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Olfactory Epithelium Amyloid-β and PHFtau Pathology in Alzheimer's Disease

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Abstract

Objectives—Olfactory dysfunction is common in Alzheimer's disease (AD) and other neurodegenerative diseases. PHFtau, α-synuclein and amyloid-β lesions occur early and severely in cerebral regions of the olfactory system and they have also been observed in olfactory epithelium (OE). However, their frequency, abundance, disease specificity, and relationships of OE pathology to brain pathology have not been established.

Methods—We investigated the pathological expression of amyloid-β, PHFtau, α-synuclein, and TDP-43 in postmortem OE of 79 cases with AD, 63 cases with various other neurodegenerative diseases, and 45 neuropathologically normal cases.

Results—Amyloid-β was present as punctate and small patchy aggregates in 71% of AD cases compared to 22% of normal cases and 14% of cases with other diseases and in greater amounts in AD than either of the other two diagnostic categories. PHFtau was evident in dystrophic neurites in 55% of cases with AD, 34% with normal brains, and 39% with other neurodegenerative diseases, also at higher densities in AD. α-Synuclein was present in dystrophic neurites in seven cases, six of whom also had cerebral Lewy bodies. Pathological TDP-43 inclusions were not observed in the OE in any cases. Amyloid-β and to a lesser degree, PHFtau ratings in OE significantly correlated with cortical Aβ and PHFtau lesion ratings in the brain.

Interpretation—These data demonstrate that AD pathology in the OE is present in the majority of cases with pathologically verified AD and correlates with brain pathology. Future work may assess the utility of amyloid-β and PHFtau measurement in OE as a biomarker for AD.

Olfactory dysfunction is an early and common sign in various neurodegenerative diseases. Microsmia or anosmia is present in approximately 90% of patients with Alzheimer's disease (AD)1 and accumulating evidence suggests that this psychophysical biomarker predicts incident mild cognitive impairment (MCI),2 conversion of MCI to AD,3 more rapid cognitive decline in older adults initially free of dementia,4 and also correlates with severity of dementia and abundance of neurodegenerative disease pathology in the brain.5, 6

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Olfactory deficits are found in other neurodegenerative disorders as well, especially Parkinson's disease[7, 8] and Huntington disease,[9] and to varying degrees in frontotemporal dementias and corticobasal syndromes,[10] pure autonomic failure,[11] multiple system atrophy,[12] Guamanian ALS-Parkinson's-Dementia Complex,[13] idiopathic ALS,[14] progressive supranuclear palsy,[15] and cerebellar degenerations.[16, 17] The neuropathological basis of olfactory dysfunction in AD and PD is thought to be due mainly to accumulations of disease-related lesions, especially tau pathology in AD and α-synuclein pathology in PD, that occur in the olfactory bulb and primary olfactory sensory cortices of the cerebrum.[18–23]

The olfactory epithelium (OE) is a pseudostratified columnar epithelium lying deep within the recesses of the superior nasal cavity (for review, see[24]). It is composed of a mixture of mulitpotential and committed stem cells (basal cells), supporting cells, and chemosensory olfactory receptor neurons. Mature neurons give rise to fine, unmyelinated axons that form bundles (olfactory fila) that ascend through the cribriform plate to synapse in the olfactory bulb. Early research on the OE in AD described tau and neurofilament immunoreactive dystrophic neurites in the lamina propria adjacent to the epithelium layer in subjects with AD but not control subjects.[25, 26] However, subsequent studies reported that dystrophic neurites, which also were found to express ubiquitin, α-, β-, and γ-synucleins, and amyloid-β, could be found in normal aging and other neurodegenerative diseases as well.[27–30] In light of this presumed non-specificity, interest in the OE for diagnostic or mechanistic research into AD, PD, and other neurodegenerative diseases waned. Still, these previous studies are relatively few in number, had small sample sizes in each diagnostic group, and may have been otherwise confounded by the limited sensitivity and specificity of reagents available at the time and a variety of other technical challenges inherent in working with OE tissues. Thus, the frequency, extent, and disease specificity of these lesions and other forms of pathological protein expression have not been established, and the degree to which they may be associated with brain pathology or olfactory dysfunction is not known.

