Sensitivity and specificity of a seed amplification assay for diagnosis of multiple system atrophy: a multicentre cohort study

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Abstract

Background: The pathological hallmarks of multiple system atrophy and Parkinson's disease are, respectively, misfolded-α-synuclein-laden glial cytoplasmic inclusions and Lewy bodies. CSF-soluble misfolded α-synuclein aggregates (seeds) are readily detected in people with Parkinson's disease by α-synuclein seed amplification assay (synSAA), but identification of seeds associated with multiple system atrophy for diagnostic purposes has proven elusive. We aimed to assess whether a novel synSAA could reliably distinguish seeds from Lewy bodies and glial cytoplasmic inclusions.

Methods: In this multicentre cohort study, a novel synSAA that multiplies and detects seeds by fluorescence was used to analyse masked CSF and brain samples from participants with either clinically diagnosed or pathology-confirmed multiple system atrophy, Parkinson's disease, dementia with Lewy bodies, isolated rapid eye movement sleep behaviour disorder (IRBD), disorders that were not synucleinopathies, or healthy controls. Participants were from eight available cohorts from seven medical centres in four countries: New York Brain Bank, New York, USA (NYBB); University of Pennsylvania, Philadelphia, PA, USA (UPENN); Paracelsus-Elena-Klinik, Kassel, Germany (DeNoPa and KAMSA); Hospital Clinic Barcelona, Spain (BARMSA); Universität Tübingen, Tübingen, Germany (EKUT); Göteborgs Universitet, Göteborgs, Sweden (UGOT); and Karolinska Institutet, Stockholm, Sweden (KIMSA). Clinical cohorts were classified for expected diagnostic accuracy as either research (longitudinal follow-up visits) or real-life (single visit). Sensitivity and specificity were estimated according to pathological (gold standard) and clinical (reference standard) diagnoses.

Findings: In 23 brain samples (from the NYBB cohort), those containing Lewy bodies were synSAA-positive and produced high fluorescence amplification patterns (defined as type 1); those containing glial cytoplasmic inclusions were synSAA-positive and produced intermediate fluorescence (defined as type 2); and those without α-synuclein pathology produced below-threshold fluorescence and were synSAA-negative. In 21 pathology-confirmed CSF samples (from the UPENN cohort), those with Lewy bodies were synSAA-positive type 1; those with glial cytoplasmic inclusions were synSAA-positive type 2; and those with four-repeat tauopathy were synSAA-negative. In the DeNoPa research cohort (which had no samples from people with multiple system atrophy), the novel synSAA had sensitivities of 95% (95% CI 88-99) for 80 participants with Parkinson's disease and 95% (76-100) for 21 participants with IRBD, and a specificity of 95% (86-99) for 60 healthy controls. Overall (combining BARMSA, EKUT, KAMSA, UGOT, and KIMSA cohorts that were enriched for cases of multiple system atrophy), the novel synSAA had 87% sensitivity for multiple system atrophy (95% CI 80-93) and specificity for type 2 seeds was 77% (67-85). For participants with multiple system atrophy just in research cohorts (BARMSA and EKUT), the novel synSAA had a sensitivity of 84% (95% CI 71-92) and a specificity for type 2 seeds of 87% (74-95), whereas cases from real-life cohorts (KAMSA, KIMSA, and UGOT) had a sensitivity of 91% (95% CI 80-97) but a decreased specificity for type 2 seeds of 68% (53-81).

Interpretation: The novel synSAA produced amplification patterns that enabled the identification of underlying α-synuclein pathology, showing two levels of fluorescence that corresponded with different pathological hallmarks of synucleinopathy. The synSAA might be useful for early diagnosis of synucleinopathies in clinical trials, and potentially for clinical use, but additional formal validation work is needed.

