# Cerebrospinal fluid α-synuclein detection by RT-QuIC in patients with isolated rapid-eye-movement sleep behaviour disorder: a longitudinal observational study

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## Summary

**Background:** Isolated rapid-eye-movement (REM) sleep behaviour disorder (IRBD) can be part of the prodromal stage of the α-synucleinopathies Parkinson disease (PD) and dementia with Lewy bodies (DLB). Cerebrospinal fluid (CSF) real-time quaking-induced conversion (RT-QuIC) analysis has high sensitivity and specificity for the detection of misfolded α-synuclein in PD and DLB. We investigated whether RT-QuIC could detect α-synuclein in the CSF of IRBD patients, and be used as a reliable biomarker of prodromal α-synucleinopathy.

**Methods:** CSF samples were obtained by lumbar puncture in polysomnographically-confirmed IRBD patients recruited at a specialized sleep disorders center in Spain and in healthy controls. CSF samples were stored until analysed using RT-QuIC assay. After lumbar puncture, participants were assessed clinically for a neurological status every 3-12 months. Rates of neurological disease-free survival were estimated using the Kaplan–Meier method.

**Findings:** Fifty-two IRBD patients and 40 controls matched for age (p=0.20), sex (p=0.15) and follow-up duration (p=0.27) underwent lumbar puncture between March 23 2008 and July 16 2017. CSF α-synuclein RT-QuIC was positive in 47 (90%) patients and in four (10%) controls. RT-QuIC had a sensitivity of 90.4% (95% CI 79.4%-95.8%) and a specificity of 90.0% (95% CI 76.9%-96.0%) for detecting α-synuclein in the CSF of IRBD patients. After lumbar puncture, patients were followed-up for a mean of 7.1 ± 2.8 years and controls for a mean follow-up of 7.7 ± 2.9 years until the end of the study in July 31 2020. During follow-up, 32 (62%) patients were diagnosed with PD or DLB 3.4 ± 2.6 years after LP, and 31 (97%) of these were positive for CSF α-synuclein RT-QuIC. Kaplan–Meier analysis showed that IRBD patients who were CSF α-synuclein RT-QuIC positive had higher risk for developing PD or DLB than those IRBD patients who were CSF α-synuclein RT-QuIC negative at 2, 4, 6, 8 and 10 years of follow-up (log rank test, p=0.028; HR=0.143; 95%CI 0.019-1.063). During follow-up, none of the controls developed an α-synucleinopathy. Kaplan–Meier method showed that participants showing CSF α-synuclein RT-QuIC positivity had higher risk of developing PD or DLB

than those participants with CSF α-synuclein RT-QuIC negativity at 2, 4, 6, 8 and 10 years from LP (log rank test, $p<0.0001$; HR:0.024; 95%CI 0.003-0.177).

**Interpretation:** In IRBD, CSF α-synuclein RT-QuIC detects misfolded α-synuclein with both sensitivity and specificity of 90% prior to clinical diagnoses of PD and DLB. Detection of α-synuclein by RT-QuIC in the CSF represents a prodromal marker of PD and DLB. If these findings are replicated in additional cohorts, detection of α-synuclein in the CSF by RT-QuIC may be used to enrich IRBD cohorts entering in future neuroprotective trials, particularly when targeting α-synuclein.

**Funding:** Department of Health and Social Care Policy Research Programme, Scottish Government and the Weston Brain Institute

## Introduction

Parkinson disease (PD) and dementia with Lewy bodies (DLB) are clinical syndromes featuring motor (e.g., parkinsonism) and non-motor (e.g., cognitive impairment, smell loss, sleep problems) symptoms that are associated with deposits of intraneuronal misfolded α-synuclein which constitute the main component of the Lewy bodies and neurites. Rapid-eye-movement (REM) sleep behaviour disorder (RBD) is characterized by dream-enactment behaviours linked to REM sleep without muscle atonia.[1] Most patients with the isolated form of RBD (IRBD) eventually evolve to an overt clinical form of α-synucleinopathy, namely PD, DLB and much more rarely multiple system atrophy (MSA).[2-4] The reliable identification of pathologic α-synuclein in IRBD could represent a valuable biomarker of the prodromal stages of the α-synucleinopathies.

Biochemical abnormalities in the brain are reflected by changes within the cerebrospinal fluid (CSF). Real-time quaking-induced conversion (RT-QuIC) is a novel ultrasensitive assay able to detect pathologic misfolded proteins.[5] This technique identifies misfolded α-synuclein in the CSF of manifest PD and DLB with a sensitivity of 90-95% and specificity of 80-100%.[5-12] The hypothesis of this study is that RT-QuIC would demonstrate the same high degree of sensitivity and specificity for detecting α-synuclein in the CSF of IRBD patients as it does in PD and DLB, and determine whether this technique could act as a reliable biomarker of prodromal α-synucleinopathy. This would provide strong evidence for IRBD as an ongoing α-synucleinopathy detected in the early stages and contribute to the development of neuroprotective trials in IRBD, focusing on interventions against α-synuclein deposition and spreading in the brain.[13]

## Methods

### Study design and participants

In this longitudinal observational study polysomnographically-confirmed IRBD patients free of parkinsonism and dementia and healthy controls underwent baseline lumbar puncture (LP) at the Hospital Clinic Barcelona, Spain, between March 23 2008 and July 16 2017, as part of previous research studies.[14-16] Aliquots of each CSF sample were frozen during these studies for use in future research projects. A first group of healthy controls comprised non-consanguineous relatives and friends of the IRBD patients who underwent video-polysomnography that excluded RBD and were enrolled in IRBD studies on CSF markers.[14] A second group of healthy controls included volunteers that participated in studies evaluating new CSF biomarkers in neurodegenerative dementias and in whom clinical history excluded the presence of RBD symptomatology.[16] At the time of LP, all healthy controls were free of neurological diseases according to unremarkable clinical history and normal neurological examination.

