Article

Is Olfactory Epithelium Biopsy Useful for Confirming Alzheimer's Disease?

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Maria Dantas Costa Lima Godoy, MD, PhD[1], Marco Aurélio Fornazieri, MD, PhD[2], Richard L. Doty, PhD[3], Fábio de Rezende Pinna, MD, PhD[1], José Marcelo Farfel, MD, PhD[1], Glaucia Bento dos Santos, BMedSci[1], Mariana Molina, MSc[1], Renata E. L. Ferretti-Rebustini, BSN, RN, CNS, PhD[1], Renata E. P. Leite, PhD[1], Claudia K. Suemoto, MD, PhD[1], Lea T. Grinberg, MD, PhD[4], Carlos A. G. Pasqualucci, MD, PhD[1], Richard Louis Voegels, MD, PhD[1], Ricardo Nitrini, MD, PhD[1], and Wilson Jacob Filho, MD, PhD[1]

Abstract

Objectives: The clinical symptoms of Alzheimer's disease (AD) are preceded by a long asymptomatic period associated with "silent" deposition of aberrant paired helical filament (PHF)-tau and amyloid-beta proteins in brain tissue. Similar depositions have been reported within the olfactory epithelium (OE), a tissue that can be biopsied in vivo. The degree to which such biopsies are useful in identifying AD is controversial. This postmortem study had 3 main goals: first, to quantify the relative densities of AD-related proteins in 3 regions of the olfactory neuroepithelium, namely, the nasal septum, middle turbinate, and superior turbinate; second, to establish whether such densities are correlated among these epithelial regions as well as with semi-quantitative ratings of general brain cortex pathology; and third, to evaluate correlations between the protein densities and measures of antemortem cognitive function.

Methods: Postmortem blocks of olfactory mucosa were obtained from 12 AD cadavers and 24 controls and subjected to amyloid-beta and PHF-tau immunohistochemistry.

Results: We observed marked heterogeneity in the presence of the biomarkers of tau and amyloid-beta among the targeted olfactory epithelial regions. No significant difference was observed between the cadavers with AD and the controls regarding the concentration of these proteins in any of these epithelial regions. Only one correlation significant was evident, namely, that between the tau protein densities of the middle and the upper turbinate ($r = .58$, $P = .002$).

Conclusion: AD-related biomarker heterogeneity, which has not been previously demonstrated, makes comparisons across studies difficult and throws into question the usefulness of OE amyloid-beta and PHF-tau biopsies in detecting AD.

Keywords

Alzheimer's disease, aging, immunohistochemistry, olfactory mucosa, olfaction, olfaction disorders

Introduction

The identification of antemortem biomarkers of Alzheimer's disease (AD), the most common neurodegenerative disease,[1-3] has been the focus of research throughout the world.[4-6] Interest in the use of biopsied olfactory mucosa, which can be sampled in living persons and contains elements of AD-like pathology, as a marker for AD largely stemmed from a study by Talamo et al.[7] These investigators found increased olfactory epithelial reactivity to antibodies raised against neurofibrillary proteins as well as the presence of dystrophic neurites typical to AD

[1]University of São Paulo, São Paulo, Brazil
[2]Department of Surgery, Londrina State University, Londrina, Brazil, and Pontifical Catholic University of Paraná, Londrina, PR, Brazil
[3]Smell and Taste Center, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA
[4]Memory and Aging Center, Department of Neurology, University of California, San Francisco, CA, USA

Corresponding Author:
Marco Aurélio Fornazieri, MD, PhD, Department of Surgery, Londrina State University, 60 Robert Koch Avenue, Londrina, PR 86038350, Brazil.
Email: marcofornazieri@uel.br

within this epithelium. Since this work, other investigators also have shown that such pathology is present within the olfactory epithelium (OE) of patients or cadavers with AD.[8-14]