Introduction

Synucleinopathies are a group of neurodegenerative diseases pathologically characterized by accumulation of misfolded αSyn aggregates. Lewy body disease (LBD) is the pathological hallmark of the Parkinson's disease (PD) and dementia with Lewy bodies (DLB) clinico-pathological spectrum[1]. Different misfolded αSyn deposits known as glial cytoplasmatic inclusions (GCI) are found in oligodendrocytes of patients with multiple system atrophy (MSA)[2]. Pure autonomic failure (PAF) and REM sleep behavior disorder (RBD) are considered prodromal symptoms for synucleinopathies, with 94% of the RBD cases presenting synucleinopathy at autopsy[3]. Clinical diagnosis of synucleinopathies is very challenging, with 42%[4], 20%[5], and 82%[6] misdiagnosis rate for early PD, DLB, and MSA, respectively. Most misdiagnosed PD subjects present either progressive supranuclear palsy (PSP) or MSA pathology[7]. Imaging and CSF biomarkers lack specificity to distinguish synucleinopathies from mimics and cannot differentiate between PD/DLB and MSA[8–10].

Recently, soluble misfolded αSyn aggregates (αSyn-seeds) have been found in CSF and demonstrated to be a robust biomarker for LBD[11–20]. The αSyn Seed Amplification Assay (αSyn-SAA) is the test used to multiply αSyn-seeds to detectable levels. During αSyn-SAA, αSyn-seeds are mechanically fragmented and then incubated to induce their elongation at the expense of recombinant αSyn (rec-αSyn). αSyn-SAA results for MSA have been inconsistent and often irreproducible, with sensitivities ranging from 6 to 80%[14,21]. The large variability in MSA detection is probably a reflection of the many αSyn-SAA conditions reported to date and the lack of understanding about the experimental conditions that increase amplification of GCI associated αSyn-seeds. Our previous αSyn-SAA conditions are highly sensitive for PD/DLB, prodromal (hyposmia, isolated-RBD (iRBD)), and LBD co-pathology in dementia cases[15,19,20,22], but not for MSA. In this multicenter study, we describe a fully standardized 24h αSyn-SAA for the accurate detection and differentiation of GCI from LBD synucleinopathies.

Methods

Subject selection and enrollment.

MSA subjects were diagnosed following the 2008 Second Consensus Statement[23] (MSA-SCS08), PD subjects were diagnosed following the 2015 Movement Disorder Society criteria[24], and DLB subjects were diagnosed following the fourth consensus report of the DLB consortium[25], unless specified otherwise. In total, 377 CSF samples collected in 5 independent centers were analyzed in this study.

DeNoPa (De Novo PD, Paracelsus-Elena-Klinik). This cohort included 175 subjects, 80PD, 21iRBD, 60HC, and 14NS, comprised of 4PSP, 5ET/DT (essential tremor with dystonia), 2RLS (restless leg syndrome), and 3vPD (vascular PD). Inclusion/exclusion criteria, clinical description, and 150h αSyn-SAA results for these cases has been reported elsewhere[19,26].

KAMSA (Kassel MSA, Paracelsus-Elena-Klinik). This sub-cohort included 43 subjects, 23MSA, 4PD, 3DLB, and 13NS, comprised of 6PSP, 3 neurodegenerative syndromes with ataxia [no definite diagnosis], 2 normal pressure hydrocephalus [NPH], 1 NPH with concomitant Alzheimer's disease, 1 dystonia-parkinsonism). PD and DLB were diagnosed as in the DeNoPa cohort, MSA was diagnosed according to the criteria issued by the international Movement Disorder Society in 2022[27] (MSA-MDS22), while NS

subjects were MSA-mimics that fit an exclusion criterion based on MSA-MDS22. Exclusion criteria were unexplained anosmia, persistent beneficial response to dopaminergic medications, fluctuating cognition with early decline in visuoperceptual abilities, visual hallucinations, dementia within 3y of disease onset, downgaze supranuclear palsy, brain MRI findings suggestive of an alternative diagnosis, and the presence of an alternative condition known to produce autonomic failure, ataxia, or parkinsonism. Olfaction was tested by blinded evaluation of sniffing-sticks and cognitive function with the mini mental state examination (MMSE)[28]. Neurogenic orthostatic hypotension diagnosis required a drop in systolic blood pressure of ≥20mmHg within 3min of standing upright[29]. Patients were classified as MSA-C or MSA-P according to the presence or absence of cerebellar signs. Detailed review of medical records was performed if patients were initially diagnosed before MSA-MDS22 release.

