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ABSTRACT

The use of ATR-FTIR spectroscopy for the diagnosis of Alzheimer's disease using oral buccal cells

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ABSTRACT

As general aging increases, the prevalence of dementia, particularly Alzheimer's disease, is anticipated to triple by 2050, posing significant socio-economic challenges. Existing biomarkers for Alzheimer's have limitations, especially in early stages, and current diagnostic methods involve invasive procedures or expensive imaging techniques. Developing a rapid, cost-effective, and noninvasive test is crucial for the early identification of individuals requiring further assessment. Oral cavity-derived samples like saliva and buccal mucosal cells are promising biomarker sources due to their correlation with peripheral changes in Alzheimer's. In this study, we explored the potential of attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy as a diagnostic tool for Alzheimer's using buccal cells. The analysis, coupled with machine learning algorithms, achieved a 76% sensitivity and 100% specificity (AUC: 88%) in distinguishing Alzheimer's patients from age-matched healthy controls. Our findings demonstrate that spectroscopic analysis of buccal cells has the potential to detect Alzheimer's disease with high diagnostic precision, offering a noninvasive and cost-effective alternative to current invasive procedures. Early diagnosis through such a test may impact disease progression by enabling timely intervention.

KEYWORDS

Alzheimer's disease; buccal mucosal cells; ATR-FTIR spectroscopy; innovative diagnostic tools

Introduction

Epidemiological studies on dementia prevalence have reported than an estimated 57 million people live with this condition worldwide and this is expected to reach 153 million by 2050.[1] Although dementia cases are annually increasing in incidence and prevalence, they remain difficult to definitively diagnose particularly in the earlier stages of the disease. The only conclusive diagnosis can be made at postmortem, after

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histological examination of brain tissue.[2] Nevertheless, current diagnosis relies on clinical features and parameters with supporting evidence from neuropsychological tests as well as laborious, costly or invasive biofluid tests (including cerebrospinal fluid (CSF)- and blood-based biomarkers) and brain imaging scans.

This limited success in both diagnosing and delivering effective treatment reflects a limited understanding of the mechanisms underlying neurodegenerative disorders and clearly denotes their complex, multifactorial nature.[3] More specifically, apart from protein misfolding and aggregation, a number of different factors have been previously associated with an increased risk of neurodegeneration, such as oxidative stress,[4] genetic factors,[5] mitochondrial dysfunction,[6] environmental factors (*e.g.,* pesticides and neurotoxic metals),[7] head injuries[8] and inflammation.[9,10] An increasing body of basic and clinical research has been focused on the study of the aforementioned risk factors and their mechanistic role(s) in relation to disease emergence and progression.

Whilst previous diagnostic studies in the field have failed to show great promise, a key finding that could potentially bring us closer to a successful diagnostic feature is the relatively recent establishment that pathologic changes in the brain begin years before symptoms manifest themselves clinically.[11] In Alzheimer's disease (AD), the commonest type of dementia, three different stages have now been suggested to characterise the disease according to its progression: preclinical (or pre-symptomatic) AD; mild cognitive impairment (MCI) due to AD; and dementia due to AD.[12] Therefore, it is anticipated that preventative and/or therapeutic strategies will be more effective in individuals with the disease at a very early stage (i.e. preclinical), before extensive brain damage occurs.[12] This would be facilitated by establishing an upstream diagnosis facilitating an early enrollment of patients into clinical trials and thus increase the potential to uncover an effective therapeutic intervention.[13] However currently-used methods and techniques are either invasive, significantly expensive and laborious or of mediocre diagnostic accuracy.[14,15]

Minimally-invasive biological fluids, such as blood plasma and serum, have emerged as a potential means for the diagnosis of neurodegenerative diseases, with some studies also reporting detection of early-stage disease.[15–18] A considerable amount of cerebrospinal fluid (CSF) is daily absorbed into the bloodstream, rendering blood an information-rich sample.[19] Following this rationale, a previous large-scale study using spectroscopic approaches for dementia detection in blood has also shown promising results.[20]