That being said, the value of OE biopsies in diagnosing AD has been questioned. The pioneering study of Talamo et al[7] was compromised by the fact that a number of the healthy controls were at an age where AD pathology and olfactory dysfunction would not be expected. Thus, 5 of the 14 controls were <40 years old, whereas all but 2 of the 9 AD cadavers were >80 years of age, with the other 2 being 70 and 77 years old. Moreover, subsequent studies have found immunohistochemical markers of AD-like pathology within the OE of healthy elderly persons as well as persons with neurodegenerative diseases other than AD, suggesting lack of specificity.[14-18] Importantly, the hallmark pathologies of AD, namely, neuritic plaques and neurofibrillary tangles, have not been found in the OE of AD cadavers,[7,11] and dystrophic neurites and proteins associated with AD-related pathology appear to occur within the OE from causes unrelated to AD.[19]

One obstacle in determining the value of olfactory biopsies in differentiating AD patients from healthy persons or persons with other neurodegenerative disorders is the difficulty in obtaining true OE. Because the OE becomes potmarked with nonsensory respiratory epithelium in older persons, a major challenge has been to obtain olfactory biopsies that yield a large enough amount of OE to allow for identifying and assessing the density of AD-related pathology. One study, for example, was able to obtain OE from only 46% of the biopsied samples.[14] Moreover, it is unclear whether OE samples from different epithelial regions yield similar degrees of tau and amyloid beta (Aβ) related pathology.

Our study sought to clarify this issue by sampling 3 different regions of the OE in cadavers with and without AD and comparing the density of these 2 AD-related proteins within the sample regions and cortex as well as between the AD and non-AD specimens. Are there sampling regions that optimize the differentiation of pathology density between the AD and non-AD subjects?

Materials and Methods

Participants

A total of 36 autopsy specimens were obtained from donors who contributed their brains to the Brain Bank of the Brazilian Aging Brain Study Group (BB-BABSG). Exclusion criteria for the use of the specimens included (a) the presence of macroscopically detectable lesions, (b) severe chronic conditions that might damage cognitive function prior to death by interfering in brain homeostasis, (c) lack of reliable collateral information related to defining the clinical and functional deficits associated with dementia, (d) indication of acidosis secondary to severe agonal status (CSF pH < 6.5), (e) age less than 50 years old, and (f) evidence of sinonasal disease (eg, nasal polyps, purulent nasal discharge).[20] This study was approved by the local ethics committee, and the family signed the informed consent.

Clinical Diagnosis of AD

A determination of the presence and degree of premorbid cognitive function was assessed using the retrospective version of Informant Questionnaire on Cognitive Decline in the Elderly (IQCODE)[21] and the Clinical Dementia Rating (CDR).[22] The BB-BABSG clinical research method with cognitive retrospective evaluation by informants was validated previously[23] and used in prior studies.[24,25]

Neuropathological Diagnosis of AD

Neuropathological analysis of the brain was performed in accordance with internationally accepted criteria and recommendations.[20] The number of neuritic plaques (amyloid-β) was classified into 4 stages—absent, sparse (mild), moderate, or frequent (severe)—using the semiquantitative criteria proposed by the Consortium to Establish a Registry for Alzheimer's Disease (CERAD).[26] The distribution of NFTs (tau protein) was classified into 6 stages—I through VI—using the criteria proposed by Braak and Braak.[27] Individuals were classified as AD positive using neuropathological criteria (CERAD worse than A and Braak and Braak higher than 2) based on the consensus of the National Institute of Aging-Reagan Institute (1997) and controls those who did not fulfill these criteria (CERAD A or 0 and Braak and Braak ⩽ 2).[28]

Collection, Processing, and Immunohistochemical Analysis of Olfactory Epithelium Samples

Previously described criteria to differentiate olfactory epithelium from the respiratory epithelium were the presence of pseudostratified epithelium, thin basement membrane, irregular cilia, cellular lamina propria, and numerous and large nerve bundles.[29,30] In this study, we used tissue location and the absence of goblet cells and presence of cilia as the main criteria to confirm that the specimen was likely OE. Metaplastic squamous epithelium within the nose does not contain cilia. It is important to note that there are descriptions of metaplastic respiratory epithelium in the olfactory region without goblet cells[31] probably related to previous infections, other toxicologic aggressions by, for example, chemicals exposure in the workplace, or even degeneration due to neurodegenerative diseases. OE samples were harvested at autopsy en bloc and subsequently separated into middle turbinate, superior turbinate, and cribriform plate along with the upper part of the nasal