Over the last twenty years, we have collected OE in many neurodegenerative disease and non-neurological control cases that have been autopsied in protocols of the University of Pennsylvania's Center for Neurodegenerative Disease Research (CNDR). At the same time, there have been steady improvements in the sensitivity and specificity of immunochemical reagents to reveal pathological proteins in tissue, as well as the discovery of new proteins, such as TDP-43, that play important roles in neurodegenerative diseases.[31] Here we present findings in a large sample of neuropathologically verified AD and control cases, as well as other neurodegenerative diseases representing a spectrum of tauopathies, synucleinopathies, and TDP-43 proteinopathies.

Materials and Methods

Subjects and Pathological Assessment

Fixed, paraffin-embedded tissue blocks of OE from 187 subjects were obtained from the CNDR Brain Bank at the University of Pennsylvania representing AD, neuropathologically normal brains, and other major neurodegenerative diseases including α-synuclein/Lewy body diseases, tauopathies, and TDP-43-related ALS and frontotemporal dementias. Consent for autopsy had been obtained from legal representatives for all subjects in accordance with local institutional review board requirements.

Subject characteristics, including diagnoses, sample sizes, ages, sex, and postmortem intervals are summarized in Table 1. The major neuropathological diagnostic groups (AD, normal, "other neurodegenerative disease") differed in age at time of death ($F_{[2,183]} = 8.9$, $p<0.001$). Decomposition of this effect revealed that the other neurodegenerative disease

subjects were younger than both normal ($F_{[1,183]}$=15.2, p<0.001) and AD ($F_{[1,183]}$=10.8, p=0.001) subjects, whereas AD subjects and normals did not differ from one another. Differences in sex distribution were also seen between the three groups (χ^2 = 9.6, df=2, p = 0.008), with the AD group having more females and the other neurodegenerative disease having more males. We therefore included age and sex as covariates in all subsequent analyses. No differences were seen among the three groups in postmortem interval (PMI) ($F_{[2,184]}$= 2.7, p = 0.065).

All cases had gross and microscopic diagnostic neuropathological examinations of their brains at the time of death, which included examination of multiple cortical and subcortical regions by trained neuropathologists, and diagnoses were established in accordance with published guidelines for neurodegenerative diseases.32–35 These examinations included thioflavin S staining in addition to immunohistochemical staining of sections with antibodies directed at a) amyloid-β for diffuse and neuritic plaques (NAB228, a mouse monoclonal antibody raised against $A\beta_{1-11}$ synthetic peptide, Santa Cruz Biotechnology, Santa Cruz, CA, 1:5000), b) hyperphosphorylated tau for neurofibrillary tangles and dystrophic neurites (PHF136, 1:800), c) α-synuclein for Lewy bodies and Lewy neurites (SYN30337, 1:4000) and d) TDP-43 for pathological inclusions using a phospho-independent antibody (Protein Tech Group, Chicago, IL, 1:4500) and a recently described rat monoclonal antibody to TDP-43 phosphorylated at residues 409/410 (1:1000).38 Semiquantitative ratings on a 0–3 ordinal scale were recorded for pathological expression of each of these proteins in hippocampus, entorhinal cortex, amygdala, superior/middle temporal gyri, angular gyrus, mid-frontal gyrus, and anterior cingulate cortex. These scores were averaged into a global brain rating for each protein for subsequent correlation analyses with OE pathological ratings.

Olfactory Epithelium Tissue Processing and Immunohistochemistry

At autopsy, the OE, bony septae, and contiguous cribriform plate were removed en bloc and fixed for 24–36 hours in 10% neutral buffered formalin, Bouin's fixative or 70% ethanol, 150 mM NaCl. As previously described,30 tissues were decalcified for 14–16 days in distilled water with sodium ethylenediaminetetraacetic acid, sodium hydroxide, and glycerol at pH 7.1 to 7.4, cut into coronal blocks, dehydrated in graded ethanols to xylene and embedded in paraffin. Six μm thick serial sections were cut from a central block of olfactory tissue in each case, and mounted on glass slides for staining.

OE sections were immunolabeled using antibodies: a) NAB228 for Aβ (1:500), b) NAB61, a monoclonal antibody that recognizes a pathologic conformation present in Aβ dimers, soluble oligomers, and higher order species of Aβ (1:100),39 c) PHF1 for hyperphosphorylated (1:500),36 d) SYN303 forα-synuclein (1:4000) and e) anti-TDP-43 (1:4500) and anti-phospho-TDP43 (1:1000) for TDP43 pathological inclusions.