The chemical composition of saliva and buccal cells has been shown in previous studies to reflect a healthy or unhealthy physiological state and dramatic systemic changes have been shown to occur with the emergence of disease.[21–23] More recently, different groups have conducted pilot studies to assess the use of buccal cells and saliva as a means to detect AD. Specifically, Yilmaz et al. performed ^{1}H NMR-based metabolomics to determine whether salivary biomarkers have the potential to distinguish MCI and AD from healthy controls.[24] Kim et al. employed antibody-conjugated magnetic particles to measure the salivary Aβ levels of AD/MCI patients and allow differentiation from controls.[25] Francois et al. demonstrated changes in the tau and Aβ protein levels of AD/MCI patients by analysing buccal mucosa cells with laser scanning cytometry. Lee et al. reported higher levels of Aβ42 in the saliva of AD cases when compared to healthy controls.[26] These smaller-scale studies indicate that easily accessible buccal cells may hold great promise as a diagnostic tool for AD.

Biospectroscopic methods have shown great promise in the field of medical diagnostics as they have the advantage of being able to investigate a number of biomolecules simultaneously, which becomes more important when dealing with multifactorial diseases.[27] Infrared spectroscopy studies the interaction of light with biological matter, upon which characteristic vibrational motions, unique for different functional groups, are induced by the absorption of infrared radiation. The spectroscopic technique that was used in this study, attenuated total reflection- Fourier transform infrared (ATR-FTIR) spectroscopy, allows the generation of a biological signature (termed "biological fingerprint") for each biological sample by providing information about different molecules including proteins, lipids, carbohydrates, and nucleic acids and other metabolites.[28,29] These signatures can then be analysed using machine learning algorithms to distinguish between a pathological and healthy sample. Previous spectroscopic studies in dementias as well as other disease arenas, have demonstrated the potential for these techniques to develop into an accurate and cost-effective diagnostic test but have also paved the way for these technologies to be implemented for point-of-care diagnostics in the clinic.[20,30–32]

Easily accessible oral buccal cells have the potential to reflect pathological changes and therefore may provide a rapid and noninvasive test for the detection of different neurological disorders. Buccal cells have been previously found to reflect changes in the brain and have been studied as potential biomarkers for dementia, since they are thought to be embryologically related to the central nervous system and share common AD-specific characteristics.[26,33–36] The primary aim of this study was to assess the performance of ATR-FTIR spectroscopy in detecting individuals with Alzheimer's disease using oral buccal mucosal cells and to unravel biological changes related to dementia.

Methods

Study design – population

This was a prospective case-control study which allowed patient recruitment during their clinic visit at the Central Lancashire Memory Assessment Service, Lancashire and South Cumbria NHS Foundation Trust between January and August 2021. All participants gave written, informed consent to participate and donated their clinical data and buccal cell samples for this research study. Ethical approval was obtained before recruitment and sample collection from patients or their appointed carers (London - Harrow REC 20/LO/0603). All methods were performed in accordance with the relevant guidelines and regulations based on the Declaration of Helsinki.

Patients with a diagnosis of AD based on the National Institute on Aging-Alzheimer's Association (NIA/AA) core clinical criteria[37] were eligible for our study. Computed tomography (CT) brain scans were performed for most patients based on the service's imaging criteria. Spouses, nearest relatives or close friends accompanying the patient to the clinic or living with the patient were also recruited to donate buccal cells and serve as the study's healthy controls, having established they were not suffering from dementia. Individuals under the age of 18 years or those not speaking English were excluded from the study. Patients unable to give informed consent for whom a

personal consultee (spouse, nearest relative or close friend) could not be identified for further consultation were not asked to participate in the study.

