshospitalet, Copenhagen, Denmark; [2]Department of Pathology, Danish Reference Center for Prion Disease, Copenhagen University Hospital—Rigshospitalet, Copenhagen, Denmark; [3]Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark; [4]Department of Otolaryngology, Head and Neck Surgery and Audiology, Copenhagen University Hospital—Rigshospitalet, Copenhagen, Denmark

*Correspondence to: Dr. Oskar Hoffmann McWilliam, Department of Neurology, Danish Dementia Research Centre, Copenhagen University Hospital—Rigshospitalet, Inge Lehmanns Vej 6, 2100 Copenhagen, Denmark; E-mail: oskar.mcwilliam@regionh.dk
Keywords: seed amplification assay, hyposmia, olfactory dysfunction, Parkinson's disease, prodromal α-synucleinopathy, dementia with Lewy bodies.

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symptoms.[1] Supporting the relationship between OD and LBD, recent studies in PD and DLB have reported higher rates of conversion to PD in participants with idiopathic OD (iOD) compared with normosmic participants[7] and a higher prevalence of αSynD in PD/DLB with OD compared with normosmic PD/DLB.[10–12] Moreover, in a study of OD, 20% of the participants developed PD within 10 years.[13] OD develops decades before the diagnosis of PD/DLB and may therefore serve as the first clinical marker for developing disease.[1]

α-Synuclein seed amplification assays (SAA) have emerged as an ultrasensitive method for detecting αSynD in various biospecimens, including skin[14–16] and olfactory mucosa.[17,18] Detection of αSynD in skin biopsies has shown an accuracy that is close to that of cerebrospinal fluid (CSF) in previous studies of manifest LBD and idiopathic RBD (iRBD).[19–23] A challenge in several studies has been the low sensitivity of SAA in CSF for LBD pathology confined to the olfactory bulb, brainstem, and amygdala.[24–26]

Various hypotheses regarding the origins and initial propagation of αSynD exist, with the olfactory bulb and the gastrointestinal tract frequently implicated as the primary sites of pathological onset.[3–5,27] Whether the pathology initiates in both sites as in the dual-hit hypothesis,[3] only in the olfactory bulb,[5] or primarily in either of the sites as the "body-first versus brain-first" hypothesis states[27] is debated. The presence of αSynD in the skin has been proposed to be associated with the "body-first" phenotype.[28,29] Consequently, employing αSynD SAA in skin and olfactory mucosa could serve as an early detection of αSynD.

Early detection of αSynD may provide critical insights into the pathophysiology of prodromal LBD, including the mechanisms underlying αSynD spread. This study aimed to compare the frequency of αSynD pathology and prodromal symptoms of LBD in individuals with iOD and normosmic healthy controls (HC).

Patients and Methods

Study Design

The alpha-synuclein Rt-quic and neurologic symptoms in persons with idiopathic anosmia study is a single-center, cross-sectional, case–control study using SAA analysis. The study was registered before recruitment at clinicaltrials.gov (NCT05740683), and regional ethical approval was obtained from the Capital Region Ethical Committee (H-22053428).

Participants

Patients with iOD were recruited from the Department of Ear, Nose, and Throat (ENT), Unit for Sense of Taste and Smell, at Rigshospitalet or private practice ENT clinics from September 2023 to November 2024 (flowchart, Fig. 1). We applied the following criteria for iOD (had to be fulfilled): (1) noncongenital OD, (2) magnetic resonance or computed tomography to exclude pathology relating to OD or progressive OD >2 years, (3) no temporal link to head trauma or infection, (4) no significant sino-nasal disease on endoscopy, and (5) no response to short-term systemic/topical corticosteroid treatment. The criteria were based on a previous study[7] except that magnetic resonance or computed tomography was unnecessary if OD had been present for more than 2 years.

The inclusion criteria for iOD participants were (1) a diagnosis of iOD as defined earlier, (2) age 55 to 75 years, and (3) Sniffin' Sticks identification test 16 (SIT16), with OD defined as ≤11 for participants aged 55 to 59 years and ≤10 for those aged >60 years, based on the lower 25th percentile of SIT16 previously reported.[30] Normosmic HCs aged 55 to 75 years were included.

The exclusion criteria for both iOD participants and HCs were (1) a diagnosis of PD/DLB, (2) a diagnosis of other major neurological or psychiatric diseases (minor stroke and mild depression/anxiety were allowed), or (3) drug or alcohol abuse.

All participants provided written informed consent.

Assessment of Olfactory Function

Olfactory function was assessed using the Danish adaptation of SIT16.[31] It is a forced-choice test where participants identify 16 suprathreshold odors from 4 options. The maximum score is 16, and a score ≤8 indicates no functional sense of smell/anosmia.[30] Furthermore, the iOD group was assessed using the Sniffin' Sticks Threshold and Discrimination tests.[30]

Assessment of Cognition

Montreal Cognitive Assessment, Danish version 8.1, was applied to assess global cognitive function (range 0–30 and <26 defined cognitive impairment),[32] the Symbol Digit Modalities Test (SDMT) was used for processing speed and attention,[33] and memory recall was evaluated using the Logical Memory subtest of the Wechsler Memory Scale, Third Edition section A + B.[34] Finally, animal fluency testing was conducted to measure semantic fluency.[35]

Assessment of Sleep

Possible RBD was screened using the REM Sleep Behavior Disorder Single-Question Screen, a single-question test with a specificity for RBD of 87%[36] and the RBD Screening Questionnaire ranges 0 to 13, with ≥5 suggestive of RBD with a specificity of 84%.[37]

Assessment of Other Motor and Nonmotor Symptoms

Movement Disorders Society-Unified Parkinson's Disease Rating Scale (MDS-UPDRS) Parts I–III were applied to evaluate parkinsonian symptoms, with part I assessing nonmotor symptoms (eg, subjective cognition, psychiatric, and autonomic symptoms),

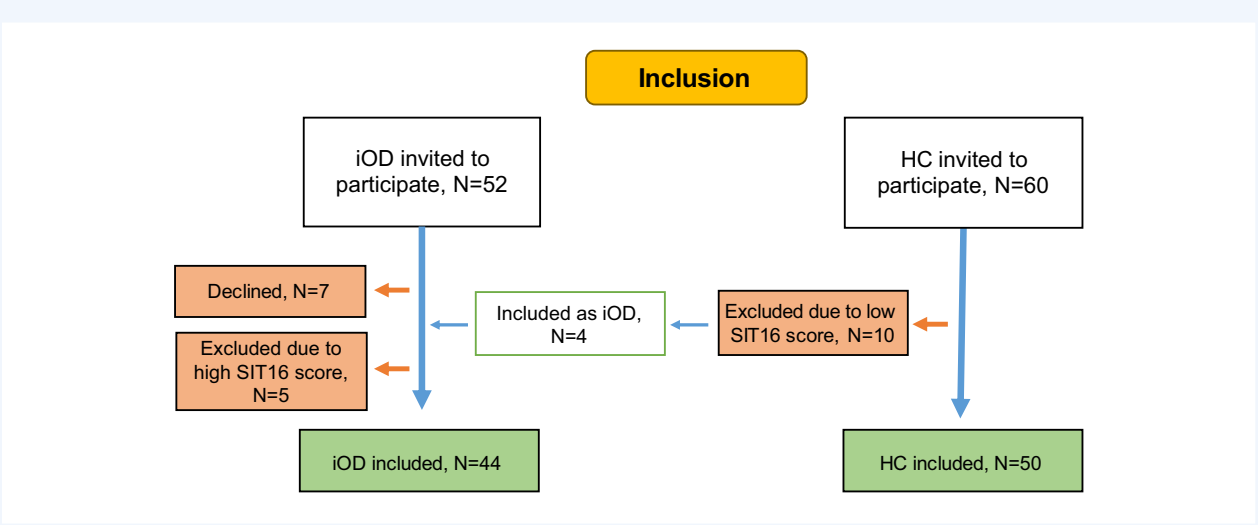

FIG. 1. Study flowchart. Flow chart of participants in the study. Ten participants screened to be included as HCs were excluded due to low olfactory score (SIT16), and 4 were referred to the Unit for Sense of Taste and Smell and diagnosed with iOD and included as such. HC, healthy controls; iOD, idiopathic olfactory dysfunction; N, number; and SIT16, Sniffin' Sticks identification test 16.

part II subjective motor symptoms, and part III objective motor performance.[38] Motor parkinsonism was rated using MDS-UPDRS III, excluding the score for action tremor and defined as positive for early signs of parkinsonism if >6.[39] For further definitions, see Supplementary Materials S1.

Color vision was assessed using the Farnsworth Munsell 100 Hue Test (FM-100)[40] on a tablet with a maximal light setting.

Prodromal PD Calculation

To estimate the likelihood of prodromal PD, we used the MDS online tool "Prodromal PD Calculator."[39] The tool calculates the likelihood ratio (LR) and the percental risk of having prodromal PD. The tool has also previously been shown to predict conversion to PD and DLB with high precision.[41] Using the tool, possible prodromal PD has previously been defined as having a percental risk of 50% to 79%, and probable prodromal PD is defined as ≥80%.[39] For parameters and definitions used in the calculations, see Supplementary Materials S1.

Sampling of Biological Tissue

Olfactory mucosa sampling was collected bilaterally from the middle turbinate and the roof of the nasal cavity (agger nasi area) using nylon (FLOQswabs Copan Diagnostics, Italy) swabs, guided by nasal endoscopy as described previously.[21] Two 3-mm skin punch biopsies were obtained 5 cm paramedially from the back at the level of C7 and homogenized together as described previously.[21]

Seed Amplification Assay

SAA detects the presence of αSynD by amplifying minute amounts of prion-like αSynD. The amplified αSynD, if present, is subsequently measured using a fluorescence technique. SAA was conducted separately on material from 2 nasal swabs per participant, whereas the material from the 2 skin biopsies was analyzed together. For 7 HCs and 4 iOD participants, only 1 swab was analyzed; in 1 iOD participant, no swabs were available due to blood contamination. Skin samples from 1 HC and 2 iOD participants were unavailable for analysis.

The SAA was based on a uniform real-time quaking-induced conversion technique performed on a FLUOStar Omega with recombinant N-terminal 6×-histidine-tagged α-synuclein as previously described by Bsoul et al.[21,42] All samples were analyzed in quadruplicate wells for 48 hours. A sample was considered positive if at least 2 of the 4 wells exceeded a predefined fluorescence threshold, set at 4 SD (standard deviation) above the mean of the negative control in CSF, as previously described.[21,42] Olfactory mucosa was deemed positive if 1 of the swabs had at least 2 positive wells.

Statistical Analysis

All analyses were performed using R (version 4.3.3). For comparisons of continuous variables between HC, iOD, and iOD SAA+/−, the unpaired *t*-test was applied for normally distributed data, whereas the Mann–Whitney test was used for non-normally distributed data. χ^2 or Fisher's exact test was employed for categorical data comparisons.

The Haldane–Anscombe correction (0.5 added to all counts in the contingency matrix) was applied in the calculation of the

odds ratio (OR) if any of the counts in the contingency matrix were 0.[43]

Both uncorrected *P*-values and *P*-values corrected for multiple comparisons using the false discovery rate method (q-value) were calculated. The significance level was set at $P < 0.05$.

Results

Demographics

We included 44 participants with iOD and 50 HCs. The groups did not differ significantly in demographics or medical history, except for a higher prevalence of anxiety in the iOD group (9% iOD vs. 0 HC, $P = 0.045$) and a numerically higher rate of depression (23% iOD vs. 10% HC, $P = 0.093$) (Table 1 and Supportive Information Table S1).

Detection of αSynD

SAA detected αSynD in the olfactory mucosa in 13 (30%) iOD participants but in none of the HCs ($q < 0.001$), corresponding to an OR of 46 (95% confidence interval [CI] 2.7–806). In 5 cases, both the nasal swabs were SAA positive, and in 8 cases, only 1 swab was positive. All but 4 swabs were positive in all 4 wells. In skin, 13 (31%) iOD participants and 1 (2%) HC ($q < 0.001$) (OR = 22 [CI 2.7–173]) were positive for αSynD. All but 1 skin sample were SAA positive in all 4 wells. Combined, αSynD was detected in at least 1 biospecimen in 18 (41%) iOD participants ($q < 0.001$) (OR = 35 [CI 4.5–280]). Of the 18 positive iODs, 8 (44%) were positive in both biospecimens.

Clinical Symptoms and Tests

Participants with iOD had more possible RBD, neuropsychiatric symptoms, and hypersomnia but not autonomic symptoms compared to HCs (Table 2; Fig. 2; Table S1).

Across all cognitive tests, participants with iOD performed worse except for animal fluency (Table 2; Table S1).

MDS-UPDRS I–III indicated more parkinsonian symptoms in iOD compared to HC, which was mostly driven by scores on part I (mean(SD): 7.6 (5.3) vs. 3.6 (2.6), $P < 0.001$), assessing nonmotor symptoms, and part III (median (IQR) 5.5 (4–9) vs. 2 (0–4), $P < 0.001$), the objective motor score (Table 2).