After LP, IRBD patients and healthy controls were followed-up at regular intervals at the hospital until July 31 2020 (end of the study).

In addition, we evaluated CSF samples from a group of neurological controls diagnosed and followed-up at the Neurological Service of Hospital Clinic Barcelona, Spain. The neurological controls included individuals with symptomatic autosomal dominant Alzheimer disease linked to mutations in the presinilin-1 gene (as brain autopsy does not show Lewy bodies in most of the cases with this condition)[17] and also patients with narcolepsy type 1 with coexistent polysomnographically-confirmed RBD (as narcolepsy is linked to RBD and this disease is thought not to have a neurodegenerative origin). At the time of lumbar puncture, clinical history and neurological examination ruled out RBD and parkinsonism in patients with autosomal dominant Alzheimer disease. At the time of lumbar puncture, clinical history and neurological examination ruled out parkinsonism and cognitive impairment in patients with

narcolepsy. Patients with autosomal dominant Alzheimer disease underwent lumbar puncture between February 26 2011 and June 23 2016. Patients with narcolepsy underwent lumbar puncture between June 28 2002 and June 27 2017.

**Procedures**

In all participants, CSF was obtained in the morning following an overnight fast using atraumatic 20 and 22G needles, as previously described.[9,14,15] In brief, 15–20 mLs of CSF were collected at room temperature, centrifuged at 2000g for 10 min, transferred to 1.5 mL pre-cooled siliconized polypropylene aliquots and immediately frozen on dry ice. No relevant adverse-events occurred related to the LP procedure. The aliquots were stored at -80°C until analyzed by RT-QuIC in November 2019 (in all IRBD patients plus the first group of healthy controls) and in July 2020 (in the second group of healthy controls plus the neurological controls).

At the time of LP, IRBD patients and the first group of healthy controls underwent an extensive clinical assessment where a number of risk and prodromal PD markers were evaluated (e.g., smoking status, constipation, depression). This baseline assessment included video-polysomnography (to exclude RBD in the healthy controls), evaluation of motor function using the Movement Disorders Society Unified Parkinson's Disease Rating Scale (UPDRS-III)[18], a comprehensive neuropsychological test battery,[3] smell function using the Spanish version of the 40-item University of Pennsylvania Smell Identification Test (UPSIT-40),[19] and nigrostriatal dopaminergic system function using $^{123}$I-2β-carbomethoxy-3β-(4-iodophenyl)-N-(3-fluoropropyl)-nortropane single photon emission computed tomography (DAT-SPECT).[20] With this information the probability of having prodromal PD at the time of LP was calculated according to the Movement Disorders Society research criteria for prodromal PD in all IRBD patients and the first group of healthy controls.[21] Prodromal PD was defined using a post-test probability ≥80%.[21]

At the time of LP, the second group of healthy controls underwent extensive assessment that included sleep

history that revealed the absence of RBD symptoms, and neurological examination, neuropsychological testing and brain MRI that were unremarkable.[16]

After LP, all the IRBD patients and all the healthy controls were clinically followed-up every 3-12 months at the Hospital Clinic Barcelona by neurologists experienced in sleep and neurodegenerative diseases using clinical history and neurological examination. When motor or cognitive impairment was detected, PD and DLB were diagnosed according to standard criteria.[22,23]

A subgroup of IRBD patients underwent serial LP performed at 6 or 12 month intervals between May 16 2014 and November 21 2018, as part of another research study.[15]

During follow-up, IRBD patients who died became brain donors to the Neurological Tissue Bank, Hospital Clinic-IDIBAPS, Barcelona, Spain. Neuropathological assessment was performed according to a standardized protocol[3] and immunohistochemical analyses included the application of a specific antibody against AS (Novocastra, mc, clone KM51, dilution 1:500) to make a definite diagnosis of PD and DLB.[23,24]

All baseline and serial aliquots of frozen CSF were shipped on dry ice from Barcelona (Spain) to Edinburgh (Scotland, UK) for RT-QuIC analysis. All CSF samples were evaluated by RT-QuIC assay blind to participant category (IRBD, healthy control, neurological control), number of individuals per category, the neurological condition of the neurological controls, and clinical data.

