The promise of amplification assays for accurate early detection of α-synucleinopathies: A review.

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Abstract

Lewy body dementia encompasses the common neurodegenerative disorders Dementia with Lewy bodies (DLB) and Parkinson's disease dementia (PDD). Lewy Body disease (LBD) is characterized by abnormal aggregates of α-synuclein (α-syn) in the brain which form Lewy bodies. LBD is commonly misdiagnosed/underdiagnosed, especially in early stages. There remains a great need for reliable biomarkers to assist with LBD diagnosis. Amplification techniques such as real-time quaking-induced conversion (RT-QuIC) and protein misfolding cyclic amplification (PMCA) represent an important advance for biomarker detection. Amplification assays detect the ability of pathogenic protein to induce conformational change in normal protein; α-syn has been shown to propagate in a prion-like manner, making it a candidate for such analysis. In this review, we describe the diagnostic potential of amplification techniques for differentiating α-synucleinopathies from other neurodegenerative disorders such as Alzheimer Disease (AD), frontotemporal dementia (FTD), progressive supranuclear palsy (PSP), corticobasal syndrome (CBS), and atypical parkinsonism, as well as α-synucleinopathies from each other. Recent studies report accurate detection of α-syn seeding activity in human tissues such as cerebrospinal fluid (CSF), submandibular gland (SMG), and posterior cervical skin. Adaptation to clinical settings may present challenges. However, the high accuracy of recent results, combined with the success of amplification assay diagnostics in clinical practice for Creutzfeldt-Jakob disease, suggest high promise for eventual clinical application.

1.1 Introduction

Lewy body dementia is an umbrella term encompassing the common neurodegenerative disorders Dementia with Lewy bodies (DLB) and Parkinson's disease dementia (PDD). Lewy body disease (LBD) is characterized by abnormal aggregates of α-synuclein (α-syn) in the brain which form into Lewy bodies. Multiple system atrophy (MSA) is also an α-synucleinopathy and can include some parkinsonian symptoms. DLB and PDD can have overlapping clinical features, but they differ in time of onset of cognitive and motor symptoms (Jellinger, 2018; Walker et al., 2015). Motor impairment precedes cognitive decline in PDD by at least one year, while in DLB motor and memory problems start together or within one year of each other (Jellinger, 2018). Although DLB and PDD differ in the sequence of onset of dementia and parkinsonism, some consider their difference to be arbitrary—

they can be seen as part of a continuum rather than entirely separate diseases (Jellinger, 2018; Walker et al., 2015).

LBD causes the second most common type of neurodegenerative dementia in older patients, comprising around 5-10% of all diagnosed cases; however, the true percentage of cases is likely higher, because LBD is commonly misdiagnosed (McKeith et al., 2017; Vann Jones and O'Brien, 2014; Walker et al., 2015). In 2861 subjects from the National Alzheimer's Coordinating Center, sensitivity of clinical diagnosis against autopsy was found to be 32% for pure DLB and 12% for AD+DLB mixed pathology, although specificity was 95% (Walker et al., 2015) (Nelson et al., 2010). Misdiagnosis/underdiagnosis is partly attributed to mixed pathology, which results in LBD cases commonly presenting with clinical profiles similar to Alzheimer disease (AD) (McKeith et al., 2017). For example, the presence of neocortical amyloid plaques and tangles in addition to Lewy bodies results in clinical profile more resembling AD (McKeith et al., 2017). Technology such as dopaminergic SPECT or amyloid and tau PET imaging can be helpful in detecting mixed pathology (McKeith et al., 2017). The fourth consensus report on International Clinical Diagnostic Criteria now require "two or more core clinical features" or "one core clinical feature, but with one or more indicative biomarkers" for the diagnosis of probable DLB, with indicative biomarkers being 1) reduced DAT uptake in basal ganglia on PET or SPECT, such as FP-CIT; 2) low MIBG uptake; and 3) the polysomnographic confirmation of rapid-eye-movement (REM) sleep behavior disorder (RBD), i.e., REM sleep without atonia (RWA) (McKeith *et al.*, 2017; Yamada *et al.*, 2020).

The development of broadly applicable genetic or fluid biomarkers, such as α-syn and tau, have been the focus of extensive research in the past decade, but results have been mostly inconsistent (McKeith et al., 2017). Oligomeric α-syn (which precedes aggregation into mature amyloid fibrils) has been found at higher concentrations in the CSF of patients with PD compared to controls, although with unsatisfactory diagnostic accuracy (71% sensitivity and 64% specificity) (Parnetti et al., 2019). α-syn measurement in the blood has been challenging, largely because the fragile and abundant nature of red blood cells (RBCs), which are a major source of α-syn in the blood, greatly increases the risk of sample contamination (Parnetti et al., 2019). Unlike detection of total α-syn in plasma and serum, oligomeric and phosphorylated α-syn may be more suitable as blood biomarkers of PD: oligomeric α-syn levels appear to be elevated in serum of patients with PD with diagnostic accuracy of 75% sensitivity and 100% specificity (results must be confirmed in larger populations), and phosphorylated α-syn levels are elevated in the plasma of patients with PD compared with controls with an AUC of 0.71 (Foulds et al., 2013; Parnetti et al., 2019; Williams et al., 2016). Although blood oligomeric and phosphorylated α-syn appear to have diagnostic potential, further studies are needed regarding biomarkers in accessible sample collection locations such as saliva, plasma, serum, and urine (Parnetti et al., 2019).

There remains a great need for highly specific biomarkers for LBD diagnosis or prognosis. Although patients may present with overlapping clinical features, their underlying pathologies may be different, and thus require different approaches to treatment. Development of a specific biomarker test is also crucial for earlier diagnosis. Precise diagnosis of DLB in prodromal phases is especially difficult because of its similarity to other neurodegenerative diseases in early stages, particularly PD, MSA, and AD (McKeith et al., 2020). The ability to reliably diagnose DLB in its prodromal stages could have profound beneficial effects on disease treatment, progression, and outcomes in patients (McKeith et al., 2020). Early detection could allow earlier intervention before symptoms become debilitating, minimize emergency visits and iatrogenic adverse events, and allow time for planning nonpharmacologic interventions (McKeith et al., 2020). Additionally, early detection would expand

populations for trials of future targeted therapies, especially where disease modification is the goal (McKeith et al., 2020).