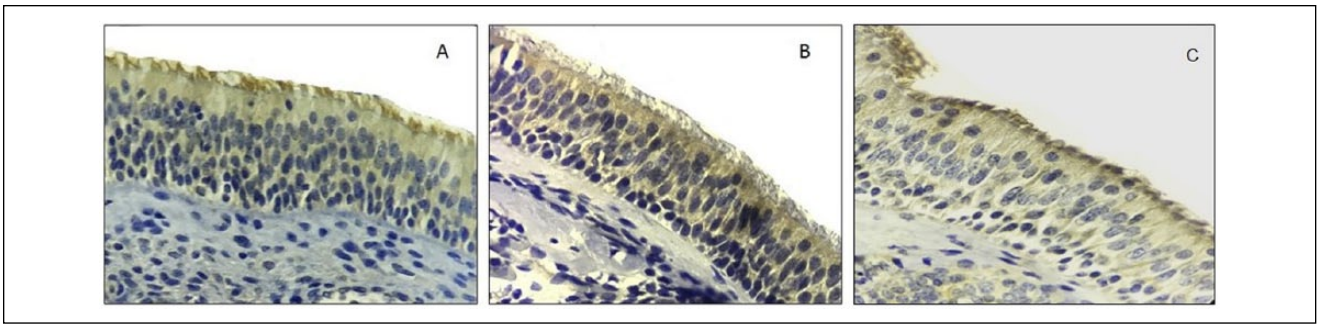

Figure 1. Olfactory epithelium from different nasal regions marked against amyloid-β. (10D5). (A) Middle turbinate. (B) Superior turbinate. (C) Nasal septum.

septum. Mucosa of the most superior part contiguous to the cribiform plate of these 3 regions was used for analysis.

Specimens were fixed in 10% neutral buffered formalin for 10 to 30 days. Afterward, tissues were decalcified with ethylenediaminetetraacetic acid (EDTA), dehydrated, and paraffin-embedded, as previously described.[8] The decalcification period lasted 15 to 45 days, depending on the structure and amount of bone tissue present in the specimen. In addition, the thickest bony component of each specimen, if resistant to decalcification, was removed manually.

Serial sections (6-μm thick) were obtained from each area of OE and mounted into histology slides for staining and immunohistochemical (IHC) analysis. IHC staining was directed at amyloid-β for neuritic plaques (a mouse monoclonal antibody against Aβ1-16, 1:10.000, Elan Pharmaceuticals, South San Francisco, California, USA), and hyperphosphorylated tau for neurofibrillary tangles (PHF-1 1:1.000, provided by Prof. Peter Davies, Albert Einstein College of Medicine, New York, New York, USA). Prior to IHC staining, the appropriate concentration of antibodies for OE sections, in addition to positive and negative controls (with and without antibodies), was determined. Test batteries with the markers were run in the minimum possible time interval to one another.

Sections were mounted on slides, and nonspecific binding was blocked by incubation in methanol with 3% H_2O_2 for 15 min, followed by 3 × 5 min washes in phosphate-buffered saline (PBS) and antigen retrieval by steam at 95°C for 40 min in 0.1 M citrate buffer (pH 6.0). Subsequently, nonspecific binding sites were blocked using 5% fat free milk powder dissolved in PBS for 30 min. Sections were then incubated with the primary antibody against Aβ (10D5) and tau protein (PHF-1) overnight at 4°C. Secondary antibodies (anti-mouse for PHF-1 and 10D5) were incubated with the sections for 1 h at 37°C in a humidified chamber at a concentration of 1:200. Primary and secondary antibodies were diluted in 5% milk/PBS solution. Immunoreactivity was detected by incubation with an avidin-biotin complex (Vectastain ABC Kit, Vector Laboratories, Burlingame, California, USA, for PHF-1 and Vectastain Elite ABC Kit for 10D5) for 1 h at 37°C followed by exposure to the chromogen diaminobenzidine tetrahydrochloride (ImmPACT DAB Peroxidase Substrate, Vector). Between each incubation step, sections were then submitted to 3 × 5 min washes in PBS. Positive and negative tissue controls sections were routinely included for quality control purposes. All sections were counterstained with hematoxylin and mounted on glass slides using synthetic resin.