There were no significant differences between αSynD+ and αSynD– iOD in the medical history or clinical scales except for a diagnosis of depression ($P = 0.033$) (Table 1). The αSynD+ iODs were numerically but nonsignificantly older, included more female, and had worse SDMT and FM-100 scores (Tables 1 and 2).

Probability of Prodromal PD

The LR and probability of prodromal PD were elevated in the iOD group compared to HCs (Fig. 3). Additionally, 12 (27%) participants with iOD met the criteria for either probable prodromal PD, and 8 (18%) iOD participants met the criteria for possible prodromal PD, compared to 1 (2%) in the HC group meeting the criteria for probable prodromal PD (Fig. 3). The 1 HC with probable prodromal PD was negative for αSyn SAA. The 1 HC with a positive αSyn SAA test in skin, but not in olfactory mucosa, had a prodromal PD risk of 18% due to orthostatic hypotension, low cognitive score, and daytime sleepiness but a normal sense of smell (SIT16 = 15).

TABLE 1 Demographics and medical history

	HC (N = 50)	iOD (N = 44)	*P*-value	iOD αSynD– (N = 26)	iOD αSynD+ (N = 18)	*P*-value
Age, mean years (SD)	67.2 (5.0)	65.4 (5.5)	0.11	64.1 (6.0)	67.3 (4.2)	0.057
Sex, N female (%)	28 (56%)	20 (45%)	0.31	9 (35%)	11 (61%)	0.083
Years of education, median years (IQR)	16 (15–17)	16 (13–17)	0.15	16 (14–17)	16 (13–17)	0.48
Years of OD, median (IQR)	NA	5 (4–8)	NA	5 (3–6)	6 (4–8)	0.23
Smoking never, N (%)	21 (42%)	21 (48%)	0.43	13 (50%)	8 (44%)	0.35
Family history of PD/dementia	26 (52%)	17 (39%)	0.34	9 (25%)	8 (44%)	0.15
Current or previous depression, N (%)	5 (10%)	10 (23%)	0.093	3 (12%)	7 (39%)	**0.033**
Current or previous anxiety, N (%)	0	4 (9%)	**0.045**	1 (4%)	3 (17%)	0.29
DM2, N (%)	3 (6%)	3 (7%)	0.87	2 (8%)	1 (6%)	0.78
TIA/minor stroke, N (%)	2 (4%)	6 (14%)	0.095	4 (15%)	2 (11%)	0.68

Note: This table presents the demographic characteristics and medical history of study participants based on the inclusion group and αSynD status in the combination of skin and olfactory mucosa of the iOD group. Significant *p*-values in bold.

Abbreviations: HC, healthy control; iOD, idiopathic olfactory dysfunction; αSynD, α-synuclein deposition; SD, standard deviation; N, number; NA, not applicable; IQR, interquartile range; PD, Parkinson's disease; DM2, diabetes mellitus type 2; TIA, transient ischemia attack.

TABLE 2 Assessments of symptoms and pathology

	HC (N = 50)	iOD (N = 44)	*P*-value	q-Value	αSynD– (N = 26)	αSynD+ (N = 18)	*P*-value
αSynD+ olfactory mucosa, N (%)	0 (0%)	13 (30%)	**<0.001**	**<0.001**	0 (0%)	13 (72%)	NA
αSynD+ skin, N (%)	1 (2%)	13 (31%)	**<0.001**	**<0.001**	0 (0%)	13 (72%)	NA
αSynD+ total, N (%)	1 (2%)	18 (41%)	**<0.001**	**<0.001**	0 (0%)	18 (100%)	NA
MDS-UPDRS I, mean (SD)	3.6 (2.6)	7.6 (5.3)	**<0.001**	**<0.001**	7.2 (5.1)	8.1 (5.6)	0.57
MDS-UPDRS II, median (IQR)	0 (0–0)	0 (0–2)	**0.003**	**0.005**	0 (0–2)	0 (0–3)	0.61
MDS-UPDRS III, median (IQR)	2 (0–4)	5.5 (4–9)	**<0.001**	**<0.001**	6 (3–9)	5 (4–7)	0.37
MDS-UPDRS I–III, median (IQR)	5.5 (3–11)	15 (7–22)	**<0.001**	**<0.001**	16 (7–21)	14 (7.5–24)	0.92
RBDSQ, median (IQR)	2.0 (1.0–4.8)	3.0 (1.0–5.0)	**<0.001**	**0.001**	4.0 (1.5–5.0)	2.0 (1.0–4.8)	0.30
MoCA, mean (SD)	27 (2)	25 (2.9)	**<0.001**	**0.001**	26 (2.1)	25 (3.8)	0.53
SDMT, mean (SD)	47 (7.8)	43 (9.9)	**<0.001**	**0.015**	45 (10)	39 (9.1)	0.068
Recall, mean (SD)	32 (6.7)	25 (8.7)	**<0.001**	**<0.001**	25 (8.5)	25 (9.2)	0.92
FM-100 Hue, median (IQR)	48 (23–77)	49 (30–75)	0.70	0.72	42 (24–69)	57 (43–114)	0.090
SIT16, mean (SD)	14.1 (1.3)	6.5 (2.4)	**<0.001**	**<0.001**	6.6 (2.5)	6.5 (2.4)	0.92
LR of prodromal PD, median (IQR)	0.3 (0.1–1)	32 (4–257)	**<0.001**	**<0.001**	38 (9.5–350)	23 (3–191)	0.36

Notes: This table presents the results of clinical assessments of symptoms and SAA (seed amplification assay). For HC versus iOD, both *P*-values and q-values are presented. For αSynD– versus αSynD+ *P*-values are presented, as there were no significant differences. Significant *p*-values in bold.
Abbreviations: HC, healthy control; iOD, idiopathic olfactory dysfunction; N, number; q-value, false discovery rate–adjusted *P*-value; αSynD, α-synuclein deposition; MDS-UPDRS, Movement Disorders Society-Unified Parkinson's Disease Rating Scale; SD, standard deviation; IQR, interquartile range; RBDSQ, REM Sleep Behavior Disorder Screening Questionnaire; MoCA, Montreal Cognitive Assessment; SDMT, Symbol Digit Modalities Test; FM-100 Hue, Farnsworth Munsell 100 Hue Test; SIT16, Sniffin' Sticks identification test 16; LR, likelihood ratio; PD, Parkinson's disease.

Recalculating the prodromal PD score without OD (Fig. 3B,D), iOD participants retained a higher probability (median % iOD vs. HC: 8.0 vs. 1.0, q = 0.003).

Across αSynD status in iOD participants, there were no significant differences in the LR (median αSynD– vs. αSynD+: 38 vs. 23, $P = 0.36$) or prodromal PD probability (median: 46% vs. 23%, $P = 0.73$). In the αSynD+ iOD group, 8 (44%) participants met the criteria for either possible or probable prodromal PD versus 12 (46%) of the αSynD– iOD group ($P = 0.91$).

Discussion

In this study, we investigated the frequency of α-synucleinopathy in participants with iOD and found a much higher prevalence compared to a normosmic control group. Specifically, we found that 41% of iOD participants had evidence of αSynD in the skin or olfactory mucosa detected by SAA versus 2% in the control group.

Participants with iOD had more prodromal symptoms of LBD, which included RBD, psychiatric symptoms (depression and anxiety), cognitive dysfunction, and parkinsonism. For most symptoms, we did not find significant differences between αSynD-positive and αSynD-negative iOD participants.

αSynD Positivity

The αSynD-positive rate in the present study closely aligns with another study that found a 48% positive rate in OD participants, although this was detected in CSF.[44] This alignment further extends to the normosmic control group, where the rates were also similar between the 2 studies. Beyond these studies, the prevalence of αSynD in iOD patients remains largely unexplored. However, a well-established model for prodromal LBD is iRBD, in which studies have found that about 67% to 90% of subjects are αSynD positive.[19,20,45] In a follow-up study of iRBD, 66% of the αSynD-positive subjects converted to PD/DLB within 7 years, as opposed to 20% of αSynD-negative iRBD subjects,[45] confirming the role of αSynD as a risk factor or a cause of disease progression. Although our results may indicate that OD is a less-sensitive clinical marker for αSynD compared to iRBD, OD is a more common condition. The prevalence of OD has been reported to be 15% in the general population, and a study found that about 21% of OD patients are idiopathic.[46] In contrast, the prevalence of definite RBD and probable RBD in the general population is somewhat lower at about 0.7% and 5.7%, respectively.[47] Despite lower positivity, iOD remains a promising marker for identifying high-risk prodromal LBD populations and potential candidates for disease-modifying treatments, particularly when combined with a specific biomarker.

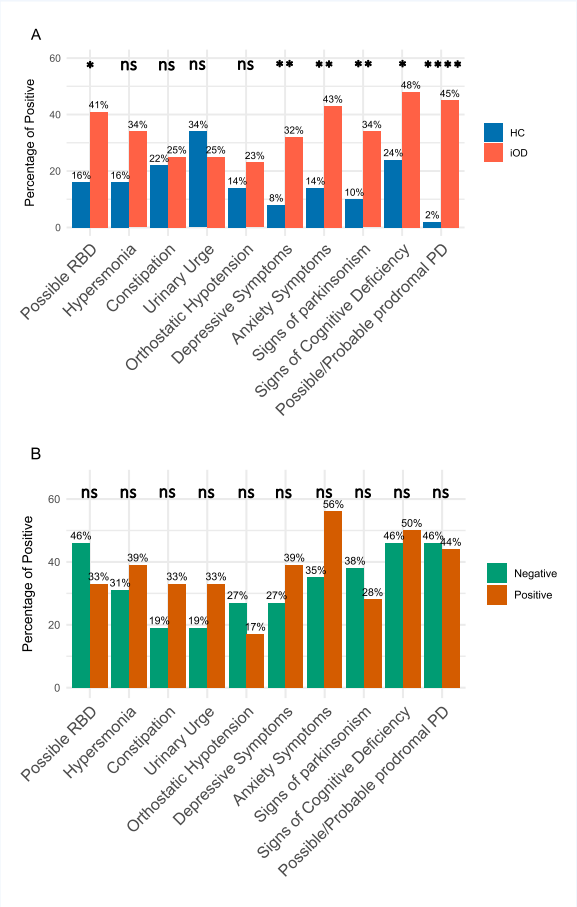

FIG. 2. Frequency of symptoms and clinical profiles across diagnostic groups. This figure presents the symptom and clinical profile frequency of participants by (**A**) group and (**B**) iOD αSynD positive versus negative. ns $P > 0.05$, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and **** $P \leq 0.0001$. αSynD, α-synuclein deposition; HC, healthy control; iOD, idiopathic olfactory dysfunction; possible RBD, possible rapid eye movement sleep behavior disorder; PD, Parkinson's disease.

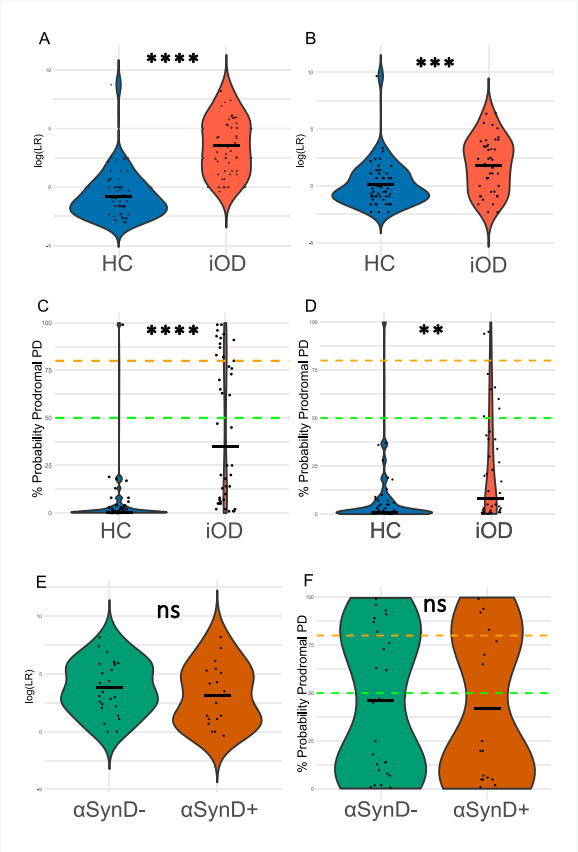

FIG. 3. Probability of prodromal Parkinson's disease. (**A**, **B**) Likelihood ratio for prodromal PD across HC and iOD (with (A)/without (B) olfaction in the calculation). (**C**, **D**) Probability of prodromal PD across groups (with (C)/without (D) olfaction in the calculation). (**E**, **F**) Likelihood ratio and probability of prodromal PD comparing iOD αSynD negative versus positive. The dashed lines indicate possible (green) and probable (orange) prodromal PD. ns $P > 0.05$, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and****$P \leq 0.0001$. αSynD, α-synuclein deposition; HC, healthy control; iOD, idiopathic olfactory dysfunction; PD, Parkinson's disease.

Prodromal LBD Symptoms

Several domains of prodromal LBD symptoms were more prevalent or pronounced in participants with iOD. Calculating the risk of prodromal PD, 2% of HCs fulfilled the criteria for probable or possible prodromal PD, which aligns with a prevalence of 1.2% found in a previous large study of prodromal PD in HCs.[48] In contrast, we found that 45% of iOD participants fulfilled the criteria for prodromal PD, indicating a substantially increased risk of LBD in participants with OD, as is also supported by other studies investigating prodromal features in OD.[7,13,49,50] The persistence of an elevated risk even after excluding OD in the calculation clearly suggests that iOD participants exhibit increased LBD symptomatology.