Immunohistochemistry was performed by means of the avidin biotin complex detection technique (Vectastain ABC kit; Vector Laboratories, Burlingame, California) with 3,3-diaminobenzidine as chromogen, using previously described methods.40 Sections were boiled in citrate antigen unmasking solution (Vector Laboratories) or pre-incubated in formic acid for antigen retrieval and counterstained with hematoxylin after immunohistochemistry. All cases, as well as positive and negative control slides (with and without primary antibody), were run simultaneously with precise timing of reactions for each run. In addition, selected cases were double immunofluorescence stained with NAB228 for Aβ and anti-β-tubulin-III (clone SDL.3D10, Sigma, St. Louis, MO, 1:500) for neurons.

Microscopy and Quantification of Pathology

OE sections were inspected for abnormal cellular immunoexpression and immunolabeled pathological lesions including intracellular inclusions, extracellular aggregates and dystrophic neurites in the epithelial layer and subjacent lamina propria. Semi-quantitative pathological expression ratings were performed in OE areas observed to have the most robust pathology using an ordinal scale (0, none; 1, mild; 2, moderate; 3, severe; see Figure 1). Sections were scored by trained technicians who were masked to diagnosis and all other case information. Ratings were validated by principal researchers (S.E.A., E.L., J.Q.T.). Digital images of stained sections were obtained using an Olympus BX 51 microscope equipped with bright-field light source using digital camera-DP71, and DP manager (Olympus, Orangeburg, NY, USA).

Statistical Analyses

Statistical analyses were conducted using the General Linear Model (GLM) as implemented in STATISTICA™(StatSoft®, Tulsa, OK). Analyses of covariance (ANCOVA) were performed with group membership as the between-subjects factor, and concentration of amyloid-β, PHFtau, α-Synuclein or TDP-43 in the OE as the dependent measure, and age at time of death and sex as covariates. Significant multivariate effects were dissected by appropriate post-hoc univariate contrasts. Chi-square analyses were utilized for categorical data and Spearman correlations were used to assess the relationships between OE pathology ratings of amyloid-β, PHFtau, α-synuclein or TDP-43 with averaged cortical pathology ratings of these variables.

Results

OE Amyloid-β

Amyloid-β expression in the OE was observed in 75 of the 187 cases and its presence was significantly more common in AD cases than in neuropathologically normal cases and cases with other neurodegenerative diseases. ($\chi^2 = 54.6$, df = 2, $p < 0.001$). As seen in Figure 1, amyloid-β was found primarily as intracellular granular or vesicular appearing cytoplasmic aggregates and occasional extracellular puncta and aggregates of varying sizes or more diffuse staining that was primarily localized in superficial portions of the OE and to a lesser degree just above the basal cell layer.

Mean OE amyloid-β abundance ratings for the major diagnostic groups are presented in Table 1. Results of ANCOVA revealed significant differences in abundance ratings between the Normal, AD, and “Other” groups ($F_{[2,181]} = 21.1$, $p < 0.001$). Posthoc analyses revealed that AD subjects had greater amyloid-β ratings in OE relative to both normal ($F_{[1,181]} = 26.3$, $p < 0.001$) and other neurodegenerative disease subjects ($F_{[1,181]} = 31.7$, $p < 0.001$). Normal and other neurodegenerative disease subjects did not differ from each other in OE amyloid-β ratings ($F_{[1,181]} = 0.03$, $p = 0.85$).

OE PHFtau

Pathological expression of PHFtau was present in 55% of cases with AD, 34% with normal brains, and 39% with other neurodegenerative diseases ($\chi^2=6.2$, df=2, $p<0.045$). PHFtau expression was mainly observed in dystrophic neurites but also as occasional intracellular staining and neurofibrillary tangles (see Figure 2).

Mean OE PHFtau abundance ratings for the major diagnostic groups are presented in Table 1. Similar to findings with amyloid-β, ANCOVA revealed significant differences in PHFtau abundance ratings in OE between the normal, AD, and other neurodegenerative disease groups ($F_{[2,172]} = 6.08$, $p = 0.003$). Posthoc analyses found that AD subjects had higher

ratings of PHFtau in OE relative to both normals ($F_{[1,172]}$=9.67, p=0.002) and other neurodegenerative disease subjects ($F_{[1,172]}$ = 6.85, p = 0.01). Neuropathologically normal and other neurodegenerative disease subjects did not differ from each other ($F_{[1,172]}$ = 0.29, p = 0.58).