EKUT (Eberhard Karls Universität Tübingen, Germany). This sub-cohort included 61 subjects, 29MSA, 10PD, 7DLB, and 15NS subjects. Some of these samples had been previously analyzed using assay conditions reported by Rossi et al.[14]

BARMSA (Barcelona MSA, Spain). This sub-cohort included 47 subjects participating in 2 internal cohorts of the Hospital Clinic Barcelona: the Catalan Multiple System Atrophy Registry (CMSAR, n=15) and the QUICK+PARK cohort (n=32)[30], both with identical enrollment clinical criteria. It included 26MSA, 2PD, and 19HC. MSA participants were followed-up every six months for 2 years[31].

KIMSA (Karolinska Institutet, Sweden). This sub-cohort included 36 subjects, 18MSA, 12PD, and 6NS.

UGOT (Göteborgs Universitet, Sweden). This sub-cohort included 15MSA subjects.

Brain homogenate preparation.
Pathologically confirmed frozen cingulate cortex samples (n=23) from the New York Brain Bank at Columbia University were homogenized to 4% in 1X PBS (Cytiva, cat#SH30256.02) with 1X protease inhibitor (Roche, cat#11873580001), using homogenization tubes containing 6 stainless steel beads by a single 15s run at 4.0m/s (FastPrep-24 homogenizer). Crude homogenate was centrifuged 800 x g for 1min at 4°C and the supernatant was collected, vortexed, aliquoted, and snap-frozen. Brain homogenates (BHs) were diluted to 10^{-8} in αSyn-SAA diluent (Amprion, cat# S2022) and tested by 24h αSyn-SAA in triplicates.

Fluorescence normalization.
Atto425 dye (MilliporeSigma, cat#56759) was resuspended in 200µL of DMSO (MilliporeSigma, cat#D8418-250ML). Concentration was determined by absorbance at 429nm (ε_{Atto}=43,000). The fluorescence signal (440nm/490nm) of 3.66µM Atto425 dye was set to 77% of the 260,000RFU maximum range of 12 fluorometers. Post-normalization performance is shown in **Supp-Figure 1**.

αSyn Seed Amplification Assay.
The final 100µL reaction mixture consisted of 100mM PIPES pH 6.50 (Sigma, cat#80635), 500mM NaCl (Lonza, cat#51202), 10µM ThT (Sigma, cat#T3516), 0.1% sarkosyl (Sigma, cat#61747-100ML), 0.3mg/mL rec-αSyn (Amprion, cat#S2020), and 40µL CSF/BH. The assay was performed using clear bottom plates (Greiner, cat#655906) with 2x 3.2mm Si_3N_4 beads per well (Tsubaki Nakashima). Plates were evaluated for 24h at 42°C with intermittent orbital shaking (800rpm) for 1min every 15min. Fluorescence was read every cycle using fluorescence normalized FLUOstar Omegas (BMG). All samples were analyzed in triplicate. Primary output included positive, negative, and inconclusive, while the secondary output included Type1, Type2, and undetermined. Detailed description of assay determination criterion in **Supplementary Material**.

Results

We modified previous 150h αSyn-SAA conditions[32] by using larger Si_3N_4 beads, higher temperature, more frequent fragmentation cycles, sarkosyl, and reducing the total reaction volume to 100µL while maintaining the same volume of CSF. The 24h αSyn-SAA amplified αSyn-seeds much faster and technical replicates presented were more reproducible, enabling more precise estimations of the maximum fluorescence (F_{max}). PD, DLB, and iRBD presented replicates with a distinct pattern characterized by high F_{max}, defined here as Type1 pattern (**Figure 1G-I**). Detection of αSyn-seed in MSA increased with the 24h αSyn-SAA, as previously negative or highly variable samples were found positive and presented more consistent amplification (**Figure 1D-E, J-K**). MSA presented two or three replicates with a distinct pattern characterized by low F_{max}, defined here as Type2 pattern. Lastly, NS was indistinguishable from background signal. We confirmed amplification patterns to be intrinsic to CSF samples and not dependent of rec-αSyn lots, as they conserved their pattern when tested with 5 different batches of rec-αSyn (**Supp-Figure 2**). To confirm that Type1 and Type2 patterns relate to brain pathology, we blindly analyzed 23 pathologically confirmed brain samples. All 5PD presented high F_{max} in all three replicates, all 12MSA presented low F_{max} in two or three replicates, and all controls displayed two or three replicates with F_{max} indistinguishable from background (**Figure 2**). Thus, high and low fluorescence patterns are directly associated to LBD and GCI pathology, respectively. Hereafter, the αSyn-seeds producing high and low F_{max} will be deemed as Type1 and Type2 αSyn-seeds, respectively.