According to the "brain-first versus body-first hypothesis," OD, dysautonomia, and RBD are key markers of the body-first phenotype.[27,29] In iOD, we observed a higher prevalence of possible RBD and parkinsonism. However, there were no significant differences in autonomic symptoms. Whereas the numerically increased RBD prevalence in iOD would be in line with the "brain-first versus body-first hypothesis," the absence of a significant difference in iOD-related dysautonomia is not. It should be noted that parkinsonism, dysautonomia, and sleep were assessed clinically rather than using imaging or electrophysiology, potentially missing preclinical cases.

It has recently been hypothesized that the presence of αSynD in the olfactory mucosa may represent the "brain-first" and presence in the skin, the "body-first" phenotype.[28,29] Examining the distribution of αSynD across the 2 biospecimens, we found an equal distribution, but a substantial number of participants were also positive in both biospecimens. This may suggest support for the dual-hit hypothesis but may also potentially reflect a later stage of LBD where aSynD has spread. These findings should be interpreted cautiously due to the limited sample size, lack of

longitudinal follow-up on αSynD distribution, CSF αSynD status, and potentially reduced sensitivity of SAA in the prodromal phase. The distribution of αSynD-positive cases and the heterogeneity of LBD symptoms observed in this study underscore the complexity of α-synucleinopathy progression and highlight the need for further research into the distinct pathways underlying prodromal phenotypes.

αSynD Positive versus Negative

Contrary to expectations, we found a few differences in Lewy body features between αSynD-positive and αSynD-negative individuals with iOD. A previous study of CSF SAA and hyposmia, in accordance with our data, did not find significant differences between αSynD-negative and αSynD-positive participants in parkinsonism, cognition, RBD symptoms, and bowel movements.[12] Several factors may explain these findings, either individually or in combination. (1) Reduced sensitivity of SAA in the prodromal phase: several studies have reported that when LB pathology is confined to the olfactory bulb, amygdala, brainstem, and some cases also limbic structures, the sensitivity of the SAA in CSF is reduced[24–26] due to assay-related factors, αSynD strain type,[51] or the amount of αSynD.[29] (2) The presence of alternative neurodegenerative pathologies driving iOD and LBD symptoms: other neurodegenerative diseases, including Alzheimer's disease, are associated with OD, albeit to a lesser extent than LBD.[8,52] This may explain neuropsychiatric and cognitive symptoms in both αSynD-positive and αSynD-negative iOD but not fully account for key LBD features like RBD and parkinsonism. (3) iOD may be an early LBD symptom, with our cohort representing varying prodromal phases: within the αSynD-positive iOD group, 2 subgroups emerged: one with high and another with low (<25%) prodromal PD probability (Fig. 3). (4) The relatively small sample size may have limited statistical power, yet LBD-related symptoms, including RBD and prodromal PD probability, were more prevalent in SAA-negative iOD. This unexpected trend warrants further investigation, potentially reflecting cohort heterogeneity or another effect of SAA's lower sensitivity in the prodromal stage, as mentioned previously.

Limitations

This study has several limitations. Most notably, the absence of longitudinal data prevents us from determining whether αSynD-positive individuals will progress to manifest LBD or remain stationary iOD. Furthermore, the absence of longitudinal data limits conclusions on the temporal spread of αSynD. The increased LBD symptoms observed in some αSynD-positive iOD cases suggest early CNS involvement. We did not measure αSynD in CSF, but previous studies of manifest and prodromal LBD have found that SAA in skin performs at the level of CSF.[19–22]

The sensitivity of SAA in olfactory mucosa has been known to vary substantially for LBD between 40% to 81% but can be positive in some CSF-negative cases.[18,21,28] The use of SAA in both olfactory mucosa and skin may have improved the αSynD detection, though olfactory mucosa αSynD could be underestimated. The absence of detailed assessments for depression, anxiety, and hypersomnia may have affected symptom characterization.

Strengths

The iOD participants underwent a thorough investigation to rule out identifiable causes of OD. Therefore, we believe our cohort represents a group of definite idiopathic cases. With access to prior medical history and a comprehensive assessment battery, we have a well-characterized cohort. We applied a blinded uniform SAA for the detection of αSynD validated in a previous study of DLB.[21] The persistence of an elevated prodromal PD risk was sustained even after excluding OD from the calculation, underscoring the increased risk in the iOD group versus HCs.

Conclusions

In this study, we found a high prevalence of αSynD and LBD symptoms in iOD, although congruence between the 2 was moderate. Nevertheless, our findings support findings from previous studies indicating that iOD is an early clinical marker of prodromal LBD. The prodromal phase is characterized by several different symptoms and is not asymptomatic. However, αSynD may not be the only driver of symptoms, and future studies should investigate the impact of co-pathology as well as whether participants with iOD will progress to manifest LBD.

Identification of early LBD may eventually open a window of opportunity for timely disease-modifying therapies and improved understanding of disease progression and aid investigations into disease causes. Because iOD is a common disorder, the combination of iOD and a sensitive biomarker such as αSynD SAA is important for the detection of the underlying α-synucleinopathy.

Author Roles

(1) Research project: A. Conception, B. Organization, C. Execution; (2) Statistical analysis: A. Design, B. Execution, C. Review and critique; (3) Manuscript: A. Writing of the first draft, B. Review and critique.

O.H.M.: 1A, 1B, 1C, 2A, 2B, 2C, 3A, 3B
R.B.: 1A, 1B, 1C, 2B, 3B
G.W.: 1A, 1B, 1C, 2C, 3B
S.G.H.: 1A, 1B, 1C, 2C, 3B
C.B.: 1A, 1B, 1C, 3B
C.K.P.: 1A, 1B, 1C, 3B
K.A.: 1A, 1B, 1C, 3B
M.B.: 1A, 1B, 1C, 3B
A.A.: 1A, 1C, 2C, 3B
E.L.L.: 1A, 1B, 1C, 2C, 3B
K.S.F.: 1A, 1B, 1C, 2A, 2C, 3A, 3B
A.H.S.: 1B, 1C, 2C, 3B
L.S.N.A.: 1B, 1C, 3B
C.H.P.: 1B, 1C, 3B

I.S.B.A.: 1B, 1C, 3B

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Disclosures

Ethical Compliance Statement: Ethical approval was obtained from the Capital Region Ethical Committee (H-22053428). All participants provided written consent. We confirm that we have read the journal's position on issues involved in ethical publication and affirm that this work is consistent with those guidelines.

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Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions. ■

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Supporting Information

Supporting information may be found in the online version of this article.

Data S1. Supporting information.

Table S1. Supplementary clinical data.

Supplementary Materials

Prodromal Lewy Body Symptoms and α-Synuclein Seeding in Idiopathic Olfactory Dysfunction

S1 Parameters and definitions for elements used in the MDS calculator for prodromal PD:

- Age.
- Sex.
- Smoking (Never, Former, or Current).
- Family history of PD: First-degree relative of a Parkinsonian diagnosis e.g. PD or DLB
- Diagnosis of Diabetes Mellitus type II.
- Results of screening for RBD: Defined as suggestive of RBD if either RBD1Q was positive or RBDSQ ≥5. The same definition is used in assessments. Note in the calculation was used: "no to exclude another differential diagnosis for RBD".
- Excessive daytime somnolence: MDS-UPDRS question 1.8 about daytime sleepiness ≥2 (mild or higher). The same definition is used in assessments.
- Olfactory loss: SIT16 ≤11 for participants aged 55–59 years and ≤10 for aged 60–75 years. All participants were tested at least one month after flu or cold symptoms to reduce the level of incidental OD in normosmic participants. The same definition is used in assessments.
- Constipation: Rated as present if participants reported taking drugs for constipation (fiber supplements or laxatives) or any points on MDS-UPDRS question 1.11. The same definition is used in assessments.
- Urinary Dysfunction: Rated as present, if participants reported taking drugs for urinary urge or any points on MDS-UPDRS question 1.10. The same definition is used in assessments.
- Severe erectile dysfunction: Rated as present if male participants reported taking drugs for erectile dysfunction. If there were no drugs for erectile dysfunction the item was rated as NA. The same definition is used in assessments.
- Orthostatic hypotension: A test was deemed positive if there was a drop in systolic blood pressure of ≥20 mmHg, a drop in diastolic blood pressure of ≥10 mmHg, or a decrease in heart rate of ≥10 beats per minute within 3 mins from the supine to the standing position. The same definition is used in assessments. When using the prodromal PD calculator, a positive orthostatic hypotension test was rated as "OH, documented, No further Diagnostic Investigation" and not "OH with no alternative cause on comprehensive expert evaluation", which induces a higher likelihood ratio.
- Depression: Classified as present if individuals exhibited a moderate or higher level of depressive symptoms according to question 1.3 on the MDS-UPDRS, or if there was a current or previous diagnosis of depression. If the patient had anxiety it was rated as NA. This definition is specific to the MDS calculator. In the other assessment, a diagnosis or any points on depressed mood or anxiety (question 1.4) was taken as a sign of depressed or anxious mood.
- Global cognitive deficit at the level of MCI: MoCA <26. The same definition is used in assessments.
- Early signs of parkinsonism: MDS-UPDRS III excluding action tremor >6[37]. The same definition is used in assessments.

Table S1 Supplementary clinical data

	HC (N=50)	*iOD (N=44)*	*p-value*	*iOD αSynD- (N=26)*	*iOD αSynD+ (N=18)*	*p-value*
RBD1Q positive. N (%)	5 (10%)	12 (27%)	0.035	7 (27%)	5 (28%)	1
Fluency animals, mean (SD)	24 (5.6)	23 (5.9)	0.32	23.3 (5.9)	22.3 (5.8)	0.57
Severe erectile dysfunction, N (% of males)	5 (24%)	4 (18%)	0.65	3 (20%)	1 (14%)	0.74
Threshold test, median (IRQ)	NA	1 (1-2)	NA	1 (1–3)	1 (1–2)	0.59
Discrimination test, mean (SD)	NA	7.8 (2.7)	NA	8.2 (2.4)	7.1 (3.1)	0.20

Table S1 presents the additional clinical data of study participants based on the inclusion group and αSynD status in the combination of skin and olfactory mucosa of the iOD group. Abbreviations: αSynD = pathological α-synuclein deposition, HC = healthy controls, iOD = idiopathic olfactory dysfunction, IQR = interquartile range, N = number, RBD1Q = REM Sleep Behavior Disorder Single-Question Screen, SD = standard deviation

8.4 Study 4: Symptoms & α-Synuclein Seeding Parameters in Olfactory Dysfunction & Lewy Body Dementia

Symptoms & α-Synuclein Seeding Parameters in Olfactory Dysfunction & Lewy Body Dementia

Running title: αSynuclein Symptoms & Seeding in Prodromal Disease

Oskar H. McWilliam MD[1#]; Remarh Bsoul Msc[2], Aušrinė Areškevičiūtė, MSc, PhD[2], Ida SB Andersen[4]; Marie Bruun, MD, PhD[1,3]; Christian von Buchwald , MD, DMSc[3,4]; Steen G. Hasselbalch , MD, DMSc[1,3]; Eva L. Lund , MD, PhD[2,3]; Christian K. Pedersen, MD[4]; Anja H. Simonsen, MSc, PhD[1]; Gunhild Waldemar, MD, DMSc[1,3]; Kasper Aanæs, MD, PhD[3,4]; and Kristian S. Frederiksen, MD, PhD[1,3]

1) Danish Dementia Research Centre, Dept. of Neurology, Copenhagen University Hospital - Rigshospitalet, Copenhagen, Denmark
2) Danish Reference Center for Prion Disease, Dept. of Pathology, Copenhagen University Hospital - Rigshospitalet, Copenhagen, Denmark
3) Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark
4) Dept. of Otolaryngology, Head & Neck Surgery and Audiology, Copenhagen University Hospital - Rigshospitalet, Copenhagen, Denmark

Keywords: seed amplification assay, real-time quaking-induced conversion, dementia with Lewy body, hyposmia, Parkinson's disease

#Corresponding author:
Oskar Hoffmann McWilliam, Inge Lehmanns Vej 6, 2100 Copenhagen. Denmark.
Tel: +4535458759, email: oskar.mcwilliam@regionh.dk