In all participants, CSF α-synuclein RT-QuIC analysis was undertaken as previously described with minor modifications.[5,9] Human recombinant full-length (1–140 aa) α-synuclein (Sigma, Poole, UK) was used as substrate. CSF samples were incubated in duplicate in a BMG OMEGA FluoSTAR plate reader at 30°C for 120 h with intermittent shaking cycles: double orbital with 1 min shake (200 rpm), 14 min rest. Thioflavin T fluorescence measurements (450 nm excitation and 480 nm emission) were taken every 15 min. A positive CSF α-synuclein RT-QuIC was defined as a relative fluorescence unit value of >2SD above the mean of the negative internal assay controls at 120 h in both of the CSF duplicates. The final fluorescence value was the mean

fluorescence value taken at 120 h. T50 was defined as the time it took to obtain 50% of the maximal relative fluorescence unit.

At the end of the study in July 31 2020, we assessed the neurological status (remained disease-free or developed a clinical-defined synucleinopathy and its type) of all participants. Healthy controls that were CSF α-synuclein RT-QuIC positive were asked to undergo complete demographic and neurological history, UPDRS-III,[18] Montreal Cognitive Assessment (MoCA) test,[25] UPSIT-40,[19] DAT-SPECT,[20] and video-polysomnography to rule out RBD.[1] With this information, the probability of prodromal PD was calculated at this final 2020 assessment in the CSF α-synuclein RT-QuIC positive controls.[21]

The study was approved by the local ethics committee of the Hospital Clinic Barcelona and all participants gave written informed consent at the start of the study. Relatives of postmortem brain autopsy patients gave consent for whole body autopsy and removal of all organs for diagnostic and research purposes.

**Statistical analysis**

Data are presented as mean and standard deviation if continuous and as percentages if discrete. Sensitivity, specificity, positive predictive value, and negative predictive value, including 95% confidence limits, of detecting CSF α-synuclein RT-QuIC were calculated in IRBD patients. The Wilson method was used to compute the confidence intervals. Differences between groups were evaluated using the chi-squared test, Student’s t test and Mann–Whitney U test, as appropriate. Rates of neurological disease-free survival were estimated using the Kaplan–Meier method. Disease-free survival rates were assessed from the date of LP to the date of PD and DLB diagnoses or to the last follow-up visit for censored observations, and comparisons between participants with positive and negative

CSF α-synuclein RT-QuIC responses were undertaken using the Log-rank test. P values less than 0.05 were considered significant. All analyses were conducted utilizing SPSS version 25.0 (Armonk, IBM Corp, NY, USA).

**Role of the funding source**

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. All the authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

## Results

The study comprised 52 IRBD patients, 40 healthy controls (nine from the first group and 31 from the second group) and 11 neurological controls (five with autosomal dominant Alzheimer disease and six with narcolepsy type 1).

IRBD patients were 43 males and nine females, with a mean age at baseline LP of 71.3 ± 6.0 years, and with a follow-up interval from baseline LP to the end of the study of 7.1 ± 2.8 years. The healthy controls were 28 males and 12 females with a mean age at LP of 69.7 ± 5.4 years and follow-up of 7.7 ± 2.9 years. IRBD patients and healthy controls were matched for age (p=0.20), sex (p=0.15) and follow-up duration (p=0.27). The mean follow-up of the 52 IRBD patients and the 40 healthy controls was 7.3 ± 2.9 years.

The autosomal dominant Alzheimer disease patients comprised four males and one female with a mean age at LP of 54.8 ± 4.8 years who were in the mild or moderate phases of dementia. The narcoleptics comprised five males and one female with a mean age at LP of 67.7 ± 5.4 years and follow-up from LP to the end of the present study of 9.2 ± 7.1 years. In all these six patients the diagnosis of narcolepsy type-1 was confirmed by ≤110 pg/mL of hypocretin in the CSF.

CSF α-synuclein RT-QuIC was positive in 47 of 52 (90%) patients and in four of 40 (10%) healthy controls, resulting in a sensitivity of 90.4% (95% CI 79.4%-95.8%) and a specificity of 90.0% (95% CI 76.9%-96.0%) (Figures 1 and 2, Table 1). At the time of LP, the prodromal PD probability was higher in those IRBD patients who were CSF α-synuclein RT-QuIC positive than those IRBD patients who were CSF α-synuclein RT-QuIC negative (89.4 ± 22.9 versus 54.7 ± 5.8, p=0.003). CSF α-synuclein RT-QuIC was negative in all patients with autosomal dominant Alzheimer disease and with narcolepsy.

At the end of the study, 32 of 52 (62%) IRBD patients developed PD (n=16) and DLB (n=16). During follow-up, 31 of 47 (66%) CSF α-synuclein RT-QuIC positive patients converted to a clinically-defined α-synucleinopathy (16 to DLB and 15 to PD) with an interval of 3.2 ± 2.5 (range, 0.4-9.1) years between LP and clinical diagnoses, and 16 (34%) remained disease-free at the end of the study. Of the five IRBD patients who were CSF α-synuclein RT-QuIC negative, one converted to PD 7.5 years after LP and four remained disease-free at the end of the study (Figure 2). Kaplan-Meier analysis showed that IRBD patients who were CSF α-synuclein RT-QuIC positive had a higher risk of incident clinically-defined α-synucleinopathy than those IRBD patients who were CSF α-synuclein RT-QuIC negative at 2, 4, 6, 8 and 10 years of follow-up (log rank test, p=0.028). The Kaplan-Meier method estimated that 50% of CSF α-synuclein RT-QuIC positive IRBD patients converted to PD or DLB after six years from LP (Figure 3A). In the IRBD patients, CSF α-synuclein RT-QuIC positivity, as a predictor of conversion to either PD or DLB, had a sensitivity of 96.9% (95% CI 84.3%-99.4%), a specificity of 20.0% (95% CI 8.1%-41.2%), a positive predictive value of 66.0% (95% CI 51.7%-77.8%), and a negative predictive value of 80.0% (95% CI 37.6%-96.4%). IRBD convertors who had positive CSF α-synuclein RT-QuIC had higher percentage of subjects with prodromal PD probability >80% than the IRBD patients who remained disease-free and were also CSF α-synuclein RT-QuIC positive (93.5% versus 62.5%, p=0.013) (Table 2).