We recently reported 95% sensitivity for PD, 93% sensitivity for iRBD, and 98% specificity for an overall 98% assay accuracy using the DeNoPA cohort and the 150h αSyn-SAA.[19] Available DeNoPa CSF samples were blindly reanalyzed with the 24h αSyn-SAA by 2 operators using 3 different substrate lots and 5 FLUOstar Omega readers to evaluate accuracy. Remarkably, both αSyn-SAAs reached the same result in 98.3% of the samples (**Table I**). The 1.7% difference was caused by 3HC negative samples that were found inconclusive (two) and positive (one) with the 24h αSyn-SAA. Inconclusives were not retested due to lack of volume. All PD, DLB, iRBD, and NS samples had the same result for both assays. The 24h αSyn-SAA reached 95% sensitivity for 80PD and 95.2% for 21iRBD, while the specificity for HC and NS was 94.8% and 85.7%, respectively. All positive PD and iRBD samples displayed F_{max} >45,000RFU in the 24h αSyn-SAA. Thus, we defined a [45,000RFU , 260,000RFU] F_{max} range for Type1 αSyn-seeds and a [3,000RFU , 45,000RFU) F_{max} range for Type2 αSyn-seeds. Results from the 3 replicates were combined to determine the result of each sample (**Supp-Methods**).

The performance of the 24h αSyn-SAA was evaluated with an MSA-enriched cohort of subjects from 5 independent sub-cohorts, including 111MSA, 28PD, 10DLB, and 53CTRL (controls, HC+NS) (**Supp-Figure 3, Supp-Table I**). Overall, 87.3% of the clinically diagnosed MSA samples were found positive, 18.8% with Type1 and 77.1% with Type2 αSyn-seeds (**Table II**). Sensitivity for MSA was sub-cohort dependent, but results were comparable for KAMSA (87%), BARMSA (84.6%), EKUT (82.8%), and UGOT (86.7%). Type2 proportion was more variable depending on the sub-cohort (90.9% for BARMSA, 83.3% for EKUT, and 80% for KAMSA, versus 69.2% for UGOT and 52.9% for KIMSA). Some of the MSA subjects were classified into the parkinsonian (MSA-P) or the cerebellar (MSA-C) variants (**Table II**). 24h αSyn-SAA detection for MSA-P was slightly higher than MSA-C in KAMSA (92.3% vs 80%) and BARMSA (90.9% vs 85.7%) sub-cohorts. MSA-C subjects presented higher Type2 proportion than MSA-P in KAMSA (87.5% vs 75%) and KIMSA (75% vs 46.2%), but lower in BARMSA (83.3% vs 90%). Interestingly, all the KIMSA positive Type1 MSA samples were MSA-P and had initial PD diagnosis until they presented dysautonomia and met criteria for MSA (n=7). In addition to clinical diagnosis based on MSA-MDS22, the KAMSA sub-cohort also had the clinical diagnosis based on the MSA-SCS08. Interestingly, sensitivity was higher (87.0% vs 72.1%) when considering MSA-MDS22 as gold standard.

Some of the sub-cohorts included subjects with PD, DLB, and CTRL. PD-EKUT samples were enriched with 50% samples that were previously found negative using alternative αSyn-SAA conditions with low detection for MSA[14]. We also found 50% of the PD samples positive, all with Type1 αSyn-seeds. Selection of PD cases in other sub-cohorts was unbiased and the 24h αSyn-SAA reached 100% sensitivity in KAMSA and BARMSA sub-cohorts, and 91.7% in the KIMSA sub-cohort (**Table II**). Like EKUT-PD samples, EKUT-DLB samples were also enriched with seeding negative samples (3/7). We found different results for 2 of the 7 samples, both with elevated NfL levels; one was negative and the other was positive Type2, consistent with MSA.[33] Results for DLB-KAMSA were the same for 24h and 150h αSyn-SAA (66% detection). The EKUT, KAMSA, and KIMSA sub-cohorts did not have HC-CSF samples, but 86%, 61%, and 75% of the NS samples were negative. The BARMSA cohort included HC-CSF samples and 95% were negative. Samples with leftover volume were also analyzed with the 150h assay and it reached lower agreement with clinical diagnosis (**Sup Table III**).