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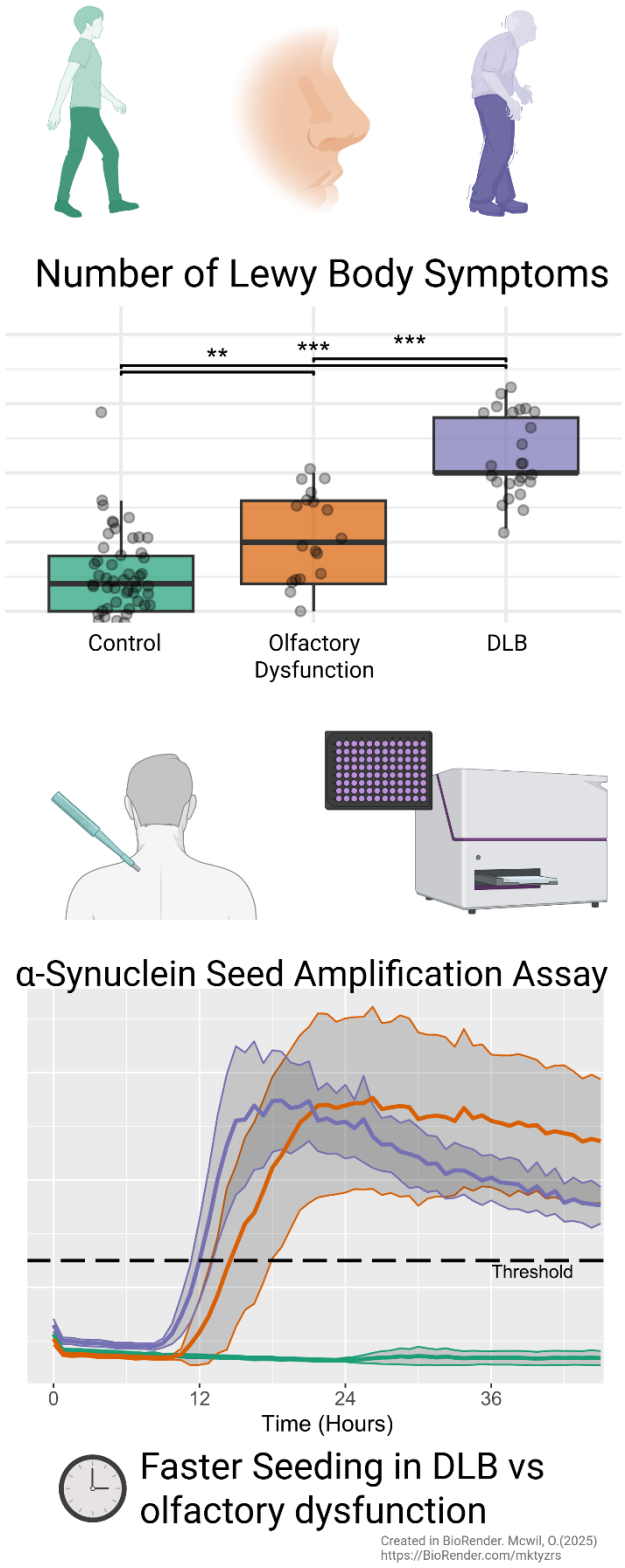
Graphical Abstract



Abstract

Objective

We investigated profiles of clinical symptoms and kinetic α-synuclein seed amplification assay (αSyn-SAA) parameters in skin across the spectrum of Lewy body disease by comparing normosmic healthy controls (HC), individuals with idiopathic olfactory dysfunction (iOD), and patients with dementia with Lewy bodies (DLB).

Methods

In this cross-sectional, case-control study, we included 49 αSyn-SAA-negative HC, 18 αSyn-SAA-positive iOD, and 25 αSyn-SAA-positive DLB patients. αSyn-SAA was performed on olfactory mucosa swabs and skin biopsies. Only the skin-derived kinetic parameters were explored for comparative kinetic analysis. Clinical assessments included the Sniffin' Sticks 16 for olfaction, Montreal Cognitive Assessment (MoCA), REM Sleep Behavior Disorder Single-Question Screen (RBD1Q), and the Movement Disorder Society-Unified Parkinson's Disease Rating Scale (MDS-UPDRS) I–III.

Results

We observed a stepwise increase in symptoms across groups from HC to iOD to DLB measured with MoCA and MDS-UPDRS I-III (all with $p<0.001$). Participants with iOD and DLB did not differ significantly in olfactory function (mean [SD]: 6.5 [2.4] vs. 6.0 [2.5], $p = 0.51$). Accelerated αSyn seeding kinetics, particularly shorter time to 50% of maximal fluorescence, were detected in DLB vs iOD (median hours [IQR] 17 [15–20] vs 14 [13–16], $p=0.017$), achieving a classification accuracy of 74% (95% CI: 57–91), and correlated with number of symptoms related to DLB without and with adjusting for age and sex ($p=0.002$, $R^2=0.605$).

Interpretation

These findings support a stepwise progression model, positioning iOD as an early detectable prodromal stage in which Lewy body symptoms and peripheral αSyn aggregation gradually increase toward manifest disease.

Introduction

Manifest Lewy body diseases, such as Parkinson's disease (PD) and dementia with Lewy bodies (DLB), are characterized by the pathological aggregation of α-synuclein (αSyn) within neurons, ultimately leading to neurodegeneration[1]. This aggregation process is thought to begin with a preclinical phase, followed by a prolonged prodromal period marked by gradually emerging symptoms, preceding the clinical onset of manifest DLB or PD[2]. Idiopathic olfactory dysfunction (iOD) has been proposed as the earliest clinical precursor, occurring decades before parkinsonism and cognitive symptoms[3,4]. Especially in DLB, it is an early and pronounced symptom[3]. Other prodromal features include REM sleep behavior disorder (RBD), psychiatric symptoms, daytime sleepiness, and autonomic dysfunction[3,5–7]. However, current understanding of the prodromal stages of Lewy body disease remains limited and is largely based on models focusing on participants with RBD, which is present in only approximately half of individuals with PD/DLB at the time of diagnosis[8]. Olfactory dysfunction, on the other hand, is present in more than 80% of de novo PD/DLB but may have a low specificity due to other etiologies affecting olfaction[8].

The αSyn seed amplification assay (SAA) has been established as a sensitive and highly specific method for detecting pathological αSyn aggregates in various tissues, including cerebrospinal fluid (CSF), skin, and olfactory mucosa, and thus has the potential as an early diagnostic, specific biomarker [9–11]. The αSyn-SAA has primarily been interpreted as a binary outcome, the development of αSyn-SAA holds promise for a more granular assessment of αSyn seeding properties, which may be more suited as a measure for tracking disease progression from the prodromal to the clinically manifest stage and may facilitate biomarker-based assessment of therapeutic response in future disease-modifying trials. However, establishing reliable continuous measurements has proven challenging. Poggiolini et al.[12] demonstrated in CSF that disease stage (RBD vs PD) and symptom clusters were correlated with kinetic parameters including time-to-threshold, maximum slope (Vmax), and maximum fluorescence (Fmax). Similarly, using CSF-based αSyn-SAA, other studies[13,14] reported an association between continuous measures and cognitive impairment and phenoconversion from prodromal to manifest Lewy body disease[15]. In skin αSyn-SAA, kinetic parameters have been investigated in PD, with one study[16] reporting correlations with disease duration and severity, whereas another found no significant associations[17]. Kuzkina et al. reported that participants with RBD showed a faster seeding profile than those with PD in skin αSyn-SAA, possibly reflecting a more extensive peripheral distribution

of pathological αSyn in the RBD phenotype[18]. However, to our knowledge, no studies have yet investigated kinetic αSyn-SAA parameters in minimally invasive peripheral tissues such as skin in a broad prodromal model like iOD versus manifest Lewy body disease.

This study aimed to explore differences in clinical symptoms and kinetic αSyn-SAA parameters in skin across the disease course of Lewy body disease by comparing αSyn-SAA negative normosmic healthy controls (HC), αSyn-SAA positive individuals with iOD, and αSyn-SAA positive patients with DLB. Moreover, we investigated the association between kinetic skin αSyn-SAA parameters and the severity of prodromal symptoms.

Methods

Study Design

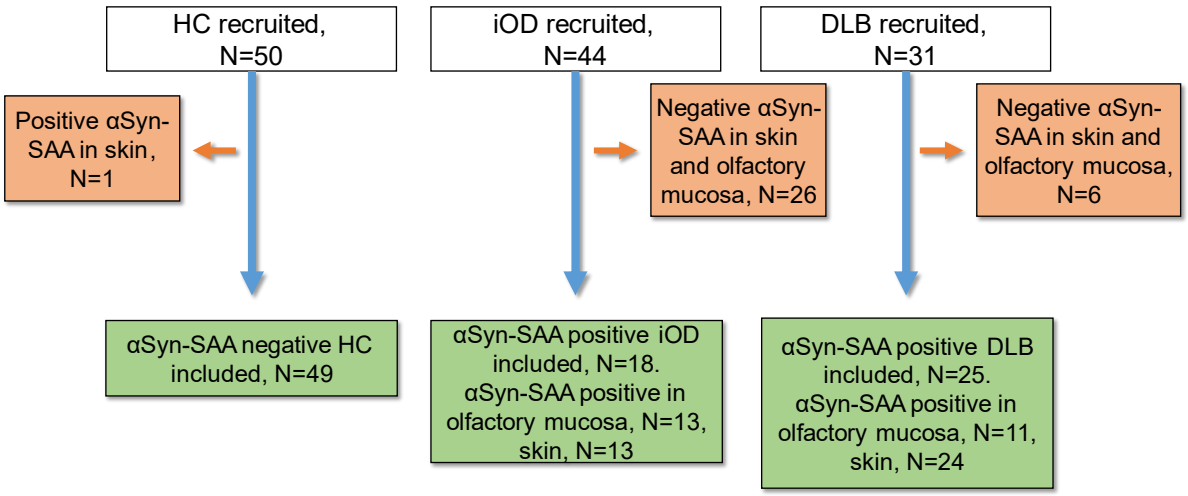
This is a cross-sectional, single-center, case-control study, which included HC, iOD, and DLB patients. A subset of findings from this cohort has previously been reported, focusing on the technical validation of the αSyn-SAA in HC and DLB[11], as well as dichotomous SAA outcomes in HC versus iOD[19]. The study was registered at clinicaltrials.gov (NCT05740683 + NCT05768425), and ethical approval was obtained by the Capital Region Ethical Committee (H-22053428 + H-22046053).

Participants

The inclusion criteria for HC and iOD were: 1) age between 55–75 years and 2) no major neurological disease 3) normal olfactory function (for HC) or idiopathic olfactory dysfunction (for iOD). Olfactory dysfunction was defined as Sniffin' Sticks identification test 16 (SIT16) $\leq$11 for participants aged 55–59 years and $\leq$10 for those aged 60+ years as described in a previous study[19], see also Supplementary Materials S1 for further details. Participants with iOD were recruited from the Unit for Sense of Taste and Smell at Rigshospitalet or private practice ear, nose, and throat specialist (ENT) clinics (Fig 1).

Participants diagnosed with probable DLB[20] or mild cognitive impairment with Lewy bodies[21] were recruited from the Memory Clinic at Copenhagen University Hospital, Rigshospitalet. In this study, we included participants with DLB and iOD, who had positive αSyn-SAA in skin and/or olfactory mucosa, and HC with negative αSyn-SAA tests (Fig 1).

Figure 1 Inclusion Flowchart



HCs were recruited from previous studies and social media posts. One HC was excluded from this study due to a positive αSyn-SAA test in skin. A total of 26 iODs were negative in olfactory mucosa and/or skin and thus excluded. In DLB, 6 patients were not positive in the olfactory mucosa and/or skin and were thus excluded.
Abbreviations: αSyn-SAA = α-Synuclein seed amplification assay, DLB = dementia with Lewy bodies, HC = healthy controls, iOD = idiopathic olfactory dysfunction, and N = number

Clinical Assessments

The olfactory function was assessed using a Danish adaptation of SIT16[22]. The Montreal Cognitive Assessment Danish (MoCA) was used to assess cognitive function, and cognitive dysfunction was defined as <26 points[23]. The REM Sleep Behavior Disorder Single-Question Screen (RBD1Q) was used to screen for possible RBD. The Movement Disorders Society-Unified Parkinson's Disease Rating Scale (MDS-UPDRS) Parts I–III were applied to evaluate Lewy body symptoms. Part I assesses non-motor symptoms (e.g., subjective cognition, psychiatric, and dysautonomia symptoms), part II subjective motor symptoms, and part III objective motor performance[24]. Parkinsonism, neuropsychiatric symptoms, and autonomic dysfunctions were evaluated by subscores of the MDS-UPDRS (see Supplementary Materials S2 for further details). We constructed a composite symptom score by counting the presence (range: 0–8) of the following Lewy body symptoms: RBD, parkinsonism, cognitive impairment, orthostatic hypotension, constipation, urinary urgency, excessive daytime sleepiness, and depressed mood.

Sampling and Seed Amplification Assay

Two 3 mm skin punch biopsies were collected 5 cm paramedially from C7, as described previously[11]. Two skin samples were analyzed per participant in the iOD and HC groups, whereas only one sample was analyzed for participants with DLB. The weight-to-volume ratio was identical across all groups.

Olfactory mucosa samples were collected via endoscopic-guided swabbing, as previously described[11,19]. The olfactory mucosa samples were excluded from quantitative analysis due to minor variations in swab type and protein extraction protocols between iOD/HC and DLB. All blood-contaminated olfactory mucosa samples were excluded from αSyn-SAA.