Microscopy and Quantitation of Tau and Aβ Protein in Olfactory Epithelium

We observed 3 to 5 olfactory epithelial fields of the middle turbinate, superior turbinate, and nasal septum using the same microscope and the same magnification (400×). The area of affected epithelium was categorized on an ordinal scale (0% or 0 = none; 1%-25% = 1, mild; 26%-75% = 2, moderate; >75% = 3, severe), based on previous publications (Figures 1 and 2).[8] Sections were scored by trained technicians, who were blinded to diagnosis and to all other case information. All assessments were validated by the principal investigator (M.D.C.L.G).

Statistical Analysis

Demographic variables were depicted using appropriate measures of central tendency and dispersion. Normality of continuous variables (age, concentration of proteins, and cognitive questionnaires) was tested by the Shapiro-Wilk test. Chi-square and Fisher's exact tests were used to compare proportions (sex and percentage of proteins presence). *t* tests as well as Kruskal-Wallis test and Spearman correlation were employed to compare and correlate the concentration of amyloid-β and tau proteins among different OE regions and brain. Multiple linear regression assessed the influences of the independent variables of diagnostic group (AD and control) and age on the total area of these proteins

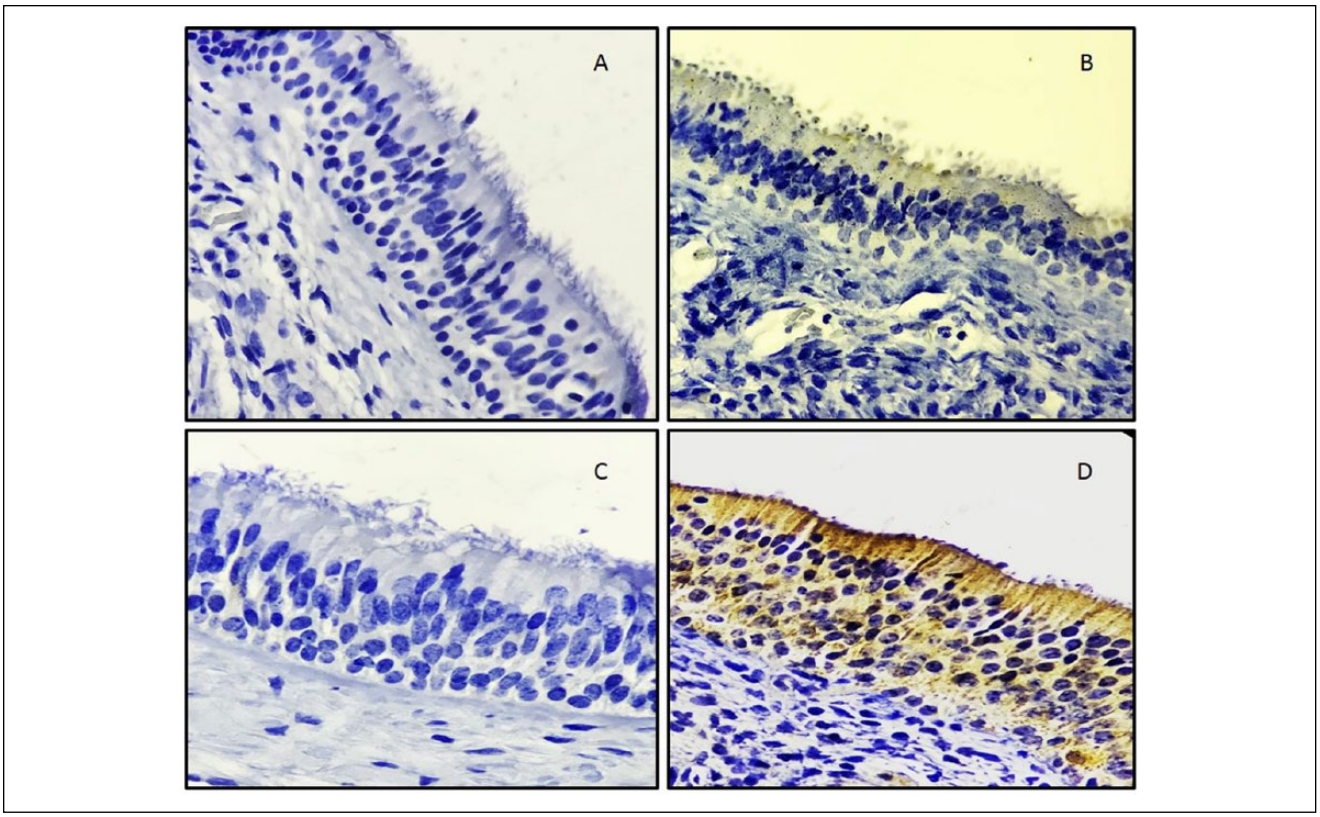

Figure 2. Olfactory epithelia from nasal septum nonmarked (0% affected) against tau and amyloid-β (A and C, respectively) and marked (B = 50% affected, category 2; D = 100% affected, category 3).

in the OE (dependent variable). The statistical significance level was set at 5% in 2-tailed tests.

Results

Demographic characteristics of the study sample, including sex, age, and presence/concentration of tau and beta-amyloid in regions of olfactory epithelium of the superior turbinate, middle turbinate, and nasal septum, are presented in Table 1. Although the controls and AD cadavers did not differ according to gender and race, the cadavers were somewhat older than the controls—none of them younger than 50 years old. Importantly, as also noted in Table 1, we took into account the influences of age in our overall multiple regression analysis and found age to have minimal influences on the control versus AD differentiations.

OE Amyloid-β and PHF-Tau