Finally, we looked at the F_{max} measurements for all CSF replicates analyzed in this study (**Figure 3**). 88.4% of the PD, DLB, and iRBD replicates presented F_{max} >45,000RFU, while 81.8% of the MSA replicates presented F_{max} values between 3,000 and 45,000RFU (61.9%) or lower than 3,000RFU (19.9%). 83.1% of the CTRL sample replicates presented F_{max} below 3,000RFU. Overall, these results demonstrate that the F_{max} from individual replicates are not normally distributed but clearly distributed into 3 groups, correctly separated by the 45,000 and 3,000RFU thresholds. Since the assay reported here includes a method to normalize fluorescence readings across instruments, these thresholds should be transferable across laboratories to enable reproducible differentiation of Type1 and Type2 αSyn-seeds.

Discussion

Given the differential sensitivity for PD and MSA seen in most αSyn-SAAs, our goal was to identify conditions that would consistently amplify αSyn-seeds from MSA-CSF, without affecting sensitivity for iRBD, PD, and DLB, or specificity. Furthermore, we developed conditions to reliably and distinctively detect Type1 and Type2 αSyn-seeds to enable *intravitam* differentiation of underlying LBD and GCI pathologies[21]. The more stringent assay conditions required for the efficient amplification of Type2 αSyn-seeds might be explained by their fibrillar structure. CryoEM studies have shown that αSyn fibrils from MSA brains have 2 protofilaments[34], while fibrils from PD and DLB brains contain single protofilaments[35]. Thus, lateral interactions between protofilaments may make Type2 αSyn-seeds more stable and difficult to fragmentate. Alternatively, sarkosyl may strip fibrils from nonspecifically bound proteins and increase interactions with the substrate to induce secondary nucleation. Stripping these nonspecific proteins may also reduce the mechanical stability of the fibrils, facilitating fragmentation. Understanding these mechanisms and their correlation with structural features of αSyn-seeds might lead to further improvements in αSyn-SAA technology.

We evaluated the 24h αSyn-SAA with the DeNoPa cohort and the largest MSA-enriched multicenter cohort tested to date by αSyn-SAA technology and demonstrated high sensitivity for all synucleinopathies. DeNoPa subjects had many years of follow-up plus DAT-SPECT in the case of PD and PSG in the case of iRBD. Thus, the diagnosis of these subjects is likely very accurate. Type1 αSyn-seeds were detected in all PD subjects, consistent with their diagnosis. Interestingly, all iRBD subjects presented Type1 αSyn-seeds as well, indicating that iRBD is a specific marker for PD/DLB. It is important to clarify that isolated-RBD presents without any other neurological symptoms. Most studies associating RBD with synucleinopathies recruited subjects with dysautonomia or other symptoms that could indicate MSA or a non-synucleinopathy. These results match their progression as some of these subjects have already phenoconverted to either PD or DLB, but not MSA[19]. In the case of the MSA-enriched cohort, diagnoses were mostly based on single visits and clinical data was rather limited. The higher sensitivity of the 24h

αSyn-SAA for PD and iRBD compared to MSA could be explained by the better characterization of the PD/iRBD cohort, the higher MSA misdiagnosis rate, or a lower analytical sensitivity for MSA. CSF from longitudinal MSA studies or with pathological confirmation would be required to refine the sensitivity estimation for MSA. Only one MSA sample in this study was pathologically confirmed and was positive Type2. Unlike other sub-cohorts, BARMSA subjects were followed-up for 2y, which could explain the higher agreement between MSA diagnosis and Type2 pattern. Given the high agreement between Type1 αSyn-seeds with LBD pathology and iRBD/PD/DLB diagnoses, it is likely that MSA subjects with Type1 αSyn-seeds have underlying LBD pathology with complex clinical presentations that overlap with MSA. Remarkably, the average disease duration for positive KAMSA-MSA subjects was 2.9y, underscoring the value of 24h αSyn-SAA as an early-stage MSA biomarker when diagnosis is known to be error-prone. These results suggest that the 24h αSyn-SAA could aid during differential diagnosis not only to differentiate MSA from iRBD/PD/DLB, but also from early stage MSA-like rapidly deteriorating neurodegenerative diseases like PSP, hereditary cerebellar ataxias, or prion diseases.