Postmortem brain examination was performed in six IRBD patients with the antemortem diagnoses of IRBD (n=1), DLB (n=4) and PD (n=1). These six patients were baseline CSF α-synuclein RT-QuIC positive and died after 5.4 ± 3.1 (range, 1.9-8.8) years from the LP. Neuropathology identified widespread immunoreactive Lewy pathology in the brain containing α-synuclein inclusions in each case.

Of the 40 healthy controls, 36 (90%) were CSF α-synuclein RT-QuIC negative and four (10%) were positive (Figure 2). At the time of LP, the prodromal PD probability was 1.6 ± 3.8 percent in the first group of nine controls.

All 40 healthy controls and the six narcoleptic type 1 patients were re-assessed at the end of this study and none were found to have developed a neurodegenerative disease.

Of the four healthy controls with baseline CSF α-synuclein RT-QuIC positivity, three remained healthy showing a prodromal PD probability of 1%, 11% and 20% at the end of the study (Table 3). The remaining control with baseline CSF α-synuclein RT-QuIC positivity developed severe herpes encephalitis eight years after LP and she was institutionalized in a nursing home and unable to be evaluated at the end of this study. On last follow-up visit prior to developing herpes encephalitis she had no motor abnormalities and no cognitive deficits.

Kaplan-Meir analysis showed that participants showing baseline CSF α-synuclein RT-QuIC positivity (47 IRBD patients plus four healthy controls) had higher risk of developing PD or DLB than those participants with CSF α-synuclein RT-QuIC negativity (five IRBD patients plus 36 healthy controls) ($p<0.0001$) at 2, 4, 6, 8 and 10 years from LP (Figure 3B).

Fourteen of the 52 (30%) IRBD patients underwent 4.1 ± 1.4 (range, 2-6) serial LP assessments. At the end of the study, four of these patients developed PD, four DLB and six remained disease-free. In 13 of these 14 (93%) IRBD patients, all baseline and serial CSF α-synuclein RT-QuIC samples were positive. In the remaining patient, CSF α-synuclein RT-QuIC was negative in 2014, 2015 and 2017, was positive in 2018 and he remained disease-free at the end of the study in July 2020 (Figure 1e).

In summary, in our cohort of 52 IRBD patients, 48 (92%) were positive for CSF α-synuclein RT-QuIC (47 at baseline and one on serial assessment).

## Discussion

We found that in IRBD 1) RT-QuIC identifies misfolded α-synuclein in CSF samples with a sensitivity and specificity of 90%, 2) these high degrees of sensitivity and specificity are similar to those found previously in manifest PD and DLB[5-12], 3) IRBD patients who were CSF α-synuclein RT-QuIC positive had a higher risk of developing PD or DLB than those who were negative, 4) CSF α-synuclein RT-QuIC was positive up to nine years prior to the clinical and pathological diagnoses of PD or DLB, 5) in the majority of IRBD patients who underwent serial LP, all CSF samples were CSF α-synuclein RT-QuIC positive over the entire course of their follow-up, and finally 6) in all the patients who were CSF α-synuclein RT-QuIC positive and underwent postmortem examination, neuropathology showed widespread Lewy-type α-synuclein pathology in the brain, the neuropathological finding seen in patients with RBD confirmed by polysomnography.[3] In addition, we found that RBD in the setting of narcolepsy type 1 is associated with CSF α-synuclein RT-QuIC negativity, as recently observed.[11] We have also found that autosomal dominant Alzheimer disease does not necessarily show underlying synucleinopathy.[17]

Forty-seven of 52 (90%) IRBD patients were CSF α-synuclein RT-QuIC positive at baseline assessment. Only five (10%) patients were CSF α-synuclein RT-QuIC negative. These five negative patients had lower prodromal PD probability and lower risk of developing a clinically-defined α-synucleinopathy compared to the IRBD patients with positive CSF α-synuclein RT-QuIC. A negative CSF α-synuclein RT-QuIC in IRBD, however, did not seem to imply an absent risk for developing a clinically-defined α-synucleinopathy since 1) one patient with baseline negative CSF α-synuclein RT-QuIC was diagnosed with PD 7.5 years after LP, and 2) one patient with baseline negative CSF α-synuclein RT-QuIC had a positive CSF α-synuclein RT-QuIC on a subsequent CSF sample. We can speculate that patients with negative CSF α-synuclein RT-QuIC may have had a smaller burden of pathology in the brain at the time of LP. It is possible that in the IRBD patients with baseline negative CSF α-synuclein RT-QuIC the performance of serial