Controls and subjects in advanced stages of Braak and Braak and CERAD classifications exhibited Aβ and PHF-tau in the OE to the same general degree (Ps $> .05$). When the percentage of positivity for the target markers was compared between the AD and control subjects using the present/nonpresent designation, no statistically significant differences were found for any of sampled regions (Table 1). This was the case even after controlling for age.

Correlation and Comparison of Amyloid- β and PHF-Tau Among Different OE Regions

Tau and amyloid-β concentrations were not uniform across the majority of the target OE regions. As shown in Table 2, only 1 significant correlation was present between the density measures, namely, that between the middle turbinate amyloid-β density and the superior turbinate tau protein density ($r = .58$; $P < .002$).

Correlation With Cerebral Pathology

In general, the concentrations of tau and amyloid-β within the OE did not correlate well with the brain pathology ratings (Table 3). Moreover, correlations among OE densities and clinical questionnaires were not significant (Ps $> .05$). Unlike OE densities, the brain densities of the 2 target protein deposits were significantly correlated with the measures of clinical function. These correlations were much stronger than the correlations between the brain and epithelial protein densities and were statistically significant in all cases.

Discussion

Our study, the first to compare the density of tau and amyloid-β markers within OE samples from the middle turbinate, superior turbinate, and cribriform plate/upper nasal

Table 1. Demographics, Marker Presence, and Ratings of Protein Concentrations.[a]

				Middle Turbinate				Superior Turbinate				Nasal Septum			
Diagnosis	No.	Age (SD)	Sex F/M	Amyloid-β (%; Mean)	Tau protein (%;Mean)	Amyloid-β and/or Tau (%)	Amyloid-β and Tau (%)	Amyloid-β (%;Mean)	Tau protein (%;Mean)	Amyloid-β and/or Tau (%)	Amyloid-β and Tau (%)	Amyloid-β (%;Mean)	Tau protein (%;Mean)	Amyloid-β and/or Tau (%)	Amyloid-β and Tau (%)
Controls	24	66.9 (10.3)	12/12	85.7; 2.6	64; 1.6	91	55	65; 2.9	53; 1.3	80	35	80.9; 2.7	30; .6	85.7	23.8
Alzheimer's disease	12	83.6 (1.9)	10/2	70; 2.5	60; 1.4	90	40	77.8; 2.5	22.2; .5	88.9	11.1	81.8; 3	30; .5	81.8	27.3
P values		<.001	.08>	>.35	>.08	1.0	.7	>.22	>.21	1.0	.37	>.07	>.36	1.0	1.0