The 24h αSyn-SAA showed different performance between the 4 centers that used the MSA-SCS08 guideline, suggesting differences in the interpretation and application of the guideline. MSA diagnosis is complex given known MSA-SCS08 shortcomings[36]. Performance of the MSA-SCS08 guideline is subpar especially in the early-disease stages, where only 18% of neuropathologically proven MSA cases met criteria for probable MSA and 41% for possible MSA[6]. The revised MSA-MDS22 criteria has a less strict definition of orthostatic hypotension to increase sensitivity and describes a well-defined exclusion criteria to increase specificity[27]. Although a direct comparison of both criteria showed that the MSA-MDS22 criteria outperformed MSA-SCS08 in overall accuracy, it showed worse sensitivity for MSA-P compared to MSA-C and only 62,1% sensitivity in early MSA (<3 years since onset). Moreover, MSA-MDS22 includes exclusion criteria that are time dependent, like dementia and hallucinations if present within 3y from onset. Therefore, it is not unusual for MSA diagnosis to change within 3y from diagnosis. Limitations in diagnostic guidelines are well known and a consequence of the lack of biomarkers that directly correlate with underlying pathology. The 24h αSyn-SAA reported here not only detects underlying synucleinopathy, but also distinguishes between LBD and GCI synucleinopathies. The implementation of this test would not only aid community and specialized neurologists to significantly reduce their misdiagnosis rates, but it would also shorten the time to final diagnosis and enable early-intervention clinical trials.

In summary, our study describes a novel 24h αSyn-SAA with high sensitivity for MSA that can differentiate GCI and LBD pathology during life, which will facilitate differential diagnosis of synucleinopathies and potentially enable clinical trials of disease modifiers.

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Conflict of interest

YM, CMF, HN, and LC-M are Amprion employees and declare employee stock option ownership and invention of patents related to SAA assigned to Amprion.
UJK is on the scientific advisory board of Amprion. MS has received consultancy honoraria from Ionis, UCB, Prevail, Orphazyme, Servier, Reata, GenOrph, AviadoBio, Biohaven, Zevra, and Lilly, all unrelated to the present manuscript. Other coauthors report no conflict of interest.
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Figure Legends.

Figure 1. Representative comparison between the 24h and 150h CSF αSyn-SAA. A-F. Fluorescence (440nm/490nm) of 3 replicates per sample collected using the 150h αSyn-SAA. Graphs show fluorescence readings every 4h, but readings are recorded every 30min. **G-L.** Fluorescence traces (440nm/490nm) of 3 replicates per sample collected using the newly developed 24h αSyn-SAA. Graphs show fluorescence readings every 1h, but readings are recorded every 15min. Parkinson's disease (PD), dementia with Lewy bodies (DLB), isolated REM sleep behavior disorder (iRBD), multiple system atrophy (MSA), non-synucleinopathy (NS).

Figure 2. Amplification patterns of LBD and GCI laden brain samples. A-C. Representative fluorescence traces produced by a PD brain sample (**A**), an MSA brain sample (**B**), and a control brain sample (**C**). **D.** F_{max} all replicates generated by the 23 pathologically confirmed brain samples tested (5PD, 12MSA, and 6CTRL).

Figure 3. Evaluation of F_{max} threshold to differentiate LBD from GCI underlying pathology. F_{max} values for all 1050 replicates analyzed in this study, including 285PD, 30DLB, 63iRBD, 291MSA, and 260CTRL. The Y-axis is broken at the 3,000RFU and 45,000RFU thresholds and each part of the axis is scaled to show dispersion of the measurements within each of the 3 groups.

Tables.

Table I. Evaluation of LBD sensitivity and specificity for the 24h and 150h assays in the DeNoPa cohort.

	24h αSyn-SAA	**150h αSyn-SAA**
Sensitivity (%)		
PD (n=80) [95% CI] Type1 (%)	95.0 [87.7 – 98.6] 100.0	95.0 [87.7 – 98.6] n.a.
iRBD (n=21) [95% CI] Type1 (%)	95.2 [76.2 – 99.9] 100.0	95.2 [76.2 – 99.9] n.a.
Specificity (%)		
HC (n=60) [95% CI]	94.8[a] [85.6 – 98.9]	96.7 [88.5 – 99.6]
NS[b] (n=14) [95% CI]	85.7 [57.2 – 98.2]	85.7 [57.2 – 98.2]
Agreement (%)	98.3	

[a]2 inconclusive samples. Not retested due to lack of sample volume, excluded from sensitivity/specificity calculations.
[b] NS included PSP, vPD, ET and/or DT, and RLS subjects.