1.2 Amplification technique background

In this review, we describe the diagnostic potential of amplification techniques for differentiating α-synucleinopathies from other neurodegenerative disorders such as AD, frontotemporal disorder (FTD), progressive supranuclear palsy (PSP), corticobasal syndrome (CBS), and atypical parkinsonism, as well as α-synucleinopathies from each other. Amplification techniques, also known as seeding aggregation assays, were first utilized for the diagnosis of Creutzfeldt-Jakob disease (CJD) and are currently implemented with high sensitivity and specificity in the diagnosis of prion diseases (Atarashi, 2022; Chatzikonstantinou *et al.*, 2021; Hermann *et al.*, 2021). These assays have also been investigated for detection of tau protein and Aβ oligomers in AD (Kraus et al., 2019; Salvadores et al., 2014; Carlomagno et al., 2021).

CJD is characterized by the accumulation of abnormally folded prion protein scrapie (PrPsc), which can be detected in human tissues such as the brain, CSF, retina, or olfactory mucosa (Hermann *et al.*, 2021). Previously, a definite diagnosis required confirmation at autopsy (Hermann *et al.*, 2021). Now, CJD is often diagnosed by in vitro misfolded protein amplification assays, such as protein misfolding cyclic amplification (PMCA) and real-time quaking-induced conversion (RT-QuIC) (Atarashi, 2022; Chatzikonstantinou *et al.*, 2021; Hermann *et al.*, 2021).

Both PMCA and RT-QuIC are assays that enable the detection of minute amounts of protein in samples of body fluids or tissues. They accomplish this by detecting the ability of pathogenic protein (in this case, PrPsc) to induce conformational change in normal protein (PrP) (Brandel et al., 2019). Pathogenic protein can act as a "seed" to convert the substrate upon contact (Brandel et al., 2019; McNulty et al., 2019). The protein is amplified to a threshold of detection and analyzed by conventional laboratory techniques such as Western blot or, more commonly, thioflavin-T fluorescence (ThT), which shows enhanced fluorescent activity when bound to B-sheet rich structures such as amyloid fibrils (Brandel et al., 2019). Amplification assays use an integrated detection system to measure this seeding activity in real-time (Becker et al., 2018; Brandel et al., 2019; McNulty et al., 2019). PMCA and RT-QuIC differ in their methods of facilitating protein amplification – PMCA utilizes sonication while RT-QuIC implements vigorous shaking (Collins and Sarros, 2016). The high specificity of PMCA is attributed to its sonication/rest cycles, which break down aggregated pathogenic protein and induce further aggregation (Brandel et al., 2019).

2.1 Amplification techniques for α-synucleinopathies

α-syn has been shown to propagate in a prion-like manner via conformational conversion and fibril formation (Chu and Kordower, 2015). Because of this seeding activity, recent research has investigated the application of PMCA and RT-QuIC assays to diagnosing α-synucleinopathies such as DLB and PD (see Figure 1) (Bongianni et al., 2017; Fairfoul et al., 2016; Rossi et al., 2020b).

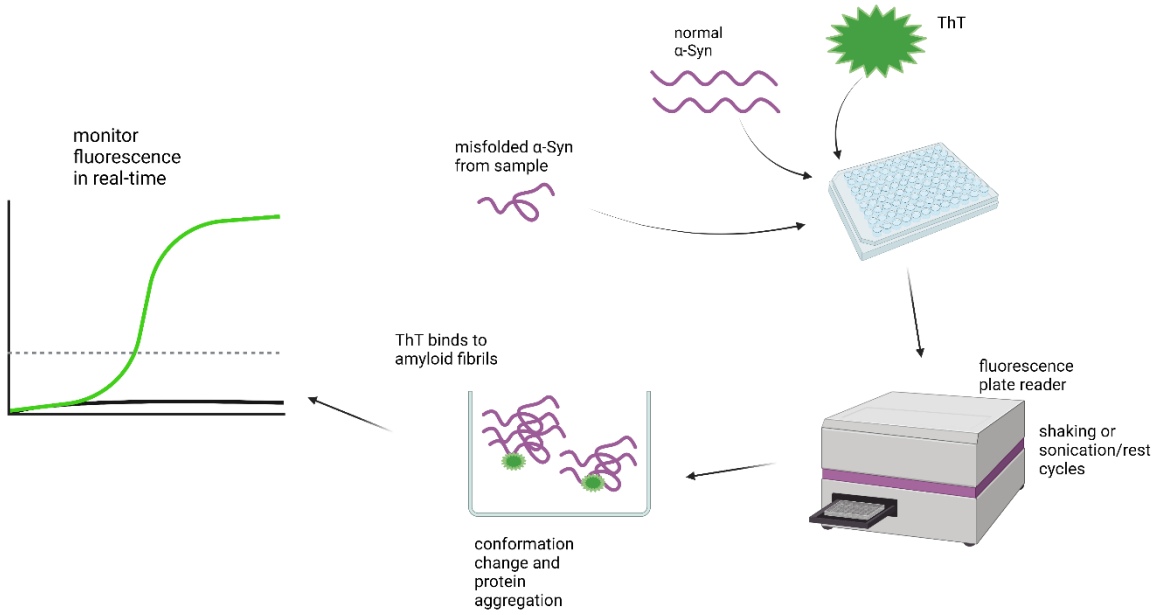

Figure 1. The PMCA/RT-QuIC process: pathogenic (misfolded) α-syn protein is combined with normal α-syn protein and ThT. Shaking (RT-QuIC) or sonication/rest (PMCA) cycles induce prion-like propagation and amyloid fibril formation, which is measured in real-time with ThT fluorescence.

A number of studies have analyzed the ability of RT-QuIC analysis of α-syn seeding activity to differentiate between DLB, PD, and AD, as well as other neurodegenerative diseases. Fairfoul et al. utilized their expertise in RT-QuIC through working with prion diseases in order to develop the first known RT-QuIC assay for α-syn (α-syn RT-QuIC). They developed the assay by initially analyzing 99 CSF samples from clinically and neuropathologically confirmed cases that included but were not limited to pure DLB, PD, DLB with AD pathology, AD with incidental LBs, pure AD, and controls (Fairfoul et al., 2016). They obtained sensitivities of 92%, 95%, and 65% for DLB, PD, and mixed DLB/AD pathology, respectfully, and specificity of 100% for both DLB and PD (Fairfoul et al., 2016). Neuropathologically confirmed PSP and corticobasal degeneration (CBD) samples gave negative α-syn RT-QuIC results (Fairfoul et al., 2016).

While previously-developed α-syn RT-QuIC assays on CSF samples required 5-13 days to complete, Groveman et al. developed an assay that required 1-2 days to complete with quantification, but preserved previously established quality (Fairfoul et al., 2016; Groveman et al., 2018; Shahnawaz et al., 2017). They used a blinded panel to obtain sensitivities of 93% for both PD and DLB; because none of the non-synucleinopathy or AD controls showed positive responses, they obtained a specificity of 100% (Groveman et al., 2018).