CSF assessments would show positivity over time as the neurodegenerative process advances. Alternatively, it might be possible that some IRBD patients with negative CSF α-synuclein RT-QuIC response represent prodromal MSA since 1) about 5% of the IRBD patients eventually develop MSA[4], and 2) patients with manifest MSA frequently show CSF α-synuclein RT-QuIC negativity,[10,11] probably because this condition has a different conformational strain of α-synuclein in the brain and CSF, than in PD and DLB.[26] Longer follow-up of our IRBD patients with CSF α-synuclein RT-QuIC negativity is needed to reveal its significance. Longer follow-up is also needed in those CSF α-synuclein RT-QuIC positive IRBD patients that were clinically disease-free at the end of the study as this explains why the specificity for conversion to clinically defined α-synucleinopathy was only 20%.

Among the IRBD patients who were CSF α-synuclein RT-QuIC positive, those who converted to a clinically-defined α-synucleinopathy had a higher frequency of prodromal PD, worse smell identification and more soft parkinsonian signs compared to those who remained disease-free. As both groups had similar follow-up periods, this finding indicates that at the time of LP the neurodegenerative process was more likely to be advanced in those who later converted compared to those who remained disease-free. This is in line with studies showing that IRBD patients showing robust prodromal PD markers such as abnormal DAT-SPECT[20] and hyposmia[4] have increased short-term risk for development of a clinically-defined α-synucleinopathy.

Serial CSF assessments in IRBD patients found that baseline α-synuclein RT-QuIC positivity remain positive during the prodromal stage of PD and DLB. In one IRBD patient with baseline negative response, the performance of serial evaluations disclosed a positive reaction when the subject was still disease-free, suggesting that the RT-QuIC assay could be used to monitor disease progression in a few initially negative cases.

Four of 40 (10%) controls, aged between 63 and 74 years at the time of the LP, were CSF α-synuclein RT-QuIC positive giving 90.0% specificity for the test. The reported specificity of CSF α-synuclein RT-QuIC assay varies from 80%-100%.[5,8-12,27] The higher specificities (96%-100%) were found in studies where CSF samples included

individuals who had subsequent neuropathological investigation and α-synuclein pathology was found to be absent in the brain, and also in living controls with a mean age less than 60 years.[5,8,11] In contrast, studies that included living healthy subjects over the age of 60 years have shown a variable but lower specificity of 80%-98%, which probably reflects the presence of incidental Lewy bodies in the brain (known to be common in this age group).[28] Autopsies in individuals without antemortem neurological diseases have identified incidental α-synuclein pathology in the brain in 10-20% cases of subjects over the age of 70 years.[28] Of note, one study evaluating 13 neuropathologically confirmed cases of sporadic Alzheimer disease with incidental Lewy bodies with a mean age of 79.8 years found CSF α-synuclein RT-QuIC positivity in 15% of cases using 15μL of CSF.[5] Following up our healthy controls with positive CSF α-synuclein RT-QuIC will help to elucidate the significance of these findings. This is important since in one study two controls who were positive for α-synuclein in the CSF using the protein misfolded cyclic amplification (PMCA) assay were diagnosed with PD one and four years after sample collection.[29]

RT-QuIC is an emerging ultrasensitive technique able to detect pathologically misfolded proteins in the CSF.[29] RT-QuIC was originally developed to identify misfolded prion proteins in the CSF, and because of its high accuracy, this test serves as a diagnostic tool for the diagnosis of sporadic Creutzfeldt-Jakob disease.[30] The RT-QuIC assay has been adapted to detect other misfolded proteins such as α-synuclein, and CSF α-synuclein RT-QuIC showed a high degree of sensitivity (90-95%) for the diagnosis of PD and DLB.[5-8,11,12] RT-QuIC is also able to detect misfolded α-synuclein in samples from brain homogenates[5,11,31] and peripheral tissues including the olfactory mucosa[32] and the submandibular gland[33] of patients with manifest PD and DLB. PMCA is another seeding aggregation assay able to detect misfolded α-synuclein in the brain. PMCA also detects and amplifies α-synuclein in the CSF of PD and DLB patients with high sensitivity and specificity showing 92% concordance with the RT-QuIC assay.[27]

In IRBD, identifying pathological α-synuclein in the CSF and peripheral organs has been the focus of previous

studies. Measuring total and oligomeric α-synuclein in the CSF using immunoassays demonstrated variable results with one study reporting no difference between IRBD patients and controls[14] and another reporting lower concentration of total α-synuclein in IRBD patients than in controls.[15] In each study there was significant overlap between IRBD patients and controls. In IRBD, detection of phosphorylated α-synuclein by immunohistochemistry in biopsies of the colon, salivary glands and skin has yielded different sensitivities ranging from 24% to 89%, and the procedures have different invasiveness.[34-39] It remains to be determined which peripheral organ is the best to detect phosphorylated α-synuclein in IRBD. In contrast to peripheral tissue biopsies performed in IRBD using immunohistochemistry, our present work in the CSF using the RT-QuIC assay demonstrated higher sensitivity to detect pathological α-synuclein, included larger numbers of IRBD patients, and participants were followed up for several years assessing the significance of the baseline findings. A recent study using RT-QuIC showed sensitivity of 44% and specificity of 90% to detect α-synuclein in the olfactory mucosa of IRBD patients.[40] CSF probably reflects more accurately a central nervous neurodegenerative process than peripheral tissues.