OE α-Synuclein

Pathological α-synuclein immunolabeling was observed in dystrophic neurites and occasional intracytoplasmic inclusions that were most often found in or near the basal cell layer (Figure 3). α–Synuclein expression was observed in only seven cases, including two cases with dementia with Lewy bodies, two with Lewy body variant of AD, and one each with AD, Parkinson's disease, and normal brain.

OE TDP-43

In brain, normal TDP-43 is seen as darkly stained heterochromatic nuclear staining in neurons, while pathological TDP-43 is seen variously as neuronal and glial cytoplasmic inclusions, neuronal intranuclear inclusions, dystrophic cellular processes or axonal swellings, and diffuse granular cytoplasmic staining ("preinclusions").41 No pathological TDP-43 or phosphorylated TDP43 lesions were observed in OE in any of the cases.

Correlation with Cerebral Pathology

Across all three groups, amyloid-β ratings in OE were highly correlated with averaged cortical amyloid-β plaque ratings (Spearman r=0.37, p<0.001) and to a lesser degree, PHFtau in OE correlated with averaged brain PHFtau neurofibrillary pathology (r=0.17, p<0.03). Contrasts between correlation effect sizes revealed that the relationship between amyloid-β ratings and cortical plaque ratings (d=0.79, 95% CI=0.57<δ<1.01) was significantly stronger than OE PHFtau ratings relative to averaged brain PHFtau pathology (d=0.34, 95% CI=0.12<δ<0.56) (QB[1]=7.96, p=0.0048).

Discussion

Pathological expression of PHFtau, amyloid-β, and α-synuclein have been described in the OE of older subjects with and without neurodegenerative diseases (for review, see42) but the frequency, extent, and disease specificity of these lesions have not been established. We found that amyloid-β and PHFtau pathological lesions are significantly more frequent and more abundant in subjects with AD than in normal elderly controls or subjects with other non-AD neurodegenerative diseases.

Pathological amyloid-β was observed in the OE principally as intracellular, cytoplasmic punctate, vesicular, and amorphous aggregates. These inclusions were immunolabeled similarly by both NAB228, an antibody directed against the N-terminus of amyloid-β, as well as NAB61, an antibody that recognizes pathological dimers, soluble oligomers, and higher order species of amyloid-β. Earlier work from our center examined expression of amyloid-β and its flanking sequences of amyloid precursor protein as well as thioflavin-S in the OE in smaller numbers of subjects.28 More diffuse labeling was noted, particularly in the basal third of the OE and was more common in AD (8/9 cases) than normal controls (2/10), but was also common in an assortment of other neurodegenerative diseases (10/12).

A number of small studies of OE obtained in biopsies of living people with AD also have investigated neurodenerative disease lesions and other phenomena that may be associated with neurodegeneration. Several early reports indicated frequent tau, amyloid, and ubiquitin immunoreactive neuritic pathologies in the OE biopsies of AD subjects,25, 26, 43 but subsequent studies did not support these.44, 45 Perry et al.46 examined OE biospy sections

from 8 subjects with probable AD and 3 controls and reported greater expression of oxidative damage markers in AD. Rawson et al.47 used freshly dissociated olfactory receptor neurons obtained by biopsy in 3 AD, 1 vascular dementia, and 14 healthy elderly subjects OE biopsies to measure intracellular calcium flux response to chemical odorant exposure. While this study importantly demonstrated the feasibility of conducting neurophysiological experiments in OE biopsy, no diagnosis-related differences were discerned in this very small sample. Finally, in a recent study of OE biopsies in 7 subjects with Parkinson's disease, Witt et al.48 reported no major histochemical differences (including α-synuclein immunolabeling) in the Parkinson's group compared to control cases.

Over the past decade, better antibodies, such as NAB228 or NAB61, and better antigen retrieval methods have been developed and we surmise that the increased sensitivity and specificity offered by these advances allowed recognition of the extensive intracellular amyloid-β deposition in OE that we report here.

The presence and nature of intracellular amyloid-β in AD has been controversial.49 Amyloid-β is predominantly described in extracellular neuritic plaques in the cerebral cortex in AD and as diffuse plaques in AD and commonly in normal cognitive aging as well.19, 50 Some of the contention about whether intracellular amyloid-β is present in the brains of people with AD may be due to technical issues including sensitivity of stains and antibodies, antigen retrieval methods, and possibly time at which pathological examination occurs in the course of the illness. Nonetheless, many investigations employing a variety of approaches provide compelling evidence for the existence and potential importance of intracellular localization amyloid-β,49 including amyloid-$\beta_{1,42}$ in familial APP mutation AD,51 Down syndrome AD,52 idiopathic AD,53 and in transgenic models of AD.54, 55

The question also arises as to whether the amyloid-β we observed was fibrillar. A previous smaller study from our group that included electron microscopy failed to find fibrillar amyloid-β in OE.28 We attempted to discern fibrillar amyloid here with Thioflavin S fluorescent labeling in select cases with abundant amyloid-β pathology (data not shown). When present, thioflavin S staining was generally similar in distribution pattern to our immunohistochemical labeling, but given the inferior sensitivity of this staining method and the degree of autofluorescence and other artifacts associated with it, the extent of pathology was lower and its nature was difficult to interpret.