Studies using Groveman et al.'s protocol supported RT-QuIC as an accurate and promising test for DLB diagnosis (Bongianni et al., 2019; Rossi et al., 2020b). Bongianni et al. analyzed 77 CSF samples from patients with initial clinical suspect of CJD who were later neuropathologically confirmed to include cases of pure DLB (n=7), MSA (n=1), LBD and AD mixed pathology (n=15), LBD with tau mixed pathology (n=2), CJD with incidental α-syn pathology (n=3), other non-α-syn

neurodegenerative (n=30) and nondegenerative (n=19) neurological diseases (Bongianni et al., 2019). This analysis resulted in 92.9% sensitivity and 95.9% specificity in differentiating between α-synucleinopathies and non-α-synucleinopathies (Bongianni et al., 2019).

They also performed analyses on CSF from 36 patients with clinical diagnosis (according to the 2017 fourth consensus report criteria) of probable DLB, possible DLB, and AD, which yielded a sensitivity range between 85 and 65%, and specificity range between 65 and 100%, depending on exclusion or inclusion of possible DLB cases in the analysis (Bongianni et al., 2019). These cases were not neuropathologically confirmed (Bongianni et al., 2019).

Ultimately, Rossi et al. used the largest cohort to date to study α-syn RT-QuIC as a means of differentiating between LBD and AD (Rossi et al., 2020). They analyzed 439 CSF samples, 122 of which had a post-mortem CNS neuropathological assessment, and 317 had a clinical diagnosis with extensive clinical follow-up (Rossi et al., 2020). They included patients with DLB, AD, PD, CJD, Frontotemporal lobar degeneration, MSA, PSP, Encephalitis, Vascular dementia, idiopathic rapid-eye-movement (REM) sleep behavior disorder (iRBD), pure autonomic failure (PAF), CBS, and a control group (patients lacking symptoms suggestive of a progressive neurodegenerative disorder) (Rossi et al., 2020). Rossi et al. obtained an overall specificity of 98% and sensitivity 95.2% (neuropathologically verified cohort), and specificity of 98.4% calculated on clinical controls. In their analysis of clinically diagnosed PD CSF samples (probable or clinically established disease for PD, PSP/CBS, MSA), Rossi et al. obtained a sensitivity of 94.4%, with specificity of 100% against patients with PSP/CBS, and specificity of 93.5% against patients with MSA (Rossi et al., 2020).

These results support recent findings from Shahnawaz et al. that showed the ability of αSyn-PMCA to discriminate between PD and MSA (Shahnawaz et al., 2020). They conducted a study using 94 CSF samples from patients with PD, 75 CSF samples from patients with MSA, and 56 control CSF samples from patients with other neurological diseases (Shahnawaz et al., 2020). The overall sensitivity for diagnosis compared to controls was 93.6% for PD and 84.6% for MSA, while specificity was 100% in both cases. Combining all samples and comparing the differential diagnosis of PD and MSA, they obtained an overall sensitivity of 95.4%, indicating that PMCA can be a highly sensitive diagnostic tool for differentiating PD and MSA (Shahnawaz et al., 2020).

The same group showed previously that PMCA provides a sensitivity for diagnosis of 88.5% for PD (96.9% specificity), 100% for DLB, and 80% for MSA (Shahnawaz et al., 2017). They had analyzed cohorts of CSF samples from patients with PD (n = 76) and individuals serving as controls affected by other neurologic disorders (n = 65), neurodegenerative diseases (n = 18), and AD (n = 14) (Shahnawaz et al., 2017).

Evidence from Singer et al. also supports the ability of PMCA to differentiate between MSA and PD/DLB (Singer et al., 2020). They analyzed seeding activity of α-syn via PMCA and neurofilament (NfL) levels via enzyme-linked immunosorbent assay (ELISA). NfL is considered to be a marker of neuronal degeneration; although not disease-specific, levels may vary between diseases depending on how fast a disease progresses (Singer et al., 2020). The authors found that NfL levels were significantly higher in early MSA compared to early PD or DLB, and analysis with NfL showed a very high ROC area under the curve (AUC) of 0.97, signifying a minimal overlap between the MSA and PD/DLB groups. They suggested that this difference may be due to the rapidly progressive nature of MSA disease (Singer et al., 2020). A separate analysis with α-syn PMCA showed that although MSA and PD/DLB are both α-synucleinopathies, their reaction kinetics are biochemically and biophysically distinct (Singer et al., 2020). PD and DLB samples showed greater maximum ThT

fluorescence than MSA samples (except for 2 samples in the DLB group with no aggregation), and yet the start of polymerization and reaction plateau formation occurred earlier in MSA samples (in less than half the time compared to PD/DLB samples) (Singer et al., 2020). Analysis with α-syn showed an ROC AUC of 0.93 (Singer et al., 2020). When using optimal THT fluorescence and NfL cutoff values and requiring BOTH markers for distinguishing MSA from PD/DLB, they obtained a sensitivity of 97% and specificity of 90%. When requiring significant results for NfL OR α-syn, sensitivity would increase to 100% but specificity would decrease to 83% (Singer et al., 2020). Overall, Singer et al. demonstrated that NfL values and α-syn PMCA could be useful biomarkers for distinguishing MSA from PD/DLB, especially when a "positive" result for both is required for diagnosis (Singer et al., 2020).

Quadalti et al. were interested in discriminating PD from atypical parkinsonism disorders (MSA, PSP, and CBS) as well as atypical parkinsonism disorders from each other by measuring NfL levels in plasma and CSF, performing α-syn RT-QuIC, and evaluating the accuracy of a combined test where samples underwent both analyses (Quadalti *et al.*, 2021). Their study population included patients with clinically diagnosed PD (n = 153), MSA (n = 80), progressive supranuclear palsy/cortico-basal syndrome (PSP/CBS) (n = 58), DLB (n = 64), and non-neurodegenerative controls (n=72) (Quadalti et al., 2021). Plasma NfL and CSF NfL levels were higher in patients with MSA or PSP/CBS compared to those with PD, with slightly higher plasma NfL values in those with MSA compared to PSP/CBS and significantly higher CSF NfL levels in the MSA group compared to PSP/CBS (Quadalti et al., 2021). They found that that the sensitivity of either test for discriminating PD from atypical parkinsonism disorders was relatively high, but most importantly, it was even higher as a combined assay (91.4% RT-QuIC, 93.9% CSF NfL, 98.3% combined) (Quadalti et al., 2021). Specificity of the combined test was higher compared to CSF NfL alone, although it was slightly decreased compared to RT-QuIC alone (97.5% RT-QuIC, 90.8% CSF NfL, 95.8% combined) (Quadalti et al., 2021). α-syn RT-QuIC detected seeding activity in 98.4% of patients with DLB (Quadalti et al., 2021).