αSyn-SAA was conducted uniformly by Real-Time Quaking-Induced Conversion with an in-house recombinant α-synuclein substrate. The assay was performed on a FLUOStar^{TM} Omega instrument for 48 hours, with fluorescence measurement every 45 min as previously described by Bsoul, McWilliam et al.,[11]. αSyn-SAA was performed in quadruple wells and considered positive when $\geq$2 wells exceeded a preset threshold of 4 standard deviations (SD) above the thioflavin T baseline of the negative controls, as described previously[11,25].

Kinetic Seed Amplification Assay Parameters

Kinetic αSyn-SAA analysis was performed on skin biopsies from all participants who tested positive for αSyn-SAA in skin tissue. Based on previous studies of αSyn-SAA in CSF[12,26], the following kinetic parameters were analyzed: (1) Maximum fluorescence (Fmax); (2) area under the curve (AUC); (3) time-to-threshold (sometimes referenced to as lag time); (4) TH50, the time in hours to reach 50% of Fmax; (5) maximum slope (Vmax), calculated as the highest rate of fluorescence increase across two time points. We also calculated the quenching ratio, which was defined as Fmax divided by the last fluorescent value. See Supplementary Materials Fig S1 for illustrated definitions of kinetic parameters.

αSyn-SAA for iOD/HC samples was conducted using a single batch of recombinant αSyn, and the DLB samples were tested with two different recombinant αSyn batches. Different brain homogenate dilutions from a single DLB patient were made uniformly and used freshly as the positive controls in both DLB and iOD/HC αSyn-SAAs. The batch used for iOD/HC demonstrated more rapid kinetics, e.g., shorter TH50 and higher Fmax and AUC than those used for DLB (Supplementary Materials Fig S3), which performed alike.

Statistical Analysis

The statistical software R with RStudio (version 4.3.0) was used for all statistical analyses. Comparisons between HC, iOD, and DLB were conducted using either ANOVA or independent-samples t-tests for normally distributed variables, and Kruskal-Wallis or Wilcoxon rank-sum tests for non-parametric data. Chi-squared ($N>5$) or Fisher's exact test ($N\leq5$) was employed for categorical data comparisons. P-values for clinical assessments were corrected post hoc pairwise with Holm's method. Associations between clinical symptoms were assessed using Spearman's rank correlation. Linear regression models were employed to evaluate the relationship between SAA kinetic parameters as predictors and clinical assessments as outcomes, with and without adjusting for age and sex as covariates. The accuracy of each parameter in distinguishing between iOD and DLB was calculated using the pROC package. The robustness was tested using Leave-One-Out Cross-Validation, and the SD was calculated with bootstrapping.

Results

One HC was αSyn-SAA positive in skin and thus excluded from analysis. A total of 18 participants with iOD were αSyn-SAA positive in olfactory mucosa (N=13) and/or skin, while 25 DLB patients were positive in olfactory mucosa (N=11) and/or skin (N=24) (Fig 1). All skin αSyn-SAA positive DLB cases, and all but one iOD case, showed positive seeding activity in all four assay wells. Participants with DLB were older than HCs and individuals with iOD (Table 1), were more often males, and reported a longer duration of subjective olfactory dysfunction compared to iOD. Possible RBD and medication for autonomic dysfunction were more common in DLB, but not significantly different between the HC and iOD groups.

Table 1 Demographics and clinical variables

	HC (N=49)	**iOD (N=18)**	**DLB (N=25)**	**p-value (HC vs iOD)**	**p-value (iOD vs DLB)**	**p-value (all)**
Sex, N female (%)	27 (55%)	11 (61%)	3 (12%)	0.66	**0.003**	**<0.001**
Age, mean years (SD)	68 (5.1)	68 (4.3)	75 (5.1)	0.72	**<0.001**	**<0.001**
Never smoker, N (%)	21 (43%)	8 (44%)	10 (40%)	0.52	0.84	0.95
Depression (current or previously), N (%)	5 (10%)	6 (33%)	4 (16%)	**0.006**	0.15	**0.028**
Hypertension, N (%)	21 (43%)	5 (28%)	13 (52%)	0.26	0.15	0.31
Olfactory Dysfunction Onset, N (%)						
≤1 year,	NA	2 (11%)	1 (5%)	NA	**0.016**	NA
>1–2 years	NA	1 (6%)	2 (10%)			
>2–5 years	NA	7 (39%)	4 (20%)			
>5–10 years	NA	6 (33%)	1 (5%)			
≥10 years	NA	2 (11%)	12 (60%)			
Use of laxatives, N (%)	5 (10%)	4 (22%)	13 (52%)	0.096	0.13	**<0.001**
Use of medication for urinary urge, N (%)	1 (2%)	0	12 (48%)	>0.99	**0.003**	**<0.001**

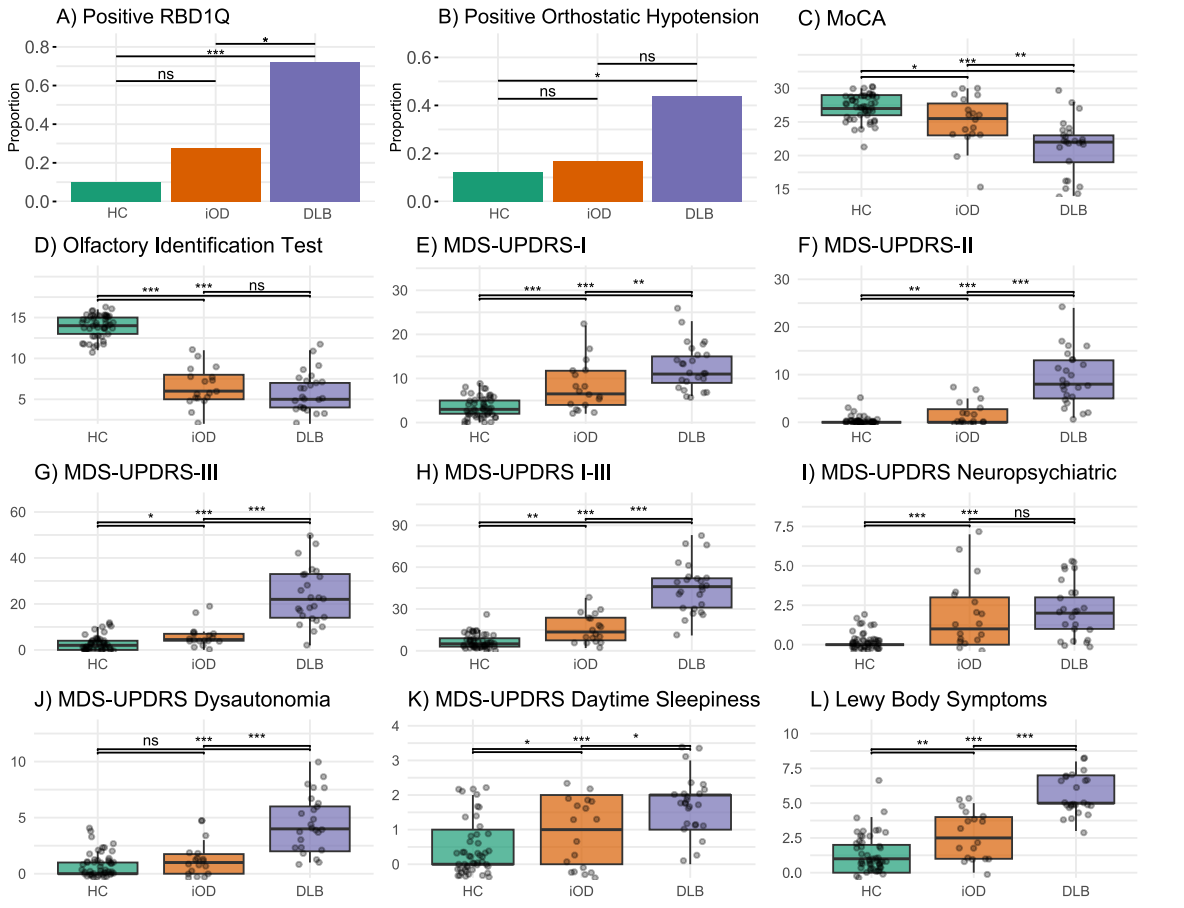
Demographics in HC, iOD, and DLB. Olfactory dysfunction onset was defined as the self-reported time at which the individual first noticed a reduced sense of smell.
Abbreviations: DLB = dementia with Lewy bodies, HC = healthy controls, iOD = idiopathic olfactory dysfunction, N = number, NA = not applicable, SD = standard deviation

As expected, Lewy body symptoms were most prominent in participants with DLB and least pronounced in HCs (Table 1 and Fig 2), whereas the results for iODs tended to be intermediate between DLB and HCs. No significant differences were observed between the iOD and DLB groups in measures of current olfactory function or neuropsychiatric symptoms (Fig 2B, I). Furthermore, symptoms of autonomic dysfunction did not differ between the iOD and HC groups (Fig 1J).

Olfactory and autonomic dysfunction were negatively correlated within the iOD and DLB groups

(Supplementary Fig S3A, B), whereas the duration of olfactory dysfunction and the degree of cognitive impairment correlated positively (Supplementary Fig S3A). In the pooled iOD and DLB group, autonomic dysfunction was positively associated with increased daytime sleepiness, parkinsonian motor symptoms, cognitive impairment, and RBD (Supplementary Fig S3C).

Figure 2 Clinical Assessments and symptoms

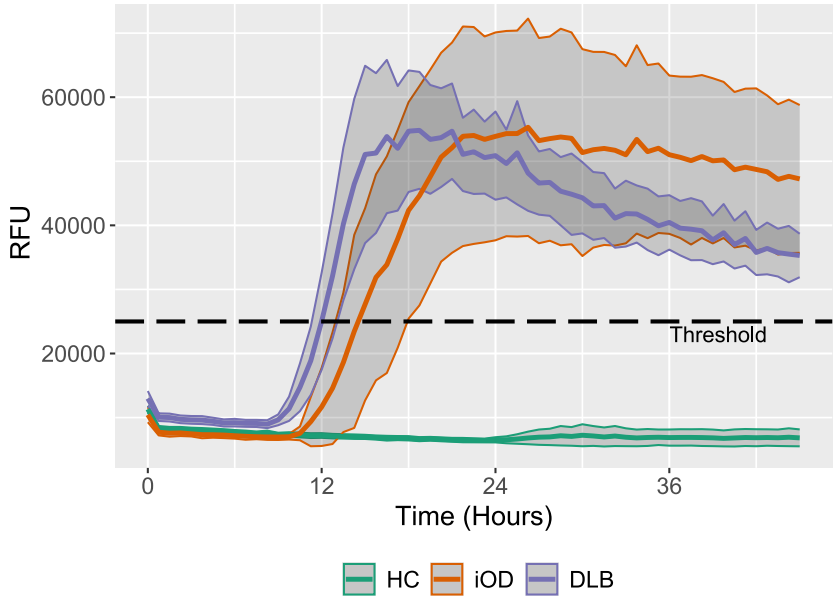


Clinical assessments across groups (HC, iOD, and DLB). The p-values are corrected pairwise with Holm's test.
*Abbreviations: ns = non-significant, *p ≤ 0.05, ** p<0.01, ***p≤0.001, **** p≤0.0001. DLB = dementia with Lewy bodies, HC = healthy controls, iOD = idiopathic olfactory dysfunction, MDS-UPDRS = Movement Disorders Society unified MoCA = Montreal Cognitive Assessment, and RBD1Q = REM sleep behavior disorder single question.*

The kinetic parameters from αSyn-SAA in skin from HC and the αSyn positive iOD and DLB are displayed in Fig 3 and Table 2. In these assays, the DLB group exhibited faster seeding kinetics compared to the iOD group, as indicated by shorter TH50 and time-to-threshold values. The AUC did not differ between iOD and DLB but was nominally higher in DLB (iOD vs DLB median (IQR)

2.4 (1.6–2.6) x 10^6 vs 3.2 (1.7–4.6) x 10^6, p=0.11) when corrected for the quenching ratio. DLB and iOD cases that were RBD1Q-positive showed a nominal but non-significant faster seeding compared with RBD1Q-negative cases (data not shown).

Figure 3 α-Synuclein Seeding in Skin over Time



A: Time plot of the seed amplification from skin sample analysis with mean and CI for each group: HC (N=48 αSyn-SAA negative in skin), iOD (N=13 αSyn-SAA positive in skin), and DLB (N=24 αSyn-SAA positive in skin). The plot is based on the mean values from the two best-performing wells out of four. Single participant data can be viewed in the Supplementary Materials Fig S4. Abbreviations: DLB = Dementia with Lewy bodies, HC = healthy controls, iOD = idiopathic olfactory dysfunction, RFU = relative fluorescence unit.