PHFtau also distinguished AD from normal control and other neurodegenerative disease cases, though not as much as did amyloid-β in this series. As in previous work of ours and others', PHFtau was especially evident in dystrophic neurites coursing through the lamina propria and in lower portions of the OE. Here, we also observed PHFtau expression in neuron somata with a morphological appearance typical of neurofibrillary tangles. While these were less frequent than dystrophic neurites, their presence is highly noteworthy as, to our knowledge, they have not heretofore been reported.

In the normal adult, the OE consists of clusters of sensory neurons interspersed among patches of metaplastic respiratory epithelium. The number of sensory neurons decreases across the lifespan, with the greatest decrease occurring after the age of 65.56 Furthermore, the proportion of respiratory epithelia increases as the olfactory neuroepithelium atrophies with the cumulative effects of environmental insult, inflammation, or disease. In this study, we did not systematically determine whether the amyloid-β, PHFtau, or α-synuclein lesions that we observed were confined to neuronal versus non-neuronal extents of OE. In several cases in which we conducted double immunofluorescence histochemistry to examine co-localization of the neuron-specific marker β-tubulin-III with amyloid-β (Figure 1C), we found amyloid-β expression in the vicinity of β-tubulin-III immunoreactive neurons, but also

in areas in which there were no β-tubulin-III neurons. We speculate that pathological expression of amyloid-β contributes to olfactory neuron loss in AD and remains in areas of the OE now devoid of olfactory receptor neurons.

Impairments in olfactory functioning in AD and other neurodegenerative diseases are likely due to degenerative changes at multiple levels of the olfactory system and the relative contributions of changes in OE, olfactory bulb, and different olfaction-related cortices are difficult to determine. Consistent with pathology in multiple levels of the olfactory system in AD, a meta-analysis found extremely large effect sizes or impairments in AD across tests of odor identification and detection threshold sensitivity.57 In AD, accumulations of PHFtau neuropil threads and neurofibrillary tangles in the olfactory bulb and nerve have been found in all cases of definite AD, and many cases with probable AD as well as MCI and cognitively normal aging (though these may represent cases at risk for subsequent progression to AD).18, 58 It is also well-established that neurofibrillary pathology is early and severe in entorhinal, perirhinal, and piriform cortices in AD,19, 59, 60 all areas that play roles in odor identification and memory.61, 62 It is notable that the presence of peripheral OE amyloid-β and PHFtau correlated with higher levels of amyloid-β plaque and PHF tau pathology in the cortex, with the strength of this relationship being stronger for amyloid-β. These data suggest that measurement of these markers in OE may have some relevance to prediction of similar pathology in brain in living patients.

Identification and validation of biochemical and neuroimaging biomarkers of AD brain pathology is of major importance for early diagnosis and monitoring of disease-modifying treatment response. Significant strides have been made in characterizing the sensitivity and specificity of cerebrospinal fluid Aβ and tau levels and amyloid-β-ligand imaging with PET for AD and MCI.63, 64 Given the early and predictive functional olfactory changes that occur in people with and at risk for AD,2 the pathological findings for AD in the current study, and the relatively easy accessibility of olfactory epithelium for biopsy,65, 66 we suggest greater attention be paid to investigating the utility of OE as a biomarker source for studies of AD.

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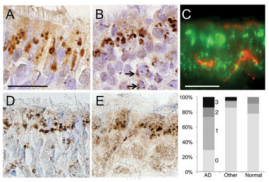

Figure 1.

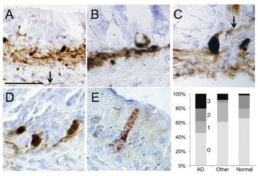

Figure 2.