Sample preparation and spectroscopic analysis

Oral buccal cells were collected from all participants (AD patients and controls) by a fully trained clinical research member of the Memory Clinic. Samples were collected from the inside of the cheek using a thin cytobrush. The clinical researcher collected samples by pressing and rotating the brush against the inside of the inner cheek for 1min, using an up and down motion, going from the front to back of each cheek to ensure maximum cellular collection. Cells were suspended in a vial containing a methanol-based preservative medium. This is a widely-used method known as liquid-based cytology (LBC), which improves cytological assessment in comparison to conventional smear testing. The vial was stored at 4 °C until spectroscopic analysis.

Buccal mucosal cells were initially washed with distilled water to remove the spectral signature from the methanol-based preservative solution. For the washing procedure, each sample was centrifuged (2000 rpm for 5 min) and the supernatant discarded; 2 ml of distilled H_2O was added to each sample, re-centrifuged and supernatant was again discarded. This procedure was repeated once more and the final cell pellet was immersed in 50 µl of distilled H_2O, placed on a suitable substrate (microscope glass slides covered with aluminum foil38] and left to air-dry at room temperature before spectroscopic analysis (Figure 1).

Spectra were collected with attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy using a Tensor 27 FTIR spectrometer with a Helios ATR attachment containing a diamond ATR crystal (Bruker Optics Ltd., Coventry, UK). In our experimental setting, the ATR crystal is on the top of the Helios attachment and the slide with the sample is placed on the platform with the sample facing up; the platform is then moved upward to ensure good contact with the crystal.[39] Spectral resolution was 8 cm^{-1} with 2× zero-filling, giving a data-spacing of 4 cm^{-1}. Thirty-two co-additions and a mirror velocity of 2.2 kHz were used for optimum signal to noise ratio. A closed-circuit television (CCTV) camera attachment was used to locate the area of interest and five spectra were acquired from each biological sample. The diamond crystal was cleaned with distilled water and dried before moving to the next sample; a background spectrum was taken after the analysis of each sample to account for changes in ambient conditions.

Spectral data analysis

Pre-processing of the acquired spectra is an essential step used to correct problems associated with spectral acquisition and instrumental noise before further analysis. The main pre-processing methods include truncation to the area of interest, spectral correction and normalisation. Spectral pre-processing and model building were performed within MATLAB R2014b (The Math-Works, Natick, EUA).

Data were pre-processed by truncating at the fingerprint region (1800-900 cm^{-1}), followed by baseline – automatic Whittaker filtering and Savitzky–Golay smoothing