[a]% represents percentage of positivity for each marker; mean represents mean of ordinal ratings (0 = 0% of epithelium marked, 1 = 1%-25%; 2 = 26%-75%; 3 ⩾75%) for the concentration of these proteins in the olfactory epithelium, variable used for comparison in the multiple linear regression.

Table 2. Spearman Correlations Between Amyloid-β and Tau Densities Among Different Olfactory Epithelium Regions.

Olfactory Epithelium Region	MT Amyloid-β *r* (*P* Value)	ST Amyloid-β *r* (*P* Value)	NS Amyloid-β *r* (*P* Value)	MT Tau *r* (*P* Value)	ST Tau *r* (*P* Value)	NS Tau *r* (*P* Value)
MT amyloid-β	1	0.31 (.18)	0.08 (.65)	0.05 (.78)	0.26 (.21)	0.14 (.45)
ST amyloid-β		1	−0.34 (.12)	0.13 (.5)	0.18 (.44)	0.08 (.71)
NS amyloid-β			1	−0.06 (.72)	−0.27 (.16)	0.22 (.20)
MT tau				1	0.58* (.002)	−0.11 (.55)
ST tau					1	−0.06 (.75)
NS tau						1

Abbreviations: MT, middle turbinate; NS, nasal septum; ST, superior turbinate.
*$P < .05$.

Table 3. Spearman Correlation Coefficients Among Clinical Questionnaire, Amyloid-β, and Tau Deposits Within the Olfactory Epithelium and Brain.

Olfactory Epithelium Region or Clinical Questionnaire	Brain Amyloid-β *r* (*P* Value)	Brain Tau *r* (*P* Value)
MT amyloid-β	−0.29 (.1)	0.05 (.7)
ST amyloid-β	−0.37 (.1)	−0.17 (.5)
NS amyloid-β	0.03 (.8)	−0.06 (.7)
MT tau	−0.27 (.1)	−0.02 (.9)
ST tau	−0.36 (.08)	−0.36 (.07)
NS tau	−0.09 (.6)	0.009 (.9)
IQCODE	0.57* (.002)	0.60* (.001)
CDR	0.56* (.001)	0.63* (<.001)

Abbreviations: CDR, Clinical Dementia Rating; IQCODE, Informant Questionnaire on Cognitive Decline in the Elderly; MT, middle turbinate; NS, nasal septum; ST, superior turbinate.
*$P < .05$.

septum, demonstrates that such density varies considerably across these regions. Moreover, it strongly suggests that such densities within the OE are not strongly related to analogous brain densities and clinical questionnaires known to be correlated with the diagnosis of AD. A number of factors are likely responsible for the heterogeneity across the OE regions, including respiratory metaplasia and cumulative damage from previous exposures to toxins, viruses, bacteria, and other infective processes.[5,32-34] This variability explains in part differences among the findings of some studies and suggests that standardization of sampling procedures are sorely needed.

In contrast to a previous study by Arnold and associates,[8] we did not find significant correlations between brain and olfactory epithelial levels of tau and beta-amyloid proteins, regardless of where the OE samples were taken. However, correlations found by these authors were small, varying between 0.17 and 0.37. In accord with other studies, our results suggest that olfactory epithelial biopsies are less accurate than other extant measures in diagnosing AD, including clinical questionnaires (accuracy up to 90%) and biomarkers in cerebrospinal fluid (CSF; sensitivity >95% and specificity >85).[4,35-40] Neuroimaging is also more accurate, with relatively high correlations being found between neuroimaging measures such as hippocampal volume imaging and 18F-FDG PET/CT AD pathology (eg, neurofibrillary tangles and amyloid plaques).[41,42] For example, the Oxford Project to Investigate Memory and Aging (OPTIMA) scanning criteria for computed tomography (CT) and single-photon emission CT (SPECT) achieved, respectively, an 80% and 83% accuracy in detecting AD; combined, they reached accuracy of 88%.[43] Correlations of CSF tau and Aβ with the density of such proteins in general brain regions range from 0.55 to 0.64.[44]