Brockmann et al. questioned whether α-syn RT-QuIC results in patients with PD would vary according to their profile of genetic mutations (Brockmann *et al.*, 2021). They utilized CSF from a cohort of patients clinically diagnosed with PD (n=236) and DLB (n=49) (Brockmann *et al.*, 2021). The PD cohort included patients carrying mutations such as GBA (n=99) and LRRK2 (n=9), as well as sporadic PD (n=108) (Brockmann et al., 2021). Patients diagnosed with DLB included those with GBA mutations (n=16) and those without (n=33). 85% of PD, 86% of DLB, and 8% of control samples were positive on α-syn RT-QuIC (Brockmann et al., 2021). Results were positive in 91% of patients with sporadic PD, 87% of patients with GBA mutations (93% of those with severe GBA mutations), and 78% of those with LRRK2 mutations (Brockmann et al., 2021). A subset of PD patients (n=100) was analyzed longitudinally with a mean time of 3.4-3.5 years between first and last lumbar puncture, with no appreciated change in number of positive RT-QuIC replicates over time (Brockmann et al., 2021). Positive a-syn seeding and higher numbers of positive RT-QuIC replicates was associated with higher Unified Parkinson's Disease Rating Scale (UPDRS-lll) and lower Montreal Cognitive Assessment (MoCA) scores (Brockmann et al., 2021).

Isolated RBD (iRBD), commonly called Idiopathic RBD, is known to be a common early-phase presentation in people who later develop an α-synucleinopathy (Stefani et al., 2021). Iranzo et al. conducted a longitudinal study, in which they obtained CSF samples from 52 patients with polysomnography-confirmed isolated RBD and 40 healthy controls; clinical follow-ups were conducted every 3-12 months to assess neurological status over the course of about 8 years (Iranzo et al., 2021). 90% of the 52 patients were positive for α-syn at baseline – this positivity was predictive

of conversion to either PD or DLB with a sensitivity of 96.9%, specificity of 20%, positive predictive value of 66%, and negative predictive value of 80% (Iranzo et al., 2021). Iranzo et al. were able to detect the seeding activity of α-syn up to 9 years before PD and DLB clinical diagnoses and show that patients with α-syn-positive isolated RBD had a higher risk of developing PD or DLB than α-syn-negative patients (Iranzo et al., 2021).

Poggiolini et al. studied the ability of α-syn RT-QuIC to help monitor for PD and MSA disease progression, stratification, and conversion from iRBD (Poggiolini *et al.,* 2021). They used CSF samples from patients with PD (n=74), MSA (n=24), iRBD (n=45), and healthy controls (n=55) (Poggiolini et al., 2021). They found a sensitivity of 89% and specificity of 96% for differentiating PD from healthy controls (Poggiolini et al., 2021). They did not observe a correlation between quantitative RT-QuIC parameters and clinical scores for PD such as the UPDRS-lll, although they did see that RT-QuIC positivity and Vmax differed according to various phenotypic clusters (Poggiolini et al., 2021). A-syn RT-QuIC was positive in 75% of patients with MSA (Poggiolini et al., 2021). Samples from MSA showed a significantly higher T50 and lower Vmax compared to PD and quantitative RT-QuIC parameters correlated with worse clinical progression of MSA on the Unified Multiple System Atrophy Rating Scale (Poggiolini et al., 2021). The authors also followed a longitudinal cohort of patients with iRBD, for which 64% of patients were a-syn RT-QuIC positive at baseline (Poggiolini et al., 2021). 14/45 (31%) of patients with iRBD converted to a synucleinopathy (9 to PD, 3 to DLB, 1 to MSA, and 1 to PAF) a mean of 2.5 years after lumbar puncture (Poggiolini et al., 2021). 9/14 of those patients had shown a-syn RT-QuIC positivity at baseline (Poggiolini et al., 2021).

Another common early-phase presentation of DLB is mild cognitive impairment (MCI) (Rossi et al., 2021). CSF α-syn RT-QuIC showed an overall accuracy of 96% (95.1% sensitivity and 96.6 specificity) in distinguishing Lewy Body MCI samples from cognitively unimpaired controls, suggesting high utility for CSF α-syn RT-QuIC as a biomarker for prodromal stages of disease (Rossi et al., 2021). They utilized 289 CSF samples from 2 independent cohorts: 81 patients with probable (clinically diagnosed) mild cognitive impairment (MCI) due to DLB (MCI-LB) and 120 patients with probable MCI due to AD (MCI-AD) (Rossi et al., 2021). They also examined samples from 30 patients with unspecified MCI, 58 patients with no cognitive decline or evidence of neurodegenerative disease, and 121 patients with absence of a-syn deposits on neuropathologic examination (Rossi et al., 2021). The 2 cohorts demonstrated consistent accuracy (97.3% and 93.7%) (Rossi et al., 2021). 13% of patients with MCI-AD and 6.7% of patients with unspecified MCI developed a positive test, although it is important to note that 44% of the positive MCI-AD samples were obtained from patients who developed 1 core or supportive clinical feature of DLB at follow-up (median of 20.3 months), suggesting underlying LB co-pathology (Rossi et al., 2021).

2.2 Other tissues

While several groups have focused on α-syn amplification in CSF, a smaller number has looked into sample collection from potentially less invasive sites, including gastrointestinal (GI) mucosa, olfactory mucosa, submandibular gland (SMG) tissue, and the skin.

Fenyi et al. examined the ability of PMCA to detect abnormal α-syn aggregation in the GI mucosa of 18 patients clinically diagnosed with PD and 11 controls (7 controls were patients undergoing colonoscopy for colorectal cancer screening; 4 had Crohn's disease; all had no PD symptoms nor cognitive deficiency) (Fenyi et al., 2019). Biopsies were taken from the antrum, sigmoid colon, or rectum and used for seeding in the assay. Fenyi et al. found that 10 out of 18 PD cases showed α-syn

seeding activity, while seeding activity was absent in 10 out of 11 controls. 3 out of 4 rectal biopsies from PD patients showed negative PMCA results, suggesting that rectum biopsies are not an appropriate sample location for PMCA analysis. (Fenyi et al., 2019)

De Luca et al. compared olfactory mucosa (OM) samples, collected by the "nasal brushing" technique, from patients diagnosed with PD and MSA with those of patients diagnosed with PSP and CBD (De Luca et al., 2019; Orrú et al., 2014). 9/11 MSA samples and 10/18 PD samples were able to induce α-syn aggregation when detected by RT-QuIC, compared with 2/12 PSP and 1/6 CBD (De Luca et al., 2019).