Table II. Sensitivity and specificity estimations for the 24h αS-SAA.

	KAMSA	**BARMSA**	**KIMSA**	**UGOT**	**EKUT**	**Total**
MSA						
n	23	26	18	15	29	111
Sensitivity (%)	87.0	84.6	100.0[a]	86.7	82.8	87.3
[95% CI]	[66.4 – 97.2]	[65 – 95.6]	[80.5 – 100]	[59.5 – 98.3]	[64.2 – 94.2]	[79.6 – 92.9]
Type1 (%)	20.0	4.5	41.2	30.8	8.3	18.8
Type2 (%)	80.0	90.9	52.9	69.2	83.3	77.1
Undetermined (%)	0.0	4.5	5.9	0.0	8.3	4.2
MSA-P						
n	13	11	14	n.a.	n.a.	38
Sensitivity (%)	92.3	90.9	100.0[a]	n.a.	n.a.	94.6
[95% CI]	[64 – 99.8]	[58.7 – 99.8]	[72.3 – 100]	n.a.	n.a.	[81.8 – 99.3]
Type1 (%)	25.0	0.0	53.8	n.a.	n.a.	42.9

Type2 (%)	75.0	90.0	46.2	n.a.	n.a.	54.3
Undetermined (%)	0.0	10.0	0.0	n.a.	n.a.	2.9
MSA-C						
n	10	7	4	n.a.	n.a.	21
Sensitivity (%)	80.0	85.7	100.0	n.a.	n.a.	85.7
[95% CI]	[44.4 – 97.5]	[42.1 – 99.6]	[39.8 – 100]	n.a.	n.a.	[63.7 – 97.0]
Type1 (%)	12.5	16.7	0.0	n.a.	n.a.	11.1
Type2 (%)	87.5	83.3	75.0	n.a.	n.a.	83.3
Undetermined (%)	0.0	0.0	25.0	n.a.	n.a.	5.6
PD						
n	4	2	12	0	10[b]	28
Sensitivity (%)	100.0	100.0	91.7	n.a.	50.0	78.6
[95% CI]	[39.8 – 100]	[15.8 – 99.9]	[61.5 – 99.8]	n.a.	[18.7 - 81.3]	[59.1 – 91.7]
Type1 (%)	75.0	100.0	81.8	n.a.	100.0	86.4
Type2 (%)	25.0	0.0	9.1	n.a.	0.0	9.1
Undetermined (%)	0.0	0.0	9.1	n.a.	0.0	4.5
DLB						
n	3	0	0	0	7[b]	10
Sensitivity (%)	66.7	n.a.	n.a.	n.a.	71.4	70.0
[95% CI]	[9.4 – 99]	n.a.	n.a.	n.a.	[29 – 96.3]	[34.8 – 93.3]
Type1 (%)	100.0	n.a.	n.a.	n.a.	80.0	85.7
Type2 (%)	0.0	n.a.	n.a.	n.a.	20.0	14.3
Undetermined (%)	0.0	n.a.	n.a.	n.a.	0.0	0.0
CTRL						
n	13	19	6	0	15	53
Specificity (%)	61.5	94.7	75.0[c]	n.a.	85.7[a]	82.0
[95% CI]	[31.6 – 86.1]	[74 – 99.9]	[19.4 – 99.4]	n.a.	[75.2 – 98.2]	[68.6 – 91.4]
Type1 (%)	80.0	100.0	100.0	n.a.	50.0	77.8
Type2 (%)	20.0	0.0	0.0	n.a.	0.0	11.1
Undetermined (%)	0.0	0.0	0.0	n.a.	50.0	11.1

[a] 1 inconclusive sample. Not retested due to lack of sample volume, excluded from sensitivity/specificity calculation.

[b] Group enriched in αSyn-SAA negative samples evaluated with alternative assay conditions.

[c] 2 inconclusive samples. Not retested due to lack of sample volume, excluded from sensitivity calculation.