There is a need for neuroprotective therapy in the α-synucleinopathies. For the design of neuroprotective trials in the prodromal stage of the α-synucleinopathies, target populations should consist of subjects at high risk of developing a clinically defined synucleinopathy, particularly if carrying the neuropathological substrate that define the condition such as misfolded α-synuclein. Patients with IRBD are a suitable population to enter in such trials.[1] Our current findings could be important in designing neuroprotective trials in IRBD, particularly using strategies against α-synuclein.

Our study has limitations. First, this is a single-centre study without independent replication. Second, we included a relatively small number of participants. The high sensitivity (90%) obtained thanks to only five CSF alpha-synuclein RT-QuIC negative IRBD patients may have a detrimental effect on the estimation of the risk of conversion in this group. Third, a small number of neurological controls were included. However, CSF α-

synuclein RT-QuIC positivity is very rare in neurological diseases not linked to α-synuclein pathology in the brain.[5,10,11] Fourth, a subgroup of healthy controls at baseline did not undergo ancillary tests. However, they underwent neurological examination, neuropsychological tests and brain MRI that were unremarkable. Moreover, healthy controls that were CSF α-synuclein RT-QuIC positive at baseline underwent complete characterization at the end of the study that included clinical history, neurological examination, neuropsychological tests, video-polysomnography, DAT-SPECT, smell test and calculation of the prodromal PD probability. Finally, not all the patients in the cohort underwent serial LP assessments and neither did our controls. Strengths of our work include the blinded analyses of all CSF samples, a long follow-up observational period of more than seven years on average in both patients and controls, and the neuropathological confirmation of α-synuclein pathology in the six IRBD patients who underwent postmortem brain examination and were CSF α-synuclein RT-QuIC positive.

In summary, the present study showed that RT-QuIC detects pathologic α-synuclein in the CSF of IRBD patients with a high degree of sensitivity and specificity, reflecting the underlying neurological aetiopathogenesis of the Lewy-type synucleinopathies at their prodromal stage. CSF α-synuclein RT-QuIC positivity represents a strong prodromal biofluid marker of PD and DLB and could be used to select IRBD patients in future neuroprotective trials.

## Data sharing

De-identified participant clinical, RT-QuIC and neuropathological data are available upon request to the corresponding authors with publication. Data underlying this report will be available only for investigators whose proposed use of the data has been approved by an institutional review board, one of the institution to which the investigator requesting the data is affiliated, or who have a certificate of exemption and agree to the terms and conditions of the data use agreement.

## STARD guidelines for studies of diagnostic accuracy

We ensure that our report conforms to STARD guidelines for studies of diagnostic accuracy.

## Research in context

### Evidence before the study

We searched Medline for articles published in any language between January 1st 1987 and November 7th 2020, with the search terms "idiopathic REM sleep behaviour disorder" "Parkinson disease", "dementia with Lewy bodies", "synuclein", and "RT-QuIC", and found no previous studies focusing on the search for misfolded α-synuclein in the CSF of IRBD patients. In one article that evaluated CSF α-synuclein RT-QuIC in 122 subjects with a variety of neurological conditions the cohort included three patients with IRBD in whom α-synuclein was detected in all three. Another study of 439 CSF samples found α-synuclein RT-QuIC positivity in 18 of 18 IRBD patients. No detailed baseline and follow-up information of these 21 IRBD patients with CSF α-synuclein RT-QuIC positivity was reported in these two studies.

### Added value of this study

In the setting of IRBD, our findings indicate that CSF α-synuclein RT-QuIC is a highly sensitive and specific assay for detecting misfolded α-synuclein. This indicates that CSF α-synuclein RT-QuIC positivity is a strong marker of prodromal PD and DLB.

### Implications of all the available evidence

As our current contribution shows that RT-QuIC detects misfolded α-synuclein in most IRBD patients this information would be useful to design neuroprotective trials in IRBD, particularly when using agents to interfere with the deposition and propagation of misfolded α-synuclein throughout the brain.

## Figure legends

### Figure 1

α-synuclein seeding activity in the cerebrospinal fluid of isolated rapid-eye-movement sleep behavior disorder patients and of healthy controls

a), b), c) and d): CSF α-synuclein RT-QuIC expressed as mean ± SD of percentage of maximal relative fluorescence unit in a) IRBD patients who converted to PD (n=16, blue) vs. CSF α-synuclein RT-QuIC negative healthy controls (n=36, purple); b) IRBD patients who converted to DLB (n=16, yellow) vs. CSF α-synuclein RT-QuIC negative healthy controls n=36, purple); c) IRBD patients who remained disease-free at the end of the study (n=20, green) vs. CSF α-synuclein RT-QuIC negative healthy controls (n=36, purple), and d) CSF α-synuclein RT-QuIC positive healthy controls (n=4, red) vs. CSF α-synuclein RT-QuIC negative healthy controls (n=36, purple). All results are expressed as the percentage of the mean ± standard deviation relative fluorescence unit of all CSF α-synuclein RT-QuIC results at each given time point.

e): Serial CSF α-synuclein RT-QuIC in a patient with IRBD. Three consecutive samples were negative for CSF α-synuclein RT-QuIC (time=3 years after IRBD diagnosis, grey; time= 3.5 years after IRBD diagnosis, green; time= 4.6 years after IRBD diagnosis, red). A subsequent sample taken 5.4 years after IRBD diagnosis and 29 months after initial lumbar puncture was positive for CSF α-synuclein RT-QuIC (blue).