Table 2 Kinetic Parameters for the α-Synuclein Seeding in Skin Samples

	iOD (N=13)	DLB (N=24)	p-value
Time-to-threshold, median, (IRQ) hours	16 (14–19)	13 (11–17)	**0.029**
TH50, median (IRQ) hours	17 (15–20)	14 (13–16)	**0.017**
Vmax, median (IRQ)×10³ RFU	11 (8.8–17)	14 (8.9–26)	0.31
Fmax, median (IRQ)×10³ RFU	66 (41–81)	65 (50–96)	0.63
AUC, median (IRQ)×10⁶	1.6 (1.1–1.9)	1.6 (1.3–1.7)	0.91
Quenching Ratio, median (IRQ)	1.4 (1.3–1.4)	2.1 (1.5–2.2)	**0.0015**

Kinetic parameters for skin SAA in SAA-positive iOD and DLB. See also Supplementary Materials Fig S1 for illustrated definitions of kinetic parameters.
Abbreviations: AUC = area under the curve, DLB = Dementia with Lewy bodies, Fmax = maximal fluorescence measured, iOD = idiopathic olfactory dysfunction, IRQ = interquartile range, RFU = relative fluorescence unit, TH50 = time to 50 of Fmax, Vmax = maximal velocity of RFU change over 1.5 hours

TH50 demonstrated the highest classification accuracy (DLB vs. iOD) at 72% (CI 55–89) (Table 3), which remained robust under cross-validation with an accuracy of 70% (SD 7.4%). Time-to-threshold yielded a comparable accuracy to TH50, whereas Vmax, Fmax, and the fluorescence AUC all showed accuracies with confidence intervals overlapping 50%, indicating limited discriminative power.

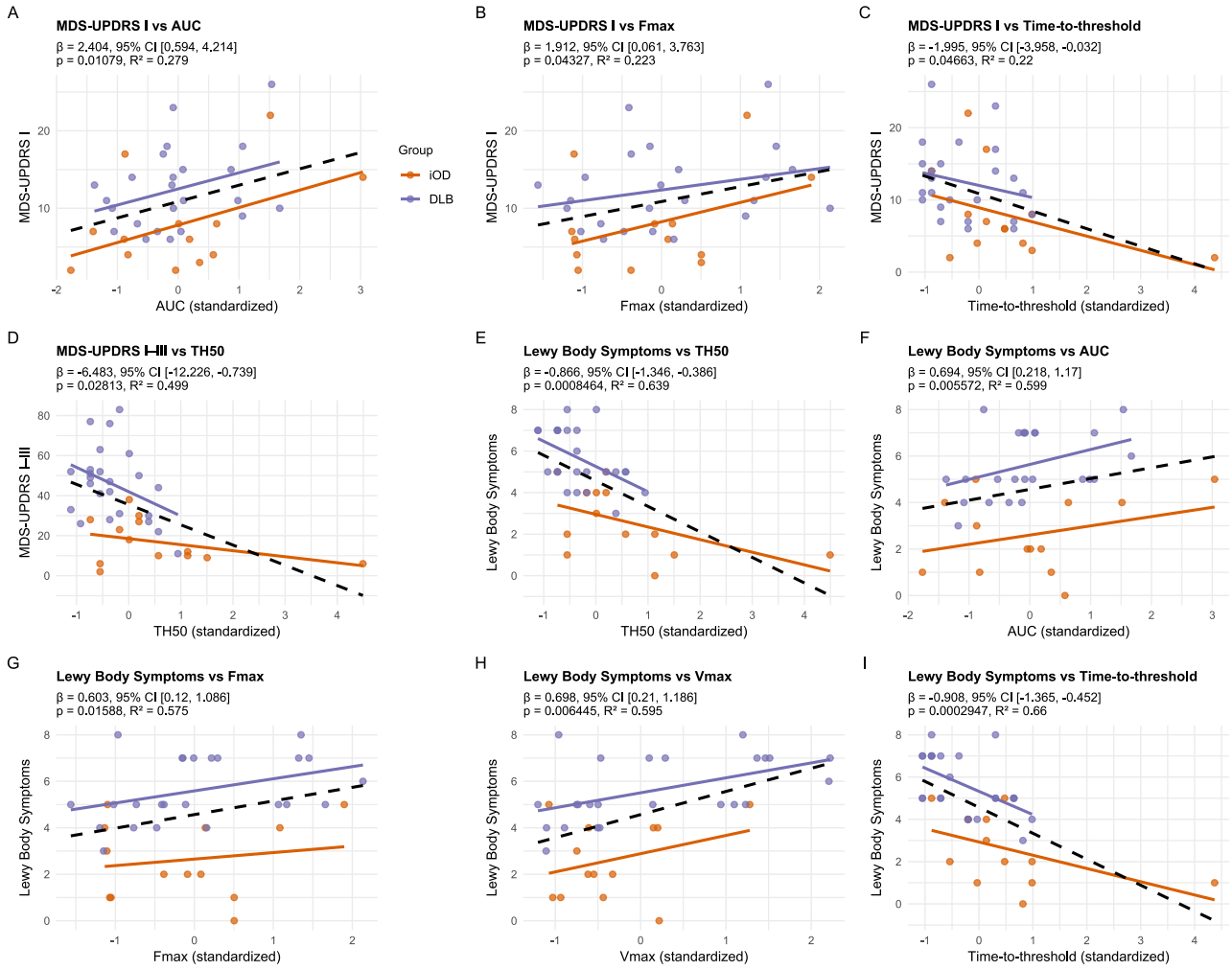
Table 3 Accuracy, sensitivity, and specificity of kinetic parameters for iOD vs DLB

	Accuracy% (CI)	Sensitivity% (CI)	Specificity% (CI)	Threshold
Time-to-threshold	72 (55–89)	54 (33–75)	84 (65–100)	14 hours
TH50	74 (57–91)	71 (50–88)	69 (46–92)	15 hours
Vmax	61 (42–79)	42 (25–63)	92 (76–100)	17 $\times 10^3$ RFU
Fmax	55 (35–75)	88 (71–100)	39 (15–69)	80 $\times 10^3$ RFU
AUC (fluorescence)	51 (29–73)	63 (48–83)	62 (38–85)	1.6 $\times 10^6$ RFU

Diagnostic performance of kinetic parameters for discrimination of DLB (case) from iOD (control). The accuracy, sensitivity, and specificity were calculated from ROC curves. See Supplementary Materials Fig. S5.
Abbreviations: AUC = area under the curve, CI = 95% confidence interval, Fmax = maximal fluorescence measured, RFU = relative fluorescence unit, TH50 = time to 50 of Fmax, Vmax = maximal velocity of RFU change over 1.5 hours.

Significant associations between kinetic parameters and symptom burden are illustrated in Figure 4 (and Supplementary Tables S1 and S2). The total number of Lewy body symptoms showed the strongest associations across kinetic measures, yielding the highest R^2 values. In contrast, cognitive dysfunction and olfactory dysfunction were not significantly associated with kinetic parameters, regardless of adjustment for age and sex.

Figure 3 Association of Symptoms and Kinetic Seeding parameters



Linear regression models between clinical assessment scale scores (outcome) and αSyn-SAA kinetic parameters (predictors) in skin samples corrected for age and sex. Orange line = iOD, purple line = DLB, and dashed line represents the combined group (iOD and DLB). Only significant associations are displayed. Full tables of uncorrected and corrected models can be viewed in the Supplementary Materials Tables S1 and S2.

Abbreviations: AUC = area under the curve, DLB = Dementia with Lewy bodies, Fmax = maximal fluorescence measured, iOD = idiopathic olfactory dysfunction, TH50 = time to 50 of Fmax, Vmax = maximal velocity of RFU change over 1.5 hours.

Discussion

This study investigated clinical symptoms and explored αSyn seeding kinetic parameters in skin across the disease course in Lewy body disease. We found increasing severity and frequency of several Lewy body symptoms across groups, from HC over iOD, to DLB. This was paralleled by accelerated αSyn seeding parameters across groups and was associated with the severity of clinical symptoms. The findings support the hypothesis of a stepwise progression with iOD as a prodromal stage where both Lewy body symptoms and peripheral αSyn aggregation gradually increase toward manifest Lewy body disease.

Lewy body symptoms

The progressive worsening of Lewy body symptoms, such as motor parkinsonism, cognitive dysfunction, and daytime sleepiness, indicates early onset and continuous symptom escalation over time. In the iOD group, cognitive dysfunction was associated with the duration of olfactory impairment (Supplementary Materials Figure S5). Similar observations were made by Weintraub et al.[27], who found that performance on specific cognitive subtests correlated with disease progression in participants with olfactory dysfunction. Notably, in individuals with RBD who later converted to manifest α-synucleinopathy, MoCA scores began to diverge significantly from non-converters approximately three years before phenoconversion to DLB/PD[3].

No significant differences were observed between the iOD and DLB groups in terms of olfactory dysfunction and neuropsychiatric symptoms such as anxiety, depression, and apathy (Fig 2D, I). This finding suggests that these symptoms may already reach a level of severity comparable to that seen in manifest Lewy body disease during the earlier, prodromal stage. The early involvement of olfaction is further supported by studies in participants with RBD, where olfactory deficits are frequently observed years before phenoconversion to manifest Lewy body disease. For example, Fereshtehnejad et al.[3] reported that olfactory impairment was already present in participants with RBD and remained relatively stable, with only a moderate, non-significant decline until phenoconversion in DLB converters. Similarly, results from a meta-analysis[28] and a review[29] of olfactory function in PD indicated similar stability in the deficiency of olfaction post-diagnosis.

Higher levels of depressive symptoms have previously been reported in iOD versus HC[30,31]. Our findings support these observations; however, we did not find more severe neuropsychiatric symptoms in DLB vs iOD, indicating a plateau of neuropsychiatric symptoms in the prodromal iOD phase.

Symptoms and treatment of autonomic dysfunction were more prevalent and pronounced in DLB compared to HC. In contrast, the iOD group compared to HC showed only a numerically increased use of laxatives (Table 1), orthostatic hypotension, and dysautonomia score (Fig 2B, J). Olfactory function was negatively correlated with autonomic dysfunction in both iOD and DLB (Supplementary Materials Table S3A+B). This unexpected inverse relationship may reflect autonomic dysfunction as a feature less prominent in iOD compared to patients with RBD.

Kinetic Seed Amplification

To our knowledge, this is the first study to demonstrate significant differences in kinetic αSyn-SAA parameters in skin between iOD and DLB. Furthermore, we showed that kinetic αSyn-SAA parameters correlate with clinical symptom burden, particularly the number of Lewy body symptoms, as well as non-motor symptoms (MDS-UPDRS I). Specifically, time-to-threshold and TH50 discriminated iOD from DLB with accuracies of 72% (CI 55–89) and 74% (CI 57–91), respectively. These findings align with previous studies showing that time-to-threshold and T50 in skin αSyn-SAA correlate with disease duration and severity in PD[16], and also in CSF-based αSyn-SAAs the parameters correlated with disease stage across Lewy body disease, particularly in participants with RBD and OD compared to those with PD[12,15]. Interestingly, Kuzkina et al. found a faster seeding in participants with RBD compared with PD, suggesting that the seeding kinetics may also be phenotype-dependent. However, iOD may encompase a broader phenotype of Lewy body disease than the RBD phenotype. Like the other studies in CSF, we did not detect in skin a significantly increased fluorescence AUC in manifest Lewy body disease compared to prodromal disease[12,15].

The link between Lewy body disease stage and αSyn pathology was further substantiated by correlations between the severity of several clinical measures and kinetic parameters derived from αSyn-SAA (Fig 4, Supplementary Materials Tables S1+S2). Notably, the number of Lewy body symptoms and non-motor symptoms showed significant correlations with kinetic αSyn-SAA parameters, even after adjusting for age and sex. In contrast to previous studies using CSF-based SAAs[13,32], we did not detect a correlation between cognitive performance (MoCA) and kinetic parameters.

A key challenge in interpreting our kinetic αSyn-SAA results was the markedly faster and stronger seeding observed in positive controls made from DLB brain homogenate, with different batches of recombinant α-synuclein used for iOD and DLB. However, without the batch-related variation, the

differences between DLB and iOD seeding kinetics in skin would likely have been even greater. Furthermore, the proximity of the results and the robust correlations with clinical parameters within and across the groups support the assay's validity (Fig 4).

The findings demonstrate that kinetic αSyn-SAA parameters derived from a minimally invasive biospecimen not only differentiate prodromal from manifest Lewy body disease but also reflect clinical symptom burden, thereby supporting skin αSyn-SAA's potential role as a stage-sensitive biomarker in Lewy body disease. These findings are explorative and should be validated in independent cohorts and larger samples with different prodromal populations, including longitudinal follow-up.

Limitations

In addition to the between-batch differences in positive controls noted above, this study has several limitations. Most notably, the limited sample size of αSyn-SAA positive iOD participants warrants caution when interpreting the results, which are exploratory and need validation in independent cohorts. Furthermore, the cross-sectional design and lack of longitudinal follow-up restrict our ability to assess symptom progression over time. We have compared iOD to DLB and not PD. It would have strengthened the study to have included PD, as some participants with iOD will likely phenoconvert to PD rather than DLB. Nonetheless, olfactory dysfunction is a more early and prominent prodromal symptom in DLB converters compared with PD[3]. Although our study did not investigate different pathological αSyn strains, previous research has shown minimal differences in seeding activity between PD and DLB when adjusted for disease severity[33,34]. In contrast, multiple system atrophy exhibits a distinct pathological αSyn strain with markedly lower seeding activity[35,36]. However, as multiple system atrophy is rarely associated with prodromal OD[37], this is unlikely to have influenced our findings.

For the clinical analyses, we included iOD and DLB participants who were SAA-positive in either the olfactory mucosa or skin, reflecting the hypothesis of distinct entry sites for pathological αSyn. iOD participants who were SAA-negative in skin but positive in the olfactory mucosa were excluded from the analyses, along with HCs, due to sample variabilities and many kinetic parameters cannot be calculated for samples that fail to reach the amplification threshold.