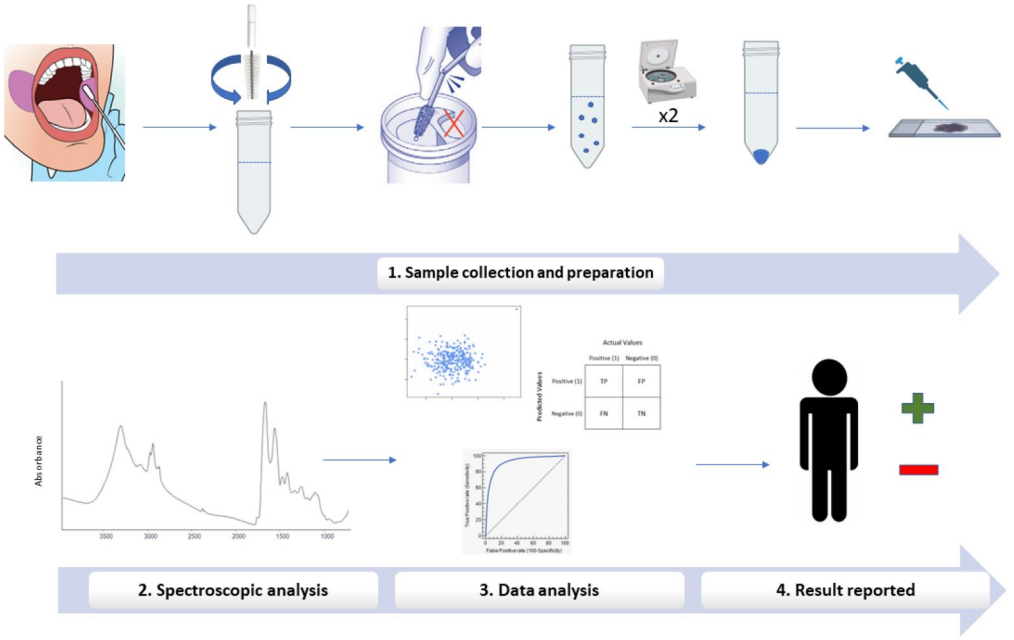

Figure 1. Diagrammatic representation of the experimental workflow. Oral buccal cells were collected from the inside of the cheeks using a thin cytobrush and suspended in a preservative methanol-based solution. The brush head was snapped off and left inside the vial until analysis. Buccal mucosal cells were washed twice with distilled water before the cell pellet was deposited on a substrate and left to air-dry before spectroscopic analysis. Following pre-processing and data analysis, the final result is reported indicating whether the participant is positive or negative for the disease. TP = true positive; FP = false positive; FN = false negative; TN = true negative.

(15-point window) (Figure 2). Samples were divided into training (70%) and test (30%) datasets before further multivariate analysis by using the Kennard-Stone uniform sample selection algorithm.[40]

The successive projection algorithm (SPA) was used in the selection of spectral variables. This technique considers variables as vectors in an iterative process. In this process, an initial vector is used, and new vectors are added with their respective projections in a subspace orthogonal to the initial vector. The criterion for selecting variables used by the SPA is the reduction of multicollinearity problems in order to eliminate redundant information and therefore the selected variables are those with the most differentiated projections.[41,42]

The variables selected by SPA were used to build a classification model based on quadratic discriminant analysis (QDA). In this supervised classification technique, training is carried out to teach the model to recognize the spectral data related to certain classes followed by a blind test that is performed to predict the class of new data without any prior information.[43] QDA calculates a function capable of discriminating the classes, called the discriminant function. The calculation used to arrive at a classification score is given in the following equation:

$$Q_{ik} = (\mathbf{x}_i - \overline{\mathbf{x}}_k)^T \sum_k^{-1} (\mathbf{x}_i - \overline{\mathbf{x}}_k) + \log_e \left| \sum_k \right| - 2\log_e \pi_k$$