CSF: cerebrospinal fluid; RT-QuIC: real-time quaking-induced conversion; IRBD: isolated REM sleep behavior disorder; PD: Parkinson disease; DLB: dementia with Lewy bodies.

**Figure 2**

Baseline cerebrospinal spinal fluid α-synuclein real-time quaking-induced conversion responses in patients with isolated rapid eye movement sleep behavior disorder and healthy controls.

After lumbar puncture, patients were followed-up clinically for a mean of 7.1 ± 2.8 years and controls for a mean follow-up of 7.7 ± 2.9 years until the end of the study.

IRBD: isolated rapid eye movement sleep behavior disorder; CSF: cerebrospinal fluid; RT-QuIC: real-time quaking-induced conversion; PD: Parkinson disease; DLB: dementia with Lewy bodies.

**Figure 3**

A. Kaplan-Meier analysis showing the rates of neurological disease-free survival according to time of baseline lumbar puncture in patients with isolated rapid eye movement sleep behavior disorder with positive and negative real-time quaking-induced conversion responses in the baseline cerebrospinal fluid.

CSF α-synuclein RT-QuIC negative: A negative response to α-synuclein detection in the cerebrospinal fluid.

CSF α-synuclein RT-QuIC positive: A positive response to α-synuclein detection in the cerebrospinal fluid.

B. Kaplan-Meier analysis showing the rates of neurological disease-free survival according to time of baseline lumbar puncture in participants (isolated rapid eye movement sleep behavior disorder patients plus healthy controls) with positive and negative real-time quaking-induced conversion responses in the baseline cerebrospinal fluid.

CSF α-synuclein RT-QuIC negative: A negative response to α-synuclein detection in the cerebrospinal fluid.

CSF α-synuclein RT-QuIC positive: A positive response to α-synuclein detection in the cerebrospinal fluid.

## Acknowledgments

AJEG and GF are funded by the Department of Health and Social Care Policy Research Programme and the Scottish Government. The views expressed in this publication are those of the authors and not necessarily those of the Department of Health or the Scottish Government. The CSF α-synuclein RT-QuIC analysis was funded by the Weston Brain Institute (Grant number R83853).

## Funding

Department of Health and Social Care Policy Research Programme, Scottish Government and the Weston Brain Institute

## Declaration of interests

AI reports personal fees for Abbvie, Roche, Takeda, Jazz and UCB outside the submitted work. RSV received fees for attending to advisory boards from Wave Pharmaceuticals and Ionis. Eduardo Tolosa received honoraria for consultancy from TEVA, Bial, Prevail, Boehringer Ingelheim, Roche and BIOGEN and has received funding for research from Spanish Network for Research on Neurodegenerative Disorders (CIBERNED) - Instituto Carlos III (ISCIII), and The Michael J. Fox Foundation for Parkinson's Research (MJFF). The other authors declare no competing interests.

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**Table 1. Cerebrospinal spinal fluid α-synuclein real-time quaking-induced conversion assay according to clinical status at the end of the study**

| Clinical status at the end of study | Positive CSF α-synuclein RT-QuIC (n, %) | T50 (*)(hours) | P value(**) | Final ThT fluorescence value (rfu)in positive RT-QuIC cases | P value (+) |
|---|---|---|---|---|---|
| **Remain IRBD (n=20)** | 16 (86%) | 63.5 ± 15.4 | 0.13 (IRBD vs. PD) | 252864 ± 26935 | 0.60 (IRBD vs. PD) <0.00001 (IRBD vs. Contr |
| **Converted to PD (n=16)** | 15 (94%) | 62.5 ± 12.5 | 0.30 (PD vs. DLB) | 252801 ± 26935 | 0.34 (PD vs. DLB) <0.00001 (PD vs. Control |
| **Converted to DLB (n=16)** | 16 (100%) | 57.9 10.1 | 0.20 (IRBD vs. DLB) | 260000 ± 0 | 0.17 (IRBD vs. DLB) <0.00001 (DLB vs. Contro |
| **Healthy controls (n=40)** | 4 (10%) | 64.8 ± 20.7 | Not applicable | 259,900 ± 200 | Not applicab |

Data are presented as number, percentage, mean and standard deviation. The mean T50 and final fluorescence were similar between IRBD patients and healthy controls (Table 1, Figure 1). CSF: cerebrospinal fluid; RT-QuIC: real-time quaking-induced conversion; IRBD= isolated rapid eye movement sleep behavior disorder; PD= Parkinson disease; DLB; dementia with Lewy bodies; T50= time that took to obtain 50% of the maximal relative fluorescence unit. Tht: thioflavin T; rfu: relative fluorescence unit; (*): time taken to reach 50% maximal relative fluorescence unit in hours; (**): comparisons of T50 between groups; (+): comparisons of thioflavin T fluorescence between groups. Statistical differences between groups were determined using Student's t test.