Our assessment of Lewy body symptoms relied mainly on clinical evaluations without the use of auxiliary investigations, such as imaging of dopaminergic function, autonomic dysfunction, or polysomnography, which could be more sensitive and precise in early stages. Moreover, diagnoses

were based on clinical criteria without neuropathological confirmation. Nevertheless, the iOD diagnoses were made in a specialized unit for smell and taste disorders, and DLB diagnoses were determined through a multidisciplinary diagnostic process. Combined with the high specificity and sensitivity of αSyn-SAAs, this provides confidence in the diagnostic classification[38].

As expected, the DLB group was older and had a higher proportion of male participants compared to the iOD and HC groups. While these demographic differences may have influenced certain clinical measures, we adjusted our linear models for age and sex and still observed significant correlations between Lewy body symptom burden and kinetic αSyn-SAA parameters.

Strengths

This study employed a comprehensive set of clinical assessments to capture the spectrum of Lewy body symptoms. Procedures such as olfactory testing, nasal swabbing, and skin biopsy are minimally invasive, time-efficient, and can be performed without the need for prior auxiliary testing. Importantly, the SAA analyses on skin and olfactory mucosa were conducted using a uniform protocol without serial dilutions, enhancing both feasibility and translational potential.

Conclusion

This study reinforces the role of iOD as a valuable prodromal model for Lewy body disease. Our findings contribute to the potential of aSyn-SAA as an early biomarker of Lewy body disease and broaden the understanding of the prodromal stage, complementing existing research focused primarily on RBD. Moreover, we demonstrate that kinetic SAA parameters, previously established in CSF, can be successfully applied to minimally invasive biospecimens such as skin, reflecting disease stage and clinical burden. These findings should be validated in larger longitudinal cohorts, as they may offer a powerful, minimally invasive tool for monitoring disease progression and evaluating treatment response to anti-α-synuclein therapies.

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Author Contributions

OHM: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology Project administration, Writing – original draft, Writing – review & editing. RB: Conceptualization, Data curation, Methodology, Project administration, Resources, Validation, Visualization, Writing – review & editing. KSF: Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – original draft Writing – review & editing. AA, GW, SGH: Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing. ELL, CvB: Conceptualization, Funding acquisition, Methodology, Resources, Writing – review & editing. AHS, MBr: Conceptualization, Methodology, Resources, Writing – review & editing. KA CKP, and ISBA: Conceptualization, Investigation, Methodology, Resources, Writing – review & editing

Potential Conflicts of Interest

The authors declare that there are no conflicts of interest relevant to this work.

Funding Sources and Conflict of Interest:

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Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supplementary Materials

Symptoms & α-Synuclein Seeding Parameters in Olfactory Dysfunction & Lewy Body Dementia

Oskar H. McWilliam MD[1#]; Remarh Bsoul Msc[2], Aušrinė Areškevičiūtė, MSc, PhD[2], Ida SB Andersen[4]; Marie Bruun, MD, PhD[1,3]; Christian von Buchwald , MD, DMSc[3,4]; Steen G. Hasselbalch , MD, DMSc[1,3]; Eva L. Lund , MD, PhD[2,3]; Christian K. Pedersen, MD[4]; Anja H. Simonsen, MSc, PhD[1]; Gunhild Waldemar, MD, DMSc[1,3]; Kasper Aanæs, MD, PhD[3,4]; and Kristian S. Frederiksen, MD, PhD[1,3]

1) Danish Dementia Research Centre, Dept. of Neurology, Copenhagen University Hospital - Rigshospitalet, Copenhagen, Denmark
2) Danish Reference Center for Prion Disease, Dept. of Pathology, Copenhagen University Hospital - Rigshospitalet, Copenhagen, Denmark
3) Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark
4) Dept. of Otolaryngology, Head & Neck Surgery and Audiology, Copenhagen University Hospital - Rigshospitalet, Copenhagen, Denmark

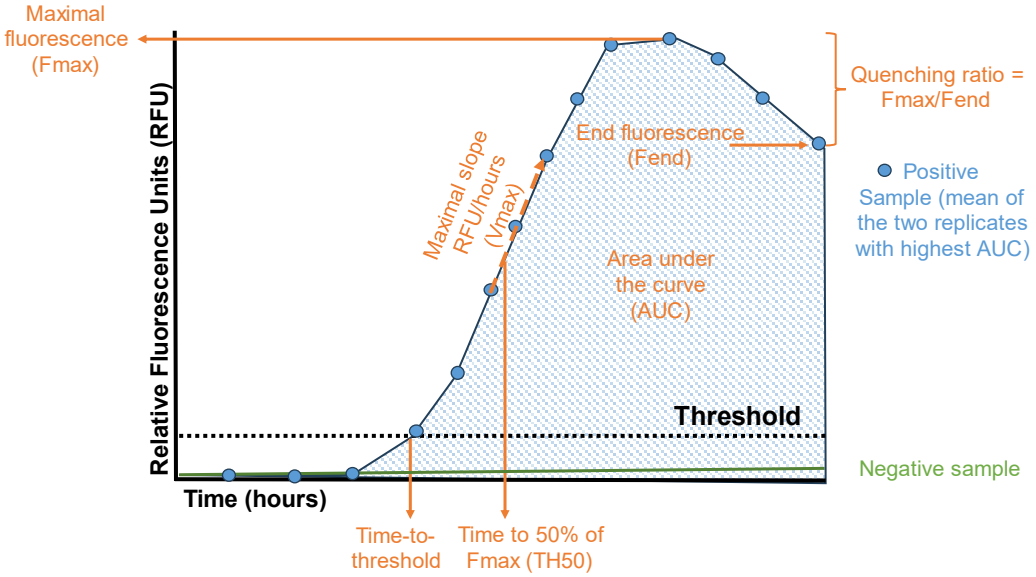
S1 Definition of iOD

Criteria for iOD: a) non-congenital olfactory dysfunction with no temporal link to infections (within 2 weeks) or head trauma, b) magnetic resonance or computed tomography to exclude pathology relating to olfactory dysfunction or progressive olfactory dysfunction >2 years, c) no significant sino-nasal disease on endoscopy, and e) no response to short-term systemic/topical corticosteroid treatment. The exclusion criteria for both iOD and HC: a) a diagnosis of PD/DLB, b) a diagnosis of other major neurological/psychiatric diseases, or c) substance or alcohol abuse.

S2 Definition of Parkinsonism, Neuropsychiatric Symptoms, and Autonomic Dysfunction

The neuropsychiatric score was calculated as the sum of values from the questions on depression, anxiety, and apathy. The dysautonomia score was calculated as the sum of values from the questions on urinary problems, constipation, lightheadedness on standing, and drooling. Parkinsonism was defined as MDS-UPDRS III score without action tremor >6.

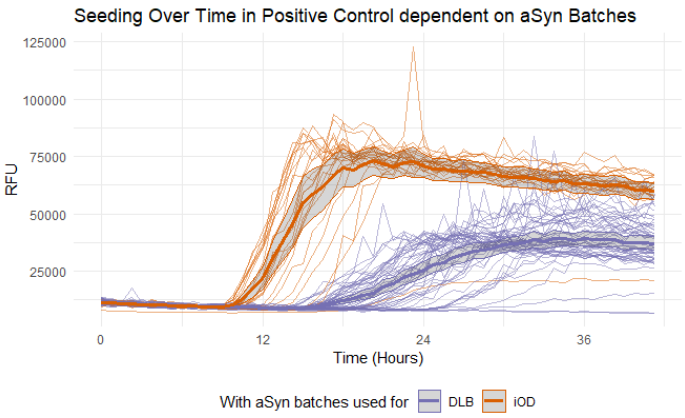
Figure S1 SAA Kinetics Parameters Definitions



Maximum fluorescence (Fmax), representing the peak intensity of the relative fluorescence units (RFU) signal; (2) area under the curve (AUC), reflecting cumulative fluorescence over time; (3) time-to-threshold (sometimes referenced to as lag time), defined as the time from 0 to when the fluorescence reached our threshold of 25,000 RFU, based on 4 SDs from a negative control; (4) TH50, the time in hours to reach 50% of Fmax; (5) maximum slope (Vmax), calculated as the highest rate of fluorescence increase across two time points (1.5 hours apart), to adjust for fluctuations in the fluorescence; and 6) the quenching ratio was defined as Fmax divided by the last fluorescent value.

Figure S2 SAA Kinetics in Different Recombinant α-Synuclein Batches

A



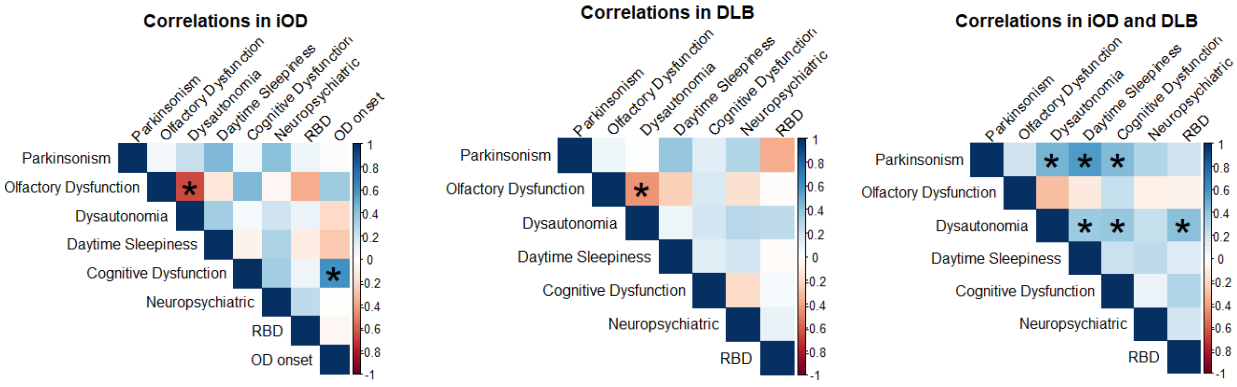
B

	Batch 1 iOD/HC (N=24)	*Batch 2+3 DLB (N=50)*	*p-value*
***TH50**, median (IRQ) hours*	14 (13–15)	23 (20–26)	**<0.0001**
***Time-to-threshold**, median (IRQ) hours*	13 (11–14)	23 (21–26.2)	**<0.0001**
***Vmax**, median (IRQ) ×10³ RFU*	16 (15–19)	7.6 (5.8–10)	**<0.0001**
***AUC**, median (IRQ) ×10⁶*	2.1 (2.0–2.1)	0.94 (0.87–1.0)	**<0.0001**
***Fmax**, median (IRQ) ×10³ RFU*	82 (80–85)	46 (42–55)	**<0.0001**
***Quenching Ratio**, median (IRQ)*	1.4 (1.3–1.4)	1.3 (1.2–1.4)	0.23

A: Time plot of the seed amplification in positive controls (brain homogenate from a DLB patient) run with different recombinant α-synuclein batches. Bath 1 was used for iOD/HC samples and Bath 1+2 (with similar kinetics) were used for DLB. The positive control sample was produced uniformly from the same brain and used fresh. B: Kinetic parameters for positive control in batches used for DLB and iOD/HC.

Abbreviations: AUC = area under the curve, DLB = Dementia with Lewy bodies, Fmax = maximal fluorescence measured, iOD = idiopathic olfactory dysfunction, IRQ = interquartile range, RFU = relative fluorescence unit, TH50 = time to 50 of Fmax, Vmax = maximal velocity of RFU change over 1.5 hours.