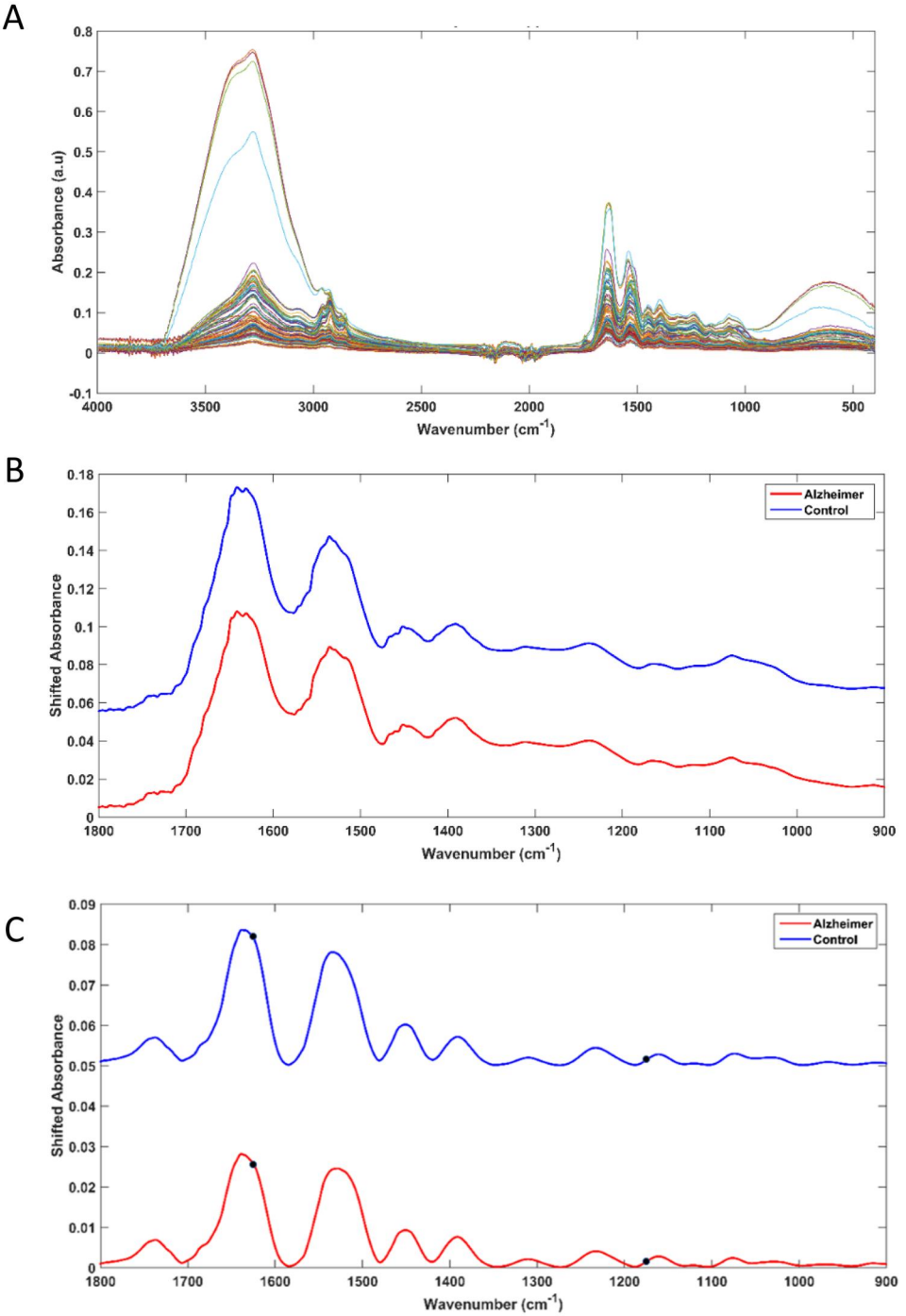

Figure 2. **A.** Raw spectra after analysis of buccal cells with ATR-FTIR. **B.** Mean spectra of Alzheimer's disease patients and controls in the fingerprint region (1800-900 cm^{-1}). **C.** Mean pre-processed spectra of Alzheimer's disease patients and controls along with the most important wavenumbers for classification selected by SPA-QDA (1628 cm^{-1}, 1181 cm^{-1}).

where $\mathbf{x}_i$ represents an unknown vector for a sample i; $\bar{\mathbf{x}}_k$ represents an average vector of class k; π_k is the previous probability of class k; $\sum_k$ is the variance-covariance matrix of class k; and $\log_e \left|\sum_k\right|$ is the natural logarithm of the k-class variance-covariance matrix.[44] Discriminant function (DF) graphs were generated to show the differences and similarities between the different classes (AD and controls).

Statistical analysis

To assess whether there were any potential baseline differences in selected clinical and demographic characteristics, we performed statistical analysis using IBM SPSS Statistics (version 26). P values were calculated using a t-test for the age and a Fisher's exact test for gender, ethnicity, education, family history of AD and other comorbidities. A P value < 0.05 was considered significant. To estimate the sample size for this pilot study, a power calculation was performed using a t-test (confidence interval of 95%) which was based on a previous spectral dataset from patients with AD. The mean and standard-deviation of the control and disease groups were respectively equal to 0.5 ± 0.2 and 0.6 ± 0.2 (Supplementary Figure S1). A total number of 34 participants (17 controls and 17 AD patients) was estimated to be statistically sufficient for achieving 80% power.