**Table 2.Comparisons between patients with positive CSF α-synuclein RT-QuIC according to clinical status at the end of study**

| | Disease-free (n=16) | PD and DLB (n=31) | P value |
|---|---|---|---|
| Age at IRBD diagnosis (y) | 67.1 ± 6.4 | 68.7 ± 6.4 | 0.42 (*) |
| Age at LP (y) | 71.0 ± 6.9 | 71.9 ± 5.6 | 0.64 (*) |
| Interval between IRBD diagnosis and LP (y) | 3.8 ± 3.5 | 3.0 ± 2.6 | 0.58 (^) |
| Interval between LP and end of the study (y) | 6.6 ± 2.4 | 7.0 ± 3.0 | 0.62 (^) |
| Interval between IRBD diagnosis and end of the study (y) | 10.4 ± 4.4 | 9.9 ± 4.2 | 0.89 (^) |
| Prodromal PD probability at the time of LP (%) | 77.9 ± 32.9 | 95.3 ± 12.5 | 0.14 (^) |
| Prodromal PD probability ≥80% at the time of LP, n (%) | 10 (62.5) | 29 (93.5) | 0.013 (=) |
| Abnormal DAT-SPECT, n (%) | 9 (56.3) | 23 (74.2) | 0.21 (=) |
| MDS-UPDRS-III, n | 1.6 ± 1.9 | 4.5 ± 4.2 | 0.012 (^) |
| UPSIT-40 score, n | 18.5 ± 5.8 | 15.2 ± 4.0 | 0.048 (^) |
| Depression, n (%) | 5 (31.3) | 15 (48.4) | 0.26 (=) |
| Constipation, n (%) | 12 (75.0) | 24 (80.0) | 0.69 (=) |

Data are presented as number, percentage, mean and standard deviation. CSF: cerebrospinal fluid; RT-QuIC: real-time quaking-induced conversion; IRBD= isolated rapid eye movement sleep behavior disorder; PD= Parkinson disease; DLB; dementia with Lewy bodies; LP= lumbar puncture; DAT-SPECT= dopamine transportar imaging single photon emission computed tomography; MDS-UPDRS III= motor part of the Movement Disorders Society Unified Parkinson Disease Rating Scale; UPSIT= Spanish version of the 40 item University of Pennsylvania Smell Identification Test. Group comparisons were made using Student t test (*), Mann-Whitney U test (^) and the chi-square test (=).

**Table 3. Assessments of the healthy controls with α-synuclein RT-QuIC positivity in the cerebrospinal fluid at the time of lumbar puncture and at the end of the study**

PD: Parkinson disease; MDS-UPDRS III: motor part of the Movement Disorders

| | Control 1 | Control 2 | Control 3 | Control 4 |
|---|---|---|---|---|
| Interval between lumbar puncture and end of the study, y | 10.6 | 10.6 | 9.6 | 3.4 |
| Assessments at the time of lumbar puncture | | | | |
| Age, y | 74 | 69 | 63 | 65 |
| Prodromal PD probability, % | 0.24 | 0.12 | Not available | Not available |
| Cognitive complaints | No | No | No | No |
| Neuropsychological examination | Normal | Normal | Normal | Normal |
| Motor complaints | No | No | No | No |
| Neurological examination | Normal | Normal | Normal | Normal |
| MDS-UPDRS-III, n | 0 | 1 | Not done | Not done |
| Abnormal DAT-SPECT | No | No | Not done | Not done |
| Subjective smell loss | No | No | No | No |
| UPSIT-40 score, n | 35 | 25 | Not done | Not done |
| Depression | No | No | No | Yes |
| Constipation | Yes | No | No | No |
| Dream-enacting behaviors by history | No | No | No | No |
| RBD at V-PSG | No | No | Not done | Not done |
| Assessments at the end of the study (2020) | | | | |
| Age, y | 84 | 80 | 72 | 68 |
| Prodromal PD probability, % | Not done | 20.3 | 10.8 | 1.0 |
| Cognitive complaints | Not done | No | No | No |
| Neuropsychological examination | Not done | Normal | Normal | Normal |
| Motor complaints | Not done | No | No | No |
| Neurological examination | Not done | Normal | Normal | Normal |
| MDS-UPDRS-III, n | Not done | 5 | 3 | 4 |
| Abnormal DAT-SPECT | Not done | Not done | Yes | Not done |
| Subjective smell loss | Not done | No | No | No |
| UPSIT-40 score, n | Not done | 18 | 31 | 33 |
| Depression | Not done | No | No | Yes |
| Constipation | Not done | No | No | No |
| Dream-enacting behaviors by history | Not done | No | No | No |
| RBD at V-PSG | Not done | No | No | No |

Society Unified Parkinson Disease Rating Scale; DAT-SPECT: dopamine transportar imaging single photon emission computed tomography; UPSIT: University of Pennsylvania Smell Identification Test that was indicative of smell loss when the score was lower than 19 points[33]; RBD: rapid eye movement sleep behaviour disorder; V-PSG: video-polysomnography.