Figure S3 Correlations of Symptoms



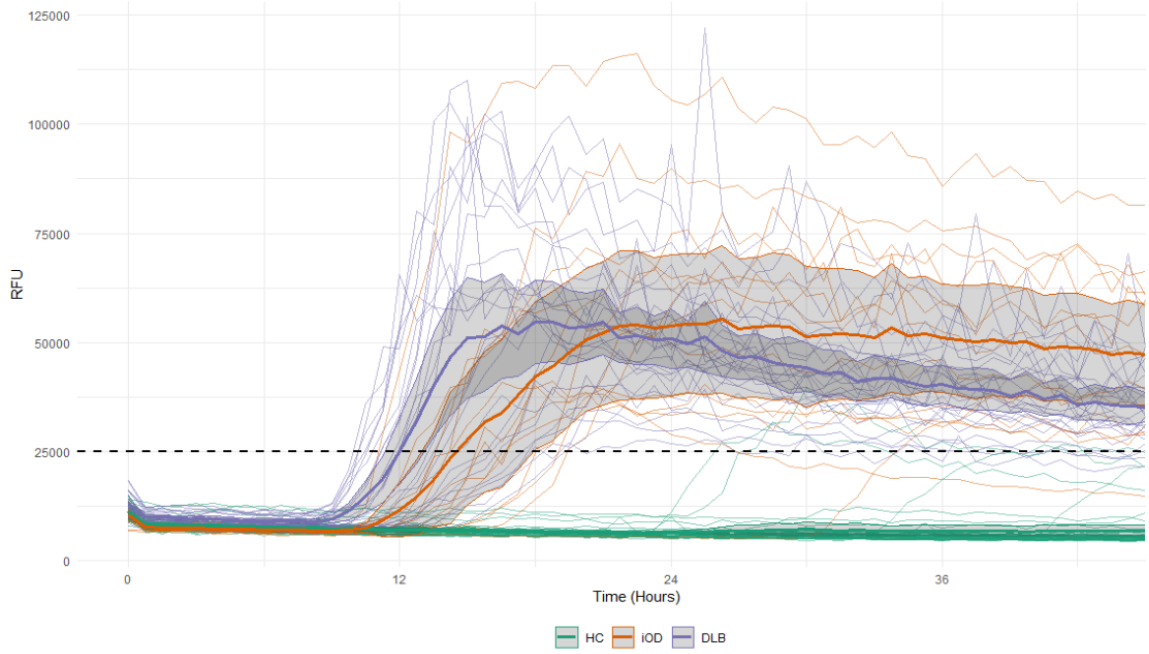
*Heatmap of Spearman's rank correlation coefficient performed on the groups: iOD, DLB, combined iOD and DLB combined. The color intensity is the correlation, and * is marked on significant results ($p<0.05$). The correlation between olfactory dysfunction and dysautonomia in the combined iOD and DLB group has a p-value of 0.050, all rho and p-values are displayed in the Supplementary Materials.*

The following variables were used: Parkinsonism is based on MDS-UPDRS III. Olfactory Function based on the inverse score of the Sniffin' Stick Identification test. Dysautonomia is based on the sum of values from MDS-UPDRS questions on urinary problems, constipation, lightheadedness on standing, and drooling. Neuropsychiatric is based on the sum of values from the MDS-UPDRS questions on depression, anxiety, and apathy. Daytime Sleepiness is based on the score of MDS-UPDRS question on daytime sleepiness. Cognitive Dysfunction is based on the inversed score of MoCA. RBD is based on the RBD1Q test.

No significant correlation was observed in the iOD, DLB, or the combined group between α-Syn in the olfactory mucosa or skin with the presence of any particular symptoms.

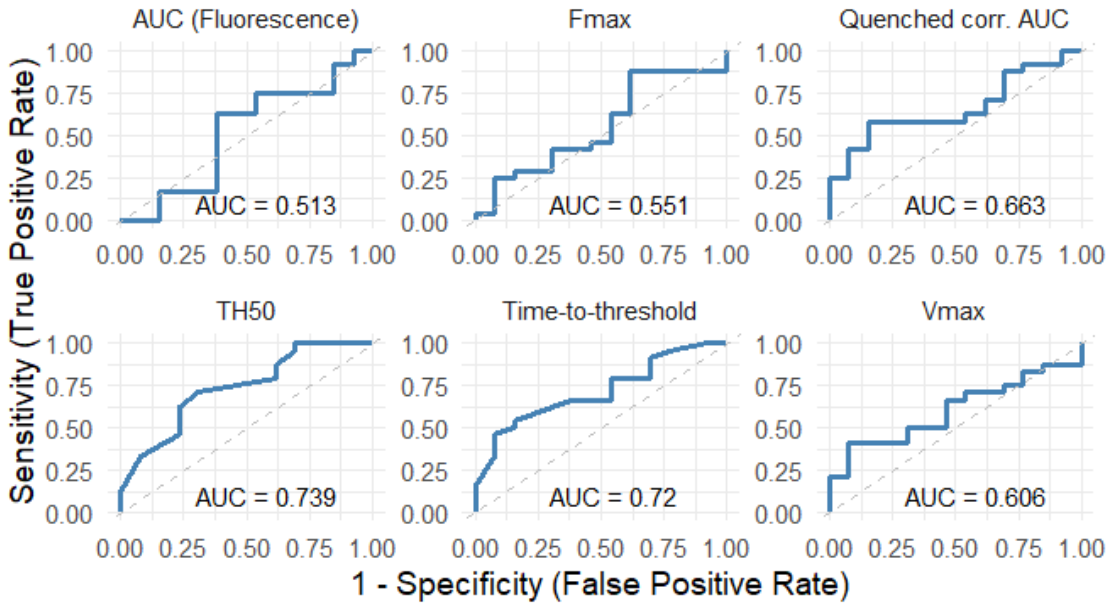
Abbreviations: DLB = Dementia with Lewy bodies, HC = healthy controls, iOD = idiopathic olfactory dysfunction, OD = Olfactory dysfunction, RBD = REM sleep behavior disorder

Figure S4 Seeding over Time in Skin with individual datapoints



Time plot with individual datapoints of the seed amplification from skin sample analysis with mean and CI for each group: HC (N=48), iOD (N=13) (αSyn positive in skin), and DLB (N=24)(αSyn positive in skin). The plot is based on the mean values from the two best-performing wells out of four. SAA in the skin from HC (N=48), iOD (N=13) (αSyn positive in skin), and DLB (N=24) (αSyn positive in skin) based on the mean of the two best-performing wells out of four. The dotted line represents the threshold.
Abbreviations: DLB = Dementia with Lewy bodies, HC = healthy controls, iOD = idiopathic olfactory dysfunction, RFU = relative fluorescence unit

Figure S5 ROC Curves for Kinetic Parameters in iOD vs DLB



ROC curves for the discrimination of DLB (case) from iOD by kinetic αSyn-SAA parameters. The Quenched corr. AUC = AUC x the quenching ratio. Abbreviations: AUC = area under the curve, DLB = Dementia with Lewy bodies, Fmax = maximal fluorescence measured, iOD = idiopathic olfactory dysfunction, IRQ = interquartile range, RFU = relative fluorescence unit, TH50 = time to 50 of Fmax, Vmax = maximal velocity of RFU change over 1.5 hours.

Table S1 The association between clinical assessments and kinetic parameters for skin SAA in iOD and DLB without correction

Response	Predictor	β Estimate	95% CI	p-value	R^2
MoCA (reversed)	TH50	-1.285	[-2.745, 0.175]	0.0826	0.084
	AUC	-0.814	[-2.314, 0.685]	0.278	0.034
	Fmax	-0.622	[-2.132, 0.888]	0.408	0.02
	Vmax	-0.164	[-1.688, 1.360]	0.829	0.001
	Time-to-threshold	-0.951	[-2.440, 0.539]	0.204	0.046
Olfactory-Identification (reversed)	TH50	0.307	[-0.499, 1.112]	0.444	0.017
	AUC	-0.599	[-1.385, 0.187]	0.131	0.064
	Fmax	-0.548	[-1.339, 0.242]	0.168	0.054
	Vmax	-0.282	[-1.089, 0.525]	0.483	0.014
	Time-to-threshold	0.407	[-0.393, 1.208]	0.309	0.03
MDS-UPDRS I	**TH50**	**-2.155**	**[-4.035, -0.274]**	**0.026**	**0.134**
	AUC	**2.113**	**[0.227, 3.999]**	**0.029**	**0.129**
	Fmax	**1.934**	**[0.025, 3.842]**	**0.047**	**0.108**
	Vmax	**2.054**	**[0.160, 3.948]**	**0.034**	**0.122**
	Time-to-threshold	**-2.427**	**[-4.268, -0.586]**	**0.011**	**0.170**
MDS-UPDRS II	**TH50**	**-2.528**	**[-4.350, -0.706]**	**0.0079**	**0.185**
	AUC	0.266	[-1.750, 2.282]	0.790	0.002
	Fmax	0.536	[-1.473, 2.546]	0.592	0.008
	Vmax	1.581	[-0.363, 3.525]	0.108	0.072
	Time-to-threshold	**-2.182**	**[-4.056, -0.308]**	**0.024**	**0.138**
MDS-UPDRS III	**TH50**	**-2.528**	**[-9.588, -1.271]**	**0.012**	**0.167**
	AUC	-1.470	[-5.999, 3.059]	0.514	0.012
	Fmax	-0.31	[-4.866, 4.246]	0.891	0.001
	Vmax	2.665	[-1.799, 7.129]	0.234	0.04
	Time-to-threshold	-4.292	[-8.604, 0.020]	0.051	0.104
MDS-UPDRS Total	**TH50**	**-5.429**	**[-9.588, -1.271]**	**0.0120**	**0.167**
	AUC	0.951	[-6.275, 8.178]	0.791	0.002
	Fmax	2.190	[-5.005, 9.385]	0.541	0.011
	Vmax	6.305	[-0.597, 13.208]	0.072	0.089
	Time-to-threshold	**-8.906**	**[-15.463, -2.349]**	**0.009**	**0.178**
Positive Symptom Count	**TH50**	**-5.429**	**[-9.588, -1.271]**	**0.0120**	**0.167**
	AUC	0.402	[-0.215, 1.018]	0.195	0.048
	Fmax	0.495	[-0.114, 1.103]	0.108	0.072
	Vmax	**0.912**	**[0.363, 1.461]**	**0.002**	**0.246**
	Time-to-threshold	**-1.044**	**[-1.564, -0.524]**	**<0.001**	**0.322**

Table of the association between standardized clinical assessments and kinetic parameters for skin SAA in iOD and DLB, with a positive αSyn-SAA in skin. Significant predictors are in bold. Table S2 displays values corrected for age and sex.

Abbreviations: AUC = Area under the curve, CI = 95% confidence interval, Fmax = maximal fluorescence measured, MDS-UPDRS = Movement Disorders Society Unified Parkinson's Rating Scale, MoCA = Montreal Cognitive Assessment, TH50 = time to 50 of Fmax, Vmax = maximal velocity of fluorescent change over 1.5 hours

Table S2 The Association between clinical assessments and kinetic parameters for skin SAA in iOD and DLB corrected for sex and age

Response	Predictor	β Estimate	95% CI	p-value	R^2
MoCA (reversed)	TH50	-0.404	-1.833 to 1.025	0.569	0.301
	AUC	-0.744	-2.069 to 0.581	0.261	0.321
	Fmax	-0.759	-2.062 to 0.545	0.245	0.323
	Vmax	-0.934	-2.274 to 0.406	0.166	0.335
	Time-to-threshold	-0.133	-1.541 to 1.275	0.849	0.295
Olfactory-Identification (reversed)	TH50	0.546	-0.330 to 1.422	0.214	0.076
	AUC	-0.634	-1.447 to 0.179	0.122	0.100
	Fmax	-0.59	-1.394 to 0.214	0.145	0.093
	Vmax	-0.434	-1.282 to 0.414	0.306	0.062
	Time-to-threshold	0.621	-0.230 to 1.473	0.147	0.092
MDS-UPDRS I	TH50	-1.637	-3.684 to 0.409	0.113	0.184
	AUC	**2.404**	**0.594 to 4.214**	**0.011**	**0.279**
	Fmax	**1.912**	**0.061 to 3.763**	**0.043**	**0.223**
	Vmax	1.631	-0.330 to 3.592	0.100	0.189
	Time-to-threshold	**-1.995**	**-3.958 to -0.032**	**0.047**	**0.220**
MDS-UPDRS II	TH50	-1.738	-3.495 to 0.018	0.052	0.397
	AUC	0.815	-0.911 to 2.541	0.344	0.342
	Fmax	0.61	-1.101 to 2.321	0.473	0.334
	Vmax	0.905	-0.855 to 2.664	0.303	0.345
	Time-to-threshold	-1.450	-3.202 to 0.302	0.102	0.377
MDS-UPDRS III	TH50	-3.087	-6.881 to 0.708	0.107	0.449
	AUC	-0.286	-3.998 to 3.426	0.876	0.403
	Fmax	-0.228	-3.886 to 3.429	0.900	0.403
	Vmax	0.71	-3.076 to 4.496	0.705	0.405
	Time-to-threshold	-2.120	-5.920 to 1.680	0.265	0.425
MDS-UPDRS Total	**TH50**	**-6.483**	**-12.23 to -0.739**	**0.028**	**0.499**
	AUC	2.966	-2.754 to 8.686	0.299	0.438
	Fmax	2.323	-3.349 to 7.994	0.411	0.431
	Vmax	3.269	-2.561 to 9.099	0.262	0.441
	Time-to-threshold	-5.593	-11.327 to 0.142	0.056	0.481
Positive Symptom Count	**TH50**	**-0.751**	**-1.197 to -0.306**	**0.002**	**0.605**
	AUC	**0.589**	**0.148 to 1.030**	**0.010**	**0.562**
	Fmax	**0.502**	**0.056 to 0.949**	**0.029**	**0.537**
	Vmax	**0.652**	**0.210 to 1.093**	**0.005**	**0.579**
	Time-to-threshold	**-0.756**	**-1.189 to -0.323**	**0.001**	**0.612**

Table of the association between standardized clinical assessments and kinetic parameters for skin SAA in iOD and DLB. Significant predictors are in bold and displayed in Figure 3.
Abbreviations: AUC = Area under the curve, CI = 95% confidence interval, Fmax = maximal fluorescence measured, MDS-UPDRS = Movement Disorders Society Unified Parkinson's Rating Scale, MoCA = Montreal Cognitive Assessment, TH50 = time to 50 of Fmax, Vmax = maximal velocity of fluorescent change over 1.5 hours.

UNIVERSITY OF COPENHAGEN

FACULTY OF HEALTH AND MEDICAL SCIENCES