Results

A total of 34 participants were prospectively recruited for this study with 5 being removed from further analysis due to insufficient cellular material. Of the remaining 29 participants included in the final analysis, 17 were patients with AD and 12 were aged-matched controls not suffering from any type of dementia. The two groups had similar age, with the mean age for AD patients being 75 years (standard deviation (SD): 11.5; range: 62-89 years) while for controls 74 years (SD: 11.6; range: 49-92 years) (P value = 0.820); there was no record for the age of four participants from the control group however these were all mature adults. The majority of the AD group was comprised of female participants (12/17, 70%) while the control group had an even distribution of females and males (6/12, 50%), although the differences were not statistically significant (P value = 0.438). All participants were of the same ethnicity (White), and education status (school, bachelor's degree or higher) between the groups did not show any statistical difference (P value= 1.000). Within the AD group, the majority reported no family history of AD (10/17, 59%) whereas 6/17 patients (35%) had someone in their family with a previous diagnosis of AD; one participant was unsure (1/17, 6%) (P value= 1.000). Other comorbidities that were reported by the participants included coronary heart disease (AD group: 2/17, 12%; control group: 1/12, 9%), anxiety and/or depression (AD group: 4/17, 23%; control group: 0/12), hypertension (AD group: 3/17, 18%; control group: 1/12, 8%) and diabetes (AD group: 2/17, 12%; control group: 1/12, 8%); 6/17 (35%) from the patient cohort and 9/12 (75%) from the control cohort had no known comorbidities (P value = 0.230). Patient characteristics are provided in detail in Table 1.

Five spectra were collected from each sample/participant resulting in a total number of 145 spectra. Before further analysis, spectra were averaged every five to account for differences between participants rather than individual spectra. Prior to multivariate

Table 1. Demographic information for the cohort included in the study.

Patient Characteristics	Alzheimer's disease (AD) ($N = 17$)	Controls ($N = 12$)	P value
Age			0.820
Mean (SD, range)	75 (11.5, 62-89)	74 (11.6, 49-92) *	
Gender, n/N (%)			0.438
Female	12/17 (70%)	6/12 (50%)	
Male	5/17 (30%)	6/12 (50%)	
Ethnicity, n/N (%)			
White	17/17 (100%)	12/12 (100%)	
Education, n/N (%)			1.000
School	10/17 (59%)	8/12 (66%)	
Bachelor's degree or higher	2/17 (12%)	2/12 (17%)	
Unknown	5/17 (29%)	2/12 (17%)	
Family history of AD			1.000
Yes	6/17 (35%)	4/12 (33%)	
No	10/17 (59%)	8/12 (67%)	
Unsure	1/17 (6%)	0/12 (0%)	
Other comorbidities			0.230
Coronary heart disease	2/17 (12%)	1/12 (9%)	
Anxiety and/or depression	4/17 (23%)	0/12 (0%)	
Hypertension	3/17 (18%)	1/12 (8%)	
Diabetes	2/17 (12%)	1/12 (8%)	
Not known comorbidities	6/17 (35%)	9/12 (75%)	

*There was no record for the age of four participants from the control group.

		Actual class	
		Alzheimer's disease	**Controls**
Predicted class	**Alzheimer's disease**	TP = 13 (sens = 76%)	FP = 0
	Controls	FN = 4	TN = 12 (spec = 100%)

Figure 3. Confusion matrix after SPA-QDA classification showing the true positive (TP), true negative (TN), false positive (FP) and false negative (FN) values alongside the sensitivity (76%) and specificity (100%) of the technique in differentiating between Alzheimer's disease patients and controls. The overall accuracy of the technique is 86% ((TP + TN)/(TP + FP + TN + FN)).

analysis and classification, spectra underwent pre-processing to correct for any non-biological difference; mean pre-processed spectra for both classes (AD and controls) are shown in Figure 2.