The "nasal brushing" technique has also been employed in patients with isolated RBD (Stefani et al., 2021). Stefani et al. recruited 63 patients with polysomnography-confirmed isolated RBD, 41 patients with clinically diagnosed PD, and 59 control subjects without parkinsonism or cognitive impairment; isolated RBD and PD patients were confirmed to exhibit similar levels of olfactory function impairment (Stefani et al., 2021). Regarding the test's diagnostic potential, Stefani et al. reported a high overall specificity of 89.8%, but lower sensitivity (45.2%) and accuracy (61.3%) (Stefani et al., 2021). The authors remarked that although RT-QuIC performed on OM samples did not show great sensitivity, the high specificity and less invasive sample collection technique could prove to be valuable for detecting early-stage α-synuclein-related neurodegeneration (Stefani et al., 2021).

Perra et al. achieved the highest diagnostic accuracy with α-syn RT-QuIC in OM to date (Perra *et al.*, 2021). They utilized OM and CSF samples from a cohort of 81 patients total: patients with clinical diagnosis of probable DLB (n=32), prodromal DLB (n=5), mixed degenerative DLB/AD dementia (n=6), and 38 non-a-syn-related neurodegenerative or non-neurodegenerative controls which consisted of patients with AD (n=10), sporadic CJD (n=10), PSP (n=8), CBS (n=1), FTD (n=3), psychosis (n=2), Down syndrome (n=1), vascular dementia (n=1), mild cognitive impairment (n=1), and Arnold-Chiari malformation type 1 (n=1) (Perra *et al.*, 2021). OM samples were collected from all 81 patients (Perra et al., 2021). CSF was available from 48 of the 81 patients: individuals with probable DLB (n=10) and mixed degenerative DLB/AD dementia (n=6), as well as 32 controls (Perra et al., 2021). The following OM samples were positive during a-syn RT-QuIC analysis: 27/32 with probable DLB, 5/5 with prodromal DLB, and 3/6 with mixed degenerative DLB/AD dementia (Perra et al., 2021). This showed an overall accuracy of 86.4% with 81.4% sensitivity and 92.1% specificity (Perra et al., 2021). 16/16 CSF samples from the DLB group and 3/32 CSF controls were positive, yielding an accuracy of 93.8% with 100% sensitivity and 90.6% specificity (Perra et al., 2021). 100% diagnostic accuracy was reached with the combination of the results from both OM and CSF a-syn RT-QuIC analysis (Perra et al., 2021).

Manne et al. tested frozen and FFPE submandibular gland (SMG) tissue from 13 PD, 3 incidental LBD (patients with Lewy bodies but not meeting diagnostic criteria for PD), and 12 control patients (controls were normal elderly subjects or those with AD-related changes insufficient for AD diagnosis) who were recruited and followed to autopsy for neuropathological examination and diagnosis (Manne et al., 2020a). SMG α-syn RT-QuIC discriminated PD and ILBD frozen samples from controls with 100% sensitivity and 94% specificity; for FFPE samples, sensitivity was 76% and specificity was 100% (Manne et al., 2020a).

The seeding concentration at which 50% of replicates turn positive during RT-QuIC, termed the median seeding dose (SD_{50}) and determined via Spearman-Kärber end-point dilution analysis, was found to be a titer of $8.8x10^8$/mg of brain homogenate and $2x10^6$/mg of SMG tissue in several

confirmed PD cases (Manne *et al.*, 2020). A separate study showed SD_{50} values of $8.9x10^5$/mg for SMG, $8.9x10^4$ for colon, $5.0x10^4$/mg for skin, and $2.8x10^2$/µl for CSF in the analysis of one neuropathologically confirmed PD case; SD_{50} values were $5.0x10^4$/mg for skin, $2.8x10^3$/µl for CSF, and $2.8x10^6$/mg for brain tissue from another neuropathologically confirmed PD case (Bargar *et al.*, 2021). These data provide evidence that compared to SD_{50} values from the brain, multiple peripheral tissue collection sites (SMG, skin, and colon) also have relatively high α-syn seeding activity (Bargar *et al.*, 2021; Manne *et al.*, 2020).

While the current literature on the presence of α-syn in skin samples has produced varying results by immunofluorescence (IF) and immunohistochemistry (IHC), Wang et al. were the first group to investigate whether α-syn seeding activity is present in skin samples and determined that both RT-QuIC and PMCA have the potential for accurate PD diagnosis (Wang et al., 2020). They analyzed a total of 130 autopsy abdominal skin samples from 47 cadavers with PD; 7 with LBD; 3 with MSA; 17 with AD; 8 with PSP; 5 with CBD; 43 non-neurodegenerative controls (Wang et al., 2020). Scalp samples were taken from 20 of the cadavers with PD and from 10 non-PD controls. All autopsy sample diagnoses were neuropathologically confirmed. Additionally, they obtained biopsy skin samples from the leg or posterior cervical region of 41 antemortem patients in total: 20 patients clinically diagnosed with PD and 21 non-PD controls, based on the Movement Disorder Society Clinical Diagnostic Criteria for PD (Wang et al., 2020). Samples were analyzed with RT-QuIC, PMCA, IHC, and IF.

IF and IHC confirmed the presence of α-syn in the skin nerve fibers of cadavers with PD; α-syn was undetectable in the skin of cadavers without PD (Wang et al., 2020). Their RT-QuIC initial analysis with 20 abdominal samples from PD cadavers and 4 non-PD controls yielded 95% sensitivity and 100% specificity; 20 scalp samples from PD cadavers and 10 non-neurodegenerative controls yielded 100% sensitivity and 100% specificity (Wang et al., 2020). They concluded that α-syn seeding activity is specifically detectable in PD autopsy samples, with scalp samples yielding higher sensitivity than abdominal samples (Wang et al., 2020). PMCA analysis of 23 abdominal skin samples from PD cadavers and 7 non-neurodegenerative controls yielded 83% sensitivity and 100% specificity.

Additional samples (27 PD, 39 non-neurodegenerative controls, as well as LBD, MSA, AD, PSP, and CBD) were tested with RT-QuIC in order to validate their initial results, where they obtained 94% sensitivity for PD, 100% sensitivity for LBD, and 67% sensitivity for MSA; sensitivity was 93% for all 3 synucleinopathies combined (Wang *et al.*, 2020). The specificity for PD was 93% when PSP, CBD, AD, and non-neurodegenerative controls were combined as a group of non-synucleinopathies and 98% when compared only to non-neurodegenerative controls (Wang et al., 2020). They obtained sensitivity of 82% and specificity of 96% with PMCA when differentiating synucleinopathies from non-synucleinopathies (Wang et al., 2020).