Our findings, as well as those of others, suggest that the deposition of AD-related proteins as the primary cause of the well-established decrease of olfactory function in patients with AD is questionable.[45] Thus, at best, most studies show "marginal" relations between olfactory test scores and the amount of aberrant Aβ in the brain, as measured by PET imaging using Pittsburgh Compound B (PiB)[46,47] or postmortem brain analyses.[48] Evidence in the literature relating olfactory function to levels of OE tau and Aβ proteins remains nonexistent. This suggests the possibility that other processes, such as damage to acetylcholine-related forebrain centers, may be more involved in the olfactory dysfunction of AD.[49]

It is important to emphasize that tau and Aβ pathologies are not present in the olfactory mucosa at the earlier stages of AD when olfactory dysfunction is present and when treatment would be most efficacious.[50,51] It is also important to note that Aβ pathology is not specific to the OE per se, as it is commonly present in the metaplastic respiratory epithelium that invades the olfactory area.[52] Thus, it is clear that for olfactory biopsies to be meaningful, the region of the epithelium that is biopsied must be carefully considered. While our findings, as well as the findings of others, imply that olfactory biopsies do not appear to be as useful as other measures in detecting AD, it is still possible that other pathological elements such as MicroRNA-206,[53] present within the OE, need to be explored in future studies to determine whether they may ultimately be useful in detecting AD.

There is no doubt that antemortem biomarkers—objective measures of a particular pathogenic process—are needed for the early diagnosis of AD and establishing and monitoring the efficacy of treatments designed to mitigate pathology before it becomes rampant or irreversible. Conceptually, if AD pathology were uniquely present in the OE of AD patients, then in vivo detection or confirmation of this disease could be made premortem using nasal biopsies, which are relatively safe and reproducible in the hands of a qualified surgeon.[53,54,55] However, our findings, along with others, suggest that tau and amyloid-β deposition within the olfactory epithelium is not a useful molecular marker for AD at any stage. This is in spite of the fact that olfactory dysfunction, as measured by psychophysical tests,[56] appears to be sensitive to the early stages of AD, including mild cognitive impairment (MCI). However, olfactory testing does not clearly distinguish AD from other types of dementia such as vascular dementia (VaD), frontotemporal lobe dementia (FTLD), or Lewy body dementia (LBD). It is entirely possible that the relative degrees of olfactory dysfunction observed among a wide range of neurological disorders is caused by a factor not specific to any given disease, such as the relative damage to forebrain neurotransmitter circuits.[49]

The present study has both strengths and weaknesses. Among its strengths are (a) the novel repeated sampling and quantification of amyloid-β and tau within multiple regions of the OE in a sizable number of AD and control specimens and (b) the examination of correlations of tau and amyloid-β levels within both olfactory epithelium and brain regions with one another as well as with measures of clinical dementia and informant assessments of cognitive decline. Its potential weaknesses include (a) the use of morphologic, rather than immunohistochemical, criteria for identifying the OE; (b) the measurement of only 2 AD-related pathologic markers, namely, tau and amyloid-β; and (c) the limited clinical application due to the difficult to obtain good quality samples from the narrow region of the olfactory cleft. However, aberrancies in the latter 2 proteins are the classical sine qua non indicators of AD and are universally considered the defining elements of AD.

The present study has clearly documented, for the first time, heterogeneity of tau and Aβ proteins among different olfactory epithelial regions. The basis of such differences is not clear but could reflect differences in the intrinsic makeup of the olfactory receptor sheet as well as other internal or external factors. Such heterogeneity is important to consider in future studies that explore more thoroughly the value of olfactory epithelial biomarkers in detecting early neurodegenerative diseases such as AD.

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Declaration of Conflicting Interests

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ORCID iD

Marco Aurélio Fornazieri https://orcid.org/0000-0001-5213-2337

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