Receiver operating characteristic (ROC) curves were used to calculate the area under the curve (AUC) and find a compromise between sensitivity and specificity.[45] Comparing AD patients with controls generated an AUC of 88% (95% confidence interval (CI): 77-97%), 76% sensitivity and 100% specificity (overall accuracy of 86%) (Figures 3 and 4). The fingerprint region (1800-900 cm^{-1}) was interrogated since it represents more closely molecules of biological interest. The most important spectral peaks that were found to be responsible for the observed differentiation between cases and controls were 1628 cm^{-1} and 1181 cm^{-1}, which correspond to the Amide I and Amide III/CH_2 regions,[46] respectively (Figure 2), with 1628 cm^{-1} being increased in AD while 1181 cm^{-1} showed a decrease in AD (Figure 5).

Discussion

Given the increasing trends in population growth and aging, the prevalence of dementia cases is also expected to rise. Dementia is already the 7th leading cause of death

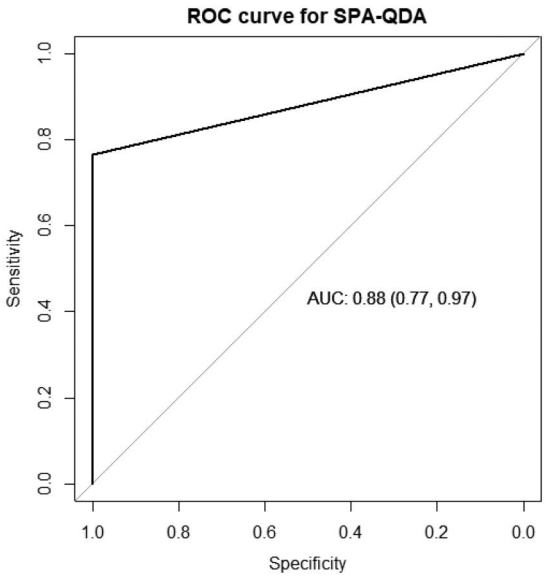

Figure 4. Receiver operating characteristic (ROC) curve displaying tradeoff between sensitivity (76%) and specificity (100%) of SPA-QDA classification of spectra derived from Alzheimer's disease patients and controls. The generated value for the area under the curve (AUC) along with 95% confidence intervals (CI) are given within the plot.

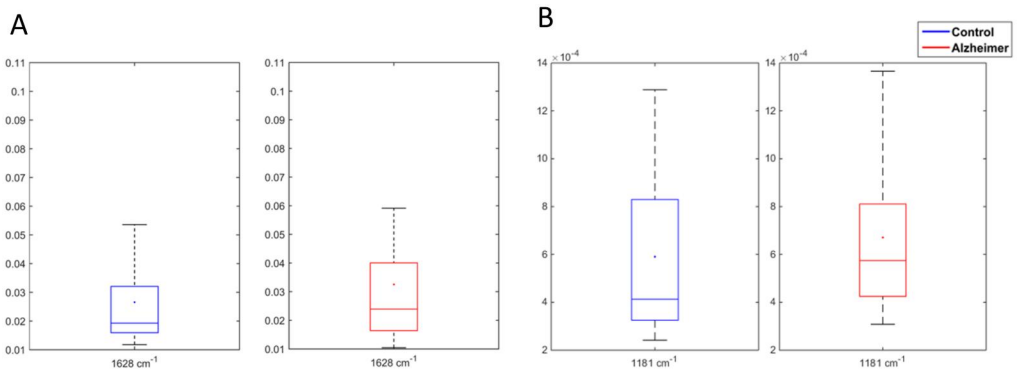

Figure 5. The two most discriminating peaks between Alzheimer's disease patients and controls detected after successive projection algorithm (SPA). Differences in absorbance levels are given as the mean ± standard deviation and were calculated after automatic baseline correction and smoothing. $p < 0.05$.

globally[47] and the number of people living with AD is predicted to almost triple by 2050[1], rendering this disease a public health crisis. An accurate diagnosis of AD at the early stages of the disease, or even before symptoms present, would potentially be a game-changer as individuals would be given the chance to enroll to clinical trials, where therapeutic interventions would be most effective before extensive neurodegeneration of the brain. Even though a definitive diagnosis for AD can only be given postmortem after histopathological assessment of the brain, current approaches for a working diagnosis necessitate a combination of different tests, including batteries of neuropsychological assessments,

neuroimaging techniques and the determination of specific biomarkers in CSF (β-amyloid, total tau, phosphorylated tau and neurofilament light).[15,48–50] Such tests present with a number of limitations, such as moderate accuracy, high cost and time-consuming laboratory tests, limited availability and invasive sample collection procedures, thus creating the need for new diagnostic tests.