Finally, Wang et al. tested skin biopsy samples from living patients – 20 patients with PD and 21 non-PD controls. RT-QuIC resulted in 95% sensitivity and 100% specificity, while PMCA (10 patients with PD and 10 non-PD controls) revealed 80% sensitivity and 90% specificity (Wang et al., 2020).

Manne et al. also examined the accuracy of RT-QuIC for detecting α-syn in the skin, although using only post-mortem samples (Manne et al., 2020b), unlike Wang et al. who had used both post-mortem and antemortem samples. Overall, Manne et al. correctly identified frozen scalp skin samples from 21 PD and 21 control subjects out of 22 in each group, and formalin-fixed paraffin-embedded (FFPE)

sections from 9 PD and 10 control subjects out of 12 in each group (75% sensitivity and 83% specificity) (Manne et al., 2020b).

Regarding optimal skin biopsy location, Wang et al. determined that skin biopsy of the posterior cervical region showed higher sensitivity and specificity than skin biopsy from abdominal regions, and that posterior cervical skin biopsy may show higher and earlier α-syn seeding activity than skin tissue biopsied from the leg. This supports findings from Donadio et al. showing a proximal-distant gradient of abnormal α-syn detected by IHC, with highest levels of deposition found at cervical sites (Donadio et al., 2017).

Several other studies have tested a-syn RT-QuIC for skin punch biopsies (Donadio *et al.*, 2021; Kuzkina *et al.*, 2021; Mammana *et al.*, 2021). Mammana et al. analyzed skin punches taken postmortem from the following neuropathologically confirmed cases: incidental Lewy Body pathology (n=7), DLB (n=1), PD (n=1), and other rapidly progressive neurological syndromes of varied etiology (n=38) (Mammana *et al*., 2021). Skin punches taken in vitam were from patients with clinical diagnosis of "probable or clinically established PD or probable DLB" (n=28) and a control group comprised of patients with other various neurological disorders (n=41) (Mammana et al., 2021). 79 patients (19 with PD or DLB and 60 controls), some diagnosed clinically and others postmortem, had both CSF and skin samples available (Mammana et al., 2021). RT-QuIC of post-mortem cervical samples with Lewy Body pathology or DLB yielded 88.9% sensitivity (8 of 9 samples showed a full 4/4 positive response) and 97.5% specificity (all but 1 control showed a negative response) (Mammana et al., 2021). Analysis of skin samples acquired in vitam yielded 89.3% sensitivity (25 out of 28 cases) and 95.1% specificity (39 out of 41 non-LBD controls) (Mammana et al., 2021). All 3 in vitam LBD samples with a negative result had been collected from the thigh (n=2) or leg (n=1) (Mammana et al., 2021). The authors reported an overall sensitivity of 89.2% and specificity of 96.3% for LBD vs. neurological controls in the whole cohort (Mammana et al., 2021). Notably, in the 79 patients with samples available from both CSF and skin, the results showed a 96.2% concordance between the positive and negative outcomes of the 2 assays (Mammana et al., 2021).

Donadio et al. compared the diagnostic accuracy of immunofluorescence, a technique which may detect the morphology of pathologic aggregates in skin nerves, to RT-QuIC of CSF and skin samples in a cohort of 31 patients clinically diagnosed with synucleinopathies: PD (n=17), DLB (n=5), probable MSA (n=8), and pure autonomic failure PAF (n=3) (Donadio *et al.*, 2021). They also included 38 patients with non-synucleinopathies, 15 of which were diagnosed with AD with typical CSF findings, and 24 controls consisting of samples from patients with non-neurodegenerative diseases, mainly various subtypes of neuropathies (Donadio *et al.*, 2021). CSF RT-QuIC showed 78% sensitivity and 100% specificity for differentiating synucleinopathies from non-synucleinopathies and controls (Donadio et al., 2021). Immunofluorescence staining of pathologic phosphorylated a-synuclein (p-syn) in skin nerves showed 90% sensitivity and 100% specificity (Donadio et al., 2021). RT-QuIC of skin biopsy samples, which were taken from proximal (cervical) and distal (thigh and leg) sites, resulted in 86% sensitivity and 80% specificity: 9/38 non-synucleinopathy samples and 3/24 controls yielded positive results (Donadio et al., 2021). Notably, authors did not report whether accuracy differed with use of proximal vs. distal biopsy sites. The authors remarked that immunofluorescence and RT-QuIC with skin samples showed good agreement in identifying synucleinopathies, which supports the use of these less-invasive diagnostic techniques instead of CSF analysis (Donadio et al., 2021).

Kuzkina et al. obtained skin punch biopsies from the neck, lower back, thigh, and lower leg of 34 patients clinically diagnosed with PD (117 biopsies) and 30 control subjects (81 biopsies) (Kuzkina *et al.*, 2021). This resulted in 198 skin biopsy samples in total, which were divided in half and analyzed by RT-QuIC at two independent laboratories (Kuzkina et al., 2021). They obtained an overall diagnostic accuracy of 88.9% with agreement of 92.2% between the two laboratories, with the best separation between PD and control groups being observed in cervical sites compared to the lower back, thigh, or lower leg (Kuzkina et al., 2021). They also calculated SD_{50} values to be fourfold higher in patients with longer duration of PD (15-28 years, n=4) compared to patients with shorter duration of disease (5-7 years, n=3); serial dilutions were performed with skin biopsies from cervical sites, lower back, and thigh (Kuzkina et al., 2021). The SD_{50} values, combined with the correlation of higher a-syn seeding activity with the presence of RBD, cognitive impairment, constipation, and lower MoCA scores, showed some potential for the implementation of a-syn RT-QuIC for indication of PD progression (Kuzkina et al., 2021).

Recently, Bargar et al. identified the need for standardization across the wide range of protocols and materials used by various research groups and developed their own streamlined assay by using exclusively commercial reagents (Bargar et al., 2021). The assay was validated with a large panel of 214 CSF samples, obtained from neuropathologically confirmed PD and DLB cases (Bargar et al., 2021). This resulted in 98% sensitivity and 100% specificity, confirming the high accuracy reported by previous studies (Bargar et al., 2021). Additionally, Bargar et al. showed that their assay could detect α-syn seeding activity in a small number of brain homogenate, OM, SMG, skin, and colon tissue samples, suggesting that RT-QuIC protocols could be standardized across different tissue types (Bargar et al., 2021).