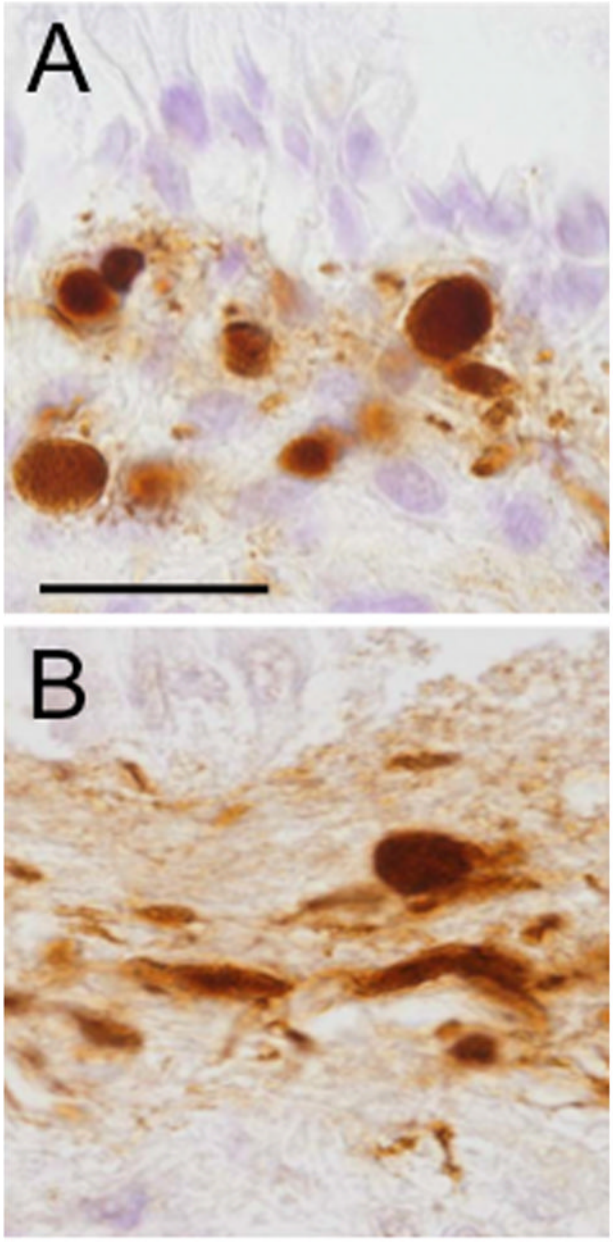

Figure 3.

Table 1

Demographics and Neuropathological Means

Neuropathological Diagnosis	n	Age (SD)	Sex (F/M)	PMI* (SD)	Amyloid-β * Mean (SD)	PHFTau * Mean (SD)
Normal	45	79.1 (10.1)	25/20	10.5 (4.0)	0.3 (0.6)	0.6 (0.9)
Alzheimer's Disease					1.1 (1.0)	1.1 (1.2)
Alzheimer's Disease	73	77.2 (9.6)	49/24	10.6 (5.1)		
Alzheimer's Disease – Lewy Body Variant	6	74.7 (6.6)	3/3	12.8 (4.8)		
Other Neurodegenerative Disease	62	71.2 (11.6)	25/38	12.6 (6.4)	0.3 (0.7)	0.6 (0.9)
α-Synucleinopathies					0.3 (0.7)	0.7 (1.1)
Dementia with Lewy Bodies	17	72.0 (14.6)	3/14	11.8 (4.8)		
Multiple System Atrophy	8	64.9 (11.9)	2/6	17.5 (11.3)		
Parkinson's Disease	7	79.5 (3.7)	2/6	9.1 (4.1)		
Tauopathies					0.4 (1.1)	0.4 (0.5)
Corticobasal Degeneration	3	73.7 (5.5)	3/0	13.5 (6.4)		
Frontotemporal Degeneration with Parkinsonism -17	2	55 (9.9)	1/1	9.0 (4.2)		
Frontotemporal Lobar Degeneration – Tau	2	67.5 (12.0)	0/2	9.5 (0.7)		
Progressive Supranuclear Palsy	8	74.8 (3.6)	6/2	13.9 (6.3)		
TDP-43 Proteinopathies					0.0 (0.0)	0.5 (0.9)
Amyotrophic Lateral Sclerosis	3	69.3 (13.6)	1/2	13.8 (7.8)		
Frontotemporal Lobar Degeneration – Ubiquitin	12	70.3 (11.5)	7/5	12.4 (5.4)		

* Mean of ordinal ratings of severity of pathology for the major neuropathological diagnostic categories