In the last decade there has been an emerging shift to the use of minimally-invasive biological fluids, such as blood and saliva, as alternative peripheral sources for AD biomarkers. Almost 500 ml of CSF are absorbed into the bloodstream daily, which renders blood an information-rich source with the potential to reflect changes occurring in the brain.[19] Also, the damage to the blood-brain barrier, caused by AD development, may enhance the leakage of important molecular information in either direction.[51] Numerous blood-based studies have so far demonstrated the promise of these samples for dementia diagnostic purposes.[16,19,20,52–55] A more recent, less explored area of interest includes the use of saliva and buccal mucosal cells in the detection of AD-associated biomarkers. Emerging evidence from numerous studies suggests that oral-derived samples also hold promise for diagnosing or monitoring AD, while exhibiting practical advantages over other biofluids, such as the ease of collection (potentially allowing for self-collected samples), low cost and completely noninvasive approaches required.[56,57] Individual biomarkers as well as panels of biomarkers have been investigated in AD using saliva samples and buccal cells, however, with some contradictory results across the different studies.[15] A number of different technological approaches have been employed using these sample types over the years, such as ELISA measurements of β-amyloid,[25,35,36,58] total tau,[34,58,59] phosphorylated tau [58,59] or lactoferrin,[60] the use of different omics techniques to evaluate the different metabolomic profiles between MCI, AD patient and controls,[24,61,62] as well as experiments to assess the oral microbiome of different cohorts,[63,64] amongst others.

In the present study, we have demonstrated that ATR-FTIR spectroscopy can be used to differentiate AD patients from controls with high diagnostic accuracy (76% sensitivity and 100% specificity) using oral buccal cells. The presence of AD-related pathological changes in orally-derived samples, such as saliva and buccal cells, has been suggested to occur after biomarker secretion by the nerves into the salivary glands due to their close proximity to the central nervous system[59] or after molecule transport from blood to saliva through ultrafiltration and passive diffusion or active transport.[65,66] To our knowledge, this is the first study of its kind demonstrating the proof-of-concept and could open new avenues for detecting AD in a rapid and inexpensive manner. The suggested approach could be used as a screening/triage test to identify individuals in primary care settings that would need referral for further testing using more invasive or expensive tests, such as CSF markers and imaging techniques.

Spectroscopic techniques are advantageous over molecular assays that investigate specific biological molecules in isolation, as they can provide a more generic, biochemical fingerprint and reflect changes of a range of biomolecules simultaneously. Future studies should focus on validating these promising results in a larger cohort, also including asymptomatic and MCI cases, to determine the clinical potential of the technology and provide a cost-effective and noninvasive diagnostic test for AD and other dementia types. Technological advancements have also allowed the advent of portable, hand-held

and miniaturised devices to permit point-of-care testing, which would enable an easier implementation into a clinical setting.

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Author contributions

The study was conceived and designed by MP, SK and SC. The samples were collected by SC. MP conducted experimental work. Data analysis was performed by MP, MS and KL. The data was interpreted, and the manuscript was drafted and revised critically for important intellectual content by all authors. All authors gave final approval of the version to be published and have contributed to the manuscript.

Availability of data

The data (raw spectra and preprocessed spectra) reported in this paper is available at the publicly accessible data repository Figshare (https://figshare.com/articles/dataset/ATR-FTIR_data_of_buccal_cells_-_OPUS/22048109).

Disclosure statement

No potential conflict of interest was reported by the authors.

Ethics approval and consent to participate

Ethical approval was obtained before recruitment and sample collection from patients or their appointed carers (London - Harrow REC 20/LO/0603).

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