Table 1 Summary of studies on α-synuclein RT-QuIC or PMCA using CSF or peripheral tissues

Author	Assay	Rec-syn source	Tissue	Disease	Sensitivity	Specificity
Fairfoul et al., 2016	RT-QuIC	purchased commercially from Stratech, Cambridge, UK	CSF	DLB	92%	100%
				PD	95%	100%
Groveman et al., 2018	RT-QuIC	synthesized in-house	CSF	DLB	93%	100%
				PD	93%	100%
Bongianni et al., 2019	RT-QuIC	synthesized in-house	CSF	DLB + MSA	93%	96%
Bongianni et al., 2019	RT-QuIC		CSF	DLB	85%	100%
Rossi et al., 2020	RT-QuIC	Synthesized in-house	CSF	DLB + PD	98%	95%
Shahnawaz et al., 2020	PMCA	Synthesized in-house	CSF	PD	94%	100%
				MSA	85%	100%
Shahnawaz et al., 2017	PMCA	synthesized in-house	CSF	PD	89%	97%
Brockmann et al., 2021	RT-QuIC	synthesized in-house	CSF	DLB	86%	92%
				PD	85%	92%
				PD, sporadic	91%	92%
				PD, GBA mutation	87%	92%
				PD, LRRK2 mutation	78%	92%
Poggiolini et al., 2021	RT-QuIC	synthesized in-house	CSF	PD	89%	96%
				MSA	75%	96%
Rossi et al., 2021	RT-QuIC	synthesized in-house	CSF	Lewy Body MCI	95%	97%

Bargar et al., 2021		purchased commercially from rPeptide, Watkinsville, GA	CSF	PD and DLB	98%	100%
Fenyi et al., 2019	PMCA	purchased commercially from Sigma, Saint-Quentin-Fallavier, France	GI mucosa	PD	56%	91%
Stefani et al., 2021	RT-QuIC	synthesized in-house	OM	iRBD + PD	45%	90%
De Luca et al., 2019		synthesized in-house	OM	PD	56%	83%
				MSA	82%	83%
Perra et al., 2021	RT-QuIC	synthesized in-house	OM	DLB + mixed DLB/AD	81%	92%
			CSF	DLB + mixed DLB/AD	100%	91%
			CSF + OM	DLB + mixed DLB/AD	100%	100%
Manne et al., 2019	RT-QuIC	synthesized in-house	brain homogenate	PD + DLB	94%	100%
			CSF	PD	100%	100%
Manne et al., 2020a	RT-QuIC	synthesized in-house	SMG, frozen	PD + ILBD	100%	94%
			SMG, FFPE	PD+ILBD	76%	100%
Wang et al., 2020	RT-QuIC	synthesized in-house AND purchased commercially from rPeptide, Watkinsville, GA	skin, abdominal, post-mortem	PD	94%	93%
				DLB	100%	93%
				MSA	67%	93%
	PMCA		skin, abdominal, post-mortem	PD	83%	100%
	RT-QuIC		skin, cervical + leg, ante-mortem	PD	95%	100%
	PMCA		skin, cervical + leg, ante-mortem	PD	80%	90%
Manne et al., 2020b	RT-QuIC	synthesized in-house	skin, scalp, FFPE	PD	75%	83%
			skin, scalp, frozen	PD	96%	96%
Mammana et al., 2021	RT-QuIC	synthesized in-house	skin, cervical	LBD	89%	95%
Donadio et al., 2021	RT-QuIC	purchased commercially from rPeptide, Watkinsville, GA	skin, cervical + thigh + leg	PD + DLB + MSA + PAF	86%	80%
			CSF	PD + DLB + MSA + PAF	78%	100%
Kuzkina et al., 2021	RT-QuIC	synthesized in-house	skin, cervical + lower back + thigh + leg	PD	91%	87%

Abbreviations: LBD Lewy body disease, *PD* Parkinson's disease, *DLB* Dementia with Lewy bodies, *MSA* Multiple system atrophy, *MCI* Mild cognitive impairment, *iRBD* Isolated rapid eye movement sleep behavior disorder, *ILBD* Incidental Lewy body disease, *GI* Gastrointestinal, *OM* Olfactory mucosa, *SMG* Submandibular gland, *FFPE* Formalin-fixed paraffin-embedded

3 Discussion

The development of a highly sensitive and specific diagnostic test for LBD is crucial in order to prevent misdiagnosis and enable diagnosis/screening at earlier stages of disease. Overall, there is consistent evidence that amplification techniques such as RT-QuIC and PMCA, which measure α-syn seeding activity, have high discriminatory ability among certain synucleinopathies (LBD vs. MSA, but not DLB vs. PD) and are highly specific and sensitive assays for discriminating synucleinopathies from non-synucleinopathies (e.g. LBD from AD). Across the articles described above, CSF RT-QuIC and PMCA sensitivities ranged from 86% to 98% roughly for PD or DLB or LBD, while specificities ranged from 92% to 100%. The high accuracy of the results so far, combined with the success of amplification assay diagnostics in clinical practice for CJD, suggest high promise for eventual clinical application as a diagnostic test for LBD.

Despite the high accuracy of amplification assays, adaptation to clinical settings may present challenges. The highly invasive nature of CSF sample collection may prevent widespread use. Although the benefits of an accurate diagnosis and appropriate treatment design may outweigh the risks of lumbar puncture in some cases, CSF collection may not be appropriate for screening or diagnosis in early/prodromal stages.

Fortunately, several groups have begun investigations into analysis of α-syn seeding activity in tissues other than CSF such as GI mucosa, OM, SMG, and skin. Although 10/11 GI control samples in a study conducted by Fenyi et al. lacked α-syn seeding activity, the test was positive for only 10/18 PD samples, suggesting an inadequately low sensitivity for PD detection (Fenyi et al., 2019). RT-QuIC detected similarly low seeding activity in the OM of PD patients (De Luca et al., 2019). According to the authors, this may be due to the constant regeneration of OM and the potential of CSF drainage from the OM, thus producing mixed or unreliable results (De Luca et al., 2019). However, Perra et al. did recently achieve an overall accuracy of 86.4% with 81.4% sensitivity and 92.1% specificity for detecting DLB via α-syn RT-QuIC with OM samples (Perra *et al.*, 2021). Considering that diagnosis of CJD using RT-QuIC has achieved 97% sensitivity and 100% specificity with nasal brushings of the olfactory mucosa, future studies should continue investigating the optimization of OM RT-QuIC (Becker et al., 2018; Bongianni et al., 2017; Orrú et al., 2014). As stated by Stefani et al., who obtained similar results to De Luca et al., but with a larger cohort and including samples from isolated RBD patients, nasal brushing may be a useful practice for screening in the early stages of disease (Stefani et al., 2021). Despite the reported low sensitivity, the minimally invasive nature of nasal brushing and high specificity make screening via OM sample collection a potentially attractive option and important focus of upcoming research.

SMG RT-QuIC resulted in high sensitivity and specificity when conducted with frozen tissue samples (Manne *et al.*, 2020). SD_{50} values for SMG were only 1 to 2 orders of magnitude lower than those for brain homogenates (Bargar *et al.*, 2021; Manne *et al.*, 2020). However, the authors remarked that SMG collection is invasive and associated with rare, but severe, adverse effects (AEs) such as hematoma, infection, and facial nerve damage (Manne et al., 2020a). These AEs may be exacerbated by multiple biopsy collections or using larger core needles, due to insufficient amount of glandular tissue captured in the needle bore (Manne et al., 2020a). Although their results suggest high accuracy of SMG RT-QuIC, future studies may have to consider methods of minimizing AE risk before SMG tissue testing can become routine.

Detection of α-syn seeding activity by RT-QuIC and PMCA in skin biopsy samples, specifically, appears to be most applicable for the clinical setting, although more studies are needed to confirm and expand on these results. As described above, several studies have reported high discriminatory ability – combined with the less invasive nature of posterior cervical skin biopsy compared to SMG biopsy or CSF collection, their results may be a crucial step towards a definitive diagnostic test for LBD. No data are available on the potential of α-syn amplification assays for samples of saliva, serum, plasma, or urine. Considering the results discussed earlier regarding oligomeric and phosphorylated α-syn as well as NfL in plasma and serum as PD biomarkers (Foulds et al., 2013; Williams et al., 2016; Quadalti et al., 2021), blood may be a good candidate for RT-QuIC or PMCA analysis and researchers should direct their attention to less invasive sample sources as listed above.

One of the most useful and unique features of amplification assays is the capability to analyze samples with minute amounts of misfolded protein (i.e. α-syn) due to the ability to capitalize on prion-like properties and amplify protein to detectable levels. This could allow clinicians to detect pathogenic protein activity in early or prodromal stages of disease before symptoms develop and before a clinical diagnosis is possible. As seen in Manne et al.'s study, RT-QuIC was able to detect pathological α-syn in SMG tissue from ILBD patients, which was undetectable by IHC, demonstrating its potential for future identification of prodromal PD (Manne et al., 2020a). Iranzo et al. were able to detect a-syn seeding activity in patients with iRBD up to 9 years before PD and DLB clinical diagnoses and Rossi et al. distinguished Lewy Body MCI samples from cognitively unimpaired controls with an overall accuracy of 96% (Iranzo et al., 2021; Rossi et al., 2021). Early detection and accurate prediction of disease progression could enable earlier treatment planning and administration, thus improving patient outcomes.

The qualities of accurate detection and real-time surveillance provided by RT-QuIC could be useful for not only early disease detection, but also for testing of therapeutics. For instance, α-syn has been shown to be detectable by RT-QuIC in microglia, which are of high interest as a therapeutic target due to being efficient scavengers of extracellular α-syn and mediators of neuroinflammation; this was determined by exposing a mouse microglial cell line to α-syn pre-formed fibrils and subsequently harvesting the cells for RT-QuIC analysis (Manne *et al.*, 2019). Kondru et al. coupled an ex vivo prion organotypic slice culture assay with RT-QuIC to create a model of prion diseases useful for investigating anti-prion therapeutics (Kondru *et al.*, 2017). Future studies should consider developing similar hybrid organotypic slice models for α-syn as well as other proteins prone to misfolding and propagation. Research should also expand on the role of RT-QuIC for detection of α-syn in cells such as neurons and microglia, which may be attractive therapeutic targets.

The success of amplification assays for monitoring prion and α-syn seeding activity should be broadened to other proteins involved in pathogenesis of neurodegenerative diseases. For example, Carlomagno et al. developed an *in vitro* RT-QuIC assay for detecting amplification of AD tau filament core (Carlomagno *et al.*, 2021). Previous assays had demonstrated seeding activity of tau *in vivo* with a RT-QuIC technique which risked impacting sensitivity because heparin was required for induction of full-length tau (Carlomagno *et al.*, 2021). The group uncovered AD tau filament core's propensity for spontaneous filament formation and showed that RT-QuIC could be used for detection of AD tau filament core propagation without the need for heparin induction (Carlomagno *et al.*, 2021). The researchers were also able to generate and demonstrate spontaneous propagation of tau filament core from the primary tauopathies CBD and Pick's Disease (PiD) (Carlomagno *et al.*, 2021). These data suggest that RT-QuIC has potential for broad application and that amplification techniques should be investigated across a wide variety of disease processes. RT-QuIC could also perhaps be combined with plasma biomarkers such as p-tau181 that could detect AD pathology with high accuracy but showed limited ability in distinguishing AD from LBD (Chouliaras *et al.*, 2022).

Some limitations exist in the presently discussed studies, which future studies should consider. Research groups worldwide have used a wide range of protocols and reagents with varying time-frames for analysis, as seen in Table 1, which discourages wide-spread use and reproduction of results. Standardization of protocols and materials is needed for further research and eventual wide-spread clinical use. Optimization and streamlining of protocols, as described by Bargar et al., should be considered by future groups in order to facilitate meta-analysis, compare outcomes, and eventually apply amplification assays as diagnostic tools (Bargar et al., 2021). Many groups also used small sample sizes; in the groups who used larger cohorts of LBD patients, small sample sizes remained for comparison groups such as AD, MSA, PSP, other neurodegenerative diseases, and non-neurodegenerative controls. In order to improve generalizability, future studies should consider ways to overcome challenges with subject recruitment, such as cross-country and international collaboration. Sensitivity and specificity determined in studies of living patients depend on clinical diagnoses of DLB and non-synucleinopathies, which could be highly variable. To validate results, neuropathological autopsy follow-ups will be required for each case, which can significantly prolong research timelines.

4 Conclusion

The use of amplification techniques such as RT-QuIC and PMCA for detection of pathological α-syn in human tissues shows great promise for the eventual development of a highly sensitive and specific diagnostic test for LBD, which may reduce misdiagnosis and allow detection of disease in prodromal stages. Amplification techniques can also facilitate the development of disease models for accurate testing of therapeutics. Recent studies report accurate detection of α-syn seeding activity in tissues such as CSF, SMG, and posterior cervical skin, with skin biopsy being the least invasive procedure and thus most promising for routine clinical application. The scientific community should increase research efforts regarding the application of amplification techniques for LBD diagnosis, with special focus on recruiting larger cohorts and investigating the use of less invasive sample collection methods such as skin biopsy or blood.

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Declarations of interest

Competing interests unrelated to this work, JTOB has received honoraria for work as DSMB chair or member for TauRx, Axon, Eisai, has acted as a consultant for Roche, and has received research support from Alliance Medical and Merck.

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Citations

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